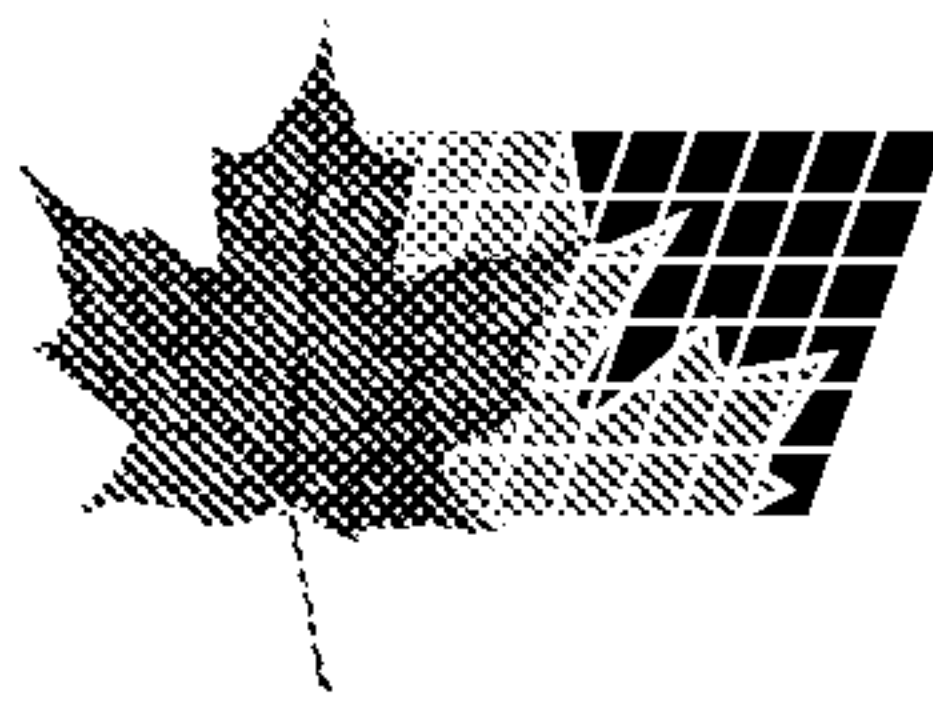




- (72) RAI, SAROJ, US
(72) CORREA, PAUL ELLIOTT, US
(72) ZHU, YONG, US
(72) GRAYCAR, THOMAS PAUL, US
(72) BOTT, RICHARD RAY, US
(71) THE PROCTER & GAMBLE COMPANY, US
(71) GENENCOR INTERNATIONAL, INC., US
(51) Int.Cl.⁷ C12N 15/57, C11D 3/386
(30) 1997/06/04 (60/048,550) US
(54) **ENZYMES PROTEASES POUR ELIMINER LES SOUILLURES
TENACES ET/OU REDUIRE LA FORMATION DE TACHES ET
DE FILMS ET COMPOSITIONS NETTOYANTES
INCORPORANT CES ENZYMES**
(54) **PROTEASE ENZYMES FOR TOUGH CLEANING AND/OR SPOT
AND FILM REDUCTION AND COMPOSITIONS
INCORPORATING SAME**

(57) Cette invention se rapporte à des compositions nettoyantes, comprenant une enzyme protéase constituée par une variante de carbonyle hydrolase ayant une séquence d'acides aminés ne se trouvant pas à l'état naturel, qui est dérivée par remplacement de plusieurs restes d'acides aminés d'une carbonyle hydrolase précurseur par différents acides aminés. Ces restes d'acides aminés remplacés dans l'enzyme précurseur correspondent à la position +210 combinée aux positions d'un ou de plusieurs restes d'acides aminés équivalentes à celles choisies dans le groupe +33, +62, +67, +76, +100, +101, +103, +104, +107, +128, +129, +130, +132, +135, +156, +158, +164, +166, +167, +170, +209, +215, +217, +218 et +222, les positions ainsi numérotées correspondant à la subtilisine existant à l'état naturel provenant du *Bacillus amyloliquefaciens* ou à des restes d'acides aminés équivalents d'autre carbonyles hydrolases ou subtilisines, telles que la subtilisine du *Bacillus lentus*.

(57) The present invention relates to cleaning compositions comprising protease enzyme which is a carbonyl hydrolase variant having an amino acid sequence not found in nature, which is derived by replacement of a plurality of amino acid residues of a precursor carbonyl hydrolase with different amino acids, wherein said plurality of amino acid residues replaced in the precursor enzyme correspond to position 210 in combination with one or more acid residue positions equivalent to those selected from the group consisting of: +33, +62, +67, +76, +100, +101, +103, +104, +107, +128, +129, +130, +132, +135, +156, +158, +164, +166, +167, +170, +209, +215, +217, +218 and +222, where the numbered positions correspond to naturally-occurring subtilisin from *Bacillus amyloliquefaciens* or to equivalent amino acid residues in other carbonyl hydrolases or subtilisins (such as *Bacillus lentus* subtilisin).

**PCT**WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ : C12N 15/57, C11D 3/386 // C12N 9/54	A1	(11) International Publication Number: WO 98/55634 (43) International Publication Date: 10 December 1998 (10.12.98)
(21) International Application Number: PCT/IB98/00853 (22) International Filing Date: 2 June 1998 (02.06.98) (30) Priority Data: 60/048,550 4 June 1997 (04.06.97) US (71) Applicants (for all designated States except US): THE PROCTER & GAMBLE COMPANY [US/US]; One Procter & Gamble Plaza, Cincinnati, OH 45202 (US). GENENCOR INTERNATIONAL, INC. [US/US]; 925 Page Mill Road, Palo Alto, CA 94304-1013 (US). (72) Inventors; and (75) Inventors/Applicants (for US only): RAI, Saroj [US/US]; 8287 Fox Knoll Drive, West Chester, OH 45069 (US). CORREA, Paul, Elliott [US/US]; 4245 Miami Trails Lane, Cincinnati, OH 45252 (US). ZHU, Yong [CN/US]; 4348 Bromyard Avenue, Cincinnati, OH 45241 (US). GRAYCAR, Thomas, Paul [US/US]; 1166 Manzanita, Pacifica, CA 94044 (US). BOTT, Richard, Ray [US/US]; 3032 Hillside Drive, Burlingame, CA 94010 (US). (74) Agents: REED, T., David et al.; The Procter & Gamble Company, 5299 Spring Grove Avenue, Cincinnati, OH 45217 (US).		(81) Designated States: BR, CA, CN, JP, MX, US, European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE). Published <i>With international search report.</i>
(54) Title: PROTEASE ENZYMES FOR TOUGH CLEANING AND/OR SPOT AND FILM REDUCTION AND COMPOSITIONS INCORPORATING SAME (57) Abstract The present invention relates to cleaning compositions comprising protease enzyme which is a carbonyl hydrolase variant having an amino acid sequence not found in nature, which is derived by replacement of a plurality of amino acid residues of a precursor carbonyl hydrolase with different amino acids, wherein said plurality of amino acid residues replaced in the precursor enzyme correspond to position 210 in combination with one or more acid residue positions equivalent to those selected from the group consisting of: +33, +62, +67, +76, +100, +101, +103, +104, +107, +128, +129, +130, +132, +135, +156, +158, +164, +166, +167, +170, +209, +215, +217, +218 and +222, where the numbered positions correspond to naturally-occurring subtilisin from <i>Bacillus amyloliquefaciens</i> or to equivalent amino acid residues in other carbonyl hydrolases or subtilisins (such as <i>Bacillus lentus</i> subtilisin).		

PROTEASE ENZYMES FOR TOUGH CLEANING AND/OR SPOT AND FILM REDUCTION AND COMPOSITIONS INCORPORATING SAME

FIELD OF THE INVENTION

The present invention relates to protease enzymes for tough cleaning and/or spot and film reduction in various compositions and methods for their use, more particularly to protease enzymes which are carbonyl hydrolase variants.

BACKGROUND OF THE INVENTION

Various types of enzymes have long been used in laundry detergents to assist in the removal of certain stains from fabrics. Each class of enzyme (amylase, protease, etc.) generally catalyzes a different chemical reaction. For example, protease enzymes are known for their ability to hydrolyze (break down a compound into two or more simpler compounds) other proteins. This ability has been taken advantage of through the incorporation of naturally occurring or engineered protease enzymes to laundry detergent compositions.

In recent years the use of enzymes has also been investigated for use in automatic dishwashing compositions. Unfortunately, many enzymes, especially protease enzymes, do not translate well into the wash environment. Specifically, thermal stability, pH stability, oxidative stability and substrate specificity need to be optimized to ensure satisfactory performance.

To optimize the characteristics of the protease enzyme, a change in the amino acid sequence is frequently employed. A change of amino acid sequence may alter the properties of the enzyme to varying degrees depending upon the location, nature, and/or magnitude of the change in the amino acid sequence. Several attempts have been made to alter the amino acid sequence of protease enzymes in an attempt to alter their properties, with the goal of increasing the efficacy of the protease for cleaning uses such as in the wash environment.

Additionally, consumers interest in automatic dishwashing compositions which deliver tough food cleaning is increasing. Baked on dairy products and eggs have long been difficult to remove via automatic dishwashing. In addition, spotting

and filming of glassware is a common problem in autodishwashing. Moreover, consumers now desire less handwashing or pre-washing of dishes and more cleaning ability delivered via the automatic dishwasher. Accordingly, the need remains for compositions which can deliver tough cleaning and/or spot and film reduction cleaning without spot/film formation. More particularly, the need remains for automatic dishwashing compositions which can deliver tough food cleaning and reduced spot/film formation via protease enzymes designed to deliver such benefits.

BACKGROUND ART

The following documents contain information which may or may not be relevant to the present invention:

WO 95/10615 to Genencor International, Inc.; WO 89/06270 to Novo Nordisk A/S; Kirk-Othmer, Encyclopedia of Chemical Technology, 4th. Ed., Vol. 9, Wiley 1994, pages 567-620, titled "Enzyme Applications-Industrial", Nielsen et al and the references therein. WO 95/10591 and WO 95/10592 to the Procter & Gamble Company.

SUMMARY OF THE INVENTION

This need is met via the present invention whereby compositions having a protease enzyme capable of tough food cleaning and reduced spotting/filming is provided. The preferred protease enzyme is a carbonyl hydrolase variant having an amino acid sequence not found in nature. The protease is engineered to deliver tough cleaning and/or spot and film reduction and reduced spotting and filming by providing the protease with trypsin-like specificity. Thus, the protease is highly effective on dairy soils such as milk and cheese and on egg yolk soils and significantly reduces the spotting and filming such soils may cause in the automatic dishwashing process. The protease is derived by replacement of a plurality of amino acid residues of a precursor carbonyl hydrolase with different amino acids. Furthermore, the preferred protease is engineered to have a higher level of bleach stability. In addition, the enzymes of the present invention may provide improved soil removal in laundry applications as well.

According to a first embodiment of the present invention, a cleaning composition is provided. The cleaning composition comprises:

- (a) an effective amount of a protease enzyme which is a carbonyl hydrolase variant having an amino acid sequence not found in nature, which is derived by replacement of a plurality of amino acid residues of a precursor carbonyl hydrolase with different amino acids, wherein said plurality of amino acid residues replaced in

the precursor enzyme correspond to position +210 in combination with one or more of the following residues: +33, +62, +67, +76, +100, +101, +103, +104, +107, +128, +129, +130, +132, +135, +156, +158, +164, +166, +167, +170, +209, +215, +217, +218 and +222, where the numbered positions correspond to naturally-occurring subtilisin from Bacillus amyloliquefaciens or to equivalent amino acid residues in other carbonyl hydrolases or subtilisins (such as Bacillus lentus subtilisin); and

(b) one or more cleaning adjunct materials compatible with the protease enzyme.

Most preferably, the protease is derived by replacement of a plurality of amino acid residues of a precursor carbonyl hydrolase with different amino acids, wherein the plurality of amino acid residues replaced in the precursor enzyme correspond to position +210 in combination with one or more of the following residues: +76, +103, +104, +156, +166, and +217, +222, and most preferably, the protease is derived from replacing amino acid residues at positions +210, +76, +103, +104, +156, and +166.

The present invention also relates to methods for cleaning items in need of cleaning by contacting the item with a protease enzyme which is a carbonyl hydrolase variant as described herein. The invention therefore encompasses a method for cleaning fabrics comprising contacting, preferably with agitation, the fabrics with an aqueous liquor containing the protease enzyme. The method can be carried out at temperatures below about 60°C but, of course, is quite effective at laundry temperatures up to the boil. The present invention also relates to a method for cleaning dishes by contacting a dish in need of cleaning with a protease enzyme as described herein. The present invention methods also include methods for personal cleansing, the methods comprising contacting the part of the human or lower animal body in need of cleaning with a protease enzyme as described herein.

Accordingly, it is an object of the present invention to provide a cleaning composition having a protease enzyme capable of tough cleaning, and/or spot and film reduction particularly an automatic dishwashing composition having tough soil or food cleaning. It is further an object of the present invention to provide methods for fabric, dish and personal cleansing via the use of the protease enzymes of the present invention. These, and other, objects, features and advantages will be clear from the following detailed description, the attached drawings and the appended claims.

All percentages, ratios and proportions herein are on a weight basis unless otherwise indicated. All documents cited herein are hereby incorporated by reference.

BRIEF DESCRIPTION OF THE DRAWINGS

Figs. 1A and 1B depict the DNA and amino acid sequence of subtilisin from *Bacillus lentus* (Seq. ID No. 6 and 7). The mature subtilisin protein is coded by the codons beginning at the codon GCG (334-336) corresponding to Ala.

Figs. 2A and 2B depict the amino acid sequence of four subtilisins. The top line represents the amino acid sequence of subtilisin from *Bacillus amyloliquefaciens* subtilisin (also sometimes referred to as subtilisin BPN') (Seq. ID No. 2). The second line depicts the amino acid sequence of subtilisin from *Bacillus subtilis* (Seq. ID No. 3). The third line depicts the amino acid sequence of subtilisin from *B. licheniformis* (Seq. ID No. 4). The fourth line depicts the amino acid sequence of subtilisin from *Bacillus lentus* (also referred to as subtilisin 309 in PCT WO 89/06276) (Seq. ID No. 5). The symbol * denotes the absence of specific amino acid residues as compared to subtilisin BPN'.

Figs. 3A-C depict the DNA and amino acid sequence for *Bacillus amyloliquefaciens* subtilisin and a partial restriction map of this gene (Seq. ID No. 1).

Fig. 4 depicts the conserved amino acid residues among subtilisins from *Bacillus amyloliquefaciens* (BPN') and *Bacillus lentus* (wild-type).

Figs. 5A and 5B depict the DNA and amino acid sequence of a preferred embodiment of the invention (P210I/S156E/S166D/N76D/S103A/V104I) (Seq. ID No. 8 and 9). The DNA in this figure has been modified by the methods described to encode aspartate at positions 76 and 166, glutamate at position 156, alanine at position 103 and isoleucine at positions 210 and 104. The mature subtilisin variant protein is coded by the codons beginning at the codon GCG (334-336) corresponding to ala.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

Protease Enzymes - The invention includes protease enzymes which are non-naturally-occurring carbonyl hydrolase variants having a different proteolytic activity, stability, substrate specificity, pH profile and/or performance characteristic as compared to the precursor carbonyl hydrolase from which the amino acid sequence of the variant is derived. As stated earlier, the protease enzymes are designed to have trypsin-like specificity and preferably also be bleach stable. The precursor carbonyl hydrolase may be a naturally-occurring carbonyl hydrolase or recombinant hydrolase. Specifically, such carbonyl hydrolase variants have an amino acid sequence not found in nature, which is derived by replacement of a plurality of amino acid residues of a precursor carbonyl hydrolase with different amino acids. The plurality of amino acid residues of the precursor enzyme

correspond to position +210 in combination with one or more of the following residues: +33, +62, +67, +76, +100, +101, +103, +104, +107, +128, +129, +130, +132, +135, +156, +158, +164, +166, +167, +170, +209, +215, +217, +218, and +222, where the numbered position corresponds to naturally-occurring subtilisin from *Bacillus amyloliquefaciens* or to equivalent amino acid residues in other carbonyl hydrolases or subtilisins, such as *Bacillus lentus* subtilisin.

The carbonyl hydrolase variants which are protease enzymes useful in the present invention compositions comprise replacement of amino acid residue +210 in combination with one or more additional modifications. While any combination of the above listed amino acid substitutions may be employed, the preferred variant protease enzymes useful for the present invention comprise the substitution, deletion or insertion of amino acid residues in the following combinations: 210/156; 210/166; 210/76; 210/103; 210/104; 210/217; 210/156/166; 210/156/217; 210/166/217; 210/76/156; 210/76/166; 210/76/217; 210/76/156/166; 210/76/156/217; 210/76/166/217; 210/76/103/156; 210/76/103/166; 210/76/103/217; 210/76/104/156; 210/76/104/166; 210/76/104/217; 210/76/103/104/156; 210/76/103/104/166; 210/76/103/104/217; 210/76/103/104/156/166; 210/76/103/104/156/217; 210/76/103/104/166/217 and/or 210/76/103/104/156/166/217; 210/76/103/104/166/222; 210/67/76/103/104/166/222; 210/67/76/103/104/166/218/222. Most preferably the variant enzymes useful for the present invention comprise the substitution, deletion or insertion of an amino acid residue in the following combination of residues: 210/156; 210/166; 210/217; 210/156/166; 210/156/217; 210/166/217; 210/76/156/166; 210/76/103/156/166 and 210/76/103/104/156/166 of *B. lentus* subtilisin with 210/76/103/104/156/166 being the most preferred.

