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(54) **Title:** BLEACHING COMPOSITIONS COMPRISING PROTEASE ENZYMES

(57) **Abstract**

The invention herein provides bleaching compositions comprising 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 +76 in combination with one or more of the following residues: +99, +101, +103, +104, +107, +123, +27, +105, +109, +126, +128, +135, +156, +166, +195, +197, +204, +206, +210, +216, +217, +218, +222, +260, +265, and/or +274, 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 a bleaching agent.

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BLEACHING COMPOSITIONS COMPRISING PROTEASE ENZYMES

This application is a continuation-in-part application of U.S. Application Serial No. 08/136,626 filed October 14, 1993 and U.S. Application Serial No. 08/237,938, filed May 2, 1994, both incorporated herein by reference in their entirety.

FIELD OF THE INVENTION

The present invention relates to bleaching compositions, especially laundry detergents, and methods which employ one or more protease enzymes which are carbonyl hydrolase variants and a bleaching system with one or more bleaching agents, especially bleach activators.

BACKGROUND OF THE INVENTION

Various types of enzymes have long been conventionally used in laundry detergents to assist in the removal of certain stains from fabrics. These stains are typically associated with lipid and protein soils. The enzymes, however, have proven less effective against other types of soils and stains.

It has also long been known that peroxygen bleaches are effective for stain and/or soil removal from fabrics, but that such bleaches are temperature dependent. At a laundry liquor temperature of 60°C, peroxygen bleaches are only partially effective. As the laundry liquor temperature is lowered below 60°C, peroxygen bleaches become relatively ineffective. As a consequence, there has been a

substantial amount of industrial research to develop effective bleaching systems.

By the present invention, it has been discovered that the combination of novel protease enzymes which are carbonyl hydrolase variants and bleaching agents, especially bleach activators, provide effective stain removal and/or dingy cleanup benefits. It is therefore an object of the present invention to provide bleaching compositions, especially laundry detergent compositions, having improved stain removal and/or dingy cleanup benefits and/or fabric cleaning benefits and/or bleaching properties.

These and other objects of the present invention will be apparent from the detailed description hereinafter.

BACKGROUND ART

U.S. Patent 4,634,551, Burns et al, issued January 6, 1987, discloses amido peroxyacid bleaching compounds and their precursors which are employed in the present invention. See also, U.S. Patent 4,852,989, Burns et al, issued August 1, 1989. U.S. Patent 5,069,809, Lagerwaard et al, issued Dec. 3, 1991 discloses the combination of NOBS bleach activators with LIPOLASE, lipase enzymes. See E.P. Patent 341,947, Lagerwaard, et al, published November 15, 1989 for a discussion of the compatibility problems of lipase enzymes with certain bleaching systems. U.S. Patent 4,545,784, Sanderson, issued October 8, 1985, discloses the absorption of activators onto sodium perborate monohydrate.

SUMMARY OF THE INVENTION

The invention herein provides bleaching compositions 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 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 +76 in combination with one or more of the following residues: +99, +101, +103, +104, +107, +123, +27, +105, +109, +126, +128, +135, +156, +166, +195, +197, +204, +206, +210, +216, +217, +218, +222, +260, +265, and/or +274, where the numbered positions 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); and

(b) a bleaching agent which either is an organic peroxyacid or is a combination of a bleach activator and a peroxygen compound capable of yielding hydrogen peroxide that can react with the activator to form an organic peroxyacid in situ in a bleaching solution formed from the composition; and

(c) one or more cleaning composition materials compatible with the protease enzyme and bleaching agent.

The invention also encompasses a method for cleaning fabrics comprising contacting, preferably with agitation, said fabrics with an aqueous liquor containing said bleaching compositions. 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 aqueous laundry liquor preferably comprises at least about 300 ppm of conventional detergent ingredients, as well as at least about 25 ppm of bleach activator and at least about 25 ppm of bleaching compound. Preferably, said aqueous liquor comprises from about 900 ppm to about 20,000 ppm of the conventional detergent ingredients, from about 100 ppm to about 25,000 ppm of bleaching compound and from about 100 ppm to about 2,500 ppm of said bleach activator.

The conventional detergent ingredients employed in said method comprise from about 1% to about 99.8%, preferably from about 5% to about 80%, of a detersive surfactant. Optionally, detersive compositions can also comprise from about 5% to about 80% of a detergent builder. Other optional detersive ingredients are also encompassed by the fully-formulated detergent/bleach compositions provided by this invention.

All percentages, ratios and proportions are by weight, unless otherwise specified. All documents cited are incorporated herein by reference.

BRIEF DESCRIPTION OF THE DRAWINGS

Figs. 1 A-C depict the DNA and amino acid sequence for *Bacillus amyloliquefaciens* subtilisin and a partial restriction map of this gene (Seq. ID No.6).

Fig. 2 depicts the conserved amino acid residues among subtilisins from *Bacillus amyloliquefaciens* (BPN)' and *Bacillus lentus* (wild-type).

Figs. 3A and 3B 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.7). The second line depicts the amino acid sequence of subtilisin from *Bacillus subtilis* (Seq. ID No.8). The third line depicts the amino acid sequence of subtilisin from *B. licheniformis* (Seq. ID No.9). The fourth line depicts the amino acid sequence of subtilisin from *Bacillus lentus* (also referred to as subtilisin 309 in PCT WO89/06276) (Seq. ID No.10). The symbol * denotes the absence of specific amino acid residues as compared to subtilisin BPN'.

Fig. 4 depicts the construction of plasmid GGA274.

Fig. 5 depicts the construction of GGT274 which is an intermediate to certain expression plasmids used in this application.

Figs. 6A and 6B depict the DNA and amino acid sequence of subtilisin from *Bacillus lentus* (Seq. ID No.11). The mature subtilisin protein is coded by the codons beginning at the codon GCG (334-336) corresponding to Ala.

Figs. 7A and 7B depict the DNA and amino acid sequence of a preferred embodiment of the invention (N76D/S103A/V104I) (Seq. ID No.12). The DNA in this figure has been modified by the methods described to encode aspartate at position 76, alanine at position 103 and isoleucine at position 104. The mature subtilisin variant protein is coded by the codons beginning at the codon GCG (334-336) corresponding to Ala.

Fig. 8 depicts the construction of vector pBCDAICAT.

Fig. 9 depicts the construction of vector pUCCATFNA.

DETAILED DESCRIPTION OF THE INVENTION

The bleaching compositions employed in the present invention provide effective and efficient cleaning of fabrics which thereby removes stains and/or soils from the fabrics. The bleaching systems in combination with the protease enzyme are particularly efficient at removing most types of soils from the fabrics, including protein and lipid

soils, dingy soils, and heavy soil loads, especially from nucleophilic and body soils.

The protease enzymes, bleaching agents (including peroxyacids and bleaching systems) and cleaning composition materials useful herein, including preferred levels, are described in detail hereinafter.

(1) Bleaching Agent

The bleaching compositions herein contain a bleaching agent, which preferably comprises from about 0.5 to about 20 wt.% of the composition. The bleaching agent is either a substantially insoluble, preferably solid, organic peroxyacid, or a bleaching system comprising a bleach activator and a peroxygen bleaching compound capable of yielding hydrogen peroxide, or a combination of both. The peracid which is in the composition, or which is formed by the combination of activator and peroxygen compound, preferably has a corresponding carboxylic acid that has a Hydrophilic-Lipophilic Balance ("H.L.B.") value which ranges from about 3 to about 6.5. Therefore, a method that can be used to characterize the preferred peroxyacids (from activators or as preformed peroxyacids) which are useful in the present invention is the "H.L.B. Scale" such as that described in Davies, J.T., Proc 2nd Internat. Congr. Surface Activity 1,. 426, Butterworths, London (1957), incorporated herein by reference. Such an H.L.B. Scale (Hydrophilic-Lipophilic Balance) has been used in the study of surface-active agents (surfactants) as a means to relate the distribution of a surface-active agent between a hydrophilic (water-like) and a lipophilic (oil-like) phase. In this manner, H.L.B. values can be used as an indication of the lipophilic (hydrophobic) character of the active bleaching species in the wash (i.e., the ability of the peroxyacid to partition out of the wash liquor and concentrate at the soil/fabric interface).

Set forth hereinafter in Table A are H.L.B. values which have been calculated for selected peroxyacids (as the corresponding carboxylic acids). The equation used to calculate the H.L.B. values can be set forth as:

HLB = Sum (Hydrophilic Group Numbers) - Sum (Hydrophobic Group Numbers) + 7.

The values for the Hydrophilic Group Numbers are [-C(O)OH & -N(H)C(O)-=2.1] and the values for the Hydrophobic Group Numbers are [aliphatic/aromatic carbon = 0.475 & aliphatic carbon atoms between polar groups are 1/2 the value of an aliphatic carbon in a hydrocarbon chain = (0.475)/2]. For reference, an H.L.B. value >7 indicates that the material is preferentially water soluble and an H.L.B. value <7 indicates increasing surface-activity and hydrophobicity.

Table A

H.L.B. Values Provided by Various Peroxyacids

Activator/Preformed Peroxyacid	Abbreviation	Peroxyacid	H.L.B. Corresponding Carboxylic Acid
Tetra Acetyl Ethylene Diamine	TAED	$CH_3C(O)OOH$	8.6
DiPeroxyDodecane Dioic Acid	DPDDA	$HOO(O)C(CH_2)_{10}C(O)OOH$	6.5
Nonyl Amide of Peroxy Succinic Acid	NAPSA	$CH_3(CH_2)_8N(H)C(O)(CH_2)_2C(O)OOH$	6.4
BenzoylOxyBenzene Sulfonate	BOBS	$C_6H_5C(O)OOH$	6.3
Nonyl Amide of Peroxy Adipic Acid	NAPAA	$CH_3(CH_2)_8N(H)C(O)(CH_2)_4C(O)OOH$	6.0
NonanoylOxyBenzene Sulfonate	NOBS	$CH_3(CH_2)_7C(O)OOH$	5.3

DecanoylOxyBenzene Sulfonate	DOBS	$CH_3(CH_2)_8C(O)OOH$	4.8
PerLauric Acid	PLA	$CH_3(CH_2)_{10}C(O)OOH$	3.9

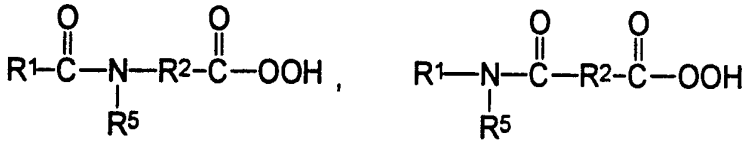
As noted hereinbefore, a preferred range of H.L.B. values (of the corresponding carboxylic acid) for the peroxyacids of the present invention (whether added directly or generated in situ) ranges from about 3.0 to about 6.5. A more preferred range of H.L.B. values (as the carboxylic acid) for the peroxyacids useful in the present invention (whether added directly or generated in situ) range from about 4.0 to 6.5. The most preferred range of H.L.B. values (as the carboxylic acid) for the peroxyacids of the present invention (whether added directly as generated in situ) ranges from about 4.0 to about 6.0.

(a) Peroxyacid

The present invention encompasses detergent compositions comprising an effective amount of the protease enzyme and a bleaching system comprising at least about 0.1%, preferably from about 0.1% to about 50%, by weight, of a substantially insoluble organic peroxyacid. The peroxyacid useful herein preferably comprises from about 0.5 to about 20, more preferably from about 1 to about 10, most preferably from about 2 to about 7, wt.% of the composition.

Preferred organic peroxyacids are selected from the group consisting of 4-nonylamino-4-oxoperoxybutyric acid; 6-(nonyl-amino)-6-oxoperoxycaproic acid; 1,12-diperoxydodecanedioic acid; heptyl sulfonylperpropionic acid; decylsulphonyl perpropionic acid; and heptyl-, octyl-, nonyl-, decyl-sulphonylperbutyric acid; and mixtures thereof.

Of the organic peroxyacids, amidoperoxyacids (amide substituted peroxycarboxylic acids) are preferred. Suitable amidoperoxyacids for use herein are described in U.S. Patents 4,634,551 and 4,686,063, both Burns et al., issued January 6, 1987 and August 11, 1987, respectively, both incorporated herein by reference. Suitable amidoperoxyacids are of the formula:



wherein R^1 is an alkyl, aryl, or alkaryl group containing from about 1 to about 14 carbon atoms (preferably R^1 is an alkyl group containing from about 6 to about 12 carbon atoms), R^2 is an alkylene, arylene or alkarylene group containing from about 1 to about 14 carbon atoms (preferably R^2 is an alkylene group containing from about 1 to about 6 carbon atoms), and R^5 is H or an alkyl, aryl, or alkaryl group containing from about 1 to about 10 carbon atoms (preferably R^5 is H). More preferably, R^1 is an alkyl group containing from about 8 to about 10 carbon atoms, and R^2 is an alkylene group containing from about 2 to about 4 carbon atoms

Also suitable for use herein are peroxyfumarates, which are described in U.S. Patent 4,852,989, Burns et al., issued August 1, 1989, incorporated herein by reference, and sulfone peroxyacids (sulfone peroxycarboxylic acids), which are described in U.S. Patents 4,758,369, 4,824,591, and 5,004,558, all Dryoff et al., issued July 19, 1988, April 25, 1989, and April 2, 1991, respectively, all incorporated herein by reference.

Example I of U.S. Patent 4,686,063 contains one description of the synthesis of NAPSA, from column 8, line 40 to column 9, line 5, and NAPAA, from column 9, line 15 to column 9, line 65. At the end of the amidoperoxyacid synthesis, the reaction is quenched with water, filtered, washed with water to remove some excess sulfuric acid (or other strong acid with which the peroxyacid was made), and filtered again.

The amidoperoxyacid wet cake thus obtained can be contacted with a phosphate buffer solution at a pH between about 3.5 and 6, preferably between about 4 and 5, according to U.S. Patent 4,909,953, Sadlowski et al., issued March 20, 1990, which is incorporated herein by reference.

Other agents for storage stabilization or exotherm control can be added to the amidoperoxyacid before incorporation into the final product. For example, boric acid, an exotherm control agent disclosed in U.S. Patent 4,686,063, Burns, issued August 11, 1987 and incorporated herein, can be mixed with the amidoperoxyacid (which has been washed in phosphate buffer) in about a 2:1 peracid:boric acid ratio. The phosphate buffer washed amidoperoxyacid can also be

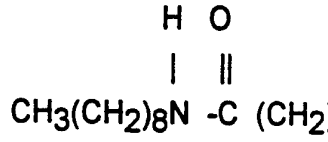
mixed with appropriate amounts of dipicolinic acid and tetrasodium pyrophosphate, a chelating stabilization system. Chelants can optionally be included in the phosphate buffer before contact with the wet cake.

The wet cake is preferably made up of particles with an average particle diameter of from about 0.1 to about 260 microns, preferably from about 10 to about 100 microns, and most preferably from about 30 to about 60 microns. Small particle size NAPAA crystals are desired herein. See U.S. Patent 5,055,218, Getty et al., issued October 8, 1991, which is incorporated herein by reference.

NAPAA filter cake herein is preferably washed twice in phosphate buffer. It has been found that two successive phosphate buffer washes lend optimal stability to NAPAA.

Particulate (solid), organic peroxyacids with a theoretical AvO (available oxygen) of between about 3 and about 12, most preferably between 5 and 7, are preferred.

Most preferred for use herein is NAPAA. Another name for the nonylamide of peroxyadipic acid ("NAPAA") is 6-(nonylamino)-6-oxoperoxycaproic acid. The chemical formula for NAPAA is:



$$CH_3(CH_2)_8N\text{-}C(CH_2)_4COOH$$

The molecular weight of NAPAA is 287.4.

Detergent compositions and bleaching compositions containing NAPAA provide extremely effective and efficient surface bleaching of textiles. Stains and/or soils are removed from the textiles. These compositions are particularly effective at removing dingy soils from textiles.

NAPAA's polar amide or substituted amide moiety results in a peroxyacid which has a very low vapor pressure and thus possesses a low odor profile as well as excellent bleaching performance. It is believed that the polarity of the amide group results in a reduction of vapor pressure of the peroxyacid, and an increase in melting point.

NAPAA can be used directly as a bleaching agent. It has a reduced vapor pressure and a good odor profile in laundry applications.

NAPAA can be prepared by, for example, first reacting NAAA (mononononyl amide of adipic acid), sulfuric acid, and hydrogen peroxide. The reaction product is quenched by addition to ice water followed by filtration, washing with distilled water, and final suction filtration to recover the wet cake. Washing can be continued until the pH of the filtrate is neutral.

It is also preferred that the NAPAA pH (10% solids in water) be between about 4.2 and 4.8. Surprisingly, this pH results in more thermally stable particles.

(b) Bleaching Systems - Bleach Activator and Peroxygen Bleaching Compound

(i) Bleach Activators

The bleach activator for the bleaching systems useful herein preferably has the following structure:

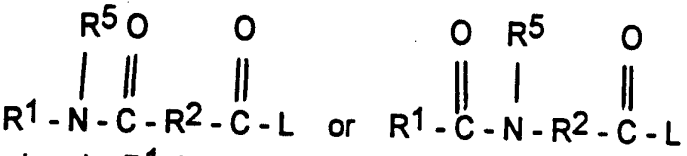


wherein R is an alkyl group containing from about 5 to about 18 carbon atoms wherein the longest linear alkyl chain extending from and including the carbonyl carbon contains from about 6 to about 10 carbon atoms and L is a leaving group, the conjugate acid of which has a pKa in the range of from about 4 to about 13, preferably from about 6 to about 11, most preferably from about 8 to about 11.

L can be essentially any suitable leaving group. A leaving group is any group that is displaced from the bleach activator as a consequence of the nucleophilic attack on the bleach activator by the perhydroxide anion. This, the perhydrolysis reaction, results in the formation of the percarboxylic acid. Generally, for a group to be a suitable leaving group it must exert an electron attracting effect. This facilitates the nucleophilic attach by the perhydroxide anion.

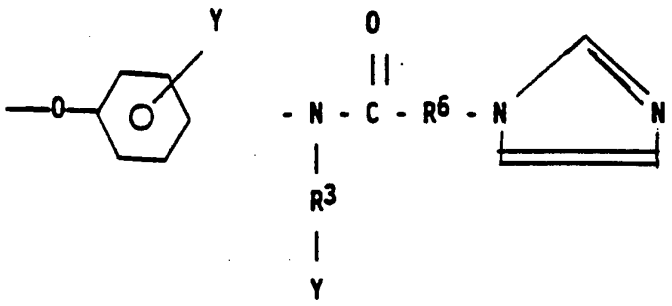
The L group must be sufficiently reactive for the reaction to occur within the optimum time frame (e.g., a wash cycle). However, if L is too reactive, this activator will be difficult to stabilize. These characteristics are generally paralleled by the pKa of the conjugate acid of the leaving group, although exceptions to this convention are known.

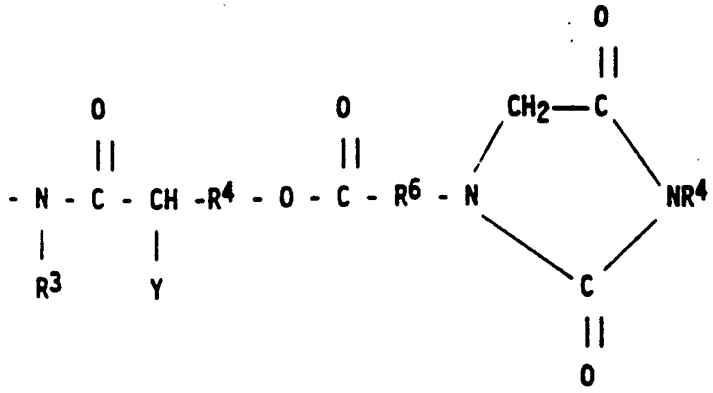
Preferred bleach activators are those of the general formula:

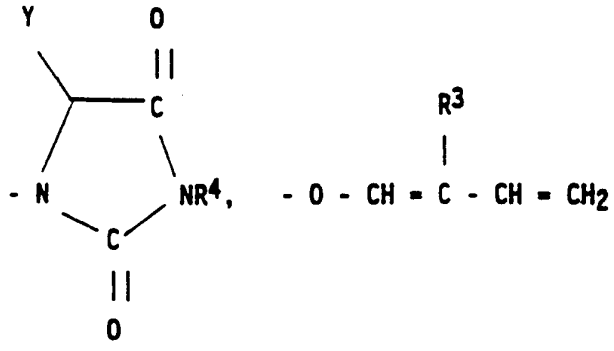


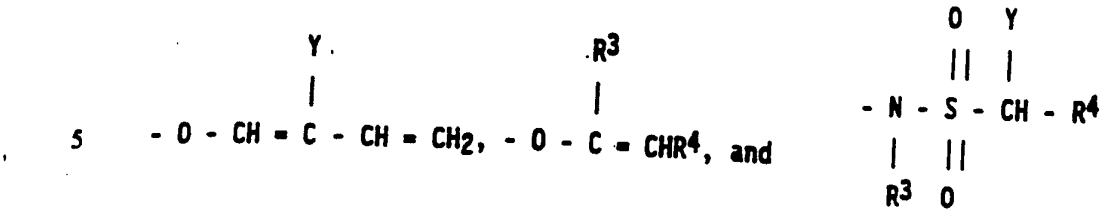
wherein R^1 is an alkyl group containing from about 6 to about 12 carbon atoms, R^2 is an alkylene containing from 1 to about 6 carbon atoms, R^5 is H or alkyl, aryl, or alkaryl containing from about 1 to about 10 carbon atoms, and L is selected from the group consisting of:



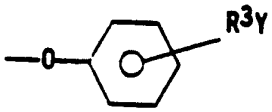






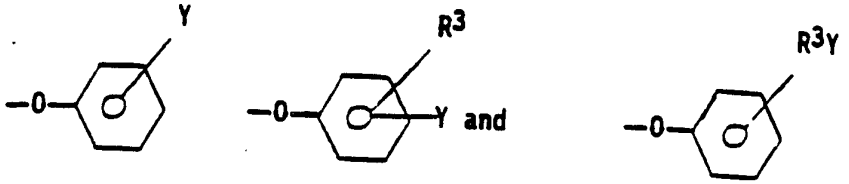


wherein R^6 is an alkylene, arylene, or alkarylene group containing from about 1 to about 14 carbon atoms, R^3 is an alkyl chain containing from about 1 to about 8 carbon atoms, R^4 is H or R^3, and Y is H or a solubilizing group. Y is preferably selected from the group consisting of $-SO_3$-M+, -COO-M+, $-SO_4$-M+, $(-N+R'_3)X-$ and $O\leftarrow N(R'_3)$, wherein R' is an alkyl chain containing from about 1 to about 4 carbon atoms, M is a cation which provides solubility to the bleach activator and X is an anion which provides solubility to the bleach activator. Preferably, M is an alkali metal, ammonium or substituted ammonium cation, with sodium and potassium being most preferred, and X is an anion selected from the group consisting of halide, hydroxide, methylsulfate and acetate anions. More preferably, Y is $-SO_3$-M+ and -COO-M+. It should be noted that bleach activators with a leaving group that does not contain a solubilizing group should be well dispersed in the bleach solution in order to assist in their dissolution. Preferred is:



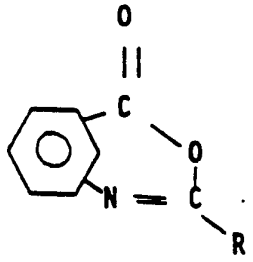
wherein R^3 is as defined above and Y is $-SO_3$-M+ or -COO-M+ wherein M is as defined above.

Especially preferred bleach activators are those wherein R^1 is a linear alkyl chain containing from about 6 to about 12 carbon atoms, R^2 is a linear alkylene chain containing from about 2 to about 6 carbon atoms, R^5 is H, and L is selected from the group consisting of:



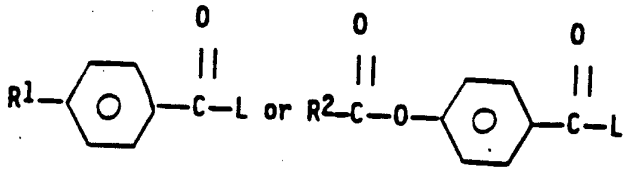
wherein R^3 is as defined above, Y is $-SO_3$-M+ or -COO-M+ and M is as defined above.

A preferred bleach activator is:



wherein R is H, alkyl, aryl or alkaryl. This is described in U.S. Patent 4,966,723, Hodge et al., incorporated by reference herein.

