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Ultrastructural Localization of an Extracellular Protease in *Pseudomonas fragi* by Using the Peroxidase-Antiperoxidase Reaction†

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An extracellular protease, which previously has been found to correlate with the appearance of bleblike evaginations on the cell wall of *Pseudomonas fragi* ATCC 4973, was purified 38-fold by ammonium sulfate precipitation and Sephadex chromatography to yield a single band by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Polyclonal rabbit antiserum raised against the purified enzyme had an enzyme-linked immunosorbent assay titer of 4×10^7. The peroxidase antiperoxidase method was used to localize the neutral protease in *P. fragi* at the ultrastructural level. Electron microscopy of cell sections of this organism revealed that high concentrations of positive immunoperoxidase reaction product were located near the cell wall, whereas control sections stained with preimmune or heterologous serum did not show similar deposits to be present. These results are consistent with the hypothesis that blebs appearing on *P. fragi* contain high concentrations of neutral protease.

The ability of pseudomonads to spoil meat and dairy products has been closely associated with their proteolytic and lipolytic activities. *Pseudomonas fragi* is one of several pseudomonads known to produce an extracellular protease during meat spoilage (2, 6, 8, 18). When *P. fragi* colonizes muscle tissue, unusual evaginations or blebs appear on the outer cell wall of *P. fragi* and in the muscle surrounding the cell (6, 20). However, *P. fragi* does not produce extracellular protease or blebs when grown on nonmeat tissue substrates such as all-purpose Tween or in simple media devoid of amino acids or protein (6, 17). It therefore has been hypothesized that blebs may function as a mechanism by which *P. fragi* concentrates, conserves, or secretes proteolytic enzymes.

The purpose of this study was to purify the extracellular neutral protease of *P. fragi*, produce polyclonal antiserum against the purified enzyme, and localize the protease in *P. fragi* at the ultrastructural level by using an immunoperoxidase technique.

MATERIALS AND METHODS

Enzyme production and purification. *P. fragi* ATCC 4973 was obtained from the Department of Food Science and Nutrition, University of Missouri-Columbia. For the production of enzyme, a 1% inoculum was transferred to a 4-liter flask containing 1,500 ml of brain heart infusion (BHI) broth (pH 7.4 ± 0.2). Flasks were shaken on a rotary shaker (Eberbach Corp., Ann Arbor, Mich.) at 10°C for 60 to 72 h at 200 to 230 rpm, and the BHI culture was centrifuged (14,000 × *g*) for 60 min. The pellet was discarded, and culture supernatant fractions were centrifuged (4,000 × *g*) for an additional 30 min. The supernatant fraction was filter sterilized through a 0.45 μm filter (Millipore Corp., Bedford, Mass.) to remove residual *P. fragi*.

Enzyme purification was a modification of the method used by Bala et al. (1). The filtered culture supernatant fraction was precipitated at room temperature by the slow addition of ammonium sulfate to 55% saturation, and the solution was stirred overnight at 10°C. The precipitate was collected by centrifugation (14,000 × *g*) for 90 min and then dissolved in 15 ml of 0.05 M Tris chloride buffer containing 5 mM $CaCl_2$ buffer (pH 7.5) per 200 ml of original culture fluid. The solution was stirred for 30 min and dialyzed against five changes of cold distilled deionized water overnight at 10°C and then against 0.05 M Tris hydrochloride buffer (pH 7.4) overnight at 10°C. The solution was concentrated by dialysis against 40% Carbowax (Union Carbide Corp., New York, N.Y.) for 8 h at 10°C and placed on a Sephadex G-100 column (2.5 by 40 cm) equilibrated with 0.05 M Tris–5 mM $CaCl_2$ (pH 7.4). The column was eluted with 0.05 M Tris–5 mM $CaCl_2$ (pH 7.4), and 5-ml fractions were collected at a flow rate of 20 ml/h. Protease activity was determined by the method of Hagihara et al. (7) as modified by Nakajima et al. (12). One unit of proteolytic activity was defined as the enzyme quantity which liberated 1 μg of tyrosine equivalent per ml of reaction mixture. Protein concentration was determined by the method of Lowry et al. (9) as modified by Cooper (4).

