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(71) Applicant(s)
DANISCO US INC.

(72) Inventor(s)
Yoon, Mee-Young

(74) Agent / Attorney
Houlihan2, Level 1 70 Doncaster Road, BALWYN NORTH, VIC, 3104

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(71) Applicant *(for all designated States except US)*: **DANISCO US INC., GENENCOR DIVISION** [US/US]; 925 Page Mill Road, Palo Alto, California 94304 (US).

(72) Inventor; and
(75) Inventor/Applicant *(for US only)*: **YOON, Mee-Young** [US/US]; c/o Danisco US Inc., Genecor Division, 925 Page Mill Road, Palo Alto, California 94304 (US).

(74) Agents: **BACSI, Steven G.** et al.; Danisco US Inc., Genecor Division, 925 Page Mill Road, Palo Alto, California 94304 (US).

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(54) **Title**: ALPHA-AMYLASE VARIANTS WITH ALTERED PROPERTIES

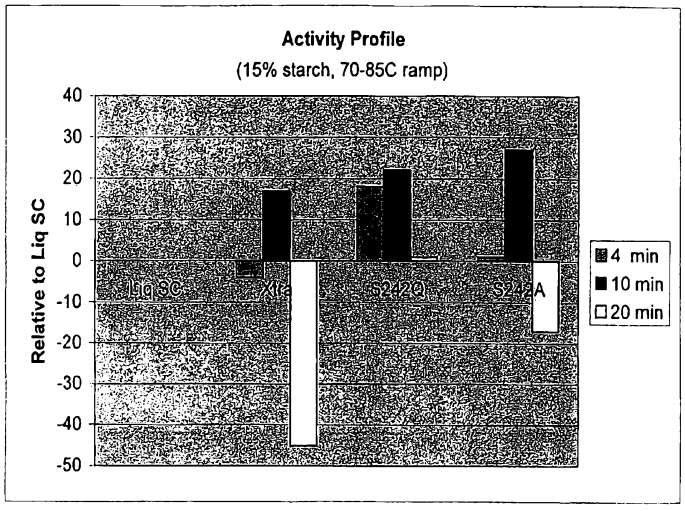

Figure 7

(57) **Abstract**: Disclosed are compositions comprising variants of alpha-amylase that have alpha-amylase activity and that exhibit altered properties relative to a parent AmyS-like alpha-amylase from which they are derived. The compositions generally comprise at least one of an additional enzyme, a detergent, a surfactant, a chelator, an oxidizing agent, an acidulant, an alkalizing agent, a source of peroxide, a source of hardness, a salt, a detergent complexing agent, a polymer, a stabilizing agent, or a fabric conditioner. Also disclosed are detergent formulations comprising the variants. Methods of using the compositions for desizing woven material and washing or cleaning items, such as dishes or laundry, are disclosed. Kits related thereto are also provided.

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Alpha-Amylase Variants With Altered Properties

SEQUENCE LISTING

Attached hereto is a sequence listing comprising SEQ ID NOS 1-30, each of which is herein incorporated by reference in its entirety.

CROSS-REFERENCE TO RELATED APPLICATIONS

This claims benefit of U.S. Provisional Applications 60/985,619, filed November 5, 2007, 61/026,579, filed February 6, 2008, 61/041,075, filed March 31, 2008, and 61/059,411, filed June 6, 2008, the disclosures of each of which are incorporated herein by reference in their entireties, for all purposes.

FIELD OF THE DISCLOSURE

This disclosure relates to novel alpha-amylases. In particular, it relates to methods of using certain variant alpha-amylase activities, and blends thereof for stain removal and as a component of detergent compositions for washing.

BACKGROUND

Alpha-amylases (alpha-1,4-glucan-4-glucanohydrolases, E.C. 3.2.1.1) constitute a group of enzymes that catalyze hydrolysis of starch and related linear or branched 1,4-glucosidic oligo- and polysaccharides.

Amylases can be used for a variety of purposes. For examples, amylases are used commercially in the initial stages of starch processing (e.g., liquefaction); in wet milling processes; and in alcohol production from carbohydrate sources. They are also used as cleaning agents or adjuncts in detergent matrices; in the textile industry for starch desizing; in baking applications; in the beverage industry; in oilfields in drilling processes; in recycling processes, e.g. for de-inking paper, and in animal feed.

Attempts have been made to construct alpha-amylase variants with improved properties for specific uses, such as starch liquefaction and textile desizing.

There is a need for the creation and improvement of amylases that provide, e.g., manufacturing and/or performance advantages over the industry standard enzymes (e.g., from *Bacillus licheniformis*), for various uses, including commercial desizing as well as cleaning/washing and stain or starch removal processes. There is also a need for detergents and cleaning aids or formulations comprising improved amylases and additional components, such as surfactant, chelators, and the like.

SUMMARY

In one aspect the present disclosure relates, inter alia, to novel α-amylolytic enzymes variants of parent α -amylase such as an AmyS-like α-amylase, in particular variants exhibiting altered properties that are advantageous in connection with the cleaning or washing processes, or the removal of starch, for example in desizing woven material.

For example, the variant is altered, as compared to a parent AmyS-like alpha-amylase or a reference amylase, in one or more of net charge, substrate specificity, substrate cleavage, substrate binding, thermal stability, activity at one or more pH's, stability at one or more pH's, stability in oxidizing conditions, Ca^{2+} requirements, specific activity, catalytic rate, catalytic efficiency, activity in the presence of a chelator, thermal or pH stability in the presence of a chelator, utility for desizing, or utility for a cleaning process, or amount of expression in a protein expression system, and other properties of interest. For instance, one or more alterations may result in a variant that has reduced Ca^{2+} dependency and/or an altered pH/activity profile and/or altered thermostability, as compared to a parent α-amylase, such as an AmyS-like amylase.

In one aspect, there is provided herein a variant of a parent *Geobacillus stearothermophilus* alpha-amylase, wherein the variant has an amino acid sequence which has at least about 95% homology to a parent *Geobacillus stearothermophilus* alpha-amylase and comprises a substitution of amino acid 242, wherein the amino acid positions in the peptide sequence are numbered relative to a reference amylase (e.g., SEQ ID NO: 1 or 2), and wherein the variant has alpha-amylase activity.

In another aspect, provided are compositions comprising: a) at least one variant alpha-amylase comprising an amino acid sequence at least about 95% identical to that of a parent AmyS-like alpha-amylase, and having a substitution at an amino acid position corresponding to position 242 of a reference alpha-amylase, said variant having detectable alpha-amylase activity, and b) at least one of an additional enzyme, a detergent, a surfactant, a chelator, an oxidizing agent, an acidulant, an alkalizing agent, a source of peroxide, a source of hardness, a salt, a detergent complexing agent, a polymer, a stabilizing agent, or a fabric conditioner. In preferred embodiments, the reference amylase is SEQ ID NO: 1 or 2, and the composition is a component of a product for use in laundry, dish, or hard-surface cleaning, desizing, or fabric or stain treatment.

In one embodiment, the composition comprises an additional enzyme is a protease, a lipase, an amylase, a cellulase, a peroxidase, an oxidase, a pectinase, a lyase, a cutinase, a laccase, or a combination thereof.

In various embodiments, the surfactant is nonionic, anionic, cationic, or zwitterionic. The variant alpha-amylase is preferably a S242Q variant. In some embodiments, the variant has altered stability to oxidation and the variant alpha-amylase further includes deletion or substitution of one or more methionine residues including residues located at amino positions 8, 9, 96, 200, 206, 284, 307, 311, 316, and 438 of a parent AmyS-like alpha-amylase, where the reference alpha-amylase in SEQ ID NO: 2.

In others, the variant alpha-amylase further comprises a sequence modification at one or more amino acid positions corresponding to amino acid positions 97, 179, 180, 193, 319, 349, 358, 416, 428, or 443 of the reference alpha-amylase. In yet other embodiments, the variant comprises one or more of substitution at positions as follows: a cysteine at 349, a cysteine at 428, a glutamic acid at 97, an arginine at 97, a glutamic acid at 319, an arginine at 319, a glutamic acid at 358, an arginine at 358, a glutamic acid at 443, or an arginine at 443.

Also useful herein are variant alpha-amylases comprising a substitution of an N193 or a V416 or both, e.g., a substitution of N193F or V416G, or both. In certain embodiments, the variants feature deletion of one or more amino acids, e.g., at positions F178, R179, G180, I181, G182 and K183.

Preferably, the variant alpha-amylase has altered metal ion dependence or altered stability or activity in an absence of added calcium or a presence of a chelator in certain embodiments.

The variant alpha-amylase preferably has at least 95%, 98%, or even 99% or more homology to SEQ ID NO: 2, and comprises a substitution of amino acid 242 relative to numbering in a reference alpha-amylase comprising SEQ ID NO: 1, and wherein the variant alpha-amylase has alpha-amylase activity.

The parent AmyS-like alpha-amylase is SEQ ID NO: 1, 2, 6, 7, 8, 9, 10, 11, 12, 15, or 16, and the reference alpha-amylase is SEQ ID NO: 1 or 2 in one embodiment.

Preferably, the variant alpha-amylase has improved performance in a wash process at a pH ≥ about 8, relative to the parent AmyS-like alpha-amylase.

The variant alpha-amylase can feature, in various embodiments, a set of substitutions of a) Q97E, Q319E, Q358E, Q443E; b) Q97E, Q319R, Q358E, Q443R; c) Q97E, Q319R, Q358E; d) Q97E, Q319R, Q443E; e) Q97E, Q319R, Q443R; f) Q97E, Q358R; g) Q97E, Q443E; h) Q319R, Q358E, Q443E; or i) Q319R, Q358R, Q443E.

In another of its several aspects, the disclosure provides compositions that are detergent or cleaning formulations comprising at least one variant amylase comprising an amino acid sequence at least about 95% identical to that of a parent AmyS-like alpha-amylase, and having a substitution at an amino acid position corresponding to position 242 of a reference alpha-amylase, wherein the variant has detectable alpha-amylase activity; wherein the reference amylase is SEQ ID NO: 1 or 2. In some embodiments, the variant is an S242 variant comprising at least a S242Q substitution.

In another of its several aspects, this disclosure provided methods of desizing a woven material subsequent to a weaving process comprising contacting the woven material with a variant alpha-amylase comprising an amino acid sequence at least about 95% identical to that of a parent AmyS-like alpha-amylase, and having a substitution at an amino acid position corresponding to position 242 of a reference alpha-amylase. The variant preferably has detectable alpha-amylase activity. The contacting is performed under conditions and for a time that are effective for at least partially removing sizing from the woven material.

In various embodiments, the variant alpha-amylase is altered, as compared to a parent AmyS-like alpha-amylase or a reference alpha-amylase, in one or more of: (a) net charge, (b) substrate specificity, (c) substrate cleavage, (d) substrate binding, (e) thermal stability, (f) activity at one or more pH's, (g) stability at one or more pH's, (h) stability in oxidizing conditions, (i) Ca^{2+} requirements, (j) specific activity, (k) catalytic rate, (l) catalytic efficiency,

(m) activity in a presence of a chelator, (n) thermal or pH stability in the presence of a chelator, (o) effectiveness for desizing, or (p) amount of expression in a protein expression system.

The parent AmyS-like alpha-amylase is SEQ ID NO: 1, 2, 6, 7, 8, 9, 10, 11, 12, 15, or 16, and the reference alpha-amylase is SEQ ID NO: 1 or 2 in various embodiments. Preferably, the variant alpha-amylase is a S242Q variant.

In certain embodiments, the variant alpha-amylase further comprises one or more of substitution at positions as follows: a cysteine at 349, a cysteine at 428, a glutamic acid at 97, an arginine at 97, a glutamic acid at 319, an arginine at 319, a glutamic acid at 358, an arginine at 358, a glutamic acid at 443, or an arginine at 443, wherein the reference alpha-amylase is SEQ ID NO: 1 or 2.

Methods of washing or cleaning are also provided. The methods comprise contacting one or more items to be washed or cleaned with a composition comprising a variant alpha-amylase comprising an amino acid sequence at least about 95% identical to that of a parent AmyS-like alpha-amylase, and having a substitution at an amino acid position corresponding to position 242 of a reference alpha-amylase. The contacting is performed under conditions and for a time effective for at least partially washing or cleaning the one or more items. The variant has detectable alpha-amylase activity. In exemplary methods, at least one item is soiled with at least one starch-containing material, the removal of which is aided by the variant amylase. In various embodiments of these methods, the composition further comprises one or more of an additional enzyme, a detergent, a surfactant, a chelator, an oxidizing agent, an acidulant, an alkalizing agent, a source of peroxide, a source of hardness, a salt, a detergent complexing agent, a polymer, a stabilizing agent, or a fabric conditioner.

In one embodiment of the methods, the parent AmyS-like alpha-amylase is SEQ ID NO: 1, 2, 6, 7, 8, 9, 10, 11, 12, 15, or 16, and the reference alpha-amylase is SEQ ID NO: 1 or 2. Preferably, the variant alpha-amylase is a S242Q variant.

In various embodiments, the variant alpha-amylase has improved performance in a wash process at a pH $\geq$ about 8, relative to the parent AmyS-like alpha-amylase.

In one embodiment, the variant alpha-amylase comprises one or more of substitution at positions as follows: a cysteine at 349, a cysteine at 428, a glutamic acid at 97, an arginine at 97, a glutamic acid at 319, an arginine at 319, a glutamic acid at 358, an arginine at 358, a glutamic acid at 443, or an arginine at 443. In others, the variant alpha-amylase comprises a set of

substitutions of a) Q97E, Q319E, Q358E, Q443E; b) Q97E, Q319R, Q358E, Q443R; c) Q97E, Q319R, Q358E; d) Q97E, Q319R, Q443E; e) Q97E, Q319R, Q443R; f) Q97E, Q358R; g) Q97E, Q443E; h) Q319R, Q358E, Q443E; or i) Q319R, Q358R, Q443E.

The method can also comprise use of variant alpha-amylases comprising deletion of one or more amino acids at positions F178, R179, G180, I181, G182, or K183.

In certain embodiments, the variant alpha-amylase has altered metal ion dependence or altered stability, or activity in an absence of added calcium or the presence of a chelator.

Also provided herein are kits comprising a) one or more variant alpha-amylases comprising an amino acid sequence at least about 95% identical to that of a parent AmyS-like alpha-amylase, and having a substitution at an amino acid position corresponding to position 242 of a reference alpha-amylase, said variant having detectable alpha-amylase activity, and b) at least one of an additional enzyme, a detergent, a surfactant, a chelator, an oxidizing agent, an acidulant, an alkalizing agent, a source of peroxide, a source of hardness, a salt, a detergent complexing agent, a polymer, a stabilizing agent, or a fabric conditioner.

In one embodiment, the kit further comprises instructions for use, e.g., for using the kit components in a process for desizing a woven material, or for washing or cleaning one or more items soiled with a starch-containing substance.

These and other features of the disclosure will described in more detail below.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows alignment of amino acid sequences among several candidate parent alpha-amylases (AmyS-like amylases) for use herein. Positions corresponding to any amino acid position (e.g., 1 through 520) of the amylase from *Geobacillus stearothermophilus* (SEQ ID NO: 1) can be readily determined. SEQ ID NO: 1, alpha-amylase from *G. stearothermophilus* "BSG"; SEQ ID NO: 2, truncated amylase from *G. stearothermophilus* (AmyS, SPEZYME XTRA); SEQ ID NO: 3, *G. stearothermophilus* (S242A variant amylase); SEQ ID NO: 4, *G. stearothermophilus* (S242Q variant amylase); SEQ ID NO: 5, *G. stearothermophilus* (S242E variant amylase); SEQ ID NO: 6, Yamane 707 amylase; SEQ ID NO: 7, mature LAT amylase; SEQ ID NO: 8, *Bacillus licheniformis* wild-type amylase [TERMAMYL (NOVOZYMES) = SEQ ID NO: 8 in WO 02/10355A2]; SEQ ID NO: 9, *B. amyloliquefaciens* amylase, BAN; SEQ ID NO: 10, STAINZYME = AA560 which is SEQ ID NO: 2 in WO 0060060 or SEQ ID NO: 24 in US 6,528,298; SEQ ID NO: 11, *B. halmapalus* amylase (NATALASE); SEQ ID NO: 12, KSM-1378 (KAO CORP., SEQ ID NO: 3 in EP1199356) ; SEQ ID NO: 13, *Bacillus* spp. KSM-K38 (KAO CORP., SEQ ID NO: 4 in US 6,403,355 B1); SEQ ID NO: 14, *Bacillus* spp. KSM-K36 (KAO CORP., SEQ ID NO: 2 in US 6,403,355 B1); SEQ ID NO: 15, LIQUOZYME SC (NOVOZYMES); and SEQ ID NO: 16, Consensus Parent Alpha-Amylase Sequence #1.

Figure 2 shows the pHPLT-AmyS plasmid.

Figure 3 shows percent residual activity of S242 variants after heat stress at 95°C for 30 minutes. Variant positions P, S, W, and Y were missing and replaced by wild-type AmyS (Spezyme® Xtra (labeled "Z")). A positive control, *G. stearothermophilus* with Δ179-180 with the C-terminus truncated by 29 amino acids (i.e., SEQ ID NO: 2) is also shown. Lines indicate 2× and 3× above the standard deviation of the percent residual

activity of the wild-type enzyme. S242A and S242Q clearly show higher residual activities than the wild-type.

Figure 4: Panels A, B, C, D, E, F, G, H, and I show pair-wise alignments and consensus sequences for several sequences from Figure 1, and feature, respectively, Consensus Sequences 2, 3, 4, 5, 6, 7, 8, 9, and 10, or SEQ ID NOs: 22, 23, 24, 25, 26, 27, 28, 29, and 30, respectively.

Figure 5 shows the thermal melting curves and the melting points for the wild-type and amylase variants without added calcium.

Figure 6 shows the thermal melting curves and the melting points in the presence of 2 mM added calcium for both the wild-type and the amylase variants.

Figure 7 shows the activity profile at 4, 10, and 20 minutes for Spezyme Xtra and two variants, relative to Liquozyme SC.

Figure 8 shows the activity profile of four variants relative to the S242Q variant for three time points.

Figure 9 is a graph depicting the performance of S242Q (filled circles) and its variants (open circles), as a function of charge, in the rice starch microswatch assay under North American laundry conditions using S242Q combinatorial charge library, rice starch microswatch cleaning in Tide 2x, at 20°C. Reference is made to Example 10.

Figure 10 is a graph depicting the performance of a truncated *Bacillus sp.* TS-23 amylase (closed circles) with the following mutations: Q98R, M201L, S243Q R309A, Q320R, Q359E, and K444E and its charge variants (open circles) (see co-pending U.S. Patent Application No. PCT/US2008/007103, filed 6 June 2008) in the rice starch microswatch assay as a function of charge under Western European laundry conditions with TS23t combinatorial charge library, rice starch microswatch cleaning in Persil at 40°C. Reference is made to Example 10.

Figure 11 is a graph depicting the performance of S242Q (closed circles) and its variants (open circles) in the BODIPY-starch assay as a function of charge. S242Q combinatorial charge library (CCL), specific activity on BODIPY-starch, standard assay conditions Reference is made to Example 10.

Figure 12: Panel A is a graph depicting the relative BODIPY-starch hydrolysis as a function of relative shake tube expression (i.e., relative BODIPY-starch hydrolysis vs. relative shake tube expression); Panel B is a graph depicting the relative microswatch-starch hydrolysis as a function of relative shake tube expression (i.e., relative microswatch-starch hydrolysis vs. relative shake tube expression). Reference is made to Example 13.

Figure 13: Panel A is a graph depicting the relative shake tube expression as a function of charge; Panel B is a graph depicting the relative BODIPY-starch hydrolysis as a function of charge. Reference is made to Example 13.

Figure 14: Panel A is a graph depicting the relative shake tube expression as a function of charge; Panel B is a graph depicting the relative microswatch cleaning activity as a function of charge. Reference is made to Example 13.

Figure 15 shows the effects of added Ca^{2+}on desizing performance of variant S242Q compared to that of Ethyl and Xtra in the LAUNDER-O-METER under conditions of 85°C, for 30 min. at 0.01 ppm active protein. The desizing was performed in the presence of 0 or 5 ppm $CaCl_2$. *See* Example 14.

Figure 16 shows the effects of added Ca^{2+}on desizing performance of variant S242Q compared to that of Ethyl and Xtra in the LAUNDER-O-METER under conditions of 97°C, for 30 min. at 0.01 ppm active protein. The desizing was performed in the presence of 0 or 5 ppm $CaCl_2$. *See* Example 14.

DETAILED DISCLOSURE

1. Definitions & Abbreviations

In accordance with this disclosure, the following abbreviations and definitions apply. It should be noted that as used herein, the singular forms "a," "an," and "the" include plural referents unless the context clearly dictates otherwise. Thus, for example, reference to "a polypeptide" includes a plurality of such polypeptides and reference to "the formulation" includes reference to one or more formulations and equivalents thereof known to those skilled in the art, and so forth.

Unless defined otherwise, all technical and scientific terms used herein have the

same meaning as commonly understood by one of ordinary skill in the art. The following terms are provided below.

1.1. Abbreviations

The following abbreviations apply unless indicated otherwise:

AATCC	American Association of Textile Chemists and Colorists;
ADW	automatic dish washing;
AE	alcohol ethoxylate;
AEO	alcohol ethoxylate;
AEOS	alcohol ethoxysulfate;
AES	alcohol ethoxysulfate;
AFAU	acid fungal alpha-amylase units;
AGU	glucoamylase activity units;
AOS	α-olefinsulfonate;
AS	alcohol sulfate;
BAA	bacterial alpha-amylase;
°C	degrees Centigrade;
CCL	combinatorial charge library;
cDNA	complementary DNA;
CMC	carboxymethylcellulose;
dE	total color difference, as defined by the CIE-LAB color space;
dH_2O	deionized water;
dIH_2O	deionized water, Milli-Q filtration;
DE	Dextrose Equivalent;
DNA	deoxyribonucleic acid;
dNTP	deoxyribonucleotide triphosphates;
DO	dissolved oxygen;
DP3	degree of polymerization with three subunits;
DPn	degree of polymerization with n subunits;
DS (or ds)	dry solids content;
DSC	differential scanning calorimetry;

	DTMPA	diethyltriaminepentaacetic acid;
	EC	enzyme commission for enzyme classification;
	EDTA	ethylenediaminetetraacetic acid;
	EDTMPA	ethylenediaminetetramethylene phosphonic acid;
5	EO	ethylene oxide;
	eq	equivalents;
	ETOH	ethanol;
	F&HC	fabric and household care;
	FTU	"fitase" units, phytate hydrolyzing unit;
10	g (or gm)	grams;
	GAU	glucoamylase unit;
	gpg	grains per gallon;
	g/l	grams per liter;
	Genencor	Danisco US Inc, Genencor Division, Palo Alto, CA;
15	H_2O	water;
	HDG	heavy duty granular detergent;
	HDL	heavy duty liquid detergent;
	HFCS	high-fructose corn syrup;
	HFSS	high-fructose starch-based syrup;
20	HPAEC-PAD	high performance anion exchange chromatography with pulsed amperometric detection;
	hr(s)	hour/hours;
	IKA	IKA Works Inc. 2635 North Chase Parkway SE, Wilmington, NC;
	IPTG	isopropyl β-D-thiogalactoside;
25	JPN	Japan;
	kg	kilograms;
	LA	Luria Agar;
	LAS	linear alkylbenezenesulfonate;
	LB	Luria Broth;
30	LU	Lipase Units;

M	molar;
MBD medium	MOPS-based defined medium;
MES	2-(*N*-morpholino)ethanesulfonic acid;
mg	milligrams;
min(s)	minute/minutes;
mL (or ml)	milliliters;
mm	millimeters;
mM	millimolar;
MOPS	3-(N-Morpholino)-propanesulfonic acid;
MW	molecular weight;
NA	North America;
Ncm	Newton centimeter;
NEO	neomycin;
ng	nanogram;
nm	nanometer;
NOBS	nonanoyloxybenzenesulfonate;
N	Normal;
NTA	nitrilotriacetic acid;
PAHBAH	p-hydroxybenzoic acid hydrazide;
PCR	polymerase chain reaction;
PEG	polyethyleneglycol;
pI	isoelectric point;
ppm	parts per million;
PVA	poly(vinyl alcohol);
PVP	poly(vinylpyrrolidone);
RAU	Reference Amylase Units;
RMS	root mean square;
RNA	ribonucleic acid;
rpm	revolutions per minute;
SAPU	spectrophotometric acid protease unit;

SAS	secondary alkane sulfonates;
1X SSC	0.15 M NaCl, 0.015 M sodium citrate, pH 7.0;
sec	seconds;
%SRI	percent stain removal index;
SSF	simultaneous saccharification and fermentation;
TAED	tetraacetylethylenediamine;
T_m	thermal midpoint for a DSC curve, or melting temperature of a protein;
TNBS	trinitrobenzenesulfonic acid;
µg	micrograms;
µl, (µL)	microliters;
µNm	microNewton meters;
µm	micrometer;
µM	micromolar;
U	units;
V/V	volume to volume;
WE	Western Europe;
wt%	weight percent;
w/v (or W/V)	weight/volume;
w/w(or W/w)	weight/weight;
wt	wild-type.

1.2. Definitions

In some aspects, the present disclosure relies on routine techniques and methods used in the field of genetic engineering and molecular biology. The following resources include descriptions of general methodology useful in accordance with what is disclosed herein: Sambrook *et al.*, MOLECULAR CLONING: A LABORATORY MANUAL (2nd Ed., 1989); Kreigler, GENE TRANSFER AND EXPRESSION; A LABORATORY MANUAL (1990) and Ausubel *et al.*, Eds. CURRENT PROTOCOLS IN MOLECULAR BIOLOGY (1994).

These general references provide definitions and methods known to those in the

art. Unless defined otherwise herein, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which the disclosure pertains. Singleton, *et al.*, DICTIONARY OF MICROBIOLOGY AND MOLECULAR BIOLOGY, 2D ED., John Wiley and Sons, New York (1994) and Hale & Markham, THE HARPER COLLINS DICTIONARY OF BIOLOGY, Harper Perennial, NY (1991) provide one of skill with general dictionaries of many of the terms used in this disclosure.

"Isolated" means that the isolated substance, e.g. a compound or a sequence, is modified by the hand of man relative to that compound or sequence as found in nature. For example, an isolated sequence is at least partially free, or even substantially free, from at least one other component with which the sequence is naturally associated as found in nature.

"Purified" when used to describe a material or substance means that the material or substance is in a relatively pure state, *e.g.*, at least about 90% pure, at least about 95% pure, at least about 98% pure, or at least about 99% pure.

As used herein, "starch" refers to any carbohydrate composition comprising complex polysaccharides, comprising amylose and/or amylopectin with the formula $(C_6H_{10}O_5)_x$, wherein "X" can be any number. Preferably, starch refers to any such carbohydrate that is naturally present in plants, including but not limited to grains, grasses, tubers, and roots, and more specifically from wheat, barley, corn, rye, rice, sorghum, cassava, millet, potato, sweet potato, and tapioca. Starch can also refer to synthetic starches or modified starches, such as chemically-modified starch for use as a detectable substrate for enzyme assays, or starches chemically- or enzymatically-modified to improve one or more properties for use.

As used herein, "phytic acid" (or inositol hexakisphosphate (IP6)), is the principle storage form of phosphorus in many plant tissues, such as bran, seeds, and the like. Phytic acid is also referred to as "phytate" herein, especially when in salt form. Various other inositol phosphates such as inositol penta- (IP5), tetra- (IP4), and triphosphate (IP3) are also referred to herein as phytates. Phytates are generally indigestible by man and most monogastric animals.

Enzymes that degrade phytates are referred to herein as "phytases" or "fytases" are generally myo-inositol-hexaphosphate phosphohydrolases. Phytase activity is defined as fytase units (FTU or U), where one FTU is defined as the quantity of enzyme that liberates 1 micromol of inorganic-P per minute from 0.0015 mol/l sodium phytate at pH 5.5, and 37 °C. This definition provides a useful measure of quantity of phytase activity and represents a simple bench mark measurement. Phytate-degrading enzymes of yeasts (e.g., *Schwanniomyces occidentalis*, *Pichia anomala*, *Arxula adeninivorans*), gram-negative bacteria (e.g., *Escherichia coli*, *Pseudomonas* spp., *Klebsiella* spp.), and gram-positive (e.g., *Bacillus* spp.) have been identified and characterized. Phytases from many plants, and from filamentous fungi such as *Penicillium* spp., *Aspergillus* spp., *Trichoderma* spp. *Mucor piriformis*, and *Cladosporium* spp., are also known. 3-phytases (EC 3.1.3.8) and 6-phytases (EC 3.1.3.26), depending on the site of initiation of hydrolysis, have been characterized. Also, phytase have been characterized, based on their pH "optima," as either acid (pH optima around 5) or alkaline (pH optima around 9). A variety of commericial phytases are available, including ROVABIO (Genencor International).

"Amylase" refers to an enzyme that is capable of catalyzing the cleavage of a starch substrate, leading to a degradation or partial degradation of the starch. Amylases are generally hydrolases that cleave glycosidic linkages in starch. As used herein amylase includes any glucoamylase, alpha-amylase, β-amylase, for example, the wild-type alpha-amylases of *Bacillus* spp., especially *B. licheniformis*. Generally, alpha-amylases (EC 3.2.1.1; α-D-(1→4)-glucan glucanohydrolase) are endo-acting enzymes defined as cleaving α-D-(1→4) O-glycosidic linkages within the starch molecule in a random fashion. In contrast, the exo-acting amylolytic enzymes, such as β-amylases (EC 3.2.1.2; α-D-(1→4)-glucan maltohydrolase) and some product-specific amylases like maltogenic alpha-amylase (EC 3.2.1.133) cleave the substrate starch molecule from the non-reducing end. β-Amylases, α-glucosidases (EC 3.2.1.20; α-D-glucoside glucohydrolase), glucoamylase (EC 3.2.1.3; α-D-(1→4)-glucan glucohydrolase), and product-specific amylases can produce malto-oligosaccharides of specific length from starch. Wild-type alpha-amylase from *Bacillus stearothermophilus* or "AmyS" amylase

is sometimes referred to herein as XTRA or SPEZYME XTRA, which are commercial AmyS products from Genencor International.

As used herein, "AmyS-like alpha-amylases" are useful as parent amylases herein. AmyS-like alpha-amylases constitute a class of alpha-amylases herein, based on the substantial homology found between them. "AmyS-like alpha-amylase" is intended to indicate the class of alpha-amylases, in particular *Bacillus* alpha-amylases, especially *Geobacillus* stearothermophilus alpha-amylases, which, at the amino acid level, exhibit a substantial identity to the alpha-amylase having the amino acid sequence shown in SEQ ID NO: 2, herein. Spezyme Xtra is commercially available from Danisco US Inc, Genencor Division. *Geobacillus stearothermophilus* has been referred to as *Bacillus stearothermophilus* in the literature and the two may be used interchangeably herein. All the alpha-amylases having the amino acid sequences provided herein as SEQ ID NOS: 1, 6, 7, 8, 9, 10, 11, 12, 15 and 16, respectively, are considered to be AmyS-like alpha-amylases and thus are suitable as parent alpha-amylases. AmyS-like alpha-amylases also include alpha-amylases i) having amino acid sequences with at least about 60% homology (identity), such as at least about 70%, at least about 75%, or at least about 80%, at least about 85%, at least about 90%, at least about 95%, at least about 96%, at least about 97%, at least about 98%, or at least about 99% identity, with at least one of the amino acid sequences shown in SEQ ID NOS: 1, 6, 7, 8, 9, 10, 11, 12, 15 and 16, and/or ii) that are encoded by a DNA sequence that hybridizes with a DNA sequence encoding any of the above-specified alpha-amylases, or those apparent from SEQ ID NOS: 9 (BAN), 5 (BSG), 3 (SP722), 1 (SP690), 7 (LAT), 11 (AA560) of WO 06/002643 or of the present specification, which encode any of the amino acid sequences shown in SEQ ID NOS: 1, 6, 7, 8, 9, 10, 11, 12, 15 and 16 herein, respectively. Still further homologous alpha-amylases useful as AmyS-like alpha-amylases and thus, as parent enzymes for producing variants herein, include the alpha-amylase produced by the *B. lichenformis* strain described in EP 0252666; (ATCC 27811), and the alpha-amylases identified in WO 91/00353 and WO 94/18314; commercial AmyS-like alpha-amylases are comprised in the products sold under the following tradenames: Spezyme® AA and ULTRAPHLOW (available from Danisco US Inc, Genencor Division), and Keistase™

(available from Daiwa) and LIQUEZYME SC (available from Novozymes, Denmark). Section 1.5 herein below provides further information regarding AmyS-like alpha-amylases. Table A therein provides a list of several useful AmyS-like alpha-amylases, as well as a convenient method of comparing amino acid sequence identities therebetween. The skilled artisan will appreciate the similar tables can be constructed for other alpha-amylases to determine their suitability for use herein as apparent enzyme.

As used herein, "spectrophotometric acid protease unit" ("SAPU") is a unit of protease enzyme activity, wherein in 1 SAPU is the amount of protease enzyme activity that liberates one micromole of tyrosine per minute from a casein substrate under conditions of the assay.

"Glucoamylase unit" ("GAU"), is a measure of amylolytic activity defined as the amount of enzyme activity that will produce 1 g of reducing sugar, calculated as glucose, per hour from a soluble starch substrate at pH 4.2 and 60°C).

As used herein, the term "variant" may be used interchangeably with the term "mutant." "Variants" can refer to either polypeptides or nucleic acids. Variants include substitutions, insertions, deletions, truncations, transversions, and/or inversions, at one or more locations relative to a reference sequence. Variant nucleic acids include sequences that are complementary to sequences that are capable of hybridizing to the nucleotide sequences presented herein. For example, a variant nucleic acid sequence herein can be at least partially complementary to a sequence capable of hybridizing under stringent conditions (*e.g.*, 50°C and 0.2X SSC {1X SSC = 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0}) to a nucleotide sequences presented herein. More preferably, the term variant encompasses sequences that are complementary to sequences that are capable of hybridizing under highly stringent conditions (*e.g.*, 65°C and 0.1X SSC) to the nucleotide sequences presented herein.

"Thermostable" when used to describe an enzyme means the enzyme is more thermostable than a reference enzyme. In the present application, an alpha-amylase variant is more thermostable than a wild-type *B. licheniformis* alpha-amylase if the variant has a relatively higher enzymatic activity after a specific interval of time under the same experimental conditions, *e.g.*, the same temperature, substrate concentration,

etc. Alternatively, a more thermostable enzyme has a higher heat capacity determined by differential scanning calorimetry, compared to a reference enzyme.

"Melting temperature" (T_m) of a polypeptide is a temperature at which the conformation of the polypeptide undergoes a measurable temperature-dependent change. Protein conformation and T_m can be analyzed, for example, by circular dichroism, one of the most general and basic tools to study protein folding. Circular dichroism spectroscopy measures the absorption of circularly polarized light. In proteins, structures such as alpha helices and beta sheets are generally chiral, and thus absorb circularly polarized light. The light absorption provides a measure of the degree of foldedness of the protein. Changes in this absorption as a function of temperature or concentration of a denaturant can be used to study equilibrium unfolding of the protein. This type of spectroscopy can also be combined with devices, such as stopped flow mixers, to measure kinetics of protein folding/unfolding.

"Calcium dependent" means that, a particular enzyme requires calcium to substantially exhibit catalytic activity. Generally as used herein, "calcium dependent" encompasses a property of any enzyme that has a strict requirement for a divalent metal ion to exhibit catalytic activity, and also includes enzymes whose catalytic activity is substantially (e.g. more than 20%) increased in the presence of calcium or another divalent cation.

As used herein, "pH stable" with respect to an enzyme can refer to the enzyme activity or the protein conformation of the enzyme. In the first sense, "pH stable" means the enzyme remains catalytically-active at a specified pH or across a specified pH range. In the second sense, an enzyme may be deemed "stable" at a pH wherein the protein is not irreversibly denatured. In such a case, the enzyme would become catalytically active when returned to a pH capable of supporting catalytic activity. pH stability may also be used in a relative or comparative manner, for example, with a reference enzyme. In the present application, an alpha-amylase variant can be more pH stable than a wild-type *B. licheniformis* alpha-amylase when the variant has a relatively higher activity than the wild-type, e.g., when held at a given pH or assayed under the same conditions, including pH. pH's of most interest are typically either the conditions of actual use, or pH's that

are at or near the boundaries or extremes of the enzyme's natural ability to remain catalytically active.

"pH range" means a range of pH values e.g., from more acid to more basic, or vice versa. With respect to an enzyme activity, a pH range indicates the upper and lower pH values at which the enzyme exhibits a specified level of activity- e.g. a minimum activity, a specified percentage of maximal activity, or a specified level of substrate conversion or product formation.

"Recombinant" when used in reference to a cell, nucleic acid, protein, or vector, indicates that the cell, nucleic acid, protein or vector, is the result of, or has been modified by, the introduction of a heterologous sequence or the alteration of a native sequence, or that the cell is derived from a cell so modified or altered. Thus, for example, recombinant cells may express genes that are not found within the native (non-recombinant) form of the cell or may express native genes that are otherwise differently expressed (e.g. under-expressed, or over-expressed), abnormally expressed, or not expressed at all.

As used herein, "nucleotide sequence" or "nucleic acid sequence" refers to any sequence of two or more nucleotides, ribonucleotides, or the like, or derivatives thereof. Nucleotide sequences include oligonucleotide and polynucleotide sequences, as well as variants, homologues, fragments and derivatives thereof. A nucleotide sequence may be single-, double-, or multi-stranded. The nucleotide sequence may be from any source or origin, e.g., genomic, synthetic, or recombinant, and includes genomic DNA, cDNA, synthetic DNA, and RNA, and the like as well as hybrids thereof. Nucleotide sequences may comprise one or more codons and may encode one or more polypeptides. Nucleotide sequences may preferentially assume one or more energetically preferred three-dimensional structures.

A "vector" refers to a nucleotide sequence frequently useful for experimental use *in vitro*, or for introduction of nucleic acids into one or more cell types. Vectors include cloning vectors, *in vivo* or *in vitro* expression vectors, shuttle vectors, plasmids, phagemids, cosmids, phage particles, cassettes and the like.

An "expression vector" as used herein means a DNA construct comprising a DNA

sequence which is operably-linked to a suitable control sequence capable of effecting expression of the DNA in a suitable host. Such control sequences may include a promoter to effect transcription, an optional operator sequence to control transcription, a sequence encoding suitable ribosome binding sites on the mRNA, enhancers and sequences which control termination of transcription and translation.

A polynucleotide or a polypeptide having a certain percent (*e.g.*, at least about 80%, 85%, 90%, 95%, or 99%) of sequence identity with another sequence means that, when aligned, that percentage of bases or amino acid residues are the same in comparing the two sequences. This alignment and the percent homology or identity can be determined using any suitable software program known in the art, for example those described in CURRENT PROTOCOLS IN MOLECULAR BIOLOGY (F. M. Ausubel *et al.* (eds) 1987, Supplement 30, section 7.7.18). Such programs may include the GCG Pileup program, FASTA (Pearson *et al.* (1988) *Proc. Natl, Acad. Sci USA* 85:2444–2448), and BLAST (BLAST Manual, Altschul *et al.*, Natl Cent. Biotechnol. Inf., Natl Lib. Med. (NCIB NLM NIH), Bethesda, Md., and Altschul *et al.*, (1997) *NAR* 25:3389–3402). Another alignment program is ALIGN Plus (Scientific and Educational Software, PA), using default parameters. Another sequence software program that finds use is the TFASTA Data Searching Program available in the Sequence Software Package Version 6.0 (Genetics Computer Group, University of Wisconsin, Madison, WI).

One skilled in the art will recognize that sequences encompassed by the disclosure are also defined by the ability to hybridize under stringent hybridization conditions with the exemplified *amyS* sequence (*e.g.*, SEQ ID NO:5 of WO 06/002643). A nucleic acid is hybridizable to another nucleic acid sequence when a single stranded form of the nucleic acid can anneal to the other nucleic acid under appropriate conditions of temperature and solution ionic strength. Hybridization and washing conditions are well known in the art (*see*, *e.g.*, Sambrook (1989) *supra*, particularly chapters 9 and 11). In some embodiments, stringent conditions correspond to a T_m of 65°C and 0.1×SSC, 0.1% SDS.

A "gene" refers to a DNA segment that is involved in producing a polypeptide and includes regions preceding and following the coding regions as well as intervening

sequences (introns) between individual coding segments (exons).

"Heterologous" with reference to a polynucleotide or protein refers to a polynucleotide or protein that does not naturally occur in a host cell. In some embodiments, the protein is a commercially important industrial protein. It is intended that the term encompass proteins that are encoded by naturally occurring genes, mutated genes, and/or synthetic genes.

"Endogenous" with reference to a polynucleotide or protein refers to a polynucleotide or protein that occurs naturally in the host cell.

As used herein, "transformed", "stably transformed", and "transgenic" used in reference to a cell means the cell comprises at least one non-native (*e.g.*, heterologous) nucleic acid sequence. A stably-transformed cell comprises at least one such nucleic acid sequence integrated into its genome, or in an episomal plasmid that is maintained through multiple generations.

As used herein, "expression" refers to the process by which a polypeptide is produced based on the nucleic acid sequence of a gene. The process includes both transcription and translation.

A "signal sequence" means a sequence of amino acids covalently-bound to the N-terminal portion of a protein, which facilitates the transport of the protein, e.g., secretion of the mature form of the protein outside the cell. The definition of a signal sequence is functional. The mature form of the extracellular protein lacks the signal sequence which is cleaved off, e.g., during the secretion process.

As used herein, the term "derived" encompasses the terms "originated from", "obtained from" or "obtainable from", and "isolated from".

The terms "protein" and "polypeptide" are used interchangeably herein. The conventional one-letter or three-letter code for amino acid residues is used herein.

A "promoter" is a regulatory sequence that is involved in binding RNA polymerase to initiate transcription of a gene. The promoter may be an inducible promoter or a constitutive promoter. For example, *cbh1* from *Trichoderma reesei*, an inducible promoter, can be used herein.

"Operably-linked" refers to juxtaposition wherein elements are in an arrangement allowing them to be functionally related, even where not in close physical proximity. For example, a promoter is operably-linked to a coding sequence if it is capable of contolling the coding sequence and does control the transcription of the sequence under conditions permissive thereof, or conducive thereto.

"Selective marker" refers to a gene capable of expression in a host, and which allows selecting those hosts expressing the marker gene. Examples of selectable markers include but are not limited to gene that provide altered resistance to an antimicrobial agent (*e.g.*, hygromycin, bleomycin, or chloramphenicol) and/or genes that confer metabolic selectivity, for example, a nutritional advantage on the host cell, such as growth on a particular substrate as a sole source of carbohydrate.

"Introduced" in the context of inserting a nucleic acid sequence into a cell, means "transfection", or "transformation" or "transduction" and includes reference to the incorporation of a nucleic acid sequence into a eukaryotic or prokaryotic cell wherein the nucleic acid sequence may be incorporated into the genome of the cell (*e.g.*, chromosome, plasmid, plastid, or mitochondrial DNA), converted into an autonomous replicon, or transiently expressed (*e.g.*, transfected mRNA).

"Host," "host strain," or "host cell" means a suitable cell in which to place an expression vector or DNA construct comprising a polynucleotide, e.g., encoding a variant alpha-amylase. Host strains are preferably bacterial cells. In a preferred embodiment, "host cell" means cells and/or protoplasts created from the cells of a microbial strain, e.g., a *Bacillus* spp.

The term "culturing" refers to growing a population of microbial cells under suitable conditions in a medium capable of supporting such growth. In one embodiment, culturing refers to fermentative bioconversion of a starch substrate containing granular starch to an end-product (typically in a vessel or reactor).

The term "enzymatic conversion" in general refers to the modification of a substrate by enzyme action. The term as used herein also refers to the modification of a starch substrate by the action of an enzyme.

As used herein the term "saccharification" refers to enzymatic conversion of starch to glucose.

The term "degree of polymerization (DP)" refers to the number (n) of anhydroglucopyranose units in a given saccharide. Examples of DP1 are the monosaccharides, such as glucose and fructose. Examples of DP2 are the disaccharides, such as maltose and sucrose. A DP>3 denotes polymers with a degree of polymerization of greater than 3. The skilled artisan will understand that compounds with greater DE are more polymeric.

"End-product" or "desired end-product" refer to any intended product of an enzymatic reaction, e.g. a starch-derived molecule that is enzymatically converted from the starch substrate.

The term "residual starch" refers to any remaining starch (soluble or insoluble) left in a composition after fermentation of a starch-containing substrate.

As used herein, "specific activity" means an enzyme unit defined as the number of moles of substrate converted to product by an enzyme preparation per unit time under specific conditions. Specific activity is expressed as units (U)/unit weight of protein, generally, U/mg protein.

"Yield" refers to the amount of end-product or desired end-products produced using the methods of the present disclosure. In some embodiments, the yield is greater than that produced using methods known in the art. In some embodiments, the term refers to the volume of the end product and in other embodiment the term refers to the concentration of the end product.

As used herein, "biologically-active" refers to a compound or sequence that has a measurable effect on a biological system, e.g., a cell, an organ, or an organism.

"ATCC" refers to American Type Culture Collection located at Manassas, VA 20108 (ATCC).

"NRRL" refers to the Agricultural Research Service Culture Collection, National Center for Agricultural Utilization Research (and previously known as USDA Northern Regional Research Laboratory), Peoria, Ill.

As used herein, "food" means any ingredient, component or composition that provides a nutritive value for an animal, including a human.

As used herein, by convention, when describing proteins and genes that encode them, the term for the gene is generally italicized, (*e.g.*, the gene that encodes amyL (*B. licheniformis* AA) may be denoted as *amyL*). The term for the protein is generally not italicized and the first letter is generally capitalized, (*e.g.*, the protein encoded by the *amyL* gene may be denoted as AmyL or amyL). Unless otherwise indicated, nucleic acids are written left to right in 5′ to 3′ orientation, and amino acid sequences are written left to right in amino to carboxy orientation, respectively.

As used herein the term "comprising" and its cognates are used in their inclusive sense; that is, equivalent to the term "including" and its corresponding cognates. Numeric ranges are inclusive of the numbers defining the range.

The headings provided herein are not limitations of the various aspects or embodiments of what is disclosed.

Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of that which is disclosed, certain presently preferred methods and materials are described with no intention to limit the practitioner to any particular methods, protocols, and reagents described, as these may be varied. All patents and publications, including all sequences disclosed within such patents and publications, referred to herein are expressly incorporated by reference.

2. Nomenclature

In the present description and claims, the conventional one-letter and three-letter codes for amino acid residues are used. For ease of reference, alpha-amylase variants are generally described by use of the following nomenclature:

Original amino acid(s): position(s): substituted amino acid(s)

According to this nomenclature, for instance the substitution of serine by an alanine in position 242 is shown as:

Ser242Ala or S242A

a deletion of alanine in position 30 is shown as:

Ala30* or A30* or ΔA30

and insertion of an additional amino acid residue, such as lysine, is shown as:

Ala30AlaLys or A30AK

A deletion of a consecutive stretch of amino acid residues, such as amino acid residues 30-33, is indicated as (30-33)* or Δ(A30-N33).

Where a specific alpha-amylase contains a "deletion" in comparison with other alpha-amylases and an insertion is made in such a position this is indicated as:

*36Asp or *36D

for insertion of an aspartic acid in position 36.

Multiple mutations are separated by plus signs, i.e.:

Ala30Asp+Glu34Ser or A30N+E34S

representing mutations in positions 30 and 34 substituting alanine and glutamic acid for asparagine and serine, respectively.

When one or more alternative amino acid residues may be inserted in a given position it is indicated as

A30N,E or alternatively, A30N or A30E

Furthermore, when a position suitable for modification is identified herein without any specific modification being suggested, it is to be understood that any amino acid residue may be substituted for the amino acid residue present in the position. Thus, for instance, when a modification of an alanine in position 30 is mentioned, but not specified, it is to be understood that the alanine may be deleted or substituted for any other amino acid, i.e., any one of:

R, N, D, A, C, Q, E, G, H, I, L, K, M, F, P, S, T, W, Y, V.

Further, "A30X" means any one of the following substitutions:A30R, A30N, A30D, A30C, A30Q, A30E, A30G, A30H, A30I, A30L, A30K, A30M, A30F, A30P, A30S, A30T, A30W, A30Y, or A30V; or in short:

A30R,N,D,C,Q,E,G,H,I,L,K,M,F,P,S,T,W,Y,V.

If the parent enzyme--used for the numbering--already has the amino acid residue in question suggested for substitution in that position the following nomenclature is used:

"X30N" or "X30N,V" in the case where, for instance, one or N or V is present in the wild-type. This indicates that other corresponding parent enzymes are substituted to an "Asn" or "Val" in position 30.

3. Characteristics of Amino Acid Residues

Charged amino acids:

Asp, Glu, Arg, Lys, His

Negatively charged amino acids (with the most negative residue first):

Asp, Glu

Positively charged amino acids (with the most positive residue first):

Arg, Lys, His

Neutral amino acids:

Gly, Ala, Val, Leu, Ile, Phe, Tyr, Trp, Met, Cys, Asn, Gln, Ser, Thr, Pro

Hydrophobic amino acid residues (with the most hydrophobic residue listed last):

Gly, Ala, Val, Pro, Met, Leu, Ile, Tyr, Phe, Trp,

Hydrophilic amino acids (with the most hydrophilic residue listed last):

Thr, Ser, Cys, His, Glu, Gln, Asn, Asp, Lys, Arg

4. Alpha-Amylases and AmyS-like Amylases

4.1 Amino Acid Identities of Various Alpha-Amylase

A number of alpha-amylases produced by *Bacillus* spp. are highly homologous (identical) on the amino acid level and may be useful as parent enzymes herein. The percent identity (based on amino acid sequence) of a number of known *Bacillus* alpha-amylases, relative to each other can be found in the below Table A:

TABLE A: Amino acid sequence identity of several known *Bacillus* alpha-amylases

	707	**AP1378**	**BAN**	**BSG**	**SP690**	**SP722**	**AA560**	**LAT**
707	100.0	86.4	66.9	66.5	87.6	86.2	95.5	68.1
AP1378	86.4	100.0	67.1	68.1	95.1	86.6	86.0	69.4
BAN	66.9	67.1	100.0	65.6	67.1	68.8	66.9	80.7
BSG	66.5	68.1	65.6	100.0	67.9	67.1	66.3	65.4
SP690	87.6	95.1	67.1	67.9	100.0	87.2	87.0	69.2

	707	AP1378	BAN	BSG	SP690	SP722	AA560	LAT
SP722	86.2	86.6	68.8	67.1	87.2	100.0	86.8	70.8
AA560	95.5	86.0	66.9	66.3	87.0	86.8	100.0	68.3
LAT	68.1	69.4	80.7	65.4	69.2	70.8	68.3	100.0

The skilled artisan will appreciate that percent identities can e determined from the literature, or by any means disclosed herein or known in the art. For instance, the *B. licheniformis* alpha-amylase (LAT) (SEQ ID NO: 7) has been found to be about 81% homologous with the *B. amyloliquefaciens* alpha-amylase (SEQ ID NO: 9), and about 65% homologous with the *G. stearothermophilus* alpha-amylase (BSG) (SEQ ID NO: 1). Additional homologous alpha-amylases include SP690 and SP722 disclosed in WO 95/26397, and the #707 alpha-amylase derived from *Bacillus* spp. (SEQ ID NO: 6), described by Tsukamoto *et al.*, *Biochemical and Biophysical Research Communications*, 151 (1988), pp. 25-31. The KSM AP1378 alpha-amylase is disclosed in WO 97/00324 (from KAO Corporation).

4.2 Parent Alpha-Amylases

AmyS-like alpha-amylases, as defined above, may be used as a parent alpha-amylase. In a preferred embodiment, the parent alpha-amylase is derived from *G. stearothermophilus*, e.g., one of those referred to above, such as the *G. stearothermophilus* alpha-amylase having the amino acid sequence shown in SEQ ID NO: 1 or 2.

4.3 Parent Hybrid Alpha-Amylases

The parent alpha-amylase (i.e., backbone alpha-amylase) may also be a hybrid alpha-amylase, i.e., an alpha-amylase that comprises a combination of partial amino acid sequences derived from at least two alpha-amylases.

The parent hybrid alpha-amylase may be one, which on the basis of amino acid homology (identity) and/or DNA hybridization (as defined above), can be determined to belong to the AmyS-like alpha-amylase family described above. In such a case, the hybrid alpha-amylase is typically composed of at least one part of a AmyS-like alpha-amylase and part(s) of one or more other alpha-amylases selected from AmyS-like alpha-

amylases or non-AmyS-like alpha-amylases of microbial (bacterial or fungal) and/or mammalian origin.

Thus, the parent hybrid alpha-amylase may comprise a combination of partial amino acid sequences deriving from at least two AmyS-like alpha-amylases, or from at least one AmyS-like and at least one non-AmyS-like bacterial alpha-amylase, or from at least one AmyS-like and at least one fungal alpha-amylase. The AmyS-like alpha-amylase from which a partial amino acid sequence derives, may be any of the specific AmyS-like alpha-amylase referred to herein.

For instance, the parent alpha-amylase may comprise a C-terminal part of an alpha-amylase derived from a strain of *B. licheniformis*, and an N-terminal part of an alpha-amylase derived from a strain of *G. stearothermophilus* or from a strain of *G. stearothermophilus* (BSG).

5. Homology (Identity)

Homology may be determined as the degree of identity between two sequences indicating a relationship therebetween, e.g. a derivation of the first sequence from the second or vice versa. The homology may be determined by visual inspection or manual calculations, but more conveniently by means of computer programs known in the art, such as GAP, a program provided in the GCG program package (described above). Thus, Gap GCG v8 may be used, for example with the default scoring matrix for identity and the following default parameters: GAP creation penalty of 5.0 and GAP extension penalty of 0.3, respectively for nucleic acidic sequence comparison, and GAP creation penalty of 3.0 and GAP extension penalty of 0.1, respectively, for protein sequence comparison. GAP uses the method of Needleman and Wunsch, (1970), *J. Mol. Biol.* 48: 443-453, to make alignments and to calculate the identity.

A structural alignment between Spezyme Xtra (SEQ ID NO: 2) and, e.g., another alpha-amylase may be used to identify equivalent/corresponding positions in other AmyS-like alpha-amylases. One method of obtaining said structural alignment is to use the Pile Up program from the GCG package using default values of gap penalties, i.e., a gap creation penalty of 3.0 and gap extension penalty of 0.1. Other structural alignment

methods include the hydrophobic cluster analysis (Gaboriaud *et al.*, *FEBS Lett.* 224: 149-155, 1987) and reverse threading (Huber, T; Torda, AE, *Protein Sci.* 7(1) 142-149, 1998).

6. Hybridization

The oligonucleotide probe used in the characterization of the AmyS-like alpha-amylase above may suitably be prepared on the basis of the full or partial nucleotide or amino acid sequence of the alpha-amylase in question.

Suitable conditions for assessing hybridization involve pre-soaking in 5X SSC and pre-hybridizing for 1 hour at 40 °C in a solution of 20% formamide, 5X Denhardt's solution, 50 mM sodium phosphate, pH 6.8, and 50 mg of denatured sonicated calf thymus DNA, followed by hybridization in the same solution supplemented with 100 mM ATP for 18 hours at 40°C, followed by three times washing of the filter in 2X SSC, 0.2% SDS at 40°C for 30 minutes (low stringency), preferred at 50°C (medium stringency), more preferably at 65°C (high stringency), even more preferably at 75°C (very high stringency). More details about the hybridization method can be found in Sambrook *et al.*, MOLECULAR CLONING: A LABORATORY MANUAL, 2nd Ed., Cold Spring Harbor, 1989.

In the present context, "derived from" is intended not only to indicate an alpha-amylase produced or producible by a strain of the organism in question, but also an alpha-amylase encoded by a DNA sequence isolated from such strain and produced in a host organism transformed with said DNA sequence. Finally, the term is intended to indicate an alpha-amylase, which is encoded by a DNA sequence of synthetic and/or cDNA origin and which has the identifying characteristics of the alpha-amylase in question. The term is also intended to indicate that the parent alpha-amylase may be a variant of a naturally occurring alpha-amylase, i.e., a variant, which is the result of a modification (insertion, substitution, deletion) of one or more amino acid residues of the naturally occurring alpha-amylase.

7. General Mutations in Variant Alpha-Amylases

A variant described herein may, in one embodiment, comprise one or more

modifications in addition to those outlined above. Thus, it may be advantageous that one or more proline residues (Pro) present in the part of the alpha-amylase variant that is modified is/are replaced with a non-proline residue which may be any of the possible, naturally-occurring non-proline residues, and which preferably is an alanine, glycine, serine, threonine, valine or leucine.

Analogously, in one embodiment, one or more cysteine residues present in the parent alpha-amylase may be replaced with a non-cysteine residue such as serine, alanine, threonine, glycine, valine or leucine.

It is to be understood that this disclosure encompasses variants incorporating two or more of the above outlined modifications.

Furthermore, it may be advantageous to introduce mutations in one or more of the following positions (using SEQ ID NO: 7 for the numbering):

M15, V128, A111, H133, W138, T149, M197, N188, A209, A210, H405, T412, in particular the following single, double or triple or multi mutations:

M15X, in particular M15T,L;

V128X, in particular V128E;

H133X, in particular H133Y;

N188X, in particular N188S,T,P;

M197X, in particular M197T,L;

A209X, in particular A209V;

M197T/W138F; M197T/138Y; M15T/H133Y/N188S;

M15N128E/H133Y/N188S; E119C/S130C; D124C/R127C;

H133Y/T149I; and/or

G475R, H133Y/S187D; H133Y/A209V.

In the case of the parent alpha-amylase having the amino acid sequence shown in SEQ ID No. 7, relevant amino acid residues which may be deleted or substituted with a view to improving the oxidation stability include the single cysteine residue (C363) and the methionine residues located in positions M8, M9, M96, M200, M206, M284, M307, M311, M316 and M438 in SEQ ID NO: 2.

With respect to increasing the thermal stability of an alpha-amylase variant relative to its parent alpha-amylase, it appears to be particularly desirable to delete at least one, and preferably two, or even three, of the following amino acid residues in the amino acid sequence shown in SEQ ID NO: 2: F178, R179, G180, I181, G182 and K183.

Particularly interesting pair-wise deletions of this type are R179*+G180*; and I181*+G182* (SEQ ID NOS: 16 or 15, respectively) (or equivalents of these pair-wise deletions in another alpha-amylase meeting the requirements of a parent alpha-amylase in the context of the present disclosure).

Other residues of interest include N193F and V416G in the amino acid sequence shown in SEQ ID NO: 2.

8. Altered Properties of Variants

8.1 General

The following section describes the relationship between mutations, which are present in a variant described herein, and desirable alterations in properties (relative to those of a parent AmyS-like alpha-amylase), which may result therefrom.

Described herein are AmyS-like alpha-amylases with altered properties. Parent alpha-amylases specifically contemplated herein are AmyS-like alpha-amylases and parent hybrid AmyS-like alpha-amylases.

In one embodiment, the *Geobacillus stearothermophilus* alpha-amylase (SEQ ID NO: 2) is used as the starting point, i.e., the parent amylase, but in other embodiments, the SP722, BLA, BAN, AA560, SP690, KSM AP1378, #707 and other *Bacillus* alpha-amylases may be used. Amino acid positions corresponding to positions in SEQ ID NO: 2 are readily determined in accordance herewith.

The skilled artisan will appreciate that while any parent alpha-amylase could be used as a reference amylase for the purpose of numbering/identifying the amino acid residues modified or to be modified in a particular variant, SEQ ID NO: 1 is presently a preferred sequence for such purpose, because it is the longest *B. stearothermophilus* sequence presently available herein.

In one aspect, this disclosure relates to variant with altered properties, e.g., as described above.

