



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁵ : C12N 9/54, 9/56, C11D 3/386	A1	(11) International Publication Number: WO 92/17579 (43) International Publication Date: 15 October 1992 (15.10.92)
(21) International Application Number: PCT/US92/02597 (22) International Filing Date: 30 March 1992 (30.03.92) (30) Priority data: 677,726 29 March 1991 (29.03.91) US (71) Applicant: GENENCOR INTERNATIONAL, INC. [US/ US]; 180 Kimball Way, South San Francisco, CA 94080 (US). (72) Inventors: BARNITZ, Joy, T. ; 26 Little Spring Run, Fairport, NY 14450 (US). FISKE, Michael, J. ; MIDDLEBROOK, Susan, M. ; 167 Wood Run, Rochester, NY 14612 (US). STEELE, Darrell, Bernie ; 815 East University Drive, Auburn, AL 36830 (US).		(74) Agent: HORN, Margaret, A.; Genencor International, Inc., 180 Kimball Way, South San Francisco, CA 94080 (US). (81) Designated States: AT (European patent), BE (European patent), CA, CH (European patent), DE (European patent), DK (European patent), ES (European patent), FR (European patent), GB (European patent), GR (European patent), IT (European patent), JP, LU (European patent), MC (European patent), NL (European patent), SE (European patent). Published <i>With international search report.</i>
(54) Title: ALKALINE PROTEASE 3733, ITS PRODUCTION AND USE IN CLEANING CONTACT LENS (57) Abstract The fermentation and isolation of a novel alkaline protease, protease 3733 is described. This enzyme has the properties of an elastase and a serine protease. This enzyme is produced by a newly discovered alkalophilic <i>Bacillus sp.</i> IAM 011105 bacterium. Protease 3733 is characterized by high activity in hydrolyzing denatured lysozyme and the use of this enzyme in cleaning contact lenses is described. The biochemical properties of the new enzyme are described and the activity of the enzyme on a variety of substrates under a variety of conditions is described and compared to other alkaline proteases.		

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ALKALINE PROTEASE 3733, ITS PRODUCTION AND
USE IN CLEANING CONTACT LENS

BACKGROUND OF THE INVENTION

FIELD OF THE INVENTION

This invention relates to a novel alkaline protease or elastase enzyme "protease 3733" having an activity capable of dissolving denatured lysozyme, a production method thereof, and the process of using protease 3733 in cleaning contact lenses.

DESCRIPTION OF RELATED ART

Users of soft contact lenses must clean and disinfect their lenses for reasons of health and comfort. In addition to cleaning lenses with detergent, it is also necessary to remove protein deposit from the surface of the lenses. The presently used method for removing deposited protein involves soaking the lenses in a protease (such as subtilisin Carlsberg) solution at room temperature, soaking in a protease in a thermal disinfecting unit, or soaking in a chemical disinfectant.

The denaturation of protein on the lens surface presents a problem in cleaning the lens, especially when thermal disinfecting is used. The predominant protein deposited on the lens is lysozyme secreted by the user's tear ducts. The removal of lysozyme from lens is difficult using conventional proteases, such as subtilisin, especially when the lysozyme

has been denatured by thermal disinfecting.

The present invention is a novel alkaline protease which overcomes the relative inefficiency of conventional proteases in removing denatured lysozyme from contact lenses.

SUMMARY OF THE INVENTION

The fermentation and isolation of a novel alkaline protease, also called an elastase, named "protease 3733" is described. The enzyme was produced by fermentation of a newly isolated Bacillus sp. strain IAM 011105. The structural and biochemical properties of protease 3733 are described and compared to other proteases. This enzyme has an unusual degree of activity in hydrolyzing denatured lysosyme. Denatured lysozyme is the prime contaminant affecting contact lenses. The new enzyme is also highly active on the insoluble fibrous protein elastin. The use of protease 3733 to clean contact lenses is described.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1a shows the effect of pH on activity of protease 3733 in hydrolyzing azocasein.

Fig. 1b shows the effect of pH on stability of protease 3733 at 4° C and at 23° C.

Fig. 1c shows the effect of temperature on activity of protease 3733 in hydrolyzing azocasein.

Fig. 1d shows the effect of 40° C at pH 8 and pH 10 on stability of protease 3733.

Fig. 1e shows the effect of 50° C at pH 8 and pH 10 on stability of protease 3733.

Fig. 2a shows the effect of chelating agent EDTA and the serine protease inhibitor PMSF on activity of protease 3733.

Fig. 2b shows the effect of EDTA at pH 10 and 40° C on stability of protease 3733

Fig. 2c shows the effect of divalent cations at pH 10 and 50° C on stability of protease 3733.

Fig. 3 shows the comparative hydrolysis of denatured lysozyme by protease 3733, subtilisin Carlsberg, subtilisin aprE, and subtilisin BPN'.

Fig. 4 shows the comparative hydrolysis of denatured human milk lysozyme by protease 3733 and subtilisin Carlsberg with and without BME.

Fig. 5 shows the comparative hydrolysis of denatured human milk lysozyme by protease 3733 and subtilisin Carlsberg with and without BME.

DESCRIPTION OF THE PREFERRED EMBODIMENT

Strain Isolation. Bacillus sp. IAM 011105 was isolated from the aeration basin of an activated sludge facility used to treat textile finishing waste. The waste temperature was 20° C and the pH was 10.4. A small volume (0.1 ml) of waste slurry was added to 5.0 ml of 4g/liter nutrient broth (Difco, Detroit, Mich.) buffered at pH 10.5 with 0.05 M 3-cyclohexylamino-1-propanesulfonic acid (CAPS; Sigma Chemical, St. Louis, MO). This culture was incubated at 30° C for 10 days. The broth was then streaked on solid medium of above composition plus 1.5% Noble agar and 10% skim milk then incubated

at 30° C for 3 days. Strain IAM 011105 was isolated as a single colony exhibiting hydrolysis of skim milk casein.

Strain IAM 011105 is a gram positive bacterium. At pH 7.5 it grows as a straight rod 0.5 - 1.0um x 5.0 - 8.0 um. At pH 9.0 it grows as long thin rods approximately 0.5 um x 5.0 to greater than 15 um. Sporulation was not observed in any cultures. Colonies on Tryptic Soy Agar pH 7.5 are cream colored, opaque, circular, and convex with an entire margin. Colonies on Tryptic Soy Agar pH 9.0 are cream colored, opaque, irregular, and flat with an erose margin.

Biochemical characteristics of Bacillus sp. IAM 011105 were determined using the Minitex Disc System (Becton Dickinson, Cockeysville, MD). The results are shown in Table 1.

Bacillus sp. IAM 011105 has been deposited under No. 55142 with the American Type Culture Collection, Rockville, Maryland.

