

Description

The recent development of various in vitro techniques to manipulate the DNA sequences encoding naturally-occurring polypeptides as well as recent developments in the chemical synthesis of relatively short sequences of single and double stranded DNA has resulted in the speculation that such techniques can be used to modify enzymes to improve some functional property in a predictable way. Ulmer, K.M. (1983) Science 219, 666-671. The only working example disclosed therein is the substitution of a single amino acid within the active site of tyrosyl-tRNA synthetase (Cys35→Ser) which lead to a reduction in enzymatic activity. See Winter, G., et al. (1982) Nature 299, 756-758; and Wilkinson, A.J., et al. (1983) Biochemistry 22, 3581-3586 (Cys35→Gly mutation also resulted in decreased activity).

When the same t-RNA synthetase was modified by substituting a different amino acid residue within the active site with two different amino acids, one of the mutants (Thr51→Ala) reportedly demonstrated a predicted moderate increase in kcat/Km whereas a second mutant (Thr51→Pro) demonstrated a massive increase in kcat/Km which could not be explained with certainty. Wilkinson, A.H., et al. (1984) Nature 307, 187-188.

Another reported example of a single substitution of an amino acid residue is the substitution of cysteine for isoleucine at the third residue of T4 lysozyme. Perry, L.J., et al. (1984) Science 226, 555-557. The resultant mutant lysozyme was mildly oxidized to form a disulfide bond between the new cysteine residue at position 3 and the native cysteine at position 97. This crosslinked mutant was initially described by the author as being enzymatically identical to, but more thermally stable than, the wild type enzyme. However, in a "Note Added in Proof", the author indicated that the enhanced stability observed was probably due to a chemical modification of cysteine at residue 54 since the mutant lysozyme with a free thiol at Cys54 has a thermal stability identical to the wild type lysozyme.

Similarly, a modified dihydrofolate reductase from E.coli has been reported to be modified by similar methods to introduce a cysteine which could be cross linked with a naturally-occurring cysteine in the reductase. Villafranca, D.E., et al. (1983) Science 222, 782-788. The author indicates that this mutant is fully reactive in the reduced state but has significantly diminished activity in the oxidized state. In addition, two other substitutions of specific amino acid residues are reported which resulted in mutants which had diminished or no activity.

EPO Publication No. 0130756 discloses the substitution of specific residues within B. amyloliquefaciens subtilisin with specific amino acids. Thus, Met222 has been substituted with all 19 other amino acids, Gly166 with 9 different amino acids and Gly169 with Ala and Ser.

As set forth below, several laboratories have also reported the use of site directed mutagenesis to produce the mutation of more than one amino acid residue within a polypeptide.

The amino-terminal region of the signal peptide of the prolipoprotein of the E. coli outer membrane was stated to be altered by the substitution or deletion of residues 2 and 3 to produce a charge change in that region of the polypeptide. Inouye, S., et al. (1982) Proc. Nat. Acad. Sci. USA 79, 3438-3441. The same laboratory also reported the substitution and deletion of amino acid residues 9 and 14 to determine the effects of such substitution on the hydrophobic region of the same signal sequence. Inouye, S., et al. (1984) J. Biol. Chem. 259, 3729-3733.

Double mutants in the active site of tyrosyl-t-RNA synthetase have also been reported. Carter, P.J., et al. (1984) Cell 38, 835-840. In this report, the improved affinity of the previously described Thr51→Pro mutant for ATP was probed by producing a second mutation in the active site of the enzyme. One of the double mutants, Gly35/Pro51, reportedly demonstrated an unexpected result in that it bound ATP in the transition state better than was expected from the two single mutants. Moreover, the author warns, at least for one double mutant, that it is not readily predictable how one substitution alters the effect caused by the other substitution and that care must be taken in interpreting such substitutions.

A mutant is disclosed in U.S. Patent No. 4,532,207, wherein a polyarginine tail was attached to the C-terminal residue of β -urogastrone by modifying the DNA sequence encoding the polypeptide. As disclosed, the polyarginine tail changed the electrophoretic mobility of the urogastrone-polyarginine hybrid permitting selective purification. The polyarginine was subsequently removed, according to the patentee, by a polyarginine specific exopeptidase to produce the purified urogastrone. Properly construed, this reference discloses hybrid polypeptides which do not constitute mutant polypeptides containing the substitution, insertion or deletion of one or more amino acids of a naturally occurring polypeptide.

Single and double mutants of rat pancreatic trypsin have also been reported. Craik, C.S., et al. (1985) Science 228, 291-297. As reported, glycine residues at positions 216 and 226 were replaced with alanine residues to produce three trypsin mutants (two single mutants and one double mutant). In the case of the single mutants, the authors stated expectation was to observe a differential effect on Km. They instead

reported a change in specificity (kcat/Km) which was primarily the result of a decrease in kcat. In contrast, the double mutant reportedly demonstrated a differential increase in Km for lysyl and arginyl substrates as compared to wild type trypsin but had virtually no catalytic activity.

The references discussed above are provided solely for their disclosure prior to the filing date of the instant case, and nothing herein is to be construed as an admission that the inventors are not entitled to antedate such disclosure by virtue of prior invention or priority based on earlier filed applications.

Based on the above references, however, it is apparent that the modification of the amino acid sequence of wild type enzymes often results in the decrease or destruction of biological activity.

Accordingly, it is an object herein to provide carbonyl hydrolase mutants which 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.

It is a further object to provide mutant DNA sequences encoding such carbonyl hydrolase mutants as well as expression vectors containing such mutant DNA sequences.

Still further, another object of the present invention is to provide host cells transformed with such vectors as well as host cells which are capable of expressing such mutants either intracellularly or extracellularly.

Summary of the Invention

The invention includes carbonyl hydrolase mutants, preferably having at least one property which is substantially different from the same property of the precursor non-human carbonyl hydrolase from which the amino acid sequence of the mutant is derived. These properties include oxidative stability, substrate, specificity catalytic activity, thermal stability, alkaline stability, pH activity profile and resistance to proteolytic degradation. The precursor carbonyl hydrolase may be naturally occurring carbonyl hydrolases or recombinant carbonyl hydrolases. The amino acid sequence of the carbonyl hydrolase mutant is derived by the substitution, deletion or insertion of one or more amino acids of the precursor carbonyl hydrolase amino acid sequence.

The invention also includes mutant DNA sequences encoding such carbonyl hydrolase mutants. Further the invention includes expression vectors containing such mutant DNA sequences as well as host cells transformed with such vectors which are capable of expressing said carbonyl hydrolase mutants.

Brief Description of the Drawings

Figure 1 shows the nucleotide sequence of the coding strand, correlated with the amino acid sequence of B. amyloliquefaciens subtilisin gene. Promoter (p) ribosome binding site (rbs) and termination (term) regions of the DNA sequence as well as sequences encoding the presequence (PRE) putative prosequence (PRO) and mature form (MAT) of the hydrolase are also shown.

Figure 2 is a schematic diagram showing the substrate binding cleft of subtilisin together with substrate.

Figure 3 is a stereo view of the S-1 binding subsite of B. amyloliquefaciens subtilisin showing a lysine P-1 substrate bound in the site in two different ways. Figure 3A shows Lysine P-1 substrate bound to form a salt bridge with a Glu at position 156. Figure 3B shows Lysine P-1 substrate bound to form a salt bridge with Glu at position 166.

Figure 4 is a schematic diagram of the active site of subtilisin Asp32, His64 and Ser221.

Figures 5A and 5B depict the amino acid sequence of subtilisin obtained from various sources. The residues directly beneath each residue of B. amyloliquefaciens subtilisin are equivalent residues which (1) can be mutated in a similar manner to that described for B. amyloliquefaciens subtilisin, or (2) can be used as a replacement amino acid residue in B. amyloliquefaciens subtilisin. Figure 5C depicts conserved residues of B. amyloliquefaciens subtilisin when compared to other subtilisin sequences.

Figures 6A and 6B depict the inactivation of the mutants Met222L and Met222Q when exposed to various organic oxidants.

Figure 7 depicts the ultraviolet spectrum of Met222F subtilisin and the difference spectrum generated after inactivation by diperiododecanoic acid (DPDA).

Figure 8 shows the pattern of cyanogen bromide digests of untreated and DPDA oxidized subtilisin Met222F on high resolution SDS-pyridine peptide gels.

Figure 9 depicts a map of the cyanogen bromide fragments of Fig. 8 and their alignment with the sequence of subtilisin Met222F.

Figure 10 depicts the construction of mutations between codons 45 and 50 of B. amyloliquefaciens subtilisin.

Figure 11 depicts the construction of mutations between codons 122 and 127 of B. amyloliquefaciens subtilisin.

Figure 12 depicts the effect of DPDA on the activity of subtilisin mutants at positions 50 and 124 in subtilisin Met222F.

5 Figure 13 depicts the construction of mutations at codon 166 of B. amyloliquefaciens subtilisin.

Figure 14 depicts the effect of hydrophobicity of the P-1 substrate side-chain on the kinetic parameters of wild-type B. amyloliquefaciens subtilisin.

Figure 15 depicts the effect of position 166 side-chain substitutions on P-I substrate specificity. Figure 15A shows position 166 mutant subtilisins containing non-branched alkyl and aromatic side-chain substitutions arranged in order of increasing molecular volume. Figure 15B shows a series of mutant enzymes progressing through β - and γ -branched aliphatic side chain substitutions of increasing molecular volume.

Figure 16 depicts the effect of position 166 side-chain volume on log kcat/Km for various P-1 substrates.

15 Figure 17 shows the substrate specificity differences between Ile166 and wild-type (Gly166) B. amyloliquefaciens subtilisin against a series of aliphatic and aromatic substrates. Each bar represents the difference in log kcat/Km for Ile166 minus wild-type (Gly166) subtilisin.

Figure 18 depicts the construction of mutations at codon 169 of B. amyloliquefaciens subtilisin.

Figure 19 depicts the construction of mutations at codon 104 of B. amyloliquefaciens subtilisin.

Figure 20 depicts the construction of mutations at codon 152 B. amyloliquefaciens subtilisin.

20 Figure 21 depicts the construction of single mutations at codon 156 and double mutations at codons 156 and 166 of B. amyloliquefaciens subtilisin.

Figure 22 depicts the construction of mutations at codon 217 for B. amyloliquefaciens subtilisin.

Figure 23 depicts the kcat/Km versus pH profile for mutations at codon 156 and 166 in B. amyloliquefaciens subtilisin.

25 Figure 23A depicts the kcat/Km versus pH profile for mutations at codon 156 and 166 in B. amyloliquefaciens subtilisin.

Figure 24 depicts the kcat/Km versus pH profile for mutations at codon 222 in B. amyloliquefaciens subtilisin.

Figure 25 depicts the constructing mutants at codons 94, 95 and 96.

30 Figures 26 and 27 depict substrate specificity of various wild type and mutant subtilisins for different substrates.

Figures 28 A, B, C and D depict the effect of charge in the P-1 binding sites due to substitutions at codon 156 and 166.

35 Figures 29 A and B are a stereoview of the P-1 binding site of subtilisin BPN' showing a lysine P-1 substrate bound in the site in two ways. In 29A, Lysine P-1 substrate is built to form a salt bridge with a Glu at codon 156. In 29B, Lysine P-1 substrate is built to form a salt bridge with Glu at codon 166.

Figure 30 demonstrates residual enzyme activity versus temperature curves for purified wild-type (Panel A), C22/C87 (Panel B) and C24/C87 (Panel C).

40 Figure 31 depicts the strategy for producing point mutations in the subtilisin coding sequence by misincorporation of α -thioldeoxynucleotide triphosphates.

Figure 32 depicts the autolytic stability of purified wild type and mutant subtilisins 170E, 107V, 213R and 107V/213R at alkaline pH.

Figure 33 depicts the autolytic stability of purified wild type and mutant subtilisins V50, F50 and F50V/107/R213 at alkaline pH.

45 Figure 34 depicts the strategy for constructing plasmids containing random cassette mutagenesis over residues 197 through 228.

Figure 35 depicts the oligodeoxynucleotides used for random cassette mutagenesis over residues 197 through 228.

Figure 36 depicts the construction of mutants at codon 204.

50 Figure 37 depicts the oligodeoxynucleotides used for synthesizing mutants at codon 204.

Detailed Description

55 The inventors have discovered that various single and multiple *in vitro* mutations involving the substitution, deletion or insertion of one or more amino acids within a non-human carbonyl hydrolase amino acid sequence can confer advantageous properties to such mutants when compared to the non-mutated carbonyl hydrolase.

Specifically, *B. amyloliquefaciens* subtilisin, an alkaline bacterial protease, has been mutated by modifying the DNA encoding the subtilisin to encode the substitution of one or more amino acids at various amino acid residues within the mature form of the subtilisin molecule. These *in vitro* mutant subtilisins have at least one property which is different when compared to the same property of the precursor subtilisin.

5 These modified properties fall into several categories including: oxidative stability, substrate specificity, thermal stability, alkaline stability, catalytic activity, pH activity profile, resistance to proteolytic degradation, Km, kcat and Km/kcat ratio.

Carbonyl hydrolases are enzymes which hydrolyze compounds containing



15 bonds in which X is oxygen or nitrogen. They include naturally-occurring carbonyl hydrolases and recombinant carbonyl hydrolases. Naturally occurring carbonyl hydrolases principally include hydrolases, e.g. lipases and peptide hydrolases, e.g. subtilisins or metalloproteases. Peptide hydrolases include α -aminoacylpeptide hydrolase, peptidylamino-acid hydrolase, acylamino hydrolase, serine carboxypeptidase, 20 metalcarboxypeptidase, thiol proteinase, carboxylproteinase and metalloproteinase. Serine, metallo, thiol and acid proteases are included, as well as endo and exoproteases.

"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 25 sequence. Suitable modification methods are disclosed herein and in EPO Publication No. 0130756 published January 9, 1985.

Subtilisins are bacterial 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 bacterial 30 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 35 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.

"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 40 more amino acids in the naturally occurring subtilisin amino acid sequence. Suitable methods to produce such modification include those disclosed herein and in EPO Publication No. 0130756. For example, the subtilisin multiple mutant herein containing the substitution of methionine at amino acid residues 50, 124 and 222 with phenylalanine, isoleucine and glutamine, respectively, can be considered to be derived from the recombinant subtilisin containing the substitution of glutamine at residue 222 (Q222) disclosed in EPO 45 Publication No. 0130756. The multiple mutant thus is produced by the substitution of phenylalanine for methionine at residue 50 and isoleucine for methionine at residue 124 in the Q222 recombinant subtilisin.

"Carbonyl hydrolases" and their genes may be obtained from many procaryotic and eucaryotic organisms. Suitable examples of procaryotic organisms include gram negative organisms such as *E. coli* or 50 *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 *S. 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 55 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 non-human eucaryotic sources.

A "carbonyl hydrolase mutant" has an amino acid sequence which is derived from the amino acid sequence of a non-human "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 *per se*. Suitable methods for such manipulation of the precursor DNA sequence include methods disclosed herein and in EPO Publication No. 0130756.

Specific residues of B. amyloliquefaciens subtilisin are identified for substitution, insertion or deletion. These amino acid position numbers refer to those assigned to the B. 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 which are "equivalent" to the particular identified residues in B. amyloliquefaciens subtilisin.

A residue (amino acid) of a precursor carbonyl hydrolase is equivalent to a residue of B. 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 B. 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 B. amyloliquefaciens subtilisin primary sequence and particularly to a set of residues known to be invariant in all subtilisins for which sequence is known (Figure 5C). 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 B. amyloliquefaciens subtilisin are defined. Alignment of conserved residues preferably should conserve 100% of such residues. However, alignment of greater than 75% or as little as 50% of conserved residues is also adequate to define equivalent residues. Conservation of the catalytic triad, Asp32/His64/Ser221 should be maintained.

For example, in Figure 5A the amino acid sequence of subtilisin from B. amyloliquefaciens B. subtilis var. I168 and B. licheniformis (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 residues are identified in Fig. 5C.

These conserved residues thus may be used to define the corresponding equivalent amino acid residues of B. amyloliquefaciens subtilisin in other carbonyl hydrolases such as thermitase derived from Thermoactinomyces. These two particular sequences are aligned in Fig. 5B to produce the maximum homology of conserved residues. As can be seen there are a number of insertions and deletions in the thermitase sequence as compared to B. amyloliquefaciens subtilisin. Thus, in thermitase the equivalent amino acid of Tyr217 in B. amyloliquefaciens subtilisin is the particular lysine shown beneath Tyr217.

In Fig. 5A, the equivalent amino acid at position 217 in B. amyloliquefaciens subtilisin is Tyr. Likewise, in B. subtilis subtilisin position 217 is also occupied by Tyr but in B. licheniformis position 217 is occupied by Leu.

Thus, these particular residues in thermitase, and subtilisin from B. subtilis and B. licheniformis may be substituted by a different amino acid to produce a mutant carbonyl hydrolase since they are equivalent in primary structure to Tyr217 in B. amyloliquefaciens subtilisin. Equivalent amino acids of course are not limited to those for Tyr217 but extend to any residue which is equivalent to a residue in B. amyloliquefaciens whether such residues are conserved or not.

Equivalent residues homologous at the level of tertiary structure for a precursor carbonyl hydrolase whose tertiary structure has been determined by x-ray crystallography, are defined as those for which the atomic coordinates of 2 or more of the main chain atoms of a particular amino acid residue of the precursor carbonyl hydrolase and B. amyloliquefaciens subtilisin (N on N, CA on CA, C on C, and O on O) are within 0.13nm and preferably 0.1nm after alignment. Alignment is achieved after the best model has been oriented and positioned to give the maximum overlap of atomic coordinates of non-hydrogen protein atoms of the carbonyl hydrolase in question to the B. amyloliquefaciens subtilisin. The best model is the crystallographic model giving the lowest R factor for experimental diffraction data at the highest resolution available.

$$R \text{ factor} = \frac{\sum_h |F_o(h)| - |F_c(h)|}{\sum_h |F_o(h)|}$$

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10 Equivalent residues which are functionally analogous to a specific residue of B. 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 B. amyloliquefaciens subtilisin as described herein. Further, they are those residues of the precursor carbonyl hydrolase (for which a tertiary structure has been obtained by x-ray crystallography), which occupy an analogous position to the extent that although the main chain atoms of the given residue may not satisfy the criteria of equivalence on the basis of occupying a homologous position, the atomic coordinates of at least two of the side chain atoms of the residue lie within 0.13nm of the corresponding side chain atoms of B. amyloliquefaciens subtilisin. The three dimensional structures would be aligned as outlined above.

20 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.

25 "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.

30 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. 0130756 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. 0130756 and further described by Yang, M.Y., et al. (1984) J. Bacteriol. 160, 15-21. Other host cells for expressing subtilisin include Bacillus subtilis 1168 (EPO Publication No. 0130756).

45 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.

50 The genes encoding the naturally-occurring precursor carbonyl hydrolase may be obtained in accord with the general methods described herein in EPO publication No. 0130756.

Once the 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 No. 0130756 and the production of carbonyl hydrolase mutants described herein.

The carbonyl hydrolase mutants of the present invention may be generated by site specific mutagenesis (Smith, M. (1985) Ann. Rev. Genet. 423; Zoeller, M.J., et al. (1982) Nucleic Acid Res. 10, 6487-6500), cassette mutagenesis (EPO Publication No. 0130756) or random mutagenesis (Shortle, D., et al. (1985) Genetics, 110, 539; Shortle, D., et al. (1986) Proteins: Structure, Function and Genetics, 1, 81; Shortle, D. (1986) J. Cell. Biochem. 30, 281; Alber, T., et al. (1985) Proc. Natl. Acad. of Sci., 82, 747; Matsumura, M., et al. (1985) J. Biochem., 260, 15298; Liao, H., et al. (1986) Proc. Natl. Acad. of Sci., 83 576) of the cloned precursor carbonyl hydrolase. Cassette mutagenesis and the random mutagenesis method disclosed herein are preferred.

The mutant carbonyl hydrolases expressed upon transformation of suitable hosts are screened for enzymes exhibiting one or more properties which are substantially different from the properties of the precursor carbonyl hydrolases, e.g., changes in substrate specificity, oxidative stability, thermal stability, alkaline stability, resistance to proteolytic degradation, pH-activity profiles and the like.

A change in substrate specificity is defined as a difference between the kcat/Km ratio of the precursor carbonyl hydrolase and that of the hydrolase mutant. The kcat/Km ratio is a measure of catalytic efficiency. Carbonyl hydrolase mutants with increased or diminished kcat/Km ratios are described in the examples. Generally, the objective will be to secure a mutant having a greater (numerically large) kcat/Km ratio for a given substrate, thereby enabling the use of the enzyme to more efficiently act on a target substrate. A substantial change in kcat/Km ratio is preferably at least 2-fold increase or decrease. However, smaller increases or decreases in the ratio (e.g., at least 1.5-fold) are also considered substantial. An increase in kcat/Km ratio for one substrate may be accompanied by a reduction in kcat/Km ratio for another substrate. This is a shift in substrate specificity, and mutants exhibiting such shifts have utility where the precursor hydrolase is undesirable, e.g. to prevent undesired hydrolysis of a particular substrate in an admixture of substrates. Km and kcat are measured in accord with known procedures, as described in EPO Publication No. 0130756 or as described herein.

Oxidative stability is measured either by known procedures or by the methods described hereinafter. A substantial change in oxidative stability is evidenced by at least about 50% increase or decrease (preferably decrease) in the rate of loss of enzyme activity when exposed to various oxidizing conditions. Such oxidizing conditions are exposure to the organic oxidant dperdodecanoic acid (DPDA) under the conditions described in the examples.

Alkaline stability is measured either by known procedures or by the methods described herein. A substantial change in alkaline stability is evidenced by at least about a 5% or greater increase or decrease (preferably increase) in the half life of the enzymatic activity of a mutant when compared to the precursor carbonyl hydrolase. In the case of subtilisins, alkaline stability was measured as a function of autoproteolytic degradation of subtilisin at alkaline pH, e.g. for example, 0.1M sodium phosphate, pH 12 at 25° or 30°C.

Thermal stability is measured either by known procedures or by the methods described herein. A substantial change in thermal stability is evidenced by at least about a 5% or greater increase or decrease (preferably increase) in the half-life of the catalytic activity of a mutant when exposed to a relatively high temperature and neutral pH as compared to the precursor carbonyl hydrolase. In the case of subtilisins, thermal stability is measured by the autoproteolytic degradation of subtilisin at elevated temperatures and neutral pH, e.g., for example 2mM calcium chloride, 50mM MOPS pH 7.0 at 59°C.

The inventors have produced mutant subtilisins containing the substitution of the amino acid residues of B. amyloliquefaciens subtilisin shown in Table I. The wild type amino acid sequence and DNA sequence of B. amyloliquefaciens subtilisin is shown in Fig. 1.

TABLE I

Residue	Replacement Amino Acid
Tyr21	F A
Thr22	C
Ser24	C
Asp32	Q S
Ser33	A T
Asp36	A G
Gly48	V
Ala48	E V R
Ser49	C L
Met50	C F V
Asn77	D
Ser87	C
Lys94	C
Val95	C
Leu96	D
Tyr104	A C D E F G H I K L M N P Q R S T V W
Ile107	V
Gly110	C R
Met124	I L
Asn155	A D H Q T
Glu156	Q S
Gly166	C E I L M P S T W Y
Gly169	C D E F H I K L M N P Q R T V W Y
Lys170	E R
Tyr171	F
Pro172	E Q
Phe189	A C D E G H I K L M N P Q R S T V W Y
Asp197	R A
Met199	I
Ser204	C R L P
Lys213	R T
Tyr217	A C D E F G H I K L M N P Q R S T V W
Ser221	A C

The different amino acids substituted are represented in Table I by the following single letter designations:

Amino acid or residue thereof	3-letter symbol	1-letter symbol
Alanine	Ala	A
Glutamate	Glu	E
Glutamine	Gln	Q
Aspartate	Asp	D
Asparagine	Asn	N
Leucine	Leu	L
Glycine	Gly	G
Lysine	Lys	K
Serine	Ser	S
Valine	Val	V
Arginine	Arg	R
Threonine	Thr	T
Proline	Pro	P
Isoleucine	Ile	I
Methionine	Met	M
Phenylalanine	Phe	F
Tyrosine	Tyr	Y
Cysteine	Cys	C
Tryptophan	Trp	W
Histidine	His	H

Except where otherwise indicated by context, wild-type amino acids are represented by the above three-letter symbols and replaced amino acids by the above single-letter symbols. Thus, if the methionine at residue 50 in *B. amyloliquefaciens* subtilisin is replaced by phenylalanine, this mutation (mutant) may be designated Met50F or F50. Similar designations are used for multiple mutants.

In addition to the amino acids used to replace the residues disclosed in Table I, other replacements of amino acids at these residues are expected to produce mutant subtilisins having useful properties. These residues and replacement amino acids are shown in Table II.

TABLE II

Residue	Replacement Amino Acid(s)
Tyr-21	L
Thr22	K
Ser24	A
Asp32	
Ser33	G
Gly46	
Ala48	
Ser49	
Met50	L K I V
Asn77	D
Ser87	N
Lys94	R Q
Val95	L I
Tyr104	
Met124	K A
Ala152	C L I T M
Asn155	
Glu156	A T M L Y
Gly166	
Gly169	
Tyr171	K R E Q
Pro172	D N
Phe189	
Tyr217	
Ser221	
Met222	

Each of the mutant subtilisins in Table I contain the replacement of a single residue of the B. amyloliquefaciens amino acid sequence. These particular residues were chosen to probe the influence of such substitutions on various properties of B. amyloliquefaciens subtilisin.

Thus, the inventors have identified Met124 and Met222 as important residues which if substituted with another amino acid produce a mutant subtilisin with enhanced oxidative stability. For Met124, Leu and Ile are preferred replacement amino acids. Preferred amino acids for replacement of Met222 are disclosed in EPO Publication No. 0130756.

Various other specific residues have also been identified as being important with regard to substrate specificity. These residues include Tyr104, Ala152, Glu156, Gly166, Gly169, Phe189 and Tyr217 for which mutants containing the various replacement amino acids presented in Table I have already been made, as well as other residues presented below for which mutants have yet to be made.

The identification of these residues, including those yet to be mutated, is based on the inventors' high resolution crystal structure of B. amyloliquefaciens subtilisin to 1.8 Å (see Table III), their experience with in vitro mutagenesis of subtilisin and the literature on subtilisin. This work and the x-ray crystal structures of subtilisin containing covalently bound peptide inhibitors (Robertus, J.D., et al. (1972) Biochemistry **11**, 2439-2449), product complexes (Robertus, J.D., et al. (1972) Biochemistry **11**, 4293-4303), and transition state analogs (Matthews, D.A., et al. (1975) J. Biol. Chem. **250**, 7120-7126; Poulos, T.L., et al. (1976) J. Biol. Chem. **251**, 1097-1103), has helped in identifying an extended peptide binding cleft in subtilisin. This substrate binding cleft together with substrate is schematically diagrammed in Fig. 2, according to the nomenclature of Schechter, I., et al. (1967) Biochem. Biophys. Res. Commun. **27**, 157. The scissile bond in the substrate is identified by an arrow. The P and P' designations refer to the amino acids which are positioned respectively toward the amino or carboxy terminus relative to the scissile bond. The S and S' designations refer to subsites in the substrate binding cleft of subtilisin which interact with the corresponding substrate amino acid residues.

