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Growth phase-dependent production of a cell wall-associated elastinolytic cysteine proteinase by *Staphylococcus epidermidis*

Aneta Oleksy[1,2], Ewa Golonka[1], Agnieszka Bańbuła[1,2], Grzegorz Szmyd[1], Jonathan Moon[2], Małgorzata Kubica[1], Doron Greenbaum[3], Matthew Bogyo[3], Timothy J. Foster[4], James Travis[2] and Jan Potempa[1,2,*]

[1] Department of Microbiology, Faculty of Biotechnology, Jagiellonian University, 30-387 Krakow, Poland
[2] Department of Biochemistry and Molecular Biology, University of Georgia, Athens, GA 30602, USA
[3] Department of Biochemistry and Biophysics, University of California, San Francisco, CA 94143, USA
[4] Department of Microbiology, Moyne Institute for Preventive Medicine, Trinity College, Dublin 2, Ireland

* Corresponding author
e-mail: potempa@uga.edu

Abstract

Staphylococcus epidermidis, a Gram-positive, coagulase-negative bacterium is a predominant inhabitant of human skin and mucous membranes. Recently, however, it has become one of the most important agents of hospital-acquired bacteriemia, as it has been found to be responsible for surgical wound infections developed in individuals with indwelling catheters or prosthetic devices, as well as in immunosupressed or neutropenic patients. Despite their medical significance, little is known about proteolytic enzymes of *S. epidermidis* and their possible contribution to the bacterium's pathogenicity; however, it is likely that they function as virulence factors in a manner similar to that proposed for the proteases of *Staphylococcus aureus*. Here we describe the purification of a cell wall-associated cysteine protease from *S. epidermidis,* its biochemical properties and specificity. A homology search using N-terminal sequence data revealed similarity to staphopain A (ScpA) and staphopain B (SspB), cysteine proteases from *S. aureus.* Moreover, the gene encoding *S. epidermidis* cysteine protease (Ecp) and a downstream gene coding for a putative inhibitor of the protease form an operon structure which resembles that of staphopain A in *S. aureus*. The active cysteine protease was detected on the bacterial cell surface as well as in the culture media and is apparently produced in a growth phase-dependent manner, with initial expression occurring in the mid-logarithmic phase. This enzyme, with elastinolytic properties, as well as the ability to cleave α_1PI, fibrinogen and fibronectin, may possibly contribute to the invasiveness and pathogenic potential of *S. epidermidis.*

Keywords: cysteine protease; elastase; staphopain; *Staphylococcus epidermidis.*

Introduction

Among members of the normal bacterial flora of human skin, skin glands and mucous membranes, *Staphylococcus epidermidis* is a predominant species, widely spread throughout the cutaneous ecosystems. This coagulase-negative bacterium, previously regarded as a commensal organism of low virulence, has recently been recognized as an emerging etiological agent of numerous clinical conditions. *S. epidermidis* is an opportunistic pathogen that requires a predisposed/susceptible host in order to change from a normal inhabitant of human skin to an infectious agent. Most of the infections are hospital-acquired and inflict those individuals with either implanted medical devices or undergoing surgical intervention, as well as immunosupressed patients (Blum and Rodvold, 1987; Kloos and Bannerman, 1994). The pathologies caused by *S. epidermidis* range from bacterial keratitis, postoperative wound infections, rejection of indwelling foreign devices (such as prosthetic joints, cardiac valves, intravascular and peritoneal dialysis catheters or intraocular lenses) to a bacteremia often developed in immunocompromised patients (Baddour et al., 1987; Blum and Rodvold, 1987; Mack, 1999; Tabbara et al., 2000). As many *S. epidermidis* isolates are multi-antibiotic resistant, such infections are very serious and can even be fatal.

The pathogenicity of *S. epidermidis* is mainly due to its ability to form biofilms on the surfaces of indwelling synthetic devices as well as damaged heart valves (Bayston and Rodgers, 1990). The hydrophobic nature of the cell surface is crucial for bacterial adherence during initial colonization of plastic or metal foreign bodies, while in the later stages a copious amount of extracellular polysaccharide material is synthesized, forming a protective multilayered biofilm preventing the clearance of bacteria by host defense mechanisms and making infection difficult to eradicate (Blum and Rodvold, 1987).

In comparison to the closely related *S. aureus*, *S. epidermidis* produces a very limited number of tissue-damaging secretory factors such as exoenzymes and toxins. The analysis of *S. epidermidis* strain ATCC 12228 genome revealed that with exception of haemolysins beta and delta, genes encoding enterotoxins, leukotoxins and hemolytic alpha and gamma toxins are absent (Zhang et al., 2003). The lack of numerous extracellular virulence factors, which are typical hallmarks of *S. aureus* infections, may explain why the infections caused by *S. epidermidis* are of subacute or chronic type (Vuong and Otto, 2002). Degradation of host connective tissues components and other proteins as well as modifications of bacterial cell surface proteins by secretory proteases is known to contribute to invasiveness of *S. aureus*. It is

possible that proteases play a similar role in *S. epidermidis* infections, although this function is highly speculative and deserves further investigation. Extracellular proteolytic enzymes produced by *S. epidermidis* include a cysteine protease (Sloot et al., 1992, Dubin et al., 2001), glutamyl endopeptidase (Moon et al., 2001; Dubin et al., 2001, Ohara-Nemoto et al., 2002), and a metalloprotease (Teufel and Götz, 1993). Overexpression and purification of *S. epidermidis* extracellular cysteine protease (Ecp), also termed staphopain, as well as some biological properties of the enzyme were reported by Dubin et al. (2001); however, the function of this protease *in vivo* as a specific virulence factor has yet to be determined. It has been suggested that the elastinolytic activity of *S. epidermidis*, correlated with the development of perifollicular macular atrophy (Varadi and Saqueton, 1968), could be linked to the presence of a metalloprotease and/or a cysteine protease in the culture media (Teufel and Götz, 1993; Sloot 1992). It has also been demonstrated that the cysteine protease is able to degrade several host matrix proteins and components of the immune system *in vitro* (Dubin et al., 2001). In this report we describe the purification and biochemical characterization of the cell wall-associated molecular form of elastinolytic cysteine protease that may possibly be important for *S. epidermidis* pathogenicity *in vivo*.

Results

Localization of the enzyme

Elastinolytic activity dependent on the presence of the reducing agents was found to be a cysteine protease localized either on the *S. epidermidis* cell surface or in a soluble form released into the culture medium. The DCG-04 labeling allowed us to monitor the presence of both enzyme forms during bacteria growth and calculate their relative concentration using densitometry analysis of the intensity of labeled bands visualized in the Western Blot.

