Identification of the Active Site Residues of *Pseudomonas aeruginosa* Protease IV

**IMPORTANCE OF ENZYME ACTIVITY IN AUTOPROCESSING AND ACTIVATION***

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Protease IV is a lysine-specific endoprotease produced by *Pseudomonas aeruginosa* whose activity has been correlated with corneal virulence. Comparison of the protease IV amino acid sequence to other bacterial proteases suggested that amino acids His-72, Asp-122, and Ser-198 could form a catalytic triad that is critical for protease IV activity. To test this possibility, site-directed mutations by alanine substitution were introduced into six selected residues including the predicted triad and identical residues located close to the triad. Mutations at any of the amino acids of the predicted catalytic triad or Ser-197 caused a loss of enzymatic activity and absence of the mature form of protease IV. In contrast, mutations at His-116 or Ser-200 resulted in normal processing into the enzymatically active mature form. A purified proenzyme that accumulated in the His-72 mutant was shown in vitro to be susceptible to cleavage by protease IV purified from *P. aeruginosa*. Furthermore, similarities of protease IV to the lysine-specific endoprotease of *Achromobacter lyticus* suggested three possible disulfide bonds in protease IV. These results identify the catalytic triad of protease IV, demonstrate that autodigestion is essential for the processing of protease IV into a mature protease, and predict sites essential to enzyme conformation.

*Pseudomonas aeruginosa* is a major opportunistic pathogen that is often associated with burns, cancers, cystic fibrosis, and immune deficiency diseases (1–6). *P. aeruginosa* is also well recognized as the most important Gram-negative bacterium causing severe keratitis, especially in contact lens wearers (7–9). This keratitis progresses rapidly and may result in permanent loss of vision (10, 11). The ability of *P. aeruginosa* to produce several extracellular enzymes, such as elastase (LasB), pseudolysin, alkaline protease (aeruginolysin), LasA (staphylolysin), and LasD (staphyloolysin), is considered to be important in its pathogenicity. Protease IV (lysyl endopeptidase; EC 3.4.24.26) has been demonstrated to correlate with corneal virulence. Protease IV has also recently been identified as the iron-regulated protein PrpL (12). Protease IV is a lysine-specific protease with molecular mass of 26 kDa that has been identified in culture supernatants of *P. aeruginosa* (13–15). Protease IV was first characterized in *P. aeruginosa* strain PA103-29, a strain reported to be deficient in alkaline protease, elastase A, and elastase B production (16). Recently, the nucleotide sequence of the protease IV gene from *P. aeruginosa* PA103-29 was determined and found to correspond to a sequence between base pairs 4,671,318 and 4,672,706 of the *P. aeruginosa* PAO1 chromosome (The *Pseudomonas* Genome Project identifies this gene as PA4175) (1). The protease IV gene is located in a 1388-bp open reading frame, encoding a protein of 463 amino acids (17). Similar to elastase B, this protein consists of three domains: signal sequence, propeptide domain, and mature protease. Protease IV is synthesized as a pre-proenzyme that is processed intracellularly and secreted into the extracellular milieu as the mature protease (33).

The 26-kDa mature protease is a unique extracellular enzyme that specifically cleaves substrates on the carboxyl side of lysine residues as determined by substrate susceptibility tests. Protease IV is also capable of degrading a variety of host defense proteins including fibrinogen, plasminogen, immunoglobulin G, and the complement proteins C1q and C3. The enzyme can be completely inhibited by a protease inhibitor, tosyl-lysyl-chloromethylk ethylene (13). Protease IV has been implicated as a virulence factor that contributes to the pathogenicity of *Pseudomonas keratitis* (13, 14, 18). Purified protease IV (50–200 ng) induced corneal epithelial damage within 3 h after injection into the corneal stroma and increased the virulence of protease IV-deficient bacteria (19). Protease IV may be involved in the processing mechanism of elastase A (LasA), because it has been reported that a lysine-specific protease of *P. aeruginosa* converts the LasA proenzyme to the active enzyme (20).

