Degradation of Human Antimicrobial Peptide LL-37 by Staphylococcus aureus-Derived Proteinases

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Cathelicidin LL-37 is one of the few human bactericidal peptides with potent antistaphylococcal activity. In this study we examined the susceptibility of LL-37 to proteolytic degradation by two major proteinases produced by Staphylococcus aureus, a metalloproteinase (aureolysin) and a glutamylendopeptidase (V8 protease). We found that aureolysin cleaved and inactivated LL-37 in a time- and concentration-dependent manner. Analysis of the generated fragments by mass spectroscopy revealed that the initial cleavage of LL-37 by aureolysin occurred between the Arg19-Ile20, Arg23-Ile24, and Leu31-Val32 peptide bonds, instantly annihilating the antibacterial activity of LL-37. In contrast, the V8 protease hydrolyzed efficiently only the Glu16-Phel7 peptide bond, rendering the C-terminal fragment refractory to further degradation. This fragment (termed LL-17-37) displayed antibacterial activity against S. aureus at a molar level similar to that of the full-length LL-37 peptide, indicating that the antibacterial activity of LL-37 resides in the C-terminal region. In keeping with LL-37 degradation by aureolysin, S. aureus strains that produce significant amounts of this metalloproteinase were found to be less susceptible to LL-17-37 than strains expressing no aureolysin activity. Taken together, these data suggest that aureolysin production by S. aureus contributes to the resistance of this pathogen to the innate immune system of humans mediated by LL-37.

Staphylococcus aureus is a major human pathogen responsible for numerous cases of community- and hospital-acquired infections, ranging from relatively minor skin and wound infections to life-threatening diseases, including toxic shock syndrome, arthritis, endocarditis, osteomyelitis, and sepsis (22). Up to 60% of healthy individuals are permanently or transiently colonized by S. aureus. Nasal carriage is an important risk factor for serious systemic and internal organ infections that have high morbidity and mortality, especially in those patients with a suppressed immune system, undergoing surgery, or with intravenous devices (18). Furthermore, the worldwide spread of multidrug-resistant strains, including the emergence of strains with greatly reduced susceptibility to vancomycin (46), typically regarded as the antibiotic of last resort, indicates the danger of untreatable staphylococcal infections. The recent outbreaks of methicillin-resistant S. aureus causing community-acquired infections emphasize the continued problems caused by this organism.

In the past decade, a broad array of virulence factors mediating adherence to host tissues, damage to host cells, and strategies for evasion of the host defense mechanisms have been thoroughly characterized (31). Neutrophils play an important role in host defense against S. aureus. They employ both oxygen-dependent and -independent mechanisms to kill the pathogen. The importance of neutrophils in innate resistance to staphylococci is supported by the fact that neutropenic patients or those with chronic granulomatous disease (CGD) are particularly susceptible to S. aureus infections (21). Oxygen-independent killing mechanisms mediated by antimicrobial peptides or proteins (34, 39) play a more important role in killing staphylococci than previously thought. However, S. aureus has evolved the means to resist effector molecules of innate immunity, such as lysozyme and cationic antibacterial peptides, including the α-defensins of human neutrophils (HNP 1 to 3) (32, 33) and the β-defensin hBD2 (14, 49). The resistance of S. aureus to α-defensins, which account for 50% of the neutrophil granule proteins, likely contributes to the inability of neutrophils to effectively kill this pathogen (25). The bacterial activity of neutrophils is reinforced by cathelicidin-derived peptides. The serine proteinases confined in the azurophil granules of neutrophils, especially elastase, may also play a major role in killing phagocytosed bacteria, including S. aureus (5, 34, 43).

