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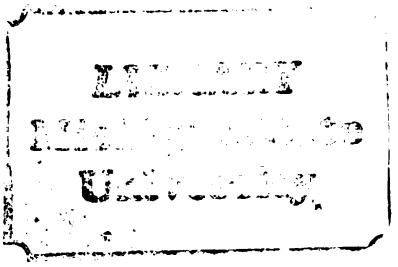

This is to certify that the

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Isolation, Purification and Immunological Localization of an Extracellular Protease Produced by *Pseudomonas fragi*

presented by

Sterling S. Thompson

has been accepted towards fulfillment
of the requirements for

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ABSTRACT

ISOLATION, PURIFICATION AND IMMUNOLOGICAL LOCALIZATION OF AN EXTRACELLULAR PROTEASE PRODUCED BY PSEUDOMONAS FRAGI

By

Sterling S. Thompson

Pseudomonas fragi ATCC 4973 is one of several pseudomonads known to produce proteolytic enzymes. This study was undertaken to determine the relationship between neutral protease production, appearance of evaginations (blebs), which have been observed on the cell wall of Pseudomonas fragi while growing in spoiled meat, and location of the protease inside the bacterial cell.

Crude protease produced by Pseudomonas fragi grown in Brain Heart Infusion broth at 10 C for 72 hours was purified 38-fold by ammonium sulfate precipitation, dialysis, and Sephadex G-100 gel exclusion chromatography. Purified enzyme was used to immunize two New Zealand white rabbits. Ten weeks after initial immunization, enzyme-linked immunosorbent assay (ELISA) indicated the anti-protease titer to be 4.0×10^7. The high titer specific antibody was subsequently used in the antiperoxidase method to localize the extracellular neutral protease in Pseudomonas fragi. Electron microscopy of cell sections stained in this manner revealed that the peroxidase-positive reaction product was located in close proximity

with the periplasmic space and with cell wall-associated evaginations. Preimmune and heterologous blood serum samples from rabbits did not show similar deposits to be present.

Blebs were not observed on Pseudomonas fragi when the organism was grown in Koser Citrate medium, nor was any proteolytic activity detected. This indicates that extracellular protease production is stimulated by meat media.

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I am grateful to my parents, my sister and other relatives for their continuous support and encouragement. Special thanks to my wife, Barbara, whose understanding, patience, encouragement, love and fortitude helped make this possible. Finally, to my sons, Garrett and Bennett, both born during the course of this project, this dissertation is dedicated.

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INTRODUCTION

Pseudomonas fragi is one of several species of *Pseudomonas* which produces proteolytic and lipolytic enzymes. Spoilage occurs when these enzymes are released by pseudomonads in meats and dairy products. Since some of the proteases and lipases produced by certain pseudomonads are heat stable, their presence in a heat processed food may eventually produce undesirable changes in the product.

The role of pseudomonads and other spoilage bacteria is well established, however the true mechanism of their action has not been completely elucidated.

The following investigation was undertaken to provide further insight into the mechanisms of bacterial spoilage of meat and meat products. The objectives of this investigation were to determine optimal conditions for the production of the neutral protease of *P. fragi*, isolate and purify the enzyme, produce antibodies to it, localize the protease in the bacterial cell at the ultrastructural level using an immunochemical technique, and correlate protease production with bleb formation and meat spoilage.

LITERATURE REVIEW

Meat Spoilage

Microorganisms Associated with Meat Spoilage

Microbial spoilage of meats continues to be a major concern to the food industry. Although complete elimination of this phenomenon is impractical and unachievable, effective control measures can be used to reduce the number and kinds of microorganisms entering meat and meat products during handling, processing and packaging. Because of its chemical properties, meat serves as an excellent substrate for microbial growth (Frazier, 1958). It is therefore essential that effective control measures be used to limit the introduction of undesirable spoilage and pathogenic microorganisms.

Spoilage of meat and other foods can be controlled by using methods that limit introduction, prevent growth and/or destroy organisms which may be present. The most effective way to control the growth of microorganisms, which enter meat during processing, is to store meat at chilling or freezing temperatures. Yet, during prolonged refrigeration meat will eventually undergo undesirable changes. Spoilage of meat is characterized by alterations in color, odor, texture, flavor and slime formation.

Spoilage of meat stored at chilling temperatures is due to the predominance of aerobic organisms on the surface of meat (Ingram and Dainty, 1971), commonly referred to as psychrotrophs (Eddy, 1960). The genus Pseudomonas is probably the most important of the genera in this group and are often associated with low temperature spoilage of meats and other products, such as poultry and fish.

Kirsch et al. (1952) examined fresh refrigerated beef for its microbial population and identified the predominant organisms as nonpigmented Pseudomonas and/or Achromobacter species. (The genus Achromobacter is now divided among the genera Acinetobacter, Alcaligenes and Moraxella.) A noticeable fading in meat surface color was also associated with an increase in the number of Pseudomonas and Achromobacter.

Pseudomonads were among the organisms identified by Lepovetsky et al. (1953) and Ayres (1955); later Halleck et al. (1958), Frazier (1958) and Weiser (1962) concluded that Pseudomonas and Achromobacter were the two predominant genera on fresh meat. Both Ayres (1955) and Lepovetsky et al. (1953) identified similar organisms in association with beef, yet their examinations of the beef for its microbial population were performed on different sections of the animal. Ayres examined the carcass surface while Lepovetsky et al. examined the lymph nodes, bone marrow and muscle tissue. Several bacterial genera were identified by

both studies as being present on beef, these included: Pseudomonas, Achromobacter, Flavobacterium, Proteus, Micrococcus, Aerobacter (Enterobacter), Escherichia, Streptococcus and Alcaligenes. A number of strains of Pseudomonas, Flavobacterium and Alcaligenes also exhibited proteolytic activity (Lepovetsky et al., 1953).

Wolin et al. (1957) compared the microflora of fresh beef prior to refrigeration with beef stored under refrigeration. Prior to refrigeration fresh beef contained Gram-positive rods as the predominant organisms. Subsequent to storage under refrigeration, the majority of the population consisted of Gram-negative rods. Pseudomonas was the dominant genus of the Gram-negative rods identified.

Brown and Weideman (1958) examined the microflora associated with chilled beef and obtained 189 isolates. Of the 189 isolates 170 were identified as pseudomonads, this represented about 90 percent of the total isolates.

Halleck et al. (1958) examined the microflora of prepackaged meat and identified two groups of bacteria during a 30 day storage period at 2 to 4 C. Nonpigmented Pseudomonas-Achromobacter type organisms were predominant during the first two to three weeks of storage, but at about day 28 P. fluorescens became the predominant organism.

Ayres (1960a) identified Achromobacter, Flavobacterium, Pseudomonas and Micrococcus as the predominant genera on

beef stored for 10 days at 5, 10 and 15 C. Some strains were theorized to be responsible for certain undesirable changes observed in the beef, such as off odor and slime formation.

Investigations concerned with establishing the identity of the microflora associated with fresh and chilled meat have concluded that Pseudomonas and Achromobacter represent the two genera most often identified. Yet the source(s) of the organisms was not well established until Ayres (1960b) presented data which suggested the origins of microflora associated with meat spoilage. According to the author these organisms originated from three primary sources: (1) entry at the time of slaughter; (2) entry via employees and cutting equipment; and (3) entry via water and air in the dressing, cooling and cutting rooms. After processing approximately 80 percent of the total microbial population consisted of sporeformers, yeasts, molds and chromogenic bacteria. Meat samples which developed undesirable changes such as off odor and surface slime were analyzed to determine which organisms were present. Gram-negative rods, most being nonpigmented, with a few producing fluorescent pigments were isolated from samples with off odor and surface slime.

Lerke et al. (1965) isolated the Pseudomonas-Achromobacter group from spoiled fish. Strains from the group were classified as either "spoilers" or "nonspoilers".

Jay's (1967) examination of the microflora in fresh and spoiled beef indicated that the genus Pseudomonas was predominant in both fresh and spoiled beef. Nine different bacterial genera were isolated from the fresh beef samples. However, only three genera other than Pseudomonas were recovered from the spoiled beef samples; these included Achromobacter, Aeromonas and Flavobacterium.

Stringer et al. (1969) investigated the changes in the microflora on beef from slaughter to retail level. The level of contaminants increased from logarithm 4.70 at slaughter to 4.78 before transport to 5.95 per square inch at the retail level. Micrococcus luteus, P. fragi and P. geniculata were the predominant organisms on the carcasses; but only the latter two were present on cuts at the retail level.

The Effect of Spoilage Bacteria on the Color of Meat

As meat undergoes microbial spoilage alterations occur in physical appearance and in chemical composition of the tissue. Jensen (1949) suggested that microorganisms oxidize meat pigments from the desirable red or bright red color to an undesirable brown color. Jensen (1945) earlier concluded that viable and nonviable microorganisms and their extracellular enzymes oxidized both fresh and cured meat pigments to methemoglobin, a dark brown compound.

Butler et al. (1953) demonstrated that there was a close correlation between meat discoloration and the number

of Pseudomonas sp. on beef cuts. Species of Pseudomonas were inoculated onto beef cuts and the inoculated samples were stored at 0, 1.1 and 4.4 C for 18 days. As the Pseudomonas population increased the percentage of metmyoglobin also increased. Discoloration was observed in all samples regardless of the storage temperature.

Costilow et al. (1955) and Robach and Costilow (1961) demonstrated that certain microorganisms and cell free extracts from some microorganisms caused discoloration of meat. Beef samples inoculated with P. fluorescens, P. aeruginosa, P. geniculata, A. liquefaciens, F. rhenanus and a yeast, Saccharomyces cerevisiae displayed surface discoloration. Samples changed from red to brown and eventually to purple. Beef samples mixed with cell free extracts from the pseudomonads exhibited similar discoloration but discoloration did not occur as rapidly as that caused by growing cultures. Since these organisms are aerobic, that is, they generate adenosine triphosphate (ATP) by aerobic respiration, the investigators concluded that as the microbial population increased on the surface of the meat the oxygen tension decreased thereby causing discoloration of the tissue. Confirmation of this theory was demonstrated when constituents such as iodoacetate and sodium malonate, which inhibit microbial respiratory activity were introduced into the system. Meat samples treated with these inhibitors showed reduced oxygen uptake

by the organisms and a low discoloration index. Greene et al. (1971) demonstrated that the addition of 30 ppm chlorotetracyline retarded meat pigment oxidation in ground meat due to inhibition of microbial growth.

Solberg (1968) also demonstrated that the growth of microorganisms on the surface of meat reduces the partial pressure of oxygen on the surface. When the partial pressure was reduced to 4 mm Hg, oxymyoglobin was rapidly oxidized to metmyoglobin. Other parameters such as pH, temperature, chemicals and light were also identified as factors which influence the color of meat.

Fellers et al. (1963) concluded that bacterial growth in meat altered fresh meat color. Beef samples stored at 32 F had a much lower microbial population after 7 days storage than samples stored at 42 F for 7 days. The samples held at 32 F also maintained the oxymyoglobin pigment as the predominant pigment during the 7 days. With the larger bacterial population more metabolic activity occurred, thereby altering muscle tissue color.

Pierson et al. (1970) observed that the number of bacteria increased substantially on beef samples stored at 3.3 C for 15 days. A population of 10^{10} organisms/cm^2 was obtained and fluorescent pseudomonads represented 80-90% of that total bacterial population. As the population increased, the color of the beef samples changed as a result of the conversion of oxymyoglobin to metmyoglobin.

Oxymyoglobin was completely oxidized to metmyoglobin on the meat surface after 5 days storage because of the activity of the aerobic bacteria.

Nicol et al. (1970) identified a green pigment in chilled beef as sulfmyoglobin. All of the isolates obtained from the samples were identified as pseudomonads. The conversion of myoglobin to sulfmyoglobin was caused by the actions of hydrogen sulfide producing bacteria. P. mephitica was suggested as the causative organism and the conditions necessary for this organism to cause the conversion included: (1) the presence of sulfur containing amino acids, (2) an oxygen tension of approximately 1 percent, and (3) meat with a pH above 6.0.

Ockerman and Cahill (1977) demonstrated that certain bacteria, under the appropriate environmental conditions, were capable of altering the normal color of beef tissue while other bacteria under similar conditions were not. Tissue samples were inoculated with P. putrefaciens, Bacillus subtilis or Leuconostoc mesenteroides and held for 21 days at 2 to 4 C. Those samples inoculated with P. putrefaciens showed the most significant change in color when compared to the other inoculated samples and the uninoculated control sample. Samples inoculated with the pseudomonad had a much lower ($P<0.05$) reflectance score than the control. The authors suggested that color deterioration was not caused by bacterial growth alone but

by pH as well. Clearly the aerobic conditions and low temperatures favored the pseudomonad, thereby contributing to the significant color alteration observed.

Bala et al. (1977a, b) reported that P. fragi altered the color of beef tissue and an aqueous beef extract at 1±1 C over a period of 20 and 10 days, respectively. Surface growth on the beef tissue increased during the 20 day storage period from log 2.2 to 7.2/cm^2. As the population increased, the pH increased from 5.5 to 6.6 and the percentages of oxymyoglobin, metmyoglobin and myoglobin also changed. Oxymyoglobin decreased from a concentration of 100 percent to 0 percent, myoglobin increased from 0 to 26.5 percent and metmyoglobin increased from 0 to about 73.5 percent. The percent concentration increases of myoglobin plus metmyoglobin equalled the percent concentration decrease of oxymyoglobin. Aqueous beef extracts showed an increase in population of log 5/ml to log 7.9/ml after 10 days storage at 1±1 C, and the pH increased from 5.5 to 6.0. The decrease in percent concentration of oxymyoglobin was about equal to the increase in the concentration of metmyoglobin, 76 percent. The authors proposed that the breakdown products, which were produced as a result of the proteolytic and lipolytic activity of P. fragi, provided a mechanism by which discoloration of meat occurred.

Contrary to the reports presented above, Lin et al. (1977) reported that neither fluorescent pseudomonads nor P. putrefaciens enhanced the oxidation of myoglobin under the conditions of their experiment. The final populations reached by these bacteria were at a high enough level whereby, based on the observations of other related studies, one would assume that alterations in the meat pigment color would have occurred on meat containing pseudomonads. No explanation was given for the lack of correlation between this investigation and the other investigations where pseudomonads were shown to be responsible for meat discoloration.

Decomposition of Muscle Proteins During Meat Spoilage

Although the role of certain bacteria in meat spoilage has been established, the exact mechanism of bacterial degradation of muscle constituents is not completely clear. Several investigations (Ockerman et al., 1963; Hasegawa et al., 1970a,b; Borton et al., 1970a) have demonstrated the most probable mechanism of microbial meat spoilage is proteolysis. Bacterial proteolytic action on muscle tissue has been demonstrated by macroscopic, microscopic, chemical and electrophoretic methods.

Jay (1966) suggested that although certain bacteria were present in spoiled meat, meat spoilage was not primarily due to microbial degradation of the primary proteins. Spoilage was apparently caused by microbial attack of

low-molecular weight components present in the sarcoplasm and/or beef cathepsins released as a result of microbial actions. Jay (1967) reported that several strains of Pseudomonas isolated from chilled beef exhibited proteolytic activity toward gelatin and litmus milk, but similar proteolytic activity was not observed when these strains were mixed with fresh beef samples. The extent to which the primary muscle proteins were attacked by those pseudomonads was minimal compared to their effect on low molecular weight substances which were present. Therefore, pseudomonads preferentially used low molecular weight constituents over larger primary proteins.

Jay and Kontou (1967) confirmed that spoilage bacteria attacked low molecular weight constituents, such as free amino acids and nucleotides in meats rather than larger primary proteins. Ground beef samples stored for 15 days at 7 C were examined for changes in protein, amino acid, nucleotide content, pH and bacterial number. Paper chromatography of samples initially inoculated with high bacterial numbers showed a dramatic decrease in amino acids; although no decrease in protein content was observed. Samples contaminated with a fluorescent pseudomonad alone or combined with an Achromobacter sp. also showed substantial decrease in amino acid content. Significant decreases were also detected in the quantity of nucleotides in samples containing the normal meat flora, the fluorescent

pseudomonad, a mixture of Pseudomonas and Achromobacter, or P. fragi. The authors concluded that the amino acids which were attacked were free amino acids and not amino acids formed as a result of protein degradation.