Atomic Coordinates for the
Apoenzyme Form of *B. Amylolyquefaciens*
Subtilisin to 1.8Å Resolution

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1	ALA N	29.434	33.195	-21.756	1	ALA CA	19.011	31.774	-21.965
1	ALA C	28.731	30.925	-21.324	1	ALA O	30.376	31.197	-20.175
1	ALA CO	23.099	31.510	-21.103	2	GLN N	30.260	49.006	-22.041
2	GLN CA	17.219	49.000	-21.634	2	GLN C	17.075	47.706	-20.992
2	GLN O	18.765	47.165	-21.691	2	GLN CO	16.125	48.760	-22.449
2	GLN CG	15.920	47.905	-21.927	2	GLN CD	13.912	47.762	-22.930
2	GLN OE1	13.023	48.612	-22.067	2	GLN NE2	14.115	46.917	-23.926
3	SER N	17.477	47.205	-19.852	3	SER CA	17.950	45.040	-19.437
3	SER C	16.735	44.910	-19.490	3	SER O	15.590	45.352	-19.229
3	SER CO	10.580	45.030	-18.069	3	SER OG	17.602	46.210	-17.049
4	VAL N	16.991	63.646	-19.725	4	VAL CA	15.946	42.619	-19.639
4	VAL C	16.124	43.934	-18.290	4	VAL O	17.123	41.170	-18.006
4	VAL CO	16.008	43.622	-20.822	4	VAL CG1	14.074	40.572	-20.761
4	VAL CG2	16.037	42.266	-22.106	5	PRO N	15.230	42.104	-17.331
5	PRO CA	19.304	41.415	-14.827	5	PRO C	15.501	39.905	-16.249
5	PRO O	16.085	39.263	-17.146	5	PRO CO	14.150	41.000	-15.263
5	PRO CG	13.841	43.215	-15.921	5	PRO CD	14.044	42.906	-17.417
6	TYR N	16.363	39.260	-15.607	6	TYR CA	16.620	37.003	-15.715
6	TYR C	15.359	36.975	-15.520	6	TYR O	15.224	35.943	-16.235
6	TYR CO	17.024	37.323	-14.034	6	TYR CG	10.021	35.047	-15.055
6	TYR CD1	10.437	35.452	-14.346	6	TYR CD2	17.096	34.908	-14.071
6	TYR CE1	10.535	34.070	-16.653	6	TYR CE2	17.015	33.539	-14.379
6	TYR C1	10.222	33.154	-15.628	6	TYR OH	10.312	31.030	-15.996
7	GLY N	14.464	37.362	-14.630	7	GLY CA	13.211	36.049	-14.376
7	GLY C	12.600	36.535	-15.670	7	GLY O	11.747	35.478	-15.003
8	VAL N	12.441	37.529	-14.541	8	VAL CA	11.777	37.523	-17.036
8	VAL C	12.363	36.433	-18.735	8	VAL O	11.639	35.716	-19.470
8	VAL CO	11.765	38.900	-18.567	8	VAL CG1	11.106	38.093	-19.943
8	VAL CG2	10.991	39.019	-17.733	9	SER N	13.661	36.310	-18.775
9	SER CA	14.419	35.342	-19.562	9	SER C	14.100	33.920	-18.965
9	SER O	14.112	33.014	-19.301	9	SER CO	15.926	35.632	-19.505
9	SER OG	16.162	36.767	-20.350	10	GLN N	14.115	35.007	-17.662
10	GLN CA	13.964	32.636	-16.076	10	GLN C	12.607	31.007	-17.277
10	GLN O	12.785	30.642	-17.613	10	GLN CO	14.125	32.005	-15.410
10	GLN CG	14.295	31.617	-14.500	10	GLN CD	14.406	31.911	-13.147
10	GLN OE1	14.554	33.060	-12.746	10	GLN NE2	14.552	30.960	-12.251
11	ILE N	11.625	32.575	-17.670	11	ILE CA	10.373	31.904	-18.102
11	ILE C	10.209	31.792	-19.605	11	ILE O	9.173	31.333	-20.100
11	ILE CO	9.132	32.469	-17.675	11	ILE CG1	9.066	34.117	-18.049
11	ILE CG2	9.162	32.655	-15.941	11	ILE CO1	7.500	34.640	-17.923
12	LYS N	11.272	32.105	-20.277	12	LYS CA	11.300	32.119	-21.722
12	LYS C	10.456	33.006	-22.522	12	LYS O	10.170	32.703	-23.606
12	LYS CO	11.257	30.646	-22.216	12	LYS CG	12.203	29.030	-21.423
12	LYS CD	12.543	28.517	-22.159	12	LYS CE	13.023	27.467	-21.166
12	LYS NZ	14.476	27.600	-20.935	13	ALA N	10.109	34.130	-21.991
13	ALA CA	9.325	35.190	-22.431	13	ALA C	10.026	35.716	-23.063
13	ALA O	9.330	35.004	-24.901	13	ALA CO	8.005	36.195	-21.565
14	PRO N	11.332	35.950	-23.093	14	PRO CA	11.905	34.430	-25.120
14	PRO C	11.706	35.557	-26.317	14	PRO O	11.770	36.047	-27.445
14	PRO CO	13.462	36.500	-24.692	14	PRO CG	13.320	34.970	-23.221
14	PRO CD	12.201	35.936	-22.750	15	ALA N	11.560	34.236	-24.129
15	ALA CA	11.379	33.450	-27.367	15	ALA C	10.002	33.795	-20.032
15	ALA O	10.000	33.710	-29.270	15	ALA CO	11.552	31.949	-27.062
16	LEU N	9.003	36.130	-27.240	16	LEU CA	7.791	34.550	-27.020
16	LEU C	7.912	35.925	-20.521	16	LEU O	7.342	36.126	-29.500
16	LEU CO	6.746	34.623	-26.690	16	LEU CG	5.790	33.665	-26.522
16	LEU CD1	5.001	33.234	-27.009	16	LEU CD2	6.094	32.207	-26.203
17	WIS N	8.665	36.020	-27.922	17	WIS CA	8.090	30.151	-28.339
17	WIS C	9.510	37.901	-29.090	17	WIS O	9.107	30.622	-30.056
17	WIS CO	9.700	39.100	-27.652	17	WIS CG	9.105	31.200	-26.262
17	WIS CD1	9.930	39.007	-23.272	17	WIS CD2	8.000	30.924	-25.694
17	WIS CE1	9.226	39.914	-24.146	17	WIS NE2	8.079	31.320	-24.301
18	SER N	10.443	37.033	-30.022	18	SER CA	11.109	36.739	-31.322

5	18	SEN C	30.139	36.123	-37.953	39	SEN D	30.947	36.112	-37.854
	19	SEN CO	32.311	35.799	-37.172	39	SEN D2	32.323	36.489	-38.299
	19	SLN M	9.990	35.493	-37.963	39	SLN CA	9.982	36.962	-37.871
	19	SLN C	7.142	36.111	-37.903	39	SLN D	8.297	39.972	-34.219
	19	SLN CO	7.221	33.849	-37.289	39	SLN CG	7.978	32.892	-37.823
	19	SLN CO2	6.923	31.797	-37.181	39	SLN CG1	9.719	31.833	-37.444
	19	SLN CG1	7.362	30.932	-37.234	39	SLN CG2	7.285	27.323	-37.297
	19	SLY CA	6.349	30.387	-37.859	39	SLY C	5.181	38.492	-37.888
	19	SLY D	4.263	39.274	-37.215	39	TYR M	9.202	37.801	-38.761
	21	TYR CA	4.118	37.831	-37.763	39	TYR C	4.879	39.932	-38.923
	21	TYR D	8.422	38.974	-37.754	39	TYR CG	3.499	24.431	-39.443
	21	TYR CG	2.973	31.784	-38.799	39	TYR CG1	1.793	36.232	-37.238
	21	TYR CG2	3.490	34.794	-37.397	39	TYR CG2	1.306	33.797	-37.444
10	21	TYR CG2	3.193	34.261	-37.888	39	TYR CG2	2.883	34.758	-37.867
	21	TYR CG2	3.501	34.241	-37.250	39	TYR CG2	3.992	39.880	-38.288
	22	TYR CA	6.262	40.927	-37.129	39	TYR C	9.991	42.922	-38.344
	22	TYR D	3.287	41.723	-37.323	39	TYR CG	3.133	41.759	-37.611
	22	TYR CG1	4.319	42.487	-37.997	39	TYR CG2	4.474	41.323	-38.229
	23	GLY M	3.939	40.293	-37.493	39	GLY CA	8.999	49.600	-37.342
	23	GLY C	-0.197	41.631	-37.110	39	GLY D	-1.013	42.899	-37.338
	24	SEN M	-0.023	41.967	-37.171	39	SEN CO	-0.897	42.937	-38.812
15	24	SEN C	-2.383	42.626	-37.864	39	SEN D	-2.613	41.898	-38.168
	24	SEN CO	-0.734	43.120	-37.320	39	SEN D2	0.963	47.632	-37.728
	24	SEN M	-3.959	43.692	-37.919	39	SEN CA	-4.519	43.697	-37.393
	24	SEN C	-9.819	42.873	-37.203	39	SEN D	-6.233	42.648	-36.189
	24	SEN CG	-8.165	43.217	-37.703	39	SEN CG	-4.969	44.170	-37.883
	24	SEN CG1	-4.963	43.767	-37.883	39	SEN CG2	-4.747	45.661	-37.994
	24	VAL M	-4.177	42.449	-37.292	39	VAL CA	-4.674	41.679	-37.143
	24	VAL C	-4.792	42.852	-37.937	39	VAL D	-3.898	43.419	-37.899
20	24	VAL CG	-3.714	40.903	-37.821	39	VAL CG1	-4.169	39.892	-37.948
	24	VAL CG2	-3.998	39.576	-37.818	39	VAL CG2	-5.910	42.613	-37.391
	27	LYS CA	-6.133	43.824	-37.179	39	LYS M	-5.816	42.872	-37.841
	27	LYS D	-6.405	41.873	-37.419	39	LYS CG	-7.390	43.981	-37.149
	27	LYS CG	-8.846	44.575	-37.690	39	LYS CO	-9.321	43.302	-37.820
	27	LYS CG1	-10.304	43.497	-37.137	39	LYS CG2	-9.686	46.253	-37.264
	28	VAL M	-4.818	43.462	-37.203	39	VAL CA	-4.637	42.958	-37.897
	28	VAL C	-4.758	43.939	-37.828	39	VAL D	-4.209	43.995	-37.817
25	28	VAL CG	-2.926	42.666	-37.932	39	VAL CG1	-2.466	42.189	-37.889
	28	VAL CG2	-2.667	41.889	-37.173	39	VAL CG2	-3.484	43.227	-37.913
	29	ALA CA	-5.747	44.330	-37.639	39	ALA C	-4.750	44.010	-37.953
	29	ALA D	-4.666	42.843	-37.194	39	ALA CG	-7.172	44.197	-37.101
	29	VAL M	-4.857	43.833	-37.872	39	VAL CA	-3.146	44.962	-37.910
	29	VAL C	-3.938	43.499	-37.881	39	VAL D	-4.129	46.648	-37.978
	29	VAL CG	-1.886	43.810	-37.149	39	VAL CG1	-0.936	43.991	-37.988
30	29	VAL CG2	-1.853	43.236	-37.207	39	VAL CG2	-4.314	44.319	-37.877
	31	ILE CA	-5.328	44.846	-37.679	39	ILE C	-4.346	44.933	-37.946
	31	ILE D	-3.823	43.913	-37.897	39	ILE CG	-6.697	43.774	-37.901
	31	ILE CG1	-7.299	43.707	-37.799	39	ILE CG2	-7.278	44.838	-37.223
	31	ILE CG2	-8.617	42.894	-37.717	39	ILE CG2	-4.844	46.193	-37.217
	32	ASP CA	-2.944	46.467	-37.255	39	ASP C	-3.871	47.889	-37.781
	32	ASP D	-4.197	48.418	-37.302	39	ASP CG	-1.693	46.129	-37.892
	32	ASP CG	-0.483	48.782	-37.273	39	ASP CG1	0.934	44.392	-37.876
	32	ASP CG2	-0.981	46.419	-37.310	39	SEN M	-1.931	48.912	-37.394
35	33	SEN CA	-1.895	49.857	-37.801	39	SEN C	-1.992	49.974	-37.988
	33	SEN D	-1.786	52.136	-37.263	39	SEN CG	-0.621	49.922	-37.939
	33	SEN CG	0.933	50.825	-37.774	39	SLY M	-2.173	50.740	-37.884
	34	GLY CA	-2.233	51.728	-37.163	39	GLY C	-1.935	51.648	-37.887
	34	GLY D	-0.144	50.931	-37.781	39	ILE M	-0.963	52.431	-37.102
	34	ILE CA	0.808	52.438	-37.993	39	ILE C	0.968	53.919	-37.163
	34	ILE D	-0.327	54.638	-37.744	39	ILE CG	-0.843	51.694	-37.367
	34	ILE CG1	-0.838	50.210	-37.897	39	ILE CG2	1.149	51.741	-37.362
40	34	ILE CG2	-0.962	49.483	-37.424	39	ASP M	1.816	54.253	-37.971
	34	ASP CA	2.359	55.618	-37.232	39	ASP C	2.281	55.958	-37.782

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36	ASP D	3.004	55.471	-13.579	36	ASP CR	3.712	55.720	-10.514
36	ASP CG	4.339	57.099	-10.804	36	ASP OD1	3.755	57.974	-11.429
36	ASP OD2	5.448	57.277	-10.243	37	SIR M	1.904	56.822	-13.111
37	SER CA	3.183	57.221	-14.512	37	SIR C	2.377	56.095	-14.949
37	SER O	2.545	58.303	-14.151	37	SER CB	-0.093	56.049	-14.788
37	SER OG	-0.090	59.133	-13.079	38	SER M	3.163	58.614	-14.001
38	SER CA	4.261	59.505	-14.487	38	SER C	3.466	58.705	-14.992
38	SER O	6.543	59.251	-15.205	38	SIR CB	4.742	60.435	-13.398
38	SER OG	5.374	59.865	-12.234	39	MIS M	5.454	57.390	-14.892
39	MIS CA	6.637	56.574	-15.291	39	MIS C	6.681	56.401	-16.778
39	MIS D	5.738	55.878	-17.419	39	MIS CB	6.637	55.203	-14.515
39	MIS CG	8.014	54.609	-14.456	39	MIS OD1	8.795	54.354	-15.561
39	MIS CD2	8.749	54.345	-13.389	39	MIS CE1	9.970	53.930	-15.130
39	MIS ME2	9.986	53.910	-13.008	40	PRO M	7.007	54.836	-17.387
40	PRO CA	7.988	56.697	-18.031	40	PRO C	8.154	55.200	-19.357
40	PRO O	8.032	55.097	-20.578	40	PRO CS	9.247	57.533	-19.161
40	PRO CG	10.053	57.405	-17.902	40	PRO CD	8.988	57.452	-16.776
41	ASP M	8.441	54.378	-18.485	41	ASP OD2	11.148	58.399	-18.668
41	ASP OD1	10.325	51.395	-20.429	41	ASP CG	10.473	51.387	-19.211
41	ASP CB	9.799	52.239	-18.224	41	ASP CA	8.645	52.959	-18.966
41	ASP C	7.311	52.163	-18.039	41	ASP O	7.396	52.947	-18.977
42	LEU M	6.185	52.003	-18.358	42	LEU CA	4.892	52.147	-18.466
42	LEU C	3.924	52.907	-19.374	42	LEU O	3.993	54.163	-19.490
42	LEU CB	4.421	52.158	-17.008	42	LEU CG	5.182	51.363	-15.946
42	LEU CD1	4.535	51.546	-14.581	42	LEU CD2	5.273	49.877	-16.350
43	LTS M	3.018	52.135	-19.044	43	LTS CA	1.093	52.085	-20.721
43	LTS C	0.637	52.156	-20.018	43	LTS O	0.584	50.920	-19.820
43	LTS CB	2.021	52.389	-22.169	43	LTS CG	0.685	52.436	-22.910
43	LTS CD	8.998	52.062	-24.339	43	LTS CE	-0.180	52.384	-25.260
43	LTS M2	0.337	51.757	-24.418	44	VAL M	-0.191	53.835	-19.490
44	VAL CA	-1.407	52.639	-18.765	44	VAL C	-2.571	52.887	-19.731
44	VAL O	-2.623	53.906	-20.434	44	VAL CB	-1.480	53.351	-17.383
44	VAL CG1	-2.724	52.941	-16.582	44	VAL CG2	-0.197	53.194	-16.553
45	ALA M	-3.494	51.951	-19.071	45	ALA CA	-4.619	51.977	-20.810
45	ALA C	-5.841	52.507	-20.053	45	ALA O	-6.703	53.085	-20.703
45	ALA CB	-4.031	50.580	-21.389	46	GLY M	-5.910	52.354	-18.768
46	GLY CA	-7.082	52.837	-18.081	46	GLY C	-6.087	52.443	-16.538
46	GLY O	-5.938	52.806	-16.035	47	GLY M	-8.092	52.658	-15.793
47	GLY CA	-8.014	52.246	-14.388	47	GLY C	-9.179	52.757	-13.572
47	GLY O	-9.988	53.481	-14.185	48	ALA M	-9.221	52.446	-12.330
48	ALA CA	-10.255	52.878	-11.382	48	ALA C	-9.790	52.675	-9.968
48	ALA O	-9.066	51.720	-9.725	48	ALA CB	-11.558	52.100	-11.617
49	SER M	-10.149	53.547	-9.037	49	SER CA	-9.752	53.355	-7.652
49	SER C	-10.947	52.986	-6.783	49	SER O	-11.972	53.677	-6.980
49	SER CB	-9.052	54.588	-7.029	49	SER OG	-8.079	54.255	-5.650
50	MET M	-10.835	52.007	-5.932	50	MET CA	-11.052	51.549	-4.974
50	MET C	-11.463	51.962	-3.561	50	MET O	-11.997	51.398	-2.575
50	MET CB	-12.012	50.018	-4.996	50	MET CG	-11.912	49.463	-6.389
50	MET SO	-13.466	49.889	-7.256	50	MET CE	-12.000	50.111	-0.903
51	VAL M	-10.427	52.740	-3.422	51	VAL CA	-9.968	53.170	-2.067
51	VAL C	-10.630	54.562	-1.907	51	VAL O	-10.237	55.437	-2.682
51	VAL CB	-8.443	53.155	-2.000	51	VAL CG1	-7.092	53.579	-0.631
51	VAL CG2	-7.764	53.015	-2.302	52	PRO M	-11.621	54.693	-1.056
52	PRO CA	-12.372	55.933	-0.021	52	PRO C	-11.490	57.123	-0.660
52	PRO O	-11.771	58.220	-0.925	52	PRO CB	-13.408	55.594	0.244
52	PRO CG	-13.583	54.183	0.085	52	PRO CO	-12.264	53.620	-0.175
53	SER M	-10.642	54.986	0.299	53	SER CA	-9.538	57.982	0.602
53	SER C	-8.420	58.245	-0.324	53	SER O	-7.679	59.224	-0.038
53	SER CB	-9.004	57.707	2.049	53	SER OG	-8.254	54.521	2.127
54	GLU M	-8.234	57.523	-1.393	54	GLU CA	-7.204	57.648	-2.421
54	GLU C	-7.767	57.303	-3.785	54	GLU O	-7.533	56.243	-4.379
54	GLU CB	-6.134	56.595	-2.154	54	GLU CG	-5.289	56.959	-0.927
54	GLU CO	-6.064	54.065	-0.070	54	GLU HPI	-7.046	55.604	-1.068

54	GLW DE2	-3.900	55.777	0.271	55	TMR H	-0.571	50.251	-4.249
55	TMR CA	-9.433	55.121	-5.441	55	TMR C	-0.764	50.139	-4.779
55	TMR O	-9.433	57.919	-7.010	55	TMR CB	-10.906	59.200	-5.303
55	TMR OC1	-9.885	60.510	-5.410	55	TMR CG2	-11.432	59.143	-4.017
56	ASN H	-7.482	58.493	-6.077	56	ASN WD2	-0.930	61.179	-9.081
56	ASN DD1	-5.075	58.967	-10.337	56	ASN CG	-5.273	59.925	-9.555
56	ASN CB	-5.075	59.694	-0.200	56	ASN CA	-6.762	58.425	-0.200
56	ASN C	-6.012	57.094	-0.305	56	ASN O	-5.104	56.866	-7.470
57	PRO H	-6.362	56.261	-9.250	57	PRO CG	-7.123	55.257	-11.177
57	PRO CO	-7.304	56.433	-10.272	57	PRO CB	-6.644	54.170	-10.235
57	PRO CA	-5.679	54.961	-9.332	57	PRO C	-4.301	55.082	-9.966
57	PRO O	-3.509	54.120	-9.945	58	PHE H	-3.990	56.262	-10.491
58	PHE CA	-2.747	56.577	-11.222	58	PHE C	-1.712	57.129	-10.253
58	PHE O	-0.635	57.497	-10.600	58	PHE CB	-2.043	57.502	-12.423
58	PHE CG	-3.903	56.940	-13.357	58	PHE CD1	-3.756	55.700	-14.059
58	PHE CD2	-5.211	57.630	-13.459	58	PHE CE1	-4.722	55.255	-14.020
58	PHE CE2	-6.194	57.095	-14.274	58	PHE C2	-5.940	55.930	-15.051
59	GLW H	-2.044	57.119	-8.990	59	GLW CA	-2.172	57.503	-7.934
59	GLW C	-0.807	56.603	-7.000	59	GLW O	-1.639	56.003	-6.119
59	GLW CB	-1.462	58.660	-7.009	59	GLW CG	-0.942	59.261	-6.034
59	GLW CO	-1.790	60.157	-5.150	59	GLW DE1	-1.404	61.200	-6.036
59	GLW HE2	-2.959	59.605	-6.742	60	ASP H	0.410	55.095	-7.211
60	ASP CA	0.051	54.792	-6.304	60	ASP C	1.631	55.267	-5.090
60	ASP O	2.027	55.550	-5.231	60	ASP CB	1.596	53.744	-7.100
60	ASP CG	2.077	52.530	-6.300	60	ASP DD1	1.746	52.337	-5.190
60	ASP DD2	2.915	51.041	-7.030	61	ASN H	0.959	55.265	-3.950
61	ASN WD2	-3.364	57.747	-2.347	61	ASN DD1	0.666	58.566	-2.075
61	ASN CG	-0.040	57.670	-2.399	61	ASN CB	0.531	56.401	-1.704
61	ASN CA	1.557	55.734	-2.700	61	ASN C	2.291	54.632	-1.960
61	ASN O	2.933	54.062	-0.902	62	ASN H	2.210	53.434	-2.460
62	ASN CA	2.077	52.340	-1.709	62	ASN C	4.124	51.093	-2.479
62	ASN O	4.951	51.313	-1.770	62	ASN CB	1.703	51.319	-1.421
62	ASN CG	2.371	50.103	-0.697	62	ASN DD1	2.633	49.077	-1.363
62	ASN WD2	2.622	50.200	0.601	63	SER H	4.152	52.104	-3.761
63	SER CA	5.109	51.694	-4.709	63	SER C	5.071	50.256	-5.209
63	SER O	5.593	49.790	-6.269	63	SER CB	6.523	51.950	-4.012
63	SER CG	6.071	50.690	-3.410	64	WIS H	4.202	49.475	-4.639
64	WIS CA	3.994	48.055	-4.935	64	WIS C	3.366	47.759	-6.261
64	WIS O	3.061	46.974	-7.100	64	WIS CB	3.104	47.501	-3.747
64	WIS CG	3.144	46.021	-3.726	64	WIS DD1	2.107	45.247	-4.261
64	WIS CD2	4.056	45.194	-3.135	64	WIS CE1	2.416	43.966	-4.054
64	WIS HE2	3.556	43.920	-3.368	65	GLY H	2.207	48.420	-6.507
65	GLY CA	1.552	48.264	-7.030	65	GLY C	2.392	48.636	-9.037
65	GLY O	2.230	48.070	-10.134	66	TMR H	3.233	49.659	-8.032
66	TMR CA	4.064	50.117	-9.954	66	TMR C	5.009	49.009	-10.291
66	TMR O	5.333	48.709	-11.463	66	TMR CB	4.764	51.511	-9.467
66	TMR OC1	3.637	52.425	-9.406	66	TMR CG2	5.536	52.070	-10.049
67	WIS H	5.605	48.443	-9.274	67	WIS CA	0.703	47.361	-9.450
67	WIS C	6.091	46.141	-10.143	67	WIS O	6.649	45.630	-11.150
67	WIS CB	7.300	47.071	-0.064	67	WIS CG	0.595	46.275	-0.140
67	WIS DD1	0.590	44.907	-0.276	67	WIS CD2	9.904	46.670	-0.076
67	WIS CE1	0.057	44.691	-0.299	67	WIS HE2	10.670	45.514	-0.100
68	VAL H	4.092	45.749	-9.731	68	VAL CA	4.142	44.607	-10.266
68	VAL C	3.056	44.060	-11.740	68	VAL O	4.114	43.942	-12.535
68	VAL CB	2.939	44.252	-9.306	68	VAL CG1	1.960	43.260	-10.020
68	VAL CG2	3.319	43.705	-8.000	69	ALA H	3.373	46.049	-12.113
69	ALA CA	3.037	46.460	-13.429	69	ALA C	0.193	46.390	-14.411
69	ALA O	4.020	45.913	-15.965	69	ALA CB	2.332	47.051	-13.306
70	GLY H	5.340	46.702	-15.914	70	GLY CA	0.595	46.005	-14.670
70	GLY C	7.046	45.370	-15.021	70	GLY O	7.604	43.154	-16.119
71	TMR H	0.820	44.431	-14.130	71	TMR CA	7.177	43.019	-14.446
71	TMR C	0.224	42.506	-15.543	71	TMR O	0.602	41.026	-16.495
71	TMR CB	7.119	42.070	-13.101	71	TMR OC1	0.191	42.592	-12.390

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71	YMR	CE2	7.274	48.983	-13.594	71	VAL	W	4.938	42.887	-18.627
72	VAL	CA	3.976	42.491	-16.684	72	VAL	C	4.312	43.004	-17.831
72	VAL	D	6.341	42.380	-18.848	72	VAL	CB	2.516	42.867	-16.885
72	VAL	CG1	1.512	42.489	-17.178	72	VAL	CG2	2.142	42.327	-16.723
73	ALA	W	4.504	44.417	-17.888	73	ALA	CA	4.987	43.891	-19.167
73	ALA	C	8.433	46.333	-19.355	73	ALA	D	3.962	47.189	-20.216
73	ALA	CB	3.107	43.443	-19.433	74	ALA	W	6.544	46.429	-18.635
74	ALA	CA	3.478	47.591	-18.959	74	ALA	C	7.740	47.648	-20.342
74	ALA	D	7.959	46.640	-21.054	74	ALA	CB	8.653	47.446	-17.925
75	LEU	W	7.650	48.784	-21.839	75	LEU	CA	7.812	48.968	-22.456
75	LEU	C	9.192	48.568	-22.966	75	LEU	D	10.162	48.750	-22.253
75	LEU	CB	7.548	50.471	-22.809	75	LEU	CG	4.123	50.913	-22.379
75	LEU	CD1	6.079	52.436	-22.308	75	LEU	CD2	5.096	50.462	-23.405
76	ASN	W	9.147	48.103	-24.169	76	ASN	WD2	12.385	46.432	-26.384
76	ASN	BD1	10.950	45.848	-27.928	76	ASN	CG	11.195	46.274	-26.882
76	ASN	CB	10.810	46.651	-25.988	76	ASN	CA	10.359	47.738	-24.938
76	ASN	C	10.783	49.848	-25.643	76	ASN	D	10.157	49.479	-26.619
77	ASN	W	11.804	49.464	-25.671	77	ASN	CA	12.220	50.957	-25.481
77	ASN	C	13.787	51.829	-25.368	77	ASN	D	14.364	49.979	-25.313
77	ASN	CB	11.335	52.076	-25.117	77	ASN	CG	11.250	52.827	-23.616
77	ASN	BD1	12.032	51.346	-22.917	77	ASN	WD2	10.294	52.741	-23.825
78	SER	W	14.125	52.267	-25.164	78	SER	CA	15.513	52.614	-24.986
78	SER	C	15.810	52.742	-23.436	78	SER	D	14.982	53.071	-23.164
78	SER	CB	15.985	53.941	-25.587	78	SER	CG	15.926	53.878	-24.999
79	ILE	W	14.858	52.565	-22.529	79	ILE	CA	15.155	52.784	-21.120
79	ILE	C	14.617	51.683	-20.230	79	ILE	D	13.843	50.841	-20.679
79	ILE	CB	14.471	54.174	-20.697	79	ILE	CG1	12.945	54.832	-20.814
79	ILE	CG2	14.997	55.328	-21.612	79	ILE	CD1	12.135	55.176	-20.155
80	GLY	W	14.995	51.768	-18.981	80	GLY	CA	14.476	58.948	-17.913
80	GLY	C	14.612	49.448	-18.219	80	GLY	D	15.719	48.994	-18.544
81	VAL	W	13.513	48.766	-17.980	81	VAL	CA	13.411	47.286	-18.061
81	VAL	C	12.511	46.919	-19.217	81	VAL	D	12.260	47.739	-20.117
81	VAL	CB	13.881	46.755	-16.677	81	VAL	CG1	14.030	47.084	-19.573
81	VAL	CG2	11.438	47.261	-16.231	82	LEU	W	12.126	49.645	-19.216
82	LEU	CA	11.312	45.828	-20.254	82	LEU	C	10.390	44.878	-19.510
82	LEU	D	10.858	43.356	-18.680	82	LEU	CB	12.286	46.719	-21.229
82	LEU	CG	11.430	43.568	-22.366	82	LEU	CD1	10.796	44.657	-23.223
82	LEU	CD2	12.359	42.675	-23.192	83	GLY	W	9.131	44.180	-19.816
83	GLY	CA	8.133	43.321	-19.114	83	GLY	C	8.827	42.811	-19.925
83	GLY	D	8.946	41.822	-21.926	84	VAL	W	7.272	41.112	-19.283
84	VAL	CA	6.973	39.807	-19.888	84	VAL	C	6.164	48.838	-21.140
84	VAL	D	6.424	39.472	-22.194	84	VAL	CB	6.256	38.920	-18.841
84	VAL	CG1	5.680	37.677	-19.557	84	VAL	CG2	7.190	38.587	-17.785
85	ALA	W	5.156	40.926	-21.824	85	ALA	CA	4.217	41.194	-22.158
85	ALA	C	4.213	42.683	-22.396	85	ALA	D	3.268	43.481	-22.838
85	ALA	CB	2.846	40.663	-21.748	86	PRO	W	5.240	43.186	-23.859
86	PRO	CA	5.413	44.435	-23.285	86	PRO	C	4.321	45.371	-23.947
86	PRO	D	4.291	46.685	-23.849	86	PRO	CB	4.822	44.784	-23.813
86	PRO	CG	7.030	43.486	-24.566	86	PRO	CD	4.377	42.440	-23.616
87	SER	W	3.548	44.476	-24.769	87	SER	CA	2.689	45.324	-25.529
87	SER	C	1.103	45.132	-24.897	87	SER	D	0.162	45.513	-25.619
87	SER	CB	2.401	44.777	-26.927	87	SER	CG	3.591	45.143	-27.183
88	ALA	W	1.817	44.564	-23.742	88	ALA	CB	-0.163	43.519	-21.828
88	ALA	CA	-0.773	44.353	-23.884	88	ALA	C	-0.898	45.717	-22.698
88	ALA	D	-0.174	46.717	-22.435	89	SER	W	-2.219	45.691	-22.678
89	SER	W	-4.146	47.182	-24.288	89	SER	CA	-4.343	46.983	-22.898
89	SER	CA	-3.881	48.867	-22.227	89	SER	C	-3.136	46.788	-28.727
89	SER	D	-3.793	45.864	-20.209	90	LEU	W	-2.446	47.654	-28.837
90	LEU	CA	-2.378	47.667	-18.593	90	LEU	C	-3.483	48.438	-17.864
90	LEU	D	-3.582	49.684	-18.215	90	LEU	CB	-0.951	48.273	-18.426
90	LEU	CG	-0.233	47.851	-17.174	90	LEU	CD1	-0.826	48.341	-17.219
90	LEU	CD2	1.168	48.524	-17.847	91	TYR	W	-4.244	47.944	-16.938
91	TYR	CA	-5.258	48.678	-16.137	91	TYR	C	-4.873	48.758	-16.685