The protease is expressed in a growth phase-dependent manner (Figure 1) with the active enzyme appearing on the cell surface of strain ATCC 14990 initially in the mid-logarithmic phase (6 hours of growth) and reaching the maximum level in the post-exponential phase (9 hours of growth). The secretory form was also detected in the media starting at 6th hour of culture and promptly its levels reached plateau and did not change during the later stages of the growth. Culture supernatants collected before 6th hour of growth were concentrated by membrane ultrafiltration using Vivaspin concentrators (Viva Science, 10 kDa cut off) and showed no trace of cysteine protease activity. A quantitative method based on the chemiluminescent detection system indicated that there was no further accumulation of any protease forms in later stages of growth.

In contrast to *S. aureus* strains Newman, 8325-4 and SH1000 (Horsburgh et al., 2002) producing only soluble enzymes, two cysteine protease forms were found in all four *S. epidermidis* strains tested, including three laboratory strains (ATCC 14990, TU 3298, TU 9142) and a clinical isolate (data not shown). The total amount of the enzyme as well as the distribution of both forms between the cell surface and the culture medium differs between strains as indicated by the DCG-04 labeling. The cell wall fraction subjected to the quantitative analysis was obtained by treatment of bacterial cells with lysostaphin in the presence of 30% raffinose in order to avoid the breakage of protoplasts. To determine the amount of protease released from the bacterial surface into solution during the experimental procedure, a cell suspension in raffinose without lysostaphin was used as a control.

The DCG-04 labeling and elastinolytic activity assay revealed that the cell-associated form was released into the medium by treatment with lysostaphin, mild deter-

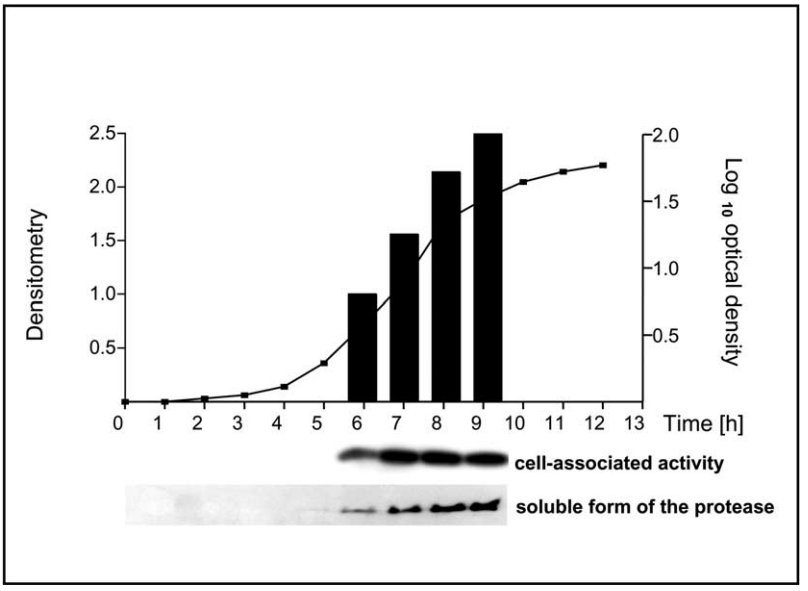

Figure 1 Growth phase-dependent production of the Ecp cysteine proteinase in the cell-associated and soluble form.
Both enzyme forms produced during growth of strain ATCC 14990 were detected in the DCG-04 binding assay (lower panels). Relative concentration of the cell-associated protease was calculated from the optical density of the labeled protein band visualized in the Western blot (solid bars on the growth curve).

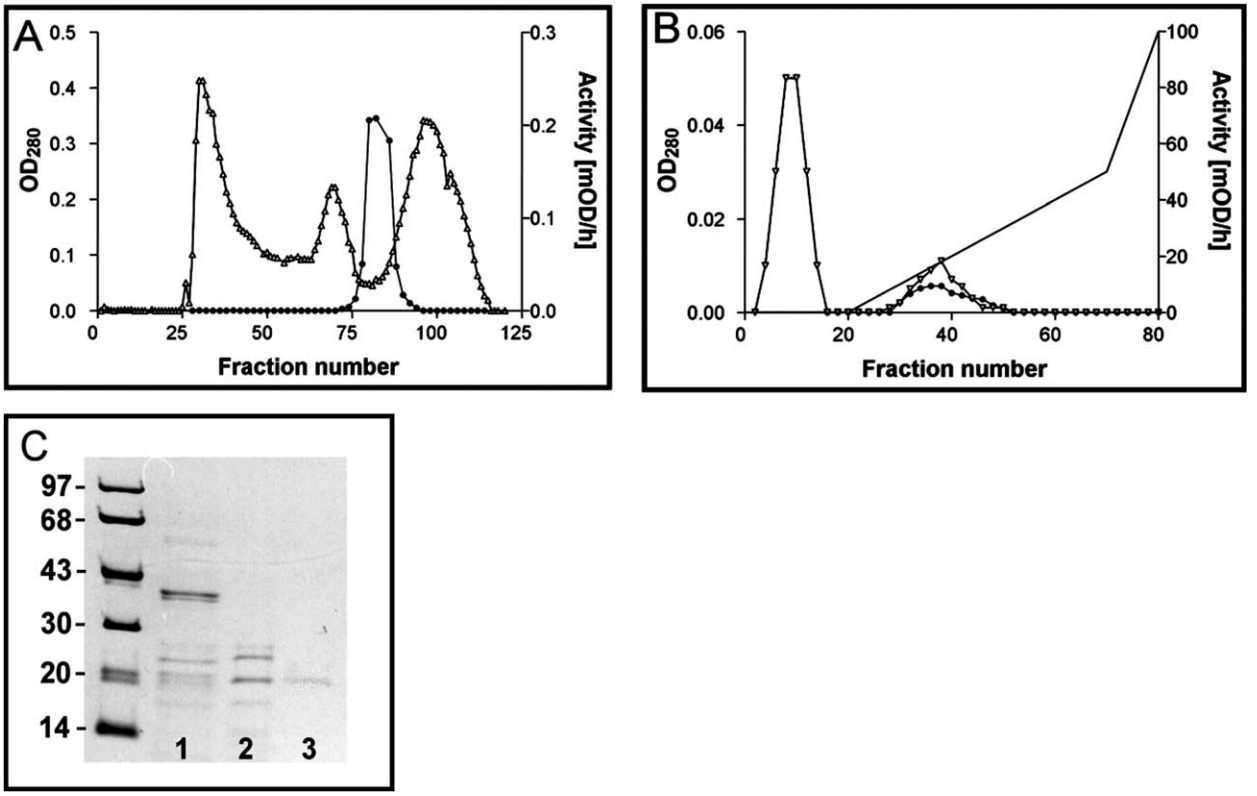

Figure 2 Purification of *S. epidermidis* cell surface-associated cysteine protease.
Purification of the enzyme released from the cell wall of *S. epidermidis* after lysostaphin treatment, monitored by the absorbance at 280 nm (triangles) and elastinolytic activity against Elastin Congo Red measured at 490 nm (circles). (A) Separation of the enzyme on Sephadex G-150 equilibrated with Sephadex G-150 buffer. (B) Separation of the enzyme from the previous step on MonoS FPLC column equilibrated with 20 mM MES, pH 6.5, 1 mM $CaCl_2$. (C) SDS-PAGE of fractions obtained during the purification steps: left lane: molecular mass markers (kDa), lane 1: lysostaphin extract of *S. epidermidis*, lane 2: Sephadex G-150 column eluate, lane 3: MonoS column eluate.

gents such as Triton X-100, CHAPS, Nonidet 40 and Brij 35 and at high concentrations of NaCl. The solubilization of the enzyme was also observed after sonication. Taken together, these results indicate that the enzyme is loosely associated with the outer cell structures.

