APPLIED MICROBIOLOGY, July 1970, p. 117-122
Vol. 20, No. 1


# Action of Bacterial Growth on the Sarcoplasmic and Urea-Soluble Proteins from Muscle

## I. Effects of *Clostridium perfringens*, *Salmonella enteritidis*, *Achromobacter liquefaciens*, *Streptococcus faecalis*, and *Kurthia zopfii*

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Received for publication 19 March 1970

Comparisons of the starch-gel patterns of uninoculated aseptic control samples from rabbit and pig muscle with similar samples inoculated and incubated with *Clostridium perfringens*, *Salmonella enteritidis*, *Achromobacter liquefaciens*, and *Kurthia zopfii* were made. Results indicated that *C. perfringens* caused extensive alteration in the proteins or enzymes, or both, of the sarcoplasmic fraction of porcine muscle, whereas *S. enteritidis* and *S. faecalis* caused complete breakdown of only myoglobin. Neither *A. liquefaciens* nor *K. zopfii* showed any measurable amount of proteolysis in the sarcoplasmic fraction from pig muscle. Although some of the bands in the starch-gel pattern of rabbit muscle decreased in size and intensity of staining, complete proteolysis of any protein fraction was absent for all test organisms. The disc-gel patterns of the 8 M urea-soluble proteins showed that *C. perfringens* caused extensive proteolysis in pig muscle and a lesser extent of proteolysis in rabbit muscle. None of the other organisms utilized in this study had any measurable effect upon the urea-soluble proteins. In addition, a simple procedure for aseptic isolation of muscle samples for studying meat spoilage is outlined. Results indicate that careful sanitation and cleanliness will give suitable samples for meat spoilage investigations.

Several workers (4, 6) have concluded that meat spoilage microorganisms are not capable of degrading and utilizing meat and fish muscle proteins for growth. Recently, Ockerman et al. (7) demonstrated that a marked decline in the sarcoplasmic protein fraction from sterile beef muscle occurred after inoculation and incubation with *Pseudomonas* and *Achromobacter* microorganisms. Similar results with pig muscle have also been recently reported by Charpentier (2). Further support for the theory that bacteria can degrade and utilize the sarcoplasmic fraction from muscle has been obtained in our laboratory for several different species and strains of microorganisms (T. Hasegawa et al., J. Food Sci. **35**: *in press*, 1970). However, corollary experiments involving the myofibrillar proteins from rabbit and pig muscle using a mixed culture of microorganisms from spoiled hamburger as well as pure culture experiments with several specific microbial strains and species have shown little or no effect on the myofibrillar proteins during storage at 3 or 10 C for as long as 20 days (J. H. Rampton, Ph.D. Thesis, Michigan State University, East Lansing, Michigan, 1969; J. H. Rampton et al., J. Food Sci. **35**: *in press*, 1970). These studies suggest that further work is needed to ascertain the effects of various meat spoilage microorganisms on the different muscle fractions.

The present investigation was undertaken to determine whether certain other species and strains of bacteria could cause proteolysis in the sarcoplasmic fraction of pig and rabbit muscle. Such studies are important in ascertaining the role different microorganisms play in meat spoilage and the mechanism whereby spoilage occurs.

### MATERIALS AND METHODS

**Sampling procedures.** Seven market weight pigs [180 to 230 lb (ca. 81 to 104 kg)] produced at the Michigan State University Swine Farm were slaughtered individually over a 7-month period in a conventional manner, except that special techniques were utilized to obtain essentially aseptic muscle samples.

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The area of the neck utilized for sticking was scrubbed thoroughly with hexachlorophene bactericidal soap. Sticking was carried out with a knife sterilized by boiling in hot water. After conventional dehairing and eviscerating, the unsplit carcass was rinsed with absolute alcohol and chilled at 1 to 3 C for 20 hr. After chilling, the alcohol rinse was repeated, and the carcass was laid on a table covered with Kraft paper in a clean room free from excessive air currents.

The shoulder and ham sections were removed and the middle portion was positioned so that the dorsal midline was accessible. A cut was then made along the dorsal midline of the backfat-loin with a sterilized knife. Subsequent cuts were then made perpendicular to the midline. The backfat was stripped off, and slices about 3 cm in thickness were aseptically excised from the exposed *M. longissimus* and placed in sterile containers. Both *longissimus* muscles were excised and handled in the same manner. The excised samples were then ground through a sterile prechilled grinder and placed in sterilized sample jars. The operator wore sterile disposable gloves during the entire sampling procedure.