5	91	TYR D	-6.496	47.749	-16.923	91	TYR C0	-6.686	48.093	-16.314
	91	TYR C0	-7.894	48.237	-17.741	91	TYR C01	-6.595	47.415	-18.755
	91	TYR C02	-7.971	49.275	-18.149	91	TYR C01	-6.905	47.872	-20.000
	91	TYR C02	-8.315	49.421	-19.492	91	TYR C2	-7.794	48.902	-20.463
	91	TYR DM	-8.102	48.752	-21.764	92	ALA M	-6.095	49.958	-14.104
	92	ALA CA	-6.549	50.109	-12.707	92	ALA C	-5.823	50.833	-11.003
	92	ALA O	-6.723	50.898	-12.050	92	ALA C0	-3.997	51.621	-12.400
	93	VAL M	-5.959	48.993	-11.129	93	VAL CA	-7.103	48.854	-19.325
	93	VAL C	-6.700	49.014	-8.099	93	VAL O	-6.101	47.993	-8.972
	93	VAL C0	-7.957	47.555	-10.631	93	VAL C01	-9.213	47.400	-9.725
	93	VAL C02	-8.195	47.378	-12.072	94	LVS M	-6.907	50.217	-8.327
	94	LVS CA	-6.378	50.464	-6.999	94	LVS C	-7.331	49.905	-5.094
10	94	LVS O	-8.458	50.480	-5.703	94	LVS C0	-6.051	51.976	-6.018
	94	LVS C0	-5.394	52.320	-5.467	94	LVS C0	-6.068	53.705	-5.502
	94	LVS CE	-6.399	54.200	-4.199	94	LVS M2	-3.735	55.544	-4.307
	95	VAL M	-6.909	49.071	-5.024	95	VAL CA	-7.644	48.457	-3.920
	95	VAL C	-6.919	48.499	-2.568	95	VAL O	-7.425	48.154	-1.501
	95	VAL C0	-8.104	47.030	-4.319	95	VAL C01	-8.060	46.852	-5.619
	95	VAL C02	-6.900	46.100	-4.332	96	LEU M	-5.476	48.974	-2.604
	96	LEU CA	-4.782	49.103	-1.486	96	LEU C	-4.331	50.559	-1.321
15	96	LEU O	-3.942	51.121	-2.336	96	LEU C0	-3.509	48.241	-1.373
	96	LEU C0	-3.593	46.799	-2.072	96	LEU C01	-2.207	46.104	-2.163
	96	LEU C02	-6.489	46.002	-1.045	97	GLY M	-4.326	50.975	-0.006
	97	GLY CA	-3.890	52.307	0.207	97	GLY C	-2.363	52.437	0.305
	97	GLY O	-1.619	51.443	0.165	98	ALA M	-1.954	53.440	0.750
	98	ALA C0	-0.420	55.470	1.510	98	ALA CA	-0.563	54.060	0.945
	98	ALA C	0.100	53.110	1.917	98	ALA O	1.393	52.921	1.663
20	99	ASP M	-0.504	52.573	2.912	99	ASP C02	-2.631	51.042	6.151
	99	ASP C01	-2.730	50.902	4.003	99	ASP C0	-2.003	51.131	5.040
	99	ASP C0	-0.640	51.603	5.175	99	ASP CA	0.101	51.610	3.055
	99	ASP C	0.146	50.165	3.320	99	ASP O	0.735	49.313	4.029
	100	GLY M	-0.424	49.803	2.140	100	GLY CA	-0.343	48.521	1.615
	100	GLY C	-1.520	47.451	2.002	100	GLY O	-1.649	46.512	1.479
	101	SER M	-2.342	48.120	2.900	101	SER CA	-3.542	47.300	3.315
	101	SER C	-4.759	47.094	2.532	101	SER O	-4.750	48.972	1.907
25	101	SER C0	-3.716	47.447	4.017	101	SER C0	-4.411	48.634	5.209
	102	GLY M	-5.821	47.092	2.577	102	GLY CA	-7.077	47.422	1.094
	102	GLY C	-0.166	46.536	2.520	102	GLY O	-7.000	45.431	3.030
	103	GLN M	-9.377	47.050	2.490	103	GLN CA	-10.535	46.297	3.020
	103	GLN C	-10.963	45.232	2.022	103	GLN	-10.779	45.402	0.017
	103	GLN C0	-11.671	47.307	3.274	103	GLN C0	-11.368	48.005	4.506
	103	GLN C0	-12.360	49.104	4.915	103	GLN C01	-12.159	49.014	5.902
	103	GLN C02	-13.419	49.197	4.112	104	TYR M	-11.611	44.141	2.451
30	104	TYR CA	-12.060	43.124	1.500	104	TYR C	-13.031	43.690	0.473
	104	TYR O	-12.939	43.276	-0.607	104	TYR C0	-12.497	41.044	2.143
	104	TYR C0	-11.629	40.029	2.472	104	TYR C01	-11.019	39.709	3.377
	104	TYR C02	-10.379	40.959	1.060	104	TYR C01	-10.803	38.805	3.707
	104	TYR C02	-9.352	40.057	2.171	104	TYR C2	-9.564	39.022	3.001
	104	TYR DM	-8.401	38.191	3.324	105	SER M	-13.909	44.972	0.903
	105	SER CA	-14.077	45.166	-0.034	105	SER C	-14.172	45.920	-1.159
35	105	SER O	-14.759	45.933	-2.258	105	SER C0	-15.000	46.121	0.601
	105	SER C0	-15.209	47.039	1.450	106	TRP M	-13.079	46.625	-0.034
	106	TRP CA	-12.421	47.391	-1.940	106	TRP C	-11.095	46.436	-3.012
	106	TRP O	-12.021	46.640	-4.245	106	TRP C0	-11.321	48.254	-1.355
	106	TRP C0	-11.645	49.131	-0.206	106	TRP C01	-12.062	49.524	0.264
	106	TRP C02	-10.650	49.012	0.501	106	TRP M01	-12.491	50.350	1.340
	106	TRP C02	-11.359	50.573	1.561	106	TRP C03	-9.275	49.952	0.576
	106	TRP C02	-10.671	51.310	2.500	106	TRP C03	-8.560	50.563	1.525
40	106	TRP C02	-9.293	51.291	2.655	107	ILE M	-11.339	45.330	-2.401
	107	ILE CA	-10.765	44.250	-3.325	107	ILE C	-11.955	45.594	-4.100
	107	ILE O	-11.695	43.474	-5.390	107	ILE C0	-9.944	43.103	-2.523
	107	ILE C01	-8.634	43.784	-1.976	107	ILE C02	-9.632	41.930	-3.301
	107	ILE C01	-8.213	42.990	-0.627	108	ILE M	-12.994	43.202	-3.577

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100	ILE CA	-14.116	42.722	-4.323	108	ILE C	-14.630	43.694	-9.386
100	ILE D	-14.894	43.320	-6.552	108	ILE CO	-15.244	42.263	-3.320
100	ILE CG1	-14.726	41.077	-2.402	108	ILE CG2	-14.960	42.024	-4.093
100	ILE CD1	-15.452	40.845	-1.131	109	ASN M	-14.791	44.958	-4.981
100	ASN CA	-15.204	40.018	-5.916	109	ASN C	-14.232	44.067	-7.004
100	ASN D	-14.668	40.272	-0.235	109	ASN CO	-15.200	47.359	-5.207
100	ASN CG	-14.528	47.406	-6.353	109	ASN CD1	-17.455	44.495	-4.646
100	ASN MD2	-14.633	40.447	-3.442	110	GLV M	-12.951	45.908	-6.774
110	GLV CA	-11.952	45.917	-7.065	110	GLV C	-12.108	44.712	-8.012
110	GLV D	-11.929	44.929	-10.034	111	ILE M	-12.379	43.539	-8.246
111	ILE CA	-12.603	42.334	-9.099	111	ILE C	-12.059	42.960	-9.942
111	ILE D	-12.921	42.304	-11.148	111	ILE CO	-12.734	40.948	-8.364
111	ILE CG1	-12.621	40.501	-7.455	111	ILE CG2	-12.122	39.791	-9.347
111	ILE CD1	-12.588	39.706	-6.336	112	GLU M	-14.093	43.075	-9.280
112	GLU CA	-14.118	43.374	-10.046	112	GLU C	-15.072	44.947	-11.171
112	GLU D	-14.467	44.130	-12.246	112	GLU CO	-17.229	43.899	-9.141
112	GLU CG	-17.047	42.917	-8.135	112	GLU CD	-10.724	41.824	-8.685
112	GLU DE1	-19.041	40.066	-8.016	112	GLU DE2	-19.123	41.928	-9.866
113	TRP M	-15.094	45.483	-10.971	113	TRP CA	-14.754	44.400	-12.000
113	TRP C	-14.076	45.663	-13.140	113	TRP D	-14.319	45.932	-14.332
113	TRP CO	-13.882	47.553	-11.434	113	TRP CG	-13.406	40.554	-12.481
113	TRP CD1	-14.148	49.736	-12.681	113	TRP CD2	-12.441	40.552	-13.463
113	TRP ME1	-13.597	50.443	-13.723	113	TRP CE2	-12.545	49.761	-14.215
113	TRP CE3	-11.651	47.645	-13.809	113	TRP CE2	-11.696	50.045	-15.274
113	TRP CE3	-10.610	47.899	-14.879	113	TRP CM2	-10.752	49.074	-15.603
114	ALA M	-13.089	44.801	-12.832	114	ALA CA	-12.333	44.065	-13.074
114	ALA C	-13.199	43.179	-14.752	114	ALA D	-12.943	43.074	-15.970
114	ALA CO	-11.299	43.192	-13.140	115	ILE M	-14.174	42.560	-14.119
115	ILE CA	-13.070	41.640	-14.097	115	ILE C	-15.928	42.405	-15.956
115	ILE D	-14.077	42.225	-17.070	115	ILE CO	-16.000	40.040	-13.922
115	ILE CG1	-15.218	39.836	-13.043	115	ILE CG2	-17.151	40.168	-14.755
115	ILE CD1	-14.004	39.411	-11.743	116	ALA M	-16.534	43.527	-15.267
116	ALA CA	-17.390	44.440	-16.050	116	ALA C	-16.706	45.069	-17.278
116	ALA D	-17.323	45.255	-18.343	116	ALA CO	-18.011	45.510	-15.151
117	ASN M	-15.423	45.390	-17.122	117	ASN CA	-14.553	45.967	-18.139
117	ASN C	-13.827	44.974	-19.034	117	ASN D	-12.997	45.436	-19.020
117	ASN CO	-13.615	44.958	-17.426	117	ASN CG	-14.400	48.177	-16.939
117	ASN MD2	-14.565	49.882	-17.773	117	ASN MD2	-14.931	48.249	-15.736
118	ASN M	-14.223	43.725	-19.967	118	ASN CA	-13.740	42.442	-19.032
118	ASN C	-12.240	42.444	-19.843	118	ASN D	-11.617	42.309	-20.932
118	ASN CO	-14.247	42.043	-21.279	118	ASN CG	-15.737	43.060	-21.995
118	ASN CD1	-14.510	42.321	-20.759	118	ASN MD2	-16.136	44.094	-22.133
119	MET M	-11.606	42.500	-18.475	119	MET CA	-10.232	42.222	-18.470
119	MET C	-10.025	40.734	-18.920	119	MET D	-10.880	39.030	-18.759
119	MET CO	-9.010	42.461	-17.055	119	MET CG	-9.880	43.083	-16.902
119	MET SO	-8.788	44.943	-17.526	119	MET CE	-9.982	40.061	-18.263
120	ASP M	-8.904	40.437	-19.504	120	ASP CA	-8.400	39.110	-20.030
120	ASP C	-7.822	34.390	-18.856	120	ASP D	-8.930	37.109	-18.690
120	ASP CO	-7.555	39.156	-21.236	120	ASP CG	-8.237	39.730	-22.454
120	ASP CD1	-7.801	40.706	-23.084	120	ASP MD2	-9.327	39.135	-22.739
121	VAL M	-7.021	39.117	-18.115	121	VAL CA	-6.224	38.401	-16.974
121	VAL C	-6.296	39.534	-15.706	121	VAL D	-6.204	40.708	-15.909
121	VAL CO	-6.755	38.507	-17.496	121	VAL CG1	-5.758	38.176	-16.427
121	VAL CG2	-4.707	37.916	-18.046	122	ILE M	-6.310	38.978	-14.390
122	ILE CA	-6.248	39.799	-13.397	122	ILE C	-5.020	39.242	-12.027
122	ILE D	-6.829	38.012	-12.469	122	ILE CO	-7.476	39.604	-12.666
122	ILE CG1	-8.684	40.392	-13.063	122	ILE CG2	-7.221	39.803	-10.954
122	ILE CD1	-9.974	39.704	-12.393	123	ASN M	-4.263	40.222	-12.110
123	ASN CA	-3.145	39.854	-11.232	123	ASN C	-3.502	40.404	-9.043
123	ASN D	-3.708	41.631	-9.933	123	ASN CO	-1.828	40.478	-11.497
123	ASN CG	-0.692	40.048	-10.777	123	ASN CD1	-0.063	39.990	-11.018
123	ASN MD2	-0.346	40.747	-9.720	124	MET M	-3.450	39.604	-8.832
124	MET CA	-3.650	39.973	-7.430	124	MET C	-2.423	39.603	-6.614

	124	NET D	-2.306	38.908	-6.892	124	NET C8	-4.963	39.387	-6.898
	124	NET C6	-6.138	40.382	-7.673	124	NET S2	-7.985	39.472	-6.138
	124	NET C8	-7.969	38.993	-7.962	123	NET M	-3.454	40.496	-6.132
	124	NET CA	-8.193	40.287	-8.769	123	NET C	-8.622	40.712	-6.326
	124	NET D	0.239	41.617	-3.005	123	NET C8	1.021	41.827	-6.326
5	124	NET D8	1.464	40.696	-7.973	124	NET M	-1.433	40.878	-2.775
	124	NET CA	-1.842	40.367	-2.386	124	NET C	-2.438	39.996	-1.807
	124	NET D	-2.944	39.136	-2.929	124	NET C8	-2.791	41.868	-2.410
	124	NET C6	-3.988	41.447	-2.233	124	NET C81	-1.278	41.131	-2.578
	124	NET C82	-6.179	42.765	-4.873	127	NET M	-2.922	39.832	-0.481
	127	NET CA	-3.833	37.871	0.199	127	NET C	-3.176	38.388	3.682
	127	NET D	-2.646	39.838	2.220	128	NET M	-4.121	37.643	2.222
	128	NET CA	-6.479	37.496	2.642	128	NET C	-6.644	36.938	6.184
	128	NET D	-4.983	39.198	3.276	129	NET M	-4.919	39.897	9.482
10	129	NET CA	-6.671	34.323	8.998	129	NET C	-6.116	34.886	6.082
	129	NET D	-6.338	32.887	6.303	129	NET C8	-4.860	34.684	7.384
	129	NET C6	-6.419	36.116	7.727	129	NET C8	-6.339	34.870	6.418
	130	NET M	-7.831	39.819	8.912	130	NET CA	-8.470	34.411	6.823
	130	NET D	-9.218	34.884	4.724	130	NET D	-8.949	35.881	6.829
	130	NET C8	-8.849	35.351	7.216	130	NET D8	-8.723	36.626	6.833
	131	NET M	-10.883	33.967	4.349	131	NET CA	-10.824	34.329	3.874
	131	NET C	-12.263	34.713	3.842	131	NET D	-12.483	34.722	4.781
15	132	NET M	-12.840	33.898	2.394	132	NET CA	-14.487	35.423	3.811
	132	NET C	-13.289	34.883	1.936	132	NET D	-14.789	34.886	0.824
	132	NET C8	-14.890	36.927	3.145	132	NET D8	-16.483	37.329	1.878
	133	NET M	-16.947	34.988	2.284	133	NET CA	-17.887	34.887	1.324
	133	NET C	-17.430	34.965	8.887	133	NET D	-17.743	34.437	-1.816
	133	NET C8	-18.866	33.828	1.996	134	NET M	-17.683	36.388	8.394
	134	NET CA	-17.872	37.259	-0.792	134	NET C	-18.633	37.369	-1.874
20	134	NET D	-14.781	37.583	-2.869	134	NET C8	-18.243	38.688	-1.887
	135	NET M	-15.478	37.229	-1.846	135	NET CA	-14.197	37.244	-1.884
	135	NET C	-14.188	36.885	-2.703	135	NET D	-15.794	36.828	-3.890
	135	NET C8	-13.838	37.328	-8.798	135	NET C8	-11.693	37.188	-1.988
	135	NET C81	-11.460	38.413	-2.192	135	NET C82	-10.982	36.887	-8.819
	136	NET M	-16.889	36.823	-2.173	136	NET CA	-14.543	33.987	-3.813
	136	NET C	-15.944	33.739	-4.180	136	NET C	-18.279	33.431	-9.388
	136	NET C8	-14.983	32.341	-2.186	136	NET C8	-14.743	31.887	-3.843
	136	NET C8	-15.883	29.812	-2.134	136	NET C8	-15.743	28.787	-2.778
25	136	NET M2	-15.388	28.411	-4.160	137	NET M	-16.744	34.260	-3.847
	137	NET CA	-17.793	34.416	-4.883	137	NET C	-17.338	35.383	-6.848
	137	NET D	-17.788	35.849	-7.208	137	NET C8	-19.894	34.943	-4.263
	138	NET M	-16.329	36.381	-3.729	138	NET CA	-14.881	37.311	-6.888
	138	NET C	-14.983	36.686	-7.937	138	NET D	-14.988	36.843	-8.762
	138	NET C8	-15.322	35.867	-3.934	139	NET M	-13.958	35.959	-7.827
	139	NET CA	-12.946	33.291	-7.837	139	NET C	-13.823	34.228	-8.728
30	139	NET D	-15.288	34.870	-9.877	139	NET C8	-11.830	34.671	-6.948
	139	NET C81	-10.919	33.854	-7.866	139	NET C82	-11.878	33.788	-6.233
	140	NET M	-14.893	33.934	-8.122	140	NET C8	-12.274	32.496	-8.929
	140	NET C	-14.823	33.131	-10.884	140	NET D	-14.988	32.579	-11.198
	140	NET C8	-14.149	31.949	-8.188	140	NET C8	-15.388	30.648	-7.184
	140	NET D81	-14.178	30.489	-7.282	140	NET D82	-14.139	30.132	-6.329
	141	NET M	-16.638	34.263	-9.820	141	NET CA	-17.373	31.886	-10.888
	141	NET C	-18.273	35.413	-11.946	141	NET D	-16.780	35.249	-13.111
	141	NET C8	-18.839	36.278	-19.323	141	NET C8	-18.884	37.886	-11.388
35	141	NET C8	-19.886	38.187	-18.536	141	NET C8	-20.972	39.881	-11.280
	141	NET M2	-21.138	40.837	-18.273	142	NET M	-19.167	37.848	-11.886
	142	NET CA	-14.173	36.192	-12.614	142	NET C	-13.818	35.918	-13.871
	142	NET D	-13.770	35.149	-14.783	142	NET C8	-12.870	36.887	-11.848
	143	NET M	-13.912	35.886	-13.832	143	NET CA	-13.168	32.788	-13.650
	143	NET C	-14.346	33.233	-14.496	143	NET D	-14.168	31.886	-13.639
	143	NET C8	-12.831	31.673	-12.716	143	NET C81	-12.388	30.370	-13.681
40	143	NET C82	-11.388	32.193	-12.814	144	NET M	-17.331	32.338	-13.873
	144	NET CA	-16.744	31.834	-14.843	144	NET C	-16.928	32.881	-13.861

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5	144	ALA C	-17.980	32.263	-16.959	144	ALA C9	-17.962	32.968	-13.700
	145	STR M	-14.907	33.948	-11.701	145	STR C9	-14.682	34.917	-16.781
	146	STR C	-13.699	34.773	-17.879	146	STR O	-13.918	35.321	-18.893
	147	STR C9	-17.916	36.376	-16.614	147	STR OC	-19.553	36.995	-19.949
	148	GLY M	-14.977	33.096	-17.965	148	GLY C9	-13.639	33.799	-19.679
	149	GLY C	-12.373	34.491	-18.385	149	GLY O	-11.628	34.386	-19.266
	150	VAL M	-12.190	35.162	-17.294	150	VAL C9	-10.874	35.896	-16.912
	151	VAL C	-9.850	34.834	-16.723	151	VAL O	-10.171	36.991	-15.436
	152	VAL C9	-11.192	36.977	-15.889	152	VAL CC1	-9.896	37.803	-15.978
	153	VAL CC2	-12.340	37.918	-16.239	153	VAL M	-8.883	35.018	-16.603
	154	VAL C9	-7.482	34.230	-16.808	154	VAL C	-7.137	34.907	-14.701
	155	VAL O	-6.845	36.132	-14.790	155	VAL C9	-6.273	34.126	-16.959
	156	VAL CC1	-5.879	33.483	-16.281	156	VAL CC2	-6.998	33.432	-18.262
	157	VAL M	-7.298	34.385	-13.531	157	VAL C9	-6.957	34.965	-11.849
	158	VAL C	-5.709	34.385	-11.613	158	VAL O	-5.624	33.173	-16.499
	159	VAL C9	-8.224	34.890	-11.313	159	VAL CC1	-7.893	35.619	-19.089
	160	VAL CC2	-9.496	35.386	-12.096	160	VAL M	-4.732	35.381	-11.494
	161	VAL C9	-3.393	34.987	-10.903	161	VAL C	-3.197	35.623	-9.939
	162	VAL O	-3.992	36.778	-9.480	162	VAL C9	-2.274	35.389	-11.951
	163	VAL CC1	-8.973	34.633	-11.461	163	VAL CC2	-2.679	34.843	-13.381
15	164	ALA M	-2.568	34.946	-8.395	164	ALA C9	-2.341	35.382	-7.287
	165	ALA C	-1.880	35.036	-6.657	165	ALA O	-0.618	33.889	-6.984
	166	ALA C9	-3.537	35.390	-4.307	166	ALA M	-0.490	35.907	-5.322
	167	ALA C9	0.714	35.638	-5.112	167	ALA C	0.304	34.320	-6.138
	168	ALA O	-8.728	34.466	-3.467	168	ALA C9	1.266	36.687	-4.294
	169	ALA M	1.125	33.302	-3.912	169	ALA C9	0.849	32.320	-2.943
	170	ALA C	0.931	32.725	-3.511	170	ALA O	0.317	32.192	-0.999
	171	ALA C9	1.750	31.038	-3.195	171	GLY M	1.627	33.693	-1.144
	172	GLY C9	2.043	34.211	0.123	172	GLY C	3.319	36.049	0.990
20	173	GLY O	4.189	33.267	-0.118	173	ASN M	3.958	34.788	1.568
	174	ASN C9	5.344	34.787	2.037	174	ASN C	5.999	34.258	3.462
	175	ASN O	6.181	34.829	4.395	175	ASN C9	6.888	34.198	1.984
	176	ASN CC	8.890	36.702	0.900	176	ASN CC1	6.123	36.865	-0.534
	177	ASN CC2	5.834	37.963	0.352	177	GLU M	4.711	33.160	3.673
	178	GLU C9	4.633	32.837	4.976	178	GLU C	8.223	31.328	5.183
	179	GLU O	3.374	30.637	0.222	179	GLU C9	3.289	31.980	3.188
	180	GLU CC	2.491	32.442	0.368	180	GLU CC	2.394	33.931	6.278
25	181	GLU CC1	1.744	34.322	9.312	181	GLU CC2	3.186	36.456	7.166
	182	GLY M	6.389	31.857	4.227	182	GLY C9	7.886	29.917	4.387
	183	GLY C	6.983	28.622	4.893	183	GLY O	5.414	28.346	4.089
	184	THR M	7.147	27.793	5.382	184	THR CC2	8.879	29.396	3.819
	185	THR CC1	8.787	25.487	6.217	185	THR C9	7.864	29.346	3.296
	186	THR C9	6.952	26.487	5.782	186	THR C	6.188	26.488	7.157
	187	THR O	6.479	27.335	7.977	187	SER M	5.338	29.441	7.497
	188	SER CC	3.141	29.904	10.515	188	SER C9	3.673	26.188	9.212
	189	SER C9	4.833	23.210	0.855	189	SER C	4.494	29.728	8.944
30	190	SER O	3.339	23.281	0.830	190	GLY M	1.874	22.847	0.833
	191	GLY C9	8.424	21.904	0.895	191	GLY C	4.376	21.845	7.738
	192	GLY O	4.888	21.326	0.955	192	SER M	3.823	28.318	6.116
	193	SER C9	2.634	19.777	7.854	193	SER C	1.477	28.788	4.788
	194	SER O	0.696	20.347	9.849	194	SER C9	2.344	28.293	7.271
	195	SER CC	1.254	18.828	8.585	195	SER M	1.583	21.841	7.499
	196	SER C9	0.147	22.728	7.113	196	SER C	0.438	23.552	8.048
35	197	SER O	1.533	23.840	8.394	197	SER C9	-0.213	23.666	8.342
	198	SER CC	8.184	23.891	9.480	198	SER M	-0.679	23.921	8.397
	199	SER C9	-0.611	24.738	3.990	199	SER C	-0.441	26.177	4.513
	200	SER O	-1.878	24.148	3.584	200	SER C9	-1.898	24.642	3.113
	201	SER CC	-1.892	23.718	2.331	201	THR M	0.387	26.952	3.837
	202	THR C9	0.899	28.340	4.312	202	THR C	0.183	29.286	2.394
	203	THR O	0.485	30.582	3.278	203	THR C9	2.899	28.318	4.818
	204	THR CC1	2.984	28.282	2.692	204	THR CC2	2.387	27.618	4.881
40	205	VAL M	-0.513	28.742	2.198	205	VAL C9	-0.989	29.342	1.818
	206	VAL C	-1.818	30.948	1.497	206	VAL O	-2.928	30.132	1.288

5	109	VAL C0	-1.399	28.624	-8.161	109	VAL C01	-1.047	29.367	-1.374
	109	VAL C02	-3.210	27.716	-8.691	109	GLY M	-1.910	31.021	1.129
	106	GLY CA	-2.949	32.779	1.520	106	GLY C	-6.999	32.899	0.617
	106	GLY D	-6.124	32.106	-8.396	107	TYR M	-1.054	33.730	0.970
	107	TYR CA	-6.221	34.044	0.113	107	TYR C	-5.993	33.300	-0.606
	107	TYR D	-6.474	36.203	0.064	107	TYR CD	-7.466	34.252	0.964
	107	TYR CG	-7.791	32.964	1.709	107	TYR CD1	-7.290	32.700	2.947
	107	TYR CD2	-8.710	32.116	1.133	107	TYR CF1	-7.567	31.920	3.610
	107	TYR CF2	-9.068	30.933	1.009	107	TYR CF	-8.406	30.471	3.066
	107	TYR D-	-8.800	29.491	3.638	108	PRO M	-6.380	33.499	-1.090
	108	PRO CG	-6.943	36.376	-3.020	108	PRO CD	-6.273	34.762	-2.624
	108	PRO C0	-7.964	33.364	-3.503	108	PRO CA	-7.134	36.497	-2.560
10	108	PRO C	-6.398	33.336	-3.270	108	PRO D	-7.807	32.520	-2.912
	109	GLY M	-9.096	33.193	-3.109	109	GLY CA	-6.446	32.077	-3.927
	109	GLY C	-6.937	30.702	-3.670	109	GLY D	-6.880	29.733	-4.249
	170	LYS M	-5.402	30.879	-2.289	170	LYS CA	-6.056	29.263	-1.748
	170	LYS C	-7.933	28.773	-2.516	170	LYS D	-7.398	27.974	-2.974
	170	LYS CD	-6.246	29.294	-8.266	170	LYS CG	-9.793	28.106	0.993
	170	LYS CD	-6.250	28.209	2.021	170	LYS CF	-5.791	27.271	3.829
	170	LYS M2	-6.259	27.463	3.213	171	TYR M	-7.838	29.616	-3.168
15	171	TYR CA	-9.812	29.043	-3.839	171	TYR C	-8.693	28.309	-8.113
	171	TYR D	-7.760	28.714	-1.920	171	TYR CD	-9.962	30.274	-4.262
	171	TYR CG	-10.497	30.004	-3.047	171	TYR CD1	-11.060	30.301	-1.062
	171	TYR CD2	-10.496	32.374	-3.026	171	TYR CF1	-11.920	31.003	-8.067
	171	TYR CF2	-10.941	33.080	-1.936	171	TYR CF	-11.920	32.393	-8.066
	171	TYR D-	-12.808	33.119	-9.170	172	PRO M	-9.297	27.204	-5.374
	172	PRO CA	-9.093	26.417	-6.966	172	PRO C	-9.233	27.196	-7.000
20	172	PRO D	-8.323	26.704	-9.861	172	PRO CD	-10.167	29.329	-6.913
	172	PRO CG	-10.670	28.271	-8.894	172	PRO CD	-10.364	28.659	-4.916
	173	SER M	-10.897	28.167	-8.019	173	SER CA	-10.320	28.810	-9.330
	173	SER C	-9.025	29.773	-9.893	173	SER D	-8.946	30.233	-10.742
	173	SER CG	-11.920	29.623	-9.491	173	SER CD	-11.993	30.946	-8.496
	174	VAL M	-8.162	29.944	-8.616	174	VAL CA	-7.033	30.091	-8.093
	174	VAL C	-8.794	29.131	-9.060	174	VAL D	-9.612	29.192	-8.944
	174	VAL CD	-6.899	31.773	-7.894	174	VAL CG1	-1.796	32.897	-7.617
25	174	VAL CG2	-8.220	32.503	-7.323	175	ILE M	-6.911	30.720	-9.003
	175	ILE CA	-3.569	30.196	-10.024	175	ILE C	-2.714	30.736	-8.096
	175	ILE D	-2.450	31.080	-8.933	175	ILE CD	-2.933	30.524	-11.010
	175	ILE CG1	-3.857	29.978	-12.924	175	ILE CG2	-1.451	30.010	-11.512
	175	ILE CF1	-3.492	30.520	-12.946	176	ALA M	-2.220	30.020	-7.929
	176	ALA CA	-1.333	30.317	-6.870	176	ALA C	0.120	30.393	-7.310
	176	ALA D	0.453	29.219	-7.039	176	ALA CD	-1.639	29.838	-5.641
	177	VAL M	0.044	31.410	-7.100	177	VAL CA	2.261	31.896	-7.636
30	177	VAL C	2.223	31.693	-6.473	177	VAL D	3.170	32.697	-5.721
	177	VAL CD	2.439	32.407	-8.765	177	VAL CG1	3.042	32.667	-9.292
	177	VAL CG2	1.374	32.532	-9.843	178	GLY M	6.077	30.494	-6.390
	178	GLY CA	5.160	30.709	-8.339	178	GLY C	6.446	31.233	-6.974
	178	GLY D	6.499	31.433	-7.296	179	ALA M	7.912	31.467	-5.287
	179	ALA CA	8.713	32.037	-9.889	179	ALA C	9.999	31.099	-5.779
	179	ALA CD	10.190	30.481	-4.719	179	ALA CD	9.023	33.251	-4.973
	180	VAL M	10.639	31.162	-6.803	180	VAL CA	11.970	30.492	-6.981
35	180	VAL C	13.048	31.993	-7.171	180	VAL D	12.712	32.691	-7.627
	180	VAL CD	12.079	29.914	-8.166	180	VAL CG1	11.271	30.291	-7.099
	180	VAL CG2	11.679	30.320	-9.509	181	ASP M	10.267	31.203	-6.800
	181	ASP CA	15.631	32.100	-7.099	181	ASP C	19.942	31.804	-5.662
	181	ASP D	15.339	31.090	-9.292	181	ASP CD	16.446	31.921	-9.014
	181	ASP CG	17.120	30.934	-8.971	181	ASP CD1	17.103	29.703	-6.972
	181	ASP CD2	17.600	30.216	-4.007	182	SER M	17.007	32.306	-8.047
40	182	SER CA	17.422	32.214	-10.191	182	SER C	20.329	30.217	-10.694
	182	SER D	18.365	30.492	-11.670	182	SER CD	20.670	33.313	-10.664
	182	SER CG	18.016	34.961	-10.475	183	SER M	18.290	30.042	-9.023
	183	SER CA	18.716	28.643	-9.464	183	SER C	17.981	27.416	-9.967
	183	SER D	17.899	28.619	-9.397	183	SER CD	19.256	28.323	-8.097