Variant DNA sequences encoding such carbonyl hydrolase or subtilisin variants are derived from a precursor DNA sequence which encodes a naturally-occurring or recombinant precursor enzyme. The variant DNA sequences are derived by modifying the precursor DNA sequence to encode the substitution of one or more specific amino acid residues encoded by the precursor DNA sequence corresponding to positions +210, +33, +62, +67, +76, +100, +101, +103, +104, +107, +128, +129, +130, +132, +135, +156, +158, +164, +166, +167, +170, +209, +215, +217, +218, and +222 in *Bacillus lentus* or any combination thereof. Although the amino acid residues identified for modification herein are identified according to the numbering applicable to *B. amyloliquefaciens* (which has become the conventional method for identifying residue positions in all subtilisins), the

preferred precursor DNA sequence useful for the present invention is the DNA sequence of *Bacillus lentus* as shown in Fig. 1.

These variant DNA sequences encode the insertion or substitution of the amino acid residue +210 in combination with one or more additional modification. Preferably the variant DNA sequences encode the substitution or insertion of amino acid residues in the following combinations: 210/156; 210/166; 210/76; 210/103; 210/104; 210/217; 210/156/166; 210/156/217; 210/166/217; 210/76/156; 210/76/166; 210/76/217; 210/76/156/166; 210/76/156/217; 210/76/166/217; 210/76/103/156; 210/76/103/166; 210/76/103/217; 210/76/104/156; 210/76/104/166; 210/76/104/217; 210/76/103/104/156; 210/76/103/104/166; 210/76/103/104/217; 210/76/103/104/156/166; 210/76/103/104/156/217; 210/76/103/104/166/217 and/or 210/76/103/104/156/166/217; 210/76/103/104/166/222; 210/67/76/103/104/166/222; 210/67/76/103/104/166/218/222. Most preferably the variant DNA sequences encode for the modification of the following combinations of residues: 210/156; 210/166; 210/217; 210/156/166; 210/156/217; 210/166/217; 210/76/156/166; 210/76/103/156/166 and 210/76/103/104/156/166. These recombinant DNA sequences encode carbonyl hydrolase variants having a novel amino acid sequence and, in general, at least one property which is substantially different from the same property of the enzyme encoded by the precursor carbonyl hydrolase DNA sequence. Such properties include proteolytic activity, substrate specificity, stability, altered pH profile and/or enhanced performance characteristics.

The protease enzymes useful herein encompass the substitution of any of the nineteen naturally occurring L-amino acids at the designated amino acid residue positions. Such substitutions can be made in any precursor subtilisin (procaryotic, eucaryotic, mammalian, etc.). Throughout this application reference is made to various amino acids by way of common one- and three-letter codes. Such codes are identified in Dale, M.W. (1989), Molecular Genetics of Bacteria, John Wiley & Sons, Ltd., Appendix B.

Preferably, the substitution to be made at each of the identified amino acid residue positions include but are not limited to substitutions at position +210 including I, V, L, and A, substitutions at positions +33, +62, +76, +100, +101, +103, +104, +107, +128, +129, +130, +132, +135, +156, +158, +164, +166, +167, +170, +209, +215, +217, and +218 of D or E, substitutions at position 76 including D, H, E, G, F, K, P and N; substitutions at position 103 including Q, T, D, E, Y, K, G, R and S; and substitutions at position 104 including S, Y, I, L, M, A, W, D, T, G and V; and substitutions at position 222 including S, C, A. The specifically preferred

amino acid(s) to be substituted at each such position are designated below in Table I. Although specific amino acids are shown in Table I, it should be understood that any amino acid may be substituted at the identified residues.

Table I

Amino Acid <u>Residue</u>	Preferred Amino Acid to <u>be Substituted/Inserted</u>
+210	I, V, L, A
+33, +62, +100, +101, +107 +128, +129, +130, +135 +156, +158, +164, +166 +167, +170, +209, +215 +217 and +218	D,E
+76	D,H
+103	A,Q,T,D,E,Y,K,G,R
+104	I,Y,S,L,A,T,G
+222	S, C, A

Carbonyl hydrolases are protease enzymes which hydrolyze compounds containing



bonds in which X is oxygen or nitrogen. They include naturally-occurring carbonyl hydrolases and recombinant carbonyl hydrolases. Naturally-occurring carbonyl hydrolases principally include hydrolases, e.g., peptide hydrolases such as subtilisins or metalloproteases. Peptide hydrolases include α -aminoacylpeptide hydrolase, peptidylamino acid hydrolase, acylamino hydrolase, serine carboxypeptidase, metallocarboxypeptidase, thiol proteinase, carboxylproteinase and metalloproteinase. Serine, metallo, thiol and acid protease's are included, as well as endo and exo-proteases.

"Recombinant carbonyl hydrolase" refers to a carbonyl hydrolase in which the DNA sequence encoding the naturally-occurring carbonyl hydrolase is modified to produce a mutant DNA sequence which encodes the substitution, insertion or deletion of one or more amino acids in the carbonyl hydrolase amino acid sequence. Suitable modification methods are disclosed herein, and in U.S. Patent 4,760,025, U.S. Patent 5,204,015 and U.S. Patent 5,185,258, the disclosure of which are incorporated herein by reference.

Subtilisins are bacterial or fungal carbonyl hydrolases which generally act to cleave peptide bonds of proteins or peptides. As used herein, "subtilisin" means a naturally-occurring subtilisin or a recombinant subtilisin. A series of naturally-occurring subtilisins is known to be produced and often secreted by various microbial species. Amino acid sequences of the members of this series are not entirely homologous. However, the subtilisins in this series exhibit the same or similar type of proteolytic activity. This class of serine proteases shares a common amino acid sequence defining a catalytic triad which distinguishes them from the chymotrypsin related class of serine proteases. The subtilisins and chymotrypsin related serine proteases both have a catalytic triad comprising aspartate, histidine and serine. In the subtilisin related proteases the relative order of these amino acids, reading from the amino to carboxy terminus, is aspartate-histidine-serine. In the chymotrypsin related proteases the relative order, however, is histidine-aspartate-serine. Thus, subtilisin herein refers to a serine protease having the catalytic triad of subtilisin related proteases. Examples include but are not limited to the subtilisins identified in Fig. 2 herein.

"Recombinant subtilisin" refers to a subtilisin in which the DNA sequence encoding the subtilisin is modified to produce a variant (or mutant) DNA sequence which encodes the substitution, deletion or insertion of one or more amino acids in the naturally-occurring subtilisin amino acid sequence. Suitable methods to produce such modification and which may be combined with those disclosed herein, include those disclosed in U.S. Patent 4,760,025, U.S. Patent 5,204,015 and U.S. Patent 5,185,258.

"Non-human carbonyl hydrolases" and the DNA encoding them may be obtained from many procaryotic and eucaryotic organisms. Suitable examples of procaryotic organisms include gram negative organisms such as *E. coli* or *Pseudomonas* and gram positive bacteria such as *Micrococcus* or *Bacillus*. Examples of eucaryotic organisms from which carbonyl hydrolase and their genes may be obtained include yeast such as *Saccharomyces cerevisiae*, fungi such as *Aspergillus* sp. and non-human mammalian sources such as, for example, *bovine* sp. from which the gene encoding the carbonyl hydrolase chymosin can be obtained. As with subtilisins, a series of carbonyl hydrolases can be obtained from various related species which have amino acid sequences which are not entirely homologous between the members of that series but which nevertheless exhibit the same or similar type of biological activity. Thus, non-human carbonyl hydrolase as used herein has a functional definition which refers to carbonyl hydrolases which are associated, directly or indirectly, with procaryotic and eucaryotic sources.

A "carbonyl hydrolase variant" has an amino acid sequence which is derived from the amino acid sequence of a "precursor carbonyl hydrolase." The precursor carbonyl hydrolases include naturally-occurring carbonyl hydrolases and recombinant carbonyl hydrolases. The amino acid sequence of the carbonyl hydrolase variant is "derived" from the precursor hydrolase amino acid sequence by the substitution, deletion or insertion of one or more amino acids of the precursor amino acid sequence. Such modification is of the "precursor DNA sequence" which encodes the amino acid sequence of the precursor carbonyl hydrolase rather than manipulation of the precursor carbonyl hydrolase enzyme *per se*. Suitable methods for such manipulation of the precursor DNA sequence include methods disclosed herein and in U.S. Patent 4,760,025.

Specific residues corresponding to position +210 in combination with one or more of the following positions +33, +62, +67, +76, +100, +101, +103, +104, +107, +128, +129, +130, +132, +135, +156, +158, +164, +166, +167, +170, +209, +215, +217, +218 and +222 of *Bacillus lentus* subtilisin are identified herein for mutation. Preferably the modified residues are selected from the following combinations: 210/156; 210/166; 210/76; 210/103; 210/104; 210/217; 210/156/166; 210/156/217; 210/166/217; 210/76/156; 210/76/166; 210/76/217; 210/76/156/166; 210/76/156/217; 210/76/166/217; 210/76/103/156; 210/76/103/166; 210/76/103/217; 210/76/104/156; 210/76/104/166; 210/76/104/217; 210/76/103/104/156; 210/76/103/104/166; 210/76/103/104/217; 210/76/103/104/156/166; 210/76/103/104/156/217; 210/76/103/104/166/217 and/or 210/76/103/104/156/166/217 with preferred combinations being: 210/156; 210/166; 210/217; 210/156/166; 210/156/217; 210/166/217; 210/76/156/166; 210/76/103/156/166 and 210/76/103/104/156/166. These amino acid position numbers refer to those assigned to the mature *Bacillus amyloliquefaciens* subtilisin sequence presented in Fig. 3. The protease enzymes useful in the present invention, however, are not limited to the mutation of this particular subtilisin but extends to precursor carbonyl hydrolases containing amino acid residues at positions which are "equivalent" to the particular identified residues in *Bacillus amyloliquefaciens* subtilisin. Preferably, the precursor subtilisin is *Bacillus lentus* subtilisin and the substitutions, deletions or insertions are made at the equivalent amino acid residue in *B. lentus* corresponding to those listed above.

A residue (amino acid) of a precursor carbonyl hydrolase is equivalent to a residue of *Bacillus amyloliquefaciens* subtilisin if it is either homologous (i.e., corresponding in position in either primary or tertiary structure) or analogous to a specific residue or portion of that residue in *Bacillus amyloliquefaciens* subtilisin

(i.e., having the same or similar functional capacity to combine, react, or interact chemically).

In order to establish homology to primary structure, the amino acid sequence of a precursor carbonyl hydrolase is directly compared to the *Bacillus amyloliquefaciens* subtilisin primary sequence and particularly to a set of residues known to be invariant in subtilisins for which sequence is known. Fig. 4 herein shows the conserved residues as between *amyloliquefaciens* subtilisin and *B. lentus* subtilisin. After aligning the conserved residues, allowing for necessary insertions and deletions in order to maintain alignment (i.e., avoiding the elimination of conserved residues through arbitrary deletion and insertion), the residues equivalent to particular amino acids in the primary sequence of *Bacillus amyloliquefaciens* subtilisin are defined. Alignment of conserved residues preferably should conserve 100% of such residues. However, alignment of greater than 75% or as little as 50% of conserved residues is also adequate to define equivalent residues. Conservation of the catalytic triad, Asp32/His64/Ser221 should be maintained.

For example, in Fig. 2 the amino acid sequence of subtilisin from *Bacillus amyloliquefaciens*, *Bacillus subtilis*, *Bacillus licheniformis* (*carlsbergensis*) and *Bacillus lentus* are aligned to provide the maximum amount of homology between amino acid sequences. A comparison of these sequences shows that there are a number of conserved residues contained in each sequence. These conserved residues (as between BPN' and *B. lentus*) are identified in Fig. 4.

These conserved residues, thus, may be used to define the corresponding equivalent amino acid residues of *Bacillus amyloliquefaciens* subtilisin in other carbonyl hydrolases such as subtilisin from *Bacillus lentus* (PCT Publication No. W089/06279 published July 13, 1989) and the preferred subtilisin precursor enzyme herein. These particular amino acid sequences are aligned in Figs. 2A and 2B with the sequence of *Bacillus amyloliquefaciens* subtilisin to produce the maximum homology of conserved residues. As can be seen, there are a number of deletions in the sequence of *Bacillus lentus* as compared to *Bacillus amyloliquefaciens* subtilisin. Thus, for example, the equivalent amino acid for Val165 in *Bacillus amyloliquefaciens* subtilisin in the other subtilisins is isoleucine for *B. lentus* and *B. licheniformis*.

Thus, for example, the amino acid at position +210 is proline (P) in both *B. amyloliquefaciens* and *B. lentus* subtilisins. In the preferred subtilisin variant useful in the invention, however, the amino acid equivalent to +210 in *Bacillus amyloliquefaciens* subtilisin is substituted with isoleucine (I). A comparison of the

preferred amino acid residues identified herein for substitution versus the preferred substitution for each such position is provided in Table II.

Table II

	<u>+210</u>	<u>+156</u>	<u>+166</u>	<u>+217</u>	<u>+76</u>	<u>+103</u>
<u>+104</u>						
<i>B. amyloliquefaciens</i> Y (wild-type)	P	E	G	Y	N	Q
<i>B. lentus</i> (wild-type) V	P	S	S	L	N	S
Most Preferred Substitution I/Y	I	E/D	E/D	E/D	D	A

Equivalent residues may also be defined by determining homology at the level of tertiary structure for a precursor carbonyl hydrolase whose tertiary structure has been determined by x-ray crystallography. Equivalent residues are defined as those for which the atomic coordinates of two or more of the main chain atoms of a particular amino acid residue of the precursor carbonyl hydrolase and *Bacillus amyloliquefaciens* subtilisin (N on N, CA on CA, C on C and O on O) are within 0.13nm and preferably 0.1nm after alignment. Alignment is achieved after the best model has been oriented and positioned to give the maximum overlap of atomic coordinates of non-hydrogen protein atoms of the carbonyl hydrolase in question to the *Bacillus amyloliquefaciens* subtilisin. The best model is the crystallographic model giving the lowest R factor for experimental diffraction data at the highest resolution available.

$$R\ factor = \frac{\sum_h |Fo(h)| - |Fc(h)|}{\sum_h |Fo(h)|}$$

Equivalent residues which are functionally analogous to a specific residue of *Bacillus amyloliquefaciens* subtilisin are defined as those amino acids of the precursor carbonyl hydrolases which may adopt a conformation such that they either alter, modify or contribute to protein structure, substrate binding or catalysis in a manner defined and attributed to a specific residue of the *Bacillus amyloliquefaciens* subtilisin. Further, they are those residues of the precursor carbonyl hydrolase (for

which a tertiary structure has been obtained by x-ray crystallography) which occupy an analogous position to the extent that, although the main chain atoms of the given residue may not satisfy the criteria of equivalence on the basis of occupying a homologous position, the atomic coordinates of at least two of the side chain atoms of the residue lie within 0.13nm of the corresponding side chain atoms of *Bacillus amyloliquefaciens* subtilisin. The coordinates of the three dimensional structure of *Bacillus amyloliquefaciens* subtilisin are set forth in EPO Publication No. 0 251 446 (equivalent to U.S. Patent Application SN 07/898,382, the disclosure of which is incorporated herein by reference) and can be used as outlined above to determine equivalent residues on the level of tertiary structure.

Some of the residues identified for substitution, insertion or deletion are conserved residues whereas others are not. In the case of residues which are not conserved, the replacement of one or more amino acids is limited to substitutions which produce a variant which has an amino acid sequence that does not correspond to one found in nature. In the case of conserved residues, such replacements should not result in a naturally-occurring sequence. The carbonyl hydrolase variants useful in the present invention include the mature forms of carbonyl hydrolase variants, as well as the pro- and prepro-forms of such hydrolase variants. The prepro-forms are the preferred construction since this facilitates the expression, secretion and maturation of the carbonyl hydrolase variants.

Methods and procedures for making the enzymes according to the present invention are known and are disclosed in PCT Publication No. WO 95/10615 the disclosure of which is herein incorporated by reference.