Enzyme purification

For the purification of the enzyme, bacterial cells from the stationary phase of growth were treated with lysostaphin at a concentration of 0.1 mg/ml and released cell-wall-associated proteins subjected to subsequent chromatography steps including gel filtration on Sephadex G-150 and ion exchange chromatography on a MonoS column (Figure 2, panels A and B). The last step yielded a homogenous enzyme as confirmed by SDS-PAGE and gelatin zymography. The elastase migrated as a single band on SDS-PAGE (Figure 2, panel C) with an apparent molecular mass of 20 kDa. Analysis of the amino-terminal sequence of the purified protease, MYAEYVNQLKNF, allowed us to identify the protein as staphopain, a cysteine protease encoded by the *ecp* gene, previously described as an *S. epidermidis* extracellular protease (Dubin et al., 2001). The protein has a calculated molecular mass of 19 832 Da.

pH optimum and enzyme stability

The purified cysteine protease was active against Elastin Congo Red over a broad pH range, with maximum activity at neutral pH (Figure 3). The enzyme was stable in 20 mM MES, pH 6.5, 1 mM $CaCl_2$, for months at 4°C or −20°C. The protease showed no appreciable loss of activity when kept at neutral pH for one hour, but after 24 hours its activity was significantly reduced or totally lost at room temperature or at 37°C, respectively. At higher temperature (42°C) the enzyme was rather unstable, losing 70 and 100% activity after incubation for 1 h and 12 h, respectively.

Effect of reducing agents

The activity of the purified protease in the absence of reducing agents was negligible. Therefore, to determine and compare the activatory effect of different reducing agents, elastinolytic activity was measured in the buffer supplemented with various concentrations of dithiothreitol (DTT), L-cysteine and reduced glutathione (see Table 1, part 'Activatory agents'). The highest activity against Elastin Congo Red was found in the assay buffer containing 10 and 100 mM cysteine. For comparative purposes the activity of the enzyme in the absence of any reducing agents and in the presence of 10 mM cysteine was assigned as 0 and 100%, respectively. DTT at the same concentration yielded much lower activity, about 40% of that in the presence of L-cysteine. Gluthatione seemed to be the least effective reducing agent, increasing the enzyme activity slightly only at 100 mM concentration. With regard to much stronger activation effect

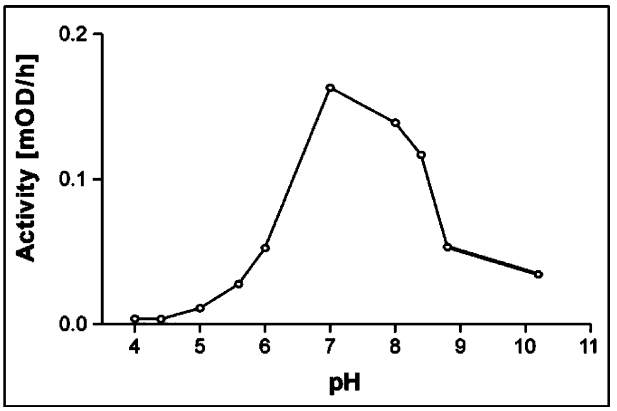

Figure 3 pH optimum of the enzyme determined with Elastin Congo Red as a substrate.
Enzyme elastinolytic activity was tested in various pH conditions.

exerted by L-cysteine in comparison to DTT, the *S. epidermidis* elastase resembles gingipain R, cysteine proteinase belonging to clan CD, which is known to prefer cysteine over DTT as the activating reducing agent (Chen et al., 1992).

Inhibition profile

All the residual activity assays were conducted in the presence of 10 mM cysteine. Based on the inhibition studies (see Table 1, part 'Inhibitory agents'), the purified *S. epidermidis* protease was classified as a cysteine protease, with the activity being completely inhibited by E-64 (10 μM) and its derivative DCG-04, iodoacetamide (10 mM) and N-ethylmaleimide (10 mM). None of the serine proteases inhibitors tested, such as diisopropylfluorophosphate (DFP), Pefabloc and 3,4-dichloroisocoumarin diminished the enzyme activity, and the metal chelator 1,10-phenanthroline did not exert any inhibitory effect either. Several chloromethyl ketones had signifi-

Table 1 Effect of different compounds on *S. epidermidis* elastinolytic activity.

Activatory agent	Concentration (mM)	Activity (%)
Ditiothreitol	0.1	4.38
	1	19.13
	10	39.49
	100	36.35
Cysteine	0.1	2.51
	1	24.13
	10	100
	100	87.45
Glutathione	0.1	0
	1	0
	10	0.62
	100	14.11

Inhibitory agent	Concentration	Residual activity (%)
Diisopropylfluorophosphate (DFP)*	1 mM	100
Pefabloc*	2 mg/ml	100
3,4-Dichloroisocoumarin (DIC)*	2 mM	10
1,10-phenanthroline	1 mM	101
Tosyl lysyl chloromethyl ketone (TLCK)	1 mM	9
Tosyl phenyl chloromethyl ketone (TPCK)	1 mM	11
Z-Phe-Lys-ck	5 mM	0
Z-Phe-Ala-ck	2 mM	0
E-64/DCG-04	1 μM	0
Iodoacetamide	1 mM	86
	10 mM	0
N-ethylmaleimide	1 mM	90
	10 mM	3
Aprotinin	0.5 mg/ml	98
Pepstatin	0.25 mg/ml	110
Bestatin	50 μg/ml	92
Leupeptin	100 μg/ml	19
Antipain	100 μg/ml	95
Cystatin C	8 μM	90
Ni^{2+}	1 mM	10
Zn^{2+}	1 mM	29
SDS	1%	16
α_2-Macroglobulin	equimolar	10

The enzyme was pretreated with various reducing agents and its elastinolytic activity was assayed against Elastin Congo Red (part 'Activatory agents'). Activity of the enzyme in the absence of any activatory agent and the highest activity measured in the buffer containing 10 mM cysteine was set as 0% and 100% of activity, respectively. The residual enzyme activity on Elastin Congo Red after preincubation with numerous inhibitory compounds was measured in the presence of 10 mM cysteine and compared to the activity of uninhibited enzyme (part 'Inhibitory agents'). Asterisks indicate compounds for which binding to the enzyme active site was estimated by their inhibitory effect on α_1PI proteolytic degradation and DCG-04 binding, because of their interaction with the dye in Elastin Congo Red assay.

