The Major Extracellular Protease of the Nosocomial Pathogen
Stenotrophomonas maltophilia

CHARACTERIZATION OF THE PROTEIN AND MOLECULAR CLONING OF THE GENE*

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Stenotrophomonas maltophilia is increasingly emerging as a multiresistant pathogen in the hospital environment. In immunosuppressed patients, these bacteria may cause severe infections associated with tissue lesions such as pulmonary hemorrhage. This suggests proteolysis as a possible pathogenic mechanism in these infections. This study describes a protease with broad specificity secreted by S. maltophilia. The gene, termed StmPr1, codes for a 63-kDa precursor that is processed to the mature protein of 47 kDa. The enzyme is an alkaline serine protease that, by sequence homology and enzymic properties, can be further classified as a new member of the family of subtilases. It differs from the classic subtilisins in molecular size, in substrate specificity, and probably in the architecture of the active site. The StmPr1 protease is able to degrade several human proteins from serum and connective tissue. Furthermore, pan-protease inhibitors such as α1-antitrypsin and α2-macroglobulin were unable to abolish the activity of the bacterial protease. The data support the interpretation that the extracellular protease of S. maltophilia functions as a pathogenic factor and thus could serve as a target for the development of therapeutic agents.

Stenotrophomonas maltophilia, formerly referred to as Xanthomonas maltophilia or Pseudomonas maltophilia (1, 2), is an aerobic nonfermentative Gram-negative bacterium of widespread occurrence. For healthy humans, it is regarded as an opportunistic germ; it has been implicated in a variety of infections without distinctive clinical features (for a review, see Ref. 3). However, in immune-compromised patients, particularly those with bone marrow aplasia or receiving intensive chemotherapy, cases of fulminant hemorrhagic pneumonia have been reported, even with fatal outcome (4–6). In patients not surviving infections with S. maltophilia, histological inspection of the lung tissue revealed massive bleeding caused by damage to the lung epithelium (4). There are further reports demonstrating involvement of this bacterium in massive hemorrhagic processes of the small intestine and the subclavian artery accompanied by severe lesions of the tissue (5, 6). These observations strongly suggest the participation of proteolytic activity, produced by the bacteria, which may damage the infected tissue. Indeed, it is known that members of the Pseudomonaceae express and secrete a variety of proteases (cf. Ref. 7). Whereas the primary function of these enzymes is to provide a source of free amino acids for bacterial survival and growth, there is accumulating evidence that bacterial proteases may play a pathogenic role in the infected host by involvement in tissue invasion and destruction, evasion of host defenses, and modulation of the host immune system (8).

The broad administration of antibiotics currently applied in cases of intensive care patients leads to selection of multiresistant S. maltophilia strains. Consequently, these bacteria are found with increasing frequency in the hospital environment. Because of the known multiresistance of this germ toward conventional antibiotics (for a review, see Ref. 9), bacterial proteases involved in the pathogenesis of human diseases are potential targets for specific drug development. This prompted us to test cultures of S. maltophilia obtained from patient material for the presence of proteolytic activity. Indeed, a highly active protease was detected as a major secretion product of the isolated bacteria.

This study describes the purification, cloning, and characterization of the S. maltophilia extracellular protease.

EXPERIMENTAL PROCEDURES

Source and Cultivation of Bacteria—S. maltophilia was isolated from a bronchoalveolar lavage performed on a patient at the Hamburg university clinic. The identity of the germ was established by standard bacteriological techniques (API 20 NE; BioMérieux, Marcy-L’Etoile, France). Bacteria were grown aerobically at 29 °C in a broth containing 5 μM MnSO4, 0.36 mM CaCl2, 0.5 mM L-methionine, 0.8 mM MgSO4, 2.2 mM K2HPO4, 3.7 mM KH2PO4, 6 mM (NH4)2HPO4, 50 mM disodium succinate, 2 glitier yeast extract (ICN Biomedicals), 40 μg/ml gentamycin, 50 μg/ml cephotaxim, and 150 μg/ml ampicillin.