The enzymes of the present invention have trypsin-like specificity. That is, the enzymes of the present invention hydrolyze proteins by preferentially cleaving the peptide bonds of charged amino acid residues, more specifically residues such as arginine and lysine, rather than preferentially cleaving the peptide bonds of hydrophobic amino acid residues, more specifically phenylalanine, tryptophan and tyrosine. Enzymes having the latter profile have a chymotrypsin-like specificity. Substrate specificity as discussed above is illustrated by the action of the enzyme on two synthetic substrates. Protease's having trypsin-like specificity hydrolyze the synthetic substrate bVGR-pNA preferentially over the synthetic substrate sucAAPF-pNA. Chymotrypsin-like protease enzymes, in contrast, hydrolyze the latter much faster than the former. For the purposes of the present invention the following procedure was employed to define the trypsin-like specificity of the protease enzymes of the present invention:

A fixed amount of a glycine buffer at a pH of 10 and a temperature of 25 °C is added to a standard 10 ml test tube. 0.5 ppm of the active enzyme to be tested is added to the test tube. Approximately, 1.25 mg of the synthetic substrate per mL of buffer solution is added to the test tube. The mixture is allowed to incubate for 15 minutes at 25 °C. Upon completion of the incubation period, an enzyme inhibitor, PMSF, is added to the mixture at a level of 0.5 mg per mL of buffer solution. The absorbency or OD value of the mixture is read at a 410 nm wavelength. The absorbence then indicates the activity of the enzyme on the synthetic substrate. The greater the absorbence, the higher the level of activity against that substrate.

To then determine the specificity of an individual enzyme, the absorbence on the two synthetic substrate proteins may be converted into a specificity ratio. For the purposes of the present invention, the ratio is determined by the formula specificity of:

$$[\text{activity on sAAPF-pNA}]/[\text{activity on bVGR-pNA}]$$

An enzyme having a ratio of less than about 10, more preferably less than about 5 and most preferably less than about 2.5 may then be considered to demonstrate trypsin-like activity.

Cleaning Adjunct Materials - The cleaning compositions of the present invention also comprise, in addition to the protease enzyme described hereinbefore, one or more cleaning adjunct materials compatible with the protease enzyme. The term "cleaning adjunct materials", as used herein, means any liquid, solid or gaseous material selected for the particular type of cleaning composition desired and the form of the product (e.g., liquid; granule; spray composition), which materials are also compatible with the protease enzyme used in the composition. The specific selection of cleaning adjunct materials are readily made by considering the surface, item or fabric to be cleaned, and the desired form of the composition for the cleaning conditions during use (e.g., through the wash detergent use). The term "compatible", as used herein, means the cleaning composition materials do not reduce the proteolytic activity of the protease enzyme to such an extent that the protease is not effective as desired during normal use situations. Specific cleaning composition materials are exemplified in detail hereinafter.

An effective amount of one or more protease enzymes described above are included in compositions useful for cleaning a variety of surfaces in need of proteinaceous stain removal. Such cleaning compositions include detergent compositions for cleaning hard surfaces, unlimited in form (e.g., liquid and

granular); detergent compositions for cleaning fabrics, unlimited in form (e.g., granular, liquid and bar formulations); dishwashing compositions (unlimited in form and including both granular and liquid automatic dishwashing); oral cleaning compositions, unlimited in form (e.g., dentifrice, toothpaste and mouthwash formulations); and denture cleaning compositions, unlimited in form (e.g., liquid, tablet). As used herein, "effective amount of protease enzyme" refers to the quantity of protease enzyme described hereinbefore necessary to achieve the enzymatic activity necessary in the specific cleaning composition. Such effective amounts are readily ascertained by one of ordinary skill in the art and is based on many factors, such as the particular enzyme variant used, the cleaning application, the specific composition of the cleaning composition, and whether a liquid or dry (e.g., granular, bar) composition is required, and the like.

Preferably the cleaning compositions of the present invention comprise from about 0.0001% to about 10% of one or more protease enzymes, more preferably from about 0.001% to about 1%, more preferably still from about 0.001% to about 0.1%. Also preferably the protease enzyme is present in the compositions in an amount sufficient to provide a ratio of mg of active protease per 100 grams of composition to ppm theoretical Available O₂ ("AvO₂") from any peroxyacid in the wash liquor, referred to herein as the Enzyme to Bleach ratio (E/B ratio), ranging from about 1:1 to about 20:1. Several examples of various cleaning compositions wherein the protease enzymes may be employed are discussed in further detail below. Also, the compositions of the present invention may include from about 1% to about 99.9% by weight of the composition of the adjunct materials.

Optional Detergent Enzymes - The detergent compositions herein may also optionally contain one or more types of detergent enzymes. Such enzymes can include proteases, amylases, cellulases and lipases. Such materials are known in the art and are commercially available under such trademarks as . They may be incorporated into the non-aqueous liquid detergent compositions herein in the form of suspensions, "marumes" or "prills". Another suitable type of enzyme comprises those in the form of slurries of enzymes in nonionic surfactants, e.g., the enzymes marketed by Novo Nordisk under the tradename "SL" or the microencapsulated enzymes marketed by Novo Nordisk under the tradename "LDP." Suitable enzymes and levels of use are described in U.S. Pat. No. 5,576,282, 5,705,464 and 5,710,115.

Enzymes added to the compositions herein in the form of conventional enzyme prills are especially preferred for use herein. Such prills will generally range in size from about 100 to 1,000 microns, more preferably from about 200 to 800 microns and will be suspended throughout the non-aqueous liquid phase of the

composition. Prills in the compositions of the present invention have been found, in comparison with other enzyme forms, to exhibit especially desirable enzyme stability in terms of retention of enzymatic activity over time. Thus, compositions which utilize enzyme prills need not contain conventional enzyme stabilizing such as must frequently be used when enzymes are incorporated into aqueous liquid detergents.

"Detergent enzyme", as used herein, means any enzyme having a cleaning, stain removing or otherwise beneficial effect in a laundry, hard surface cleaning or personal care detergent composition. Preferred detergent enzymes are hydrolases such as proteases, amylases and lipases. Preferred enzymes for laundry purposes include, but are not limited to, proteases, cellulases, lipases and peroxidases. Highly preferred for automatic dishwashing are amylases and/or proteases, including both current commercially available types and improved types which, though more and more bleach compatible through successive improvements, have a remaining degree of bleach deactivation susceptibility.

Examples of suitable enzymes include, but are not limited to, hemicellulases, peroxidases, proteases, cellulases, xylanases, lipases, phospholipases, esterases, cutinases, pectinases, keratanases, reductases, oxidases, phenoloxidases, lipooxygenases, ligninases, pullulanases, tannases, pentosanases, malanases, β -glucanases, arabinosidases, hyaluronidase, chondroitinase, laccase, and known amylases, or mixtures thereof.

Examples of such suitable enzymes are disclosed in U.S. Patent Nos. 5,705,464, 5,710,115, 5,576,282, 5,728,671 and 5,707,950

Particularly useful proteases are described in PCT publications: WO 95/30010 published November 9, 1995 by The Procter & Gamble Company; WO 95/30011 published November 9, 1995 by The Procter & Gamble Company; and WO 95/29979 published November 9, 1995 by The Procter & Gamble Company. Suitable proteases are commercially available as ESPERASE[®], ALCALASE[®], DURAZYM[®] and SAVINASE[®] all from Novo Nordisk A/S of Denmark, and as MAXATASE[®], MAXACAL[®], PROPERASE[®] and MAXAPEM[®] all from Gist-Brocades of The Netherlands.

In addition to the peroxidase enzymes disclosed in U.S. Patent Nos. 5,705,464, 5,710,115, 5,576,282, 5,728,671 and 5,707,950, other suitable peroxidase enzymes are disclosed in European Patent application EP No. 96870013.8, filed February 20, 1996. Also suitable is the laccase enzyme.

Preferred enhancers are substituted phenothiazine and phenoxazine 10-Phenothiazinepropionic acid (PPT), 10-ethylphenothiazine-4-carboxylic acid (EPC),

10-phenoxazinepropionic acid (POP) and 10-methylphenoxazine (described in WO 94/12621) and substituted syringates (C3-C5 substituted alkyl syringates) and phenols. Sodium percarbonate or perborate are preferred sources of hydrogen peroxide.

Said peroxidases are normally incorporated in the detergent composition at levels from 0.0001% to 2% of active enzyme by weight of the detergent composition.

Other preferred enzymes that can be included in the detergent compositions of the present invention include lipases. Suitable lipase enzymes for detergent usage include those produced by microorganisms of the *Pseudomonas* group, such as *Pseudomonas stutzeri* ATCC 19.154, as disclosed in British Patent 1,372,034. Suitable lipases include those which show a positive immunological cross-reaction with the antibody of the lipase, produced by the microorganism *Pseudomonas fluorescent* IAM 1057. This lipase is available from Amano Pharmaceutical Co. Ltd., Nagoya, Japan, under the trade name Lipase P "Amano," hereinafter referred to as "Amano-P". Other suitable commercial lipases include Amano-CES, lipases ex *Chromobacter viscosum*, e.g. *Chromobacter viscosum* var. *lipolyticum* NRRLB 3673 from Toyo Jozo Co., Tagata, Japan; *Chromobacter viscosum* lipases from U.S. Biochemical Corp., U.S.A. and Disoynth Co., The Netherlands, and lipases ex *Pseudomonas gladioli*. Especially suitable lipases are lipases such as M1 LIPASE[®] and LIPOMAX[®] (Gist-Brocades) and LIPOLASE[®] and LIPOLASE ULTRA[®] (Novo) which have found to be very effective when used in combination with the compositions of the present invention.

Also suitable are cutinases [EC 3.1.1.50] which can be considered as a special kind of lipase, namely lipases which do not require interfacial activation. Addition of cutinases to detergent compositions have been described in e.g. WO 88/09367 (Genencor).

In addition to the above referenced lipases, phospholipases may be incorporated into the detergent compositions of the present invention. Nonlimiting examples of suitable phospholipases included: EC 3.1.1.32 Phospholipase A1; EC 3.1.1.4 Phospholipase A2; EC 3.1.1.5 Lysopholipase; EC 3.1.4.3 Phospholipase C; EC 3.1.4.4. Phospholipase D. Commercially available phospholipases include LECITASE[®] from Novo Nordisk A/S of Denmark and Phospholipase A2 from Sigma. When phospholipases are included in the compositions of the present invention, it is preferred that amylases are also included. Without desiring to be bound by theory, it is believed that the combined action of the phospholipase and amylase provide substantive stain removal, especially on greasy/oily, starchy and

highly colored stains and soils. Preferably, the phospholipase and amylase, when present, are incorporated into the compositions of the present invention at a pure enzyme weight ratio between 4500:1 and 1:5, more preferably between 50:1 and 1:1.

Known amylases (α and/or β) can be included for removal of carbohydrate-based stains. WO 94/02597, Novo Nordisk A/S published February 03, 1994, describes cleaning compositions which incorporate mutant amylases. See also WO94/18314, Genencor, published August 18, 1994 and WO95/10603, Novo Nordisk A/S, published April 20, 1995. Other amylases known for use in detergent compositions include both α - and β -amylases. α -Amylases are known in the art and include those disclosed in US Pat. 5,003,257; EP 252,666; WO 91/00353; FR 2,676,456; EP 285,123; EP 525,610; EP 368,341; and British Patent Specification No. 1,296,839 (Novo). Other suitable amylase are stability-enhanced amylases including PURAFAC[®] OX AM[®] described in WO 94/18314, published August 18, 1994 and WO96/05295, Genencor, published February 22, 1996 and amylase variants from Novo Nordisk A/S, disclosed in WO 95/10603, published April 95.

Examples of commercial α -amylases products are TERMAMYL[®], BAN[®], FUNGAMYL[®] and DURAMYL[®], all available from Novo Nordisk A/S Denmark. WO95/26397 describes other suitable amylases : α -amylases characterized by having a specific activity at least 25% higher than the specific activity of TERMAMYL[®] at a temperature range of 25°C to 55°C and at a pH value in the range of 8 to 10, measured by the Phadebas[®] α -amylase activity assay. Other amylolytic enzymes with improved properties with respect to the activity level and the combination of thermostability and a higher activity level are described in WO95/35382.

The above-mentioned enzymes may be of any suitable origin, such as vegetable, animal, bacterial, fungal and yeast origin. Purified or non-purified forms of these enzymes may be used. Also included by definition, are mutants of native enzymes. Mutants can be obtained e.g. by protein and/or genetic engineering, chemical and/or physical modifications of native enzymes. Common practice as well is the expression of the enzyme via host organisms in which the genetic material responsible for the production of the enzyme has been cloned.

Said enzymes are normally incorporated in the detergent composition at levels from 0.0001% to 2% of active enzyme by weight of the detergent composition. The enzymes can be added as separate single ingredients (prills, granulates, stabilized liquids, etc. containing one enzyme) or as mixtures of two or more enzymes (e.g. cogramulates).

Other suitable detergent ingredients that can be added are enzyme oxidation scavengers. Examples of such enzyme oxidation scavengers are ethoxylated tetraethylene polyamines.

A range of enzyme materials and means for their incorporation into synthetic detergent compositions is also disclosed in WO 9307263 and WO 9307260 to Genencor International, WO 8908694 to Novo, and U.S. 3,553,139, January 5, 1971 to McCarty et al. Enzymes are further disclosed in U.S. 4,101,457, Place et al, July 18, 1978, and in U.S. 4,507,219, Hughes, March 26, 1985. Enzyme materials useful for liquid detergent formulations, and their incorporation into such formulations, are disclosed in U.S. 4,261,868, Hora et al, April 14, 1981.

Enzyme Stabilizers - Enzymes for use in detergents can be stabilized by various techniques. Enzyme stabilization techniques are disclosed and exemplified in U.S. 3,600,319, August 17, 1971, Gedge et al, EP 199,405 and EP 200,586, October 29, 1986, Venegas. Enzyme stabilization systems are also described, for example, in U.S. 3,519,570. A useful *Bacillus*, sp. AC13 giving proteases, xylanases and cellulases, is described in WO 9401532 to Novo. The enzymes employed herein can be stabilized by the presence of water-soluble sources of calcium and/or magnesium ions in the finished compositions which provide such ions to the enzymes. Suitable enzyme stabilizers and levels of use are described in U.S. Pat. Nos. 5,705,464, 5,710,115 and 5,576,282.

Chelating Agents - The detergent compositions herein may also optionally contain a chelating agent which serves to chelate metal ions, e.g., iron and/or manganese, within the non-aqueous detergent compositions herein. Such chelating agents thus serve to form complexes with metal impurities in the composition which would otherwise tend to deactivate composition components such as the peroxygen bleaching agent. Useful chelating agents can include amino carboxylates, phosphonates, amino phosphonates, polyfunctionally-substituted aromatic chelating agents and mixtures thereof. Further examples of suitable chelating agents and levels of use are described in U.S. Pat. Nos. 5,705,464, 5,710,115 and 5,576,282.

Organic Builders - The compositions herein also optionally, but preferably, contain up to about 50%, more preferably from about 1% to about 40%, even more preferably from about 5% to about 30%, by weight of a detergent builder material. Lower or higher levels of builder, however, are not meant to be excluded. Detergent builders can optionally be included in the compositions herein to assist in controlling mineral hardness. Inorganic as well as organic builders can be used. Builders are typically used in fabric laundering compositions to assist in the removal of particulate soils. Suitable detergent builders are described in U.S. Patent Nos.

5,705,464, 5,710,115, 5,576,282, 4,321,165 and 4,284,532. Preferred builders for use in liquid detergents herein are described in U.S. Patent Nos. 5,705,464, 5,710,115, 5,576,282 and 4,284,532.

Inorganic Builders - The detergent compositions herein may also optionally contain one or more types of inorganic detergent builders beyond those listed hereinbefore that also function as alkalinity sources. Such optional inorganic builders can include, for example, aluminosilicates such as zeolites. Aluminosilicate zeolites, and their use as detergent builders are more fully discussed in Corkill et al., U.S. Patent No. 4,605,509; Issued August 12, 1986, the disclosure of which is incorporated herein by reference. Also crystalline layered silicates, such as those discussed in this '509 U.S. patent, are also suitable for use in the detergent compositions herein. If utilized, optional inorganic detergent builders can comprise from about 2% to 15% by weight of the compositions herein. Additional examples of inorganic builders are described in U.S. Patent Nos. 5,705,464 and 5,710,115.

Surfactants - Detergent surfactants included in the fully-formulated detergent compositions afforded by the present invention comprises at least 0.01%, preferably from about 0.5% to about 50%, by weight of detergent composition depending upon the particular surfactants used and the desired effects. In a highly preferred embodiment, the detergent surfactant comprises from about 0.5% to about 20% by weight of the composition.

The detergent surfactant can be nonionic, anionic, ampholytic, zwitterionic, or cationic nonlimiting examples of which are disclosed in U.S. Patent Nos. 5,707,950 and 5,576,282. Mixtures of these surfactants can also be used. Preferred detergent compositions comprise anionic detergent surfactants or mixtures of anionic surfactants with other surfactants, especially nonionic surfactants.

Nonlimiting examples of surfactants useful herein include the conventional C₁₁-C₁₈ alkyl benzene sulfonates and primary, secondary and random alkyl sulfates, the C₁₀-C₁₈ alkyl alkoxy sulfates, the C₁₀-C₁₈ alkyl polyglycosides and their corresponding sulfated polyglycosides, C₁₂-C₁₈ alpha-sulfonated fatty acid esters, C₁₂-C₁₈ alkyl and alkyl phenol alkoxylates (especially ethoxylates and mixed ethoxy/propoxy), C₁₂-C₁₈ betaines and sulfobetaines ("sultaines"), C₁₀-C₁₈ amine oxides, and the like. Other conventional useful surfactants are listed in standard texts.

