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(54) Title: HYDROPHILIC COMPOSITION CONTAINING PROTEASE PRODUCED BY VIBRIO

(57) Abstract

Compositions and methods of use are provided for debriding and wound healing applications. The compositions contain certain proteases produced by microorganisms of the genus *Vibrio*.

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HYDROPHILIC COMPOSITION CONTAINING PROTEASE PRODUCED BY VIBRIO

Technical Field

The present invention relates to hydrophilic pharmaceutical compositions containing enzymes, particularly proteases. The composition is capable of maintaining enzyme activity at room temperature storage. More specifically, the present invention relates to hydrophilic compositions containing a protease produced by microorganisms of the genus *Vibrio*. The compositions are useful for debridement and/or wound healing. The present invention further relates to the usage of these pharmaceutical compositions for debridement and/or as wound healing agents.

Background of the Invention

The healing of wounds is a complex process which is often further complicated by the presence of non-viable, necrotic tissue in the wound area. Debridement is the process of removing the non-viable tissue from a wound to prevent infection and facilitate healing.

Considerable efforts have been made to discover materials capable of distinguishing between viable and non-viable tissue. The discovery of materials which would digest devitalized tissue while not attacking viable tissue would make it possible to remove the devitalized tissue without surgery. It would be a beneficial therapeutic agent in virtually all disease processes or injuries where topically devitalized tissue needs to be removed from the viable organism such as burns, cutaneous ulcers, pressure necroses, incisional, traumatic and pyogenic wounds, and ulcers secondary to peripheral vascular disease.

One area that has attracted considerable attention is the use of proteolytic enzymes and other chemicals to effect the early debridement of necrotic tissue from cutaneous ulcers and from burns. Such devitalized tissue is an excellent culture medium

and moreover is the principal source of the septicemia which is the proximate cause of death, for example, in the majority of severely burned patients.

Devitalized tissue, which is commonly referred to as eschar, from cutaneous ulcers or burns is a complex mixture of dried blood, purulent exudates, and denatured proteins normally found in the epidermal and dermal skin layers. The denatured proteins found in eschar are primarily collagen, elastin, fibrin, hemoglobin, and other coagulated proteins.

Collagen comprises about 75% of the skin's dry weight and is the main constituent of the necrotic debris and of eschar. Strands of semi-viable, compromised collagen, whose protective mucopolysaccharide sheath has been damaged or destroyed, anchor the necrotic tissue to the wound surface. These strands must be fully eliminated in order for the necrotic material to be separated from its base. This complete debridement then permits development of granulation tissue during the healing process.

For a proteolytic enzyme to be suitable for use as a debriding agent, it is desirable for the protease to distinguish between viable and non-viable tissue; readily and thoroughly hydrolyze a wide variety of denatured proteins found in eschar; function at physiological pH and temperature; be compatible with adjunct therapies (e.g., cleansing agents, topical antibiotics); not interfere with normal wound healing; and remain stable in various formulations and at a wide range of temperatures. Furthermore, treatment of burn wounds with proteases should not complicate skin grafting. A number of proteolytic enzyme preparations have been used as debriding agents with varying degrees of success.

However, one problem associated therewith is that obtaining stable formulations of proteolytic enzymes is often problematic. A hydrophobic formulation is a water-in-oil emulsion, whereas a hydrophilic formulation is an oil-in-water emulsion. Most proteolytic enzymes are formulated into hydrophobic formulations and must be stored at refrigerated temperatures to stabilize the enzymes. For this reason, there are definite disadvantages of hydrophobic formulations. The disadvantages include the necessity to raise temperatures of the preparation before administration, reduced accessibility of the

enzyme to the administration site, and difficulty in removing the formulation from the administration site by gentle cleansing procedures.

The composition of the invention overcomes the difficulties of the prior art by providing a hydrophilic formulation which stabilizes an enzyme, preferably a protease and more preferably a *Vibrio* protease and maintains the stability at ambient temperatures. Therefore, it is well suited for use as a therapeutic.

Summary of the Invention

This invention provides hydrophilic pharmaceutical compositions capable of maintaining stable enzyme activity at room temperature. The compositions maintain enzyme activity at greater than 80% for at least 100 days at ambient temperatures. Gyceryl cocoate appears to impart enzyme stabilizing characteristics to the hydrophilic composition.

An especially preferred aspect of the invention is a composition which includes an extracellular neutral protease produced by *Vibrio* proteolyticus ATCC 53559. A particularly preferred procedure for preparation and method of use of this protease for debridement of necrotic tissue is described in commonly assigned U.S. Patent No. 5,145,681, which is hereby incorporated by reference and relied on in its entirety. This embodiment of the invention is useful for treating wounds. Wound treatment includes debridement and wound healing.

Still another aspect of the invention is the usage of these stabilized extracellular neutral protease pharmaceutical compositions for debridement of necrotic tissue and as wound healing agents.

Brief Description of the Figure

Figure 1 compares the shelf-life stability of various hydrophilic compositions containing vibriolysin.

Detailed Description of the Invention

The proteases of this invention are characterized by a combination of properties which renders them ideal candidates for use in wound debridement and healing applications. By way of illustration and not limitation, these proteases:

i. hydrolyze components of necrotic tissue including denatured collagen, elastin and fibrin;

ii. do not substantially hydrolyze native tissue *in vivo*; and

iii. exhibit stable activity when stored at 25°C in a topical formulation.

The proteases of the invention are capable of distinguishing between viable tissue and non-viable, necrotic tissue and are also active for sustained periods in formulations which are unacceptable to other proteases.

