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(54) SUBTILISIN MUTANTS

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EP-A- 0 130 756	**WO-A-89/06279**
US-A- 4 760 025	**US-A- 4 914 031**

- **TIBS TRENDS IN BIOCHEMICAL SCIENCES. vol. 13, August 1988, CAMBRIDGE EN pages 291 - 297; J. WELLS AND D. ESTELL: "Subtilisin - an enzyme designed tobe engineered"**

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Description

Field of the Invention

The present invention relates to novel carbonyl hydrolase mutants having an amino acid sequence wherein one or more amino acid residues of a precursor carbonyl hydrolase, specifically those at positions corresponding to residues +123 and optionally +274 in Bacillus amyloliquefaciens subtilisin, have been substituted with a different amino acid. Such mutant carbonyl hydrolases, in general, are obtained by in vitro modification of a precursor DNA sequence encoding a naturally-occurring or recombinant carbonyl hydrolase to encode the substitution of one or both of these amino acid residues in a precursor amino acid sequence alone or in combination with other substitution, insertion or deletion in the precursor amino acid sequence.

Background of the Invention

Serine proteases are a subgroup of carbonyl hydrolase. They comprise a diverse class of enzymes having a wide range of specificities and biological functions. Stroud, R. M.-(1974), Sci.Amer., 131, 74-88. Despite their functional diversity, the catalytic machinery of serine proteases has been approached by at least two genetically distinct families of enzymes: the subtilisins and the mammalian chymotrypsin related and homologous bacterial serine proteases (e.g., trypsin and S.gresius trypsin). These two families of serine proteases show remarkably similar mechanisms of catalysis. Kraut, J. (1977), Ann. Rev. Biochem., 46, 331-358. Furthermore, although the primary structure is unrelated, the tertiary structure of these two enzyme families bring together a conserved catalytic triad of amino acids consisting of serine, histidine and aspartate.

Subtilisin is a serine endoprotease (MW 27,500) which is secreted in large amounts from a wide variety of Bacillus species and other microorganisms. The protein sequence of subtilisin has been determined from at least four different species of Bacillus. Markland, F.S., et al. (1983), Honne-Seyler's Z. Physiol. Chem., 364, 1537-1540. The three-dimensional crystallographic structure of Bacillus amyloliquefaciens subtilisin to 2.5A resolution has also been reported. Wright, C.S., et al. (1969), Nature, 221, 235-242; Drenth, J., et al. (1972), Eur. J. Biochem., 26, 177-181. These studies indicate that although subtilisin is genetically unrelated to the mammalian serine proteases, it has a similar active site structure. The x-ray crystal structures of subtilisin containing covalently bound peptide inhibitors (Robertus, J.D., et al. (1972), Biochemistry, 11, 2439-2449), or product complexes (Robertus, J.D., et al. (1976), J. Biol. Chem., 251, 1097-1103), have also provided information regarding the active site and putative substrate binding cleft of subtilisin. In addition, a large number of kinetic and chemical modification studies have been reported for subtilisin (Philipp, M., et al. (1983), Mol. Cell. Biochem., 51, 5-32; Svendsen, B. (1976), Carlsbera Res. Comm., 41, 237-291; Markland, F. S. Id.) as well as at least one report wherein the side chain of methione at residue 222 of subtilisin was converted by hydrogen peroxide to methionine-sulfoxide (Stauffer, D.C., et al. (1965), J. Biol. Chem., 244, 5333-5338) and the side chain of serine at residue 221 converted to cysteine by chemical modification (Polgar, et al. (1981), Biochimica et Biophysica Acta, 667, 351-354.)

U.S. Patent No. 4,760,025 and EPO Publication No. 0 130 756 published January 9, 1985 each disclose the modification of subtilisin amino acid residues corresponding to positions in Bacillus amyloliquefacien subtilisin tyrosine -1, aspartate +32, asparagine +155, tyrosine +104, methionine +222, glycine +166, histidine +64, glycine +169, phenylalanine +189, serine +33, serine +221, tyrosine +217, glutamate +156 and alanine +152. EPO Publication No. 0 251 446 published January 7, 1988 discloses other amino acid residues in Bacillus amyloliquefaciens subtilisin and their equivalents which may be modified by way of substitution, insertion or deletion and which may be combined with modifications to the residues identified in U.S. Patent No. 4,760,025 to form useful subtilisin mutants. The particular residues identified herein, however, are not identified in these references.

Similarly, PCT Publication No. WO 89/09819 and W0 89/09830 each published October 19, 1989, and PCT publication W089/06279 published on July 13, 1989, disclose subtilisin enzymes made by mutating a nucleotide sequence coding for a subtilisin. Numerous amino acid residues are identified in each of these publications which may be so modified. However, as with the previously identified references, neither identifies the residues of the present invention.

Accordingly, the present invention provides carbonyl hydrolase mutants containing the substitution of amino acid residues in a precursor carbonyl hydrolase corresponding to positions +123 and optionally + 274 in Bacillus amyloliquefaciens subtilisin. Such mutants generally have at least one property which is different from the same property of the carbonyl hydrolase precursor from which the amino acid of said mutant is derived, in particular having altered proteolytic activity compared to the precursor carbonyl hydrolase. The present invention further provides DNA sequences encoding such carbonyl hydrolase mutants as well as expression vectors containing such mutant DNA sequences.

The present invention further provides host cells transformed with such vectors as well as host cells which are capable of expressing such DNA to produce carbonyl hydrolase mutants either intracellularly or extracellularly.

Thus, the invention includes non-naturally occurring carbonyl hydrolase mutants having a different proteolytic activity, stability, and/or performance characteristic as compared to the precursor carbonyl hydrolase from which the amino acid sequence of the mutant is derived. The precursor carbonyl hydrolase may be a naturally-occurring carbonyl hydrolase or recombinant hydrolase. Specifically, such carbonyl hydrolase mutants have an amino acid sequence, not found in nature, which is derived by replacement of one or more amino acid residues of a precursor carbonyl hydrolase with one or more different amino acids. The one or more amino acid residues of the precursor enzyme correspond to position Asn +123, and optionally Ala +274, of Bacillus amyloliquefaciens subtilisin or equivalent amino acid residues in other carbonyl hydrolases or subtilisins.

The invention also includes mutant DNA sequences encoding such carbonyl hydrolase or subtilisin mutants.

These mutant DNA sequences are derived from a precursor DNA sequence which encodes a naturally-occurring or recombinant precursor enzyme. The mutant 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 position +123, and optionally +274, in Bacillus amyloliquefaciens. These recombinant DNA sequences encode carbonyl hydrolase mutants 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, stability and/or enhanced performance characteristics.

The invention also includes procaryotic and eucaryotic subtilisins with a different amino acid residue such as serine, at positions equivalent to Asn +123 in Bacillus amyloliquefaciens subtilisin and to subtilisin with different amino acid residues at positions equivalent to position +274 in Bacillus amyloliquefaciens subtilisin.

Further, the invention includes expression vectors containing such mutant carbonyl hydrolase DNA sequences as well as host cells transformed with such vectors which are capable of producing such mutants. The invention also relates to detergent compositions comprising the carbonyl hydrolase mutants of the invention.

Brief Description of the Drawings

Fig. 1 depicts the DNA and amino acid sequence for Bacillus amyloliquefaciens subtilisin and a partial restriction map of this gene.

Fig. 2 depicts the conserved amino acid residues among subtilisins from Bacillus amyloliquefaciens, Bacillus subtilis varI1668and Bacillus licheniformis (carlsbergensis).

Figs. 3A and 3B depict the amino acid sequence of subtilisin from Bacillus amyloliquefaciens, Bacillus subtilis varI168 and Bacillus licheniformis.

Fig. 4 depicts the amino acid sequence of three subtilisins. The top line represents the amino acid sequence of subtilisin from Bacillus amyloliquefaciens subtilisin (also sometimes referred to as subtilisin BPN'). The second line depicts the amino acid sequence of subtilisin from Bacillus lentus (subtilisin 309 in PCT Publication No. W0 89/06276). The bottom line represents the amino acid sequence of a preferred embodiment of the invention designated GG-RYSA. The symbol * denotes the absence of specific amino acid residues as compared to subtilisin BPN'.

Fig. 5 depicts the construction of plasmid pGG A274.

Fig. 6 depicts the construction of pGG-KVNA which is an intermediate to plasmid pGG-RYSA.

Fig. 7 depicts the oligonucleotide-duplex method used to construct a synthetic Bacillus lentus subtilisin gene.

Fig. 8 depicts the strategy for constructing a synthetic gene encoding Bacillus lentus subtilisin.

Fig. 9 depicts the cassette used to make substitutions in the DNA at codon position +123 by cassette mutagenesis. XXX represents the codon modified to encode the amino acid substitutions at position +123.

Fig. 10 depicts the DNA and amino acid sequence of a preferred embodiment of the invention wherein the DNA sequence is a synthetic DNA. The DNA in this Figure has been modified to encode arginine at position 27, serine at position 78, tyrosine at position 104, serine at position 123 and alanine at position 274.

Detailed Description of the Invention

It has been discovered that in vitro mutations in the carbonyl hydrolase subtilisin at an amino acid residue equivalent to +123 in Bacillus amyloliquefaciens subtil sin produces subtilisin mutants exhibiting altered proteolytic activity over precursor subtilisins.

It has also been discovered that in vitro mutation at residues equivalent to +274 in Bacillus amyloliquefaciens subtilisin produce subtilisin mutants exhibiting altered stability, e.g. modified autoproteolytic stability. In some instances, these latter mutants also exhibit enhanced performance when used in detergent compositions.

Carbonyl hydrolases are enzymes which hydrolyze compounds containing

$$\overset{\displaystyle O}{\overset{\|}{C}}\!-\!X$$

bonds in which X is oxygen or nitrogen. They include naturally-occurring carbonyl hydrolases and recombinant carboryl 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, in EPO Publication No. 0 130 756 published January 9, 1985 and EPO Publication No. 0 251 446 published January 7, 1988.

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 the subtilisins identified in Fig. 3 herein and as described in PCT Publication W0 89/06276 and EPO Publication No. 0 283 075.

"Recombinant subtilisin" refers to a subtilisin in which the DNA sequence encoding the subtilisin is modified to produce a 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 EPO Publication Nos. 0 130 756 and 0 251 446 and PCT Publication Nos. W0 89/06279, W0 89/09830 and W0 89/09819.

"Non-human carbonyl hydrolases" and the DNA encoding them may be obtaine 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 Saccaromycees 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 mutant" has an amino acid sequence which is derived from the amino acid sequence of a "precursor carbonyl hydrolase". The precursor carbonyl hydrolases include naturally-occurring carbonyl hydrolases and recombinant carbonyl hydrolases. The amino acid sequence of the carbonyl hydrolase mutant is "derived" from the precursor hydrolase amino acid sequence by the substitution, deletion or insertion of one or more amino acids of the precursor amino acid sequence. Such modification is of the "precursor DNA sequence" which encodes the amino acid sequence of the precursor carbonyl hydrolase rather than manipulation of the precursor carbonyl hydrolase enzyme per se· Suitable methods for such manipulation of the precursor DNA sequence include methods disclosed herein and in EPO Publication Nos. 0 130 756 and 0 251 446.

Specific residues corresponding to positions +123 and +274 of Bacillus amylolicuefaciens subtilisin are identified herein for substitution. These amino acid position numbers refer to those assigned to the mature Bacillus amyloliquefaciens subtilisin sequence presented in Fig. 1. The invention, however, is 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.

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 amylolicuefaciens subtilisin primary sequence and particularly to a set of residues known to be invariant in all subtilisins for which sequence is known (Fig. 2). 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 Bacillusamyloliquefaciens 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 var. I168 and Bacillus lichenformis (carlsbergensis) 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 are the residues 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) and the preferred subtilisin mutant herein. These particular amino acid sequences are aligned in Fig. 4 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 and in the preferred subtilisin mutant of the invention as compared to Bacillus amyloliquefaciens subtilisin. Thus, the equivalent amino acid for Val-165 in Bacillus amyloliquefaciens subtilisin in the other subtilisins is the particular isoleucine shown beneath Val-165.

