Purification and characterisation of elastase from *Staphylococcus epidermidis*

N. SLOOT, M. THOMAS, R. MARRE and S. GATERMANN

Institute for Medical Microbiology, University of Lübeck, Germany

Summary. An elastase of *Staphylococcus epidermidis* was purified by ion exchange chromatography on CM-Sepharose and characterised. Its M_r is c. 21 kDa, its optimal temperature for activity is 42°C and the pH optimum is 6.8. The enzyme is activated by cysteine and other SH-donators and inhibited by L-trans-epoxy-succinylleucylamido-(4-guanidino)butane (E64), an inhibitor of cysteine proteases, but not by 3,4-dichloroisocoumarin (3,4-DCI), an inhibitor of serine proteases. This finding suggests that the elastase of *S. epidermidis* is a cysteine protease. Because *S. epidermidis* elastase degrades human sIgA, IgM, serum albumin, fibrinogen, and fibronectin, this enzyme may be regarded as a virulence factor.

Introduction

Elastases have the unique ability to cleave elastin, a macroprotein present in skin, arteries and lung. Bacterial elastases have been isolated from several bacterial species, such as *Pseudomonas aeruginosa*^{1,2} and *Vibrio cholerae*.³ Because of their ability also to cleave several human proteins, such as IgG, IgA, IgM and transferrin, as is the case for *P. aeruginosa* elastase,^{4,5} the enzymes are considered to be virulence factors. Recently, an elastase has been described in *Staphylococcus aureus* and a role in tissue damage has been proposed for it.⁶ Unlike other known elastases,^{7,8} the enzyme from *S. aureus* was found to be a cysteine protease.⁶ The production of elastase by *S. epidermidis* was first described in 1968⁹ and the presence of the enzyme was subsequently correlated with perifollicular macular atrophy.⁹ *S. epidermidis* produces more elastase than *S. aureus*,¹⁰ but the enzyme has not yet been characterised. Therefore, the present study was done to isolate, purify and characterise it.

Materials and methods

Strains

All experiments were done with *S. epidermidis* ATCC14990. Long term storage was in PY broth (Bacto Yeast-extract 5 g/L, Bacto peptone 10 g/L, NaCl 5 g/L, glucose 1 g/L, Na_2HPO_4 1 g/L, pH 7.3)¹¹ containing glycerol 15% at –70°C. Before the experiments, the strain was grown on brain heart

infusion (BHI) agar and the identity of subcultures was confirmed with the API(STAPH)-System (Montalieu-Vercieu, France).

Preparation of elastase

A dialysis membrane technique was used.¹² Autoclaved dialysis membranes (Spectrapor, Spectrum Medical Industries, Los Angeles, CA, USA) with a cut-off of 12–14 kDa were placed on top of BHI agar plates (9 cm diameter). Strains from long-term storage were subcultured on BHI agar and 40 plates with dialysis membranes were inoculated with a suspension prepared from these cultures. Plates were incubated for 18 h at 37°C in CO_2 15% in air. After incubation, bacteria and bacterial products from each plate were harvested from the dialysis membranes in 1 ml of NaCl 0.9%. The suspension was centrifuged for 30 min at 22 100 *g* to separate bacteria from bacterial products. The supernate was filter-sterilised and the presence of elastase was checked in elastin plates—elastin (Sigma, Deisenhofen, Germany) 0.25%, 0.05 M Tris buffer, pH 7.0, agarose (Difco, USA) 1% incubated at 37°C for 18 h. The zone of clearing was determined. Elastase from porcine pancreas (Sigma) and NaCl 0.9% were used as controls, the elastase being stored at 4°C and used within 2 weeks.

Activity of elastase

Enzyme activity was assessed by incubation with Azocoll (Sigma). Samples (125 μl) were added to 1.125 ml of an Azocoll suspension (10 mg/ml in 0.1 M phosphate buffer $\text{Na}_2\text{HPO}_4/\text{NaH}_2\text{PO}_4$, pH 7.0) and incubated at 37°C with constant agitation for 15, 20 or 40 min. Uncleaved substrate was removed by centrifugation and absorbancy of the supernate was read at

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Address for correspondence: Dr N. Slood, Institut für Medizinische Mikrobiologie, Medizinische Universität zu Lübeck, Ratzeburger Allee 160, D-2400 Lübeck, Germany.

520 nm. Enzyme activity in the presence of cysteine (2–8 mM final concentration), 2 mM mercaptoethanol 2 mM dithiothreitol, 2 mM EDTA, Cd^{2+} , Pb^{2+} , Hg^{2+} , 3,4-dichloroisocoumarin (3,4-DCI, 465 nm) and L-trans-epoxysuccinylleucylamido-(4-guanidino)butane (E64, 280 nm) (all from Sigma) was also assessed.