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103	ASB	OC	29.989	29.619	-0.251	194	ASB	N	14.973	29.994	-9.001
104	ASB	CA	19.144	27.317	-0.990	194	ASB	C	24.931	29.720	-0.197
104	ASB	O	14.128	25.759	-0.097	194	ASB	CO	19.914	26.941	-10.722
104	ASB	CC	14.993	26.999	-12.074	194	ASB	CC1	14.709	28.194	-12.277
104	ASB	CC2	19.992	26.210	-13.074	193	GLN	N	15.942	27.547	-7.199
105	GLN	CA	19.274	26.444	-0.033	193	GLN	C	14.290	27.494	-0.203
105	GLN	O	14.199	29.726	-0.194	193	GLN	CO	14.999	26.969	-0.191
105	GLN	CC	16.939	26.242	-0.614	193	GLN	CO	18.911	26.102	-0.204
105	GLN	CC1	16.864	29.799	-4.061	193	GLN	CC2	19.264	26.384	-1.934
106	ASB	N	19.278	26.938	-4.448	194	ASB	CA	12.195	27.774	-0.041
106	ASB	C	12.700	29.782	-2.064	194	ASB	O	13.098	28.384	-2.093
106	ASB	CO	11.315	28.043	-2.114	194	ASB	CC	19.214	27.471	-2.161
106	ASB	CO	9.447	26.337	-1.681	194	ASB	CC	9.064	26.933	-0.117
106	ASB	CC	9.961	26.879	1.039	194	ASB	CC1	9.247	27.880	1.698
106	ASB	CC2	19.944	26.321	1.783	197	ALA	N	12.294	29.009	-2.093
107	ALA	CA	12.728	31.064	-1.095	197	ALA	C	12.162	29.604	-0.817
107	ALA	O	11.358	29.043	-0.397	197	ALA	CC	12.144	27.492	-2.344
108	SEP	N	13.091	29.770	0.549	197	SEP	CA	12.671	29.284	1.069
108	SEP	C	11.354	29.047	1.412	198	SEP	O	10.740	29.111	3.212
108	SEP	CO	13.747	29.434	2.932	198	SEP	CC	14.137	21.026	2.041
109	PME	N	10.943	22.010	1.974	199	PME	CA	9.697	22.448	2.418
109	PME	C	0.499	22.198	1.609	199	PME	O	9.389	22.556	2.911
109	PME	CO	9.747	24.217	2.243	199	PME	CC	10.117	24.698	0.047
109	PME	CC1	0.147	24.030	-0.121	199	PME	CC2	11.415	25.114	0.047
109	PME	CC2	9.483	23.187	-1.611	199	PME	CC2	11.749	25.543	-0.701
109	PME	CC2	10.786	25.884	-1.725	190	SEP	N	0.703	21.524	0.699
190	SEP	CA	7.624	21.094	-0.391	190	SEP	C	4.663	20.162	0.228
190	SEP	O	7.834	29.083	0.044	190	SEP	CO	0.181	20.990	-1.708
190	SEP	CC	7.134	29.337	-2.418	191	SEP	N	0.388	20.951	0.224
191	SEP	CA	4.341	29.694	0.987	191	SEP	C	4.261	20.339	0.223
191	SEP	O	4.343	28.269	-0.095	191	SEP	CO	9.819	20.411	0.911
191	SEP	CC	2.729	21.285	1.914	192	VAL	N	9.764	27.910	0.226
192	VAL	CA	3.629	29.932	0.391	192	VAL	C	2.284	23.291	0.084
192	VAL	O	1.994	29.498	1.998	192	VAL	CO	4.701	29.127	1.988
192	VAL	CC1	6.144	29.737	0.722	192	VAL	CC2	4.617	29.104	2.992
193	GLY	N	3.938	24.172	0.047	193	GLY	CA	0.629	23.844	0.016
193	GLY	C	0.081	22.029	-0.081	193	GLY	O	0.830	23.244	-2.015
194	PRO	N	-1.023	22.289	-0.722	194	PRO	CA	-1.662	21.651	-1.073
194	PRO	C	-2.237	22.695	-2.914	194	PRO	O	-2.003	22.244	-4.095
194	PRO	CO	-2.749	20.783	-1.210	194	PRO	CC	-2.311	20.622	0.213
194	PRO	CO	-1.633	21.954	0.578	195	GLU	N	-2.022	23.793	-2.439
195	GLU	CA	-3.148	24.090	-3.252	195	GLU	C	-2.095	25.631	-4.058
195	GLU	O	-2.916	24.398	-4.934	195	GLU	CO	-4.043	29.784	-2.479
195	GLU	CC	-4.942	25.134	-1.435	195	GLU	CO	-4.215	24.040	-0.109
195	GLU	CC1	-3.110	24.940	0.165	195	GLU	CC2	-5.130	24.120	0.703
196	LEU	N	-0.829	29.264	-3.070	196	LEU	CA	0.241	29.929	-4.664
196	LEU	C	0.228	28.374	-6.039	196	LEU	O	0.301	24.121	-6.193
196	LEU	CO	1.940	29.739	-3.054	196	LEU	CC	2.770	26.178	-4.643
196	LEU	CC1	2.739	27.716	-4.639	196	LEU	CC2	4.627	25.721	-3.011
197	ASP	N	0.140	26.209	-7.093	197	ASP	CA	0.032	25.774	-0.488
197	ASP	C	1.387	25.738	-9.293	197	ASP	O	1.033	24.734	-9.914
197	ASP	CO	-1.067	26.598	-9.191	197	ASP	CC	-2.406	26.351	-0.549
197	ASP	CC1	-2.004	25.155	-0.354	197	ASP	CC2	-3.035	27.327	-0.088
198	VAL	N	2.013	26.009	-9.364	198	VAL	CA	3.194	26.970	-10.209
198	VAL	C	4.157	27.050	-9.514	198	VAL	O	3.792	26.699	-0.587
198	VAL	CO	2.894	27.474	-11.637	198	VAL	CC1	1.938	26.774	-12.937
198	VAL	CC2	2.337	29.919	-11.484	199	MET	N	0.374	27.914	-10.016
199	MET	CA	4.439	28.802	-0.498	199	MET	C	6.045	29.510	-10.978
199	MET	O	6.694	29.118	-11.793	199	MET	CO	7.669	27.970	-0.977
199	MET	CC	7.343	24.049	-0.139	199	MET	CO	4.783	27.449	-0.568
199	MET	CC	0.227	27.755	-3.947	200	ALA	N	7.426	29.942	-10.103
200	ALA	CA	7.991	21.929	-11.095	200	ALA	C	9.088	22.066	-10.272
200	ALA	O	0.127	22.024	-9.060	200	ALA	CO	4.032	22.070	-11.638

5	201	PRC H	9.927	33.499	-10.991	201	PRC CA	11.019	34.130	-10.230
	201	PRC C	10.450	35.127	-9.230	201	PRC H	0.870	35.907	-9.692
	201	PRC CO	11.017	34.713	-11.400	201	PRC CC	11.392	34.940	-12.670
	201	PRC CO	9.941	33.610	-12.400	202	GLY H	10.920	31.804	-8.021
	202	GLY CA	10.470	34.254	-7.044	202	GLY C	11.300	34.690	-6.110
	202	GLY D	11.302	37.124	-4.970	203	VAL H	12.010	34.363	-6.013
	203	VAL CA	12.940	36.920	-9.716	203	VAL C	14.706	38.017	-6.460
	203	VAL C	13.130	37.731	-7.593	203	VAL CC	14.014	38.600	-5.391
	203	VAL CC1	14.096	36.106	-6.012	203	VAL CC2	14.070	34.741	-4.370
	204	SEP H	14.065	39.102	-9.939	204	SEP CA	10.872	40.261	-4.407
	204	SEP C	15.047	40.610	-9.872	204	SEP C	10.706	40.403	-9.009
	204	SEP CO	17.007	39.976	-9.326	204	SEP CC	17.732	41.100	-6.672
	205	ILE H	13.771	42.063	-9.000	205	ILE CA	13.000	41.234	-9.330
	205	ILE C	13.207	42.749	-9.470	205	ILE D	12.670	43.490	-9.040
	205	ILE CO	11.932	40.033	-9.144	205	ILE CC1	11.436	39.336	-9.010
	205	ILE CC2	10.090	41.251	-10.467	205	ILE CC1	12.257	40.432	-9.771
	206	GLN H	13.956	43.093	-10.400	206	GLN CA	14.204	44.017	-10.034
	206	GLN C	13.002	44.970	-11.630	206	GLN C	12.660	44.310	-11.621
	206	GLN CO	13.400	44.700	-11.740	206	GLN CC	10.004	44.163	-10.900
	206	GLN CO	17.200	45.145	-10.007	206	GLN CC1	10.320	44.036	-9.353
	206	GLN CC2	14.056	46.260	-9.057	207	SER H	12.300	40.004	-11.234
	207	SER CA	11.217	45.571	-13.907	207	SER C	11.000	40.003	-11.740
	207	SER D	11.910	43.637	-13.004	207	SER CO	9.910	40.003	-11.060
	207	SER CC	0.993	46.056	-12.613	208	THR H	10.004	40.004	-12.326
	208	THR CC2	9.171	50.330	-14.734	208	THR CC1	7.370	40.614	-13.144
	208	THR CO	0.620	50.410	-13.357	208	THR CA	9.670	50.092	-12.173
	208	THR C	9.107	50.400	-10.003	208	THR D	8.423	49.007	-10.040
	209	LEU H	9.656	51.613	-10.220	209	LEU CA	9.192	52.190	-8.950
	209	LEU C	0.670	52.610	-9.202	209	LEU D	9.160	54.227	-10.222
	209	LEU CO	10.330	52.192	-7.900	209	LEU CC	10.004	50.016	-7.416
	209	LEU CO1	11.960	51.114	-6.472	209	LEU CC2	0.007	50.202	-6.640
	210	PRO H	7.790	54.130	-8.444	210	PRO CA	7.273	53.517	-8.640
	210	PRO C	0.303	56.573	-6.030	210	PRO D	9.401	56.045	-8.104
	210	PRO CO	6.302	55.733	-7.917	210	PRO CC	6.004	54.370	-6.964
	210	PRO CC	7.103	53.491	-7.271	211	SLY H	0.077	57.065	-9.353
	211	GLY CA	9.040	54.769	-9.410	211	GLY C	10.094	58.654	-10.690
	211	GLY D	11.176	59.001	-10.200	212	ASN H	9.021	57.770	-11.007
	212	ASN CA	10.903	57.022	-12.643	212	ASN C	12.000	56.793	-12.056
	212	ASN CO	12.100	57.101	-12.620	212	ASN CO	11.224	58.090	-13.490
	212	ASN CC	11.003	58.100	-14.014	212	ASN CC1	11.003	57.054	-13.323
	212	ASN CC2	12.270	59.134	-15.376	213	LYS H	11.003	58.740	-11.247
	213	LYS CA	12.010	54.046	-10.337	213	LYS C	12.000	53.630	-10.006
	213	LYS D	11.770	53.030	-11.613	213	LYS CO	12.760	55.241	-9.050
	213	LYS CC	13.206	54.694	-8.767	213	LYS CO	13.246	57.030	-7.312
	213	LYS CC	14.100	58.210	-6.870	213	LYS CC2	10.040	58.700	-7.021
	214	TYR H	13.601	52.703	-10.444	214	TYR CA	13.003	51.246	-10.722
	214	TYR C	14.383	50.600	-9.600	214	TYR D	10.211	51.293	-9.017
	214	TYR CO	14.641	50.001	-11.904	214	TYR CC	14.130	51.421	-13.746
	214	TYR CC1	14.600	52.047	-13.670	214	TYR CC2	13.130	51.000	-14.016
	214	TYR CC1	14.230	53.470	-14.014	214	TYR CC2	12.654	51.600	-15.170
	214	TYR CC2	13.204	52.093	-15.050	214	TYR CC	12.756	53.490	-16.696
	215	GLY H	14.000	49.047	-9.100	215	GLY CA	14.622	48.772	-7.900
	215	GLY C	14.130	47.320	-7.740	215	GLY D	13.240	46.917	-8.521
	216	ALA H	14.010	46.030	-6.331	216	ALA CA	14.654	48.203	-6.703
	216	ALA C	13.602	44.922	-6.312	216	ALA D	13.940	49.527	-6.670
	216	ALA CO	15.710	46.354	-6.007	217	THR H	12.700	43.002	-5.970
	217	THR CA	11.964	43.400	-6.440	217	THR C	12.030	41.920	-6.547
	217	THR D	12.262	41.642	-9.656	217	THR CC	10.473	43.002	-6.570
	217	THR CC	10.117	45.291	-6.214	217	THR CC1	10.046	45.051	-3.236
	217	THR CC2	9.016	45.033	-6.700	217	THR CC1	10.400	47.207	-2.700
	217	THR CC2	8.654	47.210	-6.301	217	THR CC2	9.300	47.002	-3.301
	217	THR CO	0.953	45.160	-2.900	218	ASN H	11.700	41.300	-3.391
	218	ASN CA	11.640	39.942	-3.227	218	ASN C	10.204	39.636	-2.740

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210	ASL D	9.763	33.367	-1.017	218	ASL C	12.953	39.360	-2.136
210	ASL CG	16.831	39.666	-2.343	218	ASL SD1	14.612	39.789	-3.472
210	ASL WD2	16.660	39.644	-1.365	219	GLV M	0.670	38.654	-3.289
219	GLV CA	0.362	38.130	-2.669	219	GLV C	7.570	37.364	-3.681
219	GLV D	7.873	37.802	-4.876	219	YMB M	6.561	36.438	-3.105
220	YMB CA	6.697	35.736	-4.179	220	YMB C	6.879	37.064	-4.064
220	YMB D	4.417	36.742	-5.919	220	YMB CG	4.825	36.019	-3.926
220	YMB SD1	4.136	38.643	-2.691	220	YMB CG2	5.704	32.696	-2.980
221	SEB M	4.738	38.238	-4.353	221	SEB CA	5.964	37.201	-3.169
221	SEB C	6.760	39.641	-6.581	221	SEB D	6.117	48.288	-7.777
221	SEB CG	3.323	48.383	-4.546	221	SEB DC	3.435	48.282	-3.149
222	MEY M	0.040	39.389	-5.485	222	MEY CE	6.671	42.771	-9.173
222	MEY SD	7.768	41.523	-4.993	222	MEY CG	8.906	41.399	-6.602
222	MEY CD	8.331	48.023	-7.218	222	MEY CA	6.916	39.670	-7.638
222	MEY C	6.577	38.435	-8.567	222	MEY D	7.064	38.567	-9.775
223	ALA M	6.554	37.244	-8.041	223	ALA CG	6.469	36.020	-8.885
223	ALA C	5.200	36.668	-9.707	223	ALA D	5.153	35.948	-10.929
223	ALA CG	6.509	34.807	-7.923	224	SEB M	4.076	36.360	-9.833
224	SEB CA	3.798	36.488	-9.709	224	SEB C	2.641	37.163	-11.039
224	SEB D	3.145	36.593	-12.037	224	SEB CG	1.901	36.993	-8.603
224	SEB DC	0.492	36.895	-9.187	225	PRD M	3.156	38.413	-11.159
225	PRD CA	3.093	39.130	-12.439	225	PRD C	3.764	38.469	-13.626
225	PRD D	3.406	38.650	-14.804	225	PRD CG	3.653	48.313	-12.914
225	PRD CG	4.431	40.402	-10.764	225	PRD CD	3.733	38.324	-10.894
226	MIS M	4.769	37.626	-13.299	226	MIS CA	5.446	36.079	-14.062
226	MIS C	4.418	35.947	-15.061	226	MIS D	4.425	35.809	-16.293
226	MIS CG	6.008	38.066	-13.765	226	MIS CG	7.814	36.839	-13.358
226	MIS WD1	8.048	37.688	-12.170	226	MIS CD2	8.883	37.118	-16.167
226	MIS CG1	9.279	38.032	-12.236	226	MIS WD2	9.771	37.866	-13.643
227	VAL M	3.593	35.366	-14.199	227	VAL CA	2.883	34.388	-14.727
227	VAL C	3.479	35.197	-15.621	227	VAL D	1.018	34.773	-16.490
227	VAL CG	2.183	32.664	-13.619	227	VAL CG1	1.976	32.476	-14.266
227	VAL CG2	3.204	32.663	-12.891	228	ALA M	1.003	36.262	-14.814
228	ALA CA	8.811	37.189	-15.917	228	ALA C	0.343	37.338	-16.868
228	ALA D	-9.233	37.483	-17.928	228	ALA CG	-0.307	38.333	-14.663
229	GLV M	1.791	38.928	-16.943	229	GLV CA	2.332	38.408	-18.239
229	GLV C	2.420	37.197	-19.187	229	GLV D	2.189	37.373	-20.384
229	ALA M	2.711	39.988	-18.646	230	ALA CA	2.794	34.801	-19.546
229	ALA C	1.424	36.500	-20.133	230	ALA D	1.380	34.209	-21.343
229	ALA CG	3.298	32.624	-18.709	231	ALA M	0.383	34.623	-19.328
231	ALA CA	-1.010	36.416	-19.744	231	ALA C	-1.256	35.423	-20.864
231	ALA D	-1.909	35.856	-21.082	231	ALA CG	-1.932	36.664	-18.949
232	ALA M	-9.778	36.637	-20.721	232	ALA CA	-1.013	37.463	-21.792
232	ALA C	-9.281	37.284	-23.078	232	ALA D	-0.841	37.591	-24.187
232	ALA CG	-9.742	39.121	-21.377	233	LEU M	0.933	36.724	-22.967
233	LEU CA	1.617	36.293	-24.209	233	LEU C	0.821	38.149	-26.880
233	LEU D	0.696	35.231	-26.111	233	LEU CG	3.063	35.877	-23.987
233	LEU CG	3.996	36.994	-23.633	233	LEU CG1	3.239	36.342	-22.921
233	LEU CG2	4.241	37.853	-24.680	234	ILE M	0.357	34.199	-24.847
234	ILE CD1	0.306	36.604	-21.637	234	ILE CG1	0.456	31.223	-23.183
234	ILE CG	-0.811	32.014	-23.570	234	ILE CG2	-1.803	30.900	-24.891
234	ILE CA	-0.406	33.076	-24.644	234	ILE C	-1.621	33.997	-23.434
234	ILE D	-1.883	32.144	-26.544	235	LEU M	-2.390	34.463	-24.779
235	LEU CA	-3.396	35.028	-25.423	235	LEU C	-3.258	35.843	-26.672
235	LEU D	-4.109	35.914	-27.589	235	LEU CG	-4.432	35.765	-24.378
235	LEU CG	-5.140	36.899	-23.342	235	LEU CG1	-5.632	35.683	-22.145
235	LEU CG2	-6.152	34.138	-24.120	236	SEB M	-2.894	36.438	-26.798
236	SEB CA	-3.744	37.237	-27.986	236	SEB C	-1.691	36.292	-29.144
236	SEB D	-1.746	36.634	-30.290	236	SEB CG	-0.633	38.234	-27.733
236	SEB DC	0.599	37.171	-27.982	237	LYS M	-1.846	35.047	-28.882
237	LYS CA	-0.846	34.083	-29.952	237	LYS C	-2.213	33.277	-30.369
237	LYS D	-2.378	32.981	-31.444	237	LYS CG	0.272	33.112	-29.551
237	LYS CG	0.677	32.240	-30.716	237	LYS CD	2.028	31.838	-30.442

5	237	LVS	CE	2.345	30.762	-21.729	237	LVS	MT	9.020	29.040	-21.996
	238	MIS	M	-2.931	31.909	-22.312	238	MIS	CA	-4.500	32.103	-22.970
	239	MIS	C	-2.934	32.999	-22.697	239	MIS	O	-5.713	32.884	-27.042
	240	MIS	CD	-2.940	30.862	-22.511	240	MIS	CE	-2.000	29.921	-22.237
	241	MIS	CD1	-2.707	29.479	-22.035	241	MIS	CD2	-3.137	29.399	-20.394
	242	MIS	CE1	-3.004	20.001	-22.642	242	MIS	MT2	-1.040	20.000	-20.199
	243	P00	M	-2.848	32.917	-22.365	243	P00	CA	-6.000	34.770	-20.773
	244	P00	C	-2.204	34.052	-22.937	244	P00	O	-3.049	34.919	-27.662
	245	P00	CD	-2.010	35.977	-22.713	245	P00	CE	-6.666	39.294	-21.027
	246	P00	CD	-2.434	34.434	-22.660	246	AS4	M	-3.304	32.969	-20.227
	247	AS4	CA	-2.329	32.943	-22.216	247	AS4	C	-2.500	31.100	-27.980
	248	AS4	O	-20.340	30.610	-27.574	248	AS4	CD	-2.473	31.249	-20.333
	249	AS4	CE	-2.971	36.817	-22.889	249	AS4	CD1	-7.000	31.000	-21.147
	250	AS4	CD2	-2.670	29.109	-22.976	250	T0P	M	-2.354	31.006	-27.304
10	251	T0P	CA	-2.304	30.124	-22.120	251	T0P	C	-2.106	30.639	-24.936
	252	T0P	O	-2.043	31.033	-22.606	252	T0P	CD	-4.079	29.030	-22.670
	253	T0P	CE	-2.094	28.903	-22.937	253	T0P	CD1	-6.300	28.433	-27.010
	254	T0P	CD2	-4.039	28.324	-22.195	254	T0P	MT1	-2.362	27.347	-22.211
	255	T0P	CE2	-2.414	27.476	-27.210	255	T0P	CE2	-4.097	28.406	-24.981
	256	T0P	CE2	-2.199	26.706	-27.174	256	T0P	CE2	-2.912	27.067	-26.949
	257	T0P	CE2	-2.470	26.072	-22.000	257	T0P	M	-2.727	29.701	-22.142
15	258	T0P	CA	-20.430	30.119	-22.911	258	T0P	C	-2.469	30.176	-21.747
	259	T0P	O	-2.333	29.474	-22.937	259	T0P	CD	-21.079	29.032	-22.679
	260	T0P	CD1	-20.037	27.706	-22.676	260	T0P	CD2	-22.494	28.907	-22.090
	261	AS4	M	-2.946	30.439	-22.611	261	AS4	MT2	-21.707	30.406	-22.747
	262	AS4	CD1	-21.465	31.110	-22.760	262	AS4	CD	-21.093	31.331	-27.909
	263	AS4	CD	-2.700	31.030	-22.332	263	AS4	CA	-2.093	30.731	-22.646
	264	AS4	C	-2.637	29.303	-22.010	264	AS4	O	-7.593	29.136	-22.440
20	265	T0P	M	-2.364	28.362	-22.203	265	T0P	CA	-2.001	26.934	-22.059
	266	T0P	C	-2.133	26.393	-22.002	266	T0P	O	-7.324	28.707	-22.111
	267	T0P	CD	-20.003	26.000	-22.494	267	T0P	CD1	-21.730	26.679	-22.604
	268	T0P	CD2	-20.303	24.593	-22.191	268	GL4	M	-2.002	26.716	-22.073
	269	GL4	CA	-2.964	26.342	-22.962	269	GL4	C	-2.647	27.020	-22.520
	270	GL4	O	-4.572	26.393	-22.447	270	GL4	CD	-7.330	26.999	-22.397
	271	GL4	CE	-2.265	29.326	-22.919	271	GL4	CD	-2.493	29.073	-22.420
	272	GL4	CD1	-2.306	26.769	-22.727	272	GL4	MT2	-7.745	29.312	-22.370
25	273	VAL	M	-2.697	28.304	-22.210	273	VAL	CA	-4.477	29.040	-22.770
	274	VAL	C	-2.936	28.462	-22.447	274	VAL	O	-2.700	29.227	-22.941
	275	VAL	CD	-4.779	30.133	-22.671	275	VAL	CD1	-2.044	31.272	-22.027
	276	VAL	CD2	-2.169	31.230	-22.959	276	ARC	M	-4.767	29.240	-22.462
	277	ARC	CA	-4.300	27.714	-22.140	277	ARC	C	-2.770	26.292	-22.340
	278	ARC	O	-2.701	25.005	-22.764	278	ARC	CD	-2.033	27.667	-22.149
	279	ARC	CE	-4.907	27.093	-22.032	279	ARC	CD	-6.036	27.179	-22.793
	280	ARC	MT	-2.440	26.757	-22.046	280	ARC	CE	-2.093	26.006	-22.313
30	281	ARC	MT1	-7.004	27.404	-22.210	281	ARC	MT2	-2.177	26.420	-22.270
	282	SE4	M	-4.400	29.009	-22.131	282	SE4	CA	-6.039	24.131	-22.626
	283	SE4	C	-2.037	26.006	-22.072	283	SE4	O	-2.040	23.293	-22.092
	284	SE4	CD	-2.034	23.409	-22.372	284	SE4	CD	-6.146	23.090	-22.032
	285	SE4	M	-2.000	24.033	-22.126	285	SE4	CA	-1.723	24.074	-22.031
	286	SE4	C	-2.071	23.207	-22.040	286	SE4	O	-2.026	24.709	-22.049
	287	SE4	CD	-2.169	23.700	-22.000	287	SE4	CD	-2.093	25.619	-22.956
35	288	LE4	M	-2.209	26.333	-22.160	288	LE4	CD2	1.024	29.016	-22.222
	289	LE4	CD1	-2.373	20.433	-22.260	289	LE4	CE	0.332	29.430	-22.101
	290	LE4	CD	0.170	20.003	-22.903	290	LE4	CA	0.710	26.037	-22.216
	291	LE4	C	1.092	20.694	-22.760	291	LE4	C	2.393	23.421	-22.032
	292	GL4	M	0.068	23.007	-22.714	292	GL4	MT2	-2.700	29.512	-22.237
	293	GL4	MT1	-2.010	23.424	-22.933	293	GL4	CD	-2.940	24.030	-22.034
	294	GL4	CE	-2.210	24.014	-22.994	294	GL4	CD	-2.007	23.621	-22.077
	295	GL4	CA	0.001	23.941	-22.743	295	GL4	C	0.999	22.064	-22.341
	296	GL4	O	1.743	22.014	-22.610	296	AS4	M	0.693	22.394	-22.396
40	297	AS4	CA	1.002	21.204	-22.352	297	AS4	C	2.294	21.259	-22.991
	298	AS4	O	2.009	20.442	-22.760	298	AS4	CD	0.004	20.700	-22.292
	299	AS4	CE	-2.036	19.916	-22.973	299	AS4	CD1	-2.036	19.393	-22.992

5	252	254	NO2	-2.234	29.834	-19.363	253	Ym	N	3.819	22.883	-12.923
	253	Ym	CA	0.236	22.717	-19.713	253	Ym	E	9.881	22.247	-12.811
	253	Ym	D	0.368	22.733	-19.427	253	Ym	CO	4.984	22.672	-12.953
	253	Ym	DC1	3.993	24.937	-20.427	253	Ym	CC2	3.347	22.136	-12.932
	254	Ym	N	0.218	23.177	-17.351	254	Ym	CA	6.216	22.612	-12.888
	254	Ym	C	7.466	22.700	-16.612	254	Ym	D	7.482	22.980	-17.093
	254	Ym	CO	5.664	22.958	-13.131	254	Ym	DC1	3.129	22.179	-12.949
	254	Ym	CC2	4.930	24.949	-16.802	255	Ym	N	0.499	22.206	-12.876
	255	Ym	CA	9.771	22.194	-13.817	255	Ym	C	9.621	22.831	-14.414
	255	Ym	D	9.439	22.786	-13.474	255	Ym	CO	11.810	22.488	-12.897
	255	Ym	DC1	21.852	22.709	-17.321	255	Ym	CC2	22.296	22.423	-12.406
	256	LYS	N	9.686	20.702	-14.314	256	LYS	CA	9.364	20.043	-12.810
	256	LYS	C	10.320	20.333	-12.963	256	LYS	D	11.662	20.274	-12.992
	256	LYS	CO	9.074	18.890	-13.249	256	LYS	CC	9.818	17.908	-11.923
	256	LYS	CD	10.216	16.946	-11.777	256	LYS	CE	10.212	19.940	-12.623
	256	LYS	CE	9.243	14.969	-11.054	257	LEU	N	10.212	20.474	-12.824
	257	LEU	CA	11.272	21.030	-9.893	257	LEU	C	11.250	20.232	-8.614
	257	LEU	D	12.096	20.865	-7.732	257	LEU	CO	11.187	22.397	-9.822
	257	LEU	CC	11.337	23.620	-10.968	257	LEU	CD1	11.248	25.003	-9.923
	257	LEU	CC2	22.678	23.468	-11.323	258	GLY	N	10.431	24.282	-8.298
	258	GLY	CA	10.602	18.793	-6.879	258	GLY	C	9.168	16.703	-6.373
	258	GLY	D	8.283	18.956	-7.252	259	ASP	N	9.624	18.282	-8.150
	259	ASP	CA	7.787	17.896	-6.516	259	ASP	C	6.619	19.941	-4.789
	259	ASP	D	6.899	20.039	-4.214	259	ASP	CO	7.996	17.340	-3.593
	259	ASP	CC	4.781	17.128	-2.343	259	ASP	CD1	9.611	17.327	-2.394
	259	ASP	CC2	7.098	16.299	-1.321	260	SER	N	9.160	18.610	-9.312
	260	SER	CA	4.681	19.997	-9.529	260	SER	C	4.946	20.367	-6.209
	260	SER	D	2.300	21.353	-6.446	260	SER	CO	9.349	18.919	-6.219
	260	SER	CC	2.743	17.937	-3.448	261	PHE	N	4.261	19.778	-3.112
	261	PHE	CA	3.331	20.468	-1.869	261	PHE	C	4.944	21.846	-1.863
	261	PHE	D	3.944	22.848	-1.432	261	PHE	CO	4.953	19.749	-8.943
	261	PHE	CC	3.949	20.337	0.719	261	PHE	CD1	2.296	20.163	1.125
	261	PHE	CC2	4.601	21.060	1.598	261	PHE	CE	2.737	20.717	2.315
	261	PHE	CE2	3.943	21.602	2.748	261	PHE	CE	2.695	21.463	3.114
	262	TYR	N	9.778	21.788	-1.303	262	TYR	CA	4.688	22.914	-3.251
	262	TYR	C	6.820	23.689	-3.945	262	TYR	D	7.201	24.053	-3.393
	262	TYR	CO	8.122	22.419	-3.871	262	TYR	CC	8.366	21.892	-8.454
	262	TYR	CC1	9.094	20.484	-0.364	262	TYR	CC2	8.149	22.649	0.498
	262	TYR	CE1	8.062	19.373	0.842	262	TYR	CE2	8.114	22.069	1.962
	262	TYR	CE	8.069	20.672	2.018	262	TYR	CE	7.941	20.929	3.209
	263	TYR	N	6.626	23.194	-4.693	263	TYR	CA	6.812	23.488	-6.922
	263	TYR	C	8.626	23.680	-4.956	263	TYR	D	9.781	24.117	-8.111
	263	TYR	CO	7.928	22.748	-4.681	263	TYR	CC	9.279	23.839	-6.068
	263	TYR	CC1	10.064	24.046	-6.657	263	TYR	CC2	9.690	22.342	-4.956
	263	TYR	CE1	11.333	24.328	-6.169	263	TYR	CE2	11.062	22.640	-6.491
	263	TYR	CE	11.830	23.618	-8.104	263	TYR	CE	17.063	21.949	-4.897
	264	GLY	N	4.471	22.161	-6.816	264	GLY	CA	3.301	22.064	-7.412
	264	GLY	C	3.847	22.196	-8.558	264	GLY	D	4.447	21.274	-8.849
	265	LYS	N	3.636	22.477	-9.754	265	LYS	CA	3.834	21.798	-10.971
	265	LYS	C	9.188	22.232	-11.464	265	LYS	D	8.694	21.363	-12.884
	265	LYS	CO	2.759	22.071	-12.044	265	LYS	CC	1.490	21.943	-11.908
	265	LYS	CD	0.710	20.848	-12.079	265	LYS	CE	-0.672	20.496	-11.991
	265	LYS	CE	-1.678	20.757	-12.489	266	GLY	N	3.787	23.226	-10.817
	266	GLY	CA	7.120	23.612	-11.923	266	GLY	C	7.195	23.052	-11.818
	266	GLY	D	4.177	23.793	-11.648	267	LEU	N	8.262	23.336	-12.490
	267	LEU	CA	8.490	26.660	-13.097	267	LEU	C	7.804	24.771	-14.437
	267	LEU	D	7.913	25.909	-12.298	267	LEU	CO	10.010	26.058	-13.214
	267	LEU	CC	10.432	28.040	-16.058	267	LEU	CD1	18.096	29.331	-18.290
	267	LEU	CC2	11.924	27.923	-14.327	268	ILE	N	7.064	27.843	-14.672
	268	ILE	CA	4.406	18.033	-11.944	268	ILE	C	7.426	29.246	-17.061
	268	ILE	D	8.539	28.793	-16.912	268	ILE	CO	8.369	29.210	-18.899
	268	ILE	CC1	6.079	30.341	-18.752	268	ILE	CC2	6.243	28.929	-14.867
	268	ILE	CC2	8.399	31.765	-16.262	269	ASN	N	7.007	27.043	-18.237

to the wild type subtilisin. However, the mutant Ser204P demonstrated a decrease in alkaline stability.