In Fig. 4, the amino acid at position 123 is asparagine in Bacillus amyloliquefaciens subtilisin. In Bacillus lentus subtilisin the equivalent residue is the particular asparagine shown. In the preferred subtilisin mutant of the invention, however, the amino acid equivalent to +123 in Bacillus amyloliquefaciens subtilisin is an amino acid other than asparagine and is preferably the serine shown in Fig. 4. Similarly, in Fig. 4, the amino acid at position +274 Bacillus amyloliquefaciens subtilisin is alanine. As can be seen, the equivalent amino acid in Bacillus lentus subtilisin is the particular threonine shown in Fig. 4. In a particular preferred subtilisin mutant of the invention, the equivalent amino acid position 274 is occupied by the alanine shown in Fig. 4.

Thus, the positions +123 and +274 are identified by primary amino acid sequences in Fig. 4 for the subtilisin from Bacillus lentus and the preferred embodiment of the invention. However, various other amino acid residues may be modified which are equivalent to specific amino acids in Bacillus amyloliquefaciens subtilisin. Thus, in the preferred embodiment, the amino acid lysine at position 27 in Bacillus amylolicuefaciens subtilisin has an equivalent lysine at position 27 in Bacillus lentus subtilisin. As indicated in the Examples, the subtilisin comprising one of the preferred embodiments of the invention was derived by modifying a DNA sequence encoding Bacillus lentus subtilisin. Such modifications to the DNA included the modification of codons equivalent to positions 123 and 274 of Bacillus amyloliquefaciens subtilisin. However, two other modifications were made to the Bacillus lentus amino acid sequence at positions equivalent to residues 27 and 104 in Bacillus amyloliquefaciens subtilisin. Thus, as can be seen in Fig. 4, the lysine at equivalent residue 27 in Bacillus lentus subtilisin was modified to encode arginine in the preferred embodiment. Similarly, the valine residue at position 104 of Bacillus lentus, which is equivalent to tyrosine 104 in Bacillus amyloliquefaciens subtilisin, was also modified to encode tyrosine. Thus, the preferred embodiment shown in Fig. 4 contains an amino acid sequence derived from Bacillus lentus subtilisin by modifying residues of that subtilisin equivalent to positions 27, 104, 123 and 274 of Bacillus amyloliquefaciens subtilisin.

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.lnm 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 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 mutant 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 mutants of the present invention include the mature forms of carbonyl hydrolase mutants as well as the pro- and prepro-forms of such hydrolase mutants. The prepro-forms are the preferred construction since this facilitates the expression, secretion and maturation of the carbonyl hydrolase mutants.

"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 mutants, specifically subtilisin mutants, is the putative prosequence of Bacillus amyloliquefaciens subtilisin although other subtilisin prosequences may be used. In the Examples, the putative pro sequence from the subtilisin from Bacillus lentus (ATCC 21536) was 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 present invention utilizes such sequences to effect the secretion of the carbonyl hydrolase mutants as defined 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 mutant 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, the invention is intended to include 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 EPO Publication No. 0 130 756 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 EPO Publication No. 0 130 756 and further described by Yang, M.Y., et al. (1984), J. Bacteriol., 160, 15-21. Other host cells for expressing subtilisin include Bacillus subtilis I168 (EPO Publication No. 0 130 756).

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 mutants or expressing the desired carbonyl hydrolase mutant. In the case of vectors which encode the pre or prepro form of the carbonyl hydrolase mutant, such mutants, 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 described in EPO Publication Nos. 0 130 756 and 0 251 446. As can be seen from the examples disclosed therein, the methods generally comprise synthesizing labelled 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 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.

Alternatively, a synthetic gene encoding a naturally-occurring or mutant precursor carbonyl hydrolase may be produced. In such an approach, the DNA and/or amino acid sequence of the precursor hydrolase 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. This approach provides several advantages over cloning the natural gene in that restriction sites may be interposed throughout the DNA without change in the amino acid sequence encoded so as to facilitate subsequent modification to form mutant carbonyl hydrolases. Further, the synthetic approach allows for adjusting the codon usage in the synthetic gene to conform with the codon bias for the particular expression hosts to be used. An example of synthetic gene construction is set forth in the Examples.

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 EPO Publication Nos. 0 130 756 and 0 251 446 and the production of carbonyl hydrolase mutants described herein.

The following cassette mutagenesis method may be used to facilitate the construction and identification of the carbonyl hydrolase mutants of 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 enormously 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 one aspect cf the invention, the objective is to secure a mutant carbonyl hydrolase having a greater (numerically large) proteolytic activity as compared to the precursor carbonyl hydrolase, thereby enabling the use of the enzyme to more efficiently act on a target substrate. Specific amino acids useful to obtain such results in subtilisin-type carbonyl hydrolases at residues equivalent to +123 in Bacillus amyloliquefaciens subtilisin are presented in the Examples. In some instances, lower proteolytic activity may be desirable. In such cases a decrease in proteolytic activity can be produced by substituting the amino acids identified in the examples at residues equivalent to +123 in Bacillus amyloliquefaciens subtilisin.

For precursor subtilisins wherein serine is not the residue at the position equivalent to +123 in Bacillus amyloliquefaciens the greatest proteolytic activity is obtained when serine is substituted in the precursor at position +123. Further, no naturally-occurring Bacillus subtilisin is known to exist which contains serine at a position equivalent to +123 in Bacillus amyloliquefaciens subtilisin. Based on the discovery that serine at this position enhances proteolytic activity, one skilled in the art can screen naturally-occurring Bacillus subtilisin to identify and clone a natural mutant containing serine at this position. Such natural Bacillus subtilisin mutants are within the scope of the invention.

Where the carbonyl hydrolase is from other than Bacillus and a serine is present at +123 in the precursor enzyme the substitution can be one that decreases proteolytic activity. This would be useful, for example, where the synthetic activity of the carbonyl hydrolases is desired (as for synthesizing peptides). One may wish to decrease this proteolytic activity which is capable of destroying the product of such synthesis.

In another aspect of the invention, it has been determined that residues equivalent to +274 in Bacillus amyloliquefaciens subtilisin are important in modulating the overall performance characteristics of the enzyme in detergent compositions. Thus, as set forth in the Examples, the threonine in Bacillus lentus subtilisin at equivalent position +274 can be mutated to alanine in the preferred embodiment to produce enhanced performance of the mutant enzyme. As also disclosed in the Examples, substitution of this residue with an amino acid other than threonine, e.g. leucine, serine, valine and alanine results in a decrease in the stability of the mutant. Such decrease in stability is believed to be the result of autocatalytic degradation of the mutant. Thus, modifications of residues equivalent to +274 in Bacillus subtilisin are capable of enhancing the overall performance of the enzyme in a detergent composition and modulating the overall stability of the enzyme. In this aspect of the invention, the objective is to secure a mutant carbonyl hydrolase having enhanced performance when used in a detergent composition as compared to the precursor carbonyl hydrolase. As used herein, enhanced performance in a detergent is defined as increased cleaning of certain enzyme sensitive stains such as grass or blood. This cleaning is determined by visual evaluation after a standard wash cycle.

A preferred embodiment of the invention is set forth in the Examples wherein the lysine at position 27 is substituted with arginine, the valine at position 104 is substituted with tyrosine, the asparagine at position 123 substituted with serine and the threonine at residue 274 is substituted with alanine in Bacillus lentus subtilisin. Although the stability of this enzyme is somewhat reduced as compared to the precursor Bacillus lentus subtilisin, the performance level of this enzyme in a detergent composition is substantially enhanced such that the same performance of this Bacillus lentus subtilisin mutant is obtained as compared to the unmodified Bacillus lentus subtilisin when using approximately one-half the amount of enzyme.

Based on the results obtained with chis and other mutant subtilisins, it is apparent that residues in carbonyl hydrolases equivalent to positions +123 and +274 in Bacillus amyloliquefaciens are important to the proteolytic activity, performance and/or stability of these enzymes.

Many of the carbonyl hydrolase mutants of the invention, especially subtilisin, are useful ir formulating various detergent compositions. A number of known compounds are suitable surfactants useful in compositions comprising the carbonyl hydrolase mutants of the invention. These include nonionic, anionic, cationic, anionic, or zwitterionic detergents, as disclosed in U.S. 4,404,128 to Barry J. Anderson and U.S. 4,261,868 to Jiri Flora, et al. The art is familiar with the different formulations which can be used as cleaning compositions.

Subtilisins of the invention can be formulated into known powdered and liquid detergents having pH between 6.5 and 12.0 at levels of about .01 to about 5% preferably .1% to .05%) by weight. These detergent cleaning compositions can also include other enzymes such as known proteases and amylases, as well as builders and stabilizers.

The addition of subtilisins of the invention to conventional cleaning compositions does not create any special use limitation. In other words, any temperature and pH suitable for the detergent is also suitable for the present compositions as long as the pH is within the above range, and the temperature is below the subtilisins of the invention denaturing temperature. In addition, subtilisins of the invention can be used in a cleaning composition without detergents, again either alone or in combination with builders and stabilizers.

The following is presented by way of example and is not to be construed as a limitation to the scope of the claims.

EXAMPLE 1

Constructions for Expression of Bacillus lentus Subtilisin Gene in Bacillus subtilis

Plasmid pSAR, Fig. 5, carries a translational fusion via a common Sau3A restriction site at the seventh/eighth signal sequence codon of the subtilisin genes of B. subtilis and B. amyloliquefaciens. As shown in Fig. 5, this gene, on an EcoRI-BamHI 2.0 Kb fragment, was subcloned into M13mp19 in order to isolate single-stranded template DNA to be used for site-directed mutagenesis to form pSAR-Q275R. The mutagenesis protocol was essentially that of Zoller, M., et al. (1983), Methods Enzymol., 100, 468-500, (1) and used a synthetic oligonucleotide of the sequence:

```
                  *           **
5'-C-AAC-GTA-CAG-GCT-GCA-GCT-CGC-TAA-AAC-ATA-A-3'
                        Q275R
```

where the asterisks denote changes from the wild-type gene sequences and the underline represents an introduced PstI restriction endonuclease site used in screening for the particular mutant gene encoding the Q275R change. These changes were made to (1) convert the amino acid at this position to that found in Bacillus lentus subtilisin and (2) to allow hookup of the terminator in pSAR to the mature coding region of Bacillus lentus via a Pst site similarly introduced into pGG36 from Bacillus lentus (ATCC 21536).

Plasmid pGG36, Fig. 5, contains a 2.1 kb genomic DNA fragment from Bacillus lentus (ATCC 21536) encoding the complete subtilisin gene which was cloned by standard methods in the shuttle vector pBS42. Band, L., et al. (1984), DNA, 3, 17-21.

The amino acid sequence for this subtilisin is the same as that disclosed for subtilisin 309 in PCT Publication No. 89/06279. This gene was subcloned into M13 as above for site-directed mutagenesis using an oligonucleotide of the sequence:

```
                  *      * *
5'-C-AAT-GCA-GAA-GCT-GCA-GCT-CGC-TAA-TCA-A-3'
                        T274A
```

in order to 1) introduce a PstI site at the same location in this gene corresponding to the site introduced into pSAR above and 2) to substitute the threonine at position 274 with alanine to form pGG36-T274A.

The mutant pSAR-Q275R and pGG36-T274A genes were individually subcloned back into pBS42 prior to PstI/BamHI digestions, fragment isolation and ligation to produce plasmid GG-A274B.amy.term. as shown in Fig. 5, all by standard methods.

