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Isolation and Properties of the Protease from the Wild-Type and Mutant Strains of *Pseudomonas fragi*

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A simplified procedure for the purification of the extracellular protease of *Pseudomonas fragi* was developed. The enzyme was isolated from a derepressed mutant producing 40 times the enzyme level of the parental organism. It was collected from culture filtrates by ammonium sulfate precipitation, and it was obtained in pure form by single chromatography on a column of diethylaminoethyl cellulose. The protease had a molecular weight of 52,000 as estimated by sodium dodecyl sulfate-gel electrophoresis and had properties of a classical neutral endopeptidase with the exception of its substrate specificity. Mutants of *P. fragi* producing proteases of altered substrate specificities were isolated from plates containing elastin as the sole carbon source. The SP-Sephadex elution patterns of enzymes extracted from each mutant examined were complex, suggesting that either the enzyme was autodigested or several active forms could be generated from a common precursor. The substrate specificities of the mutant enzymes were different from that produced by the parental strain.

Numerous extracellular enzymes of microbial origin have been isolated and characterized. In general, these enzymes are stable, produced in relatively large quantities, and easily purified. Of the extracellular proteins investigated, the proteolytic enzymes have received considerable attention and their properties have significantly contributed to elucidate the relationship between the structure and function of enzymes. However, in spite of their widespread study, little is known concerning their mechanism(s) of secretion.

Pseudomonas fragi is a proteolytic organism which is known to be involved in low-temperature meat spoilage. This organism is of particular interest to us because it produces a single extracellular enzyme and is capable of producing the enzyme in a chemically defined medium (7), whereas the majority of microorganisms secrete more than one protease and require, in their growth media, the presence of proteins for the induction of protease synthesis. In this respect, *P. fragi* might be a suitable organism for studying enzyme transport in bacterial cells and for the isolation of mutationally altered extracellular enzymes. A fuller characterization of the basic properties of the enzyme would be a prerequisite for such an investigation. We report here the isolation of a mutant producing high levels of the enzyme. A procedure which permits the purification of large quantities of the protease and a selective medium which allows the isolation of mutationally altered enzymes have been developed.

MATERIALS AND METHODS

Bacterial strains and isolation of mutants. All of the strains used in this study were derived from *P. fragi* ATCC 4973. As this strain is prototrophic, a methionine-requiring mutant was isolated by mutagenesis with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, and this auxotrophy served as the genetic marker. This mutant strain was used in the isolation of derepressed mutants and mutants producing mutationally altered proteases.

Mutants producing high levels of the protease were isolated among colonies forming large zones of proteolysis on a milk agar medium (4) after nitrosoguanidine mutagenesis. Isolation of mutants producing a protease capable of hydrolyzing elastin was carried out in the minimal salt medium of Clark and Maaløe (1), containing methionine (4 μg/ml), elastin (Sigma Chemical Co.) (0.4%), and agar (1.5%). Washed cell suspensions in volumes of 0.1 ml containing 2×10^8 cells were spread on several petri dishes containing the elastin-agar medium, and small crystals of nitrosoguanidine were dropped on the plates. Following incubation at room temperature for 3 weeks, colonies which were surrounded by a clear zone (hydrolysis of elastin) were isolated and tested for the presence of the Met^- marker and then for elastolytic activity. The liquid medium used for the production of the protease was as described by Porzio and Pearson (7) except for the addition of 0.5% yeast extract. Growth was measured turbidimetrically with a Klett-Summerson colorimeter, using filter no. 66, following the addition of 0.1 ml of 0.1 M EDTA per ml of culture to solubilize

the calcium phosphate precipitate present in the growth medium.

Protease assay. Proteolytic activity was determined by the procedure of Kunitz, using casein (1%) and, occasionally, hemoglobin (0.5%) as substrates (3). A linear rate of increase was obtained up to an absorbance of 0.8 at 280 nm. A unit of activity was assigned as that amount of enzyme which yielded a 0.001 optical density (280 nm) unit of change per min. The specific activity is expressed as the number of units per milligram of protein. Elastase activity was assayed by the method of Shotton (8). Protein was measured by the method of Lowry et al. (5) with bovine serum albumin as a standard.