In addition, other residues, identified as being associated with the modification of other properties of subtilisin, also affect alkaline stability. These residues include Ser24, Met50, Glu156, Gly166, Gly169 and Tyr217. Specifically the following particular substitutions result in an increased alkaline stability: Ser24C, Met50F, Gly156Q or S, Gly166A, H, K, N or Q, Gly169S or A, and Tyr217F, K, R or L. The mutant Met50V, on the other hand, results in a decrease in the alkaline stability of the mutant subtilisin as compared to wild type subtilisin.

Other residues involved in alkaline stability based on the alkaline stability screen include Asp197 and Met222. Particular mutants include Asp197(R or A) and Met 222 (all other amino acids).

Various other residues have been identified as being involved in thermal stability as determined by the thermal stability screen herein. These residues include the above identified residues which effect alkaline stability and Met199 and Tyr21. These latter two residues are also believed to be important for alkaline stability. Mutants at these residues include I199 and F21.

The amino acid sequence of *B. amyloliquefaciens* subtilisin has also been modified by substituting two or more amino acids of the wild-type sequence. Six categories of multiply substituted mutant subtilisin have been identified. The first two categories comprise thermally and oxidatively stable mutants. The next three other categories comprise mutants which combine the useful properties of any of several single mutations of *B. amyloliquefaciens* subtilisin. The last category comprises mutants which have modified alkaline and/or thermal stability.

The first category comprises double mutants in which two cysteine residues have been substituted at various amino acid residue positions within the subtilisin molecule. Formation of disulfide bridges between the two substituted cysteine residues results in mutant subtilisins with altered thermal stability and catalytic activity. These mutants include A21/C22/C87 and C24/C87 which will be described in more detail in Example 11.

The second category of multiple subtilisin mutants comprises mutants which are stable in the presence of various oxidizing agents such as hydrogen peroxide or peracids. Examples 1 and 2 describe these mutants which include F50/I124/Q222, F50/I124, F50/Q222, F50/L124/Q222, I124/Q222 and L124/Q222.

The third category of multiple subtilisin mutants comprises mutants with substitutions at position 222 combined with various substitutions at positions 166 or 169. These mutants, for example, combine the property of oxidative stability of the A222 mutation with the altered substrate specificity of the various 166 or 169 substitutions. Such multiple mutants include A166/A222, A166/C222, F166/C222, K166/A222, K166/C222, V166/A222 and V166/C222. The K166/A222 mutant subtilisin, for example, has a kcat/Km ratio which is approximately two times greater than that of the single A222 mutant subtilisin when compared using a substrate with phenylalanine as the P-1 amino acid. This category of multiple mutant is described in more detail in Example 12.

The fourth category of multiple mutants combines substitutions at position 156 (Glu to Q or S) with the substitution of Lys at position 166. Either of these single mutations improve enzyme performance upon substrates with glutamate as the P-1 amino acid. When these single mutations are combined, the resulting multiple enzyme mutants perform better than either precursor. See Example 9.

The fifth category of multiple mutants contain the substitution of up to four amino acids of the *B. amyloliquefaciens* subtilisin sequence. These mutants have specific properties which are virtually identical to the properties of the subtilisin from *B. licheniformis*. The subtilisin from *B. licheniformis* differs from *B. amyloliquefaciens* subtilisin at 87 out of 275 amino acids. The multiple mutant F50/S156/A169/L217 was found to have similar substrate specificity and kinetics to the licheniformis enzyme. (See Example 13.) However, this is probably due to only three of the mutations (S156, A169 and L217) which are present in the substrate binding region of the enzyme. It is quite surprising that, by making only three changes out of the 87 different amino acids between the sequence of the two enzymes, the *B. amyloliquifaciens* enzyme was converted into an enzyme with properties similar to *B. licheniformis* enzyme. Other enzymes in this series include F50/Q156/N166/L217 and F50/S156/L217.

The sixth category of multiple mutants includes the combination of substitutions at position 107 (Ile to V) with the substitution of Lys at position 213 with Arg, and the combination of substitutions of position 204 (preferably Ser to C or L but also to all other amino acids) with the substitution of Lys at position 213 with R. Other multiple mutants which have altered alkaline stability include Q156/K166, Q156/N166, S156/K166, S156/N166 (previously identified as having altered substrate specificity), and F50/S156/A169/L217 (previously identified as a mutant of *B. amyloliquifaciens* subtilisin having properties similar to subtilisin from *B. licheniformis*). The mutant F50/V107/R213 was constructed based on the observed increase in alkaline stability for the single mutants F50, V107 and R213. It was determined that the V107/R213 mutant had an increased alkaline stability as compared to the wild type subtilisin. In this particular mutant, the increased

alkaline stability was the result of the cumulative stability of each of the individual mutations. Similarly, the mutant F50/V107/R213 had an even greater alkaline stability as compared to the V107/R213 mutant indicating that the increase in the alkaline stability due to the F50 mutation was also cumulative.

Table IV summarizes the multiple mutants which have been made including those not mentioned above.

In addition, based in part on the above results, substitution at the following residues in subtilisin is expected to produce a multiple mutant having increased thermal and alkaline stability: Ser24, Met50, Ile107, Glu156, Gly166, Gly169, Ser204, Lys213, Gly215, and Tyr217.

TABLE IV

Double Mutants	Triple, Quadruple or Other Multiple
C22/C87	F50/I124/Q222
C24/C87	F50/L124/Q222
V45/V48	F50/L124/A222
C49/C94	A21/C22/C87
C49/C95	F50/S156/N166/L217
C50/C95	F50/Q156/N166/L217
C50/C110	F50/S156/A169/L217
F50/I124	F50/S156/L217
F50/Q222	F50/Q156/K166/L217
I124/Q222	F50/S156/K166/L217
Q156/D166	F50/Q156/K166/K217
Q156/K166	F50/S156/K166/K217
Q156/N166	F50/V107/R213
S156/D166 S156/K166	[S153/S156/A158/G159/S160/ Δ 161-164/I165/S166/A169/R170]
S156/N166	L204/R213
S156/A169 A166/A222 A166/C222	R213/204A, E, Q, D, N, G, K, V, R, T, P, I, M, F, Y, W or H
F166/A222 F166/C222 K166/A222 K166/C222 V166/A222 V166/C222 A169/A222 A169/C222 A21/C22	V107/R213

In addition to the above identified amino acid residues, other amino acid residues of subtilisin are also considered to be important with regard to substrate specificity. Mutation of each of these residues is expected to produce changes in the substrate specificity of subtilisin. Moreover, multiple mutations among these residues and among the previously identified residues are also expected to produce subtilisin mutants having novel substrate specificity.

Particularly important residues are His67, Ile107, Leu126 and Leu135. Mutation of His67 should alter the S-1' subsite, thereby altering the specificity of the mutant for the P-1' substrate residue. Changes at this position could also affect the pH activity profile of the mutant. This residue was identified based on the inventor's substrate modeling from product inhibitor complexes.

Ile107 is involved in P-4 binding. Mutation at this position thus should alter specificity for the P-4 substrate residue in addition to the observed effect on alkaline stability. Ile107 was also identified by molecular modeling from product inhibitor complexes.

The S-2 binding site includes the Leu126 residue. Modification at this position should therefore affect P-2 specificity. Moreover, this residue is believed to be important to convert subtilisin to an amino peptidase.

The pH activity profile should also be modified by appropriate substitution. These residues were identified from inspection of the refined model, the three dimensional structure from modeling studies. A longer side chain is expected to preclude binding of any side chain at the S-2 subsite. Therefore, binding would be restricted to subsites S-1, S-1', S-2', S-3' and cleavage would be forced to occur after the amino terminal peptide.

Leu135 is in the S-4 subsite and if mutated should alter substrate specificity for P-4 if mutated. This residue was identified by inspection of the three-dimensional structure and modeling based on the product inhibitor complex of F222.

In addition to these sites, specific amino acid residues within the segments 97-103, 126-129 and 213-215 are also believed to be important to substrate binding.

Segments 97-103 and 126-129 form an antiparallel beta sheet with the main chain of substrate residues P-4 through P-2. Mutating residues in those regions should affect the substrate orientation through main chain (enzyme) - main chain (substrate) interactions, since the main chain of these substrate residues do not interact with these particular residues within the S-4 through S-2 subsites.

Within the segment 97-103, Gly97 and Asp99 may be mutated to alter the position of residues 101-103 within the segment. Changes at these sites must be compatible, however. In *B. amyloliquefaciens* subtilisin Asp99 stabilizes a turn in the main chain tertiary folding that affects the direction of residues 101-103. *B. licheniformis* subtilisin Asp97, functions in an analogous manner.

In addition to Gly97 and Asp99, Ser101 interacts with Asp99 in *B. amyloliquefaciens* subtilisin to stabilize the same main chain turn. Alterations at this residue should alter the 101-103 main chain direction. Mutations at Glu103 are also expected to affect the 101-103 main chain direction.

The side chain of Gly102 interacts with the substrate P-3 amino acid. Side chains of substituted amino acids thus are expected to significantly affect specificity for the P-3 substrate amino acids.

All the amino acids within the 127-129 segment are considered important to substrate specificity. Gly127 is positioned such that its side chain interacts with the S-1 and S-3 subsites. Altering this residue thus should alter the specificity for P-1 and P-3 residues of the substrate.

The side chain of Gly128 comprises a part of both the S-2 and S-4 subsites. Altered specificity for P-2 and P-4 therefore would be expected upon mutation. Moreover, such mutation may convert subtilisin into an amino peptidase for the same reasons substitutions of Leu126 would be expected to produce that result.

The Pro129 residue is likely to restrict the conformational freedom of the sequence 126-133, residues which may play a major role in determining P-1 specificity. Replacing Pro may introduce more flexibility thereby broadening the range of binding capabilities of such mutants.

The side chain of Lys213 is located within the S-3 subsite. All of the amino acids within the 213-215 segment are also considered to be important to substrate specificity. Accordingly, altered P-3 substrate specificity is expected upon mutation of this residue.

The Tyr214 residue does not interact with substrate but is positioned such that it could affect the conformation of the hair pin loop 204-217.

Finally, mutation of the Gly215 residue should affect the S-3' subsite, and thereby alter P-3' specificity.

In addition to the above substitutions of amino acids, the insertion or deletion of one or more amino acids within the external loop comprising residues 152-172 may also affect specificity. This is because these residues may play a role in the "secondary contact region" described in the model of streptomyces subtilisin inhibitor complexed with subtilisin. Hirano, *et al.* (1984) *J. Mol. Biol.* 178, 389-413. Thermitase K has a deletion in this region, which eliminates several of these "secondary contact" residues. In particular, deletion of residues 161 through 164 is expected to produce a mutant subtilisin having modified substrate specificity. In addition, a rearrangement in this area induced by the deletion should alter the position of many residues involved in substrate binding, predominantly at P-1. This, in turn, should affect overall activity against proteinaceous substrates.

The effect of deletion of residues 161 through 164 has been shown by comparing the activity of the wild type (WT) enzyme with a mutant enzyme containing this deletion as well as multiple substitutions (i.e., S153/S156/A158/G159/S160/Δ161-164/I165/S166/A169/R170). This produced the following results:

TABLE V

	kcat	Km	kcat/Km
WT	50	1.4×10^{-4}	3.6×10^5
Deletion mutant	8	5.0×10^{-6}	1.6×10^6

The WT has a k_{cat} 6 times greater than the deletion mutant but substrate binding is 28 fold tighter by the deletion mutant. The overall efficiency of the deletion mutant is thus 4.4 times higher than the WT enzyme.

All of these above identified residues which have yet to be substituted, deleted or inserted into are presented in Table VI.

TABLE VI

Substitution/Insertion/Deletion	
Residues	
His67	Ala152
Leu126	Ala153
Leu135	Gly154
Gly97	Asn155
Asp99	Gly156
Ser101	Gly157
Gly102	Gly160
Glu103	Thr158
Leu126	Ser159
Gly127	Ser161
Gly128	Ser162
Pro129	Ser163
Tyr214	Thr164
Gly215	Val165
Gly166	Gly169
Tyr167	Lys170
Pro168	Tyr171
	Pro172

The following disclosure is intended to serve as a representation of embodiments herein, and should not be construed as limiting the scope of this application. These specific examples disclose the construction of certain of the above identified mutants. The construction of the other mutants, however, is apparent from the disclosure herein and that presented in EPO Publication No. 0130756.

All literature citations are expressly incorporated by reference.

EXAMPLE 1

40. Identification of Peracid Oxidizable Residues of Subtilisin Q222 and L222

As shown in Figures 6A and 6B, organic peracid oxidants inactivate the mutant subtilisins Met222L and Met222Q (L222 and Q222). This example describes the identification of peracid oxidizable sites in these mutant subtilisins.

45 First, the type of amino acid involved in peracid oxidation was determined. Except under drastic conditions (Means, G.E., et al. (1971) Chemical Modifications of Proteins, Holden-Day, S.F., CA, pp. 160-162), organic peracids modify only methionine and tryptophan in subtilisin. Difference spectra of the enzyme over the 250nm to 350nm range were determined during an inactivation titration employing the reagent, diperoxidodecanoic acid (DPDA) as oxidant. Despite quantitative inactivation of the enzyme, no
50 change in absorbance over this wavelength range was noted as shown in Figures 7A and 7B indicating that tryptophan was not oxidized. Fontana, A., et al. (1980) Methods in Peptide and Protein Sequence Analysis - (C. Birr ed.) Elsevier, New York, p. 309. The absence of tryptophan modification implied oxidation of one or more of the remaining methionines of *B. amyloliquefaciens* subtilisin. See Figure 1.

To confirm this result the recombinant subtilisin Met222F was cleaved with cyanogen bromide (CNBr)
55 both before and after oxidation by DPDA. The peptides produced by CNBr cleavage were analyzed on high resolution SDS-pyridine peptide gels (SPG).

Subtilisin Met222F (F222) was oxidized in the following manner. Purified F222 was resuspended in 0.1 M sodium borate pH 9.5 at 10 mg/ml and was added to a final concentration of 26 diperoxidodecanoic acid

(DPDA) at 26 mg/ml was added to produce an effective active oxygen concentration of 30 ppm. The sample was incubated for at least 30 minutes at room temperature and then quenched with 0.1 volume of 1 M Tris pH 8.6 buffer to produce a final concentration of 0.1 M Tris pH 8.6). 3mM phenylmethylsulfonyl fluoride (PMSF) was added and 2.5 ml of the sample was applied to a Pharmacia PD10 column equilibrated in 10 mM sodium phosphate pH 6.2, 1 mM PMSF. 3.5 ml of 10 mM sodium phosphate pH6.2, 1mM PMSF was applied and the eluant collected.

F222 and DPDA oxidized F222 were precipitated with 9 volumes of acetone at -20°C. The samples were resuspended at 10 mg/ml in 8M urea in 88% formic acid and allowed to sit for 5 minutes. An equal volume of 200 mg/ml CNBr in 88% formic acid was added (5 mg/ml protein) and the samples incubated for 2 hours at room temperature in the dark. Prior to gel electrophoresis, the samples were lyophilized and resuspended at 2-5 mg/ml in sample buffer (1% pyridine, 5% NaDodSO₄, 5% glycerol and bromophenol blue) and disassociated at 95°C for 3 minutes.

The samples were electrophoresed on discontinuous polyacrylamide gels (Kyte, J., et al. (1953) *Anal. Bioch.* 133, 515-522). The gels were stained using the Pharmacia silver staining technique (Sammons, D.W., et al. (1981) *Electrophoresis* 2 135-141).

The results of this experiment are shown in Figure 8. As can be seen, F222 treated with CNBr only gives nine resolved bands on SPG. However, when F222 is also treated with DPDA prior to cleavage, bands X, 7 and 9 disappear whereas bands 5 and 6 are greatly increased in intensity.

In order to determine which of the methionines were effected, each of the CNBr peptides was isolated by reversed phase HPLC and further characterized. The buffer system in both Solvent A (aqueous) and Solvent B (organic) for all HPLC separations was 0.05% triethylamine/trifluoroacetic acid (TEA-TFA). In all cases unless noted, solvent A consisted of 0.05% TEA-TFA in H₂O, solvent B was 0.05% TEA-TFA in 1-propanol, and the flow rate was 0.5 ml/minute.

For HPLC analysis, two injections of 1 mg enzyme digest were used. Three samples were acetone precipitated, washed and dried. The dried 1 mg samples were resuspended at 10 mg/ml in 8M urea, 88% formic acid; an equal volume of 200 mg/ml CNBr in 88% formic acid was added (5 mg/ml protein). After incubation for 2 hours in the dark at room temperature, the samples were desalted on a 0.8 cm X 7 cm column of Tris Acryl GF05 coarse resin (IBF, Paris, France) equilibrated with 40% solvent B, 60% solvent A. 200 ul samples were applied at a flow rate of 1 ml a minute and 1.0-1.2 ml collected by monitoring the absorbance at 280nm. Prior to injection on the HPLC, each desalted sample was diluted with 3 volumes of solvent A. The samples were injected at 1.0 ml/min (2 minutes) and the flow then adjusted to 0.5 ml/min (100% A). After 2 minutes, a linear gradient to 60% B at 1.0% B/min was initiated. From each 1 mg run, the pooled peaks were sampled (50ul) and analyzed by gel electrophoresis as described above.

Each polypeptide isolated by reversed phase HPLC was further analyzed for homogeneity by SPG. The position of each peptide on the known gene sequence (Wells, J.A., et al. (1983) *Nucleic Acids Res.* 11 7911-7924) was obtained through a combination of amino acid compositional analysis and, where needed, amino terminal sequencing.

Prior to such analysis the following peptides were to rechromatographed.

1. CNBr peptides from F222 not treated with DPDA:

Peptide 5 was subjected to two additional reversed phase separations. The 10 cm C4 column was equilibrated to 80%A/ 20%B and the pooled sample applied and washed for 2 minutes. Next an 0.5% ml B/min gradient was initiated. Fractions from this separation were again rerun, this time on the 25 cm C4 column, and employing 0.05% TEA-TFA in acetonitrile/1-propanol (1:1) for solvent B. The gradient was identical to the one just described.

Peptide "X" was subjected to one additional separation after the initial chromatography. The sample was applied and washed for 2 minutes at 0.5ml/min (100%A), and a 0.5% ml B/min gradient was initiated.

Peptides 7 and 9 were rechromatographed in a similar manner to the first rerun of peptide 5.

Peptide 8 was purified to homogeneity after the initial separation.

2. CNBr Peptides from DPDA Oxidized F222:

Peptides 5 and 6 from a CNBr digest of the oxidized F222 were purified in the same manner as peptide 5 from the untreated enzyme.

Amino acid compositional analysis was obtained as follows. Samples (-1nM each amino acid) were dried, hydrolyzed in vacuo with 100 ul 6N HCl at 106°C for 24 hours and then dried in a Speed Vac. The samples were analyzed on a Beckmann 6300 AA analyzer employing ninhydrin detection.

Amino terminal sequence data was obtained as previously described (Rodriguez, H., et al. (1984) Anal. Biochem. 134, 538-547).

The results are shown in Table VII and Figure 9.

TABLE VII

Amino and COOH termini of CNBr fragments Terminus and Method		
Fragment	amino, method	COOH, method
X	1, sequence	50, composition
9	51, sequence	119, composition
7	125, sequence	199, composition
8	200, sequence	275, composition
5ox	1, sequence	119, composition
6ox	120, composition	199, composition

Peptides 5ox and 6ox refer to peptides 5 and 6 isolated from CNBr digests of the oxidized protein where their respective levels are enhanced.

From the data in Table VII and the comparison of SPG tracks for the oxidized and native protein digests in Figure 8, it is apparent that (1) Met50 is oxidized leading to the loss of peptides X and 9 and the appearance of 5; and (2) Met124 is also oxidized leading to the loss of peptide 7 and the accumulation of peptide 6. Thus oxidation of *B. amyloliquefaciens* subtilisin with the peracid, diperocecanoic acid leads to the specific oxidation of methionine at residues 50 and 124.

EXAMPLE 2

Substitution at Met50 and Met124 in Subtilisin Met222Q

The choice of amino acid for substitution at Met50 was based on the available sequence data for subtilisins from *B. licheniformis* (Smith, E.C., et al. (1968) J. Biol. Chem. 243, 2184-2191), *B. DY* (Nedkov, P., et al. (1983) Hoppe Saylor's Z. Physiol. Chem. 364 1537-1540), *B. amylosacchariticus* (Markland, F.S., et al. (1967) J. Biol. Chem. 242 5198-5211) and *B. subtilis* (Stahl, M.L., et al. (1984) J. Bacteriol. 158, 411-418). In all cases, position 50 is a phenylalanine. See Figure 5. Therefore, Phe50 was chosen for construction.

At position 124, all known subtilisins possess a methionine. See Figure 5. Molecular modelling of the x-ray derived protein structure was therefore required to determine the most probable candidates for substitution. From all 19 candidates, isoleucine and leucine were chosen as the best residues to employ. In order to test whether or not modification at one site but not both was sufficient to increase oxidative stability, all possible combinations were built on the Q222 backbone (F50/Q222, I124/Q222, F50/I124/Q222).

A. Construction of Mutations Between Codons 45 and 50

All manipulations for cassette mutagenesis were carried out on pS4.5 using methods disclosed in EPO Publication No. 0130756 and Wells, J.A., et al. (1985) Gene 34, 315-323. The pΔ50 in Fig. 10, line 4, mutations was produced using the mutagenesis primer shown in Fig. 10, line 6, and employed an approach designated as restriction-purification which is described below. Briefly, a M13 template containing the subtilisin gene, M13mp11-SUBT was used for heteroduplex synthesis (Adelman, et al (1983), DNA 2, 183-193). Following transfection of JM101 (ATCC 33876), the 1.5 kb EcoRI-BamHI fragment containing the subtilisin gene was subcloned from M13mp11 SUBT rf into a recipient vector fragment of pBS42 the construction of which is described in EPO Publication No. 0130756. To enrich for the mutant sequence (pΔ50, line 4), the resulting plasmid pool was digested with KpnI, and linear molecules were purified by polyacrylamide gel electrophoresis. Linear molecules were ligated back to a circular form, and transformed into *E. coli* MM294 cells (ATCC 31446). Isolated plasmids were screened by restriction analysis for the KpnI site. KpnI⁺ plasmids were sequenced and confirmed the pΔ50 sequence. Asterisks in Figure 11 indicate the bases that are mutated from the wild type sequence (line 4). pΔ50 (line 4) was cut with StuI and EcoRI and the 0.5 Kb fragment containing the 5' half of the subtilisin gene was purified (fragment 1). pΔ50 (line 4) was digested with KpnI and EcoRI and the 4.0 Kb fragment containing the 3' half of the subtilisin gene and vector sequences was purified (fragment 2). Fragments 1 and 2 (line 5), and duplex DNA

cassettes coding for mutations desired (shaded sequence, line 6) were mixed in a molar ratio of 1:1:10, respectively. For the particular construction of this example the DNA cassette contained the triplet TTT for codon 50 which encodes Phe. This plasmid was designated pF50. The mutant subtilisin was designated F50.

B. Construction of Mutation Between Codons 122 and 127

The procedure of Example 2A was followed in substantial detail except that the mutagenesis primer of Figure 11, line 7 was used and restriction-purification for the *EcoRV* site in pΔ124 was used. In addition, the DNA cassette (shaded sequence, Figure 11, line 6) contained the triplet ATT for codon 124 which encodes Ile and CTT for Leu. Those plasmids which contained the substitution of Ile for Met124 were designated pI124. The mutant subtilisin was designated I124.

C. Construction of Various F50/I124/Q222 Multiple Mutants

The triple mutant, F50/I124/Q222, was constructed from a three-way ligation in which each fragment contained one of the three mutations. The single mutant Q222 (pQ222) was prepared by cassette mutagenesis as described in EPO Publication No. 0130756. The F50 mutation was contained on a 2.2kb *Avall* to *PvuII* fragment from pF50; the I124 mutation was contained on a 260 bp *PvuII* to *Avall* fragment from pI124; and the Q222 mutation was contained on 2.7 kb *Avall* to *Avall* fragment from pQ222. The three fragments were ligated together and transformed into *E. coli* MM294 cells. Restriction analysis of plasmids from isolated transformants confirmed the construction. To analyze the final construction it was convenient that the *Avall* site at position 798 in the wild-type subtilisin gene was eliminated by the I124 construction.

The F50/Q222 and I124/Q222 mutants were constructed in a similar manner except that the appropriate fragment from pS4.5 was used for the final construction.

D. Oxidative Stability of Q222 Mutants

The above mutants were analyzed for stability to peracid oxidation. As shown in Fig. 12, upon incubation with diperdodecanoic acid (protein 2mg/mL, oxidant 75ppm[O]), both the I124/Q222 and the F50/I124/Q222 are completely stable whereas the F50/Q222 and the Q222 are inactivated. This indicates that conversion of Met124 to I124 in subtilisin Q222 is sufficient to confer resistance to organic peracid oxidants.

EXAMPLE 3

Subtilisin Mutants Having Altered Substrate Specificity-Hydrophobic Substitutions at Residues 166

Subtilisin contains an extended binding cleft which is hydrophobic in character. A conserved glycine at residue 166 was replaced with twelve non-ionic amino acids which can project their side-chains into the S-1 subsite. These mutants were constructed to determine the effect of changes in size and hydrophobicity on the binding of various substrates.

A. Kinetics for Hydrolysis of Substrates Having Altered P-1 Amino Acids by Subtilisin from *B. Amyloliquefaciens*

Wild-type subtilisin was purified from *B. subtilis* culture supernatants expressing the *B. amyloliquefaciens* subtilisin gene (Wells, J.A., et al. (1983) *Nucleic Acids Res.* 11, 7911-7925) as previously described (Estell, D.A., et al. (1985) *J. Biol. Chem.* 260, 6518-6521). Details of the synthesis of tetrapeptide substrates having the form succinyl-L-AlaL-AlaL-ProL-[X]-p-nitroanilide (where X is the P1 amino acid) are described by DelMar, E.G., et al. (1979) *Anal. Biochem.* 99, 316-320. Kinetic parameters, K_m (M) and k_{cat} (s⁻¹) were measured using a modified progress curve analysis (Estell, D.A., et al. (1985) *J. Biol. Chem.* 260, 6518-6521). Briefly, plots of rate versus product concentration were fit to the differential form of the rate equation using a non-linear regression algorithm. Errors in k_{cat} and K_m for all values reported are less than five percent. The various substrates in Table VIII are ranged in order of decreasing hydrophobicity. Nozaki, Y. (1971), *J. Biol. Chem.* 246, 2211-2217; Tanford C. (1978) *Science* 200, 1012).

TABLE VIII

P1 substrate Amino Acid	kcat(S ⁻¹)	1/Km(M ⁻¹)	kcat/Km (s ⁻¹ M ⁻¹)
Phe	50	7,100	380,000
Tyr	28	40,000	1,100,000
Leu	24	3,100	75,000
Met	13	9,400	120,000
His	7.9	1,800	13,000
Ala	1.9	5,500	11,000
Gly	0.003	8,300	21
Gln	3.2	2,200	7,100
Ser	2.8	1,500	4,200
Glu	0.54	32	16

The ratio of kcat/Km (also referred to as catalytic efficiency) is the apparent second order rate constant for the conversion of free enzyme plus substrate (E + S) to enzyme plus products (E + P) (Jencks, W.P., Catalysis in Chemistry and Enzymology (McGraw-Hill, 1969) pp. 321-436; Fersht, A., Enzyme Structure and Mechanism (Freeman, San Francisco, 1977) pp. 226-287). The log (kcat/Km) is proportional to transition state binding energy, $\Delta G^\ddagger$. A plot of the log kcat/Km versus the hydrophobicity of the P1 side-chain (Figure 14) shows a strong correlation ($r = 0.98$), with the exception of the glycine substrate which shows evidence for non-productive binding. These data show that relative differences between transition-state binding energies can be accounted for by differences in P-1 side-chain hydrophobicity. When the transition-state binding energies are calculated for these substrates and plotted versus their respective side-chain hydrophobicities, the line slope is 1.2 (not shown). A slope greater than unity, as is also the case for chymotrypsin (Fersht, A., Enzyme Structure and Mechanism (Freeman, San Francisco, 1977) pp. 226-287; Harper, J.W., et al. (1984) Biochemistry, 23, 2995-3002), suggests that the P1 binding cleft is more hydrophobic than ethanol or dioxane solvents that were used to empirically determine the hydrophobicity of amino acids (Nozaki, Y., et al. J. Biol. Chem. (1971) 246, 2211-2217; Tanford, C. (1978) Science 200, 1012).

For amide hydrolysis by subtilisin, kcat can be interpreted as the acylation rate constant and Km as the dissociation constant, for the Michaelis complex (E•S), Ks. Gutfreund, H., et al (1956) Biochem. J. 63, 656. The fact that the log kcat, as well as log 1/Km, correlates with substrate hydrophobicity is consistent with proposals (Robertus, J.D., et al. (1972) Biochemistry 11, 2439-2449; Robertus, J.D., et al. (1972) Biochemistry 11, 4293-4303) that during the acylation step the P-1 side-chain moves deeper into the hydrophobic cleft as the substrate advances from the Michaelis complex (E•S) to the tetrahedral transition-state complex (E•S^{*}). However, these data can also be interpreted as the hydrophobicity of the P1 side-chain effecting the orientation, and thus the susceptibility of the scissile peptide bond to nucleophilic attack by the hydroxyl group of the catalytic Ser221.