The protease IV gene has been cloned and expressed in *Pseudomonas putida* providing a more abundant supply of protease than that obtained from the naturally expressed chromosomal *P. aeruginosa* gene (33). Expression of protease IV in *P. putida* revealed a protein of 48 kDa containing the three domains of protease IV: a signal sequence, a propeptide, and a mature protease. Also detected was a 45-kDa protein (proenzyme) that contained the propeptide and mature protease domains but not the signal peptide. In an experimental model of keratitis, expression of protease IV by *P. putida* caused a 3-fold increase in the clinical score of infected eyes relative to eyes infected with *P. putida* lacking the protease IV gene (33).

The present study was undertaken to study the sequence of protease IV relative to other bacterial proteases and to determine the amino acid residues of protease IV responsible for the

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AY062882.

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enzymatic activity. Included in this study is the synthesis and testing of multiple site-directed mutants involving residues proposed to comprise the catalytic site of protease IV.

EXPERIMENTAL PROCEDURES

Bacterial Strains, Plasmids, and Growth Conditions—Escherichia coli strain DH5α was grown at 37 °C in LB broth (1% tryptone, 0.5% yeast extract, and 0.5% NaCl, pH 7.0). When appropriate, carbenicillin (100 μg/ml) was incorporated into the medium for plasmid selection. P. aeruginosa strain PA103-29 was cultivated in tryptic soy broth (Difco, Detroit, MI) or M9 minimal medium containing 50 μM monosodium glutamate, 1 mM MgSO4, and 1% glycerol. Growth of bacterial cultures was performed at 37 °C as described previously (13). E. coli strain DH5α was purchased from Invitrogen. P. aeruginosa strain PA103-29 was kindly provided by Dr. P. V. Vihbs, East Carolina University, Greenville, NC. Strain PA103-29 was originally described by Dr. D. E. Ohman (16). The plasmid pUCP20 was obtained from Dr. J. A. Hobden, Wayne State University, Detroit, MI and originally described by Dr. S. E. West (21).

Site-directed Mutagenesis—Site-directed mutagenesis was accomplished by the overlap extension method using PCR (22). The protease IV gene was obtained by amplifying the structural gene from the chromosome of P. aeruginosa strain PA103-29 using the GC-rich PCR system (Roche Molecular Biochemicals) under the following conditions: 3 min at 95 °C, followed by 30 cycles of 30 s at 95 °C for denaturing, 30 s at 60 °C for annealing, and 1 min at 72 °C for extension and ending with an incubation at 72 °C for 7 min. For generating mutations in the protease IV gene, two pairs of primers were used to direct the synthesis of two fragments, each inclusive of an overlapping region containing a replacement of His-72, His-116, Asp-122, Ser-197, Ser-198, or Ser-200 with an Ala residue (Table I). The two PCR products, with an overlap of 27 base pairs at each end of fragment, were then combined for the second round of PCR designed to amplify the entire protease IV gene. Oligonucleotide primers were designed to amplify the protease IV gene with recognition sites for the restriction enzyme EcoRI at the locus coding for the N-terminal amino acids and the restriction enzyme BamHI site at the locus coding for the C-terminal amino acids. A 1388-base pair fragment of the PCR products was ligated into a PCR cloning vector (TOPO TA cloning; Invitrogen) and transformed into chemically competent TOP10 E. coli as described by the manufacturer. Plasmid DNA was purified from transformants, and the protease IV gene was excised from the cloning vector by EcoRI/BamHI restriction digestion. The EcoRI/BamHI DNA fragment containing the protease IV gene was subcloned into pUCP20 and transformed into electroporment E. coli DH5α. The resulting plasmids with the protease IV gene and with a replacement of His-72, His-116, Asp-122, Ser-197, Ser-198, or Ser-200 were designated pIVL, pH72, PH116, pD122, pS197, pS198, and pS200, respectively. These plasmids were sequenced to verify the presence of correct mutations.

Purification of plasmid DNA was performed by the alkaline lysis method using a QiAprep Spin Miniprep kit (Qiagen Inc., Valencia, CA). Large-scale plasmid preparations were performed by Qiagen Plasmid Midi kit (Qiagen). Restriction enzymes and T4 DNA ligase were purchased from New England Biolabs, Inc. (Beverly, MA).