Lerke et al. (1967) found a similar relationship between low and high molecular weight components while examining the role of muscle protein during the spoilage of fish. Assessment of fish spoilage was based on changes in volatile reducing substances, total volatile nitrogen, trimethylamine nitrogen, protein nitrogen and nonprotein nitrogen. Analysis of two fractions, a protein and a nonprotein fraction, which were prepared from muscle press juice and inoculated with Pseudomonas spp. showed that only the nonprotein fraction exhibited characteristic spoilage. The nonprotein fraction contained low molecular weight substances which were attacked readily by the organisms whereas the protein fraction, with larger molecular weight components, did not serve as an acceptable substrate for spoilage.

Ockerman et al. (1969) compared changes in aseptically prepared beef tissue artificially contaminated with a mixed inoculum of Achromobacter sp. and Pseudomonas sp. obtained from a cutting table. Analysis of protein fractions extracted from inoculated samples revealed changes in protein concentrations while the protein composition of uninoculated samples remained the same. Sarcoplasmic,

myofibrillar and stroma proteins decreased during storage of the samples. Nonprotein nitrogen increased steadily after the first 10 days of storage.

Jay (1964a, b) suggested that measurement of the extract release volume (ERV) was a suitable mechanism for estimating the microbiological quality of beef. Beef allowed to undergo low temperature microbial spoilage consistently demonstrated a decrease in ERV. Similar decreases in ERV were detected from beef tissue mixed with two known protease enzymes. Thus, the results suggested that decline in ERV was due to the proteolytic activity of spoilage microorganisms. Jay (1964b) observed variable declines in ERV of beef tissue inoculated with either E. coli, Proteus vulgaris or B. subtilis. B. subtilis was the most proteolytic of the three organisms, under the conditions of the experiment, since greater reductions in ERV were observed in tissue contaminated with this organism. Borton et al. (1968) reported similar reductions in ERV of contaminated porcine muscle tissue. Tissue inoculated with a microbial inoculum prepared from fresh pork showed a rapid decrease in ERV during a 9 day storage period. These data again suggested that ERV may be influenced by microbial proteolytic actions.

The literature published during the 1960's did not establish agreement as to the fate of muscle proteins in the presence of certain spoilage organisms.

Hasegawa et al. (1970a, b) reported two investigations in which several bacteria were separately mixed with muscle tissue aseptically removed from pigs and rabbits. Comparing the starch-gel patterns of uninoculated and inoculated samples, the authors observed that some organisms attacked the sarcoplasmic and urea-soluble proteins while other microorganisms showed little or no detectable affect on those proteins. The organisms examined in one study (Hasegawa et al., 1970a) included: P. fragi, L. mesenteroides, Pediococcus cerevisiae and Micrococcus flavus. Electrophoretic gel patterns of samples inoculated with each organism indicated that all of these organisms were able to hydrolyze certain sarcoplasmic proteins in porcine and rabbit tissue. P. fragi caused the most extensive protein breakdown of the four microorganisms. One surprising phenomenon observed in the study was that none of the organisms attacked the same sarcoplasmic proteins in rabbit and porcine tissue. For example, P. fragi attacked specific sarcoplasmic proteins in porcine tissue, however a similar sarcoplasmic protein degradation pattern was not observed in rabbit tissue. Electrophoretic patterns also indicated that the urea-soluble proteins were hydrolyzed by P. fragi and P. cerevisiae but not by M. luteus or L. mesenteroides. In a related study, Clostridium perfringens, Salmonella enteritidis, A. liquefaciens, Streptococcus faecalis and Kurthia zophii were tested for their proteolytic activity (Hasegawa

et al., 1970b). C. perfringens attacked the sarcoplasmic and urea-soluble proteins of porcine muscle while S. enteritidis and S. faecalis altered only myoglobin in the sarcoplasmic fraction. None of the other organisms exerted demonstrable proteolytic action on the sarcoplasmic proteins.

Rampton et al. (1970) used A. liquefaciens, M. luteus, P. cerevisiae, P. fluorescens, S. faecalis and a mixed inoculum obtained from hamburger to determine their effect on the myofibrillar proteins of pig and rabbit muscle. Gel electrophoretic patterns of the tissues indicated that none of the organisms were able to significantly alter myofibrillar proteins in either rabbit or pig muscle. The investigations of Rampton et al. (1970) and Hasegawa et al. (1970a, b) suggested that certain organisms attacked sarcoplasmic, urea-soluble and myofibrillar proteins while others could not.

Borton et al. (1970a) analyzed four fractions (salt soluble, insoluble, water soluble and non-protein nitrogen) from porcine muscle, which were inoculated with either P. cerevisiae, M. luteus, L. mesenteroides or P. fragi, for changes in protein solubility. Samples inoculated with P. fragi showed an increase in the water soluble and non-protein fractions and a decrease in the salt-soluble and insoluble protein content suggesting that P. fragi is proteolytic. Borton et al. (1970b) used starch-urea gel and disc-urea gel electrophoresis to examine the proteolytic

activity of other microorganisms and to confirm their previous observation of proteolytic action of P. fragi. Gels prepared of the salt-soluble protein extracts from tissue inoculated with P. fragi showed losses of a number of protein bands. Thus, P. fragi demonstrated proteolytic hydrolysis toward the myofibrillar proteins. Although Rampton et al. (1970) published data that indicated micro-organisms had no measurable effect on the myofibrillar proteins from pig or rabbit muscles, P. fragi was not one of the test organisms.

Tarrant et al. (1971) reported data, which correlated with that of Borton et al. (1970a, b). A significant amount of proteolytic activity was detected in pork extracts which were inoculated with P. fragi and stored for 20 days at 10 C. Disc gel electrophoresis was employed to determine changes in the pattern of the myofibrillar proteins. Following the incubation period many faint bands appeared on the gels suggesting hydrolysis of the larger proteins to several smaller subunits. In addition to degradation of the myofibrillar proteins, an increase in non-protein nitrogen content, which was composed of ammonia and peptides, was observed.

The effect of microbial cultures upon the primary proteins in beef slices was determined by Dainty et al. (1975). Beef slices were inoculated with a mixed microflora prepared from beef slime, natural beef flora or 12 bacterial

pure cultures commonly isolated from beef. Off odor and slime were observed in samples inoculated with the beef slime culture on day 7, but no evidence of protein degradation was observed, however proteolysis was markedly evident after 11 days of storage. Gel electrophoretic patterns of the samples showed that several bands representing the sarcoplasmic fraction which were evident on day 0 were less evident following storage. Bands of the myofibrillar fraction were no longer visible. Samples which spoiled as a result of the activity of the natural flora did not develop off odor and slime until day 12, however proteolysis was still not noticeable. Several bands disappeared and two new bands appeared on gels prepared from this sample. Of the 12 bacteria inoculated, the pseudomonads produced the most extensive breakdown of the sarcoplasmic and myofibrillar protein fractions.

Electron micrographs prepared of pig muscle inoculated with P. fragi supported the conclusion that microbial proteolytic activity was responsible for meat spoilage (Dutson et al., 1971). The muscle tissue showed marked alterations of the myofibrils due to growth and activity of P. fragi including: (1) loss of dense material typical of the Z line; (2) substantial loss of material in the H zone; and (3) marked disruption of the A band.

Tarrant et al. (1973) treated several pig muscle protein fractions and ground muscle tissue with a proteolytic

fraction obtained from P. fragi to determine the susceptibility of the preparations to the proteolytic fraction. Myosin and G-actin preparations were degraded but not as rapidly as the salt-soluble (myofibrillar) protein preparation. Pig muscle sarcoplasmic protein fraction was only mildly effected. Electron micrographs of the treated ground tissue showed that alterations in the tissue occurred after as little as six hours incubation. As incubation time increased so did the amount of muscle tissue disruption. After 72 hours the Z line was almost completely dissolved. No other components of the myofibril appeared to be affected by the enzyme preparation.

Proteolytic activity was detected on day 11 in porcine tissue which was inoculated with a pure culture of P. perolens and incubated at 10 C (Buckley et al., 1974). Proteolytic activity coincided with changes in pH and increases in the population of the organism. Tissue samples inoculated with the culture also showed changes in protein solubility. Sarcoplasmic protein solubility decreased, myofibrillar protein solubility increased and non-protein nitrogen increased slightly. Electron micrographs of tissue treated with an enzyme preparation obtained from P. perolens showed changes in ultrastructure similar to the alterations reported by Dutson et al. (1971) and Tarrant et al. (1973). As storage time increased changes in tissue ultrastructure became more apparent. The Z line and M line

were completely dissolved and density in the A bands decreased.

More recently Bala et al. (1979) demonstrated that a purified proteolytic preparation from P. fragi caused degradation of water-soluble proteins of a beef extract. Data are presented which imply a significant correlation between non-protein nitrogen increases and absorbance at 280 nm. According to the authors these changes resulted from increases in both peptides and free amino acids which were liberated by the hydrolysis of proteins. SDS-polyacrylamide gel electrophoresis confirmed their conclusions; protein bands which were observed in the untreated control extract were missing from gels prepared of the enzyme treated extract.

Extracellular Proteolytic Enzymes

Proteases Produced by Pseudomonads

The proteases that are used in the food industry are primarily of microbial and plant origin. Isolation, purification and characterization of the proteases from various microorganisms have received a great deal of attention in recent years. Exoenzymes produced by various yeasts, molds and bacteria were reviewed by Pollock (1962) and Fogarty and Kelly (1979). Bacterial proteases produced by psychrophiles (Nunokawa and McDonald, 1968), by mesophiles (Rappaport et al., 1965; Feder and Lewis, 1967;

Fabian, 1970), and by thermophiles (Ohta *et al.*, 1966) have been described.

Keay (1971) classified microbial proteases as acid, alkaline, or neutral proteases based on the pH optima of the isolated enzyme. Hartley (1960) earlier classified proteases into four groups based on their mechanism of action: serine, thiol, acid, and metal proteases. Morihara (1974) reclassified proteases according to their side chain specificity, suggesting a classification based on specificity rather than biological activity is more acceptable.

The proteases produced by species of *Pseudomonas* are perhaps most important for both the meat and dairy industries. Pseudomonads are the most common contaminants isolated from refrigerated meat and several reports have demonstrated the undesirable effect of heat stable proteases produced by *Pseudomonas* species (Mayerhofer *et al.*, 1973; Adams *et al.*, 1975, 1976; Gebre-Egziabher *et al.*, 1980). According to Adams *et al.* (1975), these proteases survive temperatures above pasteurization and eventually cause bitter flavor, clearing, or coagulation in the milk during storage.

Extracellular proteases have been reported for several *Pseudomonas* species, such as *P. myxogenes* (Morihara, 1956), *P. putrefaciens* (Vanderzant, 1957), *P. fluorescens* (Peterson and Gunderson, 1960), *P. aeruginosa* (Morihara, 1963; Morihara et al., 1963; Morihari and Tsuzyki, 1964;

P. fragi (Juffs et al., 1968; Murayama et al., 1969; Tarrant et al., 1971; Tarrant et al., 1973; Porzio and Pearson, 1975; Bala et al., 1979), P. maltophilia (Boethling, 1975), P. perolens (Buckley, 1972). In the report presented by Juffs et al. (1968), several proteolytic Pseudomonas species are listed; proteolytic activity was influenced by various conditions of temperature and media composition. Elaboration of these extracellular proteases seemed to require the presence of organic nitrogen in the form of amino acids and/or dipeptides in the growth medium.

The proteolytic enzymes produced by Pseudomonas species are classified into two groups, either alkaline or neutral proteases. This particular classification is based on the optimum activity of the enzymes as influenced by pH. Neutral proteases are of primary concern for the meat and dairy industries because of the actions these proteases exhibit toward constituents in meat and dairy products. The pH optimum for neutral proteases is between 5.0 to 7.5 and many are activated by one or more metal ions, e.g., Zn^{++}, Ca^{++}, etc. These metal activated proteases are generally referred to as metalloenzymes; their activity depends on the presence of a metal which can be tightly or loosely bound. Removal of the metal by certain metal-chelating agents, such as ethylenediaminetetraacetate (EDTA) reduces the proteolytic activity.

There are several studies which demonstrate the activation or stabilization of a few proteases by one or more metal ions (Morihara, 1959a, b, 1960; Barach et al., 1976; Porzio and Pearson, 1975). Barach et al. (1976) reported that a protease produced by a psychrotrophic pseudomonad was stable to heating at ultra high temperature in the presence of calcium.

Most of the investigations regarding synthesis of extracellular proteases and other bacterial extracellular enzymes have been concerned with those physiological or environmental factors which trigger enzyme production; synthesis of many of these enzymes is affected by the nutritional composition of the growth medium. End product inhibition and catabolite repression appear to control the synthesis of a number of the extracellular enzymes (Glenn, 1976).

Detection, Isolation and Characterization of Protease(s) Produced by P. fragi

As previously noted, there are a number of investigations (Hasegawa et al., 1970a,b; Rampton et al., 1970; Borton et al., 1970a,b) which establish a correlation between hydrolysis of meat proteins and proteolytic enzymes produced by P. fragi and other psychrotrophic bacteria. However, relatively few articles have been published concerning isolation, purification and/or characterization of the protease(s) produced by this organism.

Juffs et al. (1968) identified P. fragi as one of several proteolytic Pseudomonas species. The proteolytic activity produced by the pseudomonads was influenced by incubation temperature and nutrient composition of the media. Proteolytic activity appeared to be more dependent upon temperature and nutrient content rather than upon growth yield of the cultures. The organisms were grown at two different temperatures, 28 or 3 C, in three different media, Peptone-Yeast Extract (P.Y.E.) broth, Koser's Citrate medium or Koser's Citrate medium plus 0.25% (w/v) Hammersten casein. More proteolytic activity was produced by P. fragi in P.Y.E. at 3 C than in the same medium at 28 C. Proteolytic activity was initiated during early logarithmic growth phase. The authors suggested that elaboration of the proteinases required the presence of organic nitrogen in the form of amino acids or peptides in the growth media. This was confirmed when no proteolytic activity was detected in a mineral salts medium containing inorganic nitrogen with either citrate or citrate and casein. The latter medium containing 0.25% casein provided a nitrogen source, however the level was probably not sufficient enough to induce proteinase production.

Tarrant et al. (1973) obtained a proteolytic preparation from a medium in which P. fragi had been grown. The medium contained 11 amino acids and 2 dipeptides. Experimental results indicated that proteolytic enzyme(s)

was excreted during the late exponential growth phase and degraded during the stationary phase. P. fragi was grown in the medium at 10 C over a time of approximately 100 hours, growth and enzyme production were followed under those conditions. When the organism was grown in Koser Citrate medium, under similar conditions, no proteolytic activity was detected which agreed with the conclusions of Juffs et al. (1968). Tarrant et al. (1973) also established two previously unknown characteristics of the enzyme(s). Enzyme activity was observed between pH 6.0 to 8.0, optimal activity was between pH 6.5 to 7.5. Enzyme activity decreased substantially at 40 C, 35 C was the optimal temperature at which proteolytic activity was detected. With an optimal activity between pH 6.5 to 7.5 the enzyme would be classified as a neutral protease. Examination of the susceptibility of casein and hemoglobin to hydrolysis by the protease indicated that casein was more susceptible than hemoglobin.

Porzio and Pearson (1975) further characterized properties of the extracellular protease produced by P. fragi in a growth medium containing casein supplemented with L-tryptophan and cysteine HCl; culture plus medium were incubated at 10 C for 50 to 60 h. Using a four-step purification scheme the enzyme was purified and several characteristics were identified. A homogenous proteolytic enzyme was obtained that was optimally active between pH

6.5 to 8.0 and up to 40 C, had a molecular weight between 40,000 to 50,000, had an isoelectric point of 5.2, was zinc activated and calcium stabilized.