Determination of substrate specificity. The procedure employed for the determination of the substrate specificity was essentially as described previously (3) by using the insulin chains as substrates. A sample of 2 mg of insulin A or B chain was dissolved in 1% ammonium bicarbonate (pH 8.0) containing 1 mM $CaCl_2$, and a sample of the protease solution was added to give an enzyme-to-substrate ratio ranging from 1:25 to 1:50. After incubation for 18 h at 37°C, the reaction was stopped by freezing and the sample was lyophilized. Peptides were purified by paper electrophoresis at pH 1.9 and, when necessary, by a combination of paper electrophoresis and chromatography. The solvent used for the chromatography contained 1-butanol–pyridine–acetic acid–water (30:20:6:24).

Other methods. The amino acid compositions of peptides from substrate digests and of the enzyme were determined with a Beckman 120C amino acid analyzer. The molecular weight of the enzyme was estimated by sodium dodecyl sulfate-gel electrophoresis as described by Weber and Osborn (10). Proteins used as standards and their molecular weights were bovine serum albumin (68,000), pepsin (35,000), chymotrypsinogen (25,700), and trypsin (23,300).

Purification of the proteolytic enzyme. Flasks of 2,000 ml containing 500 ml of medium were inoculated with 25 ml of an overnight culture grown in brain heart infusion broth (Difco Laboratories). The flasks were placed on a rotary shaker for incubation at 17°C for 24 to 36 h until the culture had reached a turbidity of 450 Klett units. The cells were removed by centrifugation, and to the spent medium was added, with stirring, 660 g of $(NH_4)_2SO_4$ per liter. The suspension was stirred for 2 additional h at 4°C, and the precipitate was collected by centrifugation. It was suspended in a small volume of 10 mM Tris-hydrochloride buffer (pH 7.5) containing 5 mM $CaCl_2$ and dialyzed for 20 h against 1 liter of the same buffer. The dialysate was centrifuged to remove insoluble material and then applied on a column of DEAE-cellulose (2.5 by 20 cm) previously equilibrated with the Tris-calcium buffer. The solution was washed in the column with 20 ml of buffer, and the enzyme was eluted with a linear gradient of 0 to 0.3 M NaCl prepared in the Tris-calcium buffer. The flow rate was set at 30 ml/h, and 10-ml fractions were collected. Fractions containing proteolytic activities were pooled, dialyzed against distilled water, and lyophilized.

Purification of the enzymes from mutants which had elastolytic activities was carried out by a similar procedure with the exception that the chromatographic step was performed on a column of SP-Sephadex. The precipitate was collected from the growth medium by ammonium sulfate precipitation and dialyzed overnight against sodium acetate buffer, 5 mM (pH 3.8), containing 5 mM $CaCl_2$. A column of SP-Sephadex C-25 previously equilibrated with the dialysis buffer was used for the chromatography. The gradient employed for the elution of the enzyme was prepared in the same buffer and contained 0 to 0.3 M NaCl.

RESULTS

Enzyme purification from the parent strain. Although a method for the purification of the protease from *P. fragi* was already available (7), we were only able to obtain low yields of the enzyme by this procedure. This problem was solved by the isolation of a mutant which produced a much larger quantity of the enzyme. As shown in Fig. 1, as many as 290 enzyme units per ml were produced in the derepressed mutant, whereas only 8 U/ml was detected in the growth medium of the parental organism. This difference in the amount of enzyme produced was not as striking, however, when the cells were grown in the same medium but without yeast extract. In this medium, 49 U of protease activity per ml was detected for the wild-type organism (results not shown). This observation is in agreement with the findings of Porzio and Pearson, who reported that yeast extract represses the synthesis of the enzyme in the wild-type organism (7).

