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Action of *Pseudomonas fragi* on the Proteins of Pig Muscle

P. J. V. TARRANT, A. M. PEARSON, J. F. PRICE, AND R. V. LECHOWICH

Department of Food Science and Human Nutrition, Michigan State University, East Lansing, Michigan 48823

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Considerable salt-soluble protein degradation was observed in pork muscle inoculated with *Pseudomonas fragi*. During a 20-day incubation period at 10 C, the samples proceeded to rank spoilage or putrefaction. There was a large decrease in the salt-soluble protein fraction and a corresponding increase in nonprotein nitrogen. Disc gel electrophoretic patterns showed that breakdown of the salt-soluble proteins had occurred after incubation for 20 days. During incubation for 10 days at 10 C, *P. fragi* produced large amounts of extracellular proteolytic activity in ground pork. Most of the proteolytic activity appeared immediately after spoilage occurred. However, a significant increase in the ability to hydrolyze casein and a slight increase in the ability to hydrolyze denatured hemoglobin occurred prior to spoilage.

Certain metabolic changes caused by bacterial growth in fresh meat which lead to spoilage have not been elucidated. In particular, there are conflicting reports concerning the role of muscle proteins in the spoilage process. According to Jay (12), low-temperature spoilage occurs in the absence of significant proteolysis. Several species of bacteria produced no alterations in the myofibrillar proteins of pork as determined by density gradient centrifugation, gel filtration, and electrophoresis (21). Metabolism of low-molecular-weight nonprotein molecules by spoilage microorganisms has been reported (14, 18). A number of strains of *Pseudomonas fragi* capable of producing strong off-odors in fish muscle were shown by Castell and Greenough (7) to be nonproteolytic. These authors noted, however, that this might be the result of the method by which the cultures were selected and that proteolytic strains could have been missed.

Although spoilage may normally be initiated by the utilization of low-molecular-weight compounds, there is evidence that proteolysis occurs with some bacteria. Protein breakdown in fish-muscle juice was observed (18) after spoilage by *Pseudomonas* species had become evident. Hydrolysis of sarcoplasmic proteins was reported by Hasegawa et al. (9, 10). These workers reported that two species of bacteria commonly associated with meat spoilage, *P. fragi* and *Leuconostoc mesenteroides*, had the highest proteolytic activity (10). Also, Borton et al. (4, 5) found that *P. fragi* caused an increase in the water soluble and nonprotein nitrogen content of pig muscle and a decrease in both the salt-soluble and insoluble protein content. The myofibrillar fraction of beef spoiled by a mixed flora was found to contain two new antigenic species (20).

A number of workers (2, 6, 13) have observed that *Pseudomonas* organisms constitute the predominant group contributing to the spoilage of fresh meat. Thus, the present investigation was undertaken to determine the extent and nature of proteolytic activity by *P. fragi*.

MATERIALS AND METHODS

Sampling procedures, inoculation, and bacterial counts. A pure culture of *P. fragi* (ATCC 4973) was used in this experiment. Aseptic sample preparation, inoculation, and bacterial counts were the same as those reported by Hasegawa et al. (10).

Extraction of proteins. The procedure for extracting sarcoplasmic and myofibrillar protein was based on that of Helander (11). Duplicate samples of ground pork were placed in cold extracting solutions and homogenized. Each sample was homogenized and extracted three times. Extraction of the salt-soluble proteins was difficult due to the high fat content of the ground pork (8 to 18%). Soluble nonprotein nitrogen was obtained after precipitation of the water-soluble proteins with an equal volume of ice-cold trichloroacetic acid (20%, w/v).

Chemical analyses. The nitrogen content of the protein and nonprotein nitrogen fractions was determined by micro-Kjeldahl analysis followed by nesslerization (17). Fat was determined gravimetrically on an ether extract of the dried tissue (1).

Trichloroacetic acid-soluble peptides were assayed in water extracts by the biuret reaction as follows. The water-soluble protein was precipitated with trichloro-

acetic acid, and the filtrate was neutralized with potassium hydroxide. A 2-ml sample of the standard or analytical solution was mixed with 0.2 ml of concentrated biuret reagent (E. M. Reagents, Brinkmann Instruments, Inc., Westbury, N.Y.). Color was allowed to develop at room temperature for 30 min, and absorbance was read on a spectrophotometer at 545 nm. The tripeptide glycylglycylglycine (Mann Research Laboratories, New York, N.Y.) was used as a standard. The intensity of the biuret reaction varied depending upon the number of peptide bonds in a molecule and upon the constituent amino acids.

