In Vivo Processing of *Staphylococcus aureus* Lipase

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The *Staphylococcus aureus* lipase gene encodes a 76-kDa protein. Extracellular lipase purified from culture supernatants is only 45 to 46 kDa, however. We show that the lipase is secreted in vivo as an 82-kDa protein with full enzymatic activity. It is then sequentially processed, both in culture and in cell-free supernatants, to a mature, 45- to 46-kDa protein. Protein sequencing demonstrates that the N-terminal region of the 82-kDa prolipase, comprising 295 amino acids, is cleaved from the central and C-terminal moieties, which contain the active site. A metalloenzyme protease is probably responsible for initiating this processing. The extremely hydrophobic, mature lipase is resistant to further protease degradation and retains the full catalytic activity of the prolipase.

Staphylococci are known to produce extracellular lipases (1,13-15). Esterase activity toward water-soluble substrates is expressed by nearly all strains, while high levels of true lipase activity (E.C. 3.1.1.3 class) for water-insoluble substrates are less common (13). The function of lipases is not fully understood, but they may be important for bacterial nutrition (4). They may also constitute a virulence factor: interaction with human leukocytes has been demonstrated (16), and free fatty acids, the end products of lipolytic activity, are known to impair several immune system functions (2,3,5).

Staphylococcal lipases have been purified (1,14), and the chromosomal *Staphylococcus aureus* (10,11) and *Staphylococcus hyicus* (6) lipase genes have been sequenced. The lipase gene from *S. aureus* encodes a protein with a calculated molecular mass of 76 kDa (including the signal peptide). However, purification of extracellular lipase has consistently yielded an enzyme of 45 to 46 kDa (1,15). Processing of the 82-kDa staphylococcal prolipase is therefore hypothesized to occur. The objective of this study was to characterize the in vivo processing of staphylococcal lipase.

MATERIALS AND METHODS

Bacterial strains. TEN 5, an *S. aureus* strain isolated from a patient suffering from toxic epidermal necrolysis and originally collected by C. G. Gemmell, Dublin, Ireland, was used throughout the study. *Escherichia coli* KSI 210, containing the structural gene for *S. aureus* lipase (stageh gene) cloned onto the pL150 vector, was kindly provided by John Iandolo, College of Veterinary Medicine, Manhattan, Kans.

**Sequencing.** Proteins were sequenced on an Applied Biosystems model 430A automated peptide synthesizer. Resin-bound peptides were cleaved and deprotected with anhydrous hydrofluoric acid, extracted with an appropriate solvent, and lyophilized. Standard reagents and conditions were employed. Based on the known amino acid sequence, a peptide representing the N-terminal region of the protein was synthesized and used for antibody production.

**Antibodies.** For Western blots (immunoblots), polyclonal rabbit antibodies raised against the TEN 5 lipase were used (15). Polyclonal rabbit antibodies against synthetic peptides were produced by Calico Biologicals, Inc. (Reamstown, Pa.). Alkaline phosphatase-conjugated antibodies were purchased from Sigma Chemical Co. (St. Louis, Mo.), and polyvinylidene difluoride membranes were from Millipore Corp. (Bedford, Mass.).

**Activity analysis.** Both for activity gels (see below) and for Western blots, samples were prepared by boiling in sodium dodecyl sulfate (SDS) with mercaptoethanol and loaded onto a 4% stacking gel and a 10% SDS-polyacrylamide gel. Western blots were performed according to standard protocols, using the alkaline phosphatase system. For activity analysis, the SDS-polyacrylamide gels were washed for 20 min each in 2.5% Triton X-100 in water, 2.5% Triton X-100 in phosphate-buffered saline (PBS), and finally pure PBS to remove SDS. The gels were then stained with Coomassie brilliant blue (Sigma) for 20 min and then washed again in PBS. Meanwhile, 1.5% tributyrin (Sigma), 0.1% gum arabic, and 100 μg of streptomycin per ml were added to melted L agar. This agar mixture was poured into petri dishes, the gels were laid onto the solidified surface, and a second layer of agar was added to embed the gels. Lipase activity was demonstrable as clearing in the opaque agar. High activity could be detected after a few hours, but clearing continued over several days. A similar system was used for analyzing enzymatic activity toward olive oil. The tributyrin was replaced by 1.5% pure olive oil (Sigma), and lipase activity was shown as bands of deposits. Using this method, the lipase activity of individual bands migrating in an SDS-polyacrylamide gel could be visualized directly, rapidly, and reproducibly.