Rabbit carcasses were handled in essentially the same way. The carcasses were washed with absolute alcohol immediately after skinning, and the muscles were removed with a sterile knife and tweezers. The muscles were minced in a presterilized grinder and placed in sterilized beakers.

**Inoculation.** Cultures of *Salmonella enteritidis* (13076) and *Kurthia zopfii* (6900) were obtained from the American Type Culture Collection, Rockville, Md., whereas the *Clostridium perfringens*, *A. liquefaciens*, and *Streptococcus faecalis* cultures came from the collection of the Food Science Department at Michigan State University. Except for *C. perfringens*, all species were grown on all purpose plus Tween (APT) broth and, after incubation at 23 to 25 C for 48 hr, were diluted either 50- (*K. zopfii*) or 100-fold in buffered dilution blanks just before addition to the meat. *C. perfringens* cultures were grown in fluid thioglycolate medium and, after 24 hr of incubation at 37 C, were diluted 100-fold in dilute peptone (0.5%) plus cysteine (0.02%) solution.

The diluted cultures were added to the muscle either as it was ground (pork) or as a preground mince (rabbit). After mixing thoroughly, the inoculated samples for each treatment were divided into three or four approximately equal portions, transferred to presterilized sample jars, and covered loosely with sterilized lids.

**Control samples.** Uninoculated control samples were handled in the same way except they were not inoculated. Thus, control samples were available for analysis at the same time as similar inoculated samples. By comparison of results of control and inoculated samples at the end of each storage period, it was possible to differentiate between the changes resulting from microbial breakdown and those resulting from incubation per se.

**Incubation.** Samples inoculated with all microorganisms, except for *C. perfringens*, were incubated at 10 C for 0, 8, and 20 days. The samples inoculated with *C. perfringens* were analyzed immediately (0 days) and again after incubation at 30 C for 4 and 8 days.

**Bacterial counts.** Plate counts were performed for all control and inoculated samples both before and after incubation. Except in the case of *C. perfringens*, for which the inoculated and control samples were incubated at 30 C, bacterial numbers for all samples were assessed by recommended techniques (1). APT agar was used for the plating medium. The plates were inoculated aseptically and incubated for 48 hr at 23 to 25 C before counting. In a few instances in which the colonies were indistinct, the plates were held for an additional 24 hr to give 72 hr of incubation.

For *C. perfringens*, both the APT method and an anaerobic plating method were used for bacterial counts on both controls and inoculated samples. The anaerobic method utilized a sterile peptone (0.5%) plus cysteine (0.02%) solution as the diluent. Plates were poured with sulfadiazine-polymyxin sulfate (SPS) agar and incubated at 35 C for 48 hr in an anaerobic incubator. The anaerobic atmosphere was obtained by vacuumizing and nitrogen flushing of a sealed incubator cabinet (National Appliance Co., Portland, Oregon) three times. All control samples were treated identically to the inoculated samples except they were not inoculated.

**Extraction of the proteins.** All extraction procedures were carried out at approximately 3 C. Four volumes of 0.3 M sucrose-0.01 M KCl-0.01 M tris(hydroxymethyl)aminomethane (Tris) buffer at *p*H 7.6 were added to a weighed sample in a Waring blendor and homogenized at high speed for 1 min. The homogenate was centrifuged at 19,400 × *g* for 15 min. The supernatant, which consisted of the sarcoplasmic proteins, was retained for starch-gel analysis (8, 9).

The remaining precipitate was washed with 12 volumes of the sucrose-KCl-Tris solution and centrifuged as before. The supernatant was discarded, and 6 volumes of Weber-Edsall solution (0.6 M KCl–0.01 M $K_2CO_3$–0.04 M $KHCO_3$) was added to the precipitate and then stored for 24 hr. Then 18 volumes of Weber-Edsall solution was added, and the suspension was mixed with the aid of a magnetic stirrer. The solution was centrifuged at 28,600 × *g* for 30 min. The supernatant, which contained the salt-soluble (myofibrillar) proteins, was discarded, and the residue was washed with 12 volumes of Weber-Edsall solution to more completely remove any residual salt-soluble proteins. After centrifuging as in the previous step, the supernatant was discarded. The precipitate was homogenized with 4 volumes of 8 M urea solution. Centrifugation was carried out at 28,600 × *g* for 30 min, and the supernatant, which contained the urea-soluble proteins, was retained for disc-gel electrophoresis. The precipitate was discarded.

**Protein analysis.** Analysis for protein was carried out before inoculation, after inoculation, and after incubation. The procedure used was the indanetrione hydrate method described by Jacobs (5). Since the quantities of proteins in the various fractions were so small, the standard deviations were relatively large and are not reported herein.

