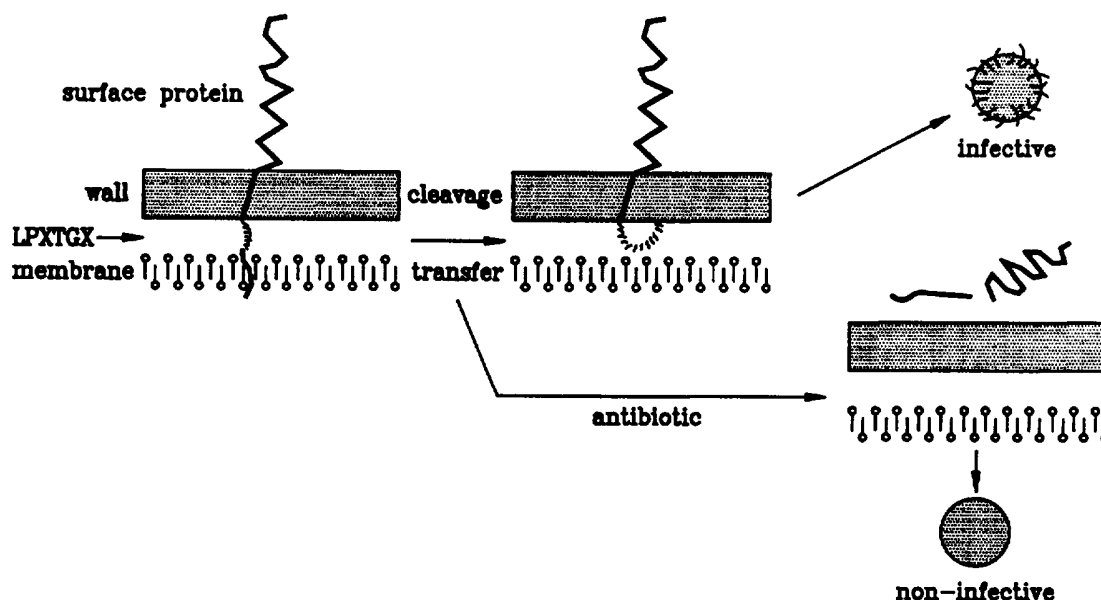




INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ : C12N 9/50, 9/52, 9/54	A1	(11) International Publication Number: WO 96/11263 (43) International Publication Date: 18 April 1996 (18.04.96)
(21) International Application Number: PCT/US95/12460 (22) International Filing Date: 6 October 1995 (06.10.95) (30) Priority Data: 08/319,540 7 October 1994 (07.10.94) US (71) Applicant: ROCKEFELLER UNIVERSITY [US/US]; 1230 York Avenue, New York, NY 10021 (US). (72) Inventors: FISCHETTI, Vincent, A.; 440 Joan Court, West Hempstead, NY 11552 (US). PANCHOLI, Vijaykumar, Apartment 26A, 500 E. 3rd Street, New York, NY 10021 (US). (74) Agent: REA, Teresa, Stanek; Burns, Doane, Swecker & Mathis, P.O. Box 1404, Alexandria, VA 22313-1404 (US).		(81) Designated States: AU, CA, CN, JP, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE). Published <i>With international search report.</i>

(54) Title: ENZYME FOR CLEAVAGE OF THE ANCHOR REGION OF SURFACE PROTEINS FROM GRAM POSITIVE BACTERIA



(57) Abstract

An enzyme which cleaves at the sequence LPXTGX (SEQ ID NO:1) of the surface proteins of gram positive bacteria is provided. The enzyme was isolated from a group A *Streptococcus*. In addition, a method of detecting the enzyme, a method of isolating the enzyme, and methods of inhibiting the enzyme are disclosed. The figure is a schematic illustrating the positioning of the surface protein in the bacterial membrane, and the cleavage step which leads to infective bacteria.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	GB	United Kingdom	MR	Mauritania
AU	Australia	GE	Georgia	MW	Malawi
BB	Barbados	GN	Guinea	NE	Niger
BE	Belgium	GR	Greece	NL	Netherlands
BF	Burkina Faso	HU	Hungary	NO	Norway
BG	Bulgaria	IE	Ireland	NZ	New Zealand
BJ	Benin	IT	Italy	PL	Poland
BR	Brazil	JP	Japan	PT	Portugal
BY	Belarus	KE	Kenya	RO	Romania
CA	Canada	KG	Kyrgyzstan	RU	Russian Federation
CF	Central African Republic	KP	Democratic People's Republic of Korea	SD	Sudan
CG	Congo	KR	Republic of Korea	SE	Sweden
CH	Switzerland	KZ	Kazakhstan	SI	Slovenia
CI	Côte d'Ivoire	LI	Liechtenstein	SK	Slovakia
CM	Cameroon	LK	Sri Lanka	SN	Senegal
CN	China	LU	Luxembourg	TD	Chad
CS	Czechoslovakia	LV	Latvia	TG	Togo
CZ	Czech Republic	MC	Monaco	TJ	Tajikistan
DE	Germany	MD	Republic of Moldova	TT	Trinidad and Tobago
DK	Denmark	MG	Madagascar	UA	Ukraine
ES	Spain	ML	Mali	US	United States of America
FI	Finland	MN	Mongolia	UZ	Uzbekistan
FR	France			VN	Viet Nam
GA	Gabon				

-1-

ENZYME FOR CLEAVAGE OF THE ANCHOR REGION OF
SURFACE PROTEINS FROM GRAM POSITIVE BACTERIA

BACKGROUND OF THE INVENTION

1. Field of the Invention

This invention relates to an enzyme for cleaving within the wall anchor region of a surface protein of a gram-positive bacterium, a method for detecting that enzyme, and a method for purifying that enzyme. In particular, the enzyme is isolated from a group A *Streptococcus*, and cleaves the anchor region of streptococcal M protein.

2. Description of the Related Art

Bacteria may be designated as gram-positive and gram-negative, based on the staining of their cell walls with Gram's stain. Within the broad division of gram-positive bacteria are the gram-positive cocci, which include the genera *Aerococcus*, *Corprococcus*, *Deinobacter*, *Deinococcus*, *Enterococcus*, *Gemella*, *Lactococcus*, *Leuconostoc*, *Marinococcus*, *Melissococcus*, *Micrococcus*, *Pediococcus*, *Peptococcus*, *Peptostreptococcus*, *Planococcus*, *Ruminococcus*, *Saccharococcus*, *Salinicoccus*, *Carcina*, *Staphylococcus*, *Stomatococcus*, *Streptococcus*, *Trichococcus*, *Vagococcus*, *Listeria* and *Actinomyces*.

In gram-positive bacteria, proteins are secreted into the surrounding medium, whereas in gram-negative bacteria, secretion occurs into the periplasmic space between the cytoplasmic and outer membranes (Model and Russel (1990) *Cell* 61:739-741; Schatz and Beckwith (1990) *Annu. Rev. Genet.* 24:215-248). Prokaryotic sorting signals are conceivable for the local assembly of supramolecular structures like pili (Strom and Lory,

-2-

(1987) *J. Bacteriol.* **169**:3181-3188), flagella (Loewy et al (1987) *Genes Dev.* **1**:626-635), and bacteriophages (Brissette and Russel (1990) *J. Mol. Biol.* **211**:565-580), or for localization of proteins in defined bacterial
5 compartments. Such compartments include the outer membrane of gram-negative bacteria (Model and Russel (1990) *Cell* **61**:739-741), the cell wall (Braun et al (1970) *Eur. J. Biochem.* **13**:336-346; *Eur. J. Biochem.* **14**:387-391; *Biochemistry* **9**:5041-5049; Shockman and
10 Barrett (1983) *Annu. Rev. Microbiol.* **37**:501-527), and the periseptal annulus (MacAlister et al (1983) *Proc. Natl. Acad. Sci.* **80**:1372-1376).

The cell wall of gram-positive bacteria can be thought of as representing a unique cell compartment,
15 which contains anchored surface proteins that require specific sorting signals. Some biologically important products are anchored in this way, including protein A and fibronectin binding proteins of *Staphylococcus aureus* and M protein from *Streptococcus pyogenes*. Studies of
20 staphylococcal Protein A and *E. coli* alkaline phosphatase show that the signal both necessary and sufficient for cell wall anchoring consists of an LPXTGX motif (SEQ ID NO:1), a C-terminal hydrophobic domain, and a charged tail. These sequence elements are conserved in many
25 surface proteins from different gram-positive bacteria.