More recently Bala et al. (1979) isolated and partially purified an extracellular protease produced by P. fragi grown in Brain Heart Infusion (BHI) broth at 21 C for 72 hours. Following a four-step purification scheme a purified fraction was obtained. Polyacrylamide disc gel electrophoresis indicated a single band. However, the authors concluded that the protease was mixed with a lipase which had similar electrophoretic mobility, accounting for only the single band on the gels. But according to Nashif and Nelson (1953) P. fragi does not produce lipolytic activity above 15 C.

Ultrastructural Studies of Bacteria

Dutson et al. (1971) used the transmission electron microscope to determine whether structural alterations occurred in the muscle proteins as a result of the presence of high numbers of P. fragi. Ultrastructural changes were observed in spoiled tissue, in addition some unusual structures were observed on the outer cell wall of P. fragi and in the area surrounding the cells. The structures were referred to as "bleb-like" evaginations or protrusions. Descriptively, the blebs contained dense granular material surrounded by a continuous membrane quite similar

to the granular material and membrane associated with the normal bacterial cell wall. In the surrounding medium, circular structures or globules which were not attached to the cell wall were observed. These globules appeared to have structural composition similar to the attached blebs. P. fragi was grown in All-Purpose Tween (APT) broth and a nonprotein containing medium to determine if bleb formation was restricted to the meat tissue. Electron micrographs of cells grown in the two media did not show blebs or globules similar to those observed attached or unattached to cells grown in meat tissue. This suggested that the organism either required meat tissue as a substrate or certain components present in the tissue, such as muscle proteins, for bleb formation. In addition, no proteolytic activity was detected when the cell free media were analyzed. Since neither blebs nor proteolytic enzyme(s) were detected in the two media, the authors concluded that the blebs may function as a mechanism which allowed P. fragi to secrete proteolytic enzymes capable of inducing myofibrillar disruption.

Recently, Wing et al. (1983) observed bleb-like structures on cell wall surfaces of P. fragi which were inoculated into bovine muscle and incubated at 21 C for greater than 5 days. Protrusions were observed on micrographs taken by both the scanning and transmission electron microscopes. Blebs were not detected on the cell during

the first 3 to 4 day incubation period. The authors also described the presence of coiling glycocalyx material. It was suggested that the glycocalyx material mediated cell-to-cell and bacterial cell-to-muscle attachment in the muscle. Since there appeared to be close association between bleb formation and glycocalyx formation it was hypothesized that these two structures might: (1) function to bring nutrients to the bacterial cell; (2) allow the organism to adhere to muscle tissue surface; and/or (3) concentrate and conserve digestive enzymes released by P. fragi.

Bleblike evaginations are not unique to P. fragi; similar structures have been observed on several bacteria which were exposed to various nutritional and/or environmental conditions. Knox et al. (1966) and Work et al. (1966) observed blebs on a lysine-requiring mutant of Escherichia coli ATCC 12408. This organism lacks the enzyme which decarboxylates diaminopimelic acid (DPA) to lysine. E. coli was grown in a salts-glycerol medium containing either suboptimal or above optimal levels of lysine; the authors were primarily interested in the response of E. coli to changes in nutritional composition of the medium. Changes in nutrient content will directly influence metabolism, different end products will be produced and this may cause structural alterations in a microorganism. When E. coli was grown in a lysine limiting

environment high levels of (DPA) and a lipopolysaccharide-lipoprotein complex were detected. Cells obtained from this environment and observed with the electron microscope showed noticeable small blebs attached to the outer cell wall surface. Globules unattached to the cell were also observed in close proximity to the cells. Bleb formation was detected after 8 hours of growth. As incubation time increased to 26 hours, the number of blebs on the cells increased correspondingly. Increases in the number of blebs were correlated to increases in extracellular end product production. Both the blebs and globules were surrounded by the outer triple-layered membranes which were continuous with the outer triple-layered membrane of the cell wall. Because there appeared to be an apparent correlation between bleb formation and production of high levels of extracellular DPA and the complex, the authors suggested that the extracellular material was produced by the blebs on the cell wall surface.

Bayer (1967) and DePetris (1967) also observed bleb-like structures on the surface of E. coli, but not the same strains. DePetris (1967) observed blebs on the surface of cells which were heated with temperatures between 75 to 100 C. Blebs were not located on the entire cell surface but rather on a limited region of the cell envelope. Bayer (1967) described finger or drumstick-like extrusions randomly attached to the cell wall surface of

E. coli when changes in osmotic pressure occurred. As a result of changing the osmolarity of the medium from 50 to 3 percent sucrose bleb formation occurred. Cells in the logarithmic growth phase contained many more blebs than cells in the stationary phase. The author suggested that bleb formation on the osmotically shocked cells was caused by the escape of cytoplasm through pores in the elastic cell wall layer. As the cytoplasm escaped through the pores it caused expansion or ballooning of the multi-layered wall.

Scheie and Ehrenspeck (1973) observed one to two very large blebs on the surface of cells of an E. coli strain which was heated to temperatures above 50 C for several minutes. However, unlike the investigation of DePetris (1967) observations were made with the phase contrast microscope rather than the electron microscope. The blebs were extremely large, representing 10 to 50 percent of the total surface area of the cell, spherical and usually no more than one or two per cell. The outer layers of the blebs were similarly composed of the outer portion of the cell envelope. Since the organism lost its ability to form colonies after heat treatments above 50 C and blebs were formed on the cells exposed to those temperatures, the authors concluded that bleb formation was related to thermal inactivation.

Wiebe and Chapman (1968a, b) compared the structure of marine pseudomonads and achromobacters isolated from waters off the Oregon-Washington coast. One distinct difference observed on the electron micrographs of the isolates was that many of the cell wall surfaces of the Pseudomonas strains contained blebs or evaginations while the Achromobacter strains did not. In order to determine whether structural characteristics were associated with the natural environment; organisms were grown in laboratory media varying in composition and incubated at different temperatures. A strain of each genus was grown at 10 and 22 C in a low nutrient and a high nutrient medium, 0.1% and 1.0% peptone, respectively. The combinations of temperature and nutrient content used were the following: 10 C, 0.1% peptone; 10 C, 1.0% peptone; 22 C, 0.1% peptone, 22 C, 1.0% peptone. Blebs were only observed on the cell wall surface of the Pseudomonas strain and they were produced only when this organism was grown at 22 C in 1.0% peptone. Structural composition of the blebs was similar to that observed in earlier investigations. The multilayer cell wall was continuous with the blebs suggesting that the cell wall was involved with bleb formation. It was concluded from experimentation that with the pseudomonads examined; bleb formation was influenced by nutritional and/or environmental parameters.

Koike et al. (1969) while examining the effect of various concentrations of three antibacterial agents, polymyxin B sulfate, colistin sulfate or colistin methanesulfonate, on E. coli B and P. aeruginosa P29, observed blebs protruding from their cell walls. E. coli cells treated with 25 µg polymyxin B sulfate per ml contained numerous blebs which appeared to originate from the multilayer cell wall. P. aeruginosa cells treated with 25 µg polymyxin sulfate B or 250 µg colistin methanesulfonate per ml contained similar blebs on the surface. As the concentration of the antibacterial agent was decreased, the number of blebs forming on the cell surface also decreased with both organisms.

Hitchins and Sadoff (1970) observed blebs or protrusions on the cell wall surface of Azotobacter vinelandii. Encystment was induced in the organism when grown in Burk's nitrogen-free medium supplemented with 0.2% β-hydroxybutyrate as a source of carbon. The authors suggested that the blebs played a role in forming the capsules of encysting cells. Oppenheim and Marcus (1970) also observed blebs attached to the cell wall surface of A. vinelandii when it was grown in Burk's medium supplemented with different nitrogen sources.

Immunocytochemical Localization of Antigens at the Electron Microscope Level

Immunocytochemistry as a research tool allows the specific examination of cellular structure within complex biological systems. Antibodies or antibody fragments labelled with specific markers can be observed by either light or electron microscopy (Short and Walker, 1976). Fluorescent-labelled proteins were used with the light microscope, but with the introduction of ferritin antibody labelling techniques, proteins can now be observed with the electron microscope. Recently, improved markers such as enzyme-conjugated antibodies and enzyme-antibody complexes have been developed which are subject to fewer limitations than those related to ferritin.

Labelled antibody techniques have helped establish some of the antigenic differences between the vegetative cells of spore-forming bacteria and the spores they produce. Walker and Batty (1964) initially used light microscopy and fluorescent labelled antibodies to follow the antigenic alterations on the surface of C. sporogenes during sporulation and germination. Later, Walker and Batty (1965) followed antigenic changes on the surface of B. cereus var terminalis during sporulation and germination. Although fluorescent labelling provided information about some structural and/or chemical differences between vegetative cells and their corresponding spores, the

method had limitations. Although it could be assumed that subcellular changes were occurring simultaneously, only surface antigenic changes were detected.

Combining electron microscopy with ferritin-labelled antibody appeared to be a promising research tool since it allowed subcellular observations. The ferritin-labelled antibody method was introduced as a method which conferred electron density upon an antibody molecule making it applicable to electron microscopy, but without inactivating the antibody (Singer, 1959). In order to combine ferritin labelling with electron microscopy two procedures were proposed (Singer and McLean, 1963). One method proposed the pre-embedding staining technique in which the specimen was mixed with ferritin labelled antibody before embedding. The second proposal used post-embedding staining, the specimen was embedded and ultra-thin sections were obtained for treatment with ferritin labelled antibody. Walker et al. (1966) were more successful with pre-embedding staining than post-embedding staining because with the latter method ferritin was absorbed nonspecifically by the embedding medium. Using the pre-embedding staining method and ferritin labelling vegetative antigens on the surface and along the cortical membrane of B. cereus var terminalis were localized. Intracellular vegetative antigens were localized in disintegrated spores of B. cereus var terminalis using the pre-embedding staining techniques (Thomson

et al., 1966). Ferritin labelling was also used to locate vegetative antigens in spores of B. subtilis. Ferritin labelled antibody to the vegetative cell was located on the surface of the vegetative cell and ferritin labelled antibody to the spore was located around the spore coat (Walker et al., 1967b). Antigens associated with C. sporogenes, C. bifermentans and C. sordelli were identified using ferritin labelling (Walker et al., 1967a). Ferritin labelled antibodies to vegetative cells of the first two clostridia were located on the surface of the vegetative cells only. Similar results were not obtained with C. sordelli. Ferritin labelled antibodies to spores of C. sporogenes and C. sordelli were located around the exosporium of the spore. Similar results were not obtained with C. bifermentans.

Walker and Thomson (1972) discussed three techniques which could be used to locate antigens with labelled antibodies. These techniques included use of heavy metal-labelled antibody, ferritin-labelled antibody and enzyme labelled antibody. The enzyme-labelled antibody technique was introduced by Nakane and Pierce (1966, 1967), Avrameas and Uriel (1966) and Avrameas (1969) as a method with several advantages over both ferritin- and fluorescent-labelled antibody techniques. Acid and alkaline phosphatase or horseradish peroxidase are coupled to antibodies and the resultant conjugates allowed to react with the antigen.

The antigen-antibody-enzyme complex is then localized by exposure to substrate. Since the enzyme-labelled antibody complex has a much lower molecular weight than ferritin-labelled antibody, it easily penetrates tissue or cells thereby allowing detection of intracellular antigens. Immunoglobulins in tissue were located using enzyme-labelled antibodies (Leduc et al., 1969). Viral antigens were located using conjugates of enzyme-labelled antibodies, light and electron microscopy (Wicker and Avrameus, 1969; Leduc et al., 1969); satisfactory and reproducible results were obtained, but at times the results were irregular. Partial success with locating bacterial antigens on thin sections was achieved, but results were inconsistent (Walker et al., 1971). Lack of consistency was attributed to a possible reduction in sensitivity due to inactivation of the antibody during labelling. The enzyme labelling technique was described as flawed because the labelling moiety was a protein therefore antigenic and it might bind indirectly to the tissue antigen when combined with its own antibody (Mason et al., 1969a). Since an unlabelled antibody technique did not have this problem, the authors suggested it may be a better method to pursue. Mason et al. (1969b) used an unlabelled antibody technique to locate human growth hormone and human chorionic gonadotrophin in the adeno-hypophsis and placenta, respectively. An enzyme,

horseradish peroxidase was combined with a tissue antigenic site via the antigen-antibody reaction of an immunoglobulin-enzyme bridge.

Several investigations have demonstrated that antigen detection could be enzymatically intensified without using labelled antibodies (Mason et al., 1969a, b; Sternberger, 1969; Sternberger and Cuculis, 1969). Sternberger et al. (1970) successfully demonstrated a method which incorporated the soluble peroxidase-anti-peroxidase (PAP) complex combined with unlabelled antibodies. Taylor et al. (1978) and Sternberger (1979) have presented two reviews discussing applications of immunochemistry, specifically the unlabelled antibody PAP method. Moriarity and Halmi (1972) modified this method and successfully located the adrenocorti-cotropin (ACTH)-secreting cell in the anterior pituitary lobe of the rat with electron microscopy. A combination of unlabelled antibody with the PAP complex provided both sensitivity and specificity. Hinton et al. (1973) illustrated that the unlabelled antibody enzyme was so sensitive and specific that it could detect the reaction of antibody with single antigenic sites on the surface of sheep erythrocytes. The sensitivity of the unlabelled antibody PAP method was compared with that of radioimmuno-assay (Moriarity et al., 1973) and under the conditions of the experiment, was more sensitive. In fact, it detected antibodies in sera of low titer and/or avidity

which were not detected by radioimmunoassay. In a similar comparative investigation, Petrali et al. (1974) evaluated the sensitivity of the unlabelled antibody PAP method. The staining intensity of the technique was evaluated in the presence or absence of the PAP complex. More sensitivity was achieved with the PAP complex present than with it absent. In addition the method was 16,000 to 100,000 times more sensitive than radioimmunoassay, confirming earlier results published by Moriarty et al. (1973).

Until the development of the peroxidase labelling technique accurate detection of subcellular bacterial antigens, without disruption of the intact cell or spore, by other methods was difficult. The PAP method was used to identify vegetative and spore antigens of B. cereus during germination and sporulation, enterotoxin in sporulating cells of C. perfringens type A, and crystalline protein antigen of B. thuringiensis during sporulation (Walker et al., 1975). Cellular disruption is unnecessary when the PAP method was used and non-specific labelling of embedding material was minimal. The PAP method has been described as a consistent, reliable and sensitive labelling technique (Hinton et al., 1973; Moriarity et al., 1973; Petrali et al., 1974; Sternberger, 1979). It has been successfully combined with electron microscopy procedures such as fixation, dehydration and embedding. Spore antigens of B. cereus var terminalis were localized in

the cytoplasm of vegetative cells during stages of sporulation. Vegetative cell antigens were located on the cell wall and in the cortical region during sporulation (Short and Walker, 1975).

EXPERIMENTAL

Chemicals and Materials

Brain Heart Infusion (BHI) broth, Plate Count Agar (PCA), Koser Citrate medium, and Special Agar-Noble were obtained from Difco Laboratories. Spectraphor membrane tubing 1 and 2, molecular weight cutoff of 6,000 to 8,000 or 12,000 to 14,000 respectively, were purchased from Fisher Scientific. The gel filtration medium, Sephadex G-100 and the electrophoresis calibration kit for molecular weight determinations were obtained from Pharmacia Fine Chemicals. Trizma Base, thimersosal, coomassie brilliant blue R-250, Freund's Adjuvant incomplete, 2,2'-azino-di-3-ethyl-benzthiazoline-6-sulfonate (ABTS), 3'3 diamino-benzidine (DAB), polyethylene glycol (Carbowax, 20,000) and polyoxyethylene sorbitan monolaurate (Tween 20) were purchased from Sigma Chemical Co.