Purification of the Protease—Cell-free supernatant (12.5 liters) was obtained from S. maltophilia cultures by centrifugation at 4 °C and mixed with 80 ml of DE-52 (Whatman) cellulose equilibrated with 10 mM Tris/HCl buffer, pH 7.4, and the mixture was stirred overnight at 4 °C. The matrix was then collected by sedimentation, transferred into a column, and washed with 10 mM Tris/HCl buffer, pH 7.4. Protein fractions were eluted by a linear gradient of 0–500 mM NaCl in the same buffer at a flow rate of 2 ml/min. 30 fractions of 24 ml were collected and assayed for proteolytic activity (see below). A single peak of activity was detected; the respective fractions were pooled and concentrated by ultrafiltration (Amicon YM 10 membrane) at 4 °C to a final volume of 4 ml. This sample was divided into two aliquots, and each was

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fractionated at a flow rate of 1 ml/min over a 310-ml column of Fractogel EMD BioSec 650 (Merek) equilibrated with phosphate-buffered saline. Fractions of 6 ml were collected, and the two fractions containing most of the proteolytic activity were pooled and served to characterize the protease. When this purified preparation was compared with the crude proteases, the similarity in their subunit patterns (930 bp, which was sequenced (Applied Biosystems 377). Optical density at 280 nm (mg/ml standard. The results were comparable with values calculated from the StmPr1 (pain and chymostatin), assays contained 1.4 mM (b/kHg a/h x ) of the substrate Suc-Ala-Ala-Pro-Phe-pNA and inhibitor over a wide range of concentration. The IC50 is obtained as the constant a/b of the inhibitor concentration (\( \log_{10} \)).

Enzyme Assays—For the initial detection of proteolytic activity in bacterial supernatants, a microassay using the nonspecific chromogenic substrate azoalbumin (Sigma) was performed as described in Ref. 12. In all other cases, a substrate specific for serine proteases was used (0.5 mM Suc-Ala-Ala-Pro-Phe-pNA, unless otherwise stated). Hydrolysis was allowed to occur in 200 μl of 20 mM sodium phosphate, pH 9.0, containing 400 mM NaCl at 37 °C. The amount of released p-nitroaniline within initial time intervals was measured at 405 nm (ε280 = 9600 M⁻¹ cm⁻¹). For determination of the IC50 of protease inhibitors (anti- p-chymotrypsin), assays contained 1.4 mM (Kc) of the substrate Suc-Ala-Ala-Pro-Phe-pNA and inhibitor over a wide range of concentration. The IC50 is obtained as the constant b of a nonlinear regression analysis of the function (a/b + 10^x) when the reaction velocity is plotted versus 10^x of the inhibitor concentration (x). Kinetic experiments with various synthetic peptide p-nitroanilides were carried out in 100 mM Tris/HCl buffer, pH 8.2, at 25 °C and in the presence of 5% dimethyl sulfoxide. The enzyme concentration was usually in the range of 1.95 × 10⁻⁶ to 9.12 × 10⁻⁸ M, and the concentration of the substrate varied between 1.6 × 10⁻⁵ and 1.2 × 10⁻⁴ M. Kinetic parameters were calculated from initial rate measurements of substrate hydrolysis using a nonlinear regression analysis based on the function \( V_{max} = a/K_c + x \), with x = the concentration of substrate.