For the purposes of this application and the appended claims, the aforementioned properties of the proteases of this invention were determined as follows: Initial in vitro efficacy studies with the proteases of this invention, constituent proteins associated with eschar (e.g., denatured collagen, fibrin, denatured elastin) and native tissue were subjected to enzymatic hydrolysis. The proteases of this invention were shown to exhibit superior activity towards these substrates compared to proteases from Travase™. Furthermore, the proteases of this invention were shown to hydrolyze eschar from partial thickness wounds.

Preparation of the Protease

The proteases of this invention may be produced by fermentation of a suitable *Vibrio* species in a nutrient medium and then recovering the protease from the resulting broth. Fermentation is conducted aerobically in, for example, a casein hydrolysate, NZ-amine B, or soy flour nutrient medium containing inorganic salts such as sea salts, sodium sulfate, potassium dihydrogen phosphate, magnesium sulfate and certain trace elements at a pH of from about 7.6 to 8.6, preferably about pH 7.8, and at a temperature of from about 25° to 30°C, e.g., about 27°C, until the culture reaches early stationary phase growth.

The enzyme may thereafter be recovered from the fermentation broth by conventional procedures. Typically, the broth is first centrifuged or filtered to separate the cell portion and insoluble material. Thereafter, the supernatant is concentrated by, e.g., ultrafiltration. The resulting ultrafiltrate may be used as is or may be precipitated with organic solvents such as acetone or inorganic salts such as ammonium sulfate, followed by centrifugation, ion-exchange chromatography or filtration in order to isolate an enzyme useful in debriding compositions. The protease is also stable when lyophilized. other procedures such as are routine to those skilled in the art may also be used to cultivate the *Vibrio* microorganism and to recover the protease of this invention therefrom.

Useful microorganisms for use as a source of the instant proteases may comprise any suitable *Vibrio, Aeromonas, Pseudomonas, Serratia* or *Bacillus* or other marine microorganism species which secretes a protease having the above properties. A particularly preferred microorganism for this purpose is *Vibrio* proteolyticus (ATCC 53559). A viable culture of this microorganism has been irrevocably deposited with the American Type Culture Collection (ATCC), 12301 Parklawn Drive, Rockville, Maryland 20852, with no restrictions as to availability, and W. R. Grace & Co.-Conn., the assignee hereof, assures permanent availability of the culture to the public through ATCC upon the grant hereof. The DNA sequence of the protease secreted by *Vibrio proteolyticus* (ATCC 53559), referred to herein as vibriolysin, is set forth in Sequence ID No. 1. While *Vibrio proteolyticus* (ATCC 53559) comprises the preferred protease source, other species of useful *Vibrio* microorganisms can readily be identified by those skilled in the art by screening the proteases produced, thereby using the procedures set forth above.

In addition to the direct cultivation of a *Vibrio* species, the proteases of this invention may also be prepared by the cultivation of recombinant host cells which have been transformed or transfected with a suitable expression vector with an insert containing the structural gene for the *Vibrio*-derived proteases of this invention or a fragment or mutant thereof which retains substantially the same protease activity as the

native protease. Such procedures may be desirable, for example, in order to increase protease yields over that obtained with the wild type *Vibrio* microorganism or in order to produce improved mutant proteases.

Techniques for the cloning of proteases are well known to those skilled in the art of recombinant DNA technology, and any suitable cloning procedure may be employed for the preparation of the proteases of this invention. Such procedures are described, for example, in U.S. Patent No. 4,468,464; European Published Patent Application No. 0 130,756; PCT Published Patent Application No. WO 87/04461; and Loffler, *Food Technology*, pages 64-70 (January 1986); the entirety of which are hereby incorporated by reference and relied on in their entirety.

A particularly preferred procedure for cloning the *Vibrio* proteases of this invention is described in commonly assigned U.S. Patent No. 4,966,846, the entirety of which is hereby incorporated by reference and relied on in its entirety. According to the procedure of this patent, a gene library is first prepared, using the DNA of *Vibrio* source cells which have been determined by the assays described above to synthesize the proteases of this invention. Chromosomal DNA is extracted from the *Vibrio* source cells and digested with restriction enzymes by known procedures to give cleavage of the DNA into large fragments. Partial digestion with Sau3A is preferred, although other restriction enzymes (e.g., *MboI, BamHI*, etc.) may be used. The DNA fragments are then ligated into vectors suitable for allowing isolation of clones which express the protease enzyme. A preferred vector for this purpose is *Bam*HI digested *E. coli* cosmid vector pHC79 (Bethesda Research Laboratories). The recombinant vectors (i.e., pHC79 cosmids containing DNA fragments from the protease-containing qenome) are then packaged into bacteriophage particles, preferably bacteriophage lambda, thereby producing a gene library in bacteriophage lambda particles. For production of a gene library in bacteriophage, a cosmid vector or lambda vector is used. In other cases, plasmid vectors may be used.

The resultant bacteriophage particles are then used to insert the gene library DNA fragments into suitable gram-negative host cells. Preferably, the recombinant

bacteriophage particles are used to transect *E. coli*, such as, for example, *E. coli* strain HB101, although other strains of *E. coli* may be used if desired. Since *E. coli* strains do not naturally synthesize an extracellular neutral protease enzyme, the *E. coli* clones easily may be evaluated for the presence and expression of the protease gene by the assays described below.

It is known that colonies of *Vibrio* which synthesize protease enzyme will produce a zone of clearing on milk agar plates due to the proteolytic hydrolysis of the casein component of milk. Non-recombinant *E. coli* colonies do not secrete a protease naturally. Thus, *E. coli* clones of this invention which contain a functional protease gene are therefore readily identified by this assay. This milk-clearing assay is preferred for use with *E. coli* and other host strains which do not naturally produce an extracellular protease. Other gram-negative and gram-positive strains may be used as hosts.

