Proteolytic Enzyme Preparation from 
*Pseudomonas fragi*: Its Action on Pig Muscle¹

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An extracellular preparation from *Pseudomonas fragi* with proteolytic enzyme activity was isolated, and its action on meat proteins and meat protein ultrastructure was studied. First, a suitable growth medium for proteolytic enzyme production was determined, and a method for partial purification of the proteolytically active fraction was developed. The enzyme preparation displayed optimal proteolytic activity at neutral pH and 35 C. Proteolytic activity was irreversibly lost by mild heat treatment. The enzyme preparation was tested for its ability to hydrolyze isolated pig muscle proteins. Myofibrillar protein was rapidly degraded, G-actin and myosin were broken down at a slower rate, and the sarcoplasmic proteins were least susceptible to hydrolysis. Electron micrographs of pork muscle showed that the proteolytic enzyme preparation caused a complete loss of dense material from the Z line. Similarities are discussed between the action of *P. fragi* extracellular proteolytic enzyme(s) on meat and normal bacterial spoilage of meat.

The extent to which meat proteins are utilized by bacteria during spoilage is controversial and has not been satisfactorily resolved. *Pseudomonas* strains constitute the predominant group among fresh meat spoilage bacteria (1, 2, 4, 6, 12, 15, 27). Recent research into the mechanism of spoilage of ground pork by *Pseudomonas fragi* has shown that the occurrence of off-odors in the meat coincided with the production of bacterial extracellular proteolytic activity (28). Ultimately, extensive breakdown of myofibrillar proteins occurred (5, 28). These findings indicate that protein breakdown may be initiated at an early stage of spoilage. This is contrary to the opinion that hydrolysis of muscle proteins by bacteria does not happen until after spoilage, i.e., until the degenerative changes accompanying putrefaction occur (11, 20).

Although the low-molecular-weight compounds in meat provide an initial source of readily available nutrients for spoilage bacteria (11, 17), there is evidence that meat proteins are hydrolyzed to provide additional energy and nitrogen for the rapid proliferation of bacteria during spoilage (5, 28). The purpose of the present investigation was to isolate the fraction containing the extracellular proteolytic activity produced by *P. fragi* and to determine its effect on meat proteins and their ultrastructure.

**MATERIALS AND METHODS**

**Organism.** A culture of *P. fragi* (ATCC 4973) was grown on all-purpose-plus Tween broth (BBL, Cockeysville, Md.) at 25 C. Two transfers were made at 24-h intervals. The cells were collected by centrifugation (3,700 × g, 20 min) and suspended in 0.85% saline solution prior to inoculation. The inoculum size used was 1% of the volume of the medium.

**Growth medium.** The medium used for cell growth and production of proteolytic enzyme activity was prepared by dissolving the following ingredients in 1 liter of distilled, deionized water: monopotassium phosphate, 1.0 g; magnesium sulfate, 0.2 g; sodium ammonium phosphate, 1.5 g; L-arginine hydrochloride, L-asparagine, L-aspartic acid, L-cystine, L-glutamic acid, L-glutamine, L-leucine, L-lysine, L-threonine, L-tryptophan, and L-tyrosine, 0.2 g each; glycyglycine and glycyl-L-aspartagine, 0.4 g each. The medium was adjusted to pH 7.5 with sodium hydroxide and was autoclaved at 121 C for 20 min.

**Cultivation.** Erlenmeyer flasks (2 liter) containing 300 ml of growth medium were inoculated and incubated at 10 C under aerobic conditions. During incubation the flasks were shaken automatically (160 excursions per min). At 12-h intervals the turbidity (E₆₅₀ₙm) of the cultures was measured. Samples of culture fluid were centrifuged (9,750 × g, 10 min, 0 C)
and the supernatant fluid was assayed for proteolytic activity.

**Assay for proteolytic activity.** A 2% solution of Hammarsten casein (Nutritional Biochemicals Corp.) in phosphate buffer (0.03 M, pH 7.5) was used as substrate. Culture fluid (1 ml) was preincubated for 1 min at 35°C in a shaking water bath (120 excursions per min). Substrate solution (1 ml) was added and the reaction was allowed to proceed for 5 min. The reaction was terminated by adding 2 ml of 5% (wt/vol) trichloroacetic acid. After 15 min the precipitated protein was removed by filtration by using Whatman no. 2 filter paper. Blanks were treated in the same manner, except the casein solution was not added to the reaction mixture until after the trichloroacetic acid. Hydrolytic products, which were not precipitated by trichloroacetic acid, were measured by the Lowry reaction (18). A solution of L-tyrosine (30 mg per liter) in trichloroacetic acid (2.5% wt/vol) was used as a standard. Results were expressed as units of proteolytic activity in 1 ml of culture supernatant fluid. A unit of proteolytic activity was defined as the amount of enzyme which liberated 1 μmol of tyrosine equivalents in 1 min at 35°C in a reaction mixture of 2 ml total volume.