Table 2 Degradation of synthetic peptides by *S. epidermidis* cysteine protease.

LMQGSNHL**E**↓FADRRPL
AL**E**↓RI**E**↓AKHRERMSQVM
ADSG**E**↓GDFLAEGGGVR
ELY**E**↓NKPRRPYIL
YLEPGPVTA
PPGFSPFR
DRVYIHPF
KGPPAALTL

Peptides incubated with the purified protease were subjected to reversed-phase HPLC using a Supelcosil™ LC-18 column, eluted with an acetonitrile gradient and subjected to subsequent identification by mass spectrometry. Arrows indicate determined cleavage sites.

cant inhibitory effect on elastinolytic activity of the protease. The enzyme was inactive in the presence of SDS and heavy metal ions including Zn^{2+} and Ni^{2+}.

Among several natural inhibitors tested, only leupeptin decreased the activity of the purified protease to about 20% (see Table 1). The human plasma inhibitor, α_2-macroglobulin, inhibited elastinolytic activity of the purified protease apparently due to the complex formation as indicated by transformation of α_2-macroglobulin from its S to F form observed on native PAGE after the incubation of macroglobulin with *S. epidermidis* elastase (data not shown). Another inhibitor derived from human plasma, α_1PI, had no inhibitory effect on the purified protease and was degraded by the enzyme as described below.

Substrate specificity

The amidolytic activity of the protease was studied with a number of synthetic substrates (see the Materials and methods section). However, no hydrolysis of any of these compounds was detected.

To determine specificity of the Epc protease, several synthetic peptides were tested as substrates for this enzyme (Table 2). Four peptides were digested by the purified enzyme with one having two distinct cleavage sites localized in close proximity. Apparently, with one significant exception, the enzyme activity was targeted against the Yaa-Glu↓Xaa sequence motif inside the polypeptide chain, where Yaa represents a large hydrophobic residue (Leu, Ile, Tyr) whereas Xaa could be any residue except proline. However, the cleavage of the peptide bond in a Gly-Glu↓Gly, but not in Ala-Glu-Gly sequence motif (peptide 3) indicates that the activity of the enzyme is affected by additional recognition of residues other than those at P1, P1′ and P2 positions. Full understanding of the enzyme specificity requires further, more detailed investigation.

In addition to the Elastin Congo Red assay, proteolytic activity of the purified protease was also checked against several proteins including collagen, laminin, albumin and immunoglobulins, fibronectin, fibrinogen and α_1PI. Among these proteins only the last three were cleaved. Therefore to further analyze concentration and time dependent proteolytic degradation of fibrinogen, the enzyme was incubated with fibrinogen for 5 min at various enzyme:substrate molar ratios (1:10–1:160) and at an enzyme:substrate molar ratio of 1:40 for various time intervals, and the samples resolved by SDS-PAGE (data not shown). Fibrinogen α-chain seemed to be the most susceptible to proteolysis as its degradation was observed at an E:S molar ratio of 1:40 already within 5 min of incubation and proceeded with time. Under the same experimental conditions, β- and γ-chains remained intact.

Cleavage efficiency of gelatin or fibrinogen incorporated in zymography gels was dependent on the amount of

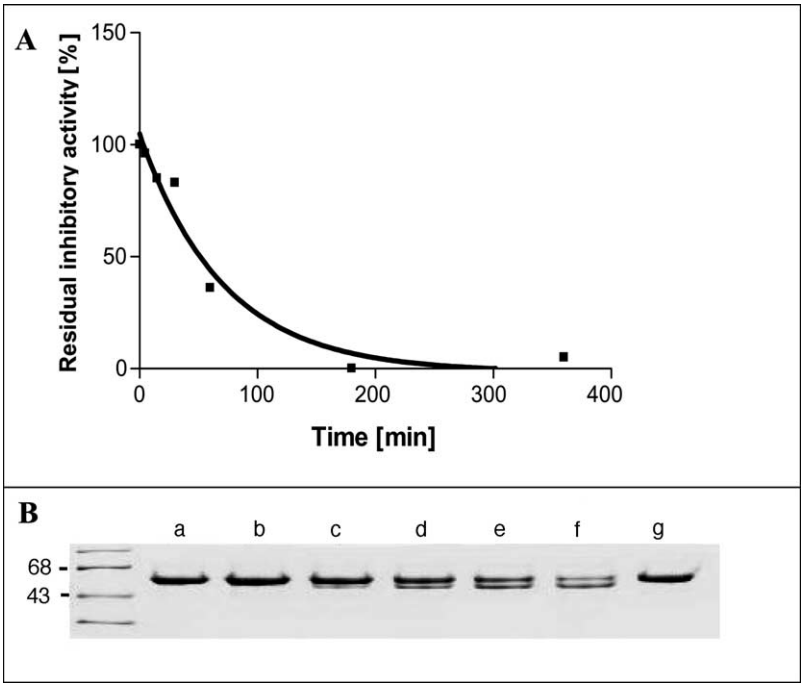

Figure 4 Effect of proteolytic degradation on α_1PI residual inhibitory activity.
Enzyme was incubated with α_1PI at enzyme:inhibitor molar ratio of 1:100 for different time intervals. Inhibitory activity of α_1PI was tested toward trypsin (upper panel) and time-dependent proteolytic degradation visualized by SDS-PAGE (lower panel). Molecular mass marker proteins are indicated (in kDa) on the left; lane a: control, lanes b-f: 5, 15, 30, 60, 180 min of incubation, respectively, lane g: enzyme preincubated with E-64 for 15 min prior to incubation with α_1PI.

enzyme accumulated in the culture media in a growth phase-dependent manner (data not shown). A weak proteolytic activity against these substrates was also observed for the purified enzyme, although clearing zones observed in zymograms were apparent after overnight incubation of gels in a buffer containing reducing agents.

Incubation of the enzyme with α_1PI (E:S ratio of 1:100) for 1 hour resulted in α_1PI cleavage as detected by SDS-PAGE (Figure 4A) and was correlated with a noticeable loss of this serpin inhibitory activity against trypsin (Figure 4B). This allowed us to assume that the cleavage of α_1PI occurs within its reactive site loop. Pretreatment of the enzyme with E-64 completely abolished its proteolytic activity.