M protein of group A streptococci, an α -helical coiled-coil fibrillar molecule found on the surface of the organism (Fischetti et al (1988) *Proteins Struct. Func. Genet.* **3**:60), is responsible for the antiphagocytic
30 property of these bacteria (Lancefield et al (1962) *J. Immunol.* **89**:307). Antigenic variation (Jones et al (1988), *Proc. Natl. Acad. Sci. USA* **85**:8271) and type-specific immunity are contingent upon epitopes located within the NH₂-terminal half of the M molecule (distal to
35 the cell wall) (Jones et al (1988) *J. Exp. Med.*

-3-

167:1114). Amino acid sequences that are conserved among different M proteins are located in the COOH-terminal half (Jones et al (1985) *J. Exp. Med.* 161:623; Hollingshead et al (1987) *Infect. Immun.* 55:3237) and
5 contain epitopes recently shown to be responsible for non-type-specific immunity against streptococcal colonization (Bessen et al (1988) *Infect. Immun.* 56:2666; Fischetti et al (1989) *Science* (Wash. DC) 244:1487).

The attachment region of the molecule, predicted
10 from DNA sequence, is located at the COOH terminal end, composed of charged amino acids at the COOH terminus, followed by 19 hydrophobic amino acids suspected to be a membrane anchor followed by a hexapeptide motif, LPXTGX. This region is adjacent to a proline and glycine-rich
15 region situated within the peptidoglycan layer of the cell wall (Fischetti (1988) *Proteins Struct. Func. Genet.* 3:60; Pancholi et al (1988) *J. Bacteriol.* 170:2618; Hollingshead et al (1986), *J. Biol. Chem.* 261:1677.)

The association of the M protein to the cytoplasmic
20 membrane of gram-positive bacteria can be examined after removing the cell wall with the muralytic enzyme lysin (Fischetti et al. (1974) *Streptococcal Disease and the Community*, M.J. Haverkorn, editor. Excerpta Medica, Amsterdam. 26.), which is active against group A
25 streptococcal cell walls over a broad pH range. M protein is released during the removal of the cell wall indicating that an endogenous factor mediates this release (Pancholi et al (1989) *J. Exp. Med.* 170:2119-2133).

30 Analysis of the released form of the M protein demonstrates that the COOH-terminal 19 hydrophobic amino acids and charged tail of the M molecule are not present (Pancholi et al (1989) *J. Exp. Med.* 170:2119-2133). This suggests that the release of M proteins from the membrane
35 and its attachment to the cell wall is in some way

-4-

associated with the cleavage of the COOH-terminal hydrophobic region.

Cleavage of the surface proteins of gram-positive bacteria in the LPXTGX region adjacent to the hydrophobic domain has been shown to occur during the anchoring process of these proteins (Schneewind et al (1992) *Cell* 70:267-281). Because interference with this cleavage prevents the proper placement of surface proteins on the bacterial cell, characterization of the enzyme responsible for this cleavage would be a critical step in antibiotic development.

Therefore, it is apparent that what is needed in this art is a method of detecting and isolating this gram-positive bacterial surface protein cleavage enzyme.

SUMMARY OF THE INVENTION

Accordingly, a major object of the present invention is to provide an isolated and purified enzyme which cleaves surface proteins of gram-positive bacteria within the LPXTGX motif.

Another object of the present invention is to provide an antibody directed to the cleaving enzyme of gram-positive bacteria.

Yet another object of the present invention is to provide a method of detecting the presence of the cleaving enzyme of gram-positive bacteria within the LPXTGX motif.

A still further object of the present invention is to provide a method of isolating an enzyme which cleaves surface proteins on gram-positive bacteria.

Briefly, the present invention features an enzyme which cleaves a surface protein on gram-positive bacteria, and a method of detecting it, including the steps of: (a) synthesizing a peptide comprising an amino acid sequence of LPXTGX; (b) labeling the peptide of step

-5-

(a) with a detectable marker; (c) covalently linking the labeled peptide to a chromatographic bead; (d) mixing the linked beads with a membrane extract of gram-positive bacteria; (e) detecting a release of label from the
5 beads; and (f) correlating the release of label with the presence of the enzyme.

The invention also features antibodies directed to that cleaving enzyme, and inhibitors of that enzyme.

The invention also features a method of isolating
10 the cleaving enzyme, including the steps of: (a) preparing a membrane extract of the gram-positive bacteria containing membrane-bound proteins; (b) fractionating the membrane-bound proteins by chromatography using a salt gradient; and (c) identifying
15 the presence of the enzyme in at least one fraction.

With the foregoing and other objects, advantages and features of the invention that will become hereinafter apparent, the nature of the invention may be more clearly understood by reference to the following detailed
20 description of the preferred embodiments of the invention and to the appended claims.

BRIEF DESCRIPTION OF THE DRAWINGS

25 Figure 1 is a dose-response curve with the dialyzed carbonate extract of D471 membranes.

Figure 2 is a time-response curve using a carbonate extract of D471 membranes.

Figure 3 shows the cleavage activity of the enzyme
30 in the presence of enzyme inhibitors and activators.

Figure 4 is a schematic illustrating the positioning of the surface protein in the bacterial membrane, and the cleavage step which leads to infective bacteria. An antibiotic directed to the cleavage step results in non-
35 infective bacteria.

-6-

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS
OF THE INVENTION

More particularly, the present invention relates to
 5 methods for detecting and purifying a cleavage enzyme
 which cleaves surface proteins of gram-positive bacteria
 at a known motif, preferably an LPXTGX (SEQ ID NO:1)
 motif, and also relates to the purified cleavage enzyme
 itself.

10 The cleavage enzyme can be isolated from any gram-
 positive bacterium. Such gram-positive bacteria include
 the genera Aerococcus, Corprococcus, Deinobacter,
 Deinococcus, Enterococcus, Gemella, Lactococcus,
 Leuconostoc, Marinococcus; Melissococcus, Micrococcus,
 15 Pediococcus, Peptococcus, Peptostreptococcus,
 Planococcus, Ruminococcus, Saccharococcus, Salinicoccus,
 Carcina, Staphylococcus, Stomatococcus, Streptococcus,
 Trichococcus, Vagococcus, Listeria and Actinomyces.
 Gram-positive bacteria known to have surface proteins
 20 containing an LPXTGX (SEQ ID NO:1) motif are the
 following:

Species	Protein	Motif
A. naeslundii	fimbriae type 1	LPLTGA (SEQ ID NO:2)
A. viscosus	fimbriae type 1	LPLTGA (SEQ ID NO:2)
25 E. faecalis	Asa1	LPQTGE (SEQ ID NO:3)
E. faecalis	Asc10 ⁺	LPKTGE (SEQ ID NO:4)
E. faecalis	Sec10	LPQTGE (SEQ ID NO:5)
Group G strep	Protein G	LPTTGE (SEQ ID NO:6)
L. monocytogenes	InlA	LPTTGD (SEQ ID NO:7)

-7-

	P. magnus	Protein L	LPKAGS (SEQ ID NO:8)
	S. aureus	Protein A	LPETGE (SEQ ID NO:9)
	S. aureus	FnBP-A	LPETGG (SEQ ID NO:10)
	S. aureus	FnBP-B	LPETGG (SEQ ID NO:10)
5	S. aureus	Cna	LPKTGM (SEQ ID NO:11)
	S. agalactiae	Bac	LPYTGTV (SEQ ID NO:12)
	S. cremoris	Wg2	LPKTGE (SEQ ID NO:4)
	S. mutans	Pac	LPNTGE (SEQ ID NO:13)
	S. mutans	SpaP	LPNTGE (SEQ ID NO:13)
10	S. mutans	WapA	LPSTGE (SEQ ID NO:14)
	S. pyogenes	Arp2	LPSTGE (SEQ ID NO:14)
	S. pyogenes	Arp4	LPSTGE (SEQ ID NO:14)
	S. pyogenes	FcRA	LPSTGE (SEQ ID NO:14)
	S. pyogenes	M2	LPSTGE (SEQ ID NO:14)
15	S. pyogenes	M5	LPSTGE (SEQ ID NO:14)
	S. pyogenes	M6	LPSTGE (SEQ ID NO:14)
	S. pyogenes	M12	LPSTGE (SEQ ID NO:14)
	S. pyogenes	M24	LPSTGE (SEQ ID NO:14)
	S. pyogenes	M49	LPSTGE (SEQ ID NO:14)

-8-

	<i>S. pyogenes</i>	M57	LPSTGE (SEQ ID NO:14)
	<i>S. pyogenes</i>	Protein H	LPSTGE (SEQ ID NO:14)
	<i>S. pyogenes</i>	SCP	LPTTND (SEQ ID NO:15)
	<i>S. pyogenes</i>	T6	LPSTGS (SEQ ID NO:16)
5	<i>S. sobrinus</i>	SpaA	LPATGD (SEQ ID NO:17)

A more preferred species for the present invention is *Streptococcus pyogenes*, and most preferred is M type 6.

