



## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification <sup>6</sup> : <b>C12N 9/50, 9/52, 9/54, 9/56, 15/57</b>	<b>A1</b>	(11) International Publication Number: <b>WO 95/23221</b>  (43) International Publication Date: 31 August 1995 (31.08.95)
<p>(21) International Application Number: PCT/US95/01937</p> <p>(22) International Filing Date: 23 February 1995 (23.02.95)</p> <p>(30) Priority Data:              08/201,120           24 February 1994 (24.02.94)    US              08/373,818           17 January 1995 (17.01.95)    US</p> <p>(71) Applicant: COGNIS, INC. [US/US]; 2331 Circadian Way, Santa Rosa, CA 95407 (US).</p> <p>(72) Inventors: CHRISTIANSON, Teresa, M.; 504 Ely Road, Petaluma, CA 94954 (US). GODDETTE, Dean, W.; 10 Marina Road, Chelmsford, MA 01824 (US). KOTTWITZ, Beatrix; Urdenbacher Allee 51, D-40593 Düsseldorf (DE). MAURER, Karl-Heinz; Dechenstrasse 5, D-40699 Erkrath (DE). PAECH, Christian, G.; 240 Second Avenue, Daly City, CA 94014 (US). POCHANDKE, Winfried; Hegelstrasse 19, D-40789 Monheim (DE). POETHKOW, Joerg; Hasseler Richtweg 79, D-40229 Düsseldorf (DE). SCHMIDT, Irmgard; Forststrasse 70a, D-42697 Solingen (DE). TANG, Maria, R.; 2043 Cliffwood Drive, Fairfield, CA 94533 (US). UPADEK, Horst; Im Sandforst 26, D-40883 Ratingen (DE). WEISS, Albrecht; Forellenweg 37, D-40764 Langenfeld (DE). WILSON, Charles, Ronald; 2323 Pacheco Place, Santa Rosa, CA 95401 (US).</p>	<p>(74) Agent: DRACH, John, E.; Henkel Corporation, Suite 150, 140 Germantown Pike, Plymouth Meeting, PA 19462 (US).</p> <p>(81) Designated States: JP, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).</p> <p><b>Published</b> <i>With international search report.</i></p>	
(54) Title: IMPROVED ENZYMES AND DETERGENTS CONTAINING THEM		
(57) Abstract		
<p>This invention relates to proteases with improved application properties in cleaners and detergents. The improvements are achieved by substituting positively charged or uncharged amino acids in the substrate binding region of the wild type subtilisin protease.</p>		

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## IMPROVED ENZYMES AND DETERGENTS CONTAINING THEM

### BACKGROUND OF THE INVENTION

#### 1. Field of the Invention

5 This invention relates to novel mutant proteolytic enzymes with improved properties relative to the wild type enzyme in cleaning and detergent formulations, to nucleotide sequences encoding the improved proteases, and to host organisms containing the nucleotide sequences encoding the novel proteases. This invention also includes within its scope new and improved detergent and cleansing compositions containing an effective cleansing amount  
10 of said enzymes.

#### 2. Description of the Related Art

Subtilisins are a family of bacterial extracellular proteases with molecular masses of 20,000 to 45,000 daltons produced by a soil bacillus e.g. *Bacillus amyloliquefaciens*. Proteases are enzymes which catalyze the hydrolysis of  
15 peptide linkages in protein and peptide substrates and of ester bonds in some terminal esters. Subtilisins belong to the group of serine proteases which initiate the nucleophilic attack on the peptide (ester) bond by a serine residue at the active site. Subtilisins are physically and chemically well characterized enzymes. The three-dimensional structure of several subtilisins has been elucidated in  
20 detail by X-ray diffraction studies (Betzel, C., Pal, G.P., and Saenger, W. (1988)

Eur. J. Biochem. **178**, 155-171; Bott, R., Ultsch, M., Kossiakoff, A., Graycar, T., Katz, B., and Power, S. (1988) J. Biol. Chem. **263**, 7895-7906; Goddette, D.W., Paech, C., Yang, S.S., Mielenz, J.R., Bystroff, C., Wilke, M., and Fletterick, R.J. (1992) J. Mol. Biol. **228**, 580-595; Heinz, D.W., Priestle, J.P., Rahuel, J., Wilson, K.S., and Grütter, M.G. (1991) J. Mol. Biol. **217**, 353-371; Kraut, J. (1977) Annu. Rev. Biochem. **46**, 331-358; Neidhart, D.J. and Petsko, G.A. (1988) Protein Eng. **2**, 271-276; Teplyakov, A.V., Kuranova, I.P., Harutyunyan, E.H., Vainshtein, B.K., Frömmel, C., Höhne, W.-E., and Wilson, K.S. (1990) J. Mol. Biol. **214**, 261-279). In spite of this wealth of information the structure/function differences between these closely related subtilisins have not been explained.

Subtilisins are widely used in commercial products (for example, in laundry and dish washing detergents, contact lens cleaners) and for research purposes (catalysts in synthetic organic chemistry). One member of the subtilisin family, a highly alkaline protease for use in detergent formulations has been described in patent application WO 91/02792. This *Bacillus lentus* alkaline protease (BLAP) can be obtained in commercial quantities from *Bacillus licheniformis* ATCC 53926 strain transformed by an expression plasmid harboring the wild type BLAP gene under the control of the *B. licheniformis* ATCC 53926 alkaline protease gene promoter. The crystal structure of BLAP has been deduced (Goddette, D.W., et al. (1992) J. Mol. Biol. **228**, 580-595; WO 92/21760), and the coordinates have been deposited with the Brookhaven Protein Data Bank. Unless otherwise noted the numbering of the amino acid positions is according to the sequence in BLAP (269 amino acids), which differs from that of subtilisin BPN' (275 amino acids). When aligned for optimal sequence homology the following pattern emerges. In BLAP positions 1 to 35, 36 to 54, 55 to 160, and 161 to 269 correspond to positions 1 to 35, 37 to 55, 57 to 162, and 167 to 275, respectively, in subtilisin BPN'.

In order to describe protease variants according to the invention, the following nomenclature is used: [Original Amino Acid; Position from N-terminus of the mature enzyme; Substituted Amino Acid]. For example, the substitution of valine with isoleucine at position 4 in BLAP is designated as V4I. The list of abbreviations for amino acids is shown in Table 1.

**TABLE 1**  
**Amino Acid Nomenclature**

	A	=	Ala	=	Alanine
	C	=	Cys	=	Cysteine
5	D	=	Asp	=	Aspartic acid
	E	=	Glu	=	Glutamic acid
	F	=	Phe	=	Phenylalanine
	G	=	Gly	=	Glycine
	H	=	His	=	Histidine
10	I	=	Ile	=	Isoleucine
	K	=	Lys	=	Lysine
	L	=	Leu	=	Leucine
	M	=	Met	=	Methionine
	N	=	Asn	=	Asparagine
15	P	=	Pro	=	Proline
	Q	=	Gln	=	Glutamine
	R	=	Arg	=	Arginine
	S	=	Ser	=	Serine
	T	=	Thr	=	Threonine
20	V	=	Val	=	Valine
	W	=	Trp	=	Tryptophan
	Y	=	Tyr	=	Tyrosine

Multiple mutations within the same protein molecule are indicated as the sum of individual mutations, i.e. S3T + V4I + A188P + V193M + V199I.

25 Protection against thermal and chemical inactivation and improvement of washing and cleaning performance are primary objectives in research and development of proteases for technical as well as for commercial applications. A large number of enzymes including subtilisins have been generated by random and site-specific mutagenesis and they provide some guidelines for a rational

30 approach to the improvement of thermal and chemical stability (Estell, D.A., Graycar, T.P., and Wells, J.A. (1985) *J. Biol. Chem.* **260**, 6518-6521; Matsumura, M., Becktel, W.J., Levitt, M., and Matthews, B.W. (1989) *Proc. Natl. Acad. Sci. USA* **86**, 6562-6566; Pantoliano, M.W., Whitlow, M., Wood, J.F., Rollence, M.L., Finzel, B.C., Gilliland, G.L., Poulos, T.L., and Bryan, P.N. (1988) *Biochemistry* **27**, 8311-8317; Russell, A.J. and Fersht, A.R. (1987) *Nature* **328**,

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496-500; Siezen, R.J., De Vos, W.M., Leunissen, J.A.M., and Dijkstra, B.W. (1991) *Protein Eng.* **4**, 719-737; van Ee, J.H. (1991) *Chimicaoggi* (7/8), 31-35; Wells, J.A. and Estell, D.A. (1988) *Trends Biochem. Sci.* **13**, 291-297). The modulation of enzymic activity, in particular rate enhancement or optimization for a subset of substrates, is a far more complex problem. EP 0260105 teaches the construction of subtilisin BPN' mutants with altered transesterification rate/hydrolysis rate ratios and nucleophile specificities by changing specific amino acid residues within 15 Å of the catalytic triad. Russell, A.J., and Fersht, A.R. (1987) *J. Mol. Biol.* **193**: 803-813, teach the isolation of a subtilisin BPN' mutant (D099S) that had a change in the surface charge 14 to 15Å from the active site. This substitution causes an effect on the pH dependence of the subtilisin's catalytic reaction. Neither of these publications teach how to predict amino acid alterations that will improve the wash performance of the protease. EP 0130756, EP 0247647, and US 4,760,025 teach a saturation mutation method where one or multiple mutations are introduced into the subtilisin BPN' at amino acid residues (BPN' numbering) Asp32, Asn155, Tyr104, Met222, Gly166, His64, Ser221, Gly169, Glu156, Ser33, Phe189, Tyr217, and/or Ala152. Using this approach mutant proteases exhibiting improved oxidative stability, altered substrate specificity, and/or altered pH activity are obtained. These publications also teach that mutations within the active site region of the protease are the most likely to influence activity. However, neither EP0130756, EP 0247647, nor US 4,760,025 teach a method for predicting amino acid alterations that will improve the wash performance of the protease.

Most of the information on the catalytic activity of subtilisins has been collected by examining the hydrolysis of small, well defined peptide substrates. Yet, little is known about interactions with large protein substrates. This is especially true for the wash performance of proteases where the substrate is attached to a textile surface and catalysis takes place in presence of interfering compounds such as bleach, tensides, and builders.

EP 0328229 teaches the isolation and characterization of PB92 subtilisin mutants with improved properties for laundry detergent applications based upon wash test results. It teaches that biochemical properties are not reliable

parameters for predicting enzyme performance in the wash. Methods for selection of mutations involve the substitution of amino acids by other amino acids in the same category (polar, nonpolar, aromatic, charged, aliphatic, and neutral), the substitution of polar amino acids asparagine and glutamine by charged amino acids, and increasing the anionic character of the protease at sites not involved with the active site. No method for identifying which specific amino acids should be altered is taught. Patent application WO 91/00345 (Novo-Nordisk) teaches a method to improve the wash performance of a subtilisin by the modification of the isoelectric point of subtilisin Carlsberg and subtilisin 309 to match the pH of the washing solution, where the enzyme is supposed to be used. However, since the highly alkaline subtilisins have pI values close to the pH of detergents under European washing conditions, this approach offers no obvious advantage. WO 91/00345 also teaches that changes in amino acids more than 15 Å from the catalytic triad can result in changes in the kinetic properties of the enzyme. A total of 116 different amino acids out of a total of 269 amino acids present in subtilisin 309 are suggested as possible sites for substitution, addition or deletion to modify the net electric charge of the enzyme.

Patent application WO 92/11357 (Novo-Nordisk) teaches increased pH-stability and improved washability of subtilisins by reduction of pH-dependent charges of the molecule. This means the introduction of mutations to approach substantial constancy of charge over a pH range. Preferably, an almost zero net charge change in the pH range from 7 to 11. European patent application No. 0 57 049 A1 discloses certain mutant proteolytic enzymes. These enzymes are said to have at least 70% homology with the amino acid sequence of PB92 serine protease and differ by at least one amino acid corresponding to 99, 102, 116, 126, 127, 128, 130, 160, 203, 211, and 212 in the PB92 serine protease. The mutant protease is prepared by growing a microorganism host strain transformed with an expression vector comprising a DNA sequence and encoding a mutant protease to produce the designed mutant protease.

The proteases hitherto proposed for use in detergents and washing aids were selected for high proteolytic activity. On account of their low

specificity, therefore, fiber damage or fiber destruction by the protease can occur in particular in the event of repeated washing of fabrics of proteinogenic fibers, for example sheet-form textiles of silk or wool. On the other hand, any reduction in protease activity results in a loss of cleaning power noticeable to the user of the detergent. Accordingly, one problem addressed by the present invention was to develop protease-containing detergents which would show reduced activity on proteinogenic fibers, more particularly wool, for substantially the same washing activity. Another problem addressed by the present invention was to develop soil-specific proteases which are proteases that remove specific stains on fabrics such as eggs stains and blood stains and detergent formulations containing such soil-specific proteases.

#### SUMMARY OF THE INVENTION

The wild-type protease from which the mutant proteases according to the invention are derived is a *Bacillus lentus* alkaline protease (BLAP) obtained from DSM 5483 having 269 amino acid residues, a molecular mass of 26,823 daltons and a calculated isoelectric point of 9.7 based on standard pK values. The BLAP gene is obtained by isolating the chromosomal DNA from the *B. lentus* strain DSM 5483, constructing DNA probes having homology to putative DNA sequences encoding regions of the *B. lentus* protease, preparing genomic libraries from the isolated chromosomal DNA and screening the libraries for the gene of interest by hybridization to the probes.

Mutants of *B. lentus* DSM 5483 protease with improved thermal and surfactant stability have been described in patent application Serial No. 07/706,691 filed May 29, 1991. In general the mutations described in this invention are introduced into wild-type BLAP with the following amino acid replacements: S3T, V4I, A188P, V193M, and V199I (numbering according to the BLAP sequence).

Research directed towards the binding of the protease to the high molecular protein substrate on the textile revealed the importance of the charged amino acids situated in the proximity of the substrate binding site. It could be shown that the removal of positively charged amino acid residues or the introduction of negatively charged amino acid residues in the region of the



substrate binding pocket of the protease leads towards better wash performance compared to the wild type.

The present invention also relates to detergent compositions for washing fabrics composed of proteinogenic fibers such as wool without causing damage to such fibers. The detergents are comprised of at least one surfactant and a proteolytically active amount of a protease having a keratinase/caseinase activity ratio of less than about 0.80.

Another aspect of the present invention relates to detergent compositions which are particularly effective in removing stains such as blood and egg stains and especially combinations of such stains from fabrics. The detergents are comprised of a combination of proteases which are *Bacillus lentus* DSM 5483 variants and at least one surfactant.

#### BRIEF DESCRIPTION OF THE FIGURES

**Figure 1** is a model of the substrate binding region of wild type BLAP with the synthetic substrate AAPF bound to the enzyme. The positions of arginine 99, serine 154 and leucine 217 in relation to the bound substrate are shown.

**Figure 2** shows the restriction map for *Escherichia coli* plasmid pCB13C which contains a hybrid gene fusion between the *Bacillus licheniformis* ATCC 53926 protease gene and the *Bacillus lentus* DSM 5483 BLAP gene. The promoter, ribosomal binding site and presequence (53926) from ATCC 53926 were fused to the pro and mature sequence of the BLAP gene. The transcription terminator of ATCC 53926 (T-53926) was appended to the BLAP coding sequence.

**Figure 3** shows the DNA sequence for the *Aval/ClaI* fragment from the N-terminal region of the ATCC 53926 alkaline protease gene discussed in Example 2. The fragment includes the putative promoter, ribosomal binding site, initiation codon, and most of the pre sequence. The 292 base pair fragment is flanked by *Aval* and *ClaI* restriction sites at its 5' and 3' ends, respectively.

**Figure 4** shows the restriction map for *E. coli* plasmid pMc13C which is derived from pMac5-8 and contains the BLAP gene. The ampicillin resistance ( $Ap^R$ ) gene present in pMc13C carries an amber mutation which renders it inactive. The plasmid still encodes resistance to chloramphenicol ( $Cm^R$ ).

**Figure 5** shows the restriction map for *Bacillus* plasmid pCB76M131 which contains the gene encoding BLAP variant M131. Included is the hybrid fusion between the ATCC 53926 protease and BLAP as described for pCB13C in Figure 2. Also present is the transcription terminator sequence from the 53926 alkaline protease gene. This is a pUB110 based plasmid which encodes resistance to kanamycin.

**Figure 6** shows a restriction map for *Bacillus* plasmid pH70 which is a derivative of plasmid pUB110 containing the ATCC 53926 alkaline protease gene. An *EcoRI/BamHI* fragment carrying the protease gene was cloned between the *EcoRI* and *BamHI* sites on pUB110. This plasmid is discussed in Example 3: Cloning of mutant protease genes. Plasmid pH70 encodes resistance to kanamycin.

**Figure 7** shows a restriction map for *Bacillus* plasmid pC51 which is a derivative of plasmid pBC16 carrying the ATCC 53926 alkaline protease gene. An *EcoRI/BamHI* carrying the protease gene was cloned between an *EcoRI* site and the *BamHI* sites on plasmid pBC16. This plasmid is discussed in Example 3: Cloning of mutant protease genes. Plasmid pC51 encodes resistance to tetracycline.

**Figure 8** shows a restriction map for *Bacillus* plasmid pBC56M131 which encodes the gene for BLAP variant M131. Included is the hybrid fusion between the ATCC 53926 alkaline protease gene and the BLAP gene as described in Figure 2 for pCB13C. Also present is the transcription terminator sequence from the ATCC 53926 alkaline protease gene. This is a pBC16 based plasmid which encodes resistance to tetracycline.

**Figure 9** shows the peptides produced by digestion of BLAP with trypsin. BLAP (11 mg/ml<sup>1</sup>) was digested in 1.6M urea, 0.16M NH<sub>4</sub>HCO<sub>3</sub> with trypsin (1%, w/w, in 1 mM HCl) for 10 min at 37 C. The sample was then acidified with 10% (v/v) Trifluoroacetic acid (TCA) to a final concentration of 1% TCA. Peptides were separated by injecting 10 ul of sample onto an HPLC column as described in Example 8. The tryptic peptides correspond to the following BLAP sequences: Fragment #1- Ala1 to Arg10; Fragment #2- Val11 to Arg19; Fragment #3- Gly20 to Lys27; Fragment #4- Val28 to Arg44; Fragment #5- Gly45 to Lys92; Fragment

#6- Val93 to Arg99; Fragment #7- Gly100 to Arg143; Fragment #8- Gly144 to Arg164; Fragment #9- Tyr165 to Arg180; Fragment #10- Ala181 to Lys229; Fragment #11- Gln230 to Lys231; Fragment #12- Asn232 to Arg241; Fragment #13- Asn242 to Lys245; and Fragment #14- Asn246 to Arg269.

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#### DEPOSIT OF MICROORGANISMS

*Bacillus licheniformis* E312 with plasmid pBC56M131 is deposited as **ATCC 68614**. *Escherichia coli* WK6 with plasmid pMC13C is deposited as **ATCC 68615**. *E. coli* GM33 with plasmid pCB13C is deposited as **ATCC 68616**. *E. coli* WK6 with plasmid pMa5-8 is deposited as **ATCC 68617**. *E. coli* WK6 with pMc5-8 is deposited as **ATCC 68618**.

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#### DESCRIPTION OF THE PREFERRED EMBODIMENTS

One aspect of the present invention relates to a method for choosing amino acid alterations which can result in a mutant protease with improved wash performance. Improved wash performance is obtained by introducing amino acid alterations within a region of the substrate binding pocket of the enzyme which results in an increased negative charge. According to the present invention, this can be achieved by increasing the number of negatively charged amino acids residues or decreasing the number of positively charged amino acid residue in the region of the substrate binding pocket of the protease within 7A of a bound substrate molecule such as AAPF. In particular, amino acid alterations at positions 99, 154 and 211 within *Bacillus lentus* Alkaline Protease (BLAP) variants M130 and M131 were shown to enhance the wash performance of the enzyme.