The dependence of kcat/Km on P-1 side chain hydrophobicity suggested that the kcat/Km for hydrophobic substrates may be increased by increasing the hydrophobicity of the S-1 binding subsite. To test this hypothesis, hydrophobic amino acid substitutions of Gly166 were produced.

Since hydrophobicity of aliphatic side-chains is directly proportional to side-chain surface area (Rose, G.D., et al. (1985) Science 229, 834-838; Reynolds, J.A., et al. (1974) Proc. Natl. Acad. Sci. USA 71, 2825-2927), increasing the hydrophobicity in the S-1 subsite may also sterically hinder binding of larger substrates. Because of difficulties in predicting the relative importance of these two opposing effects, we elected to generate twelve non-charged mutations at position 166 to determine the resulting specificities against non-charged substrates of varied size and hydrophobicity.

B. Cassette Mutagenesis of the P1 Binding Cleft

The preparation of mutant subtilisins containing the substitution of the hydrophobic amino acids Ala, Val and Phe into residue 166 has been described in EPO Publication No. 0130756. The same method was used to produce the remaining hydrophobic mutants at residue 166. In applying this method, two unique and silent restriction sites were introduced in the subtilisin genes to closely flank the target codon 166. As can be seen in Figure 13, the wild type sequence (line 1) was altered by site-directed mutagenesis in M13 using the indicated 37mer mutagenesis primer, to introduce a 13 bp deletion (dashedline) and unique SacI and XmaI sites (underlined sequences) that closely flank codon 166. The subtilisin gene fragment was subcloned back into the E. coli - B. subtilis shuttle plasmid, pBS42, giving the plasmid pΔ166 (Figure 13,

line 2). pΔ166 was cut open with SacI and XmaI, and gapped linear molecules were purified (Figure 13, line 3). Pools of synthetic oligonucleotides containing the mutation of interest were annealed to give duplex DNA cassettes that were ligated into gapped pΔ166 (underlined and overlined sequences in Figure 13, line 4). This construction restored the coding sequence except over position 166(NNN; line 4). Mutant sequences were confirmed by dideoxy sequencing. Asterisks denote sequence changes from the wild type sequence. Plasmids containing each mutant *B. amyloliquefaciens* subtilisin gene were expressed at roughly equivalent levels in a protease deficient strain of *B. subtilis*, BG2036 as previously described. EPO Publication No. 0130756; Yang, M., et al. (1984) *J. Bacteriol.* **160**, 15-21; Estell, D.A., et al (1985) *J. Biol. Chem.* **260**, 6518-6521.

C. Narrowing Substrate Specificity by Steric Hindrance

To probe the change in substrate specificity caused by steric alterations in the S-1 subsite, position 166 mutants were kinetically analyzed versus P1 substrates of increasing size (i.e., Ala, Met, Phe and Tyr). Ratios of k_{cat}/K_m are presented in log form in Figure 15 to allow direct comparisons of transition-state binding energies between various enzyme-substrate pairs.

According to transition state theory, the free energy difference between the free enzyme plus substrate ($E + S$) and the transition state complex ($E \cdot S^*$) can be calculated from equation (1),

$$(1) \quad \Delta G_T^\ddagger = -RT \ln k_{cat}/K_m + RT \ln kT/h$$

in which k_{cat} is the turnover number, K_m is the Michaelis constant, R is the gas constant, T is the temperature, k is Boltzmann's constant, and h is Planck's constant. Specificity differences are expressed quantitatively as differences between transition state binding energies (i.e., $\Delta\Delta G_T^\ddagger$), and can be calculated from equation (2).

$$(2) \quad \Delta\Delta G_T^\ddagger = -RT \ln (k_{cat}/K_m)_A / (k_{cat}/K_m)_B$$

A and B represent either two different substrates assayed against the same enzyme, or two mutant enzymes assayed against the same substrate.

As can be seen from Figure 15A, as the size of the side-chain at position 166 increases the substrate preference shifts from large to small P-1 side-chains. Enlarging the side-chain at position 166 causes k_{cat}/K_m to decrease in proportion to the size of the P-1 substrate side-chain (e.g., from Gly166 (wild-type) through W166, the k_{cat}/K_m for the Tyr substrate is decreased most followed in order by the Phe, Met and Ala P-1 substrates).

Specific steric changes in the position 166 side-chain, such as the presence of a β -hydroxyl group, β - or γ -aliphatic branching, cause large decreases in k_{cat}/K_m for larger P1 substrates. Introducing a β -hydroxyl group in going from A166 (Figure 15A) to S166 (Figure 15B), causes an 8 fold and 4 fold reduction in k_{cat}/K_m for Phe and Tyr substrates, respectively, while the values for Ala and Met substrates are unchanged. Producing a β -branched structure, in going from S166 to T166, results in a drop of 14 and 4 fold in k_{cat}/K_m for Phe and Tyr, respectively. These differences are slightly magnified for V166 which is slightly larger and isosteric with T166. Enlarging the β -branched substituents from V166 to I166 causes a lowering of k_{cat}/K_m between two and six fold toward Met, Phe and Tyr substrates. Inserting a γ -branched structure, by replacing M166 (Figure 15A) with L166 (Figure 15B), produces a 5 fold and 18 fold decrease in k_{cat}/K_m for Phe and Tyr substrates, respectively. Aliphatic γ -branching appears to induce less steric hindrance toward the Phe P-1 substrate than β -branching, as evidenced by the 100 fold decrease in k_{cat}/K_m for the Phe substrate in going from L166 to I166.

Reductions in k_{cat}/K_m resulting from increases in side chain size in the S-1 subsite, or specific structural features such as β - and γ -branching, are quantitatively illustrated in Figure 16. The k_{cat}/K_m values for the position 166 mutants determined for the Ala, Met, Phe, and Tyr P-1 substrates (top panel through bottom panel, respectively), are plotted versus the position 166 side-chain volumes (Chothia, C. (1984) *Ann. Rev. Biochem.* **53**, 537-572). Catalytic efficiency for the Ala substrate reaches a maximum for

I166, and for the Met substrate it reaches a maximum between V166 and L166. The Phe substrate shows a broad k_{cat}/K_m peak but is optimal with A166. Here, the β -branched position 166 substitutions form a line that is parallel to, but roughly 50 fold lower in k_{cat}/K_m than side-chains of similar size [i.e., C166 versus T166, L166 versus I166]. The Tyr substrate is most efficiently utilized by wild type enzyme (Gly166), and there is a steady decrease as one proceeds to large position 166 side-chains. The β -branched and γ -branched substitutions form a parallel line below the other non-charged substitutions of similar molecular volume.

The optimal substitution at position 166 decreases in volume with increasing volume of the P1 substrate [i.e., I166/Ala substrate, L166/Met substrate, A166/Phe substrate, Gly166/Tyr substrate]. The combined volumes for these optimal pairs may approximate the volume for productive binding in the S-1 subsite. For the optimal pairs, Gly166/Tyr substrate, A166/Phe substrate, L166/Met substrate, V166/Met substrate, and I166/Ala substrate, the combined volumes are 266, 295, 313, 339 and 261 $\text{\AA}^3$, respectively. Subtracting the volume of the peptide backbone from each pair (i.e., two times the volume of glycine), an average side-chain volume of $160 \pm 32 \text{\AA}^3$ for productive binding can be calculated.

The effect of volume, in excess to the productive binding volume, on the drop in transition-state binding energy can be estimated from the Tyr substrate curve (bottom panel, Figure 16), because these data, and modeling studies (Figure 2), suggest that any substitution beyond glycine causes steric repulsion. A best-fit line drawn to all the data ($r = 0.87$) gives a slope indicating a loss of roughly 3 kcal/mol in transition state binding energy per $100 \text{\AA}^3$ of excess volume. ($100 \text{\AA}^3$ is approximately the size of a leucyl side-chain.)

D. Enhanced Catalytic Efficiency Correlates with Increasing Hydrophobicity of the Position 166 Substitution

Substantial increases in k_{cat}/K_m occur with enlargement of the position 166 side-chain, except for the Tyr P-1 substrate (Figure 16). For example, k_{cat}/K_m increases in progressing from Gly166 to I166 for the Ala substrate (net of ten-fold), from Gly166 to L166 for the Met substrate (net of ten-fold) and from Gly166 to A166 for the Phe substrate (net of two-fold). The increases in k_{cat}/K_m cannot be entirely explained by the attractive terms in the van der Waals potential energy function because of their strong distance dependence ($1/r^6$) and because of the weak nature of these attractive forces (Jencks, W.P., Catalysis in Chemistry and Enzymology (McGraw-Hill, 1969) pp. 321-436; Fersht, A., Enzyme Structure and Mechanism (Freeman, San Francisco, 1977) pp. 226-287; Levitt, M. (1976) J. Mol. Biol. **104**, 59-107). For example, Levitt (Levitt, M. (1976) J. Mol. Biol. **104**, 59-107) has calculated that the van der Waals attraction between two methionyl residues would produce a maximal interaction energy of roughly -0.2 kcal/mol. This energy would translate to only 1.4 fold increase in k_{cat}/K_m .

The increases of catalytic efficiency caused by side-chain substitutions at position 166 are better accounted for by increases in the hydrophobicity of the S-1 subsite. The increase k_{cat}/K_m observed for the Ala and Met substrates with increasing position 166 side-chain size would be expected, because hydrophobicity is roughly proportional to side-chain surface area (Rose, G.D., et al. (1985) Science **229**, 834-838; Reynolds, J.A., et al. (1974) Proc. Natl. Acad. Sci. USA **71**, 2825-2927).

Another example that can be interpreted as a hydrophobic effect is seen when comparing k_{cat}/K_m for isosteric substitutions that differ in hydrophobicity such as S166 and C166 (Figure 16). Cysteine is considerably more hydrophobic than serine (-1.0 versus +0.3 kcal/mol) (Nozaki, Y., et al. (1971) J. Biol. Chem. **246**, 2211-2217; Tanford, C. (1978) Science **200**, 1012). The difference in hydrophobicity correlates with the observation that C166 becomes more efficient relative to Ser166 as the hydrophobicity of the substrates increases (i.e., Ala < Met < Tyr < Phe). Steric hindrance cannot explain these differences because serine is considerably smaller than cysteine (99 versus 118 $\text{\AA}^3$). Paul, I.C., Chemistry of the -SH Group (ed. S. Patai, Wiley Interscience, New York, 1974) pp. 111-149.

E. Production of an Elastase-Like Specificity in Subtilisin

The I166 mutation illustrates particularly well that large changes in specificity can be produced by altering the structure and hydrophobicity of the S-1 subsite by a single mutation (Figure 17). Progressing through the small hydrophobic substrates, a maximal specificity improvement over wild type occurs for the Val substrate (16 fold in k_{cat}/K_m). As the substrate side chain size increases, these enhancements shrink to near unity (i.e., Leu and His substrates). The I166 enzyme becomes poorer against larger aromatic substrates of increasing size (e.g., I166 is over 1,000 fold worse against the Tyr substrate than is Gly166). We interpret the increase in catalytic efficiency toward the small hydrophobic substrates for I166 compared to Gly166 to the greater hydrophobicity of isoleucine (i.e., -1.8 kcal/mol versus 0). Nozaki, Y., et al. (1971) J. Biol. Chem. **246**, 2211-2217; Tanford, C. (1978) Science **200**, 1012. The decrease in catalytic efficiency

toward the very large substrates for I166 versus Gly166 is attributed to steric repulsion.

The specificity differences between Gly166 and I166 are similar to the specificity differences between chymotrypsin and the evolutionary relative, elastase (Harper, J.W., et al (1984) *Biochemistry* 23, 2995-3002). In elastase, the bulky amino acids, Thr and Val, block access to the P-1 binding site for large hydrophobic substrates that are preferred by chymotrypsin. In addition, the catalytic efficiencies toward small hydrophobic substrates are greater for elastase than for chymotrypsin as we observe for I166 versus Gly166 in subtilisin.

EXAMPLE 4

Substitution of Ionic Amino Acids for Gly166

The construction of subtilisin mutants containing the substitution of the ionic amino acids Asp, Asn, Gln, Lys and Arg are disclosed in EPO Publication No. 0130756. The present example describes the construction of the mutant subtilisin containing Glu at position 166 (E166) and presents substrate specificity data on these mutants. Further data on position 166 and 156 single and double mutants is presented infra.

pΔ166, described in Example 3, was digested with SacI and XmaI. The double strand DNA cassette (underlined and overlined) of line 4 in Figure 13 contained the triplet GAA for the codon 166 to encode the replacement of Glu for Gly166. This mutant plasmid designated pQ166 was propagated in BG2036 as described. This mutant subtilisin, together with the other mutants containing ionic substituent amino acids at residue 166, were isolated as described and further analyzed for variations in substrate specificity.

Each of these mutants was analyzed with the tetrapeptide substrates, succinyl-L-AlaL-AlaProL-X-p-nitroanilide, where X was Phe, Ala and Glu.

The results of this analysis are shown in Table IX.

TABLE IX

Position 166	P-1 Substrate (kcat/Km x 10 ⁻⁴)		
	Phe	Ala	Glu
Gly (wild type)	36.0	1.4	0.002
Asp (D)	0.5	0.4	<0.001
Glu (E)	3.5	0.4	<0.001
Asn (N)	18.0	1.2	0.004
Gln (Q)	57.0	2.6	0.002
Lys (K)	52.0	2.8	1.2
Arg (R)	42.0	5.0	0.08

These results indicate that charged amino acid substitutions at Gly166 have improved catalytic efficiencies (kcat/Km) for oppositely charged P-1 substrates (as much as 500 fold) and poorer catalytic efficiency for like charged P-1 substrates.

EXAMPLE 5

Substitution of Glycine at Position 169

The substitution of Gly169 in *B. amyloliquefaciens* subtilisin with Ala and Ser is described in EPO Publication No. 0130756. The same method was used to make the remaining 17 mutants containing all other substituent amino acids for position 169.

The construction protocol is summarized in Figure 18. The overscored and underscored double stranded DNA cassettes used contained the following triplet encoding the substitution of the indicated amino acid at residue 169.

GCT	A	ATG	M
TGT	C	AAC	N
GAT	D	CCT	P
GAA	E	CAA	Q
TTC	F	AGA	R
GGC	G	AGC	S
CAC	H	ACA	T
ATC	I	GTT	V
AAA	K	TGG	W
CTT	L	TAC	Y

Each of the plasmids containing a substituted Gly169 was designated pX169, where X represents the substituent amino acid. The mutant subtilisins were similarly designated.

Two of the above mutant subtilisins, A169 and S169, were analyzed for substrate specificity against synthetic substrates containing Phe, Leu, Ala and Arg in the P-1 position. The following results are shown in Table X.

TABLE X

Effect of Serine and Alanine Mutations at Position 169 on P-1 Substrate Specificity				
Position 169	P-1 Substrate [kcat/Km x 10 ⁻⁴]			
	Phe	Leu	Ala	Arg
Gly (wild type)	40	10	1	0.4
A169	120	20	1	0.9
S169	50	10	1	0.6

These results indicate that substitutions of Ala and Ser at Gly169 have remarkably similar catalytic efficiencies against a range of P-1 substrates compared to their position 166 counterparts. This is probably because position 169 is at the bottom of the P-1 specificity subsite.

EXAMPLE 6

Substitution at Position 104

Tyr104 has been substituted with Ala, His, Leu, Met and Ser. The method used was a modification of the site directed mutagenesis method. According to the protocol of Figure 19, a primer (shaded in line 4) introduced a unique HindIII site and a frame shift mutation at codon 104. Restriction-purification for the unique HindIII site facilitated the isolation of the mutant sequence (line 4). Restriction-selection against this HindIII site using primers in line 5 was used to obtain position 104 mutants.

The following triplets were used in the primers of Figure 19, line 5 for the 104 codon which substituted the following amino acids.

GCT	A	TTC	F
ATG	M	CCT	P
CTT	L	ACA	T
AGC	S	TGG	W
CAC	H	TAC	Y
CAA	Q	GTT	V
GAA	E	AGA	R
GGC	G	AAC	N
ATC	I	GAT	D
AAA	K	TGT	C

The substrates in Table XI were used to analyze the substrate specificity of these mutants. The results obtained for H104 subtilisin are shown in Table XI.

TABLE XI

Substrate	kcat		Km		Kcat/Km	
	WT	H104	WT	H104	WT	H104
sAAPFpNA	50.0	22.0	1.4×10^{-4}	7.1×10^{-4}	3.6×10^5	3.1×10^4
sAAPpNA	3.2	2.0	2.3×10^{-4}	1.9×10^{-3}	1.4×10^4	1×10^3
sFAPFpNA	26.0	38.0	1.8×10^{-4}	4.1×10^{-4}	1.5×10^5	9.1×10^4
sFAPpNA	0.32	2.4	7.3×10^{-5}	1.5×10^{-4}	4.4×10^3	1.6×10^4

From these data it is clear that the substitution of His for Tyr at position 104 produces an enzyme which is more efficient (higher kcat/Km) when Phe is at the P-4 substrate position than when Ala is at the P-4 substrate position.

EXAMPLE 7

Substitution of Ala152

Ala152 has been substituted by Gly and Ser to determine the effect of such substitutions on substrate specificity.

The wild type DNA sequence was mutated by the V152/P153 primer (Figure 20, line 4) using the above restriction-purification approach for the new KpnI site. Other mutant primers (shaded sequences Figure 20; S152, line 5 and G152, line 6) mutated the new KpnI site away and such mutants were isolated using the restriction-selection procedure as described above for loss of the KpnI site.

The results of these substitutions for the above synthetic substrates containing the P-1 amino acids Phe, Leu and Ala are shown in Table XII.

TABLE XII

Position 152	P-1 Substrate (kcat/Km $\times 10^{-4}$)		
	Phe	Leu	Ala
Gly (G)	0.2	0.4	<0.04
Ala (wild type)	40.0	10.0	1.0
Ser (S)	1.0	0.5	0.2

These results indicate that, in contrast to positions 166 and 169, replacement of Ala152 with Ser or Gly causes a dramatic reduction in catalytic efficiencies across all substrates tested. This suggests Ala152, at the top of the S-1 subsite, may be the optimal amino acid because Ser and Gly are homologous to Ala.

EXAMPLE 8

Substitution at Position 156

Mutants containing the substitution of Ser and Gln for Glu156 have been constructed according to the overall method depicted in Figure 21. This method was designed to facilitate the construction of multiple mutants at position 156 and 166 as will be described hereinafter. However, by regenerating the wild type Gly166, single mutations at Glu156 were obtained.

The plasmid pA166 is already depicted in line 2 of Figure 13. The synthetic oligonucleotides at the top right of Figure 21 represent the same DNA cassettes depicted in line 4 of Figure 13. The plasmid p166 in Figure 21 thus represents the mutant plasmids of Examples 3 and 4. In this particular example, p166 contains the wild type Gly166.

Construction of position 156 single mutants were prepared by ligation of the three fragments (1-3) indicated at the bottom of Figure 21. Fragment 3, containing the carboxy-terminal portion of the subtilisin gene including the wild type position 166 codon, was isolated as a 610 bp SacI-BamHI fragment. Fragment 1 contained the vector sequences, as well as the amino-terminal sequences of the subtilisin gene through codon 151. To produce fragment 1, a unique KpnI site at codon 152 was introduced into the wild type subtilisin sequence from pS4.5. Site-directed mutagenesis in M13 employed a primer having the sequence 5'-TA-GTC-GTT-GCG-GTA-CCC-GGT-AAC-GAA-3' to produce the mutation. Enrichment for the mutant sequence was accomplished by restriction with KpnI, purification and self ligation. The mutant sequence containing the KpnI site was confirmed by direct plasmid sequencing to give pV152. pV152 (~1 µg) was digested with KpnI and treated with 2 units of DNA polymerase I large fragment (Klenow fragment from Boehringer-Mannheim) plus 50 µM deoxynucleotide triphosphates at 37°C for 30 min. This created a blunt end that terminated with codon 151. The DNA was extracted with 1:1 volumes phenol and CHCl₃ and DNA in the aqueous phase was precipitated by addition of 0.1 volumes 5M ammonium acetate and two volumes ethanol. After centrifugation and washing the DNA pellet with 70% ethanol, the DNA was lyophilized. DNA was digested with BamHI and the 4.6kb piece (fragment 1) was purified by acrylamide gel electrophoresis followed by electroelution. Fragment 2 was a duplex synthetic DNA cassette which when ligated with fragments 1 and 3 properly restored the coding sequence except at codon 156. The top strand was synthesized to contain a glutamine codon, and the complementary bottom strand coded for serine at 156. Ligation of heterophosphorylated cassettes leads to a large and favorable bias for the phosphorylated over the non-phosphorylated oligonucleotide sequence in the final segregated plasmid product. Therefore, to obtain Q156 the top strand was phosphorylated, and annealed to the non-phosphorylated bottom strand prior to ligation. Similarly, to obtain S156 the bottom strand was phosphorylated and annealed to the non-phosphorylated top strand. Mutant sequences were isolated after ligation and transformation, and were confirmed by restriction analysis and DNA sequencing as before. To express variant subtilisins, plasmids were transformed into a subtilisin-neutral protease deletion mutant of *B. subtilis*, BG2036, as previously described. Cultures were fermented in shake flasks for 24 h at 37°C in LB media containing 12.5 mg/mL chloramphenicol and subtilisin was purified from culture supernatants as described. Purity of subtilisin was greater than 95% as judged by SDS PAGE.

These mutant plasmids designated pS156 and pQ156 and mutant subtilisins designated S156 and Q156 were analyzed with the above synthetic substrates where P-1 comprised the amino acids Glu, Gln, Met and Lys. The results of this analyses are presented in Example 9.

EXAMPLE 9

Multiple Mutants With Altered Substrate Specificity - Substitution at Positions 156 and 166

Single substitutions of position 166 are described in Examples 3 and 4. Example 8 describes single substitutions at position 156 as well as the protocol of Figure 21 whereby various double mutants comprising the substitution of various amino acids at positions 156 and 166 can be made. This example describes the construction and substrate specificity of subtilisin containing substitutions at position 156 and 166 and summarizes some of the data for single and double mutants at positions 156 and 166 with various substrates.

K166 is a common replacement amino acid in the 156/166 mutants described herein. The replacement of Lys for Gly166 was achieved by using the synthetic DNA cassette at the top right of Figure 21 which contained the triplet AAA for NNN. This produced fragment 2 with Lys substituting for Gly166.

The 156 substituents were Gln and Ser. The Gln and Ser substitutions at Gly156 are contained within fragment 3 (bottom right Figure 21).

The multiple mutants were produced by combining fragments 1, 2 and 3 as described in Example 8. The mutants Q156/K166 and S156/K166 were selectively generated by differential phosphorylation as described. Alternatively, the double 156/166 mutants, c.f. Q156/K166 and S156/K166, were prepared by ligation of the 4.6kb SacI-BamHI fragment from the relevant p156 plasmid containing the 0.6kb SacI-BamHI fragment from the relevant p166 plasmid.

These mutants, the single mutant K166, and the S156 and Q156 mutants of Example 8 were analyzed for substitute specificity against synthetic polypeptides containing Phe or Glu as the P-1 substrate residue. The results are presented in Table XIII.

TABLE XIII

Enzymes Compared (b)	Substrate P-1 Residue	kcat		Km		kcat/Km		kcat/Km (mutant) kcat/Km (wt)	
Glu156/Gly166 (WT)	Phe	50.00		1.4x10 ⁻⁴		3.6x10 ⁵		(1)	
	Glu	0.54		3.4x10 ⁻²		1.6x10 ¹		(1)	
K166	Phe	20.00		4.0x10 ⁻⁵		5.2x10 ⁵		1.4	
	Glu	0.70		5.6x10 ⁻⁵		1.2x10 ⁴		750	
Q156/K166	Phe	30.00		1.9x10 ⁻⁵		1.6x10 ⁶		4.4	
	Glu	1.60		3.1x10 ⁻⁵		5.0x10 ⁴		3100	
S156/K166	Phe	30.00		1.8x10 ⁻⁵		1.6x10 ⁶		4.4	
	Glu	0.60		3.9x10 ⁻⁵		1.6x10 ⁴		1000	
S156	Phe	34.00		4.7x10 ⁻⁵		7.3x10 ⁵		2.0	
	Glu	0.40		1.8x10 ⁻³		1.1x10 ²		6.9	
E156	Phe	48.00		4.5x10 ⁻⁵		1.1x10 ⁶		3.1	
	Glu	0.90		3.3x10 ⁻³		2.7x10 ²		17	

As can be seen in Table XIV, either of these single mutations improve enzyme performance upon substrates with glutamate at the P-1 enzyme binding site. When these single mutations were combined, the resulting multiple enzyme mutants are better than either parent. These single or multiple mutations also alter the relative pH activity profiles of the enzymes as shown in Figure 23.

To isolate the contribution of electrostatics to substrate specificity from other chemical binding forces, these various single and double mutants were analyzed for their ability to bind and cleave synthetic substrates containing Glu, Gln, Met and Lys as the P-1 substrate amino acid. This permitted comparisons between side-chains that were more sterically similar but differed in charge (e.g., Glu versus Gln, Lys versus Met). Similarly, mutant enzymes were assayed against homologous P-1 substrates that were most sterically similar but differed in charge (Table XIV).

TABLE XIV
Kinetics of Position 156/166 Subtilisins
Determined for Different P1 Substrates

Enzyme Position (a)	Net Charge (b)	P-1 Substrate log kcat/Km (log 1/Km) (c)			
		Glu	Gln	Met	Lys
156 166					
Glu Asp	-2	n.d.	3.02 (2.56)	3.93 (2.74)	4.23 (3.00)
Glu Glu	-2	n.d.	3.06 (2.91)	3.86 (3.28)	4.48 (3.69)
Glu Asn	-1	1.62 (2.22)	3.85 (3.14)	4.99 (3.85)	4.15 (2.88)
Glu Gln	-1	1.20 (2.12)	4.36 (3.64)	5.43 (4.36)	4.10 (3.15)
Gln Asp	-1	1.30 (1.79)	3.40 (3.08)	4.94 (3.87)	4.41 (3.22)
Ser Asp	-1	1.23 (2.13)	3.41 (3.09)	4.67 (3.68)	4.24 (3.07)
Glu Met	-1	1.20 (2.30)	3.89 (3.19)	5.64 (4.83)	4.70 (3.89)
Glu Ala	-1	n.d.	4.34 (3.55)	5.65 (4.46)	4.90 (3.24)
Glu Gly (wt)	-1	1.20 (1.47)	3.85 (3.35)	5.07 (3.97)	4.60 (3.13)
Gln Gly	0	2.42 (2.48)	4.53 (3.81)	5.77 (4.61)	3.76 (2.82)
Ser Gly	0	2.31 (2.73)	4.09 (3.68)	5.61 (4.55)	3.46 (2.74)
Gln Asn	0	2.04 (2.72)	4.51 (3.76)	5.79 (4.66)	3.75 (2.74)
Ser Asn	0	1.91 (2.78)	4.57 (3.82)	5.72 (4.64)	3.68 (2.80)
Glu Arg	0	2.91 (3.30)	4.26 (3.50)	5.32 (4.22)	3.19 (2.80)
Glu Lys	0	4.09 (4.25)	4.70 (3.88)	6.15 (4.45)	4.23 (2.93)
Gln Lys	+1	4.70 (4.50)	4.64 (3.68)	5.97 (4.68)	3.23 (2.75)
Ser Lys	+1	4.21 (4.40)	4.84 (3.94)	6.16 (4.90)	3.73 (2.84)
Maximum difference:					
log kcat/Km (log 1/Km) (d)		3.5 (3.0)	1.8 (1.4)	2.3 (2.2)	-1.3 (-1.0)

Footnotes to Table XIV:

(a) *B. subtilis*, BG 2036, expressing indicated variant subtilisin were fermented and enzymes purified as previously described (Estell, *et al.* (1985) *J. Biol. Chem.* 260, 6518-6521). Wild type subtilisin is indicated (wt) containing Glu156 and Gly166.

(b) Net charge in the P-1 binding site is defined as the sum of charges from positions 156 and 166 at pH 8.6.

(c) Values for $k_{cat}(s^{-1})$ and $K_m(M)$ were measured in 0.1M Tris pH 8.6 at 25°C as previously described against P-1 substrates having the form succinyl-L-AlaL-AlaL-ProL-[X]-p-nitroanilide, where X is the indicated P-1 amino acid. Values for $\log 1/K_m$ are shown inside parentheses. All errors in determination of k_{cat}/K_m and $1/K_m$ are below 5%.

(d) Because values for Glu156/Asp166(D166) are too small to determine accurately, the maximum difference taken for GluP-1 substrate is limited to a charge range of +1 to -1 charge change.

n.d. = not determined

The k_{cat}/K_m ratios shown are the second order rate constants for the conversion of substrate to product, and represent the catalytic efficiency of the enzyme. These ratios are presented in logarithmic form to scale the data, and because $\log k_{cat}/K_m$ is proportional to the lowering of transition-state activation energy ($\Delta G^\ddagger$). Mutations at position 156 and 166 produce changes in catalytic efficiency toward Glu, Gln, Met and Lys P-1 substrates of 3100, 60, 200 and 20 fold, respectively. Making the P-1 binding-site more positively charged [e.g., compare Gln156/Lys166 (Q156/K166) versus Glu156/Met166 (Glu156/M166)] dramatically increased k_{cat}/K_m toward the Glu P-1 substrate (up to 3100 fold), and decreased the catalytic efficiency toward the Lys P-1 substrate (up to 10 fold). In addition, the results show that the catalytic efficiency of wild type enzyme can be greatly improved toward any of the four P-1 substrates by mutagenesis of the P-1 binding site.

The changes in k_{cat}/K_m are caused predominantly by changes in $1/K_m$. Because $1/K_m$ is approximately equal to $1/K_s$, the enzyme-substrate association constant, the mutations primarily cause a change in substrate binding. These mutations produce smaller effects on k_{cat} that run parallel to the effects on $1/K_m$. The changes in k_{cat} suggest either an alteration in binding in the P-1 binding site in going from the Michaelis-complex E•S to the transition-state complex (E•S[‡]) as previously proposed (Robertus, J.D., *et al.* (1972) *Biochemistry* 11, 2439-2449; Robertus, J.D., *et al.* (1972) *Biochemistry* 11, 4293-4303), or change in the position of the scissile peptide bond over the catalytic serine in the E•S complex.

Changes in substrate preference that arise from changes in the net charge in the P-1 binding site show trends that are best accounted for by electrostatic effects (Figure 28). As the P-1 binding cleft becomes more positively charged, the average catalytic efficiency increases much more for the Glu P-1 substrate than for its neutral and isosteric P-1 homolog, Gln (Figure 28A). Furthermore, at the positive extreme both substrates have nearly identical catalytic efficiencies.