Beside the defensins, cathelicidins constitute the second major family of antibacterial peptides in mammals (for reviews see references 6 and 7). Cathelicidins contain an N-terminal domain called cathelin that is strictly conserved across species, followed by a C-terminal domain that comprises an antimicrobial peptide of a highly diverse structure (38, 47). The human cathelicidin hCAP-18 or FALL-39 is constitutively present in specific granules of neutrophils (42) and the testes (1), and it is
inducibly expressed by keratinocytes (13) and in squamous epithelia (4, 10) in response to inflammatory challenge. In humans, hCAP-18 is processed by protease 3 to generate the active peptide LL-37 (42). On the other hand, the porcine and bovine cathelicidins are cleaved by neutrophil elastase-releasing antibacterial peptides referred to as protegrins and bacte-
nacin, respectively (29, 38). In contrast to most defensins, cathelin-derived bactericidal peptides, including LL-37, possess considerable antistaphylococcal activity (44, 45) and, therefore, may significantly contribute to protection of the skin and mucosal surfaces of upper airways against colonization by S. aureus and other gram-positive pathogens. Indeed, the sig-
nificance of cathelicidins for the innate host defense is appar-
ent from the following observations: the mouse cathelicidin CRAMP protects the skin from invasive bacterial infections (27); proteolytic processing of porcine cathelicidins (prote-
grins) is essential for bacterial clearance from wounds (9); improved lung clearance of Pseudomonas aeruginosa is associated with overexpression of LL-37 (2, 3); and downregulation of LL-37 in atopic dermatitis predisposes patients to skin in-
fec tion with S. aureus (28).

In addition to LL-37, human skin is also protected from S. aureus infection by the recently discovered β-defensin hBD-3 (14) as well as dermicidin, a novel antibiotic peptide secreted by sweat glands (36). Significantly, while dermicidin expression is constitutive, proinflammatory cytokines and contact with bacteria strongly induce hBD-3 expression in keratinocytes and airway epithelial cells. However, despite the formidable line of innate defense exerted by LL-37, dermicidin, and hBD-3, S. aureus and coagulase-negative staphylococci easily colonize na-
sal epithelium and skin, respectively. It is believed that the inherited resistance of Staphylococcus spp. to the bactericidal activity of α-defensins and hBD-2 is responsible for this ability (30). Here we show that proteinases from S. aureus, notably aureolysin, through degradation of LL-37 may also contribute to the ability of this pathogen to resist bactericidal peptides.

MATERIALS AND METHODS

Peptide synthesis. Peptides were synthesized on an Applied Biosystems model 433A synthesizer on a 0.1- to 0.25-mmol scale, using either tert-butoxycarbonyl or 9-fluorenylmethyloxycarbonyl chemistry as described previously (39, 40). Peptides were purified by preparative reverse-phase high-performance liquid chroma-
tography (HPLC) using C18 silica columns and were lyophilized by a SpeedVac (Savant) or 50-mmol packed with 20% trifluoroacetic acid. The purity of the peptides was deter-
mined by analytical HPLC and by mass spectrometry. The peptide concentration was determined by quantitative amino acid analysis.

Bacterial strains. The following bacterial strains were used in this study: Escherichia coli (ATCC 33694), Bacillus subtilis M168 (ATCC 23730), a clinical isolate of Pseudomonas aeruginosa, Enterococcus faecalis (ATCC 29212), S. au-
reus strains Newman and SH1000 (15), DU5977 Newman sar (23), DU5977 Newman sar (23), and SH1000 sar (this study), constructed by transducing the sarA (Kac) mutation from PC1839 (8). Proteinase purification. The S. aureus metalloproteinase (aureolysin) and V8 proteinase (glutamylendopeptidase) were purified from culture medium of the V8 strain as described by Drapeau (11). Enzyme purity was evaluated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using 10% Tricine gels. The protein concentration in each enzyme batch was determined by using a bicinchoninic acid (BCA) (Bio-Rad) kit with bovine serum albumin as a standard.

Zymography. Culture media of S. aureus strains grown to mid-exponential phase were mixed with sample buffer (0.125 M Tris-HCl, 20% glycerol, 5% sodium dodecyl sulfate, 0.03% bromphenol blue, pH 6.8) and electrophoresed on 10% polyacrylamide gels containing 0.1% bovine gelatin using the Laemmli buffer system (19). To remove SDS, gels were incubated with 2.5% Triton X-100, followed by overnight incubation at 37°C in a buffer containing 50 mM Tris-
HCl-5 mM CaCl2, pH 7.8. Aureolysin- and V8 protease-containing bands were visualized as clear zones against a dark background. To distinguish the origin of the proteolytic activity zone, media samples in some experiments were preincu-
bated with either 1 mM 3,4-dichloroisocoumarin (inhibitor of the V8 protease) or 10 mM o-phenanthroline (inhibition of aureolysin), and the zymogram was developed in buffer containing the appropriate inhibitor.