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A second aspect of the present invention relates to mutant proteolytic enzymes which have an improved wash performance relative to the wild type protease as determined by laboratory tests. The mutations described in this invention are introduced into BLAP variants M130 or M131 which have been previously described in patent application Serial No. 07/706,691 filed May 29, 1991. Both M130 and M131 have been shown to have improved stability as compared to the wild type protease. Mutant M130 contains four amino acid alterations: S3T; A188P/ V193M and V199I. Mutant M131 contains five amino acid alterations: S37; V41; A188P; V193M and V199I. The system used to

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designate preferred proteases first list the amino acid residue in the mature form of BLAP at the numbered position followed by the replacement amino acid using the accepted one letter amino acid codes. The amino acid sequences for proteases M130 and M131 are given in SEQ ID NO: 2 and SEQ ID NO: 1, respectively, contained in patent application Serial No. 07/706,691 filed May 29, 1991. Both M130 and M131 served as the basis for additional amino acid alterations to achieve proteases with improved wash performance. The mutant proteases according to the invention are those derived by the replacement of at least one amino acid residue of mutant proteases M130 or M131 wherein said amino acid residue is selected from the group consisting of arginine at position 99, serine at position 154 and leucine at position 211. Table 2 provides a description of the BLAP mutant proteases claimed in the invention which include F11, F43, F44, F45, F46, F47, F49, F54 and F55. For example F11 shown in Table 2 is derived from M130 and contains an arginine at position 99 replaced by a serine (R99S) along with the other mutations present in M130 which include: a serine at position 3 replaced by a threonine (S3T); an alanine at position 188 replaced by a proline (A188P); a valine at position 193 replaced by a methionine (V193M) and a valine at position 199 replaced by a isoleucine (V199I). Of the remaining mutant proteases, F43 through F49 are derived from M131. Mutants F54 and F55 are derived from mutant F49. The amino acid sequences of the preferred proteolytic enzymes F11, F43, F44, F45, F46, F47, F49, F54 and F55 are given in SEQ ID NO: 1 to SEQ ID NO: 9, respectively, of this application.

Another aspect of this invention relates to the genes which encode the mutant proteases listed in Table 2. The genetic construction of all mutant proteases in this invention are described in detail in Example 2. In all cases the mutations introducing the amino acid alterations are constructed using known procedures. The DNA sequences encoding the mature forms of the referred proteases F11, F43, F44, F45, F46, F47, F49, F54 and F55. Each hybrid gene encoding one of the mutant proteases listed above is comprised in the direction of transcription, a promoter, a ribosomal binding site, and initiation codon and the major portion of the pre region of the *B. licheniformis* ATCC 53926 alkaline protease gene operably linked to a portion of the pre region and all of the pro and

mature regions of a variant of the BLAP gene followed by the transcription terminator sequence for the alkaline protease gene from ATCC 53926. The hybrid gene may be integrated into the chromosome of the host or is carried on a plasmid which replicates within the *Bacillus* strain of choice. In particular, 5 plasmid pUB110 or a derivative of pUB110 is the plasmid of choice for protease production in strains of *Bacillus subtilis* and plasmid pBC16 or a derivative of pBC16 is the choice for protease production in strains of *B. licheniformis*. The mutant proteases are produced by growing the *Bacillus* strains transformed by plasmids containing the hybrid genes in a suitable medium.

10 Another aspect of the present invention relates to detergent compositions which contain at least one surfactant and a proteolytically active quantity of a protease having a keratinase/caseinase activity ratio (hereinafter referred to as the KC ratio) of less than about 0.80, preferably less than about 0.70 and more preferably in the range from about 0.2 to about 0.65.

15 The detergent preferably has a proteolytic activity in the range from about 100 PU/g to around 7500 PU/g.

The present invention also relates to the use of proteases with a KC ratio of less than about 0.80, preferably less than about 0.70 and more preferably in the range from about 0.2 to about 0.65 for washing textiles of proteinogenic fibers 20 or textiles consisting at least partly of proteinogenic fibers.

The KC ratio includes the activity of the protease with respect to two different substrates, casein and keratin.

25 The proteolytic activity of a protease solution which, in the case of casein as substrate, produces an absorption of 0.500 OD under the described measuring conditions, is defined as 10 PU (protease units) per ml. The cleaning result observed by the user of a protease-containing detergent correlates with this activity expressed in PU. The observed damage to a fabric of proteinogenic fibers correlates with the KC ratio for a constant cleaning result.

30 As expected in the light of the problem stated above, an only minimal difference in the activities towards casein and keratin was observed in the case of commercial proteases, the KC ratio being relatively close to 1. It was found that proteases with KC ratios of less than 0.80 have to be used to achieve a no

longer significantly noticeable degree of damage to proteinogenic fabric.

It has surprisingly been found that proteases having the properties mentioned above can synergistically influence the effect of certain other detergent ingredients and that, conversely, the proteolytic activity of the protease  
5 can be synergistically enhanced by certain other detergent ingredients. These effects occur in particular in the case where the surfactant component of the compositions according to the invention contain nonionic surfactants, soil-release copolyesters (more particularly those containing terephthalic acid units), water-insoluble organic builders, water-soluble inorganic and organic builders (more  
10 particularly based on oxidized carbohydrates) and synthetic anionic surfactants of the sulfate and sulfonate type, but not very strongly - if at all - in the case of alkylbenzenesulfonates. Detergents according to the invention preferably contain ingredients of which the properties are enhanced or which enhance the properties of the protease.

15 The proteases suitable for use in accordance with the invention include genetically engineered variants of *Bacillus lentus* DSM 5483. These variants can be prepared according to the method set forth in US patent application serial number 08/201,120 filed on 02/24/94, the entire contents of which is incorporated herein by reference and in US patent number 5,340,735. Preferred variants of  
20 *Bacillus lentus* DSM 5483 (BLAP) are listed in Table 2 in Example 2.

In reference to Table 2, the system used to identify the variants is the same as that used in US patent number 5,340,735 and explained therein. For example, mutant F49 is identified as S3T + V4I + A188P + V 193M + V199I + L211D. Such a protease is a mutant *Bacillus lentus* DSM 5483 variant wherein  
25 the serine residue at position 3 is replaced by threonine, the valine residue at position 4 is replaced by isoleucine, the alanine residue at position 188 is replaced by proline, the valine residue at position 193 is replaced by methionine, the valine residue at position 199 is replaced by isoleucine, and the leucine residue at position 211 is replaced by aspartic acid. The amino acid sequence of  
30 the wild-type BLAP protease is set forth as sequence ID number 52 in US patent number 5,340,735.

It has been found that some *Bacillus lentus* DSM 5483 variants showed

preferential activity toward blood stains while others showed preferential activity toward egg stains. Mutants that are particularly effective in removing blood and egg stains from fabrics are those mutants made by making the following replacements in the wild-type *Bacillus lentus* DSM 5483 protein: R99G, R99A, R99S, S154D, S154E, L221D, and L211E. Mutants having replacement amino acid residues at positions 99 and 154 are particularly effective in removing blood stains. Mutants having replacement amino acid residues at position 211 are particularly effective in removing egg stains. It has been found that a combination of mutant enzymes having replacement amino acid residues at positions 99 and 154 and those having replacement amino acid residues at position 211 are particularly effective in removing a combination of blood and egg stains.

Another aspect of the present invention relates to detergent compositions which are particularly effective in removing combinations of stains such as blood and egg stains from fabrics. The detergents are comprised of a combination of proteases which are *Bacillus lentus* DSM 5483 variants and a nonionic surfactant, an anionic surfactant, a soap, or a combination of such surfactants. Protease combinations include any two or more of the *Bacillus lentus* DSM 5483 variants listed in the Table above. A particularly preferred combination is F46 + F49 for removing stains comprised of blood and egg stains.

In one preferred embodiment, a detergent according to the invention contains nonionic surfactants selected from fatty alkyl polyglycosides, fatty alkyl polyalkoxylates, more particularly ethoxylates and/or propoxylates, fatty acid polyhydroxyamides and/or ethoxylation and/or propoxylation products of fatty alkylamines, vicinal diols, fatty acid alkyl esters and/or fatty acid amides and mixtures thereof, more particularly in a quantity of 2% by weight to 25% by weight.

In another preferred embodiment, a detergent according to the invention contains synthetic anionic surfactant of the sulfate and/or sulfonate type, more particularly fatty alkyl sulfate, fatty alkyl ether sulfate, sulfofatty acid esters and/or sulfofatty acid disalts, more particularly in a quantity of 2% by weight to 25% by weight. The anionic surfactant is preferably selected from the alkyl or alkenyl sulfates and/or the alkyl or alkenyl ether sulfates in which the alkyl or alkenyl

group contains 8 to 22 carbon atoms and, more particularly, 12 to 18 carbon atoms.

In another preferred embodiment, a detergent according to the invention contains water-soluble and/or water-insoluble builders, more particularly selected  
5 from alkali metal aluminosilicate, crystalline alkali metal silicate with a modulus of > 1, monomeric polycarboxylate, polymeric polycarboxylate and mixtures thereof, more particularly in quantities of 2.5% by weight to 60% by weight.

Another, albeit less preferred, embodiment of a detergent according to the invention intended for washing wool or silk fabrics may contain peroxygen-based  
10 bleaching agents, more particularly hydrogen peroxide, alkali metal perborate tetrahydrate, alkali metal perborate monohydrate and/or alkali metal percarbonate, more particularly in quantities of 5% by weight to 70% by weight, and optionally bleach activators, more particularly in quantities of 2% by weight to 10% by weight.

15 Finally, another embodiment of a detergent according to the invention contains soil-release agents based on copolyesters of dicarboxylic acids and glycols which may be present in particular in quantities of 0.01% by weight to 5% by weight. These soil-release agents, which are particularly effective by virtue of their chemical similarity to polyester fibers, but are also capable of developing  
20 the required effect in fabrics of other materials are copolyesters containing dicarboxylic acid units, alkylene glycol units and polyalkylene glycol units. Soil-release copolyesters of the type mentioned and their use in detergents have been known for some time.

The detergent compositions according to the invention contain at least one  
25 surfactant which includes soaps. The surfactants can be nonionic or anionic surfactants or a combination of nonionic and anionic surfactants or a combination of nonionic and anionic surfactants and one or more soaps.

Suitable nonionic surfactants include the alkoxyates, more particularly ethoxyates and/or propoxyates, of saturated or mono- to polyunsaturated linear  
30 or branched alcohols containing 10 to 22 carbon atoms and preferably 12 to 18 carbon atoms. The degree of alkoxylation of the alcohols is generally between 1 and 20 and preferably between 3 and 10. They may be obtained in known



manner by reaction of the corresponding alcohols with the corresponding alkylene oxides. Derivatives of the fatty alcohols are particularly suitable, although their branched-chain isomers, more particularly so-called oxoalcohols, may also be used for the production of useful alkoxylates. Accordingly, the

5 alkoxylates, more particularly ethoxylates, of primary alcohols containing linear groups, more particularly dodecyl, tetradecyl, hexadecyl or octadecyl groups, and mixtures thereof may be used. In addition, corresponding alkoxylation products of alkylamines, vicinal diols and carboxylic acid amides corresponding to the above-mentioned alcohols in regard to the alkyl moiety are also suitable. Alkyl

10 polyglycosides suitable for incorporation in the detergents according to the invention are compounds corresponding to the general formula  $(G)_n-OR^1$ , where  $R^1$  is an alkyl or alkenyl radical containing 8 to 22 carbon atoms, G is a glucose unit and n is a number of 1 to 10. The glycoside component  $(G)_n$  is an oligomer or polymer of naturally occurring aldose or ketose monomers, including in

15 particular glucose, mannose, fructose, galactose, talose, gulose, altrose, allose, idose, ribose, arabinose, xylose and lyxose. The oligomers consisting of such glucoside-bonded monomers are characterized not only by the type of sugars present in them, but also by their number, the so-called degree of oligomerization. As an analytically determined quantity, the degree of

20 oligomerization n is generally a broken number which may assume a value of 1 to 10 and, in the case of the glycosides preferably used, a value below 1.5 and, more particularly, between 1.2 and 1.4. Glucose is the preferred monomer unit by virtue of its ready availability. The alkyl or alkenyl substituent  $R^1$  of the glycosides also preferably emanates from readily accessible derivatives of renewable

25 raw materials, more particularly from fatty alcohols, although branched-chain isomers thereof, more particularly so-called oxoalcohols, may also be used for the production of useful glycosides. Accordingly, primary alcohols with linear octyl, decyl, dodecyl, tetradecyl, hexadecyl or octadecyl groups and mixtures thereof are particularly suitable. Particularly preferred alkyl glycosides contain

30 a cocofatty alkyl group, i.e. mixtures in which - essentially -  $R^1$  is dodecyl and  $R^1$  is tetradecyl.

The nonionic surfactant is preferably present in the detergents according

to the invention in quantities of 1% by weight to 30% by weight and more preferably in quantities of 1% by weight to 25% by weight.

In addition to or instead of the nonionic surfactant, the detergents according to the invention may contain other surfactants, preferably synthetic anionic surfactants of the sulfate or sulfonate type, in quantities of preferably not more than 20% by weight and, more preferably, in quantities of 0.1% by weight to 18% by weight, based on the detergent as a whole. Synthetic anionic surfactants particularly suitable for use in the detergents according to the invention are the alkyl and/or alkenyl sulfates containing 8 to 22 carbon atoms which contain an alkali metal, ammonium or alkyl- or hydroxyalkyl-substituted ammonium ion as counterion. Derivatives of fatty alcohols containing 12 to 18 carbon atoms and branched-chain analogs thereof, so-called oxoalcohols, are preferred. The alkyl and alkenyl sulfates may be prepared in known manner by reaction of the corresponding alcohol component with a typical sulfating agent, more particularly sulfur trioxide or chlorosulfonic acid, and subsequent neutralization with alkali metal, ammonium or alkyl- or hydroxyalkyl-substituted ammonium bases. Alkyl and/or alkenyl sulfates such as these are preferably present in the detergents according to the invention in quantities of 0.1% by weight to 20% by weight and more preferably in quantities of 0.5% by weight to 18% by weight.

Suitable surfactants of the sulfate type also include the sulfated alkoxylation products of the alcohols mentioned, so-called ether sulfates. These ether sulfates preferably contain 2 to 30 and, more particularly, 4 to 10 ethylene glycol groups per molecule. Suitable anionic surfactants of the sulfonate type include the  $\alpha$ -sulfoesters obtainable by reaction of fatty acid esters with sulfur trioxide and subsequent neutralization, more particularly the sulfonation products derived from  $C_{8-22}$  and preferably  $C_{12-18}$  fatty acids and  $C_{1-6}$  and preferably  $C_{1-4}$  linear alcohols, and the sulfofatty acids emanating from these sulfonation products through formal saponification.

Other optional surface-active ingredients include soaps, suitable soaps being saturated fatty acid soaps, such as the salts of lauric acid, myristic acid, palmitic acid or stearic acid, and soaps derived from natural fatty acid mixtures,

for example coconut oil, palm kernel oil or tallow fatty acids. Soap mixtures of which 50% by weight to 100% by weight consist of saturated C<sub>12-18</sub> fatty acid soaps and up to 50% by weight of oleic acid soap are particularly preferred. Soap is preferably present in quantities of 0.1% by weight to 5% by weight. However, larger quantities of soap of up to 20% by weight may be present in particular in liquid detergents.

A detergent according to the invention preferably contains 20% by weight to 55% by weight of water-soluble and/or water-insoluble, organic and/or inorganic builders. Water-soluble organic builders include, in particular, those from the class of polycarboxylic acids, more particularly citric acid and sugar acids, and the class of polymeric (poly)carboxylic acids, more particularly the polycarboxylates obtainable by oxidation of polysaccharides, polymeric acrylic acids, methacrylic acids, maleic acids and copolymers thereof which may even contain small amounts of polymerizable substances with no carboxylic acid functionality in copolymerized form. The relative molecular weight of the homopolymers of unsaturated carboxylic acids is generally in the range from 5,000 to 200,000 while the relative molecular weight of the copolymers is in the range from 2,000 to 200,000 and preferably in the range from 50,000 to 120,000, based on free acid. A particularly preferred acrylic acid/maleic acid copolymer has a relative molecular weight of 50,000 to 100,000. Suitable, but less preferred compounds of this class are copolymers of acrylic acid or methacrylic acid with vinyl ethers, such as vinylmethyl ethers, vinyl esters, ethylene, propylene and styrene, in which the acid makes up at least 50% by weight. Other suitable water-soluble organic builders are terpolymers containing two carboxylic acids and/or salts thereof as monomers and vinyl alcohol and/or a vinyl alcohol derivative or a carbohydrate as the third monomer. The first acidic monomer or its salt is derived from a monoethylenically unsaturated C<sub>3-8</sub> carboxylic acid and preferably from a C<sub>3-4</sub> monocarboxylic acid, more particularly from (meth)acrylic acid. The second acidic monomer or its salt may be a derivative of a C<sub>4-8</sub> dicarboxylic acid, preferably a C<sub>4-8</sub> dicarboxylic acid, maleic acid being particularly preferred. In this case, the third monomer unit is formed by vinyl alcohol and/or preferably by an esterified vinyl alcohol. Vinyl alcohol derivatives

in the form of an ester of short-chain carboxylic acids, preferably C<sub>1-4</sub> carboxylic acids, with vinyl alcohol are particularly preferred. Preferred terpolymers contain 60% by weight to 95% by weight and, more particularly, 70% by weight to 90% by weight of (meth)acrylic acid or (meth)acrylate, more preferably acrylic acid or acrylate, and maleic acid or maleate and 5% by weight to 40% by weight and preferably 10% by weight to 30% by weight of vinyl alcohol and/or vinyl acetate. Terpolymers in which the ratio by weight of (meth)acrylic acid or (meth)acrylate to maleic acid or maleate is between 1:1 and 4:1, preferably between 2:1 and 3:1 and more preferably between 2:1 and 2.5:1 are most particularly preferred (both the quantities and the ratios by weight being based on the acids). The second acidic monomer or its salt may even be a derivative of an allyl sulfonic acid substituted in the 2-position by an alkyl radical, preferably by a C<sub>1-4</sub> alkyl radical, or by an aromatic radical preferably derived from benzene or benzene derivatives. Preferred terpolymers contain 40% by weight to 60% by weight and more particularly 45% by weight to 55% by weight of (meth)acrylic acid or (meth)acrylate, more preferably acrylic acid or acrylate, 10% by weight to 30% by weight and preferably 15% by weight to 25% by weight of methallyl sulfonic acid or methallyl sulfonate and, as the third monomer, 15% by weight to 40% by weight and preferably 20% by weight to 40% by weight of a carbohydrate. This carbohydrate may be, for example, a mono-, di-, oligo- or polysaccharide, mono-, di- or oligosaccharides being preferred, sucrose being particularly preferred. Weak points responsible for the ready biodegradability of the polymer are presumably incorporated therein through the use of the third monomer. These terpolymers generally have a relative molecular weight in the range from 1,000 to 200,000, preferably in the range from 200 to 50,000 and more preferably in the range from 3,000 to 10,000. They may be used in the form of aqueous solutions, preferably 30 to 50% by weight aqueous solutions, particularly for the production of liquid detergents. All the polycarboxylic acids mentioned are generally used in the form of their water-soluble salts, more particularly their alkali metal salts.

Organic builders of the type in question are preferably present in quantities of up to 40% by weight, more preferably in quantities of up to 25% by weight and most preferably in quantities of 1% by weight to 5% by weight.

Quantities near the upper limit mentioned are preferably used in paste-form or liquid, more particularly water-containing, detergents according to the invention.