Protein Sequencing—After SDS-PAGE, the protein was blotted onto polyvinylidene difluoride membranes (Immobilon P, Millipore) and stained with Coomassie Brilliant Blue R-250. The excised band was sequenced by standard Edman degradation on an automated sequencer (Applied Biosystems 476A). To obtain internal sequence information, the Coomassie Brilliant Blue R-250-stained protein was cut out of the SDS gel and in-gel digested with the endoproteases Lys-C or Asp-N (Roche Molecular Biochemicals) in 50 mM Tris/HCl, pH 8.5, containing 1 mM EDTA (for digestion with Lys-C) or 50 mM Tris/HCl, pH 8.0 (for digestion with Asp-N), at 37 °C overnight. The peptides obtained were separated by reverse phase HPLC on a Vydac C18 column (250 × 2.1 mm) at a flow rate of 200 μl/min. The following gradient was applied: 2–80% B over a 50-min period (solvent A, 0.1% trifluoroacetic acid in water; solvent B, 0.085% trifluoroacetic acid in 70% acetonitrile). The obtained peptides were identified by mass spectrometry on a hybrid tandem mass spectrometer (Qtof II; Micromass) equipped with a nanoelectrospray source. The peptides were vacuum dried and redissolved in 5 μl of 60% methanol/5% formic acid. 1 μl of this solution was transferred into a gold-coated nanoelectrospray needle (Micromass). From the masses of the peptides determined, a tentative assignment to fragments of the insulin B chain was derived. The assignment was confirmed by subsequent tandem mass spectrometric measurements of the peptide fragmentation pattern or, in some cases, by Edman degradation.

RESULTS AND DISCUSSION

Detection of Proteolytic Activity in Bacterial Cultures

A culture of S. maltophilia was grown from a specimen of an immunocompromised patient. Proteolytic activity was detected in the cell-free growth medium of the bacterial culture using azo-albumin as an unspecific chromogenic substrate (data not shown).

To optimize bacterial cultures as a source for purification of the putative protease, the production of the enzyme during culture growth was measured. As shown in Fig. 1, the proteolytic activity is hardly detectable in the early stages of the culture; rather, protease production is up-regulated only toward the end of the exponential phase of the growth curve. Proteolytic activity reached a maximum after about 22 h and remained unchanged for at least 3 days.

Protease Purification

A culture supernatant of S. maltophilia served as a source for the protease isolation. Adsorption on an anion exchange resin was applied to concentrate and separate proteins from the bacterial broth; elution by a salt gradient yielded a single peak of proteolytic activity, which was then further fractionated by gel filtration. SDS gel electrophoresis of the protease-containing fraction revealed one major band of 47-kDa apparent molecular mass (Fig. 2A). Comparison with the electrophoretic pattern of the crude bacterial supernatant indicated that the 47-kDa protein represents the major secretory product of S. maltophilia. Amino-terminal sequencing of the 47-kDa bands yielded the sequence LAPDNPPYQQ, which turned out to be absent from protein sequence data bases. The sequence, however, showed homology with the amino termini of several known bacterial proteases, the closest of which is a serine protease from Dichelobacter nodosus (15), a member of the family of subtilisin-like proteases (cf. Ref. 7).

Covalent coupling with the radioactive inhibitor \(^{[3}H]diphosphatase confirmed that the 47-kDa protein of S. maltophilia is a serine protease (Fig. 2B). When the crude bacterial supernatant was allowed to react with the inhibitor, autoradi-
Stenotrophomonas maltophilia Protease

**Experimental Procedures**

**Growth and production of extracellular protease by S. maltophilia.** 50 ml of culture medium were inoculated with 0.1 ml of bacterial suspension (stationary phase) and grown at 29 °C. Samples were taken at intervals and tested for density (□) and, after centrifugation, enzyme activity (■). Four separate experiments of this type were performed, yielding similar results; data from one representative experiment are presented.

**Sequence Determination by Molecular Cloning**

To analyze the sequence of the protease and determine its relationship to other proteases, the gene was cloned by PCR techniques. Sequences from the amino terminus of the purified protein and from an internal peptide (PLAPAAMRT) generated by Lys-C digestion served to design degenerate primers. Using genomic DNA of *S. maltophilia* as a template, a 950-bp amplified fragment was obtained. Sequencing of the missing carboxy-terminal portion of the gene required additional steps of 3′-extension: PCR using poly(A)-tailed fragments of genomic DNA as PCR template and inverse PCR from highly diluted DNA fragments were applied until a stop codon was found (Fig. 3).