Electrophoresis. Disc gel electrophoresis was performed in 9% gels according to previously published methods (5). Gels were stained and destained as described by Chrambach et al. (3).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed in 10% gels by the method of Weber and Osborn (19). A low-molecular-weight calibration kit (Pharmacia, Uppsala, Sweden) containing phosphorylase *b*, albumin, ovalbumin, carbonic anhydrase, trypsin inhibitor, and α-lactalbumin was used for the molecular weight determination.

Immunization. Two New Zealand White rabbits were injected intramuscularly in both hind legs with 1 mg of enzyme in 5.0 ml of water-Freund incomplete adjuvant (1:1). Two weeks later these were boosted by intramuscular injections with 1 mg of enzyme in 5.0 ml of water-Freund incomplete adjuvant (1:1). At weeks 4 and 6 rabbits were again boosted intramuscularly with 0.5 mg of enzyme in 1.0

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TABLE 1. Purification of *P. fragi* neutral protease

Fraction	Vol (ml)	Total protein (mg)	Activity[a]		Sp act[a] (U/mg)	% Yield	Purification (fold)
			U/ml	Total U			
Culture supernatant	1,300	16,120	0.59	780	0.048	100	1.0
Ammonium sulfate	66	528	2.01	133	0.25	17	5.2
Sephadex G-100 gel filtration[b]	10	4.4	0.80	8	1.8	1.03	38.0

[a] One unit equals the release of 1 nmol of tyrosine equivalent per min at 21°C.
[b] The ammonium sulfate fraction (66 ml) was concentrated to 20 ml by dialysis against carbowax, and 8 ml of this concentrate was applied to the Sephadex G-100 column.

ml saline. Rabbits were bled via the marginal ear vein at regular intervals, and their sera were stored at −20°C.

Enzyme-linked immunosorbent assay. An enzyme-linked immunosorbent assay was used to determine antibody titer. For solid-phase coating, purified protease was diluted with 0.1 M phosphate-buffered saline solution (pH 7.5) (PBS) to 10 μg/ml, and 100 μl was added to each well in a 96-well microtiter plate (Immulon Removawell, Dynatech Industries, Inc., McLean, Va.). Plates were incubated at 4°C overnight, and the wells were washed twice with 250 μl of PBS-Tween per well. One percent (wt/vol) of bovine serum albumin in PBS was added in 250-μl portions to each well and incubated for 30 min at 37°C. Wells were washed twice with 250 μl of PBS-Tween per ml. Antiserum was serially diluted in 1% bovine serum albumin–PBS, and 100 μl was added to each well. Plates were incubated for 1 h at 37°C and washed five times with PBS-Tween. One hundred microliters of goat antirabbit peroxidase diluted 1:1,000 in bovine serum albumin–PBS was added to each well and incubated for 30 min at 37°C. Wells then were washed an additional five times with PBS-Tween. Bound peroxidase was determined by enzyme assay as described by Pestka et al. (14).

Electron microscopy. Cells were collected from a 60- to 72-h BHI culture (1,500 ml) by centrifugation and washed four times in distilled water, followed by a final wash in Millonig phosphate buffer (10). Washed cells were centrifuged, and the pellet was dissolved with 1.25% glutaraldehyde made in the same buffer. After incubation for 1 h at 4°C, the mixture was centrifuged and the buffer was decanted. Cells (0.1 to 0.2 ml) were mixed with 2 ml of sterile molten 2% Noble agar. The mixture was pipetted onto clear glass slides with four thin, flat pieces of glass on top, forming an empty square. After solidification, small thin cubes or strips were cut in the agar with a razor blade, and these were fixed for 2 to 4 at 4°C in 2.5% glutaraldehyde. Strips were washed for 15 to 20 min in three changes of phosphate buffer.

Samples were transferred to a 2% osmium tetroxide solution in phosphate buffer. Strips were fixed for 2 to 4 h at 4°C but allowed to come to room temperature during the final period of fixation. Samples were then rinsed five to six times in phosphate buffer. After the final wash, samples were dehydrated in a graded ethanol series at 4°C. Samples were infiltrated with a graded series of Epon-Araldite Polysciences, Inc., Warrington, Pa.) with propylene oxide as the transition solvent. A mold containing fixed cells plus Epon-Araldite was dessicated under a slight vacuum at room temperature for 2 to 3 h and then hardened at 60°C for 24 to 36 h. Ultrathin sections were cut with a diamond knife and collected on uncoated 100/, 200/, or 300/mesh copper or nickel grids.

