Characterization of an Extracellular Metalloprotease with Elastase Activity from Staphylococcus epidermidis

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The gene sepA from Staphylococcus epidermidis TÜ3298-P, encoding the extracellular neutral metalloprotease SepP1, was cloned into pT181mcs. DNA sequencing revealed an open reading frame of 1,521 nucleotides encoding a 507-amino-acid protein with an M₀ of 55,819. The sepA-containing DNA fragment did not hybridize with Staphylococcus hyicus or Staphylococcus carnosus DNA. Expression of sepA in the protease-negative S. carnosus (pT181mcsP1) resulted in overproduction of a 33-kDa protease found in the culture medium. The first 15 N-terminal amino acids of the partially purified protease completely matched the deduced DNA sequence starting at GCA (Ala-208). This finding indicated that SepP1 is synthesized as a preproenzyme with a 28-amino-acid signal peptide, a 179-amino-acid hydrophobic pro region, and a 300-amino-acid extracellular mature form with a calculated M₀ of 32,739. In activity staining, the mature protease prepared from S. carnosus (pT181mcsP1) corresponded to the extracellular S. epidermidis TÜ3298-P protease. The partially purified protease had a pH optimum between 5 and 7, and its activity could be inhibited by zinc- and metal-specific inhibitors such as EDTA and 1,10-phenanthroline, indicating that it is a neutral metalloprotease.

The protease had a low substrate specificity. Glucagon was cleaved preferentially between aromatic (Phe) and hydrophobic (Val) amino acids. The protease hydrolyzed casein and elastin. The amino acid sequence of the mature form of SepP1 revealed pronounced similarities with the thermolabile and thermostable neutral proteases of various bacilli (44 to 55% identity) and a central part of the mature form of the Pseudomonas aeruginosa elastase (31% identity). From homology comparison with the Bacillus thermoproteolyticus thermolysin, we predict that mature SepP1 binds one zinc ion at a conserved zinc-binding site.

Most Staphylococcus aureus strains secrete proteolytic enzymes; three different types of proteases have been studied in detail. Serine protease from S. aureus V8, commonly referred to as V-8 protease or protease I, is an endoprotease which cleaves peptide bonds on the carboxyl-terminal side of glutamic acid and to a lesser extent of aspartic acid (8). The enzyme, having a molecular weight of 12,000, is inhibited by diisopropyl fluorophosphate (DFP) but not by EDTA. Similar serine proteases with the same cleavage preference are also found in other S. aureus strains; however, they differ from the above-described enzyme in their size and sensitivity to DFP (2, 24). The amino acid sequence of a 27-kDa serine protease of S. aureus revealed a 43-residue C-terminal peptide composed nearly exclusively of aspartic acid, asparagine, and proline (6), proposed to be involved in the transport of the enzyme through the cytoplasmic membrane (7).

The second type of protease from S. aureus V8, thiol protease (protease II), is characterized by an isoelectric point of pH 9.4, a molecular weight of 12,500, and a very broad specificity; it also exhibits esterase activity with N-benzoyl-L-tyrosine ethyl ester as a substrate (2). The enzyme is active only in the presence of reducing agents and is inactivated by heavy metals such as Hg²⁺, Zn²⁺, or Ag⁺, indicating that protease II must possess free SH groups to be active and that it is a cysteine enzyme.

The third protease of S. aureus V8, a metalloprotease (2), is completely inactivated by EDTA and is insensitive to sulfhydryl reagents and DFP. Calcium is essential for the stability of the protease. The molecular weight is 28,000, and the pH optimum for hydrolysis of casein is 7.4. Like the thiol protease, the metalloprotease has a rather broad proteolytic specificity, cleaving preferentially at the N-terminal side of hydrophobic residues (1). A metalloprotease with a molecular weight of 38,000 and a pH optimum of 7.0 has been isolated from a mutant of S. aureus V8 (5). The protease is fully inactivated by o-phenanthroline but can be reactivated by zinc ions.

Another extracellular protease, lysostaphin, is found in Staphylococcus simulans biovar staphylolyticus (27). Lysostaphin is a zinc metalloendopeptidase (34) which hydrolyzes the glycine peptide bond of staphylococcal lysozyme, causing cell lysis. The lysostaphin gene has been recently cloned and sequenced (14, 21). According to its deduced amino acid sequence and processing, lysostaphin appears to be organized as preproenzyme.

In contrast to the well-studied proteases of S. aureus, little is known about proteases in Staphylococcus epidermidis, the predominant inhabitant of the human skin. Our interest in the role of proteases in skin colonization led to this report on the cloning and sequencing of sepA from S. epidermidis, the gene encoding the extracellular metalloprotease SepP1, and the biochemical characterization of partially purified SepP1.