In contrast, as the P-1 site becomes more positively charged the catalytic efficiency toward the Lys P-1 substrate decreases, and diverges sharply from its neutral and isosteric homolog, Met (Figure 28B). The similar and parallel upward trend seen with increasing positive charge for the Met and Glu P-1 substrates probably results from the fact that all the substrates are succinylated on their amino-terminal end, and thus carry a formal negative charge.

The trends observed in $\log k_{cat}/K_m$ are dominated by changes in the K_m term (Figures 28C and 28D). As the pocket becomes more positively charged, the $\log 1/K_m$ values converge for Glu and Gln P-1 substrates (Figure 28C), and diverge for Lys and Met P-1 substrates (Figure 28D). Although less

pronounced effects are seen in log k_{cat} , the effects of P-1 charge on log k_{cat} parallel those seen in log $1/K_m$ and become larger as the P-1 pocket becomes more positively charged. This may result from the fact that the transition-state is a tetrahedral anion, and a net positive charge in the enzyme may serve to provide some added stabilization to the transition-state.

The effect of the change in P-1 binding-site charge on substrate preference can be estimated from the differences in slopes between the charged and neutral isosteric P-1 substrates (Figure 28B). The average change in substrate preference ($\Delta \log k_{cat}/K_m$) between charged and neutral isosteric substrates increases roughly 10-fold as the complementary charge or the enzyme increases (Table XV). When comparing Glu versus Lys, this difference is 100-fold and the change in substrate preference appears predominantly in the K_m term.

TABLE XV

Differential Effect on Binding Site Charge on log k_{cat}/K_m or (log $1/K_m$) for P-1 Substrates that Differ in Charge ^(a)			
Change in P-1 Binding Site Charge ^(b)	$\Delta \log k_{cat}/K_m$ ($\Delta \log 1/K_m$)		
	GluGln	MetLys	GluLys
-2 to -1	n.d.	1.2 (1.2)	n.d.
-1 to 0	0.7 (0.6)	1.3 (0.8)	2.1 (1.4)
0 to +1	1.5 (1.3)	0.5 (0.3)	2.0 (1.5)
Avg. change in log k_{cat}/K_m or (log $1/K_m$) per unit charge change	1.1 (1.0)	1.0 (0.8)	2.1 (1.5)

^(a) The difference in the slopes of curves were taken between the P-1 substrates over the charge interval given for log (k_{cat}/K_m) (Figure 28A, B) and (log $1/K_m$) (Figure 28C, D). Values represent the differential effect a charge change has in distinguishing the substrates that are compared.

^(b) Charge in P-1 binding site is defined as the sum of charges from positions 156 and 166.

The free energy of electrostatic interactions in the structure and energetics of salt-bridge formation depends on the distance between the charges and the microscopic dielectric of the media. To dissect these structural and microenvironmental effects, the energies involved in specific salt-bridges were evaluated. In addition to the possible salt-bridges shown (Figures 29A and 29B), reasonable salt-bridges can be built between a Lys P-1 substrate and Asp at position 166, and between a Glu P-1 substrate and a Lys at position 166 (not shown). Although only one of these structures is confirmed by X-ray crystallography (Poulos, T.L., et al. (1976) *J. Mol. Biol.* 257 1097-1103), all models have favorable torsion angles (Sielecki, A.R., et al. (1979) *J. Mol. Biol.* 134, 781-804), and do not introduce unfavorable van der Waals contacts.

The change in charged P-1 substrate preference brought about by formation of the model salt-bridges above are shown in Table XVI.

TABLE XVI

Effect of Salt Bridge Formation Between Enzyme
and Substrate on P1 Substrate Preference (a)

Enzymes Compared (b)		Enzyme Position Changed	P-1 Substrates Compared	Substrate (d) Preference		Change in Substrate Preference $\Delta\Delta\log$ (kcat/Km) (1-2)
1	2			1	2	
Glu156/Asp166	Gln156/Asp166	156	LysMet	+0.30	-0.53	0.83
Glu156/Asn166	Gln156/Asn166	156	LysMet	-0.84	-2.04	1.20
Glu156/Gly166	Gln156/Gly166	156	LysMet	-0.47	-2.10	1.63
Glu156/Lys-166	Gln156/Lys166	156	LysMet	-1.92	-2.74	0.82
Ave $\Delta\Delta\log$ (kcat/Km) 1.10 $\pm$ 0.3						
Glu156/Asp166	Glu156/Asn166	166	LysMet	+0.30	-0.84	1.14
Glu156/Glu166	Glu156/Glu166	166	LysMet	+0.62	-1.33	1.95
Gln156/Asp166	Gln156/Asn166	166	LysMet	-0.53	-2.04	1.51
Ser156/Asp166	Ser156/Asn166	166	LysMet	-0.43	-2.04	1.61
Glu156/Lys166	Glu156/Met166	166	GluGln	-0.63	-2.69	2/06
Ave $\Delta\Delta\log$ (kcat/Km) 1.70 $\pm$ 0.3						

Footnotes to Table XVI:

5 (a) Molecular modeling shows it is possible to form a salt bridge between the indicated charged P-1 substrate and a complementary charge in the P-1 binding site of the enzyme at the indicated position changed.

10 (b) Enzymes compared have sterically similar amino acid substitutions that differ in charge at the indicated position.

15 (c) The P-1 substrates compared are structurally similar but differ in charge. The charged P-1 substrate is complementary to the charge change at the position indicated between enzymes 1 and 2.

20 (d) Data from Table XIV was used to compute the difference in log (kcat/Km) between the charged and the non-charged P-1 substrate (i.e., the substrate preference). The substrate preference is shown separately for enzyme 1 and 2.

25 (e) The difference in substrate preference between enzyme 1 (more highly charged) and enzyme 2 (more neutral) represents the rate change accompanying the electrostatic interaction.

30 The difference between catalytic efficiencies (i.e., $\Delta \log kcat/Km$) for the charged and neutral P-1 substrates (e.g., Lys minus Met or Glu minus Gln) give the substrate preference for each enzyme. The change in substrate preference ($\Delta \Delta \log kcat/Km$) between the charged and more neutral enzyme homologs (e.g., Glu156/Gly166 minus Gln156(Q156)/Gly166) reflects the change in catalytic efficiency that may be
35 attributed solely to electrostatic effects.

These results show that the average change in substrate preference is considerably greater when electrostatic substitutions are produced at position 166 (50-fold in kcat/Km) versus position 156 (12-fold in kcat/Km). From these $\Delta \Delta \log kcat/Km$ values, an average change in transition-state stabilization energy can be calculated of -1.5 and -2.4 kcal/mol for substitutions at positions 156 and 166, respectively. This should
40 represent the stabilization energy contributed from a favorable electrostatic interaction for the binding of free enzyme and substrate to form the transition-state complex.

EXAMPLE 1045 Substitutions at Position 217

Tyr217 has been substituted by all other 19 amino acids. Cassette mutagenesis as described in EPO publication No. 0130756 was used according to the protocol of Figure 22. The EcoRV restriction site was used for restriction-purification of p Δ 217.

50 Since this position is involved in substrate binding, mutations here effect kinetic parameters of the enzyme. An example is the substitution of Leu for Tyr at position 217. For the substrate sAAPFpNa, this mutant has a kcat of 277 5' and a Km of 4.7×10^{-4} with a kcat/Km ratio of 6×10^5 . This represents a 5.5-fold increase in kcat with a 3-fold increase in Km over the wild type enzyme.

In addition, replacement of Tyr217 by Lys, Arg, Phe or Leu results in mutant enzymes which are more
55 stable at pHs of about 9-11 than the WT enzyme. Conversely, replacement of Tyr217 by Asp, Glu, Gly or Pro results in enzymes which are less stable at pHs of about 9-11 than the WT enzyme.

EXAMPLE 11

Multiple Mutants Having Altered Thermal Stability

- 6 B. amyloliquefaciens subtilisin does not contain any cysteine residues. Thus, any attempt to produce thermal stability by Cys cross-linkage required the substitution of more than one amino acid in subtilisin with Cys. The following subtilisin residues were multiply substituted with cysteine:

Thr22/Ser87

Ser24/Ser87

Mutagenesis of Ser24 to Cys was carried out with a 5' phosphorylated oligonucleotide primer having the sequence

15 5'-pC-TAC-ACT-GGA-TGC*-AAT-GTT-AAA-G-3'.

- (Asterisks show the location of mismatches and the underlined sequence shows the position of the altered Sau3A site.) The B. amyloliquefaciens subtilisin gene on a 1.5 kb EcoRI-BAMHI fragment from pS4.5 was cloned into M13mp11 and single stranded DNA was isolated. This template (M13mp11SUBT) was double primed with the 5' phosphorylated M13 universal sequencing primer and the mutagenesis primer. Adelman, et al. (1983) DNA 2, 183-193. The heteroduplex was transfected into competent JM101 cells and plaques were probed for the mutant sequence (Zoller, M.J., et al. (1982) Nucleic Acid Res. 10, 6487-6500; Wallace, et al. (1981) Nucleic Acid Res. 9, 3647-3656) using a tetramethylammonium chloride hybridization protocol (Wood, et al. (1985) Proc. Natl. Acad. Sci. USA 82, 1585-1588). The Ser87 to Cys mutation was prepared in a similar fashion using a 5' phosphorylated primer having the sequence

30 5'-pGGC-GTT-GCG-CCA-TGC-GCA-TCA-CT-3'.

(The asterisk indicates the position of the mismatch and the underlined sequence shows the position of a new MstI site.) The C24 and C87 mutations were obtained at a frequency of one and two percent, respectively. Mutant sequences were confirmed by dideoxy sequencing in M13.

- 35 Mutagenesis of Tyr21/Thr22 to A21/C22 was carried out with a 5' phosphorylated oligonucleotide primer having the sequence

40 5'-pAC-TCT-CAA-GGC-GCT-TGT-GGC-TCA-AAT-GTT-3'.

- (The asterisks show mismatches to the wild type sequence and the underlined sequence shows the position of an altered Sau3A site.) Manipulations for heteroduplex synthesis were identical to those described for C24. Because direct cloning of the heteroduplex DNA fragment can yield increased frequencies of mutagenesis, the EcoRI-BamHI subtilisin fragment was purified and ligated into pBS42. E. coli MM 294 cells were transformed with the ligation mixture and plasmid DNA was purified from isolated transformants. Plasmid DNA was screened for the loss of the Sau3A site at codon 23 that was eliminated by the mutagenesis primer. Two out of 16 plasmid preparations had lost the wild type Sau3A site. The mutant sequence was confirmed by dideoxy sequencing in M13.

- Double mutants, C22/C87 and C24/C87, were constructed by ligating fragments sharing a common Clal site that separated the single parent cystine codons. Specifically, the 500 bp EcoRI-Clal fragment containing the 5' portion of the subtilisin gene (including codons 22 and 24) was ligated with the 4.7 kb Clal-EcoRI fragment that contained the 3' portion of the subtilisin gene (including codon 87) plus pBS42 vector sequence. E. coli MM 294 was transformed with ligation mixtures and plasmid DNA was purified from individual transformants. Double-cysteine plasmid constructions were identified by restriction site markers originating from the parent cysteine mutants (i.e., C22 and C24, Sau3A minus; Cys87, MstI plus). Plasmids from E. coli were transformed into B. subtilis BG2036. The thermal stability of these mutants as compared to wild type subtilisin are presented in Figure 30 and Tables XVII and XVIII.

TABLE XVII

Effect of DTT on the Half-Time of Autolytic Inactivation of Wild-Type and Disulfide Mutants of Subtilisin*			
Enzyme	$t_{\frac{1}{2}}$		-DTT/+ DTT
	-DDT	+ DTT	
	min		
Wild-type	95	85	1.1
C22/C87	44	25	1.8
C24/C87	92	62	1.5

(*) Purified enzymes were either treated or not treated with 25mM DTT and dialyzed with or without 10mM DTT in 2mM CaCl₂, 50mM Tris (pH 7.5) for 14 hr. at 4 °C. Enzyme concentrations were adjusted to 80μl aliquots were quenched on ice and assayed for residual activity. Half-times for autolytic inactivation were determined from semi-log plots of log₁₀ (residual activity) versus time. These plots were linear for over 90% of the inactivation.

TABLE XVIII

Effect of Mutations in Subtilisin on the Half-Time of Autolytic Inactivation at 58 °C*	
Enzyme	$t_{1/2}$
	min
Wild-type	120
C22	22
C24	120
C87	104
C22/C87	43
C24/C87	115

(*) Half-times for autolytic inactivation were determined for wild-type and mutant subtilisins as described in the legend to Table III. Unpurified and non-reduced enzymes were used directly from *B. subtilis* culture supernatants.

The disulfides introduced into subtilisin did not improve the autolytic stability of the mutant enzymes when compared to the wild-type enzyme. However, the disulfide bonds did provide a margin of autolytic stability when compared to their corresponding reduced double-cysteine enzyme. Inspection of a highly refined x-ray structure of wild-type *B. amyloliquefaciens* subtilisin reveals a hydrogen bond between Thr22 and Ser87. Because cysteine is a poor hydrogen donor or acceptor (Paul, I.C. (1974) in *Chemistry of the -SH Group* (Patai, S., ed.) pp. 111-149, Wiley Interscience, New York) weakening of 22/87 hydrogen bond may explain why the C22 and C87 single-cysteine mutant proteins are less autolytically stable than either C24 or wild-type (Table XVIII). The fact that C22 is less autolytically stable than C87 may be the result of the Tyr21A mutation (Table XVIII). Indeed, construction and analysis of Tyr21/C22 shows the mutant protein has an autolytic stability closer to that of C87. In summary, the C22 and C87 of single-cysteine mutations destabilize the protein toward autolysis, and disulfide bond formation increases the stability to a level less than or equal to that of wild-type enzyme.

EXAMPLE 12

Multiple Mutants Containing Substitutions at Position 222 and Position 166 or 169

Double mutants 166/222 and 169/222 were prepared by ligating together (1) the 2.3kb *A*cclI fragment from pS4.5 which contains the 5' portion of the subtilisin gene and vector sequences, (2) the 200bp *A*vallI fragment which contains the relevant 166 or 169 mutations from the respective 166 or 169 plasmids, and (3) the 2.2kb *A*vallI fragment which contains the relevant 222 mutation 3' and of the subtilisin genes and vector

sequence from the respective p222 plasmid.

Although mutations at position 222 improve oxidation stability they also tend to increase the K_m . An example is shown in Table XIX. In this case the A222 mutation was combined with the K166 mutation to give an enzyme with k_{cat} and K_m intermediate between the two parent enzymes.

TABLE XIX

	k_{cat}	K_m
WT	50	1.4×10^{-4}
A222	42	9.9×10^{-4}
K166	21	3.7×10^{-5}
K166/A222	29	2.0×10^{-4}
substrate sAAPFpNa		

EXAMPLE 13

Multiple Mutants Containing Substitutions at Positions 50, 156, 166, 217 and Combinations Thereof

The double mutant S156/A169 was prepared by ligation of two fragments, each containing one of the relevant mutations. The plasmid pS156 was cut with *Xma*I and treated with S1 nuclease to create a blunt end at codon 167. After removal of the nuclease by phenol/chloroform extraction and ethanol precipitation, the DNA was digested with *Bam*HI and the approximately 4kb fragment containing the vector plus the 5' portion of the subtilisin gene through codon 167 was purified.

The pA169 plasmid was digested with *Kpn*I and treated with DNA polymerase Klenow fragment plus 50 μ M dNTPs to create a blunt end codon at codon 168. The Klenow was removed by phenol/chloroform extraction and ethanol precipitation. The DNA was digested with *Bam*HI and the 590bp fragment including codon 168 through the carboxy terminus of the subtilisin gene was isolated. The two fragments were then ligated to give S156/A169.

Triple and quadruple mutants were prepared by ligating together (1) the 220bp *Pvu*II/*Hae*III fragment containing the relevant 156, 166 and/or 169 mutations from the respective p156, p166 and/or p169 double of single mutant plasmid, (2) the 550bp *Hae*III/*Bam*HI fragment containing the relevant 217 mutant from the respective p217 plasmid, and (3) the 3.9kb *Pvu*II/*Bam*HI fragment containing the F50 mutation and vector sequences.

The multiple mutant F50/S156/A169/L217, as well as *B. amyloliquefaciens* subtilisin, *B. licheniformis* subtilisin and the single mutant L217 were analyzed with the above synthetic polypeptides where the P-1 amino acid in the substrate was Lys, His, Ala, Gln, Tyr, Phe, Met and Leu. These results are shown in Figures 26 and 27.

These results show that the F50/S156/A169/L217 mutant has substrate specificity similar to that of the *B. licheniformis* enzyme and differs dramatically from the wild type enzyme. Although only data for the L217 mutant are shown, none of the single mutants (e.g., F50, S156 or A169) showed this effect. Although *B. licheniformis* differs in 88 residue positions from *B. amyloliquefaciens*, the combination of only these four mutations accounts for most of the differences in substrate specificity between the two enzymes.

EXAMPLE 14

Subtilisin Mutants Having Altered Alkaline Stability

A random mutagenesis technique was used to generate single and multiple mutations within the *B. amyloliquefaciens* subtilisin gene. Such mutants were screened for altered alkaline stability. Clones having increased (positive) alkaline stability and decreased (negative) alkaline stability were isolated and sequenced to identify the mutations within the subtilisin gene. Among the positive clones, the mutants V107 and R213 were identified. These single mutants were subsequently combined to produce the mutant V107/R213.

One of the negative clones (V50) from the random mutagenesis experiments resulted in a marked decrease in alkaline stability. Another mutant (P50) was analyzed for alkaline stability to determine the effect

of a different substitution at position 50. The F50 mutant was found to have a greater alkaline stability than wild type subtilisin and when combined with the double mutant V107/R213 resulted in a mutant having an alkaline stability which reflected the aggregate of the alkaline stabilities for each of the individual mutants.

The single mutant R204 and double mutant C204/R213 were identified by alkaline screening after random cassette mutagenesis over the region from position 197 to 228. The C204/R213 mutant was thereafter modified to produce mutants containing the individual mutations C204 and R213 to determine the contribution of each of the individual mutations. Cassette mutagenesis using pooled oligonucleotides to substitute all amino acids at position 204, was utilized to determine which substitution at position 204 would maximize the increase in alkaline stability. The mutation from Lys213 to Arg was maintained constant for each of these substitutions at position 204.

A. Construction of pB0180, an E. coli-B. subtilis Shuttle Plasmid

The 2.9 kb EcoRI-BamHI fragment from pBR327 (Covarrubias, L., et al. (1981) *Gene* 13, 25-35) was ligated to the 3.7kb EcoRI-BamHI fragment of pBD64 (Gryczan, T., et al. (1980) *J. Bacteriol.*, 141, 246-253) to give the recombinant plasmid pB0153. The unique EcoRI recognition sequence in pBD64 was eliminated by digestion with EcoRI followed by treatment with Klenow and deoxynucleotide triphosphates (Maniatis, T., et al. (eds.) (1982) in *Molecular Cloning, A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.). Blunt end ligation and transformation yielded pB0154. The unique Aval recognition sequence in pB0154 was eliminated in a similar manner to yield pB0171. pB0171 was digested with BamHI and PvuII and treated with Klenow and deoxynucleotide triphosphates to create blunt ends. The 6.4 kb fragment was purified, ligated and transformed into LE392 cells (Enquist, L.W., et al. (1977) *J. Mol. Biol.* 111, 97-120), to yield pB0172 which retains the unique BamHI site. To facilitate subcloning of subtilisin mutants, a unique and silent KpnI site starting at codon 166 was introduced into the subtilisin gene from pS4.5 (Wells, J.A., et al. (1983) *Nucleic Acids Res.*, 11, 7911-7925) by site-directed mutagenesis. The KpnI+ plasmid was digested with EcoRI and treated with Klenow and deoxynucleotide triphosphates to create a blunt end. The Klenow was inactivated by heating for 20 min at 68°C, and the DNA was digested with BamHI. The 1.5 kb blunt EcoRI-BamHI fragment containing the entire subtilisin was ligated with the 5.8 kb NruI-BamHI from pB0172 to yield pB0180. The ligation of the blunt NruI end to the blunt EcoRI end recreated an EcoRI site. Proceeding clockwise around pB0180 from the EcoRI site at the 5' end of the subtilisin gene is the unique BamHI site at the 3' end of the subtilisin gene, the chloramphenicol and neomycin resistance genes and UB110 gram positive replication origin derived from pBD64, the ampicillin resistance gene and gram negative replication origin derived from pBR327.

B. Construction of Random Mutagenesis Library

The 1.5 kb EcoRI-BamHI fragment containing the B. amyloliquefaciens subtilisin gene (Wells et al., 1983) from pB0180 was cloned into M13mp11 to give M13mp11 SUBT essentially as previously described (Wells, J.A., et al. (1986) *J. Biol. Chem.*, 261,6564-6570). Deoxyuridine containing template DNA was prepared according to Kunkel (Kunkel, T.A. (1985) *Proc. Natl. Acad. Sci. USA*, 82 488-492). Uracil containing template DNA (Kunkel, 1985) was purified by CsCl density gradients (Maniatis, T. et al. (eds.) (1982) in *Molecular Cloning, A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.). A primer (Aval⁻) having the sequence

5' GAAAAAAGACCCCTAGCGTCGCTTA

ending at codon -11, was used to alter the unique Aval recognition sequence within the subtilisin gene. (The asterisk denotes the mismatches from the wild-type sequence and underlined is the altered Aval site.)

The 5' phosphorylated Aval primer (~320 pmol) and ~40 pmol (~120µg) of uridine containing M13mp11 SUBT template in 1.88 ml of 53 mM NaCl, 7.4 mM MgCl₂ and 7.4 mM Tris.HCl (pH 7.5) were annealed by heating to 90°C for 2 min. and cooling 15 min at 24°C (Fig. 31). Primer extension at 24°C was initiated by addition of 100µL containing 1 mM in all four deoxynucleotide triphosphates, and 20µl Klenow fragment (5 units/l). The extension reaction was stopped every 15 seconds over ten min by addition of 10µl 0.25 M EDTA (pH 8) to 50µl aliquots of the reaction mixture. Samples were pooled, phenol chloroform extracted and DNA was precipitated twice by addition of 2.5 vol 100% ethanol, and washed twice with 70% ethanol.

The pellet was dried, and redissolved in 0.4 ml 1 mM EDTA, 10 mM Tris (pH 8).

Misincorporation of α -thiodeoxynucleotides onto the 3' ends of the pool of randomly terminated template was carried out by incubating four 0.2 ml solutions each containing one-fourth of the randomly terminated template mixture (~20 μ g), 0.25 mM of a given α -thiodeoxynucleotide triphosphate, 100 units AMV polymerase, 50 mM KCL, 10 mM MgCl₂, 0.4 mM dithiothreitol, and 50 mM Tris (pH 8.3) (Champoux, J.J. (1984) *Genetics*, 2, 454-464). After incubation at 37 °C for 90 minutes, misincorporation reactions were sealed by incubation for five minutes at 37 °C with 50 mM all four deoxynucleotide triphosphates (pH 8), and 50 units AMV polymerase. Reactions were stopped by addition of 25 mM EDTA (final), and heated at 68 °C for ten min to inactivate AMV polymerase. After ethanol precipitation and resuspension, synthesis of closed circular heteroduplexes was carried out for two days at 14 °C under the same conditions used for the timed extension reactions above, except the reactions also contained 1000 units T4 DNA ligase, 0.5 mM ATP and 1 mM β -mercaptoethanol. Simultaneous restriction of each heteroduplex pool with KpnI, BamHI, and EcoRI confirmed that the extension reactions were nearly quantitative. Heteroduplex DNA in each reaction mixture was methylated by incubation with 80 μ M S-adenosylmethionine and 150 units dam methylase for 1 hour at 37 °C. Methylation reactions were stopped by heating at 68 °C for 15 min.

One-half of each of the four methylated heteroduplex reactions were transformed into 2.5 ml competent *E. coli* JM101 (Messing, J. (1979) *Recombinant DNA Tech. Bull.*, 2, 43-48). The number of independent transformants from each of the four transformations ranged from 0.4-2.0 $\times 10^5$. After growing out phage pools, RF DNA from each of the four transformations was isolated and purified by centrifugation through CsCl density gradients. Approximately 2 μ g of RF DNA from each of the four pools was digested with EcoRI, BamHI and Aval. The 1.5 kb EcoRI-BamHI fragment (i.e., Aval resistant) was purified on low gel temperature agarose and ligated into the 5.5 kb EcoRI-BamHI vector fragment of pB0180. The total number of independent transformants from each α -thiodeoxynucleotide misincorporation plasmid library ranged from 1.2-2.4 $\times 10^4$. The pool of plasmids from each of the four transformations was grown out in 200 ml LB media containing 12.5 μ g/ml cmp and plasmid DNA was purified by centrifugation through CsCl density gradients.

C. Expression and Screening of Subtilisin Point Mutants

Plasmid DNA from each of the four misincorporation pools was transformed (Anagnostopoulos, C., et al. (1967), *J. Bacteriol.*, 81, 741-746) into BG2036. For each transformation, 5 μ g of DNA produced approximately 2.5 $\times 10^5$ independent BG2036 transformants, and liquid culture aliquots from the four libraries were stored in 10% glycerol at 70 °C. Thawed aliquots of frozen cultures were plated on LB/5 μ g/ml cmp/1.6% skim milk plates (Wells, J.A., et al. (1983) *Nucleic Acids Res.*, 11, 7911-7925), and fresh colonies were arrayed onto 96-well microtiter plates containing 150 l per well LB media plus 12.5 μ g/ml cmp. After 1 h at room temperature, a replica was stamped (using a matched 96 prong stamp) onto a 132 mm BA 85 nitrocellulose filter (Schleicher and Scheull) which was layered on a 140 mm diameter LB/cmp/skim milk plate. Cells were grown about 16 h at 30 °C until halos of proteolysis were roughly 5-7 mm in diameter and filters were transferred directly to a freshly prepared agar plate at 37 °C containing only 1.6% skim milk and 50 mM sodium phosphate pH 11.5. Filters were incubated on plates for 3-6 h at 37 °C to produce halos of about 5 mm for wild-type subtilisin and were discarded. The plates were stained for 10 min at 24 °C with Coomassie blue solution (0.25% Coomassie blue (R-250) 25% ethanol) and destained with 25% ethanol, 10% acetic acid for 20 min. Zones of proteolysis appeared as blue halos on a white background on the underside of the plate and were compared to the original growth plate that was similarly stained and destained as a control. Clones were considered positive that produced proportionately larger zones of proteolysis on the high pH plates relative to the original growth plate. Negative clones gave smaller halos under alkaline conditions. Positive and negative clones were restreaked to colony purify and screened again in triplicate to confirm alkaline pH results.

D. Identification and Analysis of Mutant Subtilisins

Plasmid DNA from 5 ml overnight cultures of more alkaline active *B. subtilis* clones was prepared according to Birnboim and Doly (Birnboim, H.C., et al. (1979) *Nucleic Acid Res.* 7, 1513) except that incubation with 2 mg/ml lysozyme proceeded for 5 min at 37 °C to ensure cell lysis and an additional phenol/CHCl₃ extraction was employed to remove contaminants. The 1.5 kb EcoRI-BamHI fragment containing the subtilisin gene was ligated into M13mp11 and template DNA was prepared for DNA sequencing (Messing, J., et al. (1982) *Gene*, 19 269-276). Three DNA sequencing primers ending at codon 26, +95, and +155 were synthesized to match the subtilisin coding sequence. For preliminary sequence

identification a single track of DNA sequence, corresponding to the dNTPas misincorporation library from which the mutant came, was applied over the entire mature protein coding sequence (i.e., a single dideoxyguanosine sequence track was applied to identify a mutant from the dGTPas library). A complete four track of DNA sequence was performed 200 bp over the site of mutagenesis to confirm and identify the mutant sequence (Sanger, F., et al., (1980) *J. Mol. Biol.*, **143**, 161-178). Confirmed positive and negative bacilli clones were cultured in LB media containing 12.5µg/mL cmp and purified from culture supernatants as previously described (Estell, D.A., et al. (1985) *J. Biol. Chem.*, **260**, 6518-6521). Enzymes were greater than 98% pure as analyzed by SDS-polyacrylamide gel electrophoresis (Laemmli, U.K. (1970), *Nature*, **227**, 680-685), and protein concentrations were calculated from the absorbance at 280 nm,

$$\frac{0.18}{c_{280}} = 1.17$$

(Maturbara, H., et al. (1965), *J. Biol. Chem.*, **240**, 1125-1130).

Enzyme activity was measured with 200µg/mL succinyl-L-AlaL-AlaL-ProL-Phep-nitroanilide (Sigma) in 0.1M Tris pH 8.6 or 0.1 M CAPS pH 10.8 at 25°C. Specific activity (µ moles product/min-mg) was calculated from the change in absorbance at 410 nm from production of p-nitroaniline with time per mg of enzyme (E410 = 8,480 M-lcm-l; Del Mar, E.G., et al. (1979), *Anal. Biochem.*, **99**, 316-320). Alkaline autolytic stability studies were performed on purified enzymes (200µg/mL) in 0.1 M potassium phosphate (pH 12.0) at 37°C. At various times aliquots were assayed for residual enzyme activity (Wells, J.A., et al. (1986) *J. Biol. Chem.*, **261**, 6564-6570).

E. Results

1. Optimization and analysis of mutagenesis frequency

A set of primer-template molecules that were randomly 3'-terminated over the subtilisin gene (Fig. 31) was produced by variable extension from a fixed 5'-primer (The primer mutated a unique *Ava*I site at codon 11 in the subtilisin gene). This was achieved by stopping polymerase reactions with EDTA after various times of extension. The extent and distribution of duplex formation over the 1 kb subtilisin gene fragment was assessed by multiple restriction digestion (not shown). For example, production of new *Hinf*I fragments identified when polymerase extension had proceeded past Ile110, Leu233, and Asp259 in the subtilisin gene.

Misincorporation of each dNTPas at randomly terminated 3' ends by AMV reverse transcriptase (Zakour, R.A., et al. (1982), *Nature*, **295**, 708-710; Zakour, R.A., et al. (1984), *Nucleic Acids Res.*, **12**, 6615-6628) used conditions previously described (Champoux, J.J., (1984), *Genetics*, **2**, 454-464). The efficiency of each misincorporation reaction was estimated to be greater than 80% by the addition of each dNTPas to the *Ava*I restriction primer, and analysis by polyacrylamide gel electrophoresis. Misincorporations were sealed by polymerization with all four dNTP's and closed circular DNA was produced by reaction with DNA ligase.

Several manipulations were employed to maximize the yield of the mutant sequences in the heteroduplex. These included the use of a deoxyuridine containing template (Kunkel, T.A. (1985), *Proc. Natl. Acad. Sci. USA*, **82** 488-492; Pukkila, P.J. et al. (1983), *Genetics*, **104**, 571-582), *in vitro* methylation of the mutagenic strand (Kramer, W. et al. (1982) *Nucleic Acids Res.*, **10** 6475-6485), and the use of *Ava*I restriction-selection against the wild-type template strand which contained a unique *Ava*I site. The separate contribution of each of these enrichment procedures to the final mutagenesis frequency was not determined, except that prior to *Ava*I restriction-selection roughly one-third of the segregated clones in each of the four pools still retained a wild-type *Ava*I site within the subtilisin gene. After *Ava*I restriction-selection greater than 98% of the plasmids lacked the wild-type *Ava*I site.

