



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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(21) International Application Number: PCT/US87/03113 (22) International Filing Date: 4 December 1987 (04.12.87) (31) Priority Application Number: 944,261 (32) Priority Date: 19 December 1986 (19.12.86) (33) Priority Country: US (71) Applicant: IMMUNEX CORPORATION [US/US]; Immunex Building, 51 University Street, Seattle, WA 98101 (US). (72) Inventors: HOPP, Thomas, P. ; PRICKETT, Kathryn, S. ; 4411 53rd Street S.W., Seattle, WA 98116 (US). (74) Agents: BURNAM, H., Warren, Jr.; Post Office Box 2326, Arlington, VA 22202 (US) et al.		(81) Designated States: AT (European patent), AU, BE (European patent), CH (European patent), DE (European patent), DK, FR (European patent), GB (European patent), IT (European patent), JP, KR, LU (European patent), NL (European patent), SE (European patent). Published <i>With international search report.</i>
(54) Title: IMMUNOAFFINITY PURIFICATION SYSTEM		
(57) Abstract A process for purifying a recombinant fusion protein having an N-terminal identification sequence comprising multiple anionic amino acid residues, comprising forming a complex of the protein with a divalent cation dependent monoclonal antibody specific for the sequence, isolating the complex, and dissociating antibody and protein by selectively depleting the concentration of divalent cations in contact with the complex. A particular calcium-dependent monoclonal antibody, 4E11, is useful in an embodiment of the process which employs the identification peptide DYKDDDDK.		

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TITLE

Immunoaffinity Purification System

5 BACKGROUND OF THE INVENTION

The present invention relates to immunoaffinity chromatography systems, and particularly to immunoaffinity processes employing a monoclonal antibody having divalent cation-dependent affinity for a selected peptide determinant.

10 The prior art describes recombinant fusion proteins which comprise synthetic leader peptides or protein fragments linked to independently derived polypeptides. In such fusions, the leader peptide or protein fragment can facilitate protein expression and purification by providing, for example, enzymatic activity enabling
15 identification of recombinants, an amino acid sequence recognized by cellular secretory mechanisms, or a sequence having distinctive chemical or antigenic characteristics useful in purifying the fusion protein by ion exchange, reverse phase, or affinity chromatographic media.

20 Itakura, U. S. Patent 4,571,421, describes hybrid polypeptides consisting of a somatostatin sequence and a fragment of β -galactosidase enzyme, separated by a CnBr-cleavable site permitting separation of the two protein segments. In this system, the presence of the β -galactosidase fragment permits identification of recombinants
25 bearing the somatostatin sequence. Schuman et al., J. Biol. Chem. 255:168 (1980), and Reed et al., Gene 20:255 (1982) disclose variations of this approach involving fusion of nucleotide sequences encoding biologically active fragments of β -galactosidase and newly isolated genes. The translated hybrid protein was isolated by
30 reference to the physical and enzymatic properties of β -galactosidase, and used to prepare specific antisera to the product of the newly isolated genes.

Rutter, Published European Patent Application No. 35384 (1981), discloses DNA constructions used to facilitate expression of
35 cloned DNA sequences. Among the constructions disclosed are sequences encoding fusion proteins comprising an N-terminal sequence having

distinctive physical properties useful for purification, joined to a desired C-terminal portion via a sequence which can be specifically cleaved to remove the N-terminal sequence. An example of such a cleavage sequence is the peptide sequence DDDDK recognized by enterokinase. Sequences having particular properties useful for purification include polyanionic segments and polycationic segments that will bind readily to ion exchangers, and hydrophobic segments capable of binding to reverse-phase media. This reference also discloses hybrid fusion proteins comprising fragments capable of being bound by specific antibody in an affinity chromatography step.

Brewer et al., U. S. Patent 4,532,207, disclose recombinant fusion proteins comprising a charged polymer of amino acids, for example, polyarginine, linked to a polypeptide of interest. Following expression in a microbial host, the fusion protein is purified by chromatography involving binding of the charged polymer to ion-exchange media. Following purification, the charged polymer is removed by controlled digestion with an exopeptidase. Smith et al., Gene 32:321 (1984) and Sassenfeld, Bio/Technology, January 1984, p. 76, also describe aspects of this approach to recombinant protein purification.