Because it is common for most prokaryotic extracellular proteases to be produced as larger precursor proteins (cf. Ref. 7), 5′-extension of the DNA sequence was performed to obtain the sequence of the entire gene. Applying the same techniques for extension as outlined above, a sequence was obtained that contained a stop codon within the reading frame and only one ATG coding for a methionine in position –132. However, two points argue against this ATG coding for the translation initiation of the protease precursor: (i) it is not preceded by a typical Shine-Dalgarno ribosome binding site, and (ii) the sequence 3′ to this ATG does not predict a signal peptide typical for Gram-negative bacteria (16). Therefore, we assume that, following an alternative bacterial codon usage, a GTG codes for translation initiation, resulting in a methionine in position –150. In this case, the synthax for both Shine-Dalgarno and signal sequences would be met (Fig. 3). Further evidence for this GTG to function as a start codon for the protease precursor came from the recombinant expression of the gene. When the DNA starting with the GTG in question (and not with ATG in position –132) was expressed in *Escherichia coli*, the protein was correctly processed, resulting in the mature protease with full enzymatic activity (data not shown). The DNA sequence of the gene was further established, and the amino-terminal sequences of the processed recombinant protein and of the native protease were found to be identical. Moreover, antibodies generated against the native protein also recognized the recombinant gene product (data not shown).

Taken together, the open reading frame encodes a protein with a deduced molecular mass of 63.0 kDa, corresponding to 618 amino acids in length (Fig. 3). The 27-residue stretch of the amino terminus was predicted to be the signal peptide containing a potential signal peptidase cleavage site (16) between Ala12 and Ala123.

Following the putative signal sequence and preceding the amino terminus of the mature protein, there is a pro-region of 123 residues. Finally, the sequence between the amino terminus, identified in the native protein, and the carboxyl terminus as indicated by the stop codon corresponds to a protein that encompasses 467 amino acids with a theoretical pI = 4.91 and a calculated molecular mass of 47,446 Da.

The *S. maltophilia* protease gene was termed *StmPr1* (European Molecular Biology Laboratory accession number AJ291488); the term takes into account that another protease (*StmPr2*)2 was detected in *S. maltophilia* during preparation of this manuscript.

**Comparison of the StmPr1 protease sequence with those of known bacterial serine proteases confirmed its relation to the subtilisin family of proteases (cf. Ref. 7). Alignment of the sequences indicated that Asp92, His610, and Ser708 form the putative catalytic triad characteristic for serine proteases. In the active site region, there is considerable homology with other subtilisins; in Fig. 3, conserved residues that are shared with subtilisin BPN’ (17) and proteinase K (18) are marked with open boxes as typical representatives of the “classic” subtilisins. Nevertheless, the *StmPr1* protease sequence reveals significant structural differences from these related enzymes due to large

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inserts adjacent to the catalytic His105 and Ser289. Therefore, compared with the catalytic triad formed by Asp32, His64, and Ser221 in the typical members of the subtilisin family, the architecture of the active site should be different in the StmPr1 protease. In addition, this enzyme has a longer carboxyl-terminal extension beyond the active site, which, together with the inserts in the catalytic region, makes the entire sequence almost 100 residues longer. With these structural properties, the StmPr1 protease gene and flanking DNA and the deduced amino acid sequence of the precursor molecule.

The putative Shine-Dalgarno sequence is double-underlined. The vertical arrow indicates the predicted signal peptidase cleavage site, whereas the translation-termination codon is marked with an asterisk. The amino-terminal and internal residues sequenced are underlined. Nucleotides are numbered from the putative translation initiation codon, whereas peptides are numbered from the amino terminus of the mature protein. The amino acids of the catalytic triad Asp42, His105, and Ser289 are boxed and shaded. Open boxes indicate regions of identity that result from alignment with the sequences of subtilisin BPN' (17) and proteinase K (18) using clustal W1.8 (29). The 5'-flanking sequence is not numbered; typical features of transcription promotor sequences (M. Reese, Neural Network Promoter Prediction, www.fruitfly.org/seq_tools/promoter.htm) were not detected within this stretch of 593 nucleotides sequenced.