Ammonia was determined in the nonprotein nitrogen fractions by the direct colorimetric method of Okuda et al. (22) as modified by McCullough (19). A Corning model 12 pH-meter was used to determine the *p*H on a 1–5 homogenate of meat with distilled water.

Disc gel electrophoresis. Myofibrillar proteins were prepared from the residue after aqueous extraction of the meat sample. The basic disc gel electrophoretic system of Davis (8) was used. The spacer gel and the running gel were prepared by the method of Jolley et al. (15), both containing 7 M urea. Acrylamide was added in the form of Cyanogum. The gels were stained with a solution of 0.36% Buffalo Black NBR (naphthol blue black) in methanol-acetate-water (5:5:1, v/v) and destained electrically.

Determination of spoilage. A panel of four judges was trained prior to the experiment. Two samples of pork muscle were presented to it. One of the meat samples was inoculated with *P. fragi*, and the other was an aseptic control. During spoilage of the inoculated sample, the judges became familiar with the off-odors associated with spoilage by *P. fragi*.

For the experiment, the panel of four trained judges was presented with inoculated and aseptic coded meat samples. The tests were conducted in individual booths equipped with red lights to remove the effect of color differences. Each day during the incubation period, the panel was asked to determine the acceptability of the samples by odor evaluation.

Extraction of proteolytic activity. The ground pork was extracted with 1.5 volumes of potassium phosphate buffer (0.1 M, *p*H 8.0) by stirring for 15 min and then centrifuging (7,500 × *g*, 30 min). The supernatant fluid was decanted through cheesecloth and dialyzed against potassium phosphate buffer (0.03 M, *p*H 7.5) for 20 hr at 4 C.

Assay for proteolytic activity. A 1-ml amount of meat extract was incubated with 1 ml of substrate solution at 37 C for a period of 1 to 5 hr. During this time, the reaction mixtures were shaken automatically (180 excursions per min). The reaction was terminated by adding 2 ml of 5% (w/v) trichloroacetic acid. After 15 min, the precipitated protein was removed by filtration through Whatman no. 2 filter paper. Blanks were treated in the same manner, except the trichloroacetic acid was added immediately before the meat extract. All assays were performed in duplicate. Activities were expressed as the difference in absorbance (280 nm) of the trichloroacetic acid filtrates of the blanks and assay mixtures.

The proteolytic activity of the meat extracts was tested on casein and hemoglobin substrates. A 2% Hammerstein casein solution and a 1% denatured hemoglobin solution (both supplied by Nutritional Biochemicals Corp.) were prepared in tris(hydroxymethyl)aminomethane-hydrochloride buffer (0.05 M, *p*H 7.4).

RESULTS

Protein breakdown in spoiled muscle. The growth of *P. fragi* and its effect on the nitrogenous components and on the *p*H of pork at 0, 8, and 20 days after inoculation are shown in Table 1. No significant changes were observed after 8 days in the myofibrillar, sarcoplasmic, or nonprotein nitrogen fractions. Considerable protein breakdown was evident after 20 days, at which time the amount of myofibrillar protein in the inoculated pork (5.3 mg of nitrogen per g) had decreased to about one-third of its initial value. Separation of the residual myofibrillar protein after 20 days from the water-soluble fraction required an increase in the relative centrifugal force from 1,400 × *g* to 7,500 × *g*. The quantity of

TABLE 1. *Action of Pseudomonas fragi on proteins and nonprotein nitrogen of pork muscle*[a]

Determination	Log plate count (per g)	*p*H	Protein[b]		Nonprotein nitrogen[b]	Acid-soluble peptides[b]	Ammonia[b]
			Myofibrillar	Sarcoplasmic			
P. fragi							
0 days	5.05	5.40	16.7	8.2	4.0	0.75	0.11
8 days	9.48	6.00	19.3	9.0	3.9	0.77	0.30
20 days	10.34	7.99	5.3[c]	9.5	13.9	4.37	4.43
Control							
0 days	0	5.40	15.1	7.3	4.3	0.76	0.14
8 days	0	5.53	16.8	7.5	4.5	0.75	0.16
20 days	0	5.53	14.4	7.7	4.4	0.94	0.13