A synthetic DNA linker was made by annealing complimentary single-stranded oligonucleotides of the sequences:

```
5'-G-ATC-GTC-GCG-TCG-ACC-GCA-CTA-CTC-ATT-TCT-GTT-
GCT-TTT-AGT-TCA-T-3'
```

and

```
5'-CGA-TGA-ACT-AAA-AGC-AAC-AGA-AAT-GAG-TAG-TGC-
GGT-CGA-CGC-GAC-3'
```

to give the double-stranded DNA fragment #2 shown in Fig. 6. The recessed left- and right-hand ends of this duplex linker are complimentary to the Sau3A end of fragment #1 (from pSAR) and the ClaI end of fragment #3 (from pGG-A274 B.amy.term), respectively. These 3 fragments were combined with fragment 4 from pSAR-Q275R after restriction endonuclease digestions of plasmids, fragment isolation and ligation by standard methods to produce plasmid pGG-KVNA. The designation GG-KVNA indicates that this subtilisin contains the subtilisin encoded by pGG-36 which includes lysine (K) at position 27, valine (V) at position 104, asparagine (N) at position 123 and the substitution of threonine at position 274 with alanine (A).

EXAMPLE 2

Modification of PGG-KVNA

As indicated in Fig. 6, the GG-KVNA gene (2.1 kb EcoRI-BamHI fragment) was subcloned into M13 for three successive rounds of site-directed mutagenesis using oligonucleotides having the sequence :

$$\text{(a)}\quad 5'\text{-GT-TCT-GGT-GTA-}\underset{\text{K27R}}{\text{A}\overset{*}{\text{G}}\text{A}}\text{-GTT-GCT-GT}\underline{\overset{*}{\text{T}}\text{-CT}\overset{*}{\text{A}}\text{-GA}}\text{T-ACA-GGT-}3'\text{,}$$

$$\text{(b)}\quad 5'\text{-A-GTA-TTA-GGG-}\underline{\text{GC}\overset{*}{\text{T}}\text{-AGC}}\text{-GGT-TCA-GGT-TCG-}\underset{\text{V104y}}{\overset{*}{\text{T}}\overset{*}{\text{A}}\text{C}}\text{-AGC-TCG-ATT-}3'$$

and

$$\text{(c)}\quad 5'\text{-GGG-AAC-AAT-G}\overline{\text{G}\overset{*}{\text{A}}\text{-ATG-C}}\text{AC-GTT-}\underset{\text{N123S}}{\underline{\text{GCT-A}\overset{*}{\text{G}}\overset{*}{\text{C}}}}\text{-TTG-AGT-TTA-}3'$$

The asterisks denote changes from the wild-type gene sequence. The underlines represent, in (a) an introduced XbaI site and in (b) and (c) introduced NheI sites used to screen for the presence of the linked R27, Y104 and S123 mutations, respectively. In addition, in (c), the overlined denotes a destroyed SphI site. Finally, the 2.1 kb GG-RYSA gene was subcloned back into pBS42 for expression in B. subtilis hosts.

The resultant plasmid was designated pGG-RYSA. This designation indicates that four residues were modified in the pGG-KVNA plasmid. Lysine (K) at position 27 to arginine (R), valine (V) to tyrosine (Y) at position 104 and asparagine (N) at position 123 to serine (S). The alanine previously substituted at residue 274 was not modified in this procedure.

The lysine at position 27 was substituted with arginine based upon the amino acid sequencing of subtilisin 309. As indicated in PCT Publication No. W089/06279, lysine is located at position 27. However, after independently sequencing this subtilisin protein, the initial data indicated that arginine was the residue at position 27. In the case of the substitution of tyrosine for valine at residue 104, the substitution was made to lower the pH activity profile and to increase the performance of the enzyme based on results previously obtained for Bacillus amyloliquefaciens subtilisin (sometimes referred to as BPN'). The substitution of asparagine at position 123 with serine is based on the results obtained hereinafter wherein it was determined that the substitution of serine at position 123 maximized the proteolytic activity of the enzyme in a closely related mutant.

EXAMPLE 3

Construction of Synthetic Bacillus lentus Subtilisin Gene

DNA encoding the amino acid sequence of Bacillus lentus subtilisin was also prepared by constructing a gene encoding a synthetic DNA sequence.

The 2.1 kb HindIII genomic fragment from plasmid pGG36 was sequenced. The deduced amino acid sequence of the mature gene product (GG36 subtilisin) was used to design a synthetic mature coding sequence with the following properties: (1) In general, the codons most frequently found for each amino acid in seven different B. subtilis genes (from a tabulation of codon usages, Table 2 from Maruyama, T., et al., (1986), Nucl. Acids Res., Supplement 14 pp. r151-r197) were utilized except in the cases where alternate codons resulted in conveniently located restriction enzyme recognition sites within the gene; (2) Approximately every 40-60 b.p. of the ~0.8 mature coding region, combinations of 2 or 3 specifically chosen codons were utilized which resulted in the introduction of fairly evenly spaced, unique restriction sites. These sites were chosen to facilitate (a) later cassette mutagenesis and screening studies and (b) constructions involving more than one mutation; (3) A unique Pst I recognition site was designed to cover codons 272-274 allowing hook up to the terminator sequences of a Bacillus amyloliquefaciens gene similarly modified over the same three codons and substituting threonine at position 274 with alanine; and (4) A unique NruI site was introduced to cover mature codons residues 9-10 allowing hookup to GG36's pre-pro coding sequence via a short synthetic duplex DNA linker. Based on this design, oligonucleotides ("oligos") were synthesized such that upon annealing the coding and noncoding oligos for a given ~60 b.p. coding region, the resultant duplex DNA fragment would have at it's ends single stranded regions complimentary to the end of the next duplex fragment of the gene (see, Fig. 7).

A total of 36 separate oligos (comprising 18 individual duplexes) were used in the scheme, as outlined above, resulting in an ~0.8kb duplex synthetic mature coding region (Fragment 3 in Fig. 8).

Finally, one additional pair or synthetic oligo's was synthesized, which upon annealing (to give fragment 2 in Fig. 8) has an NcoI site at it's 5' end (complimentary to GG36's NcoI site at mature codons 5-6) and an NruI site at its 3' end (complimentary to the 3's 5' end of fragment 3).

The final construction to give a complete expression unit consisting of B. subtilis promoter and the first seven amino acids of the signal sequence hooked up to GG36's sequences encoding the remainder of the signal sequence, the complete pro sequence and the first six mature amino acids (Fragment 1 from GG-KVNA), the synthetic gene encoding mature residues 7-274 (Fragments 2+3) and the terminator region (including the final mature gene codon 279) of Bacillus amyloliquefaciens (fragment 4) was done as a four-way ligation as set forth in Fig. 6.

Finally, three additional separate mutations were introduced into the mature coding region of this full length hybrid gene. The first substituted the lysine at position 27 with arginine. The second substituted the valine at position 104 with tyrosine. The third substituted the asparagine at position 123 with serine The resultant plasmid is designated pBC3-RYSA. The following example describes the method used to modify position 123 in the synthetic gene. Similar methods were used to modify positions 27 and 104 in this synthetic gene.

EXAMPLE 4

Construction of Position 123 Mutants

An Xho I site was introduced over codons 111/112 in the synthetic gene from Example 3 by making three phenotypically silent mutations via site directed mutagenesis (primer extension mutagenesis in M13). The resulting plasmid, pX123 (Fig. 9), was digested with Xho I and Ava I and the large vector-containing fragment isolated by electroelution from agarose gel. complimentary synthetic oligonucleotides were annealed, ligated with the pX123 large fragment and transformed into E· coli strain MM294. These cassettes encoded, individually, all 20 naturally-occurring amino acids at position 123, and in addition contained a silent mutation which destroyed a unique Sph I site lying between the Xho I and Ava I sites in pX123. Resulting plasmids from E. coli transformants were screened for the loss of the unique Sph I site. Positives by restriction analysis (i.e., Sph I negatives) were sequenced to confirm the presence of the desired position 123 mutations subcloned into the shuttle vector pBS42 and transformed into Bacillus subtilis BG2036 for expression.

EXAMPLE 5

Activity of Various +123 Mutants

Proteolytic activity of each of the subtilisin mutants encoded by the above modified position +123 mutants was assayed by mixing 0.04 ml of supernatant from centrifuged-culture broths with 0.56 ml of 1% w/v casein in 0.1M Tris pH8.60. After a 20 minute incubation at 37°C, reactions were quenched by precipitation with 10% trichloroacetic acid (TCA). Activity was determined from the absorbance at a wavelength of 280nm for the supernatant after precipitation with 10% TCA.

TABLE I

Relative proteolytic activity of codon 123 variants normalized to Asn-123 mutant

Codon 123	% Proteolytic Activity
Ser	116
Asn	100
Cys	22
Gly	12
Ala	9

TABLE I (continued)

Relative proteolytic activity of codon 123 variants normalized to Asn-123 mutant	
Codon 123	% Proteolytic Activity
Thr	7
Gln	7
Val	6
Glu	<5
Ile	<5
Trp	<5
Phe	<5
Asp	<5
His	<5
Leu	<5
Met	<5
Pro	<5
Tyr	<5

In the process of final confirmation of the DNA sequence of the synthetic gene coding for the enzyme BC3-RYSA, proline was found to be at position 78 instead of serine (the amino acid at this position in Bacillus lentus subtilisin). The initial properties of the position 123 mutations were tested in this enzyme, BC3-RPYA (proline at position 78). These results are shown in Table I. The amino acid at position 78 was thereafter changed back to serine to form the DNA and amino acid sequence shown in Fig. 10 by replacing the synthetic DNA duplex corresponding to that portion of the gene. As can be seen the substitution of Asn with Ser at position +123 results in a substantial increase in proteolytic activity. The relationship between the various subtilisins discussed herein are summarized for positions 27, 78, 104, 123 and 274 in Table II.

TABLE II

	position				
	27	78	104	123	274
GC36 (genomic)	Lys(K)	Ser(S)	Val(V)	Asn(N)	Thr(T)
Synthetic B. lentus gene	Lys(K)	Pro(P)	Val(V)	Asn(N)	Ala(A)
B.amyloliquefacins subtilisin (BPN)	Lys(K)	Ser(S)	Tyr(Y)	Asn(N)	Ala(A)
Subtilisin 309 as published	Lys(K)	Ser(S)	Val(V)	Asn(N)	Thr(T)
Preferred embodiment herein	Arg(R)	Ser(S)	Tyr(Y)	Ser(S)	Ala(A)

EXAMPLE 6

Stability of Position 274 Mutants

Stability of position 274 mutants in BC3-RPY (arginine at position 27, proline at position 78, and tyrosine at position 104 in Bacillus lentus subtilisin) are shown in Table III. Data are percent activity remaining following incubation at 37°C in 50mM EDTA for 60 minutes.

TABLE III

Amino Acid at Position 274	% Activity
Leucine	2%
Serine	79%
Threonine	91%
Valine	42%
Alanine	43%

Mutations at this position clearly effect stability of the enzyme. Although the alanine mutation was not as stable as serine or threonine at this position, this enzyme provided superior performance relative to Bacillus lentus subtilisin under the conditions of use described. For different applications, other amino acids at position 274 may be used.

EXAMPLE 7

Detergent Composition

A spray-dried phosphate detergent granule of the following composition was prepared:

Component	Weight %
Sodium C12 linear alkylbenzene sulfonate	8.45
Sodium Tallow Alcohol sulfate	4.23
Sodium C14~15 linear alkyl sulfate	4.23
Sodium Toluene Sulfonate	1.00
Sodium Tripolyphosphate	5.60
Sodium pyrophosphate	22.40
Silicate (1.6 r)	5.50
Sodium Sulfate	29.83
Sodium polyacrylate (4500 MW)	1.17
Brightener	0.22
Sodium Carbonate	12.30
Polyethylene Glycol (MW 80)0)	0.47
C12~13 alcohol polyethoxylate (6.5)*	0.50
Miscellaneous + Water	to 100%
Protease**	0.034

*Alcohol and monoethoxylat alcohol removed.

**mg active enzyme/g (2.0 mg active enzyme/g stock)

A 0.1 weight percent solution of this composition in water had a pH of 10.0. The composition with subtilisin mutant of the invention (Fig. 7) provided superior cleaning of enzyme-sensitive stains, when compared to Bacillus lentus at 0.068 mg active enzyme/g product, in a 95°F (35°C) wash at 6 grains per gallon (gpg) hardness (3:1 Ca/Mg).