These data indicate that strain IAM 011105 is a previously undescribed bacterial species of the genus Bacillus.

Enzyme Production and Purification. This invention relates to the culturing of a bacterium of the order Eubacteriales, family Bacillaceae, genus Bacillus, and to collecting and purifying protease 3733 from the culture medium.

A fernbach flask containing 500 ml of 30 g/l tryptic soy broth buffered to pH 9.0 with 0.05M CAPS was inoculated with 1.0 ml frozen stock of Bacillus sp. IAM 011105. The culture was shaken overnight at 200 rpm at 30° C. This was used to inoculate a 14 l Chemap fermenter containing 10 l of the same

media. Cells were grown for 48 hours at 30° C with an agitation of 1300 rpm with an airflow of 8 liters per minute. Cells were separated from the culture broth via centrifugation in a Sorvall RC-5B centrifuge and the cells discarded.

Enzyme purification. The clarified culture medium was concentrated with a YM-10 ultrafiltration membrane with a 10,000 dalton cutoff, obtained from Amicon, a division of W.R. Grace & Co., Beverly, MA. 30 ml of concentrate was passed over a 100 ml SEPHADEX G-25 column equilibrated with 0.01M sodium acetate and 1.0mM CaCl, pH 5.5. SEPHADEX is a trademark for gel separation media owned by Pharmacia, Piscataway, NJ. The desalted material was applied to a Pharmacia FPLC MonoS HR 10/10 column equilibrated with the same buffer. Protease 3733 was eluted from the column using a 90 ml linear 0 - 0.2M NaCl gradient in the above buffer.

The resulting preparation of protease 3733 was shown to be homogeneous via SDS-PAGE, IEF, and N-terminal sequencing. Purified protease is stored at 4° C as a 1.0 mg/ml solution in column elution buffer with 50% propylene glycol.

Protein concentration was determined using the Bio-Rad protein assay kit (Bio-Rad, Rockville Centre, NY).

Biochemical Properties of Protease 3733. Figs.

1a - e shows several biochemical properties of protease 3733. Unless indicated, all data were determined under the following conditions. All studies were done with protease 3733 prepared as above and stored as indicated. Enzymatic activity was

determined using azocasein (Sigma Chemical Co., St. Louis, MO) as the substrate. 0.5% azocasein was prepared in 0.05M tris (hydroxymethyl) aminomethane (Tris) buffer (Sigma Chemical Co., St. Louis, MO) plus 1.0mM CaCl adjusted to pH 8.0 with HCl. 100ul of an appropriate enzyme dilution was added to 900ul of 0.5% azocasein and incubated at 30° C for 10 minutes. The reaction was stopped by the addition of 300ul of 10% trichloroacetic acid. The reaction mixture was centrifuged at 12,000 rpm for 2 minutes in an Eppendorf microfuge. 800ul of supernatant fluid was withdrawn into a fresh tube containing 300ul of 0.5 N NaOH. The mixture was vortexed and the absorbance read at 420nm. One unit of activity is the amount of enzyme required to give an absorbance change of 1.0 in 1 minute.

In Figs. 1a - e, the Activity axis represents the % relative activity calculated by comparison of each point to the highest value, which is given a relative activity of 100.

Fig. 1a shows the effect on the activity of protease 3733 on azocasein found by varying the pH values from 4 - 12. The data in Fig. 1a indicate that protease 3733 activity against azocasein had a pH optimum of at least pH 11.

Fig. 1b shows the effect of pH on stability of protease 3733. The enzyme, at a concentration of 1mg/ml, was incubated at pH values from 4 - 12 at 4° C, dashed line; or at 23° C, solid line, for 20 hrs. and the activity was determined. The data in Fig. 1b. indicate that protease 3733 was stable for 20 hrs. at pH 5 - 9 at 4° C and stable for 20 hrs. at pH 7 -

9 at 23° C.

Fig. 1c shows the effect of temperature on activity of protease 3733. Enzymatic activity was determined at 10° - 70° C. The data in Fig. 1c indicate a temperature optimum of 50° C.

Fig. 1d shows the effect of 40° C on stability of protease 3733. The enzyme at a concentration of 1mg/ml was incubated for up to 120 min. at 40° C at a pH of 8, dashed line; or pH of 10, solid line; and the enzymatic activity determined. The data in Fig. 1d show that protease 3733 is stable at 40° C at pH 8 or pH 10.

Fig. 1e shows the effect of 50° C on stability of protease 3733. The enzyme was incubated for up to 120 min. at 50° C at a pH of 8, solid line; or pH of 10, dashed line; and the enzymatic activity determined. The data in Fig. 1d show that protease 3733 loses 50% of its activity after 15 min. at 50° C at both pH 8 and pH 10.

Figs. 2a - c show the effects of various additives on the activity of protease 3733. Unless indicated otherwise, the conditions were the same as in Figs. 1a - e.

Fig. 2a shows the effect of (1) phenylmethylsulfonyl fluoride (PMSF) and (2) ethylenediaminetetraacetic acid (EDTA) on protease 3733 activity. The data in Fig. 2a show that PMSF at 1.0mM inhibited the activity of protease 3733 while EDTA at 5.0mM had no effect on enzyme activity.

Fig. 2b shows the effect of EDTA on the stability of

protease 3733. The enzyme was incubated for up to 120 min. at pH 10 and 40° C in the presence and absence of EDTA at a concentration of 2.0mM and the activity was determined. Crosses indicated presence of EDTA points and triangles indicated control points lacking EDTA. The data in Fig. 2b indicate that EDTA drastically reduced the stability of protease 3733.

Fig. 2c shows the effect of divalent cations on stability of protease 3733. Chloride salts of a variety of cations were added to separate aliquots of enzyme solution which were held at pH 10 and 50° C for up to 120 min. At intervals indicated, samples were taken from each aliquot and the enzyme activity determined. All experimental salts were at a final concentration of 5.0mM. The experimental points on Fig. 2c are as follows: triangle, control; cross, Ba^{++} ; open square, Ca^{++} ; closed square, Co^{++} ; cross in square, Mn^{++} ; cross with hyphen, Zn^{++} ; and diamond, Mg^{++} . The data in Fig. 2c indicate that Ca^{++} and, to a lesser extent, Mg^{++} increased thermostability. The data in Figs. 2a - c are typical for Bacillus alkaline proteases.

The specific activity of protease 3733 was examined on several substrates including azocasein, N-succinyl-ala-ala-pro-phe p-nitroanilide (AAPF-pna), N-succinyl-ala-ala-ala p-nitroanilide (AAA-pna), and elastin congo red, all obtained from Sigma Chemical Co., St. Louis, MO. Specific activities were compared to those obtained with subtilisin Carlsberg and subtilisin aprE.