**Partial purification.** Bacterial cells were removed from the culture medium by centrifugation (9,750 × g, 10 min, 0°C). The supernatant fluid containing the proteolytic activity was adjusted to pH 7.5 with dilute hydrochloric acid. It was concentrated and dialyzed (Table 1) against phosphate buffer (0.01 M, pH 7.5) at 0°C in an ultrafiltration apparatus (Amicon Corp., Lexington, Mass.). The apparatus was equipped with a membrane (PM 10) designed to retain protein molecules greater than 10,000 mol wt. The complete procedure was carried out within 24 h. The enzyme solution was stored at −15°C for up to 3 weeks before use. The effects of storage on enzyme activity were not quantified; however, no major loss in proteolytic activity was observed.

**pH optimum.** The buffers used were as follows: citrate-phosphate, pH 4.0 to 7.0; phosphate, pH 5.5 to 8.0; barbital-hydrochloride, pH 7.0 to 9.0. The reaction mixtures (2 ml total volume) contained casein (1% wt/vol), buffer solution (0.04 M), and enzyme (0.3 mg). The enzyme preparation used had a sp act of 0.31 U/mg and a protein content of 1.5 mg/ml.

Reaction mixtures were incubated for 10 min at 35°C. In each case, the exact pH of the reaction mixture was determined on a replicate mixture.

**Effect of temperature.** Proteolytic activity was measured between 0 and 40°C at 5°C intervals. Each reaction mixture (2 ml total volume) contained casein (1% wt/vol) and enzyme (0.1 mg). The reaction was carried out for 5 min at each specified temperature. The enzyme preparation used had a sp act of 0.72 U/mg and a protein content of 0.57 mg/ml.

Thermal inactivation was measured by incubating the enzyme for 5 min at temperatures from 10 to 40°C. The activity of the enzyme was then assayed at 35°C for 5 min by using casein (1% wt/vol) as substrate. An assay temperature of 35°C was chosen because results indicated it to be the optimal temperature for proteolytic activity using casein as the substrate, although incubation of the enzyme without substrate at this temperature (35°C) resulted in considerable loss of enzyme activity (Fig. 3).

**Effect of enzyme concentration.** Reaction mixtures (2 ml total volume) contained enzyme ranging from 0.07 to 0.36 mg and casein (1% wt/vol). Incubation was for 5 min at 35°C. The enzyme preparation used had a sp act of 0.35 U/mg and a protein content of 0.24 mg/ml.

**Effect of substrate concentration.** The reaction mixtures (2 ml total volume) contained casein (0 to 2.4 mg) and the purified enzyme preparation (0.24 mg). Incubation was for 5 min at 35°C. The enzyme preparation used had a sp act of 0.35 U/mg and a protein content of 0.24 mg/ml.

**Preparation of proteins from pig muscle.** Myosin was prepared from the longissimus dorsi muscle by using the procedure of Kessler and Spicer (14) as modified by Kominz et al. (18). An acetone powder of longissimus dorsi was prepared by the method of Seraydarian et al. (24). A crude preparation of G-actin was extracted from the acetone powder by the method of Mommaerts (21).

Sarcoplasmic proteins were prepared from the area of the semitendinosus muscle composed predominantly of white fibers by using the aqueous extraction method of Helander (9). Nonprotein nitrogen was removed from the sarcoplasmic protein preparation by dialysis against phosphate buffer (0.03 M, pH 7.5). The myofibrillar proteins were extracted from the residue left after removing the sarcoplasmic fraction by using Weber-Edsall solution (0.6 M KCl, 0.04 M KHCO₃, 0.01 M K₂CO₃) as described by Perry (22).

Before assaying their susceptibility to proteolytic degradation, all the protein preparations from pig muscle were dialyzed against potassium phosphate buffer (0.03 M, pH 7.5) and adjusted to a concentration of 10 mg of protein/ml. Denatured hemoglobin and Hammarsten casein (Nutritional Biochemicals Corp.) were dissolved in phosphate buffer (0.05 M, pH 7.5) at a concentration of 10 mg/ml.

With the exception of myosin, the concentration of the various protein preparations from pig muscle were measured by the method of Lowry et al. (18). A solution of bovine serum albumin (200 mg/liter) was used as a standard. The concentration of myosin was measured by the biuret reaction (E. M. Reagents, Brinkman Instruments, Inc., Westbury, N.Y.).