Preferred bleach activators are:

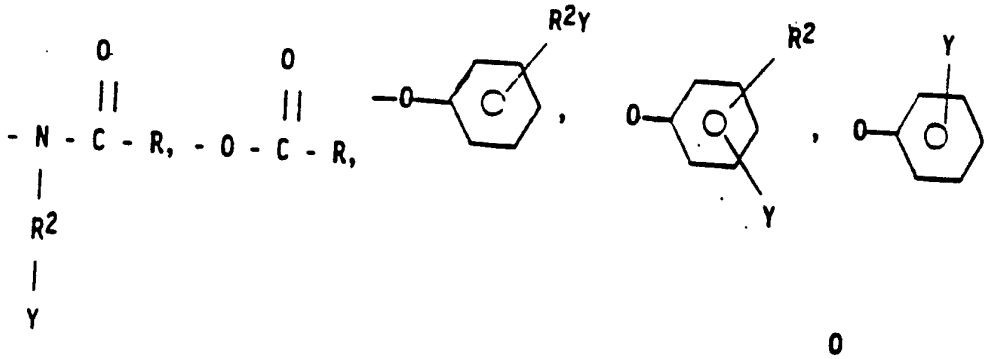


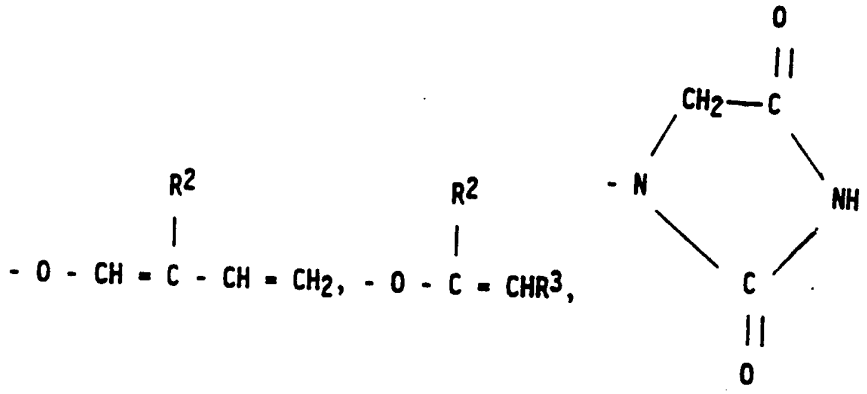
wherein R^1 is H or an alkyl group containing from about 1 to about 6 carbon atoms and R^2 is an alkyl group containing from about 1 to about 6 carbon atoms and L is as defined above.

Preferred bleach activators are also those of the above general formula wherein L is as defined in the general formula, and R^1 is H or an alkyl group containing from about 1 to about 4 carbon atoms.

Even more preferred are bleach activators of the above general formula wherein L is as defined in the general formula and R^1 is a H.

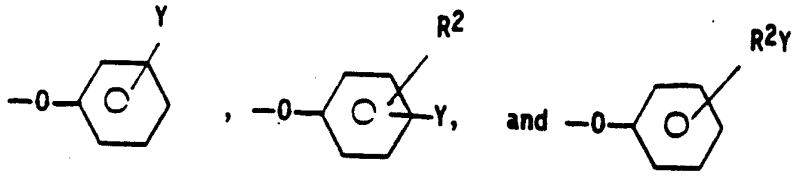
More preferred bleach activators are those of the above general formula wherein R is a linear alkyl chain containing from about 5 to about 9 and preferably from about 6 to about 8 carbon atoms and L is selected from the group consisting of:





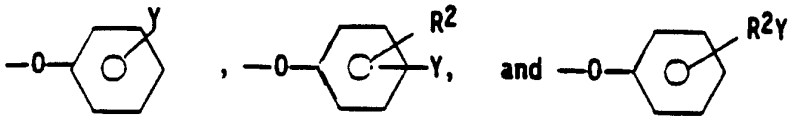
wherein R, R^2, R^3 and Y are as defined above.

Particularly preferred bleach activators are those of the above general formula wherein R is an alkyl group containing from about 5 to about 12 carbon atoms wherein the longest linear portion of the alkyl chain extending from and including the carbonyl carbon is from about 6 to about 10 carbon atoms, and L is selected from the group consisting of:



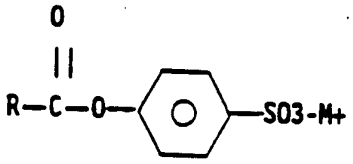
wherein R^2 is an alkyl chain containing from about 1 to about 8 carbon atoms, and Y is -SO-$_3$M+ or -COO-M+ wherein M is an alkali metal, ammonium or substituted ammonium cation.

Especially preferred bleach activators are those of the above general formula wherein R is a linear alkyl chain containing from about 5 to about 9 and preferably from about 6 to about 8 carbon atoms and L is selected from the group consisting of:



wherein R^2 is as defined above and Y is -SO-$_3$M+ or -COO-M+ wherein M is as defined above.

The most preferred bleach activators have the formula:

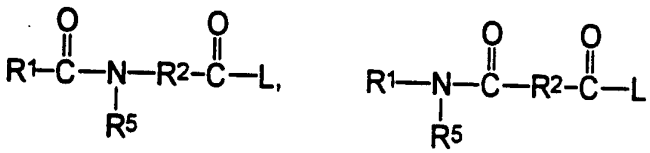


wherein R is a linear alkyl chain containing from about 5 to about 9 and preferably from about 6 to about 8 carbon atoms and M is sodium or potassium.

Preferably, the bleach activator herein is sodium nonanoyloxybenzenesulfonate (NOBS) or sodium benzoyloxybenzenesulfonate (BOBS).

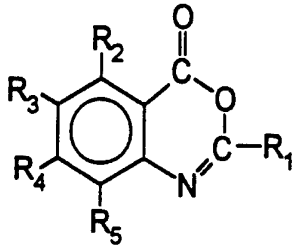
Further particularly preferred for use in the present invention bleaching compositions are the following bleach activators which are particularly safe for use with machines having natural rubber parts. This is believed to be the result of not producing oily diacylperoxide (DAP) species by the perhydrolysis reaction of these amido acid-derived bleach activators, but rather forming insoluble crystalline solid DAP's. These solids are believed to not form a coating film and thus natural rubber parts are not exposed to DAP's for extended periods of time. These preferred bleach activators are members selected from the group consisting of:

a) a bleach activator of the general formula:



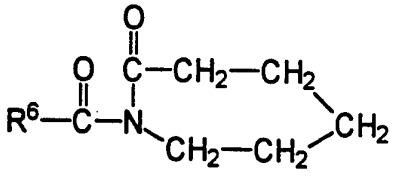
or mixtures thereof, wherein R^1 is an alkyl, aryl, or alkaryl group containing from about 1 to about 14 carbon atoms, R^2 is an alkylene, arylene or alkarylene group containing from about 1 to about 14 carbon atoms, R^5 is H or an alkyl, aryl, or alkaryl group containing from about 1 to about 10 carbon atoms, and L is a leaving group;

b) benzoxazin-type bleach activators of the general formula:



wherein R_1 is H, alkyl, alkaryl, aryl, arylalkyl, and wherein R_2, R_3, R_4, and R_5 may be the same or different substituents selected from H, halogen, alkyl, alkenyl, aryl, hydroxyl, alkoxyl, amino, alkylamino, $COOR_6$ (wherein R_6 is H or an alkyl group) and carbonyl functions;

c) N-acyl caprolactam bleach activators of the formula:



wherein R^6 is H or an alkyl, aryl, alkoxyaryl or alkaryl group containing from 1 to 12 carbons; and

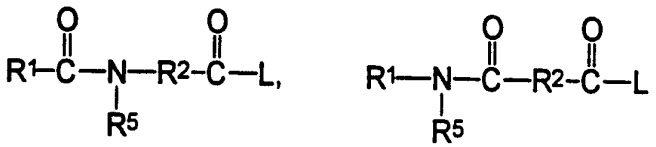
d) mixtures of a), b) and c).

Preferred bleach activators of type a) are those wherein R^1 is an alkyl group containing from about 6 to about 12 carbon atoms, R^2 contains from about 1 to about 8 carbon atoms, and R^5 is H or methyl. Particularly preferred bleach activators are those of the above general formulas wherein R^1 is an alkyl group containing from about 7 to about 10 carbon atoms and R^2 contains from about 4 to about 5 carbon atoms.

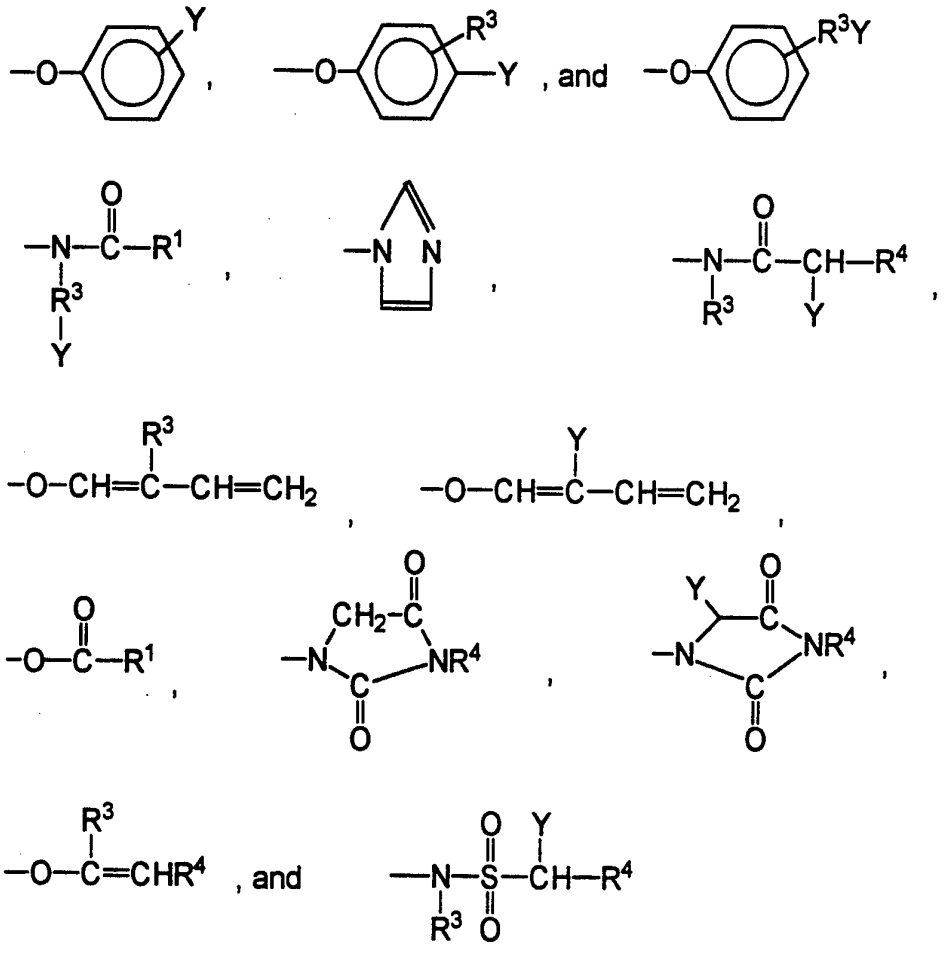
Preferred bleach activators of type b) are those wherein R_2, R_3, R_4, and R_5 are H and R_1 is a phenyl group.

The preferred acyl moieties of said N-acyl caprolactam bleach activators of type c) have the formula R^6-CO- wherein R^6 is H or an alkyl, aryl, alkoxyaryl, or alkaryl group containing from 1 to 12 carbons, preferably from 6 to 12 carbon atoms. In highly preferred embodiments, R^6 is a member selected from the group consisting of phenyl, heptyl, octyl, nonyl, 2,4,4-trimethylpentyl, decenyl and mixtures thereof.

- Amido Derived Bleach Activators - The bleach activators of type a) employed in the present invention are amide substituted compounds of the general formulas:



or mixtures thereof, wherein R^1, R^2 and R^5 are as defined above and L can be essentially any suitable leaving group. Preferred bleach activators are those of the above general formula wherein R^1, R^2 and R^5 are as defined for the peroxyacid and L is selected from the group consisting of:

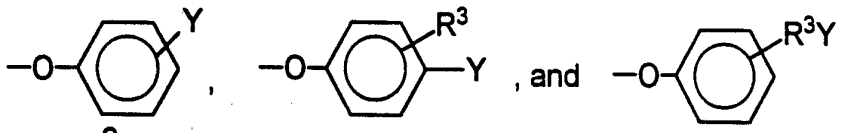


and mixtures thereof, wherein R^1 is an alkyl, aryl, or alkaryl group containing from about 1 to about 14 carbon atoms, R^3 is an alkyl chain

containing from 1 to about 8 carbon atoms, R^4 is H or R^3, and Y is H or a solubilizing group.

The preferred solubilizing groups are $-SO_3^-M^+$, $-CO_2^-M^+$, $-SO_4^-M^+$, $-N^+(R^3)_4X^-$ and $O< N(R^3)_3$ and most preferably $-SO_3^-M^+$ and $-CO_2^-M^+$ wherein R^3 is an alkyl chain containing from about 1 to about 4 carbon atoms, M is a cation which provides solubility to the bleach activator and X is an anion which provides solubility to the bleach activator. Preferably, M is an alkali metal, ammonium or substituted ammonium cation, with sodium and potassium being most preferred, and X is a halide, hydroxide, methylsulfate or acetate anion. It should be noted that bleach activators with a leaving group that does not contain a solubilizing groups should be well dispersed in the bleaching solution in order to assist in their dissolution.

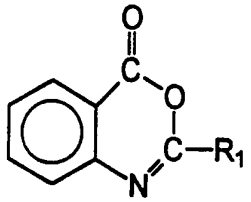
Preferred bleach activators are those of the above general formula wherein L is selected from the group consisting of:



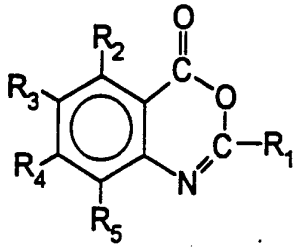
wherein R^3 is as defined above and Y is $-SO_3^-M^+$ or $-CO_2^-M^+$ wherein M is as defined above.

Another important class of bleach activators, including those of type b) and type c), provide organic peracids as described herein by ring-opening as a consequence of the nucleophilic attack on the carbonyl carbon of the cyclic ring by the perhydroxide anion. For instance, this ring-opening reaction in type c) activators involves attack at the caprolactam ring carbonyl by hydrogen peroxide or its anion. Since attack of an acyl caprolactam by hydrogen peroxide or its anion occurs preferably at the exocyclic carbonyl, obtaining a significant fraction of ring-opening may require a catalyst. Another example of ring-opening bleach activators can be found in type b) activators, such as those disclosed in U.S. Patent 4,966,723, Hodge et al, issued Oct. 30, 1990.

- Benzoxazin-type Bleach Activators - Such activator compounds disclosed by Hodge include the activators of the benzoxazin-type, having the formula:

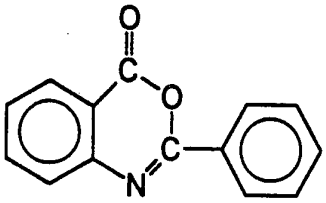


including the substituted benzoxazins of the type



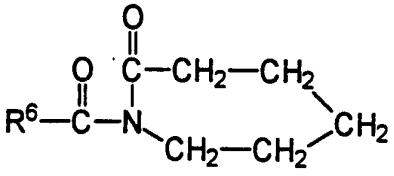
wherein R_1 is H, alkyl, alkaryl, aryl, arylalkyl, and wherein R_2, R_3, R_4, and R_5 may be the same or different substituents selected from H, halogen, alkyl, alkenyl, aryl, hydroxyl, alkoxyl, amino, alkyl amino, $COOR_6$ (wherein R_6 is H or an alkyl group) and carbonyl functions.

A preferred activator of the benzoxazin-type is:



When the activators are used, optimum surface bleaching performance is obtained with washing solutions wherein the pH of such solution is between about 8.5 and 10.5 and preferably between 9.5 and 10.5 in order to facilitate the perhydrolysis reaction. Such pH can be obtained with substances commonly known as buffering agents, which are optional components of the bleaching systems herein.

- N-Acyl Caprolactam Bleach Activators - The N-acyl caprolactam bleach activators of type c) employed in the present invention have the formula:



wherein R^6 is H or an alkyl, aryl, alkoxyaryl, or alkaryl group containing from 1 to 12 carbons. Caprolactam activators wherein the R^6 moiety

contains at least about 6, preferably from 6 to about 12, carbon atoms provide hydrophobic bleaching which affords nucleophilic and body soil clean-up, as noted above. Caprolactam activators wherein R^6 comprises from 1 to about 6 carbon atoms provide hydrophilic bleaching species which are particularly efficient for bleaching beverage stains. Mixtures of hydrophobic and hydrophilic caprolactams, typically at weight ratios of 1:5 to 5:1, preferably 1:1, can be used herein for mixed stain removal benefits.

Highly preferred N-acyl caprolactams are selected from the group consisting of benzoyl caprolactam, octanoyl caprolactam, nonanoyl caprolactam, 3,5,5-trimethylhexanoyl caprolactam, decanoyl caprolactam, undecenoyl caprolactam, and mixtures thereof. Methods for making N-acyl caprolactams are well known in the art.

Contrary to the teachings of U.S. Pat. 4,545,784, the bleach activator is preferably not absorbed onto the peroxygen bleaching compound. To do so in the presence of other organic detersive ingredients could cause safety problems.

The bleach activators of type a), b) or c) will comprise at least about 0.1%, preferably from about 0.1% to about 50%, more preferably from about 1% to about 30%, most preferably from about 3% to about 25%, by weight of bleaching system or detergent composition.

The preferred amido-derived and caprolactam bleach activators herein can also be used in combination with rubber-safe, enzyme-safe, hydrophilic activators such as TAED, typically at weight ratios of amido-derived or caprolactam activators:TAED in the range of 1:5 to 5:1, preferably about 1:1.

The bleaching mechanism generally, and the surface bleaching mechanism in particular, are not completely understood. However, it is generally believed that the bleach activator undergoes nucleophilic attack by a perhydroxide anion, which is generated from the hydrogen peroxide evolved by the peroxygen bleach, to form a peroxycarboxylic acid. This reaction is commonly referred to as perhydrolysis.

When the activators are used, optimum surface bleaching performance is obtained with washing solutions wherein the pH of such solution is between about 8.5 and 10.5 and preferably between 9.5 and 10.5 in order to facilitate the perhydrolysis reaction. Such pH can be

obtained with substances commonly known as buffering agents, which are optional components of the bleaching systems herein.

(ii) The Peroxygen Bleaching Compound

The peroxygen bleaching systems useful herein are those capable of yielding hydrogen peroxide in an aqueous liquor. These compounds are well known in the art and include hydrogen peroxide and the alkali metal peroxides, organic peroxide bleaching compounds such as urea peroxide, and inorganic persalt bleaching compounds, such as the alkali metal perborates, percarbonates, perphosphates, and the like. Mixtures of two or more such bleaching compounds can also be used, if desired.

Preferred peroxygen bleaching compounds include sodium perborate, commercially available in the form of mono-, tri-, and tetra-hydrate, sodium pyrophosphate peroxyhydrate, urea peroxyhydrate, sodium percarbonate, and sodium peroxide. Particularly preferred are sodium perborate tetrahydrate, sodium perborate monohydrate and sodium percarbonate. Percarbonate is especially preferred because it is very stable during storage and yet still dissolves very quickly in the bleaching liquor. It is believed that such rapid dissolution results in the formation of higher levels of percarboxylic acid and, thus, enhanced surface bleaching performance.

Highly preferred percarbonate can be in uncoated or coated form. The average particle size of uncoated percarbonate ranges from about 400 to about 1200 microns, most preferably from about 400 to about 600 microns. If coated percarbonate is used, the preferred coating materials include mixtures of carbonate and sulphate, silicate, borosilicate, or fatty carboxylic acids.

The peroxygen bleaching compound will comprise at least about 0.1%, preferably from about 1% to about 75%, more preferably from about 3% to about 40%, most preferably from about 3% to about 25%, by weight of bleaching system or detergent composition.

The weight ratio of bleach activator to peroxygen bleaching compound in the bleaching system typically ranges from about 2:1 to 1:5. Preferred ratios range from about 1:1 to about 1:3.

The molar ratio of hydrogen peroxide yielded by the peroxygen bleaching compound to the bleach activator is greater than about 1.0,

more preferably greater than about 1.5, and most preferably from about 2.0 to about 10. Preferably, the bleaching compositions herein comprise from about 0.5 to about 20, most preferably from about 1 to about 10, wt.% of the peroxygen bleaching compound.

The bleach activator/bleaching compound systems herein are useful per se as bleaches. However, such bleaching systems are especially useful in compositions which can comprise various detersive adjuncts such as surfactants, builders and the like.

(2) 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. 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 +76 in combination with one or more of the following residues +99, +101, +103, +104, +107, +123, +27, +105, +109, +126, +128, +135, +156, +166, +195, +197, +204, +206, +210, +216, +217, +218, +222, +260, +265, and/or +274, 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 enzyme useful in the present invention compositions comprise replacement of amino acid residue +76 in combination with one or more additional modifications. Preferably the variant protease enzymes useful for the present invention comprise the substitution, deletion or insertion of amino acid residues in the following combinations: 76/99; 76/101; 76/103; 76/104; 76/107; 76/123; 76/99/101; 76/99/103; 76/99/104; 76/101/103; 76/101/104; 76/103/104; 76/104/107; 76/104/123; 76/107/123; 76/99/101/103; 76/99/101/104; 76/99/103/104; 76/101/103/104; 76/103/104/123; 76/104/107/123; 76/99/101/103/104; 76/99/103/104/123;

76/99/101/103/104/123; 76/103/104/128; 76/103/104/260; 76/103/104/265; 76/103/104/197; 76/103/104/105; 76/103/104/135; 76/103/104/126; 76/103/104/107; 76/103/104/210; 76/103/104/126/265; and/or 76/103/104/222. 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: 76/99; 76/104; 76/99/104; 76/103/104; 76/104/107; 76/101/103/104; 76/99/101/103/104 and 76/101/104 of *B. amyloliquefaciens* subtilisin.

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 76, 99, 101, 103, 104, 107, 123, 27, 105, 109, 126, 128, 135, 156, 166, 195, 197, 204, 206, 210, 216, 217, 218, 222, 260, 265 and/or 274, in *Bacillus amyloliquefaciens* 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. 6 (Seq. ID No. 11).

These variant DNA sequences encode the insertion or substitution of the amino acid residue 76 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: 76/99; 76/101; 76/103; 76/104; 76/107; 76/123; 76/99/101; 76/99/103; 76/99/104; 76/101/103; 76/101/104; 76/103/104; 76/104/107; 76/104/123; 76/107/123; 76/99/101/103; 76/99/101/104; 76/99/103/104; 76/101/103/104; 76/103/104/123; 76/104/107/123; 76/99/101/103/104; 76/99/103/104/123; 76/99/101/103/104/123; 76/103/104/128; 76/103/104/260; 76/103/104/265; 76/103/104/197; 76/103/104/105; 76/103/104/135; 76/103/104/126; 76/103/104/107; 76/103/104/210; 76/103/104/126/265; and/or 76/103/104/222. Most preferably the variant DNA sequences encode for the modification of the

following combinations of residues: 76/99; 76/104; 76/99/104; 76/103/104; 76/104/107; 76/101/103/104; 76/99/101/103/104 and 76/101/104. 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.). Thoughout this application reference is made to various amino acids by way of common one- and three-letter codes. Such codes are identified in Dale, J.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 76 including D, H, E, G, F, K, P and N; substitutions at position 99 including D, T, N, Q, G and S; substitutions at position 101 including G, D, K, L, A, E, S and R; substitutions at position 103 including Q, T, D, E, Y, K, G, R, S, and A; substitutions at position 104 including all nineteen naturally-occurring amino acids; substitutions at position 107 including V, L, M, Y, G, E, F, T, S, A, N and I; substitutions at position 123 including N, T, I, G, A, C, and S; substitutions at position 27 including K, N, C, V and T; substitutions at position 105 including A, D, G, R and N; substitutions at position 107 including A, L, V, Y, G, F, T, S and A; substitutions at position 109 including S, K, R, A, N and D; substitutions at position 126 including A, F, I, V and G; substitutions at position 128 including G, L and A; substitutions at position 135 including A, F, I, S and V; substitutions at position 156 including D, E, A, G, Q and K; substitutions at position 166 including all nineteen naturally-occurring amino acids; substitutions at position 195 including E; substitutions at position 197 including E; substitutions at position 204 including A, G, C, S and D; substitutions at position 206 including L, Y, N, D and E;

substitutions at position 210 including L, I, S, C and F; substitutions at position 216 including V, E, T and K; substitutions at position 217 including all nineteen naturally-occurring amino acids; substitutions at position 218 including S, A, G, T and V; substitutions at position 222 including all nineteen naturally-occurring amino acids; substitutions at position 260 including P, N, G, A, S, C, K and D; substitutions at position 265 including N, G, A, S, C, K, Y and H; and substitutions at position 274 including A and S. 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 Residue	Preferred Amino Acid to be Substituted/Inserted
+76	D,H
+99	D,T,N,G
+101	R,G,D,K,L,A,E
+103	A,Q,T,D,E,Y,K,G,R
+104	I,Y,S,L,A,T,G,F,M,W,D,V,N
+107	V,L,Y,G,F,T,S,A,N
+123	S,T,I
+27	K
+105	A,D
+109	S,K,R
+126	A,I,V,F
+128	G,L
+135	I,A,S
+156	E,D,Q
+166	D,G,E,K,N,A,F,I,V,L
+195	E
+197	E
+204	A,G,C
+206	L
+210	I,S,C
+216	V

+217	H,I,Y,C,A,G,F,S,N,E,K
+218	S
+222	A,Q,S,C,I,K
+260	P,A,S,N,G
+265	N,A,G,S
+274	A,S

These protease enzymes containing *in vitro* mutations in *B. lentus* subtilisin at an amino acid residue equivalent to +76 in *Bacillus amyloliquefaciens* subtilisin produces subtilisin variants exhibiting altered stability (e.g., modified autoproteolytic stability) over precursor subtilisins. (See Tables IV and VI.)