Protoplasts may be prepared from the bacteria by any means known in the art. The culture is grown in any medium known to support the growth of the particular species, preferably Todd-Hewitt broth. The cells are then centrifuged and washed. The cells are then resuspended in a phosphate buffer, preferably 20-100 mM, more preferably about 50 mM, and preferably containing raffinose at about 10-50%, preferably about 30%, or sucrose at about 10-30%, preferably about 20%, and EDTA. An enzyme specific for degrading the cell wall, preferably a Group C streptococcal phage-associated lysin, is then added and incubated at approximately 37°C for at least 30 minutes.

Membranes can be prepared from the protoplasts using any method known in the art, but preferably by repeated freeze-thaw treatments in the presence of protease inhibitors. Membranes are then collected by centrifugation at about 100,000 x g, washed, and then resedimented in the presence of protease inhibitors.

As a method of analyzing cleavage activity of the enzyme, and especially for analyzing activity of cleavage enzyme mutants, target surface molecules can be prepared by releasing the surface molecules from the membrane using any method known in the art. Preferably, the

-9-

surface molecule can be released by treating the membranes with sodium carbonate at a pH > 9, preferably > 11, most preferably at about 11.5.

The presence of surface protein, particularly M protein, in the supernatant can be identified using any method known in the art, including separation on SDS-PAGE gel, Western blot, enzyme-linked immunosorbent assay (ELISA), capture ELISA, RIA and the like. Antibodies for the detection of M protein are available in the art and include polyclonal sera, such as that against ColiM6.1 protein (Fischetti et al (1984) *J. Exp. Med.* 159:1083); polyclonal sera to a synthetic peptide corresponding to residues of the M protein, such as residues 1-21 (Jones et al (1988) *Proc. Natl. Acad. Sci. USA* 85:8271), or anti-SM6(308-327) to residues 308-327, anti-SM6(339-352) to residues 339-352, or anti-SM6(381-398) to residues 381-398; or the monoclonal antibody 10B6 to an epitope in the conserved region of the M molecule between residues 275 and 289.

In general, monoclonals are used at a dilution of 1:100-1:10,000, preferably about 1:1000, and polyclonal sera are used at a dilution of about 1:50-1:5000, preferably about 1:500.

The cleavage enzyme is preferably detected by preparing a detectably labeled synthetic substrate. The labeled synthetic substrate preferably contains an LPXTGX (SEQ ID NO:1) sequence, and more preferably contains a LPSTGE (SEQ ID NO:14) sequence. Methods for the preparation and analysis of such a synthetic peptide are well known in the art.

The synthetic peptide can be labeled with any detectable label known in the art. Preferably, this label is an isotope, most preferably ¹²⁵I. The labeled synthetic peptide is then preferably linked to a substrate, most preferably a commercially available bead.

-10-

The bacterial strain containing the cleavage enzyme is treated to extract the bacterial membrane. Preferably, the bacterial membranes are treated with an alkaline buffer, preferably carbonate buffer, at a pH of about 9-13, preferably about 11.5. The extraction is preferably conducted at a temperature below room temperature, preferably at about 0°C. The membrane extract is then mixed with the labeled beads in a suitable buffer, and release of radiolabel from the cleaved synthetic peptide is analyzed.

The enzyme is isolated from the extract using protein purification methods well known in the art. In particular, the membrane extract containing the detected cleavage activity is subjected to chromatographic techniques which separate proteins present in the extract according to size, affinity and charge. Fractions obtained from each chromatographic step are analyzed for cleavage activity as described above, and subjected to further purification steps. A particularly preferable method for obtaining purified cleavage enzyme is high performance liquid chromatography (HPLC).

After the enzyme has been purified, its amino acid sequence can be determined using amino acid sequencing methods well known in the art. A particularly preferable method is Edman degradation. Having obtained sequence information on the cleavage enzyme, one can design oligonucleotide probes for isolating the DNA encoding the cleavage enzyme, using conventional screening methods, or amplification methods such as polymerase chain reaction (PCR). It is particularly preferable to design such oligonucleotides in a completely degenerate manner, such that oligonucleotides containing each codon encoding a particular amino acid are present in the oligonucleotide mix. Alternatively, inosine can be used at positions in the codon where degeneracies are known to be present.

-11-

In general, the procedures for isolating the DNA encoding the cleavage enzyme, subjecting it to partial digestion, isolating DNA fragments, ligating the fragments into a cloning vector, and transforming a host are well known in recombinant DNA technology. Accordingly, one of ordinary skill in the art can use or adapt the detailed protocols for such procedures as found in Sambrook et al. (1989); Molecular Cloning: A Laboratory Manual, 2nd. Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 3 volumes, or in any other manual on recombinant DNA technology.

Once the gene encoding the LPXTGX (SEQ ID NO:1) cleavage enzyme has been obtained from one species, it can serve as a hybridization probe to isolate corresponding genes from the other species by cross-hybridization under low to moderate stringency conditions. Such conditions are usually found empirically by determining the conditions wherein the probe specifically cross-hybridizes to its counterpart gene with a minimum of background hybridization. Nucleic acid hybridization is a well known technique and thoroughly detailed in Sambrook et al.

As noted above, the DNA encoding the cleavage enzyme can be originally isolated using PCR. Corresponding DNAs from other species can also be isolated using PCR, and oligonucleotides for performing these subsequent PCR reactions can be optimized using the sequence information obtained from DNA cloned from the first species.

A further aspect of the present invention provides the nucleic acids encoding the subject genes in replicable expression vectors and transformed hosts containing these vectors. The replicable expression vectors may also be used to obtain the polypeptides of the present invention by well known methods in recombinant DNA technology.

-12-

The instant replicable expression vectors comprise a nucleic acid encoding the subject gene, i.e., the coding sequence is operably linked in proper reading frame to a nucleotide sequence element which directs expression of the cleavage enzyme. In particular, the nucleotide sequence elements may include a promoter, a transcription enhancer element, a termination signal, a translation signal, or a combination of two or more of these elements, generally including at least a promoter element.

Replicable expression vectors are generally DNA molecules engineered for controlled expression of a desired gene, especially where it is desirable to produce large quantities of a particular gene product, or polypeptide. The vectors comprise one or more nucleotide sequences operably linked to a gene to control expression of that gene, the gene being expressed, and an origin of replication which is operable in the contemplated host. Preferably the vector encodes a selectable marker, for example, antibiotic resistance. Replicable expression vectors can be plasmids, bacteriophages, cosmids and viruses. Any expression vector comprising RNA is also contemplated. The replicable expression vectors of this invention can express the cleavage enzyme at high levels. Many of these vectors are based on pBR322, M13 and lambda and are well known in the art and employ such promoters as *trp*, *lac*, P_L , T7 polymerase and the like. Hence, one skilled in the art has available many choices of replicable expression vectors, compatible hosts, and well-known methods for making and using the vectors.

Moreover, peptides and fragments as well as chemically modified derivatives of the LPXTGX (SEQ ID NO:1) cleavage enzyme are also contemplated by the present invention. Briefly, any peptide fragment, derivative or analog which retains substantially the same

-13-

biological activity of the LPXTGX (SEQ ID NO:1) cleavage enzyme is contemplated. An analog may be defined herein as a peptide or fragment which exhibits LPXTGX (SEQ ID NO:1) cleaving activity, but has an amino acid

5 substitution, insertion or deletion in comparison to the wild-type cleavage enzyme. Such an analog can be prepared by the "conservative" substitution of an amino acid having similar chemical properties.

Thus, it should also be appreciated that also within
10 the scope of the present invention are DNA sequences encoding an LPXTGX (SEQ ID NO:1) cleavage enzyme having the same amino acid sequence as the wild-type enzyme, but also those DNA sequences which are degenerate to the wild-type sequence. By "degenerate to" is meant that a
15 different three-letter codon is used to specify a particular amino acid. It is well known in the art that the following codons can be used interchangeably to code for each specific amino acid:

	Phenylalanine (Phe or F)	UUU or UUC
20	Leucine (Leu or L)	UUA or UUG or CUU or CUC or CUA or CUG
	Isoleucine (Ile or I)	AUU or AUC or AUA
	Methionine (Met or M)	AUG
	Valine (Val or V)	GUU or GUC or GUA or GUG
25	Serine (Ser or S)	UCU or UCC or UCA or UCG or AGU or AGC
	Proline (Pro or P)	CCU or CCC or CCA or CCG
	Threonine (Thr or T)	ACU or ACC or ACA or ACG
	Alanine (Ala or A)	GCU or GCG or GCA or GCG
30	Tyrosine (Tyr or Y)	UAU or UAC
	Histidine (His or H)	CAU or CAC
	Glutamine (Gln or Q)	CAA or CAG
	Asparagine (Asn or N)	AAU or AAC
	Lysine (Lys or K)	AAA or AAG
35	Aspartic Acid (Asp or D)	GAU or GAC

-14-

	Glutamic Acid (Glu or E)	GAA or GAG
	Cysteine (Cys or C)	UGU or UGC
	Arginine (Arg or R)	CGU or CGC or CGA or CGG or AGA or AGG
5	Glycine (Gly or G)	GGU or GGC or GGA or GGG
	Termination codon	UAA (ochre) or UAG (amber) or UGA (opal)

It should be understood that the codons specified above are for RNA sequences. The corresponding codons for DNA have T substituted for U.