Crystalline or amorphous alkali metal aluminosilicates in particular are used as the water-insoluble, water-dispersible inorganic builders in quantities of up to 50% by weight and preferably in quantities of not more than 40% by weight and, in liquid detergents, in quantities of 1% by weight to 5% by weight. Among these aluminosilicates, crystalline sodium aluminosilicates in detergent quality, more particularly zeolite A, P and optionally X, are preferred. Quantities near the upper limit mentioned are preferably used in solid particulate detergents. Suitable aluminosilicates contain no particles larger than 30  $\mu\text{m}$  in size, at least 80% by weight consisting of particles below 10  $\mu\text{m}$  in size. Their calcium binding capacity is generally in the range from 100 to 200 mg CaO per gram.

Suitable substitutes or partial substitutes for the aluminosilicate mentioned are crystalline alkali metal silicates which may be present either on their own or in admixture with amorphous silicates. The alkali metal silicates suitable for use as builders in the detergents according to the invention preferably have a molar ratio of alkali metal oxide to  $\text{SiO}_2$  of less than 0.95 and, more particularly, in the range from 1:1.1 to 1:12 and may be present in amorphous or crystalline form. Preferred alkali metal silicates are the sodium silicates, more particularly amorphous sodium silicates, with a molar ratio of  $\text{Na}_2\text{O}$  to  $\text{SiO}_2$  of 1:2 to 1:2.8. Amorphous alkali metal silicates such as these are commercially available, for example, as PORTIL®. Those with a molar ratio of  $\text{Na}_2\text{O}$  to  $\text{SiO}_2$  of 1:1.9 to 1:2.8 are preferably used in solid form and not in the form of a solution in the production of detergents according to the invention. Preferred crystalline silicates, which may be present either on their own or in admixture with amorphous silicates, are crystalline layer silicates with the general formula  $\text{Na}_2\text{Si}_x\text{O}_{2x+1}\cdot y\text{H}_2\text{O}$ , in which x - the so-called modulus - is a number of 1.9 to 4 and y is a number of 0 to 20, preferred values for x being 2, 3 or 4. Preferred crystalline layer silicates are those in which x in the general formula assumes the value 2 or 3. Both  $\beta$ - and  $\delta$ -sodium disilicates ( $\text{Na}_2\text{Si}_2\text{O}_5\cdot y\text{H}_2\text{O}$ ) are particularly preferred.  $\delta$ -Sodium silicates with a modulus of 1.9 to 3.2 are preferred. Substantially water-free crystalline alkali metal silicates corresponding to the

above general formula, in which x is a number of 1.9 to 2.1 may also be used in detergents according to the invention. Another preferred embodiment of detergents according to the invention are crystalline sodium layer silicate having a modulus of 2 to 3. Crystalline sodium silicates with a modulus of 1.9 to 3.5 are used in another preferred embodiment of detergents according to the invention. The alkali metal silicate content of the detergents according to the invention is preferably 1% by weight to 50% by weight and more preferably 5% by weight to 35% by weight, based on anhydrous active substance. If an alkali metal aluminosilicate, more particularly zeolite, is also present as an additional builder, the alkali metal silicate content is preferably 1% by weight to 15% by weight and more preferably 2% by weight to 8% by weight, based on anhydrous active substance. In that case, the ratio by weight of aluminosilicate to silicate, based on anhydrous active substances, is preferably 4:1 to 10:1. In detergents containing both amorphous and crystalline alkali metal silicates, the ratio by weight of amorphous alkali metal silicate to crystalline alkali metal silicate is preferably 1:2 to 2:1 and more preferably 1:1 to 2:1.

In addition to the inorganic builder mentioned, other water-soluble or water-insoluble inorganic substances may be used in the detergents according to the invention. Alkali metal carbonates, alkali metal hydrogen carbonates and alkali metal sulfates and mixtures thereof are suitable in this regard. The additional inorganic material may be present in quantities of up to 70% by weight, but is preferably absent altogether.

To adjust an optionally acidic or mildly alkaline pH value of, in particular, about 8.0 to 9.5 in a 1% by weight aqueous solution, the detergents according to the invention may contain solid inorganic and/or organic acids or acidic salts, for example alkali metal hydrogen sulfates, succinic acid, adipic acid or glutaric acid and mixtures thereof. Acidic substances such as these are preferably present in the detergents according to the invention in quantities of not more than 5% by weight and, more particularly, in quantities of 0.1% by weight to 3% by weight.

The detergents according to the invention may additionally contain other typical detergent ingredients. These optional ingredients include, in particular,

complexing agents for heavy metals, for example aminopolycarboxylic acids, aminohydroxypolycarboxylic acids, polyphosphonic acids and/or aminopolyphosphonic acids, redeposition inhibitors, for example cellulose ethers, dye transfer inhibitors, for example polyvinyl pyrrolidones, foam inhibitors, for example organopolysiloxanes or paraffins, solvents and optical brighteners, for example stilbene disulfonic acid derivatives. The detergents according to the invention preferably contain up to 1% by weight and, more particularly, 0.01% by weight to 0.5% by weight of optical brighteners, more particularly compounds from the class of substituted 4,4'-bis-(2,4,6-triamino-s-triazinyl)-stilbene-2,2'-disulfonic acids, up to 5% by weight and, more particularly, 0.1% by weight to 2% by weight of complexing agents for heavy metals, more particularly aminoalkylene phosphonic acids and salts thereof, up to 3% by weight and, more particularly, 0.5% by weight to 2% by weight of deposition inhibitors and up to 2% by weight and, more particularly, 0.1% by weight to 1% by weight of foam inhibitors, the percentages by weight mentioned being based on the detergent as a whole.

Besides water, solvents which are used in particular in liquid detergents according to the invention are preferably water-miscible solvents, including lower alcohols, for example ethanol, propanol, isopropanol and isomeric butanols, glycerol, lower glycols, for example ethylene and propylene glycol, and the ethers derived from the classes of compounds mentioned.

The typical enzyme stabilizers optionally present, more particularly in liquid detergents according to the invention, include aminoalcohols, for example mono-, di-, triethanolamine and propanolamine and mixtures thereof, the lower carboxylic acids, boric acid or alkali metal borates, and boric acid/carboxylic acid combinations.

Suitable foam inhibitors include long-chain soaps, more particularly behenic soap, fatty acid amides, paraffins, waxes, microcrystalline waxes, organopolysiloxanes and mixtures thereof which, in addition, may contain microfine, optionally silanized or otherwise hydrophobicized silica. For use in particulate detergents according to the invention, foam inhibitors such as these are preferably deposited on granular water-soluble supports.

In addition, the detergent according to the invention may contain redeposition inhibitors. The function of redeposition inhibitors is to keep the soil detached from the fibers suspended in the liquor and thus to prevent discoloration of the fibers. Suitable redeposition inhibitors are water-soluble, generally organic colloids, for example the water-soluble salts of polymeric carboxylic acids, glue, gelatine, salts of ether carboxylic acids or ether sulfonic acids or starch or cellulose or salts of acidic sulfuric acid esters of cellulose or starch. Water-soluble polyamides containing acidic groups are also suitable for this purpose. Soluble starch preparations and other starch products than those mentioned above, for example partly hydrolyzed starch, may also be used. Sodium carboxymethyl cellulose, methyl cellulose, methyl hydroxyethyl cellulose and mixtures thereof are preferably used.

The bleaching agents which may be used as further ingredients of detergents according to the invention are the per compounds generally used in detergents, such as perborate which may be present as tetrahydrate or monohydrate, percarbonate, perpyrophosphate and persilicate which are generally present in the form of alkali metal salts, more particularly sodium salts. Bleaching agents such as these are preferably present in detergents according to the invention in quantities of up to 25% by weight, more preferably in quantities of up to 15% by weight and most preferably in quantities of 5% by weight to 15% by weight, based on the detergent as a whole.

The bleach activators present as an optional component include the N-acyl or O-acyl compounds typically used, for example polyacylated alkylenediamines, more particularly tetraacetyl ethylenediamine, acylated glycolurils, more particularly tetraacetyl glycoluril, N-acylated hydantoins, hydrazides, triazoles, urazoles, diketopiperazines, sulfurylamides and cyanurates, also carboxylic anhydrides, more particularly phthalic anhydride, carboxylic acid esters, more particularly sodium isononanoyl phenol sulfonate, and acylated sugar derivatives, more particularly pentaacetyl glucose. To avoid interaction with the per compounds during storage, the bleach activators may have been coated with shell-forming substances or granulated in known manner, tetraacetyl ethylenediamine granulated with carboxymethyl cellulose with



average particle sizes of 0.01 mm to 0.8 mm, and/or granulated 1,5-diacetyl-2,4-dioxohexahydro-1,3,5-triazine, being particularly preferred. The bleach activators are preferably present in detergents according to the invention in quantities of up to 8% by weight and more particularly in quantities of 2% by weight to 6% by weight, based on the detergent as a whole.

Enzymes optionally present in addition to protease include, in particular, enzymes from the class of lipases, cutinases, amylases, cellulases, pullulanases, oxidases and peroxidases and mixtures thereof. Enzymes obtained from fungi or bacterial strains are preferably used.

The amylases suitable for use in detergents according to the invention include the enzymes obtainable from bacteria or fungi which have a pH optimum preferably in the alkaline range up to about pH 10. Useful commercial products are, for example, TERMAMYL® and MAXAMYL®. Amylase is preferably used in the detergent according to the invention in such quantities that the final detergent contains 0.01 KNU/g to 2 KNU/g ("Kilo Novo Units" per gram according to the standard method of the Novo Company, 1 KNU being the quantity of enzyme which degrades 5.26 kg of starch at pH 5.6/37°C as determined by the method described by Methods in Enzymology, Vol. 1, 1955, page 149), preferably 0.015 KNU/g to 1.8 KNU/g and more preferably 0.03 KNU/g to 1.6 KNU/g.

The cellulase suitable for use in accordance with the invention also belongs to the enzymes obtainable from bacteria or fungi which have a pH optimum preferably in the almost neutral to mildly alkaline pH range from 6 to 9.5. They are preferably used in the detergent according to the invention in such quantities that the final detergent has a cellulolytic activity of 0.05 IU/g to 1.5 IU/g ("International Units" per gram, based on the enzymatic hydrolysis of Na carboxymethyl cellulose at pH 9.0/40°C, preferably 0.07 IU/g to 1.4 IU/g and more preferably 0.1 IU/g to 1.3 IU/g. Suitable commercial products are, for example, CELLUZYME®, a product of Novo Nordisk, or KAC®, a product of Kao.

The enzymes may be adsorbed in known manner onto supports, encapsulated in shell-forming substances and/or granulated in conjunction with support substances to make them easier to handle and to protect them against

premature inactivation if they are to be incorporated in particulate detergents. The enzymes optionally present in addition to protease are preferably present in detergents according to the invention in quantities of up to 2% by weight and more preferably in quantities of 0.01% by weight to 1.5% by weight, based on the  
5 detergent as a whole.

In one preferred embodiment, a detergent according to the invention is particulate and contains 20% by weight to 55% by weight of inorganic builder, up to 15% by weight and more particularly from 2% by weight to 12% by weight of water-soluble organic builder, 2.5% by weight to 20% by weight of synthetic  
10 anionic surfactant, 1% by weight to 20% by weight of nonionic surfactant, up to 25% by weight and, more particularly, from 1% by weight to 15% by weight of bleaching agent, up to 8% by weight and, more particularly, from 0.5% by weight to 6% by weight of bleach activator and up to 20% by weight and, more particularly, from 0.1% by weight to 15% by weight of inorganic salts, more  
15 particularly alkali metal carbonate and/or sulfate, and up to 2% by weight and, more particularly, from 0.4% by weight to 1.2% by weight of particulate enzymes besides protease, more particularly lipase, cutinase, amylase, cellulase, pullulanase, oxidase and/or peroxidase.

In another preferred embodiment, a powder-form detergent according to the invention intended in particular for use as a light-duty detergent contains 20%  
20 by weight to 55% by weight of inorganic builders, up to 15% by weight and, more particularly, from 2% by weight to 12% by weight of water-soluble organic builders, from 4% by weight to 24% by weight of nonionic surfactant, up to 15% by weight and, more particularly, from 1% by weight to 10% by weight of  
25 synthetic anionic surfactant, up to 65% by weight and, more particularly, from 1% by weight to 30% by weight of inorganic salts, more particularly alkali metal carbonate and/or sulfate, and neither bleaching agents nor bleach activators.

Another preferred embodiment of the detergent according to the invention is a liquid detergent containing 5% by weight to 35% by weight of water-soluble  
30 organic builders, up to 15% by weight and, more particularly, from 0.1% by weight to 5% by weight of water-insoluble inorganic builders, up to 15% by weight and, more particularly, from 0.5% by weight to 10% by weight of synthetic

anionic surfactant, from 1% by weight to 25% by weight of nonionic surfactant, up to 15% by weight and, more particularly, from 4% by weight to 12% by weight of soap and up to 30% by weight and, more particularly, from 1% by weight to 25% by weight of water and/or water-miscible solvent and also up to 10% by weight and, more particularly, from 0.01% by weight to 7.5% by weight of an enzyme stabilizing system. The protease in each of the above preferred embodiments is variant F49 having the amino acid substitutions S3T + V4I + A188P + V193M + V199I + L211D.

Particulate detergents according to the invention may be produced very easily by mixing the individual particles in a standard mixer, more particularly a drum mixer, roller mixer, ribbon mixer or free-fall mixer, other optional powder-form components and, if desired, even liquid or liquefied components, including in particular nonionic surfactants, but also dyes and fragrances, optionally being incorporated by spraying. The components capable of withstanding high temperatures are preferably converted into a powder-form product in known manner by spray drying of an aqueous slurry and the powder obtained is mixed with protease and, optionally, other enzymatic substances and other heat-sensitive components, including in particular bleaching agents. The incorporation of individual constituents by mixing in granules or an extrudate containing them is also possible and is particularly preferred for the production of detergents according to the invention with high apparent densities of, preferably, 650 g/l to 900 g/l. Flowable or liquid detergents according to the invention may be produced simply by mixing the ingredients or compounds thereof which may be present in liquid form or in the form of a solution in water or a given solvent.

### Example 1

#### Identification of Sites in BLAP for Mutagenesis

A variety of natural subtilisins and other serine proteases were tested for their washing efficacy. The results showed that washability relates to the number and distribution of charged amino acid residues in the substrate binding region. An improvement in the wash performance was seen with an increased number of

negatively charged amino acid residues or with a decrease of the number of positively charged amino acid residues in the substrate binding region. Accordingly, the BLAP mutants claimed herein have a reduced positive net charge in the substrate binding region and they show improved washability.

5 Improved wash performance was obtained by introducing amino acid alterations within the substrate binding pocket which resulted in a variation in charge. Alterations were made for amino acids residing within the substrate binding pocket of either BLAP variant M130 or BLAP variant M131. The substrate binding pocket of these enzymes is defined as the region within 7 Å of  
10 a bound substrate molecule, AAPF. Figure 1 shows the characteristic structure of the substrate binding pocket of wild type BLAP with the synthetic substrate AAPF. AAPF bound to the enzyme was modeled on crystallographic data of subtilisin-inhibitor complexes found in the literature and available from the Brookhaven Protein Data Bank. The structure of BLAP variants M130 and M131  
15 within the substrate binding pocket is essentially identical to wild type BLAP based upon Xray crystallography. The structure of wild type BLAP to a 1.4Å resolution has been published in patent application Serial No. 07/706,691 filed May 29, 1991 and the corresponding atomic coordinates deposited with the Brookhaven Protein Data Bank. In particular, amino acid alterations at positions  
20 99, 154 and 211 within BLAP variants M130 and M131 were shown to enhance the wash performance of the enzyme. The parental amino acids occupying these sites, Arginine 99, Serine 154, and Leucine 211 are depicted in Figure 1. All three amino acids are located within the specified 7Å radius of AAPF.

## Example 2

### 25 Construction of Mutant Genes of the BLAP Gene

#### A. Construction of Mutants M130 and M131

Genes which express the mutant *B. lentus* DSM 5483 proteases according to the invention are made by altering one or more codons of the wild-type *B. lentus* DSM 5483 alkaline protease gene. Protease M130 was derived  
30 from BLAP by introducing mutations S3T, A188P, V193M and V199I. Protease M131 was derived from BLAP by introducing the mutations S3T, V4I, A188P, V193M, and V199I. Genes encoding proteases M130 and M131 were

constructed using the pMac procedure (Stanssens, P., Opsomer, C., McKeown, Y.M., Kramer, W., Zabeau, M., and Fritz, H.-J. (1989) *Nucleic Acids Res.* **17**, 4441-4445). Proteases M130 and M131 have been previously described in patent application Serial No. 07/706,691 filed May 29, 1991. Proteases M130 and M131 exhibit improved thermal and surfactant stability over the wild type BLAP and served as the basis for developing proteases with improved wash performance. The genetic techniques used to modify the BLAP gene to produce the M130 and M131 proteases have also been described in patent application WO 91/02792. The description in WO 91/02792 incorporated herein by reference for purposes of indicating the state of the art.

Proteases M130 and M131 were derived from the *B. lentus* DSM 5483 alkaline protease (BLAP) by site-specific mutagenesis of DNA encoding the mature form of wild type BLAP. The DNA fragment encoding the mature form of wild type BLAP was prepared using plasmid pCB13C (Figure 2). Plasmid pCB13C contains a hybrid fusion between the *B. licheniformis* ATCC 53926 protease gene and the *B. lentus* DSM 5483 BLAP gene. Specifically, this hybrid fusion contains DNA encoding the promoter, ribosomal binding site, and 21 residues of the pre sequence from the ATCC 53926 protease gene fused to a DNA sequence encoding the last five residues of the BLAP pre sequence and all of the pro and mature residues of BLAP. This fusion is referred to as the *Cla*I fusion because this restriction site is located at the juncture between the ATCC 53926 and DSM 5483 DNA's. A new *Cla*I restriction site had to be introduced into the ATCC 53926 alkaline protease gene near to the junction of the pre and pro sequences. The *Cla*I site was introduced into the ATCC 53926 alkaline protease gene by using the polymerase chain reaction (PCR) to amplify a DNA fragment containing sequence information from the N-terminal part of the ATCC 53926 alkaline protease gene. The amplified fragment included the ATCC 53926 alkaline protease promoter, ribosomal binding site, initiation codon, and most of the pre sequence. The DNA sequence of this fragment is shown in Figure 3. This 292 bp DNA fragment was flanked by *Ava*I and *Cla*I restriction sites at its 5' and 3' ends, respectively. The BLAP gene already contained a naturally occurring *Cla*I site at the corresponding position. Analysis of the DNA sequence

across the fusion of the ATCC 53926 and BLAP genes confirmed the expected DNA and amino acid sequences.