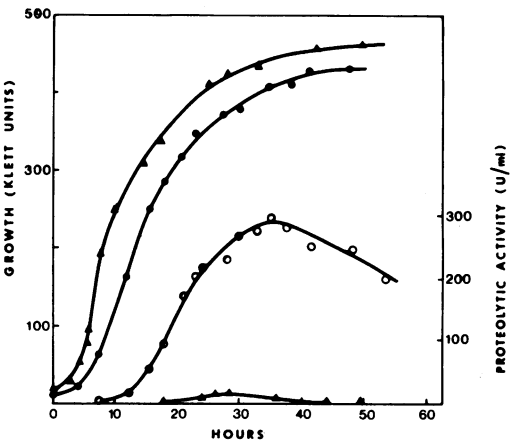

FIG. 1. *Growth and proteolytic activities of wild-type P. fragi and derepressed mutant. Flasks (2,000 ml) containing 500 ml of medium were inoculated with 15 ml of an overnight culture and incubated at 17°C on a rotary shaker. The growth was followed turbidimetrically, and the proteolytic activity was determined by using casein as substrate. Growth of* (▲) *parent and* (●) *mutant strain. Proteolytic activity of* (△) *parent and* (○) *mutant strain.*

Possibly because the enzyme is produced in larger quantities by the mutant than by the parent, it was recovered with good yields from the growth medium by ammonium sulfate precipitation. Purification was subsequently effected by a single chromatographic step. As shown in Fig. 2, the enzyme eluted in a single symmetrical peak of protein and activity suggesting homogeneity. Gel electrophoresis at pH 4.3 revealed the presence of a single band of protein (Fig. 3). The results are summarized in Table 1. Only a sevenfold purification was necessary to obtain a pure enzyme; the overall yield was 27%. The purification procedure could be readily adapted for large volumes of the growth medium. For example, as much as 850 mg of pure enzyme was recovered from a 20-liter fermentation batch.

Molecular weight and amino acid composition. The molecular weight of the enzyme was estimated by sodium dodecyl sulfate-gel electrophoresis. The enzyme migrated as a single band, and a plot of the logarithm of the molecular weights of the standard proteins against mobilities gave a straight line. By interpolation, the molecular weight of the protease was estimated to be 52,000, assuming that the enzyme is composed of a single polypeptide chain. This value is in close agreement with that determined by Porzio and Pearson (7), who estimated a molecular weight of 50,000 for the enzyme. The amino acid composition of the purified enzyme is shown in Table 2. The enzyme has a particularly high content of aspartic acid (or its amides or both), glycine, and alanine. However, it is devoid of sulfhydryl groups.

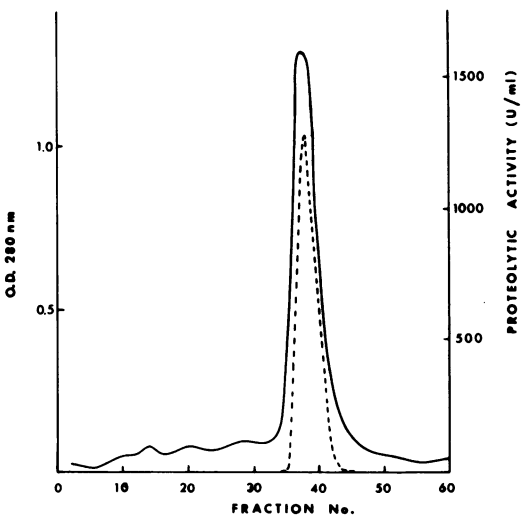

FIG. 2. *Chromatography on DEAE-cellulose. The enzyme obtained after dialysis of the ammonium sulfate precipitate was applied to the column. Flow rate, 30 ml/h. Activity (-----) was determined by using casein as the substrate. OD 280 nm, Optical density at 280 nm.*

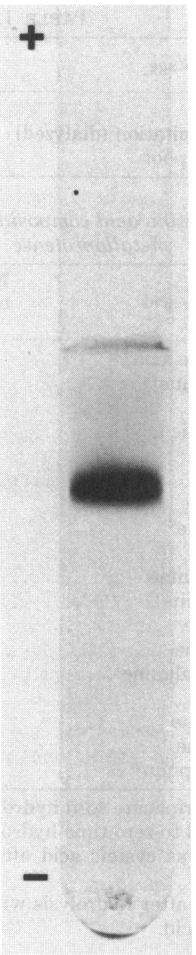

FIG. 3. *Polyacrylamide gel electrophoresis of the purified protease of P. fragi. Approximately 50 μg of the purified preparation was applied. The direction of migration is downward and the gel concentration was 10%.*

Influence of pH on proteolytic activity. The enzyme was most active at pH values near 8.0 when tested with casein as substrate and retained considerable activity at pH 6.0. The enzyme hydrolyzed hemoglobin maximally at pH 8.0, but has also another maximum at pH 6.0 with this substrate (results not shown).

