The Major Extracellular Protease of the Nosocomial Pathogen *Stenotrophomonas maltophilia*: Characterization of the Protein and Molecular Cloning of the Gene

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Running title: *Stenotrophomonas maltophilia protease*

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SUMMARY

*Stenotrophomonas maltophilia* as a multiresistant pathogen is increasingly emerging in the hospital environment. In immunosuppressed patients these bacteria may cause severe infections associated with tissue lesions like pulmonary haemorrhage. This suggests proteolysis as a possible pathogenic mechanism in these infections.

This paper describes a protease with broad specificity secreted by *S. maltophilia*. The gene, termed *StmPr1*, codes for a 63 kDa precursor which is processed to the mature protein of 47 kDa. The enzyme is an alkaline serine protease which, 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 like 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.
INTRODUCTION

*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 3). However, in immune-compromized patients, particularly 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 of the lung epithelium (4). There are further reports demonstrating involvement of this bacterium in massive hemorrhagic processes of the small intestine and of the subclavian artery accompanied by severe lesions of the tissue (5, 6). These observations strongly suggest 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. 7). While 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 towards conventional antibiotics (for a review see 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 paper 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 university clinic. The identity of the germ was established by standard bacteriological techniques (API 20 NE, BioMérieux, Marcy-L’Etoile, France). Bacteria were grown at 29 °C aerobically 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 g/liter yeast extract (ICN Biomedicals, Aurora), 40 mg/liter gentamycin, 50 mg/liter cefotaxim, and 100 mg/liter ampicillin.

*Purification of the protease.*— 12.5 liter of cell-free supernatant was obtained from *S. maltophilia* cultures by centrifugation at 4 °C, mixed with 80 ml DE-52 (Whatman) cellulose equilibrated with 10 mM Tris/HCl buffer pH 7.4, and the mixture 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 fractionated at a flow rate of 1 ml/min over a 310 ml column of Fractogel EMD BioSec 650 (Merck) 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 bacterial supernatant, similar results were obtained for enzyme action (kinetic parameters, stability, inhibitor pattern, salt dependence) indicating that the isolated protease is intact and represents the major if not only protease produced by the bacteria.

* Determination of StmPr1 protein.*— The protein concentration in preparations of the native StmPr1 protease was determined on SDS-polyacrylamide gels as described in (10). Purified recombinant StmPr1 protein, calibrated by the Biuret method (11), served as a standard. The results were comparable to values calculated from the optical density at 280 nm (mg/ml StmPr1 protease = 1.30 x OD₂₈₀).

*Electrophoresis of proteins.*— SDS-PAGE was performed as previously described (12). Protein-containing samples were denatured with 10% trichloric acid (TCA) prior to electrophoresis. Without this pretreatment, additional bands of lower
molecular weight appeared, obviously due to self-digestion of the protease. Protein precipitates were collected by centrifugation and washed with methanol to remove residual TCA.— For autofluorography with a covalent inhibitor specific for serine proteases, samples were incubated with 5 µCi of [1,3-3H]-diisopropylfluorophosphate (NEN, 8.4 Ci/mmol) for 2h at 37 °C, precipitated with 10% TCA and subjected to SDS-PAGE. Polyacrylamide gels were fixed with 10% acetic acid/30% methanol, equilibrated first with water, then with 1 M sodium salicylate, dried and exposed to Kodak X-Omat film for 90 h at –80°C.

**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 (13). 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 (ε₄₀₅ = 9600 M⁻¹ cm⁻¹). — For the IC₅₀ determination of protease inhibitors (antipain, chymostatin) assays contained 1.4 mM (= Kₘ) 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 in a non-linear regression analysis of the function (a/(b+10^x)) when the reaction velocity is plotted versus log₁₀ of the inhibitor concentration (x). — Kinetic experiments with various synthetic peptide p-nitroanilide substrates were carried out in 100 mM Tris/HCl buffer, pH 8.2, at 25 °C and in the presence of 5% dimethylformamide. The enzyme concentration was usually in the range 1.95 x 10⁻⁸ – 9.12 x 10⁻⁹ M, and that of the substrate varied between 1.6 x 10⁻³ and 1.2 x 10⁻⁴ M. Kinetic parameters were calculated from initial rate measurements of substrate hydrolysis using a nonlinear regression analysis based on the function (Vₘₐₓ *x/ (Kₐₐₚₙ+x) with x = concentration of substrate.