To perform mutagenesis the BLAP gene is subcloned into the mutagenesis vector pMa5-8 . This is accomplished by synthesizing a DNA  
5 fragment containing the *Cla*I fusion gene and the ATCC 53926 transcription terminator as a *Sa*II cassette using the PCR. The PCR was carried out using conditions as described by the manufacturer (Perkin Elmer Cetus, Norwalk, CT). In the PCR, two synthetic oligonucleotides bearing *Sa*II sites are used as primers and *Escherichia coli* vector pCB13C DNA as a template. After cutting the PCR  
10 product with *Sa*II, this fragment is cloned into the mutagenic plasmid pMc5-8 which has previously been cut with *Sa*II and dephosphorylated with bacterial alkaline phosphatase. Plasmids pMc5-8, and pMa5-8 were obtained from H.-J. Fritz and are described by Stanssens, P., et al. (1989) *Nucleic Acids Res.* **17**, 4441-4454. *Sa*II sites are chosen to allow the PCR fragment to be cloned into  
15 pMc5-8 in both orientations. The ligation mix is transformed into *E. coli* WK6. Chloramphenicol resistant ( $Cm^R$ ) transformants are screened for the presence of an insert and a correct plasmid construct pMc13C is identified as shown in Figure 4. Once the gene is cloned into the pMc5-8 vector and desirable sites for mutation are identified, the mutation(s) is introduced using synthetic DNA  
20 oligonucleotides according to a modification of a published protocol (Stanssens, P., et. al. (1989) *Nucleic Acids Res.* **17**, 4441-4454). The oligonucleotide containing the mutation(s) to be introduced is annealed to a gapped duplex (gd) structure which carries the BLAP gene on a segment of single stranded (ss) DNA. The gapped duplex can be formed by annealing linear ss DNA from  
25 pMc13C with denatured and restricted pMa5-8 DNA. Plasmid pMa5-8 contains an active ampicillin resistance gene but has an inactivating point mutation in the chloramphenicol resistance gene, whereas plasmid pMc13C contains, in addition to an intact BLAP gene, an active chloramphenicol resistance gene, but has an inactivating point mutation in the ampicillin resistance gene. The  
30 annealed product is the gd DNA which is a double stranded heteroduplex with a ss DNA gap spanning the entire cloned BLAP gene. The mutant oligonucleotide is able to anneal to homologous ss BLAP DNA within the gap and

the remaining gap is filled in by DNA polymerase I (Klenow fragment) and ligated using T4 DNA ligase (New England Biolabs Inc., Beverly, MA [NEB]). The mutagenic efficiency of such a system can be improved by the use of Exonuclease III (*Exo III*, NEB). *Exo III* is an exodeoxyribonuclease that digests double stranded DNA from the 3' end. As a free 3' end is required, closed circular ss DNA or ds DNA is unaffected by this enzyme. A subsequent treatment of the product of the fill-in reaction with *Exo III* removes any species with only partially filled gaps. This significantly improves the mutagenic efficiency and is the preferred mutagenesis method. The product of the fill-in reaction is then transformed into a repair deficient *E. coli* strain (WK6mutS) and ampicillin resistant transformants (Ap<sup>R</sup>) are selected. Replication of the transformed heteroduplex plasmid results in two different progenies. One progeny contains the wild type BLAP gene and the intact chloramphenicol resistance gene, but an inactive ampicillin resistance gene. The other progeny contains a BLAP gene carrying the mutation of interest and is resistant to ampicillin but not to chloramphenicol.

Selection of Ap<sup>R</sup>, Cm<sup>S</sup> mutant transformants with ampicillin is not sufficient to stop some background growth of the Ap<sup>S</sup>, Cm<sup>R</sup> progeny carrying the wild type BLAP gene. Therefore, it is necessary to perform a second transformation into *E. coli* using plasmid DNA prepared from the Ap<sup>R</sup> transformants of the WK6mutS strain. This second transformation uses a low plasmid concentration with a large number of recipient cells of a suppressor deficient strain of *E. coli* such as WK6. This approach decreases the likelihood of a recipient cell receiving plasmid DNA from both progeny. Ap<sup>R</sup> transformants are selected and plasmid DNA from several transformants is isolated and screened for the presence of the mutation. The pMa mutant derivative of the first mutagenesis round can be used for a second round of mutagenesis by preparing ss DNA of that species and annealing it to *Xba*I/*Hind*III restricted and denatured DNA of pMc5-8. Plasmid pMc5-8 is identical to pMa5-8 except that it contains an active chloramphenicol resistance gene and an inactive ampicillin resistance gene. The general procedure is the same as that described above. The construction of the genes encoding proteases M130 and M131 required two rounds of mutagenesis. In the first

round of mutagenesis an oligonucleotide was designed to introduce mutations A188P, V193M, and V199I. In a second round of mutagenesis an oligonucleotide was designed to introduce mutation S3T in the case of M130 and mutations S3T and V4I in the case of M131. The presence of all of these mutations was verified by DNA sequencing.

**B. Construction of Genes Encoding New Proteases with Improved Wash Performance.**

Mutations R99G, R99A, R99S, S154D, S154E and L211D were introduced into the gene encoding M131 protease using PCR mutagenesis by overlap extension. Mutation R99S was introduced into the gene encoding protease M130 using PCR mutagenesis by overlap extension. Construct pCB76M131 (Figure 5) was used to construct mutants F43, F44, F45, F46, F47, and F49 (Table 2). Construct pCB76M130 was used to construct mutant F11 and construct pCB76F49 (a newly constructed mutant) was used to construct mutants F54 and F55.



TABLE 2

## Description of BLAP Mutants

	BLAP	wild type enzyme
	M130	S3T + A188P + V193M + V199I
5	M131	S3T + V4I + A188P + V193M + V199I
	F11	S3T + R99S + A188P + V193M + V199I
	F43	S3T + V4I + R99G + A188P + V193M + V199I
	F44	S3T + V4I + R99A + A188P + V193M + V199I
	F45	S3T + V4I + R99S + A188P + V193M + V199I
10	F46	S3T + V4I + S154E + A188P + V193M + V199I
	F47	S3T + V4I + S154D + A188P + V193M + V199I
	F49	S3T + V4I + A188P + V 193M + V199I + L211D
	F54	S3T + V4I + R99G + A188P + V 193M + V199I + L211D
	F55	S3T + V4I + S154E + A188P + V193M + V199I + L211D
15		Materials required to perform this procedure include: A DNA Thermal Cycler (Perkin Elmer Cetus); GeneAmp kit (Perkin Elmer Cetus); AmpliWax (Perkin Elmer Cetus); and 0.5 ml sterile polypropylene tubes (Perkin Elmer Cetus); Microcon-100 concentrators (Amicon, Beverly, MA); TE buffer (10 mM tris-(hydroxymethyl)aminomethane [Tris], 1 mM disodium ethylenediamine
20		tetraacetic acid (EDTA), adjusted to pH 8 with 2 N HCl); Minigel electrophoresis apparatus (Hoefer Scientific, San Francisco, CA); 1% (w/v) SeaKem agarose gel (FMC, Rockland, ME) in TBE buffer (0.089 M Tris, 0.089 M boric acid, 2 mM EDTA); 0.5 $\mu\text{g}\cdot\text{ml}^{-1}$ ethidium bromide in water; restriction enzymes NheI (NEB), XbaI (NEB); and SstI (BRL, Gaithersburg, MD).
25		PCR's were carried out using the GeneAmp kit and AmpliWax as specified by the manufacturer. They were subjected to 1 cycle of denaturation (3 minutes, 95°C), annealing (2 minutes, 50°C) and extension (2 minutes, 72°C) and 30 cycles of denaturation (1 minute, 94°C), annealing (1 minutes, 50°C) and extension (1 minute, 72°C) using a DNA Thermal Cycler. Each cycle was
30		extended for 10 sec at 72°C.
		Site-directed mutagenesis by overlap extension is described by (Ho, S.N., Hunt, H.D., Horton, R.M., Pullen, J.K., and Pease, L.R. (1989) Gene 77, 51-59).

For BLAP mutants F43, F44, F45, F46, F47 and F49 10 ng of pCB76M131 plasmid DNA was used as template for the initial round of mutagenesis. For BLAP mutant F11 10 ng of pCB76M130 plasmid DNA was used as template, and for BLAP mutants F54 and F55 10 ng of pCB76F49 DNA was used as template.

5 The pUB110 forms of these plasmids were chosen because they provide higher yields of protease in the *B. subtilis* DB104 host than the pBC16 forms of the plasmids. PCR fragments were checked by agarose gel electrophoresis and cleaned using a Microcon-100 concentrator (Amicon) after each round of PCR. After the second round of PCR, the fragments were digested with either

10 *NheI/XbaI* or *NheI/SstI* (depending on the location of the intended mutation) and cloned back into pCB76M131 DNA in the case of mutants F43, F44, F45, F46, F47 and F49, pCB76M130 DNA in the case of mutant F11 and pCB76F49 in the case of mutants F54 and F55 using *B. subtilis* DB104 competent cells as previously described. Plasmid DNA isolation was accomplished using ion

15 exchange minicolumns (QIAGEN, Inc., Chatsworth, CA) and mutations were checked by Sanger ds DNA sequencing as previously described.

### Example 3

#### Cloning of Mutant Protease Genes

##### A. Cloning of Mutant Proteases Produced via the pMac System.

20 Proteases M130 and M131 can be produced by transferring the respective gene encoding either M130 or M131 from the particular *E. coli* pMc13C derivative vector into a plasmid vector which can replicate in *Bacillus*. To accomplish this, the desired mutant gene is separated from the appropriate pMc13C plasmid by digestion with the restriction endonucleases *AvaI* and *SstI*, followed by ligation

25 to the larger *AvaI/SstI* fragment from either plasmid pH70 (Figure 6) or pC51 (Figure 7). These *AvaI/SstI* fragments from pH70 and pC51 include the DNA sequences necessary for replication in *Bacillus* and encode either kanamycin resistance ( $Km^R$ ) or tetracycline resistance ( $Tc^R$ ), respectively. Plasmid pH70 is constructed by cloning the ATCC 53926 alkaline protease gene carried on a

30 *EcoRI/BamHI* DNA fragment into the  $Km^R$  plasmid pUB110 between the *EcoRI* and *BamHI* sites. Plasmid pC51 is constructed by cloning the ATCC 53926

protease gene carried on a *EcoRI/BamHI* fragment into the Tc<sup>R</sup> plasmid pBC16 between the *EcoRI* and *BamHI* sites. The larger *AvaI/SstI* fragment from either pH70 or pC51 used for cloning the DNA fragment encoding either M130 or M131 is first purified from other plasmid DNA fragments by high pressure liquid chromatography (HPLC) on an anion exchange column (Gen-Pak FAX, 4.6 mm diameter, 100 mm long; Waters, Milford, MA). Conditions for elution of the DNA are a flow rate of 0.75 ml·min<sup>-1</sup> with a gradient from 50% of Buffer A (25 mM Tris, containing 1 mM EDTA and adjusted to pH 8.0 with 2 N HCl) and Buffer B (25 mM Tris, containing 1 mM EDTA, 1 M NaCl, and adjusted to pH 8.0 with 2 N HCl) to 30% of Buffer A and 70% of Buffer B in 30 min.

The two ligated DNA's are transformed into *B. subtilis* DB104. The genes encoding the major alkaline and neutral proteases present in this strain have been inactivated (Kawamura, F. and Doi, R.A. (1984) J. Bacteriol. **160**, 442-444). Cells of *B. subtilis* DB104 transformed by these plasmids grow on a nutrient-skim milk agar in the presence of either kanamycin or tetracycline. Transformants of DB104 that manufacture mutant protease are identified by the formation of clear zones of hydrolysis in the skim milk. Confirmation that the protease-producing transformants carry a plasmid-borne M130 or M131 gene with the desired mutation(s) is accomplished by purifying plasmid DNA from a culture of each transformant. The plasmid DNA is purified away from cell protein and chromosomal DNA by SDS-salt precipitation followed by chromatography over a QIAGEN ion-exchange column (QIAGEN, Inc., Chatsworth, CA). *AvaI/SstI* digested plasmid DNAs from different transformants are compared with *AvaI/SstI*-digested derivatives of plasmid pH70 or pC51 known to carry an intact BLAP gene. Restriction digests of these plasmids are compared by agarose gel electrophoresis to identify plasmids that have the proper-sized *AvaI/SstI* DNA fragments. Selected plasmid DNAs are then sequenced across the region of the expected M130 or M131 mutations to confirm that the desired mutation(s) are present. Genes M130 and M131 cloned into the derivative of plasmid pC51(Tc<sup>R</sup>) are designated plasmids pCB56M130 and pCB56M131 respectively (Figure 8), while the same genes cloned into a derivative of plasmid pH70 are designated plasmids pCB76M130 and pCB76M131 respectively (Figure 5). One or more

clones of each BLAP mutation are stored frozen in 15% glycerol at -70°C and also cultivated in shake flasks to produce mutant protease for characterization.

#### **B. Cloning of Mutant Proteases Produced via PCR Overlap Procedure.**

5 As described above, the amplified PCR fragments are cloned back into plasmids pCB76M130, pCB76M131 or pCB76F49. For expression in the *B. licheniformis* production strain the *Ava*I/*Sst*I fragment carrying the modified M130, M131 or F49 genes is cloned back into the pC51 type vector as described previously.

10

#### **Example 4**

##### **Production of Mutant Proteases**

Wild type BLAP protein and mutant proteins were produced by transformed *Bacillus subtilis* DB 104 in shake flasks. A hot loop was used to streak each mutant strain from a frozen cryovial culture onto an LB-skim milk agar containing either 20 µg·ml<sup>-1</sup> of kanamycin or 15 µg·ml<sup>-1</sup> of tetracycline. The plates were incubated at 37°C for 20 to 24 hours. A single, isolated colony producing a good zone of hydrolysis of the skim milk was picked into a 250 ml Erlenmeyer flask containing about 50 ml Luria Broth (LB) which contained either 20 µg·ml<sup>-1</sup> kanamycin or 15 µg·ml<sup>-1</sup> of tetracycline. The broth was incubated in a New Brunswick Series 25 Incubator Shaker at 37°C with shaking at 280 rpm for 7 to 8 hours. Either 2.5 ml of the turbid preculture was transferred into 50 ml of MLBSP containing either 20 µg·ml<sup>-1</sup> kanamycin or 15 µg·ml<sup>-1</sup> of tetracycline in each of four baffled 500 ml flasks, or 5 ml of preculture was used as an inoculum for 100 ml of MLBSP broth with antibiotic contained in each of two 500 ml baffled flasks (a 5%, v/v, transfer). All flasks were incubated at 240 rpm and 37°C for 64 hours. After 64 hours of incubation the content of a set of flasks for each culture was consolidated, transferred to 50 ml centrifuge tubes, and centrifuged at 20,000 x g<sub>av</sub> for 15 minutes at 4°C. The broth was filtered through Miracloth (Calbiochem Corp., #475855) into 400 ml beakers placed on ice. The pH of the solution was adjusted to 5.8 by the addition of glacial acetic acid. After 30 minutes of stirring fine debris were removed by centrifugation at 20,000 x g<sub>av</sub> for

15 minutes. The volume of the supernatant was recorded. Aliquots of 1 ml were set aside for analysis by native and denaturing polyacrylamide gel electrophoresis (PhastSystem, Pharmacia), for determination of total proteolytic activity by the HPE method, and for active site titration. The broth was stored on ice until the protease could be purified. For long-term storage or for shipment the broth was mixed with an equal volume of propane-1,2-diol. The composition of the MLBSP media used for the production of BLAP in shake flask cultures is described in Table 3.

**TABLE 3**

10

**Composition of MLBSP Media<sup>1</sup>**

Component	Quantity (for 1 liter of media)
deionized water	750 ml
Difco Casitone	10 g
Difco Tryptone	20 g
15 Difco Yeast Extract	10 g
NaCl	5 g
Sodium Succinate	27 g

<sup>1</sup> The pH of the media was adjusted to 7.2 with 2 N NaOH, the volume was brought to 815 ml with water, and the solution was autoclaved for 15 minutes at 121°C at a pressure of 15 pounds per square inch. After cooling the sterile stock solutions described in Appendix 1 were added with stirring. Either a kanamycin or tetracycline stock solutions was added to the media just before use to a final concentration of 20 µg·ml<sup>-1</sup> and 15 µg·ml<sup>-1</sup>, respectively.

20

**APPENDIX 1****(Additions to MLBSP Broth)**

Component	Quantity (for 1 liter of media)
<del>MgSO<sub>4</sub>·7H<sub>2</sub>O</del>	<del>(100mg·ml<sup>-1</sup>stock) 1.0 ml</del>
5 CaCl <sub>2</sub> ·2H <sub>2</sub> O	(30 mg·ml <sup>-1</sup> stock) 2.5 ml
FeSO <sub>4</sub> ·7H <sub>2</sub> O	(1 mM stock) 0.5 ml
MnCl <sub>2</sub> ·4H <sub>2</sub> O	(1 mM stock) 0.5 ml
Glucose	(25% (w/v) stock) 80.0 ml
PIPES Buffer <sup>1</sup>	(pH 7.2, 1 M stock) 50.0 ml
10 Phosphate Buffer <sup>2</sup>	(pH 7.0, 1.5 M stock) 50.0 ml

<sup>1</sup> Piperazine-N,N'-bis(2-ethane sulfonic acid).

<sup>2</sup> A sufficient amount of 1.5 M dibasic phosphate (K<sub>2</sub>HPO<sub>4</sub>) was added to 200 ml of 1.5 M monobasic phosphate (KH<sub>2</sub>PO<sub>4</sub>) to adjust the pH to 6.0 using a pH meter (Beckman, model pH144) equipped with a combination electrode (Beckman, model #3952C). The final pH was adjusted to 7.0 with 4 M KOH.

15 All stock solutions were autoclaved at 121°C, 15 psi for 15 min except for the FeSO<sub>4</sub>·7H<sub>2</sub>O which was filter sterilized using a 0.22 µm filter system (Costar 8301).

**Example 5**

20

**Purification of BLAP and its Mutant Proteins**

If not mentioned otherwise, all subsequent steps were performed on ice or at 4°C. Broth samples as obtained in Example 3 were filtered through a 0.2 µm filter, and the filtrate was concentrated by ultrafiltration. The retaining membrane had a nominal molecular-weight-cut-off of 10,000 mounted in a

25 Minitan cassette (Millipore) or in a ultrafiltration cell (Amicon). For subsequent shipment or extended storage broth solutions were mixed with an equal volume of propane-1,2-diol to stabilize the protease. Otherwise, the concentrate was dialyzed for 16 hours against 20 mM sodium phosphate, pH 7.0 ('phosphate buffer'), or against 20 mM N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid) (HEPES), containing 1 mM CaCl<sub>2</sub> and adjusted to pH 7.8 with NaOH

30 ('HEPES buffer'). The dialysate was clarified by centrifugation (20,000 x g<sub>av.</sub> for

10 minutes) and the pH of the solution, if necessary, was re-adjusted. The samples dialyzed against phosphate buffer were equilibrated for 15 minutes with 5% (w/v) DEAE-cellulose previously equilibrated in the same buffer to trap negatively charged proteins. After removal of the DEAE-cellulose by centrifugation or filtration the supernatant was loaded onto a cation exchange column (S-Sepharose Fast Flow, Pharmacia; 16 mm diameter, 90 mm long, 18 ml bed volume, 50 ml·h<sup>-1</sup> flow rate) previously equilibrated with phosphate buffer. The samples dialyzed against HEPES buffer were applied directly to a cation exchange column (S-Sepharose Fast Flow, Pharmacia; 15 mm diameter, 75 mm long, 13 ml bed volume, 50 ml·h<sup>-1</sup> flow rate) previously equilibrated with HEPES buffer. When all colored by-products were eluted the column was washed with 3 to 5 column volumes of application buffer. Protease was eluted from the cation exchanger by including 1.0 M NaCl into phosphate buffer or 0.25 M NaCl into HEPES buffer. Fractions containing the active enzyme were pooled.

The enzyme purified in phosphate buffer was concentrated and desalted by ultrafiltration using Centricon tubes (molecular-weight-cut-off 10,000; Amicon). The protein concentration was determined by the bicinchoninic acid method (BCA method, Pierce Chemical Co., Rockford, IL). For stabilization of the protease 50% (v/v) propane-1,2-diol was added to the stock solution.

The pooled fractions with enzyme purified in HEPES buffer were mixed with a 5 to 8-fold volume excess of acetone at -20°C. The protein was allowed to precipitate for 4 minutes, and the mixture was centrifuged for 4 minutes at 6,600 x g<sub>av.</sub>. The supernatant was discarded, the pellet was briefly exposed to vacuum (water aspirator) to remove most of the acetone, and the pellet was dissolved in 20 mM 2-(N-morpholino)ethanesulfonic acid (MES), containing 1 mM CaCl<sub>2</sub> and adjusted to pH 5.8 with 2 N NaOH, to give an approximate protein concentration of 30 mg·ml<sup>-1</sup>. The solution was clarified by centrifugation in an Eppendorf centrifuge for 3 minutes at full speed (13,000 x g<sub>max.</sub>) and stored frozen until used. The protein concentration was determined by the biuret method (Gornall, A.G., Bardawill, C.S., and David, M.M. (1948) J. Biol. Chem. 177, 751-766).