Mutations can be made in the wild-type sequence such that a particular codon is changed to a codon which codes for a different amino acid. Such a mutation is generally made by making the fewest nucleotide changes possible. A substitution mutation of this sort can be made to change an amino acid in the resulting protein in a non-conservative manner (i.e., by changing the codon from an amino acid belonging to a grouping of amino acids having a particular size or characteristic to an amino acid belonging to another grouping) or in a conservative manner (i.e., by changing the codon from an amino acid belonging to a grouping of amino acids having a particular size or characteristic to an amino acid belonging to the same grouping). Such a conservative change generally leads to less change in the structure and function of the resulting protein. A non-conservative change is more likely to alter the structure, activity or function of the resulting protein.

The following is one example of various groupings of amino acids:

Amino acids with nonpolar R groups

Alanine
Valine
Leucine
Isoleucine

-15-

Proline
Phenylalanine
Tryptophan
Methionine

5

Amino acids with uncharged polar R groups

Glycine
Serine
Threonine

10

Cysteine
Tyrosine
Asparagine
Glutamine

15

Amino acids with charged polar R groups (negatively
charged at Ph 6.0)

Aspartic acid
Glutamic acid

20

Basic amino acids (positively charged at pH 6.0)

Lysine
Arginine
Histidine (at pH 6.0)

25

Another grouping may be those amino acids with
phenyl groups:

Phenylalanine
Tryptophan
Tyrosine

30

Another grouping may be according to molecular
weight (i.e., size of R groups):

Glycine	75
Alanine	89
Serine	105
Proline	115

35

-16-

	Valine	117
	Threonine	119
	Cysteine	121
	Leucine	131
5	Isoleucine	131
	Asparagine	132
	Aspartic acid	133
	Glutamine	146
	Lysine	146
10	Glutamic acid	147
	Methionine	149
	Histidine (at pH 6.0)	155
	Phenylalanine	165
	Arginine	174
15	Tyrosine	181
	Tryptophan	204

Particularly preferred substitutions are:

- Lys for Arg and vice versa such that a positive charge may be maintained;
- 20 - Glu for Asp and vice versa such that a negative charge may be maintained;
- Ser for Thr such that a free -OH can be maintained; and
- Gln for Asn such that a free NH₂ can be
- 25 maintained.

Amino acid substitutions may also be introduced to substitute an amino acid with a particularly preferable property. For example, a Cys may be introduced at a potential site for disulfide bridging with another Cys.

30 A His may be introduced as a particularly "catalytic" site (i.e., His can act as an acid or base and is the most common amino acid in biochemical catalysis). Pro may be introduced because of its particularly planar structure, which induces β -turns in the protein's

35 structure.

-17-

Purification of the subject LPXTGX (SEQ ID NO:1) cleavage enzyme from natural or recombinant sources can be accomplished by conventional purification means such as ammonium sulfate precipitation, gel filtration
5 chromatography, ion exchange chromatography, adsorption chromatography, affinity chromatography, chromatofocusing, HPLC, FPLC, and the like. Where appropriate purification steps can be done in batch or in columns.

10 Peptide fragments of the LPXTGX (SEQ ID NO:1) cleavage enzyme can be prepared by proteolysis or by chemical degradation. Typical proteolytic enzymes are trypsin, chymotrypsin, V8 protease, subtilisin and the like; the enzymes are commercially available, and
15 protocols for performing proteolytic digests are well known. Peptide fragments are purified by conventional means, as described above. Peptide fragments can often be identified by amino acid composition or sequence. Peptide fragments are useful as immunogens to obtain
20 antibodies against the subject LPXTGX (SEQ ID NO:1) cleavage enzyme.

The present invention also relates to antibodies to the LPXTGX (SEQ ID NO:1) cleavage enzyme. Such antibodies may be monoclonal or polyclonal and are
25 contemplated as being useful in developing detection assays (immunoassays) for cleavage enzyme proteins, monitoring cleavage enzyme levels and in purifying cleavage enzyme. Thus, in accordance with this invention, an antibody to an LPXTGX (SEQ ID NO:1)
30 cleavage enzyme encompasses monoclonal or polyclonal antibodies to said LPXTGX (SEQ ID NO:1) cleavage enzyme, or to antigenic parts thereof.

Both polyclonal and monoclonal antibodies to the LPXTGX (SEQ ID NO:1) cleavage enzyme are obtainable by
35 immunization of an animal with purified LPXTGX (SEQ ID

-18-

NO:1) cleavage enzyme, purified recombinant LPXTGX (SEQ ID NO:1) cleavage enzyme, fragments of these proteins, or purified fusion proteins of LPXTGX (SEQ ID NO:1) cleavage enzyme with another protein. In the case of monoclonal antibodies, partially purified proteins or fragments may serve as immunogens. The methods of obtaining both types of antibodies are well known in the art with excellent protocols for antibody production being found in Harlow et al. (1988) Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 726 pp.

Polyclonal sera are relatively easily prepared by injection of a suitable laboratory animal with an effective amount of the purified LPXTGX (SEQ ID NO:1) cleavage enzyme, or parts thereof, collecting serum from the animal, and isolating specific sera by any of the known immunoadsorbent techniques. Antibodies produced by this method are useful in virtually any type of immunoassay.

Monoclonal antibodies are particularly useful because they can be produced in large quantities and with a high degree of homogeneity. Hybridoma cell lines which produce monoclonal antibodies are prepared by fusing an immortal cell line with lymphocytes sensitized against the immunogenic preparation and is done by techniques which are well known to those who are skilled in the art. (See, for example, Douillard, I.Y. and Hoffman, T., "Basic Facts About Hybridomas", in Compendium of Immunology, Vol. II, L. Schwartz (Ed.) (1981); Kohler, G. and Milstein, C., Nature 256: 495-497 (1975) and European Journal of Immunology 6: 511-519 (1976); Harlow et al.; Koprowski, et al., U.S. Patent 4,172,124; Koprowski et al., U.S. Patent 4,196,265 and Wands, U.S. Patent 4,271,145, the teachings of which are herein incorporated by reference.

-19-

The presence of the LPXTGX (SEQ ID NO:1) cleavage enzyme in a sample, such as a culture supernatant and the like, in a microorganism, or in any other source suspected to contain the LPXTGX (SEQ ID NO:1) cleavage enzyme, can be detected utilizing antibodies prepared as above, either monoclonal or polyclonal, in virtually any type of immunoassay. Likewise, the present antibodies can be used to identify microorganisms which have or produce LPXTGX (SEQ ID NO:1) cleavage enzyme.

Accordingly, the present invention provides a method of detecting an LPXTGX (SEQ ID NO:1) cleavage enzyme by the steps of contacting a sample suspected of containing said LPXTGX (SEQ ID NO:1) cleavage enzyme with an antibody of the invention for a time and under conditions sufficient to form an enzyme-antibody complex and subjecting this complex to a detecting means. As well known to one skilled in the art, the time and conditions for immunodetection assays are variable and depend on the particular assay.

A wide range of detection techniques and conditions are available to one skilled in the art as can be seen by reference to U.S. Patent Nos. 4,016,043; 4,424,279 and 4,018,653 and to Harlow et al. which provides extensive protocols for immunodetection of molecules. These techniques, of course, include both single-site and two-site, or "sandwich" assays, assays of the non-competitive types as well as competitive binding assays, ELISA, radioimmunoassays, immunoprecipitation and immunoblotting (Western blotting). Sandwich assays are commonly used, a number of variations of the technique exist, and all are intended to be encompassed by the present invention.

Direct and indirect immunoassays, i.e., ELISA, immunoblotting and the like, may employ reporter molecules linked to either a primary antibody (direct assay) or a second antibody or antibody-specific protein

-20-

such as Protein A or Protein G (indirect assay). The primary antibody can be an antibody of the subject invention labelled with the desired reporter molecule.