MATERIALS AND METHODS

Bacterial strains and plasmids. S. epidermidis TÜ3298-P, the source of sepA, is a mutant of S. epidermidis TÜ3298 which has deletions in plasmid TÜ32 and is unable to produce the lantibiotic epidermin (3). Staphylococcus carnosus TM300 was used as the cloning host (10). Plasmid pT181mcs, encoding resistance to tetracycline, was used as a cloning vector (15, 23). Staphylococci were cultivated in B broth (10 g of casein hydrolysate 140 [GIBCO], 5 g of yeast extract

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Proteins were eluted with a linear gradient of 0 to 1 M NaCl. Each fraction was checked for protease activity. The purity of the protease was determined by SDS-12.5% polyacrylamide gel electrophoresis (PAGE).

Determination of pH profile and thermostability of partially purified SepP1. For pH profile determination, the following buffers were used: 100 mM sodium acetate (pH 4 to 5), 25 mM bis-Tris-HCl (pH 6 to 8.8), 100 mM Tris-HCl (pH 7 to 9), and 50 mM sodium bicarbonate (pH 10). Each assay was performed with 2.5 mM CaCl₂ and 10 μM ZnCl₂. For the determination of thermostability, a buffer containing 100 mM Tris-HCl (pH 7) with 2 mM CaCl₂ and 10 μM ZnCl₂ was used. The samples were incubated for 25 min at the indicated temperatures and cooled rapidly on ice. The residual protease activity was measured.

Other methods. SDS-PAGE was performed as described by Laemmli (16). Electrophoretic transfer of proteins to PVDF Immobilon membranes for protein sequence determination was performed according to the method of Matsudaira (18). The N-terminal sequence of SepP1 was determined by Edman degradation, using a model 477A pulsed-liquid protein sequencer (Applied Biosystems). DNA and protein sequences were analyzed by using the computer programs of Microgenie (Beckman) and PCGENE (IntelliGenetics, Inc.).

Nucleotide sequence accession number. The novel DNA sequence published here has been deposited into the EMBL sequence data bank under accession number X69957.

RESULTS

Cloning of sepA from S. epidermidis in S. carnosus. S. epidermidis Tu3298-P chromosomal DNA was partially digested with MboI. DNA fragments of 4 to 20 kb were ligated to BamHI-digested pT181mcs1. The ligated DNA was transformed into S. carnosus TM300, which has very low extracellular protease activity. By screening 8,000 tetracycline-resistant transformants on B-agar plates containing 1% skim milk and tetracycline (25 μg/ml), six positive clones forming large halos around the colonies were detected. Restriction analysis of the isolated plasmids revealed that all six positive clones carried a common restriction fragment within a 4.5-, 5.7-, or 9.5-kb DNA insert. The plasmid from the S. carnosus clone with the smallest insert (4 kb), pT181mcsP1, was used for further investigations. S. carnosus(pT181mcsP1) produced a much larger halo of proteolytic activity on skim milk agar than did S. epidermidis Tu3298-P. The control S. carnosus(pT181mcs) had no detectable proteolytic activity in this assay, rendering S. carnosus an ideal host for protease cloning and isolation.
FIG. 2. Nucleotide sequence of sepA and flanking DNA. The sequence contained only one ORF of the expected size. The deduced amino acid sequence can be subdivided into the putative pre (signal), pro, and mature sequences. The amino acids determined by Edman degradation are underlined. The SepP1 coding region is preceded by a perfect Shine-Dalgarno (SD) sequence. The putative -10 and -35 promoter regions are indicated; a typical transcription terminator sequence is missing.

The insert of another clone with a very small halo had a different restriction pattern, confirming our observation that \textit{S. epidermidis} Tü3298-P produces various exoproteases.