Confirmation may be made by using other protease assays. For example, clones may be confirmed for expression of the protease enzyme by demonstrating that the fermentation broths of these clones are capable of hydrolyzing substrates such as Hide powder azure, azocoll or N-[3-(2-furyl)acryloyl]-alanyl-phenylalanamide (FAAPA). Alternatively, these assays may be used in the first instance to identify the protease gene-containing clones.

It is significant in two respects that expression of the neutral protease gene in *E. coli* and other "non-secreting" hosts (that is, hosts which do not naturally secrete a protease) can be detected as a zone of clearing on a milk agar plate. First, this is evidence that the active, functional enzyme is being synthesized by the gram-negative host. Second, the extracellular presence of protease on the milk agar plates is evidence that the enzyme is being externalized in some manner, either by secretion or by cell lysis. Since *E. coli* and some other gram-negative bacteria normally do not secrete significant quantities of proteases into the media, this is important in terms of the ability to recover protease enzymes produced as a result of expression of *Vibrio* protease genes in these non-secreting hosts.

Sequence ID No. 1 contains the DNA sequence of the vibriolysin gene obtained from Vibrio proteolyticus ATCC 53559. This DNA sequence comprises a portion of a 6.7 kb Hind III fragment of the *Vibrio* proteolyticus gene described in U.S. Patent No. 4,966,846, which encodes vibriolysin. An open reading frame exists from approximately base 249-2078, within which the DNA region encoding vibriolysin is found.

Also contemplated for use herein are mutants and hybrids of the foregoing proteases which substantially retain the preferred performance characteristics. As used herein, the term "mutant" refers to a protease in which a change is present in the amino acid sequence as compared with wild type or parent enzymes. This includes substitution, addition and deletion modifications. Also, this will include enzyme fragments or which comprise an internal delete which possess protease activity. "Hybrid" refers to genetically engineered proteases which combine amino acid sequences from two or more parent enzymes and exhibit characteristics common to both.

Techniques for the preparation of mutant proteases are well known to those skilled in the art and include exposure of a microorganism to radiation or chemicals, site-directed mutagenesis, and cleavage with appropriate restriction enzymes. Mutagenesis by radiation or chemicals is essentially a random process and can require a tedious selection and screening to identify microorganisms which produce enzymes having the desired characteristics. Preferred mutant enzymes for the purposes of this invention are thus prepared by site directed mutagenesis. This procedure involves modification of the enzyme gene such that substitutions, deletions, and/or insertions of at least one amino acid at a predetermined site are produced in the protease enzyme. Techniques for site directed mutagenesis are well known to those skilled in the art and are described, for example, in the European Published Patent Application No. 0 130,756 and PCT Published Patent Application No. WO 87/04461, the entirety of which are hereby incorporated by reference and relied on in their entirety.

In one such procedure, known as cassette mutagenesis, silent restriction sites are introduced into the protease gene, closely flanking the target codon or codons. Duplex

synthetic oligonucleotide cassettes are then ligated into the gap between the restriction sites. The cassettes are engineered to restore the coding sequence in the gap and to introduce an altered codon at the target codon.

The use of such procedures on the parent *Vibrio* proteases may be desirable in order to improve the properties of the wild type or parent protease. For example, the methionine, histidine, cysteine or tryptophan residues in or around the active site of the protease may be replaced in order to improve stability to chemical oxidation, as suggested in Estell et al., *J. Biological Chemistry*, Vol. 160, No. 11, pages 2518-2521 (1985).

Hybrids of the parent or wild type proteases may likewise be prepared by known protein engineering procedures analogous to the above-discussed cassette mutagenesis procedure by ligating a region of the gene of one parent enzyme (which need not be derived from *Vibrio*) into the gene of a second parent enzyme.

Clinical Properties of the Protease

The proteases of this invention are well suited for use in treating wounds and are particularly useful in wound debridement and wound healing applications. The properties can be demonstrated in a number of test situations, including animal and human clinical trials. One widely used assay is a partial thickness burn wound on pigs similar to that described by Mertz et al. (*Journal Surgical Research* (1990) 48:245-248). In this assay, the formulated protease can be compared to various controls to determine effectiveness.

For wound debridement, effectiveness is determined, among other indications, by absence, softening or dissolving of eschar; non-hydrolysis of viable tissue components; and/or non-irritation of the wound. In addition, debridement can be assessed histologically; wounds can be removed with a dermatome, fixed and embedded and sections cut and stained with, for example, Gomorils Trichrome stain. Analysis of such sections with a light microscope will reveal the extent of digestion of non-viable tissue. For topical wound healing, effectiveness is determined, among other indications

by wound contracture, increased rate of healing and/or improved healing (i.e., maintain response to tactile stimulus, less scarring, improved neovascularization, etc.).

The wound healing properties of the proteases of the invention are not limited to topical applications only. The wound healing properties can include the prevention and possibly the treatment of adhesions caused by surgical or other wounds.

Adhesions are bundles of fibrin and collagen which develop initially as fibrinous formations, usually in the abdomen, after operational or other trauma. Although most adhesions do not result in clinical morbidity, their role as a cause of small bowel obstruction and infertility is well recognized.

In experimental models, adhesions may be induced by thermal or mechanical trauma, ischemia, inflammation or foreign materials. A well known model for determining the effectiveness of the protease of the invention in the reduction of adhesion formation is a rabbit uterine horn model (Doody et al., *Fertil & Steril* (1989) 51:509-512). Effectiveness is determined in this model by reduced adhesion quantity and/or reduced adhesion density as compared to controls.