Analyses of the cysteine protease coding region

Analyses of the available genomic sequences from *S. epidermidis* strains (ATCC 12228 and RP62A) allowed us to identify the gene encoding the Ecp cysteine protease in both strains. The sequence data revealed a putative bi-cistronic operon consisting of the staphopain-encoding gene *ecpA* (1185 bp) followed by a 321 bp ORF located 12 bp downstream from the *ecpA* TTA stop codon. This gene was termed *ecpB*. No evidence of an internal promoter between the two genes was found, and ribosome binding sites were evident immediately 5′ of both genes. The translated product of *ecpA* is a staphopain preproenzyme of 395 amino acid residues, with a putative signal sequence consisting of 30 residues. The proenzyme processing site has been mapped to the peptide bond between Glu^{221} and Met^{222} (relative to the methionine initiation codon) to form a 174 residues mature enzyme.

The Ecp pre-proenzyme sequence used for similarity searches against available microbial genome databases revealed a significant homology to *S. aureus* staphopain A (ScpA, SA1070 TIGRannotation) and staphopain B (SspB, SA1056) (58 and 43% identity, respectively; Figure 5). Interestingly, analysis of the deduced amino acid sequence of the EcpB protein indicated 32% and 25% identity between this protein and staphostatin A (ScpB) and staphostatin B (SspC), respectively. The *ecp* operon arrangement also resembles these of staphopain A (*scp*) and B (*ssp*). The staphopain B gene, *sspB,* is cotranscribed within the *sspABC* operon with the *sspA* and *sspC* genes encoding the V8 protease and staphostatin B, respectively. Similarly, the staphopain A gene, *scpA,* is part of the bicistronic operon with the *scpB* gene coding for staphostatin A (Rice et al., 2001; Rzychoń et al., 2003).

Discussion

In this study we have characterized a 20 kDa elastase produced by *Staphylococcus epidermidis.* The enzyme was found to be a cell wall-associated protein expressed in the mid- and late-logarithmic phase of bacterial growth. Inhibition studies and the activity dependence on reducing agents allowed us to classify the purified enzyme as a cysteine protease. Analysis of the N-terminal amino acid sequence of the purified protein revealed that the cell wall-associated enzyme is identical to a recently described extracellular cysteine protease referred to as Ecp (Dubin et al., 2001). However, to the best of our knowledge, a cell-associated elastase has never been characterized in *S. epidermidis*, only the activity found in culture filtrates was reported previously (Sloot et al., 1992; Teufel and Götz, 1993). Therefore, our goal was to cast more light on an alternative, cell-linked form of the Ecp protease, the enzyme with an ability to efficiently cleave elastin and with a potential role in *S. epidermidis* pathogenicity *in vivo*.

The Ecp protease is produced by all *S. epidermidis* strains tested in two molecular forms, cell-associated and soluble, although their ratio differs from strain to strain. The primary sequence of the Ecp protein does not contain a membrane-anchoring domain with the LPXTG motif characteristic for *S. aureus* surface proteins (Navarre and Schneewind, 1994; Mazmanian et al., 1999; Ton-That et al., 1999), and, therefore, it is not displayed on a cell surface via the sortase-mediated mechanism. The protease can be released from the bacterial cell by treatment with lysostaphin, mild detergents and high NaCl concentrations, indicating that the enzyme is only loosely attached to the cell wall by yet unidentified interactions, probably of mixed, ionic and hydrophobic character. Surface proteins in Gram-positive bacteria can be linked to the cell outer structures through various non-covalent mechanisms (Cossart and Jonquieres, 2000) such as attachment to teichoic or lipoteichoic acids in the cell wall as in the case of proteins LytA of *Streptococcus pneumoniae* or InlB of *Listeria monocytogenes*.

The soluble form of the *S. epidermidis* Ecp protease present in the media may be either a result of a direct secretion or, alternatively, a release of a cell-associated form from the bacterial surface. The mechanism of enzyme solubilization remains elusive; however, shedding by proteolytic cleavage can be excluded since the molecular mass of both forms is identical and corresponds to the size of the mature protein inferred from the DNA coding sequence. The Ecp protease is expressed as a pre-proprotein but little is known about the process of the proprotein maturation. A proposed model suggests a post-secretory cleavage of the proenzyme releasing a part of a polypeptide chain corresponding to the inhibitory profragment (Dubin et al., 2001). In this context, it is interesting that both Ecp forms detected in *S. epidermidis* are apparently fully processed and active. By analogy to involvement of the bacterial cell surface in the maturation of prostreptopain (pro-SpeB) of *Streptococcus pyogenes* (Rasmussen and Bjorck, 2002) we suggest that in the case of pro-Ecp, transient association of the proenzyme with the cell wall is crucial for correct and efficient zymogen processing. Following the auto- or exo-proteolytic cleavage of the precursor, any generated enzyme either remains associated with the wall or is released into the media. Moreover, it is probable that pro-Ecp conversion into the mature form occurs via a molecular mechanism similar to that mediating the maturation of pro-ScpA, the Ecp homolog from *S. aureus* (see below), although the agent responsible for the latter process has not yet been identified. Possible involvement of