\textbf{Nucleotide sequence of sepA.} The restriction map of the cloned 4-kb DNA fragment is shown in Fig. 1. Deletion analysis of the DNA insert indicated that HhaI and EcoRI must be located within the protease gene, which was designated \textit{sepA}. The nucleotide sequence of the \textit{HpaI} fragment revealed only one open reading frame (ORF) of the expected size, starting with ATG at nucleotide position 164 and terminating with TAA at position 1687 (Fig. 2). The 1,521-nucleotide ORF could encode a 507-amino-acid protein (\(M_r = 55,819\)). The ATG start codon is preceded by a perfect Shine-Dalgarno sequence (AGGAAGGT). The first 28 residues of the N terminus resemble a typical signal peptide sequence for secreted proteins of prokaryotic origin. A short sequence containing two positively charged residues is followed by a long hydrophobic sequence and a potential signal peptidase cleavage site (Val–Asp–Ala-28). This cleavage site conforms to the \(-3, -1\) rule (37). Normally a helix-breaking residue (Pro or Gly) or a large polar residue (Glu) occurs four to eight amino acids before the cleavage site (39). In this ORF, we found Glu in position -4. We also found Val in position -7 and Leu in position -8, in keeping with the rules of prokaryotic cleavage sites assigned by using the PC/GENE prediction of prokaryotic secretory signal sequence and by using the method of von Heijne (37). 32P-labeled \textit{sepA}-containing DNA strongly hybridized with DNA from \textit{S. epidermidis} Tü3298-P and its wild type; no hybridization was detected to \textit{S. carnosus} or \textit{S. hyicus} DNA (data not shown).

\textbf{Isolation and N-terminal sequence of SepP1 from \textit{S. epidermidis} Tü3298-P.}
carnosus(pT181mcsP1). The onset of extracellular protease production occurred very early in a batch fermentation of S. carnosus(pT181mcsP1) and correlated strongly with growth. Only in the late stationary phase did protease activity decline. The control, S. carnosus, exhibited no detectable protease activity during batch fermentation (data not shown).

The protease was isolated from 4 liters of culture supernatant of S. carnosus(pT181mcsP1) harvested at the early stationary phase. For partial purification, the protease was precipitated with ammonium sulfate (65%), and after dialysis, the sample was subjected to DEAE-Sepharose Fast Flow chromatography. Each DEAE-Sepharose fraction was checked for protease activity and analyzed by SDS-PAGE (Fig. 3). The protease-positive fractions had a 33-kDa protein band which was absent (not shown) in the similarly treated culture supernatant of S. carnosus(pT181mcsP1).

After SDS-PAGE, no protease activity was detected in the gel, even after removal of SDS and mercaptoethanol by extensive washing. However, after non-denaturing gel electrophoresis, a protein band with proteolytic activity was visible. The bands with proteolytic activity from extracts of S. carnosus(pT181mcsP1) and S. epidermidis Tu3298-P had the same electrophoretic mobility.

The 33-kDa protein on the SDS-polyacrylamide gel was electroblotted onto a PVDF Immobilon-P transfer membrane, and the N-terminal sequence was determined. The first 15 N-terminal residues, Ala-Ala-Thr-Thr-Gly-Thr-Gly-Lys-Gly-Val-Leu-Gly-Asp-Thr-Lys, completely matched that deduced from the nucleotide sequence (Fig. 2), starting at Ala-208. In combination with DNA sequence analysis, this result suggests that the protease is synthesized as a preproenzyme with a 207-amino-acid prepro sequence. If the signal peptide is cleaved at the predicted cleavage site (between Ala-28 and Lys-29), the pro sequence would be 179 amino acids long; the pro region is distinguished by high hydrophilicity. The mature extracellular SepP1 is composed of 300 amino acids with a calculated molecular weight of 32,739.

Enzymatic properties of the partially purified SepP1. The pH optimum of the protease with resorufin-labeled casein as the substrate was between 5 and 7 (Fig. 4); thus, SepP1 can be regarded as a neutral protease. The calculated isoelectric point for the proenzyme is 6.0, and that for the mature protease is 5.86. The protease was thermostable up to 50°C; at higher temperatures, stability decreased gradually. However, after incubation at 80°C for 25 min, 20% activity still remained (Fig. 4). The temperature optimum for protease activity was 37°C.

As shown in Table 1, the protease activity can be inhibited by the metal chelator EDTA and the zinc-specific chelator 1,10-phenanthroline. Although SepP1 contains no cysteine residues in its sequence, high concentrations of dithiothreitol exerted an inhibiting effect, very likely by its metal-chelating activity. The proteolytic activity of SepP1 was inhibited with Phosphoramidon, an inhibitor of thermolysin and thermolysin-related metalloproteases (40). The enzyme was resistant to phenylmethylsulfonyl fluoride and other inhibitors of serine or cysteine proteases, such as (4-amidinophenyl) methanesulfonyl fluoride, aprotinin, antipain dihydrochloride, and leupeptin. It was also resistant to bestatin, an inhibitor of aminopeptidases, and pepstatin, an inhibitor of aspartate proteases. The sensitivity of the protease to metal chelators indicates that this neutral SepP1 is a metalloprotease.