Enzyme assays with AAPF-pna and AAA-pna as substrates were

performed as follows. 190mM AAPF-pna or AAA-pna was prepared in dimethylsulfoxide. Reaction mixtures contained 980ul of 50mM Tris-HCl (pH 8.0) + 1.0mM CaCl₂, 10ul of either AAPF-pna or AAA-pna, and 10ul of an appropriate enzyme dilution. The increase in absorbance at 410nm, due to the release of p-nitroaniline, was followed continuously at 25°C. One unit of activity is the amount of enzyme required to give an absorbance change of 1.0 in 1 minute.

Enzyme assays with elastin congo red were performed as follows. 10mg of elastin congo red (Sigma Chemical Co., St. Louis, MO) was weighed into 13x100mm test tubes. 900ul of 50mM Tris-HCl (pH 8.0) + 1.0mM CaCl₂ was added followed by the addition of 100ul of an appropriate enzyme dilution. tubes were capped and incubated for 30 minutes at 37° C with shaking. the reaction was stopped by the addition of 1.0 ml of 0.7M KPO₄ (pH 5.5), the tubes centrifuged at 3000 rpm for 10 minutes, and the absorbance of the supernatant fluid read at 495nm. One unit of activity is the amount of enzyme required to give an absorbance change of 1.0 in 1 minute.

Table 2 gives the specific activities of protease 3733 on the indicated substrates in comparison to those of subtilisin Carlsberg and subtilisin aprE. Specific activities are expressed as Units/mg of enzyme. The data in Table 2 indicate that the specific activity of protease 3733 on several of the substrates examined is significantly different from that of subtilisin Carlsberg and from that of subtilisin aprE. The specific

activity of protease 3733 is similar to that of the subtilisins on azocasein. However, the specific activity of protease 3733 is higher than that of subtilisin aprE, and much lower than that of subtilisin Carlsberg on AAPF-pna. The specific activity of protease 3733 is much higher than that of either subtilisin on AAA-pna. The specific activity of protease 3733 is much higher than that of either subtilisin on Elastin congo red. This indicates that protease 3733 is a different and distinct enzyme from either subtilisin Carlsberg or subtilisin aprE.

Composition of Protease 3733. The amino acid compositions of protease 3733, subtilisin Carlsberg, and elastase Ya-B are compared in Table 3. Elastase Ya-B is an alkaline elastase isolated from Bacillus sp. Ya-B as described in Biochim Biophys Acta, 1986, 833, pages 439-447. The enzymes were hydrolyzed and the resultant component amino acids analyzed. Table 3 shows the number of amino acid residues which resulted from the hydrolysis of each enzyme. The amino acid compositions of protease 3744, subtilisin Carlsberg, and elastase Ya-B differ significantly from each other and indicate that these are different enzymes.

The N-terminal sequences of protease 3733, subtilisin Carlsberg, and elastase Ya-B were determined and are shown in the section "Sequence Listing" as SEQ ID NO:1, SEQ ID NO:2, and SEQ ID NO:3, respectively. Table 4 shows the N-terminal sequence of these enzymes. Stars in Table 4 indicate amino acids homologous to protease 3733. Protease 3733 exhibits 50%

homology with subtilisin Carlsberg and 34% homology with elastase Ya-B in the N-terminal sequence of these enzymes. This indicates that the three enzymes are distinctly different proteins.

Properties of Protease 3733 and Other Proteases. Table 5 compares several biochemical properties of pronase 3733 with those of subtilisin Carlsberg and elastase Ya-B. Unless indicated, these properties were determined as in Fig 1a - e. Data for elastase Ya-B were obtained from the above publication.

The data in Table 5 indicate that the pH optima were similar for each enzyme and all were serine proteases. Protease 3733 had a much lower pI than elastase Ya-B and subtilisin Carlsberg and protease 3733 had a lower pI than serine proteases in general. Protease 3733 exhibited some degree of cross-reactivity with subtilisin Carlsberg antibodies whereas elastase Ya-B had none. The ratio of elastin/casein degrading activity was highest for elastase Ya-B, with the ratio for protease 3733 approximately nine times that of the ratio for subtilisin Carlsberg. In addition, an elastase from Bacillus subtilis was found to have a pH optimum of 9.0 and a molecular weight of 25,000. B. subtilis elastase is an elastase isolated from B. subtilis as described in Canadian Journal of Microbiology, 1988, 34, p. 855-859.

Hydrolysis of Denatured Lysozyme. Lysozyme hydrolysis assays were performed as follows. A 1.0 mg/ml solution of chicken eggwhite lysozyme (Sigma Chemical Co., St. Louis, MO) was prepared in 50mM sodium borate (pH 8.0) and 1.0 ml of this solution was aliquoted into 13x100mm test tubes. The tubes

were capped and placed in a boiling water bath for 5 minutes resulting in denaturation of the lysozyme. After allowing the tubes to cool, 100 ul of an appropriate enzyme dilution was added and the reaction mixture was incubated at 37° C for 30 minutes. The reaction was stopped by the addition of 300 ul of 10% trichloroacetic acid followed by centrifugation at 3000 rpm for 10 minutes. The absorbance of soluble amino acids in the supernatant fluid was read at 380nm. Assays using human milk lysozyme (Sigma Chemical Co., St. Louis, MO) were performed using this same protocol. Any changes in incubation temperature or incubation time are indicated where appropriate.

Fig. 3 illustrates the enzymatic activity of protease 3733, subtilisin Carlsberg, subtilisin aprE, and subtilisin BPN' on denatured eggwhite lysozyme. Activity was determined by incubating 25ug of purified enzyme with denatured eggwhite lysozyme under the conditions described above. The data show protease 3733 to be 5 - 6 fold more active at hydrolyzing denatured eggwhite lysozyme than the other proteolytic enzymes.

Fig. 4 shows the effect of beta-mercaptoethanol (BME) on enzymatic activity of protease 3733 and subtilisin Carlsberg on denatured eggwhite lysozyme. The data show a two-fold enhancement of lysozyme hydrolysis by the addition of 0.4% BME for both protease 3733 and subtilisin Carlsberg. This presumably was due to the reduction of disulfide bonds in lysozyme making the protein more susceptible to proteolysis. Protease 3733 was clearly superior to subtilisin Carlsberg in that protease

3733 without addition of BME was 2.5 fold more active than subtilisin Carlsberg even when the activity of subtilisin Carlsberg was enhanced by addition of BME.

Fig. 5 shows the activity of protease 3733 and subtilisin Carlsberg on denatured human milk lysozyme with and without the addition of 0.4% BME. Without the addition of BME, protease 3733 exhibited a 4 - 5 fold higher activity on human milk lysozyme than did subtilisin Carlsberg. As in the case of chicken eggwhite lysozyme as substrate, the addition of BME resulted in a two-fold enhancement of enzymatic activity for both enzymes. However, protease 3733 without addition of BME was 1.5 - 2 fold more active on denatured human milk lysozyme than was subtilisin Carlsberg enhanced with BME.