The 1.5 kb *Eco*RI-*Bam*HI subtilisin gene fragment that was resistant to *Ava*I restriction digestion, from each of the four *Cs*Cl purified M13 RF pools was isolated on low melting agarose. The fragment was ligated *in situ* from the agarose with a similarly cut *E. coli*-B. subtilis shuttle vector, pB0180, and transformed directly into *E. coli* LE392. Such direct ligation and transformation of DNA isolated from agarose avoided losses and allowed large numbers of recombinants to be obtained (>100,000 per µg equivalent of input M13 pool).

The frequency of mutagenesis for each of the four dNTPas misincorporation reactions was estimated from the frequency that unique restriction sites were eliminated (Table XX). The unique restriction sites

chosen for this analysis, ClaI, PvuII, and KpnI, were distributed over the subtilisin gene starting at codons 35, 104, and 166, respectively. As a control, the mutagenesis frequency was determined at the PstI site located in the β lactamase gene which was outside the window of mutagenesis. Because the absolute mutagenesis frequency was close to the percentage of undigested plasmid DNA, two rounds of restriction-selection were necessary to reduce the background of surviving uncut wild-type plasmid DNA below the mutant plasmid (Table XX). The background of surviving plasmid from wild-type DNA probably represents the sum total of spontaneous mutations, uncut wild-type plasmid, plus the efficiency with which linear DNA can transform E. coli. Subtracting the frequency for unmutagenized DNA (background) from the frequency for mutant DNA, and normalizing for the window of mutagenesis sampled by a given restriction analysis (4-6 bp) provides an estimate of the mutagenesis efficiency over the entire coding sequence (~1000 bp).

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TABLE XX

α -thiol dNTP misincor- porated ^(b)	Restriction Site Selection	% resistant clones ^c			% resistant clones over Background ^d	% mutants per 1000bp ^e
		1st round	2nd round	Total		
None	<u>PstI</u>	0.32	0.7	0.002	0	-
G	<u>PstI</u>	0.33	1.0	0.003	0.001	0.2
T	<u>PstI</u>	0.32	<0.5	<0.002	0	0
C	<u>PstI</u>	0.43	3.0	0.013	0.011	3
None	<u>ClaI</u>	0.28	5	0.014	0	-
G	<u>ClaI</u>	2.26	85	1.92	1.91	380
T	<u>ClaI</u>	0.48	31	0.15	0.14	35
C	<u>ClaI</u>	0.55	15	0.08	0.066	17
None	<u>PvuII</u>	0.08	29	0.023	0	-
G	<u>PvuII</u>	0.41	90	0.37	0.35	88
T	<u>PvuII</u>	0.10	67	0.067	0.044	9
C	<u>PvuII</u>	0.76	53	0.40	0.38	95
None	<u>KpnI</u>	0.41	3	0.012	0	-
G	<u>KpnI</u>	0.98	35	0.34	0.33	83
T	<u>KpnI</u>	0.36	15	0.054	0.042	8
C	<u>KpnI</u>	1.47	26	0.38	0.37	93

(a) Mutagenesis frequency is estimated from the frequency for obtaining mutations that alter unique restriction sites within the mutagenized subtilisin gene (i.e., ClaI, PvuII, or KpnI) compared to mutation frequencies of the PstI site, that is outside the window of mutagenesis.

(b) Plasmid DNA was from wild-type (none) or mutagenized by dNTPs misincorporation as described.

(c) Percentage of resistant clones was calculated from the fraction of clones obtained after three fold or greater over-digestion of the plasmid with the indicated restriction enzyme compared to a

non-digested control. Restriction-resistant plasmid DNA from the first round was subjected to a second round of restriction-selection. The total represents the product of the fractions of resistant clones obtained from both rounds of selection and gives percentage of restriction-site mutant clones in the original starting pool. Frequencies were derived from counting at least 20 colonies and usually greater than 100.

(d) Percent resistant clones was calculated by subtracting the percentage of restriction-resistant clones obtained for wild-type DNA (i.e., none) from that obtained for mutant DNA.

(e) This extrapolates from the frequency of mutation over each restriction site to the entire subtilisin gene (~1 kb). This has been normalized to the number of possible bases (4-6 bp) within each restriction site that can be mutagenized by a given misincorporation event.

From this analysis, the average percentage of subtilisin genes containing mutations that result from dGTPas, dCTPas, or dTTPas misincorporation was estimated to be 90, 70, and 20 percent, respectively. These high mutagenesis frequencies were generally quite variable depending upon the dNTPas and misincorporation efficiencies at this site. Misincorporation efficiency has been reported to be both dependent on the kind of mismatch, and the context of primer (Champoux, J.J., (1984); Skinner, J.A., et al. (1986) Nucleic Acids Res., 14, 6945-6964). Biased misincorporation efficiency of dGTPas and dCTPas over dTTPas has been previously observed (Shortle, D., et al. (1985), Genetics, 110, 539-555). Unlike the dGTPas, dCTPas, and dTTPas libraries the efficiency of mutagenesis for the dATPas misincorporation library could not be accurately assessed because 90% of the restriction-resistant plasmids analyzed simply lacked the subtilisin gene insert. This problem probably arose from self-ligation of the vector when the dATPas mutagenized subtilisin gene was subcloned from M13 into pB0180. Correcting for the vector background, we estimate the mutagenesis frequency around 20 percent in the dATPas misincorporation library. In a separate experiment (not shown), the mutagenesis efficiencies for dGTPas and dTTPas misincorporation were estimated to be around 50 and 30 percent, respectively, based on the frequency of reversion of an inactivating mutation at codon 169.

The location and identity of each mutation was determined by a single track of DNA sequencing corresponding to the misincorporated α thiodioxynucleotide over the entire gene followed by a complete four track of DNA sequencing focused over the site of mutation. Of 14 mutants identified, the distribution was similar to that reported by Shortle and Lin (1985) except we did not observe nucleotide insertion or deletion mutations. The proportion of AG mutations was highest in the G misincorporation library, and some unexpected point mutations appeared in the dTTPas and dCTPas libraries.

2. Screening and Identification of Alkaline Stability Mutants of Subtilisin

It is possible to screen colonies producing subtilisin by halos of casein digestion (Wells, J.A. et al. (1983) Nucleic Acids Res., 11, 7911-7925). However, two problems were posed by screening colonies under high alkaline conditions (>pH 11). First, *B. subtilis* will not grow at high pH, and we have been unable to transform an alkylphilic strain of bacillus. This problem was overcome by adopting a replica plating strategy in which colonies were grown on filters at neutral pH to produce subtilisin and filters subsequently transferred to casein plates at pH 11.5 to assay subtilisin activity. However, at pH 11.5 the casein micells no longer formed a turbid background and thus prevented a clear observation of proteolysis halos. The problem was overcome by briefly staining the plate with Coomassie blue to amplify proteolysis zones and acidifying the plates to develop casein micell turbidity. By comparison of the halo size produced on the reference growth plate (pH 7) to the high pH plate (pH 11.5), it was possible to identify mutant subtilisins that had increased (positives) or decreased (negatives) stability under alkaline conditions.

Roughly 1000 colonies were screened from each of the four misincorporation libraries. The percentage of colonies showing a differential loss of activity at pH 11.5 versus pH 7 represented 1.4, 1.8, 1.4, and 0.6% of the total colonies screened from the thiol dGTP α s, dATP α s, dTTP α s, and dCTP α s libraries, respectively. Several of these negative clones were sequenced and all were found to contain a single base change as expected from the misincorporation library from which they came. Negative mutants included A36, E170 and V50. Two positive mutants were identified as V107 and R213. The ratio of negatives to positives was roughly 50:1.

3. Stability and Activity of Subtilisin Mutants at Alkaline pH

Subtilisin mutants were purified and their autolytic stabilities were measured by the time course of inactivation at pH 12.0 (Figs. 32 and 33). Positive mutants identified from the screen (i.e., V107 and R213) were more resistant to alkaline induced autolytic inactivation compared to wild-type; negative mutants (i.e., E170 and V50) were less resistant. We had advantageously produced another mutant at position 50 (F50) by site-directed mutagenesis. This mutant was more stable than wild-type enzyme to alkaline autolytic inactivation (Fig. 33). At the termination of the autolysis study, SDS-PAGE analysis confirmed that each subtilisin variant had autolyzed to an extent consistent with the remaining enzyme activity.

The stabilizing effects of V107, R213, and F50 are cumulative. See Table XXI. The double mutant, V107/R213 (made by subcloning the 920 bp *EcoRI*-*KpnI* fragment of pB0180V107 into the 6.6 kb *EcoRI*-*KpnI* fragment of pB0180R213), is more stable than either single mutant. The triple mutant, F50/V107/R213 (made by subcloning the 735 bp *EcoRI*-*PvuII* fragment of pF50 (Example 2) into the 6.8 kb *EcoRI*-*PvuII* fragment of pB0180/V107, is more stable than the double mutant V107/R213 or F50. The inactivation curves show a biphasic character that becomes more pronounced the more stable the mutant analyzed. This may result from some destabilizing chemical modification(s) (eg., deamidation) during the autolysis study and/or reduced stabilization caused by complete digestion of larger autolysis peptides. These alkaline autolysis studies have been repeated on separately purified enzyme batches with essentially the same results. Rates of autolysis should depend both on the conformational stability as well as the specific activity of the subtilisin variant (Wells, J.A., et al. (1986), *J. Biol. Chem.*, **261**, 6564-6570). It was therefore possible that the decreases in autolytic inactivation rates may result from decreases in specific activity of the more stable mutant under alkaline conditions. In general the opposite appears to be the case. The more stable mutants, if anything, have a relatively higher specific activity than wild-type under alkaline conditions and the less stable mutants have a relatively lower specific activity. These subtle effects on specific activity for V107/R213 and F50/V107/R213 are cumulative at both pH 8.6 and 10.8. The changes in specific activity may reflect slight differences in substrate specificity, however, it is noteworthy that only positions 170 and 107 are within 6A of a bound model substrate (Robertus, J.D., et al. (1972), *Biochemistry* **11**, 2438-2449).

TABLE XXI

Relationship between relative specific activity at pH 8.6 or 10.8 and alkaline autolytic stability			
Enzyme	Relative specific activity		Alkaline autolysis half-time (min) ^b
	pH 8.6	pH 10.8	
Wild-type	100 $\pm$ 1	100 $\pm$ 3	86
Q170	46 $\pm$ 1	28 $\pm$ 2	13
V107	126 $\pm$ 3	99 $\pm$ 5	102
R213	97 $\pm$ 1	102 $\pm$ 1	115
V107/R213	116 $\pm$ 2	106 $\pm$ 3	130
V50	66 $\pm$ 4	61 $\pm$ 1	58
F50	123 $\pm$ 3	157 $\pm$ 7	131
F50/V107/R213	126 $\pm$ 2	152 $\pm$ 3	168

^(a) Relative specific activity was the average from triplicate activity determinations divided by the wild-type value at the same pH. The average specific activity of wild-type enzyme at pH 8.6 and 10.8 was 70 μ moles/min-mg and 37 μ moles/min-mg, respectively.

^(b) Time to reach 50% activity was taken from Figs. 32 and 33.

F. Random Cassette Mutagenesis of Residues 197 through 228

Plasmid pΔ222 (Wells, et al. (1985) Gene 34, 315-323) was digested with PstI and BamHI and the 0.4 kb PstI/BamHI fragment (fragment 1, see Fig. 34) purified from a polyacrylamide gel by electroelution.

- 5 The 1.5 kb EcoRI/BamHI fragment from pS4.5 was cloned into M13mp9. Site directed mutagenesis was performed to create the A197 mutant and simultaneously insert a silent SstI site over codons 195-196. The mutant EcoRI/BamHI fragment was cloned back into pBS42. The pA197 plasmid was digested with BamHI and SstI and the 5.3 kb BamHI/SstI fragment (fragment 2) was purified from low melting agarose.

- Complimentary oligonucleotides were synthesized to span the region from SstI (codons 195-196) to PstI (codons 228-230). These oligodeoxynucleotides were designed to (1) restore codon 197 to the wild type, (2) re-create a silent KpnI site present in pΔ222 at codons 219-220, (3) create a silent SmaI site over codons 210-211, and (4) eliminate the PstI site over codons 228-230 (see Fig. 35). Oligodeoxynucleotides were synthesized with 2% contaminating nucleotides at each cycle of synthesis, e.g., dATP reagent was spiked with 2% dCTP, 2% dGTP, and 2% dTTP. For 97-mers, this 2% poisoning should give the following percentages of non-mutant, single mutants and double or higher mutants per strand with two or more misincorporations per complimentary strand: 14% non-mutant, 28% single mutant, and 57% with ≥2 mutations, according to the general formula

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$$f = \frac{\mu^n}{n!} e^{-\mu} .$$

- 25 where μ is the average number of mutations and n is a number class of mutations and f is the fraction of the total having that number of mutations. Complimentary oligodeoxynucleotide pools were phosphorylated and annealed (fragment 3) and then ligated at 2-fold molar excess over fragments 1 and 2 in a three-way ligation.

- E. coli MM294 was transformed with the ligation reaction, the transformation pool-grown up over night and the pooled plasmid DNA was isolated. This pool represented 3.4×10^4 independent transformants. This plasmid pool was digested with PstI and then used to retransform E. coli. A second plasmid pool was prepared and used to transform B. subtilis (BG2036). Approximately 40% of the BG2036 transformants actively expressed subtilisin as judged by halo-clearing on casein plates. Several of the non-expressing transformants were sequenced and found to have insertions or deletions in the synthetic cassettes. Expressing BG2036 mutants were arrayed in microtiter dishes with 150μl of LB/12.5μg/mL chloramphenicol (cmp) per well, incubated at 37°C for 3-4 hours and then stamped in duplicate onto nitrocellulose filters laid on LB 1.5% skim milk/5μg/mL cmp plates and incubated overnight at 33°C (until halos were approximately 4-8 mm in diameter). Filters were then lifted to stacks of filter paper saturated with 1 x Tide commercial grade detergent, 50 mM Na₂CO₃, pH 11.5 and incubated at 65°C for 90 min. Overnight growth plates were Commassie stained and destained to establish basal levels of expression. After this treatment, filters were returned to pH7/skim milk/20μg/mL tetracycline plates and incubated at 37°C for 4 hours to overnight.

Mutants identified by the high pH stability screen to be more alkaline stable were purified and analyzed for autolytic stability at high pH or high temperature. The double mutant C204/R213 was more stable than wild type at either high pH or high temperature (Table XXII).

- 45 This mutant was dissected into single mutant parents (C204 and R213) by cutting at the unique SmaI restriction site (Fig. 35) and either ligating wild type sequence 3' to the SmaI site to create the single C204 mutant or ligating wild type sequence 5' to the SmaI site to create the single R213 mutant. Of the two single parents, C204 was nearly as alkaline stable as the parent double mutant (C04/R213) and slightly more thermally stable. See Table XXII. The R213 mutant was only slightly more stable than wild type under both conditions (not shown).

Another mutant identified from the screen of the 197 to 228 random cassette mutagenesis was R204. This mutant was more stable than wild type at both high pH and high temperature but less stable than C204.

TABLE XXIIStability of subtilisin variants

Purified enzymes (200 μ g/mL) were incubated in 0.1M phosphate, pH 12 at 30°C for alkaline autolysis, or in 2mM CaCl₂, 50mM MOPS, pH 7.0 at 62°C for thermal autolysis. At various times samples were assayed for residual enzyme activity. Inactivations were roughly pseudo-first order, and $t_{1/2}$ gives the time it took to reach 50% of the starting activity in two separate experiments.

<u>Subtilisin variant</u>	<u>$t_{1/2}$</u> <u>(alkaline autolysis)</u>		<u>$t_{1/2}$</u> <u>(thermal autolysis)</u>	
	<u>Exp. #1</u>	<u>Exp. #2</u>	<u>Exp. #1</u>	<u>Exp. #2</u>
wild type	30	25	20	23
F50/V107/R213	49	41	18	23
R204	35	32	24	27
C204	43	46	38	40
C204/R213	50	52	32	36
L204/R213	32	30	20	21

G. Random Mutagenesis at Codon 204

Based on the above results, codon 204 was targeted for random mutagenesis. Mutagenic DNA cassettes (for codon at 204) all contained a fixed R213 mutation which was found to slightly augment the stability of the C204 mutant.

Plasmid DNA encoding the subtilisin mutant C204/R213 was digested with SstI and EcoRI and a 1.0 kb EcoRI/SstI fragment was isolated by electro-elution from polyacrylamide gel (fragment 1, see Fig. 35).

C204/R213 was also digested with SmaI and EcoRI and the large 4.7 kb fragment, including vector sequences and the 3' portion of coding region, was isolated from low melting agarose (fragment 2, see Fig. 36).

Fragments 1 and 2 were combined in four separate three-way ligations with heterophosphorylated fragments 3 (see Figs. 36 and 37). This heterophosphorylation of synthetic duplexes should preferentially drive the phosphorylated strand into the plasmid ligation product. Four plasmid pools, corresponding to the four ligations, were restricted with SmaI in order to linearize any single cut C204/R213 present from fragment 2 isolation, thus reducing the background of C204/R213. E. coli was then re-transformed with

Small-restricted plasmid pools to yield a second set of plasmid pools which are essentially free of C204/R213 and any non-segregated heterduplex material.

These second enriched plasmid pools were then used to transform *B. subtilis* (BG2036) and the resulting four mutant pools were screened for clones expressing subtilisin resistant to high pH/temperature inactivation. Mutants found positive by such a screen were further characterized and identified by sequencing.

The mutant L204/R213 was found to be slightly more stable than the wild type subtilisin. See Table XXII.

Having described the preferred embodiments of the present invention, it will appear to those ordinarily skilled in the art that various modifications may be made to the disclosed embodiments, and that such modifications are intended to be within the scope of the present invention.

Claims

1. A subtilisin mutant derived by the substitution of at least one amino acid residue of a precursor subtilisin with a different amino acid, so that the subtilisin mutant has at least one property which is different from the same property of the precursor subtilisin, characterised by the substitution at one or more of Tyr21, Thr22, Ser24, Asp36, Ala45, Gly46, Ala48, Ser49, Met50, Asn77, Ser87, Lys94, Val95, Leu96, Ile107, Gly110, Met124, Lys170, Tyr171, Pro172, Asp197, Met199, Ser204, Lys213, His67, Leu135, Gly97, Ser101, Gly102, Glu103, Gly127, Gly128, Pro129, Tyr214, and Gly215 of *Bacillus amyloliquefaciens* subtilisin and equivalent amino acid residues in other precursor subtilisins.
2. A subtilisin mutant having an amino acid sequence derived from the amino acid sequence of a precursor subtilisin by the substitution of more than one amino acid residue of said amino acid sequence of said precursor subtilisin by a different amino acid, so that the subtilisin mutant has at least one property which is different from the same property of the precursor subtilisin, characterized by substitutions at more than one of Tyr21, Thr22, Ser24, Asp32, Ser33, Asp36, Ala45, Ala48, Ser49, Met50, Ser87, Lys94, Val95, Tyr104, Ile107, Gly110, Met124, Ala152, Asn155, Glu156, Gly166, Gly169, Lys170, Tyr171, Pro172, Phe189, Asp197, Met199, Ser204, Lys213, Tyr217, Ser221, Met222, His67, Leu135, Gly97, Ser101, Gly102, Glu103, Gly127, Gly128, Pro129, Tyr214, and Gly215 of *Bacillus amyloliquefaciens* subtilisin and equivalent amino acid residues in other precursor subtilisins, with the proviso that when substitution is made at any residue in the group Asp32, Ser33, Tyr104, Ala152, Asn155, Glu156, Gly166, Gly169, Phe189, Tyr217 and Met222 a substitution is also made at at least one specified position not of that group.
3. The mutant of claim 2 wherein said combinations are selected from Thr22/Ser87, Ser24/Ser87, Ala45/Ala48, Ser49/Lys94, Ser49/Val95, Met50/Val95, Met50/Gly110, Met50/Met124, Met50/Met222, Met124/Met222, Tyr21/Thr22, Met50/Met124/Met222, Tyr21/Thr22/Ser87, Met50/Glu156/Gly166/Tyr217, Met50/Glu156/Tyr217, Ile170/Lys213, Ser204/Lys213, Met50/Ile107/Lys213 and Ser24/Met50/Ile107/Glu156/Gly166/Gly169/Ser204/Lys213/Gly215/Tyr217.
4. A subtilisin mutant derived by the deletion of one or more amino acid residues in a precursor subtilisin equivalent to 161-164 in *B. amyloliquefaciens* subtilisin, said deletion being made alone or in combination with substitutions in the amino acid sequence of the precursor subtilisin, and producing at least one property which is different from the same property of the precursor subtilisin.
5. A subtilisin mutant having altered substrate specificity when compared to a precursor subtilisin, the mutant being derived by the substitution of a different amino acid at the residue equivalent to Leu + 126 of *B. amyloliquefaciens* subtilisin, alone or in combination with other substitutions or deletions in the amino acid sequence of the precursor subtilisin.
6. A subtilisin mutant having altered substrate specificity when compared to a precursor subtilisin, the mutant being derived by the substitution of a different amino acid at the residue equivalent to Asp + 99 in *B. amyloliquefaciens* subtilisin, alone or in combination with other substitutions or deletions in the amino acid sequence of the precursor subtilisin.
7. A DNA sequence encoding the mutant of any one of the preceding claims.



8. An expression vector containing the mutant DNA sequence of claim 7.
9. A host cell transformed with the expression vector or claim 8.

5 Patentansprüche

- 10 1. Subtilisinmutante, die durch Substitution zumindest eines Aminosäurerests eines Vorläufer-Subtilisins durch eine davon verschiedene Aminosäure hergeleitet ist, sodaß die Subtilisinmutante zumindest eine Eigenschaft aufweist, die sich von der gleichen Eigenschaft des Vorläufer-Subtilisins unterscheidet, gekennzeichnet durch die Substitution an einem oder mehreren von Tyr21, Thr22, Ser24, Asp36, Ala45, Gly46, Ala48, Ser49, Met50, Asn77, Ser87, Lys94, Val95, Leu96, Ile107, Gly110, Met124, Lys170, Tyr171, Pro172, Asp197, Met199, Ser204, Lys213, His67, Leu135, Gly97, Ser101, Gly102, Glu103, Gly127, Gly128, Pro129, Tyr214 und Gly215 von *Bacillus amyloliquefaciens*-Subtilisin und äquivalenten Aminosäureresten in anderen Vorläufer-Subtilisinen.
- 15 2. Subtilisinmutante mit einer Aminosäuresequenz, die aus der Aminosäuresequenz eines Vorläufer-Subtilisins durch Substitution mehr als eines Aminosäurerests der Aminosäuresequenz des Vorläufer-Subtilisins durch eine davon verschiedene Aminosäure hergeleitet ist, sodaß die Subtilisinmutante zumindest eine Eigenschaft aufweist, die sich von der gleichen Eigenschaft des Vorläufer-Subtilisins unterscheidet, gekennzeichnet durch Substitutionen an mehr als einem von Tyr21, Thr22, Ser24, Asp32, Ser33, Asp36, Ala45, Ala48, Ser49, Met50, Ser87, Lys94, Val95, Tyr104, Ile107, Gly110, Met124, Ala152, Asn155, Glu156, Gly166, Gly169, Lys170, Tyr171, Pro172, Phe189, Asp197, Met199, Ser204, Lys213, Tyr217, Ser221, Met222, His67, Leu135, Gly97, Ser101, Gly102, Glu103, Gly127, Gly128, Pro129, Tyr214 und Gly215 von *Bacillus amyloliquefaciens*-Subtilisin und äquivalenten Aminosäureresten in anderen Vorläufer-Subtilisinen, mit der Maßgabe, daß bei einer Substitution an irgendeinem Rest in der Gruppe Asp32, Ser33, Tyr104, Ala152, Asn155, Glu156, Gly166, Gly169, Phe189, Tyr217 und Met222 eine Substitution auch an zumindest einer bestimmten Position durchgeführt wird, die nicht dieser Gruppe angehört.
- 20 3. Mutante nach Anspruch 2, worin die Kombinationen aus Thr22/Ser87, Ser24/Ser87, Ala45/Ala48, Ser49/Lys94, Ser49/Val95, Met50/Val95, Met50/Gly110, Met50/Met124, Met50/Met222, Met124/Met222, Tyr21/Thr22, Met50/Met124/Met222, Tyr21/Tyr22/Ser87, Met50/Glu156/Gly166/Tyr217, Met50/Glu156/Tyr217, Ile170/Lys213, Ser204/Lys213, Met50/Ile107/Lys213 und Ser24/Met50/Ile107/Glu156/Gly166/Gly169/Ser204/Lys213/Gly215/Tyr217 ausgewählt sind.
- 35 4. Subtilisinmutante, die durch Löschung eines oder mehrerer Aminosäurereste in einem Vorläufer-Subtilisin, das 161-164 in *B. amyloliquefaciens*-Subtilisin äquivalent ist, hergeleitet ist, wobei die Löschung entweder alleine oder in Kombination mit Substitutionen in der Aminosäuresequenz des Vorläufer-Subtilisins erfolgt, und zumindest eine Eigenschaft ergibt, die sich von der gleichen Eigenschaft des Vorläufer-Subtilisins unterscheidet.
- 40 5. Subtilisinmutante mit geänderter Substratspezifität im Vergleich zu einem Vorläufer-Subtilisin, wobei die Mutante durch Substitution einer unterschiedlichen Aminosäure am Rest, der Leu + 126 von *B. amyloliquefaciens*-Subtilisin äquivalent ist, alleine oder in Kombination mit anderen Substitutionen oder Löschungen in der Aminosäuresequenz des Vorläufer-Subtilisins hergeleitet ist.
- 45 6. Subtilisinmutante mit geänderter Substratspezifität im Vergleich zu einem Vorläufer-Subtilisin, wobei die Mutante durch Substitution einer unterschiedlichen Aminosäure am Rest, der Asp + 99 im *B. amyloliquefaciens*-Subtilisin äquivalent ist, alleine oder in Kombination mit anderen Substitutionen oder Löschungen in der Aminosäuresequenz des Vorläufer-Subtilisins hergeleitet ist.
- 50 7. DNA-Sequenz, die für die Mutante nach einem der vorhergehenden Ansprüche kodiert.
8. Expressionsvektor, der die Mutanten-DNA-Sequenz von Anspruch 7 enthält.
- 55 9. Wirtszelle, die mit dem Expressionsvektor von Anspruch 8 transformiert ist.



Revendications

1. Mutant de subtilisine dérivé par la substitution d'au moins un résidu d'acide aminé d'une subtilisine précurseur et par un acide aminé différent de manière que le mutant de subtilisine ait au moins une propriété qui est différente de la même propriété de la subtilisine précurseur, caractérisé par la substitution à un ou plusieurs de Tyr21, Thr22, Ser24, Asp36, Ala45, Gly46, Ala48, Ser49, Met50, Asn77, Ser87, Lys94, Val95, Leu96, Ile107, Gly110, Met124, Lys170, Tyr171, Pro172, Asp197, Met199, Ser204, Lys213, His67, Leu135, Gly97, Ser101, Gly102, Glu103, Gly127, Gly128, Pro129, Tyr214 et Gly215 de la subtilisine de Bacillus amyloliquefaciens et les résidus d'acides aminés équivalents dans d'autres subtilisines précurseurs.
2. Mutant de subtilisine ayant une séquence d'acides aminés dérivée de la séquence d'acides aminés d'une subtilisine précurseur par la substitution de plus d'un résidu d'acide aminé de ladite séquence d'acides aminés de ladite subtilisine précurseur par un acide aminé différent de manière que le mutant de subtilisine ait au moins une propriété qui est différente de la même propriété de la subtilisine précurseur, caractérisé par des substitutions à plus d'un de Tyr21, Thr22, Ser24, Asp32, Ser33, Asp36, Ala45, Ala48, Ser49, Met50, Ser87, Lys94, Val95, Tyr104, Ile107, Gly110, Met124, Ala152, Asn155, Glu156, Gly166, Gly169, Lys170, Tyr171, Pro172, Phe189, Asp197, Met199, Ser204, Lys213, Tyr217, Ser221, Met222, His67, Leu135, Gly97, Ser101, Gly102, Glu103, Gly127, Gly128, Pro129, Tyr214 et Gly215 de la subtilisine de Bacillus amyloliquefaciens et des résidus d'acides aminés équivalents dans d'autres subtilisines précurseurs, à condition que quand la substitution est effectuée à tout résidu dans le groupe formé de Asp32, Ser33, Tyr104, Ala152, Asn155, Glu156, Gly166, Gly169, Phe189, Tyr217 et Met222, une substitution soit également effectuée en au moins une position spécifiée ne faisant pas partie de ce groupe.
3. Mutant de la revendication 2 où lesdites associations sont choisies parmi Thr22/Ser87, Ser24/Ser87, Ala45/Ala48, Ser49/Lys94, Ser49/Val95, Met50/Val95, Met50/Gly110, Met50/Met124, Met50/Met222, Met124/Met222, Tyr21/Thr22, Met50/Met124/Met222, Tyr21/Thr22/Ser87, Met50/Glu156/Gly166/Tyr217, Met50/Glu156/Tyr217, Ile170/Lys213, Ser204/Lys213, Met50/Ile107/Lys213 et Ser24/Met50/Ile107/Glu156/Gly166/Gly169/Ser204/Lys213/Gly215/Tyr217.
4. Mutant de subtilisine dérivé par la délétion d'un ou plusieurs résidus d'acides aminés dans une subtilisine précurseur équivalente à 161-164 dans la subtilisine de B. amyloliquefaciens, ladite délétion étant effectuée seule ou en association avec des substitutions dans la séquence d'acides aminés de la subtilisine précurseur et la production d'au moins une propriété qui est différente de la même propriété de la subtilisine précurseur.
5. Mutant de subtilisine ayant une spécificité modifiée du substrat en comparaison avec une subtilisine précurseur, le mutant étant dérivé par la substitution d'un acide aminé différent au résidu équivalent à Leu + 126 de la subtilisine de B. amyloliquefaciens, seule ou en association avec d'autres substitutions ou délétions dans la séquence d'acides aminés de la subtilisine précurseur.
6. Mutant de subtilisine ayant une spécificité modifiée de substrat en comparaison avec une subtilisine précurseur, le mutant étant dérivé par la substitution d'un acide aminé différent au résidu équivalent à Asp + 99 dans la subtilisine de B. amyloliquefaciens, seule ou en association avec d'autres substitutions ou délétions dans la séquence d'acides aminés de la subtilisine précurseur.
7. Séquence d'ADN codant le mutant selon l'une quelconque des revendications précédentes.
8. Vecteur d'expression contenant la séquence d'ADN du mutant de la revendication 7.
9. Cellule hôte transformée par le vecteur d'expression de la revendication 8.