Chemicals used for electrophoresis were obtained from several companies: Acrylamide-MCB Manufacturing Chemists; N,N' methylenebisacrylamide - Miles Laboratories Inc; N,N,N',N tetramethylethylenediamine (Temed) - Canal Industrial Corp.; Ammonium persulfate - E-C. Apparatus Corp.; Dodecyl sodium sulfate (SDS) and trichloracetic

acid (TCA) - Mallinckrodt.

Chemicals used to prepare cells for observation with the electron microscope were obtained from Ernest F. Fullam, Inc. and Ladd Research Industries, Inc. The peroxidase-anti-peroxidase (PAP) kit was purchased from Cappel Laboratories, Inc. Casein Hammersten was obtained from ICN Nutritional Biochemicals. Phenol reagent Folin and Ciocalteu were acquired from Harleco,

Equipment

Koser citrate medium and BHI broth which were inoculated with P. fragi were agitated by a New Brunswick Scientific (NBS) temperature controlled gyrotory shaker and a NBS nontemperature controlled rotatory shaker. Centrifugation of both the Koser citrate medium and BHI broth was performed in a Sorvall model RC-2 centrifuge. Centrifugation of cells during preparation for transmission electron microscopy was performed in a Sorvall superspeed centrifuge type SS-1.

A Fischer and Porter 2.5x40 cm glass column was used for gel filtration. Eluates were monitored at 280 nm with a Gilson Model UV-280 IF ultraviolet Absorption Meter. Samples were collected with a LKB 7000 UltraRac fraction collector, and recorded with a Sargent recorder, model SR. A Beckman DB-G and a Bausch & Lomb Spectronic 20 were used for all spectrophotometric measurements.

A Buchler Instrument apparatus and a Bio-Rad electrophoresis cell model 155 were used for electrophoresis. Two Heathkit power supplies were used for all disc and SDS gel electrophoresis runs.

For growth studies of P. fragi, all pour plates were incubated at 25 C in a Lab-Room controlled environmental incubator manufactured by Lab-line Instruments, Inc. Pure cultures of P. fragi and purified protein solutions were lyophilized on a Virtis laboratory lyophilizer.

Agar encapsulated cells, prepared for electron microscopy, were continuously mixed in various solutions with a rotatory shaker manufactured by Ernest F. Fullam, Inc. Trimmed blocks were sectioned with a Sorvall MT-2 ultramicrotome, thin sections were observed and photographed with the Philips EM 201 electron microscope. ELISA plates were read with a Minireader model MR 590 produced by Dynatech, the strips and holder were also produced by Dynatech.

Culture Methods

A lyophilized culture of P. fragi was obtained from University of Missouri-Columbia, Department of Food Science and Nutrition. Small particles from the dried pellet were aseptically removed, suspended into 10 ml of sterile Koser Citrate medium and incubated at room temperature for 48 h during which time it became turbid. Subsequently, stock

cultures were prepared by inoculating several test tubes containing 10 ml of sterile Koser citrate with 0.1 to 0.2 ml from the growing culture, tubes were incubated at room temperature until slight turbidity was observed. The tubes were then stored at -20 C for later use if needed.

Cultures used continuously during experimentation were held at room temperature in sterile Koser Citrate. An active culture was maintained by weekly transfers. The culture was routinely checked for purity by trisector streaking one loopful of the culture to PCA, plates were observed after incubation for contamination.

Enrichment

One tenth ml of a growing culture was pipetted into a 250 or 300 ml Erlenmeyer flask containing 100 ml of sterile Koser citrate. The flask was incubated at 23-25 C for 24 h on a gyrotory shaker operating at 170 rpm. The turbid medium which was obtained was poured into a sterile 250 ml plastic centrifuge bottle and centrifuged at 4000 xg for 20 min in a Sorvall RC-2 centrifuge. The resulting pellet was resuspended in 10 ml 0.1 M Tris-HCl, 0.0045 M $CaCl_2$ buffer pH 7.4 (Tris-$CaCl_2$). A 1% inoculum from the redissolved pellet was added to a new, sterile 250 or 300 ml Erlenmeyer flask containing 100 ml of Koser Citrate and shaken for 24 h at 23-25 C. Following incubation, the bacterial cells were again separated from the growth

medium by centrifugation. The cells were resuspended in 80 ml Tris-$CaCl_2$ buffer. A 1% inoculum of this redissolved pellet was transferred aseptically to a 4 liter Erlenmeyer flask containing 1500 ml of BHI broth pH 7.4 ± 0.2. The flask was stoppered with a cheese cloth covered cotton plug, placed on a rotatory shaker which was located in a 10 C walk-in refrigerator and shaken at 200 to 230 rpm.

Subsequent to inoculation and prior to incubation at 10 C, on the shaker, an aliquot of the BHI was serially diluted using sterile 99 ml phosphate buffered dilution blanks. Duplicate PCA pour plates were prepared from appropriate dilutions. At 12 h intervals, up to 108 h an aliquot of BHI broth was serially diluted and duplicate plates prepared as above of the appropriate dilutions. All plates were incubated at 25 C for 48 h. After incubation colonies on representative plates were counted using a Quebec colony counter and the standard plate count determined according to the standard plate count procedure (Clark et al., 1978).

Enzyme Assay

Proteolytic activity was determined according to the method of Hagihara et al. (1958) with modifications as suggested by Nakajima et al. (1974). A 2% solution of Hammersten casein made up in 0.1 M Tris-HCl pH 7.2 was used as the substrate. One milliliter of casein solution

was added to 1 ml of enzyme solution, the reaction was allowed to proceed for 24 h at 21 C. The reaction was stopped by adding 2 ml of precipitation reagent - a mixture of 0.33 M acetic acid, 0.22 M sodium acetate and 0.11 M trichloroacetic acid. After standing for 30 min the solution was filtered through Whatman No. 1 filter paper. To 1 ml filtrate was added 5 ml 0.4 M sodium carbonate and 1 ml diluted (1:4 v/v) Folin Ciocalteau's Phenol reagent. After color development for 30 min, absorbance was measured at 660 nm. Assay blanks were prepared similarly, however the casein solution was not added to the enzyme solution until after the precipitation reagent. The activity was determined from a standard curve for tyrosine, converting absorbance to tyrosine equivalent, thus activity was expressed in terms of equivalent amounts of tyrosine. The difference between the amounts of tyrosine present in the assay blank and the filtrate of the digest represented the proteolytic activity of the preparation. A unit of proteolytic activity was defined as the enzyme quantity which liberated 1 µg tyrosine equivalent per milliliter of reaction mixture under the conditions previously described.

Protein Determination

Protein concentration (mg/ml) was determined by the Lowry method (1951) as described by Cooper (1977). Bovine

serum albumin (BSA) was used to set up a standard curve from a 0.30 mg/ml solution. Absorbance was measured at 660 nm.

Optimization of Enzyme Production

In order to optimize protease yield it was necessary to follow enzyme production in BHI broth. P. fragi was cultured through the enrichment procedure as outlined above, but with a minor change. In the final step, only 50 ml of Tris-$CaCl_2$ was used to redissolve the pellet after centrifugation. A 1% inoculum was inoculated into 8 separate 2 L Erlenmeyer flasks containing 500 ml of BHI broth; flasks were incubated at 10 C on the rotatory shaker. At 12, 24, 36, 48, 60, 72, 84 and 96 h a flask was removed from the shaker, centrifuged at 14,600 xg for 70 min at 10 C in a Sorvall RC-2 centrifuge. Samples obtained during the first 36 h were centrifuged only once because of the relatively low cell concentration. Remaining samples were centrifuged a second time for 30 to 45 min. After the first or second centrifugation the supernatant was filter sterilized by vacuum filtration through a 47 mm Millipore filter with a pore size of 0.45 μm. Crude preparations were then analyzed for proteolytic activity.

The pH at each 12 h preparation was also followed in order to determine whether P. fragi altered the pH of the

medium during growth. The pH was determined on the cell free supernatant fluid.

Enzyme Production and Purification

A 4 L Erlenmeyer flask containing 1500 ml of BHI broth was inoculated with a 1% inoculum of P. fragi in Tris-$CaCl_2$ and incubated at 10 C on a shaker for 60 to 72 h.

All of the steps which were previously outlined, centrifugation and filtration were performed. Filtered supernatant fraction was precipitated at room temperature by slow addition, while stirring, of ammonium sulfate to 55% saturation. The solution was continuously stirred overnight at 10 C. Precipitate was collected by centrifugation at 14,600 xg for 90 min. The precipitate obtained was redissolved in 10-15 ml of 0.05 M Tris-HCl , 5 mM $CaCl_2$ buffer pH 7.5 per centrifuge bottle. The resuspended solution was mixed by stirring for approximately 30 minutes, poured into a Spectropor-2 dialysis tubing and dialyzed at 10 C against 5 changes of cold distilled, deionized water to eliminate salts. The solution was then dialyzed against 0.05 M Tris-HCl buffer pH 7.4 overnight at 10 C. The dialyzed preparation was concentrated by dialysis against 40% carbowax for 8 to 12 h at 10 C.

Concentrated material was placed on a Sephadex G-100 column previously equilibrated with 0.05 M Tris-HCl, 5 mM $CaCl_2$ pH 7.5. The column was eluted with the same buffer and 5 ml fractions were collected with a LKB fraction collector at a flow rate of 20 ml per h. Elution was monitored at 280 nm by a UV monitor and chromatographic profiles were recorded. Fractions were examined for protein content and activity, absorbance for both was read at 660 nm with a Spectronic 20 spectrophotometer. Pooled active fractions were dialyzed against two changes of distilled, deionized water, lyophilized and stored at -20 C for later use.

Polyacrylamide Disc Gel Electrophoresis

Disc gel electrophoresis was performed according to the method of Davis (1964) and Ornstein (1964). The procedures for preparation of the different stock solutions are in Appendix A. Lyophilized enzyme was redissolved in stacking gel buffer. To 1.0 ml of sample sucrose was added to increase density and one to two drops of 1% bromophenol blue solution, which served as a tracking dye at the ion front. Gel tubes were placed in the electrophoretic cell, electrode buffer was added and 25 to 70 µl of sample was layered on top of the gels.

Electrophoresis was carried out at room temperature with a constant current of 3.0 mA/tube until the tracking

dye migrated to the bottom portion of the tubes, approximately 3-4 h. Gels were removed from the tubes by rimming, fixed for 30 min in 12.5% TCA. After fixation the gels were stained with a 1:20 dilution of 12.5% TCA and 1% solution of Coomassie Brilliant Blue R-250. Gels were stained for approximately one hour, after staining the gels were transferred to 10% TCA; band intensity increases in this solution during a 48 h storage period (Chrambach et al., 1967).

SDS-Polyacrylamide Gel Electrophoresis

Polyacrylamide, sodium dodecyl sulfate (PAGE-SDS) gels were prepared according to the method of Weber and Osborn (1969). The formulas for the various stock solutions which were prepared are in Appendix B.

For sample preparation and electrophoresis the lyophilized preparation was rehydrated with 0.01 M sodium phosphate buffer, pH 7.2 containing 2.5% (w/v) SDS and 5% (v/v) β-mercaptoethanol. This mixture was incubated at 100 C for 5 min. A Pharmacia low molecular weight (LMW) calibration kit, containing 6 proteins (phosphorylase b, albumin, ovalbumin, carbonic anhydrase, trypsin inhibitor and α-lactalbumin) was used for the standards.

After the protease preparation was heated and cooled, crystalline sucrose was added to increase the density and one to two drops of bromophenol blue solution was added as tracking dye. The upper and lower chambers of the electrophoresis unit were filled with electrode buffer; one part electrode buffer to one part water. Then 25 to 60 µl of the protease sample was layered on top of the gels; only 10 µl of the LMW solution was used per gel. Electrophoresis was performed at 3.0 mA/tube for 15 min and then 8 mA/tube until the tracking dye migrated to the bottom portion of the tube, approximately 6 to 8 h depending on gel concentration. Gels were soaked in 12% TCA for 30 min in order to remove SDS followed by staining with Coomassie Brilliant Blue R-250 at room temperature. The gels were removed from the staining solution, rinsed with distilled water then destained by diffusion (circulation) in acetic acid-methanol-water (75:50:875 ml). Gels were stored in 7.5% acetic acid.

Antibody Production

Preparation of Protease and Antiserum

Two young New Zealand white rabbits, one male, one female were used in the study. Lyophilized protein was rehydrated with sterile water in a sterile glass bottle, 5 ml of Freund's incomplete adjuvant was added to the

solution followed by 1 ml of aqueous merthiolate (1:1000 dilution). The mixtures were combined in order to prepare an initial injection containing 1 mg of the protein. Injection mixtures were prepared 48 h prior to injection and stored at 4 C. A sterility test was performed during this time, a few drops of each mixture were inoculated onto pre-poured PCA plates and the plates incubated at 37 C for 48 h. Contamination was never a problem during the entire schedule of injections. For the first and second injection, each rabbit was injected intramuscularly in the thigh muscle of each hind leg; the rabbits were not inoculated simultaneously but rather there was a two week interval between their inoculations. The rabbit receiving the first injection was closely monitored during the first week succeeding its inoculation. For the second injection, two weeks after initial, each rabbit was inoculated as indicated above with a mixture of the protein plus Freund's incomplete adjuvant; 1 mg of the protein was again injected. One and one half weeks later a third inoculation composed of 0.5 mg protein plus sterile saline was injected into one hind leg. A fourth inoculation, also composed of 0.5 mg protein and sterile saline was given two weeks later.

Each rabbit was bled via the marginal ear vein prior to the first injection and 1 week following the second and final injections. Approximately 10 cc of blood were collected in clean glass tubes following each bleeding.

Each tube was held at room temperature to allow a firm clot to develop. A small wooden applicator stick was used to detach the clot from the surface of the glass tube. The sample was then centrifuged at 3000 xg. Following centrifugation the serum was decanted and dispensed as small aliquots into small sterile tubes, aliquots were frozen at -20 C for later use.

Determination of Antibody Titer

For the determination of antibody titer the enzyme-linked immunosorbent assay (ELISA) was used. For solid phase coating the protease enzyme was diluted with 0.1 M phosphate buffered saline solution (PBS) to 10 μg/ml. See Appendix C for composition of this buffer and other systems used in this technique. A 96 well polystyrene holder containing 8 strips (12 wells/strip) was prepared. Each well was inoculated with 100 μl of the enzyme-PBS solution and the plate incubated at 4 C overnight. Procedure for ELISA was as follows:

1. Wells washed 2 x with 0.25 ml (250 μl) PBS-Tween.
2. 0.25 ml of 1.0% bovine serum albumin (BSA)-PBS was added to each well, plate incubated for 30 min at 37 C.
3. Wells washed 2 x with 0.25 ml PBS-Tween.
4. Antiserum in 1% BSA-PBS was serially diluted, 1:5 dilution and 0.1 ml of serially diluted antibody was added to the wells.
5. Plate was incubated for 60 min at 37 C.

6. Wells washed 5 x with 0.25 ml PBS-Tween.
7. 0.1 ml of 1:1000 goat antirabbit peroxidase, in BSA-PBS, was added to each well, plate incubated for 30 min at 37 C.
8. Wells washed 5 x with 0.25 ml PBS-Tween.
9. 0.1 ml ABTS was added to each well.
10. The reaction was terminated by the addition of 0.1 ml hydrofluoric acid-edetic acid stopping reagent.
11. Plates were read within 30 min with the micro ELISA minireader at 410 nm.

Electron Microscopy

Fixation and Embedding

Cells of P. fragi that were obtained from BHI broth and Koser Citrate after centrifugation were mixed with sterile distilled water to redissolve the cell pellet. Cells were then washed and centrifuged four times followed by a final centrifugation (4000 xg), after the cell pellet was dissolved in Millonig (1961) phosphate buffer (see Appendix D). Cells were centrifuged again and the pellet redissolved with 1.25% glutaraldehyde made in the same buffer. Cells were held for 1 h at 4 C, centrifuged and the buffer decanted off. A small quantity of cells was mixed with sterile molten 2% Noble agar. From this mixture a small portion was removed and pipetted onto a clean glass slide containing four thin, flat pieces of glass on top forming an empty square. The cell-agar

mixture was pipetted into this area, allowed to solidify and small thin cubes or strips were cut in the agar with a clean single edged razor blade. Cut strips were fixed for 2 to 4 h at 4 C in 2.5% glutaraldehyde. Strips were washed for 15 to 20 min in 3 changes of phosphate buffer.