In one of its several aspects, this disclosure provides a variant of a parent *G. stearothermophilus* alpha-amylase, comprising an alteration at one or more positions (using e.g., SEQ ID NO: 1 for the amino acid numbering) selected from the group of:

P17, D19, T21, N28, S51, G72, V74, A82, Q86, Q89, A93, G95, Q97, W115, D117, P123, S124, D125, N127, I130,G132, Q135, P145, G146, G148, S153,Y159, W166, S169, K171, W187, P209, N224, S242, G256, D269, N271, T278, N281, G302, A304, R308, T321, Q358, P378, S382, K383, T398, H405, T417, E418, P420, G421, P432, W437, G446, G454, S457, T459, T461, S464, G474, R483,

wherein

(a) the alteration(s) are independently (i) an insertion of an amino acid downstream of the amino acid that occupies the position; (ii) a deletion of the amino acid that occupies the position; or (iii) a substitution of the amino acid that occupies the position with a different amino acid,

(b) the variant has alpha-amylase activity, and

(c) each position corresponds to a position of the amino add sequence of the parent amylase, e.g., a *G. stearothermophilus* alpha-amylase, e.g., having the amino acid sequence shown in SEQ ID NO: 2, e.g., a truncated alpha-amylase that is available commercially as SPEZYME XTRA from Genencor.

Specifically contemplated herein are S242A, S242Q, S242N and S242E.

Additionally, residues R179, G180, I181, G182, K183 were chosen to explore the effect of mutations in the calcium-sodium binding region, and P245 was chosen because a proline in the middle of an alpha-helix is unusual.

Corresponding positions in other parent AmyS-like alpha-amylases can be found by alignment as described above, for example, as with those sequences shown in the alignment in Figure 4. Thus, variants of a parent AmyS-like alpha-amylase, comprising an alteration at one or more of the above enumerated positions (using, e.g., SEQ ID NO: 1 for comparative amino acid numbering) is contemplated herein.

8.2 Altered Properties: Stability

In the context of the variants described herein, mutations (including amino acid substitutions and deletion) of importance with respect to achieving altered stability, in particular improved stability (i.e., higher or lower), at especially high temperatures (i.e., about 70-120°C) and/or extreme pH (i.e. low or high pH, i.e., pH 4-6 or pH 8-11, respectively), in particular at free (i.e., unbound, therefore in solution) calcium concentrations below 60 ppm, include any of the mutations listed in the "Altered Properties" section. The stability may be determined as described in the "Methods" section below.

8.3 Altered Properties: Ca^{2+} Stability

Altered Ca^{2+} stability means the stability of the enzyme under Ca^{2+} depletion has been improved, i.e., higher or lower stability, relative to the parent enzyme. In the context of the presently described variants, mutations (including amino acid substitutions and deletions) of importance with respect to achieving altered Ca^{2+} stability, in particular improved Ca^{2+} stability, i.e., higher or lower stability, at especially high pH (i.e., pH 8-10.5) include any of the mutations listed in the "Altered Properties" section.

8.4 Altered Properties: Specific Activity

In a further aspect, important mutations (including amino acid substitutions and deletions) with respect to obtaining variants exhibiting altered specific activity, in particular increased or decreased specific activity, especially at temperatures from about 10-60°C, preferably about 20-50°C, especially about 0-40°C, include any of the mutations listed in the in "Altered Properties" section. The specific activity may be determined as described in the "Methods" section below.

8.5 Altered Properties: Oxidation Stability

The described variants may have altered oxidation stability, in particular higher oxidation stability, in comparison to the parent alpha-amylase. Increased oxidation stability is advantageous in, e.g., detergent compositions and decreased oxidation stability may be advantageous in compositions intended for starch liquefaction. Oxidation stability may be determined as described in the "Methods" section below.

8.6 Altered Properties: Altered pH Profile

Important positions and mutations with respect to obtaining variants with altered pH profile, in particular improved activity at especially high pH (i.e., pH 8-10.5) or low pH (i.e., pH 4-6) include mutations of amino residues located close to the active site residues.

Preferred specific mutations/substitutions include those listed above in the section "Altered Properties" for the positions in question. Suitable assays are described in the "Methods" section below.

8.7 Altered Properties: Wash Performance

Important positions and mutations with respect to obtaining variants with improved wash performance at especially high pH (i.e., pH 8.5-11) include the specific mutations/substitutions listed above in the section "Altered Properties" for the positions in question. The wash performance may be tested as described below in the "Methods" section.

9. Methods of Preparing α-Amylase Variants

Methods for introducing mutations into genes are known in the art, as are cloning methods for α-amylase-encoding DNA sequences. Such methods including methods for generating mutations at specific sites within the α-amylase-encoding sequence will be discussed below.

9.1 Cloning a DNA Sequence Encoding an α-Amylase

The DNA sequence encoding a parent α-amylase may be isolated from any cell or microorganism producing the α-amylase in question, using various methods well known in the art. First, a genomic DNA and/or cDNA library should be constructed using chromosomal DNA or messenger RNA from the organism that produces the α-amylase to be studied. If the amino acid sequence of the α-amylase is known, homologous, labeled oligonucleotide probes may be synthesized and used to identify α-amylase-encoding clones from a genomic library prepared from the organism in question. Alternatively, a labeled oligonucleotide probe containing sequences homologous to a known α-amylase

gene can be used as a probe to identify α-amylase-encoding clones, e.g., using hybridization and washing conditions of lower stringency.

Another method for identifying α-amylase-encoding clones is based on inserting fragments of genomic DNA into an expression vector, such as a plasmid, transforming α-amylase-negative bacteria with the resulting genomic DNA library, and plating the transformed bacteria onto agar containing a substrate for α-amylase, thereby allowing clones expressing the α-amylase to be readily identified.

Alternatively, the DNA sequence encoding the enzyme may be prepared synthetically by established, standard methods, e.g. the phosphoamidite method described by S. L. Beaucage and M. H. Caruthers, *Tetrahedron Letters* 22: 1859-1869 (1981) or the method described by Matthes *et al.*, *EMBO J.* 3:801-895 (1984). In the phosphoamidite method, oligonucleotides are synthesized, e.g., in an automatic DNA synthesizer, purified, annealed, ligated, and cloned in appropriate vectors.

Finally, the DNA sequence may be of mixed origin comprising e.g., genomic and synthetic sequences, synthetic and cDNA sequences, or genomic and cDNA sequences, prepared by ligating fragments of synthetic, genomic, or cDNA origin (as appropriate, the fragments corresponding to various parts of the entire DNA sequence), in accordance with standard techniques. The DNA sequence may also be prepared by polymerase chain reaction (PCR) using specific primers, for instance as described in U.S. Pat. No. 4,683,202 or R. K. Saiki *et al. EMBO J.* 3:801-895 (1988).

9.2 Site-directed Mutagenesis

Once an α-amylase-encoding DNA sequence has been isolated, and desirable sites for mutation identified, mutations may be introduced using synthetic oligonucleotides. These oligonucleotides contain nucleotide sequences flanking the desired mutation sites; mutant nucleotides are inserted during oligonucleotide synthesis. In a specific method, a single-stranded gap of DNA, bridging the α-amylase-encoding sequence, is created in a vector carrying the α-amylase gene. Then the synthetic nucleotide, bearing the desired mutation, is annealed to a homologous portion of the single-stranded DNA. The remaining gap is then filled in with DNA polymerase I (Klenow fragment) and the construct is ligated using T4 ligase. A specific example of this method is described in

Morinaga *et al.* Biotechnology 2:636-639 (1984). U.S. Pat. No. 4,760,025 discloses the introduction of oligonucleotides encoding multiple mutations by performing minor alterations of the cassette. However, an even greater variety of mutations can be introduced at any one time by the Morinaga method, because a multitude of oligonucleotides, of various lengths, can be introduced.

Another method of introducing mutations into α-amylase-encoding DNA sequences is described in Nelson and Long, *Analytical Biochem.*, 180: 147-151, 1989. It involves the 3-step generation of a PCR fragment containing the desired mutation introduced by using a chemically synthesized DNA strand as one of the primers in the PCR reactions. From the PCR-generated fragment, a DNA fragment carrying the mutation may be isolated by cleavage with restriction endonucleases and reinserted into an expression plasmid.

The skilled artisan will appreciate that many alternative methods are available for providing or obtaining variants herein. For example, gene shuffling, e.g., as described in WO 95/22625 (from Affymax Technologies N.V.) or in WO 96/00343 (from Novo Nordisk A/S), or other corresponding techniques resulting in hybrid enzymes comprising the mutation(s), e.g., substitution(s) and/or deletion(s), in question.

9.3 Expression of Alpha-Amylase Variants

A DNA sequence encoding the variant produced by methods described above, or by any alternative methods known in the art, can be expressed, in enzyme form, using an expression vector which typically includes control sequences encoding a promoter, operator, ribosome binding site, translation initiation signal, and, optionally, a repressor gene or various activator genes.

The recombinant expression vector carrying the DNA sequence encoding an alpha-amylase variant for use herein may be any vector, which may conveniently be subjected to recombinant DNA procedures, and the choice of vector will often depend on the host cell into which it is to be introduced. Thus, the vector may be an autonomously replicating vector, i.e., a vector that exists as an extrachromosomal entity, the replication of which is independent of chromosomal replication, e.g., a plasmid, a bacteriophage, an extrachromosomal element, a minichromosome, or an artificial chromosome.

Alternatively, the vector may be integrated into the host cell genome and replicated together with the chromosome(s) into which it has been integrated.

In the vector, the DNA sequence should be operably-connected to a suitable promoter sequence. The promoter may be any DNA sequence, which shows transcriptional activity in the host cell of choice and may be derived from genes encoding proteins either homologous or heterologous to the host cell. Examples of suitable promoters for directing the transcription of the DNA sequence encoding an alpha-amylase variant for use herein, especially in a bacterial host, are the promoter of the *lac* operon of *E. coli*, the *Streptomyces coelicolor* agarase gene *dag*A promoters, the promoters of the *Bacillus licheniformis* alpha-amylase gene (*amy*L), the promoters of the *Geobacillus stearothermophilus* maltogenic amylase gene (*amy*M), the promoters of the *Bacillus amyloliquefaciens* alpha-amylase (*amy*Q), the promoters of the *Bacillus subtilis* *xyl*A and *xyl*B genes etc. For transcription in a fungal host, examples of useful promoters are those derived from the gene encoding *A. oryzae* TAKA amylase, *Rhizomucor miehei* aspartic proteinase, *A. niger* neutral alpha-amylase, *A. niger* acid stable alpha-amylase, *A. niger* glucoamylase, *Rhizomucor miehei* lipase, *A. oryzae* alkaline protease, *A. oryzae* triose phosphate isomerase or *A. nidulans* acetamidase.

Expression vectors for use herein may also comprise a suitable transcription terminator and, in eukaryotes, polyadenylation sequences operably-connected to the DNA sequence encoding the alpha-amylase variant. Termination and polyadenylation sequences may suitably be derived from the same sources as the promoter.

The vector may further comprise a DNA sequence enabling the vector to replicate in the host cell in question. Examples of such sequences are the origins of replication of plasmids pUC19, pACYC177, pUB110, pE194, pAMB1 and pIJ702.

The vector may also comprise a selectable marker, e.g. a gene the product of which complements a defect in the host cell, such as the *dal* genes from *B. subtilis* or *B. licheniformis*, or one that confers antibiotic resistance such as ampicillin, kanamycin, chloramphenicol or tetracyclin resistance. Furthermore, the vector may comprise *Aspergillus* selection markers such as *amdS*, *argB*, *niaD* and *sC*, a marker giving rise to

hygromycin resistance, or the selection may be accomplished by co-transformation, e.g., as described in WO 91/17243.

While intracellular expression may be advantageous in some respects, e.g., when using certain bacteria as host cells, it is generally preferred that the expression is extracellular. In general, the *Bacillus* alpha-amylases mentioned herein comprise a pre-region permitting secretion of the expressed protease into the culture medium. If desirable, this pre-region may be replaced by a different pre-region or signal sequence, conveniently accomplished by substitution of the DNA sequences encoding the respective pre-regions.

The procedures used to ligate a DNA construct encoding an alpha-amylase variant, the promoter, terminator and other elements, respectively, and to insert them into suitable vectors containing the information necessary for replication, are well known to persons skilled in the art (cf., for instance, Sambrook *et al.*, MOLECULAR CLONING: A LABORATORY MANUAL, 2nd Ed., Cold Spring Harbor, 1989).

Cells for use herein, e.g. comprising a DNA construct or an expression vector as defined above, can be used as host cells in the recombinant production of an alpha-amylase variant. The cell may be transformed with a DNA construct encoding the variant, conveniently by integrating the DNA construct (in one or more copies) in the host chromosome. This integration is generally considered to be an advantage as the DNA sequence is more likely to be stably maintained in the cell. Integration of the DNA constructs into the host chromosome may be performed according to conventional methods, e.g., by homologous or heterologous recombination. Alternatively, the cell may be transformed with an expression vector as described above in connection with the different types of host cells.

Cells for use herein may be cells of a higher organism such as a mammal or an insect, but are preferably microbial cells, e.g., a bacterial or a fungal (including yeast) cell.

Examples of suitable bacteria are Gram-positive bacteria such as *Bacillus subtilis, Bacillus licheniformis, Bacillus lentus, Bacillus brevis, Geobacillus stearothermophilus, Bacillus alkalophilus, Bacillus amyloliquefaciens, Bacillus coagulans, Bacillus circulans,*

Bacillus lautus, Bacillus megaterium, Bacillus thuringiensis, or *Streptomyces lividans* or *Streptomyces murinus*, or gram-negative bacteria such as *E. coli*. The transformation of the bacteria may, for instance, be effected by protoplast transformation or by using competent cells in a manner known per se.

Where used for expression, a yeast may favorably be selected from a species of *Saccharomyces* or *Schizosaccharomyces*, e.g. *Saccharomyces cerevisiae*. A filamentous fungus may advantageously be selected from a species of Aspergillus, e.g., *Aspergillus oryzae* or *Aspergillus niger*. Fungal cells may be transformed by a process involving protoplast formation and transformation of the protoplasts followed by regeneration of the cell wall in a manner known per se. A suitable procedure for transformation of *Aspergillus* host cells is described in EP 238 023.

In a yet further aspect, the disclosure relates to a method of producing an alpha-amylase variant, which method comprises cultivating a host cell as described above under conditions conducive to the production of the variant and recovering the variant from the cells and/or culture medium.

The medium used to cultivate the cells may be any conventional medium suitable for growing the host cell in question and obtaining expression of the alpha-amylase variant. Suitable media are available from commercial suppliers or may be prepared according to published recipes (e.g., as described in catalogues of the ATCC).

The alpha-amylase variant secreted from the host cells may be recovered from the culture medium by known procedures, including separating the cells from the medium by centrifugation or filtration, and precipitating proteinaceous components of the medium by means of a salt such as ammonium sulphate, followed by the use of chromatographic procedures such as ion exchange chromatography, affinity chromatography, or the like.

9.4 Methods for Characterizing and Screening Variants

9.4.1 Filter Screening Assays

The below assays may be used to screening of AmyS-like alpha-amylase variants having altered stability at high or low pH and/or under Ca^{2+} depleted conditions compared to the parent enzyme and AmyS-like alpha-amylase.

9.4.2 High pH Filter Assay

Bacillus libraries are plated on a sandwich of cellulose acetate (OE 67, Schleicher & Schuell, Dassel, Germany)--and nitrocellulose filters (Protran-Ba 85, Schleicher & Schuell, Dassel, Germany) on TY agar plates with 10 μg/mL kanamycin at 37°C for at least 21 hours. The cellulose acetate layer is located on the TY agar plate.

Each filter sandwich is specifically marked with a needle after plating, but before incubation in order to be able to localize positive variants on the filter and the nitrocellulose filter with bound variants is transferred to a container with glycine-NaOH buffer, pH 8.6-10.6 and incubated at room temperature (can be altered from about 10-60°C) for 15 min. The cellulose acetate filters with colonies are stored on the TY-plates at room temperature until use. After incubation, residual activity is detected on plates containing 1% agarose, 0.2% starch in glycine-NaOH buffer, pH 8.6-10.6. The assay plates with nitrocellulose filters are marked the same way as the filter sandwich and incubated for 2 hours at room temperature. After removal of the filters the assay plates are stained with 10% Lugol solution. Starch degrading variants are detected as white spots on dark blue background and then identified on the storage plates. Positive variants are rescreened twice under the same conditions as the first screen.

9.4.3 Low Calcium Filter Assay

Bacillus libraries are plated on a sandwich of cellulose acetate (OE 67, Schleicher & Schuell, Dassel, Germany)--and nitrocellulose filters (Protran-Ba 85, Schleicher & Schuell, Dassel, Germany) on TY agar plates with a relevant antibiotic, e.g., kanamycin or chloramphenicol, at 37°C for at least 21 hours. The cellulose-acetate layer is located on the TY agar plate.

Each filter sandwich is specifically marked with a needle after plating, but before incubation in order to be able to localize positive variants on the filter and the nitrocellulose filter with bound variants is transferred to a container with carbonate/bicarbonate buffer about pH 8.5-10 and with different EDTA concentrations (about 0.001 mM to about 100 mM). The filters are incubated at room temperature for 1 hour. The cellulose acetate filters with colonies are stored on the TY-plates at room temperature until use. After incubation, residual activity is detected on plates containing

1% agarose, 0.2% starch in carbonate/bicarbonate buffer about pH 8.5-10. The assay plates with nitrocellulose filters are marked the same way as the filter sandwich and incubated for about 2 hours at room temperature. After removal of the filters, the assay plates are stained with about 10% Lugol solution. Starch degrading variants are detected as white spots on dark blue background and then identified on the storage plates. Positive variants are rescreened twice under the same conditions as the first screen.

9.4.4 Low pH Filter Assay

Bacillus libraries are plated on a sandwich of cellulose acetate (OE 67, Schleicher & Schuell, Dassel, Germany)--and nitrocellulose filters (Protran-Ba 85, Schleicher & Schuell, Dasseli Germany) on TY agar plates with 10 µg/mL chloramphenicol at 37°C for at least 21 hours. The cellulose acetate layer is located on the TY agar plate.

Each filter sandwich is specifically marked with a needle after plating, but before incubation in order to be able to localize positive variants on the filter, and the nitrocellulose filter with bound variants is transferred to a container with citrate buffer, pH 4.5 and incubated at 80°C for 20 minutes (when screening for variants in the wild-type backbone) or 85°C for 60 minutes (when screening for variants of the parent alpha-amylase). The cellulose acetate filters with colonies are stored on the TY-plates at room temperature until use. After incubation, residual activity is detected on assay plates containing 1% agarose, 0.2% starch in citrate buffer, pH 6.0. The assay plates with nitrocellulose filters are marked the same way as the filter sandwich and incubated for 2 hours at 50°C After removal of the filters the assay plates are stained with 10% Lugol solution. Starch degrading variants are detected as white spots on dark blue background and then identified on the storage plates. Positive variants are re-screened twice under the same conditions as the first screen.

9.4.5 Secondary Screening

Positive transformants after rescreening are picked from the storage plate and tested in a secondary plate assay. Positive transformants are grown for 22 hours at 37°C in 5 mL LB + chloramphenicol. The *Bacillus* culture of each positive transformant and as a control a clone expressing the corresponding backbone are incubated in citrate buffer, pH 4.5 at 90°C and samples are taken at 0, 10, 20, 30, 40, 60 and 80 minutes. A

3-μL sample is spotted on an assay plate. The assay plate is stained with 10% Lugol solution. Improved variants are seen as variants with higher residual activity (detected as halos on the assay plate) than the backbone. The improved variants are determined by nucleotide sequencing.

9.4.6 Stability Assay of Unpurified Variants

The stability of the variants may be assayed as follows: *Bacillus* cultures expressing the variants to be analyzed are grown for 21 hours at 37°C in 10 mL LB + chloramphenicol. 800 μL culture is mixed with 200 microliters citrate buffer, pH 4.5. A number of 70 μL aliquots corresponding to the number of sample time points are made in PCR tubes and incubated at 70°C or 90°C for various time points (typically 5, 10, 15, 20, 25 and 30 minutes) in a PCR machine. The 0 min sample is not incubated at high temperature. Activity in the sample is measured by transferring 20 μL to 200 μL of the alpha-amylase PNP-G_7 substrate MPR3 ((Boehringer Mannheim Cat. No. 1660730) as described below under "Assays for Alpha-Amylase Activity". Results are plotted as percentage activity (relative to the 0 time point) versus time, or stated as percentage residual activity after incubation for a certain period of time.

9.4.7 Fermentation and Purification of Alpha-Amylase Variants

A *B. subtilis* strain harboring the relevant expression plasmid may be fermented and purified as follows: The strain is streaked on a LB-agar plate with 10 μg/mL kanamycin from -80°C stock, and grown overnight at 37°C. The colonies are transferred to 100 mL PS-1 media supplemented with 10 μg/mL chloramphenicol in a 500 mL shaking flask.

Composition of PS-1 medium

Pearl sugar	100 g/L
Soy Bean Meal	40 g/L
Na_2HPO_4, 12 H_2O	10 g/L
Pluronic ™ PE 6100	0.1 g/L
$CaCO_3$	5 g/L

The culture is shaken at 37°C at 270 rpm for 5 days.

Cells and cell debris are removed from the fermentation broth by centrifugation at 4500 rpm in 20-25 minutes. Afterwards the supernatant is filtered to obtain a completely clear solution. The filtrate is concentrated and washed on a UF-filter (10000 cut off membrane) and the buffer is changed to 20 mM Acetate at pH 5.5. The UF-filtrate is applied on an S-SEPHAROSE F.F (Pharmacia) and elution is carried out by step elution with 0.2 M NaCl in the same buffer. The eluate is dialyzed against 10 mM Tris, pH 9.0 and applied on a Q-SEPHAROSE F.F. and eluted with a linear gradient from 0-0.3M NaCl over 6 column volumes. The fractions that contain the activity (measured by the PHADEBAS assay) are pooled, pH was adjusted to pH 7.5, and remaining color was removed by treatment with 0.5% w/v active charcoal in 5 minutes.

9.4.8 Specific Activity Determination

The specific activity can be determined using the PHADEBAS® assay (Magle Life Sciences) as activity/mg enzyme. The manufactures instructions are followed (see also below under "Assay for Alpha-Amylase Activity").

9.4.9 Determination of Isoelectric Point

The pI can be determined by isoelectric focusing (e.g., using Pharmacia, Ampholine, pH 3.5-9.3).

9.4.10 Stability Deter mination

The amylase stability may be measured using the method as follows:

The enzyme is incubated under the relevant conditions. Samples are taken at various time points, e.g., after 0, 5, 10, 15 and 30 minutes and diluted 25 times (same dilution for all taken samples) in assay buffer (50 mM Britton buffer pH 7.3) and the activity is measured using the PHADEBAS assay (Magle Life Sciences) under standard conditions pH 7.3, 37 °C.

The activity measured before incubation (0 minutes) is used as reference (100%). The decline in percent is calculated as a function of the incubation time. The table shows the residual activity after, e.g., 30 minutes of incubation.

9.4.11 Assays for Alpha -Amylase Activity

1. PHADEBAS Assay

Alpha-amylase activity is determined by a method employing PHADEBAS® tablets as substrate. PHADEBAS tablets (PHADEBAS® Amylase Test, supplied by Magle Life Sciences) contain a cross-linked insoluble blue-colored starch polymer, which has been mixed with bovine serum albumin and a buffer substance and tableted.

For every single measurement one tablet is suspended in a tube containing 5 mL 50 mM Britton-Robinson buffer (50 mM acetic acid, 50 mM phosphoric add, 50 mM boric acid, 0.1 mM $CaCl_2$, pH adjusted to the value of interest with NaOH). The test is performed in a water bath at the temperature of interest. The alpha-amylase to be tested is diluted in 50 mM Britton-Robinson buffer. One mL of this alpha-amylase solution is added to the 5 mL 50 mM Britton-Robinson buffer. The starch is hydrolyzed by the alpha-amylase giving soluble blue fragments. The absorbance of the resulting blue solution, measured spectrophotometrically at 620 nm, is a function of the alpha-amylase activity.

It is important that the measured 620 nm absorbance after 10 or 15 minutes of incubation (testing time) is in the range of 0.2 to 2.0 absorbance units at 620 nm. In this absorbance range there is linearity between activity and absorbance (Lambert-Beer law). The dilution of the enzyme must therefore be adjusted to fit this criterion. Under a specified set of conditions (temp., pH, reaction time, buffer conditions) 1 mg of a given alpha-amylase will hydrolyze a certain amount of substrate and a blue color will be produced. The color intensity is measured at 620 nm. The measured absorbance is directly proportional to the specific activity (activity/mg of pure alpha-amylase protein) of the alpha-amylase in question under the given set of conditions.

2. Alternative Method

Alpha-amylase activity is determined by a method employing the PNP-G_7 substrate. PNP-G_7, which is a abbreviation for p-nitrophenyl-alpha,D-maltoheptaoside, is a blocked oligosaccharide which can be cleaved by an endo-amylase. Following the cleavage, the alpha-glucosidase included in the kit digest the substrate to liberate a free

PNP molecule which has a yellow color and thus can be measured by visible spectrophotometry at λ=405 nm (400-420 nm). Kits containing PNP-G_7 substrate and alpha-Glucosidase is manufactured by Boehringer-Mannheim (Cat. No. 1054635).

To prepare the reagent solution 10 mL of substrate/buffer solution is added to 50 mL enzyme/buffer solution as recommended by the manufacturer. The assay is performed by transferring a 20 μL sample to a 96 well microtitre plate and incubating at 25°C. 200 μL reagent solution pre-equilibrated to 25°C is added. The solution is mixed and pre-incubated 1 minute and absorption is measured every 30 seconds over 4 minutes at OD 405 nm in an ELISA reader.

The slope of the time dependent absorption-curve is directly proportional to the activity of the alpha-amylase in question under the given set of conditions.

9.4.12 Det ermination of LAS Sensitivity

The variant is incubated with different concentrations of LAS (linear alkyl benzene sulphonate; Nansa 1169/P) for 10 minutes at 40°C.

The residual activity is determined using the PHADEBAS® assay method or the alternative method employing the PNP-G_7 substrate.

LAS is diluted in 0.1 M phosphate buffer pH 7.5.

The following concentrations are used: 500 ppm, 250 ppm, 100 ppm, 50 ppm, 25 ppm, and 10 ppm on no LAS.

The variant is diluted in the different LAS buffers to concentration of 0.01-5 mg/l in a total volume of 10 mL and incubated for 10 minutes in a temperature controlled water bath. The incubation is stopped by transferring a small aliquot into cold assay buffer. It is important that during activity measurement the LAS concentration is below 1 ppm, in order not to affect the activity measurement. The residual activity is determined in duplicate using the above mentioned PHADEBAS® assay or alternative method. The activity is measured after subtraction of the blank. The activity with no LAS is 100%.

10. Methods of Using the Amylase Variants: Industrial Applications

The alpha-amylase variants presented herein possess valuable properties allowing for a variety of industrial applications in cleaning processes and stain removal. One or

more of the variant enzymes or compositions described herein may also be used in detergents, in particular laundry detergent compositions and dishwashing detergent compositions, hard surface cleaning compositions. The variants can also be used in compositions for desizing of textiles, fabrics or garments, for production of pulp and paper, beer making, ethanol production, and starch conversion processes as described above.

The variants herein may also be useful for desizing of textiles, fabrics, and garments (see, e.g., WO 95/21247, U.S. Pat. No. 4,643,736, and EP 119,920 hereby incorporated by reference), beer making or brewing, and in pulp and paper production or related processes.

10.1 Pulp and Paper Production

The variant alkaline alpha-amylase may also be used in the production of lignocellulosic materials, such as pulp, paper and cardboard, from starch reinforced waste paper and cardboard, especially where re-pulping occurs at pH above about 7 and where amylases facilitate the disintegration of the waste material through degradation of the reinforcing starch. The alpha-amylase variants are especially useful in a process for producing a papermaking pulp from starch-coated printed-paper. The process may be performed as described in WO 95/14807, comprising the following steps:

a) disintegrating the paper to produce a pulp,

b) treating with a starch-degrading enzyme before, during or after step a), and

c) separating ink particles from the pulp after steps a) and b).

The alpha-amylases may also be very useful in modifying starch where enzymatically modified starch is used in papermaking together with alkaline fillers such as calcium carbonate, kaolin and clays. With the alkaline alpha-amylase variants it is possible to modify the starch in the presence of the filler thus allowing for a simpler integrated process.

10.2 Desizing of Textiles, Fabrics and Garments

An alpha-amylase variant may also be very useful in textile, fabric or garment desizing. In the textile processing industry, alpha-amylases are traditionally used as auxiliaries in the desizing process to facilitate the removal of starch-containing size,

which has served as a protective coating on weft yarns during weaving. Complete removal of the size coating after weaving is important to ensure optimum results in the subsequent processes, in which the fabric is scoured, bleached and dyed. Enzymatic starch breakdown is preferred because it does not involve any harmful effect on the fiber material. In order to reduce processing cost and increase mill throughput, the desizing processing is sometimes combined with the scouring and bleaching steps. In such cases, non-enzymatic auxiliaries such as alkali or oxidation agents are typically used to break down the starch, because traditional alpha-amylases are not very compatible with high pH levels and bleaching agents. The non-enzymatic breakdown of the starch size does lead to some fiber damage because of the rather aggressive chemicals used. Accordingly, it would be desirable to use the alpha-amylase variants as they have an improved performance in alkaline solutions. The alpha-amylases may be used alone or in combination with a cellulase when desizing cellulose-containing fabric or textile.

Desizing and bleaching processes are well known in the art. For instance, such processes are described in WO 95/21247, U.S. Pat. No. 4,643,736, and EP 119,920, which are hereby incorporated by reference.

Commercially available products for desizing include OPTISIZE® FLEX from Genencor.

10.3 Cleaning Processes and Detergent Compositions

The variant alpha-amylases described herein may be added to and thus become a component of a detergent composition for various cleaning or washing processes, including laundry and dishwashing.

The detergent composition provided for herein may for example be formulated as a hand or machine laundry detergent composition including a laundry additive composition suitable for pretreatment of stained fabrics and a rinse added fabric softener composition or be formulated as a detergent composition for use in general household hard surface cleaning operations, or be formulated for hand or machine dishwashing operations.

In a specific aspect, there is provided for herein a detergent additive comprising a variant enzyme described herein. The detergent additive as well as the detergent

composition may comprise one or more other enzymes such as a protease, a lipase, a peroxidase, another amylolytic enzyme, e.g., another alpha-amylase, glucoamylase, maltogenic amylase, CGTase and/or a cellulase mannanase (such as MANNASTAR™ from Danisco US Inc., Genencor Division)), pectinase, pectin lyase, cutinase, and/or laccase.

In general the properties of the chosen enzyme(s) should be compatible with the selected detergent, (i.e., pH-optimum, compatibility with other enzymatic and non-enzymatic ingredients, etc.), and the enzyme(s) should be present in effective amounts.

Proteases: Suitable proteases include those of animal, vegetable or microbial origin. Microbial origin is preferred. Chemically modified or protein engineered mutants are included. The protease may be a serine protease or a metalloprotease, preferably an alkaline microbial protease or a trypsin-like protease. Examples of alkaline proteases are subtilisins, especially those derived from *Bacillus*, e.g., subtilisin Novo, subtilisin Carlsberg, subtilisin 309, subtilisin 147 and subtilisin 168 (described in WO 89/06279). Examples of trypsin-like pro-teases are trypsin (e.g., of porcine or bovine origin) and the *Fusarium* protease described in WO 89/06270 and WO 94/25583. Other examples of useful proteases may be found in WO98/23732, WO99/20770, WO 92/19729, WO 98/20115, WO 98/20116, and WO 98/34946.

Preferred commercially available protease enzymes include ALCALASE®, SAVINASE®, PRIMASE®, DURALASE®, ESPERASE®, and KANNASE® (from Novozymes A/S), MAXATASE®, MAXACAL, MAXAPEM®, PROPERASE®, PURAFECT®, PURAFECT OXP®, FN2®, FN3®, FN4® (Genencor International Inc.).

Lipases: Suitable lipases include those of bacterial or fungal origin. Chemically modified or protein engineered mutants are included. Examples of useful lipases include lipases from *Humicola* (synonym *Thermomyces*), e.g., from *H. lanuginosa* (*T. lanuginosus*) as described in EP 258 068 and EP 305 216 or from *H. insolens* as described in WO 96/13580, a *Pseudomonas lipase*, e.g., from *P. alcaligenes* or *P. pseudoalcaligenes* (EP 218 272), *P. cepacia* (EP 331 376), *P. stutzeri* (GB 1,372,034), *P. fluorescens*, *Pseudomonas* spp. strain SD 705 (WO 95/06720 and WO 96/27002), *P. wisconsinensis* (WO 96/12012), a *Bacillus* lipase, e.g., from *B. subtilis* (Dartois *et al.*

(1993), *Biochemica et Biophysica Acta*, 1131, 253-360), *B. stearothermophilus* (JP 64/744992) or *B. pumilus* (WO 91/16422). Other examples are lipase variants such as those described in WO 92/05249, WO 94/01541, EP 407 225, EP 260 105, WO 95/35381, WO 96/00292, WO 95/30744, WO 94/25578, WO 95/14783, WO 95/22615, WO 97/04079 and WO 97/07202.

Preferred commercially available lipase enzymes include LIPOLASE™ and LIPOLASE ULTRA™ (Novozymes A/S).

Amylases: One or more additional amylases may also be included. Suitable amylases (alpha and/or beta) include those of bacterial or fungal origin. Chemically modified or protein engineered mutants are included. Amylases include, for example, alpha-amylases obtained from *Bacillus*, e.g., a special strain of *B. licheniformis*, described in more detail in GB 1,296,839. Examples of useful alpha-amylases are the variants described in WO 94/18314, WO 96/39528, WO 94/02597, WO 94/18314, WO 96/23873, and WO 97/43424.

Commercially available alpha-amylases are DURAMYL™, LIQUEZYME™ TERMAMY™, NATALASE™, FUNGAMYL™ and BAN™ (Novozymes A/S), RAPIDASE™ and PURASTAR™ (from Genencor).

Cellulases: Suitable cellulases include those of bacterial or fungal origin. Chemically modified or protein engineered mutants are included. Suitable cellulases include cellulases from the genera *Bacillus, Pseudomonas, Trichoderma, Humicola, Fusarium, Thielavia, Acremonium*, e.g., the fungal cellulases produced from *Humicola insolens, Myceliophthora thermophila and Fusarium oxysporum* disclosed in U.S. Pat. No. 4,435,307, U.S. Pat. No. 5,648,263, U.S. Pat. No. 5,691,178, U.S. Pat. No. 5,776,757 and WO 89/09259. The *Trichoderma reesei* cellulases are disclosed in U.S. Pat. No. 4,689,297, U.S. Pat. No. 5,814,501, U.S. Pat. No. 5,324,649, WO 92/06221 and WO 92/06165. *Bacillus* cellulases are disclosed in U.S. Pat. No. 6,562,612.

Commercially available cellulases include CELLUZYME®, and CAREZYME® (Novozymes A/S), CLAZINASE®, and PURADAX HA® (Genencor International Inc.), and KAC-500(B)® (Kao Corporation).

Peroxidases/Oxidases: Suitable peroxidases/oxidases include those of plant, bacterial or fungal origin. Chemically modified or protein engineered mutants are included. Examples of useful peroxidases include peroxidases from *Coprinus*, e.g., from *C. cinereus*, and variants thereof as those described in WO 93/24618, WO 95/10602, and WO 98/15257.

Commercially available peroxidases include GUARDZYME® (Novozymes A/S).

The detergent enzyme(s) may be included in a detergent composition by adding separate additives containing one or more enzymes, or by adding a combined additive comprising all of these enzymes. A detergent additive, e.g., a separate additive or a combined additive, can be formulated, e.g., granulate, a liquid, a slurry, etc. Preferred detergent additive formulations are granulates, in particular non-dusting granulates, liquids, in particular stabilized liquids, or slurries.

Non-dusting granulates may be produced, e.g., as disclosed in U.S. Pat. Nos. 4,106,991 and 4,661,452 and may optionally be coated by methods known in the art. Examples of waxy coating materials are poly(ethylene oxide) products (polyethyleneglycol, PEG) with mean molar weights of about 1000 to about 20000; ethoxylated nonyl-phenols having from 16 to 50 ethylene oxide units; ethoxylated fatty alcohols in which the alcohol contains from 12 to 20 carbon atoms and in which there are 15 to 80 ethylene oxide units; fatty alcohols; fatty acids; and mono- and di- and triglycerides of fatty acids. Examples of film-forming coating materials suitable for application by fluid bed techniques are given in GB 1483591. Liquid enzyme preparations may, for instance, be stabilized by adding a polyol such as propylene glycol, a sugar or sugar alcohol, lactic acid or boric acid according to established methods. Protected enzymes may be prepared according to the method disclosed in EP 238,216.

The detergent composition may be in any convenient form, e.g., a bar, a tablet, a powder, a granule, a paste or a liquid. A liquid detergent may be aqueous, typically containing up to about 70% water and 0 to about 30% organic solvent, or non-aqueous.

The detergent composition comprises one or more surfactants, which may be non-ionic, semi-polar, anionic, cationic, and/or zwitterionic. The surfactants are typically present at a level of from about 0.1% to about 60% by weight.

When included therein, the detergent will usually contain from about 1% to about 40% of an anionic surfactant such as linear alkylbenzenesulfonate, alpha-olefinsulfonate, alkyl sulfate (fatty alcohol sulfate), alcohol ethoxysulfate, secondary alkanesulfonate, alpha-sulfo fatty acid methyl ester, alkyl- or alkenylsuccinic acid or soap.

When included therein, the detergent will usually contain from about 0.2% to about 40% of a non-ionic surfactant such as alcohol ethoxylate, nonyl-phenol ethoxylate, alkylpolyglycoside, alkyldimethylamine-oxide, ethoxylated fatty acid monoethanol-amide, fatty acid monoethanolamide, polyhydroxy alkyl fatty acid amide, or N-acyl N-alkyl derivatives of glucosamine ("glucamides").

The detergent may contain 0 to about 65% of a detergent builder or complexing agent such as zeolite, diphosphate, triphosphate, phosphonate, carbonate, citrate, nitrilotriacetic acid, ethylenediaminetetraacetic acid, diethylenetriaminepentaacetic acid, alkyl- or alkenylsuccinic acid, soluble silicates or layered silicates (e.g. SKS-6 from Hoechst).

The detergent may comprise one or more polymers. Examples are carboxymethylcellulose, poly(vinyl-pyrrolidone), poly (ethylene glycol), poly(vinyl alcohol), poly(vinylpyridine-N-oxide), poly(vinylimidazole), polycarboxylates such as polyacrylates, maleic/acrylic acid copolymers and lauryl methacrylate/acrylic acid co-polymers.

The detergent may contain a bleaching system, which may comprise a H_2O_2 source such as perborate or percarbonate that may be combined with a peracid-forming bleach activator such as tetraacetylethylenediamine or nonanoyloxyben-zenesul-fonate. Alternatively, the bleaching system may comprise peroxy acids of, e.g., the amide, imide, or sulfone type.

The enzyme(s) of the detergent composition may be stabilized using conventional stabilizing agents, e.g., a polyol such as propylene glycol or glycerol, a sugar or sugar alcohol, lactic acid, boric acid, or a boric acid derivative, e.g., an aromatic borate ester, or a phenyl boronic acid derivative, such as 4-formylphenyl boronic acid, and the composition may be formulated as described in, e.g., WO 92/19709 and WO 92/19708.

The detergent may also contain other conventional detergent ingredients such as e.g. fabric conditioners including clays, foam boosters, suds suppressors, anti-corrosion agents, soil-suspending agents, anti-soil re-deposition agents, dyes, bactericides, optical brighteners, hydrotropes, tarnish inhibitors, or perfumes.

It is at present contemplated that in the detergent compositions, any enzyme, in particular, one or more of the variant enzymes described herein, may be added, e.g., at about 0.01 mg to about 100 mg of enzyme protein per liter of wash liquor. In one embodiment, about 0.055 mg of enzyme protein per liter of wash liquor are used. In other embodiments, about 0.1 mg to about 1.0 mg of enzyme protein per liter of wash liquor are used.

One or more of the variant enzymes described herein may additionally be incorporated in the detergent formulations disclosed in WO 97/07202, which is hereby incorporated as reference.

10.4 Dish Wash Detergent Compositions

The enzymes may also be used in dish wash detergent compositions, including the following:

1) POWDER AUTOMATIC DISHWASHING COMPOSITION

Nonionic surfactant	0.4-2.5%
Sodium metasilicate	0-20%
Sodium disilicate	3-20%
Sodium triphosphate	20-40%
Sodium carbonate	0-20%
Sodium perborate	2-9%
Tetraacetyl ethylene diamine (TAED)	1-4%
Sodium sulphate	5-33%
Enzymes	0.0001-0.1%

2) POWDER AUTOMATIC DISHWASHING COMPOSITION

Nonionic surfactant (e.g. alcohol ethoxylate)	1-2%
Sodium disilicate	2-30%
Sodium carbonate	10-50%
Sodium phosphonate	0-5%
Trisodium citrate dihydrate	9-30%
Nitrilotrisodium acetate (NTA)	0-20%
Sodium perborate monohydrate	5-10%

Tetraacetyl ethylene diamine (TAED)	1-2%
Polyacrylate polymer (e.g. maleic acid/acrylic acid copolymer)	6-25%
Enzymes	0.0001-0.1%
Perfume	0.1-0.5%
Water	5-10 %

3) POWDER AUTOMATIC DISHWASHING COMPOSITION

Nonionic surfactant	0.5-2.0%
Sodium disilicate	25-40%
Sodium citrate	30-55%
Sodium carbonate	0-29%
Sodium bicarbonate	0-20%
Sodium perborate monohydrate	0-15%
Tetraacetyl ethylene diamine (TAED)	0-6%
Maleic acid/acrylic acid copolymer	0-5%
Clay	1-3%
Polyamino acids	0-20%
Sodium polyacrylate	0-8%
Enzymes	0.0001-0.1%

4) POWDER AUTOMATIC DISHWASHING COMPOSITION

Nonionic surfactant	1-2%
Zeolite MAP	15-42%
Sodium disilicate	30-34%
Sodium citrate	0-12%
Sodium carbonate	0-20%
Sodium perborate monohydrate	7-15%
Tetraacetyl ethylene diamine (TAED) Polymer	0-3%
Maleic acid/acrylic acid copolymer	0-4%
Organic phosphonate	0-5%
Clay	0-4%
Enzymes	1-2%
Sodium sulphate	0.0001-0.1%
	Balance

5) POWDER AUTOMATIC DISHWASHING COMPOSITION

Nonionic surfactant	1-7%
Sodium disilicate	18-30%
Trisodium citrate	10-24%
Sodium carbonate	12-20%
Monopersulphate ($2\ KHSO_5.KHSO_4.K_2SO_4$)	15-21%
Bleach stabilizer	0.1-2%

Maleic acid/acrylic acid copolymer	0-6%
Diethylene triamine pentaacetate, pentasodium salt	0-2.5%
Enzymes	0.0001-0.1%
Sodium sulphate, water	Balance

6) POWDER AND LIQUID DISHWASHING COMPOSITION WITH CLEANING SURFACTANT SYSTEM

Nonionic surfactant	0-1.5%
Octadecyl dimethylamine N-oxide dihydrate	0-5%
80:20 wt. C18/C16 blend of octadecyl dimethylamine N-oxide dihydrate and hexadecyldimethyl amine N-oxide dihydrate	0-4%
70:30 wt. C18/C16 blend of octadecyl bis (hydroxyethyl)amine N-oxide anhydrous and hexadecyl bis (hydroxyethyl)amine N-oxide anhydrous	0-5%
C_{13}-C_{15} alkyl ethoxysulfate with an average degree of ethoxylation of 3	0-10%
C_{12}-C_{15} alkyl ethoxysulfate with an average degree of ethoxylation of 3	0-5%
C_{13}-C_{15} ethoxylated alcohol with an average degree of ethoxylation of 12	0-5%
A blend of C_{12}-C_{15} ethoxylated alcohols with an average degree of ethoxylation of 9	0-6.5%
A blend of C_{13}-C_{15} ethoxylated alcohols with an average degree of ethoxylation of 30	0-4%
Sodium disilicate	0-33%
Sodium tripolyphosphate	0-46%
Sodium citrate	0-28%
Citric acid	0-29%
Sodium carbonate	0-20%
Sodium perborate monohydrate	0-11.5%
Tetraacetyl ethylene diamine (TAED)	0-4%
Maleic acid/acrylic acid copolymer	0-7.5%
Sodium sulphate	0-12.5%
Enzymes	0.0001-0.1%

7) NON-AQUEOUS LIQUID AUTOMATIC DISHWASHING COMPOSITION

Liquid nonionic surfactant (e.g. alcohol ethoxylates)	2.0-10.0%
Alkali metal silicate	3.0-15.0%
Alkali metal phosphate	20.0-40.0%
Liquid carrier selected from higher glycols, polyglycols, polyoxides, glycolethers	25.0-45.0%
Stabilizer (e.g. a partial ester of phosphoric acid and a	0.5-7.0%

C_{16}-C_{18} alkanol)	
Foam suppressor (e.g. silicone)	0-1.5%
Enzymes	0.0001-0.1%

8) NON-AQUEOUS LIQUID DISHWASHING COMPOSITION

Liquid nonionic surfactant (e.g. alcohol ethoxylates)	2.0-10.0%
Sodium silicate	3.0-15.0%
Alkali metal carbonate	7.0-20.0%
Sodium citrate	0.0-1.5%
Stabilizing system (e.g. mixtures of finely divided silicone and low molecular weight dialkyl polyglycol ethers)	0.5-7.0%
Low molecule weight polyacrylate polymer	5.0-15.0%
Clay gel thickener (e.g. bentonite)	0.0-10.0%
Hydroxypropyl cellulose polymer	0.0-0.6%
Enzymes	0.0001-0.1%
Liquid carrier selected from higher glycols, polyglycols, polyoxides and glycol ethers	Balance

9) THIXOTROPIC LIQUID AUTOMATIC DISHWASHING COMPOSITION

C_{12}-C_{14} fatty acid	0-0.5%
Block co-polymer surfactant	1.5-15.0%
Sodium citrate	0-12%
Sodium tripolyphosphate	0-15%
Sodium carbonate	0-8%
Aluminum tristearate	0-0.1%
Sodium cumene sulphonate	0-1.7%
Polyacrylate thickener	1.32-2.5%
Sodium polyacrylate	2.4-6.0%
Boric acid	0-4.0%
Sodium formate	0-0.45%
Calcium formate	0-0.2%
Sodium n-decydiphenyl oxide disulphonate	0-4.0%
Monoethanol amine (MEA)	0-1.86%
Sodium hydroxide (50%)	1.9-9.3%
1,2-Propanediol	0-9.4%
Enzymes	0.0001-0.1%
Suds suppressor, dye, perfumes, water	Balance

10) LIQUID AUTOMATIC DISHWASHING COMPOSITION

Alcohol ethoxylate	0-20%
Fatty acid ester sulphonate	0-30%
Sodium dodecyl sulphate	0-20%
Alkyl polyglycoside	0-21%
Oleic acid	0-10%
Sodium disilicate monohydrate	18-33%

Sodium citrate dihydrate	18-33%
Sodium stearate	0-2.5%
Sodium perborate monohydrate	0-13%
Tetraacetyl ethylene diamine (TAED)	0-8%
Maleic acid/acrylic acid copolymer	4-8%
Enzymes	0.0001-0.1%

11) LIQUID AUTOMATIC DISHWASHING COMPOSITION CONTAINING PROTECTED BLEACH PARTICLES

Sodium silicate	5-10%
Tetrapotassium pyrophosphate	15-25%
Sodium triphosphate	0-2%
Potassium carbonate	4-8%
Protected bleach particles, e.g. chlorine	5-10%
Polymeric thickener	0.7-1.5%
Potassium hydroxide	0-2%
Enzymes	0.0001-0.1%
Water	Balance

12) Automatic dishwashing compositions as described in 1), 2), 3), 4), 6) and 10), wherein perborate is replaced by percarbonate.

13) Automatic dishwashing compositions as described in 1)-6) which additionally contain a manganese catalyst. The manganese catalyst may, e.g., be one of the compounds described in "Efficient manganese catalysts for low-temperature bleaching", *Nature* 369: 637-639 (1994).

14) PREMIUM HDL LIQUID DETERGENT FORMULATIONS

Bio-Soft S-101	Linear alkylbenzene sulfonic acid
Steol CS-330	Sodium Laureth sulfate
Bio-soft N25-7	Linear alkylethoxylate with 7 moles of EO
Stepanate SXS	Sodium xylene sulfonate

15) ULTRA LIQUID DETERGENT FORMULATION

Tionopal CBS-X	Fluorescent whitening agent
Alpha-step MC-48	Sodium alpha-sulfomethylester
Makon TD-6	Tridecylalcoholethoxylate

11. Compositions Comprising the Variant Alpha-Amylases

In one of its several aspects, this disclosure provides compositions comprising:

a) at least one variant alpha-amylase comprising an amino acid sequence at least 95% identical to that of a parent AmyS-like alpha-amylase, and having a substitution at an amino acid position corresponding to position 242 of a reference alpha-amylase, said variant having detectable alpha-amylase activity, and

b) at least one of an additional enzyme, a detergent, a surfactant, a chelator, an oxidizing agent, an acidulant, an alkalizing agent, a source of peroxide, a source of hardness, a salt, a detergent complexing agent, a polymer, a stabilizing agent, or a fabric conditioner.

In preferred embodiments the variant is altered, as compared to a parent AmyS-like alpha-amylase or a reference amylase, in one or more of a variety of properties that can alter it use or performance for certain applications, e.g., commercial processes described herein. The altered properties can include any property, for example, such as net charge, substrate specificity, substrate cleavage, substrate binding, thermal stability, activity at one or more pH's, stability at one or more pH's, stability in oxidizing conditions, Ca^{2+} requirements, specific activity, catalytic rate, catalytic efficiency, activity in the presence of a chelator, thermal or pH stability in the presence of a chelator, utility for desizing, or utility for a cleaning process, or amount of expression in a protein expression system. As the skilled artisan will appreciate, these altered properties preferably have utility to the end-user, or to the producer of the amylase, or both.

A number of amylases of known or readily-determined sequence can be used as the reference amylase. In various embodiments, the reference amylase is SEQ ID NOS: 1 or 2. The parent amylase and the reference amylase can be the same amylase in some embodiments.

The composition is, in certain embodiments, a component of a product for use in laundry, dish, or hard-surface cleaning, desizing, or fabric or stain treatment. For example the composition may be part of a dishwashing detergent for application as a liquid, semi-solid, solid, etc, or it can be a granular or liquid laundry detergent formulation. The composition comprises additional components as required for the

intended application. Examples of many such formulations are provided herein, and still others will be familiar to those of skill in the art.

In one embodiment, the composition comprises an additional enzyme that is a protease, a lipase, an amylase, a cellulase, a peroxidase, an oxidase, a pectinase, a lyase, a cutinase, or a laccase, or other useful enzyme. The skilled artisan will be familiar with these and other enzymes that may be useful in connection with the variant amylases provided herein. The amounts of enzyme that are useful can be determined empirically for a given application, however, guidelines are provided herein, e.g., in the examples.

In various embodiments, the composition comprises one or more surfactants. The surfactant is generally nonionic, anionic, cationic, or zwitterionic, or a combination thereof.

In one embodiment, the amylase variant is preferably a S242A, S242D, S242E, S242F, S242G, S242H, S242L, S242M, S242N, S242Q, or S242T variant. For certain uses, such as in washing and cleaning embodiments, stability to oxidation and stability to chelators or altered metal ion concentrations are useful. Accordingly in various embodiments, the variant amylase has altered stability to oxidation and the variant further includes deletion or substitution of one or more methionine residues including residues located at amino positions 8, 9, 96, 200, 206, 284, 307, 311, 316, and 438 of a parent amylase, where the reference amylase in SEQ ID NOS: 1 or 2. Variant amylases can further comprise an amino acid sequence modification at one or more amino acid positions corresponding to amino acid positions 97, 179, 180, 193, 319, 349, 358, 416, 428, or 443 of the reference amylase (which is preferably SEQ ID NOS: 1 or 2).

In various embodiments, the variant comprises or further comprises one or more of substitution at positions as follows: a cysteine at 349, a cysteine at 428, a glutamic acid at 97, an arginine at 97, a glutamic acid at 319, an arginine at 319, a glutamic acid at 358, an arginine at 358, a glutamic acid at 443, or an arginine at 443.

Moreover, the variant in one embodiment comprises a substitution of an N193 or a V416 or both, for example, a substitution that is N193F, or V416G, or both. Other embodiments include further modification such as deletion of one or more amino acids at positions F178, R179, G180, I181, G182 and K183. As described elsewhere herein, such

deletions may be even more useful when provided in pair-wise fashion or more. Preferably in such embodiments, the variant has altered metal ion dependence, or altered stability or activity in the absence of added calcium, or in the presence of a chelator, or a combination thereof. Such variants may also have excellent utility in cleaning and washing processes.

In one embodiment, the variant alpha-amylase has at least 95% homology to SEQ ID NO: 2 and comprises a substitution of amino acid 242 relative to numbering in a reference amylase comprising the amino acid sequence SEQ ID NO: 1. As with the other embodiments described herein, the variant preferably has detectable alpha-amylase activity, particularly under the conditions of use.

In certain presently preferred embodiments, the parent alpha-amylase is SEQ ID NO: 1, 2, 6, 7, 8, 9, 10, 11, 12, 15, or16, and the reference amylase is SEQ ID NO: 1 or 2.

In various embodiments, the variant amylase has improved performance in a wash process at very low and very high pH's. In one embodiment, the wash performance is improved at pH $\geq$ about 8, relative to the parent amylase. More preferred are those variants with improved wash performance above about pH 8.5 to about pH 11.

The variant in one embodiment comprises a set of substitutions of a) Q97E, Q319E, Q358E, Q443E; b) Q97E, Q319R, Q358E, Q443R; c) Q97E, Q319R, Q358E; d) Q97E, Q319R, Q443E; e) Q97E, Q319R, Q443R; f) Q97E, Q358R; g) Q97E, Q443E; h) Q319R, Q358E, Q443E; or i) Q319R, Q358R, Q443E relative to the reference amylase, e.g. a SEQ ID NO: 1 or 2 amylase sequence.

In another aspect of the disclosure, provided are detergent or cleaning formulations comprising at least one variant amylase comprising an amino acid sequence at least 95% identical to that of a parent AmyS-like alpha-amylase. The amylase variants have a substitution at an amino acid position corresponding to position 242 of a reference alpha-amylase, and have detectable alpha-amylase activity. Preferably, the reference amylase is SEQ ID NOS: 1 or 2.

The detergent or cleaning formulation preferably comprises an amylase variant that an S242 variant comprising at least a S242A, S242D, S242E, S242F, S242G, S242H, S242L, S242M, S242N, S242Q, or S242T substitution. As with the compositions

provided above, the variant can comprise any one or combination of the variant features and alterations disclosed herein.

In another of its several aspects, this disclosure provides kits. One embodiment of the kit comprises

a) one or more variant alpha-amylases comprising an amino acid sequence at least 95% identical to that of a parent AmyS-like alpha-amylase, and having a substitution at an amino acid position corresponding to position 242 of a reference alpha-amylase, said variant having detectable alpha-amylase activity, and

b) at least one of an additional enzyme, a detergent, a surfactant, a chelator, an oxidizing agent, an acidulant, an alkalizing agent, a source of peroxide, a source of hardness, a salt, a detergent complexing agent, a polymer, a stabilizing agent, or a fabric conditioner.

In one embodiment, the kits further comprise instructions for using the kit in a process for desizing a woven material or washing or cleaning one or more items soiled with a starch-containing substance.

The skilled artisan will also appreciate that kits for making the described alpha-amylases are also provided. The kits provide representative sequences e.g. amino acid sequences and/or nucleic acid derived therefrom, for use as parent alpha-amylases and reference amylases.

12. Using Amylase Variants in Desizing and Washing/Cleaning Processes

In another aspect, this disclosure provides methods of using the variant alpha-amylases in desizing of fabrics or other woven material, and in washing or cleaning processes.

In aspect, this disclosure provides methods of desizing a woven material subsequent to a weaving process. The methods generally comprise contacting the woven material with a variant alpha-amylase under conditions and for a time effective for at least partially removing sizing from the woven material. The variant comprises an amino acid sequence at least 95% identical to that of a parent AmyS-like alpha-amylase, and has

a substitution at an amino acid position corresponding to position 242 of a reference alpha-amylase. The variant has detectable alpha-amylase activity.

The variant is preferably altered in one more of its physical or enzymatic properties, as compared to a parent AmyS-like alpha-amylase or a reference amylase. In various embodiments, the amylase is altered in one or more characteristics of: net charge, substrate specificity, substrate cleavage, substrate binding, thermal stability, activity at one or more pH's, stability at one or more pH's, stability in oxidizing conditions, Ca^{2+} requirements, specific activity, catalytic rate, catalytic efficiency, activity in the presence of a chelator, thermal or pH stability in the presence of a chelator, effectiveness for desizing, or amount of expression in a protein expression system.

Reference amylases are discussed above, and in one embodiment of the method, the reference amylase is SEQ ID NOS: 1 or 2.

In one embodiment, the parent alpha-amylase is SEQ ID NOS: 1, 2, 6, 7, 8, 9, 10, 11, 12, 15, or 16, and the reference amylase is SEQ ID NOS: 1 or 2. In certain embodiments, the variant is a S242A, S242D, S242E, S242F, S242G, S242H, S242L, S242M, S242N, S242Q, or S242T variant.

The variant can further comprise one or more substitutions at positions as follows: a cysteine at 349, a cysteine at 428, a glutamic acid at 97, an arginine at 97, a glutamic acid at 319, an arginine at 319, a glutamic acid at 358, an arginine at 358, a glutamic acid at 443, or an arginine at 443, wherein the reference amylase is SEQ ID NO: 1 or 2.

In another aspect, provided herein are methods of washing or cleaning. While washing and cleaning operations can frequently benefit from the inclusion of one or more enzyme activities, the washing or cleaning processes can subject the enzymes, including amylases to extreme conditions and challenge the limits of the enzyme activity. Accordingly, the methods provided comprise contacting one or more items to be washed or cleaned with a composition comprising a variant alpha-amylase comprising an amino acid sequence at least 95% identical to that of a parent AmyS-like alpha-amylase, and having a substitution at an amino acid position corresponding to position 242 of a reference alpha-amylase. The variant preferably has detectable alpha-amylase activity, and the contacting step is under conditions and for a time effective for at least partially

washing or cleaning the one or more items. Preferably, at least one of the one or more items is soiled with at least one starch-containing material, the removal of which is aided by the variant amylase.

In one embodiment, the composition further comprises at least one component of a detergent composition or a cleaning formulation. For example, the composition comprises one or more of an additional enzyme, a detergent, a surfactant, a chelator, an oxidizing agent, an acidulant, an alkalizing agent, a source of peroxide, a source of hardness, a salt, a detergent complexing agent, a polymer, a stabilizing agent, or a fabric conditioner.

In one embodiment, the parent alpha-amylase can be any of SEQ ID NOS: 1, 2, 6, 7, 8, 9, 10, 11, 12, 15, or16, and the reference amylase is SEQ ID NOS: 1 or 2. In certain embodiments, the parent alpha-amylase is conveniently SEQ ID NOS: 1, 2, 15, or 16, while in others, the parent alpha-amylase is SEQ ID NOS: 6, 7, 8, 9, 10, 11, or 12.

In presently preferred embodiments, the variant is a S242A, S242D, S242E, S242F, S242G, S242H, S242L, S242M, S242N, S242Q, or S242T variant. The reference amylase is SEQ ID NO: 1 or 2, and the variant is a S242A, S242D, S242E, S242F, S242G, S242H, S242L, S242M, S242N, S242Q, or S242T variant in certain embodiments.

In various embodiments, e.g., where the variant is a S242A, S242D, S242E, S242F, S242G, S242H, S242L, S242M, S242N, S242Q, or S242T variant, the variant further comprises a sequence modification at one or more amino acid positions corresponding to amino acid positions 97, 179, 180, 193, 319, 349, 358, 416, 428, or 443 of the reference amylase. More particularly, the variant comprises one or more of substitution at positions as follows: a cysteine at 349, a cysteine at 428, a glutamic acid at 97, an arginine at 97, a glutamic acid at 319, an arginine at 319, a glutamic acid at 358, an arginine at 358, a glutamic acid at 443, or an arginine at 443 in various embodiments. Substitution of an N193 or a V416 or both, such as a substitution of N193F or V416G, or both are also useful in certain variants.