**Protein sequencing.—** After SDS-PAGE the protein was blotted onto PVDF membranes (Immobilon P, Millipore) and stained with Coomassie Brilliant Blue R250. The excised band was sequenced by standard Edman degradation on an automated sequencer (Applied Biosystems 476A). In order to obtain internal sequence information the Coomassie stained protein band was cut out of the SDS gel and in-gel digested with the endoproteases Lys-C or Asp-N (Roche Biochemicals, Mannheim, Germany) 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 C4 column (250 x 2.1 mm) at a flow rate of 200 µl/min. The following gradient was applied: 2%-80% B in 50 min, solvent A: 0.1% TFA in water; solvent
B: 0.085% TFA in 70% acetonitrile. The isolated peptides were sequenced by Edman degradation following standard procedures.

**Cloning of the StmPr1 gene.**—DNA oligomers were synthesized complementary to the N-terminal sequence PYYQQYQ and to the reverse complement of the sequence APAAMRT obtained by digestion of the purified protease with the endoprotease Lys-C (see above). Using these primers (40 pmoles each), amplification of chromosomal DNA (200 ng) from *S. maltophilia* with Taq polymerase (Qiagen) yielded a DNA fragment of 930 bp which was sequenced (Applied Biosystems 377). The sequence showed homology to known protease sequences and served to design gene-specific primers. The rest of the upstream and downstream portions of the gene was cloned by alternate application of inverse PCR (14), using the Eco R II and Hinf I restriction sites, and of PCR using as template dA-tailed fragments of genomic DNA generated by Aat II, Pst I, or Sph I digestion, and one gene-specific oligonucleotide plus poly-dT as primers. A final PCR product obtained, using the "Expand Long Template PCR System“ (Boehringer) and primers comprising the identified start codon and stop codon, respectively, was sequenced, cloned into the pGem-T Easy vector (Promega), and resequenced for verification. The sequence of the *StmPr1* gene has been deposited in the EMBL Nucleotide Sequence Database; the accession number is AJ291488.

**Enzyme hydrolysis of the oxidized insulin B chain.**—Hydrolysis of the oxidized insulin B chain (Sigma, St. Louis, USA) was performed in 50 mM Tris/HCl buffer, pH 8.0, at room temperature for 10 min and for 4 h. The reaction mixture (2 ml) contained the enzyme and the substrate in a ratio of 1:200. The peptides obtained after the enzymatic hydrolysis were separated by reverse phase HPLC on a Vydac C18 column (125 x 2.1 mm) at a flow rate of 200 µl/min, and the following gradient was applied: 2%-65% B in 50 min, solvent A: 0.1% TFA in water; solvent B: 0.085% TFA in 70% acetonitrile. The obtained peptides were identified by mass spectrometry on a hybrid tandem mass spectrometer (Qtof II, Micromass, Manchester) equipped with a nanoelectrospray ion source. 10 µl of the collected fractions 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, Manchester). From the masses of the peptides determined a tentative assignment to fragments of the insulin B chain was derived. It was confirmed by subsequent MS/MS experiments 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 immunocompromized patient. Proteolytic activity was detected in the cell-free growth medium of the bacterial culture using azo-albumin as an unspecific chromogenic substrate (not shown).

In order to optimize bacterial cultures as a source for purification of the putative protease, the production of the enzyme during the 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 upregulated only towards the end of the exponential phase of the growth curve. Proteolytic activity reached a maximum after about 22h 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 then was 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 band yielded the sequence LAPNDPYYQQ, which turned out to be absent in protein sequence databases. 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. 7).

Covalent coupling with the radioactive inhibitor [H^3]-difluorophosphate 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, autoradiographs also showed mainly the 47 kDa band; the faint labeling of a lower molecular mass band may indicate a degradation product or the presence of another serine protease.
in trace amounts. Thus, the 47 kDa protein seems to be the enzyme mainly responsible for the extracellular proteolytic activity of *S. maltophilia*.