Improvements in recombinant protein expression and purification technologies are of considerable interest to the biotechnology, pharmaceutical, and chemical industries.

SUMMARY OF THE INVENTION

The present invention provides a process for purifying a recombinant fusion protein having a terminal identification sequence comprising multiple anionic amino acid residues, comprising forming a complex of the protein with a divalent cation dependent monoclonal antibody specific for the sequence, isolating the complex, and dissociating antibody and protein by selectively depleting the concentration of divalent cations in contact with the complex.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides an efficient process for protein isolation, utilizing an identification peptide having multiple anionic amino acid residues in conjunction with a cation-dependent monoclonal antibody. Such a peptide provides a highly immunogenic peptide determinant, and the presence of multiple anionic amino acid residues facilitates isolation of cation-dependent antibodies. Although both natural and synthetic anionic amino acids could conceivably be incorporated into identification peptides, aspartic acid and glutamic acid are preferred. Being natural amino acids, they can be expressed as components of recombinant polypeptides in conventional protein translation systems. Generally, terminal identification peptides comprising from 3 to 6 aspartic acid or glutamic acid residues, or mixtures thereof, are useful.

A particularly preferred embodiment of this concept involves use of the amino acid sequence Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys, or DYKDDDDK, as an N-terminal identification peptide. This sequence is immunogenic and comprises an enterokinase recognition site. Fusion proteins expressed with an N-terminal DYKDDDDK "flag" can be purified using immobilized monoclonal antibody which specifically recognizes the flag determinant. If desired, the flag sequence can be cleaved from the remainder of the fusion protein using enterokinase; this cleavage step can be undertaken before or after separation of the bound flag ligand from the immobilized antibody.

Conventional recombinant DNA techniques are employed to construct DNA vectors encoding fusion proteins having N-terminal or C-terminal identification peptides, for example, the N-terminal DYKDDDDK flag, coupled to a polypeptide sequence to be isolated. Following expression in cultures of transformed organisms, the fusion proteins can be separated from crude extracts or culture supernatants in a single affinity step mediated by specific anti-flag antibody. The DYKDDDDK flag provides superior identification and purification performance due to the presence of both hydrophilic and aromatic residues. This combination renders flag constructions highly immunogenic and ensures that the flag determinant, even when conjugated to much larger protein molecules, remains accessible to

antibody in aqueous media under physiological conditions. Additional information regarding the DYKDDDDK flag system is provided in United States Patent 4,703,004, which is incorporated herein by reference.

5 The improvement which characterizes this invention is an antibody-antigen system in which binding is dependent upon the presence of divalent metal cations. In a particularly preferred embodiment, the calcium-dependent anti-flag monoclonal antibody produced by the hybridoma cell line designated 4E11, which is specific
10 for DYKDDDDK flag peptide, is employed. This cell line has been deposited with the American Type Culture Collection (ATCC), Rockville, MD, USA, under accession number HB-9259. Although calcium-dependent antibodies have previously been reported, [see. e.g., Maurer et al., J. Immun. 105:567 (1970), and Maurer et al., Methods Enzymol. 70:49
15 (1980)] their use as immunoaffinity reagents has not been disclosed.

Cation-dependent affinity media bind ligand securely only in the presence of appropriate cation, and can be induced to release ligand by selectively depleting the concentration of divalent metal cations in contact with the complex of immobilized antibody and
20 ligand. This can be achieved by simply washing the affinity media with a solution lacking the particular cation, or preferably, by eluting with a chelating agent such as an EDTA salt (ethylene diamine tetraacetic acid), or EGTA (ethylene glycol-bis(β -aminoethyl ether) N,N,N',N'-tetraacetic acid). This approach avoids use of affinity
25 elution methods employing high salt, low pH, or chaotropic agents, which may be irreversibly denaturing. Since elution by cation removal or depletion is highly specific for the flag-antibody complex, the recovered protein is less likely to be contaminated by extraneous proteins bound to the affinity column at sites other than the antibody
30 combining site.