In other embodiments, the variant comprises deletion of one or more amino acids at any of specific positions F178, R179, G180, I181, G182 and K183. In such

embodiments, the variant preferably has altered metal ion dependence or altered stability, or activity in the absence of added calcium or the presence of a chelator. As with the other modifications, the foregoing deletions of amino acids can also be used – alone or in combination with any of the foregoing alterations.

The variant generally has improved performance in a wash process relative to the parent amylase, for example under conditions such as pH ≥ about 8.

In one presently preferred embodiment, the method includes the use of a variant that comprises a set of substitutions of a) Q97E, Q319E, Q358E, Q443E; b) Q97E, Q319R, Q358E, Q443R; c) Q97E, Q319R, Q358E; d) Q97E, Q319R, Q443E; e) Q97E, Q319R, Q443R; f) Q97E, Q358R; g) Q97E, Q443E; h) Q319R, Q358E, Q443E; or i) Q319R, Q358R, Q443E.

This disclosure includes further detail in the following examples, which are not in any way intended to limit the scope of what is claimed. The attached Figures are integral parts of the specification and description provided. All references cited are herein specifically incorporated by reference for all that is described therein. The following examples are thus offered to illustrate, but not to limit what is claimed.

EXAMPLES

Example 1 - Construction of Variants

The variants at position S242 of the mature sequence of AmyS were constructed using site directed mutagenesis. The template for mutagenesis was methylated pHPLT-AmyS (*see* Figure 2) using dam-Methylase from New England Biolabs (Massachusetts). Degenerate primers (S242F(forward) and S242R(reverse), given below) were synthesized and diluted to 10 μM at Operon (Huntsville, AL) with complementary forward and reverse sequences both containing a 5' phosphate group for ligation in the reaction. The sequence of the parent alpha-amylase (SEQ ID NO: 2) is attached hereto. Libraries were created with the Stratagene Quik-Change™ Multi-site kit (Stratagene, La Jolla CA) using oligonucleotide primers randomized with NN(G/C) at the target position. The selected amino acid (i.e., S242) was randomly replaced with all 19 possible alternatives.

S242 primers for mutagenesis:

S242 F:
5'[Phos]GTCAAGCATATTAAGTTCNNSTTTTTTCCTGATTGGTTG 3' SEQ ID NO: 17

S242 R:
5'[Phos]CAACCAATCAGGAAAAAASNNGAACTTAATATGCTTGAC 3' SEQ ID NO: 18

The reaction was performed as follows:

QUIK–CHANGE reaction:

The reaction consisted of 18 µL of sterile distilled H_2O, 2.5 µL of 10x buffer from the kit, 1 µL dNTPs from the kit, 1.25 µL of the forward primers (of 10 µM stock), 1.25 µL of the reverse primers (of 10 µM stock), 1 µL of pHPLT-AmyS plasmid DNA as template (~70 ng), and 1 µL of the enzyme blend from the kit for a total of 26.5 µL.

Cycling conditions:

The cycling conditions were 95°C for 1 min once, then 95°C for 1 min, 55°C for 1min, 65°C for 10 min for 25 cycles.

One microliter *Dpn*I (10 U/µL) was added to the Multi-site Quik-Change™ reaction mixture and incubated at 37°C for 18 hours and then another 0.5 µL was added for an additional 3 hours.

One microliter of *Dpn*I digested reaction was used as template for rolling circle amplification with the TEMPLIPHI amplification kit (Amersham Biosciences, Piscataway, NJ), and the reaction was performed according to the Amersham protocol. One microliter of rolling circle DNA was transformed into 100 µL of *Bacillus subtilis* competent cells (2 protease deleted *B. subtilis* strain (Δ*aprE*, Δ*nprE*, *amyE*::*xylRPxylAcomK-phleo*)) and shaken at 37 °C for 1 hour. The entire transformation was next plated on LA + 10 ppm Neo + 1% insoluble starch plates (25 µL one plate, 75 µL on another plate) and incubated overnight at 37 °C. Ninety-six transformants were picked into 150 µL of LB + 10 ppm Neo in a micro-titer plate and grown overnight at 37°C. The overnight plate was stamped onto a large LA + 10 ppm Neo + 1% insoluble starch plate with a 96 pin replicating tool and submitted to Quintara Biosciences (Berkeley, CA) for colony PCR and sequencing.

After variant sequences were determined, the variants were picked into a 96 well micro-titer plates containing 125 µL of LB + 10 ppm Neo, arraying the variants into a quad format with controls. The arrayed micro-titer plate was grown for 6 hours at 37°C and 250 rpm. Using a replicating tool (Enzyscreen, Leiden, The Netherlands) the micro-titer culture plate was used to inoculate a new micro-titer plate (micro-titer plate and plate lids from Enzyscreen, Leiden, The Netherlands) containing 150 µL of MBD medium for protein expression (G. Vogtentanz *et al,* "A *Bacillus subtilis* fusion protein system to produce soybean Bowman-Birk protease inhibitor," *Prot. Expr. & Purif.*, 55: 40-52, 2007) and supplemented with 5 mM $CaCl_2$ for protein expression. Expression plates were grown for 64 hours at 37°C, 250 rpm, and 70% humidity. Expression cultures were next filtered through a micro-filter plate (0.22 µm, Millipore, Billerica, MA) and screened for improved thermostability (*see* Example 3).

Example 2 – Expression, Purification & Characterization of Variants

Colonies were streaked from the microtiter plates from Example 1 onto starch plates with 10 ppm Neomycin. The plates were incubated overnight at 37°C, and single colonies were picked and used to inoculate shake flasks (250 mL with 25mL media) containing media (*see* below) and 20 ppm Neomycin. The cultures were grown at 37°C, 275 rpm, for about 8 hrs (until an OD (600 nm) of 2.0 was reached). The culture broths were mixed with 50% glycerol at 2:1 ratio, put into individually-labeled culture vials and frozen at -80°C. Subsequent production of the selected alpha-amylases were made from these glycerol stocks.

Fermentations for alpha-amylases were carried out in 500 mL shake flasks grown at 37°C for 60 hours in minimal MOPS culture medium (Neidhardt *et al.*, *J. Bacteriol.* 119(3):736-747, 1974) with 1% (w/v) Soytone. Enzymes were purified from the fermentation broth using hydrophobic interaction chromatography as follows: the broth was concentrated 10-fold then diluted back to its original volume with 50 mM MES, 2 mM $CaCl_2$, pH 6.8 with 1 M ammonium sulfate, then sterile-filtered using a glass fiber filter Samples were then loaded onto PHENYL SEPHAROSE FF high density column (20 x 95 mm; Amersham, GE Healthcare Bio-Sciences, Sweden) pre-equilibrated with

the same buffer. Non-amylase proteins were removed with 10 column volumes of the same buffer without ammonium sulfate followed by 5 column volumes of water. Enzymes of interest were eluted with 50 mM MES, 2 mM $CaCl_2$, pH 6.8 containing 40% propylene glycol.

Protein concentrations were determined either with a standard quantitative SDS page gel densitometry method or using an activity assay using a standard amylase assay kit from Megazyme (Wicklow, Ireland). A standard curve generated using purified amylase (*Bacillus* 707 amylase; SEQ ID NO: 6) was used for comparing assay data.

Example 3 - Determination of Altered Properties: Thermal stress

This example shows that the variants described herein may have an altered property relative to the parent alpha-amylase. A high-throughput thermal stability screen of *G. stearothermophilus* alpha-amylase (AmyS) variants was carried out.

After an initial investigation, heat-stress conditions were chosen such that the wild-type enzyme showed approximately 40% of its initial (pre-stress) activity after the heat stress (i.e., (activity after heat stress) / (activity before heat stress) was approximately 0.4). Libraries of mutants were screened in quadruplicate, and potential winners were identified as those that showed residual activity after heat stress that was at least two standard deviations more than the average residual activity of the wild-type enzyme.

Amylase expression was approximately 100 ppm in the culture supernatants of the expression plates. After 60-65 hours of growth at 37°C in a humidified shaker (250 rpm and 70% relative humidity), the culture supernatants were clarified to remove cellular material using filter plates. The clarified supernatants were diluted 10-fold into buffer containing 50 mM NaOAc / 2.6 mM $CaCl_2$ / 0.002% Tween-20, at pH 5.8., to a final concentration of approximately 10 ppm. One aliquot of each supernatant was further diluted to 0.02 ppm, for determination of activity of the enzyme variants as described below using a fluorescently-labeled corn starch substrate. A second aliquot of each supernatant was subjected to a 30 minute heat stress at 95°C in a thermocycler then diluted to 0.02 ppm in 50 mM NaOAc / 2.6 mM $CaCl_2$ / 0.002% Tween-20, at pH 5.8 and assayed for residual activity using the fluorescent substrate and assay described below.

Amylase activity was determined using the amylase ENZCHECK ULTRA AMYLASE assay kit essentially as described by the manufacturer (Invitrogen, San Diego CA). Final concentration of the amylase in the assay was approximately 0.02 ppm. Assay buffer was 50 mM NaOAc / 2.6 mM $CaCl_2$ / 0.002% Tween-20, pH 5.8. The substrate was BODIPY fluorescence dye conjugated 100 µg/mL DQ™ starch from corn (Invitrogen - Eugene, OR). Increased fluorescence, indicating amylase activity, was measured using a SpectraMAX M2 (Molecular Devices, Sunnyvale, CA). The reaction was monitored at room temperature for 5 minutes with the instrument recording in kinetic mode. Excitation wavelength was 485 nm; emission was monitored at 520 nm with a cutoff filter at 515 nm.

The wild-type AmyS (Xtra) showed 33-43% residual activity after being subject to thermal stress for 30 minutes at 95°C. AmyS variants, S242A and S242Q, retained 55-65% and 70-80% residual activities, respectively, following the same thermal stress conditions. *See* Figure 3 and Table 3-1. These residual activity measurements indicate the two variants are more thermostable than the wild-type alpha-amylase.

Table 3-1: Percent residual activities of each variant. Wild-type (SPEZYME XTRA). Each plate includes SPEZYME ETHYL and SPEZYME XTRA as controls as indicated.

Variant	% Residual Activity				Avg	Std. Dev
A	65.0	53.4	48.5	71.1	59.5	10.4
C	35.9	24.5	27.3	29.6	29.3	4.9
D	52.2	32.6	38.5	43.3	41.6	8.3
E	40.2	53.3	33.2	51.8	44.6	9.6
F	41.7	31.8	30.1	31.7	33.8	5.3
G	34.3	27.1	27.4	37.5	31.6	5.2
H	22.6	20.5	16.2	17.8	19.3	2.8
I	36.2	26.9	19.7	25.5	27.0	6.8
K	22.3	22.6	23.3	23.0	22.8	0.5
L	26.1	29.6	30.6	27.8	28.5	2.0
M	48.8	46.6	40.5	35.9	42.9	5.9
N	32.0	29.0	24.6	35.1	30.2	4.5
P	7.2	7.7	6.4	5.7	6.7	0.9
Q	61.0	65.7	49.1	69.3	61.3	8.8
R	14.5	14.3	11.7	11.7	13.0	1.5
wildtype	44.3	27.1	29.2	35.5	34.0	7.7
T	24.6	25.4	27.7	21.5	24.8	2.5
V	17.5	25.9	22.1	23.9	22.3	3.6

Variant	% Residual Activity				Avg	Std. Dev
W	5.0	6.3	3.9	7.0	5.6	1.4
Y	18.5	13.5	14.2	16.5	15.7	2.3
Ethyl	111.8	77.3	84.3	66.7	85.0	19.2
Xtra	27.1	36.1	40.7	25.2	32.3	7.4

Example 4 - Determination of Altered Properties: DSC

Spezyme Xtra, S242A, S242E, and S242Q were purified from shake flask fermentation broth (see Example 2) using hydrophobic interaction chromatography. The protein was eluted from the column in purified form using 50 mM MES, pH 6.8, containing 40% propylene glycol and 2 mM $CaCl_2$.

Excessive heat capacity curves were measured using an μLtrasensitive scanning high-throughput microcalorimeter, VP-CAP DSC (MicroCal, Inc., Northampton, MA). The standard procedure for DSC measurements and the theory of the technique has been published (E. Freire, "Differential Scanning Calorimetry," *Methods. Mol. Biol.* 41: 191-218, 1995). Approximately 500 μL of 0.5 mg/mL wild-type *Bacillus stearothermophilus* α-amylase or variant S242A, S242E, and S242Q (both in the absence and in the presence of 2 mM calcium chloride) were scanned over a 30-120°C temperature range. The same sample was then re-scanned to check the reversibility of the process. For α-amylase, the thermal unfolding process was irreversible. The buffer used was 10 mM sodium acetate, pH 5.5. A 200°C/hr scan rate was used to minimize any artifacts that may have resulted from aggregation. The thermal midpoint (T_m) of the DSC curves was used as an indicator of the thermal stability of the tested protein. Table 4-1 shows the thermal melting points for the amylase proteins tested. The thermal melting curves and the melting points for the wild-type and variant amylases are shown in Figure 5.

The thermal unfolding for the amylase variants S242A, S242E, and S242Q in the absence and presence of 2 mM calcium chloride show considerable increase in the melting points for the variants when compared to that for the wild-type. In the absence of added calcium chloride, the wild-type amylase has a thermal melting point of 100.8°C while the T_m's for S242A, S242E, and S242Q are 106.5°C, 107.8°C, and 110.1°C, respectively. Thus, the substitution of S242 with A results in an increase in the T_m of

5.7°C; the substitution of S242 with E results in an increase in the T_m of 7.0°C; and the substitution of S242 with Q results in an increase in the T_m of 9.3°C.

In the presence of 2 mM calcium chloride, the wild-type amylase displayed a thermal melting point of 106.8°C while the T_m's for S242A, S242E, and S242Q were 111.8°C, 112.2°C, and 113.8°C, respectively. Thus, relative to measurements in the absence of calcium, in the presence of 2 mM calcium chloride, all four proteins had increased T_m values. The increase in T_m for wild-type and the S242A variants in the presence of calcium was 6°C and 5.3°C, respectively. The increase in T_m for the S242E variant was 4.4°C. The increase in T_m for the S242Q variant was 3.7°C. This suggests that the S242Q variants is stabilized less by calcium, or the variant is less dependent on calcium for stability. The increase in the T_m of the S242A, S242E, and S242Q relative to wild-type in the presence of calcium chloride was 5°C, 5.4°C, and 3°C, respectively. This suggests that the thermodynamic properties of the variants differ from those of the wild-type, or Spezyme Xtra. This observation was consistent with its enhanced performance in application studies (*see* Example 5).

Table 4-1 T_m (°C) for various amylases by DSC

	T_m (No Ca^{2+})	ΔT (°C)	T_m (w/ 2 mM Ca^{2+})	ΔT (°C)
Spezyme Xtra	100.8		106.8	
S242A	106.5	5.7	111.8	5.7
S242E	107.8	7.0	112.2	5.4
S242Q	110.1	9.3	113.8	7.0

Example 5 – Activity Profiles

This example shows that the tested variants have altered activity profiles relative not only to the parent alpha-amylase but also to an industry standard enzyme. Protein determinations were made on purified or plate samples. The variants and standard alpha-amylases were each assayed on the basis of equal protein concentration.

Either plate or purified variants were diluted to approximately 20 ppm using pH 5.6 malic acid buffer. The substrate consisted of 15% cornstarch in the same 50 mM Malic acid buffer, pH 5.6. Four hundred microliters of the starch suspension was

equilibrated to 70°C for 2.5 minutes. Seven (7) μL of the diluted enzyme was quickly added to the equilibrated starch at a final protein concentration of about 0.36 ppm. The reaction mix was then put into a pre-heated 85°C shaking heating block and mixed at 300 rpm. The reactions were quenched with 50 μL of 125 mM NaOH at predetermined time intervals. The reaction tubes were spun and the supernatant was diluted 10 fold into 10 mM NaOH, for analysis of DP profile by HPAEC-PAD.

Reactions were set up for 4, 10 and 20 minutes. The 4 min reaction provides an indication of the enzyme initial conversion f product to substrate; the 10 minute reaction provides an indication of the enzyme's thermal activity, and the 20 minute reaction provides an indication of the enzyme's thermal stability.

Total area from DP2 to the end of the HPLC run was integrated, and divided by the total protein and reaction time. The results are provided in Figures 6 and 7.

Example 6 - Additional Methods

The following assays were used in the Examples. Deviations from the protocols provided below are generally indicated in the Examples. In these experiments, a spectrophotometer was used to measure the absorbance of the products formed during the reactions.

A. Protein Content Determination

BCA (bicinchoninic acid) Assay. BCA (Pierce) assay was used to determine the protein concentration in samples on microtiter plate (MTP) scale. The chemical and reagent solutions used were: BCA protein assay reagent, and Pierce dilution buffer (50 mM MES, pH 6.5, 2 mM $CaCl_2$, 0.005% TWEEN®-80). The equipment included a SpectraMAX (type 340; Molecular Devices) MTP reader. The MTPs were obtained from Costar (type 9017).

Two-hundred (200) μL BCA Reagent was pipetted into each well, followed by 20 μL diluted protein. After thorough mixing, the MTPs were incubated for 30 minutes at 37°C. Air bubbles were removed before the optical density (OD) of the solution in the wells was read at 562 nm. To determine the protein concentration, the background reading was subtracted from the sample readings. The OD_{562} was plotted for protein

standards (purified enzyme) to produce a standard curve. The protein concentration of the samples were interpolated from the standard curve.

Bradford Assay. The Bradford dye reagent (Quick Start) assay was used to determine the protein concentration in samples on MTP scale. The chemical and reagent solutions used were: Quick Start Bradford Dye Reagent (BIO-RAD Catalog No. 500-0205), Dilution buffer (10 mM NaCl, 0.1 mM CaCl2, 0.005% TWEEN®-80). The equipment used was a Biomek FX Robot (Beckman) and a SpectraMAX (type 340) MTP reader. The MTPs were from Costar (type 9017).

Two-hundred (200) µL Bradford dye reagent was pipetted into each well, followed by 15 µL dilution buffer. Ten (10) µL of filtered culture broth were added to the wells. After thorough mixing, the MTPs were incubated for at least 10 minutes at room temperature. Air bubbles were blown away and the OD of each well was read at 595 nm. To determine the protein concentration, the background reading (*i.e.,* from un-inoculated wells) was subtracted form the sample readings. The OD_{595} values obtained provide a relative measure of the protein content in the samples.

B. Microswatch Assay for Testing Enzyme Performance

The detergents used in this assay did not contain enzymes or the enzymes present in commercial detergents had been destroyed through heat deactivation as described elsewhere in this document. The equipment used included an Eppendorf Thermomixer and a SpectraMAX (type 340) MTP reader. The MTPs were obtained from Costar (type 9017).

Detergent Preparation (AATCC HDL; US conditions). Milli-Q water was adjusted to 6 gpg water hardness (Ca/Mg=3/1), and 1.5 g/l AATCC 2003 standard reference liquid detergent without brightener was added. The detergent solution was vigorously stirred for at least 15 minutes. Then, 5 mM HEPES (free acid) was added and the pH adjusted to 8.0.

Rice Starch Microswatch Assay for testing Amylase Performance. Test detergents were prepared as described elsewhere in this document. The equipment used included a New Brunswick Innova 4230 shaker/incubator and a SpectraMAX (type 340) MTP reader. The MTPs were obtained from Corning (type 3641). Aged rice starch with

orange pigment swatches (CS-28) were obtained from Center for Test Materials (Vlaardingen, Netherlands). Before cutting 0.25-inch circular microswatches, the fabric was washed with water. Two microswatches were placed in each well of a 96-well microtiter plate. The test detergent was equilibrated at 20°C (North America) or 40°C (Western Europe). 190 μL of detergent solution were added to each well of the MTP, containing microswatches. To this mixture, 10 μL of the diluted enzyme solution was added. The MTP was sealed with adhesive foil and placed in the incubator for 1 hour with agitation at 750 rpm at the desired test temperature (typically 20°C or 40 C). Following incubation, 150 μL of the solution from each well were transferred into a fresh MTP and read at 488 nm using a SpectraMAX MTP reader to quantify cleaning. Blank controls, as well as controls containing microswatches and detergent, but no enzyme, were also included.

Calculation of Enzyme Performance. The obtained absorbance value was corrected for the blank value (*i.e.*, obtained after incubation of microswatches in the absence of enzyme). The resulting absorbance was a measure of the hydrolytic activity.

C. Amylase Concentration Determination by Antibody Titration

Alpha-amylase concentration and specific activity was determined, in some cases, by titration with an inhibitory polyclonal antibody. Polyclonal antibodies raised to *Bacillus stearothermophilus* alpha-amylase (AmyS) were found to be strongly inhibitory of AmyS and the alpha-amylase from *Bacillus sp.* TS-23 (e.g., the binding is tight enough to produce a linear titration of activity loss). Therefore, this antibody can be used to measure enzyme concentration, which, in turn, is used to calculate specific activity.

Briefly, the amount of enzyme inhibition produced by several known concentrations of antibody is measured. From this information, the concentration of antibody required for complete inhibition is extrapolated, which is equivalent to the enzyme concentration in the sample. Alpha-amylase activity and inhibition was measured using the fluorogenic BODIPY-starch assay. The buffer was 50 mM MOPS, pH 7.0, containing 0.005% Tween-80.

A polyclonal antibody directed against purified AmyS was raised in a rabbit and purified by standard methods. An empirical "apparent concentration" value of an

antibody stock solution was determined by measuring the inhibition of a sample of AmyS of known specific activity. The antibody sample was used to determine the concentration and specific activity of AmyS and TS23t variants. These values were used to create normalized 96-well enzyme stock plates, in which all of the variants were diluted to a common concentration.

D. Native Protein Gel Electrophoresis

Electrophoretic mobility of variant protein samples was measured using the PHASTGEL system (GE Healthcare) on pre-cast native polyacrylamide gels (PHASTGEL Homogeneous) at either 7.5% or 12.5% concentration. Buffer strips (PHASTGEL Native) were used and consisted of pH 8.8 in 0.88 M L-Alanine, 0.25 M Tris buffer. Typical run conditions consisted of 400 V for 12.75 minutes with an anode-to-cathode distance of 3.7 cm.

Alternatively, electrophoretic mobility of variant protein samples was measured on 1 mm-thick 0.5-1.5% agarose gels at various pH values (i.e. 5.8, 8.0 and 10.0) through a choice of a suitable buffer system. The electrophoresis was carried out under non-denaturing conditions. The Cathode–Anode length was 13.9 cm. A sample of 1-2 μg protein was mixed with 5% glycerol + 0.05% bromophenol blue and loaded on each lane. Gels were run typically for 1 hour at 100 V.

Gels were stained with Louiseville blue dye dissolved in 10% acetic acid and destained with 10% methanol and 10% acidic acid-in-water. Between 12 and 20 protein variants were loaded simultaneously, depending on native gel system used. As a consequence, the electrophoretic mobility of a protein variant can be immediately assessed, relative to charge ladder standards loaded on the same gel.

E. Detergent Heat Inactivation

Heat inactivation of commercial detergent formulas serves to destroy the enzymatic activity of any protein components while retaining the properties of non-enzymatic components. Thus, this method was suitable for preparing commercially-purchased detergents for use in testing the enzyme variants. For North American (NA) and Western European (WE) heavy duty liquid laundry (HDL) detergents, heat inactivation was performed by placing pre-weighed liquid detergent (in a glass bottle) in

a water bath at 95°C for 2 hours. The incubation time for heat inactivation of North American (NA) and Japanese (JPN) heavy duty granular laundry (HDG) detergent was 8 hours and that for Western European (WE) HDG detergent was 5 hours. The incubation time for heat inactivation of NA and WE auto dishwashing (ADW) detergents was 8 hours. The detergents were purchased from local supermarket stores. Both un-heated and heated detergents were assayed within 5 minutes of dissolving the detergent to accurately determine percentage deactivated. Enzyme activity was tested by the suc-AAPF-pNA assay.

For testing of enzyme activity in heat-inactivated detergents, working solutions of detergents were made from the heat inactivated stocks. Appropriate amounts of water hardness (6 gpg or 12 gpg) and buffer were added to the detergent solutions to match the desired conditions (Table 6-1). The solutions were mixed by vortexing or inverting the bottles.

Table 6-1. Laundry and Dish Washing Conditions

Region	Form	Dose	Detergent*	Buffer	Gpg	pH	T (°C)
Laundry (heavy duty liquid and granular)							
NA	HDL	0.78 g/l	P&G TIDE® 2X	5 mM HEPES	6	8.0	20
WE	HDL	5.0 g/L	Henkel Persil	5 mM HEPES	12	8.2	40
WE	HDG	8.0 g/L	P&G Ariel	2 mM Na_2CO_3	12	10.5	40
JPN	HDG	0.7 g/L	P&G TIDE®	2 mM Na_2CO_3	6	10.0	20
NA	HDG	1.0 g/L	P&G TIDE®	2 mM Na_2CO_3	6	10.0	20
Automatic Dish Washing							
WE	ADW	3.0 g/L	RB Calgonit	2 mM Na_2CO_3	21	10.0	40
NA	ADW	3.0 g/L	P&G Cascade	2 mM Na_2CO_3	9	10.0	40

* Abbreviations: Procter & Gamble (P&G); and Reckitt Benckiser (RB).

F. TERG-O-TOMETER Assay For Cleaning Performance Determination

A standard protocol for assessing protein and carbohydrate soil cleaning was used whereby the soil level on a fabric swatch was measured before and after cleaning under standard conditions. The fabric swatches consisted of woven cotton fabric soiled with

either maize starch, rice starch or a blood, milk, and carbon black mixture. Swatches were purchased from Testfabrics, Inc. (West Pittiston, PA). Maize Starch (EMPA 161) and Blood, Milk, Carbon Black (EMPA 116) technical soils were produced by EMPA Test materials AG (St. Gallen, Switzerland). Rice Starch (CFT CS-28) soils were produced by the Center for Testmaterials BV (Vlaardingen, Netherlands). Each stain was measured before and after treatment by optical reflectance using a Minolta Reflectometer CR-410 set to a D65 (6500°K) standard illuminant. The difference in the L, a, b values was converted to total color difference (dE), as defined by the CIE-LAB color space. Cleaning of the stains are expressed as percent stain removal index (%SRI) by taking a ratio between the color difference before and after washing and comparing it to the difference of unwashed soils (before wash) to unsoiled fabric.

Cleaning experiments were conducted in a TERG-O-TOMETER (United States Testing Co., Hoboken, NJ) equipped with 6 stainless steel 2 L pots fitted with overhead agitators. Each treatment was conducted in 1 L total volume consisting of either 6 grains per gallon 3:1 (calcium:magnesium) water hardness or 12 grains per gallon water hardness. Detergents used in the wash experiments were 1.5 g/L AATCC HDL WOB 2003 liquid detergent with 5 mM HEPES buffer at pH 8, 0.7g/L AATCC HDD WOB 1993 granular detergent, 8 g/L IEC A* 60456 granular detergent with perborate and TAED bleach, or 5 g/L Persil Power Gel liquid detergent. Enzyme was added directly into the wash solution and reactions were then initiated by addition of either 40 g/L or 200 g/L of soiled and ballast fabric. The washing reactions were agitated at 100 rpm for 10, 15, or 40 minutes at 20°C, 25°C, 30°C, 40°C, or 50°C. Following cleaning, swatches were rinsed for 3 minutes in tap water, spun in a front-loading washing machine at 1000 rpm to remove excess water, and dried in a dryer at low heat on a permanent press cycle for approximately 45 minutes. Comparison of the extent of soil removal was assessed by reflectometry and expressed as the % soil removal index (%SRI). The control condition did not contain enzyme and the positive control consisted of various doses of benchmark commercial enzymes.

G. BODIPY-Starch Assay for Determination of Amylase Activity

The BODIPY-starch assay was performed using the EnzChek® Ultra Amylase

Assay Kit (E33651, Invitrogen). A 1 mg/mL stock solution of the DQ starch substrate was prepared by dissolving the contents of the vial containing the lyophilized substrate in 100 µL of 50 mM sodium acetate buffer at pH 4.0. The vial was vortexed for about 20 seconds and left at room temperature, in the dark, with occasional mixing until dissolved. 900 µL of assay buffer (50 mM sodium acetate with 2.6 mM $CaCl_2$ at pH 5.8) was added, and the vial was mixed by vortex for about 20 seconds. The substrate solution was stored at room temperature, in the dark, until ready to use or at 4°C. For the assay, a 100 µg/mL of working solution of the DQ substrate was prepared from the 1 mg/mL substrate solution in the assay buffer. 190 µL of 100 µg/mL substrate solution was added to each well in a flat-bottom 96-well microtiter plate. 10 µL of each enzyme sample was added to a well, mixed for 30 seconds using a thermomixer at 800 rpm. A blank sample containing buffer and substrate only (no-enzyme blank) was included in the assay. The rate of change of fluorescence intensity was measured (excitation: 485 nm, emission: 520 nm) in a fluorescence microtiter plate reader at 25°C for 5 minutes.

H. Measurement of Enzyme Binding to Macromolecular Substrates

Binding assays were done to determine substrate binding of Amylase (AmyS) charge ladder variants (charge change = -12 to +12 relative to wild-type AmyS) to corn stover and bagasse. Substrates used included bagasse (sugarcane bagasse from Brazil, dilute-acid pre-treated by National Renewable Energy Laboratory, washed and buffered at pH 5), AFEX (ammonia fiber expansion corn stover), and PCS (dilute sulfuric acid pre-treated corn stover, washed and adjusted to pH 5). All substrates were brought to the desired percentage solids prior to use.

Amylase Binding: Amylase charge ladder variants were purified and diluted to 200 ppm for testing. A 1% cellulose bagasse solution was prepared in borate buffer (40mM, pH8.5, 0.016% Tween80). 150 µL of the bagasse solution was added into each well in a microtiter filtration plate. 150 µL of borate buffer was added into a set of separate wells, which served as controls. 10 µL of amylase charge ladder variants was added into the filtration plate, each condition was in duplicates. The plate was incubated at room temperature for 2 hours. The filtrate was collected and amylase activity in the supernatant was measured by BODIPY-starch assay.

Measurement of Enzyme Binding to Microswatches: Alpha-amylase variants were incubated with or without CS-28 rice starch microswatches under standard wash conditions for 30 min. The amount of free enzyme was measured by the BODIPY-starch assay. The fraction of enzyme bound to the microswatches was calculated as follows: Fraction bound = (Activity of enzyme in absence of swatch - Activity of enzyme in presence of swatch) / (Activity of enzyme in absence of swatch).

Example 7 - Amylase Production *in B. subtilis*

In this Example, production of a mutant truncated form of *Bacillus stearothermophilus* amylase alpha-amylase (having a S242Q mutation and a 29 amino acid deletion from the C-terminus; also referred to herein as S242Q) and variants thereof in *B. subtilis* are described. Transformation was performed as known in the art (*see e.g.*, WO 02/14490). Briefly, the gene encoding the parent amylases was cloned into the pHPLT expression vector, which contains the LAT promoter (PLAT), a sequence encoding the LAT signal peptide (preLAT), followed by *Pst*I and *Hpa*I restriction sites for cloning.

The coding region for the LAT signal peptide is shown below:

atgaaacaac aaaaacggct ttacgcccga ttgctgacgc tgttatttgc gctcatcttc ttgctgcctc attctgcagc ttcagca (SEQ ID NO: 19).

The amino acid sequence of the LAT signal peptide is shown below:

MKQQKRLYAR LLTLLFALIF LLPHSAASA (SEQ ID NO: 20)

The amino acid sequence of the mature truncated S242Q amylase with the substituted amino acid shown in italics was used as the basis for making the variant libraries described herein:

AAPFNGTMMQ YFEWYLPDDG TLWTKVANEA NNLSSLGITA LWLPPAYKGT SRSDVGYGVY
DLYDLGEFNQ KGTVRTKYGT KAQYLQAIQA AHAAGMQVYA DVVFDHKGGA DGTEWVDAVE
VNPSDRNQEI SGTYQIQAWT KFDFPGRGNT YSSFKWRWYH FDGVDWDESR KLSRIYKFRG
IGKAWDWEVD TENGNYDYLM YADLDMDHPE VVTELKNWGK WYVNTTNIDG FRLDAVKHIK
FQFFPDWLSY VRSQTGKPLF TVGEYWSYDI NKLHNYITKT NGTMSLFDAP LHNKFYTASK
SGGAFDMRTL MTNTLMKDQP TLAVTFVDNH DTEPGQALQS WVDPWFKPLA YAFILTRQEG
YPCVFYGDYY GIPQYNIPSL KSKIDPLLIA RRDYAYGTQH DYLDHSDIIG WTREGVTEKP

GSGLAALITD GPGGSKWMYV GKQHAGKVFY DLTGNRSDTV TINSDGWGEF KVNGGSVSVW VPRKTT (SEQ ID NO: 21).

The PCR products were purified using QIAQUIK columns from Qiagen, and resuspended in 50 µL of deionized water. 50 µL of the purified DNA was digested with *Hpa*I (Roche) and *Pst*I (Roche), and the resultant DNA resuspended in 30 µL of deionized water. 10-20 ng/µL of the DNA was cloned into plasmid pHPLT using *Pst*I and *Hpa*I cloning sites. The ligation mixtures were directly transformed into competent *B. subtilis* cells (genotype: Δ*vpr*, Δ*wpr*A, Δ*mpr-ybf*J, Δ*npr*B). The *B. subtilis* cells have a competency gene (*com*K) which is placed under a xylose inducible promoter, so xylose was used to induce competency for DNA binding and uptake (*see* Hahn *et al.*, *Mol. Microbiol.*, 21: 763-775, 1996).

The elements of plasmid pHPLT-AmyS include: pUB110 = DNA fragment from plasmid pUB110 (McKenzie *et al.*, *Plasmid* 15: 93-103, 1986). Plasmid features include: ori-pUB110 = origin of replication from pUB110; neo = neomycin resistance gene from pUB110; Plat = transcriptional promoter from *B. licheniformis* amylase; Pre LAT = signal peptide from *B. licheniformis* amylase; SAMY 425ss = the coding region for truncated *AmyE* gene sequence (replaced by the coding regions for each truncated AmyE variant expressed in this study); and Terminator = transcriptional terminator from *B. licheniformis* amylase.

Example 8 - Expression of Enzyme Variants

This Example describes the methods used to express various recombinant enzymes of the transformed *B. subtilis* of the preceding Examples.

Alpha-Amylase Expression – 2 mL scale. *B. subtilis* clones containing S242Q (or a variant thereof) expression vectors were replicated with a steel 96-well replicator from glycerol stocks into 96-well culture plates (BD, 353075) containing 150 µL of LB media + 10 µg/mL neomycin, grown overnight at 37°C, 220 rpm in a humidified enclosure. A 100 µL aliquot from the overnight culture was used to inoculate 2000 µL defined media + 10 µg/mL neomycin in 5 mL plastic culture tubes. The cultivation media was an enriched semi-defined media based on MOPS buffer, with urea as major

nitrogen source, glucose as the main carbon source, and supplemented with 1% SOYTONE and 5 mM calcium for robust cell growth. Culture tubes were incubated at 37°C, 250 rpm, for 72 hours. Following this incubation, the culture broths were centrifuged for 10 minutes at 3000 x g. The supernatant solution was decanted into 15 mL polypropylene conical tubes; 80 µL of each sample were aliquoted into 96 well plates for protein quantitation.

Example 9 - Production of Enzyme Variants

This Example describes the production of enzyme charge ladders and combinatorial charge libraries.

Enzyme Charge Ladders. Multiple protein variants spanning a range of physical properties of interest are selected from existing libraries or are generated by site-directed mutagenesis techniques as known in the art (*see e.g.*, US Pat. Appln. Ser. Nos., 10/576,331, 11/581,102, and 11/583,334, assigned to Genencor International. This defined set of probe proteins is then assayed in a test of interest.

Exemplary amylase charge ladder variants are shown in the following tables and assayed as described herein. In these tables, the charge change is relative to the parent enzyme.

Table 9-1. AmyS-S242Q Charge Ladder

AmyS-S242Q Variant	Δ Charge
Q97E-Q319E-Q358E-Q443E	-4
Q97E-Q319E-Q358E	-3
Q97E-Q319E	-2
Q97E	-1
Q97R-Q319E	0
Parent AmyS-S242Q	0
Q97R	+1
Q97R-Q319R	+2
Q97R-Q319R-Q358R	+3
Q97R-Q319R-Q358R	+4

Enzyme Combinatorial Charge Libraries (CCL)

Generation of *B. stearothermophilus* AmyS-S242Q CCL. The AmyS-S242Q plasmid DNA was isolated from a transformed *B. subtilis* strain (genotype: Δ*aprE*, Δ*nprE, amyE*::*xylRPxylAcomK-phleo*) and sent to DNA2.0 Inc. as the template for CCL construction. A request was made to DNA2.0 Inc. (Mountain View, CA) for the generation of positional libraries at each of the four sites in AmyS-S242Q (S242Q) amylase that are shown in Table 9-2. Variants were supplied as glycerol stocks in 96-well plates.

The AmyS S242Q combinatorial charge library was designed by identifying the following four residues: Gln97, Gln319, Gln358, and Gln 443. A four site, 81-member CCL was created by making all combinations of three possibilities at each site: wild-type, arginine, or aspartic acid.

Table 9-2. S242Q CCL Variants

Variant #	Q97	Q319	Q358	Q443	Δ Charge
1	Q97E	Q319E	Q358E	Q443E	-4
2	Q97E	Q319E	Q358E	Q443R	-2
3	Q97E	Q319E	Q358E	-	-3
4	Q97E	Q319E	Q358R	Q443E	-2
5	Q97E	Q319E	Q358R	Q443R	0
6	Q97E	Q319E	Q358R	-	-1
7	Q97E	Q319E	-	Q443E	-3
8	Q97E	Q319E	-	Q443R	-1
9	Q97E	Q319E	-	-	-2
10	Q97E	Q319R	Q358E	Q443E	-2
11	Q97E	Q319R	Q358E	Q443R	0
12	Q97E	Q319R	Q358E	-	-1
13	Q97E	Q319R	Q358R	Q443E	0
14	Q97E	Q319R	Q358R	Q443R	+2
15	Q97E	Q319R	Q358R	-	+1
16	Q97E	Q319R	-	Q443E	-1
17	Q97E	Q319R	-	Q443R	+1
18	Q97E	Q319R	-	-	0
19	Q97E	-	Q358E	Q443E	-3
20	Q97E	-	Q358E	Q443R	-1
21	Q97E	-	Q358E	-	-2

Table 9-2. S242Q CCL Variants

Variant #	Q97	Q319	Q358	Q443	Δ Charge
22	Q97E	-	Q358R	Q443E	-1
23	Q97E	-	Q358R	Q443R	+1
24	Q97E	-	Q358R	-	0
25	Q97E	-	-	Q443E	-2
26	Q97E	-	-	Q443R	0
27	Q97E	-	-	-	-1
28	Q97R	Q319E	Q358E	Q443E	-2
29	Q97R	Q319E	Q358E	Q443R	0
30	Q97R	Q319E	Q358E	-	-1
31	Q97R	Q319E	Q358R	Q443E	0
32	Q97R	Q319E	Q358R	Q443R	+2
33	Q97R	Q319E	Q358R	-	+1
34	Q97R	Q319E	-	Q443E	-1
35	Q97R	Q319E	-	Q443R	+1
36	Q97R	Q319E	-	-	0
37	Q97R	Q319R	Q358E	Q443E	0
38	Q97R	Q319R	Q358E	Q443R	+2
39	Q97R	Q319R	Q358E	-	+1
40	Q97R	Q319R	Q358R	Q443E	+2
41	Q97R	Q319R	Q358R	Q443R	+4
42	Q97R	Q319R	Q358R	-	+3
43	Q97R	Q319R	-	Q443E	+1
44	Q97R	Q319R	-	Q443R	+3
45	Q97R	Q319R	-	-	+2
46	Q97R	-	Q358E	Q443E	-1
47	Q97R	-	Q358E	Q443R	+1
48	Q97R	-	Q358E	-	0
49	Q97R	-	Q358R	Q443E	+1
50	Q97R	-	Q358R	Q443R	+3
51	Q97R	-	Q358R	-	+2
52	Q97R	-	-	Q443E	0
53	Q97R	-	-	Q443R	+2
54	Q97R	-	-	-	+1
55	-	Q319E	Q358E	Q443E	-3
56	-	Q319E	Q358E	Q443R	-1
57	-	Q319E	Q358E	-	-2
58	-	Q319E	Q358R	Q443E	-1
59	-	Q319E	Q358R	Q443R	+1
60	-	Q319E	Q358R	-	0
61	-	Q319E	-	Q443E	-2

Table 9-2. S242Q CCL Variants

Variant #	Q97	Q319	Q358	Q443	Δ Charge
62	-	Q319E	-	Q443R	0
63	-	Q319E	-	-	-1
64	-	Q319R	Q358E	Q443E	-1
65	-	Q319R	Q358E	Q443R	+1
66	-	Q319R	Q358E	-	0
67	-	Q319R	Q358R	Q443E	+1
68	-	Q319R	Q358R	Q443R	+3
69	-	Q319R	Q358R	-	+2
70	-	Q319R	-	Q443E	0
71	-	Q319R	-	Q443R	+2
72	-	Q319R	-	-	+1
73	-	-	Q358E	Q443E	-2
74	-	-	Q358E	Q443R	0
75	-	-	Q358E	-	-1
76	-	-	Q358R	Q443E	0
77	-	-	Q358R	Q443R	+2
78	-	-	Q358R	-	+1
79	-	-	-	Q443E	-1
80	-	-	-	Q443R	+1
81 (parent)	Q97	Q319	Q358	Q443	0

Example 10 - Enzyme Wash Performance

This Example describes the testing of S242Q variant in a microswatch assay 1.0 μg/mL in AATCC HDL detergent or 5 mM HEPES buffer under varying ionic strength. The methods provided in Example 6 were used (*See*, "Rice Starch Microswatch Assay for testing Amylase Performance" and "Corn Four Hydrolysis").

There is an optimal net charge change for cleaning performance for enzyme in AATCC HDL detergent. Performance is measured in terms of relative cleaning performance observed in a rice starch microswatch activity assay. A value of around 1.0 indicates top cleaning performance in this assay. This is an example of optimizing a protein physical property (*e.g.*, net charge) for improving a given outcome or benefit (*e.g.*, cleaning performance in a liquid laundry detergent). The charge optimum identified with this limited set of probe proteins coincides with the optimum charge

observed when measuring the entire charge combinatorial library. The use of probe proteins is therefore predictive of the behavior of the entire library.

According to the Debye-Hückel theory (Israelachivili, INTERMOLECULAR AND SURFACE FORCES, SECOND EDITION: WITH APPLICATIONS TO COLLOIDAL AND BIOLOGICAL SYSTEMS, Academic Press 2nd Ed. [1992]), electrostatic interactions are governed primarily by the strength of double-layer forces between interacting species at constant potential or constant charge (enzymes, substrates, fabric, and detergent), their size, and the dielectric constant of the surrounding medium. In order to characterize the electrostatic behavior of particles in a complex medium, such as a detergent formulation, their interaction in a reduced environment possessing the same Debye screening length is sufficient. This was accomplished by choosing a buffer of matching pH and conductivity to that of the detergent under wash conditions. An appropriate buffer for such testing is 5 mM HEPES buffer at pH 8.0 with varying amounts of indifferent electrolyte, such as NaCl. Addition of 2.5 mM NaCl to this buffer matches the pH and conductivity of typical North American wash conditions. Addition of a higher concentration of NaCl is representative of Japanese and European wash conditions, which typically are higher in ionic strength due to both increased water hardness and detergent concentrations.

Figure 10 shows that positive charge S242Q charge variants are superior for cleaning of rice starch microswatch under North American laundry conditions. Likewise, negative charge TS23t variants are superior for cleaning of rice starch microswatches in Western European laundry conditions (Figure 11).

Figure 12 demonstrates that positive S242Q variants exhibit higher specific activity for granular corn starch substrates hydrolysis.

Example 11 - Thermostability

This Example describes determining the relationship between protein charge and thermal stability. Alpha-amylase assays were based on BODIPY starch hydrolysis before and after heating the culture supernatant. The same chemical and reagent solutions used are as described in Example 6.

Thermal stability assay for alpha-amylases. The filtered culture supernatants were serially diluted in 50 mM sodium acetate + 2 mM $CaCl_2$, at pH 5.8 with 0.002% Tween. 10 µL of each diluted culture supernatant was assayed to determine the initial amylase activity by the BODIPY starch assay. 50 µL of each diluted culture supernatant was placed in a VWR low profile PCR 96 well plate. 30 µL of mineral oil was added to each well as a sealant. The plate was incubated in a BioRad DNA engine Peltier Thermal Cycler at 95°C for 30 or 60 minutes depending on the stability of the parent enzyme. Following incubation, the plate was cooled to 4°C for 5 min and then kept at room temperature. 10 µL of each sample was added to a fresh plate and assayed to determine the final amylase activity by the BODIPY starch assay as described in Example 1.

Calculation of Thermostability. The residual activity of a sample was expressed as the ratio of the final absorbance and the initial absorbance, both corrected for blanks. A higher index indicates a more thermally-stable variant. This is an example of optimizing a protein physical property, in this case net charge, for improving enzyme thermal stability for a liquid laundry application.

Thermostability Assay. Thermostability of the variants was assessed as described above. Thermostability winners are listed in Table 11-1. Winners were defined as those having a ratio of mutant residual activity to parent (i.e., S242Q) residual activity greater than 1.

Table 11-1: S242Q CCL - thermal stability winners

Variant #	97	319	358	443	Mut residual act./WT residual act.
2	Q97E	Q319E	Q358E	Q443R	1.12
10	Q97E	Q319R	Q358E	Q443E	1.12
13	Q97E	Q319R	Q358R	Q443E	1.36
14	Q97E	Q319R	Q358R	Q443R	1.16
15	Q97E	Q319R	Q358R		1.37
17	Q97E	Q319R		Q443R	1.29
18	Q97E	Q319R			1.11
27	Q97E				1.16
32	Q97R	Q319E	Q358R	Q443R	1.18
37	Q97R	Q319R	Q358E	Q443E	1.29
38	Q97R	Q319R	Q358E	Q443R	1.22
39	Q97R	Q319R	Q358E		1.21

Variant #	97	319	358	443	Mut residual act./WT residual act.
40	Q97R	Q319R	Q358R	Q443E	1.20
41	Q97R	Q319R	Q358R	Q443R	1.26
42	Q97R	Q319R	Q358R		1.48
43	Q97R	Q319R		Q443E	1.21
44	Q97R	Q319R		Q443R	1.21
45	Q97R	Q319R			1.14
50	Q97R		Q358R	Q443R	1.14
62		Q319E		Q443R	1.26
63		Q319E			1.18
64		Q319R	Q358E	Q443E	1.19
65		Q319R	Q358E	Q443R	1.28
68		Q319R	Q358R	Q443R	1.14
70		Q319R		Q443E	1.22
73			Q358E	Q443E	1.15
74			Q358E	Q443R	1.15
75			Q358E		1.18

Example 12 - Enzyme Performance

This Example demonstrates that enzyme performance may be affected by charge.

Enzyme performance was assessed using heat inactivated detergents as described above in Example 6. Winners were defined as those having Performance Index (PI) a greater than 1. PI is the ratio of mutant residual activity to parent (i.e., S242Q) residual activity. Results are shown in Tables 12-1 and 12-2.

Table 12-1: S242Q CCL - CS-28 rice starch microswatch winners, Tide 2x (North American conditions as described in Example 6).

Variant #	97	319	358	443	rel charge	PI
13	Q97E	Q319R	Q358R	Q443E	0	1.44
14	Q97E	Q319R	Q358R	Q443R	2	1.32
15	Q97E	Q319R	Q358R		1	1.40
16	Q97E	Q319R		Q443E	-1	1.33
17	Q97E	Q319R		Q443R	1	1.40
18	Q97E	Q319R			0	1.41
20	Q97E		Q358E	Q443R	-1	1.15
23	Q97E		Q358R	Q443R	1	1.21
25	Q97E			Q443E	-2	1.18

Variant #	97	319	358	443	rel charge	PI
26	Q97E			Q443R	0	1.25
27	Q97E				-1	1.16
28	Q97R	Q319E	Q358E	Q443E	-2	2.32
29	Q97R	Q319E	Q358E	Q443R	0	2.54
30	Q97R	Q319E	Q358E		-1	2.93
31	Q97R	Q319E	Q358R	Q443E	0	2.27
32	Q97R	Q319E	Q358R	Q443R	2	2.28
33	Q97R	Q319E	Q358R		1	2.34
34	Q97R	Q319E		Q443E	-1	2.31
35	Q97R	Q319E		Q443R	1	2.31
36	Q97R	Q319E			0	2.14
37	Q97R	Q319R	Q358E	Q443E	0	1.93
38	Q97R	Q319R	Q358E	Q443R	2	1.85
39	Q97R	Q319R	Q358E		1	2.14
40	Q97R	Q319R	Q358R	Q443E	2	1.92
41	Q97R	Q319R	Q358R	Q443R	4	1.37
42	Q97R	Q319R	Q358R		3	1.61
43	Q97R	Q319R		Q443E	1	1.90
44	Q97R	Q319R		Q443R	3	1.64
45	Q97R	Q319R			2	1.99
46	Q97R		Q358E	Q443E	-1	1.40
47	Q97R		Q358E	Q443R	1	1.29
48	Q97R		Q358E		0	1.60
49	Q97R		Q358R	Q443E	1	1.57
50	Q97R		Q358R	Q443R	3	1.38
51	Q97R		Q358R		2	1.37
52	Q97R			Q443E	0	1.51
54	Q97R				1	1.51
55		Q319E	Q358E	Q443E	-3	1.14
56		Q319E	Q358E	Q443R	-1	1.38
57		Q319E	Q358E		-2	1.10
58		Q319E	Q358R	Q443E	-1	1.25
59		Q319E	Q358R	Q443R	1	1.41
60		Q319E	Q358R		0	1.49
61		Q319E		Q443E	-2	1.16
62		Q319E		Q443R	0	1.45
63		Q319E			-1	1.28
64		Q319R	Q358E	Q443E	-1	1.12
65		Q319R	Q358E	Q443R	1	1.19
66		Q319R	Q358E		0	1.36
67		Q319R	Q358R	Q443E	1	1.24

Variant #	97	319	358	443	rel charge	PI
69		Q319R	Q358R		2	1.19
70		Q319R		Q443E	0	1.29
76			Q358R	Q443E	0	1.22
78			Q358R		1	1.25
79				Q443E	-1	1.24
80				Q443R	1	1.17

Table 12-2: S242Q CCL - CS-28 rice starch microswatch winners, Persil (Western European conditions)

Variant #	97	319	358	443	rel charge	PI
2	Q97E	Q319E	Q358E	Q443R	-2	1.41
3	Q97E	Q319E	Q358E		-3	1.94
4	Q97E	Q319E	Q358R	Q443E	-2	1.61
5	Q97E	Q319E	Q358R	Q443R	0	1.39
6	Q97E	Q319E	Q358R		-1	2.04
7	Q97E	Q319E		Q443E	-3	2.05
8	Q97E	Q319E		Q443R	-1	1.84
9	Q97E	Q319E			-2	2.27
10	Q97E	Q319R	Q358E	Q443E	-2	1.35
13	Q97E	Q319R	Q358R	Q443E	0	1.45
14	Q97E	Q319R	Q358R	Q443R	2	1.17
15	Q97E	Q319R	Q358R		1	1.22
16	Q97E	Q319R		Q443E	-1	1.26
17	Q97E	Q319R		Q443R	1	1.29
18	Q97E	Q319R			0	1.76
26	Q97E			Q443R	0	1.36
27	Q97E				-1	1.31
28	Q97R	Q319E	Q358E	Q443E	-2	2.21
29	Q97R	Q319E	Q358E	Q443R	0	1.96
30	Q97R	Q319E	Q358E		-1	1.94
31	Q97R	Q319E	Q358R	Q443E	0	2.11
32	Q97R	Q319E	Q358R	Q443R	2	1.87
33	Q97R	Q319E	Q358R		1	2.41
34	Q97R	Q319E		Q443E	-1	2.20
35	Q97R	Q319E		Q443R	1	2.21
36	Q97R	Q319E			0	2.07
37	Q97R	Q319R	Q358E	Q443E	0	1.86
38	Q97R	Q319R	Q358E	Q443R	2	1.83
39	Q97R	Q319R	Q358E		1	1.99
40	Q97R	Q319R	Q358R	Q443E	2	1.85

Variant #	97	319	358	443	rel charge	PI
41	Q97R	Q319R	Q358R	Q443R	4	1.36
42	Q97R	Q319R	Q358R		3	1.90
43	Q97R	Q319R		Q443E	1	1.99
44	Q97R	Q319R		Q443R	3	1.94
45	Q97R	Q319R			2	1.75
46	Q97R		Q358E	Q443E	-1	1.71
47	Q97R		Q358E	Q443R	1	1.39
48	Q97R		Q358E		0	1.85
50	Q97R		Q358R	Q443R	3	1.24
51	Q97R		Q358R		2	1.36
52	Q97R			Q443E	0	1.25
54	Q97R				1	1.88
55		Q319E	Q358E	Q443E	-3	1.12
56		Q319E	Q358E	Q443R	-1	1.17
58		Q319E	Q358R	Q443E	-1	1.16
59		Q319E	Q358R	Q443R	1	1.25
60		Q319E	Q358R		0	1.50
63		Q319E			-1	1.36
64		Q319R	Q358E	Q443E	-1	1.10
65		Q319R	Q358E	Q443R	1	1.18
66		Q319R	Q358E		0	1.25
67		Q319R	Q358R	Q443E	1	1.29
70		Q319R		Q443E	0	1.15

Activity was also measured using the BODIPY starch hydrolysis assay as provided herein. The results are shown in Table 12-3. The relative specific activity on this starch substrate (a corn starch) greater than 1 indicates the variant has higher specific activity than the S242Q parent. Relative ppm is expression titers, greater than 1 indicates higher titers (in shake tubes) than the S242Q parent.

Table 12-3: S242Q CCL - titer and/or BODIPY-starch winners

Variant #	97	319	358	443	Charge	Rel ppm	Rel Sp act
1	Q97E	Q319E	Q358E	Q443E	-4	1.27	1.29
2	Q97E	Q319E	Q358E	Q443R	-2	1.19	1.31
3	Q97E	Q319E	Q358E		-3	1.00	1.43
4	Q97E	Q319E	Q358R	Q443E	-2	1.23	1.43
5	Q97E	Q319E	Q358R	Q443R	0	0.94	1.78
6	Q97E	Q319E	Q358R		-1	0.89	1.81
7	Q97E	Q319E		Q443E	-3	1.40	1.41

Variant #	97	319	358	443	Charge	Rel ppm	Rel Sp act
8	Q97E	Q319E		Q443R	-1	1.12	1.58
9	Q97E	Q319E			-2	1.09	1.56
10	Q97E	Q319R	Q358E	Q443E	-2	1.45	1.32
11	Q97E	Q319R	Q358E	Q443R	0	1.32	1.49
12	Q97E	Q319R	Q358E		-1	1.58	1.27
13	Q97E	Q319R	Q358R	Q443E	0	0.65	1.44
14	Q97E	Q319R	Q358R	Q443R	2	0.66	1.65
15	Q97E	Q319R	Q358R		1	0.80	1.64
16	Q97E	Q319R		Q443E	-1	1.09	1.51
17	Q97E	Q319R		Q443R	1	1.00	1.42
18	Q97E	Q319R			0	0.87	1.78
19	Q97E		Q358E	Q443E	-3	1.22	0.88
21	Q97E		Q358E		-2	1.12	0.88
22	Q97E		Q358R	Q443E	-1	0.91	1.16
23	Q97E		Q358R	Q443R	1	0.78	1.25
24	Q97E		Q358R		0	1.08	1.14
25	Q97E			Q443E	-2	1.12	1.00
28	Q97R	Q319E	Q358E	Q443E	-2	0.78	1.87
29	Q97R	Q319E	Q358E	Q443R	0	0.80	1.81
30	Q97R	Q319E	Q358E		-1	0.68	2.21
31	Q97R	Q319E	Q358R	Q443E	0	0.68	1.96
32	Q97R	Q319E	Q358R	Q443R	2	0.70	2.05
33	Q97R	Q319E	Q358R		1	0.60	2.27
34	Q97R	Q319E		Q443E	-1	0.65	2.25
35	Q97R	Q319E		Q443R	1	0.70	2.15
36	Q97R	Q319E			0	0.73	2.23
37	Q97R	Q319R	Q358E	Q443E	0	0.93	2.11
38	Q97R	Q319R	Q358E	Q443R	2	0.65	2.21
39	Q97R	Q319R	Q358E		1	0.82	2.22
40	Q97R	Q319R	Q358R	Q443E	2	0.74	2.28
41	Q97R	Q319R	Q358R	Q443R	4	0.55	2.09
42	Q97R	Q319R	Q358R		3	0.67	2.48
43	Q97R	Q319R		Q443E	1	0.84	2.35
44	Q97R	Q319R		Q443R	3	0.73	2.41
45	Q97R	Q319R			2	0.76	2.45
46	Q97R		Q358E	Q443E	-1	0.79	1.45
47	Q97R		Q358E	Q443R	1	0.75	1.42
48	Q97R		Q358E		0	0.82	1.46
49	Q97R		Q358R	Q443E	1	0.67	1.69
50	Q97R		Q358R	Q443R	3	0.60	1.60
51	Q97R		Q358R		2	0.64	1.29
52	Q97R			Q443E	0	0.83	1.43

Variant #	97	319	358	443	Charge	Rel ppm	Rel Sp act
54	Q97R				1	0.72	1.49
55		Q319E	Q358E	Q443E	-3	0.99	1.15
56		Q319E	Q358E	Q443R	-1	0.77	1.40
57		Q319E	Q358E		-2	0.83	1.34
58		Q319E	Q358R	Q443E	-1	0.73	1.49
59		Q319E	Q358R	Q443R	1	0.67	1.61
60		Q319E	Q358R		0	0.80	1.67
61		Q319E		Q443E	-2	0.91	1.39
62		Q319E		Q443R	0	0.73	1.45
63		Q319E			-1	0.75	1.41
64		Q319R	Q358E	Q443E	-1	1.05	1.28
65		Q319R	Q358E	Q443R	1	0.94	1.42
66		Q319R	Q358E		0	0.96	1.39
67		Q319R	Q358R	Q443E	1	1.02	1.50
68		Q319R	Q358R	Q443R	3	0.71	1.57
69		Q319R	Q358R		2	0.71	1.58
70		Q319R		Q443E	0	0.91	1.49
72		Q319R			1	0.95	1.56
77			Q358R	Q443R	2	0.67	1.22
78			Q358R		1	0.66	1.15

Example 13 - Balancing Mutational Effects on Amylase Activity and Expression

This example illustrates that two separate enzyme properties can be simultaneously optimized by the introduction of multiple amino acid substitutions, even where the properties are negatively correlated due, for example, to oppositely linked to charge characteristics of the protein.

It was determined during experimentation that the median expression of AmyS-242Q decreased with increasing positive charge. However, specific BODIPY starch hydrolysis increased with increasing positive charge. Enhanced recombinant amylase expression and starch hydrolysis are desirable in an engineered variant of AmyS-242Q suitable for starch liquefaction in the fuel ethanol industry or cleaning in detergent applications for instance. These properties, however, are apparently conflicting properties. Using the methods provided herein, it is possible to produce a more highly expressed amylase variant without severely compromising starch hydrolysis by

selectively combining single mutations. The strategy described herein was successfully used to produce and select multiply-substituted AmyS-242Q variants having improvements in a first property (*e.g.*, expression as the primary property), while improving or not sacrificing a second property (*e.g.*, starch hydrolysis as the secondary property).

In addition, in converse to median expression of AmyS-242Q variants, corn starch microswatch cleaning increased with increasing positive charge. Enhanced recombinant amylase expression and cleaning performance are desirable in an engineered variant of AmyS-242Q. These properties, however, are also apparently conflicting properties. Using the methods disclosed herein, it is possible to produce a more highly expressed amylase variant without severely compromising cleaning performance by selectively combining single mutations. The strategy described herein was successfully used to produce and select multiply-substituted AmyS-242Q variants having improvements in a first property (*e.g.*, expression as the primary property), while improving or not sacrificing a second property (*e.g.*, rice starch microswatch cleaning as the secondary property).