Proteolytic activity assay. Proteolytic activity in growth media of all strains of S. aureus was determined by using azocasein as the substrate. Briefly, 50-μl aliquots of the medium were added to 0.2 ml of a solution of 100 mM Tris-HCl-1 mM CaCl2, pH 7.8, supplemented with either 1 mM 3,4-dichloroisocoumarin (aureolysin activity) or 10 mM o-phenanthroline (V8 protease activity). After 30 min of preincubation at 37°C, 0.125 μl of 3% azocasein (w/v, in deionized water) was added and the mixture was incubated for 3 h at 37°C. The reaction was stopped by addition of 0.35 ml of ice-cold 6% (wt/vol) trichloroacetic acid (TCA). The samples were vortexed and kept on ice for 10 min, and then the undigested precipitated azocasein was removed by centrifugation (14,000 × g, 10 min, at 4°C). From each sample, 200 μl of clear supernatant was transferred into wells of a microtiter plate. The optical density at 360 nm was measured against a blank sample containing all the reagents in the absence of substrate.

To investigate LL-37 cleavage in the presence of human plasma, the peptide solution was mixed with an equal volume of aureolysin or the V8 protease, serially diluted to the same molar concentration in 20 mM Tris-HCl-1 mM CaCl2, pH 7.8. The staphylococcal proteinases were incubated with the peptide for 0.5 and 2 h, respectively. After each time point, 10-μl aliquots were removed, diluted twofold in 0.01% acetic acid, and immediately used for assessing the residual bactericidal activity of the pep-
tide, using either a radial diffusion or colony-counting procedure (see below). The diluted samples collected after different times of incubation with various enzyme concentrations were analyzed by molecular mass analysis. Based on the determined molecular mass of LL-37 and LL-37 degradation products, the cleavage sites of the peptides by the staphylococcal proteinases were determined.

To investigate LL-37 cleavage in the presence of human plasma, the peptide solution was mixed with an equal volume of proteases contained in serially di-
luted human plasma in phosphate-buffered saline (PBS) and were incubated at 37°C. The reaction was stopped by boiling in samples in a reducing SDS-PAGE sample buffer and was analyzed for LL-37 degradation using the Western blot technique.

Western blot analysis. Samples were resolved in SDS-PAGE (peptide gels) using the Tris-Tricine discontinuous buffer system (35). The electrophoresed gels were electrophobled (Trans-Blot Semi-Dry; Bio-Rad) onto polyvinylidene diflu-
oride membranes (Amersham-Pharmacia Co.). Non-specific binding sites on the membrane were blocked overnight in 5% dry milk and then were immunoblot-
ted. The blots were probed with mouse antibodies against LL-37 (HyCulture Bio-
technology, Uden, The Netherlands). Immunoreactive peptides were detected by the chemiluminescence described in the Western Lighting Chemiluminescence Reagent Plus kit (Perkin Elmer Life Sciences, Boston, Mass.).

Antimicrobial assays. (i) Radial diffusion assays. The two-stage radial diffu-
sion assay was performed as described by Lehrer et al. (20). Briefly, the purified synthetic peptides, LL-37 and its truncated version, LL-17-37, either intact or after preincubulation with proteinases, were serially diluted in acidified water (0.01% acetic acid) that contained 0.2% human serum albumin. The target bacteria were grown to mid-exponential phase in tryptic soy broth and were washed with 10 mM phosphate buffer (pH 7.4). Approximately 2 × 10^8 CFU per ml was incorporated into an agarose gel that contained 2% (wt/vol) low-melting-
point agarose (Sigma) and 10 mM sodium phosphate buffer (pH 7.4) in a total volume of 15 ml, and it was poured into 12-cm-diameter petri dishes to form a thin (1.2 mm) underlying gel. A regularly spaced four-by-six array of wells of 3.2 mm in diameter was made in the underlying gel. Six 2.5-μl aliquots of peptide (containing 4, 3, 2, 1, 0.5, or 0.25 mg of peptide/ml) were added to the wells. After 3 h, 5 ml of overlay gel consisting of 6% tryptic soy broth agar and 10 mM sodium phosphate (pH 7.4) was poured. The plates were incubated overnight to allow surviving organisms to form microcolonies. Diameter of the zone of bacterial growth inhibition were measured to the nearest 0.1 mm before and/or after staining the gel with Coomassie brilliant blue and are expressed in units (1 U = 0.1 mm) after subtracting the diameter of the well. A linear relationship exists between the zone diameter and the base 10 logarithm of the peptide concentra-
tion. The MIC was determined by performing a least-mean-squares fit and solving for the x intercept.