[a] Determinations were made during 20 days of incubation at 10 C.
[b] Values expressed as milligrams of nitrogen per gram of wet tissue on a fat-free basis.
[c] A greater centrifugal force (7,500 × *g*) was necessary to sediment this protein.

sarcoplasmic protein in the inoculated pork did not change significantly during 20 days of incubation. However, the quantity of nonprotein nitrogen (13.9 mg of nitrogen per g) increased more than threefold. The increase of nonprotein nitrogen consisted principally of peptides (4.37 mg of nitrogen per g) and ammonia (4.43 mg of nitrogen per g). The high *p*H (7.99) of the spoiled pork after 20 days of incubation was presumably due to the high concentration of ammonia present.

The results of disc gel electrophoresis of the myofibrillar proteins at 0, 8, and 20 days are presented in Fig. 1. The patterns for the inoculated pork show that disintegration of the myofibrils occurred during incubation. The initial patterns (A) from inoculated and aseptic pork are identical. After 8 days, the myofibrillar proteins from the inoculated pork showed less distinct, lighter patterns than those from aseptic pork (B). Complete breakdown of the pattern was observed after 20 days of spoilage (C). The normal pattern for the myofibrillar proteins was replaced by numerous faint minor bands. The electrophoretic results are compatible with the quantitative measurements presented above. Both confirm the breakdown of myofibrillar proteins and a change in the physical properties of the remaining myofibrillar fraction.

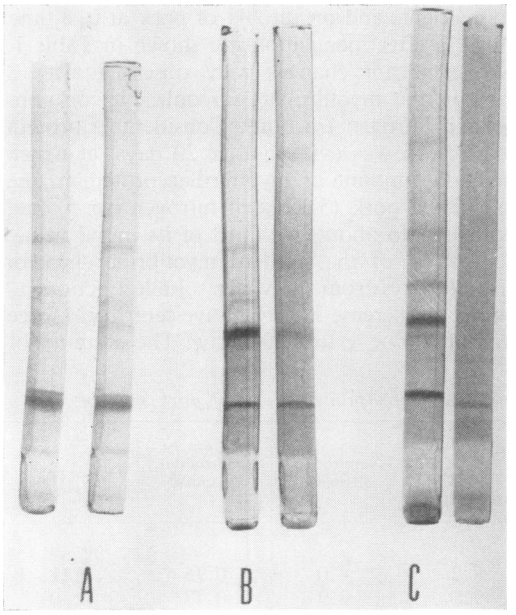

FIG. 1. *Disc gel patterns for the myofibrillar protein fractions of pork muscle. A, B, and C refer to 0, 8, and 20 days of incubation at 10 C, respectively. The left hand pattern within each pair represents aseptic pork; the right hand pattern represents pork inoculated with Pseudomonas fragi. A 200-μg amount of protein was applied to each gel.*

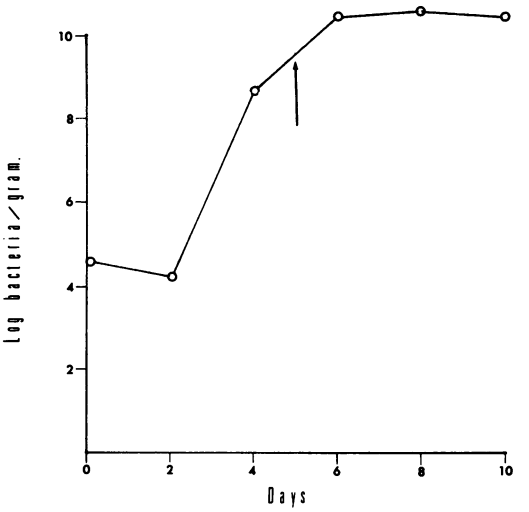

FIG. 2. *Growth of Pseudomonas fragi on pork at 10 C. The arrow indicates the time of onset of spoilage.*