In particular, an eighty member AmyS-S242Q charge combinatorial library (CCL) comprising variants having combinations of from one to four substitutions of charged residues was tested for shake tube expression, BODIPY-starch hydrolysis, and rice starch cleaning activity. AmyS-S242Q winners are shown in Tables 13-1 and 13-2. Importantly, the multiply-substituted variants of Table 13-1 have equal or improved expression and equal or improved BODIPY-starch hydrolysis as compared to the parent enzyme. Similarly, the multiply-substituted variants of Table 13-2 have equal or improved expression and equal or improved rice starch cleaning activity as compared to the parent enzyme.

Table 13-1. AmyS-S242Q Expression and BODIPY-Starch Hydrolysis Winners

Variant	97	319	358	443	Charge	Expression (PI)	BODIPY (PI)
1	Q97E	Q319E	Q358E	Q443E	-4	1.27	1.29

Table 13-1. AmyS-S242Q Expression and BODIPY-Starch Hydrolysis Winners							
Variant	97	319	358	443	Charge	Expression (PI)	BODIPY (PI)
2	Q97E	Q319E	Q358E	Q443R	-2	1.19	1.31
3	Q97E	Q319E	Q358E		-3	1.00	1.43
4	Q97E	Q319E	Q358R	Q443E	-2	1.23	1.43
7	Q97E	Q319E		Q443E	-3	1.40	1.41
8	Q97E	Q319E		Q443R	-1	1.12	1.58
9	Q97E	Q319E			-2	1.09	1.56
10	Q97E	Q319R	Q358E	Q443E	-2	1.45	1.32
11	Q97E	Q319R	Q358E	Q443R	0	1.32	1.49
12	Q97E	Q319R	Q358E		-1	1.58	1.27
16	Q97E	Q319R		Q443E	-1	1.09	1.51
17	Q97E	Q319R		Q443R	+1	1.00	1.42
24	Q97E		Q358R		0	1.08	1.14
25	Q97E			Q443E	-2	1.12	1.00
64		Q319R	Q358E	Q443E	-1	1.05	1.28
67		Q319R	Q358R	Q443E	+1	1.02	1.50

Table 13-2. AmyS-S242Q Expression and Rice-Starch Hydrolysis Winners							
Variant	97	319	358	443	Charge	Expression	CS-28
1	Q97E	Q319E	Q358E	Q443E	-4	1.27	1.01
11	Q97E	Q319R	Q358E	Q443R	0	1.32	1.18
12	Q97E	Q319R	Q358E		-1	1.58	1.13
16	Q97E	Q319R		Q443E	-1	1.09	1.43
17	Q97E	Q319R		Q443R	+1	1.00	1.55
24	Q97E		Q358R		0	1.08	1.15
25	Q97E			Q443E	-2	1.12	1.09
64		Q319R	Q358E	Q443E	-1	1.05	1.18
67		Q319R	Q358R	Q443E	+1	1.02	1.15

In sum, because enzyme activity and enzyme production have different charge dependencies (*see* FIG. 13A, 13B, 14A, and 14B) they are negatively correlated (*see* FIG. 12A and 12B). However, there are a number of variants that are improved in both expression and activity, and analyzing the library in this manner allows them to be identified.

Although demonstrated with amylases, this method is applicable to other enzyme classes such as proteases, lipases, cellulases, transferases and pectinases. Moreover any combination of two or more properties can be analyzed simultaneously such as expression, activity, binding, thermal stability, stability in the presence of one or detergents, and chelant stability.

Example 14 - Desizing performance of amylases

In this example, the desizing performance of variant S242Q was compared against Ethyl and Xtra at 85°C and 97°C at several concentrations of calcium.

$CaCl_2$ concentration was varied from 0-20 ppm per test by adding various amounts of stock $CaCl_2$ solution to Milli Q water, pH ~6.5. Ethyl, Xtra and variant S242Q were used at 0.01 ppm active protein per test. The assay was performed in a LAUNDER-O-METER using a liquor ratio of 50:1. Performance tests were conducted on rice starch-stained fabric swatches with an indicator dye bound to the starch (TestFabrics Cat. No. CS-28; TestFabrics Inc.). Three CS-28 swatches (6 cm x 8 cm) and 4 greige print cloth swatches (Testfabrics, Style 400R; 3 inches x 4 inches) were used as substrates per experiment. The temperature of the LAUNDER-O-METER with Milli Q water/Ca was pre-adjusted to 85°C or 97°C, after which the enzymes and swatches were added. The reaction was carried out for 30 min, after which the swatches were rinsed in water and dried before reading.

Measurements are made by reflectometry using the CIE L*a*b* color space. Every perceivable color can be represented by L*a*b* coordinate in the color space. "L*" represents the lightness or grey scale value on a scale of 0 to 100, pure black to pure white. "a* represents the magenta to green shift, wherein large positive values represent a very magenta hue and large negative values represent a very green hue. "b*" represents

the yellow to blue shift where large positive values represent a very yellow hue and large negative values represent a very blue hue. When both a* and b* values are 0, there is an absence of color, leaving pure grey colors with their lightness defined by the L* value.

A Minolta Chromameter CR 200 in the CIE Lab color space with a D 65 light source was utilized for measuring desizing performance. To quantify desizing performance, four CIE L* readings (i.e., 2 readings each from the front and the back of the swatch) were taken from each CS-28 swatch following the amylase treatment. Higher CIE L* values indicate better desizing performance.

As shown in Figures 15 and 16, the S242Q variant showed significantly lower calcium dependency for desizing performance compared to both Ethyl and Xtra under the conditions tested.

All publications and patents mentioned in the above specification are incorporated herein by reference. Although the disclosed methods and enzymes have in some instances been described in connection with specific or preferred embodiments, it should be understood what is covered by the appended claims is not limited to such specific or preferred embodiments. Indeed, various modifications and variations of the disclosed methods and enzymes will be apparent to those skilled in the art, and various modifications of the described modes for practicing what has been disclosed are included within the scope of the following claims.

Where the terms "comprise", "comprises", "comprised" or "comprising" are used in this specification , they are to be interpreted as specifying the presence of the stated features, integers, steps or components referred to, but not to preclude the presence or addition of one or more other feature, integer, step, component or group thereof.

Further, any prior art reference or statement provided in the specification is not to be taken as an admission that such art constitutes, or is to be understood as constituting, part of the common general knowledge in Australia.

The Claims defining the Invention are as follows:

1. A composition comprising:

a) at least one variant alpha-amylase comprising an amino acid sequence at least 95% identical to that of a parent AmyS-like alpha-amylase, and having a substitution at an amino acid position corresponding to position 242 of a reference alpha-amylase, wherein the substitution is a S242Q substitution, and said variant alpha-amylase having detectable alpha-amylase activity, wherein the reference alpha-amylase is SEQ ID NO:1 or 2; and

b) at least one of an additional enzyme, a detergent, a surfactant, a chelator, an oxidizing agent, an acidulant, an alkalizing agent, a source of peroxide, a source of hardness, a salt, a detergent complexing agent, a polymer, a stabilizing agent, or a fabric conditioner.

2. The composition of claim 1, wherein the variant alpha-amylase is altered, as compared to the parent AmyS-like alpha-amylase or the reference alpha-amylase, in one or more characteristics of: (a) net charge, (b) substrate specificity, (c) substrate cleavage, (d) substrate binding, (e) thermal stability, (f) activity at one or more pH's, (g) stability at one or more pH's, (h) stability in oxidizing conditions, (i) Ca^{2+} requirements, (j) specific activity, (k) catalytic rate, (l) catalytic efficiency, (m) activity in the presence of a chelator, (n) thermal or pH stability in the presence of a chelator, (o) utility for desizing, or utility for a cleaning process, or (p) amount of expression in a protein expression system.

3. The composition of claim 1, which is a component of a product for use in laundry, dish, or hard-surface cleaning, desizing, or fabric or stain treatment.

4. The composition of any one of claims 1 to 3, wherein the additional enzyme is a protease, a lipase, an amylase, a cellulase, a peroxidase, an oxidase, a pectinase, a lyase, a cutinase, a laccase, or a combination thereof.

5. The composition of any one of claims 1 to 4, wherein the surfactant is nonionic, anionic, cationic, or zwitterionic.

6. The composition of claim 1, wherein the variant alpha-amylase has altered stability to oxidation and the variant alpha-amylase further includes deletion or substitution of one or more methionine residues including residues located at amino positions 8, 9, 96, 200, 206, 284, 307, 311, 316, and 438 of a parent AmyS-like alpha-amylase, where the reference alpha-amylase in SEQ ID NO:2.

7. The composition of any one of claims 1 to 6, wherein the variant alpha-amylase further comprises:

i) a sequence modification at one or more amino acid positions corresponding to amino acid positions 97, 179, 180, 193, 319, 349, 358, 416, 428, or 443 of the reference alpha-amylase, wherein the modification is optionally a substitution as follows: a cysteine at 349, a cysteine at 428, a glutamic acid at 97, an arginine at 97, a glutamic acid at 319, an arginine at 319, a glutamic acid at 358, an arginine at 358, a glutamic acid at 443, or an arginine at 443, whereby the variant alpha-amylase comprises a set of substitutions selected from the group consisting of a) Q97E, Q319E, Q358E, Q443E; b) Q97E, Q319R, Q358E, Q443R; c) Q97E, Q319R, Q358E; d) Q97E, Q319R, Q443E; e) Q97E, Q319R, Q443R; f) Q97E, Q358R; g) Q97E, Q443E; h) Q319R, Q358E, Q443E; and i) Q319R, Q358R, Q443E; and optionally

ii) substitution of an N193 or a V416 or both, optionally selected from N193F and V416G, or both; and optionally

iii) deletion of one or more amino acids at positions F178, R179, G180, I181, G182 and K183.

8. The composition of claim 7, wherein the variant alpha-amylase has altered metal ion dependence or altered stability or activity in an absence of added calcium or a presence of a chelator.

9. The composition of claim 1, wherein the variant alpha-amylase has at least 95% homology to SEQ ID NO:2 and the substitution of amino acid 242 relative to numbering

in a reference alpha-amylase comprising SEQ ID NO:1, and wherein the variant alpha-amylase has alpha-amylase activity.

10. The composition of any one of claims 1 to 9, wherein the parent AmyS-like alpha-amylase is SEQ ID NO:1, 2, 6, 7, 8, 9, 10, 11, 12, 15, or 16.

11. The composition of any one of claims 1 to 10, wherein the variant alpha-amylase has improved performance in a wash process at a pH $\geq$ about 8, relative to the parent AmyS-like alpha-amylase.

12. A detergent or cleaning formulation comprising at least one variant alpha-amylase comprising an amino acid sequence at least 95% identical to that of a parent AmyS-like alpha-amylase, and having a substitution at an amino acid position corresponding to position 242 of a reference alpha-amylase, wherein the substitution is a S242Q substitution, said variant alpha-amylase having detectable alpha-amylase activity; wherein the reference amylase is SEQ ID NO:1 or 2.

13. A method of desizing a woven material subsequent to a weaving process comprising contacting the woven material with a variant alpha-amylase comprising an amino acid sequence at least 95% identical to that of a parent AmyS-like alpha-amylase, and having a substitution at an amino acid position corresponding to position 242 of a reference alpha-amylase, wherein the substitution is a S242Q substitution, said variant alpha-amylase having detectable alpha-amylase activity, under conditions and for a time effective for at least partially removing sizing from the woven material; wherein the reference amylase is SEQ ID NO: 1 or 2.

14. The method of claim 13, wherein the variant alpha-amylase is altered, as compared to the parent AmyS-like alpha-amylase or the reference alpha-amylase, in one or more of: (a) net charge, (b) substrate specificity, (c) substrate cleavage, (d) substrate binding, (e) thermal stability, (f) activity at one or more pH's, (g) stability at one or more pH's, (h) stability in oxidizing conditions, (i) Ca^{2+} requirements, (j) specific activity, (k) catalytic

rate, (l) catalytic efficiency, (m) activity in a presence of a chelator, (n) thermal or pH stability in the presence of a chelator, (o) effectiveness for desizing, or (p) amount of expression in a protein expression system.

15. The method of claim 13 or 14, wherein the parent AmyS-like alpha-amylase is SEQ ID NO:1, 2, 6, 7, 8, 9, 10, 11, 12, 15, or 16.

16. The method of any one of claims 13 to 15, wherein the variant alpha-amylase further comprises one or more of substitution at positions as follows: a cysteine at 349, a cysteine at 428, a glutamic acid at 97, an arginine at 97, a glutamic acid at 319, an arginine at 319, a glutamic acid at 358, an arginine at 358, a glutamic acid at 443, or an arginine at 443, wherein the reference alpha-amylase is SEQ ID NO: 1 or 2.

17. A method of washing or cleaning comprising contacting one or more items to be washed or cleaned with a detergent of claim 12 or a composition comprising a variant alpha-amylase comprising an amino acid sequence at least 95% identical to that of a parent AmyS-like alpha-amylase, and having a substitution at an amino acid position corresponding to position 242 of a reference alpha-amylase, wherein the substitution is a S242Q substitution, said variant alpha-amylase having detectable alpha-amylase activity, under conditions and for a time effective for at least partially washing or cleaning the one or more items; wherein the reference amylase is SEQ ID NO: 1 or 2.

18. The method of claim 17, wherein at least one item is soiled with at least one starch-containing material, removal of said starch soil is aided by the variant alpha-amylase.

19. The method of claim 17 or 18, wherein the composition further comprises one or more of an additional enzyme, a detergent, a surfactant, a chelator, an oxidizing agent, an acidulant, an alkalizing agent, a source of peroxide, a source of hardness, a salt, a detergent complexing agent, a polymer, a stabilizing agent, or a fabric conditioner.

20. The method of any one of claims 17 to 19, wherein the parent AmyS-like alpha-amylase is SEQ ID NO: 1, 2, 6, 7, 8, 9, 10, 11, 12, 15, or 16.

21. The method of any one of claims 17 to 20, wherein the variant alpha-amylase has improved performance in a wash process at a pH $\geq$ about 8, relative to the parent AmyS-like alpha-amylase.

22. The method of any one of claims 17 to 21, wherein the variant alpha-amylase comprises:
    i) one or more substitutions at positions as follows: a cysteine at 349, a cysteine at 428, a glutamic acid at 97, an arginine at 97, a glutamic acid at 319, an arginine at 319, a glutamic acid at 358, an arginine at 358, a glutamic acid at 443, or an arginine at 443, whereby the variant alpha-amylase optionally comprises a set of substitutions of a) Q97E, Q319E, Q358E, Q443E; b) Q97E, Q319R, Q358E, Q443R; c) Q97E, Q319R, Q358E; d) Q97E, Q319R, Q443E; e) Q97E, Q319R, Q443R; f) Q97E, Q358R; g) Q97E, Q443E; h) Q319R, Q358E, Q443E; or i) Q319R, Q358R, Q443E; and optionally
    ii) a deletion of one or more amino acids at positions F178, R179, G180, I181, G182, or K183.

23. The method of claim 22, wherein the variant alpha-amylase has altered metal ion dependence or altered stability, or activity in an absence of added calcium or the presence of a chelator.

24. A composition according to any one of claims 1 to 11, substantially as hereinbefore described.

```
                    1                                                  50
SEQID No 1     (1)  -AAPFNGTMMQYFEWYLPDDGTLWTKVANEANNLSSLGITALWLPPAYKG
SEQID No 2     (1)  -AAPFNGTMMQYFEWYLPDDGTLWTKVANEANNLSSLGITALWLPPAYKG
SEQID No 3     (1)  -AAPFNGTMMQYFEWYLPDDGTLWTKVANEANNLSSLGITALWLPPAYKG
SEQID No 4     (1)  -AAPFNGTMMQYFEWYLPDDGTLWTKVANEANNLSSLGITALWLPPAYKG
SEQID No 5     (1)  -AAPFNGTMMQYFEWYLPDDGTLWTKVANEANNLSSLGITALWLPPAYKG
SEQID No 6     (1)  HHNGTNGTMMQYFEWYLPNDGNHWNRLNSDASNLKSKGITAVWIPPAWKG
SEQID No 7     (1)  --ANLNGTLMQYFEWYMPNDGQHWKRLQNDSAYLAEHGITAVWIPPAYKG
SEQID No 8     (1)  --ANLNGTLMQYFEWYMPNDGQHWRRLQNDSAYLAEHGITAVWIPPAYKG
SEQID No 9     (1)  ----VNGTLMQYFEWYTPNDGQHWKRLQNDAEHLSDIGITAVWIPPAYKG
SEQID No 10    (1)  HHNGTNGTMMQYFEWYLPNDGNHWNRLRSDASNLKDKGISAVWIPPAWKG
SEQID No 11    (1)  HHNGTNGTMMQYFEWHLPNDGNHWNRLRDDASNLRNRGITAIWIPPAWKG
SEQID No 12    (1)  HHNGTNGTMMQYFEWHLPNDGNHWNRLRDDAANLKSKGITAVWIPPAWKG
SEQID No 13    (1)  --DGLNGTMMQYYEWHLENDGQHWNRLHDDAAALSDAGITAIWIPPAYKG
SEQID No 14    (1)  --DGLNGTMMQYYEWHLENDGQHWNRLHDDAEALSNAGITAIWIPPAYKG
SEQID No 15    (1)  -AAPFNGTMMQYFEWYLPDDGTLWTKVANEANNLSSLGITALWLPPAYKG
 Consensus 1   (1)    A  NGTMMQYFEWYLPNDGQHW RL NDA NLSS GITALWIPPAYKG
                    51                                                 100
SEQID No 1    (50)  TSRSDVGYGVYDLYDLGEFNQKGTVRTKYGTKAQYLQAIQAAHAAGMQVY
SEQID No 2    (50)  TSRSDVGYGVYDLYDLGEFNQKGTVRTKYGTKAQYLQAIQAAHAAGMQVY
SEQID No 3    (50)  TSRSDVGYGVYDLYDLGEFNQKGTVRTKYGTKAQYLQAIQAAHAAGMQVY
SEQID No 4    (50)  TSRSDVGYGVYDLYDLGEFNQKGTVRTKYGTKAQYLQAIQAAHAAGMQVY
SEQID No 5    (50)  TSRSDVGYGVYDLYDLGEFNQKGTVRTKYGTKAQYLQAIQAAHAAGMQVY
SEQID No 6    (51)  ASQNDVGYGAYDLYDLGEFNQKGTVRTKYGTRSQLQAAVTSLKNNGIQVY
SEQID No 7    (49)  TSQADVGYGAYDLYDLGEFHQKGTVRTKYGTKGELQSAIKSLHSRDINVY
SEQID No 8    (49)  TSQADVGYGAYDLYDLGEFHQKGTVRTKYGTKGELQSAIKSLHSRDINVY
SEQID No 9    (47)  LSQSDNGYGPYDLYDLGEFQQKGTVRTKYGTKSELQDAIGSLHSRNVQVY
SEQID No 10   (51)  ASQNDVGYGAYDLYDLGEFNQKGTIRTKYGTRNQLQAAVNALKSNGIQVY
SEQID No 11   (51)  TSQNDVGYGAYDLYDLGEFNQKGTVRTKYGTRSQLESAIHALKNNGVQVY
SEQID No 12   (51)  TSQNDVGYGAYDLYDLGEFNQKGTVRTKYGTRSQLQGAVTSLKNNGIQVY
SEQID No 13   (49)  NSQADVGYGAYDLYDLGEFNQKGTVRTKYGTKAQLERAIGSLKSNDINVY
SEQID No 14   (49)  NSQADVGYGAYDLYDLGEFNQKGTVRTKYGTKAQLERAIGSLKSNDINVY
SEQID No 15   (50)  TSRSDVGYGVYDLYDLGEFNQKGTVRTKYGTKAQYLQAIQAAHAAGMQVY
 Consensus 1  (51)  TSQSDVGYGAYDLYDLGEFNQKGTVRTKYGTKAQL  AI ALHA GIQVY
                    101                                                150
 SEQID No 1  (100)  ADVVFDHKGGADGTEWVDAVEVNPSDRNQEISGTYQIQAWTKFDFPGRGN
 SEQID No 2  (100)  ADVVFDHKGGADGTEWVDAVEVNPSDRNQEISGTYQIQAWTKFDFPGRGN
 SEQID No 3  (100)  ADVVFDHKGGADGTEWVDAVEVNPSDRNQEISGTYQIQAWTKFDFPGRGN
 SEQID No 4  (100)  ADVVFDHKGGADGTEWVDAVEVNPSDRNQEISGTYQIQAWTKFDFPGRGN
 SEQID No 5  (100)  ADVVFDHKGGADGTEWVDAVEVNPSDRNQEISGTYQIQAWTKFDFPGRGN
 SEQID No 6  (101)  GDVVMNHKGGADATEMVRAVEVNPNNRNQEVTGEYTIEAWTRFDFPGRGN
 SEQID No 7   (99)  GDVVINHKGGADATEDVTAVEVDPADRNRVISGEHLIKAWTHFHFPGRGS
 SEQID No 8   (99)  GDVVINHKGGADATEDVTAVEVDPADRNRVISGEHLIKAWTHFHFPGRGS
 SEQID No 9   (97)  GDVVLNHKAGADATEDVTAVEVNPANRNQETSEEYQIKAWTDFRFPGRGN
SEQID No 10  (101)  GDVVMNHKGGADATEMVRAVEVNPNNRNQEVSGEYTIEAWTKFDFPGRGN
SEQID No 11  (101)  GDVVMNHKGGADATENVLAVEVNPNNRNQEISGDYTIEAWTKFDFPGRGN
SEQID No 12  (101)  GDVVMNHKGGADGTEMVNAVEVNRSNRNQEISGEYTIEAWTKFDFPGRGN
SEQID No 13   (99)  GDVVMNHKMGADFTEAVQAVQVNPTNRWQDISGAYTIDAWTGFDFSGRNN
SEQID No 14   (99)  GDVVMNHKLGADFTEAVQAVQVNPSNRWQDISGVYTIDAWTGFDFPGRNN
SEQID No 15  (100)  ADVVFDHKGGADGTEWVDAVEVNPSDRNQEISGTYQIQAWTKFDFPGRGN
 Consensus 1 (101)  GDVVMNHKGGADGTE V AVEVNPSDRNQEISG Y I AWTKFDFPGRGN
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Figure 1 (1 of 4)

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                    151                                                 200
 SEQID No 1   (150) TYSSFKWRWYHFDGVDWDESRKLS-RIYKFRGIGKAWDWEVDTENGNYDY
 SEQID No 2   (150) TYSSFKWRWYHFDGVDWDESRKLS-RIYKFRGIGKAWDWEVDTENGNYDY
 SEQID No 3   (150) TYSSFKWRWYHFDGVDWDESRKLS-RIYKFRGIGKAWDWEVDTENGNYDY
 SEQID No 4   (150) TYSSFKWRWYHFDGVDWDESRKLS-RIYKFRGIGKAWDWEVDTENGNYDY
 SEQID No 5   (150) TYSSFKWRWYHFDGVDWDESRKLS-RIYKFRGIGKAWDWEVDTENGNYDY
 SEQID No 6   (151) THSSFKWRWYHFDGVDWDQSRRLNNRIYKFRGHGKAWDWEVDTENGNYDY
 SEQID No 7   (149) TYSDFKWHWYHFDGTDWDESRKLN-RIYKFQG--KAWDWEVSNENGNYDY
 SEQID No 8   (149) TYSDFKWHWYHFDGTDWDESRKLN-RIYKFQG--KAWDWEVSNENGNYDY
 SEQID No 9   (147) TYSDFKWHWYHFDGADWDESRKIS-RIFKFRGEGKAWDWEVSSENGNYDY
SEQID No 10   (151) THSNFKWRWYHFDGVDWDQSRKLNNRIYKFRGDGKGWDWEVDTENGNYDY
SEQID No 11   (151) TYSDFKWRWYHFDGVDWDQSRQFQNRIYKFRGDGKAWDWEVDSENGNYDY
SEQID No 12   (151) THSNFKWRWYHFDGTDWDQSRQLQNKIYKFRGTGKAWDWEVDIENGNYDY
SEQID No 13   (149) AYSDFKWRWFHFNGVDWDQRYQEN-HIFRFAN--TNWNWRVDEENGNYDY
SEQID No 14   (149) AYSDFKWRWFHFNGVDWDQRYQEN-HLFRFAN--TNWNWRVDEENGNYDY
SEQID No 15   (150) TYSSFKWRWYHFDGVDWDESRKLS-RIYKFRG--KAWDWEVDTEFGNYDY
 Consensus 1  (151) TYS FKWRWYHFDGVDWDESRKLN RIYKFRG GKAWDWEVDTENGNYDY
                    201                                                 250
 SEQID No 1   (199) LMYADLDMDHPEVVTELKNWGKWYVNTTNIDGFRLDAVKHIKFSFFPDWL
 SEQID No 2   (199) LMYADLDMDHPEVVTELKNWGKWYVNTTNIDGFRLDAVKHIKFSFFPDWL
 SEQID No 3   (199) LMYADLDMDHPEVVTELKNWGKWYVNTTNIDGFRLDAVKHIKFAFFPDWL
 SEQID No 4   (199) LMYADLDMDHPEVVTELKNWGKWYVNTTNIDGFRLDAVKHIKFQFFPDWL
 SEQID No 5   (199) LMYADLDMDHPEVVTELKNWGKWYVNTTNIDGFRLDAVKHIKFEFFPDWL
 SEQID No 6   (201) LMYADIDMDHPEVVNELRNWGVWYTNTLGLDGFRIDAVKHIKYSFTRDWI
 SEQID No 7   (196) LMYADIDYDHPDVAAEIKRWGTWYANELQLDGFRLDAVKHIKFSFLRDWV
 SEQID No 8   (196) LMYADIDYDHPDVAAEIKRWGTWYANELQLDGFRLDAVKHIKFSFLRDWV
 SEQID No 9   (196) LMYADVDYDHPDVVAETKKWGIWYANELSLDGFRIDAAKHIKFSFLRDWV
SEQID No 10   (201) LMYADIDMDHPEVVNELRNWGVWYTNTLGLDGFRIDAVKHIKYSFTRDWI
SEQID No 11   (201) LMYADVDMDHPEVVNELRRWGEWYTNTLNLDGFRIDAVKHIKYSFTRDWL
SEQID No 12   (201) LMYADIDMDHPEVINELRNWGVWYTNTLNLDGFRIDAVKHIKYSYTRDWL
SEQID No 13   (196) LLGSNIDFSHPEVQDELKDWGSWFTDELDLDGYRLDAIKHIPFWYTSDWV
SEQID No 14   (196) LLGSNIDFSHPEVQEELKDWGSWFTDELDLDGYRLDAIKHIPFWYTSDWV
SEQID No 15   (197) LMYADLDMDHPEVVTELKNWGKWYVNTTNIDGFRLDAVKHIKFSFFPDWL
 Consensus 1  (201) LMYADIDMDHPEVV ELKNWG WY NTLNLDGFRLDAVKHIKFSF  DWL
                    251                                                 300
 SEQID No 1   (249) SYVRSQTGKPLFTVGEYWSYDINKLHNYITKTNGTMSLFDAPLHNKFYTA
 SEQID No 2   (249) SYVRSQTGKPLFTVGEYWSYDINKLHNYITKTNGTMSLFDAPLHNKFYTA
 SEQID No 3   (249) SYVRSQTGKPLFTVGEYWSYDINKLHNYITKTNGTMSLFDAPLHNKFYTA
 SEQID No 4   (249) SYVRSQTGKPLFTVGEYWSYDINKLHNYITKTNGTMSLFDAPLHNKFYTA
 SEQID No 5   (249) SYVRSQTGKPLFTVGEYWSYDINKLHNYITKTNGTMSLFDAPLHNKFYTA
 SEQID No 6   (251) NHVRSATGKNMFAVAEFWKNDLGAIENYLQKTNWNHSVFDVPLHYNLYNA
 SEQID No 7   (246) NHVREKTGKEMFTVAEYWQNDLGALENYLNKTNFNHSVFDVPLHYQFHAA
 SEQID No 8   (246) NHVREKTGKEMFTVAEYWQNDLGALENYLNKTNFNHSVFDVPLHYQFHAA
 SEQID No 9   (246) QAVRQATGKEMFTVAEYWQNNAGKLENYLNKTSFNQSVFDVPLHFNLQAA
SEQID No 10   (251) NHVRSATGKNMFAVAEFWKNDLGAIENYLNKTNWNHSVFDVPLHYNLYNA
SEQID No 11   (251) THVRNATGKEMFAVAEFWKNDLGALENYLNKTNWNHSVFDVPLHYNLYNA
SEQID No 12   (251) THVRNTTGKPMFAVAEFWKNDLAAIENYLNKTSWNHSVFDVPLHYNLYNA
SEQID No 13   (246) RHQRNEADQDLFVVGEYWKDDVGALEFYLDEMNWEMSLFDVPLNYNFYRA
SEQID No 14   (246) RHQRSEADQDLFVVGEYWKDDVGALEFYLDEMNWEMSLFDVPLNYNFYRA
SEQID No 15   (247) SYVRSQTGKPLFTVGEYWSYDINKLHNYITKTNGTMSLFDAPLHNKFYTA
 Consensus 1  (251) SHVRS TGK LFTVGEYW  DIGALENYL KTNW MSLFDVPLHYNFY A
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                    301                                                 350
 SEQID No 1  (299) SKSGGAFDMRTLMTNTLMKDQPTLAVTFVDNHDTEPGQALQSWVDPWFKP
 SEQID No 2  (299) SKSGGAFDMRTLMTNTLMKDQPTLAVTFVDNHDTEPGQALQSWVDPWFKP
 SEQID No 3  (299) SKSGGAFDMRTLMTNTLMKDQPTLAVTFVDNHDTEPGQALQSWVDPWFKP
 SEQID No 4  (299) SKSGGAFDMRTLMTNTLMKDQPTLAVTFVDNHDTEPGQALQSWVDPWFKP
 SEQID No 5  (299) SKSGGAFDMRTLMTNTLMKDQPTLAVTFVDNHDTEPGQALQSWVDPWFKP
 SEQID No 6  (301) SKSGGNYDMRNIFNGTVVQRHPSHAVTFVDNHDSQPEEALESFVEEWFKP
 SEQID No 7  (296) STQGGGYDMRKLLNGTVVSKHPLKSVTFVDNHDTQPGQSLESTVQTWFKP
 SEQID No 8  (296) STQGGGYDMRKLLNGTVVSKHPLKSVTFVDNHDTQPGQSLESTVQTWFKP
 SEQID No 9  (296) SSQGGGYDMRRLLDGTVVSRHPEKAVTFVENHDTQPGQSLESTVQTWFKP
SEQID No 10  (301) SKSGGNYDMRQIFNGTVVQRHPMHAVTFVDNHDSQPEEALESFVEEWFKP
SEQID No 11  (301) SNSGGNYDMAKLLNGTVVQKHPMHAVTFVDNHDSQPGESLESFVQEWFKP
SEQID No 12  (301) SNSGGYFDMRNILNGSVVQKHPIHAVTFVDNHDSQPGEALESFVQSWFKP
SEQID No 13  (296) SQQGGSYDMRNILRGSLVEAHPMHAVTFVDNHDTQPGESLESWVADWFKP
SEQID No 14  (296) SKQGGSYDMRNILRGSLVEAHPIHAVTFVDNHDTQPGESLESWVADWFKP
SEQID No 15  (297) SKSGGAFDMRTLMTNTLMKDQPTLAVTFVDNHDTEPGQALQSWVDPWFKP
 Consensus 1 (301) SKSGGAYDMR LL GTLV  HP  AVTFVDNHDTQPGQALESWVD WFKP
                    351                                                 400
 SEQID No 1  (349) LAYAFILTRQEGYPCVFYGDYYGIPQYN---IPSLKSKIDPLLIARRDYA
 SEQID No 2  (349) LAYAFILTRQEGYPCVFYGDYYGIPQYN---IPSLKSKIDPLLIARRDYA
 SEQID No 3  (349) LAYAFILTRQEGYPCVFYGDYYGIPQYN---IPSLKSKIDPLLIARRDYA
 SEQID No 4  (349) LAYAFILTRQEGYPCVFYGDYYGIPQYN---IPSLKSKIDPLLIARRDYA
 SEQID No 5  (349) LAYAFILTRQEGYPCVFYGDYYGIPQYN---IPSLKSKIDPLLIARRDYA
 SEQID No 6  (351) LAYALTLTREQGYPSVFYGDYYGIPTHG---VPAMRSKIDPILEARQKYA
 SEQID No 7  (346) LAYAFILTRESGYPQVFYGDMYGTKGDSQREIPALKHKIEPILKARKQYA
 SEQID No 8  (346) LAYAFILTRESGYPQVFYGDMYGTKGDSQREIPALKHKIEPILKARKQYA
 SEQID No 9  (346) LAYAFILTRESGYPQVFYGDMYGTKGTSPKEIPSLKDNIEPILKARKEYA
SEQID No 10  (351) LAYALTLTREQGYPSVFYGDYYGIPTHG---VPAMKSKIDPILEARQKYA
SEQID No 11  (351) LAYALILTREQGYPSVFYGDYYGIPTHS---VPAMKAKIDPILEARQNFA
SEQID No 12  (351) LAYALILTREQGYPSVFYGDYYGIPTHG---VPSMKSKIDPLLQARQTYA
SEQID No 13  (346) LAYATILTREGGYPNVFYGDYYGIPNDN---ISAKKDMIDELLDARQNYA
SEQID No 14  (346) LAYATILTREGGYPNVFYGDYYGIPNDN---ISAKKDMIDELLDARQNYA
SEQID No 15  (347) LAYAFILTRQEGYPCVFYGDYYGIPQYN---IPSLKSKIDPLLIARRDYA
 Consensus 1 (351) LAYAFILTRE GYP VFYGDYYGIPQYN   IPSLKSKIDPLL ARR YA
                    401                                                 450
 SEQID No 1  (396) YGTQHDYLDHSDIIGWTREGVTEKPGSGLAALITDGPGGSKWMYVGKQHA
 SEQID No 2  (396) YGTQHDYLDHSDIIGWTREGVTEKPGSGLAALITDGPGGSKWMYVGKQHA
 SEQID No 3  (396) YGTQHDYLDHSDIIGWTREGVTEKPGSGLAALITDGPGGSKWMYVGKQHA
 SEQID No 4  (396) YGTQHDYLDHSDIIGWTREGVTEKPGSGLAALITDGPGGSKWMYVGKQHA
 SEQID No 5  (396) YGTQHDYLDHSDIIGWTREGVTEKPGSGLAALITDGPGGSKWMYVGKQHA
 SEQID No 6  (398) YGKQNDYLDHHNIIGWTREGNTAHPNSGLATIMSDGAGGSKWMFVGRNKA
 SEQID No 7  (396) YGAQHDYFDHHDIVGWTREGDSSVANSGLAALITDGPGGAKRMYVGRQNA
 SEQID No 8  (396) YGAQHDYFDHHDIVGWTREGDSSVANSGLAALITDGPGGAKRMYVGRQNA
 SEQID No 9  (396) YGPQHDYIDHPDVIGWTREGDSSAAKSGLAALITDGPGGSKRMYAGLKNA
SEQID No 10  (398) YGRQNDYLDHHNIIGWTREGNTAHPNSGLATIMSDGAGGNKWMFVGRNKA
SEQID No 11  (398) YGTQHDYFDHHNIIGWTREGNTTHPNSGLATIMSDGPGGEKWMYVGQNKA
SEQID No 12  (398) YGTQHDYFDHHDIIGWTREGDSSHPNSGLATIMSDGPGGNKWMYVGKHKA
SEQID No 13  (393) YGTQHDYFDHWDVVGWTREGSSSRPNSGLATIMSNGPGGSKWMYVGRQNA
SEQID No 14  (393) YGTQHDYFDHWDIVGWTREGTSSRPNSGLATIMSNGPGGSKWMYVGQQHA
SEQID No 15  (394) YGTQHDYLDHSDIIGWTREGGTEKPGSGLAALITDGPGGSKWMYVGKQHA
 Consensus 1 (401) YGTQHDYLDH DIIGWTREG TSKPNSGLAALITDGPGGSKWMYVGKQ A
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Figure 1 (3 of 4)

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                    451                                                 500
 SEQID No 1   (446) GKVFYDLTGNRSDTVTINSDGWGEFKVNGGSVSVWVPRKTTVSTIARPIT
 SEQID No 2   (446) GKVFYDLTGNRSDTVTINSDGWGEFKVNGGSVSVWVPRKTT---------
 SEQID No 3   (446) GKVFYDLTGNRSDTVTINSDGWGEFKVNGGSVSVWVPRKTTVSTIARPIT
 SEQID No 4   (446) GKVFYDLTGNRSDTVTINSDGWGEFKVNGGSVSVWVPRKTTVSTIARPIT
 SEQID No 5   (446) GKVFYDLTGNRSDTVTINSDGWGEFKVNGGSVSVWVPRKTTVSTIARPIT
 SEQID No 6   (448) GQVWSDITGNRTGTVTINADGWGNFSVNGGSVSIWVNK------------
 SEQID No 7   (446) GETWHDITGNRSEPVVINSEGWGEFHVNGGSVSIYVQR------------
 SEQID No 8   (446) GETWHDITGNRSEPVVINSEGWGEFHVNGGSVSIYVQR------------
 SEQID No 9   (446) GETWYDITGNRSDTVKIGSDGWGEFHVNDGSVSIYVQK------------
SEQID No 10   (448) GQVWTDITGNRAGTVTINADGWGNFSVNGGSVSIWVNK------------
SEQID No 11   (448) GQVWHDITGNKPGTVTINADGWANFSVNGGSVSIWVKR------------
SEQID No 12   (448) GQVWRDITGNRSGTVTINADGWGNFTVNGGAVSVWVKQ------------
SEQID No 13   (443) GQTWTDLTGNNGASVTINGDGWGEFFTNGGSVSVYVNQ------------
SEQID No 14   (443) GQTWTDLTGNHAASVTINGDGWGEFFTNGGSVSVYVNQ------------
SEQID No 15   (444) GKVFYDLTGNRSDTVTINSDGWGEFKVNGGSVSVWVPRKTTVS-------
 Consensus 1  (451) G VWYDLTGNRSDTVTINSDGWGEF VNGGSVSVWV R
                    501                 520
 SEQID No 1   (496) TRPWTGEFVRWTEPRLVAWP
 SEQID No 2   (487) --------------------
 SEQID No 3   (496) TRPWTGEFVRWTEPRLVAWP
 SEQID No 4   (496) TRPWTGEFVRWTEPRLVAWP
 SEQID No 5   (496) TRPWTGEFVRWTEPRLVAWP
 SEQID No 6   (486) --------------------
 SEQID No 7   (484) --------------------
 SEQID No 8   (484) --------------------
 SEQID No 9   (484) --------------------
SEQID No 10   (486) --------------------
SEQID No 11   (486) --------------------
SEQID No 12   (486) --------------------
SEQID No 13   (481) --------------------
SEQID No 14   (481) --------------------
SEQID No 15   (487) --------------------
 Consensus 1  (501)
```
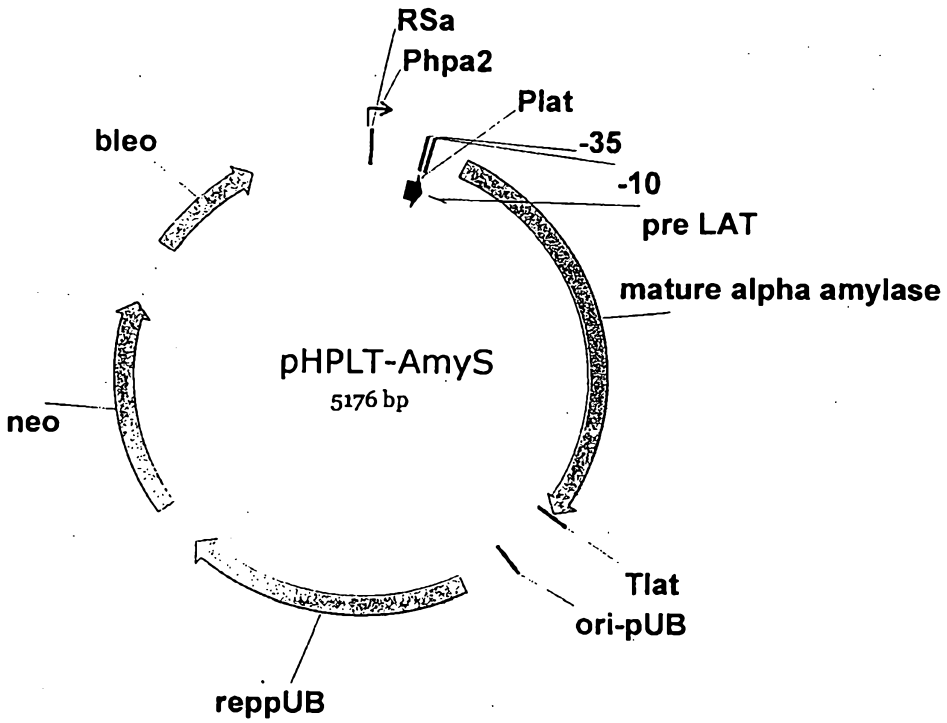

Figure 1 (4 of 4)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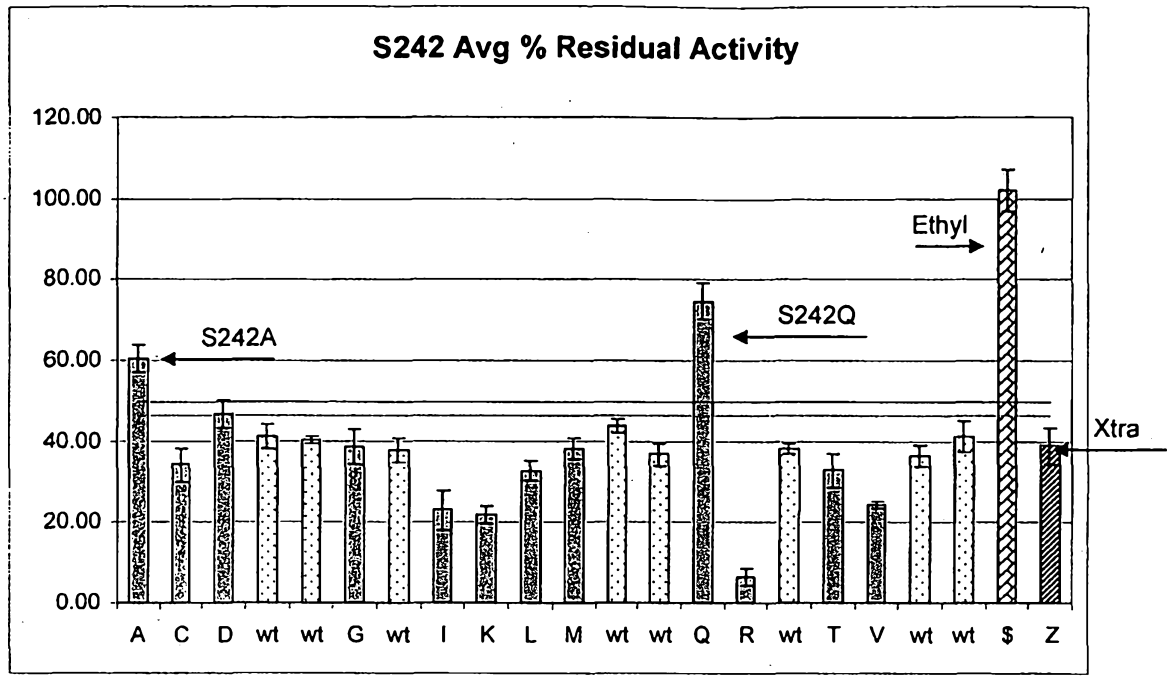
Phpa2
Plat
-35
-10
pre LAT
mature alpha amylase
bleo
pHPLT-AmyS
5176 bp
neo
Tlat
ori-pUB
reppUB

Figure 2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120.00
100.00
80.00
60.00
40.00
20.00
0.00
S242A
S242Q
Ethyl
Xtra
A C D wt wt G wt I K L M wt wt Q R wt T V wt wt $ Z

Figure 3

```
                    1                                                  50
SEQID No 1      (1) -AAPFNGTMMQYFEWYLPDDGTLWTKVANEANNLSSLGITALWLPPAYKG
SEQID No 6      (1) HHNGTNGTMMQYFEWYLPNDGNHWNRLNSDASNLKSKGITAVWIPPAWKG
 Consensus 2    (1)      NGTMMQYFEWYLP DG  W KL  DA NL S GITALWIPPAWKG
                    51                                                 100
SEQID No 1     (50) TSRSDVGYGVYDLYDLGEFNQKGTVRTKYGTKAQYLQAIQAAHAAGMQVY
SEQID No 6     (51) ASQNDVGYGAYDLYDLGEFNQKGTVRTKYGTRSQLQAAVTSLKNNGIQVY
 Consensus 2   (51)  S  DVGYG YDLYDLGEFNQKGTVRTKYGTKAQ   AI A    GIQVY
                   101                                                 150
SEQID No 1    (100) ADVVFDHKGGADGTEWVDAVEVNPSDRNQEISGTYQIQAWTKFDFPGRGN
SEQID No 6    (101) GDVVMNHKGGADATEMVRAVEVNPNNRNQEVTGEYTIEAWTRFDFPGRGN
 Consensus 2  (101) ADVV  HKGGADATE V AVEVNP  RNQEISG Y I AWTKFDFPGRGN
                   151                                                 200
SEQID No 1    (150) TYSSFKWRWYHFDGVDWDESRKLS-RIYKFRGIGKAWDWEVDTENGNYDY
SEQID No 6    (151) THSSFKWRWYHFDGVDWDQSRRLNNRIYKFRGHGKAWDWEVDTENGNYDY
 Consensus 2  (151) THSSFKWRWYHFDGVDWD SRKL  RIYKFRG GKAWDWEVDTENGNYDY
                   201                                                 250
SEQID No 1    (199) LMYADLDMDHPEVVTELKNWGKWYVNTTNIDGFRLDAVKHIKFSFFPDWL
SEQID No 6    (201) LMYADIDMDHPEVVNELRNWGVWYTNTLGLDGFRIDAVKHIKYSFTRDWI
 Consensus 2  (201) LMYADIDMDHPEVV ELKNWG WY NT  IDGFRIDAVKHIKFSF  DWI
                   251                                                 300
SEQID No 1    (249) SYVRSQTGKPLFTVGEYWSYDINKLHNYITKTNGTMSLFDAPLHNKFYTA
SEQID No 6    (251) NHVRSATGKNMFAVAEFWKNDLGAIENYLQKTNWNHSVFDVPLHYNLYNA
 Consensus 2  (251)  HVRS TGK LF VAEFW  DI  I NYI KTN   SLFD PLH   Y A
                   301                                                 350
SEQID No 1    (299) SKSGGAFDMRTLMTNTLMKDQPTLAVTFVDNHDTEPGQALQSWVDPWFKP
SEQID No 6    (301) SKSGGNYDMRNIFNGTVVQRHPSHAVTFVDNHDSQPEEALESFVEEWFKP
 Consensus 2  (301) SKSGG FDMR I   TLM   PS AVTFVDNHDS P  AL SFVD WFKP
                   351                                                 400
SEQID No 1    (349) LAYAFILTRQEGYPCVFYGDYYGIPQYNIPSLKSKIDPLLIARRDYAYGT
SEQID No 6    (351) LAYALTLTREQGYPSVFYGDYYGIPTHGVPAMRSKIDPILEARQKYAYGK
 Consensus 2  (351) LAYA  LTR  GYP VFYGDYYGIP H IPALKSKIDPIL AR  YAYG
                   401                                                 450
SEQID No 1    (399) QHDYLDHSDIIGWTREGVTEKPGSGLAALITDGPGGSKWMYVGKQHAGKV
SEQID No 6    (401) QNDYLDHHNIIGWTREGNTAHPNSGLATIMSDGAGGSKWMFVGRNKAGQV
 Consensus 2  (401) Q DYLDH  IIGWTREG T  P SGLA IISDG GGSKWMFVGKN AG V
                   451                                                 500
SEQID No 1    (449) FYDLTGNRSDTVTINSDGWGEFKVNGGSVSVWVPRKTTVSTIARPITTRP
SEQID No 6    (451) WSDITGNRTGTVTINADGWGNFSVNGGSVSIWVNK---------------
 Consensus 2  (451) F DITGNRS TVTINADGWG F VNGGSVSIWV K
                   501          517
SEQID No 1    (499) WTGEFVRWTEPRLVAWP
SEQID No 6    (486) -----------------
 Consensus 2  (501)
```

Figure 4A

```
                    1                                                  50
SEQID No 1      (1) AAPFNGTMMQYFEWYLPDDGTLWTKVANEANNLSSLGITALWLPPAYKGT
SEQID No 8      (1) -ANLNGTLMQYFEWYMPNDGQHWRRLQNDSAYLAEHGITAVWIPPAYKGT
 Consensus 3    (1)  A  NGTLMQYFEWYLP DG  W KL NDA  LA  GITALWIPPAYKGT
                    51                                                 100
SEQID No 1     (51) SRSDVGYGVYDLYDLGEFNQKGTVRTKYGTKAQYLQAIQAAHAAGMQVYA
SEQID No 8     (50) SQADVGYGAYDLYDLGEFHQKGTVRTKYGTKGELQSAIKSLHSRDINVYG
 Consensus 3   (51) S ADVGYG YDLYDLGEF QKGTVRTKYGTKA    AI A HA  INVYA
                   101                                                 150
SEQID No 1    (101) DVVFDHKGGADGTEWVDAVEVNPSDRNQEISGTYQIQAWTKFDFPGRGNT
SEQID No 8    (100) DVVINHKGGADATEDVTAVEVDPADRNRVISGEHLIKAWTHFHFPGRGST
 Consensus 3  (101) DVV  HKGGADATE V AVEV PADRN  ISG H I AWT F FPGRG T
                   151                                                 200
SEQID No 1    (151) YSSFKWRWYHFDGVDWDESRKLSRIYKFRGIGKAWDWEVDTENGNYDYLM
SEQID No 8    (150) YSDFKWHWYHFDGTDWDESRKLNRIYKFQ--GKAWDWEVSNENGNYDYLM
 Consensus 3  (151) YS FKW WYHFDG DWDESRKL RIYKF   GKAWDWEV  ENGNYDYLM
                   201                                                 250
SEQID No 1    (201) YADLDMDHPEVVTELKNWGKWYVNTTNIDGFRLDAVKHIKFSFFPDWLSY
SEQID No 8    (198) YADIDYDHPDVAAEIKRWGTWYANELQLDGFRLDAVKHIKFSFLRDWVNH
 Consensus 3  (201) YADID DHPDV  EIK WG WY N  NIDGFRLDAVKHIKFSF  DWL H
                   251                                                 300
SEQID No 1    (251) VRSQTGKPLFTVGEYWSYDINKLHNYITKTNGTMSLFDAPLHNKFYTASK
SEQID No 8    (248) VREKTGKEMFTVAEYWQNDLGALENYLNKTNFNHSVFDVPLHYQFHAAST
 Consensus 3  (251) VR  TGK LFTVAEYW  DI  L NYI KTN   SLFD PLH  FH AS
                   301                                                 350
SEQID No 1    (301) SGGAFDMRTLMTNTLMKDQPTLAVTFVDNHDTEPGQALQSWVDPWFKPLA
SEQID No 8    (298) QGGGYDMRKLLNGTVVSKHPLKSVTFVDNHDTQPGQSLESTVQTWFKPLA
 Consensus 3  (301)  GGAFDMR LL  TLM   P  AVTFVDNHDT PGQAL S V  WFKPLA
                   351                                                 400
SEQID No 1    (351) YAFILTRQEGYPCVFYGDYYGIP---QYNIPSLKSKIDPLLIARRDYAYG
SEQID No 8    (348) YAFILTRESGYPQVFYGDMYGTKGDSQREIPALKHKIEPILKARKQYAYG
 Consensus 3  (351) YAFILTR  GYP VFYGD YG     Q  IPALK KIDPIL ARK YAYG
                   401                                                 450
SEQID No 1    (398) TQHDYLDHSDIIGWTREGVTEKPGSGLAALITDGPGGSKWMYVGKQHAGK
SEQID No 8    (398) AQHDYFDHHDIVGWTREGDSSVANSGLAALITDGPGGAKRMYVGRQNAGE
 Consensus 3  (401)  QHDY DH DIIGWTREG S    SGLAALITDGPGGAK MYVGKQ AG
                   451                                                 500
SEQID No 1    (448) VFYDLTGNRSDTVTINSDGWGEFKVNGGSVSVWVPRKTTVSTIARPITTR
SEQID No 8    (448) TWHDITGNRSEPVVINSEGWGEFHVNGGSVSIYVQR--------------
 Consensus 3  (451)  FHDITGNRSD V INSDGWGEF VNGGSVSIWV R
                   501            518
SEQID No 1    (498) PWTGEFVRWTEPRLVAWP
SEQID No 8    (484) ------------------
 Consensus 3  (501)
```

Figure 4B

```
                    1                                                  50
SEQID No 1      (1) AAPFNGTMMQYFEWYLPDDGTLWTKVANEANNLSSLGITALWLPPAYKGT
SEQID No 9      (1) ---VNGTLMQYFEWYTPNDGQHWKRLQNDAEHLSDIGITAVWIPPAYKGL
 Consensus 4    (1)    NGTLMQYFEWY P DG  W KL NDA  LS IGITALWIPPAYKG
                    51                                                 100
SEQID No 1     (51) SRSDVGYGVYDLYDLGEFNQKGTVRTKYGTKAQYLQAIQAAHAAGMQVYA
SEQID No 9     (48) SQSDNGYGPYDLYDLGEFQQKGTVRTKYGTKSELQDAIGSLHSRNVQVYG
 Consensus 4   (51) S SD GYG YDLYDLGEFNQKGTVRTKYGTKA    AI A HA  MQVYA
                   101                                                 150
SEQID No 1    (101) DVVFDHKGGADGTEWVDAVEVNPSDRNQEISGTYQIQAWTKFDFPGRGNT
SEQID No 9     (98) DVVLNHKAGADATEDVTAVEVNPANRNQETSEEYQIKAWTDFRFPGRGNT
 Consensus 4  (101) DVV  HKAGADATE V AVEVNPA RNQE S  YQI AWT F FPGRGNT
                   151                                                 200
SEQID No 1    (151) YSSFKWRWYHFDGVDWDESRKLSRIYKFRGIGKAWDWEVDTENGNYDYLM
SEQID No 9    (148) YSDFKWHWYHFDGADWDESRKISRIFKFRGEGKAWDWEVSSENGNYDYLM
 Consensus 4  (151) YS FKW WYHFDG DWDESRKISRIFKFRG GKAWDWEV SENGNYDYLM
                   201                                                 250
SEQID No 1    (201) YADLDMDHPEVVTELKNWGKWYVNTTNIDGFRLDAVKHIKFSFFPDWLSY
SEQID No 9    (198) YADVDYDHPDVVAETKKWGIWYANELSLDGFRIDAAKHIKFSFLRDWVQA
 Consensus 4  (201) YADLD DHPDVV E K WG WY N   IDGFRIDA KHIKFSF  DWL
                   251                                                 300
SEQID No 1    (251) VRSQTGKPLFTVGEYWSYDINKLHNYITKTNGTMSLFDAPLHNKFYTASK
SEQID No 9    (248) VRQATGKEMFTVAEYWQNNAGKLENYLNKTSFNQSVFDVPLHFNLQAASS
 Consensus 4  (251) VR  TGK LFTVAEYW     KL NYI KT    SLFD PLH     AS
                   301                                                 350
SEQID No 1    (301) SGGAFDMRTLMTNTLMKDQPTLAVTFVDNHDTEPGQALQSWVDPWFKPLA
SEQID No 9    (298) QGGGYDMRRLLDGTVVSRHPEKAVTFVENHDTQPGQSLESTVQTWFKPLA
 Consensus 4  (301)  GGAFDMR LL  TLM   P  AVTFVDNHDT PGQAL S V  WFKPLA
                   351                                                 400
SEQID No 1    (351) YAFILTRQEGYPCVFYGDYYGIPQYN---IPSLKSKIDPLLIARRDYAYG
SEQID No 9    (348) YAFILTRESGYPQVFYGDMYGTKGTSPKEIPSLKDNIEPILKARKEYAYG
 Consensus 4  (351) YAFILTR  GYP VFYGD YG        IPSLK  IDPIL ARKDYAYG
                   401                                                 450
SEQID No 1    (398) TQHDYLDHSDIIGWTREGVTEKPGSGLAALITDGPGGSKWMYVGKQHAGK
SEQID No 9    (398) PQHDYIDHPDVIGWTREGDSSAAKSGLAALITDGPGGSKRMYAGLKNAGE
 Consensus 4  (401)  QHDYIDH DIIGWTREG S    SGLAALITDGPGGSK MY G   AG
                   451                                                 500
SEQID No 1    (448) VFYDLTGNRSDTVTINSDGWGEFKVNGGSVSVWVPRKTTVSTIARPITTR
SEQID No 9    (448) TWYDITGNRSDTVKIGSDGWGEFHVNDGSVSIYVQK--------------
 Consensus 4  (451)  FYDITGNRSDTV I SDGWGEF VN GSVSIWV K
                  501           518
SEQID No 1    (498) PWTGEFVRWTEPRLVAWP
SEQID No 9    (484) ------------------
 Consensus 4  (501)
```

Figure 4C

```
                    1                                                  50
 SEQID No 1      (1) -AAPFNGTMMQYFEWYLPDDGTLWTKVANEANNLSSLGITALWLPPAYKG
SEQID No 10      (1) HHNGTNGTMMQYFEWYLPNDGNHWNRLRSDASNLKDKGISAVWIPPAWKG
  Consensus 5    (1)      NGTMMQYFEWYLP DG  W KL  DA NL   GISALWIPPAWKG
                    51                                                 100
 SEQID No 1     (50) TSRSDVGYGVYDLYDLGEFNQKGTVRTKYGTKAQYLQAIQAAHAAGMQVY
SEQID No 10     (51) ASQNDVGYGAYDLYDLGEFNQKGTIRTKYGTRNQLQAAVNALKSNGIQVY
  Consensus 5   (51)  S  DVGYG YDLYDLGEFNQKGTIRTKYGTK Q   AINA  A GIQVY
                   101                                                 150
 SEQID No 1    (100) ADVVFDHKGGADGTEWVDAVEVNPSDRNQEISGTYQIQAWTKFDFPGRGN
SEQID No 10    (101) GDVVMNHKGGADATEMVRAVEVNPNNRNQEVSGEYTIEAWTKFDFPGRGN
  Consensus 5  (101) ADVV  HKGGADATE V AVEVNP  RNQEISG Y I AWTKFDFPGRGN
                   151                                                 200
 SEQID No 1    (150) TYSSFKWRWYHFDGVDWDESRKLS-RIYKFRGIGKAWDWEVDTENGNYDY
SEQID No 10    (151) THSNFKWRWYHFDGVDWDQSRKLNNRIYKFRGDGKGWDWEVDTENGNYDY
  Consensus 5  (151) THS FKWRWYHFDGVDWD SRKL  RIYKFRG GKAWDWEVDTENGNYDY
                   201                                                 250
 SEQID No 1    (199) LMYADLDMDHPEVVTELKNWGKWYVNTTNIDGFRLDAVKHIKFSFFPDWL
SEQID No 10    (201) LMYADIDMDHPEVVNELRNWGVWYTNTLGLDGFRIDAVKHIKYSFTRDWI
  Consensus 5  (201) LMYADIDMDHPEVV ELKNWG WY NT  IDGFRIDAVKHIKFSF  DWI
                   251                                                 300
 SEQID No 1    (249) SYVRSQTGKPLFTVGEYWSYDINKLHNYITKTNGTMSLFDAPLHNKFYTA
SEQID No 10    (251) NHVRSATGKNMFAVAEFWKNDLGAIENYLNKTNWNHSVFDVPLHYNLYNA
  Consensus 5  (251) HVRS TGK LF VAEFW  DI  I NYI KTN   SLFD PLH   Y A
                   301                                                 350
 SEQID No 1    (299) SKSGGAFDMRTLMTNTLMKDQPTLAVTFVDNHDTEPGQALQSWVDPWFKP
SEQID No 10    (301) SKSGGNYDMRQIFNGTVVQRHPMHAVTFVDNHDSQPEEALESFVEEWFKP
  Consensus 5  (301) SKSGG FDMR I   TLM   P  AVTFVDNHDS P  AL SFVD WFKP
                   351                                                 400
 SEQID No 1    (349) LAYAFILTRQEGYPCVFYGDYYGIPQYNIPSLKSKIDPLLIARRDYAYGT
SEQID No 10    (351) LAYALTLTREQGYPSVFYGDYYGIPTHGVPAMKSKIDPILEARQKYAYGR
  Consensus 5  (351) LAYA  LTR  GYP VFYGDYYGIP H IPALKSKIDPIL AR  YAYG
                   401                                                 450
 SEQID No 1    (399) QHDYLDHSDIIGWTREGVTEKPGSGLAALITDGPGGSKWMYVGKQHAGKV
SEQID No 10    (401) QNDYLDHHNIIGWTREGNTAHPNSGLATIMSDGAGGNKWMFVGRNKAGQV
  Consensus 5  (401) Q DYLDH  IIGWTREG T  P SGLA IISDG GG KWMFVGKN AG V
                   451                                                 500
 SEQID No 1    (449) FYDLTGNRSDTVTINSDGWGEFKVNGGSVSVWVPRKTTVSTIARPITTRP
SEQID No 10    (451) WTDITGNRAGTVTINADGWGNFSVNGGSVSIWVNK---------------
  Consensus 5  (451) F DITGNRA TVTINADGWG F VNGGSVSIWV K
                   501           517
 SEQID No 1    (499) WTGEFVRWTEPRLVAWP
SEQID No 10    (486) -----------------
  Consensus 5  (501)
```