Protease 3733 Treatment of Contact Lenses.

Protein-contaminated contact lenses may be cleaned by incubation in a cleansing preparation containing protease 3733. A suitable preparation is a sterile aqueous solution of 0.05 M sodium borate buffer pH 8.0 with protease 3733 at 1 mg/ml. Contaminated contact lens are immersed in the preparation and held at room temperature for 30 min. The lenses are rinsed well with sterile physiological saline before use. Protein contamination is effectively removed from contact lenses using this preparation and method.

In an optional formulation for cleaning contaminated contact lenses, 0.4% BME may be included in preparation.

Suitable cleansing preparations also may be formulated

in nonaqueous solvents, such as hexane, cyclohexane, ethanol, methanol, and dimethylsulfoxide. Such solutions may be buffered to insure a high level of enzymatic activity. The method of use is as for aqueous solutions.

The invention has been described in detail with particular reference to preferred embodiments thereof: however, it should be understood that variations and modifications can be made within the spirit and scope of the invention and the same should not be limited except as set forth in the appended claims.

TABLE 1

Mobility	+
Oxidase	+
Catalase	+
Spore Production Observed	-
Acid Produced From:	
Anaerobic Dextrose	-
Aerobic Dextrose	+
Maltose	+
Trehalose	+
Sucrose	+
Xylose	+
Lactose	+
Arabinose	+
Mannitol	+
Cellobiose	+
Adonitol	+
Inositol	+
Raffinose	+
Rhamnose	+
Sorbitol	+
Mannose	+
Galactose	+
Phenylalanine Deaminase	-

Ornithine Decarboxylase	-
Lysine Decarboxylase	-
Arginine Decarboxylase	-
Beta Galactosidase	-
Nitrate Reduction	-
Denitrification	-
Voges-Proskauer	-
Citrate (as sole carbon source)	-
Urease	-
Hydrogen Sulfide	-
Indol	-
Esculin Hydrolysis	-
Starch Hydrolysis	-
Casein Hydrolysis	+
Elastin Hydrolysis	+

TABLE 2

Substrate	Protease 3733	Subtilisin	Subtilisin
		Carlsberg	apra
Azocasein	14.4	16.7	16.2
AAPF-pna	393	4286	370
AAA-pna	22.8	10.0	1.8
Elastin congo red	2.6	0.4	0.2

Table 3

Amino Acid	Protease 3744	Subtilisin Carlsberg	Elastase Ya-B
Asx	35	28	28
Glx	18	11	14
Ser	22	32	26
Gly	40	35	37
His	9	5	8
Arg	10	4	7
Thr	13	19	17
Ala	36	41	33
Pro	13	8	8
Tyr	13	13	4
Val	23	31	21
Met	8	5	3
Cys	0	0	0
Ile	13	10	10
Leu	19	16	11
Phe	7	4	6
Lys	4	9	4
Trp	1	2	2
Total	284	273	239

TABLE 4

Residue No.	Subtilisin Carlsberg		Protease 3733		Elastase Ya-B
1	Ala				
	Gln	*	Gln		Gly
	Thr	*	Thr		Xaa
	Val	*	Val		Xaa
5	Pro	*	Pro	*	Pro
	Tyr		Trp	*	Trp
	Gly	*	Gly	*	Gly
	Ile	*	Ile	*	Ile
	Pro	*	Pro		Asn
10	Leu		Tyr		Arg
	Ile	*	Ile		Val
	Lys		Tyr		Gln
	Ala		Ser		Ala
	Asp	*	Asp		Pro
15	Lys		Val		Ile
	Val	*	Val		Ala
	Gln		Xaa		Gln
	Ala		Xaa		Ser
	Gln	*	Gln		Arg
20	Gly	*	Gly	*	Gly
	Phe		Tyr		Phe
	Lys		Phe		Ala

20

25

Gly	*	Gly	*	Gly
Ala		Asn		Ala
Asn		Gly	*	Gly
Val	*	Val	*	Val
Lys	*	Lys		Arg
Val	*	Val	*	Val
Ala	*	Ala	*	Ala

TABLE 5

Property	Protease 3733	Elastase Ya-B	Subtilisin Carlsberg
pH Optimum	11.0	11.7	10.0
Temperature Optimum ° C	50	60	60
Molecular Weight	27,000	25,000	28,000
pI	4.9	10.6	9.4
Serine Protease	+	+	+
N-terminus	Gln	Gly	Ala
X-reactivity With Subtilisin Carlsberg			
Antibody	+	-	++
Activity Ratio Elastin/Casein	0.18	0.58	0.02

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Fiske, Michael J.

Middlebrook, Susan M.

Steele, D. Bernie

Barnitz, Joy T.

(ii) TITLE OF INVENTION: Alkaline Protease 3733, Its
Production and Use In Cleaning
Contact Lens

(iii) NUMBER OF SEQUENCES: 3

(iv) CORRESPONDENCE ADDRESS:

(A) Addressee: Genencor International Inc.

(B) Street: 180 Kimball Way

(C) City: South San Francisco

(D) State: California

(E) Country: USA

(F) ZIP: 94080

(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Disc

(B) COMPUTER: IMB PS/2

(C) OPERATING SYSTEM: DOS

(D) SOFTWARE: Multimate

(viii) ATTORNEY/AGENT INFORMATION

(A) NAME: Passe, James G.

(B) REGISTRATION NUMBER: 29,966

(ix) TELECOMMUNICATION INFORMATION:

(A) TELEPHONE: (415)742-7500

(B) TELEFAX: (415)583-8269

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 28 amino acid residues

(B) TYPE: amino acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: no

(iv) ANTI-SENSE: no

(v) FRAGMENT TYPE: -N-terminal fragment

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: Bacillus sp.
- (B) INDIVIDUAL/ISOLATE: IAM 011105, ATCC 55142
- (C) CELL TYPE: unicellular bacterium
- (D) DEVELOPMENTAL STAGE: vegetative cells

(vii) IMMEDIATE SOURCE: bacteria isolated, cultured; enzyme isolated, sequenced

(ix) FEATURE

- (A) NAME/KEY: N-terminal sequence of alkaline protease, protease 3733
- (C) IDENTIFICATION METHOD: Biochemical assay
- (D) OTHER INFORMATION: active as alkaline protease especially active on denatured lysozyme and elastin, useful for cleaning contact lens

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

Gln Thr Val Pro Trp Gly Ile Pro Tyr Ile Tyr Ser Asp Val Val
1 5 10 15

Xaa Xaa Gln Gly Tyr Phe Gly Asn Gly Val Lys Val Ala
20 25

(3) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 29 amino acid residues
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: no