Particularly preferred surfactants in the preferred automatic dishwashing compositions (ADD) of the present invention are low foaming nonionic surfactants (LFNI) which are described in U.S. Patent Nos. 5,705,464 and 5,710,115. LFNI may be present in amounts from 0.01% to about 10% by weight, preferably from

about 0.1% to about 10%, and most preferably from about 0.25% to about 4%. LFNI's are most typically used in ADDs on account of the improved water-sheeting action (especially from glass) which they confer to the ADD product. They also encompass non-silicone, nonphosphate polymeric materials further illustrated hereinafter which are known to defoam food soils encountered in automatic dishwashing.

Preferred LFNI's include nonionic alkoxyated surfactants, especially ethoxylates derived from primary alcohols, and blends thereof with more sophisticated surfactants, such as the polyoxypropylene/polyoxyethylene/polyoxypropylene (PO/EO/PO) reverse block polymers as described in U.S. Patent Nos. 5,705,464 and 5,710,115.

Highly preferred ADDs herein wherein the LFNI is present make use of ethoxylated monohydroxy alcohol or alkyl phenol and additionally comprise a polyoxyethylene, polyoxypropylene block polymeric compound as described in U.S. Patent Nos. 5,705,464 and 5,710,115.

LFNI's which may also be used include those POLY-TERGENT® SLF-18 nonionic surfactants from Olin Corp., and any biodegradable LFNI having the melting point properties discussed hereinabove.

These and other nonionic surfactants are well known in the art, being described in more detail in Kirk Othmer's Encyclopedia of Chemical Technology, 3rd Ed., Vol. 22, pp. 360-379, "Surfactants and Detergent Systems", incorporated by reference herein.

Preferred are ADD compositions comprising mixed surfactants wherein the sudsing (absent any silicone suds controlling agent) is less than 2 inches, preferably less than 1 inch, as determined by the disclosure below.

The equipment useful for these measurements are: a Whirlpool Dishwasher (model 900) equipped with clear plexiglass door, IBM computer data collection with Labview and Excel Software, proximity sensor (Newark Corp. - model 95F5203) using SCXI interface, and a plastic ruler.

The data is collected as follows. The proximity sensor is affixed to the bottom dishwasher rack on a metal bracket. The sensor faces downward toward the rotating dishwasher arm on the bottom of the machine (distance approximately 2 cm. from the rotating arm). Each pass of the rotating arm is measured by the proximity sensor and recorded. The pulses recorded by the computer are converted to rotations per minute (RPM) of the bottom arm by counting pulses over a 30 second interval. The rate of the arm rotation is directly proportional to the amount of suds in the

machine and in the dishwasher pump (i.e., the more suds produced, the slower the arm rotation).

The plastic ruler is clipped to the bottom rack of the dishwasher and extends to the floor of the machine. At the end of the wash cycle, the height of the suds is measured using the plastic ruler (viewed through the clear door) and recorded as suds height.

The following procedure is followed for evaluating ADD compositions for suds production as well as for evaluating nonionic surfactants for utility. (For separate evaluation of nonionic surfactant, a base ADD formula, such as Cascade powder, is used along with the nonionic surfactants which are added separately in glass vials to the dishwashing machine.)

First, the machine is filled with water (adjust water for appropriate temperature and hardness) and proceed through a rinse cycle. The RPM is monitored throughout the cycle (approximately 2 min.) without any ADD product (or surfactants) being added (a quality control check to ensure the machine is functioning properly). As the machine begins to fill for the wash cycle, the water is again adjusted for temperature and hardness, and then the ADD product is added to the bottom of the machine (in the case of separately evaluated surfactants, the ADD base formula is first added to the bottom of the machine then the surfactants are added by placing the surfactant-containing glass vials inverted on the top rack of the machine). The RPM is then monitored throughout the wash cycle. At the end of the wash cycle, the suds height is recorded using the plastic ruler. The machine is again filled with water (adjust water for appropriate temperature and hardness) and runs through another rinse cycle. The RPM is monitored throughout this cycle.

An average RPM is calculated for the 1st rinse, main wash, and final rinse. The %RPM efficiency is then calculated by dividing the average RPM for the test surfactants into the average RPM for the control system (base ADD formulation without the nonionic surfactant). The RPM efficiency and suds height measurements are used to dimension the overall suds profile of the surfactant.

Bleaching Agents - Hydrogen peroxide sources are described in detail in the herein incorporated Kirk Othmer's Encyclopedia of Chemical Technology, 4th Ed (1992, John Wiley & Sons), Vol. 4, pp. 271-300 "Bleaching Agents (Survey)", and include the various forms of sodium perborate and sodium percarbonate, including various coated and modified forms. An "effective amount" of a source of hydrogen peroxide is any amount capable of measurably improving stain removal (especially of tea stains) from soiled dishware compared to a hydrogen peroxide source-free

composition when the soiled dishware is washed by the consumer in a domestic automatic dishwasher in the presence of alkali.

More generally a source of hydrogen peroxide herein is any convenient compound or mixture which under consumer use conditions provides an effective amount of hydrogen peroxide. Levels may vary widely and are usually in the range from about 0.1% to about 70%, more typically from about 0.5% to about 30%, by weight of the ADD compositions herein.

The preferred source of hydrogen peroxide used herein can be any convenient source, including hydrogen peroxide itself. For example, perborate, e.g., sodium perborate (any hydrate but preferably the mono- or tetra-hydrate), sodium carbonate peroxyhydrate or equivalent percarbonate salts, sodium pyrophosphate peroxyhydrate, urea peroxyhydrate, or sodium peroxide can be used herein. Also useful are sources of available oxygen such as persulfate bleach (e.g., OXONE, manufactured by DuPont). Sodium perborate monohydrate and sodium percarbonate are particularly preferred. Mixtures of any convenient hydrogen peroxide sources can also be used.

A preferred percarbonate bleach comprises dry particles having an average particle size in the range from about 500 micrometers to about 1,000 micrometers, not more than about 10% by weight of said particles being smaller than about 200 micrometers and not more than about 10% by weight of said particles being larger than about 1,250 micrometers. Optionally, the percarbonate can be coated with a silicate, borate or water-soluble surfactants. Percarbonate is available from various commercial sources such as FMC, Solvay and Tokai Denka.

While not preferred for ADD compositions of the present invention which comprise deterative enzymes, the present invention compositions may also comprise as the bleaching agent a chlorine-type bleaching material. Such agents are well known in the art, and include for example sodium dichloroisocyanurate ("NaDCC").

While effective ADD compositions herein may comprise only the nonionic surfactant and builder, fully-formulated ADD compositions typically will also comprise other automatic dishwashing detergent adjunct materials to improve or modify performance. These materials are selected as appropriate for the properties required of an automatic dishwashing composition. For example, low spotting and filming is desired -- preferred compositions have spotting and filming grades of 3 or less, preferably less than 2, and most preferably less than 1, as measured by the standard test of The American Society for Testing and Materials ("ASTM") D3556-85 (Reapproved 1989) "Standard Test Method for Deposition on Glassware During Mechanical Dishwashing".

(a) Bleach Activators - Preferably, the peroxygen bleach component in the composition is formulated with an activator (peracid precursor). The activator is present at levels of from about 0.01% to about 15%, preferably from about 0.5% to about 10%, more preferably from about 1% to about 8%, by weight of the composition. Preferred activators are selected from the group consisting of tetraacetyl ethylene diamine (TAED), benzoylcaprolactam (BzCL), 4-nitrobenzoylcaprolactam, 3-chlorobenzoylcaprolactam, benzoyloxybenzenesulphonate (BOBS), nonanoyloxybenzenesulphonate (NOBS), phenyl benzoate (PhBz), decanoyloxybenzenesulphonate (C₁₀-OBS), benzoylvalerolactam (BZVL), octanoyloxybenzenesulphonate (C₈-OBS), perhydrolyzable esters and mixtures thereof, most preferably benzoylcaprolactam and benzoylvalerolactam. Particularly preferred bleach activators in the pH range from about 8 to about 9.5 are those selected having an OBS or VL leaving group.

Preferred bleach activators are those described in U.S. Patent Nos. 5,698,504, 5,695,679, 5,686,014, 5,130,045 and 4,412,934, and copending patent applications U. S. Serial Nos. 08/064,624, 08/064,623, 08/064,621, 08/064,562, 08/064,564, 08/082,270 and copending application to M. Burns, A. D. Willey, R. T. Hartshorn, C. K. Ghosh, entitled "Bleaching Compounds Comprising Peroxyacid Activators Used With Enzymes" and having U.S. Serial No. 08/133,691 (P&G Case 4890R), all of which are incorporated herein by reference.

The mole ratio of peroxygen bleaching compound (as AvO) to bleach activator in the present invention generally ranges from at least 1:1, preferably from about 20:1 to about 1:1, more preferably from about 10:1 to about 3:1.

Quaternary substituted bleach activators may also be included. The present detergent compositions preferably comprise a quaternary substituted bleach activator (QSBA) or a quaternary substituted peracid (QSP); more preferably, the former. Preferred QSBA structures are further described in copending U.S. Serial No. 08/298,903, 08/298,650, 08/298,906 and 08/298,904 filed August 31, 1994, now respectively U.S. Patent Nos. 5,686,015, 5,460,747, 5,584,888 and 5,578,136, incorporated herein by reference.

Highly preferred bleach activators useful herein are amide-substituted as described in U.S. Patent Nos. 5,698,504, 5,695,679 and 5,686,014. Preferred examples of such bleach activators include: (6-octanamidocaproyl)oxybenzenesulfonate, (6-nonanamidocaproyl)oxybenzenesulfonate, (6-decanamidocaproyl)oxybenzenesulfonate and mixtures thereof.

Other useful activators, disclosed in U.S. Patent Nos. 5,698,504, 5,695,679, 5,686,014 and 4,966,723, include benzoxazin-type activators, such as a C₆H₄ ring to which is fused in the 1,2-positions a moiety --C(O)OC(R¹)=N-.

Depending on the activator and precise application, good bleaching results can be obtained from bleaching systems having with in-use pH of from about 6 to about 13, preferably from about 9.0 to about 10.5. Typically, for example, activators with electron-withdrawing moieties are used for near-neutral or sub-neutral pH ranges. Alkalis and buffering agents can be used to secure such pH.

Acyl lactam activators, as described in U.S. Patent Nos. 5,698,504, 5,695,679 and 5,686,014, are very useful herein, especially the acyl caprolactams (see for example WO 94-28102 A) and acyl valerolactams (see U.S. Patent No. 5,503,639).

(b) Organic Peroxides, especially Diacyl Peroxides - These are extensively illustrated in Kirk Othmer, Encyclopedia of Chemical Technology, Vol. 17, John Wiley and Sons, 1982 at pages 27-90 and especially at pages 63-72, all incorporated herein by reference. If a diacyl peroxide is used, it will preferably be one which exerts minimal adverse impact on spotting/filming.

(c) Metal-containing Bleach Catalysts - The present invention compositions and methods utilize metal-containing bleach catalysts that are effective for use in ADD compositions. Preferred are manganese and cobalt-containing bleach catalysts.

One type of metal-containing bleach catalyst is a catalyst system comprising a transition metal cation of defined bleach catalytic activity, such as copper, iron, titanium, ruthenium tungsten, molybdenum, or manganese cations, an auxiliary metal cation having little or no bleach catalytic activity, such as zinc or aluminum cations, and a sequester having defined stability constants for the catalytic and auxiliary metal cations, particularly ethylenediaminetetraacetic acid, ethylenediaminetetra (methylenephosphonic acid) and water-soluble salts thereof. Such catalysts are disclosed in U.S. Pat. 4,430,243.

If desired, the compositions herein can be catalyzed by means of a manganese compound. Such compounds and levels of use are well known in the art and include, for example, the manganese-based catalysts disclosed in U.S. Pat. No. 5,576,282, U.S. Pat. 5,246,621, U.S. Pat. 5,244,594; U.S. Pat. 5,194,416; U.S. Pat. 5,114,606; and European Pat. App. Pub. Nos. 549,271A1, 549,272A1, 544,440A2, and 544,490A1; Preferred examples of these catalysts include Mn^{IV}₂(u-O)₃(1,4,7-trimethyl-1,4,7-triazacyclononane)₂(PF₆)₂, Mn^{III}₂(u-O)₁(u-OAc)₂(1,4,7-trimethyl-1,4,7-triazacyclononane)₂(ClO₄)₂, Mn^{IV}₄(u-O)₆(1,4,7-

triazacyclononane)₄(ClO₄)₄, Mn^{III}Mn^{IV}₄(u-O)₁(u-OAc)₂-(1,4,7-trimethyl-1,4,7-triazacyclononane)₂(ClO₄)₃, Mn^{IV}(1,4,7-trimethyl-1,4,7-triazacyclononane)-(OCH₃)₃(PF₆), and mixtures thereof. Other metal-based bleach catalysts include those disclosed in U.S. Pat. 4,430,243 and U.S. Pat. 5,114,611. The use of manganese with various complex ligands to enhance bleaching is also reported in the following United States Patents: 4,728,455; 5,284,944; 5,246,612; 5,256,779; 5,280,117; 5,274,147; 5,153,161; and 5,227,084.

Cobalt bleach catalysts useful herein are known, and are described, for example, in U.S. Patent Nos. 5,597,936, 5,595,967, 5,703,030 and M. L. Tobe, "Base Hydrolysis of Transition-Metal Complexes", Adv. Inorg. Bioinorg. Mech., (1983), 2, pages 1-94. The most preferred cobalt catalyst useful herein are cobalt pentaamine acetate salts having the formula [Co(NH₃)₅OAc] T_y, wherein "OAc" represents an acetate moiety and "T_y" is an anion, and especially cobalt pentaamine acetate chloride, [Co(NH₃)₅OAc]Cl₂; as well as [Co(NH₃)₅OAc](OAc)₂; [Co(NH₃)₅OAc](PF₆)₂; [Co(NH₃)₅OAc](SO₄); [Co(NH₃)₅OAc](BF₄)₂; and [Co(NH₃)₅OAc](NO₃)₂ (herein "PAC").

These cobalt catalysts are readily prepared by known procedures, such as taught for example in U.S. Patent Nos. 5,597,936, 5,595,967, 5,703,030, in the Tobe article and the references cited therein, and in U.S. Patent 4,810,410, to Diakun et al, issued March 7, 1989, J. Chem. Ed. (1989), 66 (12), 1043-45; The Synthesis and Characterization of Inorganic Compounds, W.L. Jolly (Prentice-Hall; 1970), pp. 461-3; Inorg. Chem., 18, 1497-1502 (1979); Inorg. Chem., 21, 2881-2885 (1982); Inorg. Chem., 18, 2023-2025 (1979); Inorg. Synthesis, 173-176 (1960); and Journal of Physical Chemistry, 56, 22-25 (1952).

The bleach catalysts useful in automatic dishwashing compositions and concentrated powder detergent compositions may also be selected as appropriate for the present invention. For examples of suitable bleach catalysts see U.S. Pat. 4,246,612 and U.S. Pat. 5,227,084.

As a practical matter, and not by way of limitation, the compositions and cleaning processes herein can be adjusted to provide on the order of at least one part per hundred million of the active bleach catalyst species in the aqueous washing medium, and will preferably provide from about 0.01 ppm to about 25 ppm, more preferably from about 0.05 ppm to about 10 ppm, and most preferably from about 0.1 ppm to about 5 ppm, of the bleach catalyst species in the wash liquor. In order to obtain such levels in the wash liquor of an automatic washing process, typical compositions herein will comprise from about 0.0005% to about 0.2%, more

preferably from about 0.004% to about 0.08%, of bleach catalyst, especially manganese or cobalt catalysts, by weight of the cleaning compositions.

pH and Buffering Variation - Many detergent compositions herein will be buffered, i.e., they are relatively resistant to pH drop in the presence of acidic soils. However, other compositions herein may have exceptionally low buffering capacity, or may be substantially unbuffered. Techniques for controlling or varying pH at recommended usage levels more generally include the use of not only buffers, but also additional alkalis, acids, pH-jump systems, dual compartment containers, etc., and are well known to those skilled in the art.

The preferred ADD compositions herein comprise a pH-adjusting component selected from water-soluble alkaline inorganic salts and water-soluble organic or inorganic builders as described in U.S. Patent Nos. 5,705,464 and 5,710,115.

Water-Soluble Silicates - The present automatic dishwashing detergent compositions may further comprise water-soluble silicates as described in U.S. Patent Nos. 5,705,464 and 5,710,115.

Material Care Agents - The preferred ADD compositions may contain one or more material care agents which are effective as corrosion inhibitors and/or anti-tarnish aids as described in U.S. Patent Nos. 5,705,464, 5,710,115 and 5,646,101. When present, such protecting materials are preferably incorporated at low levels, e.g., from about 0.01% to about 5% of the ADD composition.