Effects of various inhibitors. The activity of the protease was not affected by the presence of phenylmethylsulfonyl fluoride but was strongly inhibited by the addition of *o*-phenanthroline (Table 3). The inhibition by *o*-phenan-

TABLE 1. *Purification of the metalloprotease of P. fragi*

Stage	Vol (ml)	Total units	Protein (mg/ml)	Sp act	Yield (%)
Culture medium	800	144,000	1.70	106	100
$(NH_4)_2SO_4$ precipitation (dialyzed)	26	91,806	6.35	556	64
DEAE-cellulose pool	70	39,140	0.74	753	27

TABLE 2. *Amino acid composition of P. fragi metalloprotease*

Amino acid	No. of amino acid residues per molecule[a]
Aspartic acid	85
Threonine[b]	37
Serine[b]	43
Glutamic acid	26
Proline	9
Glycine	76
Alanine	59
Cysteine[c]	0
Valine	40
Methionine	4
Isoleucine	24
Leucine	34
Tyrosine	18
Phenylalanine	27
Lysine	11
Histidine	9
Arginine	9
Tryptophan[d]	4

[a] Average of triplicate 48-h hydrolyses.
[b] Extrapolated to zero time hydrolysis.
[c] Determined as cysteic acid after oxidation with performic acid.
[d] Determined after hydrolysis with 3 N mercaptoethanesulfonic acid.

TABLE 3. *Reactivation of o-phenanthroline-inhibited protease by metals*

Metal	Concn (mM)	Activity[a] (%)
None		0
$Zn(C_4H_6O_4)$	0.1	44
	1.0	100
$CoCl_2$	0.1	28
	1.0	128
$CaCl_2$	1.0	0
$MgCl_2$	1.0	0

[a] Assays performed in the presence of 1 mM *o*-phenanthroline. Activity was measured by determining the amount of trichloroacetic acid-soluble peptides by using the method of Lowry et al. (5).

throline suggested that the enzyme requires Zn^{2+} for activity. Addition of equimolar amounts of Zn^{2+} to a solution of enzyme inhibited by *o*-phenanthroline restored activity. Co^{2+} ions may be substituted for Zn^{2+}, producing an activity which corresponded to 128% of that of the native enzyme. The addition of Ca^{2+} and Mg^{2+} had no effect. These results are in agreement with those reported previously for this enzyme and thus clearly demonstrate that the protease of *P. fragi* is a typical neutral protease (6).

Substrate specificity. The substrate specificity of the protease was elucidated by determining the amino acid compositions of peptides produced by hydrolysis of insulin chains. The results are shown in Fig. 4. The pseudomonad enzyme did not exhibit a well-delineated specificity except that it appeared to have a preference for bonds on the *N*-terminal side of hydrophilic residues such as those of aminoethylcysteine, serine, threonine, and glutamine, and the bonds of smaller residues, particularly glycine. It is clear that the specificity of the protease of *P. fragi* differs markedly from that of other metalloproteases which are known to cleave exclusively on the *N*-terminal side of large hydrophobic residues (6).