(ii) Colony reduction assay. Bacteria were grown as described above for the radial diffusion assays, washed twice in 10 mM sodium phosphate buffer (pH 7.4), and diluted to a final concentration of 2 × 10^7 to 5 × 10^7 CFU/ml in 10 mM 18
sodium phosphate buffer (pH 7.4) with 0.2% (wt/vol) tryptic soy broth powder. Twofold serial dilutions of LL-37 or LL-17-37, either intact or after preincubation with aureolysin or the V8 protease, were prepared in acidified water (0.01% acetic acid), and 90-μl aliquots were transferred to a low-protein-binding polypropylene round-bottom microtiter plate (Costar, Cambridge, United Kingdom). To each of the wells, 10 μl of the bacterial suspension was added. The plates were incubated on a rotary shaker (300 rpm) at 37°C. After a 1-h incubation, 10-μl aliquots were removed and diluted 1:100 with the medium, and 10-μl samples were plated onto tryptic soy agar plates. Alternatively, 5-μl samples were spotted in duplicate on plates. The bactericidal activity was assessed next after counting colonies on the agar plates (CFU) and by visual inspection of the growth. The 50% lethal dose (LD₅₀) and the minimal bactericidal concentrations (MBC) were defined as the concentration of the peptide at which 50% of the inoculum survived after the 1-h exposure or no visible colonies were seen on the plate, respectively. All experiments were performed at least in triplicate.

RESULTS

Degradation and inactivation of LL-37 by staphylococcal proteases. LL-37 was incubated with purified aureolysin and V8 protease, either at a constant enzyme concentration over various time intervals or with varied concentrations of the proteinases for 45 min, and the peptide bactericidal and/or bacteriostatic activity was then determined by using the agar diffusion assay with E. coli and S. aureus (Newman strain) as the target bacteria. The data from these experiments indicated that aureolysin efficiently inactivated the antibacterial activity of LL-37 in a time- and concentration-dependent manners (Fig. 1A and B). In contrast, the antibacterial activity of LL-37 was not only resistant to inactivation by the V8 proteinase but, paradoxically, was enhanced (Fig. 1C and D).

The rate of inactivation of the bactericidal activity by aureolysin correlated well with the rate of LL-37 degradation monitored by mass spectroscopy analysis. The peptide degradation apparently occurred by simultaneous hydrolysis of the Arg19-Ile20, Arg23-Ile24, and Leu31-Val32 peptide bonds (Fig. 2). In contrast, the V8 proteinase only hydrolyzed efficiently the

FIG. 1. Concentration-dependent effect of staphylococcal proteinases on the antibacterial activity of LL-37. LL-37 (3 mg/ml) was mixed with an equal volume of aureolysin (A and B) or the V8 protease (C and D) in 20 mM Tris, pH 7.8, with 1 mM CaCl₂, to yield indicated concentrations of both proteinases. The digested samples were tested for antibacterial action against S. aureus strain Newman (A and C) and E. coli ATCC 33694 (B and D). The diameter of the bacterial growth inhibition zones was measured and converted into units (0.1 mm = 1 U of inhibition growth). Each point in the figure represents a mean ± standard deviation of at least two independent triplicate assays. Insets in panels C and D show representative results of bacterial growth inhibition by LL-37 alone and after preincubation with two different concentrations of the V8 protease.