Formulation and Administration

Formulations of the debriding protease using available excipients and carriers are prepared according to standard methods known to those in the art. The protease can be formulated in ointments, lotions, gels, pastes, foams, aerosols, or immobilized on beads. The protease can also be immobilized in a wound dressing, tape or gauze. The enzyme formulations can be either hydrophilic or hydrophobic. Examples of hydrophobic bases include paraffin-mineral oil, and hydrophilic bases include petrolatum-propylene water bases. Hydrophilic formulations are preferred, particularly if the enzyme is stable in the formulation during storage at room temperature. Reasons for the preference include the convenience of not having to raise the temperature of the preparation before administering to the wound. More importantly, enzymes in a hydrophilic ointment should be more accessible for hydrolysis of necrotic tissue, and in contrast to a hydrophobic base, the ointment can be easily removed from the wound by washing with

saline. Additional active ingredients, including antibiotics, humectants, deoxyribonucleases, fibronectin, growth factors such as fibroblast growth factor (FGF), epidermal growth factor (EGF) , the transforming growth factors (TGF) , insulin- like growth factors (IGF-1 and IGF-2), and/or platelet-derived growth factor (PDGF) and the like, can be included in the formulation, if desired.

Topical administration is most appropriate for wound debridement, although other routes of administration may be desirable under certain conditions. Standard topical formulations are employed using, for example, 0.01-10% protease by weight. Such formulations are usually repeatedly applied, e.g., about 1-6 times per day to the affected area. However, the number of applications, type of application, and concentration of the ointment or other formulation depends, of course, on the severity and type of the wound and nature of the subject.

Topical administration is also appropriate in order to stimulate vascularization and healing of traumatized tissue. Substrates include burns, bone fractures, surgical abrasions such as those of plastic surgery, cuts, lacerations, bed sores, slow-healing ulcers, tendinitis, bursitis, vaginitis, cervicitis, circumcisions, episiotomy, pilonidal cyst wounds, carbuncles, sunburn, frostbite.

Local, or possibly systemic, administration is appropriate for the prevention, or possibly treatment, of adhesions caused by surgical or other wounds. Local administration can be by injection, subcutaneous implant or slow release formulation implanted directly proximal the target. Implantation is directly practical especially under surgical conditions. Slow-release forms can be formulated in polymers as is well within the skill of the art. The concentration of protease in the formulation depends on a number of factors, including the severity of the condition and the rate of protease release from the polymer.

The following abbreviations have been used throughout in describing the invention:

$HB0_3$ - boric acid

$CaCl_2$ - calcium chloride

$CaSO_4$	-	calcium sulfate
cm	-	centimeter
$CUSO_4$	-	copper sulfate
°C	-	degrees Centigrade
g	-	gram(s)
I.M.	-	intramuscular
kb	-	kilobase pair
$MgSO_4$	-	magnesium sulfate
$MnCl_2$	-	manganese chloride
mg	-	milligram(s)
ml	-	milliliter(s)
mm	-	millimeter(s)
mM	-	millimolar
mS	-	milli semen
nm	-	nanometer(s)
O.D.	-	optical density
%	-	percent
K_2HPO_4	-	potassium phosphate
NaOH	-	sodium hydroxide
Na_2MoO_3	-	sodium molybdate
Na_2SO_4	-	sodium sulfate
H_20	-	water
w/v	-	weight to volume
$ZnSO_4$	-	zinc sulfate

EXAMPLES

The following examples serve to give specific illustration of the practice of this invention, but they are not intended in any way to act to limit the scope of the invention.

Example 1

Preparation of Vibriolysin

V. proteolyticus ATCC 53559 was cultured in a medium with the following composition (g or ml per liter): NZ-amine B, 40; Na_2SO_4, 25; dextrose, 10; K_2HP0_4, 4; $MgSO_4.7H_20$, 0.4; Darastil-8270 (Dearborn) 0.1 ml and 6.1 ml of trace elements solution. The trace element solution comprises (grams per liter) the following: $ZnSO_4.7H_20$, 18.29; $MnC1_2.4H_20$, 18.86; $CaSO_4.2H_20$, 0.91 g, HBO_3, 0.07; and $Na_2MOO_4.2H_20$, 0.04. Prior to sterilization, pH was adjusted to 7.0.

V. proteolyticus was cultured in either 1.5- or 10-liter fermentors. Fermentors containing the aforementioned medium were inoculated with 1% (v/v) culture obtained by growing *V. proteolyticus* in shake flasks containing medium of the same composition for 20 hours. The fermentations were performed at 28°C, 1,000-1,250 rpm and an aeration of 1.0 volume of air per volume of medium per minute. The pH of the fermentation was maintained at pH 7.8 by the automatic addition of an acid and base titrant.

Growth of *V. proteolyticus* was monitored by measuring optical density at 640 nm, and protease activity was monitored by quantifying the hydrolysis of azocasein. Azocasein hydrolysis is determined by incubating a sample of protease for ten minutes at 37°C in 50 mM tris-HC1 buffer (pH 7.4) containing 1.0 mg/ml of azocasein (sulfanilamideazocasein, Sigma Corp., St. Louis, Missouri) with a final volume of 0.5 ml. At the end of this incubation period, 0.5 ml of 10% w/v trichloroacetic acid are added and immediately mixed and the resulting mixture is then stored on ice for 10 minutes. The mixture is then centrifuged and the optical density of the resulting supernatant is determined at 420 nm against a blank that contains either no enzyme or inactivated enzyme in the buffered azocasein solution. one unit of activity is defined as the amount of enzyme required to cause a change in absorbance of 2.5 at 420 nm. During the early stationary growth phase of the fermentation, the product protease reaches titers of approximately 85,200 to 127,800 azocasein units/liter as measured by

the azocasein assay described earlier. The broth was harvested by centrifugation to separate the cell portion.