**Treatment of pig muscle with proteolytic enzyme.** The longissimus dorsi muscle from a market weight pig was excised and ground under aseptic conditions (7). A total of 3 ml of the proteolytic enzyme preparation isolated from *P. fragi* and dissolved in phosphate buffer (0.1 M, pH 7.6) was added, with thorough mixing, to a series of test tubes, each of which contained 1.5 g of ground muscle tissue. The enzyme preparation contained 0.5 mg of protein/ml and had a sp act of 0.61. Control samples were prepared similarly, except that phosphate buffer (0.1 M, pH 7.6) was used in place of the enzyme preparation. In all cases the final pH of the incubation mixture was 7.0. The preparations were incubated at 10°C, and tissue samples for electron microscopy were taken at intervals between 0 and 72 h.
Electron microscopy. On removal from the incubator, the muscle tissue was separated from the enzyme solution by centrifugation (3,000 × g, 10 min, 0 C). The supernatant fluid was decanted and the tissue was immersed in 1.25% glutaraldehyde solution (Fisher Scientific Co., Pittsburgh, Pa., biological grade 50%) buffered with 0.07 M NaH₂PO₄ and 0.041 M Na₂HPO₄. The solution had a pH of 7.3 and contained 0.043 M NaCl (25). The tissue pellets were transferred to petri dishes containing fresh fixative. Six to eight small bundles of fibers (0.5 mm in thickness and 2 to 3 mm in length) were removed from each pellet for fixation (1 h) in 1.25% glutaraldehyde solution at 4 C. Subsequent to washing, samples were postfix (1 h, 4 C) in 1% osmium tetroxide (25). Dehydration was carried out in ethanol (25 and 50% at 4 C; 70, 90, and 100% × 3 at room temperature). Dehydrated samples were washed in three changes of propylene oxide, embedded in Epon 812 (19), and sectioned on an LKB 4801 A ultramicrotome. The sections were cut 10⁻⁴ m in thickness. They were mounted on Formvar-coated copper grids and stained in uranyl acetate (23) and lead citrate (23). A Philips EM-300 transmission electron microscope, operated at an accelerating voltage of 80 kV, was used to observe the sections.

RESULTS
Production of proteolytic activity. P. fragi grew rapidly in Koser citrate medium (26) but did not produce extracellular proteolytic activity. When citrate was replaced by a mixture of 11 amino acids and 2 dipeptides, growth was unimpaired and extracellular proteolytic activity was produced (Fig. 1). The proteolytic enzyme(s) was excreted into the medium during the late exponential growth phase and degraded during the stationary phase.

Partial purification of the proteolytic activity. Typical data for concentration and purification of P. fragi proteolytic activity are shown in Table 1. The use of a protein-free growth medium greatly facilitated purification. An 18-fold concentration of proteolytic activity was achieved by a simple 24-h procedure. Bacterial cells were removed by centrifugation, and the constituents of the growth medium were removed by ultrafiltration followed by dialysis. The low yield (18%) was not accounted for in this study. However, the filtrate obtained upon ultrafiltration was practically negative for proteolytic enzyme activity.

Properties of the proteolytic enzyme preparation. The effect of pH on activity is shown in Fig. 2. The enzyme preparation was active from pH 6.0 to 8.0, with optimal activity between pH 6.5 to 7.5. At pH values less than 6.0 and greater than 8.0, the enzyme activity decreased rapidly. The failure of the buffer curves to overlap between pH 6 and 8 was not due to a breakdown in the buffering capacity of the phosphate buffer, since the final pH values were closely checked on replicate samples.

The optimal temperature for proteolytic activity was 35 C (Fig. 3). Enzyme activity was rapidly and irreversibly lost by mild heat treatment. Preincubation of the enzyme only for 5 min at 35 C caused a 50% decrease in proteolytic activity; preincubation at 40 C caused a 90% loss in activity. Considerable heat denaturation of the enzyme was observed at the optimal reaction temperature and suggests that the enzyme(s) was stabilized by the presence of the substrate (casein).

The proteolytic preparation displayed normal enzyme kinetics with respect to the effect of enzyme and substrate concentrations, and time, on the reaction velocity. A linear relationship was observed between enzyme concentration and the rate of proteolysis within the range studied (Fig. 4). The effect of substrate concentration on proteolytic activity is shown in Fig. 5. To ensure substrate saturation of the enzyme, a concentration of 10 mg of casein/ml reaction mixture was used in all standardized assays. The influence of time on reaction velocity (Fig. 6) showed that proteolysis proceeded at a constant rate for the first 20 min of reaction time.