Also, *in vitro* mutation at residues equivalent to +99, +101, +103, +104, +107, +123, +27, +105, +109, +126, +128, +135, +156, +166, +195, +197, +204, +206, +210, +216, +217, +218, +222, +260, +265, and/or +274 in *Bacillus amyloliquefaciens* subtilisin, alone or in combination with each other and in any combination with +76 mutations, produce subtilisin variants exhibiting altered proteolytic activity, altered thermal stability, altered pH profile, altered substrate specificity and/or altered performance characteristics.

Carbonyl hydrolases are protease enzymes which hydrolyze compounds containing

O
||
C-X

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 proteases 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 (RE 34,606), 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. 3 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 (RE 34,606), 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 (such as a subtilisin) include naturally-occurring carbonyl hydrolases (subtilisin) and recombinant carbonyl hydrolases (subtilisin). 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 (subtilisin) rather than manipulation of the precursor carbonyl hydrolase (subtilisin) enzyme *per se*. Suitable methods for such manipulation of the precursor DNA sequence include methods disclosed herein, as well as methods known to those skilled in the art (see, for example, EP 0 328299, WO89/06279 and the US patents and applications already referenced herein).

Specific residues corresponding to position +76 in combination with one or more of the following positions +99, +101, +103, +104, +107, +123, +27, +105, +109, +126, +128, +135, +156, +166, +195, +197, +204, +206, +210, +216, +217, +218, +222, +260, +265 and/or +274 of *Bacillus amyloliquefaciens* subtilisin are identified herein for mutation. Preferably the modified residues are selected from the following combinations: 76/99; 76/101; 76/103; 76/104; 76/107; 76/123; 76/99/101; 76/99/103; 76/99/104; 76/101/103; 76/101/104; 76/103/104; 76/104/107; 76/104/123; 76/107/123; 76/99/101/103; 76/99/101/104;

76/99/103/104; 76/101/103/104; 76/103/104/123; 76/104/107/123; 76/99/101/103/104; 76/99/103/104/123; 76/99/101/103/104/123; 76/103/104/128; 76/103/104/260; 76/103/104/265; 76/103/104/197; 76/103/104/105; 76/103/104/135; 76/103/104/126; 76/103/104/107; 76/103/104/210; 76/103/104/126/265; and/or 76/103/104/222; and most preferably are 76/99; 76/104; 76/99/104; 76/103/104; 76/104/107; 76/101/103/104; 76/99/101/103/104 and 76/101/104. These amino acid position numbers refer to those assigned to the mature *Bacillus amyloliquefaciens* subtilisin sequence presented in Fig. 1. 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. 2 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. 3 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. 2.

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), the preferred subtilisin precursor enzyme herein, or the subtilisin referred to as PB92 (EP O 328 299), which is highly homologous to the preferred *Bacillus lentus* subtilisin. The amino acid sequences of certain of these subtilisins are aligned in Figs. 3A and 3B 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 +76 is asparagine (N) in both *B. amyloliquefaciens* and *B. lentus* subtilisins. In the preferred subtilisin variant useful in the invention, however, the amino acid equivalent to +76 in *Bacillus amyloliquefaciens* subtilisin is substituted with aspartate (D). A comparison of all the amino acid residues identified herein for substitution versus the preferred substitution for each such position is provided in Table II for illustrative purposes.

Table II

	+76	+99	+101	+103	+104	+107	+123
B. amyloliquefaciens (wild-type)	N	D	S	Q	Y	I	N

B. lentus (wild-type)	N	S	S	S	V	I	N
Most Preferred Substitution	D	D	R	A	I/Y	V	S

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.

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

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 with 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 08/212,291, 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.

"Prosequence" refers to a sequence of amino acids bound to the N-terminal portion of the mature form of a carbonyl hydrolase which when removed results in the appearance of the "mature" form of the carbonyl hydrolase. Many proteolytic enzymes are found in nature as translational proenzyme products and, in the absence of post-translational processing, are expressed in this fashion. A preferred prosequence for producing carbonyl hydrolase variants, specifically subtilisin variants, is the putative prosequence of *Bacillus amyloliquefaciens* subtilisin, although other subtilisin prosequences may be used. In the Examples, the putative prosequence from the subtilisin from *Bacillus lentus* (ATCC 21536) is used.

A "signal sequence" or "presequence" refers to any sequence of amino acids bound to the N-terminal portion of a carbonyl hydrolase or to the N-terminal portion of a prohydrolase which may participate in the secretion of the mature or pro forms of the hydrolase. This definition of signal sequence is a functional one, meant to include all those amino acid sequences encoded by the N-terminal portion of the subtilisin gene or other secretable carbonyl hydrolases which participate in the effectuation of the secretion of subtilisin or other carbonyl hydrolases under native conditions. The protease enzymes useful for the present invention utilize such sequences to effect the secretion of the carbonyl hydrolase variants as described herein. A preferred signal sequence used in the Examples comprises the first seven amino acid residues of

the signal sequence from *Bacillus subtilis* subtilisin fused to the remainder of the signal sequence of the subtilisin from *Bacillus lentus* (ATCC 21536).

A "prepro" form of a carbonyl hydrolase variant consists of the mature form of the hydrolase having a prosequence operably linked to the amino terminus of the hydrolase and a "pre" or "signal" sequence operably linked to the amino terminus of the prosequence.

"Expression vector" refers to a DNA construct containing a DNA sequence which is operably linked to a suitable control sequence capable of effecting the expression of said DNA in a suitable host. Such control sequences include a promoter to effect transcription, an optional operator sequence to control such transcription, a sequence encoding suitable mRNA ribosome binding sites and sequences which control termination of transcription and translation. The vector may be a plasmid, a phage particle, or simply a potential genomic insert. Once transformed into a suitable host, the vector may replicate and function independently of the host genome, or may, in some instances, integrate into the genome itself. In the present specification, "plasmid" and "vector" are sometimes used interchangeably as the plasmid is the most commonly used form of vector at present. However, included herein are such other forms of expression vectors which serve equivalent functions and which are, or become, known in the art.

The "host cells" used in the present invention generally are procaryotic or eucaryotic hosts which preferably have been manipulated by the methods disclosed in U.S. Patent 4,760,025 (RE 34,606) to render them incapable of secreting enzymatically active endoprotease. A preferred host cell for expressing subtilisin is the *Bacillus* strain BG2036 which is deficient in enzymatically active neutral protease and alkaline protease (subtilisin). The construction of strain BG2036 is described in detail in U.S. Patent 5,264,366. Other host cells for expressing subtilisin include *Bacillus subtilis* I168 (also described in U.S. Patent 4,760,025 (RE 34,606) and U.S.Patent 5,264,366, the disclosure of which are incorporated herein by reference), as well as any suitable *Bacillus* strain such as *B. licheniformis, B. lentus,* etc.

Host cells are transformed or transfected with vectors constructed using recombinant DNA techniques. Such transformed host cells are

capable of either replicating vectors encoding the carbonyl hydrolase variants or expressing the desired carbonyl hydrolase variant. In the case of vectors which encode the pre- or prepro-form of the carbonyl hydrolase variant, such variants, when expressed, are typically secreted from the host cell into the host cell medium.

"Operably linked, " when describing the relationship between two DNA regions, simply means that they are functionally related to each other. For example, a presequence is operably linked to a peptide if it functions as a signal sequence, participating in the secretion of the mature form of the protein most probably involving cleavage of the signal sequence. A promoter is operably linked to a coding sequence if it controls the transcription of the sequence; a ribosome binding site is operably linked to a coding sequence if it is positioned so as to permit translation.

The genes encoding the naturally-occurring precursor carbonyl hydrolase may be obtained in accord with the general methods known to those skilled in the art. The methods generally comprise synthesizing labeled probes having putative sequences encoding regions of the hydrolase of interest, preparing genomic libraries from organisms expressing the hydrolase, and screening the libraries for the gene of interest by hybridization to the probes. Positively hybridizing clones are then mapped and sequenced. The *B. lentus* gene used in the Examples is cloned as described in Example 1 of U.S. Patent 5,185,258, the disclosure of which is incorporated herein. The BPN' gene used in the Examples is cloned as described in Example 1 in RE 34,606, the disclosure of which is incorporated herein.

The cloned carbonyl hydrolase is then used to transform a host cell in order to express the hydrolase. The hydrolase gene is then ligated into a high copy number plasmid. This plasmid replicates in hosts in the sense that it contains the well-known elements necessary for plasmid replication: a promoter operably linked to the gene in question (which may be supplied as the gene's own homologous promotor if it is recognized, i.e., transcribed, by the host), a transcription termination and polyadenylation region (necessary for stability of the MRNA transcribed by the host from the hydrolase gene in certain eucaryotic host cells) which is exogenous or is supplied by the endogenous terminator region

of the hydrolase gene and, desirably, a selection gene such as an antibiotic resistance gene that enables continuous cultural maintenance of plasmid-infected host cells by growth in antibiotic-containing media. High copy number plasmids also contain an origin of replication for the host, thereby enabling large numbers of plasmids to be generated in the cytoplasm without chromosomal limitations. However, it is within the scope herein to integrate multiple copies of the hydrolase gene into host genome. This is facilitated by procaryotic and eucaryotic organisms which are particularly susceptible to homologous recombination.

The genes used in the present examples are a natural *B. lentus* gene and a natural *B. amyloliquefaciens* gene. Alternatively, a synthetic gene encoding a naturally-occurring or mutant precursor carbonyl hydrolase (subtilisin) may be produced. In such an approach, the DNA and/or amino acid sequence of the precursor hydrolase (subtilisin) is determined. Multiple, overlapping synthetic single-stranded DNA fragments are thereafter synthesized, which upon hybridization and ligation produce a synthetic DNA encoding the precursor hydrolase. An example of synthetic gene construction is set forth in Example 3 of U.S. Patent 5,204,015, the disclosure of which is incorporated herein by reference.

Once the naturally-occurring or synthetic precursor carbonyl hydrolase gene has been cloned, a number of modifications are undertaken to enhance the use of the gene beyond synthesis of the naturally-occurring precursor carbonyl hydrolase. Such modifications include the production of recombinant carbonyl hydrolases as disclosed in U.S. Patent 4,760,025 (RE 34,606) and EPO Publication No. 0 251 446 and the production of carbonyl hydrolase variants described herein.

The following cassette mutagenesis method may be used to facilitate the construction and identification of the carbonyl hydrolase variants useful in the present invention, although other methods including site-directed mutagenesis may be used. First, the naturally-occurring gene encoding the hydrolase is obtained and sequenced in whole or in part. Then the sequence is scanned for a point at which it is desired to make a mutation (deletion, insertion or substitution) of one or more amino acids in the encoded enzyme. The sequences flanking this point are evaluated for the presence of restriction sites for replacing a short

segment of the gene with an oligonucleotide pool which when expressed will encode various mutants. Such restriction sites are preferably unique sites within the hydrolase gene so as to facilitate the replacement of the gene segment. However, any convenient restriction site which is not overly redundant in the hydrolase gene may be used, provided the gene fragments generated by restriction digestion can be reassembled in proper sequence. If restriction sites are not present at locations within a convenient distance from the selected point (from 10 to 15 nucleotides), such sites are generated by substituting nucleotides in the gene in such a fashion that neither the reading frame nor the amino acids encoded are changed in the final construction. Mutation of the gene in order to change its sequence to conform to the desired sequence is accomplished by M13 primer extension in accord with generally known methods. The task of locating suitable flanking regions and evaluating the needed changes to arrive at two convenient restriction site sequences is made routine by the redundancy of the genetic code, a restriction enzyme map of the gene and the large number of different restriction enzymes. Note that if a convenient flanking restriction site is available, the above method need be used only in connection with the flanking region which does not contain a site.

Once the naturally-occurring DNA or synthetic DNA is cloned, the restriction sites flanking the positions to be mutated are digested with the cognate restriction enzymes and a plurality of end termini-complementary oligonucleotide cassettes are ligated into the gene. The mutagenesis is simplified by this method because all of the oligonucleotides can be synthesized so as to have the same restriction sites, and no synthetic linkers are necessary to create the restriction sites.

As used herein, proteolytic activity is defined as the rate of hydrolysis of peptide bonds per milligram of active enzyme. Many well known procedures exist for measuring proteolytic activity (K. M. Kalisz, "Microbial Proteinases," Advances in Biochemical Engineering/Biotechnology, A. Fiechter ed., 1988). In addition to or as an alternative to modified proteolytic activity, the variant enzymes of the present invention may have other modified properties such as K_m, K_{cat}, K_{cat}/K_m ratio and/or modified substrate specificity and/or modified pH activity profile. These enzymes can be tailored for the particular

substrate which is anticipated to be present, for example, for hydrolytic processes such as laundry uses.

One objective can be to secure a variant carbonyl hydrolase having altered proteolytic activity as compared to the precursor carbonyl hydrolase, since increasing such activity (numerically larger) enables the use of the enzyme to more efficiently act on a target substrate. Also of interest are variant enzymes having altered thermal stability and/or altered substrate specificity as compared to the precursor. Preferably the carbonyl hydrolase to be mutated is a subtilisin. Specific amino acids useful to obtain such results in subtilisin-type carbonyl hydrolases at residues equivalent to +76, +99, +101, +103, +104, +107, +123, +27, +105, +109, +126, +128, +135, +156, +166, +195, +197, +204, +206, +210, +216, +217, +218, +222, +260, +265 and/or +274 or any combination thereof in *Bacillus amyloliquefaciens* subtilisin are presented in the Examples. In some instances, lower proteolytic activity may be desirable. Conversely, in some instances it may be desirable to increase the proteolytic activity of the variant enzyme versus its precursor. Additionally, increases or decreases (alteration) of the stability of the variant, whether alkaline or thermal stability, may be desirable. Increases or decreases in K_{cat}, K_m or K_{cat}/K_m are specific to the substrate used to determine these kinetic parameters.

Also, it has been determined that residues equivalent to +76 in combination with a number of other modifications in subtilisin are important in modulating overall stability and/or proteolytic activity of the enzyme. Thus, as set forth in the Examples, the Asparagine (N) in *Bacillus lentus* subtilisin at equivalent position +76 can be substituted with Aspartate (D) in the preferred protease enzymes in combination with modification of one or more of the following amino acid residues +99, +101, +103, +104, +107, +123, +27, +105, +109, +126, +128, +135, +156, +166, +195, +197, +204, +206, +210, +216, +217, +218, +222, +260, +265 and/or +274 to produce enhanced stability and/or enhanced activity of the resulting mutant enzyme.

The most preferred protease enzymes useful in this invention are set forth in the Examples. These include the following specific combinations of substituted residues: N76D/S99D; N76D/V104I; N76D/S99D/V104I; N76D/S103A/V104I; N76D/V104I/I107V;

N76D/V104Y/I107V and N76D/S101R/S103A/V104I. These substitutions are preferably made in *Bacillus lentus* (recombinant or native-type) subtilisin, although the substitutions may be made in any *Bacillus* subtilisin.

Based on the results obtained with this and other variant subtilisins, it is apparent that residues in carbonyl hydrolases (preferably subtilisin) equivalent to positions +76, +99, +101, +103, +104, +107, +123, +27, +105, +109, +126, +128, +135, +156, +166, +195, +197, +204, +206, +210, +216, +217, +218, +222, +260, +265 and/or +274 in *Bacillus amyloliquefaciens* are important to the proteolytic activity, performance and/or stability of these enzymes and the cleaning or wash performance of such variant enzymes.

The following is presented by way of example for manufacturing protease enzymes useful in the present invention compositions.

Protease Manufacture Example

Construction for the Expression of GG36 Gene in *B. subtilis*

The cloning and the construction for expression of the subtilisin gene from *B. lentus* is performed essentially the same as that described in U.S. Patent 5,185,258. The plasmid GGA274 (described in Fig. 4 herein) is further modified in the following manner, as shown in Fig. 5. The PstI site that is introduced during the construction of the GGA274 plasmid is removed by the oligonucleotide directed mutagenesis described below, with an oligonucleotide having the following sequence: 5' GAAGCTGCAACTCGTTAAA 3' (Seq. ID No.1). The underlined "A" residue eliminates the recognition sequence of restriction enzyme PstI and changes the corresponding amino acid residue from alanine to threonine at position 274. Threonine at position 274 is the wild-type residue originally found in the cloned *B. lentus* subtilisin gene sequences. The DNA segment encoding subtilisin is excised from the plasmid GGA274 or its derivatives (GGT274 shown in Fig. 5) by EcoRI and BamHI digest. The DNA fragment is subcloned back into Bacteriophage M13-based vectors, such as MP19, for mutagenesis. After mutagenesis, the EcoRI and HindIII digest, followed by cloning, are performed to move the mutated subtilisin gene back into an expression plasmid like GGA274 for the expression and the recovery of mutated subtilisin proteins.

Oligonucleotide-Directed Mutagenesis

Oligonucleotide-directed mutagenesis is performed as described in Zoller, M. et al. (1983), Methods Enzymol., **100**:468-500. As an example, a synthetic oligonucleotide of the sequence 5' GCTGCTCTAGACAATTCG 3' (Seq. ID No.2) is used to change the amino acid residue at position 76 from asparagine (N) to aspartic acid (D), or N76D. The underlined "G" and "C" residues denote changes from the wild-type gene sequence. The CA keeps the leucine at position +75 and changes the amino acid sequence to introduce an XbaI recognition site of the XbaI restriction enzyme (TCTAGA), while the change at GAC changes asparagine at +76 to aspartate.

For mutagenesis at positions 99, 101, 103 and 104, different oligonucleotides can be used depending on the combination of mutations desired. For example, an oligonucleotide of the sequence 5' GTATTAGGGGCGGACGGTCGAGGCGCCATCAGCTCGATT 3'(Seq. ID No.3) is used to simultaneously make the following changes: S99D; S101R; S103A and V104I in a single subtilisin molecule. Similarly, oligonucleotides of the sequence 5' TCAGGTTCGGTCTCGAGCGTTGCCCAAGGATTG 3' (Seq. ID No.4) and 5' CACGTTGCTAGCTTGAGTTTAG 3' (Seq. ID No.5) are utilized to generate I107V and N123S, respectively. Again, the underlined residues denote changes from wild-type sequences which produce desired changes either in amino acid sequences or restriction enzyme recognition sequences.

Proteolytic Activity of Subtilisin Variants

Following the methods of Oligonucleotide-Directed Mutagenesis hereinbefore, the variants listed in Table III are made. Proteolytic activity of each of these subtilisin variants is shown in Table III. The kinetic parameters k_{cat}, K_M, and k_{cat}/K_M are measured for hydrolysis of the synthetic peptide substrate succinyl-L-Ala-L-Ala-L-Pro-L-Phe-p-nitroanilide using the method described in P. Bonneau et al. (1991) J. Am. Chem. Soc., Vol. 113, No. 3, p. 1030. Briefly, a small aliquot of subtilisin variant stock solution is added to a 1 cm cuvette containing substrate dissolved in 0.1M Tris-HCL buffer, pH 8.6, and thermostated at 25°C. The reaction progress is followed spectrophotometrically by monitoring the absorbance of the reaction product p-nitroaniline at 410

nm. Kinetic parameters are obtained by using a non-linear regression algorithm to fit the reaction velocity and product concentration for each reaction to the Michaelis-Menten equation.

Table III

Kinetic Parameters k_{cat}, K_M and k_{cat}/K_M
Measured for Bacillus lentus Subtilisin and Variants

Protease #	Enzyme Variants	k_{cat} (s^{-1})	K_M (M)	k_{cat}/K_M ($s^{-1}M^{-1}$)
-	B. lentus Subtilisin	170	0.00078	$2.18x10^5$
-	N76D	219	0.0008	$2.74x10^5$
1	N76D/S99D	88	0.00061	$1.44x10^5$
2	N76D/S101R	371	0.0013	$2.85x10^5$
3	N76D/S103A	400	0.0014	$2.86x10^5$
4	N76D/V104I	459	0.0011	$4.17x10^5$
5	N76D/I107V	219	0.0011	$1.99x10^5$
6	N76D/N123S	115	0.0018	$6.40x10^4$
7	N76D/S99D/S101R	146	0.00038	$3.84x10^5$
8	N76D/S99D/S103A	157	0.0012	$1.31x10^5$
9	N76D/S99D/V104I	247	0.00097	$2.55x10^5$
10	N76D/S101R/S103A	405	0.00069	$5.90x10^5$
11	N76D/S101R/V104I	540	0.00049	$1.10x10^6$
12	N76D/S103A/V104I	832	0.0016	$5.20x10^5$
13	N76D/V104I/I107V	497	0.00045	$1.10x10^6$
14	N76D/V104Y/I107V	330	0.00017	$1.90x10^6$
15	N76D/V104I/N123S	251	0.0026	$9.65x10^4$
16	N76D/I107V/N123S	147	0.0035	$4.20x10^4$
17	N76D/S99D/S101R/S103A	242	0.00074	$3.27x10^5$
18	N76D/S99D/S101R/V104I	403	0.00072	$5.60x10^5$
19	N76D/S99D/S103A/V104I	420	0.0016	$2.62x10^5$
20	N76D/S101R/S103A/V104I	731	0.00065	$1.12x10^6$
21	N76D/S103A/V104I/N123S	321	0.0026	$1.23x10^5$
22	N76D/V104I/I107V/N123S	231	0.003	$7.70x10^4$
23	N76D/S99D/S101R/S103A/V104I	624	0.00098	$6.37x10^5$
24	N76D/S99D/S103A/V104I/N123S	194	0.0043	$4.51x10^4$
25	N76D/S99D/S101R/S103A/V104I/N123S	311	0.0023	$1.35x10^5$

The results listed in Table III indicate that all of the subtilisin variants tested retain proteolytic activity. Further, detailed analysis of the data reveal that proteolytic activity is significantly altered for *Bacillus lentus* subtilisin by the various combinations of substitutions at amino

acid residues equivalent to positions 76, 99, 101, 103, 104, 107 and 123 in *Bacillus amyloliquefaciens*.

Thermal Stability of Subtilisin Variants

A comparison of thermal stability observed for *Bacillus lentus* subtilisin and the variants of the present invention made by the process of Oligonucleotide-Directed Mutagenesis hereinbefore is shown in Table IV. Purified enzyme, 15 ug/ml in 0.1 M glycine 0.01% Tween-80 pH 10.0, with or without 50 mM $CaCl_2$, is aliquotted into small tubes and incubated at 10°C for 5 minutes, 10°C to 60°C over 1 minute, and 60°C for 20 minutes. Tubes are then placed on ice for 10 minutes. Aliquots from the tubes are assayed for enzyme activity by addition to 1 cm cuvettes containing 1.2 mM of the synthetic peptide substrate succinyl-L-ala-L-Ala-L-Pro-L-Phe-p-nitroanilide dissolved in 0.1 M tris-HCL buffer, pH 8.6, thermostatted at 25°C. The initial linear reaction velocity is followed spectrophotometrically by monitoring the absorbance of the reaction product p-nitroaniline at 410 nm as a function of time. Data are presented as percent activity prior to heating. The results listed in Table IV indicate that a vast majority of variants exhibit thermal stability comparable to *Bacillus lentus* subtilisin (24 out of 26) in the test condition with 50mM $CaCl_2$ added. In the test condition without 50mM $CaCl_2$ added, a vast majority of variants (19 out of 26) are significantly more stable than *Bacillus lentus* subtilisin. Further, the variants N76D/S99D, N76D/V104I, N76D/S99D/V104I, N76D/S103A/V104I, N76D/V104I/I107V, N76D/V104Y/I107V and N76D/S101R/S103A/V104I are significantly more stable than the single substitution variant N76D in the test condition without 50mM $CaCl_2$ added.