Sections were stained by floating the grids, sections down, on a saturated solution of uranyl acetate and lead citrate. Grids containing stained sections were observed in a Philips

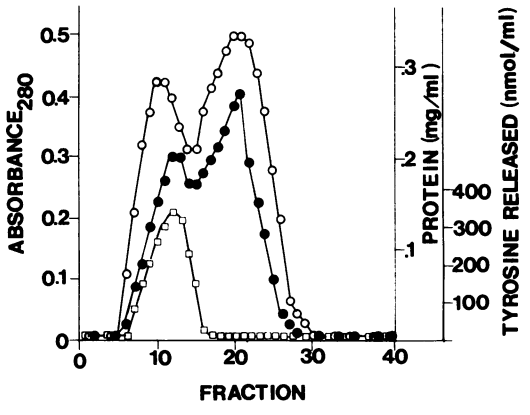

FIG. 1. Elution pattern of Sephadex G-100 gel chromatography. Protein solution was loaded on a column (2.5 by 40 cm) equilibrated with 0.05 M Tris hydrochloride–5 mM (pH 7.5). The enzyme was eluted at 20 ml/h with 5-ml fractions per tube. Symbols: ○, absorbance at 280 nm; ●, protein concentration; □, protease activity.

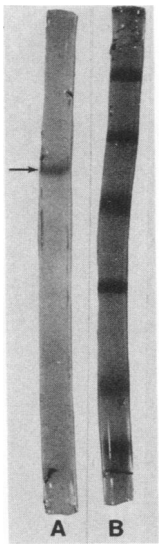

FIG. 2. Sodium dodecyl sulfate-polyacrylamide gel (10% total concentration) electrophoretogram of the neutral protease (22 μg) (A) and standard proteins (B). The lower limit of detectable protein by the staining procedure was 10 μg.

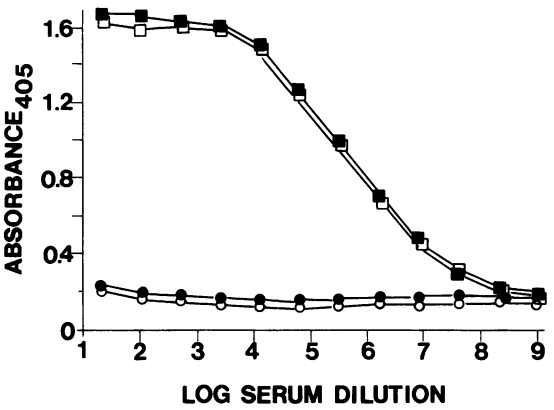

FIG. 3. Enzyme-linked immunosorbent assay titration of protease antisera. Symbols: ■, R-1 antiserum; □, R-2 antiserum; ●, R-1 preimmune serum; ○, R-2 preimmune serum.

EM-201 electron microscope at an accelerating voltage of 60 kV.

PAP localization of protease. Fixed sections were stained with a peroxidase-antiperoxidase (PAP) kit (Cappell Laboratories, West Chester, Pa.) as described previously (11, 16). They were etched for 20 min prior to initiating immunochemical staining by floating them on 10% aqueous hydrogen peroxide. After each staining solution (except after 3'3-diaminobenzidine [DAB] and osmium tetroxide), grids were rinsed in 0.5 M Tris hydrochloride-buffered (pH 7.6) saline (TBS) for 3 min. After etching with H_2O_2, sections were floated on normal goat serum diluted 1:30 in TBS for 3 min and rinsed. They then were floated on rabbit antiprotease antiserum diluted 1:10, 1:50, 1:100, and 1:1,000 in TBS for 2, 4, 10, and 45 min, respectively. Grids then were floated on goat antirabbit immunoglobulin G diluted 1:5 in TBS for 3 min. They then were floated on the PAP complex diluted 1:10 for 3 min. After mixing and filtering, grids were immersed in the peroxidase substrate for 2 to 3 min. The substrate consisted of 170 ml of TBS, 22 mg of DAB, and 1.5 ml of 0.3% H_2O_2 and was continuously stirred by a magnetic stirrer to prevent nonspecific adsorption of the reaction product to the embedding medium.