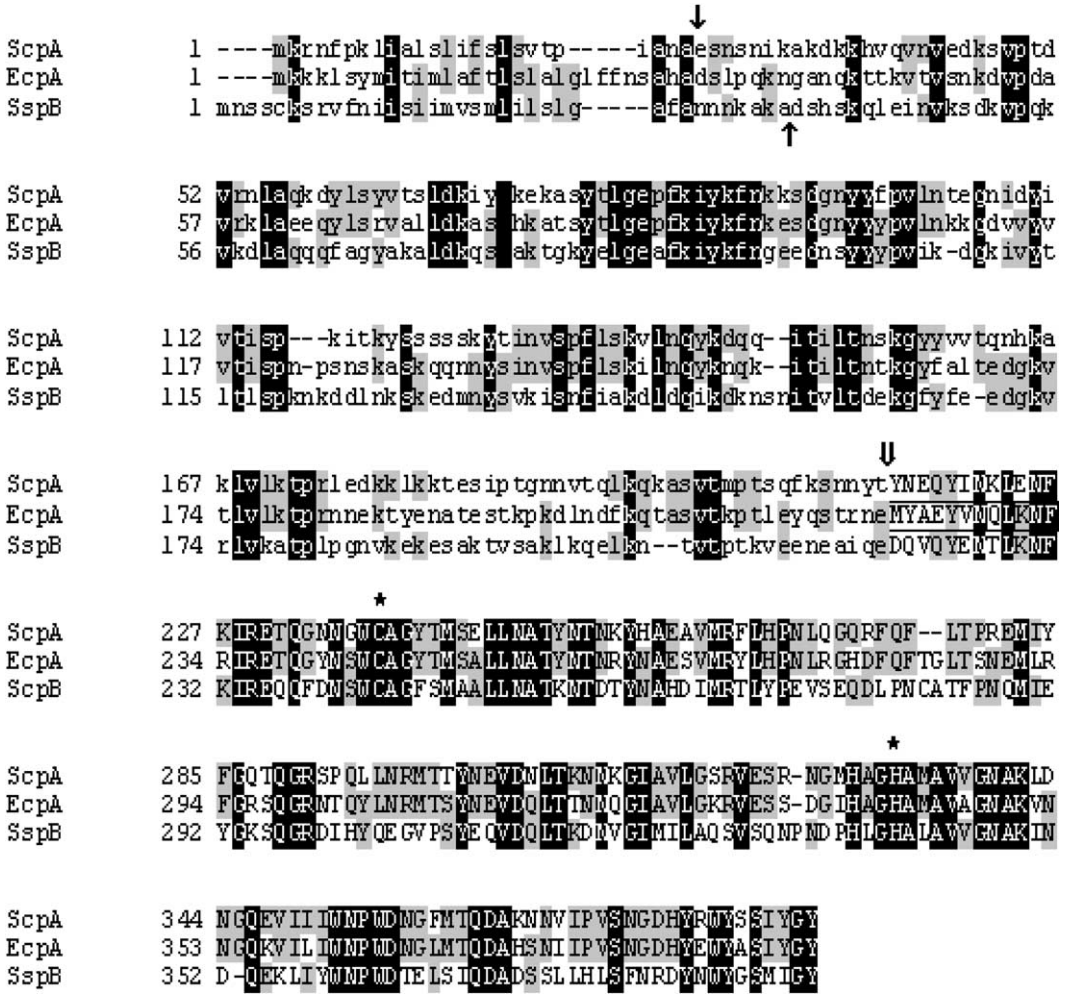

Figure 5 Multiple sequence alignment of *S. epidermidis* cysteine protease and its putative homologs from *S. aureus*. EcpA, *S. epidermidis* elastase; ScpA, *S. aureus* staphopain A; and SspB, *S. aureus* staphopain B. Identical amino acid residues are shaded. Lower case letters represent amino acids of the signal sequence and the profragment, the mature enzyme sequence is shown in capital letters. The predicted signal peptide cleavage sites are indicated by a downward-pointing (for ScpA and EcpA) or upward-pointing (for SspB) arrows. The proteolytic processing site of pro-EcpB at Glu^{221} is shown by a double downward-pointing arrow and the N-terminal sequence determined in this study is boxed. The asterisk indicates an active site cysteine and histidine residues, determined by crystallography analysis of staphopain A inhibited by E-64 (Hoffman et al., 1993).

metalloprotease and/or *S. epidermidis* glutamyl endopeptidase in the processing of Ecp definitely deserves further investigation.

The expression of the Ecp protease occurs in a growth phase-dependent manner and, interestingly, both molecular forms are detectable at the same time during bacterial growth with the levels reaching maxima in the post-exponential phase. As no accumulation of the protease is observed, it can be concluded that either gene expression occurs for a relatively short period of time during the mid-log phase or there is a constant turnover of the enzyme with equilibrium between its production and degradation. Such a regulation pattern resembles that of the *S. aureus* extracellular cysteine proteases (Shaw et al., 2004). This observation is also consistent with recent data describing in *S. epidermidis* the presence of global regulatory loci *sar*, *agr* and *sigB*, which are known to coordinate temporally, spatially and environmentally the expression of virulence factors in *S. aure*us (Fluckinger et al., 1998; Vuong et al., 2000; Otto, 2001; Kies et al., 2001).

The predicted amino acid sequence of *S. epidermidis* Ecp pre-proenzyme of 395 residues shows significant similarity to both *S. aureus* cysteine proteases, with 59% and 42% identity of the primary structure with staphopain A (ScpA) and B (SspB), respectively. Significantly, the mature forms of Ecp and ScpA, comprising 174 residues each, are 75% identical. In addition to similarity at the protein level, the gene arrangement within the *ecp* operon resembles the staphopain A and B operons, in which the staphopain gene is followed by an ORF coding for a highly specific staphopain inhibitor, termed staphostatin (Rice et al., 2001; Massimi et al., 2002; Rzychoń et al., 2003; Shaw et al., 2004). The operon structure found in *S. epidermidis* encompasses the *ecp* (*ecpA*) gene encoding a cysteine protease and the downstream ORF (*ecpB*) coding for a protein homologous to staphostatins A and B (Dubin, 2003), but there is no second cysteine proteinase operon in the *S. epidermidis* genome. Based on structural similarity, it can be inferred that the function of EcpA/EcpB system in *S. epidermidis* is analogous to ScpA/ScpB and/or SspB/SspC in *S.*

aureus. Apparently, this genetic arrangement of enzymes co-expressed with their specific inhibitors is highly conserved since they are present in genomes of all staphylococcal species and strains which have been sequenced to date. It suggests that staphylococcal cysteine proteases must be strictly controlled at the active enzyme level, and this tight regulation of expression and activity could imply an essential role in staphylococcal virulence and/or survival *in vivo*.

The similarities between ScpA and EcpA regarding their biochemical properties also advocate for their close relation. Both enzymes are characterized by strong elastinolytic activity and very limited ability to cleave chromogenic *p*-nitroanilide substrates (Potempa et al., 1988; Dubin et al., 2001). The ability to cleave elastin fibers, the main component of extracellular matrix in tissues, was observed in many bacteria such as *Pseudomonas aeruginosa, Flavobacterium menningosepticum, Bacterioides nodus* and a positive correlation between elastase production and pathogenic potential has been established. Degradation of elastin by ScpA and EcpA might be important for bacterial spreading from the sites of initial colonization. In addition to elastin, the Ecp protease was also shown to digest fibrinogen and fibronectin. In the case of fibrinogen, the α-chain was shown to be exceptionally susceptible to Ecp-mediated degradation. This feature could possibly facilitate bacterial invasion by detachment from host tissues or surfaces of implanted biomedical devices. It could also play an important role in the development of wound infections, as fibrinogen is a key factor in wound healing processes.

The Ecp protease inactivates α_1-protease inhibitor, which seems to be of pathological importance since the presence of *S. epidermidis* on biomaterial surfaces induces activation of polymorphonuclear granulocytes. In this manner, the Ecp protease may indirectly contribute to uncontrolled degradation of surrounding connective tissues by human neutrophil elastase released from PMNs at infection sites, enhancing the inflammation processes and interfering with tissue repair mechanisms (Potempa et al., 1886).