### Example 6

#### Determination of Active Protease

The active enzyme concentration was determined by active site titration with the inhibitor phenylmethyl sulfonylfluoride. A protease solution was prepared in 10 mM sodium phosphate, pH 6.5, at an approximate concentration of 100  $\mu$ M based on protein determination. Inhibitor concentrations were equivalent to an estimated molar ratio of 0.25, 0.5, 0.75, 1.0 and 1.25. The mixtures were allowed to react for one hour at room temperature. Residual enzyme activity was measured spectrophotometrically by the AAPF-pNA method (see below).

### Example 7

#### Determination of Proteolytic Activity

Two different protease assays were used. With the HPE method protease activity was established at a single concentration of casein as substrate. In the AAPF-pNA assay initial rates of succinyl-L-alanyl-L-alanyl-L-prolyl-L-phenylalanyl-p-nitroanilide (AAPF-pNA; Bachem Bioscience, Philadelphia, PA) supported catalysis were used to determine the kinetic parameters  $K_m$ ,  $k_{cat}$ , and  $k_{cat}/K_m$ .

##### A. Protease Assay by the HPE Method

Proteolytic activity was determined by a discontinuous assay using casein as a substrate. The final concentrations of the substrate solution were 12 mg·ml<sup>-1</sup> of casein (prepared according to Hammarsten; Merck, Darmstadt, #2242) and 30 mM Tris in synthetic tap water. Synthetic tap water is a solution of 0.029% (w/v) CaCl<sub>2</sub>·2H<sub>2</sub>O, 0.014% (w/v) MgCl<sub>2</sub>·6H<sub>2</sub>O, and 0.021% (w/v) NaHCO<sub>3</sub> with a hardness of 15°dH (deutsche Härte, German hardness). The substrate solution is heated to 70°C and pH is adjusted to 8.5 at 50°C using 0.1 N NaOH. The protease solution is prepared with 2% (w/v) anhydrous pentasodium tripolyphosphate in synthetic tap water, adjusted to pH 8.5 with hydrochloric acid. To 600  $\mu$ l of casein substrate solution 200  $\mu$ l of enzyme solution is added. The mixture is incubated at 50°C for 15 minutes. The reaction is terminated by the addition of 600  $\mu$ l of 0.44 M trichloroacetic acid (TCA), 0.22 M sodium acetate in 3% (v/v) glacial acetic acid. After cooling on ice for 15 minutes the TCA-insoluble protein is removed by centrifugation, an aliquot of



900  $\mu$ l is mixed with 300  $\mu$ l of 2N NaOH and the absorbance of this mixture containing TCA-soluble peptides is recorded at 290 nm. Control values are produced by adding 600  $\mu$ l of TCA solution to 600  $\mu$ l of casein solution followed by 200  $\mu$ l of enzyme solution.

5 A protease solution which produces under the conditions of this assay an absorbance change of 0.500 OD at 290 nm is declared to have an activity 10 HPE per ml. Data for BLAP and BLAP mutant proteins are summarized below (Table 4).

**B. Protease Assay With the Chromogenic Substrate AAPF-pNA**

10 Protease samples were diluted with 50% (v/v) 1,2-propanediol in 100 mM Tris, adjusted with 2 N HCl to pH 8.6 at 25°C ('Tris-propanediol buffer'), in which they were stable for at least 6 h at room temperature. A stock solution of 160 mM AAPF-pNA was prepared in dimethylsulfoxide dried over molecular sieve beads (Aldrich; 4 Å, 4-8 mesh) for at least 24 h prior to use. Fixed point  
15 assays were performed at 25°C with 1.6 mM AAPF-pNA in 100 mM Tris, adjusted with 2 N HCl to pH 8.6 at 25°C, in a total volume of 1.020 ml. The substrate was added to the assay buffer 1 minute prior to the assay initiation and the reaction was started by addition of enzyme at a final concentration of 20 ng to 1.3  $\mu$ g of protein per ml (0.75 to 48.5 nM enzyme) depending on specific  
20 activity. Release of *p*-nitroanilide was monitored at 410 nm, and a molar extinction coefficient of 8,480  $M^{-1}cm^{-1}$  was used to calculate amount and concentration of product formed (DelMar, E.G., Largman, C., Brodrick, J.W., and Geokas, M.C. (1979) *Anal Biochem.* **99**, 316-320).

Kinetic parameters were calculated from a velocity vs. substrate  
25 concentration plot constructed from initial rates measured once each at 12 different AAPF-pNA concentrations ranging from 0.16 to 3.2 mM. Data were fitted to a hyperbolic curve and proportionally weighted using the program ENZFITTER (Leatherbarrow, R.J. (1987) *ENZFITTER*. Biosoft, Cambridge, UK). A nominal molecular weight of 26.8 kDa was used in all calculations that required  
30 the interconversion of protein concentration and molarity of protease enzyme (Table 4).

**TABLE 4**  
**Kinetic Data of BLAP and BLAP Mutant Proteases**

Protease	Specific	$k_{cat}$	$K_m$	$k_{cat} \cdot K_m^{-1}$	
Label	Activity				
	(HPE·mg <sup>-1</sup> )	(s <sup>-1</sup> )	(mM)	(s <sup>-1</sup> ·mM <sup>-1</sup> )	
5	BLAP	4000	312	1.05	297
	M131	3670	153	0.98	156
	F11	3760	174	1.88	92
	F43	3780	171	1.73	99
10	F44	3750	257	2.49	103
	F45	3710	208	3.05	68
	F46	3180	358	2.0	179
	F47	3610	249	2.0	124
	F49	4610	38	3.5	11
15	F54	2510	7	5.5	1.3
	F55	3200	11	4.3	2.6

### Example 8

#### Protein Structure Verification

Mutations introduced into the BLAP were verified by DNA sequence analysis through the immediate vicinity of the point of mutation. However, spontaneous mutations outside the region of site-directed mutagenesis are known to occur and it was imperative to establish that the determined property of a mutant protease indeed resides with the amino acid sequence deduced from the nucleotide sequence. Therefore, a tryptic map of BLAP was produced (Figure 9) which was verified by amino acid sequence analysis of the individual peaks and to which all mutant BLAP proteins were compared. Not only in peptides with  $\leq 17$  amino acid residues, but also in peptides 5T, 7T, and 10T (Figure 9) with 48, 44, and 49 amino acid residues, a single (even conservative) substitution resulted in a significant change in retention time under the conditions of the separation. Particularly close calls were resolved by co-digestion of equal quantities of the reference (BLAP) and the mutant protein.

An aliquot of up to 5 mg of protease from a stock solution was placed on ice in a 2.2-ml Eppendorf tube, then mixed with 1.0 ml of 0.15 N HCl and water (both chilled) to give a final concentration of 3.33 mg · ml<sup>-1</sup> protein and 0.1 N HCl in a total volume of 1.5 ml. After the mixture had been incubated for 30 minutes, protein was precipitated by the addition of 165 µl of chilled 50% (w/v) trichloroacetic acid (TCA). The precipitate was allowed to stand on ice for 5 minutes and then pelleted by centrifugation for 4 minutes at 13,000 × g<sub>max</sub> (Eppendorf centrifuge). The pellet was washed once with 1 ml of 80% (v/v) acetone and briefly dried *in vacuo*.

All reagent solutions and water needed for the tryptic digest were passed through a 0.45 µm filter (Ultrafree-MC, Millipore Products) prior to use. The pellet of the denatured protein (5 mg; 185 nmol) was dissolved in 90 µl of 0.8 M ammonium bicarbonate, containing 8 M urea. This solution was slowly diluted with 360 µl of water and then passed by centrifugation through a 0.45 µm filter. Subsequent steps were carried out in 0.5-ml siliconized microtubes (Phenix Res. Products). An aliquot of 300 µl was mixed with 13 µl of 2.5 mg·ml<sup>-1</sup> trypsin in 1 mM HCl (mass ratio of BLAP:trypsin = 100:1). For a control, 100 µl of the protein solution was mixed with 4.5 µl of 1 mM HCl. The remaining 50 µl aliquot of protein solution was mixed with 5 µl of 10% (v/v) trifluoroacetic acid (TFA) and used as control of the starting material. The two other solutions were incubated for 10 minutes at 37°C. The reactions were terminated by adding 30 µl and 10 µl of 10% (v/v) TFA to the digest and the control, respectively. The peptide mixture was separated by reverse-phase HPLC.

The HPLC equipment was from Waters and consisted of an autosampler (model 715 Ultra Wisp), a dual pump system (model 600E) and a diode array detector (model 990). Sampling and gradient formation was governed by Waters' software program '990+ Powerline'. Tryptic peptides were separated on a C<sub>18</sub> column (Vydac model 218TP54; 4.6 x 250 mm; 5 µ particle size; 300 Å pore size). In line with the separation column was a C<sub>18</sub> guard column (Vydac model 218FSK104, 10 µ particle size). Separation column and guard column were housed in a column heater set to 30±1°C. The solvent system used was: Solvent A = 0.1% (v/v) TFA in water; Solvent B = 0.08% (v/v) TFA in acetonitrile. After sample loading the C<sub>18</sub> column was developed for 3 minutes with Solvent A

followed by a gradient from 0 to 35% (v/v) of Solvent B in Solvent A in 70 minutes. At 70 minutes the gradient increased to 100% Solvent B in 15 minutes and then returned to 100% Solvent A in 15 minutes. Prior to the next injection, the column was equilibrated for at least 20 minutes with Solvent A.

5 Absorbance changes were recorded at 215 nm and at 280 nm. Quantities of peptides mixtures separated for analytical and preparative purposes ranged from 11  $\mu\text{g}$  (0.4 nmol) to 500  $\mu\text{g}$  (18 nmol) in a volume of 5 to 50  $\mu\text{l}$  at concentrations from 2.2 to 10  $\text{mg}\cdot\text{ml}^{-1}$ .

10 For peptide sequencing the eluate was hand-fractionated according to absorbance changes observed on the attached recorder. Previous studies with  $\beta$ -mercaptoethanol showed that the delay time from the time of recording to the time of elution from the system was less than 2 seconds. Fractions were concentrated *in vacuo* in a Speed Vac Concentrator (Savant, Hicksville, NY) to less than 100  $\mu\text{l}$  and brought to 100  $\mu\text{l}$  with aqueous TFA to achieve a final TFA  
15 concentration of 1%. Care was taken that during concentration the samples did not reach complete dryness.

### Example 9

#### Characterization of the Proteases by Tenside Stability

20 Stability of the protease mutants to tensides was tested with SDS as typical anionic detergent. Stability was tested in 50 mM sodium carbonate, pH 10.5 at 50°C, containing 1% (w/v) SDS. Protease proteins were incubated at a final protein concentration of 0.25  $\text{mg}\cdot\text{ml}^{-1}$ . Periodically, an aliquot was removed from the incubation mixture and diluted into Tris-propanediol buffer chilled on ice. Residual protease activity was determined by the AAPF-pNA assay at  
25 a substrate concentration of 1.1 mM. Stability is expressed as half-life ( $t_{1/2}$ ) of activity determined from semi-logarithmic plots of residual activity as function of time.

### Example 10

#### Wash Performance of the Protease Mutants

30 The wash performance was tested in a specially developed washing test using cotton swatches soiled with egg and soot (ER) and with blood, milk and soot (BMR). The washing tests were performed in an Atlas launderometer (type LP 2), equipped with stainless steel test vessels each containing a defined

detergent composition plus the protease to be tested.

The pre-washed cotton was soiled with a defined amount of soil and air-dried for 6 days. The launderometer beakers were filled with 1 swatch of soiled and 3 swatches of unsoiled cotton. Ten metal balls (10 mm diameter) were added for mechanical treatment. The washing time was 30 minutes with a final temperature of 30°C reached after 4 minutes of heating.

Laundry detergents for these tests were of typical composition for European usage (Jakobi, G. and Löhr, A. (1987) *Detergents and Textile Washing*, VCH, Weinheim, Germany). The concentrations of a delicate fabric detergent for easy-care and colored fabrics (5-15% (w/w) anionic surfactants, 1-5% (w/w) nonionic surfactants), a heavy duty compact detergent (8% (w/w) anionic surfactants, 6% (w/w) nonionic surfactants, with tetraacetythylenediamine (TAED) and perborate bleach) and a super compact detergent concentrate (18% (w/w) anionic surfactants, 2.5% (w/w) nonionic surfactants, with TAED and perborate bleach) were 0.5 g, 0.5 g, and 0.4 g, respectively, in 100 ml of water at 16°dH (deutsche Härte, German hardness). The super compact detergent is an extruded detergent granulate, described by a number of patent applications (WO 91/02047, WO 91/13678, WO 93/02176, WO 93/15180, WO 94/01526). In all cases the pH was 10.4. A protease was added to washing solutions on the basis of its enzymatic activity measured in HPE at a ratio of 0, 50, 100, 200, 300, 400, 500, 700 and 1000 HPE per gram of detergent.

Subsequent to washing, the fabric was rinsed with running tap water, air-dried and ironed. The enzymatic washing effect was determined by the change ( $\Delta\text{Rem}$ ) of the remission (%Rem) at 440 nm (400-500 nm) measured on a Dr. Lange color difference measuring instrument (Micro Color).  $\Delta\text{Rem}$  is the difference in remission after washing with added protease and the remission after washing without added protease. Results from the wash performance tests are presented in Tables 5 and 6.

The improvement in washing performance was determined by the ratio of wild type enzyme necessary to achieve a standard  $\Delta\text{Rem}$ , versus the amount of mutant enzyme to achieve an identical effect. Thus an improvement of 2 indicates that half of the mutant enzyme is needed to get the same effect as with

the wild type enzyme.

**TABLE 5**

**Washing Effect of BLAP and BLAP Mutant Proteins on Blood-Milk-Soot-Stains**

5	Enzyme	Detergent		
		A <sup>1</sup>	B	C
		Improvement Ratio <sup>2</sup>	Improvement Ratio	Improvement Ratio
	BLAP	1	1	1
10	M131	1	1	1
	F43	2.8	1.8	0.7
	F44	2.0	1.8	<0.7
	F45	2.6	1.1	<0.7
	F46	2.9	1.9	1
15	F47	1.5	1.8	0.8
	F49	1.8	1	2.5

<sup>1</sup> Detergent **A** is a Heavy Duty Compact Detergent, Detergent **B** is a Heavy Duty Super Compact Detergent and Detergent **C** is a Delicate Fabric Detergent.

20 <sup>2</sup> For definition see text.

TABLE 6

**Washing Effect of BLAP and BLAP Mutant Proteins on Egg-Soot-Stains**

	Enzyme	Detergent	
		Heavy Duty Compact Detergent	Heavy Duty Super Compact Detergent
5			
		Improvement Ratio <sup>1</sup>	Improvement Ratio
	BLAP	1	1
	M131	1	1
	F46	1.5	1
10	F47	1	1
	F49	4.0	1.7

<sup>1</sup> For definition see text.

**Example 11**

An enzymatic detergent composition is prepared by mixing about 30 wt% of a concentrated preparation of a protease mutant as defined in claim 6, with about 5 wt% of cellulose, 5 wt% of saccharose, 20 wt% of wheat flour, 30 wt% of starch, 5 wt% of carboxymethylcellulose and 5% of polyethylene glycol (mw 20,000). The resulting mixture is granulated by extrusion granulation. After drying, the granulate has an activity of 70,000 to 250,000 HPE/g. The granulate is coated with polyethylene glycol (mw 6000) containing TiO<sub>2</sub>. The granulated protease enzyme is then mixed with 100g Heavy Duty Compact Detergent (Porsil supra) containing sodium perborate\TAED resulting in a standard protease activity of 1200 HPE/g.

**Example 12**

An enzymatic detergent composition is prepared according to Example 11 except the protease is replaced by the protease as defined in claim 7.

**Example 13**

A liquid enzymatic detergent composition is prepared by mixing together at room temperature about 13 wt% C10-C13 linear alkylbenzene-sulfonic acid, about 5 wt% alkylpolyglycoside(C12-C14), about 10 wt% of a C13 alcohol polyethoxylate having 7 EO units, about 6 wt% lauric acid, about 7 wt% of oleic acid, about 5 wt% triethanolamine, about 5 wt% propanediol 1,2, about 2 wt sodium hydroxide, about 1 wt% citric acid, about 7 wt% ethanol, about 1 wt% citric acid about 7 wt% ethanol, about 1-hydroxyethane-1,1-diphosphonic acid and the remainder being water.

The pH of the resulting solution is 8.1 (measured as a 10% aqueous solution). A sufficient amount of the protease as defined in claim 6 is added to yield a composition having about 0.5 wt% of liquid protease concentrate (1250 HPE/g).

**Example 14:**

The KC ratio of protease F49 and, for comparison, the KC ratios of three commercial proteases were determined by the method described above. The following results were obtained:

<u>Protease</u>	<u>KC ratio</u>
MAXACAL® <sup>a</sup>	0.92
SAVINASE® <sup>b</sup>	0.80
BLAP®	0.83
F49	0.63

a) Manufacturer: Gist Brocades, Netherlands

b) Manufacturer: Novo Nordisk, Denmark

**Example 15**

1 g of wool yarn was added to 50 ml of an F 49-containing sodium carbonate buffer solution (pH 10.5) with a proteolytic activity of 5.6 PU/ml and the whole was left to react for 1 hour at room temperature. The quantity of amino acids released and peptides was then determined with TNBS (control determination in buffer solution without protease). The wool damage value obtained under these conditions for the protease BLAP was put at 100%. Under these conditions, the protease F 49 had a value of 31%.

**Example 16**



The test described in Example 2 was repeated using 1 g of silk yarn. The following silk damage values were obtained:

	Protease	Silk damage
	BLAP	100 %
5	ESPERASE® <sup>a</sup>	165 %
	SAVINASE® <sup>a</sup>	89 %
	F 49	28 %

a) Manufacturer: Novo Nordisk, Denmark

### Example 17

10 In a MIELE® Electronic W 793 washing machine, articles of clothing of wool were washed 9 times at 40°C (water hardness 16°d, liquor ratio 1:15) in the presence of ballast washing of cotton (total load 1.2 kg). 98 g of an enzyme-free coloreds detergent (control test), to which 49 ml of a BLAP 140 solution (comparison test) or an equally active quantity, expressed in PU of F 49  
15 (invention) had been added, were used in the wash liquor. The washed woollen fabrics were dried and pieces of specific dimensions were cut out and weighed. The reduction in weight, expressed in % of the value before washing, is shown in the following Table:

a) Blend of 80% Merino wool and 20% polyamide

20

### Example 18

Example 4 was repeated using grey socks of pure wool, the proteases again being used in quantities of equal activity (1080 PU/g of the detergent); in contrast to Example 4, 5 washes were carried out at 60°C. The reduction in weight was only 10.7% where the protease F 49 was used and 40.5% where  
25 BLAP® was used.

### Example 19

A solution containing 12 g/l of the substrate (casein or keratin) and 30 mM of sodium triphosphate in water with a hardness of 15°dH (containing 0.058% by weight of CaCl<sub>2</sub>·2H<sub>2</sub>O, 0.028% by weight of MgCl<sub>2</sub>·6H<sub>2</sub>O and 0.042%  
30 by weight of NaHCO<sub>3</sub>) is heated to 70°C and the pH is adjusted to a value of 8.5 by addition of 0.1 N NaOH at 50°C. 200 µl of a solution of the enzyme to be

tested in 2% by weight sodium tripolyphosphate buffer solution (pH 8.5) are added to 600  $\mu$ l of the substrate solution. The reaction mixture is incubated for 15 minutes at 50°C. The reaction is then terminated by addition of 500  $\mu$ l of TCA solution (0.44 M trichloroacetic acid and 0.22 M sodium acetate in 3% by volume acetic acid) and cooling (ice bath at 0°C, 15 minutes). The protein insoluble in TCA is removed by centrifugation and 900  $\mu$ l of the supernatant phase are diluted with 300  $\mu$ l of 2 N NaOH. The absorption of this solution at 290 nm is determined with an absorption spectrometer, the absorption zero value having to be determined by measurement of a centrifuged solution prepared by mixing 600  $\mu$ l of the above-mentioned TCA solution with 600  $\mu$ l of the above-mentioned substrate solution and subsequent addition of the enzyme solution.