**Lipase activity in broth cultures** was kindly measured by Mark Lowe, Washington University School of Medicine, St. Louis, Mo., using a method based on radiolabeled triolein.

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substrates, emulsified by sonication. A liquid-liquid partition system was used for isolation of released $^3$H-labeled free fatty acids, and the radioactivity was measured in a liquid scintillation counter.

The protease inhibitors used were all purchased from Boehringer Mannheim Biochemicals (Indianapolis, Ind.).

RESULTS

The lipase gene, sequenced by Lee and Iandolo (10, 11), encodes a 76-kDa preproprotein. Henceforth, we will denote the protein including signal peptide as preprolipase and call it prolipase after the signal peptide has been cleaved. When E. coli KSI 210 was transformed with a pl150 vector carrying the lipase gene, small amounts of an 82-kDa protein were found in lysed whole cells and in the periplasm by Western blots with a polyclonal anti-S. aureus lipase antibody (data not shown). This 82-kDa band probably represents the prolipase with a calculated molecular mass of 73 kDa, which migrates more slowly through the gel because of its physicochemical properties.

The lipase produced by E. coli was not secreted into the medium, and no processing of the protein to its mature form of 45 to 46 kDa could be observed.

A 12-amino-acid N-terminal sequence, from a 45- to 46-kDa lipase purified from S. aureus culture supernatants as described elsewhere (14), was determined. The sequence corresponded precisely to amino acids 296 through 314 of the sequence published by Lee and Iandolo (10, 11) (Fig. 1). The molecular mass of a protein extending from the cleavage site to the C terminus would be calculated to be 44 kDa, in accordance with the mass of the purified staphylococcal lipase. However, the apparent mass of the mature lipase was somewhat larger (45 to 46 kDa) because of its reduced mobility through polyacrylamide gels (in parallel to the prolipase). The mass of the remaining N-terminal region of the protein would be calculated to be 32 kDa.

Since the 45- to 46-kDa lipase purified from staphylococcal culture supernatants has a strong lipolytic activity, this part of the gene product should contain the active site. Analysis of the known amino acid sequence readily identified this site. A serine located in position 412 is the center of the typical lipase motif of Gly-X$_1$-Ser-X$_2$-Gly (X$_1$ is most often a histidine or tyrosine, and X$_2$ is a methionine, leucine, or glutamine) (Fig. 1), identical or similar to that found in several other bacterial, fungal (8), and even mammalian (12) lipases.

When S. aureus was cultured in broth at 37°C, an extracellular protein of approximately 82 kDa could be detected in sterile supernatant after 6 h. The supernatants were analyzed in Western blots, using an anti-S. aureus lipase antiserum. The amount of 82-kDa protein present reached its maximum after 8 h, at which time the 45- to 46-kDa protein could also be detected. With prolonged incubation in both whole-cell-containing and filter-sterilized supernatants, a spontaneous degradation of the larger protein occurred, finally yielding only the mature lipase of 45 to 46 kDa and trace amounts of the 82-kDa protein (Fig. 2A). A diffuse and broad band between the prolipase and the mature lipase was consistently present for a transient period. This region both reacted with the antibodies and demonstrated lipase activity, suggesting that randomly generated intermediate-length proteins are produced during processing of the prolipase.