Throughout this application reference is made to various amino acids by way of common one-and three-letter codes. Such codes are identified in Proteins: Structures and Molecular Proteases, Thomas E. Creighton, eds. W.N. Freeman, N.Y., N.Y. (1983), p.3.

Although the preferred form of the invention has been described above, it will be obvious to those skilled in the art to which the invention pertains, that, after understanding the invention as a whole, various changes and equivalent modifications may be made without departing from the scope of the invention as defined by the appended claims.

All publications are expressly incorporated herein by reference.

Claims

Claims for the following Contracting States : AT, BE, CH, DE, DK, FR, GB, GR, IT, LU, NL, SE

1. A carbonyl hydrolase mutant having an amino acid sequence not found in nature which is derived from a precursor carbonyl hydrolase by substituting a different amino acid for the amino acid residue at a position in said precursor equivalent to +123 in Bacillus amyloliquefaciens subtilisin wherein the mutant has an altered proteolytic activity compared to the precursor carbonyl hydrolase.

2. The carbonyl hydrolase mutant according to claim 1 having further substitution at a position equivalent to +274 in Bacillus amyloliquefaciens.

3. The carbonyl hydrolase mutant of claim 1 or claim 2 wherein said precursor carbonyl hydrolase is a subtilisin.

4. The carbonyl hydrolase mutant according to claim 3 wherein said precursor carbonyl hydrolase is a Bacillus subtilisin.

5. The carbonyl hydrolase mutant of any one of claims 1 to 4 wherein the amino acid residue in said mutant at position +123 is serine and the amino acid residue in said mutant at position +274 is alanine.

6. The carbonyl hydrolase mutant of any one of claims 1 to 5 wherein the proteolytic activity of the mutant is increased relative to the precursor carbonyl hydrolase.

7. A mutant Bacillus subtilisin having the amino acid sequence:

AQSVPWGISRVQAPAAHNRGLTGSGVRVAVLDTGISTHPDLNIRGGASFVPGE
PSTQDGNGHGTHVAGTIAALNNSIGVLGVAPSAELYAVKVLGASGSGSYSSIA
QGLEWAGNNGMHVASLSLGSPSPSATLEQAVNSATSRGVLVVAASGNSGAGSI
SYPARYANAMAVGATDQNNNRASFSQYGAGLDIVAPGVNVQSTYPGSTYASLN
GTSMATPHVAGAAALVKQKNPSWSNVQIRNHLKNTATSLGSTNLYGSGLVNAE

AAAR

8. DNA encoding a carbonyl hydrolase mutant of any one of claims 1 to 7.

9. An expression vector including the DNA of claim 8.

10. Host cells transformed with the expression vector of claim 9.

11. A method of producing a carbonyl hydrolase mutant having an amino acid sequence not found in nature which is derived from a precursor carbonyl hydrolase by substituting a different amino acid for the amino acid residue at a position in said precursor equivalent to +123 in Bacillus amyloliquefaciens subtilisin, the method comprising culturing the host cells of claim 10 and separating the carbonyl hydrolase mutant produced.

12. An enzymatic cleaning composition capable of degrading proteins comprising:

(a) a surfactant; and,
(b) a carbonyl hydrolase mutant having an amino acid sequence not found in nature which is derived from a precursor carbonyl hydrolase by substituting a different amino acid for the amino acid residue at a position in said precursor equivalent to +123 in Bacillus amyloliquefaciens subtilisin.

13. The composition of claim 12 wherein said carbonyl hydrolase enzyme is a subtilisin.

14. The composition of claim 12 or claim 13 wherein said subtilisin has the following amino acid sequence:

AQSVPWGISRVQAPAAHNRGLTGSGVRVAVLDTGISTHPDLNIRGGASFVPGE
PSTQDGNGHGTHVAGTIAALNNSIGVLGVAPSAELYAVKVLGASGSGSYSSIA

QGLEWAGNNGMHVASLSLGSPSPSATLEQAVNSATSRGVLVVAASGNSGAGSI
SYPARYANAMAVGATDQNNNRASFSQYGAGLDIVAPGVNVQSTYPGSTYASLN
GTSMATPHVAGAAALVKQKNPSWSNVQIRNHLKNTATSLGSTNLYGSGLVNAE
AAAR

15. The composition of any one of claims 12 to 14 wherein the surfactant comprises a detergent.

16. A composition according to any one of claims 12 to 15 formulated as a spray-dried detergent granule.

Claims for the following Contracting State : ES

1. A process which comprises preparing a carbonyl hydrolase mutant having an amino acid sequence not found in nature which is derived from a precursor carbonyl hydrolase by substituting a different amino acid for the amino acid residue at a position in said precursor equivalent to +123 in Bacillus amyloliquefaciens subtilisin wherein the mutant has an altered proteolytic activity compared to the precursor carbonyl hydrolase.

2. The process according to claim 1 wherein the carbonyl hydrolase mutant has further substitution at a position equivalent to +274 in Bacillus amyloliquefaciens.

3. The process of claim 1 or claim 2 wherein said precursor carbonyl hydrolase is a subtilisin.

4. The process according to claim 3 wherein said precursor carbonyl hydrolase is a Bacillus subtilisin.

5. The process of any one of claims 1 to 4 wherein the amino acid residue in said mutant at position +123 is serine and the amino acid residue in said mutant at position +274 is alanine.

6. The process of any one of claims 1 to 5 wherein the proteolytic activity of the mutant is increased relative to the precursor carbonyl hydrolase.

7. A process which comprises preparing a mutant Bacillus subtilisin having the amino acid sequence:

AQSVPWGISRVQAPAAHNRGLTGSGVRVAVLDTGISTHPDLNIRGGASFVPGE
PSTQDGNGHGTHVAGTIAALNNSIGVLGVAPSAELYAVKVLGASGSGSYSSIA
QGLEWAGNNGMHVASLSLGSPSPSATLEQAVNSATSRGVLVVAASGNSGAGSI
SYPARYANAMAVGATDQNNNRASFSQYGAGLDIVAPGVNVQSTYPGSTYASLN
GTSMATPHVAGAAALVKQKNPSWSNVQIRNHLKNTATSLGSTNLYGSGLVNAE

AAAR

8. DNA encoding a carbonyl hydrolase mutant produced by the process of any one of claims 1 to 7.

9. An expression vector including the DNA of claim 8.

10. Host cells transformed with the expression vector of claim 9.

11. A method of producing a carbonyl hydrolase mutant having an amino acid sequence not found in nature which is derived from a precursor carbonyl hydrolase by substituting a different amino acid for the amino acid residue at a position in said precursor equivalent to +123 in Bacillus amyloliquefaciens subtilisin, the method comprising culturing the host cells of claim 10 and separating the carbonyl hydrolase mutant produced.

12. A process which comprises preparing an enzymatic cleaning composition capable of degrading proteins comprising:

(a) a surfactant; and,
(b) a carbonyl hydrolase mutant having an amino acid sequence not found in nature which is derived from a precursor carbonyl hydrolase by substituting a different amino acid for the amino acid residue at a position in said precursor equivalent to +123 in Bacillus amyloliquefaciens subtilisin.

13. The process of claim 12 wherein said carbonyl hydrolase enzyme is a subtilisin.

14. The process of claim 12 or claim 13 wherein said subtilisin has the following amino acid sequence:

AQSVPWGISRVQAPAAHNRGLTGSGVRVAVLDTGISTHPDLNIRGGASFVPGE
PSTQDGNGHGTHVAGTIAALNNSIGVLGVAPSAELYAVKVLGASGSGSYSSIA

QGLEWAGNNGMHVASLSLGSPSPSATLEQAVNSATSRGVLVVAASGNSGAGSI
SYPARYANAMAVGATDQNNNRASFSQYGAGLDIVAPGVNVQSTYPGSTYASLN
GTSMATPHVAGAAALVKQKNPSWSNVQIRNHLKNTATSLGSTNLYGSGLVNAE
AAAR

15. The process of any one of claims 12 to 14 wherein the surfactant comprises a detergent.

16. A process according to any one of claims 12 to 15 formulated as a spray-dried detergent granule.

Patentansprüche

Patentansprüche für folgende Vertragsstaaten : AT, BE, CH, DE, DK, FR, GB, GR, IT, LU, NL, SE

1. Carbonylhydrolasemutante mit einer Aminosäuresequenz, die in der Natur nicht gefunden wird und die von einer Vorläufer-Carbonylhydrolase abgeleitet ist, indem der Aminosäurerest an einer Position in dem Vorläufer, die äquivalent ist zu +123 in Bacillus amyloliquefaciens-Subtilisin, durch eine unterschiedliche Aminosäure ausgetauscht wird, wobei die Mutante eine veränderte proteolytische Aktivität im Vergleich zu der Vorläufer-Carbonylhydrolase aufweist.

2. Carbonylhydrolasemutante nach Anspruch 1, die eine weitere Substitution an einer Position, die äquivalent ist zu +274 in Bacillus amyloliquefaciens, aufweist.

3. Carbonylhydrolasemutante nach Anspruch 1 oder Anspruch 2, wobei die Vorläufer-Carbonylhydrolase ein Subtilisin ist.

4. Carbonylhydrolasemutante nach Anspruch 3, wobei die Vorläufer-Carbonylhydrolase ein Bacillus-Subtilisin ist.

5. Carbonylhydrolasemutante nach einem der Ansprüche 1 bis 4, wobei der Aminosäurerest in der Mutante an Position +123 Serin ist und der Aminosäurerest in der Mutante an Position +274 Alanin ist.

6. Carbonylhydrolasemutante nach einem der Ansprüche 1 bis 5, wobei die proteolytische Aktivität der Mutante gegenüber der Vorläufer-Carbonylhydrolase erhöht ist.

7. Mutiertes Bacillus-Subtilisin mit der Aminosäuresequenz:

AQSVPWGISRVQAPAAHNRGLTGSGVRVAVLDTGISTHPDLNIRGGASFVPGE
PSTQDGNGHGTHVAGTIAALNNSIGVLGVAPSAELYAVKVLGASGSGSYSSIA
QGLEWAGNNGMHVASLSLGSPSPSATLEQAVNSATSRGVLVVAASGNSGAGSI
SYPARYANAMAVGATDQNNNRASFSQYGAGLDIVAPGVNVQSTYPGSTYASLN
GTSMATPHVAGAAALVKQKNPSWSNVQIRNHLKNTATSLGSTNLYGSGLVNAE
AAAR

8. DNA, die eine Carbonylhydrolasemutante nach einem der Ansprüche 1 bis 7 kodiert.

9. Expressionsvektor, der die DNA nach Anspruch 8 enthält.

10. Wirtszellen, die mit dem Expressionsvektor nach Anspruch 9 transformiert worden sind.

11. Verfahren zur Herstellung einer Carbonylhydrolasemutante mit einer Aminosäuresequenz, die in der Natur nicht gefunden wird und die von einer Vorläufer-Carbonylhydrolase abgeleitet ist, indem der Aminosäurerest an einer Position in dem Vorläufer, die äquivalent ist zu +123 in Bacillus amyloliquefaciens-Subtilisin, durch eine unterschiedliche Aminosäure ausgetauscht wird, wobei das Verfahren umfaßt, die Wirtszellen nach Anspruch 10 zu züchten und die produzierte Carbonylhydrolasemutante abzutrennen.

12. Enzymatische Reinigungszusammensetzung, die in der Lage ist, Proteine abzubauen, umfassend:

(a) ein grenzflächenaktives Mittel und
(b) eine Carbonylhydrolasemutante mit einer Aminosäuresequenz, die in der Natur nicht gefunden wird und die von einer Vorläufer-Carbonylhydrolase abgeleitet ist, indem der Aminosäurerest an einer Position in dem Vorläufer, die äquivalent ist zu +123 in Bacillus amyloliquefaciens-Subtilisin, durch eine unterschiedliche Aminosäure ausgetauscht wird.