Production of proteolytic enzymes by Pseudomonas fragi. In this experiment, sampling was carried out daily from 0 to 10 days. Bacterial numbers did not increase in the inoculated meat until after the second day (Fig. 2), with the maximum count being reached on day 6. Bacterial growth of the inoculated samples was considerably faster in this experiment than in the previous one. There was no bacterial growth in the uninoculated meat samples. The pork was considered spoiled when the panel decided unanimously that it was unacceptable due to the off-odors liberated. The inoculated meat was first considered objectionable on the fifth day of incubation, which is considered as the onset of spoilage and is indicated by an arrow in Fig. 2–4. The uninoculated meat did not spoil during the experiment.

Results demonstrate production of one or more proteolytic enzymes in pork undergoing spoilage by *P. fragi*. Extracts of inoculated meat were found to contain high proteolytic activity. Although hemoglobin was less susceptible to hydrolysis than casein, the production of proteolytic activity followed a similar pattern with both substrates. Figure 3 shows the activity with casein as a substrate. One unit of activity is defined as the amount of enzyme which liberates hydrolysis products, giving an extinction of 0.001 at 280 nm in 1 hr at 37 C. Hydrolysis of casein was first observed after 4 days of incubation of the inoculated sample. At the onset of spoilage (day 5), there was considerable proteolytic activity

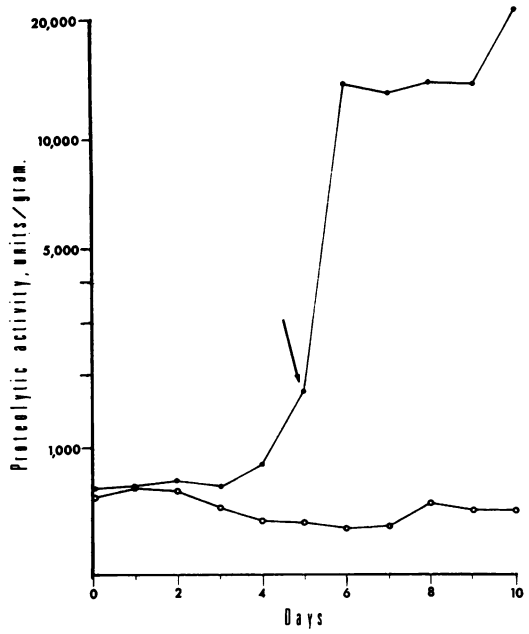

FIG. 3. *Proteolytic activity of pork extracts with casein as substrate. Symbols: ●, pork inoculated with Pseudomonas fragi; ○, aseptic pork. One unit equals 0.001 ΔA_{280} per hour at 37 C. The arrow indicates the time of onset of spoilage.*

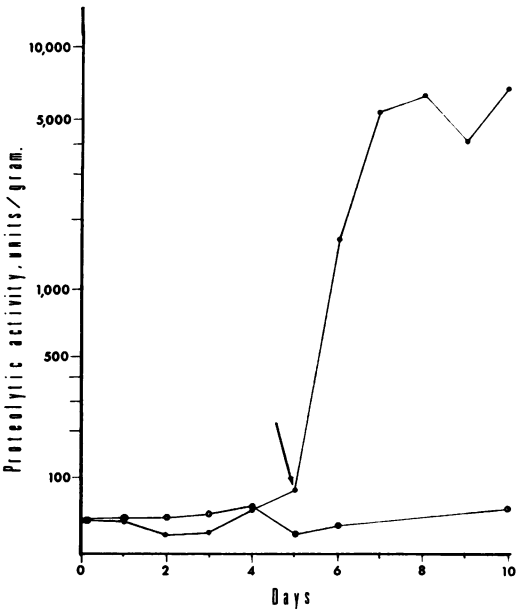

FIG. 4. *Proteolytic activity of pork extracts with hemoglobin as substrate. Symbols: ●, pork inoculated with Pseudomonas fragi; ○, aseptic pork. One unit equals 0.001 ΔA_{280} per hour at 37 C. The arrow indicates the time of onset of spoilage.*

present. However, immediately after spoilage, a very high level of proteolytic activity was reached. None of the extracts of aseptic meat showed any increase in the ability to hydrolyze casein during the 10-day period of the experiment. The low level of proteolysis in the aseptic samples may be due to the residual activity of the muscle cathepsins at the neutral *p*H of the assay medium.