The supernatant containing the proteolytic activity was concentrated using an Amicon SlOY10 spiral wound filter (Amicon Corp., Lexington, Massachusetts). The concentrate was diafiltered with 50 nM Tris buffer, pH 7.5, containing 1 mM $CaCl_2$ until the conductivity of the rententate was approximately 1 mS and the pH was neutral. This material was lyophilized and stored at -20°C until used or formulated.

Example 2

Hydrophilic Cream Composition

A preferred hydrophilic cream composition was prepared as follows. The cream contains the following ingredients at the indicated levels.

Ingredient	Weight Percent
glyceryl cocoate	34
glyceryl trilaurate	5
glycerin	13
antimicrobial agent	0.2
phosphate buffer (pH 7.0)	46
Vibriolysin	1.8

Glyceryl cocoate, glyceryl trilaurate and glycerin were mixed together and heated to 60°C. In a separate container, the anti-microbial, Cosmocil™ CQ (ICI Americas, Inc.) and phosphate buffer (0.3 M Na_2HP0_4, pH 7.0) were combined and heated to 60°C. The buffer solution was then added to the glyceryl-containing solution and cooled with mixing to 40°C. The vibriolysin prepared as in Example 1 was then slowly added with mixing and allowed to cool to room temperature.

Example 3

Shelf-life Stability

Enzyme activity extracted from the hydrophilic composition of Example 2 was monitored over time after storage at either 4°C or 25°C (ambient room temperature). One-tenth gram of composition was removed periodically, extracted with one ml of 100 mM TES (N-Tris [hydroxymethyl] methyl-2-amino ethanesulfonic acid) buffer, pH 7.5, containing 0.9% NaCl and 0.5 mM $CaCl_2$. The mixture was agitated thoroughly with a vortex, diluted 1:10 and residual proteolytic activity was determined by hydrolysis of azocasein as described in Example 1.

The residual proteolytic activity recovered from this composition is shown in Figure 1 and is compared with other standard hydrophilic compositions containing vibriolysin. The stability of the enzyme in the subject composition is significantly better than prior art compositions.

Example 4

Releasibility of Protease from Compositions

One purported advantage of a hydrophilic composition is the accessibility of the therapeutic agent (e.g., protease) to the wound site. The releasibility of vibriolysin from various compositions was determined as follows: milk casein agar (1.5%) plates were prepared, and 6 mm circular wells were punched out of the agar. Each well was filled with either a vibriolysin composition or a vibriolysin buffer solution and the plates were incubated at 37°C. As the protease migrated from each well into the milk casein agar, it hydrolyzes the casein, leaving a zone of clearing of halo of hydrolysis around each well. Zones of hydrolysis were measured at a function of time and are shown below:

Composition	Zones (mm)- of Hydrolysis at 7.5 h
Vibriolysin/buffer	18.5 (100)[a]
Vibriolysin/pB-0135-157	16.2 (88)
Vibriolysin/plastibase	14.5 (78)
Vibriolysin/silvadene	17 (92)

[a] Percent releasibility of enzyme.

These data indicate that vibriolysin is released more readily from hydrophilic compositions (e.g., pB-0135-157 and silvadene) than hydrophobic compositions (e.g., plastibase).

Example 5

In vitro Activity of Composition

The vibriolysin-containing composition of Example 2 is useful for treatment of wounds. Native porcine skin is an excellent source of collagen (-70%), which is the principal component of necrotic tissue. To monitor debridement activity of the composition, a simple assay was devised that allows qualitative visualization of skin digestion. Briefly, the method consists of denaturing 3 CM2 of Mediskin-I (porcine skin) (Bioplasty, Inc.) by boiling for 20 seconds. The denatured skin was blotted dry and mounted onto Petri dishes with surgical tape. The test composition ($\sim$1 g) was applied to the denatured skin, a solution of phosphate buffered saline (PBS) was added to the bottom of the dish to prevent desiccation of the skin. The dishes were covered and incubated at 37°C. After 6 to 24 hours incubation, the composition was removed from the skin using a gentle stream of phosphate buffered saline (PBS). Using this method, the vibriolysin composition was shown to completely hydrolyze the skin

directly beneath the location where the enzyme composition was applied. It was further shown that this composition was more active than a vibriolysin/hydrophobic composition (e.g., plastibase) and that the composition was superior to commercial products (e.g., Travase, Elase, Santyl, Granulex and Varidase) in hydrolyzing denatured pig skin collagen.

Example 6

In vivo Activity of the Composition

To assess the effectiveness of enzymatic digestion of eschar following either one or two treatments with the enzyme composition of Example 2, a third degree burn (full-thickness) injury was selected as the model. After appropriate anesthesia and shaving, three rows of steam burns were created on a pig. Six wounds were steamed for 30 sec, six wounds (40 sec), six wounds (50 sec). Wounds appeared white after injury with no apparent blood flow. The margins were red indicating a thin (2 mm) rim of second degree injury. The wounds were covered with an occlusive dressing (Op-Site). The pig was observed 24-hours later, and the dressing changed without anesthesia. At 48-hours post wounding, the pig was anesthetized. The wounds were washed with sterile saline. All wounds remained white with firm eschar. The formulations were applied and all wounds were covered with an occlusive bandage. The formulations were the Vibriolysin composition of Example 2, an equivalent vehicle composition (without vibriolysin) and an untreated control. Twenty-four hours post treatment, the pig was anesthetized and the Op-Site was removed. The wounds were gently cleansed with sterile saline and gauze. Gross observations were recorded and photographs were made. A second application of formulations was applied and the pig was again wrapped in occlusive bandage. The percentage of eschar digestion is shown in Table I.