13. Zusammensetzung nach Anspruch 12, wobei das Carbonylhydrolaseenzym ein Subtilisin ist.

14. Zusammensetzung nach Anspruch 12 oder Anspruch 13, wobei das Subtilisin die folgende Aminosäuresequenz hat:

AQSVPWGISRVQAPAAHNRGLTGSGVRVAVLDTGISTHPDLNIRGGASFVPGE
PSTQDGNGHGTHVAGTIAALNNSIGVLGVAPSAELYAVKVLGASGSGSYSSIA
QGLEWAGNNGMHVASLSLGSPSPSATLEQAVNSATSRGVLVVAASGNSGAGSI
SYPARYANAMAVGATDQNNNRASFSQYGAGLDIVAPGVNVQSTYPGSTYASLN
GTSMATPHVAGAAALVKQKNPSWSNVQIRNHLKNTATSLGSTNLYGSGLVNAE
AAAR

15. Zusammensetzung nach einem der Ansprüche 12 bis 14, wobei das grenzflächenaktive Mittel ein Detergens umfaßt.

16. Zusammensetzung nach einem der Ansprüche 12 bis 15, die als ein sprühgetrocknetes Detergensgranulat formuliert ist.

Patentansprüche für folgenden Vertragsstaaten : ES

1. Verfahren, umfassend die Herstellung einer Carbonylhydrolasemutante mit einer Aminosäuresequenz, die in der Natur nicht gefunden wird und die von einer Vorläufer-Carbonylhydrolase abgeleitet ist, indem der Aminosäurerest an einer Position in dem Vorläufer, die äquivalent ist zu +123 in Bacillus amyloliquefaciens-Subtilisin, durch eine unterschiedliche Aminosäure ausgetauscht wird, wobei die Mutante eine veränderte proteolytische Aktivität im Vergleich zu der Vorläufer-Carbonylhydrolase aufweist.

2. Verfahren nach Anspruch 1, wobei die Carbonylhydrolasemutante eine weitere Substitution an einer Position, die äquivalent ist zu +274 in Bacillus amyloliquefaciens, aufweist.

3. Verfahren nach Anspruch 1 oder Anspruch 2, wobei die Vorläufer-Carbonylhydrolase ein Subtilisin ist.

4. Verfahren nach Anspruch 3, wobei die Vorläufer-Carbonylhydrolase ein Bacillus-Subtilisin ist.

5. Verfahren nach einem der Ansprüche 1 bis 4, wobei der Aminosäurerest in der Mutante an Position +123 Serin ist und der Aminosäurerest in der Mutante an Position +274 Alanin ist.

6. Verfahren nach einem der Ansprüche 1 bis 5, wobei die proteolytische Aktivität der Mutante gegenüber der Vorläufer-Carbonylhydrolase erhöht ist.

7. Verfahren, umfassend die Herstellung eines Mutierten Bacillus-Subtilisins mit der Aminosäuresequenz:

AQSVPWGISRVQAPAAHNRGLTGSGVRVAVLDTGISTHPDLNIRGGASFVPGE
PSTQDGNGHGTHVAGTIAALNNSIGVLGVAPSAELYAVKVLGASGSGSYSSIA
QGLEWAGNNGMHVASLSLGSPSPSATLEQAVNSATSRGVLVVAASGNSGAGSI
SYPARYANAMAVGATDQNNNRASFSQYGAGLDIVAPGVNVQSTYPGSTYASLN
GTSMATPHVAGAAALVKQKNPSWSNVQIRNHLKNTATSLGSTNLYGSGLVNAE
AAAR

8. DNA, die eine Carbonylhydrolasemutante kodiert, welche nach dem Verfahren gemäß einem der Ansprüche 1 bis 7 hergestellt worden ist.

9. Expressionsvektor, der die DNA nach Anspruch 8 enthält.

10. Wirtszellen, die mit dem Expressionsvektor nach Anspruch 9 transformiert worden sind.

11. Verfahren zur Herstellung einer Carbonylhydrolasemutante mit einer Aminosäuresequenz, die in der Natur nicht gefunden wird und die von einer Vorläufer-Carbonylhydrolase abgeleitet ist, indem der Aminosäurerest an einer Position in dem Vorläufer, die äquivalent ist zu +123 in Bacillus amyloliquefaciens-Subtilisin, durch eine unterschiedliche Aminosäure ausgetauscht wird, wobei das Verfahren umfaßt, die Wirtszellen nach Anspruch 10 zu züchten und die produzierte Carbonylhydrolasemutante abzutrennen.

12. Verfahren, umfassend die Herstellung einer Enzymatischen Reinigungszusammensetzung, die in der Lage ist, Proteine abzubauen, umfassend:

(a) ein grenzflächenaktives Mittel und
(b) eine Carbonylhydrolasemutante mit einer Aminosäuresequenz, die in der Natur nicht gefunden wird und die von einer Vorläufer-Carbonylhydrolase abgeleitet ist, indem der Aminosäurerest an einer Position in dem Vorläufer, die äquivalent ist zu +123 in Bacillus amyloliquefaciens-Subtilisin, durch eine unterschiedliche Aminosäure ausgetauscht wird.

13. Verfahren nach Anspruch 12, wobei das Carbonylhydrolaseenzym ein Subtilisin ist.

14. Verfahren nach Anspruch 12 oder Anspruch 13, wobei das Subtilisin die folgende Aminosäuresequenz hat:

AQSVPWGISRVQAPAAHNRGLTGSGVRVAVLDTGISTHPDLNIRGGASFVPGE
PSTQDGNGHGTHVAGTIAALNNSIGVLGVAPSAELYAVKVLGASGSGSYSSIA
QGLEWAGNNGMHVASLSLGSPSPSATLEQAVNSATSRGVLVVAASGNSGAGSI
SYPARYANAMAVGATDQNNNRASFSQYGAGLDIVAPGVNVQSTYPGSTYASLN
GTSMATPHVAGAAALVKQKNPSWSNVQIRNHLKNTATSLGSTNLYGSGLVNAE
AAAR

15. Verfahren nach einem der Ansprüche 12 bis 14, wobei das grenzflächenaktive Mittel ein Detergens umfaßt.

16. Verfahren nach einem der Ansprüche 12 bis 15, die als ein sprühgetrocknetes Detergensgranulat formuliert ist.

Revendications

Revendications pour les Etats contractants suivants : AT, BE, CH, DE, DK, FR, GB, GR, IT, LU, NL, SE

1. Carbonyle hydrolase mutée présentant une séquence d'acides aminés ne se trouvant pas dans la nature qui est obtenue à partir d'une carbonyle hydrolase précurseur par substitution du résidu d'acide aminé occupant la position dans ledit précurseur équivalente à +123 dans la subtilisine de *Bacillus amyloliquefaciens* par un acide aminé différent, dans laquelle le mutant présente une activité protéolytique altérée par rapport à la carbonyle hydrolase précurseur.

2. Carbonyle hydrolase mutée selon la revendication 1, présentant une substitution supplémentaire dans une position équivalente à +274 dans *Bacillus amyloliquefaciens.*

3. Carbonyle hydrolase mutée selon la revendication 1 ou la revendication 2, dans laquelle ladite carbonyle hydrolase précurseur est une subtilisine.

4. Carbonyle hydrolase mutée selon la revendication 3, dans laquelle ladite carbonyle hydrolase précurseur est une subtilisine de *Bacillus.*

5. Carbonyle hydrolase mutée selon l'une quelconque des revendications 1 à 4, dans laquelle le résidu d'acide aminé en position +123 dans ledit mutant est la sérine et le résidu d'acide aminé en position +274 dans ledit mutant est l'alanine.

6. Carbonyle hydrolase mutée selon l'une quelconque des revendications 1 à 5, dans laquelle l'activité protéolytique du mutant est augmentée par rapport à la carbonyle hydrolase précurseur.

7. Subtilisine de *Bacillus* mutée présentant la séquence d'acides aminés :

AQSVPWGISRVQAPAAHNRGLTGSGVRVAVLDTGISTHPDLNIRGGASFVPGE
PSTQDGNGHGTHVAGTIAALNNSIGVLGVAPSAELYAVKVLGASGSGSYSSIA
QGLEWWAGNNGMHVASLSLGSPSPATLEQAVNSATSRGVLVVAASGNSHAGSI
SYPARYANAMAVGATDQNNNRASFSQYGAGLDIVAPGVNVQSTYPGSTYASLN

GTSMATPHVAGAAALVKQKNPSWSNVQIRNHLKNTATSLGSTNLYGSGLVNAE
AAAR.

8. ADN codant pour une carbonyle hydrolase mutée selon l'une quelconque des revendications 1 à 7.

9. Vecteur d'expression comprenant l'ADN selon la revendication 8.

10. Cellules hôtes transformées par le vecteur d'expression selon la revendication 9.

11. Procédé de production d'une carbonyle hydrolase mutée présentant une séquence d'acides aminés ne se trouvant pas dans la nature qui est obtenue à partir d'une carbonyle hydrolase précurseur par substitution du résidu d'acide aminé occupant une position dans ledit précurseur équivalente à +123 dans la subtilisine de *Bacillus amyloliquefaciens* par un acide aminé différent, le procédé comprenant la mise en culture des cellules hôtes selon la revendication 10 et la séparation de la carbonyle hydrolase mutée ainsi produite.

12. Composition de lavage enzymatique capable de dégrader des protéines comprenant :

(a) un agent tensio-actif ; et
(b) une carbonyle hydrolase mutée présentant une séquence d'acides aminés ne se trouvant pas dans la nature qui est obtenue à partir d'une carbonyle hydrolase précurseur par substitution du résidu d'acide aminé occupant une position dans ledit précurseur équivalente à +123 dans la subtilisine de *Bacillus amyloliquefaciens* par un acide aminé différent.

13. Composition selon la revendication 12, dans laquelle ladite enzyme carbonyle hydrolase est une subtilisine.

14. Composition selon la revendication 12 ou la revendication 13, dans laquelle ladite subtilisine présente la séquence d'acides aminés suivante :

AQSVPWGISRVQAPAAHNRGLTGSGVRVAVLDTGISTHPDLNIRGGASFVPGE

PSTQDGNGHGTHVAGTIAALNNSIGVLGVAPSAELYAVKVLGASGSGSYSSIA
QGLEWWAGNNGMHVASLSLGSPSPATLEQAVNSATSRGVLVVAASGNSHAGSI
SYPARYANAMAVGATDQNNNRASFSQYGAGLDIVAPGVNVQSTYPGSTYASLN
GTSMATPHVAGAAALVKQKNPSWSNVQIRNHLKNTATSLGSTNLYGSGLVNAE
AAAR.

15. Composition selon l'une quelconque des revendications 12 à 14, dans laquelle l'agent tensio-actif comprend un détergent.

16. Composition selon l'une quelconque des revendications 12 à 15, formulée sous la forme de granulés détergents séchés par pulvérisation.

Revendications pour l'Etat contractant suivant : ES

1. Procédé comprenant la préparation d'une Carbonyle hydrolase mutée présentant une séquence d'acides aminés ne se trouvant pas dans la nature qui est obtenue à partir d'une carbonyle hydrolase précurseur par substitution du résidu d'acide aminé occupant la position dans ledit précurseur équivalente à +123 dans la subtilisine de *Bacillus amyloliquefaciens* par un acide aminé différent, dans laquelle le mutant présente une activité protéolytique altérée par rapport à la carbonyle hydrolase précurseur.

2. Procédé selon la revendication 1, où la Carbonyle hydrolase mutée présente une substitution supplémentaire dans une position équivalente à +274 dans *Bacillus amyloliquefaciens.*

3. Procédé selon la revendication 1 ou la revendication 2, dans laquelle ladite carbonyle hydrolase précurseur est une subtilisine.

4. Procédé selon la revendication 3, dans laquelle ladite carbonyle hydrolase précurseur est une subtilisine de *Bacillus.*

5. Procédé selon l'une quelconque des revendications 1 à 4, dans laquelle le résidu d'acide aminé en position +123 dans ledit mutant est la sérine et le résidu d'acide aminé en position +274 dans ledit mutant est l'alanine.