Purification of Protease IV—For purification of the intracellular wild-type and mutant protease IV, plasmid-containing E. coli was grown at 37 °C in 2 liters of LB medium. Cells were pelleted, washed three times, and resuspended in 50 ml of 10 mM Tris-HCl, pH 7.0. Cells were ruptured by sonication (Branson Sonifier 250; Branson Ultrasonics Corp., Danbury, CT). Intact cells were removed by centrifugation at 8,000 × g for 20 min at 4 °C. The extracts were dialyzed overnight against 10 mM ammonium acetate buffer, pH 6.4. The dialyzed supernatant was applied to a CM cation exchange column (Bio-Rad, Hercules, CA) and was washed with 10 mM ammonium acetate buffer, pH 6.4. A pH gradient, generated by mixing 10 mM ammonium acetate buffer at pH 9.0 with 10 mM ammonium acetate buffer at pH 6.4, was used to elute the protein. The eluted fractions containing protease IV were assayed by Western blotting with rabbit polyclonal antiserum prepared against a recombinant form of mature protease IV (33). The reactive fractions were pooled and then concentrated to 1.0 ml using a stirred ultrafiltration cell with a 10-kDa cutoff filter membrane (YM10; Amicon Inc., Beverly, MA) and loaded onto a Sephacyr S-300 molecular sieve matrix (Amersham Biosciences). The fractions were eluted with 10 mM Tris-HCl, pH 7.0, and assayed by immunoblotting. Positive fractions were pooled and concentrated to 1.0 ml. Total protein was determined using the biochiconic acid assay (Sigma). The purity of the sample was determined by 12% SDS-PAGE and visualized by silver staining (Bio-Rad). The purification of extracellular protease IV was performed using the method of Engel et al. (13), as modified by Traidej et al. (33).

Colorimetric Substrate Assay for Protease IV Activity—Protease IV activity was determined by the hydrolysis of the chromogenic substrate, Chromozym PL (toxyl-Gly-Pro-Lys-p-nitroanilide; Sigma), as described by O’Callaghan et al. (14). End point reactions were incubated at 37 °C for 30 min or as stated in each experiment. Kinetic analysis was performed at 25 °C by reading the optical density every 2 min for 30 min to measure the increase in optical density per min (ΔA/min). One unit of activity was defined as the amount of enzyme that caused an optical density increase at 410 nm of 1 A/min under the assay conditions.

SDS-PAGE, Immunoblotting, and N-terminal Sequence Analysis—Proteins were separated by 12% SDS-PAGE under reducing conditions and electrobotted by standard laboratory methods (23). Rabbit polyclonal antiserum prepared against a recombinant form of mature protease IV was the primary antibody, and detection of immune complexes was done with ECL Western blotting detection reagents (Amersham Biosciences).

For N-terminal sequence analysis, proteins were electrotransferred from SDS-polyacrylamide gel to Sequi-blot polyvinylidene difluoride membrane (Bio-Rad). N-terminal amino acids were determined by Core Laboratories, Louisiana State University Health Sciences Center, New Orleans, LA.

RESULTS

Comparison of Amino Acid Sequences of Pseudomonas Protease IV with Other Serine Proteases—The amino acid sequence of mature protease IV was subjected to a Blastp search against the Microbial Genomes database (www.ncbi.nlm.nih.gov/BLAST/) for related proteins. The three proteases found to be related to protease IV were endopeptidase Arg-C (44% identity) of Lysobacter enzymogenes, lysyl endopeptidase (31% identity) of L. enzymogenes, and Achromobacter protease I (30% identity) of Achromobacter lyticus (Fig. 1). These three proteins are classified as serine proteases and, like protease IV, cleave substrates containing a lysine residue (12, 23–26). Both protease IV and these related proteases are synthesized as proenzymes that are subsequently processed to mature extraacellular forms (24, 27, 29).