**Starch-gel electrophoresis.** The sarcoplasmic protein fractions were subjected to starch-gel electrophoresis by using a modification of the methods described by Scopes (8, 9). The outer solution was made up of 60 mM Tris and 50 mM boric acid (*p*H 8.6 at 5 C), whereas the inner gel contained 12 mM Tris and 2 mM diethylenetriamine-pentaacetic acid (*p*H 8.25 at 5 C). Electrophoresis was carried out on the horizontal axis at 400 v (approx 36 v/cm) for 6.0 to 6.5 hr at 8 to 12 ma in a cold (3 C) room. After slicing, the gels were stained with 0.18% Buffalo Black (NBR-naphthol blue black) in methanol–acetic acid–water (5:5:1, v/v), washed with glycerin–water–methanol–acetic acid (1:5:5:1, v/v), and photographed.

**Disc-gel electrophoresis.** Acrylamide disc-gel electrophoresis was performed on the 8 M urea-soluble protein fraction by using minor modifications of the method of Davis (3). The gels were generally made in tubes having an inner diameter of 5 mm. The 6.5% running gel and the 5.0% spacer gel both contained 8.1 M urea. The samples were applied with a syringe underneath the buffer directly on the surface of the spacer gel. The spacer gel (7.5 mm) was added upon the acrylamide gel (50 mm).

Electrophoresis was carried out at 200 v (approximately 2.5 ma/tube) in 5 mM Tris-40 mM glycine buffer for 2 hr at room temperature and at 13 to 34 ma. The gels were stained with a solution of 0.18% Buffalo Black in methanol-acetate-water (5:5:1, v/v) for 1 hr. They were then kept overnight (18 to 20 hr) in 7% acetic acid solution and destained electrically. Densitometer tracings of the gels were made with a Canalco model F Microdensitometer.

**Detection of enzymes.** Methods for detecting certain enzymes on the starch-gel patterns were based upon the reduction of nitroblue tetrazolium by reduced nicotinamide adenine dinucleotide as described by Scopes (8, 9). Exact identifications are given in another paper from our laboratory (T. Hasegawa et al., J. Food Sci. **35:** *in press*, 1970).

## RESULTS AND DISCUSSION

**Changes in bacterial numbers and pH.** The changes in log bacterial numbers and *p*H for both rabbit and pig muscle are shown in Table 1. The control samples were essentially free from contamination at 0 days, except in the case of the control pig muscle used in the *C. perfringens* trial. This indicates that it is possible to obtain aseptic samples by using careful sampling procedures without the use of aseptic isolators as utilized by Ockerman et al. (7). Obviously, the procedures described herein are simpler, easier to

TABLE 1. *Log bacterial numbers and pH from control and inoculated rabbit and porcine muscle held 0, 4, and 8 days at 30 C or at 0, 8, and 20 days at 10 C*

| Organism | Storage temp | Days | Rabbit | | | | | | Porcine | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Bacteria[a] | | | | *p*H | | Bacteria[a] | | | | *p*H | |
| | | | Control | | Inoculated | | | | Control | | Inoculated | | | |
| | | | Aerobic | Anaerobic | Aerobic | Anaerobic | Control | Inoculated | Aerobic | Anaerobic | Aerobic | Anaerobic | Control | Inoculated |
| | *C* | | | | | | | | | | | | | |
| *Clostridium perfringens* | 30 | 0 | 0 | 0 | 0 | <1.00 | 5.5 | 5.5 | 0 | 0 | 0 | 1.17 | 5.3 | 5.3 |
| | | 4 | 0 | 0 | 0 | 5.50 | 5.6 | 6.2 | 2.72 | 2.83 | 0 | 4.84 | 5.4 | 5.9 |
| | | 8 | 0 | 0 | 0 | 3.74 | 5.7 | 6.5 | 0 | 1.00 | 0 | 4.75 | 5.4 | 6.2 |
| *Salmonella enteriditis* | 10 | 0 | 0 | | 5.39 | | 5.5 | 5.5 | 0 | | 5.03 | | 5.3 | 5.3 |
| | | 8 | 0 | | 8.12 | | 5.6 | 5.6 | 0 | | 6.04 | | 5.4 | 5.4 |
| | | 20 | 0 | | 8.46 | | 5.7 | 5.6 | 0 | | 7.15 | | 5.4 | 5.4 |
| *Achromobacter liquifaciens* | 10 | 0 | 0 | | 6.40 | | 5.5 | 5.5 | 0 | | 5.91 | | 5.3 | 5.4 |
| | | 8 | 0 | | 6.62 | | 5.6 | 5.6 | 0 | | 5.90 | | 5.4 | 5.3 |
| | | 20 | 0 | | 6.38 | | 5.7 | 6.4 | 0 | | 5.74 | | 5.4 | 5.4 |
| *Kurthia zopfii* | 10 | 0 | 0 | | 2.38 | | 5.5 | 5.5 | 0 | | 1.00 | | 5.3 | 5.3 |
| | | 8 | 0 | | 4.94 | | 5.6 | 5.7 | 0 | | 3.52 | | 5.4 | 5.4 |
| | | 20 | 0 | | 7.00 | | 5.7 | 5.7 | 0 | | 4.96 | | 5.4 | 5.3 |
| *Streptococcus faecalis* | 10 | 0 | 0 | | 5.40 | | 5.5 | 5.5 | 0 | | 5.60 | | 5.3 | 5.4 |
| | | 8 | 0 | | 6.19 | | 5.6 | 5.6 | 0 | | 5.55 | | 5.4 | 5.5 |
| | | 20 | 0 | | 6.19 | | 5.7 | 5.6 | 0 | | 5.22 | | 5.4 | 5.4 |