Materials and methods

Cultivation conditions for bacteria

S. epidermidis laboratory strains included in this study (ATCC 14990, TU 3298, TU 9142) were obtained from the collection of Department of Microbiology, Trinity College, Dublin, Ireland. The clinical *S. epidermidis* isolate from marginal and apical periodontitis sites was obtained through the courtesy of Dr. J. Mayo (Department of Biochemistry and Molecular Biology, University of Georgia, Athens, USA). Unless stated otherwise, the bacteria were routinely grown overnight (until the late stationary phase, OD_{600}>10) in Tryptic Soy Broth (TSB) medium (Difco, Detroit, USA), supplemented with 10 g/l β-glycerophosphate (Sigma, St. Louis, USA) and 0.74 g/l $CaCl_2$ and adjusted to pH 7.5, at 37°C with vigorous agitation (250 rpm).

To determine the amounts of soluble and cell wall-associated forms of cysteine protease during *S. epidermidis* growth, a single colony inoculum was grown overnight in TSB. The cells were washed twice with fresh media (5000 *g*, 5 min, 4°C) and subcultured into TSB medium to achieve an initial optical density at 600 nm (OD_{600}) of 0.05. The batch culture was sampled at different phases of growth.

For comparative purposes culture volumes before centrifugation were adjusted to represent an equivalent OD_{600} value based on the cell density of each collected culture. Cell-free culture media obtained by centrifugation (5000 *g*, 30 min, 4°C) were sterilized using 0.2 μm pore size filters (Costar Corp., Kennebunk, USA) and collected bacterial pellets washed and resuspended in PBS to obtain an identical optical density value at 600 nm (OD_{600}).

Subcellular localization of the enzyme

After centrifugation of the bacterial culture, harvested cells were washed and fractionated to obtain cytoplasmic and cell wall fractions as described previously by McAleese et al. (2001). Briefly, the cells were suspended to OD_{600} of 10 in 50 mM Tris-HCl, pH 7.6, 20 mM $MgCl_2$, 30% raffinose (Sigma) and to each sample lysostaphin was added to a final concentration of 0.1 mg/ml. Following incubation at 37°C for 30 min, protoplasts were removed by centrifugation (12 000 *g*, 15 min, 4°C) and supernatants containing wall-associated proteins were collected. In addition to lysostaphin treatment, mild detergents such as Triton X-100 and CHAPS (0.5%, 0.1% or 0.02%), Nonidet P40 and Brij 35 (0.5% or 0.1%) and high concentrations of NaCl (0.25 M, 0.75 M) were used to check the release of cell wall-associated proteins from the bacterial surface. During these procedures no cell lysis was observed. The fractions were assayed for elastinolytic activity against Elastin Congo Red (Sigma, Poznan, Poland; see below). In addition, the presence of cysteine protease in the culture media and the cell wall-associated form was confirmed by DCG-04 labeling (Greenbaum et al., 2000), as described below. All experiments were repeated at least three times using independently grown strains and the same results were obtained. Therefore, only the representative data of selected experiments are presented here.

Enzyme purification

All purification steps were performed at 4°C except for lysostaphin treatment and final separation of the enzyme using MonoS FPLC chromatography. The cells were collected by centrifugation (5000 *g*, 30 min), washed three times with PBS and resuspended in 30% raffinose in 20 mM $MgCl_2$ to a final OD_{600} value of 100. Lysostaphin, at a concentration of 0.1 mg/ml, was added in order to solubilize peptidoglycan and remove the cell wall-associated proteins. After 30 min of gentle stirring at 37°C the protoplasts were removed by centrifugation (12 000 *g*, 60 min). The supernatant was extensively dialyzed against 0.1 M Tris-HCl, pH 7.6, 0.15 M NaCl, 1 mM $CaCl_2$ and subjected to gel filtration on a Sephadex G-150 column (2.6×100 cm) equilibrated with the same buffer, at a flow rate of 6 ml/h. Collected fractions were assayed for elastinolytic activity using Elastin Congo Red. Activity-containing fractions were pooled and, following dialysis against 20 mM MES, pH 6.5, 1 mM $CaCl_2$, loaded onto a MonoS HR 5/5 FPLC column (Amersham Pharmacia, Freiburg, Germany) equilibrated with the same buffer. The column was washed until the A_{280} value reached the baseline and proteins bound to the matrix were eluted with a 0–300 mM NaCl gradient.

Enzyme activity assay

Elastinolytic activity of the protease was assayed with Elastin Congo Red (Sigma) as a substrate. The enzyme was activated for 10 min at 37°C in 0.1 M Tris-HCl, pH 7.6, 1 mM $CaCl_2$, 10 mM L-cysteine and then Elastin Congo Red suspended in water was added to a final concentration of 10 mg/ml. The mixture was incubated for 3–12 hours at 37°C with shaking, and after remov-

al of undigested substrate by centrifugation (10 000 *g*, 5 min, 4°C) the amount of dye released into solution was measured at 490 nm.

Electrophoretic separations, zymography, Western blotting, N-terminal sequencing

SDS-PAGE separations were performed as described essentially by Schägger and von Jagow (1987) and Laemmli (1970) to monitor the steps of enzyme purification procedure and estimate its molecular mass. Native PAGE was conducted according to the modified method of Schägger and von Jagow (1991).

For zymographic analysis samples were incubated in a non-reducing SDS-PAGE sample buffer and resolved at 4°C on 10% SDS-PAGE gels containing either 0.2% gelatin or 0.1% fibrinogen. The gels were developed following the modified method of Heussen and Dowdle (1980). Briefly, separated proteins were renaturated by washing with 2.5% (w/v) Triton X-100, incubated in 0.2 M Tris-HCl, pH 7.6, 1 mM $CaCl_2$, 10 mM cysteine, overnight at 37°C, then stained in 0.1% amido black and destained to reveal zones of proteolytic activity.

Western blots were conducted according to the method of Towbin et al. (1979). For N-terminal sequence analysis, proteins resolved by SDS-PAGE were electrotransferred onto a polyvinylidene difluoride (PVDF) membrane using CAPS buffer by the method of Matsudaira (1987). The membrane was washed thoroughly with water, briefly stained with 0.1% Coomassie Brilliant Blue G-250, destained and air-dried. Protein bands were cut out and subjected to N-terminal sequence analysis by automatic Edman degradation using an Applied Biosystems 491 protein sequencer (Applied Biosystems, Foster City, USA) at the University of Georgia core facility.