Results using hemoglobin as a substrate are shown in Fig. 4. Immediately after spoilage, there was a very large increase in proteolytic activity. Prior to spoilage, the inoculated meat extract showed a small increase in the ability to hydrolyze hemoglobin. This occurred between days 3 and 5. The aseptic meat extract had a low, constant level of activity.

DISCUSSION

These results show that growth of *P. fragi* on pork was accompanied by considerable proteolysis. The loss of myofibrillar protein was compensated for by an equivalent increase in nonprotein nitrogen, composed mainly of peptides and ammonia. Results indicated that major proteolysis did not occur before spoilage, because quantitative changes in the protein fraction could not be detected until after 8 days of incubation, at which time spoilage was evident. Large scale proteolysis was evident at an advanced stage of spoilage, in agreement with the findings of Lerke et al. (18) and Borton et al. (4). Growth and spoilage by *P. fragi*, an aerobe, occurred on the surface, and only at later stages did the effects of spoilage penetrate the entire meat sample. It is conceivable that proteolysis may have occurred at an early stage but was not readily detectable until the entire mass of meat was affected.

This study did not indicate preferential breakdown of a particular myofibrillar protein. However, electron micrographs taken in this laboratory have indicated an absence of myosin in the myofilaments of pork muscle spoiled by *P. fragi* (T. R. Dutson, Ph.D. Thesis, Michigan State Univ., East Lansing, Mich., 1971). Also, it has been reported (3) that the structural proteins of muscle contain about 54% by weight of myosin. Taking these two observations into consideration, it is probable that the quantitative loss of myofibrillar protein observed in the present study may be caused by the breakdown of myosin. Also, the changes observed in the sedimentation and electrophoretic properties of the myofibrillar proteins may be due to a loss of myosin.

No significant change was observed in the sarcoplasmic protein fraction from the inoculated pork. Hasegawa et al. (10) reported substantial degradation of specific sarcoplasmic proteins of pork by *P. fragi*. Similar degradation presumably

went undetected in the present study because the loss of sarcoplasmic protein was compensated for by the release of water-soluble fragments from the myofibrillar protein fraction.

The demonstration of proteolytic enzyme production in pork by *P. fragi* indicates the mechanism by which protein hydrolysis occurs. The appearance of proteolytic activity was associated with the onset of spoilage. The development of proteolytic activity before the onset of spoilage may be of considerable significance. Although the quantity of enzyme produced at this stage was relatively small, its concentration on the surface of the meat presumably influenced bacterial growth and the production of off-odors.

Immediately after spoilage there was a large increase in proteolytic activity, as indicated by the greatly increased ability of the meat extracts to hydrolyze casein and hemoglobin. At this time, the bacterial proteolytic enzyme(s) were present in high concentration and penetrated deep into the meat. The total sample was then considered rankly spoiled or putrid. It is not surprising that maintenance of a large number of viable cells would necessitate utilization of the prime source of energy and nitrogen in the medium, that is, the proteins.

Results suggest that the proteolytic enzyme(s) are extracellular because the method used to extract the proteolytic activity involved mild treatments, insufficient to disrupt bacterial cells. Liberation of extracellular proteolytic enzyme(s) by *P. fragi* was previously reported (16). However, from the present data, it was not possible to decide whether the enzymes were secreted by living cells or released by autolysis of dead cells. Evidence to indicate that the living cell secreted the enzyme(s) has been obtained (T. R. Dutson, Ph.D. Thesis, Michigan State Univ., East Lansing, Mich., 1971). Electron micrographs have shown myosin breakdown to occur at a distance from the bacterial cells, whereas the cells themselves formed and released globules into the medium. The globules may have contained the proteolytic enzyme(s). However, further work is necessary to establish the method by which the enzymes are released into the medium.