Determination of temperature and pH optima

The Azocoll technique was used as described above and the samples were incubated at 6°C, 23°C, 32°C, 37°C, 42°C and 50°C for 30 and 60 min. Elastase samples were also incubated on elastin plates as described above and the pH of the Tris-buffer was adjusted to 5.0, 6.0, 6.5, 7.0, 7.5 and 8.0. The activity of elastase was measured as the diameter of the zones of elastin digestion.

Elastase purification

Purification was achieved by ion-exchange chromatography with a CM-Sepharose CL-6B column (1.6 × 20 cm, Pharmacia LKB, Freiburg, Germany), packed and equilibrated with 50 mM sodium phosphate buffer, pH 6.0, according to the specifications of the manufacturer; 10 ml of crude preparation (2.5 mg/ml) were applied to the column and eluted at 35 ml/h with a NaCl gradient (0–0.6 M, 400 ml). Elastase activity of all fractions (3.5 ml) was checked by the plate assay, and peak fractions were analysed by SDS-PAGE (see below).

Protein content was assessed with the BioRad assay (BioRad, München, Germany) with bovine serum albumin as the standard.

SDS-PAGE

Electrophoresis of proteins in the presence of sodium dodecyl sulphate (SDS) was done in poly-

acrylamide gels (PAGE) according to the method of Laemmli¹³ and the concentrations of running and stacking gels were 12 and 6%, respectively. Samples containing 5–6 µg of protein were heated at 100°C for 3 min in sample buffer (62.5 mM Tris buffer, pH 6.8, SDS 2%, 2-mercaptoethanol 5%, glycerol 10%, bromophenol blue 0.001%) and applied to the gel. Proteins were stained with silver according to the method of Blum *et al.*¹⁴

Cleavage of human IgG, IgM, sIgA, fibronectin, fibrinogen, and human serum albumin (all from Sigma) was assessed in solutions which contained 1 mg/ml in distilled water; 10 µl of each solution was mixed with 40 µl of purified elastase (275 µg/ml) and, after incubation for 18 h at 37°C, cleavage products were analysed by SDS-PAGE.

Cleavage of collagen was examined with collagen plates prepared by the same means as the elastase plates but containing collagen (collagen type I from bovine achilles tendon, Sigma) 0.25%. Again, the zone of clearing was determined and compared with that for NaCl 0.9% after incubation at 37°C for 18 h.

The hydrolysis of carbobenzoxy-L-phenylalanyl-L-leucyl- α -glutamyl-4-nitranilid (CBZ-Phe-Leu-Glu-pNA; Boehringer, Mannheim, Germany) was tested at a final concentration of 0.17 mM as described by Potempa *et al.*⁶ The generation of *p*-nitroanilin was measured at 410 nm after incubation for 130 and 180 min. For quantitative assessment, a molar extinction coefficient of 8800 was used for *p*-nitroanilin.¹⁵

All experiments with human immunoglobulins were repeated at least five times, with other substrates at least twice with enzyme from several preparations.

Results

The activity of *S. epidermidis* elastase was increased four-fold by the presence of SH-donators (cysteine,

Fig. 1. Proteolytic activity of *S. epidermidis* elastase; 125 µl of crude elastase (480 mg of protein/ml) was incubated with 12.5 mg of Azocoll in phosphate buffer, pH 7.0, for 20 min at 37°C. A: *S. epidermidis* crude elastase; B: with 2 mM cysteine, C: with 2 mM mercaptoethanol, D: with 2 mM EDTA. E: with 2 mM cysteine and 2 mM EDTA.

Fig. 2. Effect of temperature on the activity of *S. epidermidis* elastase; 125 μ l of crude elastase was incubated with 12.5 mg of Azocoll in phosphate buffer pH 7.0 for 30 (■) and 60 (●) min.

Fig. 3. Purification of *S. epidermidis* elastase with CM-Sepharose CL-6B; 10 ml of crude elastase in sodium phosphate buffer, pH 6.0, was applied to the column (1.6 $\times$ 20 cm), which had been equilibrated with sodium phosphate buffer (pH 6.0) and eluted with a gradient from 0 to 0.6 M NaCl. Bar indicates fractions with elastolytic activity.

mercaptoethanol, dithiothreitol) and the addition of 2 mM EDTA, alone or in combination with 2 mM cysteine, had no effect on enzyme activity (fig. 1).

The presence of E64 inhibited elastase activity and at a concentration of 280 nM there was a complete loss of proteolysis; the degree of inhibition depended on the concentration of E64. The addition of 3,4-DCI had no significant effect on elastase activity. The enzyme showed no significant activity below 23°C; proteolysis was most efficient at 42°C (fig. 2), with a distinct loss at 50°C. Proteolytic activity was determined at a range of pH values (5.0–8.0) and maximum activity was observed at pH 6.8.

Purification to homogeneity was achieved by ion exchange chromatography on CM-Sepharose and fractions with peak activity yielded a single band in SDS-PAGE of c. 21 kDa (fig. 5). Elastase eluted at a NaCl concentration of 0.25–0.28 M (fig. 3) and no additional proteolytic fractions were found.