**Sequence determination by molecular cloning**

In order to analyse the sequence of the protease and to determine its relationship to other proteases, the gene was cloned by PCR techniques. Sequences from the N-terminus of the purified protein and from an internal peptide (PLAPAAAMRT), generated by Lys-C digestion, served to design degenerate primers. Using genomic DNA of *S. maltophilia* as a template, a 930 bp amplified fragment was obtained. Sequencing of the missing C-terminal portion of the gene required further steps of 3′-extension: PCR using polyA-tailed fragments of genomic DNA as PCR template as well as inverse PCR from highly diluted DNA fragments, were applied until a stop codon was found (Fig. 3).

Since it is a common feature of most prokaryotic extracellular proteases to be produced as larger precursor proteins (cf. 7), 5′-extension of the DNA sequence was performed in order to obtain the sequence of the entire gene. Applying the same techniques for extension, as outlined above, a sequence was obtained which 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 preceeded by a typical Shine-Dalgarno ribosome binding site; (ii) the sequence 3′ to this ATG does not predict a signal peptide typical for gram-(-) 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 *E. coli*, the protein was correctly processed, resulting in the mature protease with full enzymatic activity (not shown). The DNA sequence of the gene was further established: N-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 (see Experimental Procedures).

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).
residue stretch of the N-terminus was predicted to be the signal peptide containing a potential signal peptidase cleavage site (16) between Ala\textsuperscript{124} and Ala\textsuperscript{123}.

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

The gene of the \textit{S. maltophilia} protease was termed \textit{StmPr1} (EMBL accession number AJ291488); the term takes into account that another protease gene (\textit{StmPr2}) was detected in \textit{S. maltophilia} during preparation of this manuscript (to be published elsewhere).

Comparison of the \textit{StmPr1} protease sequence with those of other bacterial serine proteases confirmed its relation to the subtilisin family of proteases (cf. 7). Alignment of the sequences indicated that Asp\textsuperscript{42}, His\textsuperscript{105}, and Ser\textsuperscript{289} form the putative catalytic triade characteristic for serine proteases. In the active site region there is considerable homology with other subtilisins; in Fig. 3 conserved residues are marked which are shared with subtilisin BPN\textsuperscript{e} (17) and with proteinase K (18) as typical representatives of the “classic” subtilisins. Nevertheless, the \textit{StmPr1} protease sequence reveals significant structural differences from theses related enzymes due to large inserts adjacent to the catalytic His\textsuperscript{105} and Ser\textsuperscript{289}. Therefore, compared to the catalytic triade formed by Asp\textsuperscript{32}, His\textsuperscript{64}, and Ser\textsuperscript{221} in the typical members of the subtilisin family, the architecture of the active site should be different in the \textit{StmPr1} protease. In addition, this enzyme has a longer C-terminal extension beyond the active site which, together with the inserts in the catalytic region, makes the entire sequence almost one hundred residues longer. With these structural properties the \textit{StmPr1} protease is similar to the extracellular proteases of \textit{Xanthomonas campestris} (19), \textit{Dichelobacter nodosus} (15), and \textit{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 C-terminal extensions, and no homology can be seen between the pro-sequences. The sequence homology between the \textit{StmPr1} protease and the classic subtilisins 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 pH optimum 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 maximum tested). The stimulating effect of NaCl has also been reported for several other proteases of the subtilisin family (21). Enzyme activity was found to be stimulated also by calcium which was effective at low concentrations: a maximum of 3.5-fold increase of the 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 (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 towards 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. 22). By contrast, the mamalian serine protease chymotrypsin tested under the same conditions completely lost its activity at a concentration of 0.1% dodecyl sulfate.

To get more information on the type of protease, the effect of a series of protease inhibitors on the *StmPr1* enzyme activity was tested. The enzyme was effectively
inhibited by antipain, chymostatin, and PMSF, 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 uneffected. 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 Pseudomonas 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 ineffective (25). Moreover, both enzymes differ in molecular size from the protein reported here. Obviously, at that time Pseudomonas maltophilia was a heterogenous species due to other differentiation criteria applied. Therefore, it is strongly suggestive that the bacteria used at that time were not identical with the strain of S. maltophilia which served as a source of the StmPr1 protease.