Samples were transferred from phosphate buffer to a 2% osmium tetroxide (OsO_4) solution in phosphate buffer. Samples were fixed for 2 to 4 h at 4 C but allowed to come to room temperature during the final period of fixation.

After fixation, samples were rinsed 5 to 6 times in phosphate buffer. Following the final wash, samples were dehydrated by placing them in 25, 50, 75 and 95% ethanol for 10 to 15 min each at 4 C. They were then held for 20 to 30 min each in two changes of 100% alcohol. Alcohol was removed and replaced with a 1:1 mixture of propylene oxide to ethanol, two changes held for 30 min at 4 C. Samples were then mixed with two 30 min changes of 100% propylene oxide at 4 C. Propylene oxide was removed and replaced with a 1:1 solution of propylene oxide to epon-araldite, and the samples were held overnight with continuous agitation. The propylene oxide-epon-araldite mixture was replaced with a 100% epon-araldite mixture and mixed continuously for 2 to 6 h.

Agar encapsulated cells were embedded using the epon-araldite mixture given in Appendix E. One cube or strip was placed into a flat embedding mold containing a

small sample of the epon-araldite mixture. Additional epon-araldite was added until the area was filled. The specimens were relocated toward the front and placed in a desiccator under a slight vacuum at room temperature for 2 to 3 h. The mold was removed, specimen relocated and placed in an oven at 60 C. After approximately 30 min each specimen was relocated, if necessary, placed back in the oven, and allowed to harden for 24 to 36 h. Hardened epon-araldite blocks containing the cells were stored under vacuum in a desiccator at room temperature for later use.

Sectioning and Staining

Blocks were trimmed with a single edged razor blade and sectioned on a diamond knife using a Sorvall ultra-microtome. Sections were picked up from the knife boat on uncoated 100, 200 or 300-meshed copper or nickle grids.

Sections were stained by applying a drop or floating the grids, sections down, on a saturated solution of uranyl acetate for 30 min followed by a distilled water rinse. Sections were then stained for 5 min with lead citrate followed by washing with 0.02 N sodium hydroxide then distilled water, see Appendix F for description of these solutions. Grids were placed on Whatman filter paper to dry. After drying, the grids were stored in a multiple grid holder until observations were made.

Observation and Photography

Grids containing stained sections were observed in a Philips EM-201 electron microscope at an accelerating voltage of 60 kilovolts. Two to five grids were observed for each sample and representative photographs were taken. Film was developed for 4 min in Kodak D-19 developer, washed for 2 min in running water, fixed for 8 min in Kodak fixer, washed in running water for 30 min, dipped in Kodak Photo-Flo solution for 30 sec and air dried.

Selected Kodak photographic paper was exposed from the negatives using a Simmon model D3 Automega enlarger. Prints were processed and dried for later use.

Peroxidase-Antiperoxidase Localization of Protease on Ultrathin Sections

Sections fixed in glutaraldehyde and embedded in an epon-araldite mixture were stained by the immunochemical staining procedure developed by Sternberger et al. (1970), modified by Moriarity and Halmi (1972). Thin sections were cut on a Sorvall ultramicrotome, collected on 100, 200 or 300-mesh nickel grids and floated sections down on the various staining solutions.

All sections were etched for 20 min prior to initiating immunochemical staining by floating them on 10% aqueous hydrogen peroxide. Following each staining solution, except the DAB and osmium tetroxide solutions, grids were

rinsed in 0.5 M Tris-HCl buffered (pH 7.6) saline for 3 min. After etching with H_2O_2, sections were floated on normal goat serum diluted 1:30 for 3 min and rinsed. They were then floated on rabbit anti-protease antiserum diluted 1:10, 1:50, 1:100 and 1:1000 for 2, 4, 10 and 45 min, respectively. Grids were floated on goat antirabbit IgG diluted 1:5 for 3 min. They were then floated on the PAP complex diluted 1:10 for 3 min. After mixing and filtering, the peroxidase substrate which consisted of 170 ml of 0.5 M Tris buffered saline (pH 7.6), 22 mg DAB and 1.5 ml 0.3% H_2O_2, the grids were immersed in this solution for 2 to 3 min; grids were held tightly by forceps that were used to lower the grids into the solution. This substrate solution was continuously stirred by a magnetic stirrer in order to prevent non-specific adsorption of the reaction product to the embedding medium.

Following 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 fumed hood. Osmium deposits in sites of oxidized, precipitated DAB. Polymers of the DAB reaction product are visible with the electron microscope as black deposits. Grids were washed in one change of distilled water and examined in a Philips EM 201 electron microscope.

Cellular detail was improved if grids were stained in saturated uranyl acetate for 30 min and lead citrate for 5 min following osmication.

Controls for this procedure included use of preimmune or normal rabbit sera as a substitute for the protease antiserum. Other controls included omission of either PAP, DAB or antirabbit IgG. Two grids were observed for each control sample.

RESULTS AND DISCUSSION

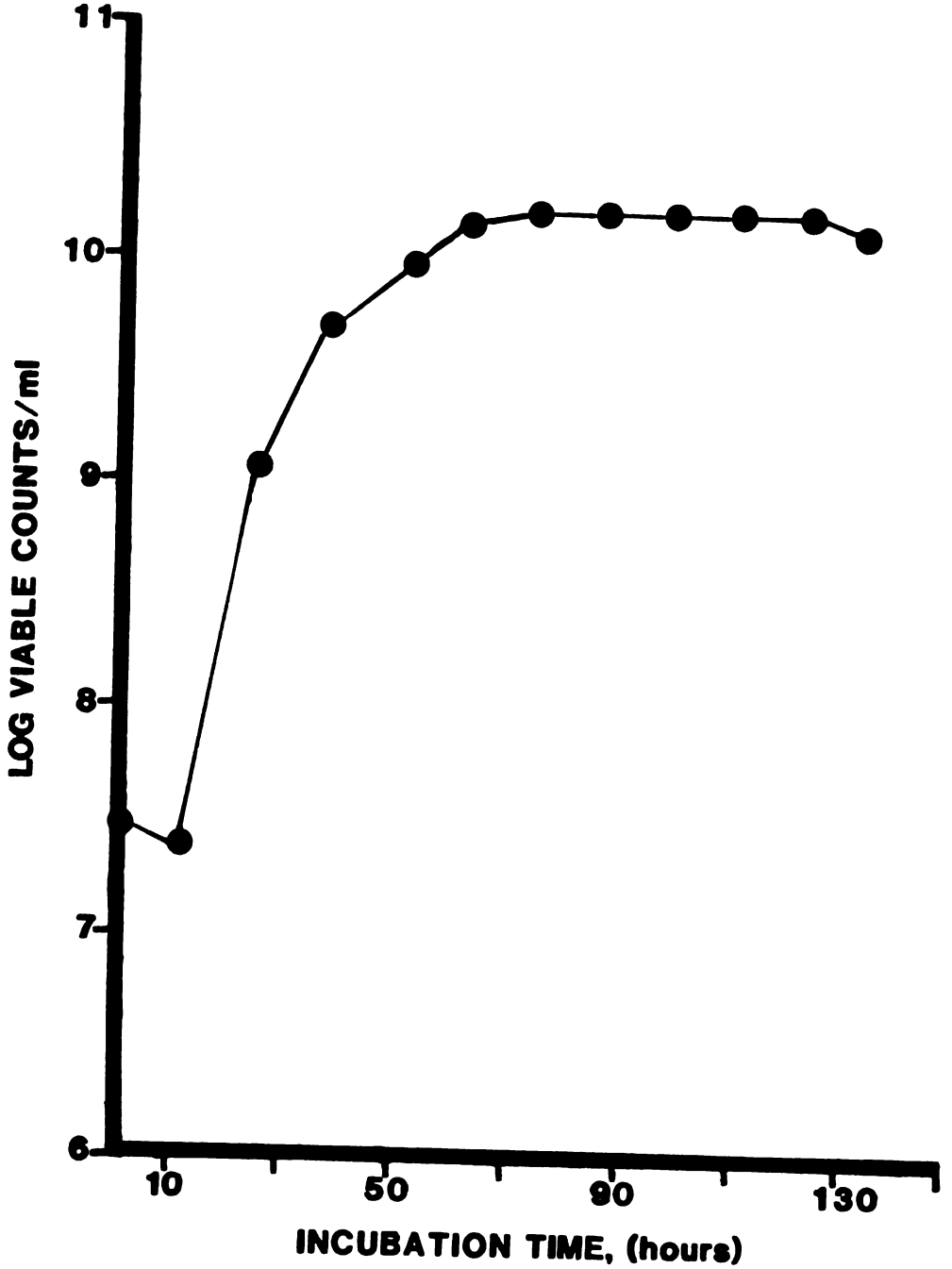
Growth of P. fragi in BHI Broth

Monitoring Growth of P. fragi

The enrichment procedure described herein provided an initial high population and an active culture for inoculation of the BHI broth. Beginning with a large actively growing P. fragi culture, the incubation period was shortened and resulted in sufficient cell numbers to insure adequate protease production.

A typical growth curve obtained when P. fragi was grown in BHI broth at 10 C is illustrated (Figure 1). The initial population was over $1x10^7$ colony forming units (C.F.U.)/ml. All data points represent an average of 4 different runs. This growth curve is similar to a growth curve illustrated by Juffs et al. (1968) when P. fragi was grown in P.Y.E. broth at 28 C. Exponential growth was initiated at approximately 12 h and continued until 72 h where the stationary phase began and continued without a noticeable decline in population even after 168 h incubation. Juffs et al. (1968) also observed several other different growth patterns for P. fragi when grown at varying temperatures and media composition. Temperature and substrate composition are

Figure 1. Growth of *Pseudomonas fragi* ATCC 4973 in Brain Heart Infusion broth at 10 C on a rotatory shaker.


11
10
9
8
7
6
LOG VIABLE COUNTS/ml
10
50
90
130
INCUBATION TIME, (hours)

parameters which must be considered in order to achieve optimum growth and microbial activity.

Tarrant et al. (1973) described the growth of P. fragi in a medium consisting of amino acids and dipeptides. Lag phase corresponded to the first 20 h of incubation, exponential phase corresponded to the time following the lag phase until approximately 80 h. There was a dramatic decline in population immediately following the exponential growth phase; no significant stationary phase was observed.

Exponential growth phase for P. fragi growing in BHI broth was also initiated after 12 h, however it was complete at 60 h, not at 72 as described by Juffs et al. (1968). Stationary phase was continuous through 120 h followed by a slight decline in population. Although Figure 1 does not indicate results beyond 132 h, plating continued through 168 h indicated a further decline in population. At 168 h incubation the population decreased to 8.9×10^9 C.F.U./ml. The results of this experiment indicated that the BHI broth and the temperature of incubation (10 C) provided suitable conditions for growth of P. fragi.

The growth pattern for P. fragi in BHI broth differs from growth patterns observed by other investigators (Tarrant et al., 1973; Juffs et al., 1968) probably because media and/or temperature conditions were different. Either of these parameters can enhance, decrease or inhibit microbial growth rate and/or metabolism.

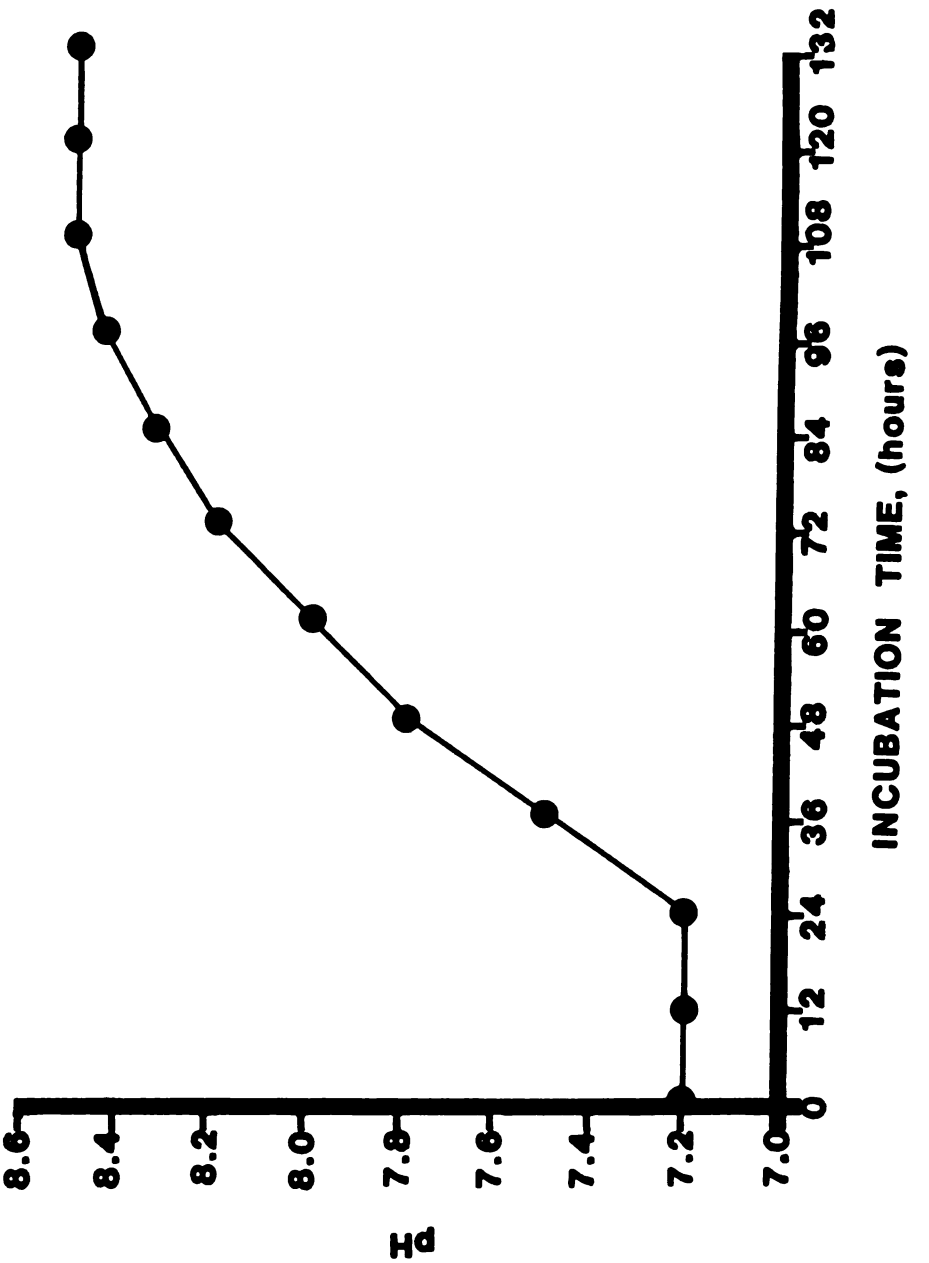
Influence of P. fragi on the pH of BHI broth

The metabolic activity of microorganisms can alter the pH of a substrate. Such pH changes were detected in BHI broth inoculated with P. fragi (Figure 2). The pH of the medium did not change during the first 24 h of incubation. However, at 36 hr the first change in pH was observed and it continually changed until 108 h, reaching a final pH of 8.5, where it remained constant through 132 h incubation; changes in pH were not monitored after 132 h. This initial change in pH at 36 h also corresponded to the time at which initial proteolytic activity was detected in the crude medium.

Changes in pH occurred in shorter incubation time in BHI broth than has been observed in muscle tissue inoculated with P. fragi. Hasegawa et al. (1970) observed pH changes in rabbit and porcine muscle inoculated with P. fragi, from 5.5 to 8.72 and 5.41 to 8.49 in 20 days, respectively. Borton et al. (1971) observed similar changes in the pH of porcine samples during storage for 20 days, pH increased from 5.4 to 7.8. Several other studies have also shown similar results (Tarrant et al., 1971; Bala et al., 1977; Yada and Skura, 1981; Wing et al., 1983).