Other Materials - Detergent ingredients or adjuncts optionally included in the instant compositions can include one or more materials for assisting or enhancing cleaning performance, treatment of the substrate to be cleaned, or designed to improve the aesthetics of the compositions. Adjuncts which can also be included in compositions of the present invention, at their conventional art-established levels for use (generally, adjunct materials comprise, in total, from about 30% to about 99.9%, preferably from about 70% to about 95%, by weight of the compositions), include other active ingredients such as non-phosphate builders, chelants, enzymes, suds suppressors, dispersant polymers (e.g., from BASF Corp. or Rohm & Haas), color speckles, silvercare, anti-tarnish and/or anti-corrosion agents, dyes, fillers, germicides, alkalinity sources, hydrotropes, anti-oxidants, enzyme stabilizing agents, perfumes, solubilizing agents, carriers, processing aids, pigments, and pH control agents as described in U.S. Patent Nos. 5,705,464, 5,710,115, 5,698,504, 5,695,679, 5,686,014 and 5,646,101.

The following nonlimiting examples further illustrate the ADD compositions of the present invention.

EXAMPLE 1

<u>Ingredients:</u>	<u>Weight%</u>	
	<u>A</u>	<u>B</u>
Sodium Tripolyphosphate (STPP)	24.0	45
Sodium carbonate	20.0	13.5
Hydrated 2.0r silicate	15	13.5
nonionic surfactants	2.0	2.0
Polymer ¹	4.0	--
Protease ² (4% active)	0.83	0.83
Amylase (0.8% active)	0.5	0.5
Perborate monohydrate (15.5% Active AvO) ³	14.5	14.5
Cobalt catalyst ⁴	0.008	--
Dibenzoyl Peroxide (18% active)	4.4	4.4
Water, sodium sulfate and misc.	Balance	Balance

¹ Terpolymer selected from either 60% acrylic acid/20% maleic acid/20% ethyl acrylate, or 70% acrylic acid/10% maleic acid/20% ethyl acrylate.

² A carbonyl hydrolase variant of *B. amyloliquefaciens* subtilisin with the amino acid substitutions 210I/76D/103A/104I/156E/166D.

³ Available from DeGussa Corp. The AvO level of the above formula is 2.2%.

⁴ Pentaammineacetatocobalt(III) nitrate prepared as described hereinbefore; may be replaced by MnTACN.

The ADD's of the above dishwashing detergent composition examples are used to wash milk-soiled glasses, starch, cheese, egg or babyfood- soiled flatware, by loading the soiled dishes in a domestic automatic dishwashing appliance and washing using either cold fill, 60°C peak, or uniformly 45-50°C wash cycles with a product concentration of the exemplary compositions of from about 1,000 to about 8,000 ppm, with excellent results.

EXAMPLE 2

Light-duty liquid dishwashing detergent formulae are prepared as follows:

<u>Ingredient</u>	<u>A</u>	<u>Composition</u>	
		<u>B</u>	<u>C</u>
		<u>% Weight</u>	
Surfactant	32.00	29.50	30.75
Ethanol	4.00	4.00	4.00
Ammonium citrate	0.06	0.06	0.06
Magnesium chloride	3.32	3.32	3.32
Ammonium sulfate	0.08	0.08	0.08
Hydrogen peroxide	200 ppm	200 ppm	200 ppm
Perfume	0.18	0.18	0.18
Protease ¹	0.50	0.50	0.50
Water and minors	----- Balance -----		

¹ A carbonyl hydrolase variant of *B. amyloliquefaciens* subtilisin with the amino acid substitutions 210I/76D/103A/104I/156E/166D.

Having described the invention in detail with reference to preferred embodiments and the examples, it will be clear to those skilled in the art that various changes and modifications may be made without departing from the scope of the invention and the invention is not to be considered limited to what is described in the specification.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

- (i) APPLICANT: Rai, Saroj
Correa, Paul E.
Zhu, Yong
Graycar, Thomas P.
Bott, Richard R.

(ii) TITLE OF INVENTION: Protease Enzymes for Tough Cleaning and/or
Spot and Film Reduction and
Compositions Incorporating Same

(iii) NUMBER OF SEQUENCES: 9

(iv) CORRESPONDENCE ADDRESS:

- (A) ADDRESSEE: The Procter & Gamble Company
- (B) STREET: 11810 East Miami River Road
- (C) CITY: Ross
- (D) STATE: OH
- (E) COUNTRY: USA
- (F) ZIP: 45061

(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: June 4, 1997
- (C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:

- (A) NAME: Bolam, Brian M.
- (B) REGISTRATION NUMBER: 37,513
- (C) REFERENCE/DOCKET NUMBER: 6689P

(ix) TELECOMMUNICATION INFORMATION:

- (A) TELEPHONE: 513/627-2457
- (B) TELEFAX: 513/627-0318

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1497 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

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

```

GGTCTACTAA AATATTATTC CATACTATAC AATTAATACA CAGAATAATC TGTCTATTGG      60
TTATTCTGCA AATGAAAAAA AGGAGAGGAT AAAGAGTGAG AGGCAAAAAA GTATGGATCA      120
GTTTGCTGTT TGCTTTAGCG TTAATCTTTA CGATGGCGTT CGGCAGCACA TCCTCTGCCC      180
AGGCGGCAGG GAAATCAAAC GGGGAAAAGA AATATATTGT CGGGTTTAAA CAGACAATGA      240
GCACGATGAG CGCCGCTAAG AAGAAAGATG TCATTTCTGA AAAAGGCGGG AAAGTGCAAA      300
AGCAATTCAA ATATGTAGAC GCAGCTTCAG TCACATTAAA CGAAAAAGCT GTAAAAGAAT      360
TGAAAAAAGA CCGGAGCGTC GCTTACGTTG AAGAAGATCA CGTAGCACAT GCGTACGCGC      420
AGTCCGTGCC TTACGGCGTA TCACAAATTA AAGCCCCTGC TCTGCACTCT CAAGGCTACA      480
CTGGATCAAA TGTAAAGTA GCGGTTATCG ACAGCGGTAT CGATTCTTCT CATCCTGATT      540
TAAAGGTAGC AAGCGGAGCC AGCATGGTTC CTTCTGAAAC AAATCCTTTC CAAGACAACA      600
ACTCTCACGG AACTCACGTT GCCGGCACAG TTGCGGCTCT TAATAACTCA ATCGGTGTAT      660
TAGGCGTTGC GCCAAGCGCA TCACTTTACG CTGTAAAAGT TCTCGGTGCT GACGGTTCCG      720
GCCAATACAG CTGGATCATT AACGGAATCG AGTGGGCGAT CGCAAACAAT ATGGACGTTA      780
TTAACATGAG CCTCGGCGGA CCTTCTGGTT CTGCTGCTTT AAAAGCGGCA GTTGATAAAG      840
CCGTTGCATC CGGCGTCGTA GTCGTTGCGG CAGCCGGTAA CGAAGGCACT TCCGGCAGCT      900
CAAGCACAGT GGGCTACCCT GGTAATACC CTTCTGTCAT TGCAGTAGGC GCTGTTGACA      960
GCAGCAACCA AAGAGCATCT TTCTCAAGCG TAGGACCTGA GCTTGATGTC ATGGCACCTG     1020
GCGTATCTAT CCAAAGCACG CTTCCTGGAA ACAAATACGG GCGGTACAAC GGTACGTCAA     1080
TGGCATCTCC GCACGTTGCC GGAGCGGCTG CTTTGATTCT TTCTAAGCAC CCGAACTGGA     1140
CAAACACTCA AGTCCGCAGC AGTTTAGAAA ACACCACTAC AAAACTTGGT GATTCTTTGT     1200
ACTATGGAAG AGGGCTGATC AACGTACAAG CGGCAGCTCA GTAAAACATA AAAAACCAGC     1260
CTTGGCCCCG CCGGTTTTTT ATTATTTTTC TTCCTCCGCA TGTTCAATCC GCTCCATAAT     1320
CGACGGATGG CTCCCTCTGA AAATTTTAAC GAGAAACGGC GGGTTGACCC GGCTCAGTCC     1380
CGTAACGGCC AACTCCTGAA ACGTCTCAAT CGCCGCTTCC CGGTTTCCGG TCAGCTCAAT     1440
GCCATAACGG TCGGCGGCGT TTTCCTGATA CCGGGAGACG GCATTCGTAA TCGGATC      1497

```

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 275 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

WO 98/55634

PCT/IB98/00853

31

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

Ala	Gln	Ser	Val	Pro	Tyr	Gly	Val	Ser	Gln	Ile	Lys	Ala	Pro	Ala	Leu	1	5	10	15
His	Ser	Gln	Gly	Tyr	Thr	Gly	Ser	Asn	Val	Lys	Val	Ala	Val	Ile	Asp	20	25	30	
Ser	Gly	Ile	Asp	Ser	Ser	His	Pro	Asp	Leu	Lys	Val	Ala	Gly	Gly	Ala	35	40	45	
Ser	Met	Val	Pro	Ser	Glu	Thr	Asn	Pro	Phe	Gln	Asp	Asn	Asn	Ser	His	50	55	60	
Gly	Thr	His	Val	Ala	Gly	Thr	Val	Ala	Ala	Leu	Asn	Asn	Ser	Ile	Gly	65	70	75	80
Val	Leu	Gly	Val	Ala	Pro	Ser	Ala	Ser	Leu	Tyr	Ala	Val	Lys	Val	Leu	85	90	95	
Gly	Ala	Asp	Gly	Ser	Gly	Gln	Tyr	Ser	Trp	Ile	Ile	Asn	Gly	Ile	Glu	100	105	110	
Trp	Ala	Ile	Ala	Asn	Asn	Met	Asp	Val	Ile	Asn	Met	Ser	Leu	Gly	Gly	115	120	125	
Pro	Ser	Gly	Ser	Ala	Ala	Leu	Lys	Ala	Ala	Val	Asp	Lys	Ala	Val	Ala	130	135	140	
Ser	Gly	Val	Val	Val	Val	Ala	Ala	Ala	Gly	Asn	Glu	Gly	Thr	Ser	Gly	145	150	155	160
Ser	Ser	Ser	Thr	Val	Gly	Tyr	Pro	Gly	Lys	Tyr	Pro	Ser	Val	Ile	Ala	165	170	175	
Val	Gly	Ala	Val	Asp	Ser	Ser	Asn	Gln	Arg	Ala	Ser	Phe	Ser	Ser	Val	180	185	190	
Gly	Pro	Glu	Leu	Asp	Val	Met	Ala	Pro	Gly	Val	Ser	Ile	Gln	Ser	Thr	195	200	205	
Leu	Pro	Gly	Asn	Lys	Tyr	Gly	Ala	Tyr	Asn	Gly	Thr	Ser	Met	Ala	Ser	210	215	220	
Pro	His	Val	Ala	Gly	Ala	Ala	Ala	Leu	Ile	Leu	Ser	Lys	His	Pro	Asn	225	230	235	240
Trp	Thr	Asn	Thr	Gln	Val	Arg	Ser	Ser	Leu	Glu	Asn	Thr	Thr	Thr	Lys	245	250	255	
Leu	Gly	Asp	Ser	Phe	Tyr	Tyr	Gly	Lys	Gly	Leu	Ile	Asn	Val	Gln	Ala	260	265	270	
Ala	Ala	Gln														275			

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 275 amino acids

WO 98/55634

PCT/IB98/00853

32

(B) TYPE: amino acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

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

Ala	Gln	Ser	Val	Pro	Tyr	Gly	Ile	Ser	Gln	Ile	Lys	Ala	Pro	Ala	Leu	1	5	10	15
His	Ser	Gln	Gly	Tyr	Thr	Gly	Ser	Asn	Val	Lys	Val	Ala	Val	Ile	Asp	20	25	30	
Ser	Gly	Ile	Asp	Ser	Ser	His	Pro	Asp	Leu	Asn	Val	Arg	Gly	Gly	Ala	35	40	45	
Ser	Phe	Val	Pro	Ser	Glu	Thr	Asn	Pro	Tyr	Gln	Asp	Gly	Ser	Ser	His	50	55	60	
Gly	Thr	His	Val	Ala	Gly	Thr	Ile	Ala	Ala	Leu	Asn	Asn	Ser	Ile	Gly	65	70	75	80
Val	Leu	Gly	Val	Ser	Pro	Ser	Ala	Ser	Leu	Tyr	Ala	Val	Lys	Val	Leu	85	90	95	
Asp	Ser	Thr	Gly	Ser	Gly	Gln	Tyr	Ser	Trp	Ile	Ile	Asn	Gly	Ile	Glu	100	105	110	
Trp	Ala	Ile	Ser	Asn	Asn	Met	Asp	Val	Ile	Asn	Met	Ser	Leu	Gly	Gly	115	120	125	
Pro	Thr	Gly	Ser	Thr	Ala	Leu	Lys	Thr	Val	Val	Asp	Lys	Ala	Val	Ser	130	135	140	
Ser	Gly	Ile	Val	Val	Ala	Ala	Ala	Ala	Gly	Asn	Glu	Gly	Ser	Ser	Gly	145	150	155	160
Ser	Thr	Ser	Thr	Val	Gly	Tyr	Pro	Ala	Lys	Tyr	Pro	Ser	Thr	Ile	Ala	165	170	175	
Val	Gly	Ala	Val	Asn	Ser	Ser	Asn	Gln	Arg	Ala	Ser	Phe	Ser	Ser	Ala	180	185	190	
Gly	Ser	Glu	Leu	Asp	Val	Met	Ala	Pro	Gly	Val	Ser	Ile	Gln	Ser	Thr	195	200	205	
Leu	Pro	Gly	Gly	Thr	Tyr	Gly	Ala	Tyr	Asn	Gly	Thr	Ser	Met	Ala	Thr	210	215	220	
Pro	His	Val	Ala	Gly	Ala	Ala	Ala	Leu	Ile	Leu	Ser	Lys	His	Pro	Thr	225	230	235	240
Trp	Thr	Asn	Ala	Gln	Val	Arg	Asp	Arg	Leu	Glu	Ser	Thr	Ala	Thr	Tyr	245	250	255	
Leu	Gly	Asn	Ser	Phe	Tyr	Tyr	Gly	Lys	Gly	Leu	Ile	Asn	Val	Gln	Ala	260	265	270	

WO 98/55634

PCT/IB98/00853

33

Ala Ala Gln
275

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 274 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Ala	Gln	Thr	Val	Pro	Tyr	Gly	Ile	Pro	Leu	Ile	Lys	Ala	Asp	Lys	Val	1	5	10	15
Gln	Ala	Gln	Gly	Phe	Lys	Gly	Ala	Asn	Val	Lys	Val	Ala	Val	Leu	Asp	20	25	30	
Thr	Gly	Ile	Gln	Ala	Ser	His	Pro	Asp	Leu	Asn	Val	Val	Gly	Gly	Ala	35	40	45	
Ser	Phe	Val	Ala	Gly	Glu	Ala	Tyr	Asn	Thr	Asp	Gly	Asn	Gly	His	Gly	50	55	60	
Thr	His	Val	Ala	Gly	Thr	Val	Ala	Ala	Leu	Asp	Asn	Thr	Thr	Gly	Val	65	70	75	80
Leu	Gly	Val	Ala	Pro	Ser	Val	Ser	Leu	Tyr	Ala	Val	Lys	Val	Leu	Asn	85	90	95	
Ser	Ser	Gly	Ser	Gly	Ser	Tyr	Ser	Gly	Ile	Val	Ser	Gly	Ile	Glu	Trp	100	105	110	
Ala	Thr	Thr	Asn	Gly	Met	Asp	Val	Ile	Asn	Met	Ser	Leu	Gly	Gly	Ala	115	120	125	
Ser	Gly	Ser	Thr	Ala	Met	Lys	Gln	Ala	Val	Asp	Asn	Ala	Tyr	Ala	Arg	130	135	140	
Gly	Val	Val	Val	Val	Ala	Ala	Ala	Gly	Asn	Ser	Gly	Asn	Ser	Gly	Ser	145	150	155	160
Thr	Asn	Thr	Ile	Gly	Tyr	Pro	Ala	Lys	Tyr	Asp	Ser	Val	Ile	Ala	Val	165	170	175	
Gly	Ala	Val	Asp	Ser	Asn	Ser	Asn	Arg	Ala	Ser	Phe	Ser	Ser	Val	Gly	180	185	190	
Ala	Glu	Leu	Glu	Val	Met	Ala	Pro	Gly	Ala	Gly	Val	Tyr	Ser	Thr	Tyr	195	200	205	
Pro	Thr	Asn	Thr	Tyr	Ala	Thr	Leu	Asn	Gly	Thr	Ser	Met	Ala	Ser	Pro	210	215	220	

WO 98/55634

PCT/IB98/00853

34

His Val Ala Gly Ala Ala Ala Leu Ile Leu Ser Lys His Pro Asn Leu
 225 230 235 240
 Ser Ala Ser Gln Val Arg Asn Arg Leu Ser Ser Thr Ala Thr Tyr Leu
 245 250 255
 Gly Ser Ser Phe Tyr Tyr Gly Lys Gly Leu Ile Asn Val Glu Ala Ala
 260 265 270
 Ala Gln

(2) INFORMATION FOR SEQ ID NO:5:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 269 amino acids
 - (B) TYPE: amino acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