After exposure to the substrate solution, sections were washed in three changes of distilled water. A drop of aqueous 2% osmium tetroxide was placed on each grid for 60 min at room temperature in a fume hood. Osmium will deposit on sites of oxidized, precipitated DAB. Polymers of the DAB reaction product are visible with the electron microscope as black deposits (11, 16). Grids were washed in one change of distilled water and examined in a Philips EM-201 electron microscope. Controls included use of preimmune, normal rabbit or antiaflatoxin (13) sera as a substitute for the protease antiserum. Other controls included omission of PAP, DAB, or antirabbit immunoglobulin G.

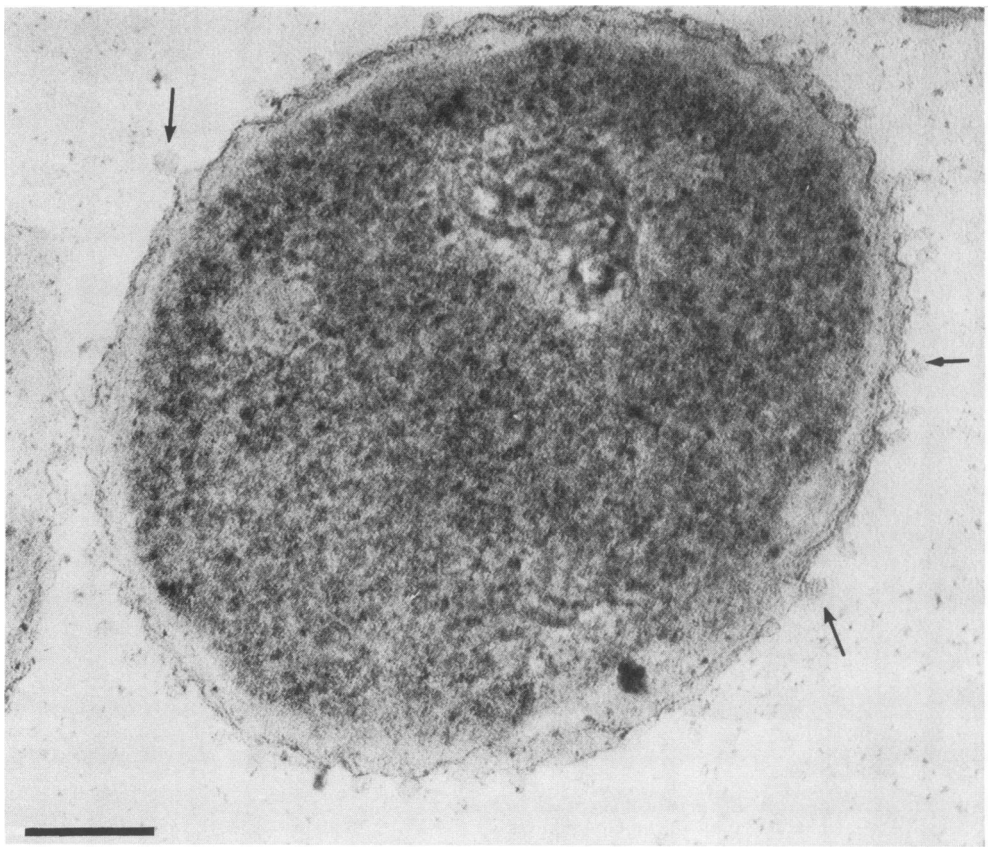

FIG. 4. Electron micrograph of *P. fragi* cells grown in BHI broth at 10°C for 60 to 72 h. Arrows point to bleblike evaginations. Bar, 0.25 μm.