Antisera were raised against a synthetic peptide derived from the N-terminal region of the known amino acid sequence. These antisera failed to detect a conceivable 32-kDa N-terminal peptide portion of the processed lipase in supernatants, cell walls, or lysed whole cells (data not shown). An assay was developed to monitor lipolytic activity of proteins separated on SDS-polyacrylamide gels (see Materials and Methods). With this method, it could be demonstrated that the 82-kDa protein had no less lipolytic activity than did the mature 45- to 46-kDa protein (Fig. 3). This was true for both tributylin and olive oil as substrates. The incompletely processed proteins in the range between 82 and 45 to 46 kDa also demonstrated activity.

To characterize the processing of prolipase, we added different protease inhibitors to growing bacteria. With the addition of E 64, a cysteine protease inhibitor, at a final concentration of 10 μM, a marked shift toward retention of the 82-kDa protein could be seen (Fig. 2B), even after incubations of more than 24 h. As shown in Fig. 2B, a shift was observed in both cell-free supernatants and whole-cell preparations. A similar shift was noted when the metalloprotease inhibitor phosphoramidon was added at a concentra-
The proline could theoretically be split in the bacterial cell, on the cell surface, or outside the cell by secreted proteases. To investigate this further, we also studied the effect of protease inhibitors in a cell-free system. Filter-sterilized supernatants from pure broth cultures were harvested after 8 h of incubation, at which time a significant amount of the 82-kDa protein was still present. A control aliquot was immediately frozen at −20°C, and other aliquots were incubated at 37°C overnight both with and without protease inhibitors. For aliquots without protease inhibitors, both Western blots and activity plates showed a processing of the 82-kDa protein to the mature 45- to 46-kDa protein. In the frozen sample, the 82-kDa protein remained intact. Protease inhibitors had the same effect as they did in growing bacterial cultures, i.e., E 64 and phosphoramidon inhibited the processing markedly, while PMSF mainly affected the distribution of intermediate products. The same pattern was found when the activity of these aliquots was analyzed in tributyrin plates. The frozen aliquot, containing mainly the 82-kDa protein, and samples in which the lipase was processed to the mature 43-kDa protein showed identical lipolytic activity toward triolein.

**FIG. 3.** Lipolytic activity of lipase from cultures grown with E. coli (10 μM final concentration) and pure L broth. The bands were separated on an SDS-polyacrylamide gel, washed as indicated in the text, and then baked into agar containing 1.5% tributyrin. Purified 46- to 46-kDa lipase served as a control. When grown with E. coli, the main lipase activity is seen to correspond to the 82-kDa proline, while the mature 45- to 46-kDa lipase dominates when cells are grown in L broth without addition of the protease inhibitor. A lesser region of tributyrin clearing is seen between the 82- and 45- to 46-kDa proteins, indicating that the incompletely processed lipase retains lipolytic activity.

**DISCUSSION**

When the _S. aureus_ lipase gene was cloned into _E. coli_, a protein corresponding in size to the full-length gene sequence was produced. The protein could be found in whole-cell lysates and in the periplasm but was not secreted. Based on the amino acid sequence, a protein migrating at 76 kDa (or somewhat smaller without the signal peptide) would be expected. Consistently, the lipase produced was found to migrate more slowly than that, a fact previously observed for other lipases (6). The combination of lipophilic and hydrophobic regions in the protein may account for its reduced electrophoretic mobility. Probably, the signal peptide is cleaved in _E. coli_, splitting the prolipase to lipolipase. However, no further processing of prolipase to mature lipase takes place in _E. coli_, indicating that the protein does not have an autocatalytic activity and that _E. coli_ lacks the proteases required to initiate or complete processing of the staphylococcal prolipase. In _staphyloccoci_, the protein is secreted as an approximately 82-kDa prolipase but is then processed in a stepwise fashion. The processing takes place outside the bacteria since it can be demonstrated in filter-sterilized supernatants. The prolipase has full lipolytic activity. We do not intermediate, incompletely processed products. With time, virtually no 82-kDa prolipase is found, leaving only the mature 45- to 46-kDa lipase in the supernatant.