#### Example 20

Pre-washed cotton fabrics were soiled with equal amounts of soil and air dried for 3 days. Launderometer beakers were filled with 6 swatches of soiled and unsoiled cotton and 10 metal balls (1 cm in diameter) and washed in an Atlas LP-2 type laundrometer for 30 minutes with a final temperature of 30°C, which was reached after 4 minutes of heating. A fine specialty detergent formulation was used for easy-care and colored fabrics (0.75 g/100 ml). A heavy duty compact detergent (0.5 g/100 ml) and a super compact detergent concentrate (0.4 g/100 ml) were also used. The composition of the detergents is described in "Detergents and Textile Washing", G. Jacobi & A. Löhner, VCH, Weinheim, ISBN 0-89573-687-X, 1987. The water used was 16° dH (German Hardness) and the pH was 4. Enzyme activities of the detergents were 0, 50, 100, 200, 300, 400, 500, 700, and 1000 HPE/g detergent. After washing, the test swatches were rinsed in tap water, air dried, and ironed. The enzymatic washing effect was determined by the change ( $\Delta$ Rem) in the remission (%Rem) at 440 nm measured using a Dr. Lange color difference measuring instrument Micro Color,  $\Delta$ Rem being the difference in remission after wash with protease added and the remission after wash without protease. The results of washing performance for various enzyme-containing detergents and detergent compositions containing a combination of enzymes are given in Tables 2 and 3 below. Each entry is a ratio of the amount of mutant enzyme achieving a standard  $\Delta$ Rem, versus the amount of the wild-type enzyme to achieve an

identical cleaning effect. A entry of 2 indicates that twice as much of the wild-type enzyme was needed as a particular mutant to achieve the same cleaning effect. The data also show that a detergent composition containing a combination of mutants F46 and F49 produced a greater cleaning effect than the average of  
 5 the individual enzymes.

Table 2  
 Washing Effect of Enzymes on  
 Blood-Milk-Soot Stains

Enzyme	Detergent	
	HeavyDuty	Heavy Duty Compact
BLAP/M131	1	1
F46	2.9	1.9
F49	1.8	1.0
F46+F49	2.6	2.2

Table 3  
 Washing Effect of Enzymes on  
 Egg-Soot Stains

Enzyme	Detergent	
	HeavyDuty	Heavy Duty Compact
BLAP/M131	1	1
F46	1.5	1.3
F49	4.0	1.7
F46+F49	3.3	2.2

25

### Example 21

A similar composition as described in example 13 is prepared except the protease is replaced by the protease as defined in claim 7.

## SEQUENCE LISTING

## (1) GENERAL INFORMATION:

(i) APPLICANT: Wilson, Charles R

(ii) TITLE OF INVENTION: Proteases with Improved Wash  
5 Performance

(iii) NUMBER OF SEQUENCES: 18

(iv) CORRESPONDENCE ADDRESS:

(A) ADDRESSEE: Henkel Corporation

10 (B) STREET: 140 Germantown Pike, Suite 150

(C) CITY: Plymouth Meeting

(D) STATE: Pennsylvania

(E) COUNTRY: USA

(F) ZIP: 19462

15 (v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk

(B) COMPUTER: IBM PC compatible

(C) OPERATING SYSTEM: PC-DOS/MS-DOS

(D) SOFTWARE: PatentIn Release #1.0, Version

#1.25

20 (vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER:

(B) FILING DATE:

(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:

25 (A) NAME: Drach, John E.

(B) REGISTRATION NUMBER: 32891

(C) REFERENCE/DOCKET NUMBER: H0587

(ix) TELECOMMUNICATION INFORMATION:

(A) TELEPHONE: 215-832-2215

5 (B) TELEFAX: 215-941-6067

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 807 base pairs

(B) TYPE: nucleic acid

10 (C) STRANDEDNESS: double

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

15 (vi) ORIGINAL SOURCE:

(B) STRAIN: Bacillus lentus DSM 5483

(vii) IMMEDIATE SOURCE:

(B) CLONE: S3T, R99S, A188P, V193M, V199I

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

20 GCGCAAACAG TGCCATGGGG AATTAGCCGT GTGCAAGCCC CGGCTGCCCA TAACCGTGGA 60

TTGACAGGTT CTGGTGTAAA AGTTGCTGTC CTCGATACAG GTATTTCCAC TCATCCAGAC 120  
 TTAAATATTC GTGGTGGCGC TAGCTTTGTA CCAGGGGAAC CATCCACTCA AGATGGGAAT 180  
 GGGCATGGCA CGCATGTGGC CGGGACGATT GCTGCTTTAA ACAATTCGAT TGGCGTTCTT 240  
 GGCCTAGCGC CTAGTGCGGA ACTATACGCT GTTAAAGTTT TAGGAGCCGA CGGTAGCGGT 300  
 5 GCAATCAGCT CGATTGCCCA AGGGTTGGAA TGGGCAGGGA ACAATGGCAT GCACGTTGCT 360  
 AATTTGAGTT TAGGAAGCCC TTCGCCAAGT GCCACACTTG AGCAAGCTGT TAATAGCGCG 420  
 ACTTCTAGAG GCGTTCTTGT TGTAGCGGCA TCTGGGAATT CAGGTGCAAG CTCAATCAGC 480  
 TATCCGGCCC GTTATGCGAA CGCAATGGCA GTCGGAGCTA CTGACCAAAA CAACAACCGC 540  
 GCCAGCTTTT CACAGTATGG CCCAGGGCTT GACATTATGG CACCAGGGGT AACATTCAG 600  
 10 AGCACATACC CAGGTTCAAC GTATGCCAGC TTAAACGGTA CATCGATGGC TACTCCTCAT 660  
 GTTGCAAGTG CAGCAGCCCT TGTTAAACAA AAGAACCCAT CTTGGTCCAA TGTAACAATC 720  
 CGCAACCATC TAAAGAATAC GGCAACGAGC TTAGGAAGCA CGAACTTGTA TGGAAGCGGA 780  
 CTTGTCAATG CAGAAGCGGC AACACGC 807

## (2) INFORMATION FOR SEQ ID NO:2:

## 15 (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 807 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

## 20 (ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

(B) STRAIN: *Bacillus lentus* DSM 5483

(vii) IMMEDIATE SOURCE:

5 (B) CLONE: S3T, V4I, R99G, A188P, V193M, V199I

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

GCGCAAACAA TCCCATGGGG AATTAGCCGT GTGCAAGCCC CGGCTGCCCA TAACCGTGGA 60  
TTGACAGGTT CTGGTGTAAG AGTTGCTGTC CTCGATACAG GTATTTCCAC TCATCCAGAC 120  
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10 GGGCATGGCA CGCATGTGGC CGGGACGATT GCTGCTTTAA ACAATTCGAT TGGCGTTCTT 240  
GGCGTAGCGC CTAGTGC GGA ACTATACGCT GTTAAAGTTT TAGGAGCCGA CGGTGGAGGT 300  
GCAATCAGCT CGATTGCCCA AGGTTGGAA TGGGCAGGGA ACAATGGCAT GCACGTTGCT 360  
AATTTGAGTT TAGGAAGCCC TCGCCAAGT GCCACACTTG AGCAAGCTGT TAATAGCGCG 420  
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15 TATCCGGCCC GTTATGCGAA CGCAATGGCA GTCGGAGCTA CTGACCAAAA CAACAACCGC 540  
GCCAGCTTTT CACAGTATGG CCCAGGGCTT GACATTATGG CACCAGGGGT AAACATTCAG 600  
AGCACATACC CAGGTTCAAC GTATGCCAGC TTAACGGTA CATCGATGGC TACTCCTCAT 660  
GTTGCAGGTG CAGCAGCCCT TGTTAAACAA AAGAACCCAT CTTGGTCCAA TGACAAATC 720

CGCAACCATC TAAAGAATAC GGCAACGAGC TTAGGAAGCA CGAACTTGTA TGGAAGCGGA 780

CTTGTCAATG CAGAAGCGGC AACACGC 807

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

- 5 (A) LENGTH: 807 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: double  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

10 (iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

(B) STRAIN: Bacillus lentus DSM 5483

(vii) IMMEDIATE SOURCE:

15 (B) CLONE: S3T, V4I, R99A, A188P, V193M, V199I

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

GCGCAAACAA TCCCATGGGG AATTAGCCGT GTGCAAGCCC CGGCTGCCCA TAACCGTGGA 60

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TTAAATATTC GTGGTGCGC TAGCTTTGTA CCAGGGGAAC CATCCACTCA AGATGGGAAT 180

20 GGGCATGGCA CGCATGTGGC CGGGACGATT GCTGCTTTAA ACAATTCGAT TGGCGTTCTT 240

GGCGTAGCGC CTAGTGCGGA ACTATACGCT GTTAAAGTTT TAGGAGCCGA CGGTGCAGGT 300



GCAATCAGCT CGATTGCCCA AGGGTTGGAA TGGGCAGGGA ACAATGGCAT GCACGTTGCT 360  
AATTTGAGTT TAGGAAGCCC TTCGCCAAGT GCCACACTTG AGCAAGCTGT TAATAGCGCG 420  
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TATCCGGCCC GTTATGCGAA CGCAATGGCA GTCGGAGCTA CTGACCAAAA CAACAACCGC 540  
5 GCCAGCTTTT CACAGTATGG CCCAGGGCTT GACATTATGG CACCAGGGGT AAACATTCAG 600  
AGCACATACC CAGGTTCAAC GTATGCCAGC TTAACCGGTA CATCGATGGC TACTCCTCAT 660  
GTTGCAGGTG CAGCAGCCCT TGTTAAACAA AAGAACCCAT CTTGGTCCAA TGTACAAATC 720  
CGCAACCATC TAAAGAATAC GGCAACGAGC TTAGGAAGCA CGAACTTGTA TGGAAGCGGA 780  
CTTGTCAATG CAGAAGCGGC AACACGC 807

10 (2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 807 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: double

15 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

20 (B) STRAIN: Bacillus lentus DSM 5483

(vii) IMMEDIATE SOURCE:

(B) CLONE: S3T, V4I, R99S, A188P, V193M, V199I

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

GCGCAAACAA TCCCATGGGG AATTAGCCGT GTGCAAGCCC CGGCTGCCCA TAACCGTGGA 60  
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 5 GGGCATGGCA CGCATGTGGC CGGGACGATT GCTGCTTTAA ACAATTCGAT TGGCGTTCTT 240  
 GGCGTAGCGC CTAGTGC GGA ACTATACGCT GTTAAAGTTT TAGGAGCCGA CGGTAGCGGT 300  
 GCAATCAGCT CGATTGCCCA AGGGTTGGAA TGGGCAGGGA ACAATGGCAT GCACGTTGCT 360  
 AATTTGAGTT TAGGAAGCCC TTCGCCAAGT GCCACACTTG AGCAAGCTGT TAATAGCGCG 420  
 ACTTCTAGAG GCGTTCCTGT TGTAGCGGCA TCTGGGAATT CAGGTGCAAG CTCAATCAGC 480  
 10 TATCCGGCCC GTTATGCGAA CGCAATGGCA GTCGGAGCTA CTGACCAAAA CAACAACCGC 540  
 GCCAGCTTTT CACAGTATGG CCCAGGGCTT GACATTATGG CACCAGGGGT AACATTCAG 600  
 AGCACATACC CAGGTTCAAC GTATGCCAGC TTAAACGGTA CATCGATGGC TACTCCTCAT 660  
 GTTGCAGGTG CAGCAGCCCT TGTTAAACAA AAGAACCCAT CTTGGTCCAA TGTACAAATC 720  
 CGCAACCATC TAAAGAATAC GGCAACGAGC TTAGGAAGCA CGAACTTGTA TGGAAGCGGA 780  
 15 CTTGTCAATG CAGAAGCGGC AACACGC 807

## (2) INFORMATION FOR SEQ ID NO:5:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 807 base pairs  
 (B) TYPE: nucleic acid  
 20 (C) STRANDEDNESS: double  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

5 (B) STRAIN: Bacillus lentus DSM 5483

(vii) IMMEDIATE SOURCE:

(B) CLONE: S3T, V4I, S154E, A188P, V193M, V199I

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

GCGCAAACAA TCCCATGGGG AATTAGCCGT GTGCAAGCCC CGGCTGCCCA TAACCGTGGA 60  
 10 TTGACAGGTT CTGGTGAAA AGTTGCTGTC CTCGATACAG GTATTTCCAC TCATCCAGAC 120  
 TAAATATTC GTGGTGCGC TAGCTTTGTA CCAGGGGAAC CATCCACTCA AGATGGGAAT 180  
 GGGCATGGCA CGCATGTGGC CGGGACGATT GCTGCTTTAA ACAATTCGAT TGGCGTTCTT 240  
 GCGGTAGCGC CTAGTGCGGA ACTATACGCT GTTAAAGTTT TAGGAGCCGA CGGTAGAGGT 300  
 GCAATCAGCT CGATTGCCCA AGGGTTGGAA TGGGCAGGGA ACAATGGCAT GCACGTTGCT 360  
 15 AATTTGAGTT TAGGAAGCCC TTCGCCAAGT GCCACACTTG AGCAAGCTGT TAATAGCGCG 420  
 ACTTCTAGAG GCGTTCTTGT TGTAGCGGCA TCTGGGAATG AAGGTGCAAG CTCAATCAGC 480  
 TATCCGGCCC GTTATGCGAA CGCAATGGCA GTCGGAGCTA CTGACCAAAA CAACAACCGC 540  
 GCCAGCTTTT CACAGTATGG CCCAGGGCTT GACATTATGG CACCAGGGGT AAACATTGAG 600  
 AGCACATACC CAGGTTCAAC GTATGCCAGC TTAACGGTA CATCGATGGC TACTCCTCAT 660  
 20 GTTGCAGGTG CAGCAGCCCT TGTTAAACAA AAGAACCCAT CTTGGTCCAA TGACAAATC 720

CGCAACCATC TAAAGAATAC GGCAACGAGC TTAGGAAGCA CGAACTTGTA TGGAAGCGGA 780

CTTGTCAATG CAGAAGCGGC AACACGC 807

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:

- 5 (A) LENGTH: 807 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: double  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

- 10 (iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

(B) STRAIN: Bacillus lentus DSM 5483

(vii) IMMEDIATE SOURCE:

- 15 (B) CLONE: S3T, V4I, S154D, A188P, V193M, V199I

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

GCGCAAACAA TCCCATGGGG AATTAGCCGT GTGCAAGCCC CGGCTGCCCA TAACCGTGGA 60

TTGACAGGTT CTGGTGTAAG AGTTGCTGTC CTCGATACAG GTATTTCCAC TCATCCAGAC 120

TTAAATATTC GTGGTGGCGC TAGCTTTGTA CCAGGGGAAC CATCCACTCA AGATGGGAAT 180

20 GGGCATGGCA CGCATGTGGC CGGGACGATT GCTGCTTTAA ACAATTCGAT TGGCGTTCTT 240

GGCGTAGCGC CTAGTGCGGA ACTATACGCT GTTAAAGTTT TAGGAGCCGA CGGTAGAGGT 300

GCAATCAGCT CGATTGCCCA AGGGTTGGAA TGGGCAGGGA ACAATGGCAT GCACGTTGCT 360  
AATTTGAGTT TAGGAAGCCC TTCGCCAAGT GCCACACTTG AGCAAGCTGT TAATAGCGCG 420  
ACTTCTAGAG GCGTTCCTTGT TGTAGCGGCA TCTGGGAATG ACGGTGCAAG CTCAATCAGC 480  
TATCCGGCCC GTTATGCGAA CGCAATGGCA GTCGGAGCTA CTGACCAAAA CAACAACCGC 540  
5 GCCAGCTTTT CACAGTATGG CCCAGGGCTT GACATTATGG CACCAGGGGT AACATTCAG 600  
AGCACATACC CAGGTTCAAC GTATGCCAGC TTAACGGTA CATCGATGGC TACTCCTCAT 660  
GTTGCAGGTG CAGCAGCCCT TGTTAAACAA AAGAACCCAT CTTGGTCAA TGTAACAATC 720  
CGCAACCATC TAAAGAATAC GGCAACGAGC TTAGGAAGCA CGAACTTGTA TGGAAGCGGA 780  
CTTGCAATG CAGAAGCGGC AACACGC 807

## 10 (2) INFORMATION FOR SEQ ID NO:7:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 807 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: double  
15 (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: DNA (genomic)

## (iii) HYPOTHETICAL: NO

## (iv) ANTI-SENSE: NO

## (vi) ORIGINAL SOURCE:

- 20 (B) STRAIN:
- Bacillus lentus*
- DSM 5483

## (vii) IMMEDIATE SOURCE:

- (B) CLONE: S3T, V4I, A188P, V193M, V199I, L211D

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

GCGCAAACAA TCCCATGGGG AATTAGCCGT GTGCAAGCCC CGGCTGCCCA TAACCGTGGA 60  
TTGACAGGTT CTGGTGTAAG AGTTGCTGTC CTCGATACAG GTATTTCCAC TCATCCAGAC 120  
TTAAATATTC GTGGTGGCGC TAGCTTTGTA CCAGGGGAAC CATCCACTCA AGATGGGAAT 180  
5 GGGCATGGCA CGCATGTGGC CGGGACGATT GCTGCTTTAA ACAATTCGAT TGGCGTCTT 240  
GGCGTAGCGC CTAGTGCGGA ACTATACGCT GTTAAAGTTT TAGGAGCCGA CGGTAGAGGT 300  
GCAATCAGCT CGATTGCCCA AGGGTTGGAA TGGGCAGGGA ACAATGGCAT GCACGTTGCT 360  
AATTTGAGTT TAGGAAGCCC TTCGCCAAGT GCCACACTTG AGCAAGCTGT TAATAGCGCG 420  
ACTTCTAGAG GCGTCTTGT TGTAGCGGCA TCTGGGAATT CAGGTGCAAG CTCAATCAGC 480  
10 TATCCGGCCC GTTATGCGAA CGCAATGGCA GTCGGAGCTA CTGACCAAAA CAACAACCGC 540  
GCCAGCTTTT CACAGTATGG CCCAGGGCTT GACATTATGG CACCAGGGGT AACATTTCAG 600  
AGCACATACC CAGGTTCAAC GTATGCCAGC GACAACGGTA CATCGATGGC TACTCCTCAT 660  
GTTGCAGGTG CAGCAGCCCT TGTTAAACAA AAGAACCCAT CTTGGTCCAA TGACAAATC 720  
CGCAACCATC TAAAGAATAC GGCAACGAGC TTAGGAAGCA CGAACTTGTA TGGAAGCGGA 780  
15 CTTGTCAATG CAGAAGCGGC AACACGC 807

## (2) INFORMATION FOR SEQ ID NO:8:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 807 base pairs  
(B) TYPE: nucleic acid  
20 (C) STRANDEDNESS: double  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

5 (B) STRAIN: Bacillus lentus DSM 5483

(vii) IMMEDIATE SOURCE:

(B) CLONE: S3T, V4I, R99G, A188P, V193M, V199I, L211D

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

GCGCAAACAA TCCCATGGGG AATTAGCCGT GTGCAAGCCC CGGCTGCCCA TAACCGTGGA 60  
 10 TTGACAGGTT CTGGTGAAA AGTTGCTGTC CTCGATACAG GTATTTCCAC TCATCCAGAC 120  
 TTAAATATTC GTGGTGGCGC TAGCTTTGTA CCAGGGGAAC CATCCACTCA AGATGGGAAT 180  
 GGGCATGGCA CGCATGTGGC CGGGACGATT GCTGCTTTAA ACAATTCGAT TGGCGTTCTT 240  
 GGCGTAGCGC CTAGTGCGGA ACTATACGCT GTTAAAGTTT TAGGAGCCGA CGGTGGAGGT 300  
 GCAATCAGCT CGATTGCCCA AGGGTTGAA TGGGCAGGGA ACAATGGCAT GCACGTTGCT 360  
 15 AATTTGAGTT TAGGAAGCCC TTCGCCAAGT GCCACACTTG AGCAAGCTGT TAATAGCGCG 420  
 ACTTCTAGAG GCGTTCTTGT TGTAGCGGCA TCTGGGAATT CAGGTGCAAG CTCAATCAGC 480  
 TATCCGGCCC GTTATGCGAA CGCAATGGCA GTCGGAGCTA CTGACCAAAA CAACAACCGC 540  
 GCCAGCTTTT CACAGTATGG CCCAGGGCTT GACATTATGG CACCAGGGGT AAACATTCAG 600  
 AGCACATACC CAGGTTCAAC GTATGCCAGC GACAACGGTA CATCGATGGC TACTCCTCAT 660  
 20 GTTGCAAGTG CAGCAGCCCT TGTTAAACAA AAGAACCCAT CTTGGTCCAA TGTAACAATC 720

CGCAACCATC TAAAGAATAC GGCAACGAGC TTAGGAAGCA CGAACTTGTA TGGAAGCGGA 780

CTTGTCAATG CAGAAGCGGC AACACGC 807

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:

- 5 (A) LENGTH: 807 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: double  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

10 (iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

(B) STRAIN: Bacillus lentus DSM 5483

(vii) IMMEDIATE SOURCE:

15 (B) CLONE: S3T, V4I, S154E, A188P, V193M, V199I, L211D

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

GCGCAAACAA TCCCATGGGG AATTAGCCGT GTGCAAGCCC CGGCTGCCCA TAACCGTGGA 60

TTGACAGGTT CTGGTGAAA AGTTGCTGTC CTCGATACAG GTATTTCCAC TCATCCAGAC 120

TTAAATATTC GTGGTGGCGC TAGCTTTGTA CCAGGGGAAC CATCCACTCA AGATGGGAAT 180

20 GGGCATGGCA CGCATGTGGC CGGGACGATT GCTGCTTTAA ACAATTCGAT TGGCGTTCTT 240

GGCGTAGCGC CTAGTGCGGA ACTATACGCT GTTAAAGTTT TAGGAGCCGA CGGTAGAGGT 300



GCAATCAGCT CGATTGCCCA AGGGTTGGAA TGGGCAGGGA ACAATGGCAT GCACGTTGCT 360  
 AATTTGAGTT TAGGAAGCCC TTCGCCAAGT GCCACACTTG AGCAAGCTGT TAATAGCGCG 420  
 ACTTCTAGAG GCGTTCTTGT TGTAGCGGCA TCTGGGAATG AAGGTGCAAG CTCAATCAGC 480  
 TATCCGGCCCC GTTATGCGAA CGCAATGGCA GTCGGAGCTA CTGACCAAAA CAACAACCGC 540  
 5 GCCAGCTTTT CACAGTATGG CCCAGGGCTT GACATTATGG CACCAGGGGT AACATTGAG 600  
 AGCACATACC CAGGTTCAAC GTATGCCAGC GACAACGGTA CATCGATGGC TACTCCTCAT 660  
 GTTGCAGGTG CAGCAGCCCT TGTTAAACAA AAGAACCCAT CTTGGTCCAA TGACAAAATC 720  
 CGCAACCATC TAAAGAATAC GGCAACGAGC TTAGGAAGCA CGAACTTGTA TGGAAGCGGA 780  
 CTTGTCAATG CAGAAGCGGC AACACGC 807

10 (2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 269 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: unknown

15 (ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: Serine Protease
- 20 (B) STRAIN: Bacillus lentus DSM 5843

(vii) IMMEDIATE SOURCE:

## (B) CLONE: F11

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

Ala Gln Thr Val Pro Trp Gly Ile Ser Arg Val Gln Ala Pro Ala Ala  
 1            5            10            15

5 His Asn Arg Gly Leu Thr Gly Ser Gly Val Lys Val Ala Val Leu Asp  
           20            25            30

Thr Gly Ile Ser Thr His Pro Asp Leu Asn Ile Arg Gly Gly Ala Ser  
           35            40            45

10 Phe Val Pro Gly Glu Pro Ser Thr Gln Asp Gly Asn Gly His Gly Thr  
       50            55            60

His Val Ala Gly Thr Ile Ala Ala Leu Asn Asn Ser Ile Gly Val Leu  
       65            70            75            80

Gly Val Ala Pro Ser Ala Glu Leu Tyr Ala Val Lys Val Leu Gly Ala  
           85            90            95

15 Asp Gly Ser Gly Ala Ile Ser Ser Ile Ala Gln Gly Leu Glu Trp Ala  
           100            105            110

Gly Asn Asn Gly Met His Val Ala Asn Leu Ser Leu Gly Ser Pro Ser  
           115            120            125

20 Pro Ser Ala Thr Leu Glu Gln Ala Val Asn Ser Ala Thr Ser Arg Gly  
       130            135            140

Val Leu Val Val Ala Ala Ser Gly Asn Ser Gly Ala Ser Ser Ile Ser  
       145            150            155            160

Tyr Pro Ala Arg Tyr Ala Asn Ala Met Ala Val Gly Ala Thr Asp Gln  
           165            170            175

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

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

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

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

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

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

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 269 amino acids
- (B) TYPE: amino acid
- 15    (D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

- 20    (A) ORGANISM: Serine Protease
- (B) STRAIN: Bacillus lentus DSM 5843

(vii) IMMEDIATE SOURCE:

- (B) CLONE: F43

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

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

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

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

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

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

(2) INFORMATION FOR SEQ ID NO:12:

10            (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 269 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: protein

15            (iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Serine Protease

(B) STRAIN: Bacillus lentus DSM 5843

20            (vii) IMMEDIATE SOURCE:

(B) CLONE: F44

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

Ala Gln Thr Ile Pro Trp Gly Ile Ser Arg Val Gln Ala Pro Ala Ala

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

225            230            235            240  
 Arg Asn His Leu Lys Asn Thr Ala Thr Ser Leu Gly Ser Thr Asn Leu  
                  245            250            255  
 Tyr Gly Ser Gly Leu Val Asn Ala Glu Ala Ala Thr Arg  
 5                   260            265

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 269 amino acids
- (B) TYPE: amino acid
- 10 (D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

- 15 (A) ORGANISM: Serine Protease
- (B) STRAIN: Bacillus lentus DSM 5843

(vii) IMMEDIATE SOURCE:

- (B) CLONE: F45

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

20 Ala Gln Thr Ile Pro Trp Gly Ile Ser Arg Val Gln Ala Pro Ala Ala  
 1            5            10            15  
 His Asn Arg Gly Leu Thr Gly Ser Gly Val Lys Val Ala Val Leu Asp  
                  20            25            30

Thr Gly Ile Ser Thr His Pro Asp Leu Asn Ile Arg Gly Gly Ala Ser  
 35 40 45

Phe Val Pro Gly Glu Pro Ser Thr Gln Asp Gly Asn Gly His Gly Thr  
 50 55 60

5 His Val Ala Gly Thr Ile Ala Ala Leu Asn Asn Ser Ile Gly Val Leu  
 65 70 75 80

Gly Val Ala Pro Ser Ala Glu Leu Tyr Ala Val Lys Val Leu Gly Ala  
 85 90 95

10 Asp Gly Ser Gly Ala Ile Ser Ser Ile Ala Gln Gly Leu Glu Trp Ala  
 100 105 110

Gly Asn Asn Gly Met His Val Ala Asn Leu Ser Leu Gly Ser Pro Ser  
 115 120 125

Pro Ser Ala Thr Leu Glu Gln Ala Val Asn Ser Ala Thr Ser Arg Gly  
 130 135 140

15 Val Leu Val Val Ala Ala Ser Gly Asn Ser Gly Ala Ser Ser Ile Ser  
 145 150 155 160

Tyr Pro Ala Arg Tyr Ala Asn Ala Met Ala Val Gly Ala Thr Asp Gln  
 165 170 175

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

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

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

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

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



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

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:

- 5            (A) LENGTH: 269 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: NO

10           (iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: Serine Protease
- (B) STRAIN: Bacillus lentus DSM 5843

(vii) IMMEDIATE SOURCE:

15           (B) CLONE: F46

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

Ala Gln Thr Ile Pro Trp Gly Ile Ser Arg Val Gln Ala Pro Ala Ala  
 1            5                      10                      15

20                      20                      25                      30  
 His Asn Arg Gly Leu Thr Gly Ser Gly Val Lys Val Ala Val Leu Asp

Thr Gly Ile Ser Thr His Pro Asp Leu Asn Ile Arg Gly Gly Ala Ser  
 35                      40                      45

Phe Val Pro Gly Glu Pro Ser Thr Gln Asp Gly Asn Gly His Gly Thr  
50            55            60

His Val Ala Gly Thr Ile Ala Ala Leu Asn Asn Ser Ile Gly Val Leu  
65            70            75            80

5 Gly Val Ala Pro Ser Ala Glu Leu Tyr Ala Val Lys Val Leu Gly Ala  
85            90            95

Asp Gly Arg Gly Ala Ile Ser Ser Ile Ala Gln Gly Leu Glu Trp Ala  
100           105           110

10 Gly Asn Asn Gly Met His Val Ala Asn Leu Ser Leu Gly Ser Pro Ser  
115           120           125

Pro Ser Ala Thr Leu Glu Gln Ala Val Asn Ser Ala Thr Ser Arg Gly  
130           135           140

Val Leu Val Val Ala Ala Ser Gly Asn Glu Gly Ala Ser Ser Ile Ser  
145           150           155           160

15 Tyr Pro Ala Arg Tyr Ala Asn Ala Met Ala Val Gly Ala Thr Asp Gln  
165           170           175

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

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

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

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

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

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

## (2) INFORMATION FOR SEQ ID NO:15:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 269 amino acids

(B) TYPE: amino acid

5 (D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

## (vi) ORIGINAL SOURCE:

10 (A) ORGANISM: Serine Protease

(B) STRAIN: Bacillus lentus DSM 5843

## (vii) IMMEDIATE SOURCE:

(B) CLONE: F47

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

15 Ala Gln Thr Ile Pro Trp Gly Ile Ser Arg Val Gln Ala Pro Ala Ala

1 5 10 15

His Asn Arg Gly Leu Thr Gly Ser Gly Val Lys Val Ala Val Leu Asp

20 25 30

Thr Gly Ile Ser Thr His Pro Asp Leu Asn Ile Arg Gly Gly Ala Ser

20 35 40 45

Phe Val Pro Gly Glu Pro Ser Thr Gln Asp Gly Asn Gly His Gly Thr

50 55 60

His Val Ala Gly Thr Ile Ala Ala Leu Asn Asn Ser Ile Gly Val Leu

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

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 269 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: protein

5 (iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: Serine Protease
- (B) STRAIN: Bacillus lentus DSM 5843

10 (vii) IMMEDIATE SOURCE:

(B) CLONE: F49

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

Ala Gln Thr Ile Pro Trp Gly Ile Ser Arg Val Gln Ala Pro Ala Ala  
 1            5                    10                    15

15 His Asn Arg Gly Leu Thr Gly Ser Gly Val Lys Val Ala Val Leu Asp  
           20                    25                    30

Thr Gly Ile Ser Thr His Pro Asp Leu Asn Ile Arg Gly Gly Ala Ser  
           35                    40                    45

20 Phe Val Pro Gly Glu Pro Ser Thr Gln Asp Gly Asn Gly His Gly Thr  
           50                    55                    60

His Val Ala Gly Thr Ile Ala Ala Leu Asn Asn Ser Ile Gly Val Leu  
 65            70                    75                    80

Gly Val Ala Pro Ser Ala Glu Leu Tyr Ala Val Lys Val Leu Gly Ala  
           85                    90                    95

Asp Gly Arg Gly Ala Ile Ser Ser Ile Ala Gln Gly Leu Glu Trp Ala  
 100 105 110

Gly Asn Asn Gly Met His Val Ala Asn Leu Ser Leu Gly Ser Pro Ser  
 115 120 125

5 Pro Ser Ala Thr Leu Glu Gln Ala Val Asn Ser Ala Thr Ser Arg Gly  
 130 135 140

Val Leu Val Val Ala Ala Ser Gly Asn Ser Gly Ala Ser Ser Ile Ser  
 145 150 155 160

10 Tyr Pro Ala Arg Tyr Ala Asn Ala Met Ala Val Gly Ala Thr Asp Gln  
 165 170 175

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

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

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

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

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

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

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:

- 25 (A) LENGTH: 269 amino acids  
 (B) TYPE: amino acid  
 (D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

- 5 (A) ORGANISM: Serine Protease
- (B) STRAIN: Bacillus lentus DSM 5843

(vii) IMMEDIATE SOURCE:

(B) CLONE: F54

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

10 Ala Gln Thr Ile Pro Trp Gly Ile Ser Arg Val Gln Ala Pro Ala Ala  
 1 5 10 15

His Asn Arg Gly Leu Thr Gly Ser Gly Val Lys Val Ala Val Leu Asp  
 20 25 30

15 Thr Gly Ile Ser Thr His Pro Asp Leu Asn Ile Arg Gly Gly Ala Ser  
 35 40 45

Phe Val Pro Gly Glu Pro Ser Thr Gln Asp Gly Asn Gly His Gly Thr  
 50 55 60

His Val Ala Gly Thr Ile Ala Ala Leu Asn Asn Ser Ile Gly Val Leu  
 65 70 75 80

20 Gly Val Ala Pro Ser Ala Glu Leu Tyr Ala Val Lys Val Leu Gly Ala  
 85 90 95

Asp Gly Gly Gly Ala Ile Ser Ser Ile Ala Gln Gly Leu Glu Trp Ala  
 100 105 110

Gly Asn Asn Gly Met His Val Ala Asn Leu Ser Leu Gly Ser Pro Ser

115            120            125  
 Pro Ser Ala Thr Leu Glu Gln Ala Val Asn Ser Ala Thr Ser Arg Gly  
 130            135            140  
 Val Leu Val Val Ala Ala Ser Gly Asn Ser Gly Ala Ser Ser Ile Ser  
 5    145            150            155            160  
 Tyr Pro Ala Arg Tyr Ala Asn Ala Met Ala Val Gly Ala Thr Asp Gln  
 165            170            175  
 Asn Asn Asn Arg Ala Ser Phe Ser Gln Tyr Gly Pro Gly Leu Asp Ile  
 180            185            190  
 10 Met Ala Pro Gly Val Asn Ile Gln Ser Thr Tyr Pro Gly Ser Thr Tyr  
 195            200            205  
 Ala Ser Asp Asn Gly Thr Ser Met Ala Thr Pro His Val Ala Gly Ala  
 210            215            220  
 Ala Ala Leu Val Lys Gln Lys Asn Pro Ser Trp Ser Asn Val Gln Ile  
 15    225            230            235            240  
 Arg Asn His Leu Lys Asn Thr Ala Thr Ser Leu Gly Ser Thr Asn Leu  
 245            250            255  
 Tyr Gly Ser Gly Leu Val Asn Ala Glu Ala Ala Thr Arg  
 260            265

20 (2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 269 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: unknown

25 (ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: NO



(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Serine Protease

(B) STRAIN: Bacillus lentus DSM 5843

5 (vii) IMMEDIATE SOURCE:

(B) CLONE: F55

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

Ala Gln Thr Ile Pro Trp Gly Ile Ser Arg Val Gln Ala Pro Ala Ala  
 1            5            10            15

10 His Asn Arg Gly Leu Thr Gly Ser Gly Val Lys Val Ala Val Leu Asp  
           20            25            30

Thr Gly Ile Ser Thr His Pro Asp Leu Asn Ile Arg Gly Gly Ala Ser  
           35            40            45

15 Phe Val Pro Gly Glu Pro Ser Thr Gln Asp Gly Asn Gly His Gly Thr  
           50            55            60

His Val Ala Gly Thr Ile Ala Ala Leu Asn Asn Ser Ile Gly Val Leu  
           65            70            75            80

Gly Val Ala Pro Ser Ala Glu Leu Tyr Ala Val Lys Val Leu Gly Ala  
           85            90            95

20 Asp Gly Arg Gly Ala Ile Ser Ser Ile Ala Gln Gly Leu Glu Trp Ala  
           100            105            110

Gly Asn Asn Gly Met His Val Ala Asn Leu Ser Leu Gly Ser Pro Ser  
           115            120            125

25 Pro Ser Ala Thr Leu Glu Gln Ala Val Asn Ser Ala Thr Ser Arg Gly  
           130            135            140

Val Leu Val Val Ala Ala Ser Gly Asn Glu Gly Ala Ser Ser Ile Ser  
145            150            155            160

Tyr Pro Ala Arg Tyr Ala Asn Ala Met Ala Val Gly Ala Thr Asp Gln  
                 165            170            175

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

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

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

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

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

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

What is claimed is:

1. A mutant subtilisin protease, characterized in that it carries at least one mutation of its amino acid sequence resulting in a reduced positive charge or an increased negative charge in the proximity of the substrate binding region of the molecule.  
5
2. A mutant *Bacillus lentus* DSM 5483 protease derived by replacement of at least one amino acid residue of the mature form of the *Bacillus lentus* DSM 5483 alkaline protease shown in SEQ ID NO:52 in patent application Serial No. 07/706,691 filed May 29, 1991 wherein the position for said amino acid replacement of at least one amino acid residue is selected from the group  
10 consisting of arginine-99, serine-154, and leucine-211 is replaced by a glutamic acid or an aspartic acid residue.
3. A mutant *Bacillus lentus* DSM 5483 protease derived by replacement of at least one amino acid residue of the mutant alkaline protease M131 (S3T + V4I  
15 + A188P + V193M + V199I) shown in SEQ ID No:1 in patent application Serial No. 07/706,691 filed May 29, 1991 wherein the position for said amino acid replacement of at least one amino acid residue is selected from the group consisting of serine-154, and leucine-211 is replaced by a glutamic acid or an aspartic acid residue.
- 20 4. A mutant *Bacillus lentus* DSM 5483 protease derived by replacement of at least one amino acid residue of the mature form of the *Bacillus lentus* DSM 5483 alkaline protease shown in SEQ ID No:52 in patent application Serial No. 07/706,691 filed May 29, 1991 wherein the amino acid residue arginine-99 is replaced by a glycine, alanine or serine residue.

5. A mutant *Bacillus lentus* DSM 5483 protease derived by replacement of at least one amino acid residue of the mutant alkaline protease M131 (S3T + V4I + A188P + V193M + V199I) shown in SEQ ID NO:1 patent application Serial No. 07/706,691 filed May 29, 1991 wherein the amino acid residue arginine-99 is replaced by a glycine, alanine or serine residue.
6. An enzymatic detergent composition, comprising a mutated subtilisin protease, characterized in that the protease is a mutated subtilisin protease comprising one or more of the mutations: R99G, R99A, R99S, R99E, L211D, L211E, S154D, S154E.
7. An enzymatic detergent composition, comprising a mutated *Bacillus lentus* DSM 5483 alkaline protease characterized in that the protease is a mutated protease comprising one or more of the following mutations: S3T, V4I, R99G, R99A, R99S, S154D, S154E, A188P, V193M, V199I, L211D, L211E.
8. A mutant *Bacillus lentus* DSM 5483 protease derived by replacement of at least one amino acid residue of the mutant alkaline protease M130 (S3T + A188P + V193M + V199I) shown in SEQ ID NO:2 in patent application Serial No. 07/706,691 filed May 29, 1991 wherein the amino acid residue arginine-99 is replaced by a glycine, alanine or serine residue.
9. A mutant gene which encodes for a mutant *Bacillus lentus* DSM 5483 alkaline protease (BLAP) comprising in the direction of transcription, a promoter, a ribosomal binding site, an initiation codon, the major portion of the pre region of the *Bacillus licheniformis* ATCC 53926 alkaline protease gene operably linked to a portion of the pre region and all of the pro and mature regions of the gene encoding BLAP variant M130 followed by the ATCC 53926 alkaline protease transcription terminator, wherein codons of said BLAP gene are altered to

produce a mutant gene which encodes for a protease derived by the replacement of amino acids at positions Ser3, Ala188, Val193, Val199 and Arg99.