Agitation of a supportive growth medium, such as BHI broth, containing an aerobic organism will greatly enhance growth and metabolism, thus causing changes to occur more rapidly. Because similar constituents in meat may also be

Figure 2. Changes in the pH of Brain Heart Infusion broth inoculated with Pseudomonas fragi and incubated at 10 C on a rotatory shaker.


pH
8.6
8.4
8.2
8.0
7.8
7.6
7.4
7.2
7.0
0
12
24
36
48
60
72
84
96
108
120
132
INCUBATION TIME, (hours)

present in BHI broth, metabolic products may have been produced which caused similar pH changes to occur in the medium.

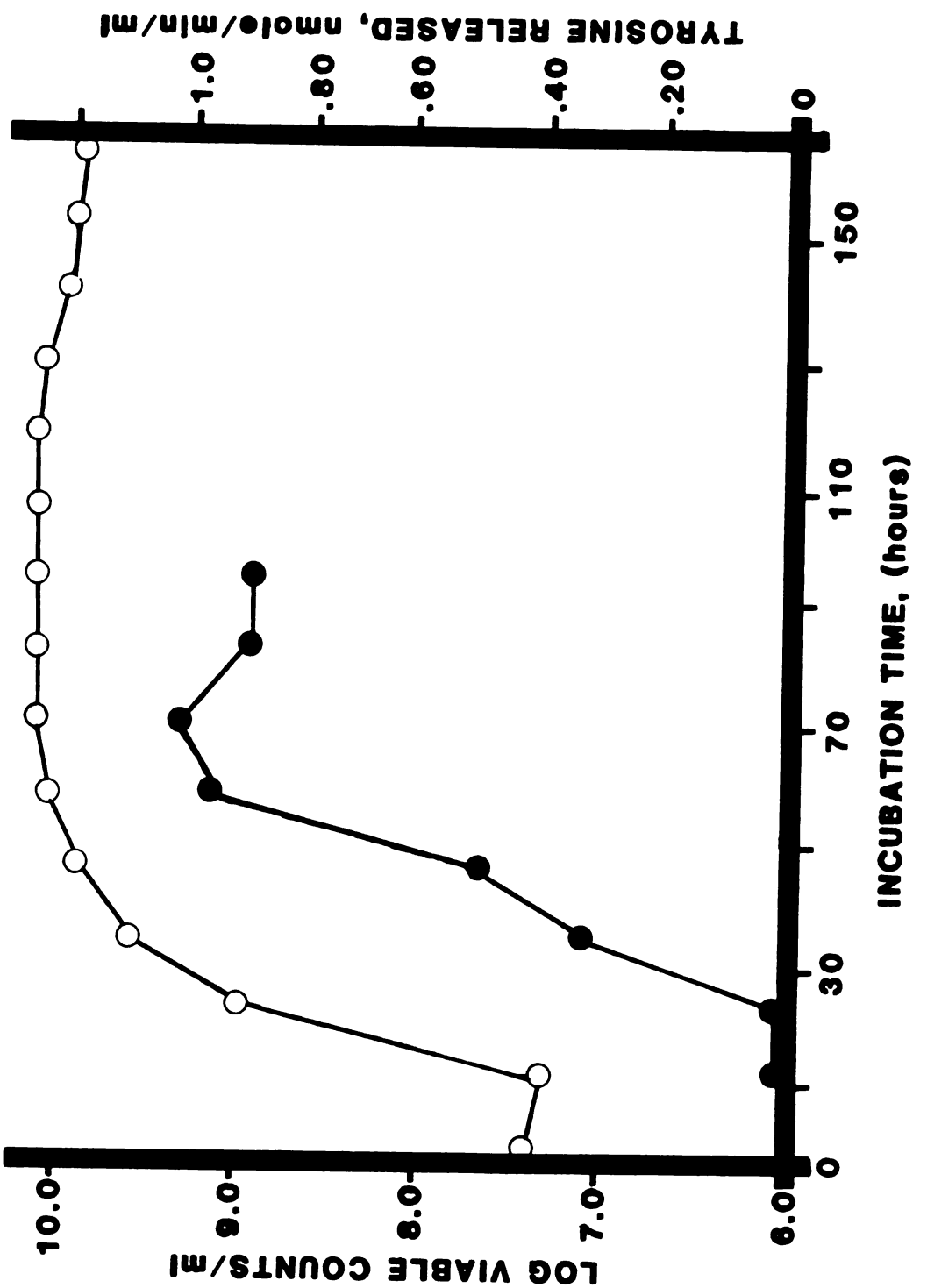
Since the pH was altered from near neutrality to the alkaline range, it can be concluded that these changes resulted from metabolism of nitrogenous containing constituents such as proteins, peptides and free amino acids; similar constituents are in muscle tissue. Degradation of these constituents will produce ammonia, amines, indole and mercaptans, all of which can be responsible for the type of pH changes observed.

Production of Proteolytic Activity

Not only will incubation temperature and composition of a medium influence the rate of microbial growth, but they will also influence enzyme production. Like growth rate, enzyme production may be enhanced, decreased or inhibited by these two parameters.

The protease activity was monitored in this study in order to estimate the time at which the enzyme was maximally produced (Figure 3). This time was then designated as the time at which protease isolation and purification steps initiated. Proteolytic activity was measured through 96 h incubation, while growth, as indicated earlier, was monitored for a longer incubation period. Proteolytic activity was first detected at 36 h, during late exponential growth; the same time

Figure 3. Growth of Pseudomonas fragi ATCC 4973 (○) and proteolytic activity (●) in Brain Heart Infusion broth at 10 C.


TYROSINE RELEASED, nmole/min/ml
1.0
.80
.60
.40
.20
0
LOG VIABLE COUNTS/ml
10.0
9.0
8.0
7.0
6.0
0
30
70
110
150
INCUBATION TIME, (hours)

at which an initial change in pH was detected. Maximum proteolytic activity was reached between 60 and 72 h, during early stationary phase, and began to decline, consequently incubation of the growing culture in BHI broth was stopped at this time.

Tarrant et al. (1973) first observed proteolytic activity in a medium consisting of known amino acids and dipeptides inoculated with P. fragi after incubation for approximately 40 h. This corresponds to about 4 to 5 h beyond the time at which initial proteolytic activity was detected in BHI broth. Proteolytic activity was first detected during late exponential growth, as was observed in this investigation, and maximum activity was achieved at 60 h followed by a substantial decline in activity. Maximum proteolytic activity obtained was reported as approximately 0.08 units/ml culture supernatant fluid.

Results presented earlier by Juffs et al. (1968) were different than those presented by Tarrant et al. (1973) in that rapid production of proteolytic activity was observed when P. fragi was grown in P.Y.E. broth at 28 C. Maximum proteoltyic activity was reached at 24 h followed by as rapid a decline in activity. At 3 C in P.Y.E. broth, maximum proteolytic activity was not detected until between 60 to 70 h, a decline in activity was not observed until after 170 to 180 h incubation. Maximum proteolytic activity reported was approximately 25 and 80 μg tyrosine liberated

at 28 and 3 C, respectively. Growth at 3 C was slow because of the low temperature and the population was low at the time in which maximum activity was detected. Good growth was observed at 28 C, a much higher bacterial population was reached than at 3 C, however, at the time maximum proteolytic activity was detected the population was low. Thus, the data suggest that activity was not proportional to growth. Juffs et al. (1968) also detected a second relatively small increase in proteolytic activity at 200 h incubation, perhaps due to autolysis. The authors also determined that P. fragi did not produce significant levels of proteolytic activity in Koser Citrate medium or Koser Citrate medium containing 0.25% casein at temperatures identical to those used to grow P. fragi in P.Y.E. broth. A similar investigation where P. fragi was grown in Koser Citrate at 10 C was done and no proteolytic activity was detected, confirming their results. This suggested that a specific constituent(s) was necessary for protease production which the two media did not evidently contain.

Enzyme Purification

The proteolytic enzyme was obtained with a 38-fold purification of specific activity (Table 1). The elution pattern obtained during gel chromatography is similar to one illustrated by Porzio and Pearson (1975), however

Table 1. Purification of *Pseudomonas fragi* neutral protease.

Step	Volume (ml)	Protein (mg/ml)	Total Protein (mg)	Activity (unit/ml)	Activity (total units*)	Specific Activity (unit/mg)	Yield (%)	Purification (fold)
Culture supernatant	1300	12.40	16,120	0.59	780	0.048	100	1.0
1) 55% $(NH_4)_2SO_4$ precipitation	66	8.0	528	2.01	133	0.251	17	5.2
2) Dialysis concentration	20	13.2	264	2.2	44	0.17	5.6	3.5
3) Sephadex G-100 gel filtration	10	0.44	4.4	0.80	8	1.8	1.03	38.0

*1 unit equals release of 1 nmole tyrosine equivalent per min at 21 C.

Figure 4. Elution pattern of Sephadex G-100 gel chromatography. Protein solution was loaded on 2.5 x 40 cm column equilibrated with 0.05M Tris HCl, $CaCl_2$ 5 mM (pH 7.5). The enzyme was eluted at 20 ml/h with 5 ml fractions per tube. Absorbance at 280 nm○——○, protein concentration●——●, protease activity□——□.

TYROSINE RELEASED (nmole/ml)
400
300
200
100
PROTEIN (mg/ml)
.30
.20
.10
ABSORBANCE 280nm
.50
.40
.30
.20
.10
0
10
20
30
40
FRACTION NUMBER (5ml/tube)

protein content was higher in fractions comprising the second peak than in fractions making up the first peak (Figure 4). Fractions which comprise the first peak contained the proteolytic activity, therefore these fractions were combined and used for the remaining studies.

Polyacrylamide Disc Gel Electrophoresis

The lyophilized sample was rehydrated with the desired sample buffer, the gels prepared according to the procedure outlined, electrophoresed and stained. Regardless of the quantity of sample applied to the gels, 20, 30, 40, 50 or 60 µl, each gel showed a single protein band located several millimeters above the migration point of the tracking dye (Figure 5). Bala *et al*. (1979) detected a single protein band from a purified preparation obtained from the growth of *P. fragi* in BHI broth.

Presence of a single protein band on gels suggests that the preparation is homogenous. However, it should be noted that the presence of a single band may not always indicate definite homogeneity since it is possible that more than one protein specie may have similar electrophoresis mobility, thereby yielding a single band.

Figure 5. Polyacrylamide disc gel (9% total concentration) electrophoretogram of the neutral protease.

SDS-Polyacrylamide Gel Electrophoresis

The molecular weight of the isolated protease was determined from a standard curve of log molecular weight vs. mobility (Figure 6). Molecular weight was estimated as 48,000 ± 1,200. Points on the graph represent the average of 5 duplicate gels. The electrophoretogram obtained when sample was applied to 10% SDS gel shows one protein band (Figure 7).

Porzio and Pearson (1975) estimated the molecular weight of P. fragi protease, using SDS-PAGE, gel permeation chromatography, and zinc microassay, molecular weight ranged from 40,000 to 50,000. With SDS-PAGE molecular weight was estimated as 50,000 while gel permeation chromatography and zinc microassay gave a value of 40,000. The molecular weight estimated in this study is within the range proposed by Porzio and Pearson (1975).

Antibody Titration by Enzyme Linked Immunosorbent Assay (ELISA)

The ELISA technique was devised by Engvall and Perlmann (1971) as a viable procedure for determining immunoglobulin G (IgG) from a rabbit, which was used as antigen. The authors concluded that ELISA was similar to radioimmunosorbent techniques (RIST) in sensitivity and precision. Engvall and Perlmann (1972) detected less than 1.0 nonogram/ml of

Figure 6. Standard curve for determination of molecular weight by SDS-PAGE (10%). A. phosphorylase B; B. bovine serum albumin; C. ovalbumin; D. carbonic anhydrase; E. soybean trypsin inhibitor; F. α-lactalbumin. Protease indicated by arrow.

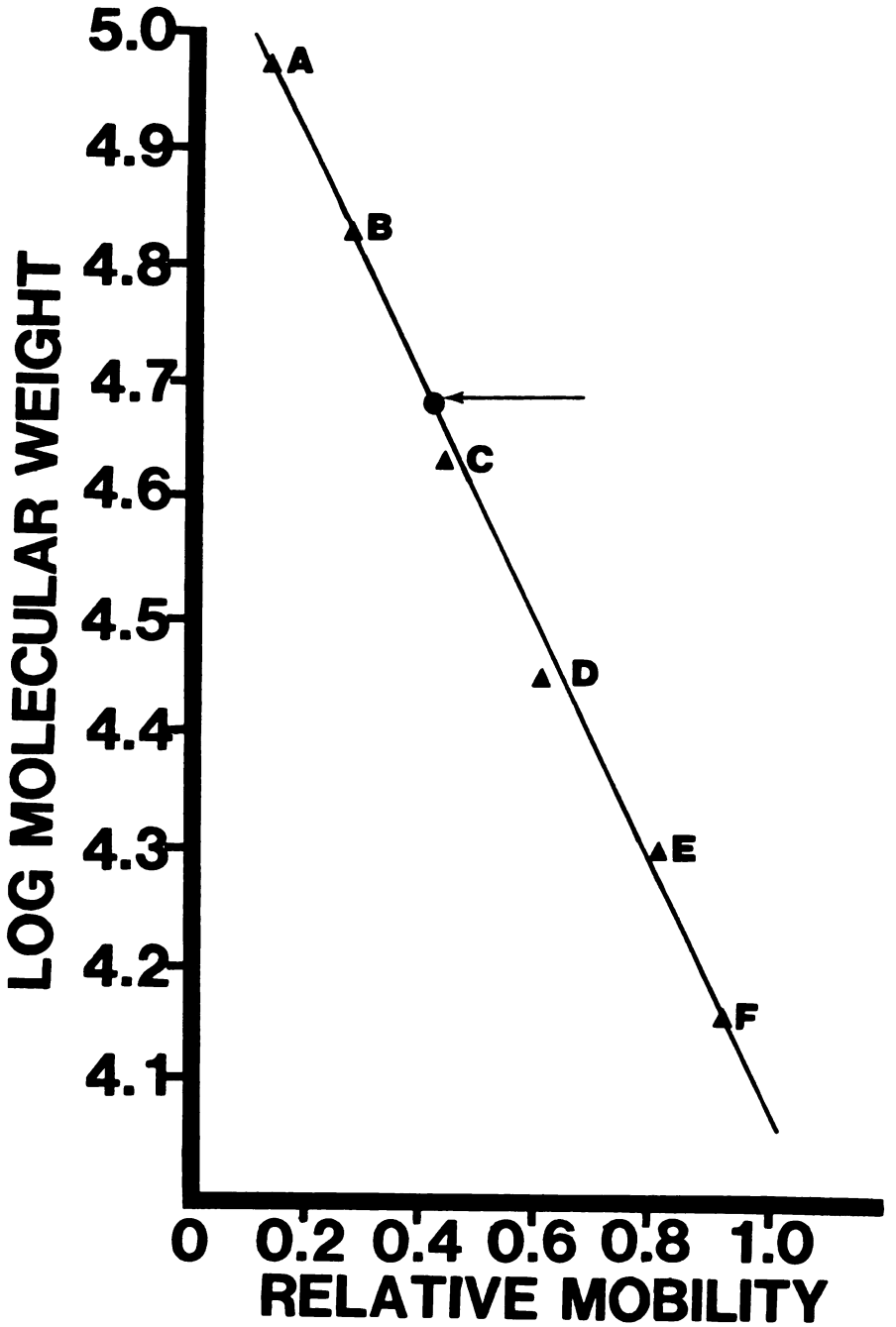
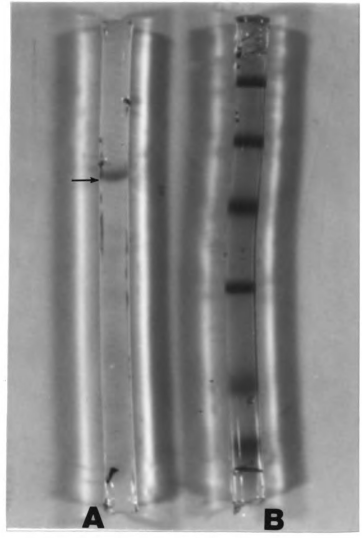

LOG MOLECULAR WEIGHT
5.0
4.9
4.8
4.7
4.6
4.5
4.4
4.3
4.2
4.1
A
B
C
D
E
F
0
0.2
0.4
0.6
0.8
1.0
RELATIVE MOBILITY

Figure 7. Polyacrylamide-SDS gel (10% total concentration) electrophoretogram of (A) neutral protease; (B) standard proteins.