The media employed to immobilize the cation-dependent antibody can be agarose, Sepharose, acrylamide, cellulosic materials, or other suitable matrices known to those of skill in the art. Preferably, antibody is covalently bonded to media, for example, by
35 coupling to Sepharose (Pharmacia, Uppsala, Sweden) or Affi-Gel-10 (Bio Rad Laboratories, Richmond, CA, USA) using a procedure involving

reaction of free amino groups with N-hydroxysuccinimide esters. Kits for this purpose are commercially available; a convenient method is disclosed by Wilchek et al., Biochemistry 10:2828 (1971).

Immunoaffinity purification procedures employed in connection with the system disclosed herein can be by batch or column chromatographic methods. In addition, numerous immunoassays for expressed fusion proteins can be devised, relying upon the specificity of the divalent cation-dependent antibody for the flag determinant.

Depending upon the particular characteristics of the monoclonal antibody which is isolated, various divalent cations may be suitable in effecting binding of antibody to antigen. Such cations could include Ca^{++} , Mg^{++} , Mn^{++} , Cu^{++} , Ni^{++} , Zn^{++} , Cd^{++} , Ba^{++} or Sr^{++} . Of the foregoing, Ca^{++} is more commonly found to mediate cation-dependent binding sensitivity and is therefore preferred. Generally, cation concentrations should be at least 0.3 to 1 mM to ensure effective binding. Use of an EDTA solution of at least 0.5 mM as eluant, or alternatively, a concentration greater than the effective concentration of cation, effectively reduces cation concentration to a level enabling protein release.

Synthesis of Peptide Immunogen

Synthetic identification peptides can be conjugated to fatty acid-derivatized amino acids to prepare peptide immunogens, which are presented to mice to raise anti-peptide antibodies. The fatty acid-derivatized amino acids are added to the C-terminal region of the peptides, in order that the resulting peptides form micelles in aqueous media. The linear, hydrophobic fatty acid chains form the micelle nucleus, while the more hydrophilic N-terminal portions of the ligand peptides are presented to antibody on the micelle periphery.

One or more amino acids can be used as spacers to separate the hydrophilic residues from the derivatized residues. Preferably, one to six neutral amino acids, selected from the group consisting of Gly, Pro, or Ser, are employed for this purpose. The amino acids to be derivatized to fatty acids are preferably selected from the group consisting of lysine and ornithine, and can be from 1 to 3 in number. Palmitic, oleic, and stearic acids are useful fatty acids; palmitic acid is preferred. The peptide antigen can be synthesized by any

suitable method, for example, the Merrifield solid phase method widely employed in connection with automated peptide synthesizers. A suitable method is detailed in U. S. Patent 4,703,004.

Alternatively, the identification peptide can be conjugated to a carrier polypeptide or protein to provide a flag immunogen. Suitable carrier proteins include globulin fractions, the serum albumins of various species, hemocyanin, ovalbumin, lactalbumin, thyroglobulin, fibrinogen, or synthetic polypeptides, for example poly(L-lysine). The number of haptens bound to the carrier protein can vary from 2 to 50, depending upon the conditions of conjugation. Preferably, a given carrier has, on average, at least five peptide haptens covalently attached. Generally, higher antibody titers are obtained using conjugates having higher epitope densities. Suitable methods for hapten-carrier conjugation are disclosed by Bauminger et al., Methods in Enzymology 70:151 (1980); Reichlin, Methods in Enzymology 70:159 (1980); Kitagawa et al., J. Biochem. 94:1165 (1983); and various references reviewed by Lerner, Nature 299:592 (1982).

Preparation of Monoclonal Antibodies

The derivatized or conjugated peptide immunogen is employed to generate monoclonal antibodies against the peptide hapten using conventional techniques, for example, those disclosed in U. S. Patent 4,411,993. In this method, the immunogen is emulsified in complete Freund's adjuvant and injected in amounts ranging from 10-100 µg subcutaneously into Balb/c mice. Ten to twelve days later, the immunized animals are boosted with additional immunogen emulsified in incomplete Freund's adjuvant and periodically boosted thereafter on a weekly to biweekly immunization schedule. Serum samples are periodically taken by retro-orbital bleeding or tail-tip excision for testing by dot-blot assay (antibody sandwich) or ELISA (enzyme-linked immunosorbent assay). Other assay procedures are also suitable. Following detection of an appropriate antibody titer, positive animals are given an intravenous injection of antigen in saline. Three to four days later, the animals are sacrificed, splenocytes harvested, and fused to the murine myeloma cell line NS1. Hybridoma cell lines generated by this procedure are plated in multiple microtiter plates in a HAT selective medium (hypoxanthine, aminopterin, and thymidine)

to inhibit proliferation of non-fused cells, myeloma hybrids, and spleen cell hybrids.