Fig. 3. Nucleotide sequence of the StmPr1 protease gene and flanking DNA and the deduced amino acid sequence of the precursor molecule. The putative Shine-Dalgarno sequence is double-underlined. The vertical arrow indicates the predicted signal peptidase cleavage site, whereas the translation-termination codon is marked with an asterisk. The amino-terminal and internal residues sequenced are underlined. Nucleotides are numbered from the putative translation initiation codon, whereas peptides are numbered from the amino terminus of the mature protein. The amino acids of the catalytic triad Asp32, His105, and Ser289 are boxed and shaded. Open boxes indicate regions of identity that result from alignment with the sequences of subtilisin BPN' (17) and proteinase K (18) using clustal W1.8 (29). The 5'-flanking sequence is not numbered; typical features of transcription promotor sequences (M. Reese, Neural Network Promoter Prediction, www.fruitfly.org/seq_tools/promoter.html) were not detected within this stretch of 593 nucleotides sequenced.

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protease is similar to the extracellular proteases of Xanthomonas campestris (19), D. nodosus (15), and Alteromonas sp. (20); the homology with these proteases is 49%, 40%, and 38% identity, respectively, for the mature proteins. On the other hand, there is only low homology within the region of the carboxyl-terminal extensions, and no homology can be seen between the sequences. The sequence homology between the characterized subtilisins and the StmPr1 protease is also lower, e.g. 23% identity with proteinase K (18) within the region of the mature enzymes.

Properties of the Enzyme

In view of the sequence differences between the well-characterized subtilisins and the StmPr1 protease, it was important to analyze the enzymatic activity of the new protease in detail.

The StmPr1 protease hydrolyzes the widely used chromogenic substrate Suc-Ala-Ala-Pro-Phe-pNA with a $K_m$ of 1.4 mM. This substrate was used for characterization of the enzyme purified from the native source.

Effect of pH

The enzyme activity of the purified StmPr1 protease was measured in the pH range 4–11 (Fig. 4A) and showed a typical bell shape. The optimum pH was 9.0, classifying the StmPr1 protein as an alkaline protease. Pre-exposure of the protease to extreme pH (0.1 M acetic acid, pH 3) for 10 min on ice resulted in a 68% loss of enzyme activity.

Modulation of Enzyme Activity

A study of the salt requirement for enzyme activity was conducted by assaying the enzyme at pH 9. Raising the final NaCl concentration to 0.4 M increased activity about 4-fold (Fig. 4B). No further increase in enzyme activity was observed at higher salt concentrations (1 M NaCl was the maximum concentration tested). The stimulating effect of NaCl has also been reported for several other proteases of the subtilisin family (21). Enzyme activity was also found to be stimulated by calcium, which was effective at low concentrations; a maximum 3.5-fold increase in activity was observed at 50 mM calcium chloride. The effects of Na$^+$ and Ca$^{2+}$ were not additive. Ca$^{2+}$ can be replaced by Mg$^{2+}$ to achieve the same activating effect (data not shown). Thus, the StmPr1 protease is an enzyme dependent on bivalent metal ions; the strong activation effect of the cations can be explained by a conformational change leading to a catalytically more active conformation. Na$^+$ possibly binds to the same site and may substitute for Ca$^{2+}$ at higher concentrations.

A remarkable property of the StmPr1 protease is its relative stability toward the anionic detergent sodium dodecyl sulfate (Fig. 4C). The enzyme preserved 85% of its activity in the presence of 0.1% detergent, and even at a concentration of 1% dodecyl sulfate, 45% of proteolytic activity was retained. Similar results indicating a particular conformational stability have been reported for some, mainly microbial, proteases (cf. Ref. 22). By contrast, the mammalian serine protease chymotrypsin tested under the same conditions completely lost its activity at a concentration of 1% detergent.