6. Procédé selon l'une quelconque des revendications 1 à 5, dans laquelle l'activité protéolytique du mutant est augmentée par rapport à la carbonyle hydrolase précurseur.

7. Procédé comprenant la préparation d'une subtilisine de *Bacillus* mutée présentant la séquence d'acides aminés :

AQSVPWGISRVQAPAAHNRGLTGSGVRVAVLDTGISTHPDLNIRGGASFVPGE
PSTQDGNGHGTHVAGTIAALNNSIGVLGVAPSAELYAVKVLGASGSGSYSSIA
QGLEWWAGNNGMHVASLSLGSPSPATLEQAVNSATSRGVLVVAASGNSHAGSI
SYPARYANAMAVGATDQNNNRASFSQYGAGLDIVAPGVNVQSTYPGSTYASLN

GTSMATPHVAGAAALVKQKNPSWSNVQIRNHLKNTATSLGSTNLYGSGLVNAE
AAAR.

8. ADN codant pour une carbonyle hydrolase mutée produite par le procédé selon l'une quelconque des revendications 1 à 7.

9. Vecteur d'expression comprenant l'ADN selon la revendication 8.

10. Cellules hôtes transformées par le vecteur d'expression selon la revendication 9.

11. Procédé de production d'une carbonyle hydrolase mutée présentant une séquence d'acides aminés ne se trouvant pas dans la nature qui est obtenue à partir d'une carbonyle hydrolase précurseur par substitution du résidu d'acide aminé occupant une position dans ledit précurseur équivalente à +123 dans la subtilisine de *Bacillus amyloliquefaciens* par un acide aminé différent, le procédé comprenant la mise en culture des cellules hôtes selon la revendication 10 et la séparation de la carbonyle hydrolase mutée ainsi produite.

12. Procédé comprenant la préparation d'une composition de lavage enzymatique capable de dégrader des protéines comprenant :

(a) un agent tensio-actif ; et
(b) une carbonyle hydrolase mutée présentant une séquence d'acides aminés ne se trouvant pas dans la nature qui est obtenue à partir d'une carbonyle hydrolase précurseur par substitution du résidu d'acide aminé occupant une position dans ledit précurseur équivalente à +123 dans la subtilisine de *Bacillus amyloliquefaciens* par un acide aminé différent.

13. Procédé selon la revendication 12, dans laquelle ladite enzyme carbonyle hydrolase est une subtilisine.

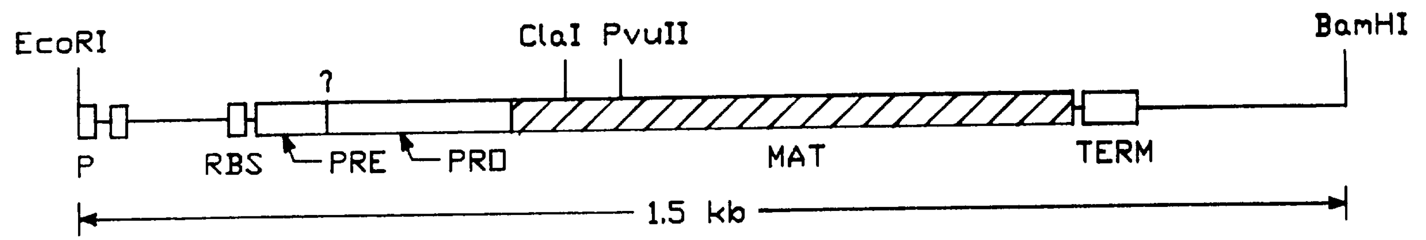
14. Procédé selon la revendication 12 ou la revendication 13, dans laquelle ladite subtilisine présente la séquence d'acides aminés suivante :

AQSVPWGISRVQAPAAHNRGLTGSGVRVAVLDTGISTHPDLNIRGGASFVPGE

PSTQDGNGHGTHVAGTIAALNNSIGVLGVAPSAELYAVKVLGASGSGSYSSIA
QGLEWWAGNNGMHVASLSLGSPSPATLEQAVNSATSRGVLVVAASGNSHAGSI
SYPARYANAMAVGATDQNNNRASFSQYGAGLDIVAPGVNVQSTYPGSTYASLN
GTSMATPHVAGAAALVKQKNPSWSNVQIRNHLKNTATSLGSTNLYGSGLVNAE
AAAR.

15. Procédé selon l'une quelconque des revendications 12 à 14, dans laquelle l'agent tensio-actif comprend un détergent.

16. Procédé selon l'une quelconque des revendications 12 à 15, formulée sous la forme de granulés détergents séchés par pulvérisation.



```
   ⑤                        ③                        ④                                   -107
   ↓                        ↓                        ↓                            RBS        Met
1  GGTCTACTAAAATATTATTCCATACTATACAATTAATACACAGAATAATCTGTCTATTGGTTATTCTGCAAATGAAAAAAAGGAGAGGATAAAGA GTG

                          -100          PRE                         -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

        -80                              -70    PRO                                 -60
    Ser Ala Gln Ala Ala Gly Lvs 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 Lvs Gln Phe Lvs Tvr Val Asp Ala
249 AGC GCC GCT AAG 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 Lvs 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                                     18υ                                     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  AACATAAAAAACCGGCCTTGGCCCCGCCGGTTTTTATTATTTTTCTTCCTCCGCATGTTCAATCCGCTCC

1316 ATAATCGACGGATGGCTCCCTCTGAAAATTTTAACGAGAAACGGCGGGTTGACCCGGCTCAGTCCCGTAACGGCCAACTCCTGAAACGTCTCAATCGCCG

1416 CTTCCCGGTTTCCGGTCAGCTCAATGCCATAACGGTCGGCGGCGTTTTCCTGATACCGGGAGACGGCATTCGTAATCGGATC
```

FIG.-1C

```
TOTALLY CONSERVED RESIDUES IN SUBTILISINS
1                          10                            20
.  .  .  .  P  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .

21                         30                            40
.  .  G  .  .  .  .  .  .  .  .  D  .  G  .  .  .  .  H  .

41                         50                            60
.  .  .  .  .  G  .  .  .  .  V  .  .  .  .  .  .  .  .  .

61                         70                            80
.  .  .  H  G  T  H  .  .  G  .  .  .  .  .  .  .  .  .  .

81                         90                            100
   .  G  .  .  .  .  .  .  .  .  .  .  .  V  L  .  .  .  G

 01                        110                           120
.  G  .  .  .  .  .  .  .  G  .  .  .  .  .  .  .  .  .  .

121                        130                           140
.  .  .  .  .  L  G  .  .  .  .  .  .  .  .  .  .  .  .  .

141                        150                           160
.  .  .  .  .  G  .  .  .  .  .  .  .  G  N  .  .  .  .  .

161                        170                           180
.  .  .  .  .  .  V  P  .  .  .  .  .  .  .  .  V  .  .  .

181                        190                           200
.  .  .  .  .  .  .  S  F  S  .  .  .  .  .  .  .  .  .  .

201                        210                           220
P  G  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  G  T

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

241                        250                           260
.  .  .  .  .  .  R  .  .  .  .  .  .  .  .  .  .  .  .  .

261                        270
.  .  .  .  .  .  .  .  N  .  .  .  .  .  .
```

FIG.-2

Homology of Baclllur proteases

1.Baclllus amyloliqulfaclens
2.Baclllus subtllls var.I168
3.Baclllus llchenlformls (carlsbergensls)

1									10										20
A	Q	S	V	P	Y	G	V	S	Q	I	K	A	P	A	L	H	S	Q	G
A	Q	S	V	P	Y	G	I	S	Q	I	K	A	P	A	L	H	S	Q	G
A	Q	T	V	P	Y	G	I	P	L	I	K	A	D	K	V	Q	A	Q	G

21									30										40
Y	T	G	S	N	V	K	V	A	V	I	D	S	G	I	D	S	S	H	P
Y	T	G	S	N	V	K	V	A	V	I	D	S	G	I	D	S	S	H	P
F	K	G	A	N	V	K	V	A	V	L	D	T	G	I	Q	A	S	H	P

41									50										60
D	L	K	V	A	G	G	A	S	M	V	P	S	E	T	N	P	F	Q	D
D	L	N	V	R	G	G	A	S	F	V	P	S	E	T	N	P	Y	Q	D
D	L	N	V	V	G	G	A	S	F	V	A	G	E	A	Y	N	T	.	D

61									70										80
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	N	G	H	G	T	H	V	A	G	T	V	A	A	L	D	N	T	T	G

81									90										100
V	L	G	V	A	P	S	A	S	L	Y	A	V	K	V	L	G	A	D	G
V	L	G	V	S	P	S	A	S	L	Y	A	V	K	V	L	D	S	T	G
V	L	G	V	A	P	S	V	S	L	Y	A	V	K	V	L	N	S	S	G

101									110										120
S	G	Q	Y	S	W	I	I	N	G	I	E	W	A	I	A	N	N	M	D
S	G	Q	Y	S	W	I	I	N	G	I	E	W	A	I	S	N	N	M	D
S	G	S	Y	S	G	I	V	S	G	I	E	W	A	T	T	N	G	M	D

FIG.-3A

```
121                        130                           140
V  I  N  M  S  L  G  G  P  S  G  S  A  A  L  K  A  A  V  D
V  I  N  M  S  L  G  G  P  T  G  S  T  A  L  K  T  V  V  D
V  I  N  M  S  L  G  G  A  S  G  S  T  A  M  K  Q  A  V  D

141                        150                           160
K  A  V  A  S  G  V  V  V  V  A  A  A  G  N  E  G  T  S  G
K  A  V  S  S  G  I  V  V  A  A  A  A  G  N  E  G  S  S  G
N  A  Y  A  R  G  V  V  V  V  A  A  A  G  N  S  G  N  S  G

161                        170                           180
S  S  S  T  V  G  Y  P  G  K  Y  P  S  V  I  A  V  G  A  V
S  T  S  T  V  G  Y  P  A  K  Y  P  S  T  I  A  V  G  A  V
S  T  N  T  I  G  Y  P  A  K  Y  D  S  V  I  A  V  G  A  V

181                        190                           200
D  S  S  N  Q  R  A  S  F  S  S  G  V  P  E  L  D  V  M  A
N  S  S  N  Q  R  A  S  F  A  S  A  G  S  E  L  D  V  M  A
D  S  N  S  N  R  A  S  F  S  S  V  G  A  E  L  E  V  M  A

201                        210                           220
P  G  V  S  I  Q  S  T  L  P  G  N  K  Y  G  A  Y  N  G  T
P  G  V  S  I  Q  S  T  L  P  G  G  T  Y  G  A  Y  N  G  T
P  G  A  V  G  Y  S  T  Y  P  T  N  T  Y  A  T  L  N  G  T

221                        230                           240
S  M  S  A  P  H  V  A  G  A  A  A  L  I  L  S  K  H  P  N
S  M  A  T  P  H  V  A  G  A  A  A  L  I  L  S  K  H  P  T
S  M  A  S  P  H  V  A  G  A  A  A  L  I  L  S  K  N  P  N

241                        250                           260
W  T  N  T  Q  V  R  S  S  L  E  N  T  T  T  K  L  G  D  S
W  T  N  A  Q  V  R  D  R  L  E  S  T  A  T  Y  L  G  N  S
L  S  A  S  Q  V  R  N  R  L  S  S  T  A  T  Y  L  G  S  S

261                        270
F  Y  Y  G  K  G  L  I  N  V  Q  A  A  A  Q
F  Y  Y  G  K  G  L  I  N  V  Q  A  A  A  Q
F  Y  Y  G  K  G  I  L  N  V  E  A  A  A  Q
```