Figure 4D

```
                    1                                                  50
 SEQID No 1      (1) -AAPFNGTMMQYFEWYLPDDGTLWTKVANEANNLSSLGITALWLPPAYKG
SEQID No 11      (1) HHNGTNGTMMQYFEWHLPNDGNHWNRLRDDASNLRNRGITAIWIPPAWKG
  Consensus 6    (1)      NGTMMQYFEWHLP DG  W KL  DA NL   GITAIWIPPAWKG
                    51                                                 100
 SEQID No 1     (50) TSRSDVGYGVYDLYDLGEFNQKGTVRTKYGTKAQYLQAIQAAHAAGMQVY
SEQID No 11     (51) TSQNDVGYGAYDLYDLGEFNQKGTVRTKYGTRSQLESAIHALKNNGVQVY
  Consensus 6   (51) TS  DVGYG YDLYDLGEFNQKGTVRTKYGTKAQ   AI A    GMQVY
                   101                                                 150
 SEQID No 1    (100) ADVVFDHKGGADGTEWVDAVEVNPSDRNQEISGTYQIQAWTKFDFPGRGN
SEQID No 11    (101) GDVVMNHKGGADATENVLAVEVNPNNRNQEISGDYTIEAWTKFDFPGRGN
  Consensus 6  (101) ADVV  HKGGADATE V AVEVNP  RNQEISG Y I AWTKFDFPGRGN
                   151                                                 200
 SEQID No 1    (150) TYSSFKWRWYHFDGVDWDESR-KLSRIYKFRGIGKAWDWEVDTENGNYDY
SEQID No 11    (151) TYSDFKWRWYHFDGVDWDQSRQFQNRIYKFRGDGKAWDWEVDSENGNYDY
  Consensus 6  (151) TYS FKWRWYHFDGVDWD SR    RIYKFRG GKAWDWEVDSENGNYDY
                   201                                                 250
 SEQID No 1    (199) LMYADLDMDHPEVVTELKNWGKWYVNTTNIDGFRLDAVKHIKFSFFPDWL
SEQID No 11    (201) LMYADVDMDHPEVVNELRRWGEWYTNTLNLDGFRIDAVKHIKYSFTRDWL
  Consensus 6  (201) LMYADLDMDHPEVV ELK WG WY NT NIDGFRIDAVKHIKFSF  DWL
                   251                                                 300
 SEQID No 1    (249) SYVRSQTGKPLFTVGEYWSYDINKLHNYITKTNGTMSLFDAPLHNKFYTA
SEQID No 11    (251) THVRNATGKEMFAVAEFWKNDLGALENYLNKTNWNHSVFDVPLHYNLYNA
  Consensus 6  (251) SHVR  TGK LF VAEFW  DI  L NYI KTN   SLFD PLH   Y A
                   301                                                 350
 SEQID No 1    (299) SKSGGAFDMRTLMTNTLMKDQPTLAVTFVDNHDTEPGQALQSWVDPWFKP
SEQID No 11    (301) SNSGGNYDMAKLLNGTVVQKHPMHAVTFVDNHDSQPGESLESFVQEWFKP
  Consensus 6  (301) S SGG FDM  LL  TLM   P  AVTFVDNHDS PG AL SFV  WFKP
                   351                                                 400
 SEQID No 1    (349) LAYAFILTRQEGYPCVFYGDYYGIPQYNIPSLKSKIDPLLIARRDYAYGT
SEQID No 11    (351) LAYALILTREQGYPSVFYGDYYGIPTHSVPAMKAKIDPILEARQNFAYGT
  Consensus 6  (351) LAYA ILTR  GYP VFYGDYYGIP H IPALKAKIDPIL AR  FAYGT
                   401                                                 450
 SEQID No 1    (399) QHDYLDHSDIIGWTREGVTEKPGSGLAALITDGPGGSKWMYVGKQHAGKV
SEQID No 11    (401) QHDYFDHHNIIGWTREGNTTHPNSGLATIMSDGPGGEKWMYVGQNKAGQV
  Consensus 6  (401) QHDY DH  IIGWTREG T  P SGLA IISDGPGG KWMYVG N AG V
                   451                                                 500
 SEQID No 1    (449) FYDLTGNRSDTVTINSDGWGEFKVNGGSVSVWVPRKTTVSTIARPITTRP
SEQID No 11    (451) WHDITGNKPGTVTINADGWANFSVNGGSVSIWVKR----------------
  Consensus 6  (451) FHDITGNK  TVTINADGWA F VNGGSVSIWV R
                   501          517
 SEQID No 1    (499) WTGEFVRWTEPRLVAWP
SEQID No 11    (486) -----------------
  Consensus 6  (501)
```

Figure 4E

```
                    1                                                  50
 SEQID No 1      (1) -AAPFNGTMMQYFEWYLPDDGTLWTKVANEANNLSSLGITALWLPPAYKG
SEQID No 12      (1) HHNGTNGTMMQYFEWHLPNDGNHWNRLRDDAANLKSKGITAVWIPPAWKG
  Consensus 7    (1)      NGTMMQYFEWHLP DG  W KL  DA NL S GITALWIPPAWKG
                    51                                                 100
 SEQID No 1     (50) TSRSDVGYGVYDLYDLGEFNQKGTVRTKYGTKAQYLQAIQAAHAAGMQVY
SEQID No 12     (51) TSQNDVGYGAYDLYDLGEFNQKGTVRTKYGTRSQLQGAVTSLKNNGIQVY
  Consensus 7   (51) TS  DVGYG YDLYDLGEFNQKGTVRTKYGTKAQ   AI A    GIQVY
                   101                                                 150
 SEQID No 1    (100) ADVVFDHKGGADGTEWVDAVEVNPSDRNQEISGTYQIQAWTKFDFPGRGN
SEQID No 12    (101) GDVVMNHKGGADGTEMVNAVEVNRSNRNQEISGEYTIEAWTKFDFPGRGN
  Consensus 7  (101) ADVV  HKGGADGTE V AVEVN S RNQEISG Y I AWTKFDFPGRGN
                   151                                                 200
 SEQID No 1    (150) TYSSFKWRWYHFDGVDWDESR-KLSRIYKFRGIGKAWDWEVDTENGNYDY
SEQID No 12    (151) THSNFKWRWYHFDGTDWDQSRQLQNKIYKFRGTGKAWDWEVDIENGNYDY
  Consensus 7  (151) THS FKWRWYHFDG DWD SR    KIYKFRG GKAWDWEVD ENGNYDY
                   201                                                 250
 SEQID No 1    (199) LMYADLDMDHPEVVTELKNWGKWYVNTTNIDGFRLDAVKHIKFSFFPDWL
SEQID No 12    (201) LMYADIDMDHPEVINELRNWGVWYTNTLNLDGFRIDAVKHIKYSYTRDWL
  Consensus 7  (201) LMYADIDMDHPEVI ELKNWG WY NT NIDGFRIDAVKHIKFSF  DWL
                   251                                                 300
 SEQID No 1    (249) SYVRSQTGKPLFTVGEYWSYDINKLHNYITKTNGTMSLFDAPLHNKFYTA
SEQID No 12    (251) THVRNTTGKPMFAVAEFWKNDLAAIENYLNKTSWNHSVFDVPLHYNLYNA
  Consensus 7  (251) SHVR  TGKPLF VAEFW  DI  I NYI KT    SLFD PLH   Y A
                   301                                                 350
 SEQID No 1    (299) SKSGGAFDMRTLMTNTLMKDQPTLAVTFVDNHDTEPGQALQSWVDPWFKP
SEQID No 12    (301) SNSGGYFDMRNILNGSVVQKHPIHAVTFVDNHDSQPGEALESFVQSWFKP
  Consensus 7  (301) S SGG FDMR IL  SLM   P  AVTFVDNHDS PG AL SFV  WFKP
                   351                                                 400
 SEQID No 1    (349) LAYAFILTRQEGYPCVFYGDYYGIPQYNIPSLKSKIDPLLIARRDYAYGT
SEQID No 12    (351) LAYALILTREQGYPSVFYGDYYGIPTHGVPSMKSKIDPLLQARQTYAYGT
  Consensus 7  (351) LAYA ILTR  GYP VFYGDYYGIP H IPSLKSKIDPLL AR  YAYGT
                   401                                                 450
 SEQID No 1    (399) QHDYLDHSDIIGWTREGVTEKPGSGLAALITDGPGGSKWMYVGKQHAGKV
SEQID No 12    (401) QHDYFDHHDIIGWTREGDSSHPNSGLATIMSDGPGGNKWMYVGKHKAGQV
  Consensus 7  (401) QHDY DH DIIGWTREG S  P SGLA IISDGPGG KWMYVGK  AG V
                   451                                                 500
 SEQID No 1    (449) FYDLTGNRSDTVTINSDGWGEFKVNGGSVSVWVPRKTTVSTIARPITTRP
SEQID No 12    (451) WRDITGNRSGTVTINADGWGNFTVNGGAVSVWVKQ---------------
  Consensus 7  (451) F DITGNRS TVTINADGWG F VNGGAVSVWV
                   501           517
 SEQID No 1    (499) WTGEFVRWTEPRLVAWP
SEQID No 12    (486) -----------------
  Consensus 7  (501)
```

Figure 4F

```
                    1                                                  50
 SEQID No 1      (1) AAPFNGTMMQYFEWYLPDDGTLWTKVANEANNLSSLGITALWLPPAYKGT
SEQID No 13      (1) -DGLNGTMMQYYEWHLENDGQHWNRLHDDAAALSDAGITAIWIPPAYKGN
  Consensus 8    (1)     NGTMMQYFEWHL  DG  W KL  DA  LS  GITAIWIPPAYKG
                    51                                                 100
 SEQID No 1     (51) SRSDVGYGVYDLYDLGEFNQKGTVRTKYGTKAQYLQAIQAAHAAGMQVYA
SEQID No 13     (50) SQADVGYGAYDLYDLGEFNQKGTVRTKYGTKAQLERAIGSLKSNDINVYG
  Consensus 8   (51) S ADVGYG YDLYDLGEFNQKGTVRTKYGTKAQ   AI A  A  INVYA
                   101                                                 150
 SEQID No 1    (101) DVVFDHKGGADGTEWVDAVEVNPSDRNQEISGTYQIQAWTKFDFPGRGNT
SEQID No 13    (100) DVVMNHKMGADFTEAVQAVQVNPTNRWQDISGAYTIDAWTGFDFSGRNNA
  Consensus 8  (101) DVV  HK GAD TE V AV VNPS R QDISG Y I AWT FDF GR N
                   151                                                 200
 SEQID No 1    (151) YSSFKWRWYHFDGVDWDESRKLSRIYKFRGIGKAWDWEVDTENGNYDYLM
SEQID No 13    (150) YSDFKWRWFHFNGVDWDQRYQENHIFRFANTN--WNWRVDEENGNYDYLL
  Consensus 8  (151) YS FKWRWFHF GVDWD       IFKF      W W VD ENGNYDYLL
                   201                                                 250
 SEQID No 1    (201) YADLDMDHPEVVTELKNWGKWYVNTTNIDGFRLDAVKHIKFSFFPDWLSY
SEQID No 13    (198) GSNIDFSHPEVQDELKDWGSWFTDELDLDGYRLDAIKHIPFWYTSDWVRH
  Consensus 8  (201)  A ID  HPEV  ELK WG WF     IDGFRLDAIKHI F F  DWL H
                   251                                                 300
 SEQID No 1    (251) VRSQTGKPLFTVGEYWSYDINKLHNYITKTNGTMSLFDAPLHNKFYTASK
SEQID No 13    (248) QRNEADQDLFVVGEYWKDDVGALEFYLDEMNWEMSLFDVPLNYNFYRASQ
  Consensus 8  (251)  R      LF VGEYW  DI  L  YI   N  MSLFD PL   FY AS
                   301                                                 350
 SEQID No 1    (301) SGGAFDMRTLMTNTLMKDQPTLAVTFVDNHDTEPGQALQSWVDPWFKPLA
SEQID No 13    (298) QGGSYDMRNILRGSLVEAHPMHAVTFVDNHDTQPGESLESWVADWFKPLA
  Consensus 8  (301)  GGAFDMR IL  SLM   P  AVTFVDNHDT PG AL SWV  WFKPLA
                   351                                                 400
 SEQID No 1    (351) YAFILTRQEGYPCVFYGDYYGIPQYNIPSLKSKIDPLLIARRDYAYGTQH
SEQID No 13    (348) YATILTREGGYPNVFYGDYYGIPNDNISAKKDMIDELLDARQNYAYGTQH
  Consensus 8  (351) YA ILTR  GYP VFYGDYYGIPN NI A K  ID LL AR  YAYGTQH
                   401                                                 450
 SEQID No 1    (401) DYLDHSDIIGWTREGVTEKPGSGLAALITDGPGGSKWMYVGKQHAGKVFY
SEQID No 13    (398) DYFDHWDVVGWTREGSSSRPNSGLATIMSNGPGGSKWMYVGRQNAGQTWT
  Consensus 8  (401) DY DH DIIGWTREG S KP SGLA IIS GPGGSKWMYVGKQ AG  F
                   451                                                 500
 SEQID No 1    (451) DLTGNRSDTVTINSDGWGEFKVNGGSVSVWVPRKTTVSTIARPITTRPWT
SEQID No 13    (448) DLTGNNGASVTINGDGWGEFFTNGGSVSVYVNQ----------------
  Consensus 8  (451) DLTGN   SVTIN DGWGEF  NGGSVSVWV
                   501         515
 SEQID No 1    (501) GEFVRWTEPRLVAWP
SEQID No 13    (481) ---------------
  Consensus 8  (501)
```

Figure 4G

```
                    1                                                  50
 SEQID No 1      (1) AAPFNGTMMQYFEWYLPDDGTLWTKVANEANNLSSLGITALWLPPAYKGT
SEQID No 14      (1) -DGLNGTMMQYYEWHLENDGQHWNRLHDDAEALSNAGITAIWIPPAYKGN
  Consensus 9    (1)     NGTMMQYFEWHL  DG  W KL  DA  LS  GITAIWIPPAYKG
                    51                                                 100
 SEQID No 1     (51) SRSDVGYGVYDLYDLGEFNQKGTVRTKYGTKAQYLQAIQAAHAAGMQVYA
SEQID No 14     (50) SQADVGYGAYDLYDLGEFNQKGTVRTKYGTKAQLERAIGSLKSNDINVYG
  Consensus 9   (51) S ADVGYG YDLYDLGEFNQKGTVRTKYGTKAQ   AI A  A  INVYA
                   101                                                 150
 SEQID No 1    (101) DVVFDHKGGADGTEWVDAVEVNPSDRNQEISGTYQIQAWTKFDFPGRGNT
SEQID No 14    (100) DVVMNHKLGADFTEAVQAVQVNPSNRWQDISGVYTIDAWTGFDFPGRNNA
  Consensus 9  (101) DVV  HK GAD TE V AV VNPS R QDISG Y I AWT FDFPGR N
                   151                                                 200
 SEQID No 1    (151) YSSFKWRWYHFDGVDWDESRKLSRIYKFRGIGKAWDWEVDTENGNYDYLM
SEQID No 14    (150) YSDFKWRWFHFNGVDWDQRYQENHLFRFANTN--WNWRVDEENGNYDYLL
  Consensus 9  (151) YS FKWRWFHF GVDWD       IFKF      W W VD ENGNYDYLL
                   201                                                 250
 SEQID No 1    (201) YADLDMDHPEVVTELKNWGKWYVNTTNIDGFRLDAVKHIKFSFFPDWLSY
SEQID No 14    (198) GSNIDFSHPEVQEELKDWGSWFTDELDLDGYRLDAIKHIPFWYTSDWVRH
  Consensus 9  (201)  A ID  HPEV  ELK WG WF     IDGFRLDAIKHI F F  DWL H
                   251                                                 300
 SEQID No 1    (251) VRSQTGKPLFTVGEYWSYDINKLHNYITKTNGTMSLFDAPLHNKFYTASK
SEQID No 14    (248) QRSEADQDLFVVGEYWKDDVGALEFYLDEMNWEMSLFDVPLNYNFYRASK
  Consensus 9  (251)  RS     LF VGEYW  DI  L  YI   N  MSLFD PL   FY ASK
                   301                                                 350
 SEQID No 1    (301) SGGAFDMRTLMTNTLMKDQPTLAVTFVDNHDTEPGQALQSWVDPWFKPLA
SEQID No 14    (298) QGGSYDMRNILRGSLVEAHPIHAVTFVDNHDTQPGESLESWVADWFKPLA
  Consensus 9  (301)  GGAFDMR IL  SLM   P  AVTFVDNHDT PG AL SWV  WFKPLA
                   351                                                 400
 SEQID No 1    (351) YAFILTRQEGYPCVFYGDYYGIPQYNIPSLKSKIDPLLIARRDYAYGTQH
SEQID No 14    (348) YATILTREGGYPNVFYGDYYGIPNDNISAKKDMIDELLDARQNYAYGTQH
  Consensus 9  (351) YA ILTR  GYP VFYGDYYGIPN NI A K  ID LL AR  YAYGTQH
                   401                                                 450
 SEQID No 1    (401) DYLDHSDIIGWTREGVTEKPGSGLAALITDGPGGSKWMYVGKQHAGKVFY
SEQID No 14    (398) DYFDHWDIVGWTREGTSSRPNSGLATIMSNGPGGSKWMYVGQQHAGQTWT
  Consensus 9  (401) DY DH DIIGWTREG S KP SGLA IIS GPGGSKWMYVG QHAG  F
                   451                                                 500
 SEQID No 1    (451) DLTGNRSDTVTINSDGWGEFKVNGGSVSVWVPRKTTVSTIARPITTRPWT
SEQID No 14    (448) DLTGNHAASVTINGDGWGEFFTNGGSVSVYVNQ----------------
  Consensus 9  (451) DLTGN A SVTIN DGWGEF  NGGSVSVWV
                   501         515
 SEQID No 1    (501) GEFVRWTEPRLVAWP
SEQID No 14    (481) ---------------
  Consensus 9  (501)
```

Figure 4H

```
                    1                                                  50
 SEQID No 1      (1) AAPFNGTMMQYFEWYLPDDGTLWTKVANEANNLSSLGITALWLPPAYKGT
SEQID No 15      (1) AAPFNGTMMQYFEWYLPDDGTLWTKVANEANNLSSLGITALWLPPAYKGT
 Consensus 10    (1) AAPFNGTMMQYFEWYLPDDGTLWTKVANEANNLSSLGITALWLPPAYKGT
                    51                                                 100
 SEQID No 1     (51) SRSDVGYGVYDLYDLGEFNQKGTVRTKYGTKAQYLQAIQAAHAAGMQVYA
SEQID No 15     (51) SRSDVGYGVYDLYDLGEFNQKGTVRTKYGTKAQYLQAIQAAHAAGMQVYA
 Consensus 10   (51) SRSDVGYGVYDLYDLGEFNQKGTVRTKYGTKAQYLQAIQAAHAAGMQVYA
                   101                                                 150
 SEQID No 1    (101) DVVFDHKGGADGTEWVDAVEVNPSDRNQEISGTYQIQAWTKFDFPGRGNT
SEQID No 15    (101) DVVFDHKGGADGTEWVDAVEVNPSDRNQEISGTYQIQAWTKFDFPGRGNT
 Consensus 10  (101) DVVFDHKGGADGTEWVDAVEVNPSDRNQEISGTYQIQAWTKFDFPGRGNT
                   151                                                 200
 SEQID No 1    (151) YSSFKWRWYHFDGVDWDESRKLSRIYKFRGIGKAWDWEVDTENGNYDYLM
SEQID No 15    (151) YSSFKWRWYHFDGVDWDESRKLSRIYKFR--GKAWDWEVDTEFGNYDYLM
 Consensus 10  (151) YSSFKWRWYHFDGVDWDESRKLSRIYKFR  GKAWDWEVDTE GNYDYLM
                   201                                                 250
 SEQID No 1    (201) YADLDMDHPEVVTELKNWGKWYVNTTNIDGFRLDAVKHIKFSFFPDWLSY
SEQID No 15    (199) YADLDMDHPEVVTELKNWGKWYVNTTNIDGFRLDAVKHIKFSFFPDWLSY
 Consensus 10  (201) YADLDMDHPEVVTELKNWGKWYVNTTNIDGFRLDAVKHIKFSFFPDWLSY
                   251                                                 300
 SEQID No 1    (251) VRSQTGKPLFTVGEYWSYDINKLHNYITKTNGTMSLFDAPLHNKFYTASK
SEQID No 15    (249) VRSQTGKPLFTVGEYWSYDINKLHNYITKTNGTMSLFDAPLHNKFYTASK
 Consensus 10  (251) VRSQTGKPLFTVGEYWSYDINKLHNYITKTNGTMSLFDAPLHNKFYTASK
                   301                                                 350
 SEQID No 1    (301) SGGAFDMRTLMTNTLMKDQPTLAVTFVDNHDTEPGQALQSWVDPWFKPLA
SEQID No 15    (299) SGGAFDMRTLMTNTLMKDQPTLAVTFVDNHDTEPGQALQSWVDPWFKPLA
 Consensus 10  (301) SGGAFDMRTLMTNTLMKDQPTLAVTFVDNHDTEPGQALQSWVDPWFKPLA
                   351                                                 400
 SEQID No 1    (351) YAFILTRQEGYPCVFYGDYYGIPQYNIPSLKSKIDPLLIARRDYAYGTQH
SEQID No 15    (349) YAFILTRQEGYPCVFYGDYYGIPQYNIPSLKSKIDPLLIARRDYAYGTQH
 Consensus 10  (351) YAFILTRQEGYPCVFYGDYYGIPQYNIPSLKSKIDPLLIARRDYAYGTQH
                   401                                                 450
 SEQID No 1    (401) DYLDHSDIIGWTREGVTEKPGSGLAALITDGPGGSKWMYVGKQHAGKVFY
SEQID No 15    (399) DYLDHSDIIGWTREGGTEKPGSGLAALITDGPGGSKWMYVGKQHAGKVFY
 Consensus 10  (401) DYLDHSDIIGWTREG TEKPGSGLAALITDGPGGSKWMYVGKQHAGKVFY
                   451                                                 500
 SEQID No 1    (451) DLTGNRSDTVTINSDGWGEFKVNGGSVSVWVPRKTTVSTIARPITTRPWT
SEQID No 15    (449) DLTGNRSDTVTINSDGWGEFKVNGGSVSVWVPRKTTVS------------
 Consensus 10  (451) DLTGNRSDTVTINSDGWGEFKVNGGSVSVWVPRKTTVS
                   501         515
 SEQID No 1    (501) GEFVRWTEPRLVAWP
SEQID No 15    (487) ---------------
 Consensus 10  (501)
```

Figure 4I

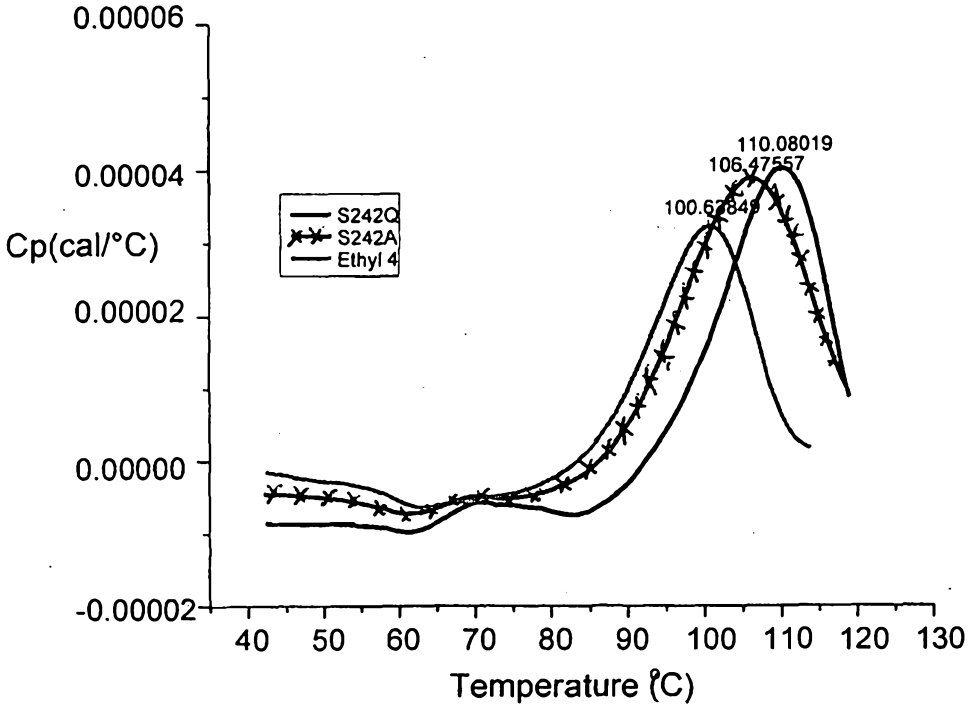
0.00006
0.00004
0.00002
0.00000
-0.00002
Cp(cal/°C)
S242Q
S242A
Ethyl 4
110.08019
106.47557
100.62849
40
50
60
70
80
90
100
110
120
130
Temperature (°C)

Figure 5

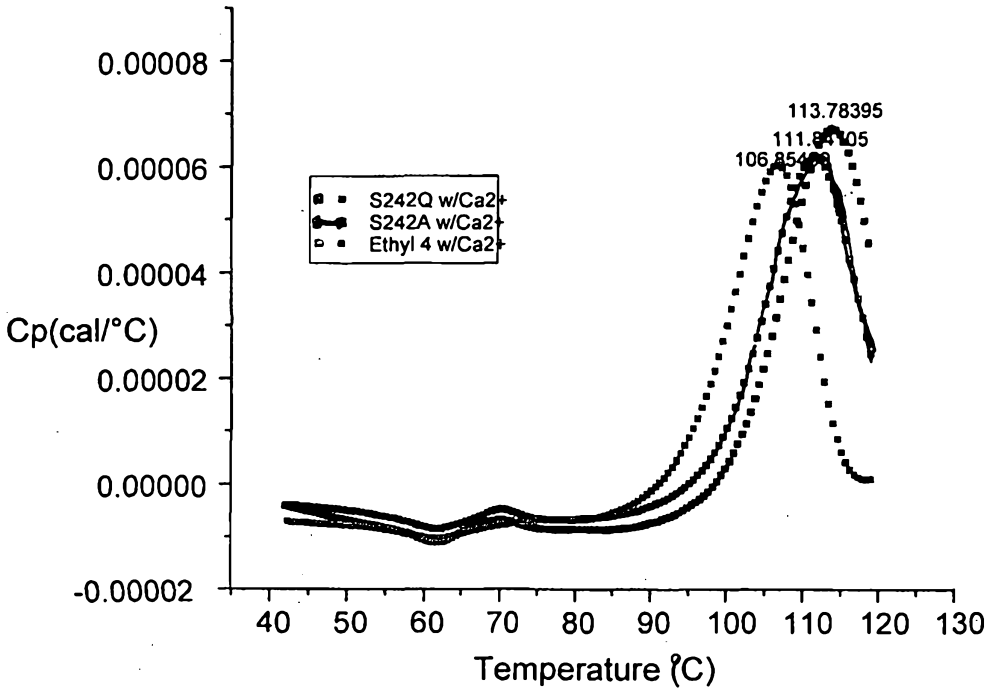
0.00008
0.00006
0.00004
0.00002
0.00000
-0.00002
Cp(cal/°C)
40
50
60
70
80
90
100
110
120
130
Temperature (°C)
S242Q w/Ca2+
S242A w/Ca2+
Ethyl 4 w/Ca2+
113.78395
111.84105
106.85409

Figure 6

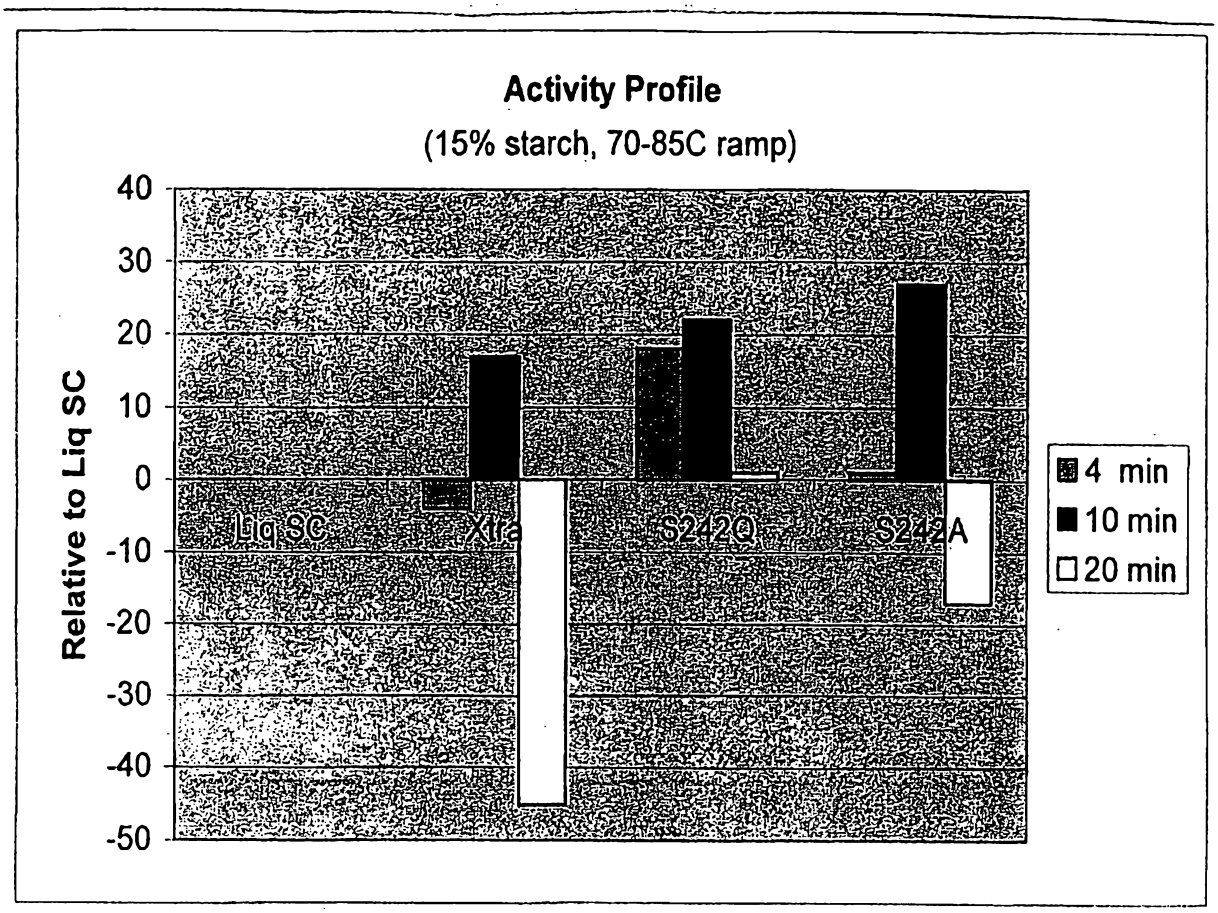
Activity Profile
(15% starch, 70-85C ramp)
Relative to Liq SC
40
30
20
10
0
-10
-20
-30
-40
-50
Liq SC
Xtra
S242Q
S242A
4 min
10 min
20 min

Figure 7

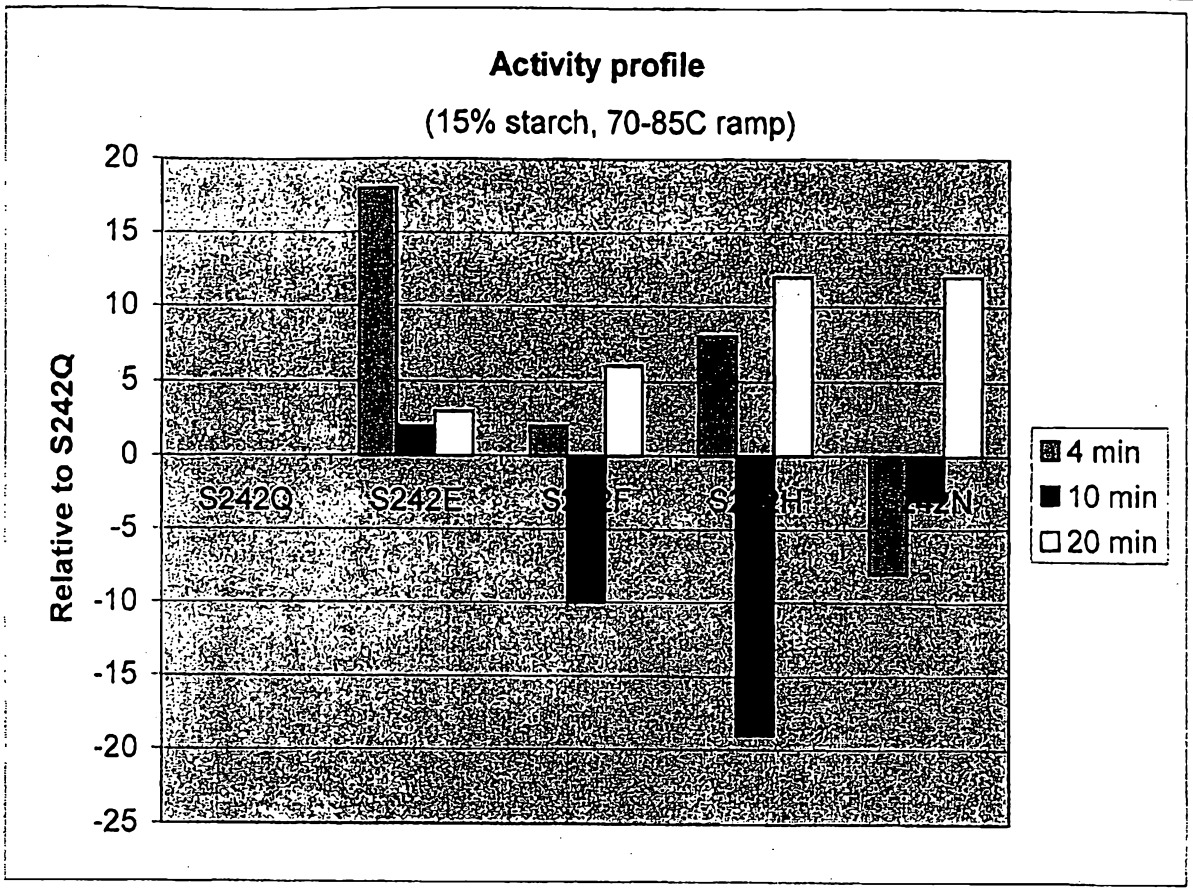
Activity profile
(15% starch, 70-85C ramp)
Relative to S242Q
20
15
10
5
0
-5
-10
-15
-20
-25
S242Q
S242E
4 min
10 min
20 min

Figure 8

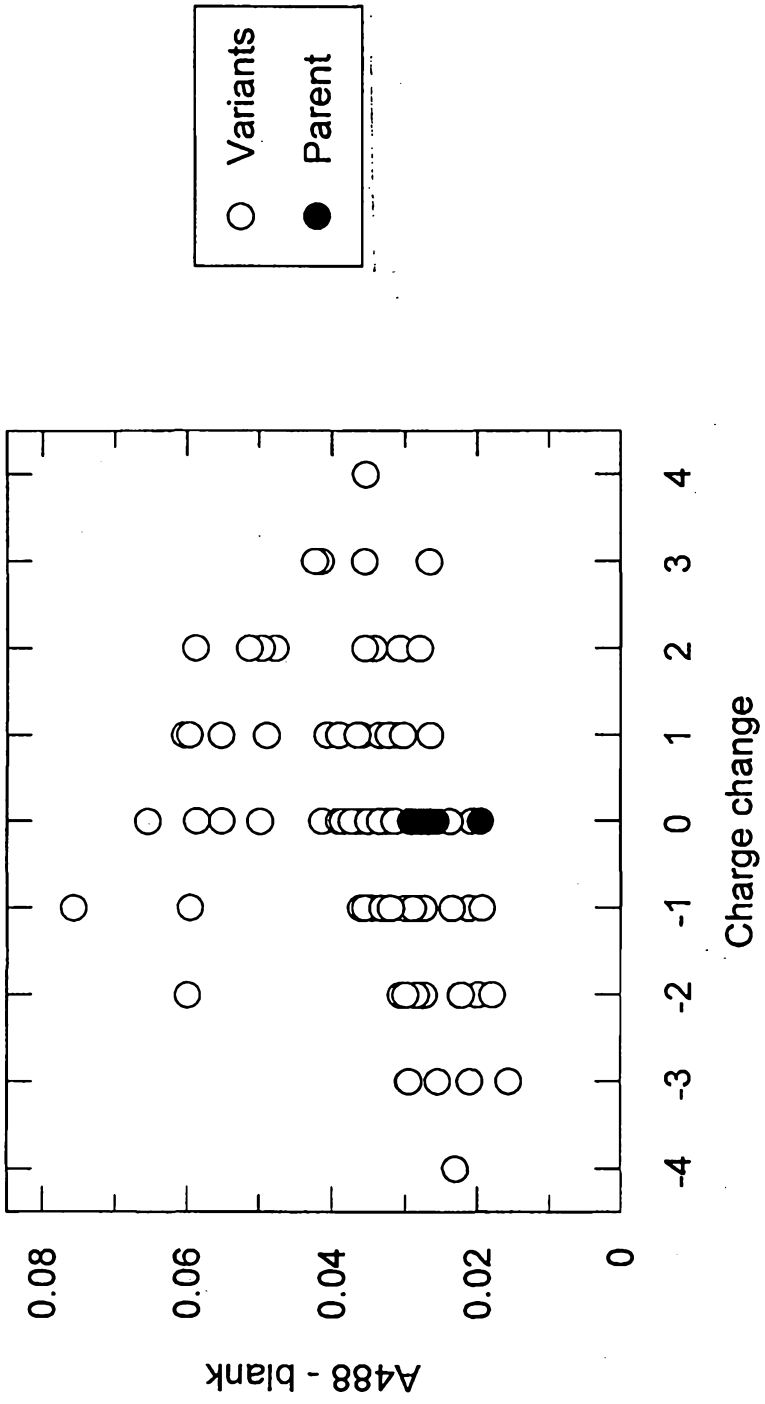
Variants
Parent
A488 - blank
0.08
0.06
0.04
0.02
0
Charge change
-4
-3
-2
-1
0
1
2
3
4

Figure 9

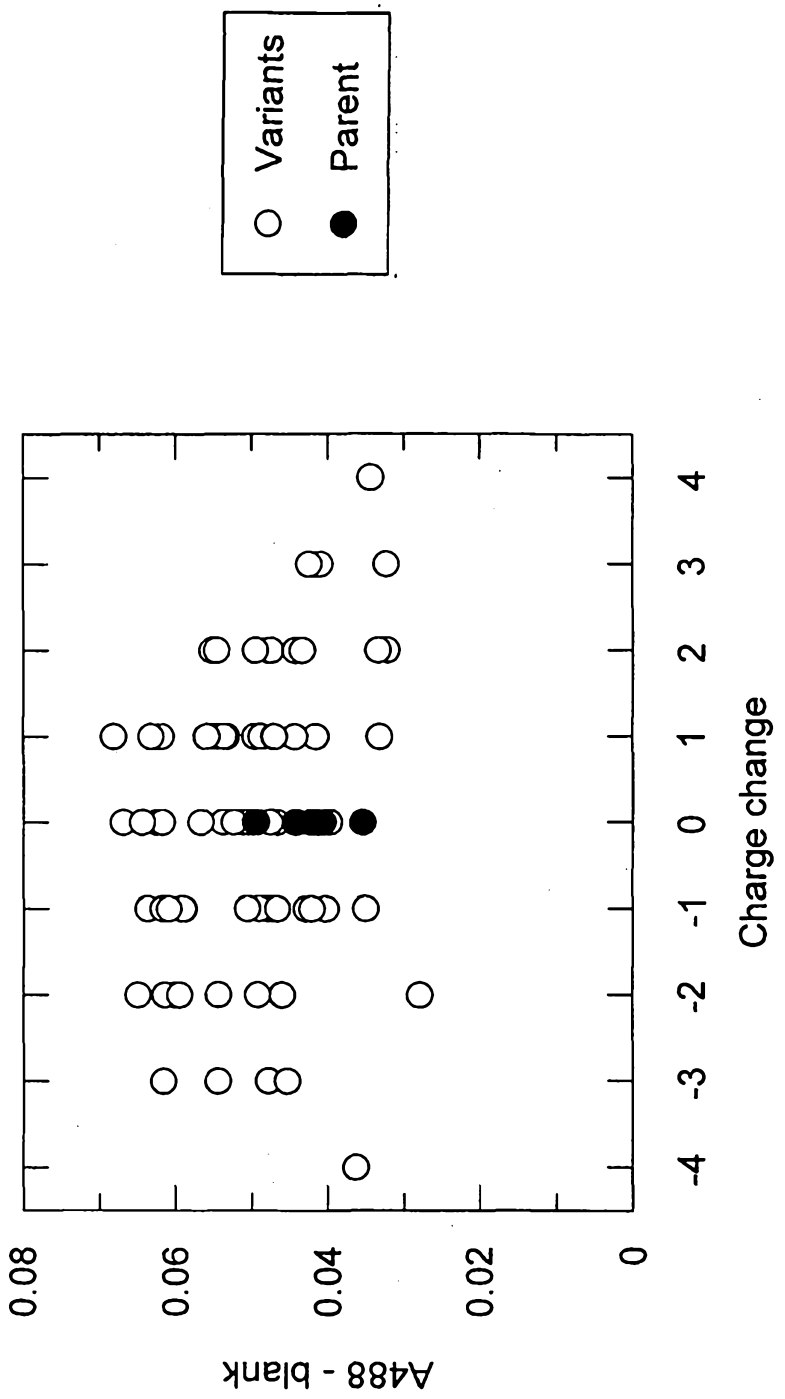
Variants
Parent
A488 - blank
0.08
0.06
0.04
0.02
0
Charge change
-4
-3
-2
-1
0
1
2
3
4

Figure 10

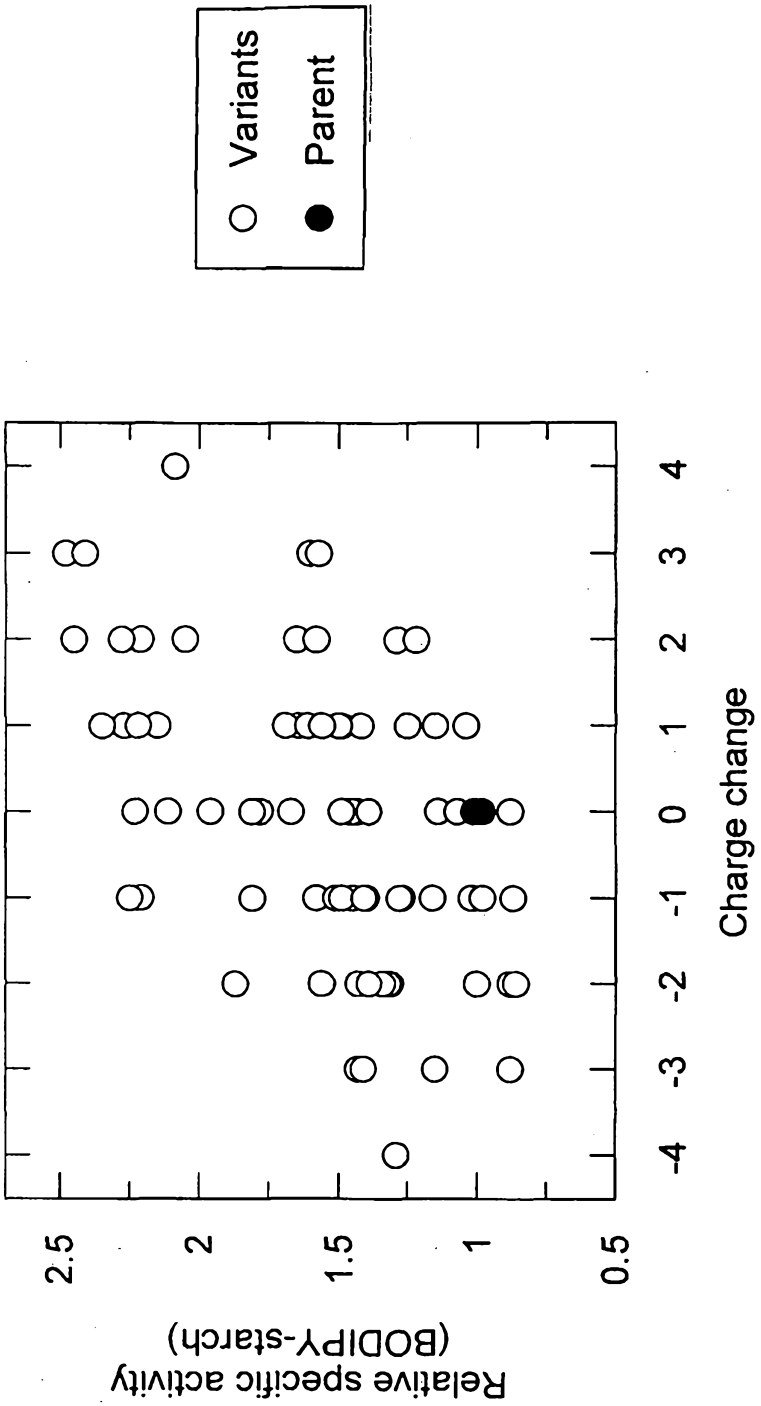
Variants
Parent
Relative specific activity
(BODIPY-starch)
2.5
2
1.5
1
0.5
Charge change
-4
-3
-2
-1
0
1
2
3
4

Figure 11

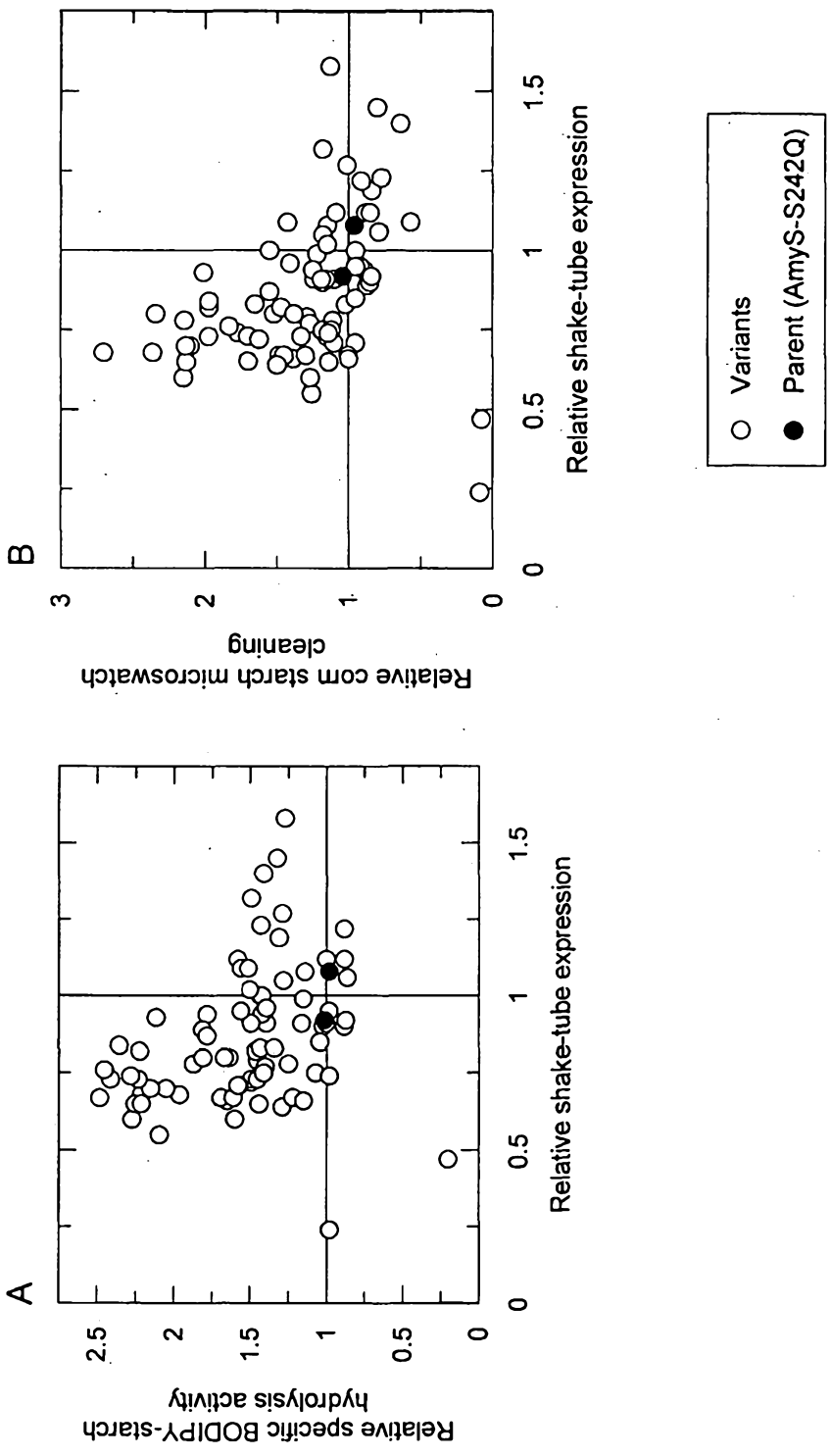
A
Relative specific BODIPY-starch hydrolysis activity
Relative shake-tube expression
B
Relative corn starch microswatch cleaning
Relative shake-tube expression
Variants
Parent (AmyS-S242Q)

Figure 12

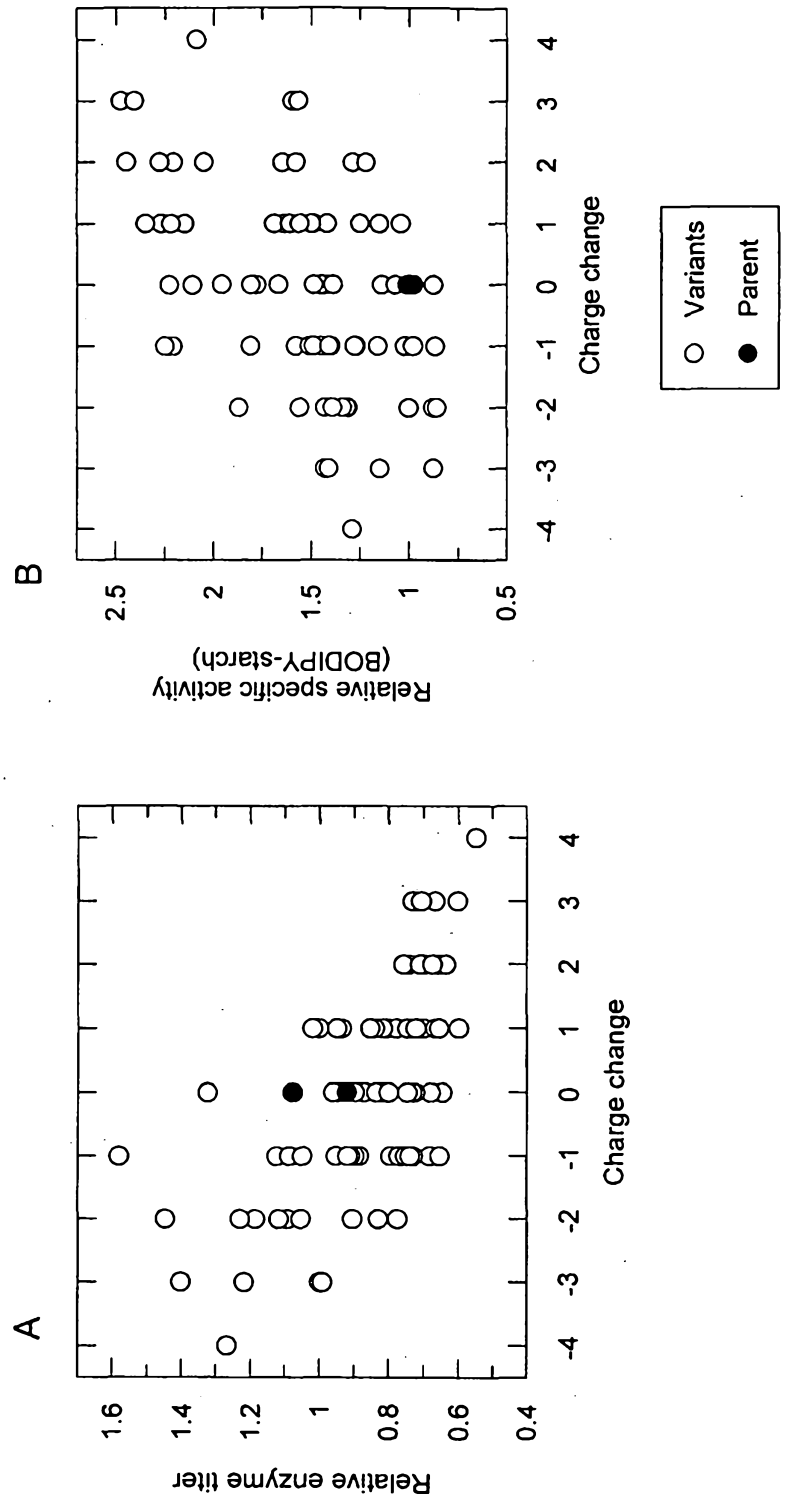
A
Relative enzyme titer
1.6
1.4
1.2
1
0.8
0.6
0.4
-4
-3
-2
-1
0
1
2
3
4
Charge change
B
Relative specific activity
(BODIPY-starch)
2.5
2
1.5
1
0.5
-4
-3
-2
-1
0
1
2
3
4
Charge change
Variants
Parent

Figure 13

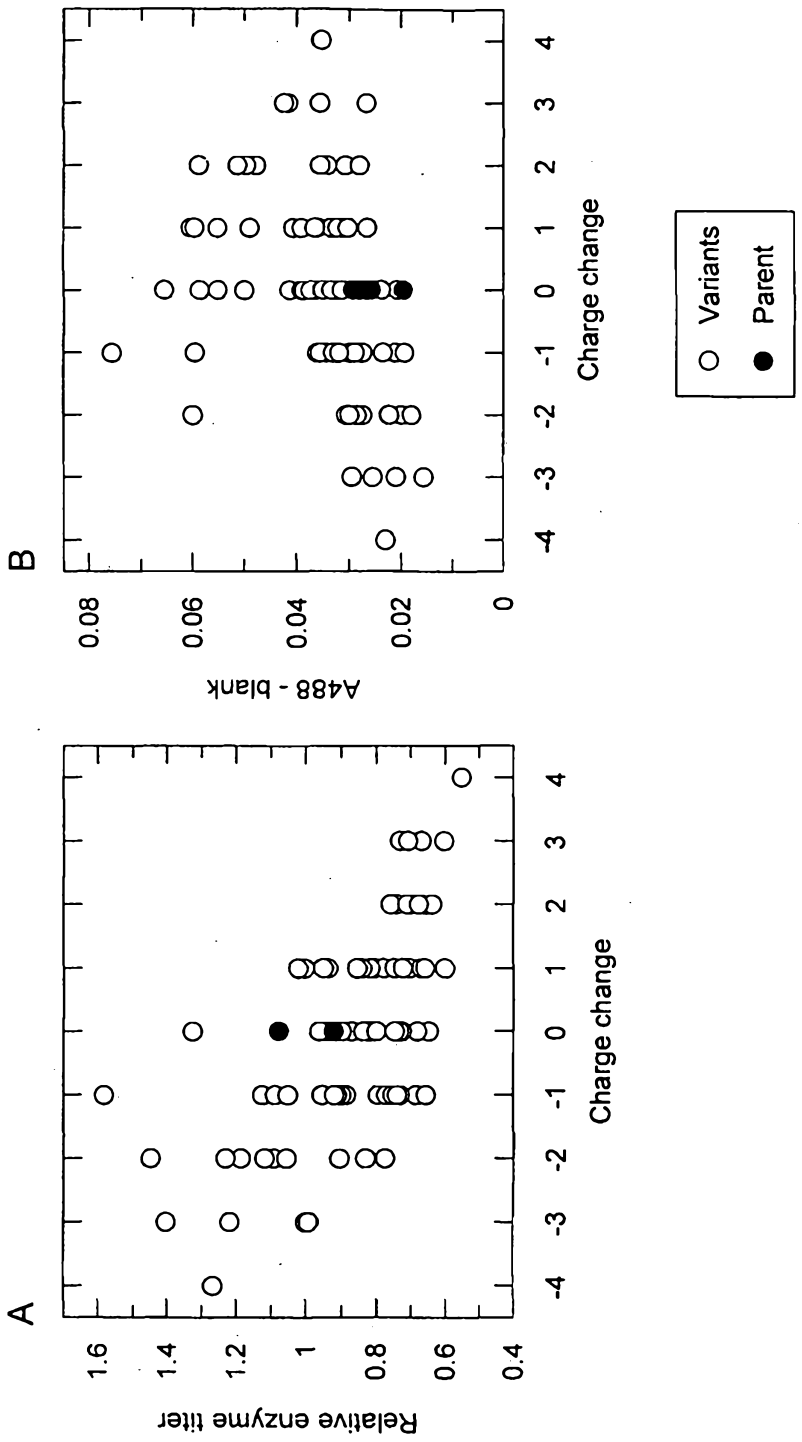
A
B
Relative enzyme titer
A488 - blank
Charge change
Charge change
Variants
Parent

Figure 14

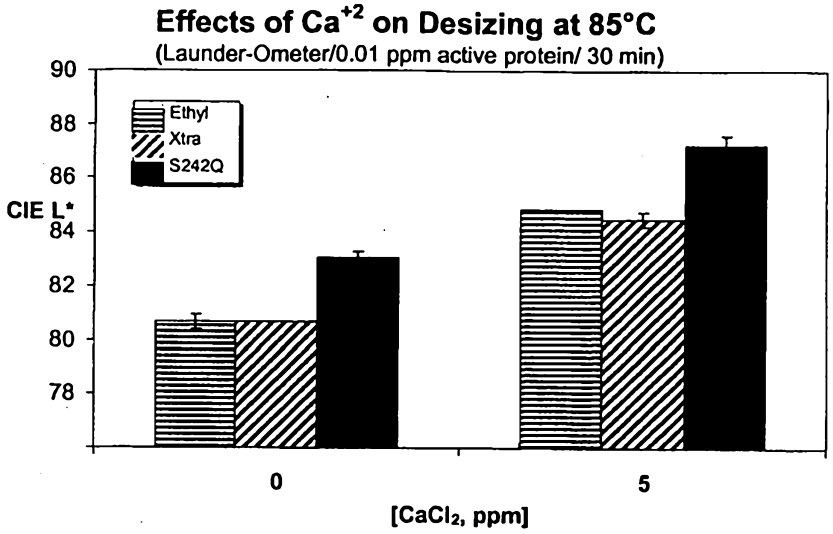
Effects of Ca+2 on Desizing at 85°C
(Launder-Ometer/0.01 ppm active protein/ 30 min)
90
88
86
CIE L*
84
82
80
78
Ethyl
Xtra
S242Q
0
5
[CaCl2, ppm]