Table I

WOUND ESCHAR DIGESTION			
24 Hours	30 Seconds	40 Seconds	50 Seconds
Vibriolysin Composition	50%	50%	50%
Vehicle	0%	0%	0%
Untreated	0%	0%	0%
48 Hours			
Vibriolysin Composition	80%	90%	95%
Vehicle	0%	0%	0%
Untreated	0%	0%	0%

After two treatments with the vibriolysin composition, 80-95% of the eschar was removed. The remaining base of the wounds were pink, and in several instances, subcutaneous blood vessels were observed through the fat. No spontaneous bleeding was noted. By comparison, the wounds that were untreated or treated with vehicle had firm eschar remaining.

Histological examination of harvested wounds corroborated visual, subjective assessments. Burn wounds were excised, fixed in 10% neutral buffered formalin for 48 hours and embedded in paraffin wax. Representative sections (7μ) were stained with Gomori's Trichrome and photographed with an Olympus Vanox AH light microscope. Analysis of the sections of wounds treated with vibriolysin showed that hydrolysis proceeded downward throughout the non-viable epidermis and dermis to a level just above the subcutaneous fat. Sections of untreated wounds or wounds receiving vehicle revealed no hydrolysis.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Fortney, Donald Zane
Durham, Donald Richard
Yang, Kang

(ii) TITLE OF INVENTION: HYDROPHILIC COMPOSITION CONTAINING PROTEASE PRODUCED BY VIBRIO

(iii) NUMBER OF SEQUENCES: 1

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: W. R. Grace & Co.-Conn.
(B) STREET: 7379 Route 32
(C) CITY: Columbia
(D) STATE: Maryland
(E) COUNTRY: United States
(F) ZIP: 21044

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:

(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Teskin, Robin L.
(B) REGISTRATION NUMBER: 35,030
(C) REFERENCE/DOCKET NUMBER: 010440-068

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: (703) 836-6620
(B) TELEFAX: (703) 836-2021

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 2000 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 61..1890

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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ATG AAT AAA ACA CAA CGT CAC ATC AAC TGG CTG CTG GCT GTT AGC GCG 108
Met Asn Lys Thr Gln Arg His Ile Asn Trp Leu Leu Ala Val Ser Ala
1 5 10 15

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Ala Thr Ala Leu Pro Val Thr Ala Ala Glu Met Ile Asn Val Asn Asp
            20                  25                  30

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Gly Ser Leu Leu Asn Gln Pro Leu Lys Ala Gln Ser Gln Ser Val Ala
        35                  40                  45

CCG GTG GAA ACC GGA TTC AAA CAA ATG AAA CGA GTT GTT TTG CCA AAT      252
Pro Val Glu Thr Gly Phe Lys Gln Met Lys Arg Val Val Leu Pro Asn
    50                  55                  60

GGC AAA GTG AAA GTT CGT TAT CAA CAA ACT CAC CAC GGT CTA CCG GTT      300
Gly Lys Val Lys Val Arg Tyr Gln Gln Thr His His Gly Leu Pro Val
65                  70                  75                  80

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Phe Asn Thr Ser Val Val Ala Thr Glu Ser Lys Ser Gly Ser Ser Glu
                85                  90                  95

GTG TTC GGT GTG ATG GCT CAG GGT ATC GCA GAC GAC GTG TCT ACA CTG      396
Val Phe Gly Val Met Ala Gln Gly Ile Ala Asp Asp Val Ser Thr Leu
            100                 105                 110

ACG CCA TCC GTT GAG ATG AAG CAG GCC ATT TCA ATT GCT AAA TCG CGT      444
Thr Pro Ser Val Glu Met Lys Gln Ala Ile Ser Ile Ala Lys Ser Arg
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TTC CAA CAG CAA GAA AAA ATG GTT GCG GAA CCT GCA ACG GAA AAC GAA      492
Phe Gln Gln Gln Glu Lys Met Val Ala Glu Pro Ala Thr Glu Asn Glu
    130                 135                 140

AAA GCC GAG TTG ATG GTT CGT CTG GAC GAC AAC AAT CAA GCG CAA CTA      540
Lys Ala Glu Leu Met Val Arg Leu Asp Asp Asn Asn Gln Ala Gln Leu
145                 150                 155                 160
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180 185 190

GGT CTG AAC CAT GCA CAA GCT GAC GGT ACT GGC CCT GGC GGT AAC ACC 684
Gly Leu Asn His Ala Gln Ala Asp Gly Thr Gly Pro Gly Gly Asn Thr
195 200 205

AAA ACA GGT CGT TAT GAA TAC GGT TCT GAC TTT CCT CCG TTT GTC ATC 732
Lys Thr Gly Arg Tyr Glu Tyr Gly Ser Asp Phe Pro Pro Phe Val Ile
210 215 220

GAT AAA GTC GGC ACT AAG TGT TCA ATG AAC AAC AGC GCG GTA AGA ACG 780
Asp Lys Val Gly Thr Lys Cys Ser Met Asn Asn Ser Ala Val Arg Thr
225 230 235 240