Proteolysis of meat proteins. The susceptibility of various proteins, from meat and other sources, to the P. fragi extracellular proteolytic enzyme preparation is illustrated in Fig. 7. The conditions of temperature and pH used in this
**TABLE 1. Typical data for the proteolytic enzyme purification procedure**

| Procedure      | Volume (ml) | Activity (U/ml) | Total U | Protein concn* (mg/ml) | Sp act (U/mg of protein) | Yield (%) |
|----------------|-------------|-----------------|---------|------------------------|--------------------------|-----------|
| Centrifugation | 1,800       | 0.036           | 65.4    | 1.15                   | 0.04                     | 100       |
| Ultrafiltration| 180         | 0.102           | 18.4    | 1.13                   | 0.12                     | 28        |
| Dialysis       | 100         | 0.119           | 11.9    | 0.17                   | 0.70                     | 18        |

*Measured by the Lowry method; includes dipeptides and amino acid.

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assay were those previously found to give optimal activity by using casein as the substrate. Of the proteins tested, casein was the most susceptible to hydrolysis. A myofibrillar protein preparation from pig muscle was degraded more rapidly than either myosin or G-actin preparations from pig muscle. Hemoglobin was broken down more slowly than these salt-soluble muscle proteins. Finally, a pig muscle sarcoplasmic protein preparation was least susceptible to hydrolysis. Equal concentrations of protein sub-

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**FIG. 2. Effect of pH on proteolytic enzyme activity.** The buffering systems used were: citrate-phosphate, Δ; potassium phosphate, O; barbital, ●.

**FIG. 3. Effect of temperature on proteolytic enzyme activity.** Proteolytic activity as a function of reaction temperatures, ---O--; effect of preincubating the enzyme on its activity, ---O---.

**FIG. 4. Effect of proteolytic enzyme concentration on the rate of reaction.**

**FIG. 5. Effect of substrate (casein) concentration on the rate of reaction.**
strates were used in all assays. These results are subject to the limitations of the Lowry assay, i.e., proteolysis is measured by the tyrosine equivalents liberated (18). Since the relative tyrosine content of all the proteins are not available, it was impossible to correct the values shown in Fig. 7. However, the relative tyrosine values (highest to lowest) are 4.67 for casein, 3.94 for actin, 2.13 for hemoglobin, and 1.81 for myosin. Correction of these values would decrease the rate of hydrolysis for casein and actin while increasing those for myosin and hemoglobin.

Electron microscopy of enzyme-treated muscle. Figure 8 shows control muscle fragments without added proteolytic enzyme(s), whereas Fig. 9 shows similar muscle fragments after the addition of added enzyme(s) but before incubation. Comparison of Fig. 8 and 9 demonstrates that addition of the enzyme preparation did not alter the structure of the muscle at 0 h. Figures 10 and 11 show representative electron micrographs of muscle fragments after 48 and 72 h of incubation with the enzyme preparation. Electron micrographs of similar muscle fragments incubated with the enzyme preparation were also examined after incubation for 6 and 24 h. After 6 h of incubation with the added enzyme(s), only small areas of the dense Z line material showed any dissolution, but by 24 h a considerable amount of Z line material had been uniformly removed. After 48 h of incubation with the added enzyme(s), the only remaining portion of the Z line was a darkened area in the middle of the I band (Fig. 10). After 72 h in the presence of the enzyme(s), almost all of the Z line was dissolved (Fig. 11). Other parts of the myofibril did not appear to be affected by the enzyme(s).

To demonstrate that the dissolution of the Z line was not due to autolysis per se, control muscle fragments were incubated without the enzyme preparation. Figures 12 and 13 show these samples after incubation for 48 and 72 h, respectively. Neither samples show any appreciable amount of Z line dissolution, which was borne out by examination of other similar preparations with the electron microscope. These results clearly demonstrate that the added enzyme preparation was responsible for the dissolution of the Z line in muscle.

**DISCUSSION**

It has been reported (13) that elaboration of extracellular proteolytic enzymes by *P. fragi* requires the presence of organic nitrogen in the
Fig. 8. Myofibrils from control muscle fragments incubated without added proteolytic enzyme in 0.1 M phosphate buffer at 10 C for 0 h. Z, Z line; M, M line. ×24,600.

Fig. 9. Myofibrils from muscle fragments incubated with proteolytic enzyme preparation at 10 C for 0 h. Z, Z line; M, M line. ×34,900.
Fig. 10. Myofibrils from muscle fragments incubated in proteolytic enzyme preparation at 10°C for 48 h. Z, Z line; M, M line. ×32,300.