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LITERATURE CITED

1. Association of Official Agricultural Chemists. 1965. Official methods of analysis of the A.O.A.C., 10th ed., p. 346. Ass. Offic. Agr. Chemists, Washington, D.C.
2. Ayres, J. C. 1960. Temperature relationships and some other characteristics of the microbial flora developing on refrigerated beef. Food Res. 25:1–18.
3. Bendall, J. R. 1969. Muscles, molecules and movement, p. 18. Heinemann Educational Books Ltd., London.
4. Borton, R. J., L. J. Bratzler, and J. F. Price. 1970. Effects of four species of bacteria on porcine muscle. 1. Protein solubility and emulsifying capacity. J. Food Sci. 35:779–782.
5. Borton, R. J., L. J. Bratzler, and J. F. Price. 1970. Effects of four species of bacteria on porcine muscle. 2. Electrophoretic patterns of extracts of salt soluble protein. J. Food Sci. 35:783–786.
6. Brown, A. D., and J. F. Weidman. 1958. The taxonomy of the psychrophilic meat spoilage bacteria: a reassessment. J. Appl. Bacteriol. 21:11–17.
7. Castell, C. H., and M. F. Greenough. 1959. The action of *Pseudomonas* on fish muscle. IV. Relation between substrate composition and the development of odours by *Pseudomonas fragi*. J. Fisheries Res. Board Can. 16:21–31.
8. Davis, B. J. 1964. Disc electrophoresis. II. Method and application to human serum proteins. Ann. N. Y. Acad. Sci. 121:404–423.
9. Hasegawa, T., A. M. Pearson, J. F. Price, and R. V. Lechowich. 1970. Action of bacterial growth on the sarcoplasmic and urea-soluble proteins from muscle. 1. Effects of *Clostridium perfringens*, *Salmonella enteritidis*, *Achromobacter liquefaciens*, *Streptococcus faecalis*, and *Kurthia zopfii*. Appl. Microbiol. 20:117–122.
10. Hasegawa, T., A. M. Pearson, J. F. Price, J. H. Rampton, and R. V. Lechowich. 1970. Effect of microbial growth upon sarcoplasmic and urea-soluble proteins from muscle. J. Food Sci. 35:720–724.
11. Helander, E. 1957. On quantitative muscle protein determination: Sarcoplasmic and myofibril protein content of normal and atrophic skeletal muscle. Acta Physiol. Scand. 41: Suppl. 141.
12. Jay, J. M. 1966. Influence of *post-mortem* conditions on muscle microbiology, p. 387–402. *In* E. J. Briskey, R. G. Cassens, and J. C. Trautman (ed.), The physiology and biochemistry of muscle as a food. Univ. of Wisconsin Press, Madison.
13. Jay, J. M. 1967. Nature, characteristics, and proteolytic properties of beef spoilage bacteria at low and high temperatures. Appl. Microbiol. 15:943–944.
14. Jay, J. M., and K. S. Kontou. 1967. Fate of free amino acids and nucleotides in spoiling beef. Appl. Microbiol. 15:759–764.
15. Jolley, W. B., H. W. Allen, and O. M. Griffith. 1967. Ultracentrifugation using acrylamide gel. Anal. Biochem. 21: 454–461.
16. Juffs, H. S., A. C. Hayward, and H. W. Doelle. 1968. Growth and proteinase production in *Pseudomonas* spp. cultivated under various conditions of temperature and nutrition. J. Dairy Res. 35:385–393.
17. Lang, C. A. 1958. Simple microdetermination of Kjeldahl nitrogen in biological materials. Anal. Chem. 30:1692–1694.
18. Lerke, P., L. Farber, and R. Adams. 1967. Bacteriology of spoilage of fish muscle. IV. Role of protein. Appl. Microbiol. 15:770–776.
19. McCullough, H. 1967. The determination of ammonia in whole blood by a direct colorimetric method. Clin. Chim. Acta 17:297–304.
20. Margitic, S., and J. M. Jay. 1970. Antigenicity of salt-soluble beef muscle proteins held from freshness to spoilage at low temperatures. J. Food Sci. 35:252–255.
21. Rampton, J. H., A. M. Pearson, J. F. Price, T. Hasegawa, and R. V. Lechowich. 1970. Effect of microbial growth upon myofibrillar proteins. J. Food Sci. 35:510–513.
22. Okuda, H., S. Fujii, and Y. Kawashima. 1965. A direct colorimetric determination of blood ammonia. Tokushima J. Exp. Med. 12:11–23.