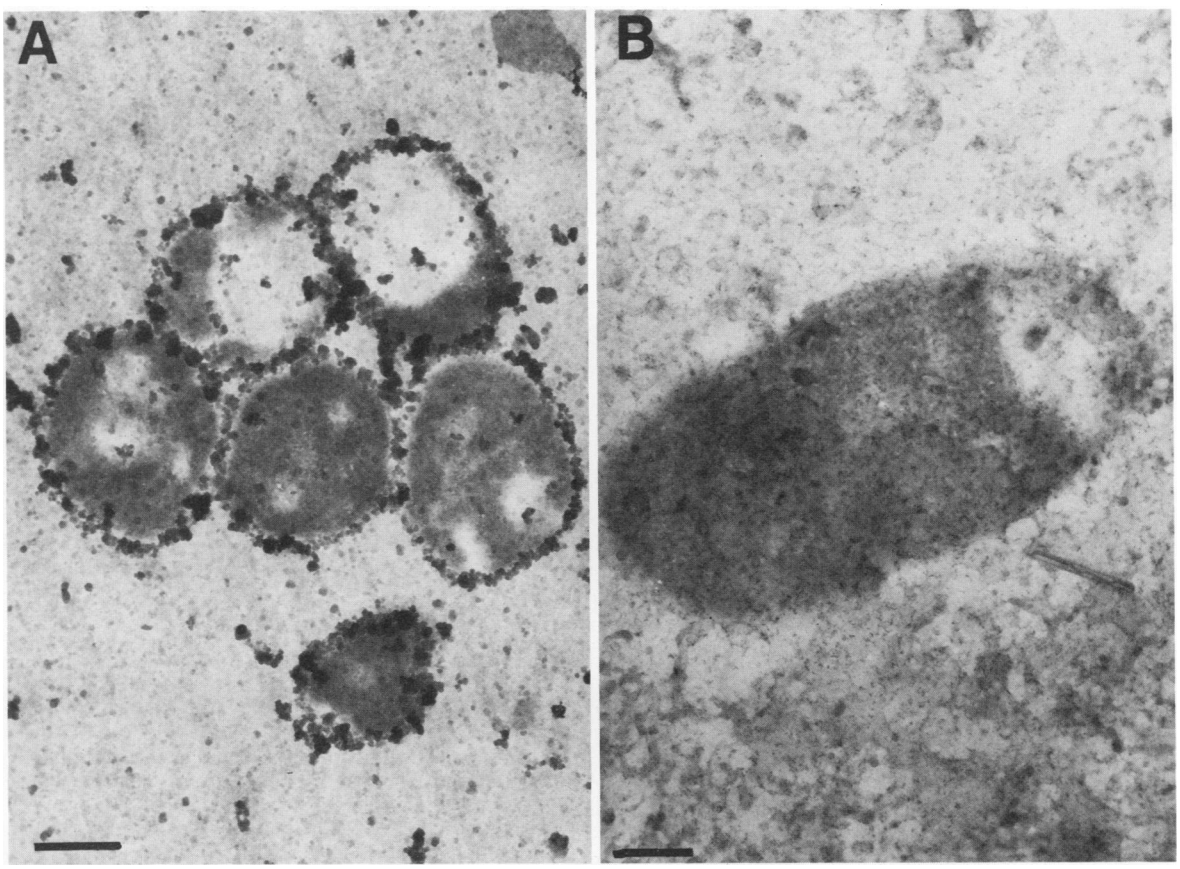

FIG. 5. (A) Electron micrograph of *P. fragi* cells from BHI broth stained with protease antiserum (1:100). Note the dark deposits in the periplasmic space, blebs, and globules. (B) Electron micrograph of *P. fragi* cells from BHI broth stained with normal rabbit serum. Note the absence of stain deposits. Bars, 0.5 μm.

RESULTS AND DISCUSSION

Enzyme production and purification. The initial population after inoculation of BHI broth was approximately 10^7 CFU/ml. At 60 to 72 h a maximal population of 10^{10} CFU/ml was reached concurrently with an optimal protease activity of 1 nmol of tyrosine released per min/ml.

The protease was purified 38-fold by the purification schedule (Table 1). Elution on Sephadex G-100 resulted in a protease peak between fractions 6 and 16 (Fig. 1). Fractions containing peak activity were combined and used for the remaining studies. The purity of this enzyme was verified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, which yielded only a single protein band whose molecular weight was estimated as 48,000 ± 1,200 (Fig. 2). This value was consistent with the molecular weight previously reported for *P. fragi* neutral protease as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, gel permeation, or zinc microassay (15). A single protein band was also observed by disc gel electrophoresis.