Ala Gln Ser Val Pro Trp Gly Ile Ser Arg Val Gln Ala Pro Ala Ala
 1 5 10 15
 His Asn Arg Gly Leu Thr Gly Ser Gly Val Lys Val Ala Val Leu Asp
 20 25 30
 Thr Gly Ile Ser Thr His Pro Asp Leu Asn Ile Arg Gly Gly Ala Ser
 35 40 45
 Phe Val Pro Gly Glu Pro Ser Thr Gln Asp Gly Asn Gly His Gly Thr
 50 55 60
 His Val Ala Gly Thr Ile Ala Ala Leu Asn Asn Ser Ile Gly Val Leu
 65 70 75 80
 Gly Val Ala Pro Ser Ala Glu Leu Tyr Ala Val Lys Val Leu Gly Ala
 85 90 95
 Ser Gly Ser Gly Ser Val Ser Ser Ile Ala Gln Gly Leu Glu Trp Ala
 100 105 110
 Gly Asn Asn Gly Met His Val Ala Asn Leu Ser Leu Gly Ser Pro Ser
 115 120 125
 Pro Ser Ala Thr Leu Glu Gln Ala Val Asn Ser Ala Thr Ser Arg Gly
 130 135 140
 Val Leu Val Val Ala Ala Ser Gly Asn Ser Gly Ala Gly Ser Ile Ser
 145 150 155 160
 Tyr Pro Ala Arg Tyr Ala Asn Ala Met Ala Val Gly Ala Thr Asp Gln
 165 170 175

35

```

Asn Asn Asn Arg Ala Ser Phe Ser Gln Tyr Gly Ala Gly Leu Asp Ile
      180                      185                      190

Val Ala Pro Gly Val Asn Val Gln Ser Thr Tyr Pro Gly Ser Thr Tyr
      195                      200                      205

Ala Ser Leu Asn Gly Thr Ser Met Ala Thr Pro His Val Ala Gly Ala
      210                      215                      220

Ala Ala Leu Val Lys Gln Lys Asn Pro Ser Trp Ser Asn Val Gln Ile
      225                      230                      235                      240

Arg Asn His Leu Lys Asn Thr Ala Thr Ser Leu Gly Ser Thr Asn Leu
      245                      250                      255

Tyr Gly Ser Gly Leu Val Asn Ala Glu Ala Ala Thr Arg
      260                      265

```

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1140 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: 1..1140

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

```

ATG AAG AAA CCG TTG GGG AAA ATT GTC GCA AGC ACC GCA CTA CTC ATT      48
Met Lys Lys Pro Leu Gly Lys Ile Val Ala Ser Thr Ala Leu Leu Ile
  1              5              10              15

TCT GTT GCT TTT AGT TCA TCG ATC GCA TCG GCT GCT GAA GAA GCA AAA      96
Ser Val Ala Phe Ser Ser Ser Ile Ala Ser Ala Ala Glu Glu Ala Lys
      20              25              30

GAA AAA TAT TTA ATT GGC TTT AAT GAG CAG GAA GCT GTC AGT GAG TTT      144
Glu Lys Tyr Leu Ile Gly Phe Asn Glu Gln Glu Ala Val Ser Glu Phe
      35              40              45

GTA GAA CAA GTA GAG GCA AAT GAC GAG GTC GCC ATT CTC TCT GAG GAA      192
Val Glu Gln Val Glu Ala Asn Asp Glu Val Ala Ile Leu Ser Glu Glu
      50              55              60

GAG GAA GTC GAA ATT GAA TTG CTT CAT GAA TTT GAA ACG ATT CCT GTT      240
Glu Glu Val Glu Ile Glu Leu Leu His Glu Phe Glu Thr Ile Pro Val
      65              70              75              80

TTA TCC GTT GAG TTA AGC CCA GAA GAT GTG GAC GCG CTT GAA CTC GAT      288
Leu Ser Val Glu Leu Ser Pro Glu Asp Val Asp Ala Leu Glu Leu Asp
      85              90              95

```

CCA	GCG	ATT	TCT	TAT	ATT	GAA	GAG	GAT	GCA	GAA	GTA	ACG	ACA	ATG	GCG	336
Pro	Ala	Ile	Ser	Tyr	Ile	Glu	Glu	Asp	Ala	Glu	Val	Thr	Thr	Met	Ala	
			100					105						110		
CAA	TCA	GTG	CCA	TGG	GGA	ATT	AGC	CGT	GTG	CAA	GCC	CCA	GCT	GCC	CAT	384
Gln	Ser	Val	Pro	Trp	Gly	Ile	Ser	Arg	Val	Gln	Ala	Pro	Ala	Ala	His	
		115					120					125				
AAC	CGT	GGA	TTG	ACA	GGT	TCT	GGT	GTA	AAA	GTT	GCT	GTC	CTC	GAT	ACA	432
Asn	Arg	Gly	Leu	Thr	Gly	Ser	Gly	Val	Lys	Val	Ala	Val	Leu	Asp	Thr	
	130					135					140					
GGT	ATT	TCC	ACT	CAT	CCA	GAC	TTA	AAT	ATT	CGT	GGT	GGC	GCT	AGC	TTT	480
Gly	Ile	Ser	Thr	His	Pro	Asp	Leu	Asn	Ile	Arg	Gly	Gly	Ala	Ser	Phe	
145					150					155					160	
GTA	CCA	GGG	GAA	CCA	TCC	ACT	CAA	GAT	GGG	AAT	GGG	CAT	GGC	ACG	CAT	528
Val	Pro	Gly	Glu	Pro	Ser	Thr	Gln	Asp	Gly	Asn	Gly	His	Gly	Thr	His	
				165				170						175		
GTG	GCC	GGG	ACG	ATT	GCT	GCT	TTA	AAC	AAT	TCG	ATT	GGC	GTT	CTT	GGC	576
Val	Ala	Gly	Thr	Ile	Ala	Ala	Leu	Asn	Asn	Ser	Ile	Gly	Val	Leu	Gly	
			180					185					190			
GTA	GCG	CCG	AGC	GCG	GAA	CTA	TAC	GCT	GTT	AAA	GTA	TTA	GGG	GCG	AGC	624
Val	Ala	Pro	Ser	Ala	Glu	Leu	Tyr	Ala	Val	Lys	Val	Leu	Gly	Ala	Ser	
		195					200					205				
GGT	TCA	GGT	TCG	GTC	AGC	TCG	ATT	GCC	CAA	GGA	TTG	GAA	TGG	GCA	GGG	672
Gly	Ser	Gly	Ser	Val	Ser	Ser	Ile	Ala	Gln	Gly	Leu	Glu	Trp	Ala	Gly	
	210					215					220					
AAC	AAT	GGC	ATG	CAC	GTT	GCT	AAT	TTG	AGT	TTA	GGA	AGC	CCT	TCG	CCA	720
Asn	Asn	Gly	Met	His	Val	Ala	Asn	Leu	Ser	Leu	Gly	Ser	Pro	Ser	Pro	
225					230					235					240	
AGT	GCC	ACA	CTT	GAG	CAA	GCT	GTT	AAT	AGC	GCG	ACT	TCT	AGA	GGC	GTT	768
Ser	Ala	Thr	Leu	Glu	Gln	Ala	Val	Asn	Ser	Ala	Thr	Ser	Arg	Gly	Val	
			245					250					255			
CTT	GTT	GTA	GCG	GCA	TCT	GGG	AAT	TCA	GGT	GCA	GGC	TCA	ATC	AGC	TAT	816
Leu	Val	Val	Ala	Ala	Ser	Gly	Asn	Ser	Gly	Ala	Gly	Ser	Ile	Ser	Tyr	
			260				265						270			
CCG	GCC	CGT	TAT	GCG	AAC	GCA	ATG	GCA	GTC	GGA	GCT	ACT	GAC	CAA	AAC	864
Pro	Ala	Arg	Tyr	Ala	Asn	Ala	Met	Ala	Val	Gly	Ala	Thr	Asp	Gln	Asn	
		275				280						285				
AAC	AAC	CGC	GCC	AGC	TTT	TCA	CAG	TAT	GGC	GCA	GGG	CTT	GAC	ATT	GTC	912
Asn	Asn	Arg	Ala	Ser	Phe	Ser	Gln	Tyr	Gly	Ala	Gly	Leu	Asp	Ile	Val	
	290					295					300					
GCA	CCA	GGT	GTA	AAC	GTG	CAG	AGC	ACA	TAC	CCA	GGT	TCA	ACG	TAT	GCC	960
Ala	Pro	Gly	Val	Asn	Val	Gln	Ser	Thr	Tyr	Pro	Gly	Ser	Thr	Tyr	Ala	
305					310					315					320	
AGC	TTA	AAC	GGT	ACA	TCG	ATG	GCT	ACT	CCT	CAT	GTT	GCA	GGT	GCA	GCA	1008
Ser	Leu	Asn	Gly	Thr	Ser	Met	Ala	Thr	Pro	His	Val	Ala	Gly	Ala	Ala	
			325					330					335			

WO 98/55634

PCT/IB98/00853

37

GCC CTT GTT AAA CAA AAG AAC CCA TCT TGG TCC AAT GTA CAA ATC CGC	1056
Ala Leu Val Lys Gln Lys Asn Pro Ser Trp Ser Asn Val Gln Ile Arg	
340 345 350	
AAT CAT CTA AAG AAT ACG GCA ACG AGC TTA GGA AGC ACG AAC TTG TAT	1104
Asn His Leu Lys Asn Thr Ala Thr Ser Leu Gly Ser Thr Asn Leu Tyr	
355 360 365	
GGA AGC GGA CTT GTC AAT GCA GAA GCG GCA ACA CGC	1140
Gly Ser Gly Leu Val Asn Ala Glu Ala Ala Thr Arg	
370 375 380	

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 380 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

Met	Lys	Lys	Pro	Leu	Gly	Lys	Ile	Val	Ala	Ser	Thr	Ala	Leu	Leu	Ile
1				5					10					15	
Ser	Val	Ala	Phe	Ser	Ser	Ser	Ile	Ala	Ser	Ala	Ala	Glu	Glu	Ala	Lys
			20					25					30		
Glu	Lys	Tyr	Leu	Ile	Gly	Phe	Asn	Glu	Gln	Glu	Ala	Val	Ser	Glu	Phe
		35					40					45			
Val	Glu	Gln	Val	Glu	Ala	Asn	Asp	Glu	Val	Ala	Ile	Leu	Ser	Glu	Glu
	50					55					60				
Glu	Glu	Val	Glu	Ile	Glu	Leu	Leu	His	Glu	Phe	Glu	Thr	Ile	Pro	Val
65				70						75				80	
Leu	Ser	Val	Glu	Leu	Ser	Pro	Glu	Asp	Val	Asp	Ala	Leu	Glu	Leu	Asp
				85					90					95	
Pro	Ala	Ile	Ser	Tyr	Ile	Glu	Glu	Asp	Ala	Glu	Val	Thr	Thr	Met	Ala
			100					105					110		
Gln	Ser	Val	Pro	Trp	Gly	Ile	Ser	Arg	Val	Gln	Ala	Pro	Ala	Ala	His
		115				120					125				
Asn	Arg	Gly	Leu	Thr	Gly	Ser	Gly	Val	Lys	Val	Ala	Val	Leu	Asp	Thr
	130					135					140				
Gly	Ile	Ser	Thr	His	Pro	Asp	Leu	Asn	Ile	Arg	Gly	Gly	Ala	Ser	Phe
145				150					155					160	
Val	Pro	Gly	Glu	Pro	Ser	Thr	Gln	Asp	Gly	Asn	Gly	His	Gly	Thr	His
			165					170						175	
Val	Ala	Gly	Thr	Ile	Ala	Ala	Leu	Asn	Asn	Ser	Ile	Gly	Val	Leu	Gly
			180					185						190	

WO 98/55634

PCT/IB98/00853

38

Val Ala Pro Ser Ala Glu Leu Tyr Ala Val Lys Val Leu Gly Ala Ser
 195 200 205

Gly Ser Gly Ser Val Ser Ser Ile Ala Gln Gly Leu Glu Trp Ala Gly
 210 215 220

Asn Asn Gly Met His Val Ala Asn Leu Ser Leu Gly Ser Pro Ser Pro
 225 230 235 240

Ser Ala Thr Leu Glu Gln Ala Val Asn Ser Ala Thr Ser Arg Gly Val
 245 250 255

Leu Val Val Ala Ala Ser Gly Asn Ser Gly Ala Gly Ser Ile Ser Tyr
 260 265 270

Pro Ala Arg Tyr Ala Asn Ala Met Ala Val Gly Ala Thr Asp Gln Asn
 275 280 285

Asn Asn Arg Ala Ser Phe Ser Gln Tyr Gly Ala Gly Leu Asp Ile Val
 290 295 300

Ala Pro Gly Val Asn Val Gln Ser Thr Tyr Pro Gly Ser Thr Tyr Ala
 305 310 315 320

Ser Leu Asn Gly Thr Ser Met Ala Thr Pro His Val Ala Gly Ala Ala
 325 330 335

Ala Leu Val Lys Gln Lys Asn Pro Ser Trp Ser Asn Val Gln Ile Arg
 340 345 350

Asn His Leu Lys Asn Thr Ala Thr Ser Leu Gly Ser Thr Asn Leu Tyr
 355 360 365

Gly Ser Gly Leu Val Asn Ala Glu Ala Ala Thr Arg
 370 375 380

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1140 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: 1..1140

(ix) FEATURE:

- (A) NAME/KEY: mat_peptide
- (B) LOCATION: 334..1140

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

ATG AAG AAA CCG TTG GGG AAA ATT GTC GCA AGC ACC GCA CTA CTC ATT
 Met Lys Lys Pro Leu Gly Lys Ile Val Ala Ser Thr Ala Leu Leu Ile
 -111-110 -105 -100

48

WO 98/55634

PCT/IB98/00853

39

TCT GTT GCT TTT AGT TCA TCG ATC GCA TCG GCT GCT GAA GAA GCA AAA Ser Val Ala Phe Ser Ser Ser Ile Ala Ser Ala Ala Glu Glu Ala Lys -95 -90 -85 -80	96
GAA AAA TAT TTA ATT GGC TTT AAT GAG CAG GAA GCT GTC AGT GAG TTT Glu Lys Tyr Leu Ile Gly Phe Asn Glu Gln Glu Ala Val Ser Glu Phe -75 -70 -65	144
GTA GAA CAA GTA GAG GCA AAT GAC GAG GTC GCC ATT CTC TCT GAG GAA Val Glu Gln Val Glu Ala Asn Asp Glu Val Ala Ile Leu Ser Glu Glu -60 -55 -50	192
GAG GAA GTC GAA ATT GAA TTG CTT CAT GAA TTT GAA ACG ATT CCT GTT Glu Glu Val Glu Ile Glu Leu Leu His Glu Phe Glu Thr Ile Pro Val -45 -40 -35	240
TTA TCC GTT GAG TTA AGC CCA GAA GAT GTG GAC GCG CTT GAA CTC GAT Leu Ser Val Glu Leu Ser Pro Glu Asp Val Asp Ala Leu Glu Leu Asp -30 -25 -20	288
CCA GCG ATT TCT TAT ATT GAA GAG GAT GCA GAA GTA ACG ACA ATG GCG Pro Ala Ile Ser Tyr Ile Glu Glu Asp Ala Glu Val Thr Thr Met Ala -15 -10 -5 1	336
CAA TCA GTG CCA TGG GGA ATT AGC CGT GTG CAA GCC CCA GCT GCC CAT Gln Ser Val Pro Trp Gly Ile Ser Arg Val Gln Ala Pro Ala Ala His 5 10 15	384
AAC CGT GGA TTG ACA GGT TCT GGT GTA AAA GTT GCT GTC CTC GAT ACA Asn Arg Gly Leu Thr Gly Ser Gly Val Lys Val Ala Val Leu Asp Thr 20 25 30	432
GGT ATT TCC ACT CAT CCA GAC TTA AAT ATT CGT GGT GGC GCT AGC TTT Gly Ile Ser Thr His Pro Asp Leu Asn Ile Arg Gly Gly Ala Ser Phe 35 40 45	480
GTA CCA GGG GAA CCA TCC ACT CAA GAT GGG AAT GGG CAT GGC ACG CAT Val Pro Gly Glu Pro Ser Thr Gln Asp Gly Asn Gly His Gly Thr His 50 55 60 65	528
GTG GCC GGG ACG ATT GCT GCT TTA GAC AAC TCG ATT GGC GTT CTT GGC Val Ala Gly Thr Ile Ala Ala Leu Asp Asn Ser Ile Gly Val Leu Gly 70 75 80	576
GTA GCG CCG AGC GCG GAA CTA TAC GCT GTT AAA GTA TTA GGG GCG AGC Val Ala Pro Ser Ala Glu Leu Tyr Ala Val Lys Val Leu Gly Ala Ser 85 90 95	624
GGT TCA GGC GCC ATC AGC TCG ATT GCC CAA GGA TTG GAA TGG GCA GGG Gly Ser Gly Ala Ile Ser Ser Ile Ala Gln Gly Leu Glu Trp Ala Gly 100 105 110	672
AAC AAT GGC ATG CAC GTT GCT AAT TTG AGT TTA GGA AGC CCT TCG CCA Asn Asn Gly Met His Val Ala Asn Leu Ser Leu Gly Ser Pro Ser Pro 115 120 125	720
AGT GCC ACA CTT GAG CAA GCT GTT AAT AGC GCG ACT TCT AGA GGC GTT Ser Ala Thr Leu Glu Gln Ala Val Asn Ser Ala Thr Ser Arg Gly Val 130 135 140 145	768