Hybridoma clones thus generated can be screened by ELISA for reactivity with the immunogen in the presence and absence of calcium or other divalent metal cations. Generally, metal cation concentrations in the range 0.1 - 10 mM are suitable. Methods for ELISA are disclosed by Engvall et al., Immunochemistry 8:871 (1971) and in U. S. Patent 4,703,004. Positive clones are then injected into the peritoneal cavities of syngeneic Balb/c mice to produce ascites containing high concentrations (>1 mg/ml) of anti-peptide monoclonal antibody. The resulting monoclonal antibody can be purified by ammonium sulfate precipitation followed by gel exclusion chromatography, and/or affinity chromatography based on binding of antibody to Protein A of Staphylococcus aureus, or binding to immobilized identification peptide.

The following examples illustrate particular aspects of the present invention:

Example 1: Preparation of 4E11 Antibody Affinity Media

A murine hybridoma clone, designated 4E11, was isolated by screening for monoclonal antibody capable of specifically binding the DYKDDDDK flag fused to the N-terminus of human interleukin-1 (IL-2). Antibody-containing ascites produced by injection of the 4E11 hybridoma into syngeneic mice were purified and concentrated by standard methods including ammonium sulfate precipitation, affinity chromatography, and ultrafiltration. 10 ml of a purified, concentrated protein solution containing about 2.6 mg/ml 4E11 monoclonal antibody were dialyzed against 4 liters 0.1 M HEPES buffer, pH 7.5, at 4°C. After about 48 hr and three changes of dialysis buffer, the contents of the dialysis tubing (about 7.5 ml) were transferred to a 50 ml polypropylene tube and held at 4°C. Affi-gel-10 (ω -aminoethyl agarose; Biorad, Richmond, CA, USA) was transferred to a sintered glass funnel and washed extensively with isopropanol followed by deionized water. Approximately 10 ml of the washed gel were added to the tube containing the antibody solution and reacted overnight on a rotator at 4°C, in accordance with the manufacturer's instructions. The following day, unreacted sites on

the affinity media were blocked by adding 100 μ l of 1 M glycine ethyl ester, pH 8, to the media, and gently agitating for 1.5 H at 4°C.

The resulting antibody-coupled gel, containing approximately 3.2 mg antibody/ml of gel, was washed with phosphate-buffered saline (PBS; 0.9 M NaCl, 0.1 M KHPO_4 , pH 7.0) followed by PBS/0.02% sodium azide, and then stored at 4°C.

Example 2: Purification of Flag-BPA Fusion Protein

A yeast expression plasmid, pBC65, was constructed, comprising DNA sequences from pBR322 for selection and replication in *E. coli* (Ap^r gene and origin of replication) and yeast DNA sequences including the TRP1 gene as a selectable marker and the yeast 2 μ origin of replication. pBC65 also includes a glucose-repressible alcohol dehydrogenase 2 (ADH2) promoter, and an α -factor leader sequence enabling secretion of heterologous proteins from a yeast host. Fused in-frame to the α -factor leader was an inserted DNA sequence encoding a flag fusion protein comprising the DYKDDDDK sequence linked to a cDNA encoding putative human BPA (burst-promoting factor) protein. *S. cerevisiae* strains 79 and XV2181 (Trp^-) were transformed with pBC65 by the method of Hinnen et al., Proc. Natl. Acad. Sci. USA 75:1929 (1978), selecting for Trp^+ transformants. Transformants of each yeast strain were cloned and grown for expression in a rich medium consisting of 1% yeast extract, 2% peptone, and 1% glucose supplemented with 80 μ g/ml adenine and 80 μ g/ml uracil. Derepression of the ADH2 promoter and expression of the fusion protein was induced upon exhaustion of medium glucose. Crude yeast-conditioned supernatant was collected from the fermentation mixtures by filtration and held at 4° until needed.