FIG. 2. Cleavage sites of LL-37 and its N-terminally truncated form (LL-17-37) by aureolysin (A and B) and the V8 protease (C). Solid and dotted arrows indicate primary and secondary cleavage sites, respectively.
Glu16-Phe17 peptide bond, releasing the C-terminal fragment which was refractory to further cleavage even after prolonged incubation (5 h) in the presence of the V8 protease. However, under this condition the N-terminal 16-mer peptide was further cleaved at the Glu5-Phe6 and Glu11-Lys12 peptide bonds. These data indicated that the bactericidal activity of LL-37 apparently resides in the C-terminal part of the peptide. To confirm this assumption, the peptide encompassing amino acid residues 17 to 37 of LL-37 was synthesized and tested for biological activity. Indeed, on a weight basis its bactericidal activity against E. coli, Bacillus subtilis, Pseudomonas aeruginosa, and Enterococcus faecalis was from 3- to 1.5-fold higher than that of intact LL-37 (Table 1). Similar results were obtained when the antistaphyloccocal activity of the peptides was compared using the agarose diffusion assay (Fig. 3). However, because the truncated peptide is nearly one-half the molecular mass of the full-length peptide, the two peptides are essentially equivalent in activity on a molar basis. Thus, we conclude that the majority (if not total) bactericidal capacity of LL-37 can be localized to its C-terminal region.

In accordance with the cleavage pattern of full-length LL-37, the LL-17-37 peptide was resistant to degradation by V8 protease and its antistaphyloccocal activity remained intact even after 5 h of incubation with a high concentration of enzyme (1.4 μM). In contrast, the antibacterial activity of LL-17-37 was lost during incubation with aureolysin, and the kinetics of degradation were similar to those of the inactivation of intact LL-37 (data not shown). Mass spectroscopic analysis confirmed that the same peptide bonds were cleaved (Fig. 2B).

LL-37 was also susceptible to proteolytic degradation by aureolysin and the V8 protease in the presence of human plasma, as determined by the Western blot analysis (data not shown). In this case, the concentration of both V8 protease and aureolysin necessary to achieve complete cleavage of the peptide was somehow higher than that of buffer alone. This is apparently due to inhibition of the staphyloccocal protease activity by human plasma inhibitors. Nevertheless, this result suggests that the LL-37 cleavage can occur in vivo.

**Proteolytic activity produced by different isogenic mutants of S. aureus.** Metalloprotease activity released by S. aureus into the environment may constitute one mechanism by which this pathogen defends itself against antimicrobial peptides. Because the expression of aureolysin is negatively regulated (8, 48) by the product of the staphyloccocal accessory regulator gene (sarA), we used mutants of strains Newman and SH1000 deficient in this regulator. Before assessing the susceptibility of the S. aureus strains to the antistaphyloccocal activity of LL-37 and LL-17-37, the proteolytic activity in media from the late-exponential cultures was analyzed by gelatin zymography (Fig. 4) and in an assay with azocasein as the substrate. The wild-type Newman strain produced very weak proteolytic activity which was visible on the gelatin zymography gels only after overnight incubation. In agreement with previously published data (23), this proteolytic activity was strongly enhanced in the

![](image)

**FIG. 3.** Comparison of bacteriostatic and bactericidal activity of LL-37 and LL-17-37. The Newman strain of S. aureus in the logarithmic phase was mixed with basic agar and poured on the sterile petri dishes. After agar solidification, the wells were punched and 2.5-μl aliquots of the peptide at concentrations of 4, 3, 2, 1, 0.5, and 0.25 mg/ml were added. Plates were incubated for 3 h at 37°C, and then nutritive TSA was poured on the basic agar. Plates were incubated overnight at 37°C, dried, and stained with Coomassie brilliant blue, and the diameters of zones of inhibition growth were measured (inset) and plotted against the log of peptide concentration (in micrograms/milliliter). Each point represents a mean ± standard deviation of three independent duplicate assays. From the intercept with the x-axis, the MIC was calculated at 30 and 152 μg/ml for LL-17-37 and LL-37, respectively. The inset shows representative results of bacterial growth inhibition by different concentrations of LL-37 and LL-17-37.
sarA mutant (Fig. 4, lane 2). With this mutant, bands related to V8 proteinase and aureolysin activity, as distinguished by gelatin-zymography analysis (A) or was quantified using azocasein as the substrate (B). The contribution of V8 proteinase (full arrowhead in panel A and stippled bars in panel B) and aureolysin (empty arrowhead and crossed bars) to total proteolytic activity was determined with the specific inhibitors 3,4-dichloroisocoumarin and o-phenanthroline, respectively.