Table IV

Thermal Stability Measured for *Bacillus lentus* Subtilisin and Variants At pH 10, 60°C, +/- 50mM $CaCl_2$ Added

	% Initial Activity Remaining	
Enzyme	-$CaCl_2$	+$CaCl_2$
B. lentus Subtilisin	2	96
N76D	34	97
N76D/S99D	49	98
N76D/S101R	0	82
N76D/S103A	26	92

N76D/V104I	58	98
N76D/I107V	32	96
N76D/N123S	0	97
N76D/S99D/S101R	30	100
N76D/S99D/S103A	36	100
N76D/S99D/V104I	48	97
N76D/S101R/S103A	26	100
N76D/S101R/V104I	38	100
N76D/S103A/V104I	58	100
N76D/V104I/I107V	60	97
N76D/V104Y/I107V	48	74
N76D/V104I/N123S	0	98
N76D/I107V/N123S	16	100
N76D/S99D/S101R/S103A	38	100
N76D/S99D/S101R/V104I	33	100
N76D/S99D/S103A/V104I	38	98
N76D/S101R/S103A/V104I	40	99
N76D/S103A/V104I/N123S	1	98
N76D/V104I/I107V/N123S	3	99
N76D/S99D/S101R/S103A/V104I	36	99
N76D/S99D/S103A/V104I/N123S	2	95
N76D/S99D/S101R/S103A/V104I/N123S	0	100

Oligonucleotide-Directed Mutagenesis with Single-Stranded DNA Template Generated from Phagemid

A. Construction of *B. lentus* Variants

The mutagenesis protocol is essentially the same as described above in Oligonucleotide-Directed Mutagenesis. The single-stranded DNA template is generated by phagemid method. To construct the phagemid vector for generating the single-stranded DNA template we first construct the vector pBCDAICAT. The flow chart of vector construction is outlined in Figure 8. First, the *C1al* to *C1al* fragment encoding the CAT gene from pC194 plasmid (Horinouchi, S. and Weisblum, B., J. Bacteriol., **150**:8-15, 1982) is cloned into the *Accl* site of polylinker region of pUC19 (New England BioLabs, Beverly, MA) to make plasmid pUCCHL and the *EcoRI-DraI* 0.6 KB fragment from the 5' end of the GG36DAI encoding DNA is cloned into the *EcoRI* and *EcoRV* sites of

pBSKS plasmid (Stratagene, Inc., San Diego, CA) to make pBC2SK5. The single *EcoRI* site of the plasmid pBC2SK5 is eliminated by *EcoRI* digestion, followed by filling in catalyzed-by-T4 DNA polymerase, and religation to generate the plasmid pBC2SK-5R which does not have the *EcoRI* site. The *EcoRI-DraI* fragment which is cloned into pBCSK-5R is isolated as a *PstI-HindIII* fragment and cloned into the *PstI-HindIII* site of the pUCCHL (part of the polylinker of pUC19) to generate plasmid pUCCHL5R. The encoding sequence of GG36DAI gene is excised as an *EcoRI-BamHI* fragment and cloned into the *EcoRI-BamHI* sites of pUCCHL5R to make pUCCAT. The large *EcoRI-HindIII* fragment of pUCCAT is then cloned into the *EcoRI* and *HindIII* sites of BS2KS+ to generate the plasmid pBCDAICAT.

To generate single-stranded DNA, *E. coli*-containing pBCDAICAT are infected with phage R408 (obtained from Stratagene, San Diego, CA) following the protocol described in Russel, M., Kidd, S. and Kelley, M.R., GENE **45**:333-338, 1986. Once the single-stranded DNA template is available, standard mutagenesis methods as described above in Oligonucleotide-Directed Mutogenesis are carried out. The construction of certain mutants is detailed below for illustrative purposes.

For the construction of *B. lentus* (GG36) N76D/S103A/V104I/L217H, an *EcoRI-BamHI* DNA fragment encoding GG36 N76D/S103A/V104I is used in the construction of pUCCAT (see Fig. 8) to generate the plasmid pBCDAICAT. After the single-stranded DNA template is made following the protocol described above, a mutagenesis primer with the following sequence

* *** ** x *C1aI*

5' TAT GCC AGC CAC AAC GGT ACT TCG ATG GCT 3' (Seq. ID No.13)

is used to make the L217H. As before, the underlined residues denote the nucleotide changes that are made and the x *C1aI* indicates that the existing *C1aI* site is eliminated after the mutagenesis. The mutagenesis protocol is as described in Oligonucleotide-Directed Mutogenesis hereinbefore. After the mutagenesis, plasmid DNA is first screened for the elimination of the *C1aI* site and those clones missing the *C1aI* site are subjected to DNA sequence analysis to verify the DNA sequence which made the L to H change at the 217th amino acid residue.

B. Construction of BPN' Variants and their Expression in *B. subtilis*

The construction of *B. amyloliquefaciens* (BPN') N76D/Q103A/Y104I/Y217L is done in a similar fashion except in two consecutive steps. N76D is first introduced into BPN' Y217L to make BPN' N76D/Y217L and a second mutagenesis is done to convert BPN' N76D/Y217L to BPN' N76D/Q103A/Y104I/Y217L. To generate the single-stranded DNA template for the first mutagenesis, an *EcoRI-BamHI* fragment encoding BPN' Y217L subtilisin (derived from the Y217L plasmid described in Wells, J., et al., PNAS, 84, 5167, 1087) is used to construct a plasmid pUCCATFNA (see Fig. 9). The pUCCATFNA plasmid containing BPN' Y217L is used to construct the pBCFNACAT plasmid (Fig. 9). Single-stranded DNA is generated as described above. To generate BPN' N76D/Y217L, an oligonucleotide primer with the sequence

* *** ** *XbaI*

5' C ACA GTT GCG GCT CTA GAT AAC TCA ATC GGT G 3'
(Seq. ID No.14)

is used to generate the change N76D. Single-stranded DNA is then prepared from the pBCFNACAT plasmid containing the BPN' N76D/Y217L (the pBCFNACAT plasmid after N76D mutagenesis) and mutagenized with another oligonucleotide with the sequence

* *** ** x *PvuII*

5' GCT GAC GGT TCC GGC GCT ATT AGT TGG ATC ATT 3'
(Seq. ID No.15)

to obtain BPN' N76D/Q103A/Y104I/Y217L. All steps involved in the cloning, the single-stranded DNA preparation, the mutagenesis, and the screening for mutants are carried out as described above. Expression of the BPN' gene and its variants are achieved by integrating plasmid DNA (pBCFNACAT containing the different variants' BPN' gene) directly into a protease-deficient strain of *Bacillus subtilis* as described in RE 34,606.

Numerous variants are made as per the teachings of these Protease Manufacture Examples. Kinetics data and stability data are generated for such variants. The kinetics data are generated using the methods described hereinbefore and are provided in Table V. The stability data are generated as detailed herein. Results are shown in Table VI.

Thermal Stability Assay Procedure

Purified enzyme is buffer-exchanged into 0.1 M glycine pH 10.0, 0.01% Tween-80 by applying the enzyme to a column consisting of

Sephadex G-25 equilibrated with this buffer and eluting the enzyme from the column using the same buffer.

To a tube containing 0.1 M glycine, 0.01% Tween-80 pH 10.0 thermostatted at 60°C, the buffer-exchanged enzyme is added to give a final enzyme concentration of 15 ug/ml.

Aliquots are removed from the 60°C incubation at various times and immediately assayed for enzyme activity by addition to a 1 cm cuvette containing 1.2 mM of the synthetic peptide substrate succinyl-L-Ala-L-Ala-L-Pro-L-Phe-p-nitroanilide dissolved in 0.1 M tris-HCL buffer, pH 8.6, thermostatted at 25°C. The initial linear reaction velocity is followed spectrophotometrically by monitoring the absorbance of the reaction product p-nitroaniline at 410 nm as a function of time.

Half-life, which is the length of time required for 50% enzyme inactivation, is determined from the first-order plot of reaction velocity as a function of the time of incubation at 60°C.

The data are presented in Table VI as percent of the half-life determined for *Bacillus lentus* subtilisin (GG36) under identical conditions.

Table V

Enzyme	kcat (s^{-1})	KM (mM)	kcat/KM ($s^{-1}M^{-1}$)
B. lentus subtilisin	170	0.78	2.20E+05
N76D/S103G/V104I*	380	1.4	2.70E+05
N76D/S103A/V104F	730	0.33	2.20E+06
N76D/S103A/V104N	790	2.8	2.80E+05
N76D/S103A/V104S	170	0.83	2.00E+05
N76D/S103A/V104T	370	1.9	2.00E+05
N76D/S103A/V104W	880	0.31	2.80E+06
N76D/S103A/V104Y	690	0.5	1.40E+06
K27R/N76D/V104Y/N123S	500	1.2	4.20E+05
N76D/S101G/S103A/V104I*	620	1.3	4.80E+05
N76D/S103A/V104I/S105A*	550	1.3	4.20E+05
N76D/S103A/V104I/S105D*	440	1.7	2.60E+05
N76D/S103A/V104T/I107A*	120	5.7	2.10E+04
N76D/S103A/V104T/I107L*	310	3.2	9.70E+04
N76D/S103A/V104I/L126A	90	2.2	4.10E+04
N76D/S103A/V104I/L126F	180	1.9	9.50E+04
N76D/S103A/V104I/L126I	100	2.4	4.20E+04
N76D/S103A/V104I/L126V	64	3.2	2.00E+04
N76D/S103A/V104I/S128G*	560	1.7	3.30E+05
N76D/S103A/V104I/S128L*	430	3.8	1.10E+05

N76D/S103A/V104I/L135A	140	0.76	1.80E+05
N76D/S103A/V104I/L135F	390	0.69	5.70E+05
N76D/S103A/V104I/L135I	110	0.73	1.50E+05
N76D/S103A/V104I/L135V	140	0.86	1.60E+05
N76D/S103A/V104I/S156E*	170	2.6	6.50E+04
N76D/S103A/V104I/S166D*	160	3.5	4.60E+04
N76D/S103A/V104I/D197E	510	1.4	3.60E+05
N76D/S103A/V104I/N204A*	530	1.1	4.80E+05
N76D/S103A/V104I/N204G*	580	1.4	4.10E+05
N76D/S103A/V104I/N204C*	370	1.3	2.90E+05
N76D/S103A/V104I/P210I*	500	1.2	4.20E+05
N76D/S103A/V104I/L217H*	80	0.63	1.30E+05
N76D/S103A/V104I/M222A	70	3.1	2.30E+04
N76D/S103A/V104I/M222S	80	3.1	2.60E+04
N76D/S103A/V104I/T260P	660	1.5	4.40E+05
N76D/S103A/V104I/S265N	590	1.3	4.50E+05
K27R/N76D/V104Y/I107V/N123S	220	1.4	1.60E+05
K27R/N76D/V104Y/N123S/D197E	430	1.1	3.90E+05
K27R/N76D/V104Y/N123S/N204C	400	1.1	3.60E+05
K27R/N76D/V104Y/N123S/Q206L	440	1.2	3.70E+05
K27R/N76D/V104Y/N123S/S216V	440	1.2	3.70E+05
K27R/N76D/V104Y/N123S/N218S	760	0.98	7.80E+05
K27R/N76D/V104Y/N123S/T260P	410	1.2	3.40E+05
K27R/N76D/V104Y/N123S/T274A	390	1	3.90E+05
N76D/S103A/V104I/L126F/S265N	170	2.1	8.10E+04
N76D/S103A/V104I/S156E/S166D*	40	6.3	6.40E+03
K27R/N76D/V104Y/N123S/G195E/G197E	410	0.98	4.20E+05
K27R/N76D/V104Y/N123S/G195E/N218S	540	0.66	8.20E+05
K27R/N76D/V104Y/N123S/D197E/N218S	770	0.79	9.80E+05
K27R/N76D/V104Y/N123S/N204C/N218S	610	0.99	6.20E+05
K27R/N76D/V104Y/N123S/Q206L/N218S	580	0.78	7.40E+05
K27R/N76D/V104Y/N123S/N218S/T260P	660	1	6.60E+05
K27R/N76D/V104Y/N123S/N218S/T274A	590	0.89	6.60E+05
K27R/N76D/V104Y/Q109S/N123S/N218S/T274A	520	1	5.20E+05
K27R/N76D/V104Y/N123S/G195E/D197E/N218S	460	0.65	7.10E+05
B. amyloliquefaciens subtilisin (BPN')	50	0.14	3.60E+05
BPN'-N76D/Y217L*	380	0.46	8.30E+05

* These mutants are made as per Oligonucleotide-Directed Mutagenesis with Single-Stranded DNA Template Generated from Phagemid, all others made as per Oligonucleotide-Directed Mutagenesis, hereinbefore.

Table VI

Enzyme	Thermal Stability (% half-life of native enzyme)
B. lentus subtilisin	100
N76D	590
N76D/S99D	840
N76D/S103A	390
N76D/V104I	660
N76D/I107V	710
N76D/N123S	70
N76D/S99D/S101R	610
N76D/S99D/S103A	590
N76D/S99D/V104I	910
N76D/S101R/S103A	930
N76D/S101R/V104I	500
N76D/S103A/V104I	460
N76D/S103G/V104I*	370
N76D/S103A/V104F	480
N76D/S103A/V104N	230
N76D/S103A/V104S	230
N76D/S103A/V104T	370
N76D/S103A/V104W	280
N76D/S103A/V104Y	400
N76D/V104I/I107V	940
N76D/V104Y/I107V	820
N76D/V104I/N123S	80
N76D/I107V/N123S	150
K27R/N76D/V104Y/N123S	100
N76D/S99D/S101R/S103A	570
N76D/S99D/S101R/V104I	1000
N76D/S99D/S103A/V104I	680
N76D/S101G/S103A/V104I*	390
N76D/S101R/S103A/V104I	470
N76D/S103A/V104I/S105A*	360
N76D/S103A/V104I/S105D*	370
N76D/S103A/V104T/I107A*	270
N76D/S103A/V104T/I107L*	230
N76D/S103A/V104I/N123S	110
N76D/V104I/I107V/N123S	220
N76D/S103A/V104I/L126A	270
N76D/S103A/V104I/L126F	950
N76D/S103A/V104I/L126I	410
N76D/S103A/V104I/L126V	320
N76D/S103A/V104I/S128G*	640
N76D/S103A/V104I/S128L*	760
N76D/S103A/V104I/L135A	230
N76D/S103A/V104I/L135F	200
N76D/S103A/V104I/L135I	510

N76D/S103A/V104I/L135V	500
N76D/S103A/V104I/S156E*	120
N76D/S103A/V104I/S166D*	590
N76D/S103A/V104I/D197E	460
N76D/S103A/V104I/N204A*	230
N76D/S103A/V104I/N204G*	240
N76D/S103A/V104I/N204C*	500
N76D/S103A/V104I/P210I*	1370
N76D/S103A/V104I/L217H*	60
N76D/S103A/V104I/M222A	520
N76D/S103A/V104I/M222S	490
N76D/S103A/V104I/T260P	490
N76D/S103A/V104I/S265N	360
K27R/N76D/V104Y/I107V/N123S	210
K27R/N76D/V104Y/N123S/D197E	120
K27R/N76D/V104Y/N123S/N204C	110
K27R/N76D/V104Y/N123S/Q206L	380
K27R/N76D/V104Y/N123S/S216V	140
K27R/N76D/V104Y/N123S/N218S	270
K27R/N76D/V104Y/N123S/T260P	40
K27R/N76D/V104Y/N123S/T274A	60
N76D/S99D/S101R/S103A/V104I	590
N76D/S99D/S103A/V104I/N123S	110
N76D/S103A/V104I/L126F/S265N	810
N76D/S103A/V104I/S156E/S166D*	220
K27R/N76D/V104Y/N123S/G195E/G197E	90
K27R/N76D/V104Y/N123S/G195E/N218S	250
K27R/N76D/V104Y/N123S/D197E/N218S	270
K27R/N76D/V104Y/N123S/N204C/N218S	460
K27R/N76D/V104Y/N123S/Q206L/N218S	1400
K27R/N76D/V104Y/N123S/N218S/T260P	310
K27R/N76D/V104Y/N123S/N218S/T274A	180
N76D/S99D/S101R/S103A/V104I/N123S	90
K27R/N76D/V104Y/Q109S/N123S/N218S/T274	230
K27R/N76D/V104Y/N123S/G195E/D197E/N21	240
B. amyloliquefaciens subtilisin (BPN')	100
BPN'-N76D/Y217L*	420

* These mutants are made as per Oligonucleotide-Directed Mutagenesis with Single-Stranded DNA Template Generated from Phagemid, all others made as per Oligonucleotide-Directed Mutagenesis, hereinbefore.

(3) Cleaning Composition Materials:

The cleaning compositions of the present invention also comprise, in addition to the bleaching agent and protease enzyme described hereinbefore, one or more cleaning composition materials compatible with the protease enzyme. The term "cleaning composition 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 composition 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); 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 O_2 ("AvO_2") from the 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. All parts, percentages and ratios used herein are by weight unless otherwise specified.

(i) Optional Detersive Enzymes

The compositions and methods herein are effective with all manner of detersive enzymes in addition to the specified protease enzymes. Optional detersive enzymes useful in the present invention may be included for a wide variety of fabric laundering purposes, including removal of protein-based, carbohydrate-based, or triglyceride-based stains, for example, and for the prevention of fugitive dye transfer. The enzymes to be incorporated include other proteases, amylases, lipases, cellulases, and peroxidases, as well as mixtures thereof. Other types of enzymes may also be included. They may be of any suitable origin, such as vegetable, animal, bacterial, fungal and yeast origin. However, their choice is governed by several factors such as pH-activity and/or stability optima, thermostability, stability versus active detergents, builders and so on. In this respect bacterial or fungal enzymes are preferred, such as bacterial amylases and proteases, and fungal cellulases.

Enzymes are normally incorporated at levels sufficient to provide up to about 50 mg by weight, more typically about 0.01 mg to about 10 mg, of active enzyme per gram of detergent composition. Stated otherwise, an effective amount of the optional enzymes employed in the present invention will typically comprise at least about 0.001%, preferably from about 0.001% to about 5%, more preferably from about 0.001% to about 1%, most preferably from about 0.01% to about 1%, by weight of detergent composition.

Suitable examples of optional proteases are the subtilisins which are obtained from particular strains of B.subtilis and B.licheniforms. Another suitable protease is a modified bacterial serine protease enzyme obtained from Bacillus subtilis or Bacillus licheniformis, having maximum activity throughout the pH range of 8-12, developed and sold

by Novo Industries A/S under the registered trade name ESPERASE. The preparation of this enzyme and analogous enzymes is described in British Patent Specification No. 1,243,784 of Novo. Other proteolytic enzymes that are commercially available include those sold under the tradenames ALCALASE and SAVINASE by Novo Industries A/S (Denmark) and MAXATASE by International Bio-Synthetics, Inc. (The Netherlands). Still other proteases include Protease A (see European Patent Application 130,756, published January 9, 1985) and Protease B (see European Patent Application Serial No. 87303761.8, filed April 28, 1987, and European Patent Application 130,756, Bott et al, published January 9, 1985), and what is called herein "Protease C", which is a triple variant of an alkaline serine protease from Bacillus in which tyrosine replaced valine at position 104, serine replaced asparagine at position 123, and alanine replaced threonine at position 274. Protease C is described in EP 90915958.4, corresponding to WO 91/06637, published May 16, 1991, which is incorporated herein by reference. Genetically modified variants, particularly of Protease C, are also included herein.

Amylases include, for example, a-amylases described in British Patent Specification No. 1,296,839 (Novo), RAPIDASE, International Bio-Synthetics, Inc. and TERMAMYL, Novo Industries.

The cellulases usable in the present invention include both bacterial or fungal cellulase. Preferably, they will have a pH optimum of between 5 and 9.5. Suitable cellulases are disclosed in U.S. Patent 4,435,307, Barbesgoard et al, issued March 6, 1984, which discloses fungal cellulase produced from Humicola insolens and Humicola strain DSM1800 or a cellulase 212-producing fungus belonging to the genus Aeromonas, and cellulase extracted from the hepatopancreas of a marine mollusk (Dolabella Auricula Solander). Suitable cellulases are also disclosed in GB-A-2.075.028; GB-A-2.095.275 and DE-OS-2.247.832.

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. See also lipases in Japanese Patent Application 53-20487, laid open to public inspection on February 24, 1978. 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 commercial lipases include Amano-CES, lipases ex Chromobacter viscosum, e.g. Chromobacter viscosum var. lipolyticum NRRLB 3673, commercially available from Toyo Jozo Co., Tagata, Japan; and further Chromobacter viscosum lipases from U.S. Biochemical Corp., U.S.A. and Disoynth Co., The Netherlands, and lipases ex Pseudomonas gladioli. The LIPOLASE enzyme, derived from the fungus Humicola lanuginosa and expressed in Aspergillus oryzae as host and commercially available from Novo (see also E.P. Patent 341,947) is a preferred lipase for use herein.

Peroxidase enzymes are used in combination with oxygen sources, e.g., percarbonate, perborate, persulfate, hydrogen peroxide, etc. They are used for "solution bleaching," i.e. to prevent transfer of dyes or pigments removed from sub-strates during wash operations to other substrates in the wash solution. Peroxidase enzymes are known in the art, and include, for example, horseradish peroxidase, ligninase, and haloperoxidase such as chloro- and bromo-peroxidase. Peroxidase-containing detergent compositions are disclosed, for example, in PCT International Application WO 89/099813, published October 19, 1989, by O. Kirk, assigned to Novo Industries A/S.

A wide range of enzyme materials and means for their incorporation into synthetic detergent granules is also disclosed in U.S. Patent 3,553,139, issued January 5, 1971 to McCarty et al. Enzymes are further disclosed in U.S. Patent 4,101,457, Place et al, issued July 18, 1978, and in U.S. Patent 4,507,219, Hughes, issued March 26, 1985, both. Enzyme materials useful for liquid detergent formulations, and their incorporation into such formulations, are disclosed in U.S. Patent 4,261,868, Hora et al, issued April 14, 1981. Enzymes for use in detergents can be stabilized by various techniques. Enzyme stabilization techniques are disclosed and exemplified in U.S. Patent 4,261,868, issued April 14, 1981 to Horn, et al, U.S. Patent 3,600,319, issued August 17, 1971 to Gedge, et al, and European Patent Application Publication No. 0199405, Application No. 86200586.5, published October 29, 1986, Venegas. Enzyme stabilization systems

are also described, for example, in U.S. Patents 4,261,868, 3,600,319, and 3,519,570.

(ii) Enzyme Stabilizers

The enzymes employed herein can be stabilized by the presence of water-soluble sources of calcium ions in the finished compositions which provide calcium ions to the enzymes. Additional stability can be provided by the presence of various other art-disclosed stabilizers, especially borate species: see Severson, U.S. 4,537,706, cited above. Typical detergents, especially liquids, will comprise from about 1 to about 30, preferably from about 2 to about 20, more preferably from about 5 to about 15, and most preferably from about 8 to about 12, millimoles of calcium ion per liter of finished composition. This can vary somewhat, depending on the amount of enzyme present and its response to the calcium ions. The level of calcium ion should be selected so that there is always some minimum level available for the enzyme, after allowing for complexation with builders, fatty acids, etc., in the composition. Any water-soluble calcium salt can be used as the source of calcium ion, including, but not limited to, calcium chloride, calcium sulfate, calcium malate, calcium hydroxide, calcium formate, and calcium acetate. A small amount of calcium ion, generally from about 0.05 to about 0.4 millimoles per liter, is often also present in the composition due to calcium in the enzyme slurry and formula water. In solid detergent compositions the formulation may include a sufficient quantity of a water-soluble calcium ion source to provide such amounts in the laundry liquor. In the alternative, natural water hardness may suffice.

The compositions herein may also optionally, but preferably, contain various additional stabilizers including silicate coatings and, especially borate-type stabilizers. Typically, such stabilizers will be used at levels in the compositions from about 0.25% to about 10%, preferably from about 0.5% to about 5%, more preferably from about 0.75% to about 3%, by weight of boric acid or other borate compound capable of forming boric acid in the composition (calculated on the basis of boric acid). Boric acid is preferred, although other compounds such as boric oxide, borax and other alkali metal borates (e.g., sodium ortho-, meta- and pyroborate, and sodium pentaborate) are suitable.