The active site of Achromobacter protease I, like that of trypsin and chymotrypsin (30), is described as a catalytic triad.
The Active Site Residues of Pseudomonas Protease IV

consisting of histidine, aspartic acid, and serine (31). The active site sequences of these proteases are similar to sequences in the mature protease IV. Based on these sequence similarities, the catalytic triad of protease IV is suggested to be comprised of His-72, Asp-122, and Ser-198. In addition, six cysteines of the protease IV are also aligned with those of the other related proteases. Based on similarities to the A. lyticus protease I positioning of disulfide bonds, the disulfide bonds present in protease IV are anticipated to align as indicated in Fig. 1.

Fig. 1. Alignment of the amino acid sequence of the mature protease IV of *P. aeruginosa* with other serine proteases. Amino acids identical to *Pseudomonas* protease IV (PIV) are boxed and shaded lightly. His-72, Asp-122, and Ser-198 of protease IV, corresponding to His, Asp, and Ser of other serine proteases, are proposed as components of the protease IV catalytic triad and are in bold and in boxes. The six cysteines are in bold and in dotted boxes. The cysteines for possible sites of three disulfide bonds are labeled with *, ‡, or † above each of the paired cysteine residues. Arg-C is the designation for endoprotease Arg-C of *L. enzymogenes*, Lys is for lysyl endopeptidase of *L. enzymogenes*, and AP-1 is for *Achromobacter* protease I of *A. lyticus*.

Effect of Active Site Mutations on Enzymatic Activity—To analyze the protease IV enzyme in terms of the amino acid residues comprising its active site, site-directed mutations were introduced into cloned copies of the protease IV gene for each of the following amino acids: His-72, His-116, Asp-122, Ser-197, Ser-198, and Ser-200. The amino acids (histidine and serine, as well as aspartic acid) were substituted with alanine to eliminate functional groups from the enzyme at the selected positions. Successful mutation of the protease IV gene at each chosen site was verified by DNA sequencing (data not shown).

To examine the effect of active site mutations on the enzymatic activity of protease IV, culture supernatants of *E. coli* expressing wild-type and mutated forms of protease IV were analyzed for their ability to hydrolyze a lysine-containing substrate, Chromomyl PL. *E. coli* expressing wild-type protease IV possessed high proteolytic activity as demonstrated by chromogenic substrate cleavage (Table II). Mutants with protease IV sequence substitutions of alanine at residues His-116 or Ser-200 showed similar proteolytic activity comparable with that of *E. coli* expressing wild-type protease IV. In contrast, mutants with an alanine substitution at His-72, Asp-122, Ser-197, or Ser-198 displayed an insignificant amount of proteolytic activity. These findings suggest that the proposed catalytic triad constituents (His-72, Asp-122, and Ser-198), as well as Ser-197, are essential for the proteolytic activity of protease IV.

Effect of Active Site Mutations on the Protease IV Processing Mechanism—To determine the effect of alanine substitutions on protease IV processing, the presence of wild-type and mutated protease IV gene products in cell lysates was examined by immunoblotting using rabbit anti-mature protease IV antibodies. For the wild-type protease IV gene expressed in *E. coli*, the findings were similar to those detected previously for protease IV expression in *P. putida*; that is, the mature protease (26 kDa), as well as intracellular proteins of 48 and 45 kDa, were present (Fig. 2) (33). The 48-kDa protein has been reported to be the full-length protease IV gene product (pre-proenzyme), and the 45-kDa protein is a proenzyme inclusive of the propeptide and the 26-kDa mature protease domains but lacking the signal sequence (33). Unlike the wild-type protease IV, each mutated protease IV gene product with an alanine substitution at an amino acid proposed to be part of the catalytic triad (His-72, Asp-122, or Ser-198) was not processed intracellularly to the 26-kDa mature form but instead remained in the 48-kDa pre-proenzyme and 45-kDa protein is a proenzyme inclusive of the propeptide and the 26-kDa mature protease domains. Percent activity was calculated as described in the text. Specific activity assays were performed in triplicate.