![Figure 4](image)

**DISCUSSION**

Cationic peptides with antimicrobial activity constitute a formidable innate immunity defense barrier against infections (6). Nevertheless, some bacterial species have developed various mechanisms to circumvent this barrier. Through extensive modification of cell membrane phospholipids and cell wall teichoic and lipoteichoic acids that neutralize the cell surface negative charge (32, 33), *S. aureus* has gained a significant mechanism to protect itself against cationic peptides. In addition to these structural changes in the bacterial surface or membrane, it was recently shown that staphylokinase, a protein commonly released by *S. aureus*, binds and counteracts the bactericidal effect of α-defensins (16). A high frequency of staphylokinase-producing strains among isolates from the upper respiratory tract of healthy individuals may indicate that this mechanism is important in colonization of these mucosal membranes containing antimicrobial peptides. In this study we noted that an additional level of protection against antimicrobial peptides, at least LL-37, may depend on the proteolytic activity secreted by *S. aureus*.

The mass spectroscopy analysis of the LL-37 incubation mixture with *S. aureus* metalloproteinase (aureolysin) and the V8 protease indicated that the peptide is efficiently cleaved by both enzymes. The cleavage occurred even in the presence of human plasma, suggesting that LL-37 proteolytic processing by
S. aureus proteases may take place in vivo. Surprisingly, however, only in the case of aureolysin was the cleavage of LL-37 correlated with the loss of bactericidal activity. In stark contrast, the V8 protease did not destroy the bactericidal action of LL-37 against both gram-negative and gram-positive bacteria. This was apparently related to the generation of the LL-17-37 C-terminal fragment of the intact peptide, which was refractory to further degradation by the V8 protease. N-terminally truncated LL-37 derivatives with increased bactericidal activity were recently described as being generated in human sweat by endogenous serine proteinases (26). Interestingly, the V8-like protease is commonly produced by Staphylococcus epidermidis (12, 24), which constitutes a normal human bacterial flora. We suggest that this activity may augment the LL-37-dependent antibacterial potential of human skin, protecting this niche from colonization by other bacterial species, including S. aureus.

Although S. aureus would remain sensitive to the bactericidal activity exerted by the truncated LL-17-37 peptide, it could still in theory defend itself against this peptide through the action of using aureolysin. This metalloproteinase efficiently inactivates LL-37 bactericidal activity through the cleavage of the peptide bonds located in the C-terminal portion of the peptide, and it would still be able to cleave LL-17-37 because this truncated peptide was inactivated at the same rate as intact peptide. Our results also suggest that the level of secreted aureolysin correlated with levels of S. aureus susceptibility to bactericidal activity of both LL-37 and LL-17-37. Thus, both the Newman and SH1000 strains with relatively weak proteolytic activity were more susceptible to LL-17-37 than their respective sarA mutants that produce greater amounts of aureolysin. To learn whether loss of aureolysin production by S. aureus altered bacterial susceptibility to LL-37, we also examined an aureolysin-deficient mutant of strain Newman sarA. We found that this mutant displayed a level of LL-37 susceptibility that was identical to that of wild-type strain Newman (data not shown). Thus, loss of aureolysin production by an overproducing strain (Newman sarA) and a concomitant increased susceptibility to LL-37 lends further support to our hypothesis that the action of aureolysin on LL-37 is yet another mechanism by which S. aureus resists antimicrobial peptides.

A protease-dependent deterrence of host defense based on cathelicidin peptides has been long suggested as an important mechanism in bacterial virulence (17), but it was only experimentally verified relatively recently (37). Thus, the P. aeruginosa elastase, Proteus mirabilis metalloprotease, and Enterococcus faecalis gelatinase can degrade and inactivate LL-37. In the case of P. aeruginosa infection, degradation of LL-37 in human wound fluid enhanced bacterial survival. Interestingly, aureolysin, elastase, and gelatinase belong to family M4 (http://merops.sanger.ac.uk) of structurally related metalloproteinases of similar specificity, which in addition encompasses enzymes of other well-known human pathogens, including Vibrio cholerae, Listeria monocytogenes, Legionella pneumophila, and Clostridium perfringens. This may indicate that antibiotic peptide inactivation through proteolysis is widespread among pathogenic bacteria.

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