By "reporter molecule," as used herein, is meant a molecule which, by its chemical nature, provides an identifiable signal to detect antigen-antibody complexes. Detection may be either qualitative or quantitative. The most commonly used reporter molecules are either enzymes, fluorophores, or radionuclide containing molecules (i.e., radioisotopes). In the case of an enzyme immunoassay, an enzyme is conjugated to the antibody, generally by means of glutaraldehyde or periodate. As will be readily recognized, however, a wide variety of different conjugation techniques exist, which are readily available to the skilled artisan. Commonly used enzymes include horseradish peroxidase, glucose oxidase, β -galactosidase, and alkaline phosphatase among others. The substrate to be used with a particular enzyme is generally chosen for the production of a detectable color change upon reaction. For example, p-nitrophenyl phosphate is suitable for use with alkaline phosphatase conjugates; for peroxidase conjugates, 1,2-phenylenediamine, 5-aminosalicylic acid, or toluidine are commonly used. It is also possible to employ fluorogenic substrates, which yield a fluorescent product rather than the chromogenic substrates noted above. After binding an enzyme-labeled antibody to an antigen or antigen-antibody complex, as appropriate, the excess labeled antibody is washed away, and a solution containing the appropriate substrate is added. The substrate reacts with the enzyme, i.e., the reporter molecule, to give a qualitative visual signal or a quantitative signal which can be assessed to indicate the amount of antigen present in the sample.

Alternately, fluorescent compounds, such as fluorescein and rhodamine, may be chemically coupled to

-21-

antibodies without altering their binding capacity. As used in immunofluorescence, when activated by illumination with light of a specific wavelength, a fluorophore-labeled antibody absorbs the light energy, inducing the fluorophore into an excited state which is followed by emission of light having a characteristic wavelength. Generally, the emitted light is a characteristic color in the visible range and is detectable with a light microscope equipped for immunofluorescence. Fluorescent antibodies are used in sandwich assays, direct and indirect immunoassays as described above, except after washing, the immune complex is exposed to light of the appropriate wavelength, and the fluorescence is observed. Immunofluorescence and enzyme-based immunoassay techniques are both well established in the art and are particularly preferred. However, other reporter molecules, such as radioisotopes, chemiluminescent or bioluminescent molecules, may also be employed. It will be readily apparent to the skilled technician how to vary the procedure to suit the required purpose.

Another aspect of the invention provides a means of purifying an LPXTGX (SEQ ID NO:1) cleavage enzyme by affinity selection. This method involves contacting a sample containing the LPXTGX (SEQ ID NO:1) cleavage enzyme with an antibody of the invention, and separating the antigen-antibody complex, e.g., the enzyme-antibody complex from the remainder of the sample and recovering the enzyme in a form free from the antibody. Typically the complex-containing sample is fractionated and the fraction(s) containing the enzyme are identified by a convenient biochemical, enzymatic, immunological or other detection means. To facilitate fractionation, the subject antibodies can be bound to a chromatography resin before or after binding to the cleavage enzyme. This

-22-

method can yield purified cleavage enzyme in large amounts and in pure form.

Accordingly, the present invention is also directed to a kit for the rapid and convenient assay of an LPXTGX (SEQ ID NO:1) cleavage enzyme, in samples suspected of containing the enzyme. The kit may contain either an antibody directed to the LPXTGX (SEQ ID NO:1) cleavage enzyme, and a secondary detectable antibody thereto, or may contain a labelled substrate for the enzyme, such that a labelled cleavage product is detected in the presence of the cleavage enzyme.

Another aspect of the present invention is directed to a method of detecting the DNA or RNA encoding the subject LPXTGX (SEQ ID NO:1) cleavage enzyme by nucleic acid hybridization techniques such as Southern blotting, Northern blotting and the like, or by the polymerase chain reaction (PCR). Accordingly, a method of detecting a cleavage enzyme is provided which comprises contacting a sample suspected of containing said cleavage enzyme-encoding DNA with a first nucleic acid sufficiently complementary to hybridize to a second nucleic acid which encodes said cleavage enzyme in said sample for a time and under conditions sufficient to effect said hybridization and thereby form a complex of said first and second nucleic acids and subjecting said complex to a detecting means. In this method, the first nucleic acid may have a reporter group attached thereto. Reporter groups can include radioisotopes, enzymatically detected groups such as biotin or fluorophores such as rhodamine and fluorescein. Detailed methods for hybridization and blotting is found in Sambrook et al.

For PCR, the present method of detecting a gene encoding the LPXTGX (SEQ ID NO:1) cleavage enzyme comprises subjecting a sample suspected of containing the cleavage enzyme to a polymerase chain reaction (PCR)

-23-

using at least two oligonucleotide primers sufficiently complementary to hybridize to a nucleic acid in said sample which encodes said cleavage enzyme, and thereby producing at least one amplified nucleic acid segment and identifying said segment. PCR has been described in U.S. Patent Nos. 4,683,195; 4,683,202; and 4,800,159 which are incorporated herein by reference as well as described extensively in the literature, see for example Saiki et al. (1988), Science 239: 487-491. The segment may be detected by gel electrophoresis or blotting, for example.

Also encompassed by the present invention are inhibitors of the cleavage enzyme which can be routinely screened using the cleavage assay described above.

The following examples are presented in order to more fully illustrate the preferred embodiments of the invention. They should in no way be construed, however, as limiting the broad scope of the invention.

EXAMPLE 1

Preparation of a synthetic substrate for the detection of cleavage activity.

A synthetic peptide was designed having the following amino acid sequence (SEQ ID NO:18):

K R Q L P S T G E T A N P F Y

This peptide was prepared by the solid-phase method of Barany and Merrifield (1979) *In* Gross and J. Meienhofer, (ed.), Academic Press, Inc., New York, p. 1-284, which is hereby incorporated by reference in its entirety, and purified by high pressure liquid chromatography on a Brownlee C8 reverse-phase column (Brownlee Laboratories, Santa Clara, CA) with a gradient of acetonitrile in 0.05% trifluoroacetic acid. The sequence was verified by amino acid composition and sequence analysis as follows:

For amino acid analysis, peptides were hydrolyzed in 6 N HCl at 110°C for 22 hours and derivatized with

-24-

ethanol-triethylamine-water-phenylisothiocyanate (7:1:1:1) in a Picotag Work Station (Waters Associates, Inc., Milford, MA) and analyzed with a Novapak C18 column (Waters) and a Waters 840 Data Module.

5 Amino acid sequence analysis was performed by automated Edman degradation in a model 470A gas phase sequencer (Applied Biosystems, Foster City, CA). The phenylhydantoin amino acids were identified by high-pressure liquid chromatography on a C18 column with
10 either a 1084B analyzer (Hewlett-Packard Co, Rockville, MD) or a model 120A PTH analyzer (Applied Biosystems). For amino sugar analysis, peptides were hydrolyzed in 4 N HCl at 100°C for 7 hours. They were then derivatized and analyzed as described above for amino acid analysis with
15 a Novapak C18 column (Waters).

The purified peptide contained the LPSTGE (SEQ ID NO:14) sequence flanked on either side by amino acids found in this position in the streptococcal M protein molecule. As a marker, the peptide was sequenced with a
20 tyrosine (Y) at the C-terminal end so that it could be labeled with ^{125}I .

The peptide was radiolabeled with ^{125}I using Iodobeads and then the labeled peptide was purified on a Sephadex G10 column and covalently linked by its N-
25 terminal end by EDC (ethyl-3-(3 diethylaminopropyl)carbodiimide HCl) to an extended arm on a commercially available bead (3M Emphase Biospheres AB1 from Pierce, Rockford, IL), and the excess radiolabel removed by washing the beads with 1 M NaCl buffer.

30

-25-

EXAMPLE 2Detection of cleavage activity in streptococcal membrane extracts.

5 M type 6 streptococcal strain D471 was treated with 0.1 M sodium carbonate (pH 11.5) and incubated for 30 minutes at 0°C to extract the streptococcal membranes.

10 The resulting extract was mixed with the labeled beads of Example 1 (50 μ l sample, 50 μ l 50 mM Tris HCl (pH 8.0) with 10 mM DTT). Radiolabel was released in a time- and dose-dependent manner, as seen in Figures 1 and 2. Controls using nonionic detergent, and buffers containing a high concentration of salt were unable to release the enzymatic activity suggesting that the cleaving enzyme activity was firmly associated with the streptococcal membrane. However, membranes extracted with 0.1 M sodium carbonate pH 11.5 demonstrated cleavage activity.

EXAMPLE 3

20 Isolation of the cleaving enzyme.

The carbonate extract was chromatographed on a 20 HQ perfusion chromatography column, using a Biocad Sprint instrument (Perceptive Biosystem), employing a NaCl gradient from 0 mM to 500 mM (24.9 ml) followed by a 500 mM to 1000 mM (33.2 ml) gradient. To measure the enzyme activity found in the fractions, a 50 μ l sample from each fraction was mixed in a reaction buffer in a final volume of 100 μ l containing 5 mM DTT and 5 μ l of the bead-substrate as described in Example 1. Control samples containing only buffer and the substrate were also mixed and incubated at 37°C for 4 hours under constant slow rotation. The released radioactivity was then measured in 25 μ l of the supernatant obtained after centrifugation. The specific activity was measured after subtracting the control values from the test samples.