![FIG. 2. Detection of wild-type and mutated protease IV gene product expressed in transformed *E. coli*.](http://www.jbc.org/)

<table>
<thead>
<tr>
<th>Culture supernatants</th>
<th>Protease IV</th>
<th>Specific activity</th>
<th>Relative activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>PIV (wild-type)</td>
<td>128.6</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>Vector alone</td>
<td>&lt;1.0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>&lt;0.8&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>His-72</td>
<td>&lt;1.0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>&lt;0.8&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>His-116</td>
<td>115.3</td>
<td>91.1</td>
<td></td>
</tr>
<tr>
<td>Asp-122</td>
<td>&lt;1.0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>&lt;0.8&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Ser-197</td>
<td>&lt;1.0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>&lt;0.8&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Ser-198</td>
<td>&lt;1.0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>&lt;0.8&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Ser-200</td>
<td>121.8</td>
<td>96.2</td>
<td></td>
</tr>
</tbody>
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<sup>a</sup> Significantly different from *E. coli*/pPIV (*p* < 0.0001).

![TABLE II](http://www.jbc.org/)
not only essential for proteolytic activation of protease IV but also play a crucial role in autoprocessing of the proenzyme.

Characterization of Alanine-substituted Protease IV—To further characterize the alanine-substituted protease IV mutations, *E. coli* carrying pH72, which lacked protease activity, and *E. coli* harboring pH116, whose protease activity was retained, were used as sources for purification of intracellular protease IV. *E. coli* carrying pH72 contained a protein of 45 kDa whereas cells carrying pH116 contained a protein of 26 kDa (Fig. 3). The 26-kDa protein of pH116 had an N-terminal amino acid sequence of AGYRDGFGAS, which was identical to that of the mature protease IV of *P. aeruginosa*. Furthermore, the N-terminal amino acid sequence obtained from the purified 45-kDa protein of pH72 was APGASE, matching the amino acid sequence of protease IV beginning at position 25 of the full-length protease IV. The APGASE sequence suggests that the cleavage site for signal peptidase is located between amino acid residues 24 and 25. The purified proteins of 45 and 26 kDa were analyzed for protease activity by the Chromozym PL assay, and only the 26-kDa mature protease was enzymatically active (data not shown).

In Vitro Processing of Protease IV—There was a possibility that mutations at His-72, Asp-122, Ser-197, or Ser-198 caused a conformational change in the proenzyme form of protease IV that rendered it resistant to proteolytic processing from the proenzyme form to the mature 26-kDa form. To test this possibility, the 45-kDa proenzyme of the His-72 mutant was purified and then incubated *in vitro* for 1 h with the 26-kDa mature protease IV (0.1 μg) purified from *P. aeruginosa* PA103-29 culture supernatants. The samples were analyzed by immunoblotting, and the proteolytic activity was determined by using the Chromozym PL substrate.

Incubation of the His-72 proenzyme alone did not induce protein processing into the mature protease form as evidenced by the 45-kDa band that was present after incubation (Fig. 4). Incubation of the His-72 proenzyme in the presence of active protease IV resulted in the disappearance of the 45-kDa proenzyme. Furthermore, incubation of the His-72 proenzyme with heat-inactivated protease IV failed to cause the disappearance of the His-72 45-kDa protein. The liberation of the His-72 mature protease domain (with alanine substituted for histidine at position 72) caused no increase in the total protease IV activity (data not shown). These results on the His-72 mutant suggest that the 45-kDa proenzyme with an alanine substitution was not resistant to the proteolytic cleavage that naturally occurs in the production of the mature protease IV domain. The ability of this proenzyme to be cleaved into the 26-kDa protein lacking proteolytic activity is consistent with the proposal that the mutated amino acid is part of the active site of protease IV.

**DISCUSSION**

This study determined sequence similarities between protease IV and other bacterial proteases specific for lysine residues and, based on sequence similarities, predicted three amino acids, histidine 72, aspartic acid 122, and serine 198, that likely comprise the catalytic triad of protease IV. This prediction was supported by site-directed mutagenesis that demonstrated a dramatic loss of enzymatic activity resulting from mutation in any of these three residues. These results also revealed that the junction of amino acids 24–25 is the site of cleavage by a signal peptidase that removes the signal sequence from the full-length pre-proenzyme, leaving the proenzyme intact. The results also demonstrate that protease IV undergoes autodigestion to liberate the mature protease from the 45-kDa proenzyme containing both the propeptide and mature protease domains.