5 ml of the 4E11 antibody-coupled affinity gel slurry prepared as described in Example 1, above, were added to a polypropylene column to provide a bed volume of about 1.5 ml. The column was then flushed with 15 ml PBS, followed by 15 ml 0.1 M glycine-HCl, pH 3.0, and finally 25 ml PBS.

70 ml of the 79:pBC65 yeast-conditioned supernatant were treated by adding 7 ml 10x PBS, raising the pH of the extract to 7.12. The resulting buffered yeast supernatant was applied to the 4E11

immunoaffinity column in two 10 ml aliquots. Following sample application, the column was washed with 9 ml PBS, and stored at 4°C overnight. The column was eluted with five 1 ml additions of 0.1 M glycine-HCl, while collecting 1 ml fractions. After elution, the
5 column was extensively washed with PBS and stored at 4°C in PBS/0.02% sodium azide.

Samples of the wash and elution fractions were analyzed by SDS-PAGE in conjunction with Western immunoblots using silver staining of gels and 4E11 antibody. The Western transfer indicated the
10 presence of 4E11-reactive material having an apparent molecular weight of about 28,000 daltons in the wash fractions, indicating lack of binding.

The 4E11 immunoaffinity column was then prepared for a second trial in which the wash buffers used in connection with one of the
15 yeast supernatants was supplemented to contain 0.5 mM MgCl₂ and 1.0 mM CaCl₂. In this second experiment, approximately 112 ml of the 79:pBC65 yeast supernatant (pH 7.04) was applied to the column in 2 10 ml aliquots. The column was washed with PBS containing 0.5 mM MgCl₂/1.0 mM CaCl₂. The column was eluted with 0.1 M glycine-HCl as
20 before. SDS-PAGE/Western analysis of the collected fractions indicated that the 4E11-reactive material was bound until elution with glycine-HCl. The majority of the yeast proteins were removed from the column in the wash fractions.

25 Example 3: Purification of Flag-GM-CSF Fusion Protein

A yeast expression vector, comprising DNA coding for the DYKDDDDK sequence fused to DNA encoding mature human granulocyte-macrophage colony stimulating factor (GM-CSF), was constructed as follows.

30 A 417 base pair AhaII-NcoI fragment containing the majority of the coding region and part of the 3' flanking region of the wild-type human GM-CSF gene was excised from plasmid pYαfHuGM to provide a fragment lacking the sequence corresponding to the N-terminal 24 amino acids of the mature protein. This portion of the
35 gene was reconstituted using an oligonucleotide which provides a revised 5' nucleotide sequence encoding an amino acid sequence

coincident with the first 22 amino acids of the mature protein, but containing a 5' KpnI terminal restriction site, a BglIII site at amino acid codon 4, a second NcoI site at amino acid codon 12, HpaI and HindIII sites at codons 16 and 21, respectively, and a codon substitution to provide a leucine residue at position 23. The sequence of the linker appears below:

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                    Ala Pro Ala Arg Ser Pro
5'-CT TTG GAT AAA AGA GCT CCA GCT AGA TCT CCA
10 3'-CAT GGA AAC CTA TTT TCT CGA GGT CGA TCT AGA GGT
    KpnI                                BglIII

Ser Pro Ser Thr Glu Pro Trp Glu His Val Asn Ala Ile
TCT CCA TCT ACT CAA CCA TGG GAA CAC GTT AAC GCT ATT
AGA GGT AGA TGA GTT GGT ACC CTT GTG CAA TTG CGA TAA
                    NcoI                HpaI

15 Glu Glu Ala Leu
    CAA GAA GCT TTG-3'
    GTT CTT CGA AAC GC-5'
        HindIII      AhaII->
    
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The resulting construct was cloned into KpnI and NcoI-cut plasmid pBC11 (a pBR322 derivative) to generate plasmid pBC25. This plasmid is a S. cerevisiae expression vector substantially similar to pYαfHuGM, except for substitution of the glucose-repressible alcohol dehydrogenase 2 (ADH2) promoter for the α-factor promoter of pYαfHuGM. pBC25 was then cut with KpnI and BglIII and ligated to the following oligonucleotide, which provides a DNA sequence encoding a 3' fragment of the yeast α-factor leader sequence, fused in-frame to an S. cerevisiae KEX2 protease recognition site, the flag identification peptide, and the first 3-5 amino acids of mature human GM-CSF:

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                    |←-----flag determinant-----→|
30      Leu Asp Lys Arg Asp Tyr Lys Asp Asp Asp Asp Lys
        5'-CT TTG GAT AAA AGA GAC TAC AAG GAC GAC GAT GAC AAG-
        3'-CAT GGA AAC CTA TTT TCT CTG ATG TTC CTG CTG CTA CTG TTC-
            KpnI                                ↑ KEX2      Enterokinase ↑
                                           Cleavage      Cleavage

        Ala Pro Ala
        GCT CCA GCT A-3'
        CGA GGT CGA TCT AG-5'
35                                BglIII
    
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Using this vector, a fusion protein comprising the flag determinant linked to the N-terminal alanine of mature human GM-CSF is expressed and secreted by a yeast host. The α -factor secretory mechanism cleaves the translated polypeptide following the Lys-Arg KEX2
5 recognition site. This vector was used to transform yeast strain XV2181 by standard methods, selecting for Trp⁺ transformants in a selective medium consisting of 0.67% yeast nitrogen base, 0.5% casamino acids, 2% glucose, 10 μ g/ml adenine and 20 μ g/ml uracil.

10 Transformed yeast were grown in rich medium (1% yeast extract, 2% peptone, and 1% glucose supplemented with 80 μ g/ml adenine and 80 μ g/ml uracil) at 30°C. After removing yeast by centrifugation, the resulting conditioned medium was prepared for assay by filtering through a 0.45 μ cellulose acetate filter.

15 5 ml of the 4E11 antibody immunoaffinity gel slurry, prepared as described in Example 1, above, were transferred to a polypropylene column, providing a bed volume of about 1.5 ml. The column was flushed with 15 ml PBS, followed by 15 ml 0.1 M glycine-HCl, pH 3, and then an additional 30 ml PBS.

20 11 ml of 10x PBS were added to 100 ml of yeast extract, raising the pH to about 6.9. 30 ml of the resulting diluted yeast extract were applied to the immunoaffinity column, which was then washed with five 1 ml aliquots of PBS/1 mM CaCl₂, with 5 minute intervals between aliquots. Fractions were collected from each application. The column was then eluted with 1.5 ml PBS, followed by
25 four additional aliquots, each 1 ml, of PBS added hourly. 1.5 ml of PBS containing 10 mM EDTA was then added, followed by four additional 1 ml aliquots of PBS/10 mM EDTA at hourly intervals.

30 Finally, 4 ml glycine-HCl, pH 3, were added and collected as one fraction, which was neutralized by addition of 50 μ l 1 M Tris-HCl, pH 7.0. All fractions were then analyzed by SDS-PAGE followed by silver staining. The results indicated that the majority of the proteins and other contaminants present in the yeast supernatant eluted from the column in the initial wash employing PBS/1.0 mM CaCl₂, while the flag-GM-CSF fusion protein remained bound. The fusion
35 protein eluted in a relatively sharp peak in the initial wash with PBS lacking Ca⁺⁺ ion. A small amount of additional material of the proper

molecular weight was observed to elute from the column when the PBS/EDTA solution was applied.

The foregoing experiment was repeated substantially as described above, except that the fusion protein was eluted from the column by application of a PBS/10 mM EDTA solution following washing with PBS/1 mM CaCl_2 . In this experiment, all detectable 4E11-reactive material eluted from the column in a sharp peak upon application of the PBS/EDTA eluant, free of other protein contaminants.