(iv) ANTI-SENSE: no

(v) FRAGMENT TYPE: -N-terminal fragment

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: Bacillus subtilis
- (B) INDIVIDUAL/ISOLATE: Var. Carlsberg
- (C) CELL TYPE: unicellular organism

(vii) IMMEDIATE SOURCE: enzyme obtained commercially,
sequenced

(ix) FEATURE

- (A) NAME/KEY: N-terminal sequence of alkaline protease,
subtilisin Carlsberg
- (C) IDENTIFICATION METHOD: Biochemical assay

(D) OTHER INFORMATION: active as alkaline protease

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Ala Gln Thr Val Pro Tyr Gly Ile Pro Leu Ile Lys Ala Asp Lys

1 5 10 15

Val Gln Ala Gln Gly Phe Lys Gly Ala Asn Val Lys Val Ala

20 25

(4) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 28 amino acid residues

(B) TYPE: amino acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: no

(iv) ANTI-SENSE: no

(v) FRAGMENT TYPE: -N-terminal fragment

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: Bacillus sp.
(B) INDIVIDUAL/ISOLATE: Ya-B
(D) DEVELOPMENTAL STAGE: vegetative cells
(G) CELL TYPE: unicellular bacterium

(vii) IMMEDIATE SOURCE: enzyme isolated from culture, sequenced

(ix) FEATURE

- (A) NAME/KEY: N-terminal sequence of alkaline protease,
elastase Ya-B
- (C) IDENTIFICATION METHOD: biochemical assay
- (D) OTHER INFORMATION: active as alkaline protease

(x) PUBLICATION INFORMATION

- (C) JOURNAL: Biochim Biophys Acta
(D) VOLUME: 833
(F) PAGES: 439-447
(G) DATE: 1986

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Gly Xaa Xaa Pro Trp Gly Ile Asn Arg Val Gln Ala Pro Ile Ala
1 5 10 15

Gln Ser Arg Gly Phe Ala Gly Ala Gly Val Arg Val Ala
20 25

CLAIMS

We claim:

1. A Bacillus sp. alkaline protease "protease 3733" enzyme characterized by activity in hydrolyzing denatured lysozyme, as a serine protease, with a molecular weight of about 27,000 daltons based on SDS-PAGE analysis, an amino acid composition as set out in Table 3, a N-terminal sequence as set out in SEQ ID NO:1, a pH optimum of at least pH 11.0 with azocasein, a temperature optimum of about 50° C, a pI of about 4.9, and an activity ratio on elastin/casein of about 0.18.
2. A culture of the microorganism Bacillus sp. strain IAM 011105, said culture being capable of producing the alkaline protease enzyme "protease 3733" in a recoverable quantity upon fermentation in an aqueous nutrient medium containing assimilable sources of carbon, nitrogen, and inorganic substances.
3. A biologically pure culture of the microorganism Bacillus sp. strain IAM 011105, said culture being capable of producing the alkaline protease enzyme "protease 3733" in a recoverable quantity upon fermentation in an aqueous nutrient medium containing assimilable sources of carbon, nitrogen, and inorganic substances.
4. A process for producing a novel alkaline protease

"protease 3733", as recited in claim 1, which comprises the steps:

culturing a protease 3733-producing bacterium belonging to the genus of Bacillus in a culture medium, and collecting protease 3733 from the culture medium.

5. A process according to claim 4, wherein the protease 3733-producing bacterium is Bacillus sp. IAM 011105 strain.

6. A process according to claim 4, wherein the culture medium is tryptic soy broth buffered with CAPS buffer at pH 9.0.

7. A process according to claim 4, wherein culturing is conducted aerobically at about 30° C.

8. A process for cleaning protein contaminated contact lenses comprising the step:

soaking the contact lenses in a solution of alkaline protease 3733.

9. The process of claim 8 wherein the solution of alkaline protease 3733 is an aqueous solution.

10. A cleansing preparation for contact lens comprising alkaline protease 3733 in a buffered solution at an alkaline pH.

11. The cleansing preparation of claim 10 wherein the pH is about 7.0 to about 13.0

12. The cleansing preparation of claim 10 wherein the buffer is sodium borate.

13. The cleansing preparation of claim 10 wherein the

pH is about 8.0.

14. The cleansing preparation of claim 10 further comprising beta-mercaptoethanol at a concentration adequate to enhance the activity of the alkaline protease 3733.

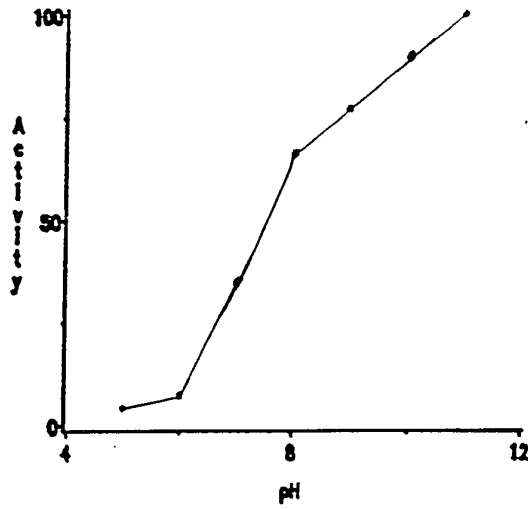
15. The cleansing preparation of claim 14 wherein the beta-mercaptoethanol is at a concentration of about 0.4%.

16. The cleansing preparation of claim 10 wherein the solution of alkaline protease 3733 is a nonaqueous solvent.

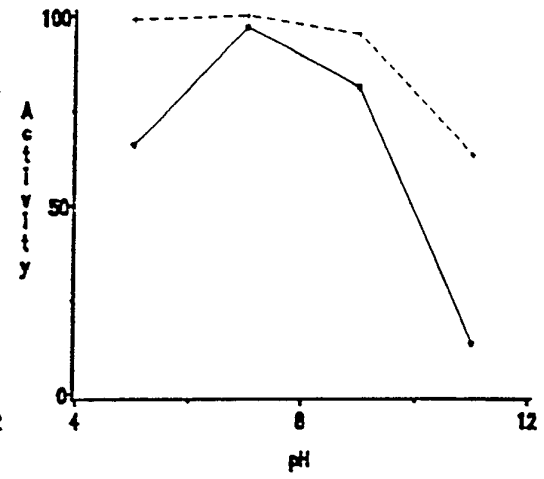
17. The cleansing preparation of claim 16 wherein the nonaqueous solvent is chosen from the group consisting of hexane, cyclohexane, ethanol, methanol, and dimethylsulfoxide.