To get more information on the type of protease, the effect of a series of protease inhibitors on StmPr1 enzyme activity was tested (Table I). The enzyme was effectively inhibited by antipain, chymostatin, and phenylmethylsulfonyl fluoride, whereas other serine protease inhibitors such as leupeptin, TLCK, and TPCK were not effective. The lack of inhibitory
activity of TPCK is in contrast to the reported effect of this compound on subtilisin (21, 23). The StmPr1 protease is not inhibited by EDTA, presumably because the calcium bound to the enzyme cannot be chelated, and the protein remains structurally unaffected. This result demonstrates that metal ions are not directly involved in the catalytic mechanism, which is characteristic for subtilisins and other serine proteases.

Of interest with respect to the pathogenic potential of the StmPr1 protease was the observation that human plasma protease inhibitors α1-antitrypsin and α2-macroglobulin could not abolish the proteolytic activity of the enzyme; as shown below (Fig. 6), these two polypeptide inhibitors themselves are subject to proteolytic digestion through the bacterial protease.

The properties of the StmPr1 protease clearly show that this enzyme is different from proteases isolated from S. maltophilia in 1975 (24) and in 1985 (25). These enzymes are strongly inhibited by EDTA, whereas antipain, which is a potent inhibitor of the StmPr1 protease, was found to be ineffective (25). Moreover, both enzymes differ in molecular size from the protein reported here. Obviously, at that time, S. maltophilia was a heterogenous species due to other differentiation criteria applied. Therefore, these observations strongly suggest that the bacteria used at that time are not identical with the strain of S. maltophilia that served as a source of the StmPr1 protease.

Substrate Specificity

In view of the pathogenic effect that the StmPr1 protease may exert in infected patients, the substrate specificity of this enzyme was studied in detail. This is a prerequisite for the development of specific inhibitors to be tested as therapeutic agents.

Proteolytic Activity toward the Oxidized Insulin B Chain—
Proteolytic specificity of the StmPr1 protease was determined using the oxidized insulin B chain as a substrate with a known sequence. The proteolytic fragments were analyzed by HPLC and mass spectroscopy. A total of eight bonds were cleaved (Fig. 5). The results characterized the protease as an endopeptidase with broad specificity.

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The best interaction is realized with the side chain of lysine, at the bottom of which negatively charged group(s) is (are) located.

Dulbecco's bacterial supernatant dialyzed against statin without protease (data not shown) showed a strong preference for the positively charged side chain of lysine. The high efficiency of the enzyme is derived from both greater binding (lower $K_m$) and increased turnover (higher $k_{cat}$). The enzyme efficiently hydrolyzed substrates containing aromatic or aliphatic groups in position P1, but with a lower efficiency. The S1 subsite accepted the negatively charged side chains of glutamic and aspartic acid very poorly. The following order of specificity, characterized by the ratio $k_{cat}/K_m$, was observed:

P1-P4 Specificity—P1 specificity of StmPr1 protease was investigated with a series of 10 tetrapeptide 4-nitroanilides in which only the amino acid residue in position P1 was varied (Table II). Determination of the specificity constant $k_{cat}/K_m$ showed a strong preference for the positively charged side chain of lysine. The high efficiency of the enzyme is derived from both greater binding (lower $K_m$) and increased turnover (higher $k_{cat}$). The enzyme efficiently hydrolyzed substrates containing aromatic or aliphatic groups in position P1, but with a lower efficiency. The S1 subsite accepted the negatively charged side chains of glutamic and aspartic acid very poorly. The following order of specificity, characterized by the ratio $k_{cat}/K_m$, was observed:

Lys > Phe > Leu = Nle > Ala = Arg > Orn > Asp > Glu > Val (Eq. 1)

This order differs considerably from those of other subtilases like BPN', Savinase, Esperase, and so forth (see Refs. 26 and 27 and the references therein). The results of the kinetic investigations described here lead to the conclusions below.