Figure 15

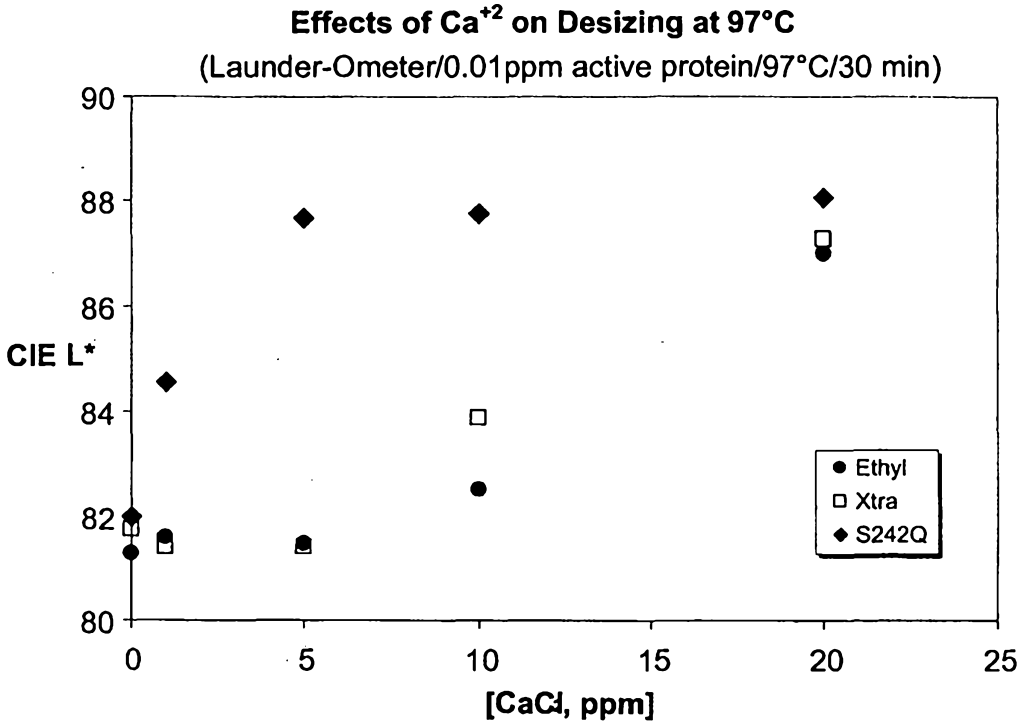
Effects of Ca+2 on Desizing at 97°C
(Launder-Ometer/0.01ppm active protein/97°C/30 min)
CIE L*
90
88
86
84
82
80
0
5
10
15
20
25
[CaCl, ppm]
Ethyl
Xtra
S242Q

Figure 16

2008325249 17 Sep 2013

SEQUENCE LISTING

<110> DANISCO US INC., GENENCOR DIVISION

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<151> 2008-06-06

<150> 61/041,075
<151> 2008-03-31

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<151> 2008-02-06

<150> 60/985,619
<151> 2007-11-05

<160> 30

<170> PatentIn version 3.5

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<212> PRT
<213> Geobacillus stearothermophilus

<400> 1

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1               5                  10                  15

Pro Asp Asp Gly Thr Leu Trp Thr Lys Val Ala Asn Glu Ala Asn Asn
            20                  25                  30

Leu Ser Ser Leu Gly Ile Thr Ala Leu Trp Leu Pro Pro Ala Tyr Lys
        35                  40                  45

Gly Thr Ser Arg Ser Asp Val Gly Tyr Gly Val Tyr Asp Leu Tyr Asp
    50                  55                  60

Leu Gly Glu Phe Asn Gln Lys Gly Thr Val Arg Thr Lys Tyr Gly Thr
65                  70                  75                  80

Lys Ala Gln Tyr Leu Gln Ala Ile Gln Ala Ala His Ala Ala Gly Met
                85                  90                  95
```

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Gln Val Tyr Ala Asp Val Val Phe Asp His Lys Gly Gly Ala Asp Gly
            100                 105                 110

Thr Glu Trp Val Asp Ala Val Glu Val Asn Pro Ser Asp Arg Asn Gln
        115                 120                 125

Glu Ile Ser Gly Thr Tyr Gln Ile Gln Ala Trp Thr Lys Phe Asp Phe
    130                 135                 140

Pro Gly Arg Gly Asn Thr Tyr Ser Ser Phe Lys Trp Arg Trp Tyr His
145                 150                 155                 160

Phe Asp Gly Val Asp Trp Asp Glu Ser Arg Lys Leu Ser Arg Ile Tyr
                165                 170                 175

Lys Phe Arg Gly Ile Gly Lys Ala Trp Asp Trp Glu Val Asp Thr Glu
            180                 185                 190

Asn Gly Asn Tyr Asp Tyr Leu Met Tyr Ala Asp Leu Asp Met Asp His
        195                 200                 205

Pro Glu Val Val Thr Glu Leu Lys Asn Trp Gly Lys Trp Tyr Val Asn
    210                 215                 220

Thr Thr Asn Ile Asp Gly Phe Arg Leu Asp Ala Val Lys His Ile Lys
225                 230                 235                 240

Phe Ser Phe Phe Pro Asp Trp Leu Ser Tyr Val Arg Ser Gln Thr Gly
                245                 250                 255

Lys Pro Leu Phe Thr Val Gly Glu Tyr Trp Ser Tyr Asp Ile Asn Lys
            260                 265                 270

Leu His Asn Tyr Ile Thr Lys Thr Asn Gly Thr Met Ser Leu Phe Asp
        275                 280                 285

Ala Pro Leu His Asn Lys Phe Tyr Thr Ala Ser Lys Ser Gly Gly Ala
    290                 295                 300

Phe Asp Met Arg Thr Leu Met Thr Asn Thr Leu Met Lys Asp Gln Pro
305                 310                 315                 320

Thr Leu Ala Val Thr Phe Val Asp Asn His Asp Thr Glu Pro Gly Gln
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325 330 335

Ala Leu Gln Ser Trp Val Asp Pro Trp Phe Lys Pro Leu Ala Tyr Ala
340 345 350

Phe Ile Leu Thr Arg Gln Glu Gly Tyr Pro Cys Val Phe Tyr Gly Asp
355 360 365

Tyr Tyr Gly Ile Pro Gln Tyr Asn Ile Pro Ser Leu Lys Ser Lys Ile
370 375 380

Asp Pro Leu Leu Ile Ala Arg Arg Asp Tyr Ala Tyr Gly Thr Gln His
385 390 395 400

Asp Tyr Leu Asp His Ser Asp Ile Ile Gly Trp Thr Arg Glu Gly Val
405 410 415

Thr Glu Lys Pro Gly Ser Gly Leu Ala Ala Leu Ile Thr Asp Gly Pro
420 425 430

Gly Gly Ser Lys Trp Met Tyr Val Gly Lys Gln His Ala Gly Lys Val
435 440 445

Phe Tyr Asp Leu Thr Gly Asn Arg Ser Asp Thr Val Thr Ile Asn Ser
450 455 460

Asp Gly Trp Gly Glu Phe Lys Val Asn Gly Gly Ser Val Ser Val Trp
465 470 475 480

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485 490 495

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500 505 510

Ala Trp Pro
515

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1               5                  10                  15

Pro Asp Asp Gly Thr Leu Trp Thr Lys Val Ala Asn Glu Ala Asn Asn
            20                  25                  30

Leu Ser Ser Leu Gly Ile Thr Ala Leu Trp Leu Pro Pro Ala Tyr Lys
        35                  40                  45

Gly Thr Ser Arg Ser Asp Val Gly Tyr Gly Val Tyr Asp Leu Tyr Asp
    50                  55                  60

Leu Gly Glu Phe Asn Gln Lys Gly Thr Val Arg Thr Lys Tyr Gly Thr
65                  70                  75                  80

Lys Ala Gln Tyr Leu Gln Ala Ile Gln Ala Ala His Ala Ala Gly Met
                85                  90                  95

Gln Val Tyr Ala Asp Val Val Phe Asp His Lys Gly Gly Ala Asp Gly
            100                 105                 110

Thr Glu Trp Val Asp Ala Val Glu Val Asn Pro Ser Asp Arg Asn Gln
        115                 120                 125

Glu Ile Ser Gly Thr Tyr Gln Ile Gln Ala Trp Thr Lys Phe Asp Phe
    130                 135                 140

Pro Gly Arg Gly Asn Thr Tyr Ser Ser Phe Lys Trp Arg Trp Tyr His
145                 150                 155                 160

Phe Asp Gly Val Asp Trp Asp Glu Ser Arg Lys Leu Ser Arg Ile Tyr
                165                 170                 175

Lys Phe Arg Gly Ile Gly Lys Ala Trp Asp Trp Glu Val Asp Thr Glu
            180                 185                 190

Asn Gly Asn Tyr Asp Tyr Leu Met Tyr Ala Asp Leu Asp Met Asp His
        195                 200                 205

Pro Glu Val Val Thr Glu Leu Lys Asn Trp Gly Lys Trp Tyr Val Asn
    210                 215                 220

Thr Thr Asn Ile Asp Gly Phe Arg Leu Asp Ala Val Lys His Ile Lys
```

```
225                 230                 235                 240

Phe Ser Phe Phe Pro Asp Trp Leu Ser Tyr Val Arg Ser Gln Thr Gly
                245                 250                 255

Lys Pro Leu Phe Thr Val Gly Glu Tyr Trp Ser Tyr Asp Ile Asn Lys
            260                 265                 270

Leu His Asn Tyr Ile Thr Lys Thr Asn Gly Thr Met Ser Leu Phe Asp
        275                 280                 285

Ala Pro Leu His Asn Lys Phe Tyr Thr Ala Ser Lys Ser Gly Gly Ala
    290                 295                 300

Phe Asp Met Arg Thr Leu Met Thr Asn Thr Leu Met Lys Asp Gln Pro
305                 310                 315                 320

Thr Leu Ala Val Thr Phe Val Asp Asn His Asp Thr Glu Pro Gly Gln
                325                 330                 335

Ala Leu Gln Ser Trp Val Asp Pro Trp Phe Lys Pro Leu Ala Tyr Ala
            340                 345                 350

Phe Ile Leu Thr Arg Gln Glu Gly Tyr Pro Cys Val Phe Tyr Gly Asp
        355                 360                 365

Tyr Tyr Gly Ile Pro Gln Tyr Asn Ile Pro Ser Leu Lys Ser Lys Ile
    370                 375                 380

Asp Pro Leu Leu Ile Ala Arg Arg Asp Tyr Ala Tyr Gly Thr Gln His
385                 390                 395                 400

Asp Tyr Leu Asp His Ser Asp Ile Ile Gly Trp Thr Arg Glu Gly Val
                405                 410                 415

Thr Glu Lys Pro Gly Ser Gly Leu Ala Ala Leu Ile Thr Asp Gly Pro
            420                 425                 430

Gly Gly Ser Lys Trp Met Tyr Val Gly Lys Gln His Ala Gly Lys Val
        435                 440                 445

Phe Tyr Asp Leu Thr Gly Asn Arg Ser Asp Thr Val Thr Ile Asn Ser
    450                 455                 460
```

Asp Gly Trp Gly Glu Phe Lys Val Asn Gly Gly Ser Val Ser Val Trp
465 470 475 480

Val Pro Arg Lys Thr Thr
485

<210> 3
<211> 515
<212> PRT
<213> Geobacillus stearothermophilus

<400> 3
Ala Ala Pro Phe Asn Gly Thr Met Met Gln Tyr Phe Glu Trp Tyr Leu
1 5 10 15

Pro Asp Asp Gly Thr Leu Trp Thr Lys Val Ala Asn Glu Ala Asn Asn
20 25 30

Leu Ser Ser Leu Gly Ile Thr Ala Leu Trp Leu Pro Pro Ala Tyr Lys
35 40 45

Gly Thr Ser Arg Ser Asp Val Gly Tyr Gly Val Tyr Asp Leu Tyr Asp
50 55 60

Leu Gly Glu Phe Asn Gln Lys Gly Thr Val Arg Thr Lys Tyr Gly Thr
65 70 75 80

Lys Ala Gln Tyr Leu Gln Ala Ile Gln Ala Ala His Ala Ala Gly Met
85 90 95

Gln Val Tyr Ala Asp Val Val Phe Asp His Lys Gly Gly Ala Asp Gly
100 105 110

Thr Glu Trp Val Asp Ala Val Glu Val Asn Pro Ser Asp Arg Asn Gln
115 120 125

Glu Ile Ser Gly Thr Tyr Gln Ile Gln Ala Trp Thr Lys Phe Asp Phe
130 135 140

Pro Gly Arg Gly Asn Thr Tyr Ser Ser Phe Lys Trp Arg Trp Tyr His
145 150 155 160

Phe Asp Gly Val Asp Trp Asp Glu Ser Arg Lys Leu Ser Arg Ile Tyr

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                165                 170                 175

Lys Phe Arg Gly Ile Gly Lys Ala Trp Asp Trp Glu Val Asp Thr Glu
            180                 185                 190

Asn Gly Asn Tyr Asp Tyr Leu Met Tyr Ala Asp Leu Asp Met Asp His
        195                 200                 205

Pro Glu Val Val Thr Glu Leu Lys Asn Trp Gly Lys Trp Tyr Val Asn
    210                 215                 220

Thr Thr Asn Ile Asp Gly Phe Arg Leu Asp Ala Val Lys His Ile Lys
225                 230                 235                 240

Phe Ala Phe Phe Pro Asp Trp Leu Ser Tyr Val Arg Ser Gln Thr Gly
                245                 250                 255

Lys Pro Leu Phe Thr Val Gly Glu Tyr Trp Ser Tyr Asp Ile Asn Lys
            260                 265                 270

Leu His Asn Tyr Ile Thr Lys Thr Asn Gly Thr Met Ser Leu Phe Asp
        275                 280                 285

Ala Pro Leu His Asn Lys Phe Tyr Thr Ala Ser Lys Ser Gly Gly Ala
    290                 295                 300

Phe Asp Met Arg Thr Leu Met Thr Asn Thr Leu Met Lys Asp Gln Pro
305                 310                 315                 320

Thr Leu Ala Val Thr Phe Val Asp Asn His Asp Thr Glu Pro Gly Gln
                325                 330                 335

Ala Leu Gln Ser Trp Val Asp Pro Trp Phe Lys Pro Leu Ala Tyr Ala
            340                 345                 350

Phe Ile Leu Thr Arg Gln Glu Gly Tyr Pro Cys Val Phe Tyr Gly Asp
        355                 360                 365

Tyr Tyr Gly Ile Pro Gln Tyr Asn Ile Pro Ser Leu Lys Ser Lys Ile
    370                 375                 380

Asp Pro Leu Leu Ile Ala Arg Arg Asp Tyr Ala Tyr Gly Thr Gln His
385                 390                 395                 400
```

```
Asp Tyr Leu Asp His Ser Asp Ile Ile Gly Trp Thr Arg Glu Gly Val
                405                 410                 415

Thr Glu Lys Pro Gly Ser Gly Leu Ala Ala Leu Ile Thr Asp Gly Pro
            420                 425                 430

Gly Gly Ser Lys Trp Met Tyr Val Gly Lys Gln His Ala Gly Lys Val
        435                 440                 445

Phe Tyr Asp Leu Thr Gly Asn Arg Ser Asp Thr Val Thr Ile Asn Ser
    450                 455                 460

Asp Gly Trp Gly Glu Phe Lys Val Asn Gly Gly Ser Val Ser Val Trp
465                 470                 475                 480

Val Pro Arg Lys Thr Thr Val Ser Thr Ile Ala Arg Pro Ile Thr Thr
                485                 490                 495

Arg Pro Trp Thr Gly Glu Phe Val Arg Trp Thr Glu Pro Arg Leu Val
            500                 505                 510

Ala Trp Pro
        515


<210> 4
<211> 515
<212> PRT
<213> Geobacillus stearothermophilus

<400> 4
Ala Ala Pro Phe Asn Gly Thr Met Met Gln Tyr Phe Glu Trp Tyr Leu
1               5                   10                  15

Pro Asp Asp Gly Thr Leu Trp Thr Lys Val Ala Asn Glu Ala Asn Asn
            20                  25                  30

Leu Ser Ser Leu Gly Ile Thr Ala Leu Trp Leu Pro Pro Ala Tyr Lys
        35                  40                  45

Gly Thr Ser Arg Ser Asp Val Gly Tyr Gly Val Tyr Asp Leu Tyr Asp
    50                  55                  60

Leu Gly Glu Phe Asn Gln Lys Gly Thr Val Arg Thr Lys Tyr Gly Thr
```

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65 70 75 80

Lys Ala Gln Tyr Leu Gln Ala Ile Gln Ala Ala His Ala Ala Gly Met
85 90 95

Gln Val Tyr Ala Asp Val Val Phe Asp His Lys Gly Gly Ala Asp Gly
100 105 110

Thr Glu Trp Val Asp Ala Val Glu Val Asn Pro Ser Asp Arg Asn Gln
115 120 125

Glu Ile Ser Gly Thr Tyr Gln Ile Gln Ala Trp Thr Lys Phe Asp Phe
130 135 140

Pro Gly Arg Gly Asn Thr Tyr Ser Ser Phe Lys Trp Arg Trp Tyr His
145 150 155 160

Phe Asp Gly Val Asp Trp Asp Glu Ser Arg Lys Leu Ser Arg Ile Tyr
165 170 175

Lys Phe Arg Gly Ile Gly Lys Ala Trp Asp Trp Glu Val Asp Thr Glu
180 185 190

Asn Gly Asn Tyr Asp Tyr Leu Met Tyr Ala Asp Leu Asp Met Asp His
195 200 205

Pro Glu Val Val Thr Glu Leu Lys Asn Trp Gly Lys Trp Tyr Val Asn
210 215 220

Thr Thr Asn Ile Asp Gly Phe Arg Leu Asp Ala Val Lys His Ile Lys
225 230 235 240

Phe Gln Phe Phe Pro Asp Trp Leu Ser Tyr Val Arg Ser Gln Thr Gly
245 250 255

Lys Pro Leu Phe Thr Val Gly Glu Tyr Trp Ser Tyr Asp Ile Asn Lys
260 265 270

Leu His Asn Tyr Ile Thr Lys Thr Asn Gly Thr Met Ser Leu Phe Asp
275 280 285

Ala Pro Leu His Asn Lys Phe Tyr Thr Ala Ser Lys Ser Gly Gly Ala
290 295 300

```
Phe Asp Met Arg Thr Leu Met Thr Asn Thr Leu Met Lys Asp Gln Pro
305                 310                 315                 320

Thr Leu Ala Val Thr Phe Val Asp Asn His Asp Thr Glu Pro Gly Gln
                325                 330                 335

Ala Leu Gln Ser Trp Val Asp Pro Trp Phe Lys Pro Leu Ala Tyr Ala
            340                 345                 350

Phe Ile Leu Thr Arg Gln Glu Gly Tyr Pro Cys Val Phe Tyr Gly Asp
        355                 360                 365

Tyr Tyr Gly Ile Pro Gln Tyr Asn Ile Pro Ser Leu Lys Ser Lys Ile
    370                 375                 380

Asp Pro Leu Leu Ile Ala Arg Arg Asp Tyr Ala Tyr Gly Thr Gln His
385                 390                 395                 400

Asp Tyr Leu Asp His Ser Asp Ile Ile Gly Trp Thr Arg Glu Gly Val
                405                 410                 415

Thr Glu Lys Pro Gly Ser Gly Leu Ala Ala Leu Ile Thr Asp Gly Pro
            420                 425                 430

Gly Gly Ser Lys Trp Met Tyr Val Gly Lys Gln His Ala Gly Lys Val
        435                 440                 445

Phe Tyr Asp Leu Thr Gly Asn Arg Ser Asp Thr Val Thr Ile Asn Ser
    450                 455                 460

Asp Gly Trp Gly Glu Phe Lys Val Asn Gly Gly Ser Val Ser Val Trp
465                 470                 475                 480

Val Pro Arg Lys Thr Thr Val Ser Thr Ile Ala Arg Pro Ile Thr Thr
                485                 490                 495

Arg Pro Trp Thr Gly Glu Phe Val Arg Trp Thr Glu Pro Arg Leu Val
            500                 505                 510

Ala Trp Pro
        515
```

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<210> 5
<211> 515
<212> PRT
<213> Geobacillus stearothermophilus

<400> 5

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Ala Ala Pro Phe Asn Gly Thr Met Met Gln Tyr Phe Glu Trp Tyr Leu
1               5                  10                  15

Pro Asp Asp Gly Thr Leu Trp Thr Lys Val Ala Asn Glu Ala Asn Asn
            20                  25                  30

Leu Ser Ser Leu Gly Ile Thr Ala Leu Trp Leu Pro Pro Ala Tyr Lys
        35                  40                  45

Gly Thr Ser Arg Ser Asp Val Gly Tyr Gly Val Tyr Asp Leu Tyr Asp
    50                  55                  60

Leu Gly Glu Phe Asn Gln Lys Gly Thr Val Arg Thr Lys Tyr Gly Thr
65                  70                  75                  80

Lys Ala Gln Tyr Leu Gln Ala Ile Gln Ala Ala His Ala Ala Gly Met
                85                  90                  95

Gln Val Tyr Ala Asp Val Val Phe Asp His Lys Gly Gly Ala Asp Gly
            100                 105                 110

Thr Glu Trp Val Asp Ala Val Glu Val Asn Pro Ser Asp Arg Asn Gln
        115                 120                 125

Glu Ile Ser Gly Thr Tyr Gln Ile Gln Ala Trp Thr Lys Phe Asp Phe
    130                 135                 140

Pro Gly Arg Gly Asn Thr Tyr Ser Ser Phe Lys Trp Arg Trp Tyr His
145                 150                 155                 160

Phe Asp Gly Val Asp Trp Asp Glu Ser Arg Lys Leu Ser Arg Ile Tyr
                165                 170                 175

Lys Phe Arg Gly Ile Gly Lys Ala Trp Asp Trp Glu Val Asp Thr Glu
            180                 185                 190

Asn Gly Asn Tyr Asp Tyr Leu Met Tyr Ala Asp Leu Asp Met Asp His
        195                 200                 205
```

```
Pro Glu Val Val Thr Glu Leu Lys Asn Trp Gly Lys Trp Tyr Val Asn
    210                 215                 220

Thr Thr Asn Ile Asp Gly Phe Arg Leu Asp Ala Val Lys His Ile Lys
225                 230                 235                 240

Phe Glu Phe Phe Pro Asp Trp Leu Ser Tyr Val Arg Ser Gln Thr Gly
                245                 250                 255

Lys Pro Leu Phe Thr Val Gly Glu Tyr Trp Ser Tyr Asp Ile Asn Lys
            260                 265                 270

Leu His Asn Tyr Ile Thr Lys Thr Asn Gly Thr Met Ser Leu Phe Asp
        275                 280                 285

Ala Pro Leu His Asn Lys Phe Tyr Thr Ala Ser Lys Ser Gly Gly Ala
    290                 295                 300

Phe Asp Met Arg Thr Leu Met Thr Asn Thr Leu Met Lys Asp Gln Pro
305                 310                 315                 320

Thr Leu Ala Val Thr Phe Val Asp Asn His Asp Thr Glu Pro Gly Gln
                325                 330                 335

Ala Leu Gln Ser Trp Val Asp Pro Trp Phe Lys Pro Leu Ala Tyr Ala
            340                 345                 350

Phe Ile Leu Thr Arg Gln Glu Gly Tyr Pro Cys Val Phe Tyr Gly Asp
        355                 360                 365

Tyr Tyr Gly Ile Pro Gln Tyr Asn Ile Pro Ser Leu Lys Ser Lys Ile
    370                 375                 380

Asp Pro Leu Leu Ile Ala Arg Arg Asp Tyr Ala Tyr Gly Thr Gln His
385                 390                 395                 400

Asp Tyr Leu Asp His Ser Asp Ile Ile Gly Trp Thr Arg Glu Gly Val
                405                 410                 415

Thr Glu Lys Pro Gly Ser Gly Leu Ala Ala Leu Ile Thr Asp Gly Pro
            420                 425                 430
```

```
Gly Gly Ser Lys Trp Met Tyr Val Gly Lys Gln His Ala Gly Lys Val
        435                 440                 445

Phe Tyr Asp Leu Thr Gly Asn Arg Ser Asp Thr Val Thr Ile Asn Ser
    450                 455                 460

Asp Gly Trp Gly Glu Phe Lys Val Asn Gly Gly Ser Val Ser Val Trp
465                 470                 475                 480

Val Pro Arg Lys Thr Thr Val Ser Thr Ile Ala Arg Pro Ile Thr Thr
                485                 490                 495

Arg Pro Trp Thr Gly Glu Phe Val Arg Trp Thr Glu Pro Arg Leu Val
            500                 505                 510

Ala Trp Pro
        515
```

<210> 6
<211> 485
<212> PRT
<213> Bacillus sp. 707

<400> 6

```
His His Asn Gly Thr Asn Gly Thr Met Met Gln Tyr Phe Glu Trp Tyr
1               5                   10                  15

Leu Pro Asn Asp Gly Asn His Trp Asn Arg Leu Asn Ser Asp Ala Ser
            20                  25                  30

Asn Leu Lys Ser Lys Gly Ile Thr Ala Val Trp Ile Pro Pro Ala Trp
        35                  40                  45

Lys Gly Ala Ser Gln Asn Asp Val Gly Tyr Gly Ala Tyr Asp Leu Tyr
    50                  55                  60

Asp Leu Gly Glu Phe Asn Gln Lys Gly Thr Val Arg Thr Lys Tyr Gly
65                  70                  75                  80

Thr Arg Ser Gln Leu Gln Ala Ala Val Thr Ser Leu Lys Asn Asn Gly
                85                  90                  95

Ile Gln Val Tyr Gly Asp Val Val Met Asn His Lys Gly Gly Ala Asp
            100                 105                 110
```

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```
Ala Thr Glu Met Val Arg Ala Val Glu Val Asn Pro Asn Asn Arg Asn
        115                 120                 125

Gln Glu Val Thr Gly Glu Tyr Thr Ile Glu Ala Trp Thr Arg Phe Asp
    130                 135                 140

Phe Pro Gly Arg Gly Asn Thr His Ser Ser Phe Lys Trp Arg Trp Tyr
145                 150                 155                 160

His Phe Asp Gly Val Asp Trp Asp Gln Ser Arg Arg Leu Asn Asn Arg
                165                 170                 175

Ile Tyr Lys Phe Arg Gly His Gly Lys Ala Trp Asp Trp Glu Val Asp
            180                 185                 190

Thr Glu Asn Gly Asn Tyr Asp Tyr Leu Met Tyr Ala Asp Ile Asp Met
        195                 200                 205

Asp His Pro Glu Val Val Asn Glu Leu Arg Asn Trp Gly Val Trp Tyr
    210                 215                 220

Thr Asn Thr Leu Gly Leu Asp Gly Phe Arg Ile Asp Ala Val Lys His
225                 230                 235                 240

Ile Lys Tyr Ser Phe Thr Arg Asp Trp Ile Asn His Val Arg Ser Ala
                245                 250                 255

Thr Gly Lys Asn Met Phe Ala Val Ala Glu Phe Trp Lys Asn Asp Leu
            260                 265                 270

Gly Ala Ile Glu Asn Tyr Leu Gln Lys Thr Asn Trp Asn His Ser Val
        275                 280                 285

Phe Asp Val Pro Leu His Tyr Asn Leu Tyr Asn Ala Ser Lys Ser Gly
    290                 295                 300

Gly Asn Tyr Asp Met Arg Asn Ile Phe Asn Gly Thr Val Val Gln Arg
305                 310                 315                 320

His Pro Ser His Ala Val Thr Phe Val Asp Asn His Asp Ser Gln Pro
                325                 330                 335
```

```
Glu Glu Ala Leu Glu Ser Phe Val Glu Glu Trp Phe Lys Pro Leu Ala
            340                 345                 350

Tyr Ala Leu Thr Leu Thr Arg Glu Gln Gly Tyr Pro Ser Val Phe Tyr
        355                 360                 365

Gly Asp Tyr Tyr Gly Ile Pro Thr His Gly Val Pro Ala Met Arg Ser
    370                 375                 380

Lys Ile Asp Pro Ile Leu Glu Ala Arg Gln Lys Tyr Ala Tyr Gly Lys
385                 390                 395                 400

Gln Asn Asp Tyr Leu Asp His His Asn Ile Ile Gly Trp Thr Arg Glu
                405                 410                 415

Gly Asn Thr Ala His Pro Asn Ser Gly Leu Ala Thr Ile Met Ser Asp
            420                 425                 430

Gly Ala Gly Gly Ser Lys Trp Met Phe Val Gly Arg Asn Lys Ala Gly
        435                 440                 445

Gln Val Trp Ser Asp Ile Thr Gly Asn Arg Thr Gly Thr Val Thr Ile
    450                 455                 460

Asn Ala Asp Gly Trp Gly Asn Phe Ser Val Asn Gly Gly Ser Val Ser
465                 470                 475                 480

Ile Trp Val Asn Lys
                485
```

<210> 7
<211> 483
<212> PRT
<213> Bacillus sp. 707

<400> 7

```
Ala Asn Leu Asn Gly Thr Leu Met Gln Tyr Phe Glu Trp Tyr Met Pro
1               5                   10                  15

Asn Asp Gly Gln His Trp Lys Arg Leu Gln Asn Asp Ser Ala Tyr Leu
            20                  25                  30

Ala Glu His Gly Ile Thr Ala Val Trp Ile Pro Pro Ala Tyr Lys Gly
        35                  40                  45
```

```
Thr Ser Gln Ala Asp Val Gly Tyr Gly Ala Tyr Asp Leu Tyr Asp Leu
    50                  55                  60

Gly Glu Phe His Gln Lys Gly Thr Val Arg Thr Lys Tyr Gly Thr Lys
65                  70                  75                  80

Gly Glu Leu Gln Ser Ala Ile Lys Ser Leu His Ser Arg Asp Ile Asn
                85                  90                  95

Val Tyr Gly Asp Val Val Ile Asn His Lys Gly Gly Ala Asp Ala Thr
            100                 105                 110

Glu Asp Val Thr Ala Val Glu Val Asp Pro Ala Asp Arg Asn Arg Val
        115                 120                 125

Ile Ser Gly Glu His Leu Ile Lys Ala Trp Thr His Phe His Phe Pro
    130                 135                 140

Gly Arg Gly Ser Thr Tyr Ser Asp Phe Lys Trp His Trp Tyr His Phe
145                 150                 155                 160

Asp Gly Thr Asp Trp Asp Glu Ser Arg Lys Leu Asn Arg Ile Tyr Lys
                165                 170                 175

Phe Gln Gly Lys Ala Trp Asp Trp Glu Val Ser Asn Glu Asn Gly Asn
            180                 185                 190

Tyr Asp Tyr Leu Met Tyr Ala Asp Ile Asp Tyr Asp His Pro Asp Val
        195                 200                 205

Ala Ala Glu Ile Lys Arg Trp Gly Thr Trp Tyr Ala Asn Glu Leu Gln
    210                 215                 220

Leu Asp Gly Phe Arg Leu Asp Ala Val Lys His Ile Lys Phe Ser Phe
225                 230                 235                 240

Leu Arg Asp Trp Val Asn His Val Arg Glu Lys Thr Gly Lys Glu Met
                245                 250                 255

Phe Thr Val Ala Glu Tyr Trp Gln Asn Asp Leu Gly Ala Leu Glu Asn
            260                 265                 270
```

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```
Tyr Leu Asn Lys Thr Asn Phe Asn His Ser Val Phe Asp Val Pro Leu
        275                 280                 285

His Tyr Gln Phe His Ala Ala Ser Thr Gln Gly Gly Gly Tyr Asp Met
    290                 295                 300

Arg Lys Leu Leu Asn Gly Thr Val Val Ser Lys His Pro Leu Lys Ser
305                 310                 315                 320

Val Thr Phe Val Asp Asn His Asp Thr Gln Pro Gly Gln Ser Leu Glu
                325                 330                 335

Ser Thr Val Gln Thr Trp Phe Lys Pro Leu Ala Tyr Ala Phe Ile Leu
            340                 345                 350

Thr Arg Glu Ser Gly Tyr Pro Gln Val Phe Tyr Gly Asp Met Tyr Gly
        355                 360                 365

Thr Lys Gly Asp Ser Gln Arg Glu Ile Pro Ala Leu Lys His Lys Ile
    370                 375                 380

Glu Pro Ile Leu Lys Ala Arg Lys Gln Tyr Ala Tyr Gly Ala Gln His
385                 390                 395                 400

Asp Tyr Phe Asp His His Asp Ile Val Gly Trp Thr Arg Glu Gly Asp
                405                 410                 415

Ser Ser Val Ala Asn Ser Gly Leu Ala Ala Leu Ile Thr Asp Gly Pro
            420                 425                 430

Gly Gly Ala Lys Arg Met Tyr Val Gly Arg Gln Asn Ala Gly Glu Thr
        435                 440                 445

Trp His Asp Ile Thr Gly Asn Arg Ser Glu Pro Val Val Ile Asn Ser
    450                 455                 460

Glu Gly Trp Gly Glu Phe His Val Asn Gly Gly Ser Val Ser Ile Tyr
465                 470                 475                 480

Val Gln Arg
```

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<210> 8
<211> 483
<212> PRT
<213> Bacillus licheniformis

<400> 8

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Ala Asn Leu Asn Gly Thr Leu Met Gln Tyr Phe Glu Trp Tyr Met Pro
1               5                  10                  15

Asn Asp Gly Gln His Trp Arg Arg Leu Gln Asn Asp Ser Ala Tyr Leu
            20                  25                  30

Ala Glu His Gly Ile Thr Ala Val Trp Ile Pro Pro Ala Tyr Lys Gly
        35                  40                  45

Thr Ser Gln Ala Asp Val Gly Tyr Gly Ala Tyr Asp Leu Tyr Asp Leu
    50                  55                  60

Gly Glu Phe His Gln Lys Gly Thr Val Arg Thr Lys Tyr Gly Thr Lys
65                  70                  75                  80

Gly Glu Leu Gln Ser Ala Ile Lys Ser Leu His Ser Arg Asp Ile Asn
                85                  90                  95

Val Tyr Gly Asp Val Val Ile Asn His Lys Gly Gly Ala Asp Ala Thr
            100                 105                 110

Glu Asp Val Thr Ala Val Glu Val Asp Pro Ala Asp Arg Asn Arg Val
        115                 120                 125

Ile Ser Gly Glu His Leu Ile Lys Ala Trp Thr His Phe His Phe Pro
    130                 135                 140

Gly Arg Gly Ser Thr Tyr Ser Asp Phe Lys Trp His Trp Tyr His Phe
145                 150                 155                 160

Asp Gly Thr Asp Trp Asp Glu Ser Arg Lys Leu Asn Arg Ile Tyr Lys
                165                 170                 175

Phe Gln Gly Lys Ala Trp Asp Trp Glu Val Ser Asn Glu Asn Gly Asn
            180                 185                 190

Tyr Asp Tyr Leu Met Tyr Ala Asp Ile Asp Tyr Asp His Pro Asp Val
        195                 200                 205
```

```
Ala Ala Glu Ile Lys Arg Trp Gly Thr Trp Tyr Ala Asn Glu Leu Gln
    210                 215                 220

Leu Asp Gly Phe Arg Leu Asp Ala Val Lys His Ile Lys Phe Ser Phe
225                 230                 235                 240

Leu Arg Asp Trp Val Asn His Val Arg Glu Lys Thr Gly Lys Glu Met
                245                 250                 255

Phe Thr Val Ala Glu Tyr Trp Gln Asn Asp Leu Gly Ala Leu Glu Asn
            260                 265                 270

Tyr Leu Asn Lys Thr Asn Phe Asn His Ser Val Phe Asp Val Pro Leu
        275                 280                 285

His Tyr Gln Phe His Ala Ala Ser Thr Gln Gly Gly Gly Tyr Asp Met
    290                 295                 300

Arg Lys Leu Leu Asn Gly Thr Val Val Ser Lys His Pro Leu Lys Ser
305                 310                 315                 320

Val Thr Phe Val Asp Asn His Asp Thr Gln Pro Gly Gln Ser Leu Glu
                325                 330                 335

Ser Thr Val Gln Thr Trp Phe Lys Pro Leu Ala Tyr Ala Phe Ile Leu
            340                 345                 350

Thr Arg Glu Ser Gly Tyr Pro Gln Val Phe Tyr Gly Asp Met Tyr Gly
        355                 360                 365

Thr Lys Gly Asp Ser Gln Arg Glu Ile Pro Ala Leu Lys His Lys Ile
    370                 375                 380

Glu Pro Ile Leu Lys Ala Arg Lys Gln Tyr Ala Tyr Gly Ala Gln His
385                 390                 395                 400

Asp Tyr Phe Asp His His Asp Ile Val Gly Trp Thr Arg Glu Gly Asp
                405                 410                 415

Ser Ser Val Ala Asn Ser Gly Leu Ala Ala Leu Ile Thr Asp Gly Pro
            420                 425                 430
```

```
Gly Gly Ala Lys Arg Met Tyr Val Gly Arg Gln Asn Ala Gly Glu Thr
        435                 440                 445

Trp His Asp Ile Thr Gly Asn Arg Ser Glu Pro Val Val Ile Asn Ser
    450                 455                 460

Glu Gly Trp Gly Glu Phe His Val Asn Gly Gly Ser Val Ser Ile Tyr
465                 470                 475                 480

Val Gln Arg
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<210> 9
<211> 483
<212> PRT
<213> Bacillus amyloliquefaciens

<400> 9

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Val Asn Gly Thr Leu Met Gln Tyr Phe Glu Trp Tyr Thr Pro Asn Asp
1               5                   10                  15

Gly Gln His Trp Lys Arg Leu Gln Asn Asp Ala Glu His Leu Ser Asp
            20                  25                  30

Ile Gly Ile Thr Ala Val Trp Ile Pro Pro Ala Tyr Lys Gly Leu Ser
        35                  40                  45

Gln Ser Asp Asn Gly Tyr Gly Pro Tyr Asp Leu Tyr Asp Leu Gly Glu
    50                  55                  60

Phe Gln Gln Lys Gly Thr Val Arg Thr Lys Tyr Gly Thr Lys Ser Glu
65                  70                  75                  80

Leu Gln Asp Ala Ile Gly Ser Leu His Ser Arg Asn Val Gln Val Tyr
                85                  90                  95

Gly Asp Val Val Leu Asn His Lys Ala Gly Ala Asp Ala Thr Glu Asp
            100                 105                 110

Val Thr Ala Val Glu Val Asn Pro Ala Asn Arg Asn Gln Glu Thr Ser
        115                 120                 125

Glu Glu Tyr Gln Ile Lys Ala Trp Thr Asp Phe Arg Phe Pro Gly Arg
    130                 135                 140
```

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```
Gly Asn Thr Tyr Ser Asp Phe Lys Trp His Trp Tyr His Phe Asp Gly
145                 150                 155                 160

Ala Asp Trp Asp Glu Ser Arg Lys Ile Ser Arg Ile Phe Lys Phe Arg
                165                 170                 175

Gly Glu Gly Lys Ala Trp Asp Trp Glu Val Ser Ser Glu Asn Gly Asn
            180                 185                 190

Tyr Asp Tyr Leu Met Tyr Ala Asp Val Asp Tyr Asp His Pro Asp Val
        195                 200                 205

Val Ala Glu Thr Lys Lys Trp Gly Ile Trp Tyr Ala Asn Glu Leu Ser
    210                 215                 220

Leu Asp Gly Phe Arg Ile Asp Ala Ala Lys His Ile Lys Phe Ser Phe
225                 230                 235                 240

Leu Arg Asp Trp Val Gln Ala Val Arg Gln Ala Thr Gly Lys Glu Met
                245                 250                 255

Phe Thr Val Ala Glu Tyr Trp Gln Asn Asn Ala Gly Lys Leu Glu Asn
            260                 265                 270

Tyr Leu Asn Lys Thr Ser Phe Asn Gln Ser Val Phe Asp Val Pro Leu
        275                 280                 285

His Phe Asn Leu Gln Ala Ala Ser Ser Gln Gly Gly Gly Tyr Asp Met
    290                 295                 300

Arg Arg Leu Leu Asp Gly Thr Val Val Ser Arg His Pro Glu Lys Ala
305                 310                 315                 320

Val Thr Phe Val Glu Asn His Asp Thr Gln Pro Gly Gln Ser Leu Glu
                325                 330                 335

Ser Thr Val Gln Thr Trp Phe Lys Pro Leu Ala Tyr Ala Phe Ile Leu
            340                 345                 350

Thr Arg Glu Ser Gly Tyr Pro Gln Val Phe Tyr Gly Asp Met Tyr Gly
        355                 360                 365
```

```
Thr Lys Gly Thr Ser Pro Lys Glu Ile Pro Ser Leu Lys Asp Asn Ile
    370                 375                 380

Glu Pro Ile Leu Lys Ala Arg Lys Glu Tyr Ala Tyr Gly Pro Gln His
385                 390                 395                 400

Asp Tyr Ile Asp His Pro Asp Val Ile Gly Trp Thr Arg Glu Gly Asp
                405                 410                 415

Ser Ser Ala Ala Lys Ser Gly Leu Ala Ala Leu Ile Thr Asp Gly Pro
            420                 425                 430

Gly Gly Ser Lys Arg Met Tyr Ala Gly Leu Lys Asn Ala Gly Glu Thr
        435                 440                 445

Trp Tyr Asp Ile Thr Gly Asn Arg Ser Asp Thr Val Lys Ile Gly Ser
    450                 455                 460

Asp Gly Trp Gly Glu Phe His Val Asn Asp Gly Ser Val Ser Ile Tyr
465                 470                 475                 480

Val Gln Lys
```

<210> 10
<211> 485
<212> PRT
<213> Bacillus sp. 707

<400> 10

```
His His Asn Gly Thr Asn Gly Thr Met Met Gln Tyr Phe Glu Trp Tyr
1               5                   10                  15

Leu Pro Asn Asp Gly Asn His Trp Asn Arg Leu Arg Ser Asp Ala Ser
            20                  25                  30

Asn Leu Lys Asp Lys Gly Ile Ser Ala Val Trp Ile Pro Pro Ala Trp
        35                  40                  45

Lys Gly Ala Ser Gln Asn Asp Val Gly Tyr Gly Ala Tyr Asp Leu Tyr
    50                  55                  60

Asp Leu Gly Glu Phe Asn Gln Lys Gly Thr Ile Arg Thr Lys Tyr Gly
65                  70                  75                  80
```

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```
Thr Arg Asn Gln Leu Gln Ala Ala Val Asn Ala Leu Lys Ser Asn Gly
                85                  90                  95

Ile Gln Val Tyr Gly Asp Val Val Met Asn His Lys Gly Gly Ala Asp
            100                 105                 110

Ala Thr Glu Met Val Arg Ala Val Glu Val Asn Pro Asn Asn Arg Asn
        115                 120                 125

Gln Glu Val Ser Gly Glu Tyr Thr Ile Glu Ala Trp Thr Lys Phe Asp
    130                 135                 140

Phe Pro Gly Arg Gly Asn Thr His Ser Asn Phe Lys Trp Arg Trp Tyr
145                 150                 155                 160

His Phe Asp Gly Val Asp Trp Asp Gln Ser Arg Lys Leu Asn Asn Arg
                165                 170                 175

Ile Tyr Lys Phe Arg Gly Asp Gly Lys Gly Trp Asp Trp Glu Val Asp
            180                 185                 190

Thr Glu Asn Gly Asn Tyr Asp Tyr Leu Met Tyr Ala Asp Ile Asp Met
        195                 200                 205

Asp His Pro Glu Val Val Asn Glu Leu Arg Asn Trp Gly Val Trp Tyr
    210                 215                 220

Thr Asn Thr Leu Gly Leu Asp Gly Phe Arg Ile Asp Ala Val Lys His
225                 230                 235                 240

Ile Lys Tyr Ser Phe Thr Arg Asp Trp Ile Asn His Val Arg Ser Ala
                245                 250                 255

Thr Gly Lys Asn Met Phe Ala Val Ala Glu Phe Trp Lys Asn Asp Leu
            260                 265                 270

Gly Ala Ile Glu Asn Tyr Leu Asn Lys Thr Asn Trp Asn His Ser Val
        275                 280                 285

Phe Asp Val Pro Leu His Tyr Asn Leu Tyr Asn Ala Ser Lys Ser Gly
    290                 295                 300
```

```
Gly Asn Tyr Asp Met Arg Gln Ile Phe Asn Gly Thr Val Val Gln Arg
305                 310                 315                 320

His Pro Met His Ala Val Thr Phe Val Asp Asn His Asp Ser Gln Pro
                325                 330                 335

Glu Glu Ala Leu Glu Ser Phe Val Glu Glu Trp Phe Lys Pro Leu Ala
            340                 345                 350

Tyr Ala Leu Thr Leu Thr Arg Glu Gln Gly Tyr Pro Ser Val Phe Tyr
        355                 360                 365

Gly Asp Tyr Tyr Gly Ile Pro Thr His Gly Val Pro Ala Met Lys Ser
    370                 375                 380

Lys Ile Asp Pro Ile Leu Glu Ala Arg Gln Lys Tyr Ala Tyr Gly Arg
385                 390                 395                 400

Gln Asn Asp Tyr Leu Asp His His Asn Ile Ile Gly Trp Thr Arg Glu
                405                 410                 415

Gly Asn Thr Ala His Pro Asn Ser Gly Leu Ala Thr Ile Met Ser Asp
            420                 425                 430

Gly Ala Gly Gly Asn Lys Trp Met Phe Val Gly Arg Asn Lys Ala Gly
        435                 440                 445

Gln Val Trp Thr Asp Ile Thr Gly Asn Arg Ala Gly Thr Val Thr Ile
    450                 455                 460

Asn Ala Asp Gly Trp Gly Asn Phe Ser Val Asn Gly Gly Ser Val Ser
465                 470                 475                 480

Ile Trp Val Asn Lys
                485
```

<210> 11
<211> 485
<212> PRT
<213> Bacillus halmapalus

<400> 11

```
His His Asn Gly Thr Asn Gly Thr Met Met Gln Tyr Phe Glu Trp His
1               5                   10                  15
```

```
Leu Pro Asn Asp Gly Asn His Trp Asn Arg Leu Arg Asp Asp Ala Ser
            20                  25                  30

Asn Leu Arg Asn Arg Gly Ile Thr Ala Ile Trp Ile Pro Pro Ala Trp
        35                  40                  45

Lys Gly Thr Ser Gln Asn Asp Val Gly Tyr Gly Ala Tyr Asp Leu Tyr
    50                  55                  60

Asp Leu Gly Glu Phe Asn Gln Lys Gly Thr Val Arg Thr Lys Tyr Gly
65                  70                  75                  80

Thr Arg Ser Gln Leu Glu Ser Ala Ile His Ala Leu Lys Asn Asn Gly
                85                  90                  95

Val Gln Val Tyr Gly Asp Val Val Met Asn His Lys Gly Gly Ala Asp
            100                 105                 110

Ala Thr Glu Asn Val Leu Ala Val Glu Val Asn Pro Asn Asn Arg Asn
        115                 120                 125

Gln Glu Ile Ser Gly Asp Tyr Thr Ile Glu Ala Trp Thr Lys Phe Asp
    130                 135                 140

Phe Pro Gly Arg Gly Asn Thr Tyr Ser Asp Phe Lys Trp Arg Trp Tyr
145                 150                 155                 160

His Phe Asp Gly Val Asp Trp Asp Gln Ser Arg Gln Phe Gln Asn Arg
                165                 170                 175

Ile Tyr Lys Phe Arg Gly Asp Gly Lys Ala Trp Asp Trp Glu Val Asp
            180                 185                 190

Ser Glu Asn Gly Asn Tyr Asp Tyr Leu Met Tyr Ala Asp Val Asp Met
        195                 200                 205

Asp His Pro Glu Val Val Asn Glu Leu Arg Arg Trp Gly Glu Trp Tyr
    210                 215                 220

Thr Asn Thr Leu Asn Leu Asp Gly Phe Arg Ile Asp Ala Val Lys His
225                 230                 235                 240
```

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```
Ile Lys Tyr Ser Phe Thr Arg Asp Trp Leu Thr His Val Arg Asn Ala
                245                 250                 255

Thr Gly Lys Glu Met Phe Ala Val Ala Glu Phe Trp Lys Asn Asp Leu
            260                 265                 270

Gly Ala Leu Glu Asn Tyr Leu Asn Lys Thr Asn Trp Asn His Ser Val
        275                 280                 285

Phe Asp Val Pro Leu His Tyr Asn Leu Tyr Asn Ala Ser Asn Ser Gly
    290                 295                 300

Gly Asn Tyr Asp Met Ala Lys Leu Leu Asn Gly Thr Val Val Gln Lys
305                 310                 315                 320

His Pro Met His Ala Val Thr Phe Val Asp Asn His Asp Ser Gln Pro
                325                 330                 335

Gly Glu Ser Leu Glu Ser Phe Val Gln Glu Trp Phe Lys Pro Leu Ala
            340                 345                 350

Tyr Ala Leu Ile Leu Thr Arg Glu Gln Gly Tyr Pro Ser Val Phe Tyr
        355                 360                 365

Gly Asp Tyr Tyr Gly Ile Pro Thr His Ser Val Pro Ala Met Lys Ala
    370                 375                 380

Lys Ile Asp Pro Ile Leu Glu Ala Arg Gln Asn Phe Ala Tyr Gly Thr
385                 390                 395                 400

Gln His Asp Tyr Phe Asp His His Asn Ile Ile Gly Trp Thr Arg Glu
                405                 410                 415

Gly Asn Thr Thr His Pro Asn Ser Gly Leu Ala Thr Ile Met Ser Asp
            420                 425                 430

Gly Pro Gly Gly Glu Lys Trp Met Tyr Val Gly Gln Asn Lys Ala Gly
        435                 440                 445

Gln Val Trp His Asp Ile Thr Gly Asn Lys Pro Gly Thr Val Thr Ile
    450                 455                 460

Asn Ala Asp Gly Trp Ala Asn Phe Ser Val Asn Gly Gly Ser Val Ser
```

465 470 475 480

Ile Trp Val Lys Arg
485

<210> 12
<211> 485
<212> PRT
<213> Bacillus sp.

<400> 12
His His Asn Gly Thr Asn Gly Thr Met Met Gln Tyr Phe Glu Trp His
1 5 10 15

Leu Pro Asn Asp Gly Asn His Trp Asn Arg Leu Arg Asp Asp Ala Ala
20 25 30

Asn Leu Lys Ser Lys Gly Ile Thr Ala Val Trp Ile Pro Pro Ala Trp
35 40 45

Lys Gly Thr Ser Gln Asn Asp Val Gly Tyr Gly Ala Tyr Asp Leu Tyr
50 55 60

Asp Leu Gly Glu Phe Asn Gln Lys Gly Thr Val Arg Thr Lys Tyr Gly
65 70 75 80

Thr Arg Ser Gln Leu Gln Gly Ala Val Thr Ser Leu Lys Asn Asn Gly
85 90 95

Ile Gln Val Tyr Gly Asp Val Val Met Asn His Lys Gly Gly Ala Asp
100 105 110

Gly Thr Glu Met Val Asn Ala Val Glu Val Asn Arg Ser Asn Arg Asn
115 120 125

Gln Glu Ile Ser Gly Glu Tyr Thr Ile Glu Ala Trp Thr Lys Phe Asp
130 135 140

Phe Pro Gly Arg Gly Asn Thr His Ser Asn Phe Lys Trp Arg Trp Tyr
145 150 155 160

His Phe Asp Gly Thr Asp Trp Asp Gln Ser Arg Gln Leu Gln Asn Lys
165 170 175

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```
Ile Tyr Lys Phe Arg Gly Thr Gly Lys Ala Trp Asp Trp Glu Val Asp
            180                 185                 190

Ile Glu Asn Gly Asn Tyr Asp Tyr Leu Met Tyr Ala Asp Ile Asp Met
        195                 200                 205

Asp His Pro Glu Val Ile Asn Glu Leu Arg Asn Trp Gly Val Trp Tyr
    210                 215                 220

Thr Asn Thr Leu Asn Leu Asp Gly Phe Arg Ile Asp Ala Val Lys His
225                 230                 235                 240

Ile Lys Tyr Ser Tyr Thr Arg Asp Trp Leu Thr His Val Arg Asn Thr
                245                 250                 255

Thr Gly Lys Pro Met Phe Ala Val Ala Glu Phe Trp Lys Asn Asp Leu
            260                 265                 270

Ala Ala Ile Glu Asn Tyr Leu Asn Lys Thr Ser Trp Asn His Ser Val
        275                 280                 285

Phe Asp Val Pro Leu His Tyr Asn Leu Tyr Asn Ala Ser Asn Ser Gly
    290                 295                 300

Gly Tyr Phe Asp Met Arg Asn Ile Leu Asn Gly Ser Val Val Gln Lys
305                 310                 315                 320

His Pro Ile His Ala Val Thr Phe Val Asp Asn His Asp Ser Gln Pro
                325                 330                 335

Gly Glu Ala Leu Glu Ser Phe Val Gln Ser Trp Phe Lys Pro Leu Ala
            340                 345                 350

Tyr Ala Leu Ile Leu Thr Arg Glu Gln Gly Tyr Pro Ser Val Phe Tyr
        355                 360                 365

Gly Asp Tyr Tyr Gly Ile Pro Thr His Gly Val Pro Ser Met Lys Ser
    370                 375                 380

Lys Ile Asp Pro Leu Leu Gln Ala Arg Gln Thr Tyr Ala Tyr Gly Thr
385                 390                 395                 400

Gln His Asp Tyr Phe Asp His His Asp Ile Ile Gly Trp Thr Arg Glu
```

405 410 415

Gly Asp Ser Ser His Pro Asn Ser Gly Leu Ala Thr Ile Met Ser Asp
420 425 430

Gly Pro Gly Gly Asn Lys Trp Met Tyr Val Gly Lys His Lys Ala Gly
435 440 445

Gln Val Trp Arg Asp Ile Thr Gly Asn Arg Ser Gly Thr Val Thr Ile
450 455 460

Asn Ala Asp Gly Trp Gly Asn Phe Thr Val Asn Gly Gly Ala Val Ser
465 470 475 480

Val Trp Val Lys Gln
485

<210> 13
<211> 480
<212> PRT
<213> Bacillus sp.

<400> 13
Asp Gly Leu Asn Gly Thr Met Met Gln Tyr Tyr Glu Trp His Leu Glu
1 5 10 15

Asn Asp Gly Gln His Trp Asn Arg Leu His Asp Asp Ala Ala Ala Leu
20 25 30

Ser Asp Ala Gly Ile Thr Ala Ile Trp Ile Pro Pro Ala Tyr Lys Gly
35 40 45

Asn Ser Gln Ala Asp Val Gly Tyr Gly Ala Tyr Asp Leu Tyr Asp Leu
50 55 60

Gly Glu Phe Asn Gln Lys Gly Thr Val Arg Thr Lys Tyr Gly Thr Lys
65 70 75 80

Ala Gln Leu Glu Arg Ala Ile Gly Ser Leu Lys Ser Asn Asp Ile Asn
85 90 95

Val Tyr Gly Asp Val Val Met Asn His Lys Met Gly Ala Asp Phe Thr
100 105 110

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```
Glu Ala Val Gln Ala Val Gln Val Asn Pro Thr Asn Arg Trp Gln Asp
        115                 120                 125

Ile Ser Gly Ala Tyr Thr Ile Asp Ala Trp Thr Gly Phe Asp Phe Ser
    130                 135                 140

Gly Arg Asn Asn Ala Tyr Ser Asp Phe Lys Trp Arg Trp Phe His Phe
145                 150                 155                 160

Asn Gly Val Asp Trp Asp Gln Arg Tyr Gln Glu Asn His Ile Phe Arg
                165                 170                 175

Phe Ala Asn Thr Asn Trp Asn Trp Arg Val Asp Glu Glu Asn Gly Asn
            180                 185                 190

Tyr Asp Tyr Leu Leu Gly Ser Asn Ile Asp Phe Ser His Pro Glu Val
        195                 200                 205

Gln Asp Glu Leu Lys Asp Trp Gly Ser Trp Phe Thr Asp Glu Leu Asp
    210                 215                 220

Leu Asp Gly Tyr Arg Leu Asp Ala Ile Lys His Ile Pro Phe Trp Tyr
225                 230                 235                 240

Thr Ser Asp Trp Val Arg His Gln Arg Asn Glu Ala Asp Gln Asp Leu
                245                 250                 255

Phe Val Val Gly Glu Tyr Trp Lys Asp Asp Val Gly Ala Leu Glu Phe
            260                 265                 270

Tyr Leu Asp Glu Met Asn Trp Glu Met Ser Leu Phe Asp Val Pro Leu
        275                 280                 285

Asn Tyr Asn Phe Tyr Arg Ala Ser Gln Gln Gly Gly Ser Tyr Asp Met
    290                 295                 300

Arg Asn Ile Leu Arg Gly Ser Leu Val Glu Ala His Pro Met His Ala
305                 310                 315                 320

Val Thr Phe Val Asp Asn His Asp Thr Gln Pro Gly Glu Ser Leu Glu
                325                 330                 335

Ser Trp Val Ala Asp Trp Phe Lys Pro Leu Ala Tyr Ala Thr Ile Leu
```

```
            340                 345                 350

Thr Arg Glu Gly Gly Tyr Pro Asn Val Phe Tyr Gly Asp Tyr Tyr Gly
        355                 360                 365

Ile Pro Asn Asp Asn Ile Ser Ala Lys Lys Asp Met Ile Asp Glu Leu
    370                 375                 380

Leu Asp Ala Arg Gln Asn Tyr Ala Tyr Gly Thr Gln His Asp Tyr Phe
385                 390                 395                 400

Asp His Trp Asp Val Val Gly Trp Thr Arg Glu Gly Ser Ser Ser Arg
                405                 410                 415

Pro Asn Ser Gly Leu Ala Thr Ile Met Ser Asn Gly Pro Gly Gly Ser
            420                 425                 430

Lys Trp Met Tyr Val Gly Arg Gln Asn Ala Gly Gln Thr Trp Thr Asp
        435                 440                 445

Leu Thr Gly Asn Asn Gly Ala Ser Val Thr Ile Asn Gly Asp Gly Trp
    450                 455                 460

Gly Glu Phe Phe Thr Asn Gly Gly Ser Val Ser Val Tyr Val Asn Gln
465                 470                 475                 480


<210> 14
<211> 480
<212> PRT
<213> Bacillus sp.

<400> 14
Asp Gly Leu Asn Gly Thr Met Met Gln Tyr Tyr Glu Trp His Leu Glu
1               5                   10                  15

Asn Asp Gly Gln His Trp Asn Arg Leu His Asp Asp Ala Glu Ala Leu
            20                  25                  30

Ser Asn Ala Gly Ile Thr Ala Ile Trp Ile Pro Pro Ala Tyr Lys Gly
        35                  40                  45

Asn Ser Gln Ala Asp Val Gly Tyr Gly Ala Tyr Asp Leu Tyr Asp Leu
    50                  55                  60
```

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```
Gly Glu Phe Asn Gln Lys Gly Thr Val Arg Thr Lys Tyr Gly Thr Lys
65                  70                  75                  80

Ala Gln Leu Glu Arg Ala Ile Gly Ser Leu Lys Ser Asn Asp Ile Asn
                85                  90                  95

Val Tyr Gly Asp Val Val Met Asn His Lys Leu Gly Ala Asp Phe Thr
            100                 105                 110

Glu Ala Val Gln Ala Val Gln Val Asn Pro Ser Asn Arg Trp Gln Asp
        115                 120                 125

Ile Ser Gly Val Tyr Thr Ile Asp Ala Trp Thr Gly Phe Asp Phe Pro
    130                 135                 140

Gly Arg Asn Asn Ala Tyr Ser Asp Phe Lys Trp Arg Trp Phe His Phe
145                 150                 155                 160

Asn Gly Val Asp Trp Asp Gln Arg Tyr Gln Glu Asn His Leu Phe Arg
                165                 170                 175

Phe Ala Asn Thr Asn Trp Asn Trp Arg Val Asp Glu Glu Asn Gly Asn
            180                 185                 190

Tyr Asp Tyr Leu Leu Gly Ser Asn Ile Asp Phe Ser His Pro Glu Val
        195                 200                 205

Gln Glu Glu Leu Lys Asp Trp Gly Ser Trp Phe Thr Asp Glu Leu Asp
    210                 215                 220