Cysteine protease active site labeling with DCG-04

The cell suspensions and media samples pretreated with 1 mM DTT for 10 min at 37°C were incubated with 0.5 μM DCG-04, biotinylated derivative of E-64 (Greenbaum et al., 2000) for 30 min at 37°C. As a control for DCG-04 binding specificity, parallel samples were preincubated with 1 μM E-64 for 30 min at room temperature before adding DCG-04. Labeling reactions were stopped by addition of a hot reducing SDS-PAGE treatment buffer and samples boiled for 5 min. Proteins resolved by SDS-PAGE were electrotransferred onto a nitrocellulose membrane using standard Tris transfer buffer (Towbin, 1979). Nonspecific binding sites were blocked with T-TBS (20 mM Tris-HCl, pH 7.5, 0.5 M NaCl, 0.05% w/v Tween 20) containing 1% BSA for 1 h at room temperature, and the membrane was incubated for 1 h with streptavidin-alkaline phosphatase conjugate (1:2000 dilution in 1% BSA/T-TBS). After extensive washing with T-TBS the blots were developed using an AP substrate kit (BioRad, Hercules, USA) according to the instruction provided by the manufacturer.

Determination of enzyme activity and stability

The effect of the pH on elastinolytic activity of the purified protease was studied in the presence of 10 mM cysteine using following buffers: 0.2 M sodium acetate (pH 4–5), 0.2 M MES (pH 5.5–6.5), 0.2 M Tris-HCl (pH 7–10). In order to determine the pH stability, the enzyme was preincubated for 12 hours in buffers without cysteine at room temperature or 37°C and the residual activity determined in the standard assay. To test the temperature stability, the enzyme preincubated in a standard buffer (0.1 M Tris-HCl, pH 7.6, 1 mM $CaCl_2$) without cysteine for 1 h or 12 h at room temperature, 37°C and 42°C was assayed for elastinolytic activity.

Effect of reducing agents

Reducing agents tested for the effect on the purified enzyme elastinolytic activity included dithiothreitol, L-cysteine and reduced glutathione (Fluka, Poznan, Poland). The assay was performed in 0.1 M Tris-HCl, pH 7.6, 1 mM $CaCl_2$, containing a reducing agent at a concentration range from 0.1 mM to 100 mM (see Table 1) to determine a full scope of proteolytic activity dependence on a reducing agent concentration. Activity of the enzyme in the buffer without any reducing agents was used as a control.

Inhibition profile

The purified protease was preincubated for 10 min at room temperature with various metallo-, serine and cysteine protease inhibitors, at indicated concentrations (see Table 1), followed by residual activity assay with Elastin Congo Red (in the presence of 10 mM cysteine). Several natural inhibitors including aprotinin, pepstatin, bestatin, leupeptin, antipain and cystatin C (recombinant protein kindly provided by Dr. Magnus Abrahamson, Department of Clinical Chemistry, University of Lund, Sweden) were also tested for inhibitory effects on the purified enzyme. Activity of non-inhibited enzyme assayed under standard conditions was used as a control.

Enzyme specificity studies

Proteolytic activity of the enzyme was tested with the following chromogenic substrates used at 1 mM final concentration: Boc-Val-Leu-Gly-pNA, H-Ala-Ala-Phe-pNA, H-Ala-Gly-Arg-pNA, H-Leu-Thr-Arg-pNA, H-Ala-Phe-Pro-pNA, H-Ala-Pro-pNA, H-Ala-pNA, H-Gly-Arg-pNA, H-Gly-Arg-pNA, H-Gly-Gly-pNA, H-Gly-Phe-pNA, H-Gly-pNA, H-Ile-pNA, H-Leu-pNA, H-Lys-pNA, H-Phe-pNA, N-α-benzoyl-DL-arginine-pNA, N-methoxysuccinyl-Ala-Pro-Val-pNA, N-Suc-Ala-Ala-pNA, N-Suc-Ala-Ala-Pro-Glu-pNA, N-Suc-Ala-Ala-Pro-Leu-pNA, N-Suc-Ala-Ala-Val-Ala-pNA, Z-Ala-Ala-pNA, Z-Lys-pNA, Z-Arg-pNA, Z-Glu-Glu-pNA, Z-Leu-Leu-Glu-pNA, Z-Lys-Arg-pNA, Z-Phe-Arg-pNA, Z-Phe-Val-Arg-pNA, and Z-Tyr-Lys-Arg-pNA (from Sigma or Bachem), in 0.1 M Tris-HCl, pH 7.6, at 37°C. The assay was performed on microplates in a total volume of 0.2 ml, using a microplate reader (SpectraMax, Molecular Devices, Sunnyvale, USA). Initial turnover rate was recorded at 405 nm.

To further determine specificity of the enzyme, several synthetic peptides (Table 2) were incubated with the purified protease at an enzyme:substrate ratio of 1:50 in 0.1 M Tris-HCl, pH 7.6 for 12 hours at 37°C. The peptides were prepared for a purpose of other projects and were used here as substrates. The reaction was stopped by addition of 1 μl of trifluoroacetic acid, sample centrifuged (10 000 *g*, 15 min, 4°C) and subjected to reversed-phase high pressure liquid chromatography using a Supelcosil™ LC-18 column (250×4.6 mm). Peptides were eluted with acetonitrile gradient (0–80% in 0.075% TFA in 50 min) and each peak detected at 220 nm was manually collected and subjected to subsequent identification by electrospray mass spectroscopy at the Chemical and Biological Sciences Mass Spectrometry Facility, University of Georgia.

The activity of the purified protease toward native proteins was determined using α_1PI (E:S molar ratio of 1:100), fibrinogen (1:10), fibronectin, collagen, laminin, albumin, IgA, IgM, IgG1 and IgG2 (in each case 0.185 μg of enzyme:5 μg of substrate, in a final volume of 20 μl) for time intervals in the range from 0 to 360 min. The reaction was stopped by the addition of E-64 to a final concentration of 1 μM, followed by boiling in the reducing SDS-PAGE sample buffer for 5 min.

Effect of elastase on α_1PI inhibitory activity and inhibition by α_2-macroglobulin

The purified protease was incubated with α_1PI at an enzyme:inhibitor molar ratio of 1:100 at 37°C. At given time intervals aliquots were removed and tested for inhibitory activity of uncleaved α_1PI towards human neutrophil elastase (Athens Research Inc., Athens, USA). Residual elastase amidolytic activity was determined using N-methoxysuccinyl-Ala-Ala-Pro-Val-pNA as a substrate. To test the effect of α_2-macroglobulin on the elastinolytic activity of the protease, the purified enzyme was incubated with the inhibitor at a 1:1 molar ratio at 37°C for 15 min and the mixture analyzed for proteolytic activity and for α_2-macroglobulin-protease complex formation using native PAGE.

Sequence analyses

Nucleotide sequence of cysteine protease coding region used to identify the *ecp* operon in *S. epidermidis* ATCC 12228 was retrieved from The Institute for Genomic Research genome sequence database (http://www.tigr.org/tigr-scripts/CMR2/CMRGenomes.spl, TIGR assignation SE0184). Protein sequence analyses were performed using programs provided by the ExPasy Proteomics Tools molecular biology server at http://www.expasy.ch/tools. Homology searches were conducted using the BLAST algorithms provided by the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov).

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