(i) The S1 subsite of StmPr1 protease is negatively charged. It can be supposed that a carboxylic group(s) is (are) located in this site. This can explain the high affinity for the side chain of lysine and the very low efficiency toward substrates with aspartic or glutamic acid in P1. S1 can also accommodate residues containing nonpolar side chains but with lower affinity.

(ii) Most probably, S1 is a deep and narrow “cavity” at the bottom of which negatively charged group(s) is (are) located. The best interaction is realized with the side chain of lysine (four methylene groups). Shortening of the chain by one CH$_2$ group, as in the case of ornitine, drastically decreased the catalytic efficiency. The distorted binding of the bulky side chain of valine seems to be due to a steric repulsion and the narrow entrance of the cavity.

(iii) In general, the StmPr1 protease exhibits a mixed type of P1 specificity (trypsin-like and, to a lesser extent, subtilisin-like activity). This is unusual for subtilases.

The subsite S2 prefers Pro instead of Leu in position P2 (Table II). The low efficiency toward Suc-Phe-Leu-Phe-pNA is due to a decreased turnover number. Some subtilases, like Esperase, Savinase, and subtilisin BPN' exhibit an opposite preference (26, 27).

StmPr1 protease efficiently hydrolyzes tetrapeptide $p$-nitroanilides with different P3 residues. The enzyme definitely prefers Leu and Gly in position P3 (Table II). The absence of a considerable discrimination between the P3 residues with different nature can be explained by a location of the subsite S3 at or near to the surface of the protein globule. The following decreasing order of P3 specificity was observed: Leu $>$ Gly $>$ Phe = Ala $>$ Glu. This order is completely different from those of other proteases of this family.

Subtilases exhibit a preference for the aromatic group of Phe in P4 because hydrophobic forces predominate in the S4-P4 interactions. As a result, Suc-Phe-Ala-Ala-Phe-pNA is one of the most favorable substrates for this group of proteases. However, the catalytic efficiency of the StmPr1 protease is 2

![Fig. 6. Reactivity of the StmPr1 protease toward native substrates.](Image)

Various proteins (10 $\mu$g) were incubated with purified enzyme (0.2 $\mu$g) containing 50 mM phosphate (pH 9)/150 mM NaCl at 37 °C for 16 h. Samples (18 $\mu$l) were heated with 6 $\mu$l of sample buffer and electrophoresed on SDS-polyacrylamide gels (8% with 12% g) containing 50 mM phosphate (pH 9)/150 mM NaCl at 37 °C. Protein bands were stained with Coomassie Brilliant Blue R-250. $-$, proteins incubated without enzyme; $+$, proteins incubated with enzyme. All substrate proteins except IgG were from Sigma (catalogue numbers are indicated in parentheses): collagen type VIII (C7774), fibronectin (F2006), fibrinogen (F3879), $\alpha_1$-antitrypsin (A6150), and $\alpha_2$-macroglobulin (M6159). A mouse monoclonal anti-epidermal growth factor receptor IgG1 (monoclonal antibody E30; P. Nobis and W. Weber, unpublished observations; Merck Nr. 120050.0001) was used for the immunoglobulin incubation.

![Fig. 7. Effect of the StmPr1 protease on cultures of human fibroblasts.](Image)

Confluent cultures of human fibroblasts (A) were incubated for 30 min at 37 °C with 12 $\mu$g/ml purified protease in Dulbecco's modified Eagle's medium (B) or bacterial supernatant dialyzed against Dulbecco's modified Eagle's medium (C and D). In D, 50 $\mu$g chymostatin was added before the bacterial supernatant. A control incubation in Dulbecco's modified Eagle's medium containing 50 $\mu$g chymostatin without protease (data not shown) remained unchanged.

![Image 1](Image)

![Image 2](Image)
orders of magnitude lower than those of typical subtilases (Table II and Ref. 26). The low efficiency is due mainly to a decreased turnover number. This result again demonstrates the specific active site structure of the investigated protease, which is somewhat different from those of typical subtilases.