Leu Asp Gly Tyr Arg Leu Asp Ala Ile Lys His Ile Pro Phe Trp Tyr
225                 230                 235                 240

Thr Ser Asp Trp Val Arg His Gln Arg Ser Glu Ala Asp Gln Asp Leu
                245                 250                 255

Phe Val Val Gly Glu Tyr Trp Lys Asp Asp Val Gly Ala Leu Glu Phe
            260                 265                 270

Tyr Leu Asp Glu Met Asn Trp Glu Met Ser Leu Phe Asp Val Pro Leu
        275                 280                 285

Asn Tyr Asn Phe Tyr Arg Ala Ser Lys Gln Gly Gly Ser Tyr Asp Met
```

```
    290                 295                 300

Arg Asn Ile Leu Arg Gly Ser Leu Val Glu Ala His Pro Ile His Ala
305                 310                 315                 320

Val Thr Phe Val Asp Asn His Asp Thr Gln Pro Gly Glu Ser Leu Glu
                325                 330                 335

Ser Trp Val Ala Asp Trp Phe Lys Pro Leu Ala Tyr Ala Thr Ile Leu
            340                 345                 350

Thr Arg Glu Gly Gly Tyr Pro Asn Val Phe Tyr Gly Asp Tyr Tyr Gly
        355                 360                 365

Ile Pro Asn Asp Asn Ile Ser Ala Lys Lys Asp Met Ile Asp Glu Leu
    370                 375                 380

Leu Asp Ala Arg Gln Asn Tyr Ala Tyr Gly Thr Gln His Asp Tyr Phe
385                 390                 395                 400

Asp His Trp Asp Ile Val Gly Trp Thr Arg Glu Gly Thr Ser Ser Arg
                405                 410                 415

Pro Asn Ser Gly Leu Ala Thr Ile Met Ser Asn Gly Pro Gly Gly Ser
            420                 425                 430

Lys Trp Met Tyr Val Gly Gln Gln His Ala Gly Gln Thr Trp Thr Asp
        435                 440                 445

Leu Thr Gly Asn His Ala Ala Ser Val Thr Ile Asn Gly Asp Gly Trp
    450                 455                 460

Gly Glu Phe Phe Thr Asn Gly Gly Ser Val Ser Val Tyr Val Asn Gln
465                 470                 475                 480


<210> 15
<211> 486
<212> PRT
<213> Geobacillus stearothermophilus

<400> 15
Ala Ala Pro Phe Asn Gly Thr Met Met Gln Tyr Phe Glu Trp Tyr Leu
1               5                   10                  15
```

```
Pro Asp Asp Gly Thr Leu Trp Thr Lys Val Ala Asn Glu Ala Asn Asn
            20                  25                  30

Leu Ser Ser Leu Gly Ile Thr Ala Leu Trp Leu Pro Pro Ala Tyr Lys
        35                  40                  45

Gly Thr Ser Arg Ser Asp Val Gly Tyr Gly Val Tyr Asp Leu Tyr Asp
    50                  55                  60

Leu Gly Glu Phe Asn Gln Lys Gly Thr Val Arg Thr Lys Tyr Gly Thr
65                  70                  75                  80

Lys Ala Gln Tyr Leu Gln Ala Ile Gln Ala Ala His Ala Ala Gly Met
                85                  90                  95

Gln Val Tyr Ala Asp Val Val Phe Asp His Lys Gly Gly Ala Asp Gly
            100                 105                 110

Thr Glu Trp Val Asp Ala Val Glu Val Asn Pro Ser Asp Arg Asn Gln
        115                 120                 125

Glu Ile Ser Gly Thr Tyr Gln Ile Gln Ala Trp Thr Lys Phe Asp Phe
    130                 135                 140

Pro Gly Arg Gly Asn Thr Tyr Ser Ser Phe Lys Trp Arg Trp Tyr His
145                 150                 155                 160

Phe Asp Gly Val Asp Trp Asp Glu Ser Arg Lys Leu Ser Arg Ile Tyr
                165                 170                 175

Lys Phe Arg Gly Lys Ala Trp Asp Trp Glu Val Asp Thr Glu Phe Gly
            180                 185                 190

Asn Tyr Asp Tyr Leu Met Tyr Ala Asp Leu Asp Met Asp His Pro Glu
        195                 200                 205

Val Val Thr Glu Leu Lys Asn Trp Gly Lys Trp Tyr Val Asn Thr Thr
    210                 215                 220

Asn Ile Asp Gly Phe Arg Leu Asp Ala Val Lys His Ile Lys Phe Ser
225                 230                 235                 240

Phe Phe Pro Asp Trp Leu Ser Tyr Val Arg Ser Gln Thr Gly Lys Pro
```

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```
                245                 250                 255

Leu Phe Thr Val Gly Glu Tyr Trp Ser Tyr Asp Ile Asn Lys Leu His
            260                 265                 270

Asn Tyr Ile Thr Lys Thr Asn Gly Thr Met Ser Leu Phe Asp Ala Pro
        275                 280                 285

Leu His Asn Lys Phe Tyr Thr Ala Ser Lys Ser Gly Gly Ala Phe Asp
    290                 295                 300

Met Arg Thr Leu Met Thr Asn Thr Leu Met Lys Asp Gln Pro Thr Leu
305                 310                 315                 320

Ala Val Thr Phe Val Asp Asn His Asp Thr Glu Pro Gly Gln Ala Leu
                325                 330                 335

Gln Ser Trp Val Asp Pro Trp Phe Lys Pro Leu Ala Tyr Ala Phe Ile
            340                 345                 350

Leu Thr Arg Gln Glu Gly Tyr Pro Cys Val Phe Tyr Gly Asp Tyr Tyr
        355                 360                 365

Gly Ile Pro Gln Tyr Asn Ile Pro Ser Leu Lys Ser Lys Ile Asp Pro
    370                 375                 380

Leu Leu Ile Ala Arg Arg Asp Tyr Ala Tyr Gly Thr Gln His Asp Tyr
385                 390                 395                 400

Leu Asp His Ser Asp Ile Ile Gly Trp Thr Arg Glu Gly Gly Thr Glu
                405                 410                 415

Lys Pro Gly Ser Gly Leu Ala Ala Leu Ile Thr Asp Gly Pro Gly Gly
            420                 425                 430

Ser Lys Trp Met Tyr Val Gly Lys Gln His Ala Gly Lys Val Phe Tyr
        435                 440                 445

Asp Leu Thr Gly Asn Arg Ser Asp Thr Val Thr Ile Asn Ser Asp Gly
    450                 455                 460

Trp Gly Glu Phe Lys Val Asn Gly Gly Ser Val Ser Val Trp Val Pro
465                 470                 475                 480
```

Arg Lys Thr Thr Val Ser
485

<210> 16
<211> 520
<212> PRT
<213> Artificial sequence

<220>
<221> source
<223> /note="Description of artificial sequence: Synthetic consensus sequence"

<220>
<221> VARIANT
<222> (1)..(1)
<223> /replace=" "

<220>
<221> VARIANT
<222> (2)..(2)
<223> /replace="His" or " "

<220>
<221> misc_feature
<222> (2)..(2)
<223> /note="Residues given in the sequence have no preference with respect to those in the annotation for said positions"

<220>
<221> VARIANT
<222> (4)..(4)
<223> /replace="Gly" or "Asn" or " "

<220>
<221> VARIANT
<222> (5)..(5)
<223> /replace="Thr" or "Leu" or "Val"

<220>
<221> misc_feature
<222> (4)..(5)
<223> /note="Residues given in the sequence have no preference with respect to those in the annotation for said positions"

<220>
<221> VARIANT
<222> (25)..(25)
<223> /replace="Asn" or "Lys" or "Arg"

<220>
<221> misc_feature
<222> (25)..(25)

<223> /note="Residue given in the sequence has no preference
with respect to those in the annotation for said position"

<220>
<221> VARIANT
<222> (28)..(28)
<223> /replace="Asn" or "Gln" or "Arg" or "His"

<220>
<221> misc_feature
<222> (28)..(28)
<223> /note="Residue given in the sequence has no preference
with respect to those in the annotation for said position"

<220>
<221> VARIANT
<222> (32)..(32)
<223> /replace="Ser" or "Ala" or "Glu"

<220>
<221> misc_feature
<222> (32)..(32)
<223> /note="Residue given in the sequence has no preference
with respect to those in the annotation for said position"

<220>
<221> VARIANT
<222> (37)..(37)
<223> /replace="Lys" or "His" or "Ile" or "Arg" or "Ala"

<220>
<221> misc_feature
<222> (37)..(37)
<223> /note="Residue given in the sequence has no preference
with respect to those in the annotation for said position"

<220>
<221> VARIANT
<222> (86)..(86)
<223> /replace="Gln" or "Glu"

<220>
<221> VARIANT
<222> (87)..(87)
<223> /replace="Ala" or "Ser" or "Asp" or "Gly" or "Arg"

<220>
<221> misc_feature
<222> (86)..(87)
<223> /note="Residues given in the sequence have no preference
with respect to those in the annotation for said positions"

<220>
<221> VARIANT
<222> (90)..(90)
<223> /replace="Thr" or "Lys" or "Gly" or "Asn" or "His"

<220>
<221> misc_feature
<222> (90)..(90)
<223> /note="Residue given in the sequence has no preference
with respect to those in the annotation for said position"

<220>
<221> VARIANT
<222> (95)..(95)
<223> /replace="Asn" or "Arg"

<220>
<221> misc_feature
<222> (95)..(95)
<223> /note="Residue given in the sequence has no preference
with respect to those in the annotation for said position"

<220>
<221> VARIANT
<222> (116)..(116)
<223> /replace="Met" or "Asp" or "Asn" or "Ala"

<220>
<221> misc_feature
<222> (116)..(116)
<223> /note="Residue given in the sequence has no preference
with respect to those in the annotation for said position"

<220>
<221> VARIANT
<222> (118)..(118)
<223> /replace="Arg" or "Thr" or "Leu" or "Asn" or "Gln"

<220>
<221> misc_feature
<222> (118)..(118)
<223> /note="Residue given in the sequence has no preference
with respect to those in the annotation for said position"

<220>
<221> VARIANT
<222> (134)..(134)
<223> /replace="Glu" or "Asp" or "Ala" or "Val"

<220>
<221> misc_feature
<222> (134)..(134)
<223> /note="Residue given in the sequence has no preference
with respect to those in the annotation for said position"

<220>
<221> VARIANT
<222> (136)..(136)
<223> /replace="Thr" or "Leu"

<220>
<221> misc_feature

<222> (136)..(136)
<223> /note="Residue given in the sequence has no preference
with respect to those in the annotation for said position"

<220>
<221> VARIANT
<222> (138)..(138)
<223> /replace="Glu" or "Lys" or "Asp"

<220>
<221> misc_feature
<222> (138)..(138)
<223> /note="Residue given in the sequence has no preference
with respect to those in the annotation for said position"

<220>
<221> VARIANT
<222> (154)..(154)
<223> /replace="Asp" or "Asn"

<220>
<221> misc_feature
<222> (154)..(154)
<223> /note="Residue given in the sequence has no preference
with respect to those in the annotation for said position"

<220>
<221> VARIANT
<222> (175)..(175)
<223> /replace=" "

<220>
<221> VARIANT
<222> (183)..(183)
<223> /replace="His" or "Glu" or "Asp" or "Thr" or " "

<220>
<221> misc_feature
<222> (183)..(183)
<223> /note="Residue given in the sequence has no preference
with respect to those in the annotation for said position"

<220>
<221> VARIANT
<222> (215)..(215)
<223> /replace="Asn" or "Ala" or "Asp" or "Glu"

<220>
<221> misc_feature
<222> (215)..(215)
<223> /note="Residue given in the sequence has no preference
with respect to those in the annotation for said position"

<220>
<221> VARIANT
<222> (222)..(222)
<223> /replace="Val" or "Thr" or "Ile" or "Glu" or "Ser"

<220>
<221> misc_feature
<222> (222)..(222)
<223> /note="Residue given in the sequence has no preference
with respect to those in the annotation for said position"

<220>
<221> VARIANT
<222> (225)..(225)
<223> /replace="Thr" or "Ala"

<220>
<221> misc_feature
<222> (225)..(225)
<223> /note="Residue given in the sequence has no preference
with respect to those in the annotation for said position"

<220>
<221> VARIANT
<222> (246)..(246)
<223> /replace="Thr" or "Leu"

<220>
<221> misc_feature
<222> (246)..(246)
<223> /note="Residues given in the sequence have no preference
with respect to those in the annotation for said positions"

<220>
<221> VARIANT
<222> (247)..(247)
<223> /replace="Pro" or "Ser"

<220>
<221> VARIANT
<222> (256)..(256)
<223> /replace="Ala" or "Lys" or "Thr" or "Glu"

<220>
<221> misc_feature
<222> (256)..(256)
<223> /note="Residue given in the sequence has no preference
with respect to those in the annotation for said position"

<220>
<221> VARIANT
<222> (260)..(260)
<223> /replace="Asn" or "Glu" or "Asp"

<220>
<221> misc_feature
<222> (260)..(260)
<223> /note="Residue given in the sequence has no preference
with respect to those in the annotation for said position"

<220>

<221> VARIANT
<222> (269)..(269)
<223> /replace="Lys" or "Gln"

<220>
<221> misc_feature
<222> (269)..(269)
<223> /note="Residue given in the sequence has no preference
with respect to those in the annotation for said position"

<220>
<221> VARIANT
<222> (270)..(270)
<223> /replace="Tyr" or "Asp"

<220>
<221> VARIANT
<222> (280)..(280)
<223> /replace="Gln" or "Asn" or "Asp"

<220>
<221> misc_feature
<222> (280)..(280)
<223> /note="Residue given in the sequence has no preference
with respect to those in the annotation for said position"

<220>
<221> VARIANT
<222> (285)..(285)
<223> /replace="Thr" or "Glu"

<220>
<221> VARIANT
<222> (299)..(299)
<223> /replace="Asn" or "Ala" or "Arg"

<220>
<221> misc_feature
<222> (299)..(299)
<223> /note="Residue given in the sequence has no preference
with respect to those in the annotation for said position"

<220>
<221> VARIANT
<222> (311)..(311)
<223> /replace="Asn" or "Lys" or "Arg" or "Gln"

<220>
<221> misc_feature
<222> (311)..(311)
<223> /note="Residue given in the sequence has no preference
with respect to those in the annotation for said position"

<220>
<221> VARIANT
<222> (314)..(314)
<223> /replace="Asn" or "Asp" or "Arg"

<220>
<221> misc_feature
<222> (314)..(314)
<223> /note="Residue given in the sequence has no preference
with respect to those in the annotation for said position"

<220>
<221> VARIANT
<222> (319)..(319)
<223> /replace="Gln" or "Ser" or "Glu"

<220>
<221> VARIANT
<222> (320)..(320)
<223> /replace="Arg" or "Lys" or "Ala"

<220>
<221> misc_feature
<222> (319)..(320)
<223> /note="Residues given in the sequence have no preference
with respect to those in the annotation for said positions"

<220>
<221> VARIANT
<222> (323)..(323)
<223> /replace="Ser" or "Leu" or "Glu" or "Met" or "Ile"

<220>
<221> VARIANT
<222> (324)..(324)
<223> /replace="His" or "Lys"

<220>
<221> misc_feature
<222> (323)..(324)
<223> /note="Residues given in the sequence have no preference
with respect to those in the annotation for said positions"

<220>
<221> VARIANT
<222> (346)..(346)
<223> /replace="Glu" or "Thr" or "Ser" or "Asp"

<220>
<221> misc_feature
<222> (346)..(346)
<223> /note="Residue given in the sequence has no preference
with respect to those in the annotation for said position"

<220>
<221> VARIANT
<222> (361)..(361)
<223> /replace="Gln" or "Ser" or "Gly"

<220>
<221> misc_feature

<222> (361)..(361)
<223> /note="Residue given in the sequence has no preference
with respect to those in the annotation for said position"

<220>
<221> VARIANT
<222> (365)..(365)
<223> /replace="Ser" or "Gln" or "Asn"

<220>
<221> misc_feature
<222> (365)..(365)
<223> /note="Residue given in the sequence has no preference
with respect to those in the annotation for said position"

<220>
<221> VARIANT
<222> (379)..(379)
<223> /replace="Pro" or " "

<220>
<221> VARIANT
<222> (380)..(380)
<223> /replace="Lys" or " "

<220>
<221> VARIANT
<222> (381)..(381)
<223> /replace=" "

<220>
<221> VARIANT
<222> (394)..(394)
<223> /replace="Glu" or "Lys" or "Gln" or "Asp"

<220>
<221> misc_feature
<222> (394)..(394)
<223> /note="Residue given in the sequence has no preference
with respect to those in the annotation for said position"

<220>
<221> VARIANT
<222> (398)..(398)
<223> /replace="Lys" or "Gln" or "Glu" or "Asn" or "Thr"

<220>
<221> misc_feature
<222> (398)..(398)
<223> /note="Residue given in the sequence has no preference
with respect to those in the annotation for said position"

<220>
<221> VARIANT
<222> (411)..(411)
<223> /replace="His" or "Pro" or "Trp"

<220>
<221> misc_feature
<222> (411)..(411)
<223> /note="Residue given in the sequence has no preference
with respect to those in the annotation for said position"

<220>
<221> VARIANT
<222> (421)..(421)
<223> /replace="Asn" or "Asp" or "Ser" or "Thr" or "Gly"

<220>
<221> misc_feature
<222> (421)..(421)
<223> /note="Residue given in the sequence has no preference
with respect to those in the annotation for said position"

<220>
<221> VARIANT
<222> (449)..(449)
<223> /replace="Lys" or "Asn"

<220>
<221> misc_feature
<222> (449)..(449)
<223> /note="Residue given in the sequence has no preference
with respect to those in the annotation for said position"

<220>
<221> VARIANT
<222> (452)..(452)
<223> /replace="Gln" or "Glu"

<220>
<221> misc_feature
<222> (452)..(452)
<223> /note="Residue given in the sequence has no preference
with respect to those in the annotation for said position"

<220>
<221> VARIANT
<222> (476)..(476)
<223> /replace="Ser" or "His" or "Thr" or "Phe"

<220>
<221> misc_feature
<222> (476)..(476)
<223> /note="Residue given in the sequence has no preference
with respect to those in the annotation for said position"

<220>
<221> VARIANT
<222> (487)..(487)
<223> /replace="Asn" or "Gln" or "Lys"

<220>
<221> misc_feature

<222> (487)..(487)
<223> /note="Residue given in the sequence has no preference
with respect to those in the annotation for said position"

<220>
<221> VARIANT
<222> (489)..(489)
<223> /replace=" "

<220>
<221> VARIANT
<222> (490)..(491)
<223> /replace=" "

<220>
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<222> (492)..(492)
<223> /replace=" "

<220>
<221> VARIANT
<222> (493)..(493)
<223> /replace=" "

<220>
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<222> (494)..(494)
<223> /replace=" "

<220>
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<223> /replace=" "

<220>
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<222> (496)..(496)
<223> /replace=" "

<220>
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<223> /replace=" "

<220>
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<222> (498)..(498)
<223> /replace=" "

<220>
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<222> (499)..(499)
<223> /replace=" "

<220>
<221> VARIANT
<222> (500)..(501)

<223> /replace=" "

<220>
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<222> (502)..(502)
<223> /replace=" "

<220>
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<222> (503)..(503)
<223> /replace=" "

<220>
<221> VARIANT
<222> (504)..(504)
<223> /replace=" "

<220>
<221> VARIANT
<222> (505)..(505)
<223> /replace=" "

<220>
<221> VARIANT
<222> (506)..(506)
<223> /replace=" "

<220>
<221> VARIANT
<222> (507)..(507)
<223> /replace=" "

<220>
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<222> (508)..(508)
<223> /replace=" "

<220>
<221> VARIANT
<222> (509)..(509)
<223> /replace=" "

<220>
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<222> (510)..(510)
<223> /replace=" "

<220>
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<222> (511)..(511)
<223> /replace=" "

<220>
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<222> (512)..(512)
<223> /replace=" "

<220>
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<222> (513)..(513)
<223> /replace=" "

<220>
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<222> (514)..(514)
<223> /replace=" "

<220>
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<222> (515)..(515)
<223> /replace=" "

<220>
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<222> (516)..(516)
<223> /replace=" "

<220>
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<222> (517)..(517)
<223> /replace=" "

<220>
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<222> (518)..(518)
<223> /replace=" "

<220>
<221> VARIANT
<222> (519)..(519)
<223> /replace=" "

<220>
<221> VARIANT
<222> (520)..(520)
<223> /replace=" "

<220>
<221> misc_feature
<222> (489)..(520)
<223> /note="Residues given in the sequence have no preference
with respect to those in the annotation for said positions"

<400> 16
His Ala Ala Pro Phe Asn Gly Thr Met Met Gln Tyr Phe Glu Trp Tyr
1 5 10 15