35

-26-

The enzyme activity was eluted in one peak obtained between 0.6 M to 0.7 M NaCl (i.e., 37.5-43.2 mSiemens of conductivity) gradient in a total of 6 fractions of 1 ml each. The specific released radioactivity in these
5 fractions was found to be between 6,000 to 11,000 cpm as compared to other fractions where the values varied from 0 to 1000 cpm.

EXAMPLE 4

10 Properties of the cleavage enzyme.

Figure 3 illustrates the enzymatic activity of the carbonate extract of the membranes (as the enzyme source) in the presence of various inhibitors and activators.

Activity was found to be enhanced in the presence of
15 5 mM DTT and divalent cations such as calcium.

1 mM each of parahydroxymercuribenzoic acid (PHMB) and parahydroxymercuriphenylsulfonic acid (PHMPS) inhibited cleavage activity.

These results indicate that the enzyme is sulfhydryl
20 dependant.

EXAMPLE 5

Determination of the site of cleavage.

Cleaved labeled synthetic peptide is analyzed using
25 C-terminal sequencing and/or amino acid analysis to determine the exact site of cleavage, and optimal flanking regions to the target LPXTGX (SEQ ID NO:1) site.

While the invention has been described and illustrated herein by references to various specific
30 material, procedures and examples, it is understood that the invention is not restricted to the particular material combinations of material, and procedures selected for that purpose. Numerous variations of such details can be implied as will be appreciated by those
35 skilled in the art.

-27-

WHAT IS CLAIMED IS:

1. An isolated and purified enzyme which cleaves surface proteins of gram-positive bacteria.
- 5 2. The enzyme of Claim 1, wherein the bacteria is *Streptococcus*.
- 10 3. The enzyme of Claim 1, wherein the *Streptococcus* is a group A *Streptococcus*.
4. The enzyme of Claim 3, wherein the enzyme cleaves an M protein.
- 15 5. The enzyme of Claim 4, wherein the enzyme cleaves a peptide comprising the amino acid sequence LPXTGX (SEQ ID NO:1).
- 20 6. The enzyme of Claim 5, wherein the amino acid sequence is LPSTGE (SEQ ID NO:14).
7. The enzyme of Claim 6, wherein the enzyme's cleavage activity is enhanced in the presence of a reducing agent.
- 25 8. The enzyme of Claim 7, wherein the reducing agent is dithiothreitol.
9. The enzyme of Claim 6, wherein the enzyme is inhibited by parahydroxymercuribenzoic acid or
30 parahydroxymercuriphenylsulfonic acid.
10. An antibody directed to the enzyme of Claim 1.
11. The antibody of Claim 10, wherein the antibody is
35 polyclonal.

-28-

12. The antibody of Claim 10, wherein the antibody is monoclonal.

13. A method for detecting the presence of the enzyme of Claim 1, comprising the steps of:

(a) synthesizing a peptide comprising an amino acid sequence of LPXTGX (SEQ ID NO:1);

(b) labeling the peptide of step (a) with a detectable marker;

(c) covalently linking the labeled peptide to a chromatographic bead;

(d) mixing the linked beads with a membrane extract of gram-positive bacteria;

(e) detecting a release of label from the beads;

and

(f) correlating the release of label with the presence of the enzyme.

14. The method of Claim 13, wherein the peptide comprises the sequence LPSTGE (SEQ ID NO:14).

15. The method of Claim 13, wherein the bacteria is *Streptococcus*.

16. The method of Claim 15, wherein the *Streptococcus* is a group A *Streptococcus*.

17. The method of Claim 13, wherein the label is radioactive.

18. The method of Claim 13, wherein the chromatographic bead is from 30-100 μm in diameter.

19. The method of Claim 13, wherein the chromatographic bead is from 50-80 μm in diameter.

-29-

20. The method of Claim 13, wherein the membrane extract is made using carbonate buffer.

21. The method of Claim 20, wherein the carbonate buffer
5 is from 0.05-2.0 M sodium carbonate.

22. The method of Claim 21, wherein the carbonate buffer is 0.1 M sodium carbonate.

10 23. The method of Claim 20, wherein the carbonate buffer is at a pH of 9-14.

24. The method of Claim 23, wherein the carbonate buffer is at a pH of approximately 11.5.

15

25. A method of isolating an enzyme which cleaves surface proteins on gram-positive bacteria, comprising the steps of:

- 20 (a) preparing a membrane extract of the gram-positive bacteria containing membrane-bound proteins;
(b) fractionating the membrane-bound proteins by chromatography using a salt gradient; and
(c) identifying the presence of the enzyme in a fraction using the method of Claim 13.

25

26. The method of Claim 25, wherein the gram-positive bacteria is *Streptococcus*.

27. The method of Claim 26, wherein the *Streptococcus* is
30 a group A *Streptococcus*.

28. The method of Claim 25, wherein the membrane extract is made using carbonate buffer.

-30-

29. The method of Claim 28, wherein the carbonate buffer is from 0.05-0.2 M sodium carbonate.

30. The method of Claim 29, wherein the carbonate buffer is 0.1 M sodium carbonate.

31. The method of Claim 28, wherein the carbonate buffer is at a pH of 9-14.

32. The method of Claim 31, wherein the carbonate buffer is at a pH of approximately 11.5.

33. A method for assessing the activity of putative inhibitors of the enzyme of Claim 1, comprising the steps of:

- (a) synthesizing a peptide comprising an amino acid sequence of LPXTGX (SEQ ID NO:1);
- (b) labeling the peptide of step (a) with a detectable marker;
- (c) covalently linking the labeled peptide to a chromatographic bead;
- (d) mixing the linked beads with a membrane extract of gram-positive bacteria in the presence of the putative inhibitor;
- (e) detecting a release of label from the beads; and
- (f) comparing the release of label in the presence of the inhibitor with the release of label in the absence of the putative inhibitor.

30

34. A method for inhibiting the activity of the enzyme of Claim 1, comprising treating the enzyme with a compound which oxidizes the sulfhydryl groups of the cystein residues in the enzyme to form cystine, in an

-31-

amount sufficient to inactivate the binding of the enzyme to its substrate.

35. A method for inhibiting the activity of the enzyme
5 of Claim 1, comprising treating the enzyme with a heavy metal-containing compound which reacts with the sulfhydryl groups of the cystein residues in the enzyme to form mercaptides, in an amount sufficient to inactivate the binding of the enzyme to its substrate.
10
36. The method of claim 35, wherein the heavy metal-containing compound is selected from the group consisting of parahydroxymercuribenzoic acid and parahydroxymercuriphenylsulfonic acid.
15
37. A compound which inhibits the enzyme of claim 1.