10. The mutant gene of claim 9 which encodes for said mutant protease wherein the serine at position 3 is substituted by threonine, the alanine at  
5 position 188 is substituted by proline, the valine at position 193 is substituted by methionine, the valine at position 199 is substituted by isoleucine and the arginine at position 99 is substituted by glycine, alanine or serine.

11. A mutant gene which encodes for a mutant *Bacillus lentus* DSM 5483 alkaline protease (BLAP) comprising in the direction of transcription, a promoter,  
10 a ribosomal binding site, an initiation codon, the major portion of the pre region of the *Bacillus licheniformis* ATCC 53926 alkaline protease gene operably linked to a portion of the pre region and all of the pro and mature regions of the gene encoding BLAP variant M131 followed by the ATCC 53926 alkaline protease transcription terminator, wherein codons of said BLAP gene are altered to  
15 produce a mutant gene which encodes for a protease derived by the replacement of amino acids at position Ser3, Val4, Ala188, Val193 and Val199 combined with replacement of at least one amino acid at position Arg99, Ser154, and Leu211.

12. The mutant gene of claim 11 encodes for said mutant protease wherein the serine at position 3 is substituted by threonine, the valine at position 4 is  
20 substituted by isoleucine, the alanine at position 188 is substituted by proline, the valine at position 193 is substituted by methionine, the valine at position 199 substituted by isoleucine and the arginine at position 99 is substituted by glycine, alanine or serine.

13. The mutant gene of claim 11 which encodes for said mutant protease  
25 wherein the serine at position 3 is substituted by threonine, the valine at position

4 is substituted by isoleucine, the alanine at position 188 is substituted by proline, the valine at position 193 is substituted by methionine, the valine at position 199 is substituted by isoleucine and the serine at position 154 is substituted by aspartic acid or glutamic acid.

5 14. The mutant gene of claim 11 which encodes for said mutant protease wherein the serine at position 3 substituted by threonine, the valine at position 4 is substituted by isoleucine, the alanine at position 188 is substituted by proline, the valine at position 193 is substituted by methionine, the valine at position 199 is substituted by isoleucine, and the leucine at position 211 is substituted by  
10 aspartic acid or glutamic acid.

15 15. The mutant gene of claim 11 which encodes for said mutant protease wherein the serine at position 3 is substituted by threonine, the valine at position 4 is substituted by isoleucine, the alanine at position 188 is substituted by proline, the valine at position 193 is substituted by methionine, the valine at position 199  
15 is substituted by isoleucine, the arginine at position 99 is substituted by glycine, alanine or serine and the leucine at position 211 is substituted by aspartic acid or glutamic acid.

20 16. The mutant gene of claim 11 which encodes for said mutant protease wherein the serine at position 3 is substituted by threonine, the valine at position 4 is substituted by isoleucine, the alanine at position 188 is substituted by proline, the valine at position 193 is substituted by methionine, the valine at position 199 is substituted by isoleucine, the serine at position 154 is substituted by aspartic or glutamic acid and the leucine at position 211 is substituted by aspartic acid or glutamic acid.

17. Plasmids capable of replication in *Bacillus* sp. which carry the mutant protease genes described in claim 9.
18. Plasmids capable of replication in *Bacillus* sp. which carry the mutant protease genes described in claim 10.
- 5 19. Plasmids capable of replication in *Bacillus* sp. which carry the mutant protease genes described in claim 11.
20. Plasmids capable of replication in *Bacillus* sp. which carry the mutant protease genes described in claim 12.
21. Plasmids capable of replication in *Bacillus* sp. which carry the mutant  
10 protease genes described in claim 13.
22. Plasmids capable of replication in *Bacillus* sp. which carry the mutant protease genes described in claim 14.
23. Plasmids capable of replication in *Bacillus* sp. which carry the mutant protease genes described in claim 15.
- 15 24. Plasmids capable of replication in *Bacillus* species which carry the mutant protease gene described in claim 16.
25. A transformed *Bacillus* host comprising a plasmid carrying a mutant protease gene described in claim 9.
26. A transformed *Bacillus* host comprising a plasmid carrying a mutant  
20 protease gene described in claim 10.
27. A transformed *Bacillus* host comprising a plasmid carrying a mutant protease gene described in claim 11.
28. A transformed *Bacillus* host comprising a plasmid carrying a mutant protease gene described in claim 12.

29. A transformed *Bacillus* host comprising a plasmid carrying a mutant protease gene described in claim 13.
30. A transformed *Bacillus* host comprising a plasmid carrying a mutant protease gene described in claim 14.
- 5 31. A transformed *Bacillus* host comprising a plasmid carrying a mutant protease gene described in claim 15.
32. A transformed *Bacillus* host comprising a plasmid carrying a mutant protease gene described in claim 16.
33. The transformed *Bacillus* host cell of claim 17 wherein said host cell is  
10 *Bacillus licheniformis* ATCC 53926.
34. The transformed *Bacillus* host cell of claim 17 wherein said host cell is *Bacillus subtilis*.
35. The transformed *Bacillus* host strain of claim 19 wherein said host cell is *Bacillus licheniformis* ATCC 53926.
- 15 36. The transformed *Bacillus* host strain of claim 19 wherein said host cell is *Bacillus subtilis*.
37. A detergent composition for washing fabrics composed of proteinogenic fibers comprising at least one surfactant and a proteolytically active amount of a protease having a keratinase/caseinase activity ratio of less than about 0.80.
- 20 38. The detergent of claim 37 wherein said protease has a KC ratio of less than about 0.70.
39. The detergent of claim 37 wherein said ratio is from about 0.2 to about 0.65.
40. The detergent of claim 37 wherein said protease has a proteolytic activity  
25 of from about 100 PU/g to about 7500 PU/g.



41. The detergent of claim 37 wherein said protease is mutant *Bacillus lentus* DSM 5483 variant F49.

42. A detergent composition for washing fabrics composed of proteinogenic fibers comprising a proteolytically active amount of a protease having a  
5 keratinase/caseinase activity ratio of less than about 0.80 and a nonionic surfactant selected from the group consisting of a fatty alkyl polyglycoside, a fatty alkyl polyalkoxylate, a fatty acid polyhydroxyamide, an ethoxylated fatty alkyl-amine, a propoxylated fatty alkylamine, an ethoxylated vicinal diol, a propoxy-  
lated vicinal diol, an ethoxylated fatty acid alkyl ester, a propoxylated fatty acid  
10 alkyl ester, an ethoxylated fatty acid amide, a propoxylated fatty acid amide, and mixtures thereof.

43. The detergent of claim 42 wherein said nonionic surfactant is present in an amount of from about 2% to about 25% by weight.

44. The detergent of claim 42 further comprising an anionic surfactant  
15 selected from the group consisting of a fatty alkyl sulfate, a fatty alkyl ether sulfate, a sulfofatty acid ester, a sulfofatty acid disalt, and combinations thereof.

45. A detergent composition for washing fabrics composed of proteinogenic fibers comprising a proteolytically active amount of a protease having a  
keratinase/caseinase activity ratio of less than about 0.80 and an anionic  
20 surfactant selected from the group consisting of a fatty alkyl sulfate, a fatty alkyl ether sulfate, a sulfofatty acid ester, a sulfofatty acid disalt, and combinations thereof.

46. The detergent of claim 45 wherein said anionic surfactant is present in an amount of from about 2% to about 25% by weight.

47. The detergent of claim 45 wherein said anionic surfactant is selected from the group consisting of an alkyl sulfate, an alkenyl sulfate, an alkyl ether sulfate, an alkenyl ether sulfate in wherein the alkyl or alkenyl group contains from about 8 to about 22 carbon atoms.

5 48. The detergent of claim 47 wherein said alkyl or alkenyl group contains from about 12 to about 18 carbon atoms.

49. The detergent of claim 37 further comprising a builder selected from the group consisting of an alkali metal aluminosilicate, a crystalline alkali metal silicate with a modulus above 1, a monomeric polycarboxylate, a polymeric  
10 polycarboxylate and mixtures thereof.

50. The detergent of claim 37 wherein said builder is present in an amount of from about 2.5% to about 60% by weight.

51. A detergent composition for washing fabrics composed of proteinogenic fibers comprising from about 20% to about 55% by weight of an inorganic builder;  
15 up to about 15% by weight of a water-soluble organic builder; from about 2.5% to about 20% by weight of an anionic surfactant; from about 1% to about 20% by weight of a nonionic surfactant; up to about 25% by weight of a bleaching agent; up to about 8% by weight of a bleach activator; up to about 20% by weight of an inorganic salt; from about 0.4% to about 1.2% by weight of a lipase,  
20 cutinase, amylase, cellulase, pullulanase, oxidase or peroxidase and a proteolytically active amount of a protease having a keratinase/caseinase activity ratio of less than about 0.80.

52. The detergent of claim 51 wherein the amount of said organic builder is from about 2% to about 12% by weight; the amount of said bleaching agent is  
25 from about 1% to about 15% by weight; the amount of said bleach activator is

from about 0.5% to about 6% by weight; the amount of said inorganic salt is from about 0.1% to about 15% by weight; the amount of said lipase, cutinase, amylase, cellulase, pullulanase, oxidase or peroxidase is from about 0.4% to about 1.2% by weight.

5 53. The detergent of claim 51 wherein said inorganic salt is an alkali metal carbonate, an alkali metal hydrogen carbonate, or an alkali metal sulfate.

54. A detergent composition for washing fabrics composed of proteinogenic fibers comprising from about 20% to about 55% by weight of an inorganic builder; up to about 15% by weight of a water-soluble organic builder; up to about 15%  
10 by weight of an anionic surfactant; from about 4% to about 24% by weight of a nonionic surfactant; up to about 65% by weight of an inorganic salt; and a proteolytically active amount of a protease having a keratinase/caseinase activity ratio of less than about 0.80.

55. The detergent of claim 54 wherein the amount of said organic builder is  
15 from about 2% to about 12% by weight; the amount of said anionic surfactant is from about 1% to about 10% by weight; the amount of said inorganic salt is from about 1% to about 30% by weight.

56. A composition comprising first and second mutant proteases wherein in  
said first mutant protease at least one of the amino acid residues at positions 99  
20 and 154 are different from those in *Bacillus lentus* DSM 5483 and wherein in said second mutant protease at least the amino acid residue at position 211 is different from that in *Bacillus lentus* DSM 5483.

57. The composition of claim 56 wherein said first mutant protease is F46.

58. The composition of claim 56 wherein said second mutant protease is F49.

59. A detergent composition comprising at least one surfactant and at least a first and second mutant protease wherein in said first mutant protease at least one of the amino acid residues at positions 99 and 154 are different from those in *Bacillus lentus* DSM 5483 and wherein in said second mutant protease at least  
5 the amino acid residue at position 211 is different from that in *Bacillus lentus* DSM 5483.

60. A method for washing fabrics composed of proteinogenic fibers comprising contacting said fabric with a composition comprised of at least one surfactant and a proteolytically active amount of a protease having a  
10 keratinase/caseinase activity ratio of less than about 0.80.

61. The method of claim 60 wherein said protease has a KC ratio of less than about 0.70.

62. The method of claim 60 wherein said ratio is from about 0.2 to about 0.65.

63. The method of claim 60 wherein said protease has a proteolytic activity  
15 of from about 100 PU/g to about 7500 PU/g.

64. The method of claim 60 wherein said protease is mutant *Bacillus lentus* DSM 5483 variant F49.

65. The method of claim 60 wherein said surfactant is a nonionic surfactant selected from the group consisting of a fatty alkyl polyglycoside, a fatty alkyl  
20 polyalkoxylate, a fatty acid polyhydroxyamide, an ethoxylated fatty alkylamine, a propoxylated fatty alkylamine, an ethoxylated vicinal diol, a propoxylated vicinal diol, an ethoxylated fatty acid alkyl ester, a propoxylated fatty acid alkyl ester, an ethoxylated fatty acid amide, a propoxylated fatty acid amide, and mixtures thereof.

66. The method of claim 60 wherein said nonionic surfactant is present in an amount of from about 2% to about 25% by weight.
67. The method of claim 60 wherein said surfactant is further comprised of an anionic surfactant selected from the group consisting of a fatty alkyl sulfate, a fatty alkyl ether sulfate, a sulfofatty acid ester, a sulfofatty acid disalt, and combinations thereof.
68. A method for washing fabrics composed of proteinogenic fibers comprising contacting said fabric with a proteolytically active amount of a protease having a keratinase/caseinase activity ratio of less than about 0.80 and an anionic surfactant selected from the group consisting of a fatty alkyl sulfate, a fatty alkyl ether sulfate, a sulfofatty acid ester, a sulfofatty acid disalt, and combinations thereof.
69. The method of claim 68 wherein said anionic surfactant is present in an amount of from about 2% to about 25% by weight.
70. The method of claim 69 wherein said anionic surfactant is selected from the group consisting of an alkyl sulfate, an alkenyl sulfate, an alkyl ether sulfate, an alkenyl ether sulfate in wherein the alkyl or alkenyl group contains from about 8 to about 22 carbon atoms.
71. The method of claim 70 wherein said alkyl or alkenyl group contains from about 12 to about 18 carbon atoms.
72. The method of claim 60 wherein said composition is further comprised of a builder selected from the group consisting of an alkali metal aluminosilicate, a crystalline alkali metal silicate with a modulus above 1, a monomeric polycarboxylate, a polymeric polycarboxylate and mixtures thereof.

73. The method of claim 72 wherein said builder is present in an amount of from about 2.5% to about 60% by weight.

74. A method for washing fabrics composed of proteinogenic fibers comprising contacting said fabric with a detergent composition comprising from  
5 about 20% to about 55% by weight of an inorganic builder; up to about 15% by weight of a water-soluble organic builder; from about 2.5% to about 20% by weight of an anionic surfactant; from about 1% to about 20% by weight of a nonionic surfactant; up to about 25% by weight of a bleaching agent; up to about 8% by weight of a bleach activator; up to about 20% by weight of an inorganic  
10 salt; from about 0.4% to about 1.2% by weight of a lipase, cutinase, amylase, cellulase, pullulanase, oxidase or peroxidase and a proteolytically active amount of a protease having a keratinase/caseinase activity ratio of less than about 0.80.

75. The method of claim 74 wherein the amount of said organic builder is from about 2% to about 12% by weight; the amount of said bleaching agent is  
15 from about 1% to about 15% by weight; the amount of said bleach activator is from about 0.5% to about 6% by weight; the amount of said inorganic salt is from about 0.1% to about 15% by weight; the amount of said lipase, cutinase, amylase, cellulase, pullulanase, oxidase or peroxidase is from about 0.4% to about 1.2% by weight.

20 76. The method of claim 74 wherein said inorganic salt is an alkali metal carbonate, an alkali metal hydrogen carbonate, or an alkali metal sulfate.

77. A method for washing fabrics composed of proteinogenic fibers comprising contacting said fabric with a detergent composition comprising from  
25 about 20% to about 55% by weight of an inorganic builder; up to about 15% by weight of a water-soluble organic builder; up to about 15% by weight of an

anionic surfactant; from about 4% to about 24% by weight of a nonionic surfactant; up to about 65% by weight of an inorganic salt; and a proteolytically active amount of a protease having a keratinase/caseinase activity ratio of less than about 0.80.

5 78. The method of claim 77 wherein the amount of said organic builder is from about 2% to about 12% by weight; the amount of said anionic surfactant is from about 1% to about 10% by weight; the amount of said inorganic salt is from about 1% to about 30% by weight.

79. A composition comprising at least one surfactant and a proteolytically  
10 active amount of first and second mutant proteases wherein in said first mutant protease at least one of the amino acid residues at positions 99 and 154 are different from those in *Bacillus lentus* DSM 5483 and wherein in said second mutant protease at least the amino acid residue at position 211 is different from that in *Bacillus lentus* DSM 5483.

15 80. The composition of claim 79 wherein said first mutant protease is F46.

81. The composition of claim 79 wherein said second mutant protease is F49.

82. A composition comprising at least one surfactant and a proteolytically active amount of first and second mutant proteases wherein each of said protease has an amino acid substitution selected from the group consisting of  
20 R99G, R99A, R99S, S154D, S154E, L221D, L211E, and a combination thereof.

83. A method for removing stains from cloth which comprises contacting a cloth having both blood and egg stains with a composition comprising at least one surfactant and a proteolytically active amount of first and second mutant proteases wherein in said first mutant protease at least one of the amino acid  
25 residues at positions 99 and 154 are different from those in *Bacillus lentus* DSM

5483 and wherein in said second mutant protease at least the amino acid residue at position 211 is different from that in *Bacillus lentus* DSM 5483.

84. The method of claim 83 wherein said first mutant protease is F46.

85. The method of claim 83 wherein said second mutant protease is F49.

5 86. A method for removing stains from cloth which comprises contacting a cloth having both blood and egg stains with a composition comprising at least one surfactant and a proteolytically active amount of first and second mutant proteases wherein each of said protease has an amino acid substitution selected from the group consisting of R99G, R99A, R99S, S154D, S154E, L221D, L211E,  
10 and a combination thereof.



INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US95/01937

**A. CLASSIFICATION OF SUBJECT MATTER**  
 IPC(6) :C12N 9/50, 9/52, 9/54, 9/56, 15/57  
 US CL :435/220, 221, 222, 320.1, 252.3, 252.8; 536/23.2  
 According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**  
 Minimum documentation searched (classification system followed by classification symbols)  
 U.S. : 435/220, 221, 222, 320.1, 252.3, 252.8; 536/23.2

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched  
 NONE

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

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES USA, VOLUME 84, ISSUED AUGUST 1987, WELLS ET AL., "RECRUITMENT OF SUBSTRATE SPECIFICITY PROPERTIES FROM ONE ENZYME INTO A RELATED ONE BY PROTEIN ENGINEERING", PAGES 5167-5171, SEE ENTIRE DOCUMENT.	1-86
Y	JOURNAL OF MOLECULAR BIOLOGY, VOLUME 228, ISSUED 1992, GODDETTE ET AL., "THE CRYSTAL STRUCTURE OF THE <i>BACILLUS LENTUS</i> ALKALINE PROTEASE, SUBTILISIN BL, AT 1.4 ANGSTROM RESOLUTION", PAGES 580-595, SEE ENTIRE DOCUMENT.	1-86
Y,P	US, A, 5,340,735 (CHRISTIANSON ET AL.) 23 AUGUST 1994, SEE ENTIRE DOCUMENT.	1-86

Further documents are listed in the continuation of Box C.  See patent family annex.

* Special categories of cited documents:	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A" document defining the general state of the art which is not considered to be of particular relevance	"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E" earlier document published on or after the international filing date	"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&" document member of the same patent family
"O" document referring to an oral disclosure, use, exhibition or other means	
"P" document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search 26 MAY 1995	Date of mailing of the international search report 07 / 06 / 95
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Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer KEITH D. HENDRICKS Telephone No. (703) 308-0196
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## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US95/01937

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y,P	US, A, 5,352,604 (WILSON ET AL.) 04 OCTOBER 1994, SEE ENTIRE DOCUMENT.	9-86