Our studies with protease inhibitors indicate that a cysteine protease, possibly a metalloprotease protease, is necessary for initiation of the processing. Since PMSF and leupeptin also affect the pattern of partially processed lipase, more than one type of protease may contribute to further degradation. Antiserum raised against the mature protein would not be expected to identify the N-terminal region of the protein, but antisera raised against a synthetic peptide from the N-terminal part also failed to demonstrate a 32-kDa degradation product. This agrees with the presence of intermediate, incompletely processed proteins demonstrated by Western blot and on activity plates. We therefore hypothesize that the enzyme is processed in a stepwise manner. The prolipase has full lipolytic activity against both short- and long-chain fatty acid substrates, so it is unlikely that the processing represents the cleavage of an inactive prolipase to an active form.

Why do the bacteria elaborate a large N-terminal region of the lipase which is not necessary for lipolytic activity and which is ultimately degraded? Lipases are extremely hydrophobic proteins. The N-terminal region of the prolipase could theoretically be necessary to allow secretion of the enzyme across the cell membrane and cell wall. A system for protein secretion involving proteolysis has been described in gram-negative bacteria; a beta-domain of the _Neisseria_ immunoglobulin A protease is necessary for its transport across the outer membrane (7). The _Neisseria_ immunoglobulin A protease is processed by autocleavage, in contrast to the staphyloccoccal lipase.

Supporting the hypothesis of a similar system in _staphylococci_, a hydrophilicity plot based on the amino acid sequence (9) shows that the N-terminal end of the prolipase, to approximately the cleavage site at amino acid 295, is markedly hydrophilic. This is also true for the known sequence of _S. hyicus_ (6), in which the N-terminal region is markedly more hydrophilic than are the central and C-terminal regions.

To purify _S. aureus_ lipases, culture supernatants have typically been harvested after 8 h of growth, at which time a large amount of the prolipase should remain. Still, the
purification procedures based on affinity chromatography consistently yield the mature 45- to 46-kDa protein only. If the prolipase is more hydrophilic, this result can be fully understood, since the prolipase would not bind as strongly to the hydrophobic octyl-Sepharose as would the mature lipase.

Alignments between the lipase amino acid sequences from S. aureus and S. hyicus show significant homology in the portion corresponding to the mature 45- to 46-kDa protein, while the N-terminal regions seem less conserved (11). Sequence analyses by polymerase chain reaction amplification, using oligonucleotides derived from different parts of the nucleotide sequence, also indicate greater dissimilarity in the N-terminal end of the gene than in the central and C-terminal regions among different S. aureus strains (unpublished data). Amino acid composition analysis also demonstrates considerable differences among various staphylococcal species and strains (6, 15). This variation could be assumed to occur in the N-terminal region of the enzyme, since the central and C-terminal portions are conserved. If the N-terminal part is only necessary for secretion, no strong selection against variation would be expected, provided that it is hydrophobic.

On the other hand, the stepwise degradation of the prolipase, probably mediated by several different proteases, is not typical of a specific secretory mechanism. The possibility that the processing represents a fortuitous event in the protease-rich supernatant must therefore be considered.

In conclusion, we have shown that the S. aureus lipase is secreted as an 82-kDa prolipase, which is subsequently processed outside the bacteria. The protein, with an apparent molecular size of 82 kDa, most probably represents the full gene product (calculated to be 73 kDa), which is slowed in gels because of its physicochemical properties. A metalloenzyme protease seems to be responsible for the initial processing, while several different proteases may contribute to the further stepwise degradation. The 82-kDa protein, as well as the intermediate products which result from this successive processing, has full lipolytic activity against both short- and long-chain substrates. Because it has no role in enzymatic function, considerable variation is allowed in the ultimately degraded N-terminal region of the protein. The hydrophilic N-terminal portion of the prolipase may be necessary for transport of this extremely hydrophobic protein across the cell membrane and cell wall, but the processing may also be the result of unspecific proteolytic activity in the supernatant.

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REFERENCES