GTT GAC CTG AAC GGC TCA ACT TCA GGT AAC ACC ACT TAC AGC TAT ACC 828
Val Asp Leu Asn Gly Ser Thr Ser Gly Asn Thr Thr Tyr Ser Tyr Thr
245 250 255

TGT AAC GAC TCA ACC AAC TAC AAC GAT TAC AAA GCC ATT AAC GGC GCG 876
Cys Asn Asp Ser Thr Asn Tyr Asn Asp Tyr Lys Ala Ile Asn Gly Ala
260 265 270

TAC TCG CCA CTG AAC GAT GCC CAC TAC TTC GGT AAA GTG GTT TTC GAT 924
Tyr Ser Pro Leu Asn Asp Ala His Tyr Phe Gly Lys Val Val Phe Asp
275 280 285

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290 295 300

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305                 310                 315                 320

TCA TCC ATG ACC TTC GGT GAT GGC TAC AGC ACC TTC TAC CCG CTG GTG      1068
Ser Ser Met Thr Phe Gly Asp Gly Tyr Ser Thr Phe Tyr Pro Leu Val
                325                 330                 335

GAT ATT AAC GTT AGT GCC CAC GAA GTG AGC CAC GGT TTC ACC GAA CAA      1116
Asp Ile Asn Val Ser Ala His Glu Val Ser His Gly Phe Thr Glu Gln
            340                 345                 350

AAC TCG GGT CTG GTG TAC GAG AAT ATG TCT GGT GGT ATG AAC GAA GCG      1164
Asn Ser Gly Leu Val Tyr Glu Asn Met Ser Gly Gly Met Asn Glu Ala
        355                 360                 365

TTC TCT GAT ATT GCA GGT GAA GCA GCA GAG TTC TAC ATG AAA GGC AGC      1212
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    370                 375                 380

GTT GAC TGG GTT GTC GGT GCG GAT ATC TTC AAA TCA TCC GGC GGT CTG      1260
Val Asp Trp Val Val Gly Ala Asp Ile Phe Lys Ser Ser Gly Gly Leu
385                 390                 395                 400

CGT TAC TTT GAT CAG CCT TCG CGT GAC GGC CGT TCT ATC GAC CAT GCG      1308
Arg Tyr Phe Asp Gln Pro Ser Arg Asp Gly Arg Ser Ile Asp His Ala
                405                 410                 415

TCT GAC TAC TAC AAT GGC CTG AAT GTT CAC TAC TCA AGT GGT GTA TTC      1356
Ser Asp Tyr Tyr Asn Gly Leu Asn Val His Tyr Ser Ser Gly Val Phe
            420                 425                 430

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        435                 440                 445
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Asn Ser Thr Phe Asp Glu Gly Gly Cys Gly Val Val Lys Ala Ala Ser
465                 470                 475                 480

GAC ATG GGT TAC AGC GTT GCA GAC GTA GAA GAT GCG TTT AAC ACG GTA      1548
Asp Met Gly Tyr Ser Val Ala Asp Val Glu Asp Ala Phe Asn Thr Val
                485                 490                 495

GGC GTT AAC GCG TCT TGT GGT GCA ACT CCT CCT CCG TCT GGC GAT GTA      1596
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            500                 505                 510

CTG GAA ATC GGT AAA CCG CTG GCG AAC CTT TCA GGT AAC CGC AAT GAC      1644
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        515                 520                 525

ATG ACT TAC TAC ACG TTC ACA CCA AGC AGC TCA TCT AGC GTA GTG ATT      1692
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    530                 535                 540

AAG ATC ACT GGC GGT ACA GGT GAT GCA GAC CTT TAC GTG AAA GCG GGT      1740
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545                 550                 555                 560

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                565                 570                 575

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Asn Glu Glu Gln Cys Ser Ile Ser Ala Gln Ala Gly Thr Thr Tyr His
            580                 585                 590
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GTT ATG CTG CGT GGT TAC AGC AAT TAC GCT GGT GTA ACT TTG CGT GCT      1884
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        595                 600                 605

GAC TAA ACTCAGAATG GAACCAGTGA AGGCGCACCT TAAGGTCGCC TTTTTTGTAT      1940
Asp  *
    610

CAGGCGATCT GTGTAAACGT GACCTGATCG AAGTGAGGAT TGGCCGCCAG CGCTTGCATG   2000
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WE CLAIM:

1. A hydrophilic pharmaceutical composition comprising a pharmaceutically effective amount of an enzyme and glyceryl cocoate in an amount effective to maintain enzyme activity at greater than 80 per cent for at least 100 days at room temperature.

2. The composition of Claim 1, wherein said enzyme is a protease.

3. The composition of Claim 2, wherein said protease is an extracellular neutral protease produced by Vibrio.

4. The composition of Claim 3, wherein said Vibrio is a Vibrio proteolyticus stream.

5. The composition of Claim 1, which is useful for debriding wounds.

6. The composition of Claim 1, which is useful for promoting wound healing.

7. The composition of Claim 1, wherein said protease is encoded by a DNA sequence having Sequence ID No. 1.

8. The composition of Claim 1, which further comprises glycerin.

9. The composition of Claim 1, which further comprises an anti-microbial agent.

10. The composition of Claim 1, which further comprises

about 0.5 to about 2.0% protease

about 10.0 to about 70.0% glyceryl cocoate

about 0 to about 30.0% glyceryl trilaurate
about 0 to about 40.0% glycerin
about 0.05 to about 0.5% antimicrobial
about 30.0 to about 80.0% buffer.

11. A method of therapy which provides for the removal of necrotic and/or non-viable tissue from a subject in need of such treatment comprising administering a hydrophilic pharmaceutical composition according to Claim 1.