WO 98/55634

PCT/IB98/00853

40

CTT GTT GTA GCG GCA TCT GGG AAT GAA GGT GCA GGC TCA ATC GAC TAT	816
Leu Val Val Ala Ala Ser Gly Asn Glu Gly Ala Gly Ser Ile Asp Tyr	
150 155 160	
CCG GCC CGT TAT GCG AAC GCA ATG GCA GTC GGA GCT ACT GAC CAA AAC	864
Pro Ala Arg Tyr Ala Asn Ala Met Ala Val Gly Ala Thr Asp Gln Asn	
165 170 175	
AAC AAC CGC GCC AGC TTT TCA CAG TAT GGC GCA GGG CTT GAC ATT GTC	912
Asn Asn Arg Ala Ser Phe Ser Gln Tyr Gly Ala Gly Leu Asp Ile Val	
180 185 190	
GCA CCA GGT GTA AAC GTG CAG AGC ACA TAC CCA ATT TCA ACG TAT GCC	960
Ala Pro Gly Val Asn Val Gln Ser Thr Tyr Pro Ile Ser Thr Tyr Ala	
195 200 205	
AGC TTA AAC GGT ACA TCG ATG GCT ACT CCT CAT GTT GCA GGT GCA GCA	1008
Ser Leu Asn Gly Thr Ser Met Ala Thr Pro His Val Ala Gly Ala Ala	
210 215 220 225	
GCC CTT GTT AAA CAA AAG AAC CCA TCT TGG TCC AAT GTA CAA ATC CGC	1056
Ala Leu Val Lys Gln Lys Asn Pro Ser Trp Ser Asn Val Gln Ile Arg	
230 235 240	
AAT CAT CTA AAG AAT ACG GCA ACG AGC TTA GGA AGC ACG AAC TTG TAT	1104
Asn His Leu Lys Asn Thr Ala Thr Ser Leu Gly Ser Thr Asn Leu Tyr	
245 250 255	
GGA AGC GGA CTT GTC AAT GCA GAA GCG GCA ACA CGC	1140
Gly Ser Gly Leu Val Asn Ala Glu Ala Ala Thr Arg	
260 265	

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 380 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

Met Lys Lys Pro Leu Gly Lys Ile Val Ala Ser Thr Ala Leu Leu Ile	
-111 -110 -105 -100	
Ser Val Ala Phe Ser Ser Ser Ile Ala Ser Ala Ala Glu Glu Ala Lys	
-95 -90 -85 -80	
Glu Lys Tyr Leu Ile Gly Phe Asn Glu Gln Glu Ala Val Ser Glu Phe	
-75 -70 -65	
Val Glu Gln Val Glu Ala Asn Asp Glu Val Ala Ile Leu Ser Glu Glu	
-60 -55 -50	
Glu Glu Val Glu Ile Glu Leu Leu His Glu Phe Glu Thr Ile Pro Val	
-45 -40 -35	
Leu Ser Val Glu Leu Ser Pro Glu Asp Val Asp Ala Leu Glu Leu Asp	
-30 -25 -20	

WO 98/55634

PCT/IB98/00853

41

Pro Ala Ile Ser Tyr Ile Glu Glu Asp Ala Glu Val Thr Thr Met Ala
 -15 -10 -5 1
 Gln Ser Val Pro Trp Gly Ile Ser Arg Val Gln Ala Pro Ala Ala His
 5 10 15
 Asn Arg Gly Leu Thr Gly Ser Gly Val Lys Val Ala Val Leu Asp Thr
 20 25 30
 Gly Ile Ser Thr His Pro Asp Leu Asn Ile Arg Gly Gly Ala Ser Phe
 35 40 45
 Val Pro Gly Glu Pro Ser Thr Gln Asp Gly Asn Gly His Gly Thr His
 50 55 60 65
 Val Ala Gly Thr Ile Ala Ala Leu Asp Asn Ser Ile Gly Val Leu Gly
 70 75 80
 Val Ala Pro Ser Ala Glu Leu Tyr Ala Val Lys Val Leu Gly Ala Ser
 85 90 95
 Gly Ser Gly Ala Ile Ser Ser Ile Ala Gln Gly Leu Glu Trp Ala Gly
 100 105 110
 Asn Asn Gly Met His Val Ala Asn Leu Ser Leu Gly Ser Pro Ser Pro
 115 120 125
 Ser Ala Thr Leu Glu Gln Ala Val Asn Ser Ala Thr Ser Arg Gly Val
 130 135 140 145
 Leu Val Val Ala Ala Ser Gly Asn Glu Gly Ala Gly Ser Ile Asp Tyr
 150 155 160
 Pro Ala Arg Tyr Ala Asn Ala Met Ala Val Gly Ala Thr Asp Gln Asn
 165 170 175
 Asn Asn Arg Ala Ser Phe Ser Gln Tyr Gly Ala Gly Leu Asp Ile Val
 180 185 190
 Ala Pro Gly Val Asn Val Gln Ser Thr Tyr Pro Ile Ser Thr Tyr Ala
 195 200 205
 Ser Leu Asn Gly Thr Ser Met Ala Thr Pro His Val Ala Gly Ala Ala
 210 215 220 225
 Ala Leu Val Lys Gln Lys Asn Pro Ser Trp Ser Asn Val Gln Ile Arg
 230 235 240
 Asn His Leu Lys Asn Thr Ala Thr Ser Leu Gly Ser Thr Asn Leu Tyr
 245 250 255
 Gly Ser Gly Leu Val Asn Ala Glu Ala Ala Thr Arg
 260 265

WHAT IS CLAIMED IS:

1. A cleaning composition comprising:
 - (a) an effective amount of protease enzyme which is a carbonyl hydrolase variant having an amino acid sequence not found in nature, which is derived from a precursor carbonyl hydrolase by substituting a different amino acid for a plurality of amino acid residues at a position in said precursor carbonyl hydrolase equivalent to position +210 in combination with one or more amino acid residue position equivalent to those selected from the group consisting of +33, +62, +67, +76, +100, +101, +103, +104, +107, +128, +129, +130, +132, +135, +156, +158, +164, +166, +167, +170, +209, +215, +217, +218, and +222 in *Bacillus amyloliquefaciens* subtilisin; and
 - (b) one or more cleaning adjunct materials compatible with the protease enzyme.
2. A cleaning composition according to Claim 1 wherein said cleaning composition is a dishwashing detergent composition comprising:
 - (a) from about 5% to about 90% by weight of the composition of a builder ;
 - (b) from about 0.1% to about 15% by weight of the composition of deterative surfactant;
 - (c) from about 0.1% to about 10% by weight of the composition of a protease enzyme which is a carbonyl hydrolase variant having an amino acid sequence not found in nature, which is derived from a precursor carbonyl hydrolase by substituting a different amino acid for a plurality of amino acid residues at a position in said precursor carbonyl hydrolase equivalent to position +210 in combination with one or more amino acid residue position equivalent to those selected from the group consisting of +33, +62, +67, +76, +100, +101, +103, +104, +107, +128, +129, +130, +132, +135, +156, +158, +164, +166, +167, +170, +209, +215, +217, +218 and +222 in *Bacillus amyloliquefaciens* subtilisin;
 - (d) optionally, from about 0.1% to about 40% by weight of the composition of a bleaching agent; and
 - (e) cleaning adjunct materials.
3. The compositions according to any of Claims 1-2 wherein the cleaning adjunct materials are selected from the group consisting of surfactants, solvents, buffers, enzymes, soil release agents, clay soil removal agents, dispersing agents, brighteners, suds suppressors, fabric softeners, suds boosters, enzyme stabilizers, builders, bleaching agents, dyes, perfumes, and mixtures thereof.

4. The composition according to any of Claims 1-3 further comprising from about 5% to about 50% of a builder selected from the group consisting of zeolites, polycarboxylates, layered silicates, phosphates, and mixtures thereof.
5. The compositions according to any of Claims 1-4 wherein the cleaning adjunct materials comprise at least one bleaching agent.
6. The compositions according to Claim 5 wherein the bleaching agent is selected from the group consisting of percarbonates, perborates, and mixtures thereof, and optionally further comprising at least one bleach activator.
7. The compositions according to any of Claims 1-6 wherein the composition further includes at least one detergent enzyme selected from the group consisting of cellulases, lipases, amylases, phospholipases, proteases, peroxidases and mixtures thereof.
8. The compositions according to any of Claims 1-7 wherein the precursor carbonyl hydrolase for the protease enzyme is a subtilisin, and the protease enzyme is a subtilisin variant wherein a combination of substitutions is made at the positions equivalent to those selected from the group consisting of 210/156; 210/166; 210/76; 210/103; 210/104; 210/217; 210/156/166; 210/156/217; 210/166/217; 210/76/156; 210/76/166; 210/76/217; 210/76/156/166; 210/76/156/217; 210/76/166/217; 210/76/103/156; 210/76/103/166; 210/76/103/217; 210/76/104/156; 210/76/104/166; 210/76/104/217; 210/76/103/104/156; 210/76/103/104/166; 210/76/103/104/217; 210/76/103/104/156/166; 210/76/103/104/156/217; 210/76/103/104/166/217 and 210/76/103/104/156/166/217; 210/76/103/104/166/222; 210/67/76/103/104/166/222; 210/67/76/103/104/166/218/222.
9. The composition according to any of Claims 1-8 wherein the protease enzyme is a subtilisin variant selected from the group consisting of 210/156; 210/166; 210/217; 210/156/166; 210/156/217; 210/166/217; 210/76/156/166; 210/76/103/156/166 and 210/76/103/104/156/166.
10. A method for cleaning fabric, said method comprising contacting a fabric in need of cleaning with the composition as claimed in any of Claim 1 and 3-9.

11. A method for cleaning dishes, said method comprising contacting a dish in need of cleaning with a composition according to any of Claims 1-9.
12. A method for personal cleansing, said method comprising contacting the part of the human or lower animal body in need of cleaning with a composition according to any of Claims 1 and 3-9.
13. A method for providing improved spotting and filming benefits in automatic dishwashing comprising contacting a dish in need of cleaning with a composition according to any of Claims 1-9.

10 30 50
ATGAAGAAACCGTTGGGGAAAATTGTCGCAAGCACCGCACTACTCATTCTGTTGCTTTT
MetLysLysProLeuGlyLysIleValAlaSerThrAlaLeuLeuIleSerValAlaPhe

70 90 110
AGTTCATCGATCGCATCGGCTGCTGAAGAAGCAAAAGAAAAATATTTAATTGGCTTTAAT
SerSerSerIleAlaSerAlaAlaGluGluAlaLysGluLysTyrLeuIleGlyPheAsn

130 150 170
GAGCAGGAAGCTGTCAGTGAGTTTGTAGAACAAGTAGAGGCAAATGACGAGGTGCGCATT
GluGlnGluAlaValSerGluPheValGluGlnValGluAlaAsnAspGluValAlaIle

190 210 230
CTCTCTGAGGAAGAGGAAGTCGAAATTGAATTGCTTCATGAATTTGAAACGATTCTCTGT
LeuSerGluGluGluGluValGluIleGluLeuLeuHisGluPheGluThrIleProVal

250 270 290
TTATCCGTTGAGTTAAGCCCAGAAGATGTGGACGCGCTTGAACCTCGATCCAGCGATTCT
LeuSerValGluLeuSerProGluAspValAspAlaLeuGluLeuAspProAlaIleSer

310 330 350
TATATTGAAGAGGATGCAGAAGTAACGACAATGGCGCAATCAGTGCCATGGGGAATTAGC
TyrIleGluGluAspAlaGluValThrThrMetAlaGlnSerValProTrpGlyIleSer

370 390 410
CGTGTGCAAGCCCCAGCTGCCCATAACCGTGGATTGACAGGTTCTGGTGTAAGTTGCT
ArgValGlnAlaProAlaAlaHisAsnArgGlyLeuThrGlySerGlyValLysValAla

430 450 470
GTCCTCGATACAGGTATTTCCACTCATCCAGACTTAAATATTCGTGGTGGCGCTAGCTTT
ValLeuAspThrGlyIleSerThrHisProAspLeuAsnIleArgGlyGlyAlaSerPhe

490 510 530
GTACCAGGGGAACCATCCACTCAAGATGGGAATGGGCATGGCACGCATGTGGCCGGGACG
ValProGlyGluProSerThrGlnAspGlyAsnGlyHisGlyThrHisValAlaGlyThr

550 570 590
ATTGCTGCTTTAAACAATTGATTGGCGTTCTTGGCGTAGCGCCGAGCGCGGAACCTATAC
IleAlaAlaLeuAsnAsnSerIleGlyValLeuGlyValAlaProSerAlaGluLeuTyr

610 630 650
GCTGTTAAAGTATTAGGGGCGAGCGGTTTCAGGTTTCGGTCAGCTCGATTGCCCAAGGATTG
AlaValLysValLeuGlyAlaSerGlySerGlySerValSerSerIleAlaGlnGlyLeu

670 690 710
GAATGGGCAGGGAACAATGGCATGCACGTTGCTAATTTGAGTTTAGGAAGCCCTTCGCCA
GluTrpAlaGlyAsnAsnGlyMetHisValAlaAsnLeuSerLeuGlySerProSerPro

730 750 770
AGTGCCACACTTGAGCAAGCTGTTAATAGCGCGACTTCTAGAGGCGTTCTTGTTGTAGCG
SerAlaThrLeuGluGlnAlaValAsnSerAlaThrSerArgGlyValLeuValValAla

790 810 830
GCATCTGGGAATTCAGGTGCAGGCTCAATCAGCTATCCGGCCCCGTTATGCGAACGCAATG
AlaSerGlyAsnSerGlyAlaGlySerIleSerTyrProAlaArgTyrAlaAsnAlaMet

850 870 890
GCAGTCGGAGCTACTGACCAAACAACAACCGCGCCAGCTTTTCACAGTATGGCGCAGGG
AlaValGlyAlaThrAspGlnAsnAsnAsnArgAlaSerPheSerGlnTyrGlyAlaGly

FIG. 1A

SUBSTITUTE SHEET (RULE 26)

910 930 950
CTTGACATTGTCGCACCAGGTGTAAACGTGCAGAGCACATACCCAGGTTCAACGTATGCC
LeuAspIleValAlaProGlyValAsnValGlnSerThrTyrProGlySerThrTyrAla

970 990 1010
AGCTTAAACGGTACATCGATGGCTACTCCTCATGTTGCAGGTGCAGCAGCCCTTGTTAAA
SerLeuAsnGlyThrSerMetAlaThrProHisValAlaGlyAlaAlaAlaLeuValLys

1030 1050 1070
CAAAGAACCCATCTTGGTCCAATGTACAAATCCGCAATCATCTAAAGAATACGGCAACG
GlnLysAsnProSerTrpSerAsnValGlnIleArgAsnHisLeuLysAsnThrAlaThr

1090 1110 1130
AGCTTAGGAAGCACGAACTTGTATGGAAGCGGACTTGTCAATGCAGAAGCGGCAACACGC
SerLeuGlySerThrAsnLeuTyrGlySerGlyLeuValAsnAlaGluAlaAlaThrArg

FIG. 2A

Comparison of subtilisin sequences from:

B. amyloliquefaciens
B. subtilis
B. licheniformis
B. lentus

01	A	Q	S	V	P	Y	G	V	S	Q	I	K	A	P	A	L	H	S	Q	G	Y	T	G	S	N	V	K	V	A	V	I	D	S	G	I	D	S	S	H	P	
	A	Q	S	V	P	Y	G	I	S	Q	I	K	A	P	A	L	H	S	Q	G	Y	T	G	S	N	V	K	V	A	V	I	D	S	G	I	D	S	S	H	P	
	A	Q	T	V	P	Y	G	I	P	L	I	K	A	D	K	V	Q	A	Q	G	F	K	G	A	N	V	K	V	A	V	L	D	T	G	I	Q	A	S	H	P	
	A	Q	S	V	P	W	G	I	S	R	V	Q	A	P	A	A	H	N	R	G	L	T	G	S	G	V	K	V	A	V	L	D	T	G	I	S	T	*	H	P	
										10									20										30												
41	D	L	K	V	A	G	G	A	S	M	V	P	S	E	T	N	P	F	Q	D	N	N	S	H	G	T	H	V	A	G	T	V	A	A	L	N	N	S	I	G	
	D	L	N	V	R	G	G	A	S	F	V	P	S	E	T	N	P	Y	Q	D	G	S	S	H	G	T	H	V	A	G	T	I	A	A	L	N	N	S	I	G	
	D	L	N	V	V	G	G	A	S	F	V	A	G	E	*	A	Y	N	*	T	D	G	N	G	H	G	T	H	V	A	G	T	V	A	A	L	N	N	T	T	G
	D	L	N	I	R	G	G	A	S	F	V	P	G	E	*	P	S	T	Q	D	G	N	G	H	G	T	H	V	A	G	T	I	A	A	L	N	N	S	I	G	
										50									60										70												
81	V	L	G	V	A	P	S	A	S	L	Y	A	V	K	V	L	G	A	D	G	S	G	Q	Y	S	W	I	I	N	G	I	E	W	A	I	A	N	N	M	D	
	V	L	G	V	S	P	S	A	S	L	Y	A	V	K	V	L	D	S	T	G	S	G	Q	Y	S	W	I	I	N	G	I	E	W	A	I	S	N	N	M	D	
	V	L	G	V	A	P	S	V	S	L	Y	A	V	K	V	L	N	S	S	G	S	G	S	Y	S	G	I	V	S	G	I	E	W	A	T	N	G	M	D		
	V	L	G	V	A	P	S	A	E	L	Y	A	V	K	V	L	G	A	S	G	S	G	S	V	S	I	A	Q	G	L	E	W	A	G	N	N	G	M	H		
										90									100										110												
121	V	I	N	M	S	L	G	G	P	S	G	S	A	A	L	K	A	A	V	D	K	A	V	A	S	G	V	V	V	A	A	A	A	G	N	E	G	T	S	G	
	V	I	N	M	S	L	G	G	P	T	G	S	T	A	L	K	T	V	V	D	K	A	V	S	S	G	I	V	V	A	A	A	A	G	N	E	G	S	S	G	
	V	I	N	M	S	L	G	G	A	S	G	S	T	A	M	K	Q	A	V	D	N	A	Y	A	S	R	G	V	V	V	A	A	A	G	N	S	G	N	S	G	
	V	A	N	L	S	L	G	S	P	S	P	S	A	T	L	E	Q	A	V	N	S	A	T	S	R	G	V	L	V	V	A	A	S	G	N	S	G	A	G	S	
										130									140										150												