Reactivity toward Relevant Human Proteins

After having demonstrated with synthetic substrates the broad specificity of StmPr1 protease, it was important to test some human proteins that could be substrates in vivo. As shown in Fig. 6, the enzyme degrades protein components of connective tissue such as collagen and fibronectin. This property of the bacterial protease may contribute to the tissue destruction seen in infected patients. Also, the serum component fibrinogen was completely degraded, indicating that the StmPr1 protease may interfere with the process of blood clotting. It has been shown above that the physiological protease inhibitors α1-antitrypsin and α2-macroglobulin present in serum at high concentrations are unable to abolish the StmPr1 proteolytic activity; Fig. 6, E and F, now demonstrates that these protein inhibitors, too, are subject to degradation. It is noteworthy that when immunoglobulin G was incubated with StmPr1 protease, the heavy chain appears to be cleaved at a specific site, giving raise to two smaller fragments; Fig. 6F shows the result obtained with a mouse monoclonal IgGl. Polyclonal IgG from human serum principally yielded the same result but with more diffuse bands (data not shown) due to the heterogeneity of the immunoglobulin fraction. Taken together, the StmPr1 protease appears to be associated not only with tissue destruction but may also possess the ability to inactivate components of the host defense mechanism.

Cell-damaging Activity

To verify the biological significance of the obtained data, cultures of human fibroblasts were exposed to supernatants of S. maltophilia (Fig. 7). After application of the cell-free bacterial medium, significant changes in cell morphology were observed: the cell layer partially condensed, forming cell-free areas, and finally detached from the culture plate. The same cell-damaging effect was achieved by addition of the purified StmPr1 protein to the fibroblast culture (Fig. 7C). The destruc
tive effect of both bacterial supernatant and purified enzyme could be prevented by preincubation with chymostatin, which has been shown above to be a potent inhibitor of the protease. This experiment demonstrates that secretions of S. maltophilia are able to destroy living cells and that the StmPr1 protease is the major factor responsible for this effect. Therefore, it seems likely that the tissue lesions seen in infected patients are a consequence of StmPr1 action.

Conclusions

A new bacterial protease with broad specificity has been characterized that is important from both a biochemical and a medical point of view. The sequence and the enzymatic properties demonstrate that this protease is a new member of the family of subtilases. It differs from the classic subtilisins by its larger molecular size and, presumably, by the architecture of the catalytic site. In this respect, the StmPr1 protease is homologous to the extracellular proteases of X. campestris, a plant pathogen causing black rot in crucifers, and D. nodosus, which is the causative pathogen of ovine foot rot, a disease characterized by separation of the hoof from the epidermal tissue. In both cases, the pathological situation seems to be associated with proteolytic tissue damage. Consequently, the StmPr1 protease is likely to function as a pathogenic factor as well.

Broad spectrum antibiotic treatments causing bacterial selection combined with the multiresistance of S. maltophilia force the development of new therapeutic strategies. A possible approach to this problem is to interfere with the pathogenic mechanisms of the bacteria (in the case of S. maltophilia, to suppress protease-mediated tissue damage and destruction). In this context, inhibitors of the StmPr1 protease should be of therapeutic value. It seems important, however, that such inhibitors do not affect host proteases. Fortunately, little structural relationship seems to exist between the prokaryotic and eukaryotic proteases, despite similar mechanisms of action (cf. Ref. 8). Therefore, it should be possible to design inhibitors with the required specificity. The development of such discriminating inhibitors is not without precedence: human immunodeficiency virus protease inhibitors, designed on the basis of crystal structures of the target protein, have been successfully introduced into therapy of AIDS. The data presented here should pave the way toward determination of the StmPr1 protease structure. Crystallization of the protein will be facilitated (cf. Ref. 28) by complexing with inhibitor molecules as developed on the basis of the enzyme kinetics presented.

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