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1a pH vs. activity

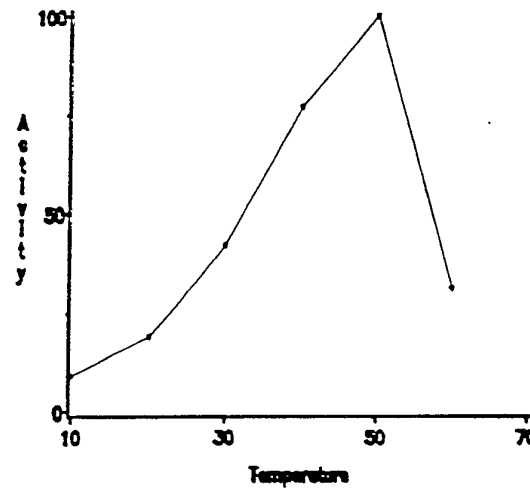


1b pH stability : 20 hrs
— 23 C --- 4 C



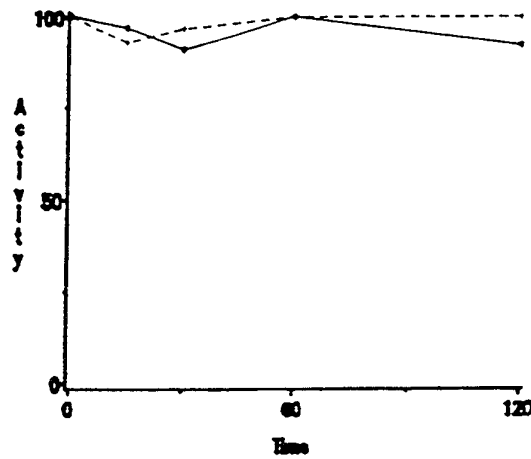
temperature vs activity

1c



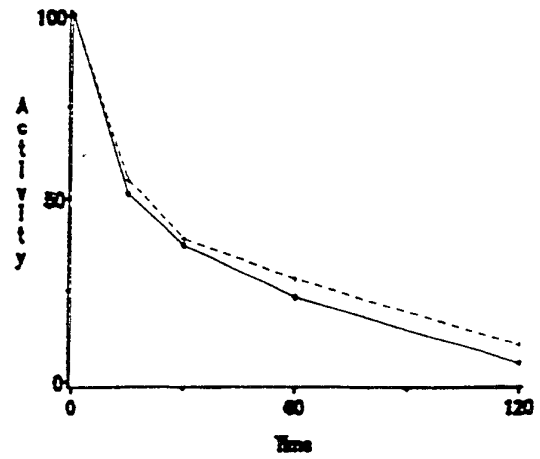
temperature stability : 40 C
— pH 8 --- pH 10

1d



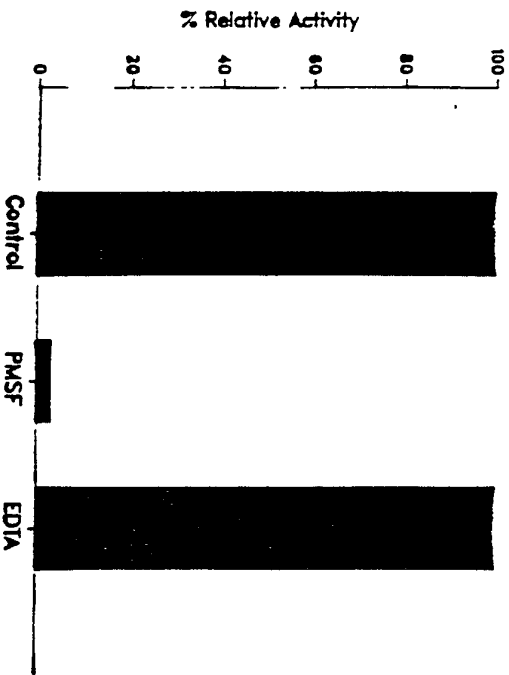
temperature stability : 50 C
— pH 8 --- pH 10