FIG. 2A

161 S S T S T N * * * * *
170 G K Y P S V I A V G A V D S S N Q R A S F S S V G P E L D V M A
180 P P A K Y P S T I A V G A V D S S N Q R A S F S S V G P E L D V M A
190 P P A K Y P S T I A V G A V D S S N Q R A S F S S V G P E L D V M A
201 P G V S S I Q S T L P G N K Y G A Y N G T S M A S P H V A G A A A L I L S S K H P P N
210 P G V S S I Q S T L P G N K Y G A Y N G T S M A S P H V A G A A A L I L S S K H P P N
220 P G V S S I Q S T L P G N K Y G A Y N G T S M A S P H V A G A A A L I L S S K H P P N
230 P G V S S I Q S T L P G N K Y G A Y N G T S M A S P H V A G A A A L I L S S K H P P N
241 W T N A S V T Q Q V V R R S S L E N T T A T T K L G D S F Y Y Y G K G L I N V Q A A A Q
250 W T N A S V T Q Q V V R R S S L E N T T A T T K L G D S F Y Y Y G K G L I N V Q A A A Q
260 W T N A S V T Q Q V V R R S S L E N T T A T T K L G D S F Y Y Y G K G L I N V Q A A A Q
270 W T N A S V T Q Q V V R R S S L E N T T A T T K L G D S F Y Y Y G K G L I N V Q A A A Q

FIG. 2B

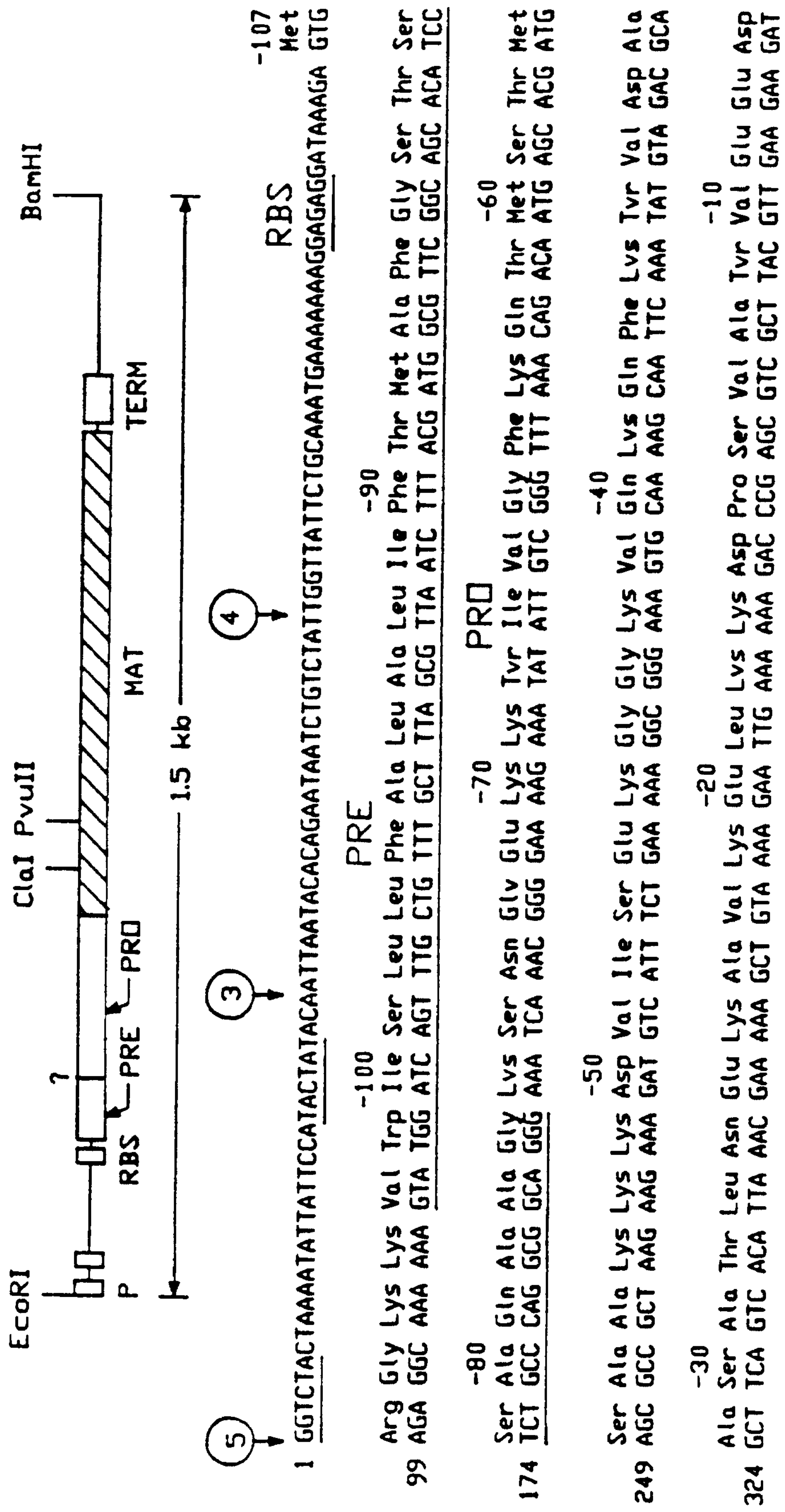


FIG. 3A

[illegible]

FIG. 3B

170
Lvs Tvr Pro Ser Val Ile Ala Val Glv Ala Val Asp Ser Ser Asn Gln Arp Ala Ser Phe Ser Ser Vsl Glv Pro
924 AAA TAC CCT TCT GTC ATT GCA ATT GCA GTA GGC GCT GCT GAC AGC AGC AGC AAG AAC CAA AGA GCA TCT TTC TCA AGC GTA GGA CCT

180
Glu Leu Asp Val Met Ala Pro Gly Val Ser Ile Gln Ser Thr Leu Pro Glv Asn Lvs Tvr Gly Ala Tvr Asn Glv
999 GAG CTT GAT GTC ATG GCA CCT GCA CCT GGC GTA TCT ATC CAA AGC AGC GCT CCT GGA AAC AAA TAC GGG GCG TAC AAC GGT

200
Thr Ser Met Ala Ser Pro His Val Ala Gly Aal Ala Ala Leu Ile Leu Ser Lvs His Pro Asn Trp Thr Asn Thr
1074 ACG TCA ATG GCA TCT CCG CAC GCT GGT GCC GGA GCG GCT GCT TTG ATT CTT TCT AAG CAC CCG AAC TGG ACA AAC ACT

220
Gln Val Aro Ser Ser Leu Glu Asn Thr Thr Thr Lvs Leu Gly Asp Ser Phe Tyr Tyr Glv Lvs Glv Leu Ile Asn
1149 CAA GTC CGC AGC AGT TTA GAA AAC ACC ACT ACA AAA CTT GGT GAT TCT TTG TAC TAT GGA AAA GGG CTG ATC AAC

240
Val Gln Ala Ala Ala Gln DC
1224 GTA CAA GCG GCA GCT CAG TAA AACATAAAACCGCCTTGGCCCGCGGTTTTTATTTATTTTCTCCGCAITCAATCCGCTCC

250 Gln
Gln Val Aro Ser Ser Leu Glu Asn Thr Thr Thr Lvs Leu Gly Asp Ser Phe Tyr Tyr Glv Lvs Glv Leu Ile Asn
1316 ATAATCGACGGATGGCTCCCTCTGAAAATTTTACGAGAAACGGGGTTGACCCGGCTCAGTCCCGTACGGCCAACTCCIGAAACGGTCTCAATCGCCG

260
Val Gln Ala Ala Ala Gln DC
1416 CTTCCTGGTTTCCGGTCAGCTCAATGCCATAACGGTCCGGCGGTTTTTCCIGATACGGGAGACGGCATTCGTAATCGGATC

FIG. 3C

CONSERVED RESIDUES IN SUBTILISINS FROM
BACILLUS AMYLOLIQUEFACIENS

1	A	Q	S	V	P	.	G	.	.	.	.	.	.	A	P	A	.	H	.	.	G	20
21	.	T	G	S	.	V	K	V	A	V	.	D	.	G	.	.	.	.	.	H	P	40
41	D	L	.	.	.	G	G	A	S	.	V	P	.	.	.	.	.	.	.	Q	D	60
61	.	N	.	H	G	T	H	V	A	G	T	.	A	A	L	N	N	S	I	G	80	
81	V	L	G	V	A	P	S	A	.	L	Y	A	V	K	V	L	G	A	.	G	100	
101	S	G	.	.	S	.	L	.	.	G	.	E	W	A	.	N	.	.	.	.	120	
121	V	.	N	.	S	L	G	.	P	S	.	S	.	.	.	.	.	A	.	.	140	
141	.	.	.	.	.	G	V	.	V	V	A	A	.	G	N	.	G	.	.	.	160	
161	.	.	.	.	.	.	Y	P	.	.	Y	.	.	.	.	A	V	G	A	.	180	
181	D	.	.	N	.	.	A	S	F	S	.	.	G	.	.	L	D	.	.	A	200	
201	P	G	V	.	.	Q	S	T	.	P	G	.	.	Y	.	.	.	N	G	T	220	
221	S	M	A	.	P	H	V	A	G	A	A	A	L	.	.	.	K	.	.	.	240	
241	W	.	.	.	Q	.	R	.	.	L	.	N	T	.	.	.	L	G	.	.	260	
261	.	.	Y	G	.	G	L	.	N	.	.	A	A	.	.	.	.	.	.	.	270	

FIG. 4

ATG AAG AAA CCG TTG GGG AAA ATT GTC GCA AGC ACC GCA CTA CTC ATT Met Lys Lys Pro Leu Gly Lys Ile Val Ala Ser Thr Ala Leu Leu Ile -111-110 -105 -100	48
TCT GTT GCT TTT AGT TCA TCG ATC GCA TCG GCT GCT GAA GAA GCA AAA Ser Val Ala Phe Ser Ser Ser Ile Ala Ser Ala Ala Glu Glu Ala Lys -95 -90 -85 -80	96
GAA AAA TAT TTA ATT GGC TTT AAT GAG CAG GAA GCT GTC AGT GAG TTT Glu Lys Tyr Leu Ile Gly Phe Asn Glu Gln Glu Ala Val Ser Glu Phe -75 -70 -65	144
GTA GAA CAA GTA GAG GCA AAT GAC GAG GTC GCC ATT CTC TCT GAG GAA Val Glu Gln Val Glu Ala Asn Asp Glu Val Ala Ile Leu Ser Glu Glu -60 -55 -50	192
GAG GAA GTC GAA ATT GAA TTG CTT CAT GAA TTT GAA ACG ATT CCT GTT Glu Glu Val Glu Ile Glu Leu Leu His Glu Phe Glu Thr Ile Pro Val -45 -40 -35	240
TTA TCC GTT GAG TTA AGC CCA GAA GAT GTG GAC GCG CTT GAA CTC GAT Leu Ser Val Glu Leu Ser Pro Glu Asp Val Asp Ala Leu Glu Leu Asp -30 -25 -20	288
CCA GCG ATT TCT TAT ATT GAA GAG GAT GCA GAA GTA ACG ACA ATG GCG Pro Ala Ile Ser Tyr Ile Glu Glu Asp Ala Glu Val Thr Thr Met Ala -15 -10 -5 1	336
CAA TCA GTG CCA TGG GGA ATT AGC CGT GTG CAA GCC CCA GCT GCC CAT Gln Ser Val Pro Trp Gly Ile Ser Arg Val Gln Ala Pro Ala Ala His 5 10 15	384
AAC CGT GGA TTG ACA GGT TCT GGT GTA AAA GTT GCT GTC CTC GAT ACA Asn Arg Gly Leu Thr Gly Ser Gly Val Lys Val Ala Val Leu Asp Thr 20 25 30	432
GGT ATT TCC ACT CAT CCA GAC TTA AAT ATT CGT GGT GGC GCT AGC TTT Gly Ile Ser Thr His Pro Asp Leu Asn Ile Arg Gly Gly Ala Ser Phe 35 40 45	480
GTA CCA GGG GAA CCA TCC ACT CAA GAT GGG AAT GGG CAT GGC ACG CAT Val Pro Gly Glu Pro Ser Thr Gln Asp Gly Asn Gly His Gly Thr His 50 55 60 65	528
GTG GCC GGG ACG ATT GCT GCT TTA GAC AAC TCG ATT GGC GTT CTT GGC Val Ala Gly Thr Ile Ala Ala Leu Asp Asn Ser Ile Gly Val Leu Gly 70 75 80	576
GTA GCG CCG AGC GCG GAA CTA TAC GCT GTT AAA GTA TTA GGG GCG AGC Val Ala Pro Ser Ala Glu Leu Tyr Ala Val Lys Val Leu Gly Ala Ser 85 90 95	624
GGT TCA GGC GCC ATC AGC TCG ATT GCC CAA GGA TTG GAA TGG GCA GGG Gly Ser Gly Ala Ile Ser Ser Ile Ala Gln Gly Leu Glu Trp Ala Gly 100 105 110	672

FIG. 5A

SUBSTITUTE SHEET (RULE 26)

WO 98/55634

PCT/IB98/00853

10/10

AAC AAT GGC ATG CAC GTT GCT AAT TTG AGT TTA GGA AGC CCT TCG CCA Asn Asn Gly Met His Val Ala Asn Leu Ser Leu Gly Ser Pro Ser Pro 115 120 125	720
AGT GCC ACA CTT GAG CAA GCT GTT AAT AGC GCG ACT TCT AGA GGC GTT Ser Ala Thr Leu Glu Gln Ala Val Asn Ser Ala Thr Ser Arg Gly Val 130 135 140 145	768
CTT GTT GTA GCG GCA TCT GGG AAT GAA GGT GCA GGC TCA ATC GAC TAT Leu Val Val Ala Ala Ser Gly Asn Glu Gly Ala Gly Ser Ile Asp Tyr 150 155 160	816
CCG GCC CGT TAT GCG AAC GCA ATG GCA GTC GGA GCT ACT GAC CAA AAC Pro Ala Arg Tyr Ala Asn Ala Met Ala Val Gly Ala Thr Asp Gln Asn 165 170 175	864
AAC AAC CGC GCC AGC TTT TCA CAG TAT GGC GCA GGG CTT GAC ATT GTC Asn Asn Arg Ala Ser Phe Ser Gln Tyr Gly Ala Gly Leu Asp Ile Val 180 185 190	912
GCA CCA GGT GTA AAC GTG CAG AGC ACA TAC CCA ATT TCA ACG TAT GCC Ala Pro Gly Val Asn Val Gln Ser Thr Tyr Pro Ile Ser Thr Tyr Ala 195 200 205	960
AGC TTA AAC GGT ACA TCG ATG GCT ACT CCT CAT GTT GCA GGT GCA GCA Ser Leu Asn Gly Thr Ser Met Ala Thr Pro His Val Ala Gly Ala Ala 210 215 220 225	1008
GCC CTT GTT AAA CAA AAG AAC CCA TCT TGG TCC AAT GTA CAA ATC CGC Ala Leu Val Lys Gln Lys Asn Pro Ser Trp Ser Asn Val Gln Ile Arg 230 235 240	1056
AAT CAT CTA AAG AAT ACG GCA ACG AGC TTA GGA AGC ACG AAC TTG TAT Asn His Leu Lys Asn Thr Ala Thr Ser Leu Gly Ser Thr Asn Leu Tyr 245 250 255	1104
GGA AGC GGA CTT GTC AAT GCA GAA GCG GCA ACA CGC Gly Ser Gly Leu Val Asn Ala Glu Ala Ala Thr Arg 260 265	1140

FIG. 5B