Incubation of purified elastase with human sIgA, IgM (fig. 4) and human serum albumin (HSA) (fig. 5) showed an incomplete degradation with the appearance of new bands in SDS-PAGE, but no subunit of sIgA was completely cleaved. In the case of IgM a

Fig. 4. Cleavage of human immunoglobulins by *S. epidermidis* elastase. Substrate proteins (5 μ g) were incubated (18 h, 37°C) with crude or purified elastase (5 μ g). Products were analysed by SDS-PAGE and the gel was stained with silver. Lane 1: human immunoglobulin; 2: human immunoglobulin and crude elastase; 3: human immunoglobulin and purified elastase; elastase (◄) and digestion products (◄) are indicated.

Fig. 5. Cleavage of human proteins by *S. epidermidis* elastase. Substrate proteins (5 µg) were incubated (18 h, 37°C) with purified elastase (5 µg). Products were analysed by SDS-PAGE and the gel was stained with silver. M: marker proteins; E: purified elastase; HSA: human serum albumin; Fg: fibrinogen, Fn: fibronectin; purified elastase was present in the second lane for each of the substrates.

single new band of *c.* 31 kDa was found when crude elastase was used (fig. 4), whilst a number of new bands resulted from degradation of HSA (fig. 5). In contrast fibrinogen (67, 56 and 47 kDa) were completely digested, with the production of at least five degradation products (94, 64, 56, 28 and 24 kDa) with the former and two break-down products with the latter (both 55 kDa) (fig. 5). Elastase generated 0.11 mM of *p*-nitroanilin after incubation with CBZ-Phe-Leu-Glu-*p*Na for 2 h and 0.12 mM after 3 h, indicating cleavage of 65 and 70% of the substrate. These experiments were done in triplicate and results deviated by less than 1%. Degradation of collagen was not observed in any experiment.

Discussion

Production of elastase by *S. epidermidis* was first described in 1968⁹ and the distribution and biochemical properties of the enzyme in clinical isolates were investigated later.^{16,17} A role for *S. epidermidis* elastase as a virulence factor was postulated in these reports, but cleavage of human proteins was not demonstrated.

In this investigation, we purified and characterised elastase from *S. epidermidis* and identified human proteins that are cleaved by the enzyme. The enzyme has an apparent *M_r* of *c.* 21 kDa, which is larger than

that reported for the *S. aureus* enzyme (13 kDa).⁶ *S. epidermidis* elastase is activated by SH-donors, such as cysteine or mercaptoethanol, and inactivated by heavy metal ions such as Cd²⁺, Pb²⁺ or Hg²⁺ but not by EDTA. Furthermore, the enzyme is inhibited by E64, a specific inhibitor of cysteine proteases, but not by 3,4-DCI, which inhibits serine proteases. Thus, *S. epidermidis* elastase can be regarded as a cysteine proteinase, as is the case for the *S. aureus* enzyme⁶ but in contrast to most other bacterial elastases so far described which are serine or metallo-proteinases.

In addition to the biochemical characterisation, we have shown that *S. epidermidis* elastase is able to cleave several human proteins, including immunoglobulins. This finding is in contrast to that of Plaut,¹⁸ who reported that staphylococci did not produce IgA-proteases. In our cleavage experiments, crude elastase digested human proteins more efficiently than purified elastase and, in the case of sIgA, complete digestion was observed only with the former. As no other proteolytic fraction was detected during column chromatography, it is likely that cleavage was due to the elastase. Purification of the enzyme appeared to reduce elastase activity, probably by removing unknown auxiliary substances.

Elastase may be involved in the pathogenicity of *S. epidermidis* in a number of ways. Both sIgA and IgM are degraded by the enzyme and inactivation of these immunoglobulins may assist bacteria in the early phase of infection by depressing the host defence mechanisms.^{19,20} *S. epidermidis* can adhere to plastic surfaces, and subsequent colonisation often leads to bacteraemia.²¹ As these surfaces are covered with albumin, fibrinogen and fibronectin *in vivo*, it is possible that elastase removes or modifies protein surface films on indwelling devices and thus helps the organism to adhere more effectively. Fibrinogen and fibronectin are also important in wound healing since they supply an essential network for blood coagulation. Thus, the ability of elastase to cleave fibrinogen and fibronectin may play a role in the development of wound infection.²¹

The elastase of *S. aureus* is known to cleave only CBZ-Phe-Leu-Glu-*p*Na from a range of synthetic substrates,⁶ and in this investigation *S. epidermidis* elastase produced a similar result. *S. epidermidis* elastase cleaved 65% and 70% of the synthetic substrate after incubation for 1 and 2 h respectively. The apparent loss of activity may be due to instability of the protein in the incubation mixture or a consequence of substrate depletion. In addition, it cannot be excluded that one of the breakdown products is inhibitory to the enzyme.

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