Substituted boric acids (e.g., phenylboronic acid, butane boronic acid, and p-bromo phenylboronic acid) can also be used in place of boric acid.

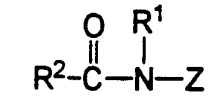
(iii) Detersive Surfactant

The amount of detersive surfactant included in the fully-formulated detergent compositions afforded by the present invention can vary from about 1% to about 99.8% depending upon the particular surfactants used and the effects desired. Preferably, the detersive surfactants comprise from about 5% to about 80% by weight of the detergent ingredients.

The detersive surfactant can be nonionic, anionic, ampholytic, zwitterionic, or cationic. Mixtures of these surfactants can also be used. Preferred detergent compositions comprise anionic detersive surfactants or mixtures of anionic surfactants with other surfactants, especially nonionic surfactants.

Nonlimiting examples of surfactants useful herein include the conventional C_{11}-C_{18} alkyl benzene sulfonates and primary, secondary, and random alkyl sulfates, the C_{10}-C_{18} alkyl alkoxy sulfates, the C_{10}-C_{18} alkyl polyglycosides and their corresponding sulfated polyglycosides, C_{12}-C_{18} alpha-sulfonated fatty acid esters, C_{12}-C_{18} alkyl and alkyl phenol alkoxylates (especially ethoxylates and mixed ethoxy/propoxy), C_{12}-C_{18} betaines and sulfobetaines ("sultaines"), C_{10}-C_{18} amine oxides, C_8-C_{24} sarcosinates (especially oleoyl sarcosinate) and the like. Other conventional useful surfactants are listed in standard texts.

One particular class of adjunct nonionic surfactants especially useful herein comprises the polyhydroxy fatty acid amides of the formula:

(I) 

wherein: R^1 is H, C_1-C_8 hydrocarbyl, 2-hydroxyethyl, 2-hydroxypropyl, or a mixture thereof, preferably C_1-C_4 alkyl, more preferably C_1 or C_2 alkyl, most preferably C_1 alkyl (i.e., methyl); and R^2 is a C_5-C_{32} hydrocarbyl moiety, preferably straight chain C_7-C_{19} alkyl or alkenyl, more preferably straight chain C_9-C_{17} alkyl or alkenyl, most preferably

straight chain C_{11}-C_{19} alkyl or alkenyl, or mixture thereof; and Z is a polyhydroxyhydrocarbyl moiety having a linear hydrocarbyl chain with at least 2 (in the case of glyceraldehyde) or at least 3 hydroxyls (in the case of other reducing sugars) directly connected to the chain, or an alkoxylated derivative (preferably ethoxylated or propoxylated) thereof. Z preferably will be derived from a reducing sugar in a reductive amination reaction; more preferably Z is a glycityl moiety. Suitable reducing sugars include glucose, fructose, maltose, lactose, galactose, mannose, and xylose, as well as glyceraldehyde. As raw materials, high dextrose corn syrup, high fructose corn syrup, and high maltose corn syrup can be utilized as well as the individual sugars listed above. These corn syrups may yield a mix of sugar components for Z. It should be understood that it is by no means intended to exclude other suitable raw materials. Z preferably will be selected from the group consisting of $-CH_2-(CHOH)_n-CH_2OH$, $-CH(CH_2OH)-(CHOH)_{n-1}-CH_2OH$, $-CH_2-(CHOH)_2(CHOR')(CHOH)-CH_2OH$, where n is an integer from 1 to 5, inclusive, and R' is H or a cyclic mono- or poly-saccharide, and alkoxylated derivatives thereof. Most preferred are glycityls wherein n is 4, particularly $-CH_2-(CHOH)_4CH_2OH$.

In Formula (I), R^1 can be, for example, N-methyl, N-ethyl, N-propyl, N-isopropyl, N-butyl, N-isobutyl, N-2-hydroxy ethyl, or N-2-hydroxy propyl. For highest sudsing, R^1 is preferably methyl or hydroxyalkyl. If lower sudsing is desired, R^1 is preferably C_2-C_8 alkyl, especially n-propyl, iso-propyl, n-butyl, iso-butyl, pentyl, hexyl and 2-ethyl hexyl.

R^2-CO-N< can be, for example, cocamide, stearamide, oleamide, lauramide, myristamide, capricamide, palmitamide, tallowamide, etc.

Another class of nonionic surfactants particularly useful in the present invention are condensates of ethylene oxide with a hydrophobic moiety to provide a surfactant having an average hydrophilic-lipophilic balance (HLB) in the range from 5 to 17, preferably from 6 to 14, more preferably from 7 to 12. The hydrophobic (lipophilic) moiety may be aliphatic or aromatic in nature and the length of the polyoxyethylene group which is condensed with any particular hydrophobic group can be readily adjusted to yield a water-soluble

compound having the desired degree of balance between hydrophilic and hydrophobic elements.

Especially preferred nonionic surfactants of this type are the C_9-C_{15} primary alcohol ethoxylates containing 3-8 moles of ethylene oxide per mole of alcohol, particularly the C_{14}-C_{15} primary alcohols containing 6-8 moles of ethylene oxide per mole of alcohol, the C_{12}-C_{15} primary alcohols containing 3-5 moles of ethylene oxide per mole of alcohol, and mixtures thereof.

(iv) Detersive Builders

Optional detergent ingredients employed in the present invention contain inorganic and/or organic detersive builders to assist in mineral hardness control. If used, these builders comprise from about 5% to about 80% by weight of the detergent compositions.

Inorganic detersive builders include, but are not limited to, the alkali metal, ammonium and alkanolammonium salts of polyphosphates (exemplified by the tri-polyphosphates, pyrophosphates, and glassy polymeric meta-phosphates), phos-phonates, phytic acid, silicates, carbonates (including bicarbonates and sesqui-carbonates), sulphates, and aluminosilicates. However, nonphosphate builders are required in some locales.

Examples of silicate builders are the alkali metal silicates, particularly those having a SiO_2:Na_2O ratio in the range 1.6:1 to 3.2:1 and layered silicates, such as the layered sodium silicates described in U.S. Patent 4,664,839, issued May 12, 1987 to H. P. Rieck, available from Hoechst under the trademark "SKS"; SKS-6 is an especially preferred layered silicate builder.

Carbonate builders, especially a finely ground calcium carbonate with surface area greater than 10 m^2/g, are preferred builders that can be used in granular compositions. The density of such alkali metal carbonate built detergents can be in the range of 450-850 g/l with the moisture content preferably below 4%.

Examples of carbonate builders are the alkaline earth and alkali metal carbonates as disclosed in German Patent Application No. 2,321,001 published on November 15, 1973.

Aluminosilicate builders are especially useful in the present invention. Preferred aluminosilicates are zeolite builders which have the formula:

$$Na_z[(AlO_2)_z(SiO_2)_y]\cdot xH_2O$$

wherein z and y are integers of at least 6, the molar ratio of z to y is in the range from 1.0 to about 0.5, and x is an integer from about 15 to about 264.

Useful aluminosilicate ion exchange materials are commercially available. These aluminosilicates can be crystalline or amorphous in structure and can be naturally-occurring aluminosilicates or synthetically derived. Methods for producing aluminosilicate ion exchange materials are disclosed in U.S. Patent 3,985,669, Krummel, et al, issued October 12, 1976, and U.S. Patent 4,605,509, Corkill, et al, issued Aug. 12, 1986. Preferred synthetic crystalline aluminosilicate ion exchange materials useful herein are available under the designations Zeolite A, Zeolite P (B) (including those disclosed in EPO 384,070), and Zeolite X. Preferably, the aluminosilicate has a particle size of about 0.1-10 microns in diameter.

Organic detersive builders suitable for the purposes of the present invention include, but are not restricted to, a wide variety of polycarboxylate compounds, such as ether polycarboxylates, including oxydisuccinate, as disclosed in Berg, U.S. Patent 3,128,287, issued April 7, 1964, and Lamberti et al, U.S. Patent 3,635,830, issued January 18, 1972. See also "TMS/TDS" builders of U.S. Patent 4,663,071, issued to Bush et al, on May 5, 1987. Suitable ether polycarboxylates also include cyclic compounds, particularly alicyclic compounds, such as those described in U.S. Patents 3,923,679; 3,835,163; 4,158,635; 4,120,874 and 4,102,903.

Other useful detersive builders include the ether hydroxy-polycarboxylates, copolymers of maleic anhydride with ethylene or vinyl methyl ether, 1, 3, 5-trihydroxy benzene-2, 4, 6-trisulphonic acid, and carboxymethyl-oxysuccinic acid, the various alkali metal, ammonium and substituted ammonium salts of polyacetic acids such as ethylenediamine tetraacetic acid and nitrilotriacetic acid, as well as polycarboxylates such as mellitic acid, succinic acid, oxydisuccinic

acid, polymaleic acid, benzene 1,3,5-tricarboxylic acid, carboxymethyloxysuccinic acid, and soluble salts thereof.

Citrate builders, e.g., citric acid and soluble salts thereof (particularly sodium salt), are preferred polycarboxylate builders that can also be used in granular compositions, especially in combination with zeolite and/or layered silicate builders.

Also suitable in the detergent compositions of the present invention are the 3,3-dicarboxy-4-oxa-1,6-hexanedioates and the related compounds disclosed in U.S. Patent 4,566,984, Bush, issued January 28, 1986.

In situations where phosphorus-based builders can be used, and especially in the formulation of bars used for hand-laundering operations, the various alkali metal phosphates such as the well-known sodium tripolyphosphates, sodium pyrophosphate and sodium orthophosphate can be used. Phosphonate builders such as ethane-1-hydroxy-1,1-diphosphonate and other known phosphonates (see, for example, U.S. Patents 3,159,581; 3,213,030; 3,422,021; 3,400,148 and 3,422,137) can also be used.

(v) Optional Detersive Adjuncts

As a preferred embodiment, the conventional detergent ingredients employed herein can be selected from typical detergent composition components such as detersive surfactants and detersive builders. Optionally, the detergent ingredients can include one or more other detersive adjuncts or other materials for assisting or enhancing cleaning performance, treatment of the substrate to be cleaned, or to modify the aesthetics of the detergent composition. Usual detersive adjuncts of detergent compositions include the ingredients set forth in U.S. Pat. No. 3,936,537, Baskerville et al, are incorporated herein by reference. Such adjuncts which can be included in detergent compositions employed in the present invention, in their conventional art-established levels for use (generally from 0% to about 20% of the detergent ingredients, preferably from about 0.5% to about 10%), include color speckles, suds boosters, suds suppressors, antitarnish and/or anticorrosion agents, soil-suspending agents, soil release agents, dyes, fillers, optical brighteners, germicides, alkalinity sources, hydrotropes, antioxidants, perfumes, solvents, solubilizing agents, clay

soil removal/anti-redeposition agents, polymeric dispersing agents, processing aids, fabric softening components (e.g., smectite clays), dye transfer inhibiting agents (e.g., polyvinyl pyrrolidone), static control agents, etc.

Bleach systems optionally, but preferably, will also comprise a chelant which not only enhances bleach stability by scavenging heavy metal ions which tend to decompose bleaches, but also assists in the removal of polyphenolic stains such as tea stains, and the like. Various chelants, including the aminophosphonates, available as DEQUEST from Monsanto, the nitrilotriacetates, the hydroxyethyl-ethylenediamine triacetates, and the like, are known for such use. Preferred biodegradable, non-phosphorus chelants include ethylene-diamine disuccinate ("EDDS"; see U.S. Patent 4,704,233, Hartman and Perkins), ethylenediamine-N,N'-diglutamate (EDDG) and 2-hydroxypropylenediamine-N,N'-disuccinate (HPDDS) compounds. Such chelants can be used in their alkali or alkaline earth metal salts, typically at levels from about 0.1% to about 10% of the present compositions.

The present invention compositions are especially useful as conventional laundry detergent compositions such as those typically found in granular detergents or laundry bars. U.S. Patent 3,178,370, Okenfuss, issued April 13, 1965, describes laundry detergent bars and processes for making them. Philippine Patent 13,778, Anderson, issued Sept. 23, 1980, describes synthetic detergent laundry bars. Methods for making laundry detergent bars by various extrusion methods are well known in the art.

The following examples are given to further illustrate the present invention, but are not intended to be limiting thereof.

EXAMPLE 1

A granular detergent composition is prepared comprising the following ingredients.

Component	Weight %
C_{12} linear alkyl benzene sulfonate	22
Phosphate (as sodium tripolyphosphate)	30
Sodium carbonate	14
Sodium silicate	3

Protease 12	0.3
Sodium percarbonate	5
Ethylenediamine disuccinate chelant (EDDS)	0.4
Sodium sulfate	5.5
Nonanoyl caprolactam	5
Filler* and water	Balance to 100%

*Can be selected from convenient materials such as $CaCO_3$, talc, clay, silicates, and the like.

Aqueous crutcher mixes of heat and alkali stable components of the detergent compositions are prepared and spray-dried and the other ingredients are admixed so that they contain the ingredients tabulated at the levels shown.

In this example the Protease #'s 1-11 and 13-25 recited in Table III, among others including the additional proteases useful in the present invention described in Tables V and VI, are substituted for Protease #12, with substantially similar results. Also in this example, any combination of the proteases useful in the present invention recited in Tables III, V and VI among others, are substituted for Protease # 12 with substantially similar results.

EXAMPLE 2

A granular detergent composition is prepared comprising the following ingredients.

Component	Weight %
Anionic alkyl sulfate	7
Nonionic surfactant	5
Zeolite (0.1-10 micron)	10
Trisodium citrate	2
SKS-6 silicate builder	10
Acrylate maleate polymer	4
Nonanoyl caprolactam	5
Sodium percarbonate*	15
Sodium carbonate	5
Ethylenediamine disuccinate chelant (EDDS)	0.4
Suds suppressor	2
Protease 12	0.3
Lipase	0.3

Soil release agent	0.2
Minors, filler** and water	Balance to 100%

*Average particle size of 400 to 1200 microns.

**Can be selected from convenient materials such as $CaCO_3$, talc, clay, silicates, and the like.

Aqueous crutcher mixes of heat and alkali stable components of the detergent composition are prepared and spray-dried, and the other ingredients are admixed so that they contain the ingredients tabulated at the levels shown.

In this example the Protease #'s 1-11 and 13-25 recited in Table III, among others including the additional proteases useful in the present invention described in Tables V and VI, are substituted for Protease #12, with substantially similar results. Also in this example, any combination of the proteases useful in the present invention recited in Tables III, V and VI among others, are substituted for Protease # 12 with substantially similar results.

EXAMPLE 3

A detergent composition is prepared by a procedure identical to that of Example 2, with the exceptions that 15% of a 1:1:1 mixture of benzoyl caprolactam, nonanoyl caprolactam and (6-Nonanamidocaproyl)oxybenzenesulfonate is substituted for the nonanoyl caprolactam and the amount of sodium percarbonate is 30%.

In this example the Protease #'s 1-11 and 13-25 recited in Table III, among others including the additional proteases useful in the present invention described in Tables V and VI, are substituted for Protease #12, with substantially similar results. Also in this example, any combination of the proteases useful in the present invention recited in Tables III, V and VI among others, are substituted for Protease # 12 with substantially similar results.

EXAMPLE 4

A detergent composition is prepared by a procedure identical to that of Example 1, with the exceptions that 20% of a 1:1 mixture of benzoyl caprolactam and (6-Nonanamidocaproyl)oxybenzenesulfonate is substituted for the nonanoyl caprolactam, the amount of sodium percarbonate is 20%, and the amount of phosphate is 0%.

In this example the Protease #'s 1-11 and 13-25 recited in Table III, among others including the additional proteases useful in the present invention described in Tables V and VI, are substituted for Protease #12, with substantially similar results. Also in this example, any combination of the proteases useful in the present invention recited in Tables III, V and VI among others, are substituted for Protease # 12 with substantially similar results.

EXAMPLE 5

A detergent composition is prepared by a procedure identical to that of Example 2, with the single exception that an equivalent amount of a benzoxazin-type activator is substituted for the nonanoyl caprolactam.

In this example the Protease #'s 1-11 and 13-25 recited in Table III, among others including the additional proteases useful in the present invention described in Tables V and VI, are substituted for Protease #12, with substantially similar results. Also in this example, any combination of the proteases useful in the present invention recited in Tables III, V and VI among others, are substituted for Protease # 12 with substantially similar results.

EXAMPLE 6

A detergent composition is prepared by a procedure identical to that of Example 2, with the exceptions that 10% of a 1:1 mixture of a benzoxazin-type activator and tetraacetyl ethylene diamine is substituted for the nonanoyl caprolactam and the amount of sodium percarbonate is 25%.

In this example the Protease #'s 1-11 and 13-25 recited in Table III, among others including the additional proteases useful in the present invention described in Tables V and VI, are substituted for Protease #12, with substantially similar results. Also in this example, any combination of the proteases useful in the present invention recited in Tables III, V and VI among others, are substituted for Protease # 12 with substantially similar results.

EXAMPLE 7

A laundry bar suitable for hand-washing soiled fabrics is prepared by standard extrusion processes and comprises the following:

Component	Weight %

C_{12} linear alkyl benzene sulfonate	30
Phosphate (as sodium tripolyphosphate)	7
Sodium carbonate	25
Sodium pyrophosphate	7
Coconut monoethanolamide	2
Zeolite A (0.1-10 micron)	5
Carboxymethylcellulose	0.2
Polyacrylate (m.w. 1400)	0.2
(6-Nonanamidocaproyl)oxybenzenesulfonate	5
Sodium percarbonate	5
Brightener, perfume	0.2
Protease 12	0.3**
Lipase	0.3
$CaSO_4$	1
$MgSO_4$	1
Water	4
Filler*	Balance to 100%

*Can be selected from convenient materials such as $CaCO_3$, talc, clay, silicates, and the like.

**Denotes mg of active enzyme per gram of composition.

The detergent laundry bars are processed in conventional soap or detergent bar making equipment as commonly used in the art.

In this example the Protease #'s 1-11 and 13-25 recited in Table III, among others including the additional proteases useful in the present invention described in Tables V and VI, are substituted for Protease #12, with substantially similar results. Also in this example, any combination of the proteases useful in the present invention recited in Tables III, V and VI among others, are substituted for Protease # 12 with substantially similar results.

EXAMPLE 8

A detergent composition is prepared by a procedure identical to that of Example 7, with the single exception that an equivalent amount of benzoyl caprolactam is substituted for the (6-Nonanamidocaproyl)oxybenzenesulfonate.

In this example the Protease #'s 1-11 and 13-25 recited in Table III, among others including the additional proteases useful in the present

invention described in Tables V and VI, are substituted for Protease #12, with substantially similar results. Also in this example, any combination of the proteases useful in the present invention recited in Tables III, V and VI among others, are substituted for Protease # 12 with substantially similar results.

EXAMPLE 9

A detergent composition is prepared by a procedure identical to that of Example 7, with the single exception that an equivalent amount of nonanoyl caprolactam is substituted for the (6-Nonanamidocaproyl)oxybenzenesulfonate.

In this example the Protease #'s 1-11 and 13-25 recited in Table III, among others including the additional proteases useful in the present invention described in Tables V and VI, are substituted for Protease #12, with substantially similar results. Also in this example, any combination of the proteases useful in the present invention recited in Tables III, V and VI among others, are substituted for Protease # 12 with substantially similar results.

EXAMPLE 10

A granular detergent composition is prepared comprising the following ingredients.

Component	Weight %
Anionic alkyl sulfate	7
Nonionic surfactant	5
Zeolite (0.1-10 micron)	10
Trisodium citrate	2
SKS-6 silicate builder	10
Acrylate maleate polymer	4
Nonanoyl caprolactam	5
Sodium percarbonate*	15
Sodium carbonate	5
Ethylenediamine disuccinate chelant (EDDS)	0.4
Suds suppressor	2
Protease 12	0.5
Soil release agent	0.2
Minors, filler** and water	Balance to 100%

*Average particle size of 400 to 1200 microns.

**Can be selected from convenient materials such as $CaCO_3$, talc, clay, silicates, and the like.

Aqueous crutcher mixes of heat and alkali stable components of the detergent composition are prepared and spray-dried, and the other ingredients are admixed so that they contain the ingredients tabulated at the levels shown.

In this example the Protease #'s 1-11 and 13-25 recited in Table III, among others including the additional proteases useful in the present invention described in Tables V and VI, are substituted for Protease #12, with substantially similar results. Also in this example, any combination of the proteases useful in the present invention recited in Tables III, V and VI among others, are substituted for Protease # 12 with substantially similar results.

EXAMPLE 11

A detergent composition is prepared by a procedure identical to that of Example 10, with the single exception that an equivalent amount of benzoyl caprolactam is substituted for the nonanoyl caprolactam.

In this example the Protease #'s 1-11 and 13-25 recited in Table III, among others including the additional proteases useful in the present invention described in Tables V and VI, are substituted for Protease #12, with substantially similar results. Also in this example, any combination of the proteases useful in the present invention recited in Tables III, V and VI among others, are substituted for Protease # 12 with substantially similar results.

EXAMPLE 12

A detergent composition is prepared by a procedure identical to that of Example 10, with the exceptions that 15%, by weight, of (6-Nonanamido-caproyl)oxybenzenesulfonate is substituted for the nonanoyl caprolactam and the amount of sodium percarbonate is 30%.

In this example the Protease #'s 1-11 and 13-25 recited in Table III, among others including the additional proteases useful in the present invention described in Tables V and VI, are substituted for Protease #12, with substantially similar results. Also in this example, any combination of the proteases useful in the present invention recited in Tables III, V and VI among others, are substituted for Protease # 12 with substantially similar results.

EXAMPLE 13

A detergent composition is prepared by a procedure identical to that of Example 10, with the exceptions that 15%, by weight, of a benzoxazin-type activator is substituted for the nonanoyl caprolactam and the amount of sodium percarbonate is 30%.

In this example the Protease #'s 1-11 and 13-25 recited in Table III, among others including the additional proteases useful in the present invention described in Tables V and VI, are substituted for Protease #12, with substantially similar results. Also in this example, any combination of the proteases useful in the present invention recited in Tables III, V and VI among others, are substituted for Protease # 12 with substantially similar results.

EXAMPLE 14

Wash Performance Test

The wash performance of the variants useful in the present invention compositions is evaluated by measuring the removal of stain from EMPA 116 (blood/milk/carbon black on cotton) cloth swatches (Testfabrics, Inc., Middlesex, NJ 07030).

Six EMPA 116 swatches, cut to 3 X 4-1/2 inches with pinked edges, are placed in each pot of a Model 7243S Terg-O-Tometer (United States Testing Co., Inc., Hoboken, NJ) containing 1000 ml of water, 15 gpg hardness (Ca^{++}:Mg^{++}::3:1::w:w), 7 g of detergent, and enzyme as appropriate. The detergent base is WFK1 detergent from *wfk* - Testgewebe GmbH, Adlerstrasse 42, Postfach 13 07 62, D-47759 Krefeld, Germany:

Component	% of Final Formulation
Zeolite A	25%
Sodium sulfate	25%
Soda Ash	10%

Linear alkylbenzenesulfonate	8.8%
Alcohol ethoxylate (7-8 EO)	4.5%
Sodium soap	3%
Sodium silicate (SiO_2:Na_2O::3.3:1)	3%

To this base detergent, the following additions are made:

Component	% of Final Formulation
Sodium perborate monohydrate	13%
Copolymer (Sokalan CP5)	4%
TAED (Mykon ATC Green)	3%
Enzyme	0.5%
Brightener (Tinopal AMS-GX)	0.2%

Sodium perborate monohydrate is obtained from Degussa Corporation, Ridgefield-Park, NJ 07660. Sokalan CP5 is obtained from BASF Corporation, Parsippany, NJ 07054. Mykon ATC Green (TAED, tetraacetylethylenediamine) is obtained from Warwick International, Limited, Mostyn, Holywell, Clwyd CH8 9HE, England. Tinopal AMS GX is obtained from Ciba-Geigy Corporation, Greensboro, NC 27419.

Six EMPA 116 swatches are washed in detergent with enzyme for 30 min at 60°C and are subsequently rinsed twice for 5 minutes each time in 1000 ml water. Enzymes are added at final concentrations of 0.05 to 1 ppm for standard curves, and 0.25 ppm for routine analyses. Swatches are dried and pressed, and the reflectance from the swatches is measured using the L value on the L*a*b* scale of a Minolta Chroma Meter, Model CR-200 (Minolta Corporation, Ramsey, NJ 07446). Performance is reported as a percentage of the performance of *B. lentus* (GG36) protease and is calculated by dividing the amount of *B. lentus* (GG36) protease by the amount of variant protease that is needed to provide the same stain removal performance X 100. The data are shown in Table VII.