A
B

specific dinitrophenol (DPN) and human serum albumin (HSA) antibodies using ELISA. In this later investigation it was concluded that ELISA was as sensitive as corresponding RIST. Two additional advantages of ELISA over RIST were that more stable reagents were used and less specialized equipment was required. Another very important advantage of ELISA is that it incorporates enzyme rather than isotope labelling of antigen.

Because of the high degree of sensitivity, use of limited equipment, ease of preparation, relatively rapid time and small sample requirement, ELISA was chosen over other more commonly used immunological procedures.

Antisera specific for the protease were obtained from the rabbits one week after the second and final immunizations. Antibody was determined on each antiserum as denoted by green color development in the wells of the microtest plate (Figure 8). Dilutions of the preimmune control serum showed negligible binding of the protease-peroxidase conjugate. The last well in a dilution scheme to give color visually distinct from the preimmune control serum represented the titer endpoint (Figure 9). Antibody titer endpoints of both rabbits were approximately 4×10^7.

Lawellin *et al*. (1977) detected less than 10 picograms (pg) aflatoxin B_1 per ml using ELISA. Pestka *et al*. (1980)

Figure 8. Enzyme-linked immunosorbent assay microtest plate determining antibody titer.

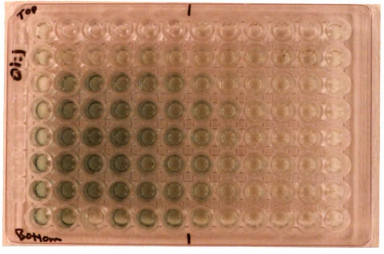
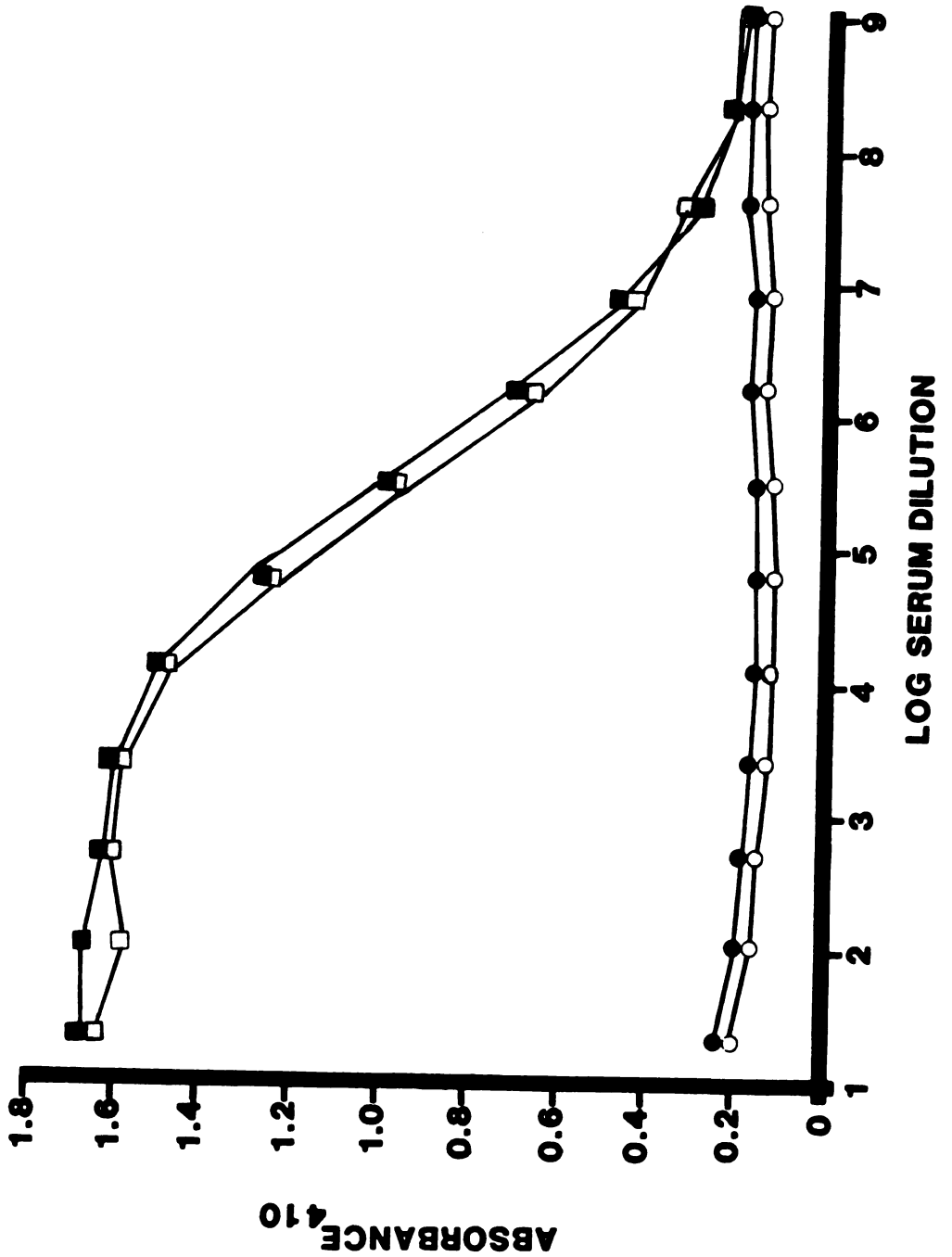

Top
1
Bottom
1

Figure 9. Enzyme-linked immunosorbent assay titration of protease antisera. Symbols: (■) R-1 antiserum; (□) R-2 antiserum; (●) R-1 preimmune serum; (○) R-2 preimmune serum.


ABSORBANCE 410
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
1
2
3
4
5
6
7
8
9
LOG SERUM DILUTION

developed an ELISA microtest plate technique which quantitated aflatoxin B_1 and aflatoxin B_1 antibody; as low as 25 pg aflatoxin B_1 per assay were quantitated. Pestka et al. (1981) developed an ELISA technique which detected as little as 0.25 nanograms (ng) aflatoxin M_1/ml in raw, whole and skim milk artificially contaminated with known levels of M_1. El-Nakib et al. (1981) also used ELISA to detect aflatoxin B_1 in corn, wheat and peanut butter artificially contaminated with the toxin.

ELISA also shows potential as a new method for analyzing foods for the presence of toxins and other proteins produced by microorganisms. Shah presented three potential procedures in which ELISA techniques can be used to detect C. perfringens enterotoxin, E. coli heat labile toxin, and E. coli heat stable toxin. The methods described included the double antibody sandwich, nitrocellulose blot, and competitive, respectively (Personal communications, AOAC 1983).

With the recent problem of survival of heat stable proteases in heat treated milk, ELISA could be used to successfully detect these enzymes thereby creating a useful screening procedure. The double antibody sandwich technique should be investigated further as a potential screening method.

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 hr (Figures 10, 11, 12, 13 and 14). The blebs as well as the globules were surrounded by multiple layered membranes identical to those of the cell wall. This result is significant in that it represents the first time that blebs have been detected on the surface of *P. fragi* cells grown in a laboratory medium. A fiber-like glycocalyx material was occasionally found to be attached to the blebs (Figure 11). This is similar to the coiling glycocalyx fibers observed by Wing *et al.* (1983). Blebs were not observed on the surface of cells grown in Koser Citrate medium incubated at similar conditions (Figure 15).

Several investigations have demonstrated the presence of blebs or protrusions on the surface of *P. fragi*, however in each of those studies *P. fragi* was observed while growing in spoiled pork or beef (Dutson *et al.*, 1971; Yada and Skura, 1981; Wing *et al.*, 1983). The authors in each investigation have suggested a connection between bleb formation and proteolytic activity. When Dutson *et al.* (1971) grew *P. fragi* in a nonprotein medium no blebs were observed on the surface of the cells. Tarrant *et al.* (1973) also indicated that proteolytic activity was not detected in Koser Citrate medium used

Figure 10. Electron micrograph showing a cell of *Pseudomonas fragi* that was present in Brain Heart Infusion broth incubated at 10 C for 60 to 72 h. B = bacterial cell, E = evaginations, G = globules. Negative stain. x 52,000.

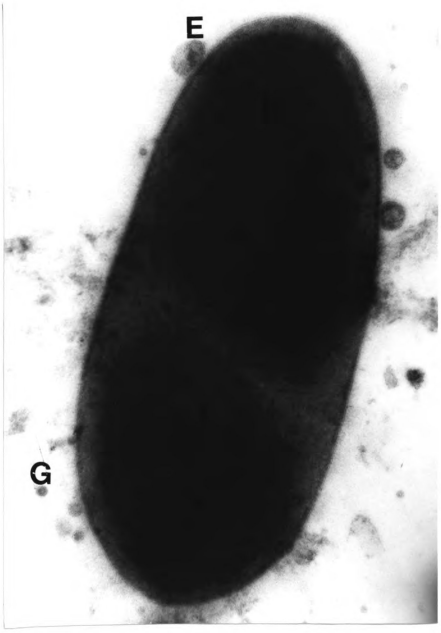

E
G

Figure 11. Electron micrograph of a Pseudomonas fragi cell showing a fiber-like glycocalyx material. Cell obtained from Brain Heart Infusion broth incubated at 10 C for 72 h. G = glycocalyx. B = bacterial cell. x 52,000.

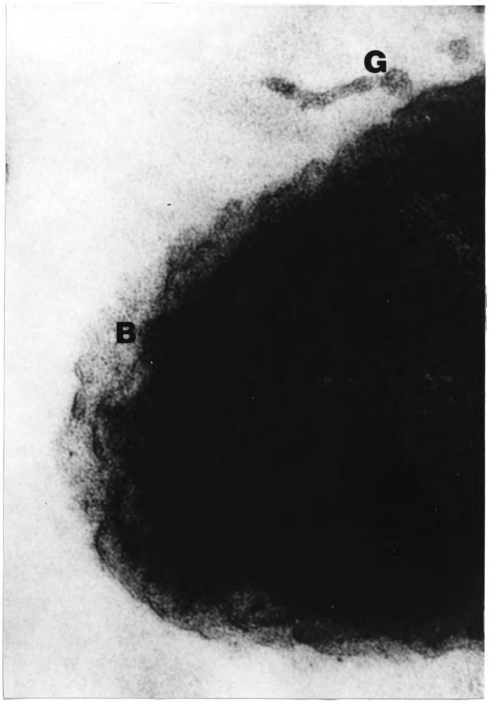

G
B

Figure 12. Electron micrograph showing a Pseudomonas fragi cell grown in Brain Heart Infusion broth at 10 C for 60 to 72 h. B = bacterial cell, arrows point to bleblike evagination extending from the cell wall. x 30,000.

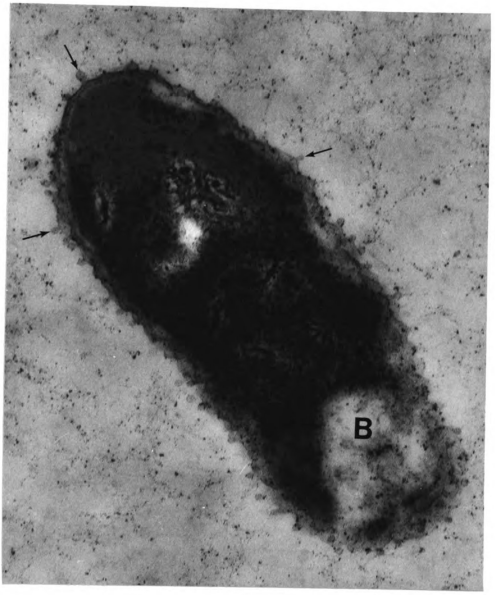

B

Figure 13. Electron micrograph showing Pseudomonas fragi cells grown in Brain Heart Infusion broth at 10 C for 60 to 72 hr. Arrows point to bleblike evaginations. x 70,000.

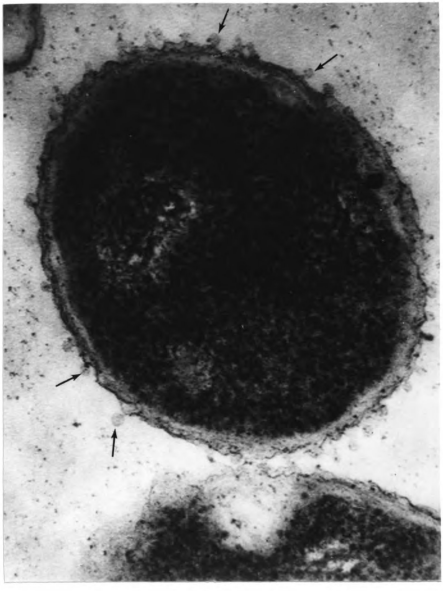


Figure 14. Electron micrograph showing a cell of *Pseudomonas fragi* from Brain Heart Infusion broth incubated at 10 C for 60 to 72 h. G = globules, E = evaginations. x 45,000.

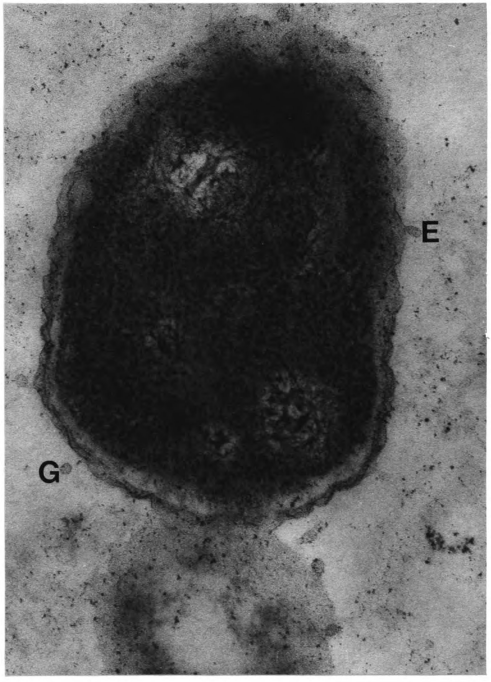
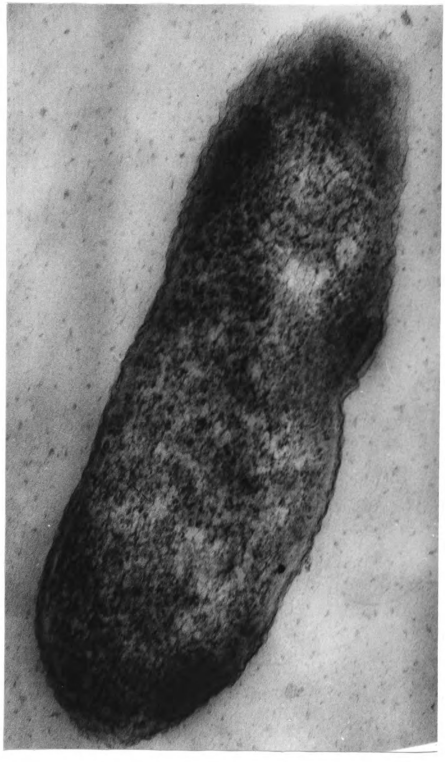

E
G

Figure 15. Electron micrograph showing cells of Pseudomonas fragi grown in Koser Citrate medium at 10 C for 72 h. x 45,000.



to grow P. fragi. It may be concluded that nonprotein media, such as Koser Citrate readily support growth of P. fragi but do not contain constituents which support production of proteolytic enzymes. In this study neither proteolytic activity nor bleb formation was detected in cells grown in Koser Citrate medium. Since proteolytic activity was detected in BHI broth and blebs were observed on the surface of cells grown in BHI broth it can be concluded that this medium is suitable for supporting production of both proteolytic enzymes and blebs.