**Substrate specificity**

In view of the pathogenic effect which 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 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. StmPr1 protease attacks peptide bonds comprising the carboxylic groups of both hydrophobic and hydrophilic residues. Comparison with the alkaline protease from
D. nodosus and with subtilisin BPN’ showed that none of the specificity patterns is identical with that of StmPr1 protease.

**P1 – P4 specificity.**— P1 specificity of StmPr1 protease was investigated with a series of ten tetrapeptide 4-nitroanilides in which only the amino acid residue in position P1 was varied (Table 2). 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 very poorly the negatively charged side chains of glutamic and aspartic acid. 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.

This order differs considerably from those of other subtilases like BPN’, Savinase, Esperase etc. (26, 27 and citations therein). The results of kinetic investigations, described here, lead to the following conclusions:

- S1 subsite of StmPr1 protease is negatively charged. It can be supposed that 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 towards substrates with aspartic or glutamic acid in P1. S1 can also accomodate residues containing nonpolar side chains but with lower affinity.

- 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 (4 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.

- In general, StmPr1 protease exhibits a mixed type of P1 specificity: trypsin-like and in a lower extent subtilisin-like activity. This is unusual for subtilases.

The subsite S2 prefers Pro instead of Leu in position P2 (Table 2). 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 prefers definitely Leu and Gly in position P3 (Table 2). 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-Phe-pNA is one of the most favorable substrates for this group of proteases. However, the catalytic efficiency of the StmPr1 protease is two orders of magnitude lower than those of typical subtilases (Table 2 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 towards relevant human proteins.**—After having demonstrated with synthetic substrates the broad specificity of StmPr1 protease, it was important to test some human proteins which could be substrates in vivo. As shown in Fig. 6, the enzyme degrades protein components of connective tissue like 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 in high concentrations are unable to abolish the StmPr1 proteolytic activity; Fig. 6E,F now demonstrates that these protein inhibitors, too, are subject to degradation. Noteworthy, when immunoglobulin G was incubated with StmPr1 protease, the heavy chain appears to be cleaved at a specific site giving rise to two smaller fragments; Fig. 6F shows the result obtained with a mouse monoclonal IgG1. Polyclonal IgG from human serum principally yielded the same result although with more diffuse bands (not shown) due to the heterogeneity of the immunoglobulin fraction. Taken together, the StmPr1 protease appears to be associated not only with tissue destruction; it may also possess the ability to inactivate components of the host defense mechanism.

**Cell damaging activity.**—In order 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 of 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. 7 C). The destructive effect of both, bacterial supernatant or
purified enzyme, could be prevented by preincubation with chymostatin which has
been shown above as 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 which is
important from both a biochemical as well as a medical point of view. The sequence
and the enzymic properties demonstrate that this protease is a new member of the
family of subtilases. It differs from the classic subtilisins by the 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 *Xanthomonas campestris*, a
plant pathogen causing black rot in crucifers, and of *Dichelobacter 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 pathogenic
mechanisms of the bacteria - in the case of *S. maltophilia* to suppress protease-
mediated tissue invasion 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, there seems to exist only little
structural relationship between the prokaryotic and eukaryotic proteases, despite
similar mechanisms of action (cf. 8). Therefore, it should be possible to design
inhibitors with the required specificity. The development of such discriminating
inhibitors is not without precedence: HIV 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 towards
determination of the StmPr1 protease structure. Crystallization of the protein will be facilitated (cf. 28) by complexing with inhibitor molecules as developed on the basis of the enzyme kinetics presented.

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Table 1. **Effects of various substances on the activity of StmPr1 protease.** – The purified enzyme was preincubated with the substances for 20 min prior to reaction at pH 9 with Suc-Ala-Ala-Pro-Phe-pNA as substrate. IC$_{50}$ determinations were performed as described in *Experimental Procedures (Enzyme assays)*; values are presented as means of two (antipain) or four separate experiments (chymostatin; ± 0.2 µM SEM), respectively. The (covalently binding) inhibitor PMSF completely abolished protease activity at a concentration of 1 mM.