1/4

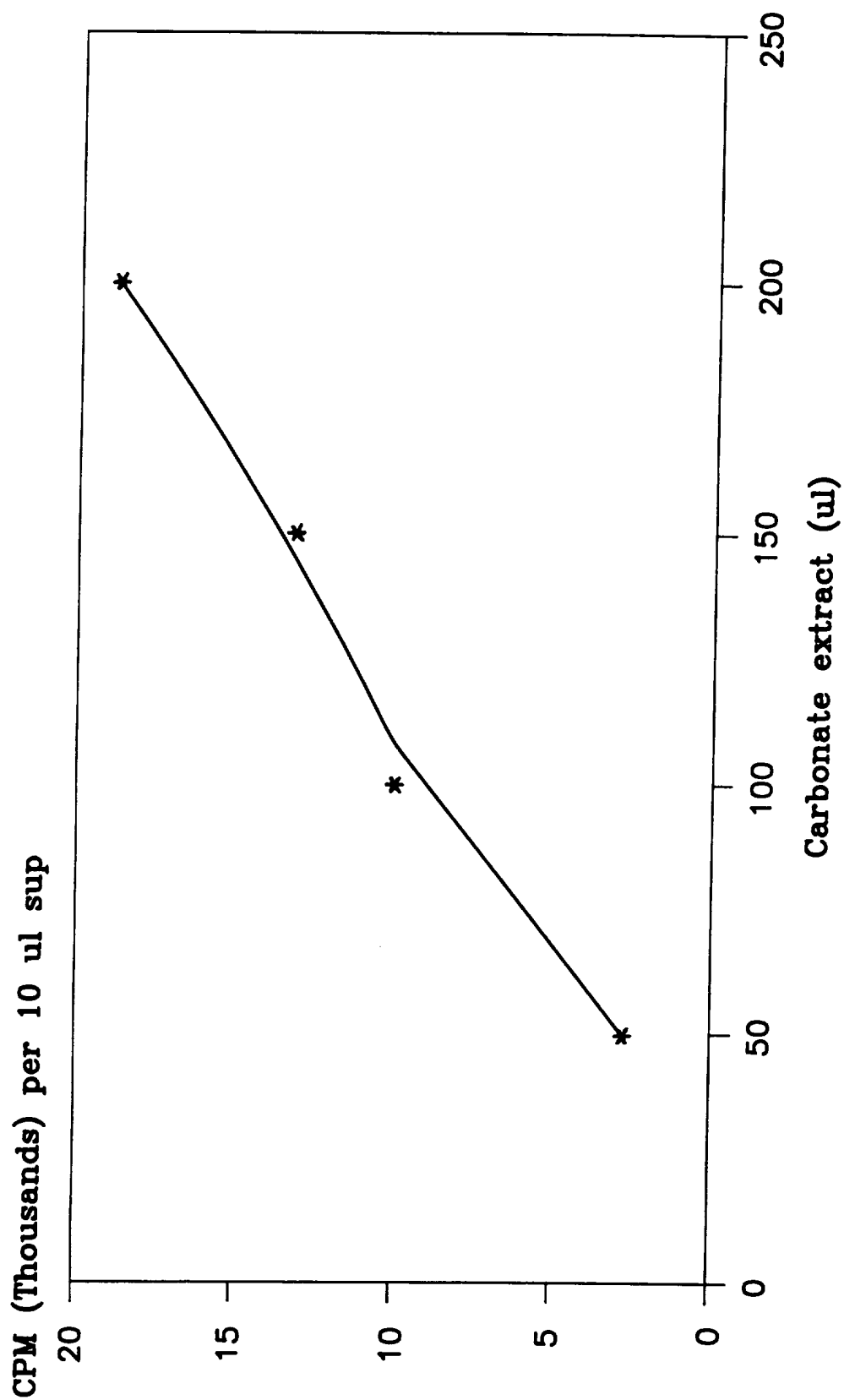


FIG. 1

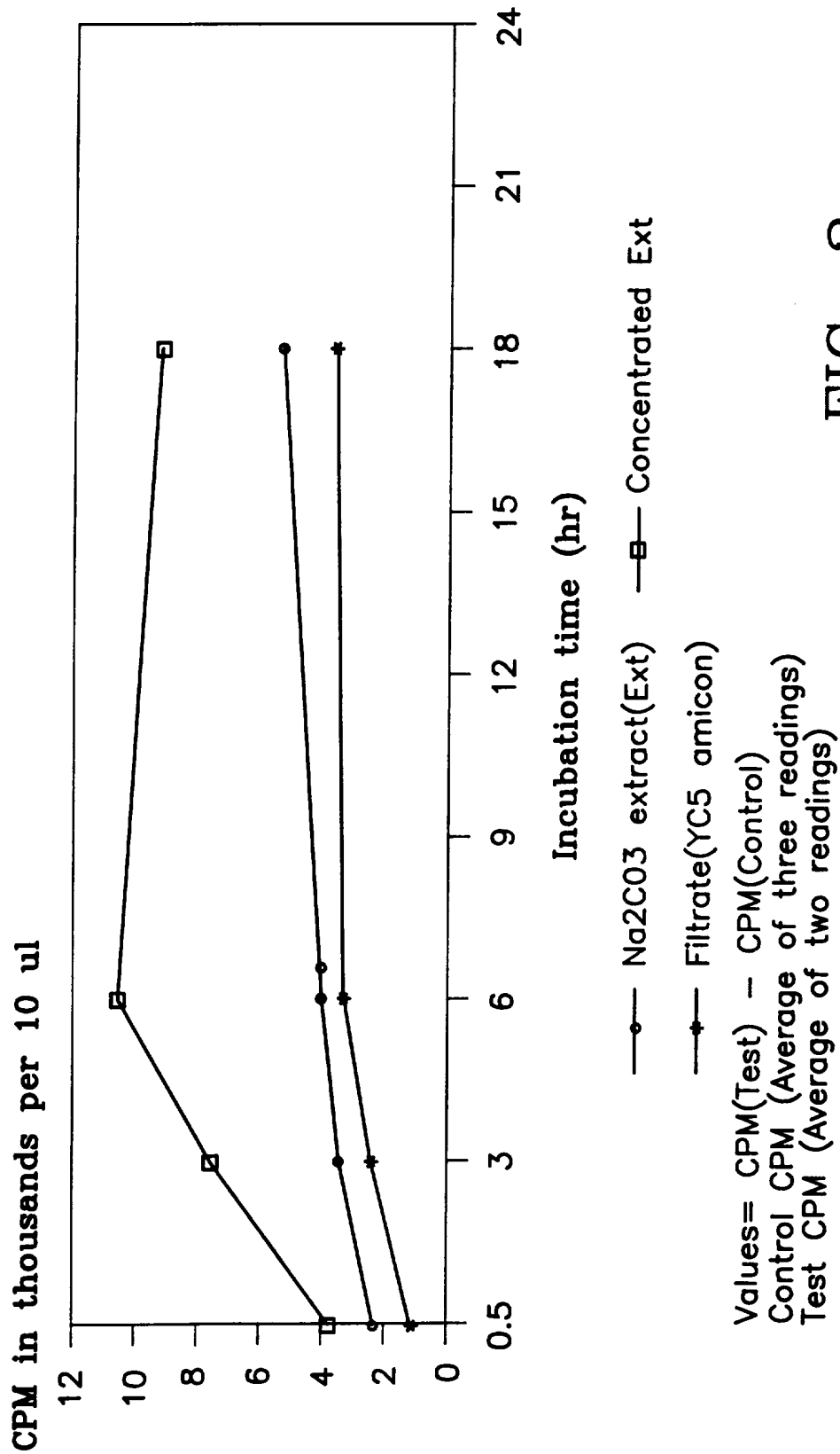
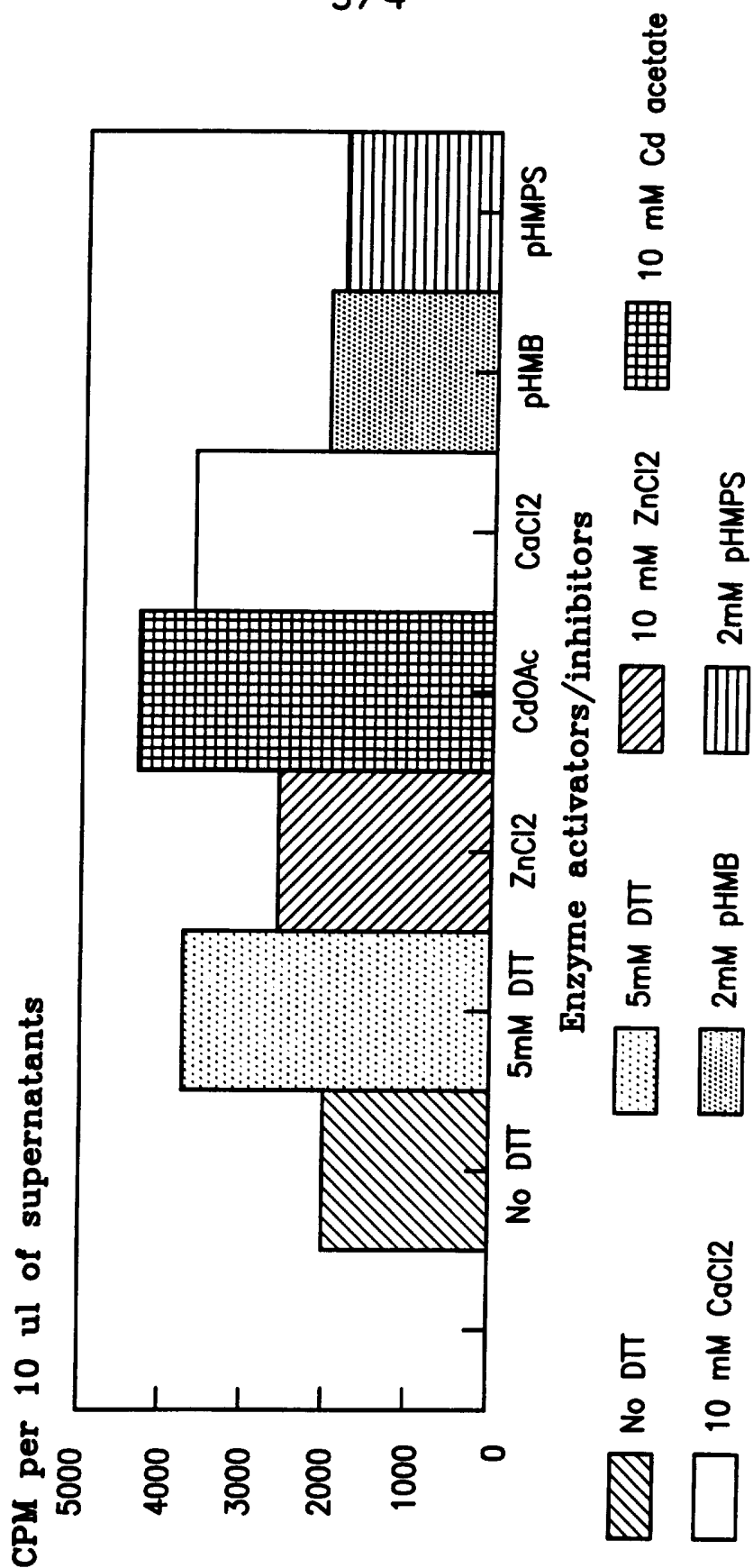