SepP1 was also tested for collagenase and elastase activity. We found proteolytic activity only with elastin in the culture supernatants of S. carnosus(pT181mcsP1) and S. epidermidis.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Conc (mM)</th>
<th>Remaining protease activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>-</td>
<td>100</td>
</tr>
<tr>
<td>Phenylmethylsulfonyl fluoride</td>
<td>1</td>
<td>86</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>74</td>
</tr>
<tr>
<td>EDTA</td>
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<tr>
<td>EGTA</td>
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<td>72</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>0.5</td>
</tr>
<tr>
<td>1,10-Phenanthroline</td>
<td>0.1</td>
<td>89</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>12</td>
</tr>
<tr>
<td>Dithiothreitol</td>
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<td>30</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>27</td>
</tr>
<tr>
<td>3,4-Dichloroisoumarin</td>
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<td>93</td>
</tr>
<tr>
<td>Phosphoramidon</td>
<td>0.2</td>
<td>29</td>
</tr>
</tbody>
</table>

* No inhibition was observed with (4-amidinophenyl)methanesulfonyl fluoride (40 μg/ml), aprotinin (10 μg/ml), leupeptin (0.5 μg/ml), bestatin (40 μg/ml), and pepstatin (0.7 μg/ml).


protease has various lengths and locations in the precursor protein; their suggested role is to maintain the protease in an inactive state and to mediate the folding of the protease (38). Synthesis as an inactive precursor is a common feature of all extracellular proteases studied so far. The thermolabile proteases from \textit{B. thermoproteolyticus} (thermolysin) (32) and the thermolysin-related neutral proteases from \textit{B. stearothermophilus} (31) and \textit{B. cereus} (28, 42) are synthesized as preproenzymes, as are the thermolabile neutral proteases A (44) and B (33) from \textit{B. subtilis} and \textit{B. amyloliquefaciens} (36) and all other proteases from \textit{B. subtilis}, such as subtilisin (30) and bacillopeptidase F (43).

Propeptides are also found in extracellular proteins of various staphylococci, for example, in lysostaphin of \textit{S. simulans} biovar staphyloyticus (14) and the lipase of \textit{S. hyicus} (41). The lengths of the propeptides vary, ranging from 10 to over 200 amino acids. In general, the propeptide region is hydrophilic and lacks an extended hydrophobic core. The pro region of SepP1 is also distinguished by a pronounced hydrophilicity.

The mature form of SepP1 was shown here to be a neutral metalloprotease with proteolytic and elastolytic activity. It has similarity to a central region of the \textit{P. aeruginosa} elastase (LasB), although the amino and carboxy termini of the two molecules are quite different (Fig. 5). LasB (4) is a 33-kDa metalloprotease which cleaves elastin, collagen, immunoglobulin G, serum α1-protease inhibitor, and several complement components. The lasB nucleotide sequence revealed that the protein is also organized as a preproelastase; the propeptide comprises 150 amino acids (4). However, there is a lasA gene product (26) required for elastase activation. From the studies presented here, it is unlikely that a second gene is involved in elastase activation of the \textit{S. epidermidis} metalloprotease SepP1. If a second activator protein is involved, it must be produced by both \textit{Staphylococcus} species, because the enzyme shows elastolytic activity when prepared from \textit{S. epidermidis} as well as from \textit{S. carnosus}.

We found no homology of SepP1 with any of the published staphylococcal protease sequences. However, the mature form of SepP1 shows considerable similarities with the primary structures of various \textit{Bacillus} proteases. Sequence homologies of neutral proteases produced by \textit{Bacillus} spp. were compared by using the thermolysin sequence as a reference. The thermolysin protein sequence (32), three-dimensional structure (20), and catalytic mechanism (13, 19) have been determined. Thermolysin requires a zinc ion for catalysis and has four calcium-binding sites (22). The zinc-binding site (His-142, His-146, and Glu-166 in the mature thermolysin) is conserved in all \textit{Bacillus} neutral proteases and in the elastase enzyme of \textit{P. aeruginosa} (4). These residues are also conserved in SepP1 (His-144, His-148, and Glu-168) (Fig. 5). On the basis of this homology and the inhibition by zinc- and metal-specific inhibitors, we predict that mature SepP1 binds one zinc ion at this conserved zinc-binding site.

In thermolysin, five residues (Asn-112, Ala-113, Glu-143, Tyr-157, and His-231) are essential for the full catalytic activity and the positioning of the substrate backbone in the active site (13). These residues are found at homologous positions in the \textit{Bacillus} neutral proteases, in LasB (4), and
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