Fig. 11. Myofibrils from muscle fragments incubated in proteolytic enzyme preparation at 10°C for 72 h. ×27,600.
FIG. 12. Myofibrils from control muscle fragments after incubation at 10 C for 48 h without added proteolytic enzyme. Z, Z line; M, M line. ×17,200.

FIG. 13. Myofibrils from control muscle fragments after incubation at 10 C for 72 h without added proteolytic enzyme preparation. Z, Z line; M, M line. ×17,200.
form of amino acids or peptides in the growth medium. In the present work, proteolytic activity was induced when a mixture of amino acids and dipeptides was substituted for carbohydrate as a source of energy. Proteins were excluded from the medium to facilitate isolation of the fraction containing the proteolytic activity.

The proteolytic activity was considered to be extracellular, since it was present in a cell-free extract of the growth medium, which was prepared under mild conditions. The rapid increase in proteolytic activity during the growth phase suggested that the enzymes were secreted by living cells rather than being released into the medium by autolysis of dead cells.

Isolation and concentration of the fraction containing proteolytic activity gave successive preparations varying considerably (twofold) in specific activity. This was probably caused by fluctuations in the amount of proteolytic activity produced initially in the growth medium together with the presence of various amounts of non-enzyme protein in the preparation. Results do not indicate whether one, or more than one, extracellular proteolytic enzyme was present in the preparation.

The temperature and pH range within which the enzyme preparation displayed proteolytic activity corresponded to conditions of temperature and pH prevailing during meat spoilage. Considerable proteolytic activity was observed at 10 C, which is within the usual temperature range at which meat is frequently held. Mild heat treatment caused irreversible enzyme denaturation. It has been observed that many enzymes from psychrophilic bacteria are sensitive to heat (4). Although the proteolytic enzyme preparation was inactive at pH values associated with fresh pork (pH <5.5), it has been established that the metabolic action of P. fragi slowly raises the pH of the tissue during spoilage and that it ultimately attains an alkaline value (26). In the initial stages of bacterial growth, the pH at the meat surface, where most growth occurs, may rapidly exceed pH 6.0, thus allowing proteolytic activity to proceed. In many instances the ultimate pH of fresh meat is sufficiently high (pH >6.0) to allow P. fragi extracellular proteolytic activity to proceed without inhibition.

The salt-soluble proteins of pork muscle were rapidly hydrolyzed by the P. fragi proteolytic preparation. The reaction was carried out in a dilute phosphate buffer medium in which the salt-soluble proteins were in the precipitated or native state. The water-soluble proteins of pork muscle were not attacked as readily as the salt-soluble proteins, and of all the proteins tested, they were least susceptible to hydrolysis. Earlier observations (5, 28) showed that P. fragi caused extensive breakdown of the salt-soluble proteins of pork muscle. The present results confirm the previous findings and also demonstrate the extracellular enzymatic mechanism by which the proteins of pork are degraded during spoilage.

An increased amount of ultrastructural damage to muscle fragments incubated in the P. fragi proteolytic preparation over the 72-h period indicates that the enzyme(s) is capable of action on intact muscle proteins. These results are in agreement with previous findings where bacteria were incubated with ground muscle fragments (5). These results also support previous reports (5, 28) suggesting that P. fragi causes proteolysis of intact muscle proteins by secretion of extracellular enzymes. However, the results on purified muscle proteins indicate a greater amount of proteolysis than has been reported for ground muscle (5). This may be due to the inaccessibility of the proteins to the enzyme(s) in whole muscle. Furthermore, 10 C is not the optimal temperature for the enzyme(s). The Z line and M line have been shown to be the most susceptible portions of the myofibril to the proteolytic breakdown (8, 10), which is in agreement with the results of this study.

Dutson et al. (5) found a greater amount of proteolysis in the H zone (probably myosin) than was found in the present study. This could be due to different enzymes being added to the muscle fragments in the present study, or to the longer incubation period used by Dutson et al. (5) resulting in greater proteolysis, or both. The more prominent and distinct M line in muscle fragments incubated for 48 and 72 h (Fig. 10, 11) indicates that the proteins in the H zone were altered, allowing a greater binding of the electron-dense substances used in electron microscopy.

The onset of spoilage in pork by P. fragi has previously been shown to coincide with the production of extracellular proteolytic activity (28). In the present study, the proteolytic enzyme(s) isolated from P. fragi was shown to be active at pH and temperatures similar to those found in fresh meat, and the myofibrillar proteins from pig muscle were rapidly degraded by their action. The proteolytic enzyme(s) also caused disruption of the ultrastructure of pork muscle similar to that caused by P. fragi during spoilage (5).

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