Table VII

Enzyme	**Wash Performance**
B. lentus subtilisin	100
N76D	310
N76D/S103A	230
N76D/V104I	130
N76D/V107V	160
N76D/S99D/S101R	370
N76D/S99D/S103A	290
N76D/S101R/S103A	130
N76D/S101R/V104I	300

N76D/S103A/V104I	320
N76D/S103G/V104I	160
N76D/S103A/V104F	210
N76D/S103A/V104N	110
N76D/S103A/V104T	170
N76D/V104I/I107V	210
N76D/S99D/S101R/S103A	220
N76D/S99D/S101R/V104I	140
N76D/S101G/S103A/V104I	170
N76D/S101R/S103A/V104I	150
N76D/S103A/V104I/S105A	170
N76D/S103A/V104T/I107A	120
N76D/S103A/V104T/I107L	110
N76D/S103A/V104I/L126F	110
N76D/S103A/V104I/S128G	280
N76D/S103A/V104I/L135I	160
N76D/S103A/V104I/L135V	160
N76D/S103A/V104I/D197E	170
N76D/S103A/V104I/N204A	160
N76D/S103A/V104I/N204G	150
N76D/S103A/V104I/P210I	470
N76D/S103A/V104I/M222A	100
N76D/S103A/V104I/T260P	280
N76D/S103A/V104I/S265N	190

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Ghosh, Chanchal K.
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DiGiulio, David N.
Getty, Edward E.
Hartshorn, Richard T.
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Rubingh, Donn N.

(ii) TITLE OF INVENTION: Bleaching Compositions Comprising Protease Enzymes

(iii) NUMBER OF SEQUENCES: 15

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(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Diskette
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE: 13-OCT-1994
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:
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(C) REFERENCE/DOCKET NUMBER: 5041R

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(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

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

GAAGCTGCAA CTCGTTAAA 19

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

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

GCTGCTCTAG ACAATTCG 18

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 39 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

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

GTATTAGGGG CGGACGGTCG AGGCGCCATC AGCTCGATT 39

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 33 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

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

TCAGGTTCGG TCTCGAGCGT TGCCCAAGGA TTG 33

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

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

CACGTTGCTA GCTTGAGTTT AG 22

(2) INFORMATION FOR SEQ ID NO:6:

(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:6:

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 CCCGAGCGTC GCTTACGTTG AAGAAGATCA CGTAGCACAT GCGTACGCGC   420
AGTCCGTGCC TTACGGCGTA TCACAAATTA AAGCCCCTGC TCTGCACTCT CAAGGCTACA   480
CTGGATCAAA TGTTAAAGTA 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 GGTAAATACC CTTCTGTCAT TGCAGTAGGC GCTGTTGACA   960
GCAGCAACCA AAGAGCATCT TTCTCAAGCG TAGGACCTGA GCTTGATGTC ATGGCACCTG  1020
GCGTATCTAT CCAAAGCACG CTTCCTGGAA ACAAATACGG GGCGTACAAC GGTACGTCAA  1080
TGGCATCTCC GCACGTTGCC GGAGCGGCTG CTTTGATTCT TTCTAAGCAC CCGAACTGGA  1140
CAAACACTCA AGTCCGCAGC AGTTTAGAAA ACACCACTAC AAAACTTGGT GATTCTTTGT  1200
ACTATGGAAA AGGGCTGATC AACGTACAAG CGGCAGCTCA GTAAAACATA AAAAACCGGC  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:7:

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

(ii) MOLECULE TYPE: protein

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

```
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:8:

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

(ii) MOLECULE TYPE: protein

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

```
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

Ala Ala Gln
        275
```

(2) INFORMATION FOR SEQ ID NO:9:

(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:9:

```
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

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:10:

(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:10:

```
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

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:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1140 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

ATGAAGAAAC CGTTGGGGAA AATTGTCGCA AGCACCGCAC TACTCATTTC TGTTGCTTTT 60

AGTTCATCGA TCGCATCGGC TGCTGAAGAA GCAAAAGAAA AATATTTAAT TGGCTTTAAT 120

GAGCAGGAAG CTGTCAGTGA GTTTGTAGAA CAAGTAGAGG CAAATGACGA GGTCGCCATT 180

CTCTCTGAGG AAGAGGAAGT CGAAATTGAA TTGCTTCATG AATTTGAAAC GATTCCTGTT 240

TTATCCGTTG AGTTAAGCCC AGAAGATGTG GACGCGCTTG AACTCGATCC AGCGATTTCT 300

TATATTGAAG AGGATGCAGA AGTAACGACA ATGGCGCAAT CAGTGCCATG GGGAATTAGC 360

CGTGTGCAAG CCCCAGCTGC CCATAACCGT GGATTGACAG GTTCTGGTGT AAAAGTTGCT 420

GTCCTCGATA CAGGTATTTC CACTCATCCA GACTTAAATA TTCGTGGTGG CGCTAGCTTT 480

GTACCAGGGG AACCATCCAC TCAAGATGGG AATGGGCATG GCACGCATGT GGCCGGGACG 540

ATTGCTGCTT TAAACAATTC GATTGGCGTT CTTGGCGTAG CGCCGAGCGC GGAACTATAC 600

GCTGTTAAAG TATTAGGGGC GAGCGGTTCA GGTTCGGTCA GCTCGATTGC CCAAGGATTG 660

GAATGGGCAG GGAACAATGG CATGCACGTT GCTAATTTGA GTTTAGGAAG CCCTTCGCCA 720

AGTGCCACAC TTGAGCAAGC TGTTAATAGC GCGACTTCTA GAGGCGTTCT TGTTGTAGCG 780

GCATCTGGGA ATTCAGGTGC AGGCTCAATC AGCTATCCGG CCCGTTATGC GAACGCAATG 840

GCAGTCGGAG CTACTGACCA AAACAACAAC CGCGCCAGCT TTTCACAGTA TGGCGCAGGG 900

CTTGACATTG TCGCACCAGG TGTAAACGTG CAGAGCACAT ACCCAGGTTC AACGTATGCC 960

AGCTTAAACG GTACATCGAT GGCTACTCCT CATGTTGCAG GTGCAGCAGC CCTTGTTAAA 1020

CAAAAGAACC CATCTTGGTC CAATGTACAA ATCCGCAATC ATCTAAAGAA TACGGCAACG 1080

AGCTTAGGAA GCACGAACTT GTATGGAAGC GGACTTGTCA ATGCAGAAGC GGCAACACGC 1140

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1140 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

ATGAAGAAAC CGTTGGGGAA AATTGTCGCA AGCACCGCAC TACTCATTTC TGTTGCTTTT 60

AGTTCATCGA TCGCATCGGC TGCTGAAGAA GCAAAAGAAA AATATTTAAT TGGCTTTAAT 120

GAGCAGGAAG CTGTCAGTGA GTTTGTAGAA CAAGTAGAGG CAAATGACGA GGTCGCCATT 180

CTCTCTGAGG AAGAGGAAGT CGAAATTGAA TTGCTTCATG AATTTGAAAC GATTCCTGTT 240

TTATCCGTTG AGTTAAGCCC AGAAGATGTG GACGCGCTTG AACTCGATCC AGCGATTTCT 300

TATATTGAAG AGGATGCAGA AGTAACGACA ATGGCGCAAT CAGTGCCATG GGGAATTAGC 360

CGTGTGCAAG CCCCAGCTGC CCATAACCGT GGATTGACAG GTTCTGGTGT AAAAGTTGCT 420

GTCCTCGATA CAGGTATTTC CACTCATCCA GACTTAAATA TTCGTGGTGG CGCTAGCTTT 480

GTACCAGGGG AACCATCCAC TCAAGATGGG AATGGGCATG GCACGCATGT GGCCGGGACG 540

ATTGCTGCTT TAGACAACTC GATTGGCGTT CTTGGCGTAG CGCCGAGCGC GGAACTATAC 600

GCTGTTAAAG TATTAGGGGC GAGCGGTTCA GGCGCCATCA GCTCGATTGC CCAAGGATTG 660

GAATGGGCAG GGAACAATGG CATGCACGTT GCTAATTTGA GTTTAGGAAG CCCTTCGCCA 720

AGTGCCACAC TTGAGCAAGC TGTTAATAGC GCGACTTCTA GAGGCGTTCT TGTTGTAGCG 780

GCATCTGGGA ATTCAGGTGC AGGCTCAATC AGCTATCCGG CCCGTTATGC GAACGCAATG 840

GCAGTCGGAG CTACTGACCA AAACAACAAC CGCGCCAGCT TTTCACAGTA TGGCGCAGGG 900

CTTGACATTG TCGCACCAGG TGTAAACGTG CAGAGCACAT ACCCAGGTTC AACGTATGCC 960

AGCTTAAACG GTACATCGAT GGCTACTCCT CATGTTGCAG GTGCAGCAGC CCTTGTTAAA 1020

CAAAAGAACC CATCTTGGTC CAATGTACAA ATCCGCAATC ATCTAAAGAA TACGGCAACG 1080

AGCTTAGGAA GCACGAACTT GTATGGAAGC GGACTTGTCA ATGCAGAAGC GGCAACACGC 1140

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid

(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

TATGCCAGCC ACAACGGTAC TTCGATGGCT 30

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 31 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

CACAGTTGCG GCTCTAGATA ACTCAATCGG T 31

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 33 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

GCTGACGGTT CCGGCGCTAT TAGTTGGATC ATT 33

What is claimed is:

1. A bleaching 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 comprising a substitution of a different amino acid for a plurality of amino acid residues at a position in said precursor carbonyl hydrolase equivalent to position +76 in Bacillus amyloliquefaciens subtilisin, in combination with one or more amino acid residue positions equivalent to those selected from the group consisting of +99, +101, +103, +104, +107, +123, +27, +105, +109, +126, +128, +135, +156, +166, +195, +197, +204, +206, +210, +216, +217, +218, +222, +260, +265, and/or +274 in Bacillus amyloliquefaciens subtilisin; and

(b) a bleaching agent which either is an organic peroxyacid or is a combination of a bleach activator and a peroxygen compound capable of yielding hydrogen peroxide that can react with the activator to form an organic peroxyacid in situ in a bleaching solution formed from the composition; and

(c) one or more cleaning composition materials compatible with the protease enzyme and bleaching agent.

2. The bleaching compositions according to Claim 1 wherein the cleaning composition 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, dyes, perfumes, and mixtures thereof.

3. The bleaching compositions according to Claim 2 in a form useful as a fabric cleaning composition comprising at least one surfactant.

4. The bleaching compositions according to Claim 3 comprising at least about 5% surfactant selected from the group consisting of alkyl benzene sulfonates, primary alkyl sulfates, secondary alkyl sulfates, alkyl alkoxy sulfates, alkyl alkoxy carboxylates, alkyl polyglycosides and their corresponding sulfated polyglycosides, alpha-sulfonated fatty acid esters,

alkyl and alkyl phenol alkoxylates, betaines and sulfobetaines, amine oxides, N-methyl glucamides, nonionic primary alcohol ethoxylates, nonionic primary alcohol mixed ethoxy/propoxy, and mixtures thereof.

5. The bleaching compositions according to Claim 4 further comprising at least about 5% builder selected from the group consisting of zeolites, polycarboxylates, layered silicates, phosphates, and mixtures thereof.

6. The bleaching compositions according to Claim 4 in the form of a concentrated granular fabric cleaning composition comprising at least about 30% surfactant.

7. The bleaching compositions according to Claim 1 wherein the cleaning composition materials comprise at least one enzyme selected from the group consisting of cellulases, lipases, amylases, proteases, peroxidases and mixtures thereof.

8. The bleaching compositions according to Claim 1 in a form useful as a liquid fabric cleaning composition comprising at least one solvent and at least one surfactant.

9. The bleaching compositions according to Claim 8 comprising water and at least 5% surfactant.

10. The bleaching compositions according to Claim 1 wherein the cleaning composition materials comprise at lease one fabric softener.

11. The cleaning compositions according to Claim 1 wherein the precursor carbonyl hydrolase for the protease enzyme is a subtilisin, and the protease enzyme is a subtilisin variant wherein a combination of subtitutions is made at the positions equivalent to 76/99, 76/101, 76/103, 76/104, 76/107, 76/123, 76/99/101, 76/99/103, 76/99/104, 76/101/103, 76/101/104, 76/103/104, 76/104/107, 76/104/123, 76/107/123, 76/99/101/103, 76/99/101/104, 76/99/103/104, 76/101/103/104, 76/103/104/123, 76/104/107/123, 76/99/101/103/104, 76/99/103/104/123,

76/99/101/103/104/123, 76/103/104/126, 76/103/104/135, 76/103/104/197, 76/103/104/222, 76/103/104/260, 76/103/104/265, 76/103/104/126/265, 27/76/104/123/274, 27/76/104/109/123/274, 27/76/104/123/218/274, 27/76/104/123, 27/76/104/107/123, 27/76/104/109/123, 27/76/104/109/123/218/274, 27/76/104/123/197, 27/76/104/123/204, 27/76/104/123/206, 27/76/104/123/216, 27/76/104/123/218, 27/76/104/123/260, 27/76/104/123/195/197, 27/76/104/123/195/218, 27/76/104/123/197/218, 27/76/104/123/204/218, 27/76/104/123/206/218, 27/76/104/123/218/260, 27/76/104/123/195/197/218, 76/103/104/217, 76/103/104/156, 76/103/104/166, 76/103/104/105, 76/101/103/104, 76/103/104/128, 76/103/104/210, 76/103/104/107, 76/103/104/204, 76/217, 76/103/104/156/166 and 76/103/104/128.

12. The cleaning compositions according to Claim 1 wherein the protease enzyme is a subtilisin variant selected from the group consisting of 76/99, 76/101, 76/103, 76/104, 76/107, 76/123, 76/99/101, 76/99/103, 76/99/104, 76/101/103, 76/101/104, 76/103/104, 76/104/107, 76/104/123, 76/107/123, 76/99/101/103, 76/99/101/104, 76/99/103/104, 76/101/103/104, 76/103/104/123, 76/104/107/123, 76/99/101/103/104, 76/99/103/104/123, 76/99/101/103/104/123, 76/103/104/128; 76/103/104/260; 76/103/104/265; 76/103/104/197; 76/103/104/105; 76/103/104/135; 76/103/104/126; 76/103/104/107; 76/103/104/210; 76/103/104/126/265; and/or 76/103/104/222.

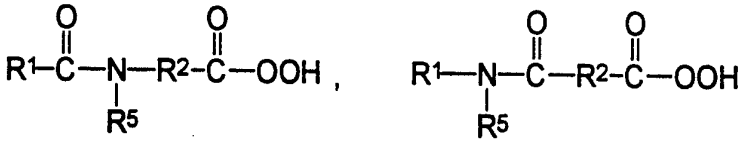
13. A fabric cleaning composition comprising:

(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 subtilisin precursor carbonyl hydrolase, and wherein the protease enzyme is a subtilisin variant selected from N76D/S99D; N76D/S101R; N76D/S103A; N76D/V104I; N76D/I107V; N76D/N123S; N76D/S99D/S101R; N76D/S99D/S103A; N76D/S99D/V104I; N76D/S101R/S103A; N76D/S101R/V104I; N76D/S103A/V104I; N76D/V104I/I107V; N76D/V104Y/I107V; N76D/V104I/N123S; N76D/I107V/N123S; N76D/S99D/S101R/S103A; N76D/S99D/S101R/V104I; N76D/S99D/S103A/V104I;

N76D/S101R/S103A/V104I; N76D/S103A/V104I/N123S; N76D/V104I/I107V/N123S; N76D/S99D/S101R/S103A/V104I; N76D/S99D/S103A/V104I/N123S; N76D/S99D/S101R/S103A/V104I/N123S; N76D/S103A/V104I/S128G; N76D/S103A/V104I/T260P; N76D/S103A/V104I/S265N; N76D/S103A/V104I/D197E; N76D/S103A/V104I/S105A; N76D/S103A/V104I/L135I; N76D/S103A/V104I/L126F; N76D/S103A/V104T/L107T; N76D/S103A/V104I/L126F/S265N, and N76D/S103A/V104I/M222A and mixtures thereof; and

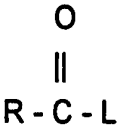
(b) from about 0.5% to about 20% of a bleaching agent selected from the group consisting of:

(i) an organic peroxyacid of the formula:



wherein R^1 is an alkyl, aryl, or alkaryl group containing from about 1 to about 14 carbon atoms, R^2 is an alkylene, arylene or alkarylene group containing from about 1 to about 14 carbon atoms, and R^5 is H or an alkyl, aryl, or alkaryl group containing from about 1 to about 10 carbon atoms, and

(ii) a combination of a bleach activator and a peroxygen compound capable of yielding hydrogen peroxide that can react with the activator to form an organic peroxyacid in situ in a bleaching solution formed from the composition, wherein said bleach activator has the general formula:



wherein R is an alkyl group containing from about 5 to about 18 carbon atoms wherein the longest linear alkyl chain extending from and including the carbonyl carbon contains from about 6 to about 10 carbon atoms and L is a leaving group, the conjugate acid of which has a pKa in the range of from about 4 to about 13; and

(c) one or more cleaning composition materials compatible with the protease enzyme and bleaching agent comprising at least about 5% surfactant and at least about 5% builder, by weight of the composition.

14. The fabric cleaning composition according to Claim 13 further comprising cleaning composition materials selected from the group consisting of solvents, buffers, enzymes, soil release agents, clay soil removal agents, dispersing agents, brighteners, suds suppressors, fabric softeners, suds boosters, enzyme stabilizers, dyes, perfumes, and mixtures thereof.

15. The fabric cleaning composition according to Claim 13 further comprising at least one enzyme selected from the group consisting of cellulases, lipases, amylases, proteases, peroxidases, and mixtures thereof.

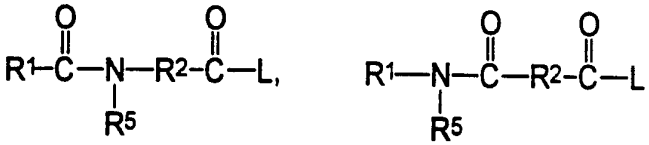
16. The fabric cleaning composition according to Claim 13 in the form of a liquid, granule or bar.

17. The fabric cleaning composition according to Claim 13 wherein the protease enzyme is a subtilisin variant selected from N76D/S99D, N76D/V104I, N76D/S99D/V104I, N76D/S103A/V104I, N76D/V104I/I107V, N76D/V104Y/I107V, N76D/S101R/S103A/V104I, N76D/S99D/S101R/S103A/V104I, N76D/S101R/V104I, and mixtures thereof.

18. The fabric cleaning composition according to Claim 13 wherein the protease enzyme is a Bacillus subtilisin variant selected from N76D/V104I, N76D/S103A/V104I, and mixtures thereof.

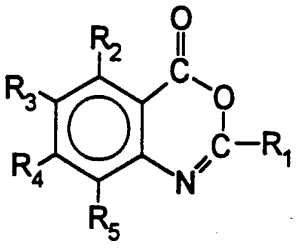
19. The fabric cleaning compositions according to Claim 13 comprising a bleaching agent comprising at least about 0.1% by weight of a peroxygen bleaching compound capable of yielding hydrogen peroxide in an aqueous liquor and at least 0.1% by weight of one or more bleach activators, wherein said bleach activators are members selected from the group consisting of:

a) a bleach activator of the general formula:



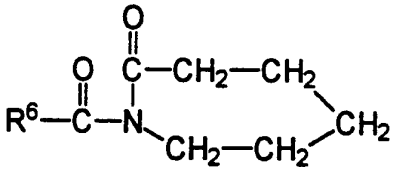
or mixtures thereof,wherein R^1 is an alkyl, aryl, or alkaryl group containing from about 1 to about 14 carbon atoms, R^2 is an alkylene, arylene or alkarylene group containing from about 1 to about 14 carbon atoms, R^5 is H or an alkyl, aryl, or alkaryl group containing from about 1 to about 10 carbon atoms, and L is a leaving group;

b) a benzoxazin-type bleach activator of the formula:



wherein R_1 is H, alkyl, alkaryl, aryl, arylalkyl, and wherein R_2, R_3, R_4, and R_5 may be the same or different substituents selected from H, halogen, alkyl, alkenyl, aryl, hydroxyl, alkoxyl, amino, alkylamino, $-COOR_6$, wherein R_6 is H or an alkyl group and carbonyl functions;

c) a N-acyl caprolactam bleach activator of the formula:



wherein R^6 is H or an alkyl, aryl, alkoxyaryl, or alkaryl group containing from 1 to 12 carbons; and

d) mixtures of a), b) and c).

20. The fabric cleaning composition according to Claim 13 wherein the corresponding carboxylic acid of the organic peroxyacid bleaching agent has a Hydrophilic-Lipophilic Balance value within the range of from about 3 to about 6.5.

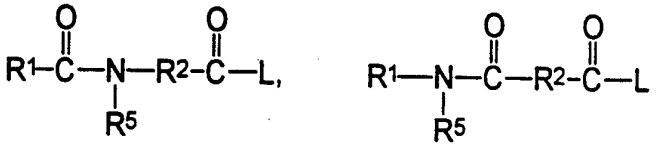
21. The fabric cleaning composition according to Claim 20 wherein the ratio of mg protease enzyme per 100 grams of composition to ppm theoretical Available O_2 of the organic peroxyacid is within the range of from about 1:1 to about 20:1.

22. A fabric cleaning composition comprising:

(a) from about 0.0001% to about 10% protease enzyme which is an N76D/S103A/V104I subtilisin variant derived from Bacillus lentus subtilisin;

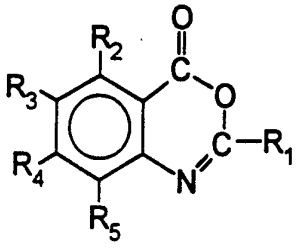
(b) from about 0.5% to about 20% of a bleaching system comprising at least about 0.1% by weight of a peroxygen bleaching compound capable of yielding hydrogen peroxide in an aqueous liquor and at least 0.1% by weight of one or more bleach activators, wherein said bleach activators are members selected from the group consisting of:

i) a bleach activator of the general formula:



or mixtures thereof,wherein R^1 is an alkyl, aryl, or alkaryl group containing from about 1 to about 14 carbon atoms, R^2 is an alkylene, arylene or alkarylene group containing from about 1 to about 14 carbon atoms, R^5 is H or an alkyl, aryl, or alkaryl group containing from about 1 to about 10 carbon atoms, and L is a leaving group;

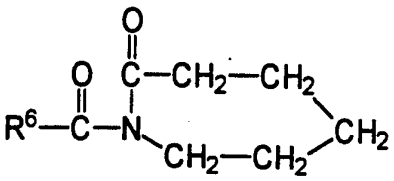
ii) a benzoxazin-type bleach activator of the formula:



wherein R_1 is H, alkyl, alkaryl, aryl, arylalkyl, and wherein R_2, R_3, R_4, and R_5 may be the same or different substituents selected from H, halogen, alkyl, alkenyl, aryl, hydroxyl, alkoxyl, amino,

alkylamino, -$COOR_6$, wherein R_6 is H or an alkyl group and carbonyl functions;

iii) a N-acyl caprolactam bleach activator of the formula:



wherein R^6 is H or an alkyl, aryl, alkoxyaryl, or alkaryl group containing from 1 to 12 carbons; and

iv) mixtures of i), ii) and iii);

(c) at least about 5% surfactant;

(d) at least about 5% builder; and

(e) optionally, one or more cleaning composition materials compatible with the protease enzyme and bleaching system selected from the group consisting of solvents, buffers, enzymes, soil release agents, clay soil removal agents, dispersing agents, brighteners, suds suppressors, fabric softeners, suds boosters, enzyme stabilizers, dyes, perfumes, and mixtures thereof.

23. The fabric cleaning composition according to Claim 22 wherein the surfactant is selected from the group consisting of alkyl benzene sulfonates, primary alkyl sulfates, secondary alkyl sulfates, alkyl alkoxy sulfates, alkyl alkoxy carboxylates, alkyl polyglycosides and their corresponding sulfated polyglycosides, alpha-sulfonated farry acid esters, alkyl and alkyl phenol alkoxylates, betaines and sulfobetaines, amine oxides, N-methyl glucamides, nonionic primary alcohol ethoxylates, nonionic primary alcohol mixed ethoxy/propoxy, and mixtures thereof; and wherein further the builder is selected from the group consisting of zeolites, polycarboxylates, layered silicates, phosphates, and mixtures thereof.

24. A composition according to Claim 23 further comprising at least about 0.001%, by weight of said cleaning composition, of at least one enzyme selected from the group consisting of proteases, amylases, lipases, cellulases, peroxidases and mixtures thereof.

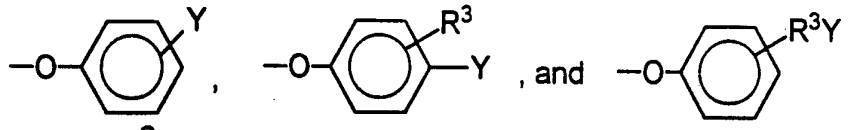
25. A composition according to Claim 22 wherein said bleach activator is selected from the group consisting of benzoyl caprolactam, nonanoyl caprolactam, (6-nonanamidocaproyl)oxybenzenesulfonate, and mixtures thereof.

26. A composition according to Claim 22 wherein the peroxygen bleaching compound is selected from the group consisting of sodium perborate monohydrate, sodium perborate tetrahydrate, sodium pyrophosphate peroxyhydrate, urea peroxyhydrate, sodium percarbonate, sodium peroxide and mixtures thereof.

27. A composition according to Claim 26 wherein the molar ratio of hydrogen peroxide to bleach activator is greater than about 1.0.

28. A composition according to Claim 22 wherein R^1 is an alkyl group containing from about 6 to about 12 carbon atoms, R^2 contains from about 1 to about 8 carbon atoms, and R^5 is H or methyl.

29. A composition according to Claim 22 wherein R^1 is an alkyl group containing from about 7 to about 10 carbon atoms and R^2 contains from about 4 to about 5 carbon atoms, and wherein L is selected from the group consisting of:



, , and

wherein R^3 is an alkyl chain containing from about 1 to about 8 carbon atoms, and Y is $-SO_3^-M^+$ or $-CO_2^-M^+$ wherein M is sodium or potassium.

30. A composition according to Claim 22 comprising an N-acyl caprolactam selected from the group consisting of benzoyl caprolactam, octanoyl caprolactam, nonanoyl caprolactam, 3,5,5-trimethylhexanoyl capro-lactam, decanoyl caprolactam, undecenoyl caprolactam, and mixtures thereof.

31. A method for cleaning fabric, said method comprising contacting a fabric in need of cleaning with a wash solution containing an effective amount of a composition according to Claim 1.

32. A method for cleaning fabric, said method comprising contacting a fabric in need of cleaning with a wash solution containing an effective amount of a composition according to Claim 22.

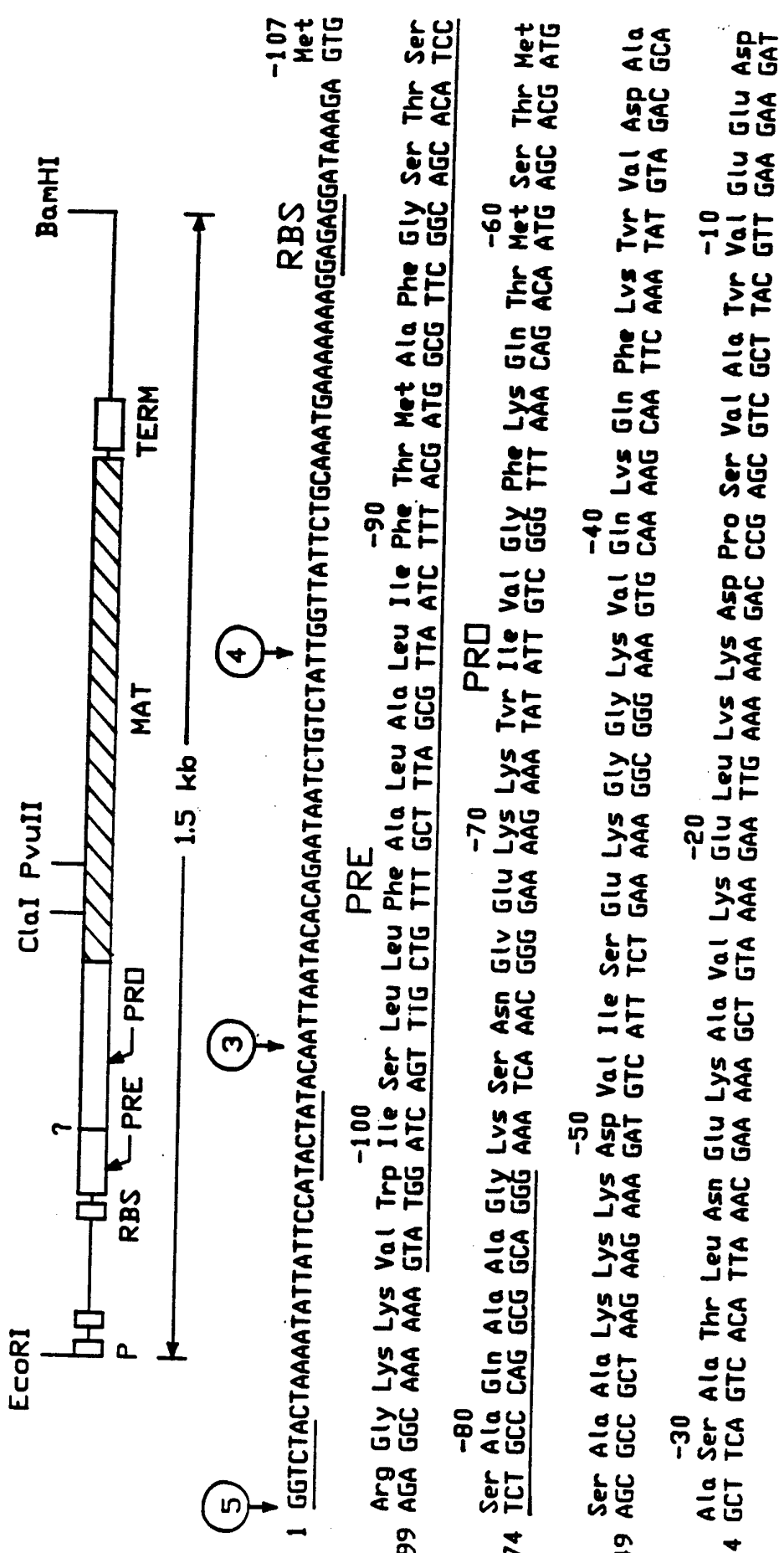

EcoRI
P
RBS
PRE
PRO
?
ClaI PvuII
BamHI
TERM
MAT
1.5 kb
RBS
-107 Met
1 GGTCTACTAAAATATTATTCCATACTATACAATTAATACACAGAATAATCTGTCTATTGGTTATTCTGCAAATGAAAAAAAGGAGAGGATAAAGA GTG
PRE
-100 -90
Arg Gly Lys Lys Val Trp Ile Ser Leu Leu Phe Ala Leu Ala Leu Ile Phe Thr Met Ala Phe Gly Ser Thr Ser
99 AGA GGC AAA AAA GTA TGG ATC AGT TTG CTG TTT GCT TTA GCG TTA ATC TTT ACG ATG GCG TTC GGC AGC ACA TCC
PRO
-80 -70 -60
Ser Ala Gln Ala Ala Gly Lys Ser Asn Glv Glu Lys Lys Tvr Ile Val Gly Phe Lys Gln Thr Met Ser Thr Met
174 TCT GCC CAG GCG GCA GGG AAA TCA AAC GGG GAA AAG AAA TAT ATT GTC GGG TTT AAA CAG ACA ATG AGC ACG ATG
-50 -40
Ser Ala Ala Lys Lys Lys Asp Val Ile Ser Glu Lys Gly Gly Lys Val Gln Lys Gln Phe Lys Tvr Val Asp Ala
249 AGC GCC GCT AAG AAA GAT GTC ATT TCT GAA AAA GGC GGG AAA GTG CAA AAG CAA TTC AAA TAT GTA GAC GCA
-30 -20 -10
Ala Ser Ala Thr Leu Asn Glu Lys Ala Val Lys Glu Leu Lys Lys Asp Pro Ser Val Ala Tvr Val Glu Glu Asp
324 GCT TCA GTC ACA TTA AAC GAA AAA GCT GTA AAA GAA TTG AAA AAA GAC CCG AGC GTC GCT TAC GTT GAA GAA GAT

FIG.-1A

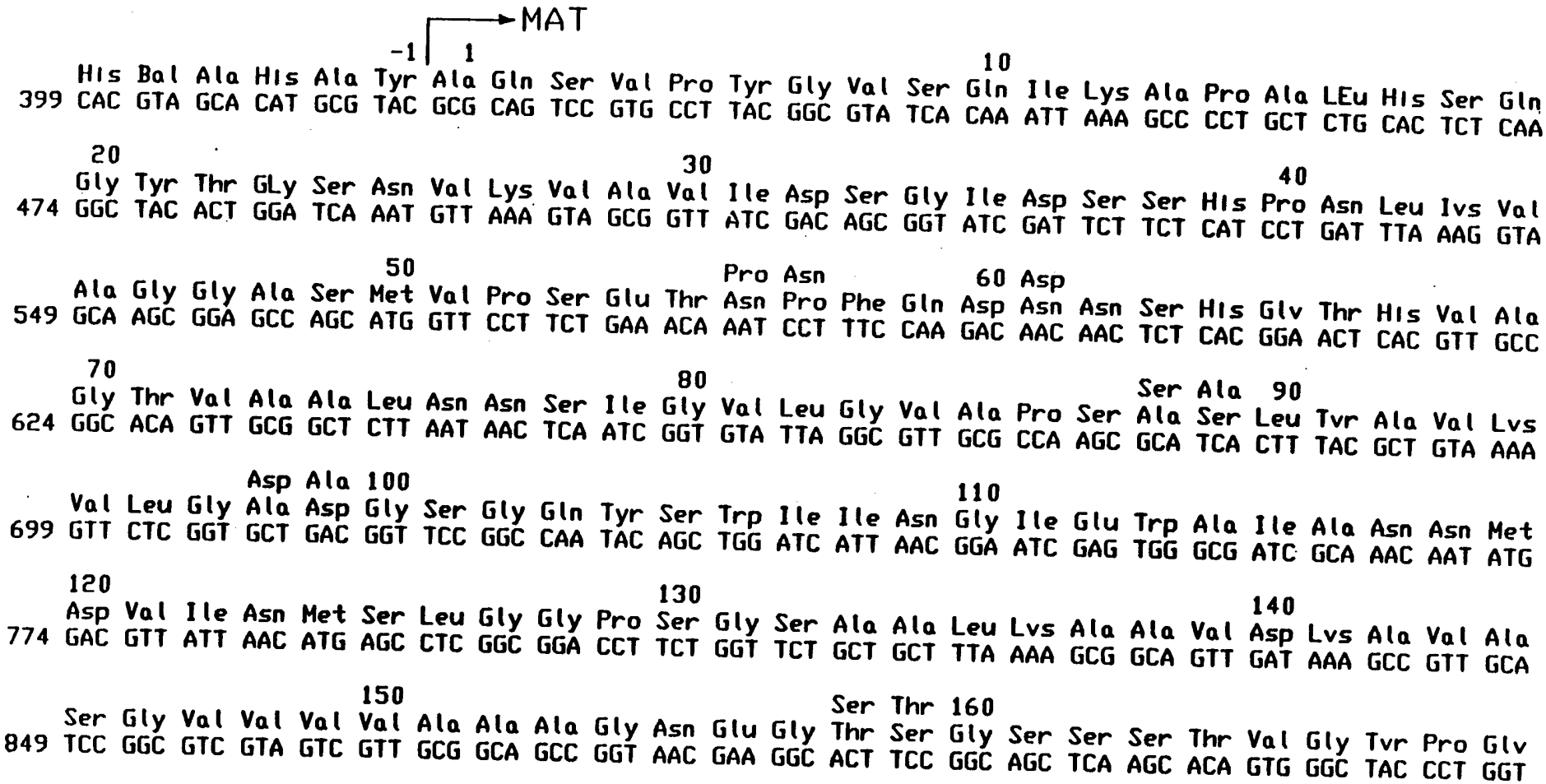

MAT
-1 1
10
His Bal Ala His Ala Tyr Ala Gln Ser Val Pro Tyr Gly Val Ser Gln Ile Lys Ala Pro Ala LEu His Ser Gln
399 CAC GTA GCA CAT GCG TAC GCG CAG TCC GTG CCT TAC GGC GTA TCA CAA ATT AAA GCC CCT GCT CTG CAC TCT CAA
20
30
40
Gly Tyr Thr GLy Ser Asn Val Lys Val Ala Val Ile Asp Ser Gly Ile Asp Ser Ser His Pro Asn Leu Ivs Val
474 GGC TAC ACT GGA TCA AAT GTT AAA GTA GCG GTT ATC GAC AGC GGT ATC GAT TCT TCT CAT CCT GAT TTA AAG GTA
50
Pro Asn
60 Asp
Ala Gly Gly Ala Ser Met Val Pro Ser Glu Thr Asn Pro Phe Gln Asp Asn Asn Ser His Glv Thr His Val Ala
549 GCA AGC GGA GCC AGC ATG GTT CCT TCT GAA ACA AAT CCT TTC CAA GAC AAC AAC TCT CAC GGA ACT CAC GTT GCC
70
80
Ser Ala 90
Gly Thr Val Ala Ala Leu Asn Asn Ser Ile Gly Val Leu Gly Val Ala Pro Ser Ala Ser Leu Tvr Ala Val Lvs
624 GGC ACA GTT GCG GCT CTT AAT AAC TCA ATC GGT GTA TTA GGC GTT GCG CCA AGC GCA TCA CTT TAC GCT GTA AAA
Asp Ala 100
110
Val Leu Gly Ala Asp Gly Ser Gly Gln Tyr Ser Trp Ile Ile Asn Gly Ile Glu Trp Ala Ile Ala Asn Asn Met
699 GTT CTC GGT GCT GAC GGT TCC GGC CAA TAC AGC TGG ATC ATT AAC GGA ATC GAG TGG GCG ATC GCA AAC AAT ATG
120
130
140
Asp Val Ile Asn Met Ser Leu Gly Gly Pro Ser Gly Ser Ala Ala Leu Lvs Ala Ala Val Asp Lvs Ala Val Ala
774 GAC GTT ATT AAC ATG AGC CTC GGC GGA CCT TCT GGT TCT GCT GCT TTA AAA GCG GCA GTT GAT AAA GCC GTT GCA
150
Ser Thr 160
Ser Gly Val Val Val Val Ala Ala Ala Gly Asn Glu Gly Thr Ser Gly Ser Ser Ser Thr Val Gly Tvr Pro Glv
849 TCC GGC GTC GTA GTC GTT GCG GCA GCC GGT AAC GAA GGC ACT TCC GGC AGC TCA AGC ACA GTG GGC TAC CCT GGT

FIG-1B