Isolation of mutant producing mutationally altered proteases. Cells of *P. fragi* grow well on a medium containing purified casein as sole carbon source, presumably because the secreted enzyme can provide the amino acids from casein to sustain the growth of the organism. However, if elastin is substituted for casein no growth can occur because the enzyme cannot hydrolyze this protein. Fifteen mutants capable of growth on elastin as the sole carbon source were isolated. Each of the mutants was grown in the liquid medium as described for the parental strain. The enzyme was concentrated from the medium by ammonium sulfate precipitation and then purified by column chromatography. While the protease from the parental strain readily adsorbed to DEAE-cellulose (see Fig. 2), the enzyme from the 15 mutants did not. On chromatography on columns of SP-Sephadex, the proteolytic activities eluted in several peaks, from 3 to 5, depending on the mutant used. The elution profile of the enzymes from one of the mutants (Mel) is shown in Fig. 5. Several protein peaks were obtained and nearly all had proteolytic activities. The enzymes, designated protease I to III according to their orders of elution from the column, had elastinolytic activities. The substrate specificities of these three proteases were essentially identical (Fig. 6). It can be seen that the enzymes hydrolyzed several bonds of the insulin B chain, but none of the

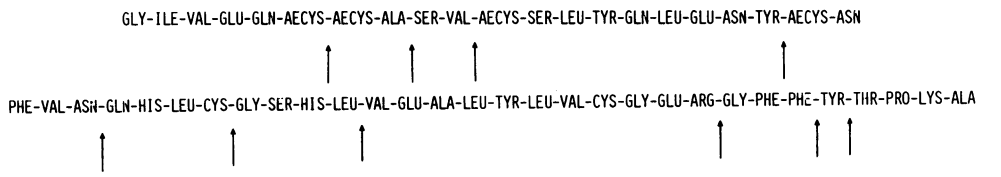

FIG. 4. *Substrate specificity of the protease from P. fragi. Substrates, aminoethylated insulin A chain, and performic-oxidized B chain were digested in 1% bicarbonate and 5 mM $CaCl_2$ for 18 h. Arrows indicate the bonds cleaved.*

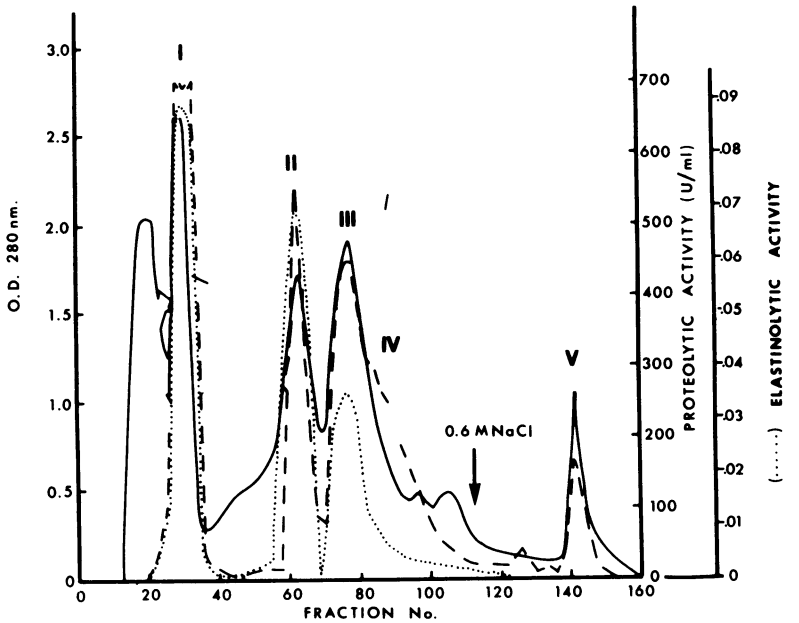

FIG. 5. *Chromatography on SP-Sephadex. The ammonium sulfate precipitate from 20 liters of growth medium of mutant Mel was dialyzed and applied to the column (5 by 20 cm). The gradient bottles contained 0 and 0.3 M NaCl, respectively, in 900 ml of sodium acetate buffer, 5 mM (pH 3.8), and $CaCl_2$, 5 mM. The flow rate was 60 ml/h. Protein concentration is shown as a continuous line.*