FIG.-3B

```
                          PREFERRED MUNTANT
                            BACILLUS LENTUS
                 BACILLUS AMYLOLIQUIFACIENS

01        10        20        30
AQSVPYGVSQIKAPALHSQGYTGSNVKVAVIDSGIDSSHP
AQSVPWGISRVQAPAAHNRGLTGSGVKVAVLDTGI*STHP
AQSVPWGISRVQAPAAHNRGLTGSGVRVAVLDTGI*STHP


41        50        60        70
DLKVAGGASMVPSETNPFQDNNSHGTHVAGTVAALNNSIG
DLNIRGGASFVPGE*PSTQDGNGHGTHVAGTIAALNNSIG
DLNIRGGASFVPGE*PSTQDGNGHGTHVAGTIAALNNSIG


81        90        100       110
VLGVAPSASLYAVKVLGADGSGQYSWIINGIEWAIANNMD
VLGVAPSAELYAVKVLGASGSGSVSSIAQGLEWAGNNGMH
VLGVAPSAELYAVKVLGASGSGSYSSIAQGLEWAGNNGMH


121       130       140       150
VINMSLGGPSGSSAALKAAVDKAVSGVVVVAAAGNEGTSG
VANLSLGSPSPSATLEQAVNSATSRGVLVVAASGNSGAGS
VASLSLGSPSPSATLEQAVNSATSRGVLVVAASGNSGAGS


161       170       180       190
SSSTVGYPGKYPSVIAVGAVDSSNQRASFSSVGPELDVMA
****ISYPARYANAMAVGATDQNNNRASFSQYGAGLDIVA
****ISYPARYANAMAVGATDQNNNRASFSQYGAGLDIVA


201       210       220       230
PGVSIQSTLPGNKYGAYNGTSMASPHVAGAAALILSKHPN
PGVNVQSTYPGSTYASLNGTSMATPHVAGAAALVKQKNPS
PGVNVQSTYPGSTYASLNGTSMATPHVAGAAALVKQKNPS


241       250       260       270
WTNTQVRSSLENTITKLGDSFYYGKGLINVQAAAQ
WSNVQIRNHLKNTATSLGSTNLYGSGLVNAEAATR
WSNVQIRNHLKNTATSLGSTNLYGSGLVNAEAAAR
```

FIG.-4

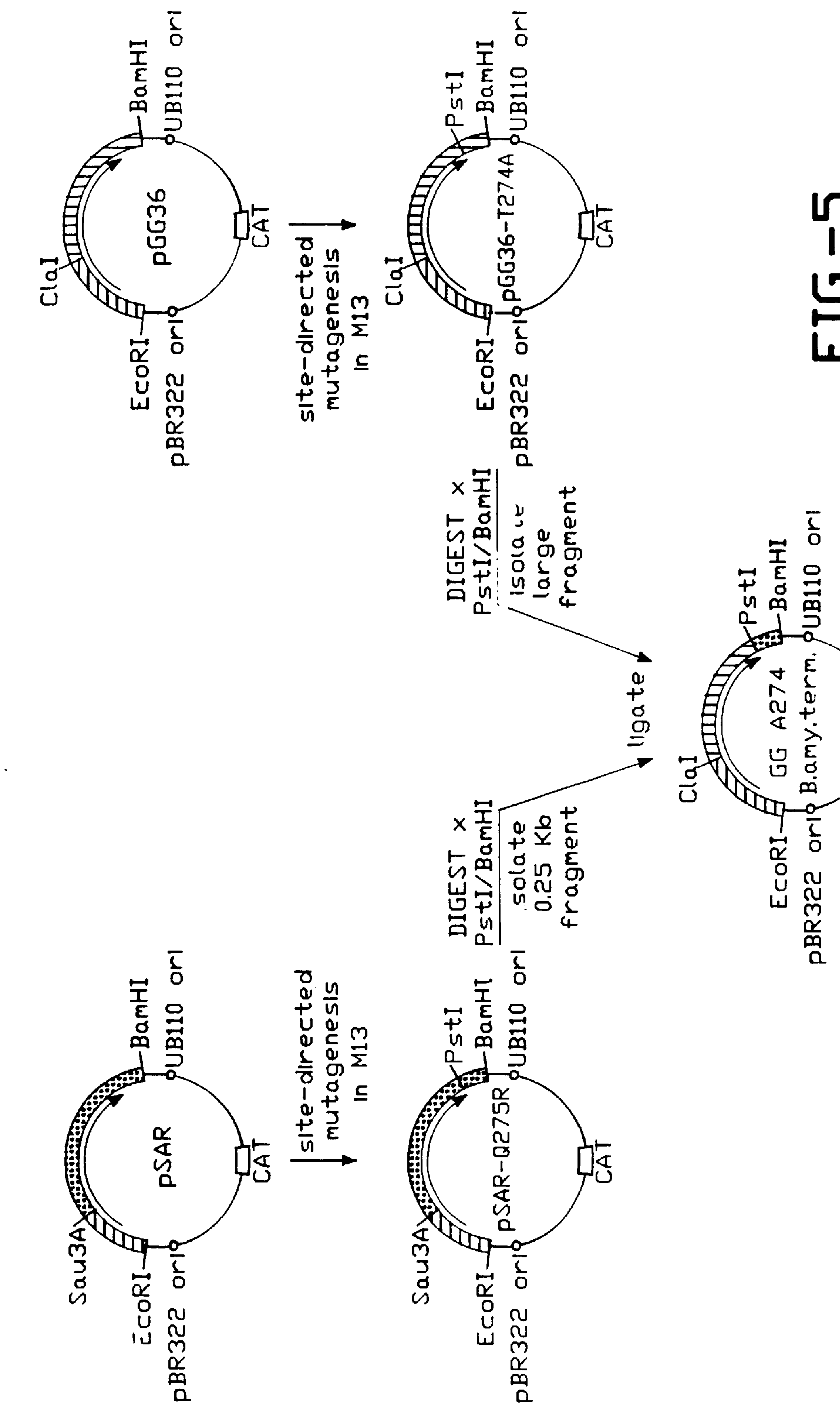
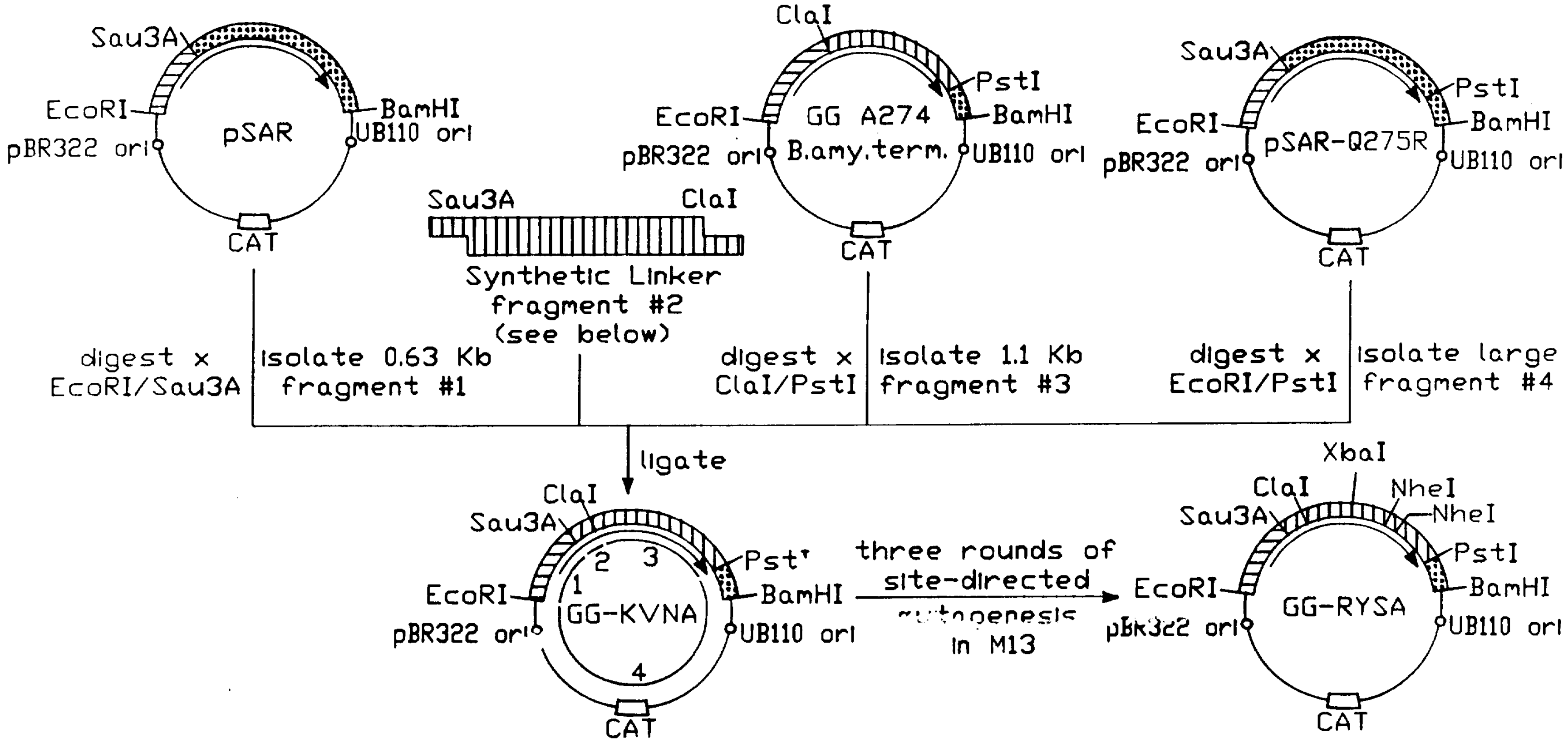

Sau3A
EcoRI
pBR322 ori
pSAR
BamHI
UB110 ori
CAT
site-directed mutagenesis in M13
Sau3A
EcoRI
pBR322 ori
pSAR-Q275R
PstI
BamHI
UB110 ori
CAT
DIGEST x PstI/BamHI
isolate 0.25 Kb fragment
ClaI
EcoRI
pBR322 ori
pGG36
BamHI
UB110 ori
CAT
site-directed mutagenesis in M13
ClaI
EcoRI
pBR322 ori
pGG36-T274A
PstI
BamHI
UB110 ori
CAT
DIGEST x PstI/BamHI
isolate large fragment
ligate
ClaI
EcoRI
pBR322 ori
GG A274
B.amy.term.
PstI
BamHI
UB110 ori
CAT

FIG.-5



Sequence of synthetic DNA linker (fragment #2)

5'-G-ATC-GTC-GCG-TCG-ACC-GCA-CTA-CTC-ATT-TCT-GTT-GCT-TTT-AGT-TCA-T-3'
3'-CAG-CGC-AGC-TGG-CGT-GAT-GAG-TAA-AGA-CAA-CGA-AAA-TCA-AGT-AGC-5'