```
     170                                     180                                     190
     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 GTA GGC GCT GTT GAC AGC AGC AAC CAA AGA GCA TCT TTC TCA AGC GTA GGA CCT

                         200                                     210
     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 GGC GTA TCT ATC CAA AGC ACG CTT CCT GGA AAC AAA TAC GGG GCG TAC AAC GGT

     220                                     230                                     240
     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 GTT GCC GGA GCG GCT GCT TTG ATT CTT TCT AAG CAC CCG AAC TGG ACA AAC ACT

                         250 Gln                                 260
     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

     270                 275
     Val Gln Ala Ala Ala Gln DC                 TERM
1224 GTA CAA GCG GCA GCT CAG TAA  AACATAAAAAACCGGCCTTGGCCCCGCCGGTTTTTTATTATTTTTCTTCCTCCGCATGTTCAATCCGCTCC

1316 ATAATCGACGGATGGCTCCCTCTGAAAATTTTAACGAGAAACGGCGGGTTGACCCGGCTCAGTCCCGTAACGGCCAACTCCTGAAACGTCTCAATCGCCG

1416 CTTCCCGGTTTCCGGTCAGCTCAATGCCATAACGGTCGGCGGCGTTTTCCTGATACCGGGAGACGGCATTCGTAATCGGATC
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FIG.-1C

CONSERVED RESIDUES IN SUBTILISINS FROM *BACILLUS AMYLOLIQUEFACIENS*

```
1                   10                  20
A Q S V P . G . . . . . A P A . H . . G

21                  30                  40
. T G S . V K V A V . D . G . . . . H P

41                  50                  60
D L . . . G G A S . V P . . . . . Q D

61                  70                  80
. N . H G T H V A G T . A A L N N S I G

81                  90                  100
V L G V A P S A . L Y A V K V L G A . G

101                 110                 120
S G . . S . L . . G . E W A . N . . . .

121                 130                 140
V . N . S L G . P S . S . . . . . A . .

141                 150                 160
. . . . . G V . V V A A . G N . G . . .

161                 170                 180
. . . . . Y P . . Y . . . . A V G A .

181                 190                 200
D . . N . . A S F S . . G . . L D . . A

201                 210                 220
P G V . . Q S T . P G . . Y . . . N G T

221                 230                 240
S M A . P H V A G A A A L . . . K . . .

241                 250                 260
W . . . Q . R . . L . N T . . . L G . .

261                 270
. . Y G . G L . N . . A A . .
```

FIG. 2

Comparison of subtilisin sequences from:

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

```
01                  10                  20                  30
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

41                  50                  60                  70
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 D 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

81                  90                  100                 110
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 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 S I A Q G L E W A G N N G M H

121                 130                 140                 150
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 V 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 R G V 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
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FIG 3/A

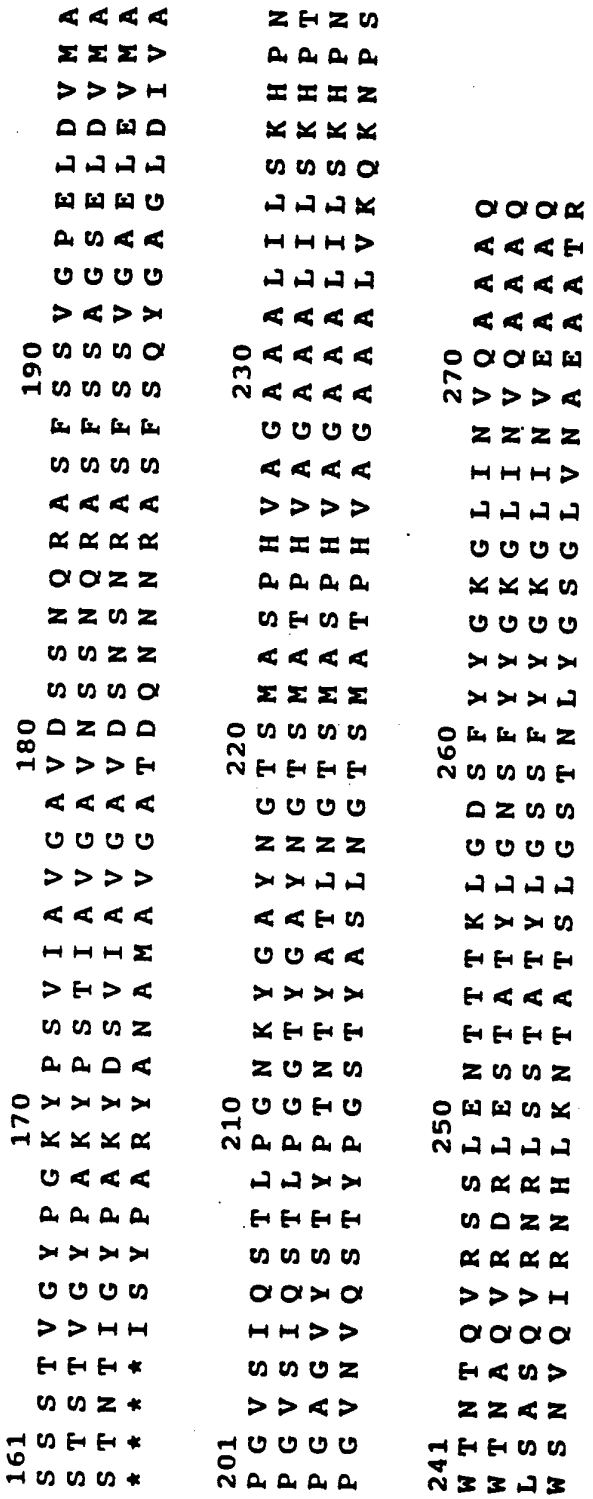


FIG 3/B

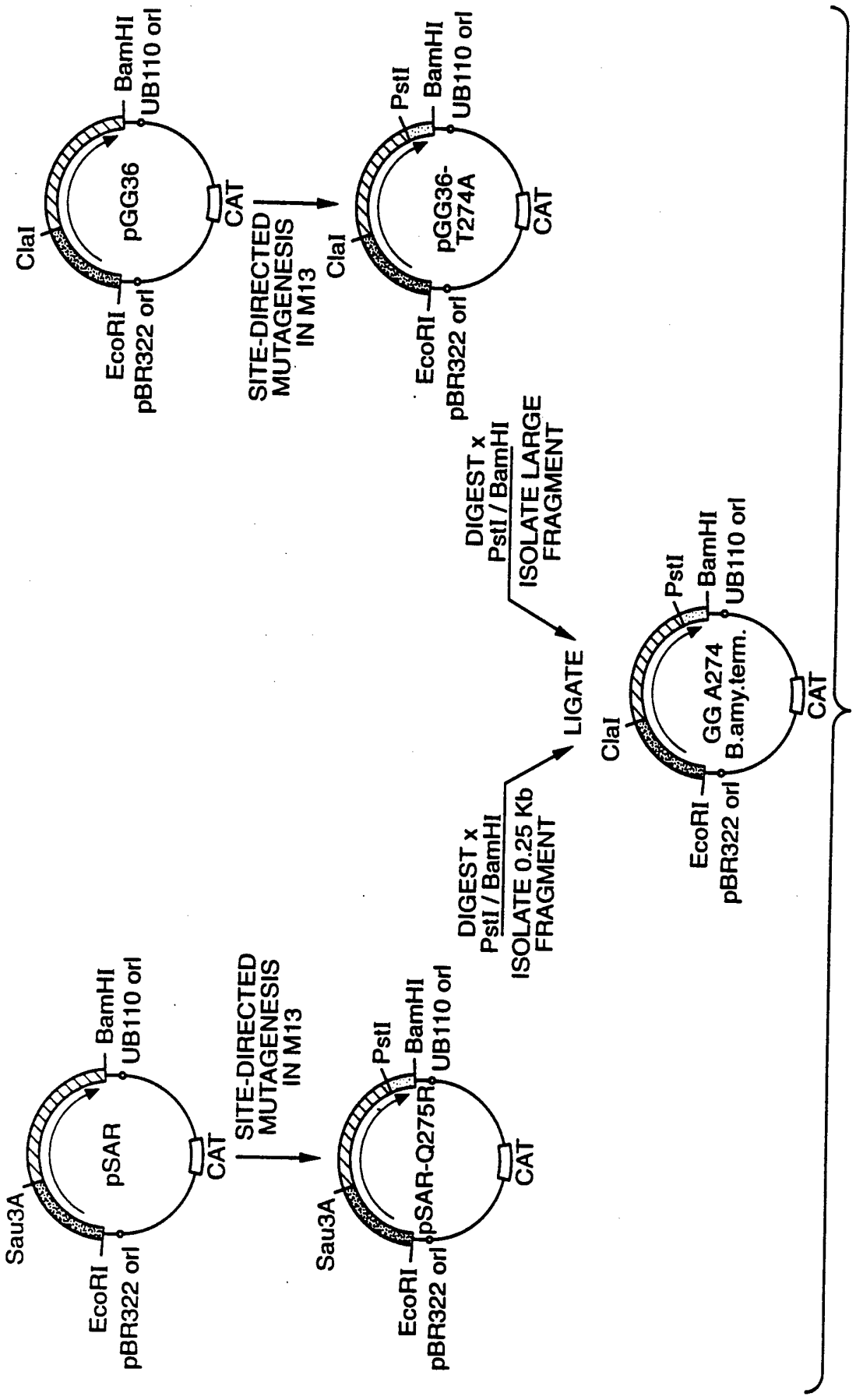
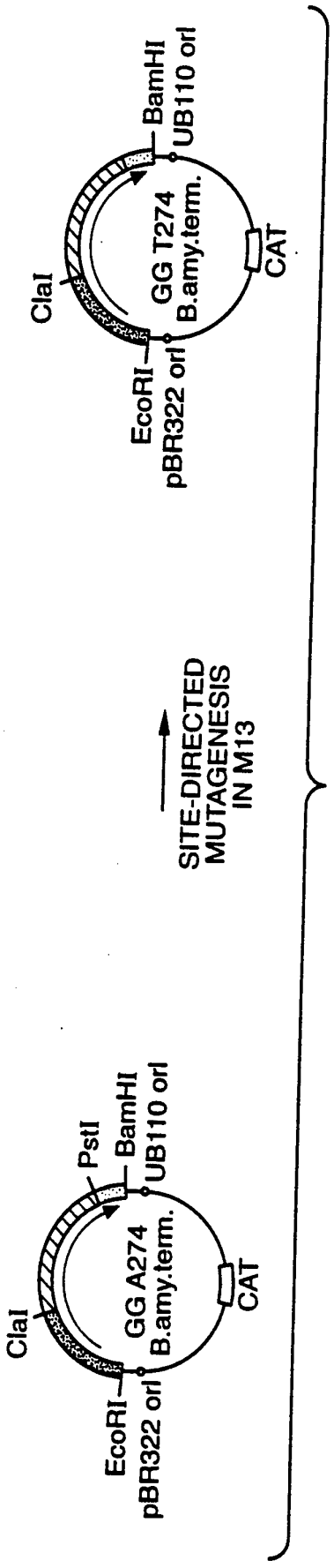

pSAR
Sau3A
EcoRI
pBR322 ori
BamHI
UB110 ori
CAT
SITE-DIRECTED MUTAGENESIS IN M13
pSAR-Q275R
PstI
DIGEST x PstI / BamHI
ISOLATE 0.25 Kb FRAGMENT
pGG36
ClaI
pGG36-T274A
ISOLATE LARGE FRAGMENT
LIGATE
GG A274 B.amy.term.

FIG._4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GG A274
B.amy.term.
PstI
BamHI
UB110 ori
CAT
SITE-DIRECTED
MUTAGENESIS
IN M13
ClaI
EcoRI
pBR322 ori
GG T274
B.amy.term.
BamHI
UB110 ori
CAT

FIG._5