The protease IV gene mutated at any of the proposed active site amino acids produced both the pre-proenzyme (48 kDa) and the proenzyme (45 kDa) that were not processed to the mature protease (26 kDa). Pre-proenzyme and proenzyme were produced in cells with the wild-type protease IV gene, but these native proteins, unlike the mutant proteins, were processed naturally into the enzymatically active protease IV. The failure of the mutated proenzymes to be processed into mature proteases is apparently because of their loss of the functional catalytic site and not to the inaccessibility of the lysine cleavage site to the mature protease IV. Protease IV purified from *P. aeruginosa* appeared to be capable of digesting the mutated proenzyme into a 26-kDa protein that, unlike wild-type mature protease, lacked enzyme activity because of its alanine substitution at position 72. These results demonstrate that a mutation at a single amino acid needed for the proposed catalytic site did not cause the proenzyme to misfold into an unfavorable conformation for proteolytic cleavage at the position between the propeptide and mature protease domains. These findings...
inferred that the natural separation of the propeptide from the mature protease is a result of autoprocessing involving the cleavage of the 45-kDa proenzyme at the lysine residue present at the junction between the propeptide and the mature protease domains. The significant role of the proposed catalytic triad of protease IV in proteolytic activity is in agreement with findings for related serine proteases (24, 30, 31).

In addition to the catalytic triad, serine at position 197 of protease IV also showed a crucial role in processing and enzyme activity. This amino acid is located adjacent to Ser-198, a constituent of the proposed catalytic triad. To date, the three-dimensional structure of protease IV has not been resolved. Our prediction that the catalytic triad of protease IV consisting of His-72, Asp-192, and Ser-198 was originally based on an alignment of the primary structure of protease IV with other lysine-specific endoproteases. A possibility is that Ser-197 could act as a component of the catalytic triad instead of Ser-198. However, when compared with the mammalian serine proteases (trypsin and chymotrypsin), Ser-197 and Ser-198 of protease IV aligned with Asp-194 and Ser-195 of trypsin and chymotrypsin, respectively. X-ray crystallography revealed that Asp-194 of trypsin has an important role in the structural conformation whereas Ser-195 was part of the catalytic triad of trypsin and chymotrypsin (28, 32). The Asp-194 residue forms a salt bridge with Ile-16 near the N terminus that helps establish the proper conformation for the enzymatic activity of trypsin and chymotrypsin. Ser-197 of protease IV, like Asp-194 of trypsin and chymotrypsin, could be responsible for the proper folding of protease IV. Therefore, an amino acid substitution at Ser-197 could result in a structural change that would alter the folding of the tertiary structure of protease IV into the correct active form and could prevent enzymatic activity.

Based on the similarity between protease IV and other bacterial serine proteases, speculation can be offered regarding the disulfide bonds in protease IV. The positions of all six cysteines of protease IV are aligned to cysteines in the amino acid sequences of other related proteases. Critical to the structure of *Achromobacter* protease I is the disulfide bond that forms between the most distant cysteines (i.e., Cys-6 and Cys-216) (24). Protease IV has cysteins in similar positions (Cys-13 and Cys-220) that could function to stabilize protease IV in an enzymatically active form. This possibility is consistent with the sensitivity of protease IV activity to the reducing agent, β-mercaptoethanol (13).

In summary, we have determined the essential amino acid residues of protease IV that affect the catalytic activity. The processing of protease IV from a 45-kDa proenzyme to the secreted mature protease IV was dependent upon the catalytic activity of protease IV, demonstrating that protease IV undergoes autoprocessing in the *P. putida* system presented here. The processing of protease IV could be augmented by other proteases in a *P. aeruginosa* system. We have also identified the cleavage site for the signal peptidase that converts the 48-kDa pre-proenzyme to the 45-kDa proenzyme. The determination of the three-dimensional structure of protease IV is still needed to gain a better understanding of the structure/function relationship of protease IV.

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REFERENCES

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