FIG.-6

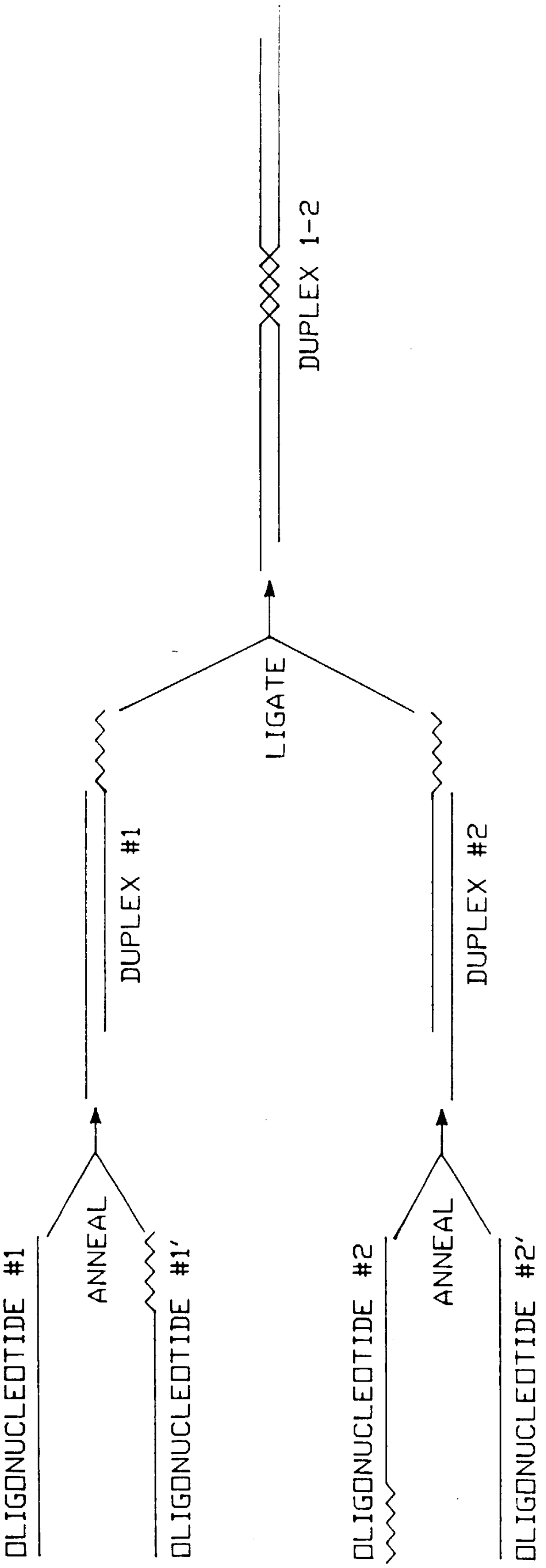

OLIGONUCLEOTIDE #1
ANNEAL
OLIGONUCLEOTIDE #1'
OLIGONUCLEOTIDE #2
ANNEAL
OLIGONUCLEOTIDE #2'
DUPLEX #1
DUPLEX #2
LIGATE
DUPLEX 1-2

FIG.-7

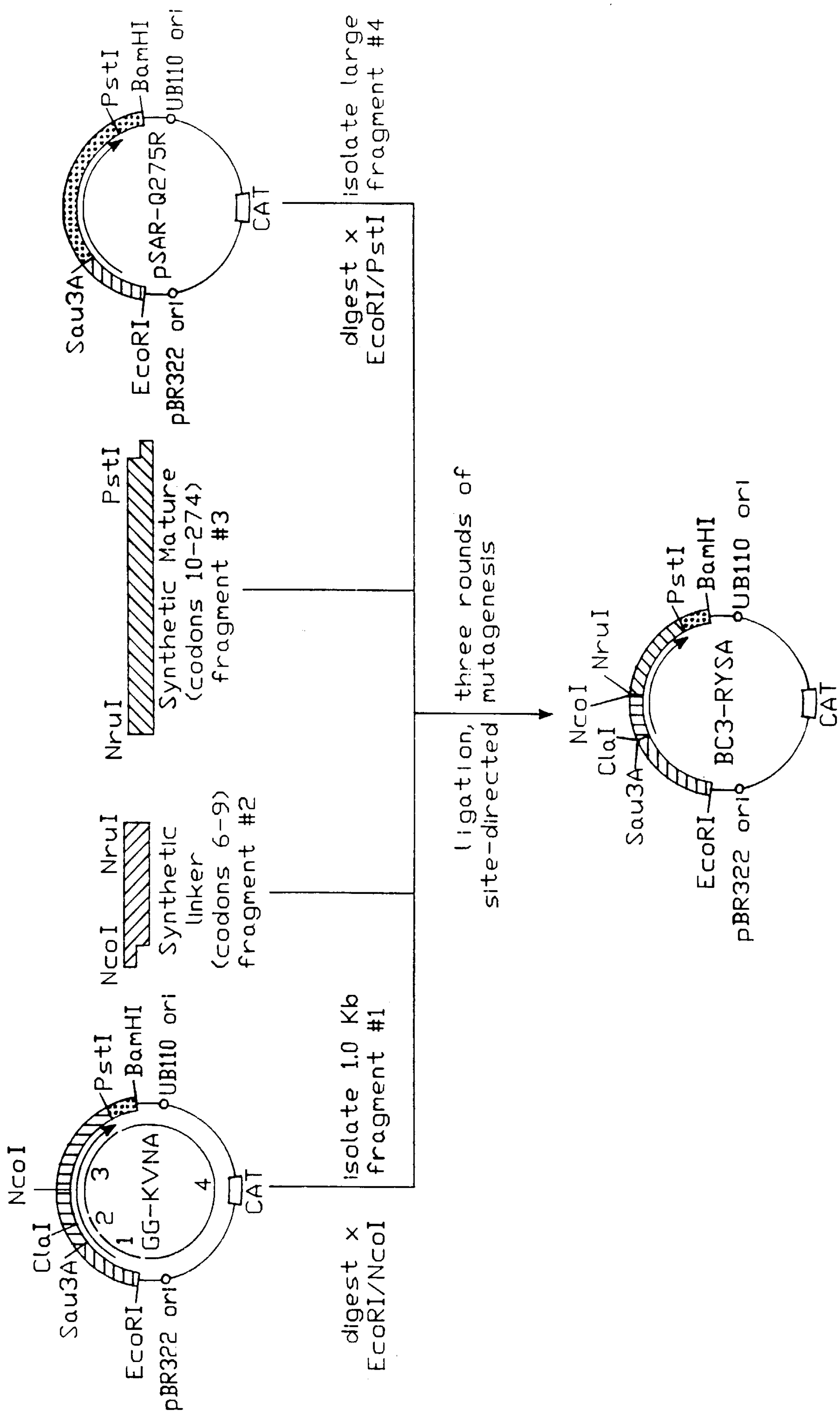

pBR322 ori
EcoRI
Sau3A
ClaI
NcoI
PstI
BamHI
UB110 ori
GG-KVNA
1
2
3
4
CAT
digest x
EcoRI/NcoI
isolate 1.0 Kb
fragment #1
NcoI
NruI
Synthetic
linker
(codons 6-9)
fragment #2
NruI
PstI
Synthetic Mature
(codons 10-274)
fragment #3
pBR322 ori
EcoRI
Sau3A
PstI
BamHI
UB110 ori
pSAR-Q275R
CAT
digest x
EcoRI/PstI
isolate large
fragment #4
ligation,
site-directed
three rounds of
mutagenesis
pBR322 ori
EcoRI
Sau3A
ClaI
NcoI
NruI
PstI
BamHI
UB110 ori
BC3-RYSA
CAT

FIG.-8

```
                110                 115                 120                 125
W.T. A.A.:      Gly Leu Glu Trp Ala Gly Asn Asn Gly Met His Val Ala Asn Leu Ser Leu Gly

W.T. DNA :      GGC TTA GAA TGG GCT GGC AAC AAC GGC ATG CAC GTT GCT AAC TTA AGC CTC GGG-3'
                CCG AAT CTT ACC CGA CCG TTG TTG CCG TAC GTG CAA CGA TTG AAT TCG GAG CCC-5'
                                                 SphI                            AvaI
                    *   *   *
pX123 DNA:      GGC CTC GAG TGG GCT GGC AAC AAC GGC ATG CAC GTT GCT AAC TTA AGC CTC GGG-3'
                CCG GAG CTC ACC CGA CCG TTG TTG CCG TAC GTG CAA CGA TTG AAT TCG GAG CCC-5
                     XhoI                        SphI                           AvaI

pX123 cut       GGC C                                                           pTC GGG-3'
w/ XhoI         CCG GAG CTp                                                          C-5'
and AvaI :

                                                  *
Cut pX1213      GGC CTC GAG TGG GCT GGC AAC AAC GGT ATG CAC GTT GCT XXX TTA AGC CTC GGG-3'
ligated w/      CCG GAG CTC ACC CGA CCG TTG TTG CCA TAC GTG CAA CGA XXX AAT TCG GAG CCC-5'
duplex DNA            XhoI                     SphI  destroyed          123
cassettes:
```

FIG.-9

```
  1 GCG CAA TCA GTG CCA TGG GGC ATC TCG CGA GTT CAA GCT CCT GCT GCT CAC AAC CGC GGC
  1 Ala Gln Ser Val Pro Trp Gly Ile Ser Arg Val Gln Ala Pro Ala Ala His Asn Arg Gly

 61 TTA ACA GGC AGC GGC GTT AGA GTT GCT GTT TTA GAT ACA GGC ATC AGC ACA --- CAC CCA
 21 Leu Thr Gly Ser Gly Val Arg Val Ala Val Leu Asp Thr Gly Ile Ser Thr  -  His Pro

121 GAT CTT AAT ATT AGA GGC GGC GCG AGC TTC GTT CCC GGC GAA --- CCG TCG ACA CAA GAT
 41 Asp Leu Asn Ile Arg Gly Gly Ala Ser Phe Val Pro Gly Glu  -  Pro Ser Thr Gln Asp

181 GGC AAC GGC CAC GGC ACA CAC GTT GCC GGC ACA ATC GCT GCT TTA AAC AAC TCG ATC GGA
 61 Gly Asn Gly His Gly Thr His Val Ala Gly Thr Ile Ala Ala Leu Asn Asn Ser Ile Gly

241 GTT TTA GGC GTT GCT CCT TCG GCC GAA TTA TAC GCT GTT AAA GTT TTA GGC GCT AGC GGC
 81 Val Leu Gly Val Ala Pro Ser Ala Glu Leu Tyr Ala Val Lys Val Leu Gly Ala Ser Gly

301 AGC GGC AGC TAC AGC TCT ATC GCT CAA GGC CTC GAG TGG GCT GGC AAC AAC GGT AGT CAC
101 Ser Gly Ser Tyr Ser Ser Ile Ala Gln Gly Leu Glu Trp Ala Gly Asn Asn Gly Met His

361 GTT GCT AGC TTA AGC CTC GGG AGC CCT AGC CCT AGC GCT ACA TTA GAA CAA GCT GTT AAC
121 Val Ala Ser Leu Ser Leu Gly Ser Pro Ser Pro Ser Ala Thr Leu Glu Gln Ala Val Asn
```

FIG.-10A

```
421 AGC GCT ACA TCT AGA GGC GTT TTA GTT GTT GCT GCG AGC GGC AAC AGC GGC GCT GGA TCG
141 Ser Ala Thr Ser Arg Gly Val Leu Val Val Ala Ala Ser Gly Asn Ser Gly Ala Gly Ser

481 --- --- --- --- ATC AGC CTA CCC TGC TAG ATA CGC TAA TGC CAT GGC TGT TGG CGC ACA
161  -   -   -   -  Ile Ser tyr Pro Ala Arg Tyr Ala Asn Ala Met Ala Val Gly Ala Thr

541 GAT CAA AAC AAC AAC AGA GCA AGC TTC AGT CAA TAC GGC GCT GGC TTA GAT ATC GTG GCG
181 Asp Gln Asn Asn Asn Arg Ala Ser Phe Ser Gln Tyr Gly Ala Gly Leu Asp Ile Val Ala

601 CCT GGC GTT AAC GTT CAA AGC ACA TAC CCT GGC AGC ACA TAC GCC AGC TTG AAC GGT ACA
201 Pro Gly Val Asn Val Gln Ser Thr Tyr Pro Gly Ser Thr Tyr Ala Ser Leu Asn Gly Thr

651 TCG ATG GCG ACA CCT CAC GTT GCC GGA GCG GCT GCA CTA GTT AAA CAA AAA AAC CCT TCA
221 Ser Met Ala Thr Pro His Val Ala Gly Ala Ala Ala Leu Val Lys Gln Lys Asn Pro Ser

721 TGG AGC AAC GTT CAA ATC CGC AAC CAC TTA AAA AAC ACA GCT ACT AGC TTA GGC AGT ACT
241 Trp Ser Asn Val Gln Ile Arg Asn His Leu Lys Asn Thr Ala Thr Ser Leu Gly Ser Thr

731 AAC TTA TAC GGC AGC GGC TTA GTT AAC GCT GAA GCT GCA GCT CGT
261 Asn Leu Tyr Gly Ser Gly Leu Val Asn Ala Glu Ala Ala Ala Arg
```

FIG.-10B