```
          10                  30                  50
ATGAAGAAACCGTTGGGGAAAATTGTCGCAAGCACCGCACTACTCATTTCTGTTGCTTTT
MetLysLysProLeuGlyLysIleValAlaSerThrAlaLeuLeuIleSerValAlaPhe

          70                  90                 110
AGTTCATCGATCGCATCGGCTGCTGAAGAAGCAAAAGAAAAATATTTAATTGGCTTTAAT
SerSerSerIleAlaSerAlaAlaGluGluAlaLysGluLysTyrLeuIleGlyPheAsn

         130                 150                 170
GAGCAGGAAGCTGTCAGTGAGTTTGTAGAACAAGTAGAGGCAAATGACGAGGTCGCCATT
GluGlnGluAlaValSerGluPheValGluGlnValGluAlaAsnAspGluValAlaIle

         190                 210                 230
CTCTCTGAGGAAGAGGAAGTCGAAATTGAATTGCTTCATGAATTTGAAACGATTCCTGTT
LeuSerGluGluGluGluValGluIleGluLeuLeuHisGluPheGluThrIleProVal

         250                 270                 290
TTATCCGTTGAGTTAAGCCCAGAAGATGTGGACGCGCTTGAACTCGATCCAGCGATTTCT
LeuSerValGluLeuSerProGluAspValAspAlaLeuGluLeuAspProAlaIleSer

         310                 330                 350
TATATTGAAGAGGATGCAGAAGTAACGACAATGGCGCAATCAGTGCCATGGGGAATTAGC
TyrIleGluGluAspAlaGluValThrThrMetAlaGlnSerValProTrpGlyIleSer

         370                 390                 410
CGTGTGCAAGCCCCAGCTGCCCATAACCGTGGATTGACAGGTTCTGGTGTAAAAGTTGCT
ArgValGlnAlaProAlaAlaHisAsnArgGlyLeuThrGlySerGlyValLysValAla

         430                 450                 470
GTCCTCGATACAGGTATTTCCACTCATCCAGACTTAAATATTCGTGGTGGCGCTAGCTTT
ValLeuAspThrGlyIleSerThrHisProAspLeuAsnIleArgGlyGlyAlaSerPhe

         490                 510                 530
GTACCAGGGGAACCATCCACTCAAGATGGGAATGGGCATGGCACGCATGTGGCCGGGACG
ValProGlyGluProSerThrGlnAspGlyAsnGlyHisGlyThrHisValAlaGlyThr

         550                 570                 590
ATTGCTGCTTTAAACAATTCGATTGGCGTTCTTGGCGTAGCGCCGAGCGCGGAACTATAC
IleAlaAlaLeuAsnAsnSerIleGlyValLeuGlyValAlaProSerAlaGluLeuTyr

         610                 630                 650
GCTGTTAAAGTATTAGGGGCGAGCGGTTCAGGTTCGGTCAGCTCGATTGCCCAAGGATTG
AlaValLysValLeuGlyAlaSerGlySerGlySerValSerSerIleAlaGlnGlyLeu

         670                 690                 710
GAATGGGCAGGGAACAATGGCATGCACGTTGCTAATTTGAGTTTAGGAAGCCCTTCGCCA
GluTrpAlaGlyAsnAsnGlyMetHisValAlaAsnLeuSerLeuGlySerProSerPro

         730                 750                 770
AGTGCCACACTTGAGCAAGCTGTTAATAGCGCGACTTCTAGAGGCGTTCTTGTTGTAGCG
SerAlaThrLeuGluGlnAlaValAsnSerAlaThrSerArgGlyValLeuValValAla

         790                 810                 830
GCATCTGGGAATTCAGGTGCAGGCTCAATCAGCTATCCGGCCCGTTATGCGAACGCAATG
AlaSerGlyAsnSerGlyAlaGlySerIleSerTyrProAlaArgTyrAlaAsnAlaMet

         850                 870                 890
GCAGTCGGAGCTACTGACCAAAACAACAACCGCGCCAGCTTTTCACAGTATGGCGCAGGG
AlaValGlyAlaThrAspGlnAsnAsnAsnArgAlaSerPheSerGlnTyrGlyAlaGly
```

FIG. 6A

```
       910                 930                 950
CTTGACATTGTCGCACCAGGTGTAAACGTGCAGAGCACATACCCAGGTTCAACGTATGCC
LeuAspIleValAlaProGlyValAsnValGlnSerThrTyrProGlySerThrTyrAla

       970                 990                1010
AGCTTAAACGGTACATCGATGGCTACTCCTCATGTTGCAGGTGCAGCAGCCCTTGTTAAA
SerLeuAsnGlyThrSerMetAlaThrProHisValAlaGlyAlaAlaAlaLeuValLys

      1030                1050                1070
CAAAAGAACCCATCTTGGTCCAATGTACAAATCCGCAATCATCTAAAGAATACGGCAACG
GlnLysAsnProSerTrpSerAsnValGlnIleArgAsnHisLeuLysAsnThrAlaThr

      1090                1110                1130
AGCTTAGGAAGCACGAACTTGTATGGAAGCGGACTTGTCAATGCAGAAGCGGCAACACGC
SerLeuGlySerThrAsnLeuTyrGlySerGlyLeuValAsnAlaGluAlaAlaThrArg
```

FIG. 6B

```
          10                  30                  50
ATGAAGAAACCGTTGGGGAAAATTGTCGCAAGCACCGCACTACTCATTTCTGTTGCTTTT
MetLysLysProLeuGlyLysIleValAlaSerThrAlaLeuLeuIleSerValAlaPhe

          70                  90                  110
AGTTCATCGATCGCATCGGCTGCTGAAGAAGCAAAAGAAAAATATTTAATTGGCTTTAAT
SerSerSerIleAlaSerAlaAlaGluGluAlaLysGluLysTyrLeuIleGlyPheAsn

          130                 150                 170
GAGCAGGAAGCTGTCAGTGAGTTTGTAGAACAAGTAGAGGCAAATGACGAGGTCGCCATT
GluGlnGluAlaValSerGluPheValGluGlnValGluAlaAsnAspGluValAlaIle

          190                 210                 230
CTCTCTGAGGAAGAGGAAGTCGAAATTGAATTGCTTCATGAATTTGAAACGATTCCTGTT
LeuSerGluGluGluGluValGluIleGluLeuLeuHisGluPheGluThrIleProVal

          250                 270                 290
TTATCCGTTGAGTTAAGCCCAGAAGATGTGGACGCGCTTGAACTCGATCCAGCGATTTCT
LeuSerValGluLeuSerProGluAspValAspAlaLeuGluLeuAspProAlaIleSer

          310                 330                 350
TATATTGAAGAGGATGCAGAAGTAACGACAATGGCGCAATCAGTGCCATGGGGAATTAGC
TyrIleGluGluAspAlaGluValThrThrMetAlaGlnSerValProTrpGlyIleSer

          370                 390                 410
CGTGTGCAAGCCCCAGCTGCCCATAACCGTGGATTGACAGGTTCTGGTGTAAAAGTTGCT
ArgValGlnAlaProAlaAlaHisAsnArgGlyLeuThrGlySerGlyValLysValAla

          430                 450                 470
GTCCTCGATACAGGTATTTCCACTCATCCAGACTTAAATATTCGTGGTGGCGCTAGCTTT
ValLeuAspThrGlyIleSerThrHisProAspLeuAsnIleArgGlyGlyAlaSerPhe

          490                 510                 530
GTACCAGGGGAACCATCCACTCAAGATGGGAATGGGCATGGCACGCATGTGGCCGGGACG
ValProGlyGluProSerThrGlnAspGlyAsnGlyHisGlyThrHisValAlaGlyThr

          550                 570                 590
ATTGCTGCTTTAGACAACTCGATTGGCGTTCTTGGCGTAGCGCCGAGCGCGGAACTATAC
IleAlaAlaLeuAspAsnSerIleGlyValLeuGlyValAlaProSerAlaGluLeuTyr

          610                 630                 650
GCTGTTAAAGTATTAGGGGCGAGCGGTTCAGGCGCCATCAGCTCGATTGCCCAAGGATTG
AlaValLysValLeuGlyAlaSerGlySerGlyAlaIleSerSerIleAlaGlnGlyLeu

          670                 690                 710
GAATGGGCAGGGAACAATGGCATGCACGTTGCTAATTTGAGTTTAGGAAGCCCTTCGCCA
GluTrpAlaGlyAsnAsnGlyMetHisValAlaAsnLeuSerLeuGlySerProSerPro

          730                 750                 770
AGTGCCACACTTGAGCAAGCTGTTAATAGCGCGACTTCTAGAGGCGTTCTTGTTGTAGCG
SerAlaThrLeuGluGlnAlaValAsnSerAlaThrSerArgGlyValLeuValValAla

          790                 810                 830
GCATCTGGGAATTCAGGTGCAGGCTCAATCAGCTATCCGGCCCGTTATGCGAACGCAATG
AlaSerGlyAsnSerGlyAlaGlySerIleSerTyrProAlaArgTyrAlaAsnAlaMet

          850                 870                 890
GCAGTCGGAGCTACTGACCAAAACAACAACCGCGCCAGCTTTTCACAGTATGGCGCAGGG
AlaValGlyAlaThrAspGlnAsnAsnAsnArgAlaSerPheSerGlnTyrGlyAlaGly
```

FIG. 7A

```
                                930                 950
CTTGACATTGTCGCACCAGGTGTAAACGTGCAGAGCACATACCCAGGTTCAACGTATGCC
LeuAspIleValAlaProGlyValAsnValGlnSerThrTyrProGlySerThrTyrAla

      970                 990                 1010
AGCTTAAACGGTACATCGATGGCTACTCCTCATGTTGCAGGTGCAGCAGCCCTTGTTAAA
SerLeuAsnGlyThrSerMetAlaThrProHisValAlaGlyAlaAlaAlaLeuValLys

     1030                1050                1070
CAAAAGAACCCATCTTGGTCCAATGTACAAATCCGCAATCATCTAAAGAATACGGCAACG
GlnLysAsnProSerTrpSerAsnValGlnIleArgAsnHisLeuLysAsnThrAlaThr

     1090                1110                1130
AGCTTAGGAAGCACGAACTTGTATGGAAGCGGACTTGTCAATGCAGAAGCGGCAACACGC
SerLeuGlySerThrAsnLeuTyrGlySerGlyLeuValAsnAlaGluAlaAlaThrArg
```

FIG. 7B

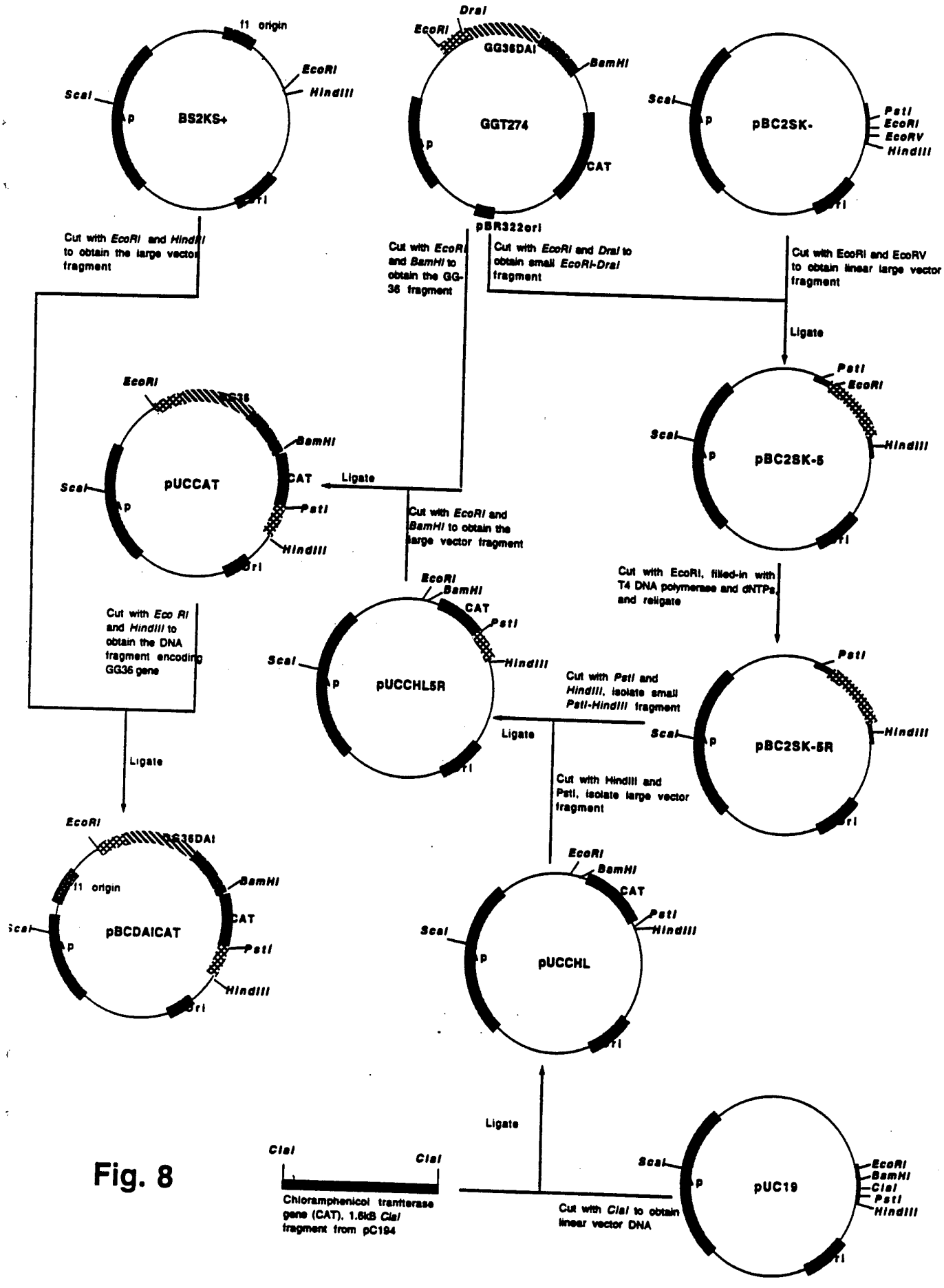
f1 origin
EcoRI
HindIII
ScaI
BS2KS+
DraI
EcoRI
GG36DAI
BamHI
GGT274
CAT
pBR322ori
pBC2SK-
PstI
EcoRI
EcoRV
HindIII
Cut with EcoRI and HindIII to obtain the large vector fragment
Cut with EcoRI and BamHI to obtain the GG-36 fragment
Cut with EcoRI and DraI to obtain small EcoRI-DraI fragment
Cut with EcoRI and EcoRV to obtain linear large vector fragment
Ligate
pUCCAT
Ligate
Cut with EcoRI and BamHI to obtain the large vector fragment
pBC2SK-5
Cut with EcoRI, filled-in with T4 DNA polymerase and dNTPs, and religate
Cut with Eco RI and HindIII to obtain the DNA fragment encoding GG36 gene
pUCCHL5R
Cut with PstI and HindIII, isolate small PstI-HindIII fragment
Ligate
pBC2SK-5R
Cut with HindIII and PstI, isolate large vector fragment
Ligate
pBCDAICAT
pUCCHL
Ligate
ClaI
ClaI
Chloramphenicol tranferase gene (CAT), 1.6kB ClaI fragment from pC194
Cut with ClaI to obtain linear vector DNA
pUC19

Fig. 8

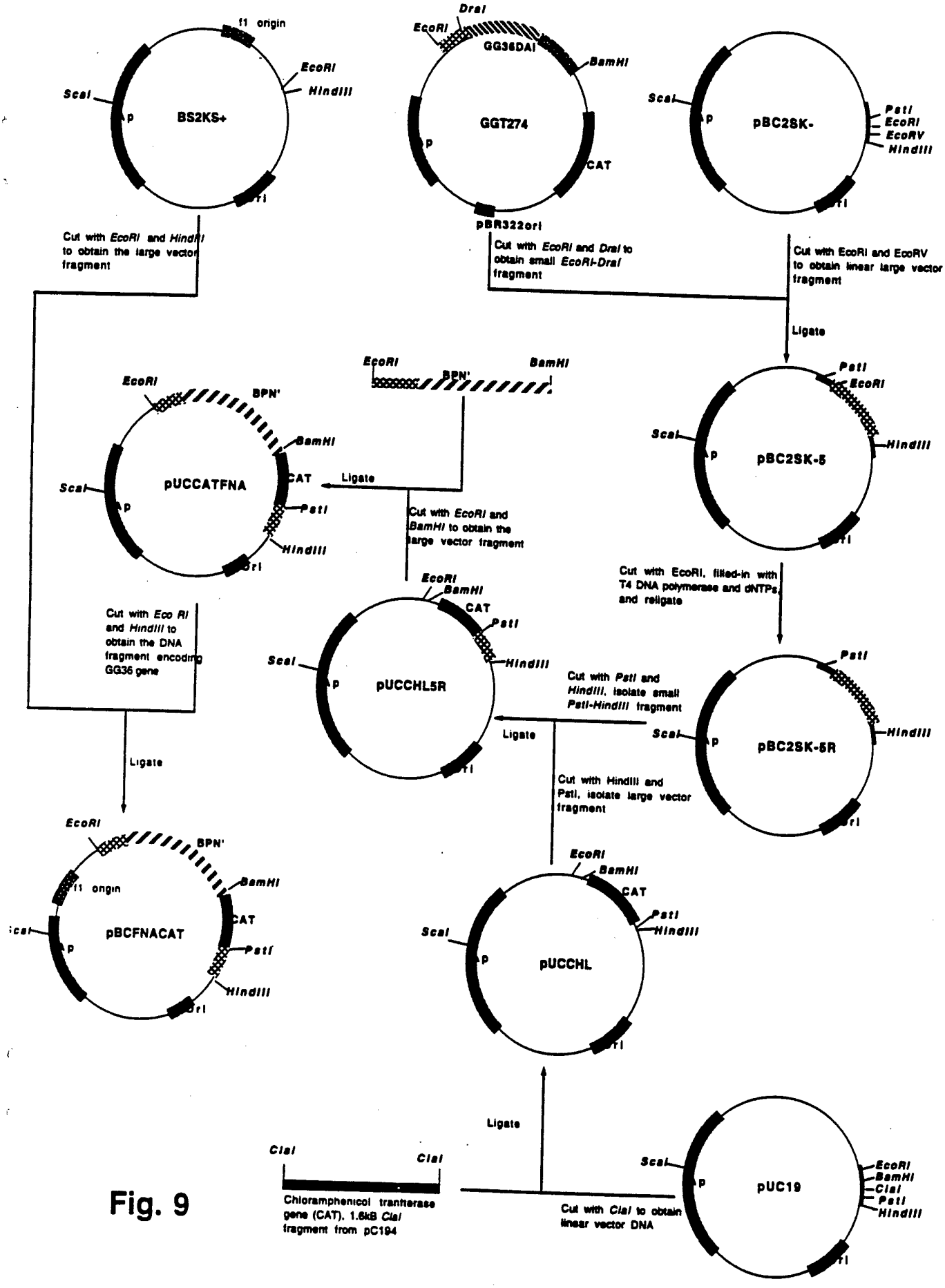
f1 origin
EcoRI
HindIII
ScaI
BS2KS+
Cut with EcoRI and HindIII to obtain the large vector fragment
DraI
EcoRI
GG36DAI
BamHI
GGT274
CAT
pBR322ori
Cut with EcoRI and DraI to obtain small EcoRI-DraI fragment
pBC2SK-
PstI
EcoRI
EcoRV
HindIII
Cut with EcoRI and EcoRV to obtain linear large vector fragment
Ligate
EcoRI
BPN'
BamHI
pUCCATFNA
CAT
PstI
HindIII
Ligate
Cut with EcoRI and BamHI to obtain the large vector fragment
pBC2SK-5
Cut with EcoRI, filled-in with T4 DNA polymerase and dNTPs, and religate
Cut with Eco RI and HindIII to obtain the DNA fragment encoding GG36 gene
pUCCHL5R
Cut with PstI and HindIII, isolate small PstI-HindIII fragment
pBC2SK-5R
Ligate
Cut with HindIII and PstI, isolate large vector fragment
Ligate
pBCFNACAT
pUCCHL
ClaI
Chloramphenicol tranferase gene (CAT), 1.6kB ClaI fragment from pC194
Ligate
Cut with ClaI to obtain linear vector DNA
pUC19
EcoRI
BamHI
ClaI
PstI
HindIII

Fig. 9

INTERNATIONAL SEARCH REPORT

Internat l Application No
PCT/US 94/11777

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C11D3/386 A61K7/48 C12N15/57 C11D3/39

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C11D C12N

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

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

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP,A,0 405 901 (UNILEVER) 2 January 1991 see page 30, line 25 - page 35, line 47; claims; examples	1-5,7-9, 11,12
X	EP,A,0 398 539 (AMGEN INC.) 22 November 1990 see page 7, line 14 - line 43; claims 1-23; examples	1-5,7-9, 11,12
X	WO,A,88 08033 (AMGEN INC.) 20 October 1988 see page 14, line 1 - page 15, line 30; claims	1-5,7
A	WO,A,89 09830 (GENEX CORP.) 19 October 1989 see claims 11-16	1,11

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
24 February 1995	- 8. 03. 95

Name and mailing address of the ISA	Authorized officer
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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	EP,A,0 516 200 (UNILEVER) 2 December 1992 see page 12, line 10 - page 13, line 50; claims 1-10	1-5,7-9, 31
A	EP,A,0 170 386 (PROCTER & GAMBLE) 5 February 1986 see claims	1-7, 13-16, 19,22,24
P,A	WO,A,94 10284 (PROCTER & GAMBLE) 11 May 1994 see the whole document	1-7, 13-16, 19,22, 31,32

INTERNATIONAL SEARCH REPORT

Information on patent family members

Interna' l Application No
PCT/US 94/11777

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
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		JP-T-	4500385	23-01-92
		EP-A-	0405902	02-01-91
		WO-A-	9100335	10-01-91
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		AU-A-	5558090	18-12-90
		CA-A-	2016211	17-11-90
		JP-T-	3505976	26-12-91
		WO-A-	9014420	29-11-90
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		AU-A-	1701688	04-11-88
		CA-A-	1316473	20-04-93
		EP-A-	0309565	05-04-89
		JP-T-	1502957	12-10-89
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		JP-T-	3503602	15-08-91
		US-A-	4990452	05-02-91
		US-A-	5246849	21-09-93
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EP-A-0170386	05-02-86	AU-B-	584621	01-06-89
		AU-A-	4388685	02-01-86
		AU-B-	585762	22-06-89
		AU-A-	4388785	02-01-86
		CA-A,C	1254581	23-05-89
		DE-A-	3586957	18-02-93
		DE-D-	3587678	20-01-94
		DE-T-	3587678	30-06-94
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		EP-A-	0484324	06-05-92
		IE-B-	59250	26-01-94
		JP-A-	61081498	25-04-86
		JP-A-	61081499	25-04-86

INTERNATIONAL SEARCH REPORT

Information on patent family members

Internal Application No
PCT/US 94/11777

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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