Leu Pro Asn Asp Gly Gln His Trp Thr Arg Leu Ala Asn Asp Ala Asn
20 25 30

Asn Leu Ser Ser Leu Gly Ile Thr Ala Leu Trp Ile Pro Pro Ala Tyr
35 40 45

```
Lys Gly Thr Ser Gln Ser Asp Val Gly Tyr Gly Ala Tyr Asp Leu Tyr
    50                  55                  60

Asp Leu Gly Glu Phe Asn Gln Lys Gly Thr Val Arg Thr Lys Tyr Gly
65                  70                  75                  80

Thr Lys Ala Gln Leu Leu Gln Ala Ile Gln Ala Leu His Ala Ala Gly
                85                  90                  95

Ile Gln Val Tyr Gly Asp Val Val Met Asn His Lys Gly Gly Ala Asp
            100                 105                 110

Gly Thr Glu Trp Val Asp Ala Val Glu Val Asn Pro Ser Asp Arg Asn
        115                 120                 125

Gln Glu Ile Ser Gly Thr Tyr Gln Ile Gln Ala Trp Thr Lys Phe Asp
    130                 135                 140

Phe Pro Gly Arg Gly Asn Thr Tyr Ser Ser Phe Lys Trp Arg Trp Tyr
145                 150                 155                 160

His Phe Asp Gly Val Asp Trp Asp Glu Ser Arg Lys Leu Asn Asn Arg
                165                 170                 175

Ile Tyr Lys Phe Arg Gly Ile Gly Lys Ala Trp Asp Trp Glu Val Asp
            180                 185                 190

Thr Glu Asn Gly Asn Tyr Asp Tyr Leu Met Tyr Ala Asp Ile Asp Met
        195                 200                 205

Asp His Pro Glu Val Val Thr Glu Leu Lys Asn Trp Gly Lys Trp Tyr
    210                 215                 220

Val Asn Thr Leu Asn Leu Asp Gly Phe Arg Leu Asp Ala Val Lys His
225                 230                 235                 240

Ile Lys Phe Ser Phe Phe Arg Asp Trp Leu Ser His Val Arg Ser Gln
                245                 250                 255

Thr Gly Lys Pro Leu Phe Thr Val Gly Glu Tyr Trp Ser Asn Asp Ile
            260                 265                 270
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```
Gly Ala Leu Glu Asn Tyr Leu Thr Lys Thr Asn Trp Asn Met Ser Leu
        275                 280                 285

Phe Asp Val Pro Leu His Tyr Asn Phe Tyr Thr Ala Ser Lys Ser Gly
    290                 295                 300

Gly Ala Tyr Asp Met Arg Thr Leu Leu Thr Gly Thr Leu Val Lys Asp
305                 310                 315                 320

His Pro Thr Leu Ala Val Thr Phe Val Asp Asn His Asp Thr Gln Pro
                325                 330                 335

Gly Gln Ala Leu Glu Ser Trp Val Asp Pro Trp Phe Lys Pro Leu Ala
            340                 345                 350

Tyr Ala Phe Ile Leu Thr Arg Glu Glu Gly Tyr Pro Cys Val Phe Tyr
        355                 360                 365

Gly Asp Tyr Tyr Gly Ile Pro Gln Tyr Asn Gln Arg Glu Ile Pro Ser
    370                 375                 380

Leu Lys Ser Lys Ile Asp Pro Leu Leu Ile Ala Arg Arg Asp Tyr Ala
385                 390                 395                 400

Tyr Gly Thr Gln His Asp Tyr Leu Asp His Ser Asp Ile Ile Gly Trp
                405                 410                 415

Thr Arg Glu Gly Val Thr Ser Lys Pro Asn Ser Gly Leu Ala Ala Leu
            420                 425                 430

Ile Thr Asp Gly Pro Gly Gly Ser Lys Trp Met Tyr Val Gly Lys Gln
        435                 440                 445

His Ala Gly Lys Val Trp Tyr Asp Leu Thr Gly Asn Arg Ser Asp Thr
    450                 455                 460

Val Thr Ile Asn Ser Asp Gly Trp Gly Glu Phe Lys Val Asn Gly Gly
465                 470                 475                 480

Ser Val Ser Val Trp Val Pro Arg Lys Thr Thr Val Ser Thr Ile Ala
                485                 490                 495
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Arg Pro Ile Thr Thr Arg Pro Trp Thr Gly Glu Phe Val Arg Trp Thr
500 505 510

Glu Pro Arg Leu Val Ala Trp Pro
515 520

<210> 17
<211> 39
<212> DNA
<213> Artificial sequence

<220>
<221> source
<223> /note="Description of artificial sequence: Synthetic oligonucleotide"

<220>
<221> modified_base
<222> (19)..(20)
<223> a, c, t, g, unknown or other

<400> 17
gtcaagcata ttaagttcnn stttttcct gattggttg 39

<210> 18
<211> 39
<212> DNA
<213> Artificial sequence

<220>
<221> source
<223> /note="Description of artificial sequence: Synthetic oligonucleotide"

<220>
<221> modified_base
<222> (20)..(21)
<223> a, c, t, g, unknown or other

<400> 18
caaccaatca ggaaaaaasn ngaacttaat atgcttgac 39

<210> 19
<211> 87
<212> DNA
<213> Artificial sequence

<220>
<221> source
<223> /note="Description of artificial sequence: Synthetic oligonucleotide"

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<400> 19
atgaaacaac aaaaacggct ttacgcccga ttgctgacgc tgttatttgc gctcatcttc 60

ttgctgcctc attctgcagc ttcagca 87

<210> 20
<211> 29
<212> PRT
<213> Artificial sequence

<220>
<221> source
<223> /note="Description of artificial sequence: Synthetic peptide"

<400> 20
Met Lys Gln Gln Lys Arg Leu Tyr Ala Arg Leu Leu Thr Leu Leu Phe
1 5 10 15

Ala Leu Ile Phe Leu Leu Pro His Ser Ala Ala Ser Ala
20 25

<210> 21
<211> 486
<212> PRT
<213> Artificial sequence

<220>
<221> source
<223> /note="Description of artificial sequence: Synthetic polypeptide"

<400> 21
Ala Ala Pro Phe Asn Gly Thr Met Met Gln Tyr Phe Glu Trp Tyr Leu
1 5 10 15

Pro Asp Asp Gly Thr Leu Trp Thr Lys Val Ala Asn Glu Ala Asn Asn
20 25 30

Leu Ser Ser Leu Gly Ile Thr Ala Leu Trp Leu Pro Pro Ala Tyr Lys
35 40 45

Gly Thr Ser Arg Ser Asp Val Gly Tyr Gly Val Tyr Asp Leu Tyr Asp
50 55 60

Leu Gly Glu Phe Asn Gln Lys Gly Thr Val Arg Thr Lys Tyr Gly Thr
65 70 75 80

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```
Lys Ala Gln Tyr Leu Gln Ala Ile Gln Ala Ala His Ala Ala Gly Met
                85                  90                  95

Gln Val Tyr Ala Asp Val Val Phe Asp His Lys Gly Gly Ala Asp Gly
            100                 105                 110

Thr Glu Trp Val Asp Ala Val Glu Val Asn Pro Ser Asp Arg Asn Gln
        115                 120                 125

Glu Ile Ser Gly Thr Tyr Gln Ile Gln Ala Trp Thr Lys Phe Asp Phe
    130                 135                 140

Pro Gly Arg Gly Asn Thr Tyr Ser Ser Phe Lys Trp Arg Trp Tyr His
145                 150                 155                 160

Phe Asp Gly Val Asp Trp Asp Glu Ser Arg Lys Leu Ser Arg Ile Tyr
                165                 170                 175

Lys Phe Arg Gly Ile Gly Lys Ala Trp Asp Trp Glu Val Asp Thr Glu
            180                 185                 190

Asn Gly Asn Tyr Asp Tyr Leu Met Tyr Ala Asp Leu Asp Met Asp His
        195                 200                 205

Pro Glu Val Val Thr Glu Leu Lys Asn Trp Gly Lys Trp Tyr Val Asn
    210                 215                 220

Thr Thr Asn Ile Asp Gly Phe Arg Leu Asp Ala Val Lys His Ile Lys
225                 230                 235                 240

Phe Gln Phe Phe Pro Asp Trp Leu Ser Tyr Val Arg Ser Gln Thr Gly
                245                 250                 255

Lys Pro Leu Phe Thr Val Gly Glu Tyr Trp Ser Tyr Asp Ile Asn Lys
            260                 265                 270

Leu His Asn Tyr Ile Thr Lys Thr Asn Gly Thr Met Ser Leu Phe Asp
        275                 280                 285

Ala Pro Leu His Asn Lys Phe Tyr Thr Ala Ser Lys Ser Gly Gly Ala
    290                 295                 300

Phe Asp Met Arg Thr Leu Met Thr Asn Thr Leu Met Lys Asp Gln Pro
```

```
305                 310                 315                 320

Thr Leu Ala Val Thr Phe Val Asp Asn His Asp Thr Glu Pro Gly Gln
                325                 330                 335

Ala Leu Gln Ser Trp Val Asp Pro Trp Phe Lys Pro Leu Ala Tyr Ala
            340                 345                 350

Phe Ile Leu Thr Arg Gln Glu Gly Tyr Pro Cys Val Phe Tyr Gly Asp
        355                 360                 365

Tyr Tyr Gly Ile Pro Gln Tyr Asn Ile Pro Ser Leu Lys Ser Lys Ile
    370                 375                 380

Asp Pro Leu Leu Ile Ala Arg Arg Asp Tyr Ala Tyr Gly Thr Gln His
385                 390                 395                 400

Asp Tyr Leu Asp His Ser Asp Ile Ile Gly Trp Thr Arg Glu Gly Val
                405                 410                 415

Thr Glu Lys Pro Gly Ser Gly Leu Ala Ala Leu Ile Thr Asp Gly Pro
            420                 425                 430

Gly Gly Ser Lys Trp Met Tyr Val Gly Lys Gln His Ala Gly Lys Val
        435                 440                 445

Phe Tyr Asp Leu Thr Gly Asn Arg Ser Asp Thr Val Thr Ile Asn Ser
    450                 455                 460

Asp Gly Trp Gly Glu Phe Lys Val Asn Gly Gly Ser Val Ser Val Trp
465                 470                 475                 480

Val Pro Arg Lys Thr Thr
                485
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<210> 22
<211> 517
<212> PRT
<213> Artificial sequence

<220>
<221> source
<223> /note="Description of artificial sequence: Synthetic consensus sequence"

<220>
<221> VARIANT
<222> (1)..(1)
<223> /replace=" "

<220>
<221> VARIANT
<222> (2)..(2)
<223> /replace="His"

<220>
<221> VARIANT
<222> (3)..(3)
<223> /replace="Asn"

<220>
<221> VARIANT
<222> (4)..(4)
<223> /replace="Gly"

<220>
<221> VARIANT
<222> (5)..(5)
<223> /replace="Thr"

<220>
<221> misc_feature
<222> (1)..(5)
<223> /note="Residues given in the sequence have no preference
with respect to those in the annotation for said positions"

<220>
<221> VARIANT
<222> (19)..(19)
<223> /replace="Asn"

<220>
<221> misc_feature
<222> (19)..(19)
<223> /note="Residues given in the sequence have no preference
with respect to those in the annotation for said positions"

<220>
<221> VARIANT
<222> (22)..(22)
<223> /replace="Asn"

<220>
<221> VARIANT
<222> (23)..(23)
<223> /replace="His"

<220>
<221> misc_feature
<222> (22)..(23)
<223> /note="Residues given in the sequence have no preference

with respect to those in the annotation for said positions"

<220>
<221> VARIANT
<222> (25)..(25)
<223> /replace="Asn"

<220>
<221> misc_feature
<222> (25)..(25)
<223> /note="Residue given in the sequence has no preference
with respect to those in the annotation for said position"

<220>
<221> VARIANT
<222> (28)..(28)
<223> /replace="Asn"

<220>
<221> VARIANT
<222> (29)..(29)
<223> /replace="Ser"

<220>
<221> misc_feature
<222> (28)..(29)
<223> /note="Residues given in the sequence have no preference
with respect to those in the annotation for said positions"

<220>
<221> VARIANT
<222> (32)..(32)
<223> /replace="Ser"

<220>
<221> misc_feature
<222> (32)..(32)
<223> /note="Residue given in the sequence has no preference
with respect to those in the annotation for said position"

<220>
<221> VARIANT
<222> (35)..(35)
<223> /replace="Lys"

<220>
<221> misc_feature
<222> (35)..(35)
<223> /note="Residue given in the sequence has no preference
with respect to those in the annotation for said position"

<220>
<221> VARIANT
<222> (37)..(37)
<223> /replace="Lys"

<220>

<221> misc_feature
<222> (37)..(37)
<223> /note="Residue given in the sequence has no preference
with respect to those in the annotation for said position"

<220>
<221> VARIANT
<222> (51)..(51)
<223> /replace="Ala"

<220>
<221> misc_feature
<222> (51)..(51)
<223> /note="Residue given in the sequence has no preference
with respect to those in the annotation for said position"

<220>
<221> VARIANT
<222> (53)..(53)
<223> /replace="Gln"

<220>
<221> VARIANT
<222> (54)..(54)
<223> /replace="Asn"

<220>
<221> misc_feature
<222> (53)..(54)
<223> /note="Residues given in the sequence have no preference
with respect to those in the annotation for said positions"

<220>
<221> VARIANT
<222> (60)..(60)
<223> /replace="Ala"

<220>
<221> misc_feature
<222> (60)..(60)
<223> /note="Residue given in the sequence has no preference
with respect to those in the annotation for said position"

<220>
<221> VARIANT
<222> (85)..(85)
<223> /replace="Leu"

<220>
<221> VARIANT
<222> (86)..(86)
<223> /replace="Gln"

<220>
<221> VARIANT
<222> (87)..(87)
<223> /replace="Ala"

<220>
<221> misc_feature
<222> (85)..(87)
<223> /note="Residues given in the sequence have no preference
with respect to those in the annotation for said positions"

<220>
<221> VARIANT
<222> (90)..(90)
<223> /replace="Thr"

<220>
<221> misc_feature
<222> (90)..(90)
<223> /note="Residue given in the sequence has no preference
with respect to those in the annotation for said position"

<220>
<221> VARIANT
<222> (92)..(92)
<223> /replace="Leu"

<220>
<221> VARIANT
<222> (93)..(93)
<223> /replace="Lys"

<220>
<221> VARIANT
<222> (94)..(95)
<223> /replace="Asn"

<220>
<221> misc_feature
<222> (92)..(95)
<223> /note="Residues given in the sequence have no preference
with respect to those in the annotation for said positions"

<220>
<221> VARIANT
<222> (105)..(105)
<223> /replace="Met"

<220>
<221> VARIANT
<222> (106)..(106)
<223> /replace="Asn"

<220>
<221> misc_feature
<222> (105)..(106)
<223> /note="Residues given in the sequence have no preference
with respect to those in the annotation for said positions"

<220>
<221> VARIANT

<222> (116)..(116)
<223> /replace="Met"

<220>
<221> misc_feature
<222> (116)..(116)
<223> /note="Residue given in the sequence has no preference
with respect to those in the annotation for said position"

<220>
<221> VARIANT
<222> (118)..(118)
<223> /replace="Arg"

<220>
<221> misc_feature
<222> (118)..(118)
<223> /note="Residue given in the sequence has no preference
with respect to those in the annotation for said position"

<220>
<221> VARIANT
<222> (125)..(125)
<223> /replace="Asn"

<220>
<221> VARIANT
<222> (126)..(126)
<223> /replace="Asn"

<220>
<221> misc_feature
<222> (125)..(126)
<223> /note="Residues given in the sequence have no preference
with respect to those in the annotation for said positions"

<220>
<221> VARIANT
<222> (134)..(134)
<223> /replace="Glu"

<220>
<221> misc_feature
<222> (134)..(134)
<223> /note="Residue given in the sequence has no preference
with respect to those in the annotation for said position"

<220>
<221> VARIANT
<222> (136)..(136)
<223> /replace="Thr"

<220>
<221> misc_feature
<222> (136)..(136)
<223> /note="Residue given in the sequence has no preference
with respect to those in the annotation for said position"

<220>
<221> VARIANT
<222> (138)..(138)
<223> /replace="Glu"

<220>
<221> misc_feature
<222> (138)..(138)
<223> /note="Residue given in the sequence has no preference
with respect to those in the annotation for said position"

<220>
<221> VARIANT
<222> (169)..(169)
<223> /replace="Gln"

<220>
<221> misc_feature
<222> (169)..(169)
<223> /note="Residue given in the sequence has no preference
with respect to those in the annotation for said position"

<220>
<221> VARIANT
<222> (174)..(174)
<223> /replace="Asn"

<220>
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<222> (174)..(175)
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<222> (183)..(183)
<223> /replace="His"

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<223> /replace="Leu"

<220>
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<222> (229)..(229)
<223> /replace="Gly"

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<223> /replace="Thr"

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<223> /replace="Arg"

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<220>
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<220>
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<220>
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<222> (274)..(274)
<223> /replace="Ala"

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<220>
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<223> /replace="Trp"

<220>
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<223> /replace="Asn"

<220>
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<223> /replace="His"

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<222> (284)..(286)
<223> /note="Residues given in the sequence have no preference
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<223> /replace="Val"

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<223> /replace="Tyr"

<220>
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<222> (296)..(296)
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<223> /replace="Leu"

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<223> /replace="Asn"

<220>
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<223> /replace="Phe"

<220>
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<223> /replace="Asn"

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<223> /replace="Gly"

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<220>
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<223> /replace="His"

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<223> /replace="Glu"

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<223> /replace="Glu"

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<223> /note="Residue given in the sequence has no preference

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<222> (355)..(355)
<223> /replace="Leu"

<220>
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<222> (356)..(356)
<223> /replace="Thr"

<220>
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<222> (355)..(356)
<223> /note="Residues given in the sequence have no preference
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<222> (360)..(360)
<223> /replace="Glu"

<220>
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<222> (361)..(361)
<223> /replace="Gln"

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<223> /replace="Ser"

<220>
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<222> (365)..(365)
<223> /note="Residue given in the sequence has no preference
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<223> /replace="Thr"

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<222> (376)..(376)
<223> /note="Residue given in the sequence has no preference
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<222> (378)..(378)
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<222> (394)..(394)
<223> /replace="Gln"

<220>
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<222> (395)..(395)
<223> /replace="Lys"

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<223> /note="Residues given in the sequence have no preference
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<223> /replace="Lys"

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<222> (400)..(400)
<223> /note="Residue given in the sequence has no preference
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<223> /replace="Asn"

<220>
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<222> (402)..(402)
<223> /note="Residue given in the sequence has no preference

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<220>
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<222> (408)..(408)
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<220>
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<222> (409)..(409)
<223> /replace="Asn"

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<222> (408)..(409)
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<222> (452)..(452)
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<223> /replace="Gly"

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<223> /replace=" "

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<223> /replace=" "

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His Ala Ala Pro Phe Asn Gly Thr Met Met Gln Tyr Phe Glu Trp Tyr
1 5 10 15

Leu Pro Asp Asp Gly Thr Leu Trp Thr Lys Leu Ala Asn Asp Ala Asn
20 25 30

Asn Leu Ser Ser Leu Gly Ile Thr Ala Leu Trp Ile Pro Pro Ala Trp
35 40 45

Lys Gly Thr Ser Arg Ser Asp Val Gly Tyr Gly Val Tyr Asp Leu Tyr
50 55 60

Asp Leu Gly Glu Phe Asn Gln Lys Gly Thr Val Arg Thr Lys Tyr Gly
65 70 75 80

Thr Lys Ala Gln Tyr Leu Gln Ala Ile Gln Ala Ala His Ala Ala Gly
85 90 95

Ile Gln Val Tyr Ala Asp Val Val Phe Asp His Lys Gly Gly Ala Asp
100 105 110

Ala Thr Glu Trp Val Asp Ala Val Glu Val Asn Pro Ser Asp Arg Asn
115 120 125

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Gln Glu Ile Ser Gly Thr Tyr Gln Ile Gln Ala Trp Thr Lys Phe Asp
    130                 135                 140

Phe Pro Gly Arg Gly Asn Thr His Ser Ser Phe Lys Trp Arg Trp Tyr
145                 150                 155                 160

His Phe Asp Gly Val Asp Trp Asp Glu Ser Arg Lys Leu Ser Asn Arg
                165                 170                 175

Ile Tyr Lys Phe Arg Gly Ile Gly Lys Ala Trp Asp Trp Glu Val Asp
            180                 185                 190

Thr Glu Asn Gly Asn Tyr Asp Tyr Leu Met Tyr Ala Asp Ile Asp Met
        195                 200                 205

Asp His Pro Glu Val Val Thr Glu Leu Lys Asn Trp Gly Lys Trp Tyr
    210                 215                 220

Val Asn Thr Thr Asn Ile Asp Gly Phe Arg Ile Asp Ala Val Lys His
225                 230                 235                 240

Ile Lys Phe Ser Phe Phe Pro Asp Trp Ile Ser His Val Arg Ser Gln
                245                 250                 255

Thr Gly Lys Pro Leu Phe Thr Val Ala Glu Phe Trp Ser Tyr Asp Ile
            260                 265                 270

Asn Lys Ile His Asn Tyr Ile Thr Lys Thr Asn Gly Thr Met Ser Leu
        275                 280                 285

Phe Asp Ala Pro Leu His Asn Lys Phe Tyr Thr Ala Ser Lys Ser Gly
    290                 295                 300

Gly Ala Phe Asp Met Arg Thr Ile Met Thr Asn Thr Leu Met Lys Asp
305                 310                 315                 320

Gln Pro Ser Leu Ala Val Thr Phe Val Asp Asn His Asp Ser Glu Pro
                325                 330                 335

Gly Gln Ala Leu Gln Ser Phe Val Asp Pro Trp Phe Lys Pro Leu Ala
            340                 345                 350
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Tyr Ala Phe Ile Leu Thr Arg Gln Glu Gly Tyr Pro Cys Val Phe Tyr
        355                 360                 365

Gly Asp Tyr Tyr Gly Ile Pro Gln His Asn Ile Pro Ala Leu Lys Ser
    370                 375                 380

Lys Ile Asp Pro Ile Leu Ile Ala Arg Arg Asp Tyr Ala Tyr Gly Thr
385                 390                 395                 400

Gln His Asp Tyr Leu Asp His Ser Asp Ile Ile Gly Trp Thr Arg Glu
                405                 410                 415

Gly Val Thr Glu Lys Pro Gly Ser Gly Leu Ala Ala Ile Ile Ser Asp
            420                 425                 430

Gly Pro Gly Gly Ser Lys Trp Met Phe Val Gly Lys Asn His Ala Gly
        435                 440                 445

Lys Val Phe Tyr Asp Ile Thr Gly Asn Arg Ser Asp Thr Val Thr Ile
    450                 455                 460

Asn Ala Asp Gly Trp Gly Glu Phe Lys Val Asn Gly Gly Ser Val Ser
465                 470                 475                 480

Ile Trp Val Pro Lys Lys Thr Thr Val Ser Thr Ile Ala Arg Pro Ile
                485                 490                 495

Thr Thr Arg Pro Trp Thr Gly Glu Phe Val Arg Trp Thr Glu Pro Arg
            500                 505                 510

Leu Val Ala Trp Pro
        515
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<210> 23
<211> 518
<212> PRT
<213> Artificial sequence

<220>
<221> source
<223> /note="Description of artificial sequence: Synthetic consensus sequence"

<220>

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<223> /replace=" "

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with respect to those in the annotation for said position"

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<222> (3)..(3)
<223> /replace="Asn"

<220>
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<222> (4)..(4)
<223> /replace="Leu"

<220>
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<223> /note="Residues given in the sequence have no preference
with respect to those in the annotation for said positions"

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<223> /replace="Asn"

<220>
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<222> (22)..(22)
<223> /replace="His"

<220>
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<222> (21)..(22)
<223> /note="Residues given in the sequence have no preference
with respect to those in the annotation for said positions"

<220>
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<223> /replace="Arg"

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<223> /replace="Gln"

<220>
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<222> (27)..(27)
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<220>
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<222> (32)..(32)
<223> /replace="Tyr"

<220>
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<222> (31)..(32)
<223> /note="Residues given in the sequence have no preference
with respect to those in the annotation for said positions"

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<223> /replace="Glu"

<220>
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<222> (36)..(36)
<223> /replace="His"

<220>
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with respect to those in the annotation for said positions"

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<223> /replace="Gln"

<220>
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<222> (52)..(52)
<223> /note="Residue given in the sequence has no preference
with respect to those in the annotation for said position"

<220>
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<222> (59)..(59)
<223> /replace="Ala"

<220>
<221> misc_feature
<222> (59)..(59)
<223> /note="Residue given in the sequence has no preference
with respect to those in the annotation for said position"

<220>
<221> VARIANT
<222> (69)..(69)
<223> /replace="His"

<220>
<221> misc_feature
<222> (69)..(69)
<223> /note="Residue given in the sequence has no preference
with respect to those in the annotation for said position"

<220>
<221> VARIANT
<222> (83)..(83)
<223> /replace="Glu"

<220>
<221> VARIANT
<222> (84)..(84)
<223> /replace="Leu"

<220>
<221> VARIANT
<222> (85)..(85)
<223> /replace="Gln"

<220>
<221> VARIANT
<222> (86)..(86)
<223> /replace="Ser"

<220>
<221> misc_feature
<222> (83)..(86)
<223> /note="Residues given in the sequence have no preference
with respect to those in the annotation for said positions"

<220>
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<222> (89)..(89)
<223> /replace="Lys"

<220>
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<222> (89)..(89)
<223> /note="Residue given in the sequence has no preference
with respect to those in the annotation for said position"

<220>
<221> VARIANT
<222> (91)..(91)
<223> /replace="Leu"

<220>
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<223> /note="Residue given in the sequence has no preference
with respect to those in the annotation for said position"

<220>
<221> VARIANT
<222> (94)..(94)
<223> /replace="Arg"

<220>
<221> VARIANT
<222> (95)..(95)
<223> /replace="Asp"

<220>
<221> misc_feature
<222> (94)..(95)
<223> /note="Residues given in the sequence have no preference
with respect to those in the annotation for said positions"

<220>
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<222> (104)..(104)
<223> /replace="Ile"

<220>
<221> VARIANT
<222> (105)..(105)
<223> /replace="Asn"

<220>
<221> misc_feature
<222> (104)..(105)
<223> /note="Residues given in the sequence have no preference
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<220>
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<222> (115)..(115)
<223> /replace="Asp"

<220>
<221> misc_feature
<222> (115)..(115)

<223> /note="Residue given in the sequence has no preference
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<220>
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<222> (117)..(117)
<223> /replace="Thr"

<220>
<221> misc_feature
<222> (117)..(117)
<223> /note="Residue given in the sequence has no preference
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<220>
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<222> (122)..(122)
<223> /replace="Asp"

<220>
<221> misc_feature
<222> (122)..(122)
<223> /note="Residue given in the sequence has no preference
with respect to those in the annotation for said position"

<220>
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<222> (128)..(128)
<223> /replace="Arg"

<220>
<221> VARIANT
<222> (129)..(129)
<223> /replace="Val"

<220>
<221> misc_feature
<222> (128)..(129)
<223> /note="Residues given in the sequence have no preference
with respect to those in the annotation for said positions"

<220>
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<222> (133)..(133)
<223> /replace="Glu"

<220>
<221> misc_feature
<222> (133)..(133)
<223> /note="Residue given in the sequence has no preference
with respect to those in the annotation for said position"

<220>
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<222> (135)..(135)
<223> /replace="Leu"

<220>
<221> misc_feature
<222> (135)..(135)
<223> /note="Residue given in the sequence has no preference
with respect to those in the annotation for said position"

<220>
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<222> (137)..(137)
<223> /replace="Lys"

<220>
<221> misc_feature
<222> (137)..(137)
<223> /note="Residue given in the sequence has no preference
with respect to those in the annotation for said position"

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<222> (141)..(141)
<223> /replace="His"

<220>
<221> misc_feature
<222> (141)..(141)
<223> /note="Residue given in the sequence has no preference
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<220>
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<222> (143)..(143)
<223> /replace="His"

<220>
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<222> (143)..(143)
<223> /note="Residue given in the sequence has no preference
with respect to those in the annotation for said position"

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<222> (149)..(149)
<223> /replace="Ser"

<220>
<221> misc_feature
<222> (149)..(149)
<223> /note="Residue given in the sequence has no preference
with respect to those in the annotation for said position"

<220>
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<222> (153)..(153)
<223> /replace="Asp"

<220>
<221> misc_feature

<222> (153)..(153)
<223> /note="Residue given in the sequence has no preference
with respect to those in the annotation for said position"

<220>
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<222> (157)..(157)
<223> /replace="His"

<220>
<221> misc_feature
<222> (157)..(157)
<223> /note="Residue given in the sequence has no preference
with respect to those in the annotation for said position"

<220>
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<222> (164)..(164)
<223> /replace="Thr"

<220>
<221> misc_feature
<222> (164)..(164)
<223> /note="Residue given in the sequence has no preference
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<220>
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<222> (173)..(173)
<223> /replace="Asn"

<220>
<221> misc_feature
<222> (173)..(173)
<223> /note="Residue given in the sequence has no preference
with respect to those in the annotation for said position"

<220>
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<222> (179)..(179)
<223> /replace="Gln"

<220>
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<222> (180)..(180)
<223> /replace=" "

<220>
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<222> (181)..(181)
<223> /replace=" "

<220>
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<222> (179)..(181)
<223> /note="Residues given in the sequence have no preference
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<220>
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<222> (190)..(190)
<223> /replace="Ser"

<220>
<221> VARIANT
<222> (191)..(191)
<223> /replace="Asn"

<220>
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<222> (190)..(191)
<223> /note="Residues given in the sequence have no preference
with respect to those in the annotation for said positions"

<220>
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<222> (206)..(206)
<223> /replace="Tyr"

<220>
<221> misc_feature
<222> (206)..(206)
<223> /note="Residue given in the sequence has no preference
with respect to those in the annotation for said position"

<220>
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<222> (212)..(212)
<223> /replace="Ala"

<220>
<221> VARIANT
<222> (213)..(213)
<223> /replace="Ala"

<220>
<221> misc_feature
<222> (212)..(213)
<223> /note="Residues given in the sequence have no preference
with respect to those in the annotation for said positions"

<220>
<221> VARIANT
<222> (217)..(217)
<223> /replace="Arg"

<220>
<221> misc_feature
<222> (217)..(217)
<223> /note="Residue given in the sequence has no preference
with respect to those in the annotation for said position"

<220>
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<222> (220)..(220)
<223> /replace="Thr"

<220>
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<222> (220)..(220)
<223> /note="Residue given in the sequence has no preference
with respect to those in the annotation for said position"

<220>
<221> VARIANT
<222> (223)..(223)
<223> /replace="Ala"

<220>
<221> misc_feature
<222> (223)..(223)
<223> /note="Residue given in the sequence has no preference
with respect to those in the annotation for said position"

<220>
<221> VARIANT
<222> (225)..(225)
<223> /replace="Glu"

<220>
<221> VARIANT
<222> (226)..(226)
<223> /replace="Leu"

<220>
<221> misc_feature
<222> (225)..(226)
<223> /note="Residues given in the sequence have no preference
with respect to those in the annotation for said positions"

<220>
<221> VARIANT
<222> (244)..(244)
<223> /replace="Leu"

<220>
<221> VARIANT
<222> (245)..(245)
<223> /replace="Arg"

<220>
<221> misc_feature
<222> (244)..(245)
<223> /note="Residues given in the sequence have no preference
with respect to those in the annotation for said positions"

<220>
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<222> (249)..(249)
<223> /replace="Asn"

<220>
<221> misc_feature
<222> (249)..(249)
<223> /note="Residue given in the sequence has no preference
with respect to those in the annotation for said position"

<220>
<221> VARIANT
<222> (253)..(253)
<223> /replace="Glu"

<220>
<221> VARIANT
<222> (254)..(254)
<223> /replace="Lys"

<220>
<221> misc_feature
<222> (253)..(254)
<223> /note="Residues given in the sequence have no preference
with respect to those in the annotation for said positions"

<220>
<221> VARIANT
<222> (258)..(258)
<223> /replace="Glu"

<220>
<221> misc_feature
<222> (258)..(258)
<223> /note="Residue given in the sequence has no preference
with respect to those in the annotation for said position"

<220>
<221> VARIANT
<222> (267)..(267)
<223> /replace="Gln"

<220>
<221> VARIANT
<222> (268)..(268)
<223> /replace="Asn"

<220>
<221> misc_feature
<222> (267)..(268)
<223> /note="Residues given in the sequence have no preference
with respect to those in the annotation for said positions"

<220>
<221> VARIANT
<222> (271)..(271)
<223> /replace="Gly"

<220>
<221> VARIANT
<222> (272)..(272)

<223> /replace="Ala"

<220>
<221> misc_feature
<222> (271)..(272)
<223> /note="Residues given in the sequence have no preference
with respect to those in the annotation for said positions"

<220>
<221> VARIANT
<222> (274)..(274)
<223> /replace="Glu"

<220>
<221> misc_feature
<222> (274)..(274)
<223> /note="Residue given in the sequence has no preference
with respect to those in the annotation for said position"

<220>
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<222> (278)..(278)
<223> /replace="Asn"

<220>
<221> misc_feature
<222> (278)..(278)
<223> /note="Residue given in the sequence has no preference
with respect to those in the annotation for said position"

<220>
<221> VARIANT
<222> (282)..(282)
<223> /replace="Phe"

<220>
<221> VARIANT
<222> (283)..(283)
<223> /replace="Asn"

<220>
<221> VARIANT
<222> (284)..(284)
<223> /replace="His"

<220>
<221> misc_feature
<222> (282)..(284)
<223> /note="Residues given in the sequence have no preference
with respect to those in the annotation for said positions"

<220>
<221> VARIANT
<222> (289)..(289)
<223> /replace="Val"

<220>

<221> misc_feature
<222> (289)..(289)
<223> /note="Residue given in the sequence has no preference
with respect to those in the annotation for said position"

<220>
<221> VARIANT
<222> (293)..(293)
<223> /replace="Tyr"

<220>
<221> VARIANT
<222> (294)..(294)
<223> /replace="Gln"

<220>
<221> misc_feature
<222> (293)..(294)
<223> /note="Residues given in the sequence have no preference
with respect to those in the annotation for said positions"

<220>
<221> VARIANT
<222> (297)..(297)
<223> /replace="Ala"

<220>
<221> misc_feature
<222> (297)..(297)
<223> /note="Residue given in the sequence has no preference
with respect to those in the annotation for said position"

<220>
<221> VARIANT
<222> (300)..(300)
<223> /replace="Thr"

<220>
<221> VARIANT
<222> (301)..(301)
<223> /replace="Gln"

<220>
<221> misc_feature
<222> (300)..(301)
<223> /note="Residues given in the sequence have no preference
with respect to those in the annotation for said positions"

<220>
<221> VARIANT
<222> (309)..(309)
<223> /replace="Lys"

<220>
<221> misc_feature
<222> (309)..(309)
<223> /note="Residue given in the sequence has no preference

with respect to those in the annotation for said position"

<220>
<221> VARIANT
<222> (312)..(312)
<223> /replace="Asn"

<220>
<221> VARIANT
<222> (313)..(313)
<223> /replace="Gly"

<220>
<221> misc_feature
<222> (312)..(313)
<223> /note="Residues given in the sequence have no preference
with respect to those in the annotation for said positions"

<220>
<221> VARIANT
<222> (317)..(317)
<223> /replace="Ser"

<220>
<221> VARIANT
<222> (318)..(318)
<223> /replace="Lys"

<220>
<221> VARIANT
<222> (319)..(319)
<223> /replace="His"

<220>
<221> misc_feature
<222> (317)..(319)
<223> /note="Residues given in the sequence have no preference
with respect to those in the annotation for said positions"

<220>
<221> VARIANT
<222> (321)..(321)
<223> /replace="Leu"

<220>
<221> VARIANT
<222> (322)..(322)
<223> /replace="Lys"

<220>
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<222> (321)..(322)
<223> /note="Residues given in the sequence have no preference
with respect to those in the annotation for said positions"

<220>
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<222> (333)..(333)
<223> /replace="Gln"

<220>
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<222> (333)..(333)
<223> /note="Residue given in the sequence has no preference
with respect to those in the annotation for said position"

<220>
<221> VARIANT
<222> (339)..(339)
<223> /replace="Glu"

<220>
<221> misc_feature
<222> (339)..(339)
<223> /note="Residue given in the sequence has no preference
with respect to those in the annotation for said position"

<220>
<221> VARIANT
<222> (341)..(341)
<223> /replace="Thr"

<220>
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<222> (341)..(341)
<223> /note="Residue given in the sequence has no preference
with respect to those in the annotation for said position"

<220>
<221> VARIANT
<222> (343)..(343)
<223> /replace="Gln"

<220>
<221> VARIANT
<222> (344)..(344)
<223> /replace="Thr"

<220>
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<222> (343)..(344)
<223> /note="Residues given in the sequence have no preference
with respect to those in the annotation for said positions"

<220>
<221> VARIANT
<222> (358)..(358)
<223> /replace="Glu"

<220>
<221> VARIANT
<222> (359)..(359)
<223> /replace="Ser"

<220>
<221> misc_feature
<222> (358)..(359)
<223> /note="Residues given in the sequence have no preference
with respect to those in the annotation for said positions"

<220>
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<222> (363)..(363)
<223> /replace="Gln"

<220>
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<222> (363)..(363)
<223> /note="Residue given in the sequence has no preference
with respect to those in the annotation for said position"

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<222> (369)..(369)
<223> /replace="Met"

<220>
<221> misc_feature
<222> (369)..(369)
<223> /note="Residue given in the sequence has no preference
with respect to those in the annotation for said position"

<220>
<221> VARIANT
<222> (372)..(372)
<223> /replace="Thr"

<220>
<221> VARIANT
<222> (373)..(373)
<223> /replace="Lys"

<220>
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<222> (374)..(374)
<223> /replace=" "

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<222> (375)..(375)
<223> /replace=" "

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<222> (376)..(376)
<223> /replace=" "

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<222> (378)..(378)
<223> /replace="Arg"

<220>
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<222> (379)..(379)
<223> /replace="Glu"

<220>
<221> misc_feature
<222> (372)..(379)
<223> /note="Residues given in the sequence have no preference
with respect to those in the annotation for said positions"

<220>
<221> VARIANT
<222> (385)..(385)
<223> /replace="His"

<220>
<221> misc_feature
<222> (385)..(385)
<223> /note="Residue given in the sequence has no preference
with respect to those in the annotation for said position"

<220>
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<222> (392)..(392)
<223> /replace="Lys"

<220>
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<222> (392)..(392)
<223> /note="Residue given in the sequence has no preference
with respect to those in the annotation for said position"

<220>
<221> VARIANT
<222> (396)..(396)
<223> /replace="Gln"

<220>
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<222> (396)..(396)
<223> /note="Residue given in the sequence has no preference
with respect to those in the annotation for said position"

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<222> (401)..(401)
<223> /replace="Ala"

<220>
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<222> (401)..(401)
<223> /note="Residue given in the sequence has no preference
with respect to those in the annotation for said position"

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<221> VARIANT
<222> (406)..(406)
<223> /replace="Phe"

<220>
<221> misc_feature
<222> (406)..(406)
<223> /note="Residue given in the sequence has no preference
with respect to those in the annotation for said position"

<220>
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<222> (409)..(409)
<223> /replace="His"

<220>
<221> misc_feature
<222> (409)..(409)
<223> /note="Residue given in the sequence has no preference
with respect to those in the annotation for said position"

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<222> (419)..(419)
<223> /replace="Asp"

<220>
<221> misc_feature
<222> (419)..(419)
<223> /note="Residue given in the sequence has no preference
with respect to those in the annotation for said position"

<220>
<221> VARIANT
<222> (421)..(421)
<223> /replace="Ser"

<220>
<221> VARIANT
<222> (422)..(422)
<223> /replace="Val"

<220>
<221> VARIANT
<222> (423)..(423)
<223> /replace="Ala"

<220>
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<222> (424)..(424)
<223> /replace="Asn"

<220>
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<222> (421)..(424)
<223> /note="Residues given in the sequence have no preference
with respect to those in the annotation for said positions"

<220>
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<222> (440)..(440)
<223> /replace="Arg"

<220>
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<222> (440)..(440)
<223> /note="Residue given in the sequence has no preference
with respect to those in the annotation for said position"

<220>
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<222> (447)..(447)
<223> /replace="Asn"

<220>
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<222> (447)..(447)
<223> /note="Residue given in the sequence has no preference
with respect to those in the annotation for said position"

<220>
<221> VARIANT
<222> (450)..(450)
<223> /replace="Glu"

<220>
<221> VARIANT
<222> (451)..(451)
<223> /replace="Thr"

<220>
<221> misc_feature
<222> (450)..(451)
<223> /note="Residues given in the sequence have no preference
with respect to those in the annotation for said positions"

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<222> (462)..(462)
<223> /replace="Pro"

<220>
<221> misc_feature
<222> (462)..(462)
<223> /note="Residue given in the sequence has no preference
with respect to those in the annotation for said position"

<220>
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<222> (464)..(464)
<223> /replace="Val"

<220>
<221> misc_feature

<222> (464)..(464)
<223> /note="Residue given in the sequence has no preference
with respect to those in the annotation for said position"

<220>
<221> VARIANT
<222> (474)..(474)
<223> /replace="His"

<220>
<221> misc_feature
<222> (474)..(474)
<223> /note="Residue given in the sequence has no preference
with respect to those in the annotation for said position"

<220>
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<222> (485)..(485)
<223> /replace="Gln"

<220>
<221> misc_feature
<222> (485)..(485)
<223> /note="Residue given in the sequence has no preference
with respect to those in the annotation for said position"

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<222> (487)..(487)
<223> /replace=" "

<220>
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<222> (488)..(489)
<223> /replace=" "

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<223> /replace=" "

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<223> /replace=" "

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<223> /replace=" "

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<223> /replace=" "

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<223> /replace=" "

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<222> (495)..(495)
<223> /replace=" "

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<223> /replace=" "

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<223> /replace=" "

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<223> /replace=" "

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<223> /replace=" "

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<223> /replace=" "

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<223> /replace=" "

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<223> /replace=" "

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<223> /replace=" "

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<223> /replace=" "

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<223> /replace=" "

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<223> /replace=" "

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<223> /replace=" "

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<223> /replace=" "

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<223> /replace=" "

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<223> /replace=" "

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<223> /replace=" "

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<222> (513)..(513)
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Ala Ala Pro Phe Asn Gly Thr Leu Met Gln Tyr Phe Glu Trp Tyr Leu
1               5                  10                  15

Pro Asp Asp Gly Thr Leu Trp Thr Lys Leu Ala Asn Asp Ala Asn Asn
            20                  25                  30

Leu Ala Ser Leu Gly Ile Thr Ala Leu Trp Ile Pro Pro Ala Tyr Lys
        35                  40                  45

Gly Thr Ser Arg Ala Asp Val Gly Tyr Gly Val Tyr Asp Leu Tyr Asp
    50                  55                  60

Leu Gly Glu Phe Asn Gln Lys Gly Thr Val Arg Thr Lys Tyr Gly Thr
65                  70                  75                  80

Lys Ala Gln Tyr Leu Gln Ala Ile Gln Ala Ala His Ala Ala Gly Ile
                85                  90                  95

Asn Val Tyr Ala Asp Val Val Phe Asp His Lys Gly Gly Ala Asp Ala
            100                 105                 110

Thr Glu Trp Val Asp Ala Val Glu Val Asn Pro Ala Asp Arg Asn Gln
        115                 120                 125

Glu Ile Ser Gly Thr His Gln Ile Gln Ala Trp Thr Lys Phe Asp Phe
    130                 135                 140

Pro Gly Arg Gly Asn Thr Tyr Ser Ser Phe Lys Trp Arg Trp Tyr His
145                 150                 155                 160

Phe Asp Gly Val Asp Trp Asp Glu Ser Arg Lys Leu Ser Arg Ile Tyr
                165                 170                 175

Lys Phe Arg Gly Ile Gly Lys Ala Trp Asp Trp Glu Val Asp Thr Glu
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            180                 185                 190

Asn Gly Asn Tyr Asp Tyr Leu Met Tyr Ala Asp Ile Asp Met Asp His
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Pro Asp Val Val Thr Glu Ile Lys Asn Trp Gly Lys Trp Tyr Val Asn
    210                 215                 220

Thr Thr Asn Ile Asp Gly Phe Arg Leu Asp Ala Val Lys His Ile Lys
225                 230                 235                 240

Phe Ser Phe Phe Pro Asp Trp Leu Ser His Val Arg Ser Gln Thr Gly
                245                 250                 255

Lys Pro Leu Phe Thr Val Ala Glu Tyr Trp Ser Tyr Asp Ile Asn Lys
            260                 265                 270

Leu His Asn Tyr Ile Thr Lys Thr Asn Gly Thr Met Ser Leu Phe Asp
        275                 280                 285

Ala Pro Leu His Asn Lys Phe His Thr Ala Ser Lys Ser Gly Gly Ala
    290                 295                 300

Phe Asp Met Arg Thr Leu Leu Thr Asn Thr Leu Met Lys Asp Gln Pro
305                 310                 315                 320

Thr Leu Ala Val Thr Phe Val Asp Asn His Asp Thr Glu Pro Gly Gln
                325                 330                 335

Ala Leu Gln Ser Trp Val Asp Pro Trp Phe Lys Pro Leu Ala Tyr Ala
            340                 345                 350

Phe Ile Leu Thr Arg Gln Glu Gly Tyr Pro Cys Val Phe Tyr Gly Asp
        355                 360                 365

Tyr Tyr Gly Ile Pro Gly Asp Ser Gln Tyr Asn Ile Pro Ala Leu Lys
    370                 375                 380

Ser Lys Ile Asp Pro Ile Leu Ile Ala Arg Lys Asp Tyr Ala Tyr Gly
385                 390                 395                 400

Thr Gln His Asp Tyr Leu Asp His Ser Asp Ile Ile Gly Trp Thr Arg
                405                 410                 415
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Glu Gly Val Ser Glu Lys Pro Gly Ser Gly Leu Ala Ala Leu Ile Thr
            420                 425                 430

Asp Gly Pro Gly Gly Ala Lys Trp Met Tyr Val Gly Lys Gln His Ala
        435                 440                 445

Gly Lys Val Phe His Asp Ile Thr Gly Asn Arg Ser Asp Thr Val Thr
    450                 455                 460

Ile Asn Ser Asp Gly Trp Gly Glu Phe Lys Val Asn Gly Gly Ser Val
465                 470                 475                 480

Ser Ile Trp Val Pro Arg Lys Thr Thr Val Ser Thr Ile Ala Arg Pro
                485                 490                 495

Ile Thr Thr Arg Pro Trp Thr Gly Glu Phe Val Arg Trp Thr Glu Pro
            500                 505                 510

Arg Leu Val Ala Trp Pro
        515


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<223> /replace="Leu"

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Ala Ala Pro Phe Asn Gly Thr Leu Met Gln Tyr Phe Glu Trp Tyr Leu
1               5                  10                  15

Pro Asp Asp Gly Thr Leu Trp Thr Lys Leu Ala Asn Asp Ala Asn Asn
            20                  25                  30

Leu Ser Ser Ile Gly Ile Thr Ala Leu Trp Ile Pro Pro Ala Tyr Lys
        35                  40                  45

Gly Thr Ser Arg Ser Asp Val Gly Tyr Gly Val Tyr Asp Leu Tyr Asp
    50                  55                  60

Leu Gly Glu Phe Asn Gln Lys Gly Thr Val Arg Thr Lys Tyr Gly Thr
65                  70                  75                  80

Lys Ala Gln Tyr Leu Gln Ala Ile Gln Ala Ala His Ala Ala Gly Met
                85                  90                  95

Gln Val Tyr Ala Asp Val Val Phe Asp His Lys Ala Gly Ala Asp Ala
            100                 105                 110

Thr Glu Trp Val Asp Ala Val Glu Val Asn Pro Ala Asp Arg Asn Gln
        115                 120                 125

Glu Ile Ser Gly Thr Tyr Gln Ile Gln Ala Trp Thr Lys Phe Asp Phe
    130                 135                 140

Pro Gly Arg Gly Asn Thr Tyr Ser Ser Phe Lys Trp Arg Trp Tyr His
145                 150                 155                 160

Phe Asp Gly Val Asp Trp Asp Glu Ser Arg Lys Ile Ser Arg Ile Phe
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                165                 170                 175

Lys Phe Arg Gly Ile Gly Lys Ala Trp Asp Trp Glu Val Asp Ser Glu
            180                 185                 190

Asn Gly Asn Tyr Asp Tyr Leu Met Tyr Ala Asp Leu Asp Met Asp His
        195                 200                 205

Pro Asp Val Val Thr Glu Leu Lys Asn Trp Gly Lys Trp Tyr Val Asn
    210                 215                 220

Thr Thr Asn Ile Asp Gly Phe Arg Ile Asp Ala Val Lys His Ile Lys
225                 230                 235                 240

Phe Ser Phe Phe Pro Asp Trp Leu Ser Tyr Val Arg Ser Gln Thr Gly
                245                 250                 255

Lys Pro Leu Phe Thr Val Ala Glu Tyr Trp Ser Tyr Asp Ile Asn Lys
            260                 265                 270

Leu His Asn Tyr Ile Thr Lys Thr Asn Gly Thr Met Ser Leu Phe Asp
        275                 280                 285

Ala Pro Leu His Asn Lys Phe Tyr Thr Ala Ser Lys Ser Gly Gly Ala
    290                 295                 300

Phe Asp Met Arg Thr Leu Leu Thr Asn Thr Leu Met Lys Asp Gln Pro
305                 310                 315                 320

Thr Leu Ala Val Thr Phe Val Asp Asn His Asp Thr Glu Pro Gly Gln
                325                 330                 335

Ala Leu Gln Ser Trp Val Asp Pro Trp Phe Lys Pro Leu Ala Tyr Ala
            340                 345                 350

Phe Ile Leu Thr Arg Gln Glu Gly Tyr Pro Cys Val Phe Tyr Gly Asp
        355                 360                 365

Tyr Tyr Gly Ile Pro Gln Tyr Asn Pro Lys Glu Ile Pro Ser Leu Lys
    370                 375                 380

Ser Lys Ile Asp Pro Ile Leu Ile Ala Arg Lys Asp Tyr Ala Tyr Gly
385                 390                 395                 400
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Thr Gln His Asp Tyr Ile Asp His Ser Asp Ile Ile Gly Trp Thr Arg
                405                 410                 415

Glu Gly Val Ser Glu Lys Pro Gly Ser Gly Leu Ala Ala Leu Ile Thr
            420                 425                 430

Asp Gly Pro Gly Gly Ser Lys Trp Met Tyr Val Gly Lys Gln His Ala
        435                 440                 445

Gly Lys Val Phe Tyr Asp Ile Thr Gly Asn Arg Ser Asp Thr Val Thr
    450                 455                 460

Ile Asn Ser Asp Gly Trp Gly Glu Phe Lys Val Asn Gly Gly Ser Val
465                 470                 475                 480

Ser Ile Trp Val Pro Lys Lys Thr Thr Val Ser Thr Ile Ala Arg Pro
                485                 490                 495

Ile Thr Thr Arg Pro Trp Thr Gly Glu Phe Val Arg Trp Thr Glu Pro
            500                 505                 510

Arg Leu Val Ala Trp Pro
        515


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<220>
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<223> /replace="Lys"

<220>
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<223> /replace="Asp"

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<223> /replace="Lys"

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<223> /replace="Leu"

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<222> (86)..(86)
<223> /replace="Gln"

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<222> (87)..(87)
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<223> /replace="Lys"

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<223> /replace=" "

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<220>
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<222> (247)..(247)
<223> /replace="Arg"

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<223> /replace="Asn"

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<223> /replace="Gly"

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<223> /replace="Ala"

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<223> /replace="Glu"

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<223> /note="Residue given in the sequence has no preference
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<220>
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<223> /replace="Trp"

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<223> /replace="Asn"

<220>
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<223> /replace="His"

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<222> (284)..(286)
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<223> /replace="Val"

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with respect to those in the annotation for said position"

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<223> /replace="Tyr"

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<222> (314)..(314)
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<223> /replace="Lys"

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<222> (409)..(409)
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1 5 10 15

Leu Pro Asp Asp Gly Thr Leu Trp Thr Lys Leu Ala Asn Asp Ala Asn
20 25 30

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Asn Leu Ser Ser Leu Gly Ile Ser Ala Leu Trp Ile Pro Pro Ala Trp
        35                  40                  45

Lys Gly Thr Ser Arg Ser Asp Val Gly Tyr Gly Val Tyr Asp Leu Tyr
    50                  55                  60

Asp Leu Gly Glu Phe Asn Gln Lys Gly Thr Ile Arg Thr Lys Tyr Gly
65                  70                  75                  80

Thr Lys Ala Gln Tyr Leu Gln Ala Ile Asn Ala Ala His Ala Ala Gly
                85                  90                  95

Ile Gln Val Tyr Ala Asp Val Val Phe Asp His Lys Gly Gly Ala Asp
            100                 105                 110

Ala Thr Glu Trp Val Asp Ala Val Glu Val Asn Pro Ser Asp Arg Asn
        115                 120                 125

Gln Glu Ile Ser Gly Thr Tyr Gln Ile Gln Ala Trp Thr Lys Phe Asp
    130                 135                 140

Phe Pro Gly Arg Gly Asn Thr His Ser Ser Phe Lys Trp Arg Trp Tyr
145                 150                 155                 160

His Phe Asp Gly Val Asp Trp Asp Glu Ser Arg Lys Leu Ser Asn Arg
                165                 170                 175

Ile Tyr Lys Phe Arg Gly Ile Gly Lys Ala Trp Asp Trp Glu Val Asp
            180                 185                 190

Thr Glu Asn Gly Asn Tyr Asp Tyr Leu Met Tyr Ala Asp Ile Asp Met
        195                 200                 205

Asp His Pro Glu Val Val Thr Glu Leu Lys Asn Trp Gly Lys Trp Tyr
    210                 215                 220

Val Asn Thr Thr Asn Ile Asp Gly Phe Arg Ile Asp Ala Val Lys His
225                 230                 235                 240

Ile Lys Phe Ser Phe Phe Pro Asp Trp Ile Ser His Val Arg Ser Gln
                245                 250                 255
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Thr Gly Lys Pro Leu Phe Thr Val Ala Glu Phe Trp Ser Tyr Asp Ile
            260                 265                 270

Asn Lys Ile His Asn Tyr Ile Thr Lys Thr Asn Gly Thr Met Ser Leu
        275                 280                 285

Phe Asp Ala Pro Leu His Asn Lys Phe Tyr Thr Ala Ser Lys Ser Gly
    290                 295                 300

Gly Ala Phe Asp Met Arg Thr Ile Met Thr Asn Thr Leu Met Lys Asp
305                 310                 315                 320

Gln Pro Thr Leu Ala Val Thr Phe Val Asp Asn His Asp Ser Glu Pro
                325                 330                 335

Gly Gln Ala Leu Gln Ser Phe Val Asp Pro Trp Phe Lys Pro Leu Ala
            340                 345                 350

Tyr Ala Phe Ile Leu Thr Arg Gln Glu Gly Tyr Pro Cys Val Phe Tyr
        355                 360                 365

Gly Asp Tyr Tyr Gly Ile Pro Gln His Asn Ile Pro Ala Leu Lys Ser
    370                 375                 380

Lys Ile Asp Pro Ile Leu Ile Ala Arg Arg Asp Tyr Ala Tyr Gly Thr
385                 390                 395                 400

Gln His Asp Tyr Leu Asp His Ser Asp Ile Ile Gly Trp Thr Arg Glu
                405                 410                 415

Gly Val Thr Glu Lys Pro Gly Ser Gly Leu Ala Ala Ile Ile Ser Asp
            420                 425                 430

Gly Pro Gly Gly Ser Lys Trp Met Phe Val Gly Lys Asn His Ala Gly
        435                 440                 445

Lys Val Phe Tyr Asp Ile Thr Gly Asn Arg Ala Asp Thr Val Thr Ile
    450                 455                 460

Asn Ala Asp Gly Trp Gly Glu Phe Lys Val Asn Gly Gly Ser Val Ser
465                 470                 475                 480
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Ile Trp Val Pro Lys Lys Thr Thr Val Ser Thr Ile Ala Arg Pro Ile
485 490 495

Thr Thr Arg Pro Trp Thr Gly Glu Phe Val Arg Trp Thr Glu Pro Arg
500 505 510

Leu Val Ala Trp Pro
515

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<220>
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<223> /replace="His"

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<222> (3)..(3)
<223> /replace="Asn"

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<220>
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<222> (5)..(5)
<223> /replace="Thr"

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<223> /replace="Asn"

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<223> /replace="His"

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<222> (29)..(29)
<223> /replace="Asp"

<220>
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<223> /note="Residues given in the sequence have no preference
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<223> /replace="Ser"

<220>

<221> misc_feature
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<223> /replace="Arg"

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<222> (36)..(36)
<223> /replace="Asn"

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<223> /replace="Arg"

<220>
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<223> /replace="Gln"

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<222> (54)..(54)
<223> /replace="Asn"

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<223> /note="Residues given in the sequence have no preference
with respect to those in the annotation for said positions"

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<223> /replace="Ala"

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<223> /replace="Leu"

<220>
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<223> /replace="Glu"

<220>
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<223> /replace="Ser"

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<222> (90)..(90)
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<223> /replace="Leu"

<220>
<221> VARIANT
<222> (93)..(93)
<223> /replace="Lys"

<220>
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<222> (94)..(95)
<223> /replace="Asn"

<220>
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<222> (105)..(105)
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<223> /replace="Asn"

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<220>
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<223> /note="Residue given in the sequence has no preference
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<223> /replace="Leu"

<220>
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<223> /replace="Asn"

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<220>
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<223> /replace="Gln"

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<220>

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<220>
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<220>
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<220>
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<222> (320)..(320)
<223> /replace="Lys"

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<223> /note="Residues given in the sequence have no preference
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<223> /replace="Glu"

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<223> /replace="Gln"

<220>
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<222> (346)..(346)
<223> /replace="Glu"

<220>
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<223> /note="Residues given in the sequence have no preference
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<223> /replace="Leu"

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<223> /note="Residue given in the sequence has no preference
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<222> (360)..(360)
<223> /replace="Glu"

<220>
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<222> (361)..(361)
<223> /replace="Gln"

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<223> /note="Residues given in the sequence have no preference
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<223> /replace="Ser"

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<222> (365)..(365)
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<222> (376)..(376)
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<223> /replace="Ser"

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<222> (378)..(378)
<223> /note="Residue given in the sequence has no preference
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<223> /replace="Glu"

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<223> /note="Residue given in the sequence has no preference
with respect to those in the annotation for said position"

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<222> (394)..(394)
<223> /replace="Gln"

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<222> (395)..(395)
<223> /replace="Asn"

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<222> (394)..(395)
<223> /note="Residues given in the sequence have no preference
with respect to those in the annotation for said positions"

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<222> (405)..(405)
<223> /replace="Phe"

<220>
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<222> (405)..(405)
<223> /note="Residue given in the sequence has no preference
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<220>
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<222> (408)..(408)
<223> /replace="His"

<220>
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<222> (409)..(409)
<223> /replace="Asn"

<220>
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<222> (408)..(409)
<223> /note="Residues given in the sequence have no preference
with respect to those in the annotation for said positions"

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<223> /replace="Asn"

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<223> /note="Residue given in the sequence has no preference
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<222> (420)..(420)
<223> /replace="Thr"

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<222> (421)..(421)
<223> /replace="His"

<220>
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<222> (420)..(421)
<223> /note="Residues given in the sequence have no preference
with respect to those in the annotation for said positions"

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<223> /replace="Asn"

<220>
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<223> /note="Residue given in the sequence has no preference
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<220>
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<223> /note="Residue given in the sequence has no preference
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<223> /replace="Glu"

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<223> /note="Residue given in the sequence has no preference
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<223> /note="Residue given in the sequence has no preference
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<223> /replace="Lys"

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<222> (446)..(446)
<223> /note="Residue given in the sequence has no preference
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<223> /replace="Gln"

<220>
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<222> (449)..(449)
<223> /note="Residue given in the sequence has no preference
with respect to those in the annotation for said position"

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<222> (459)..(459)
<223> /replace="Pro"

<220>
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<222> (460)..(460)
<223> /replace="Gly"

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<222> (459)..(460)
<223> /note="Residues given in the sequence have no preference
with respect to those in the annotation for said positions"

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<222> (471)..(471)
<223> /replace="Asn"

<220>
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<222> (471)..(471)
<223> /note="Residue given in the sequence has no preference
with respect to those in the annotation for said position"

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<222> (473)..(473)
<223> /replace="Ser"

<220>
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<223> /note="Residue given in the sequence has no preference
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<223> /replace="Lys"

<220>
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<223> /note="Residue given in the sequence has no preference
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<223> /replace=" "

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<223> /replace=" "

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<223> /replace=" "

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<223> /replace=" "

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<223> /replace=" "

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<223> /replace=" "

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<223> /replace=" "

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<223> /replace=" "

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<223> /replace=" "

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<223> /replace=" "

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<223> /replace=" "

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<223> /replace=" "

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<223> /replace=" "

<220>
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<223> /replace=" "

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<223> /replace=" "

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<223> /replace=" "

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<223> /replace=" "

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<223> /replace=" "

<220>

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<221> misc_feature
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His Ala Ala Pro Phe Asn Gly Thr Met Met Gln Tyr Phe Glu Trp His
1               5                  10                  15

Leu Pro Asp Asp Gly Thr Leu Trp Thr Lys Leu Ala Asn Asp Ala Asn
            20                  25                  30

Asn Leu Ser Ser Leu Gly Ile Thr Ala Ile Trp Ile Pro Pro Ala Trp
        35                  40                  45

Lys Gly Thr Ser Arg Ser Asp Val Gly Tyr Gly Val Tyr Asp Leu Tyr
    50                  55                  60

Asp Leu Gly Glu Phe Asn Gln Lys Gly Thr Val Arg Thr Lys Tyr Gly
65                  70                  75                  80

Thr Lys Ala Gln Tyr Leu Gln Ala Ile Gln Ala Ala His Ala Ala Gly
                85                  90                  95

Met Gln Val Tyr Ala Asp Val Val Phe Asp His Lys Gly Gly Ala Asp
            100                 105                 110

Ala Thr Glu Trp Val Asp Ala Val Glu Val Asn Pro Ser Asp Arg Asn
        115                 120                 125

Gln Glu Ile Ser Gly Thr Tyr Gln Ile Gln Ala Trp Thr Lys Phe Asp
    130                 135                 140

Phe Pro Gly Arg Gly Asn Thr Tyr Ser Ser Phe Lys Trp Arg Trp Tyr
145                 150                 155                 160

His Phe Asp Gly Val Asp Trp Asp Glu Ser Arg Gln Lys Leu Ser Arg
                165                 170                 175

Ile Tyr Lys Phe Arg Gly Ile Gly Lys Ala Trp Asp Trp Glu Val Asp
            180                 185                 190

Ser Glu Asn Gly Asn Tyr Asp Tyr Leu Met Tyr Ala Asp Leu Asp Met
        195                 200                 205
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Asp His Pro Glu Val Val Thr Glu Leu Lys Asn Trp Gly Lys Trp Tyr
    210                 215                 220

Val Asn Thr Thr Asn Ile Asp Gly Phe Arg Ile Asp Ala Val Lys His
225                 230                 235                 240

Ile Lys Phe Ser Phe Phe Pro Asp Trp Leu Ser His Val Arg Ser Gln
                245                 250                 255

Thr Gly Lys Pro Leu Phe Thr Val Ala Glu Phe Trp Ser Tyr Asp Ile
            260                 265                 270

Asn Lys Leu His Asn Tyr Ile Thr Lys Thr Asn Gly Thr Met Ser Leu
        275                 280                 285

Phe Asp Ala Pro Leu His Asn Lys Phe Tyr Thr Ala Ser Lys Ser Gly
    290                 295                 300

Gly Ala Phe Asp Met Arg Thr Leu Leu Thr Asn Thr Leu Met Lys Asp
305                 310                 315                 320

Gln Pro Thr Leu Ala Val Thr Phe Val Asp Asn His Asp Ser Glu Pro
                325                 330                 335

Gly Gln Ala Leu Gln Ser Phe Val Asp Pro Trp Phe Lys Pro Leu Ala
            340                 345                 350

Tyr Ala Phe Ile Leu Thr Arg Gln Glu Gly Tyr Pro Cys Val Phe Tyr
        355                 360                 365

Gly Asp Tyr Tyr Gly Ile Pro Gln His Asn Ile Pro Ala Leu Lys Ala
    370                 375                 380

Lys Ile Asp Pro Ile Leu Ile Ala Arg Arg Asp Phe Ala Tyr Gly Thr
385                 390                 395                 400

Gln His Asp Tyr Leu Asp His Ser Asp Ile Ile Gly Trp Thr Arg Glu
                405                 410                 415

Gly Val Thr Glu Lys Pro Gly Ser Gly Leu Ala Ala Ile Ile Ser Asp
            420                 425                 430
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Gly Pro Gly Gly Ser Lys Trp Met Tyr Val Gly Lys Asn His Ala Gly
        435                 440                 445

Lys Val Phe His Asp Ile Thr Gly Asn Lys Ser Asp Thr Val Thr Ile
    450                 455                 460

Asn Ala Asp Gly Trp Ala Glu Phe Lys Val Asn Gly Gly Ser Val Ser
465                 470                 475                 480

Ile Trp Val Pro Arg Lys Thr Thr Val Ser Thr Ile Ala Arg Pro Ile
                485                 490                 495

Thr Thr Arg Pro Trp Thr Gly Glu Phe Val Arg Trp Thr Glu Pro Arg
            500                 505                 510

Leu Val Ala Trp Pro
        515


<210> 27
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<212> PRT
<213> Artificial sequence

<220>
<221> source
<223> /note="Description of artificial sequence: Synthetic
      consensus sequence"


<220>
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<223> /replace=" "

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<223> /replace="His"

<220>
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<222> (3)..(3)
<223> /replace="Asn"

<220>
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<222> (4)..(4)
<223> /replace="Gly"

<220>
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<222> (5)..(5)
<223> /replace="Thr"

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<223> /note="Residues given in the sequence have no preference
with respect to those in the annotation for said positions"

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<223> /replace="Asn"

<220>
<221> misc_feature
<222> (19)..(19)
<223> /note="Residue given in the sequence has no preference
with respect to those in the annotation for said position"

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<223> /replace="Asn"

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<222> (23)..(23)
<223> /replace="His"

<220>
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<222> (22)..(23)
<223> /note="Residues given in the sequence have no preference
with respect to those in the annotation for said positions"

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<223> /replace="Asn"

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<223> /note="Residue given in the sequence has no preference
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<223> /replace="Arg"

<220>
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<222> (29)..(29)
<223> /replace="Asp"

<220>
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<222> (28)..(29)
<223> /note="Residues given in the sequence have no preference
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<220>
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<223> /replace="Ala"

<220>
<221> misc_feature
<222> (32)..(32)
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<223> /replace="Lys"

<220>
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<222> (35)..(35)
<223> /note="Residue given in the sequence has no preference
with respect to those in the annotation for said position"

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<223> /replace="Lys"

<220>
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<222> (37)..(37)
<223> /note="Residue given in the sequence has no preference
with respect to those in the annotation for said position"

<220>
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<223> /replace="Gln"

<220>
<221> VARIANT
<222> (54)..(54)
<223> /replace="Asn"

<220>
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Leu Pro Asp Asp Gly Thr Leu Trp Thr Lys Leu Ala Asn Asp Ala Asn
            20                  25                  30

Asn Leu Ser Ser Leu Gly Ile Thr Ala Leu Trp Ile Pro Pro Ala Trp
        35                  40                  45

Lys Gly Thr Ser Arg Ser Asp Val Gly Tyr Gly Val Tyr Asp Leu Tyr
    50                  55                  60

Asp Leu Gly Glu Phe Asn Gln Lys Gly Thr Val Arg Thr Lys Tyr Gly
65                  70                  75                  80

Thr Lys Ala Gln Tyr Leu Gln Ala Ile Gln Ala Ala His Ala Ala Gly
                85                  90                  95

Ile Gln Val Tyr Ala Asp Val Val Phe Asp His Lys Gly Gly Ala Asp
            100                 105                 110

Gly Thr Glu Trp Val Asp Ala Val Glu Val Asn Pro Ser Asp Arg Asn
        115                 120                 125

Gln Glu Ile Ser Gly Thr Tyr Gln Ile Gln Ala Trp Thr Lys Phe Asp
    130                 135                 140

Phe Pro Gly Arg Gly Asn Thr His Ser Ser Phe Lys Trp Arg Trp Tyr
145                 150                 155                 160
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His Phe Asp Gly Val Asp Trp Asp Glu Ser Arg Gln Lys Leu Ser Lys
                165                 170                 175

Ile Tyr Lys Phe Arg Gly Ile Gly Lys Ala Trp Asp Trp Glu Val Asp
            180                 185                 190

Thr Glu Asn Gly Asn Tyr Asp Tyr Leu Met Tyr Ala Asp Ile Asp Met
        195                 200                 205

Asp His Pro Glu Val Ile Thr Glu Leu Lys Asn Trp Gly Lys Trp Tyr
    210                 215                 220

Val Asn Thr Thr Asn Ile Asp Gly Phe Arg Ile Asp Ala Val Lys His
225                 230                 235                 240

Ile Lys Phe Ser Phe Phe Pro Asp Trp Leu Ser His Val Arg Ser Gln
                245                 250                 255

Thr Gly Lys Pro Leu Phe Thr Val Ala Glu Phe Trp Ser Tyr Asp Ile
            260                 265                 270

Asn Lys Ile His Asn Tyr Ile Thr Lys Thr Asn Gly Thr Met Ser Leu
        275                 280                 285

Phe Asp Ala Pro Leu His Asn Lys Phe Tyr Thr Ala Ser Lys Ser Gly
    290                 295                 300

Gly Ala Phe Asp Met Arg Thr Ile Leu Thr Asn Ser Leu Met Lys Asp
305                 310                 315                 320

Gln Pro Thr Leu Ala Val Thr Phe Val Asp Asn His Asp Ser Glu Pro
                325                 330                 335

Gly Gln Ala Leu Gln Ser Phe Val Asp Pro Trp Phe Lys Pro Leu Ala
            340                 345                 350

Tyr Ala Phe Ile Leu Thr Arg Gln Glu Gly Tyr Pro Cys Val Phe Tyr
        355                 360                 365

Gly Asp Tyr Tyr Gly Ile Pro Gln His Asn Ile Pro Ser Leu Lys Ser
    370                 375                 380
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Lys Ile Asp Pro Leu Leu Ile Ala Arg Arg Asp Tyr Ala Tyr Gly Thr
385                 390                 395                 400

Gln His Asp Tyr Leu Asp His Ser Asp Ile Ile Gly Trp Thr Arg Glu
                405                 410                 415

Gly Val Ser Glu Lys Pro Gly Ser Gly Leu Ala Ala Ile Ile Ser Asp
            420                 425                 430

Gly Pro Gly Gly Ser Lys Trp Met Tyr Val Gly Lys Gln His Ala Gly
        435                 440                 445

Lys Val Phe Tyr Asp Ile Thr Gly Asn Arg Ser Asp Thr Val Thr Ile
    450                 455                 460

Asn Ala Asp Gly Trp Gly Glu Phe Lys Val Asn Gly Gly Ala Val Ser
465                 470                 475                 480

Val Trp Val Pro Arg Lys Thr Thr Val Ser Thr Ile Ala Arg Pro Ile
                485                 490                 495

Thr Thr Arg Pro Trp Thr Gly Glu Phe Val Arg Trp Thr Glu Pro Arg
            500                 505                 510

Leu Val Ala Trp Pro
        515


<210> 28
<211> 515
<212> PRT
<213> Artificial sequence

<220>
<221> source
<223> /note="Description of artificial sequence: Synthetic
      consensus sequence"


<220>
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<223> /replace="Asp"
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<220>
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<223> /replace="Leu"

<220>
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<223> /note="Residues given in the sequence have no preference
with respect to those in the annotation for said positions"

<220>
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<223> /replace="Glu"

<220>
<221> VARIANT
<222> (18)..(18)
<223> /replace="Asn"

<220>
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with respect to those in the annotation for said positions"

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<222> (21)..(21)
<223> /replace="Gln"

<220>
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<222> (22)..(22)
<223> /replace="His"

<220>
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<223> /replace="Asn"

<220>
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<223> /note="Residue given in the sequence has no preference
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<222> (27)..(27)
<223> /replace="His"

<220>
<221> VARIANT
<222> (28)..(28)
<223> /replace="Asp"

<220>
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<223> /replace="Ala"

<220>
<221> misc_feature
<222> (31)..(32)
<223> /note="Residue given in the sequence has no preference
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<220>
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<222> (35)..(35)
<223> /replace="Asp"

<220>
<221> VARIANT
<222> (36)..(36)
<223> /replace="Ala"

<220>
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<223> /note="Residues given in the sequence have no preference
with respect to those in the annotation for said positions"

<220>
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<223> /replace="Asn"

<220>
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<222> (50)..(50)
<223> /note="Residue given in the sequence has no preference
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<220>
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<222> (52)..(52)
<223> /replace="Gln"

<220>
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<222> (52)..(52)
<223> /note="Residue given in the sequence has no preference
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<223> /replace="Ala"

<220>
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<223> /note="Residue given in the sequence has no preference
with respect to those in the annotation for said position"

<220>
<221> VARIANT
<222> (84)..(84)
<223> /replace="Leu"

<220>
<221> VARIANT
<222> (85)..(85)
<223> /replace="Glu"

<220>
<221> VARIANT
<222> (86)..(86)
<223> /replace="Arg"

<220>
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<222> (89)..(89)
<223> /replace="Gly"

<220>
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<222> (89)..(89)
<223> /note="Residue given in the sequence has no preference
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<220>
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<222> (91)..(91)

<223> /replace="Leu"

<220>
<221> VARIANT
<222> (92)..(92)
<223> /replace="Lys"

<220>
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<222> (91)..(92)
<223> /note="Residues given in the sequence have no preference
with respect to those in the annotation for said positions"

<220>
<221> VARIANT
<222> (94)..(94)
<223> /replace="Asn"

<220>
<221> VARIANT
<222> (95)..(95)
<223> /replace="Asp"

<220>
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<222> (94)..(95)
<223> /note="Residues given in the sequence have no preference
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<220>
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<222> (104)..(104)
<223> /replace="Met"

<220>
<221> VARIANT
<222> (105)..(105)
<223> /replace="Asn"

<220>
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<222> (104)..(105)
<223> /note="Residues given in the sequence have no preference
with respect to those in the annotation for said positions"

<220>
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<222> (108)..(108)
<223> /replace="Met"

<220>
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<222> (108)..(108)
<223> /note="Residue given in the sequence has no preference
with respect to those in the annotation for said position"

<220>

<221> VARIANT
<222> (112)..(112)
<223> /replace="Phe"

<220>
<221> misc_feature
<222> (112)..(112)
<223> /note="Residue given in the sequence has no preference
with respect to those in the annotation for said position"

<220>
<221> VARIANT
<222> (115)..(115)
<223> /replace="Ala"

<220>
<221> misc_feature
<222> (115)..(115)
<223> /note="Residue given in the sequence has no preference
with respect to those in the annotation for said position"

<220>
<221> VARIANT
<222> (117)..(117)
<223> /replace="Gln"

<220>
<221> misc_feature
<222> (117)..(117)
<223> /note="Residue given in the sequence has no preference
with respect to those in the annotation for said position"

<220>
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<222> (120)..(120)
<223> /replace="Gln"

<220>
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<222> (120)..(120)
<223> /note="Residue given in the sequence has no preference
with respect to those in the annotation for said position"

<220>
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<222> (125)..(125)
<223> /replace="Asn"

<220>
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<222> (125)..(125)
<223> /note="Residue given in the sequence has no preference
with respect to those in the annotation for said position"

<220>
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<222> (127)..(127)

<223> /replace="Trp"

<220>
<221> misc_feature
<222> (127)..(127)
<223> /note="Residue given in the sequence has no preference
with respect to those in the annotation for said position"

<220>
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<222> (133)..(133)
<223> /replace="Ala"

<220>
<221> misc_feature
<222> (133)..(133)
<223> /note="Residue given in the sequence has no preference
with respect to those in the annotation for said position"

<220>
<221> VARIANT
<222> (135)..(135)
<223> /replace="Thr"

<220>
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<222> (135)..(135)
<223> /note="Residue given in the sequence has no preference
with respect to those in the annotation for said position"

<220>
<221> VARIANT
<222> (137)..(137)
<223> /replace="Asp"

<220>
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<222> (137)..(137)
<223> /note="Residue given in the sequence has no preference
with respect to those in the annotation for said position"

<220>
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<222> (141)..(141)
<223> /replace="Gly"

<220>
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<222> (141)..(141)
<223> /note="Residue given in the sequence has no preference
with respect to those in the annotation for said position"

<220>
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<222> (145)..(145)
<223> /replace="Ser"

<220>
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<222> (145)..(145)
<223> /note="Residue given in the sequence has no preference
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<220>
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<222> (148)..(148)
<223> /replace="Asn"

<220>
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<222> (148)..(148)
<223> /note="Residue given in the sequence has no preference
with respect to those in the annotation for said position"

<220>
<221> VARIANT
<222> (150)..(150)
<223> /replace="Ala"

<220>
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<222> (150)..(150)
<223> /note="Residue given in the sequence has no preference
with respect to those in the annotation for said position"

<220>
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<222> (153)..(153)
<223> /replace="Asp"

<220>
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<222> (153)..(153)
<223> /note="Residue given in the sequence has no preference
with respect to those in the annotation for said position"

<220>
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<222> (162)..(162)
<223> /replace="Asn"

<220>
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<222> (162)..(162)
<223> /note="Residue given in the sequence has no preference
with respect to those in the annotation for said position"

<220>
<221> VARIANT
<222> (168)..(168)
<223> /replace="Gln"

<220>
<221> VARIANT

<222> (169)..(169)
<223> /replace="Arg"

<220>
<221> VARIANT
<222> (170)..(170)
<223> /replace="Tyr"

<220>
<221> VARIANT
<222> (171)..(171)
<223> /replace="Gln"

<220>
<221> VARIANT
<222> (172)..(172)
<223> /replace="Glu"

<220>
<221> VARIANT
<222> (173)..(173)
<223> /replace="Asn"

<220>
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<222> (174)..(174)
<223> /replace="His"

<220>
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<222> (168)..(174)
<223> /note="Residues given in the sequence have no preference
with respect to those in the annotation for said positions"

<220>
<221> VARIANT
<222> (179)..(179)
<223> /replace="Ala"

<220>
<221> VARIANT
<222> (180)..(180)
<223> /replace="Asn"

<220>
<221> VARIANT
<222> (181)..(181)
<223> /replace="Thr"

<220>
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<222> (182)..(182)
<223> /replace="Asn"

<220>
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<222> (183)..(183)

<223> /replace=" "

<220>
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<222> (184)..(184)
<223> /replace=" "

<220>
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<222> (179)..(184)
<223> /note="Residues given in the sequence have no preference
with respect to those in the annotation for said positions"

<220>
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<222> (186)..(186)
<223> /replace="Asn"

<220>
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<222> (186)..(186)
<223> /note="Residue given in the sequence has no preference
with respect to those in the annotation for said position"

<220>
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<222> (188)..(188)
<223> /replace="Arg"

<220>
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<222> (188)..(188)
<223> /note="Residue given in the sequence has no preference
with respect to those in the annotation for said position"

<220>
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<222> (191)..(191)
<223> /replace="Glu"

<220>
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<222> (191)..(191)
<223> /note="Residue given in the sequence has no preference
with respect to those in the annotation for said position"

<220>
<221> VARIANT
<222> (201)..(201)
<223> /replace="Gly"

<220>
<221> misc_feature
<222> (201)..(201)
<223> /note="Residue given in the sequence has no preference
with respect to those in the annotation for said position"

<220>
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<222> (203)..(203)
<223> /replace="Asn"

<220>
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<222> (203)..(203)
<223> /note="Residue given in the sequence has no preference
with respect to those in the annotation for said position"

<220>
<221> VARIANT
<222> (206)..(206)
<223> /replace="Phe"

<220>
<221> VARIANT
<222> (207)..(207)
<223> /replace="Ser"

<220>
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<222> (206)..(207)
<223> /note="Residues given in the sequence have no preference
with respect to those in the annotation for said positions"

<220>
<221> VARIANT
<222> (212)..(212)
<223> /replace="Gln"

<220>
<221> VARIANT
<222> (213)..(213)
<223> /replace="Asp"

<220>
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<222> (212)..(213)
<223> /note="Residues given in the sequence have no preference
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<220>
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<222> (217)..(217)
<223> /replace="Asp"

<220>
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<223> /replace="Ser"

<220>
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<222> (220)..(220)
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with respect to those in the annotation for said position"

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<223> /replace="Thr"

<220>
<221> VARIANT
<222> (224)..(224)
<223> /replace="Asp"

<220>
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<222> (225)..(225)
<223> /replace="Glu"

<220>
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<222> (226)..(226)
<223> /replace="Leu"

<220>
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<222> (227)..(227)
<223> /replace="Asp"

<220>
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<222> (223)..(227)
<223> /note="Residues given in the sequence have no preference
with respect to those in the annotation for said positions"

<220>
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<222> (240)..(240)
<223> /replace="Pro"

<220>
<221> misc_feature
<222> (240)..(240)
<223> /note="Residue given in the sequence has no preference
with respect to those in the annotation for said position"

<220>
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<222> (242)..(242)
<223> /replace="Trp"

<220>
<221> misc_feature

<222> (242)..(242)
<223> /note="Residue given in the sequence has no preference
with respect to those in the annotation for said position"

<220>
<221> VARIANT
<222> (244)..(244)
<223> /replace="Thr"

<220>
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<222> (245)..(245)
<223> /replace="Ser"

<220>
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<222> (244)..(245)
<223> /note="Residues given in the sequence have no preference
with respect to those in the annotation for said positions"

<220>
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<222> (249)..(249)
<223> /replace="Arg"

<220>
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<222> (249)..(249)
<223> /note="Residue given in the sequence has no preference
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<220>
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<222> (251)..(251)
<223> /replace="Gln"

<220>
<221> misc_feature
<222> (251)..(251)
<223> /note="Residue given in the sequence has no preference
with respect to those in the annotation for said position"

<220>
<221> VARIANT
<222> (253)..(253)
<223> /replace="Asn"

<220>
<221> VARIANT
<222> (254)..(254)
<223> /replace="Glu"

<220>
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<222> (255)..(255)
<223> /replace="Ala"

<220>
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<222> (256)..(256)
<223> /replace="Asp"

<220>
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<223> /replace="Gln"

<220>
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<223> /replace="Asp"

<220>
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<223> /note="Residues given in the sequence have no preference
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<220>
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<222> (261)..(261)
<223> /replace="Val"

<220>
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<222> (261)..(261)
<223> /note="Residue given in the sequence has no preference
with respect to those in the annotation for said position"

<220>
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<222> (267)..(267)
<223> /replace="Lys"

<220>
<221> VARIANT
<222> (268)..(268)
<223> /replace="Asp"

<220>
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<222> (267)..(268)
<223> /note="Residues given in the sequence have no preference
with respect to those in the annotation for said positions"

<220>
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<222> (271)..(271)
<223> /replace="Gly"

<220>
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<222> (272)..(272)
<223> /replace="Ala"

<220>
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<222> (271)..(272)
<223> /note="Residues given in the sequence have no preference
with respect to those in the annotation for said positions"

<220>
<221> VARIANT
<222> (274)..(274)
<223> /replace="Glu"

<220>
<221> VARIANT
<222> (275)..(275)
<223> /replace="Phe"

<220>
<221> misc_feature
<222> (274)..(275)
<223> /note="Residues given in the sequence have no preference
with respect to those in the annotation for said positions"

<220>
<221> VARIANT
<222> (278)..(278)
<223> /replace="Asp"

<220>
<221> VARIANT
<222> (279)..(279)
<223> /replace="Glu"

<220>
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<222> (280)..(280)
<223> /replace="Met"

<220>
<221> misc_feature
<222> (278)..(280)
<223> /note="Residues given in the sequence have no preference
with respect to those in the annotation for said positions"

<220>
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<222> (282)..(282)
<223> /replace="Trp"

<220>
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<222> (283)..(283)
<223> /replace="Glu"

<220>
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<222> (282)..(283)

<223> /note="Residues given in the sequence have no preference
with respect to those in the annotation for said positions"

<220>
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<222> (289)..(289)
<223> /replace="Val"

<220>
<221> misc_feature
<222> (289)..(289)
<223> /note="Residue given in the sequence has no preference
with respect to those in the annotation for said position"

<220>
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<222> (292)..(292)
<223> /replace="Asn"

<220>
<221> VARIANT
<222> (293)..(293)
<223> /replace="Tyr"

<220>
<221> VARIANT
<222> (294)..(294)
<223> /replace="Asn"

<220>
<221> misc_feature
<222> (292)..(294)
<223> /note="Residues given in the sequence have no preference
with respect to those in the annotation for said positions"

<220>
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<222> (297)..(297)
<223> /replace="Arg"

<220>
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<222> (297)..(297)
<223> /note="Residue given in the sequence has no preference
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<220>
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<222> (300)..(300)
<223> /replace="Gln"

<220>
<221> VARIANT
<222> (301)..(301)
<223> /replace="Gln"

<220>

<221> misc_feature
<222> (300)..(301)
<223> /note="Residues given in the sequence have no preference
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<222> (309)..(309)
<223> /replace="Asn"

<220>
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<222> (309)..(309)
<223> /note="Residue given in the sequence has no preference
with respect to those in the annotation for said position"

<220>
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<222> (312)..(312)
<223> /replace="Arg"

<220>
<221> VARIANT
<222> (313)..(313)
<223> /replace="Gly"

<220>
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<222> (312)..(313)
<223> /note="Residues given in the sequence have no preference
with respect to those in the annotation for said positions"

<220>
<221> VARIANT
<222> (317)..(317)
<223> /replace="Glu"

<220>
<221> VARIANT
<222> (318)..(318)
<223> /replace="Ala"

<220>
<221> VARIANT
<222> (319)..(319)
<223> /replace="His"

<220>
<221> misc_feature
<222> (317)..(319)
<223> /note="Residues given in the sequence have no preference
with respect to those in the annotation for said positions"

<220>
<221> VARIANT
<222> (321)..(321)
<223> /replace="Met"

<220>
<221> VARIANT
<222> (322)..(322)
<223> /replace="His"

<220>
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<222> (321)..(322)
<223> /note="Residues given in the sequence have no preference
with respect to those in the annotation for said positions"

<220>
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<222> (333)..(333)
<223> /replace="Gln"

<220>
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<222> (333)..(333)
<223> /note="Residue given in the sequence has no preference
with respect to those in the annotation for said position"

<220>
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<222> (336)..(336)
<223> /replace="Glu"

<220>
<221> misc_feature
<222> (336)..(336)
<223> /note="Residue given in the sequence has no preference
with respect to those in the annotation for said position"

<220>
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<222> (339)..(339)
<223> /replace="Glu"

<220>
<221> misc_feature
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Pro Asp Asp Gly Thr Leu Trp Thr Lys Leu Ala Asn Asp Ala Asn Asn
            20                  25                  30

Leu Ser Ser Leu Gly Ile Thr Ala Ile Trp Ile Pro Pro Ala Tyr Lys
        35                  40                  45

Gly Thr Ser Arg Ala Asp Val Gly Tyr Gly Val Tyr Asp Leu Tyr Asp
    50                  55                  60

Leu Gly Glu Phe Asn Gln Lys Gly Thr Val Arg Thr Lys Tyr Gly Thr
65                  70                  75                  80

Lys Ala Gln Tyr Leu Gln Ala Ile Gln Ala Ala His Ala Ala Gly Ile
                85                  90                  95

Asn Val Tyr Ala Asp Val Val Phe Asp His Lys Gly Gly Ala Asp Gly
            100                 105                 110

Thr Glu Trp Val Asp Ala Val Glu Val Asn Pro Ser Asp Arg Asn Gln
        115                 120                 125

Asp Ile Ser Gly Thr Tyr Gln Ile Gln Ala Trp Thr Lys Phe Asp Phe
    130                 135                 140

Pro Gly Arg Gly Asn Thr Tyr Ser Ser Phe Lys Trp Arg Trp Phe His
145                 150                 155                 160

Phe Asp Gly Val Asp Trp Asp Glu Ser Arg Lys Leu Ser Arg Ile Phe
                165                 170                 175

Lys Phe Arg Gly Ile Gly Lys Ala Trp Asp Trp Glu Val Asp Thr Glu
            180                 185                 190

Asn Gly Asn Tyr Asp Tyr Leu Leu Tyr Ala Asp Ile Asp Met Asp His
        195                 200                 205

Pro Glu Val Val Thr Glu Leu Lys Asn Trp Gly Lys Trp Phe Val Asn
    210                 215                 220
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Thr Thr Asn Ile Asp Gly Phe Arg Leu Asp Ala Ile Lys His Ile Lys
225                 230                 235                 240

Phe Ser Phe Phe Pro Asp Trp Leu Ser His Val Arg Ser Gln Thr Gly
                245                 250                 255

Lys Pro Leu Phe Thr Val Gly Glu Tyr Trp Ser Tyr Asp Ile Asn Lys
            260                 265                 270

Leu His Asn Tyr Ile Thr Lys Thr Asn Gly Thr Met Ser Leu Phe Asp
        275                 280                 285

Ala Pro Leu His Asn Lys Phe Tyr Thr Ala Ser Lys Ser Gly Gly Ala
    290                 295                 300

Phe Asp Met Arg Thr Ile Leu Thr Asn Ser Leu Met Lys Asp Gln Pro
305                 310                 315                 320

Thr Leu Ala Val Thr Phe Val Asp Asn His Asp Thr Glu Pro Gly Gln
                325                 330                 335

Ala Leu Gln Ser Trp Val Asp Pro Trp Phe Lys Pro Leu Ala Tyr Ala
            340                 345                 350

Phe Ile Leu Thr Arg Gln Glu Gly Tyr Pro Cys Val Phe Tyr Gly Asp
        355                 360                 365

Tyr Tyr Gly Ile Pro Asn Tyr Asn Ile Pro Ala Leu Lys Ser Lys Ile
    370                 375                 380

Asp Pro Leu Leu Ile Ala Arg Arg Asp Tyr Ala Tyr Gly Thr Gln His
385                 390                 395                 400

Asp Tyr Leu Asp His Ser Asp Ile Ile Gly Trp Thr Arg Glu Gly Val
                405                 410                 415

Ser Glu Lys Pro Gly Ser Gly Leu Ala Ala Ile Ile Ser Asp Gly Pro
            420                 425                 430

Gly Gly Ser Lys Trp Met Tyr Val Gly Lys Gln His Ala Gly Lys Val
        435                 440                 445
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Phe Tyr Asp Leu Thr Gly Asn Arg Ser Asp Ser Val Thr Ile Asn Ser
    450                 455                 460

Asp Gly Trp Gly Glu Phe Lys Val Asn Gly Gly Ser Val Ser Val Trp
465                 470                 475                 480

Val Pro Arg Lys Thr Thr Val Ser Thr Ile Ala Arg Pro Ile Thr Thr
                485                 490                 495

Arg Pro Trp Thr Gly Glu Phe Val Arg Trp Thr Glu Pro Arg Leu Val
            500                 505                 510

Ala Trp Pro
        515
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1               5                  10                  15

Pro Asp Asp Gly Thr Leu Trp Thr Lys Leu Ala Asn Asp Ala Asn Asn
            20                  25                  30

Leu Ser Ser Leu Gly Ile Thr Ala Ile Trp Ile Pro Pro Ala Tyr Lys
        35                  40                  45

Gly Thr Ser Arg Ala Asp Val Gly Tyr Gly Val Tyr Asp Leu Tyr Asp
    50                  55                  60

Leu Gly Glu Phe Asn Gln Lys Gly Thr Val Arg Thr Lys Tyr Gly Thr
65                  70                  75                  80

Lys Ala Gln Tyr Leu Gln Ala Ile Gln Ala Ala His Ala Ala Gly Ile
                85                  90                  95

Asn Val Tyr Ala Asp Val Val Phe Asp His Lys Gly Gly Ala Asp Gly
            100                 105                 110
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Thr Glu Trp Val Asp Ala Val Glu Val Asn Pro Ser Asp Arg Asn Gln
        115                 120                 125

Asp Ile Ser Gly Thr Tyr Gln Ile Gln Ala Trp Thr Lys Phe Asp Phe
    130                 135                 140

Pro Gly Arg Gly Asn Thr Tyr Ser Ser Phe Lys Trp Arg Trp Phe His
145                 150                 155                 160

Phe Asp Gly Val Asp Trp Asp Glu Ser Arg Lys Leu Ser Arg Ile Phe
                165                 170                 175

Lys Phe Arg Gly Ile Gly Lys Ala Trp Asp Trp Glu Val Asp Thr Glu
            180                 185                 190

Asn Gly Asn Tyr Asp Tyr Leu Leu Tyr Ala Asp Ile Asp Met Asp His
        195                 200                 205

Pro Glu Val Val Thr Glu Leu Lys Asn Trp Gly Lys Trp Phe Val Asn
    210                 215                 220

Thr Thr Asn Ile Asp Gly Phe Arg Leu Asp Ala Ile Lys His Ile Lys
225                 230                 235                 240

Phe Ser Phe Phe Pro Asp Trp Leu Ser His Val Arg Ser Gln Thr Gly
                245                 250                 255

Lys Pro Leu Phe Thr Val Gly Glu Tyr Trp Ser Tyr Asp Ile Asn Lys
            260                 265                 270

Leu His Asn Tyr Ile Thr Lys Thr Asn Gly Thr Met Ser Leu Phe Asp
        275                 280                 285

Ala Pro Leu His Asn Lys Phe Tyr Thr Ala Ser Lys Ser Gly Gly Ala
    290                 295                 300

Phe Asp Met Arg Thr Ile Leu Thr Asn Ser Leu Met Lys Asp Gln Pro
305                 310                 315                 320

Thr Leu Ala Val Thr Phe Val Asp Asn His Asp Thr Glu Pro Gly Gln
                325                 330                 335

Ala Leu Gln Ser Trp Val Asp Pro Trp Phe Lys Pro Leu Ala Tyr Ala
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Phe Ile Leu Thr Arg Gln Glu Gly Tyr Pro Cys Val Phe Tyr Gly Asp
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Tyr Tyr Gly Ile Pro Asn Tyr Asn Ile Pro Ala Leu Lys Ser Lys Ile
    370                 375                 380

Asp Pro Leu Leu Ile Ala Arg Arg Asp Tyr Ala Tyr Gly Thr Gln His
385                 390                 395                 400

Asp Tyr Leu Asp His Ser Asp Ile Ile Gly Trp Thr Arg Glu Gly Val
                405                 410                 415

Ser Glu Lys Pro Gly Ser Gly Leu Ala Ala Ile Ile Ser Asp Gly Pro
            420                 425                 430

Gly Gly Ser Lys Trp Met Tyr Val Gly Lys Gln His Ala Gly Lys Val
        435                 440                 445

Phe Tyr Asp Leu Thr Gly Asn Arg Ala Asp Ser Val Thr Ile Asn Ser
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Asp Gly Trp Gly Glu Phe Lys Val Asn Gly Gly Ser Val Ser Val Trp
465                 470                 475                 480

Val Pro Arg Lys Thr Thr Val Ser Thr Ile Ala Arg Pro Ile Thr Thr
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Arg Pro Trp Thr Gly Glu Phe Val Arg Trp Thr Glu Pro Arg Leu Val
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Ala Trp Pro
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Leu Ser Ser Leu Gly Ile Thr Ala Leu Trp Leu Pro Pro Ala Tyr Lys
        35                  40                  45

Gly Thr Ser Arg Ser Asp Val Gly Tyr Gly Val Tyr Asp Leu Tyr Asp
    50                  55                  60

Leu Gly Glu Phe Asn Gln Lys Gly Thr Val Arg Thr Lys Tyr Gly Thr
65                  70                  75                  80

Lys Ala Gln Tyr Leu Gln Ala Ile Gln Ala Ala His Ala Ala Gly Met
                85                  90                  95

Gln Val Tyr Ala Asp Val Val Phe Asp His Lys Gly Gly Ala Asp Gly
            100                 105                 110

Thr Glu Trp Val Asp Ala Val Glu Val Asn Pro Ser Asp Arg Asn Gln
        115                 120                 125

Glu Ile Ser Gly Thr Tyr Gln Ile Gln Ala Trp Thr Lys Phe Asp Phe
    130                 135                 140

Pro Gly Arg Gly Asn Thr Tyr Ser Ser Phe Lys Trp Arg Trp Tyr His
145                 150                 155                 160

Phe Asp Gly Val Asp Trp Asp Glu Ser Arg Lys Leu Ser Arg Ile Tyr
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Lys Phe Arg Gly Ile Gly Lys Ala Trp Asp Trp Glu Val Asp Thr Glu
            180                 185                 190

Asn Gly Asn Tyr Asp Tyr Leu Met Tyr Ala Asp Leu Asp Met Asp His
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Pro Glu Val Val Thr Glu Leu Lys Asn Trp Gly Lys Trp Tyr Val Asn
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Thr Thr Asn Ile Asp Gly Phe Arg Leu Asp Ala Val Lys His Ile Lys
225                 230                 235                 240

Phe Ser Phe Phe Pro Asp Trp Leu Ser Tyr Val Arg Ser Gln Thr Gly
                245                 250                 255

Lys Pro Leu Phe Thr Val Gly Glu Tyr Trp Ser Tyr Asp Ile Asn Lys
            260                 265                 270

Leu His Asn Tyr Ile Thr Lys Thr Asn Gly Thr Met Ser Leu Phe Asp
        275                 280                 285

Ala Pro Leu His Asn Lys Phe Tyr Thr Ala Ser Lys Ser Gly Gly Ala
    290                 295                 300

Phe Asp Met Arg Thr Leu Met Thr Asn Thr Leu Met Lys Asp Gln Pro
305                 310                 315                 320

Thr Leu Ala Val Thr Phe Val Asp Asn His Asp Thr Glu Pro Gly Gln
                325                 330                 335

Ala Leu Gln Ser Trp Val Asp Pro Trp Phe Lys Pro Leu Ala Tyr Ala
            340                 345                 350

Phe Ile Leu Thr Arg Gln Glu Gly Tyr Pro Cys Val Phe Tyr Gly Asp
        355                 360                 365

Tyr Tyr Gly Ile Pro Gln Tyr Asn Ile Pro Ser Leu Lys Ser Lys Ile
    370                 375                 380

Asp Pro Leu Leu Ile Ala Arg Arg Asp Tyr Ala Tyr Gly Thr Gln His
385                 390                 395                 400

Asp Tyr Leu Asp His Ser Asp Ile Ile Gly Trp Thr Arg Glu Gly Val
                405                 410                 415

Thr Glu Lys Pro Gly Ser Gly Leu Ala Ala Leu Ile Thr Asp Gly Pro
            420                 425                 430

Gly Gly Ser Lys Trp Met Tyr Val Gly Lys Gln His Ala Gly Lys Val
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         435                 440                 445

Phe Tyr Asp Leu Thr Gly Asn Arg Ser Asp Thr Val Thr Ile Asn Ser
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Asp Gly Trp Gly Glu Phe Lys Val Asn Gly Gly Ser Val Ser Val Trp
465                 470                 475                 480

Val Pro Arg Lys Thr Thr Val Ser Thr Ile Ala Arg Pro Ile Thr Thr
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Arg Pro Trp Thr Gly Glu Phe Val Arg Trp Thr Glu Pro Arg Leu Val
            500                 505                 510

Ala Trp Pro
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