12. The method of Claim 10, wherein the therapy is effected for the treatment of a condition selected from burns, bone fractures, surgical abrasions, bed sores, slow healing ulcers, tendinitis, bursitis, vaginitis, cervicitis, circumcision, episiotomy, pilonidal cyst warts, carbuncles, sunburn and frostbite.

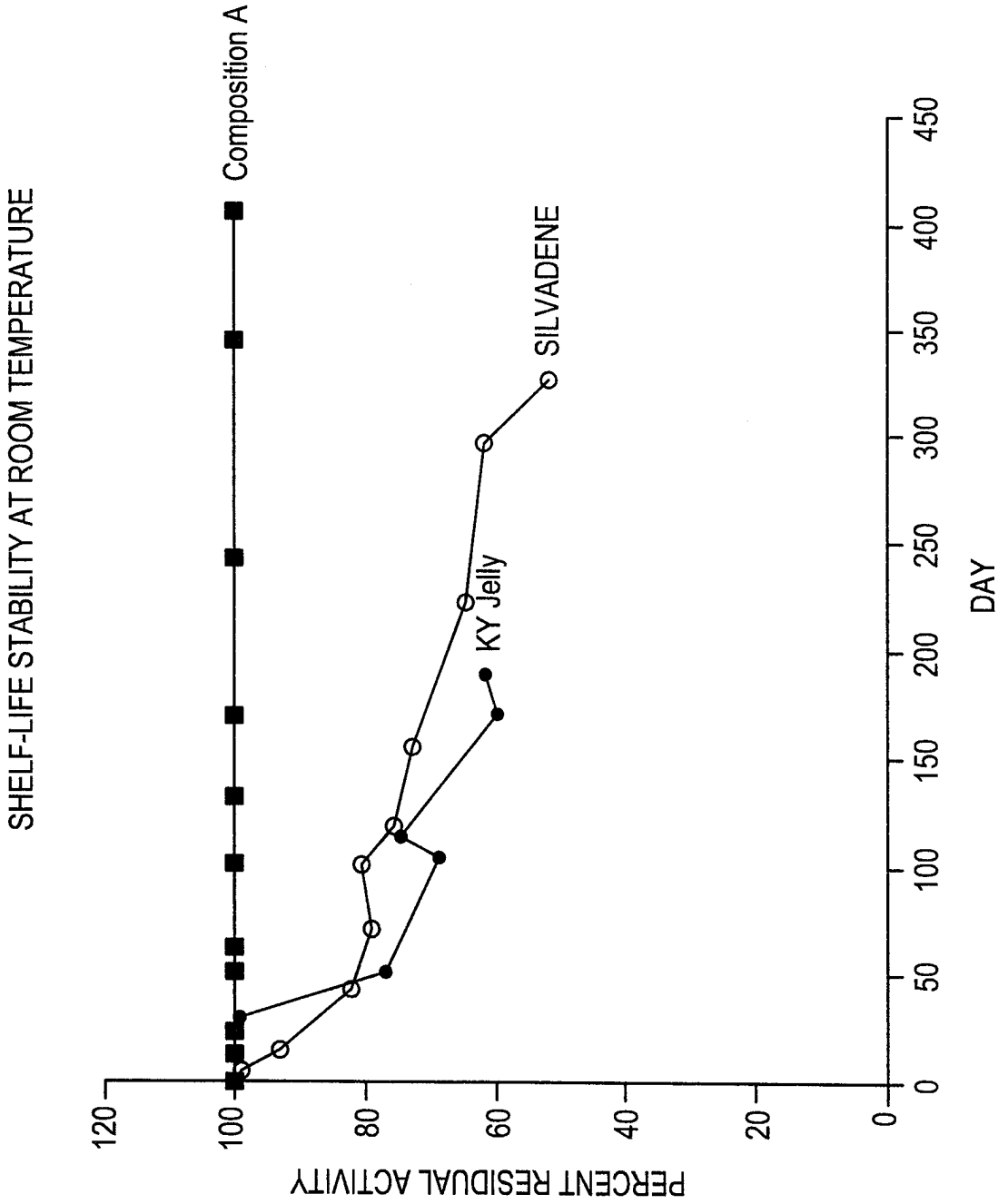
13. The method of Claim 10, wherein the protease is a neutral protease produced by Vibrio.

14. The method of Claim 12, wherein the Vibrio is a Vibrio proteolyticus strain.

15. The method of Claim 11, wherein the composition is topically applied.

16. The method of Claim 15, wherein the composition is topically applied about 1 to 6 times daily.

FIG. 1


SHELF-LIFE STABILITY AT ROOM TEMPERATURE
Composition A
SILVADENE
KY Jelly
PERCENT RESIDUAL ACTIVITY
120
100
80
60
40
20
0
0
50
100
150
200
250
300
350
400
450
DAY

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/10698

A. CLASSIFICATION OF SUBJECT MATTER
IPC(6) :C12N 9/50, 9/52; A 61K 38/46, 38/48, 47/06
US CL : 424/94.63; 435/212, 219, 220, 909
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/94.63; 435/212, 219, 220, 909

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, BIOSIS, MEDLINE, EMBASE, SCISEARCH
search terms: Vibrio, extracellular neutral protease, vibriolysin, glyceryl cocoate

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 5,145,681 A (FORTNEY et al) 08 September 1992, see entire document.	1-16
A	US 5,104,656 A (SETH et al) 14 April 1992, see entire document.	1-16

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 09 JULY 1998	Date of mailing of the international search report 03 SEP 1998
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer DIAN JACOBSON Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)★