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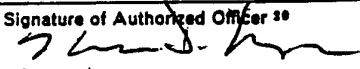
CLAIMS

What is claimed is:

- 5 1. A process for purifying a recombinant fusion protein having a terminal peptide identification sequence comprising multiple anionic amino acid residues, comprising forming a complex of the protein with a divalent cation dependent monoclonal antibody specific for the sequence, isolating the complex, and dissociating antibody and protein by selectively depleting the concentration of divalent cations in contact with the complex.
- 10 2. A process according to Claim 1, wherein the divalent cation is selected from the group consisting of Ca^{++} , Mg^{++} , Mn^{++} , Cu^{++} , Ni^{++} , Zn^{++} , Cd^{++} , Ba^{++} and Sr^{++} .
3. A process according to Claim 1, wherein antibody and protein are dissociated by depleting cations with a chelating agent.
- 15 4. A process according to Claim 3, wherein the chelating agent is EDTA.
5. A process according to Claim 2, wherein the divalent cation is Ca^{++} .
- 20 6. A process according to Claim 5, wherein the identification sequence is N-terminal DYKDDDDK.
7. A process according to Claim 6, wherein the monoclonal antibody is that secreted by the murine hybridoma 4E11 (ATCC HB 9259).
- 25 8. A process for producing a polypeptide encoded by a structural gene inserted in a prokaryotic or eukaryotic expression vector, comprising inserting adjacent to the structural gene a DNA sequence encoding a terminal identification peptide comprising multiple anionic amino acid residues; expressing the structural gene as a fusion protein comprising the identification peptide covalently linked to the polypeptide; and separating the fusion protein from contaminants using a cation-dependent monoclonal antibody specific for the identification peptide.
- 30 9. Murine hybridoma 4E11 (ATCC HB 9259).
10. Monoclonal antibody secreted by murine hybridoma 4E11 (ATCC HB 9259).

INTERNATIONAL SEARCH REPORT

International Application No PCT/US87/03113

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) ³		
According to International Patent Classification (IPC) or to both National Classification and IPC IPC(4): C12P 21/00; C12N 15/00; C12N 5/00; C07K 13/00 U.S.Cl.: 435/68; 435/172.3; 435/240.27; 530/387		
II. FIELDS SEARCHED		
Minimum Documentation Searched ⁴		
Classification System	Classification Symbols	
U.S.	435/68, 172.3, 240.27 530/387 935/95, 104, 108	
Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched ⁴		
Chemical Abstracts Data Base (CAS) 1967-1988; Biological Abstracts Data Base (BIOSIS) 1967-1988; Keywords: Monoclonal; Immunoaffinity; fusion peptide		
III. DOCUMENTS CONSIDERED TO BE RELEVANT ¹⁴		
Category ⁵	Citation of Document, ¹⁶ with indication, where appropriate, of the relevant passages ¹⁷	Relevant to Claim No. ¹⁸
Y, P	US, A, 4,703,004 (HOPP ET AL) 27 October 1987. See columns 10-12.	1-18
Y, P	US, A, 4,681,848 (TSUKAMOTO ET AL) 21 July 1987. See columns 1-3.	1-18
Y	<u>GENE</u> (AMSTERDAM, NL) Volume 38, issued 1985 (ITAMURA ET AL) "Expression of the gag gene of human T-cell Leukemia virus type I in <u>Escherichia coli</u> and its diagnostic use". See pages 57-64.	1-18
<p>⁵ Special categories of cited documents: ¹⁵</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"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)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> <p>"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</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"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.</p> <p>"&" document member of the same patent family</p>		
IV. CERTIFICATION		
Date of the Actual Completion of the International Search ¹	Date of Mailing of this International Search Report ²	
1 MARCH 1988	28 MAR 1988	
International Searching Authority ¹	Signature of Authorized Officer ¹⁹	
ISA/US	 Thomas D. Mays	

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

Y	JOURNAL OF IMMUNOLOGY (BALTIMORE, MARYLAND USA) Volume 105, issued September 1970 (MAURER ET AL) "Antigenicity of polypeptides (poly alpha amino acids): Calcium-dependent and independent antibodies". See pages 567-573.	1-18
Y	METHODS IN ENZYMOLOGY (NEW YORK) Volume 70, issued 1980 (MAURER ET AL) "Proteins and polypeptides as antigens". See pages 49-69.	17&18

V. OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE ¹⁰

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. Claim numbers _____, because they relate to subject matter ¹² not required to be searched by this Authority, namely:

2. Claim numbers _____, because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out ¹³, specifically:

VI. OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING ¹¹

This International Searching Authority found multiple inventions in this international application as follows:

1. As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

The additional search fees were accompanied by applicant's protest.

No protest accompanied the payment of additional search fees.