1e



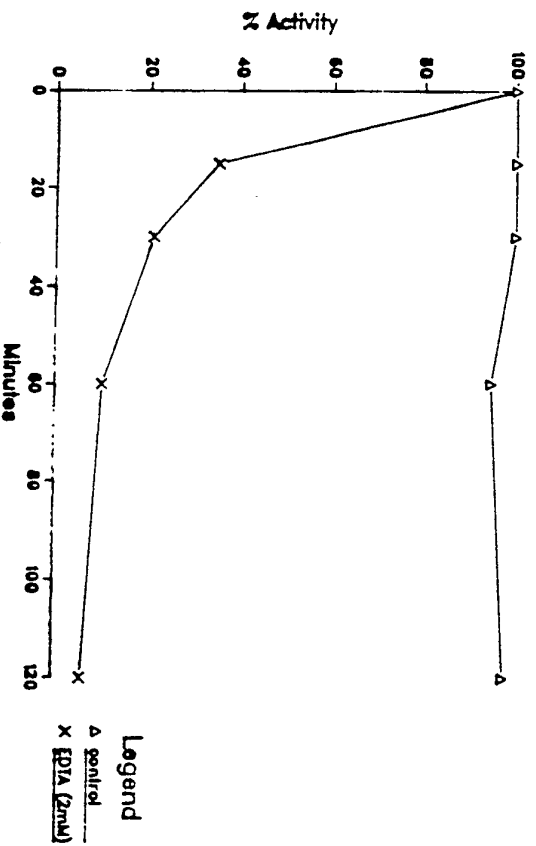
2a

Effect of PMSF + EDIA on Protease Activity



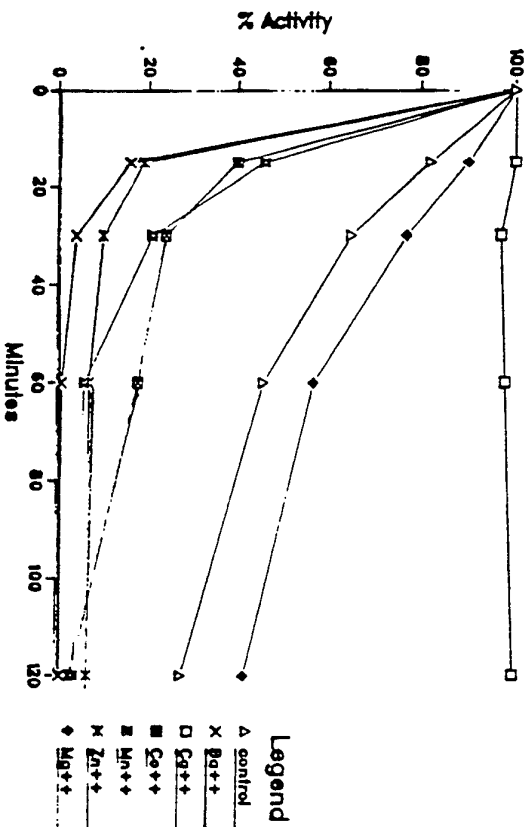
2b

Stability: EDIA / pH 10 / 40°C



2c

Effects of Divalent Cations on Stability at pH 10 / 50°C



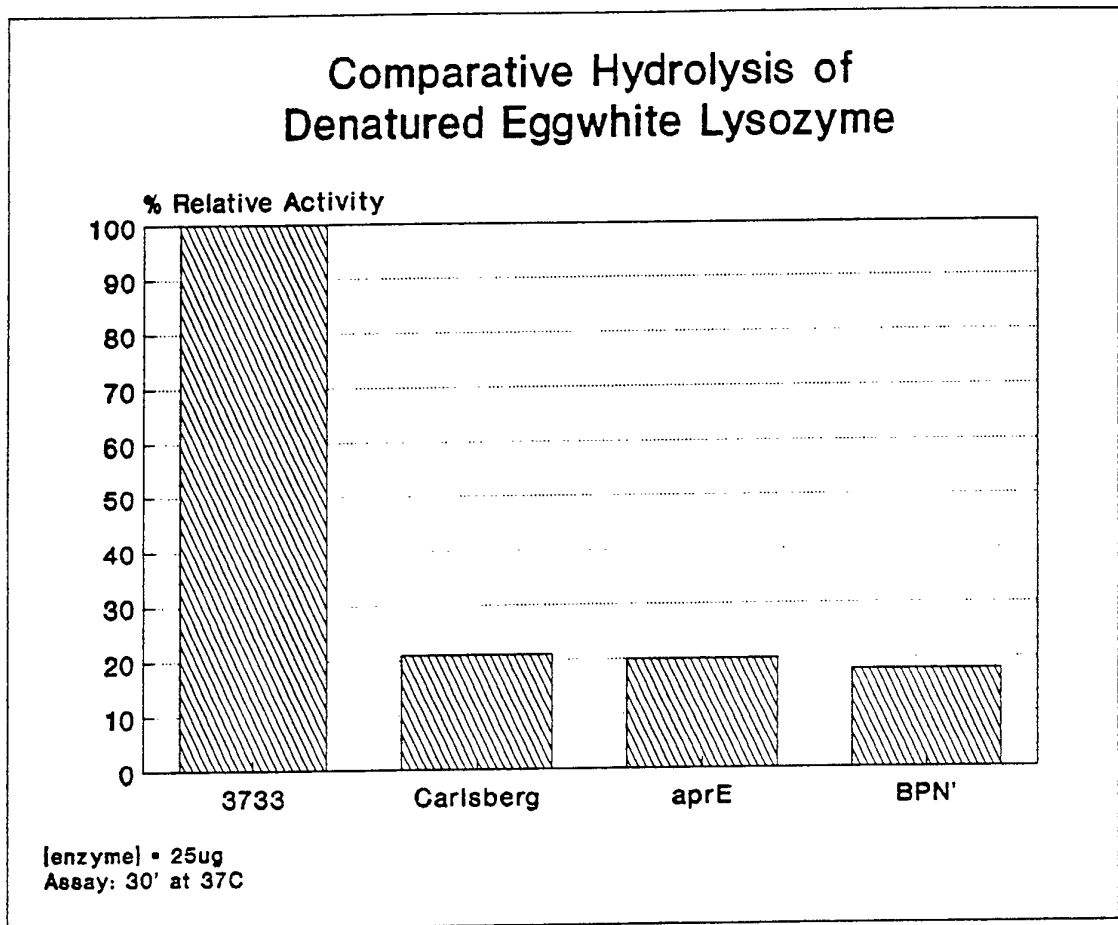


Figure 3

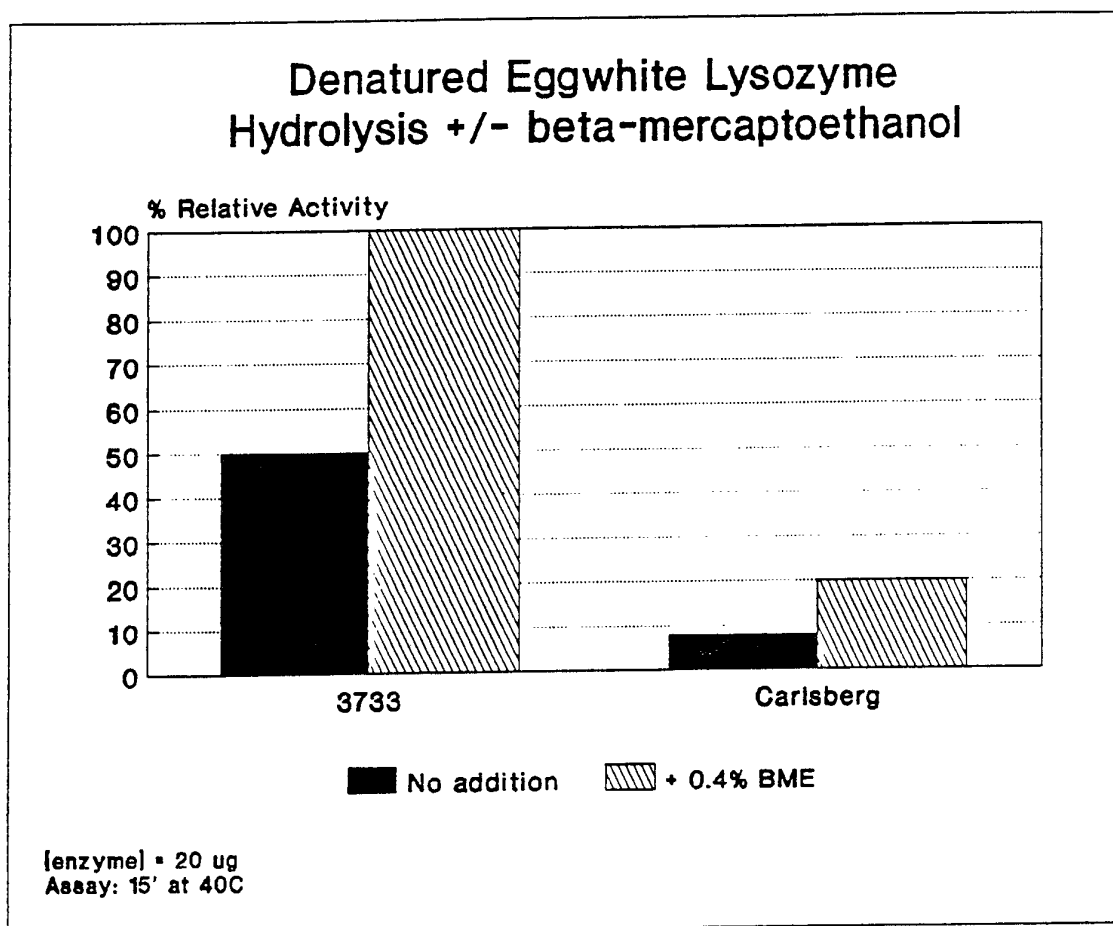


Figure 4

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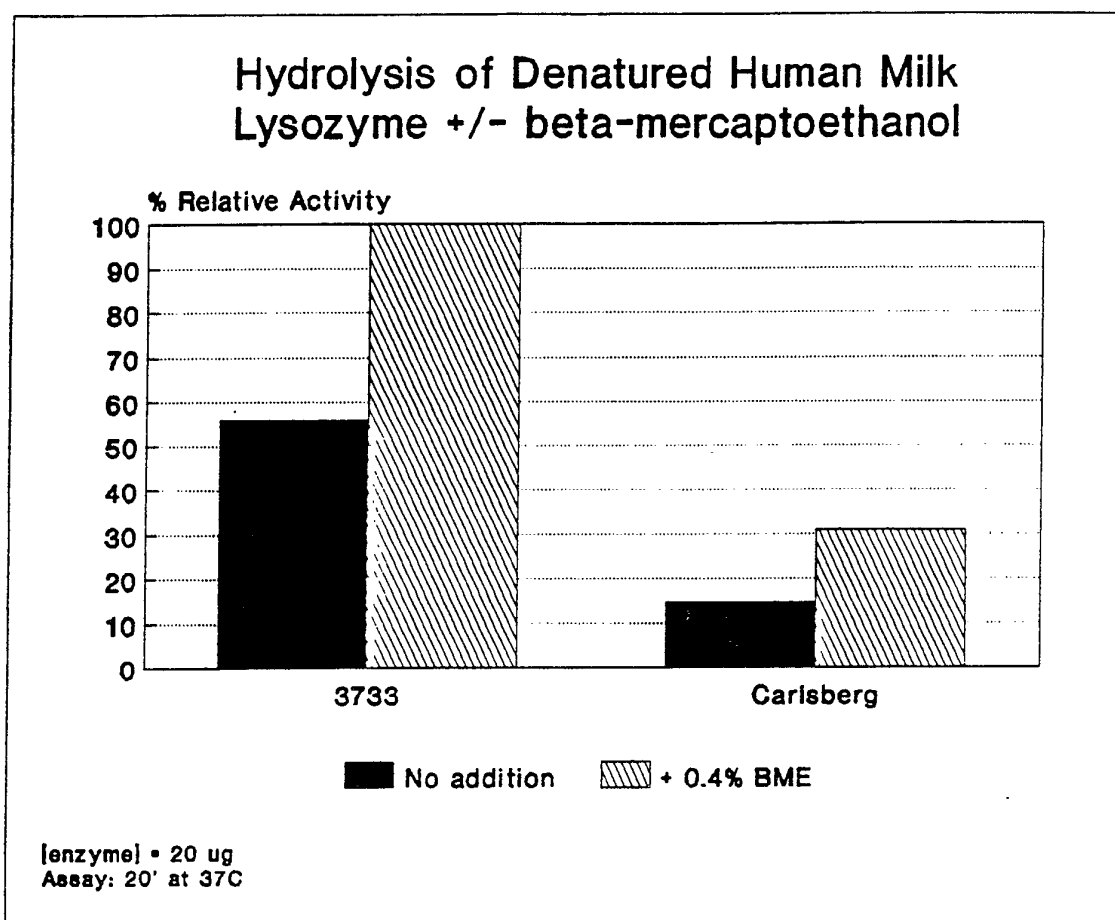
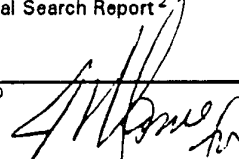


Figure 5

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US92/02597

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 (5): C12N 9/54, 9/56; C11D 3/386 US CL : 252/174.12; 435/221, 222; 514/840		
II. FIELDS SEARCHED		
Minimum Documentation Searched ⁴		
Classification System	Classification Symbols	
U.S.	252/174.12; 435/221, 222; 514/840	
Documentation Searched other than Minimum Documentation to the extent that such Documents are included in the Fields Searched ⁶		
APS		
III. DOCUMENTS CONSIDERED TO BE RELEVANT ¹⁴		
Category*	Citation of Document, ¹⁸ with indication, where appropriate, of the relevant passages ¹⁷	Relevant to Claim No. ¹⁸
Y	US, A, 4,771,003 (STELLWAG ET AL.) 13 SEPTEMBER 1988, SEE ESPECIALLY CLAIM 7.	1-17
Y	US, A, 4,511,490 (STANISLOWSKI ET AL.) 16 APRIL 1985, SEE ESPECIALLY COLUMN 13, LINES 9-25).	10-17
Y	US, A, 3,838,009 (FUKUMOTO ET AL.) 24 SEPTEMBER 1974, SEE ESPECIALLY CLAIM 1.	1-17
Y	US, A, 4,670,178 (HUTH ET AL.) 02 JUNE 1987, SEE ENTIRE DOCUMENT.	10-17
Y, P	US, A, 5,096,607 (MOWREY-MCKEE ET AL.) 17 MARCH 1992, SEE ENTIRE DOCUMENT.	10-17
A	US, A, 3,905,869 (HIDAKA ET AL.) 16 SEPTEMBER 1975, SEE CLAIMS 1-10.	1-17
A	US, A 4,002,572 (de NIJENHUIS) 11 JANUARY 1977, SEE ENTIRE DOCUMENT.	1-17
A	US, A, 4,052,262 (HORIKOSHI ET AL.) 04 OCTOBER 1977, SEE ESPECIALLY CLAIM 1.	1-17
A	US, A, 4,480,037 (ICHISHIMA ET AL.) 30 OCTOBER 1984, SEE ESPECIALLY ABSTRACT.	1-17
* Special categories of cited documents:¹⁶ "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier document but published on or after the international filing date "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) "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 "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 "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step "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 "&" document member of the same patent family		
IV. CERTIFICATION		
Date of the Actual Completion of the International Search ²	Date of Mailing of this International Search Report ²	
30 JUNE 1992	7 JUL 1992	
International Searching Authority ¹	Signature of Authorized Officer ²⁰	
ISA/US	JON P. WEBER, PH.D. 	

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

A	US, A, 4,764,470 (DURHAM ET AL.) 16 AUGUST 1988, SEE ESPECIALLY CLAIM 6.	1-17
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V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE¹

1. ☐ Claim numbers , because they relate to subject matter (1) 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 (1), specifically:
3. ☐ Claim numbers , because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING²

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 Search 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.