| inhibitor    | max. conc. tested (µM) | inhibition | IC$_{50}$ (µM) |
|--------------|------------------------|------------|---------------|
| Antipain     | 100                    | +          | 19            |
| Aprotinin    | 0.3                    | –          |               |
| Bestatin     | 130                    | –          |               |
| Chymostatin  | 100                    | +          | 0.4           |
| E-64         | 28                     | –          |               |
| EDTA-Na$_2$  | 10000                  | –          |               |
| Leupeptin    | 1.1                    | –          |               |
| Pepstatin    | 0.7                    | –          |               |
| Phosphoramidon | 500                | –          |               |
| PMSF         | 2000                   | +          |               |
| TLCK         | 150                    | –          |               |
| TPCK         | 300                    | –          |               |
Table 2.— Kinetic constants for the *StmPr1* proteinase–catalyzed hydrolysis of peptidyl substrates.- Reactions were performed as described in *Experimental Procedures*. The various substrates were assayed in two separate determinations yielding similar results. $K_m$ and $k_{cat}$ parameters were calculated from one representative saturation curve with standard errors resulting from the non-linear regression analysis. Errors of the quotient $k_{cat}/K_m$ are presented as root of the variance calculated according to (33).

| Substrate                  | $K_m$ (mM) | $k_{cat}$ (s$^{-1}$) | $k_{cat}/K_m$ (s$^{-1}$ mM$^{-1}$) |
|----------------------------|------------|----------------------|-----------------------------------|
| Suc-Ala-Ala-Pro-Phe-pNA    | 1.4 ± 0.20 | 60 ± 4.1             | 44 ± 9.4                          |
| Suc-Ala-Ala-Pro-Leu-pNA    | 0.94 ± 0.097 | 37 ± 1.6           | 39 ± 5.7                          |
| Suc-Ala-Ala-Pro-Nle-pNA    | 1.2 ± 0.14 | 43 ± 2.4             | 35 ± 5.9                          |
| Suc-Ala-Ala-Pro-Ala-pNA    | 1.0 ± 0.25 | 26 ± 2.8             | 25 ± 8.7                          |
| Suc-Ala-Ala-Pro-Asp-pNA    | 1.1 ± 0.15 | 0.66 ± 0.32          | < 1                               |
| Suc-Ala-Ala-Pro-Val-pNA    | 3.3 ± 0.50 | 0.97 ± 0.08          | < 1                               |
| Suc-Ala-Ala-Pro-Glu-pNA    | 16 ± 4.5   | 6.7 ± 1.3            | < 1                               |
| Suc-Ala-Ala-Pro-Lys-pNA    | 0.52 ± 0.09 | 92 ± 4.6            | 178 ± 39                          |
| Suc-Ala-Ala-Pro-Arg-pNA    | 4.3 ± 1.8  | 100 ± 27             | 23 ± 16                           |
| Suc-Ala-Ala-Pro-Orn-pNA    | 0.74 ± 0.11 | 4.5 ± 0.23         | 6.1 ± 1.2                         |
| Suc-Phe-Leu-Phe-pNA        | 0.19 ± 0.037 | 0.09 ± 0.002      | < 1                               |
| Suc-Phe-Pro-Phe-pNA        | 0.14 ± 0.017 | 3.2 ± 0.04          | 23 ± 3.0                          |
| Suc-Ala-Ala-Pro-Phe-pNA    | 1.4 ± 0.20 | 60 ± 4.1             | 44 ± 9.4                          |
| Suc-Ala-Gly-Pro-Phe-pNA    | 0.95 ± 0.063 | 112 ± 2.6          | 118 ± 10                          |
| Suc-Ala-Phe-Pro-Phe-pNA    | 0.58 ± 0.10 | 27 ± 1.6            | 46 ± 11                           |
| Suc-Ala-Leu-Pro-Phe-pNA    | 0.49 ± 0.080 | 104 ± 3.8          | 213 ± 42                          |
| Suc-Ala-Glu-Pro-Phe-pNA    | 1.0 ± 0.11 | 27 ± 0.77            | 26 ± 3.3                          |
| Suc-Phe-Ala-Ala-Phe-pNA    | 0.15 ± 0.014 | 4.1 ± 0.08         | 27 ± 2.9                          |
FIGURE LEGENDS

Figure 1. **Growth and production of extracellular protease by *S. maltophilia***.— 50 ml culture medium was inoculated with 0.1 ml bacterial suspension (stationary phase) and grown at 29°C. Samples were taken at intervals and were tested for density (open squares) and, after centrifugation, for enzyme activity (closed squares). Four separate experiments of this type were performed yielding similar results; data of one representative experiment are presented.