Antibody titration. High-titer antisera specific for the protease were obtained from the rabbits 1 week after the final immunization. The titer was determined by incubating the rabbit antiserum over a purified protease solid phase and then determining total bound rabbit antibody with a goat antirabbit peroxidase conjugate. The last well in a dilution scheme to give color visually distinct from the preimmune control serum represented the titer endpoint. The antibody titer endpoints of both rabbits were approximately 4×10^7, whereas dilutions of preimmune control serum showed negligible binding to the protease solid phase (Fig. 3).

Ultrastructure of *P. fragi*. Blebs were observed on the surface of *P. fragi* cells grown in BHI broth at 10°C for 60 to 72 h (Fig. 4). Blebs as well as unattached globules were surrounded by multiple-layered membranes identical to those of the cell wall. A fiberlike glycocalyx material, similar to that described previously by Wing et al. (20), was occasionally observed attached to the blebs. We also have observed blebs on *P. fragi* grown in 1% peptone, Casamino Acids (Difco Laboratories, Detroit, Mich.), or protease peptone (unpublished data).

Immunoperoxidase localization of the neutral protease. Thin sections were stained with several dilutions (1:10, 1:50, 1:100, or 1:1,000) of protease antiserum. The 1:100 dilution of antiserum produced optimal peroxidase staining under the conditions described in Materials and Methods. Ultrathin sections of *P. fragi* stained with 1:100 protease antiserum contained discrete packets of peroxidase-positive reaction product close to the cell wall (Fig. 5A). In some cases, these were observed inside blebs which were attached externally to the cell wall as well as in globular structures unattached to the cell. Peroxidase-positive deposits were not detected in cell sections stained with normal serum controls (Fig. 5B). Relative intensities of the cells as determined by the standard immunoperoxidase protocol and by various control protocols were also compared; (Table 2). Sections treated

TABLE 2. Relative immunoperoxidase staining intensities of *P. fragi* cells

Serum		PAP[a]	DAB[a]	Relative staining intensity[b]
Antibody specificity	Dilution			
Protease	1:10	+	+	+
Protease	1:50	+	+	+
Protease	1:100	+	+	+ + +
Protease	1:1,000	+	+	+ +
Preimmune	1:100	+	+	−
Normal	1:100	+	+	−
Aflatoxin B_1	1:50	+	+	−
Protease	1:100	−	+	−
Protease	1:100	+	−	−

[a] PAP or DAP present (+) or absent (−).
[b] Relative intensities: +, degree of positive staining; −, negative staining.

with preimmune serum, normal serum, or aflatoxin B_1 antiserum were negative along with those sections in which PAP or DAB was omitted.

Several investigations have demonstrated the presence of blebs or protrusions on the surface of *P. fragi*. In each of those studies the organism was observed while growing in spoiled pork or beef (6, 20, 21), and a correlation between bleb formation and proteolytic activity was hypothesized. We have previously determined that neither proteolytic activity nor bleb formation occurred when *P. fragi* was grown in koser citrate broth (S. S. Thompson, Ph.D. thesis, Michigan State University, East Lansing, 1983). In this report, we determined that both blebs and proteolytic activity were associated with *P. fragi* grown in BHI broth.

The results of our immunoperoxidase procedure suggests that neutral protease of *P. fragi* is present in concentrated packets near the cell wall but is absent from the cytoplasm. The protease is most probably synthesized at the cell membrane and secreted directly into the bleb. Since immunoperoxidase-positive deposits correlating to the protease were associated with the gram-negative cell wall and blebs, it also can be concluded that these latter sites are likely to be closely associated with the release of the neutral protease. Since proteins and other large molecules are too large to penetrate the cell wall and membrane, these macromolecules must be broken down to smaller units which can be transported through these barriers. Excretion of the protease from blebs by *P. fragi* into the growth medium or meat tissue would result in hydrolysis of proteins, thereby providing a source of requisite amino acids for cellular metabolism.

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