[a] Log bacterial numbers per gram.

utilize, and require only careful attention to sanitation.

A rapid increase in the numbers of *C. perfringens* occurred under anaerobic conditions during the first 4 days of incubation at 30 C, but from 4 to 8 days there appeared to be some decline in numbers (Table 1), indicating maximum growth had been reached and a decline in numbers may have occurred before 8 days of incubation. The exact point of maximum growth was not determined in this study, as it could have occurred either before or after 4 days of incubation. *S. enteritidis* (Table 1) also showed a rapid increase in numbers between 0 and 8 days of storage but little further increase between 8 and 20 days. *K. zopfii* (Table 1) showed continued growth throughout storage, with maximum numbers occurring at 20 days of incubation for both pig and rabbit muscle. *A. liquefaciens* and *S. faecalis* both showed little increase in numbers during incubation, but apparently reproduced slowly or remained constant since numbers were essentially the same after 20 days of storage (Table 1).

The *p*H values were relatively stable during storage of controls at both 10 and 30 C. Incubation with *C. perfringens* resulted in some increase in *p*H for both rabbit and porcine muscle. Rabbit muscle inoculated with *A. liquefaciens* and incubated for 20 days showed some increase in *p*H, but the *p*H of pig muscle did not change during storage. The *p*H values for samples inoculated and incubated with *S. enteritidis*, *K. zopfii*, and *S. faecalis* remained essentially unchanged during storage (Table 1).

**Sarcoplasmic proteins.** The data show that incubation of control samples of rabbit muscle at both 10 and 30 C resulted in complete disappearance of myoglobin (MB), apparently due to autolysis. Similarly, at 30 C, hemoglobin (HB) disappeared from control samples during incubation for 8 days but was still present in control rabbit muscle after storage for 20 days at 10 C. This suggests that MB is more susceptible to autolysis than HB. This is not too surprising since it is well known that the molecular weight of MB is only about one-fourth of HB. However, none of the organisms studied herein caused complete disappearance of HB, MB, phosphofructokinase, aldolase (ALD), glyceraldehyde phosphate dehydrogenase (GAPDH), $\alpha$-glycerophosphate dehydrogenase, phosphoglycerate kinase, phosphoglycerate mutase, phosphopyruvate hydratase, pyruvate kinase, lactate dehydrogenase (LDH), creatine kinase, or albumin during incubation of rabbit muscle.

Although none of the proteins or enzymes, or both in rabbit sarcoplasm disappeared as a consequence of microbial growth, this does not prove