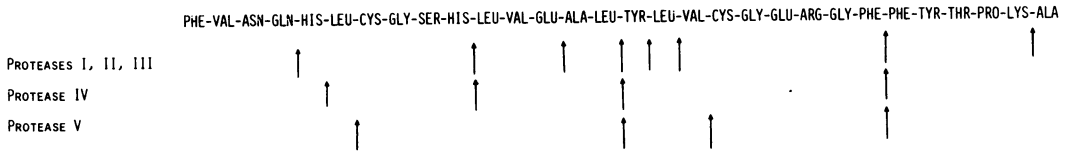

FIG. 6. *Substrate specificities of the proteases obtained from mutant Mel. Oxidized insulin B chain was the substrate. Other conditions were as described in the legend to Fig. 4.*

bonds cleaved by these proteases corresponded to those cleaved by the enzyme from the parental strain (see Fig. 4). The fourth peak of protein in Fig. 6 was found to contain, on its trailing edge, a second proteolytic enzyme in addition to protease III. This enzyme can be purified further by a second chromatography on a column of SP-Sephadex (results not shown). The eluted enzyme, protease IV, had very little if any elastinolytic activity. This protease had a similar but narrower specificity, cleaving only a phenylalanine, a tyrosine, and two leucine bonds (Fig. 6). These four proteases, therefore, have substrate specificities which resemble that of metalloproteases (6). The specificity of protease V is rather unique. While it cleaved the tyrosine and phenylalanine bonds hydrolyzed by the other enzymes, it also split the bonds of cysteic acid residues at positions 7 and 19, respectively. The proteases from five other mutants tested were found to exhibit similar specificities, although differences in the rate of hydrolysis of some

bonds were noted. None of these proteases hydrolyzed the peptide bonds which were cleaved by the enzyme from the parental strain.

DISCUSSION

A simplified procedure for the purification of the extracellular protease from *P. fragi* is reported. The enzyme is secreted in large quantities from a derepressed mutant which produces up to 40 times the amount of protease present in the culture medium of the parental organism. Furthermore, the procedure can be readily adapted for the purification of the enzyme from large volumes of growth medium. As reported previously by others (7), the enzyme is a metalloprotease which requires Zn^{2+} for activity and has a pH optimum of about 8.0 with proteins as substrates. Its molecular weight is estimated to be 52,000.

The protease of *P. fragi* differs from other metalloproteases by its substrate specificity. Whereas all metalloproteases have marked preferences for the *N*-terminal bonds of large hydrophobic residues, the pseudomonad enzyme appears to cleave only bonds of small and hydrophilic residues. In view of this difference in specificity in comparison with other metalloproteases, the pseudomonad enzyme cannot solubilize elastin, a property which was used advantageously in the selection of mutants capable of secreting proteases with modified substrate specificities. The enzymes produced by the mutants were found to differ markedly from that of the parental enzyme not only in their substrate specificities but also in their chromatographic properties. Furthermore, more than one protease was secreted by all mutants examined.

In the present study, the chemical and physical properties of the mutant enzymes were not investigated. It is therefore not possible to account for the presence of the multiple enzyme species in the growth media of the mutants. However, the possibility that these enzymes represent different active fragments generated by autodigestion is probable. In fact, a protease which has undergone, as a result of mutation, a structural modification, even subtle, could be expected to be more susceptible to autodigestion, particularly if a change in the specificity of the enzyme has also taken place. Alternatively, if the pseudomonad enzyme is secreted in a precursor form, as has been found to occur in staphylococci (2), processing or activation of such a precursor could have taken place by cleavage at different locations in the amino acid sequence of the precursor chain, thus generating fragments of different properties such as molecular weights.

Since nothing is known concerning the structure of the binding site of the enzyme from *P. fragi*, one can only postulate concerning the modification of substrate specificities observed for the mutant enzymes. The differences in the substrate specificities of the mammalian pancreatic enzymes have been explained by the substitution of one or more amino acids in the substrate binding site (9). Therefore, it is probable that such amino acid changes, in the pseudomonad enzyme, might have occurred as a result of mutagenesis. The difference in the substrate specificity of protease V would imply that the geometry of the binding site of this enzyme differs even more significantly such that the side chain of cysteic acid which has different physical and chemical properties can be recognized.

Our observation that mutants producing proteases of altered substrate specificities can be isolated offers new possibilities for studying relationships between structure and specificity. In the present study, only one protein, elastin, was used in the isolation of mutants. Thus, it is probable that other proteins or peptides which cannot be hydrolyzed by the parent enzyme could also be tested in the selection of new mutants.

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