FIG. 2

3 / 4



Values = CPM(test) - CPM(Control)
 All values are average of three
 independent readings. SD < 5%

FIG. 3

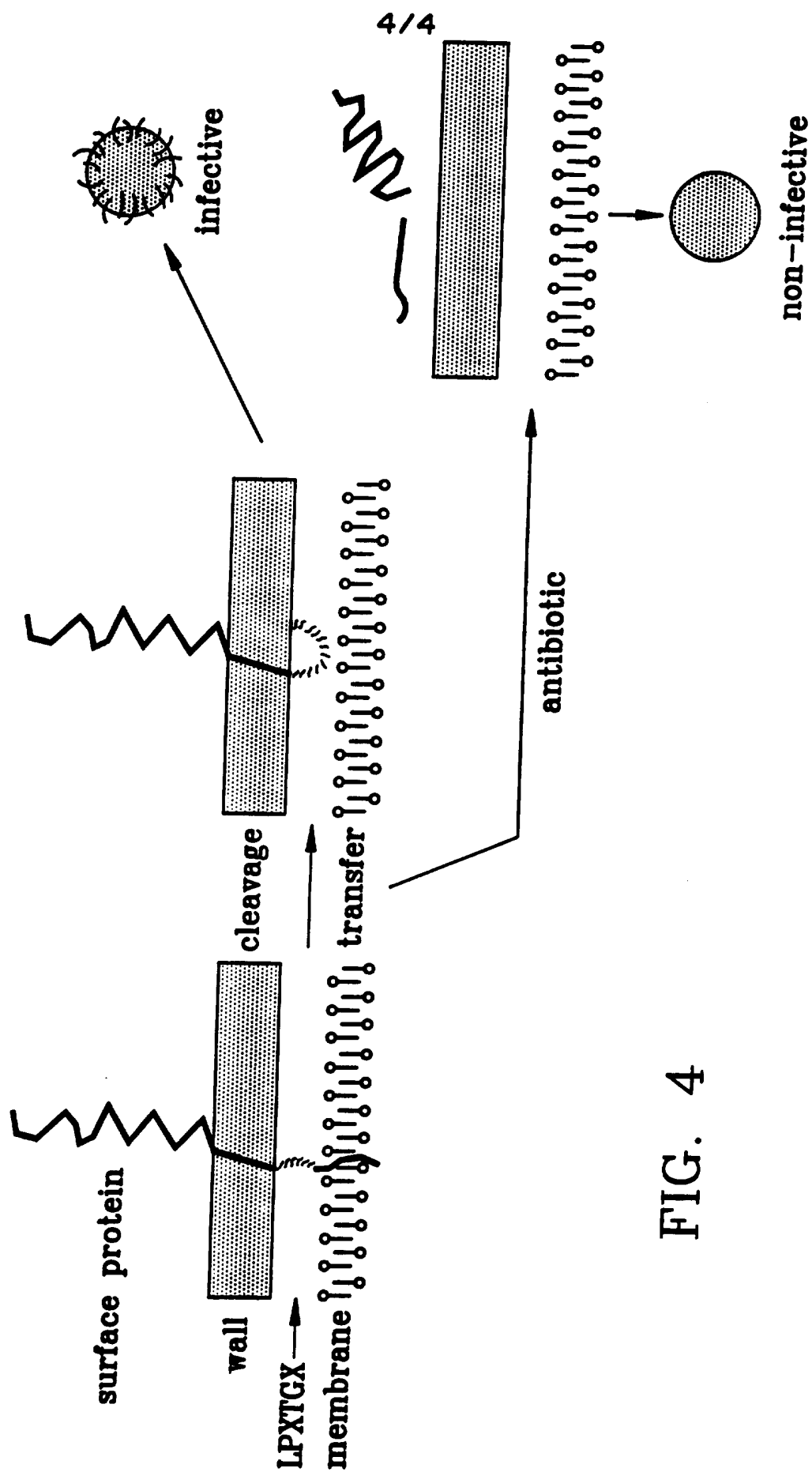


FIG. 4

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/12460

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C12N 9/50, 9/52, 9/54

US CL :435/219,220, 221

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. : 435/219, 220, 221

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

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

APS, MEDLIN, SCISEARCH

search terms: protease#, proteinase#, cleav?, hydroly?, fischetti, pancholi v

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A,P	TRENDS MICROBIOL., Volume 3, Number 9, issued September 1995, H.F. Jenkinson, "Anchorage and Release of Gram-Positive Bacterial Cell-Surface Polypeptides", pages 333-335.	1-37
A	MOLEC. MICROBIOL., Volume 4, Number 9, issued 1990, V.A. Fischetti et al., "Conservation of a Hexapeptide Sequence in the Anchor Region of Surface Proteins form Gram-Positive Cocci", pages 1603-1605.	1-37



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)	*G*	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

15 DECEMBER 1995

Date of mailing of the international search report

29 JAN 1996

Name and mailing address of the ISA/US
Commissioner of Patents and Trademarks
Box PCT
Washington, D.C. 20231

Authorized officer

JON P. WEBER, PH.D.

Facsimile No. (703) 305-3230

Telephone No. (703) 308-0196

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/12460

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	J. EXP. MED., Volume 170, Number 6, issued December 1989, V. Pancholi et al., "Identification of an Endogenous Membrane Anchor-Cleaving Enzyme for Group A Streptococcal M Protein", pages 2119-2133, see entire document. (Also attached is a correction page published in Ibid., Volume 171, Number 2, at page 593).	1-9, 34-37 ----- 10-33
A, P	MOLEC. MICROBIOL., Volume 14, Number 1, issued October 1994, W.W. Navarre et al., "Proteolytic Cleavage and Cell Wall Anchoring at the LPXTG Motif of Surface Proteins in Gram-Positive Bacteria", pages 115-121.	1-37
Y	EMBO J., Volume 12, Number 12, issued 01 December 1993, O. Schneewind et al., "Cell Wall Sorting Signals in Surface Proteins of Gram-Positive Bacteria", pages 4603-4611, see entire document.	1-37
A, Y	MOLEC. MICROBIOL., Volume 9, Number 5, issued September 1993, S. Dramsi et al., "Common Features of Gram-Positive Bacterial Proteins Involved in Cell Recognition", pages 1119-1122, see entire document.	1-37
X --- Y	INFECT. IMMUN., Volume 60, Number 10, issued October 1992, S.F. Lee, "Identification and Characterization of a Surface Protein-Releasing Activity in Streptococcus mutans and Other Pathogenic Streptococci", pages 4032-4039, see entire document.	1-3, 7-9 ----- 4-6, 10-37
A	CELL, Volume 70, issued 24 July 1992, O. Schneewind et al. "Sorting of Protein A to the Staphylococcal Cell Wall", pages 267-281.	1-37
A,P	SCIENCE, Volume 268, Number 5207, issued 07 April 1995, O. Schneewind et al., "Structure of the Cell Wall Anchor of Surface Proteins in Staphylococcus aureus", pages 103-106.	1-37
Y	US, A, 4,598,044 (KRICKA ET AL.) 01 July 1986, see especially column 8, lines 15-24.	13-37
Y	US, A, 5,235,039 (HEATH, JR., ET AL.) 10 August 1993, see especially the abstract, for example.	13-37

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/12460

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/12460

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING

This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

I. a) Claims 1-9, drawn to a first product, an anchor protease.

b) Claims 13-24, and 33-36 drawn to a methods of use of the first product: a method of assaying anchor protease, a method of finding inhibitors to anchor protease, and a method of inhibiting anchor protease.

c) Claims 25-32, drawn to a first method of making a first product, a method of isolating anchor protease.

II. Claims 10-12, drawn to a second product, an antibody to anchor protease.

III. Claim 37, drawn to a third product, inhibitors of anchor protease.

The inventions listed as Groups I, II, and III do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

Inventions Groups I, II, and III are each drawn to distinct products: an enzyme, an antibody, and inhibitors of unknown nature. The antibody and inhibitors both bind to the enzyme, but so do ions, substrates, and modifiers. None of these products shares any structural similarity.