Figure 2. **SDS-PAGE of the StmPr1 protease.**— (A) Purified StmPr1 protease (4 µg) analyzed on a 12% gel and stained with Coomassie Blue. (B) Autofluorogram of [3H]-DFP labeled proteins (see Experimental Procedures): left lane, 200 µl crude supernatant of *S. maltophilia*; right lane, preparation of purified StmPr1 protease (0.5 µg).

Figure 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 shown double-underlined. The vertical arrow indicates the predicted signal peptidase cleavage site while the translation-termination codon is marked with an asterix. The amino terminal and internal residues sequenced are underlined. Nucleotides are numbered from the putative translation initiation codon while peptides are numbered from the N-terminus of the mature protein. The amino acids of the catalytic triad, D\textsuperscript{42}, H\textsuperscript{105} and S\textsuperscript{289} are boxed and inverted. Open boxes indicate regions of identity which 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 (30) were not detected within this stretch of 593 nucleotides sequenced.

Figure 4. **(A) Effect of pH.**— Protease activity was measured in the pH range 3–10.5 in 50 mM of citrate (triangles), phosphate (circles), or ethylendiamine (squares) buffer. **(B) Effect of salt.**— Enzyme activity was determined in the presence of increasing concentrations of sodium chloride (circles) or calcium chloride (squares) in 50 mM Tris/HCl buffer pH 9. **(C) Effect of SDS.**— StmPr1 protease
(open squares) and chymotrypsin (closed squares) were assayed for enzyme activity in the presence of increasing concentrations of sodium dodecyl sulfate.

Plotted values represent means of three or four (StmPr1 activity in C) determinations, respectively. Error bars indicate SEM ranges.

Figure 5. Cleavage points of oxidized insulin B chain by StmPr1 protease and various subtilases.—— Insulin B chain was incubated with enzyme, and proteolytic fragments were analyzed as described in Experimental Procedures. C indicates CysSO₃H. The bold/thin arrows represent the proteolytic peptides identified in the reaction in major or trace amounts, respectively. Bold lines indicate major sites of cleavage. The results for the Dichelobacter nodosus basic protease (31) and subtilisin BPN’ (32) were taken from the literature.

Figure 6. Reactivity of the StmPr1 protease towards native substrates.—— Various proteins (10 µg) were incubated with purified enzyme (0.2 µg) containing 50 mM phosphate pH 9/150 mM NaCl at 37°C for 16h. Samples (18 µl) were heated with 6 µl sample buffer and electrophoresed on SDS polyacrylamide gels (8–13% depending on the size of the protein substrate). Protein bands were stained with Coomassie Blue. Lanes marked (-) represent proteins incubated without enzyme and (+) lanes are proteins incubated with enzyme. All substrate proteins except IgG were from Sigma (catalogue numbers indicated): collagen type VIII (C7774), fibronectin (F2006), fibrinogen (F3879), α₁-antitrypsin (A6150), α₂-macroglobulin (M6159). A mouse monoclonal anti EGF receptor IgG1 (mab E30, P. Nobis & W. Weber, unpublished; Merck Nr. 120050.0001) was used for the immunoglobulin incubation.

Figure 7. Effect of the StmPr1 protease on cultures of human fibroblasts.—— Confluent cultures of human fibroblasts (A) were incubated for 30 min at 37°C with 12 µg/ml purified protease in DME medium (B), or bacterial supernatant dialysed against DME medium (C and D). In D 50 µM chymostatin was added prior to the bacterial supernatant. A control incubation in DME medium containing 50 µM chymostatin without protease (not shown) remained unchanged.
Fig. 5

| 5 | 10 | 15 | 20 | 25 |
|---|----|----|----|----|
| F-V-N-Q-H-L-C-G-S-H-L-V-E-A-L-Y-L-V-C-G-E-R-G-F-F-Y-T-P-K-A |
| St. maltophilia StmPr1 | | | | | |
| D. nodosus basic protease | | | | | |
| Subtilisin BPN' | | | | | |

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