Immunoperoxidase Localization of the Protease on Ultrathin Sections

Sections were stained with several dilutions of antiserum, 1:10, 1:50, 1:100 or 1:1000. Controls were as follows: 1. Preimmune rabbit serum; 2. Normal rabbit serum, which was obtained with the peroxidase kit; 3. Specific aflatoxin B_1 antibody; and 4. Omission of PAP or DAB from the staining procedure. The 1:100 dilution of antiserum yielded optimum peroxidase staining under the conditions of the assay. Lower relative intensities observed in the 1:10 and 1:50 dilutions may be related to increased background. This may be due to nonspecific binding of protease antibody to the cell section thereby causing decreased contrast. According to Sternberger (1979) excessive concentrations of primary antiserum may produce negative results. An excess

of primary antiserum appears to inhibit the bifunctional reactivity of the link antibody.

Ultrathin sections of P. fragi stained 1:100 and 1:1000 protease antiserum had dark deposits of stain in the cell in close proximity to the cell wall (Figures 16, 17). Dark deposits were also observed inside the blebs which were attached externally to the cell wall as well as in globular or circular structures unattached to the cell (Figures 16, 17). These globules probably represent blebs detached from the cell due to as yet unknown causes as has been suggested by Dutson et al. (1971). Peroxidase positive deposits were not detected in the controls (Figures 18, 19). Sections treated with preimmune serum, normal serum, or aflatoxin B_1 antiserum were negative along with those sections in which specific reagents were omitted.

Since dark deposits were not confined to the cell interior but were also observed in the blebs, it can be concluded that bleb formation is associated with production and/or release of the proteolytic enzyme.

Relative intensities of the cells as determined by the standard immunoperoxidase protocol and by various control protocols are illustrated (Table 2).

No significant concentration of peroxidase endproduct was detected in the cell cytoplasm of experimental sections. Primary staining was concentrated at the periphery of the

Figure 16. Electron micrograph showing cells of Pseudomonas fragi from Brain Heart Infusion broth stained with protease antiserum, 1:100. Note the dark deposits in the periplasmic space, blebs and globules. x 20,000.

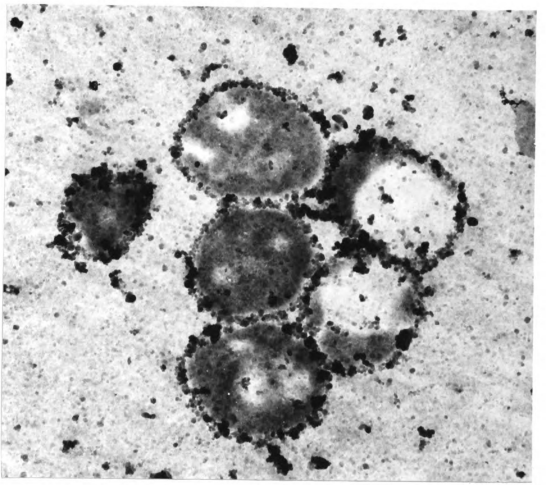


Figure 17. Electron micrograph showing cells of Pseudomonas fragi from Brain Heart Infusion broth, stained with protease antiserum, 1:1000 dilution. Note the stain deposits in the periplasmic space, and the blebs. x 30,000.

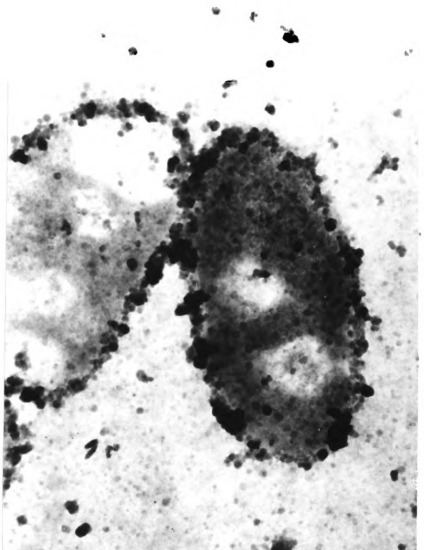


Figure 18. Electron micrograph showing cells of Pseudomonas fragi from Brain Heart Infusion broth, stained with normal rabbit serum. Note the absence of stain deposits. x 30,000.

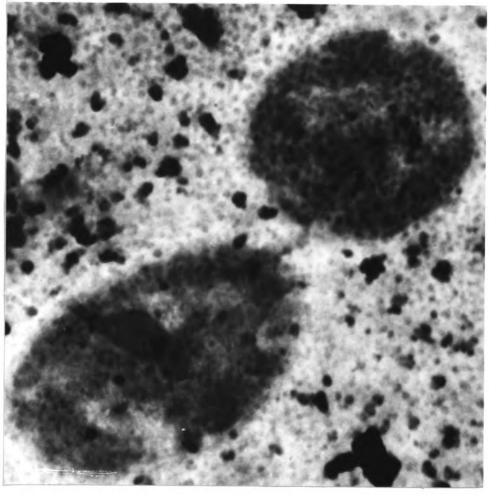


Figure 19. Electron micrograph showing a cell of Pseudomonas fragi from Brain Heart Infusion broth, stained with preimmune rabbit serum. Note the absence of stain deposits. x 30,000.

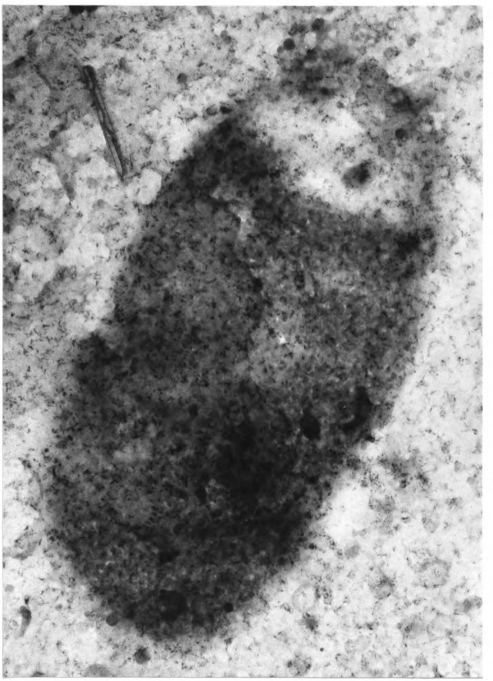


Table 2. Relative intensities of antiprotease staining of *Pseudomonas fragi*.

Serum		PAP[1]	DAB[1]	Relative staining intensity[2]
Specificity	Dilution			
Protease	1:10	+	+	+
Protease	1:50	+	+	+
Protease	1:100	+	+	+++
Protease	1:1000	+	+	++
Preimmune	1:100	+	+	-
Normal	1:100	+	+	-
Aflatoxin B_1	1:50	+	+	-
Protease	1:100	-	+	-
Protease	1:100	+	-	-

[1]PAP or DAP present (+), absent (-).

[2]Relative intensities (+) positive staining
(-) negative staining.

cytoplasm and beneath the cell wall. This area known as the periplasmic space has been previously demonstrated to contain several degradative enzymes and binding proteins. A classic example of a periplasmic degradative enzyme is the inducible alkaline phosphatase of E. coli. Binding proteins are associated with transport of nutrients, such as amino acids, into the bacterial cell. In order to transport nutrients across the cell membrane, binding proteins combine with the nutrients and concentrate them in the periplasmic region where they are eventually carried into the cell.

Since proteins and other large molecules are much too large to enter the cell via the cytoplasmic membrane they must be broken down to smaller units which can be transported through the cytoplasmic membrane. Excretion of the protease into the growth medium by P. fragi results in hydrolysis of proteins thereby providing amino acids for cellular metabolism.

The immunoperoxidase procedure clearly indicates that neutral protease of P. fragi is present in the periplasmic space and absent in the cytoplasm. Two possible conclusions might be drawn from this observation. First, the protease may be synthesized at the cell membrane and secreted directly into the periplasmic space. Alternatively, a precursor or inactive form of the enzyme exists in the cytoplasm that can not be detected by antiserum prepared against the active form. This may be converted to a functional enzyme

upon release into the periplasmic space. An analogous situation may exist for alkaline phosphatase which is synthesized and released by whole cells of E. coli. Spheroplasts of E. coli do not produce active alkaline phosphatase, but a protein antigenetically related to alkaline phosphatase is synthesized and secreted into the medium.

Either of the two previously mentioned models would explain why the P. fragi protease would not hydrolyze cellular protein within the organism. Formation of bleb structures might serve as a mechanism for protease release. With the release of the proteolytic enzyme in meats, degradation of muscle tissue occurs.

CONCLUSIONS

A proteolytic enzyme was isolated and purified from BHI broth inoculated with P. fragi. Ammonium sulfate precipitation, dialysis, concentration by dialysis and gel permeation chromatography yielded a single band when disc gel electrophoresis was performed. Molecular weight calculated from the relative mobility on SDS-PAGE electrophoresis was 48,000 ± 1,200 for the single band detected.

Antibody to the protease was produced in two New Zealand white rabbits during a 10 week immunization schedule. Antibody titer at the end of this period, determined by enzyme linked immunosorbent assay was 4.0×10^7.

The protease specific antibody was used to localize the extracellular protease in P. fragi using a soluble peroxidase-antiperoxidase complex and unlabeled antibody. Electron micrographs demonstrated heavy black deposits in the periplasmic space as well as in the blebs, thereby confirming a relationship between protease production and bleb formation. Similar deposits were not observed in cells treated as controls. No proteolytic activity was detected in Koser Citrate medium inoculated with P. fragi and incubated under identical conditions as the BHI broth.

Blebs were not observed on cells obtained from Koser Citrate medium.

Since the protease was localized in the periplasmic space and the blebs but not in the cytoplasm, it can be concluded that the enzyme is synthesized near the cell membrane and secreted into the periplasmic space or a precursor or inactive form of the enzyme is formed in the cytoplasm. This protein would then be converted into an active enzyme once released into the periplasmic space.

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APPENDICES

Appendix A

Preparation of solutions for disc gel electrophoresis

Stock Solutions

A. Running gel buffer, 0.38 M Tris-HCl pH 8.9
   48.018 g Tris
   950 ml distilled water
   Adjust pH to 8.9 with HCl
   Add distilled water to make 1 liter

B. Stacking gel buffer, 0.062 M Tris-HCl pH 6.7
   7.508 g Tris
   950 ml distilled water
   Adjust pH to 6.7 with HCl
   Add distilled water to make 1 liter

C. Running gel solution, 25% (w/v) acrylamide solution
   0.64 g BIS
   24.83 g acrylamide monomer
   100 ml running gel buffer

D. Stacking gel solution, 6.2% (w/v) acrylamide solution
   1.25 g BIS
   5.00 g acrylamide monomer
   100 ml stacking gel buffer

E. Ammonium persulfate solution, 5.0% (w/v) ammonium persulfate solution
   0.50 g ammonium persulfate
   10 ml distilled water

F. TEMED

G. Saturated sucrose solution

H. Bromophenol blue solution 1% (w/v) bromophenol blue solution.

Final concentration of acrylamide (% w/v) for 9% gels

Working solution	(ml)
Running gel solution	9.0
Running gel buffer	15.5
Ammonium persulfate	0.3
TEMED	0.02

Appendix B

Solutions prepared for SDS-PAGE

Stock Solutions

A. Gel buffer, 0.2 M sodium phosphate plus 0.2% (w/v) SDS pH 7.2
   7.8 g sodium phosphate monobasic
   38.6 g sodium phosphate dibasic
   1.0 L distilled water

B. Sample buffer 0.01 M sodium phosphate with 2.5% (w/v) SDS and 5.0% (v/v) mercaptoethanol
   5 ml gel buffer
   5 ml mercaptoethanol
   2.5 g SDS
   100 ml distilled water

C. 26.7% (w/v) acrylamide solution
   26.0 g acrylamide monomer
   0.70 g BIS
   100 ml distilled water

D. Electrode buffer, 0.1 M sodium phosphate with 0.1% (w/v) SDS pH 7.2 diluted 1:1
   3.9 g sodium phosphate monobasic
   10.23 g sodium phosphate dibasic
   1.0 g SDS

E. 1% (w/v) ammonium persulfate solution
   0.10 g ammonium persulfate
   10 ml distilled water

F. TEMED

G. Saturated sucrose

H. 1% (w/v) bromophenol blue solution

	Final concentration of acrylamide % (w/v)
Solution (ml)	10
Acrylamide	11.3
Gel buffer	15.0
Water	2.2
Ammonium persulfate	1.5
TEMED	0.03

Appendix C

Preparation of solutions for ELISA

Solutions

A. Phosphate buffered saline solution (PBS), 0.1 M phosphate buffer, pH 7.5 with 0.15 M sodium chloride
   80 ml 0.2 M NaH_2PO_4 (27.6 g $NaH_2PO_4 \cdot H_2O$/L
   420 ml 0.2 M NaH_2PO_4 (53.65 g $Na_2HPO_4 \cdot 7H_2O$/L
   8.7 g NaCl/L
   Adjust to 1 L with distilled water

B. PBS-Tween, 0.05% Tween in PBS
   0.5 ml Tween 20
   1 L PBS

C. BSA-PBS solution
   1% TBS in PBS

D. Citrate buffer (CB)
   9.6 g citric acid
   500 ml distilled water
   Adjust pH to 4.0 with 1.0 M NaOH
   Adjust volume to 1 L with distilled water

E. Enzyme substrate solution (ABTS), make fresh daily
   0.22 g ABST/10 ml distilled water
   0.03 ml 30% H_2O_2
   1 ml of this solution added to 100 ml CB pH 4.0

F. Hydrofloric acid-EDTA solution (HF-EDTA)-Stopping reagent
   0.4 g EDTA/ml distilled water
   3.47 ml 48% HF plus 6 ml 1N NaOH/L HF
   Add 100 µl EDTA to 100 ml HF solution.
   Make HF-EDTA on day of use.

Appendix D

Preparation of Millonig phosphate buffer (1961) for fixation of agar encapsulated cells

Stock Solutions

A. 2.26% $NaH_2PO_4 \cdot H_2O$

B. 2.52% NaOH

C. 5.4% glucose

D. 1.0% $CaCl_2$

Fixative solution (washing buffer)

41.5 ml 2.26% $NaH_2PO_4 \cdot H_2O$
8.5 ml 2.52% NaOH
5.0 ml 5.4% glucose
0.25 ml 1.0% $CaCl_2$

Appendix E

Preparation of 1.25 and 2.25% gluteraldehyde fixatives

Stock Solutions

A. Millonig phosphate buffer

B. 25% commercial glutaraldehyde

Fixative Solutions	Amount (ml)	
	Phosphate buffer	Glutaraldehyde
A. 1.25% glutaraldehyde	95	5
B. 2.25% glutaraldehyde	91	9

Appendix F

Preparation of epon-araldite mixture (Mollenhauer, 1963)

	Amount (g or ml)
Epon 812	62
Araldite 506	81
DDSA[1]	100
DBP[2]	4-7
DMP-30[3]	1.5-3% depending on age

[1]Dodecenyl Succinic Anhydride

[2]Dibutylphthalate

[3]Dimethyl Amino Methyl Phenol

Appendix G

Preparation of uranyl acetate and lead citrate stains

Uranyl acetate

50% ethanol saturated with uranyl acetate; store in a dark bottle at room temperature

Lead citrate

Lead Nitrate ($Pb(NO_3)_2$)	1.33 g
Sodium citrate	1.76 g
Distilled water	30 ml

Shake continuously for 30 min, add 8.0 ml 1N NaOH and dilute to 50 ml with water.