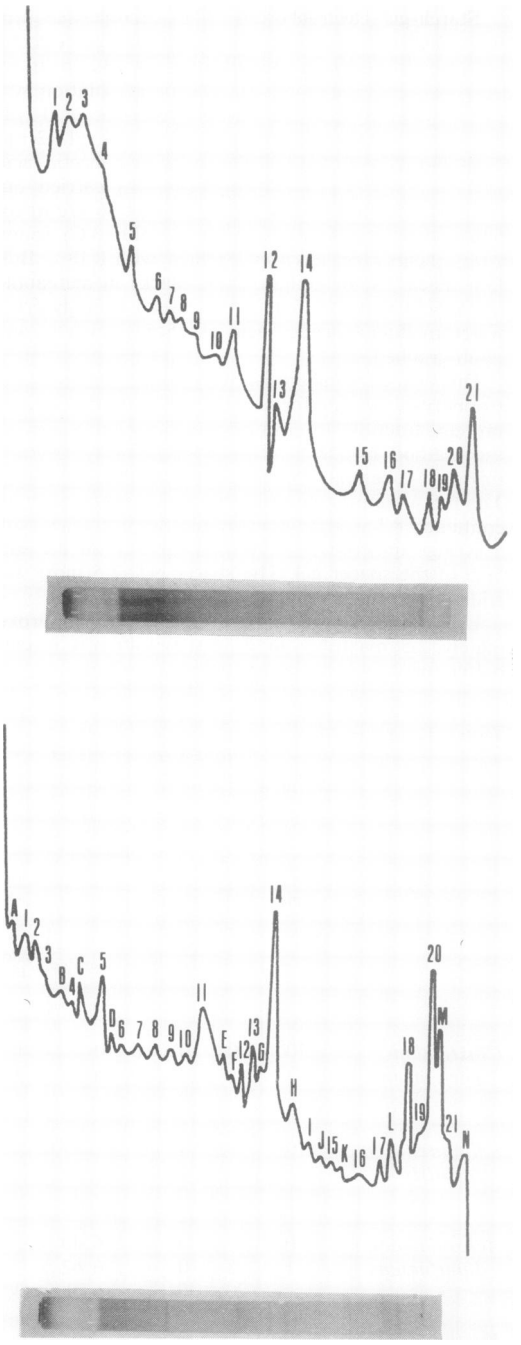

FIG. 1. *Disc-gel patterns and densitometer tracings for 8 M urea-soluble proteins from rabbit muscle after incubation at 30 C for 8 days. Top, uninoculated control. Bottom, inoculated with C. perfringens. Numbers for all peaks correspond for control and inoculated samples. The absence of any number indicates that the peak disappeared from the pattern, whereas a letter indicates the presence of a new peak.*

that the organisms studied did not cause some proteolysis of the sarcoplasmic fraction. It does, however, demonstrate that complete proteolysis of individual proteins did not occur under the experimental conditions imposed during this study. The fact that some protein bands stained appreciably lighter after inoculation and incubation suggests that minor proteolysis did in fact take place during storage.

In agreement with the study on rabbit muscle, MB disappeared from uninoculated control pig muscle during incubation at 30 C, but, in contrast, HB did not disappear from the control. In addition, the growth of *C. perfringens* at 30 C completely removed ALD, GAPDH, and LDH from the starch-gel pattern of porcine muscle, indicating considerable proteolysis.

MB was still present in the uninoculated control pig muscle incubated at 10 C as was HB, indicating some resistance to autolysis. Both *S. faecalis* and *S. enteritidis* completely broke down MB, although neither of these organisms caused complete proteolysis of the other sarcoplasmic proteins or enzymes, or both, in pig muscle. Neither *A. liquefaciens* nor *K. zopfii* caused the complete breakdown of any of the sarcoplasmic proteins or enzymes, or both.

**Urea-soluble proteins.** The disc-gel patterns for the 8 M urea-soluble proteins are shown in Fig. 1 for rabbit muscle and in Fig. 2 for porcine muscle. The disc-gel patterns for control rabbit muscle after storage for 8 days at 30 C showed 21 distinct bands (Fig. 1). After incubation with *C. perfringens* for 8 days at 30 C, there was considerable protein breakdown (Fig. 1). Although all 21 bands were still evident, the size and distinctness of 5 bands were definitely altered. In addition, 14 new bands were present in the pattern. This suggests that some proteolysis of the urea-soluble fraction from rabbit muscle had occurred as a result of the growth of *C. perfringens*.

Although the disc-gel patterns were obviously different for rabbit and pig muscle, the latter also showed 21 distinct bands in control samples after incubation at 30 C for 8 days (Fig. 2). After incubation with *C. perfringens* for 8 days at 30 C, 6 of the bands in the disc-gel pattern had completely disappeared and 10 new bands were apparent. This demonstrates that *C. perfringens* caused marked protein breakdown in the 8 M urea-soluble fraction of pig muscle. Examination of disc-gel patterns of samples incubated with *C. perfringens* showed that proteolysis of both rabbit and pig muscle was almost as extensive after storage for 4 as for 8 days.

Disc-gel patterns for samples inoculated and incubated with *S. enteritidis*, *A. liquefaciens*, *K. zopfii*, and *S. faecalis* showed no evidence of

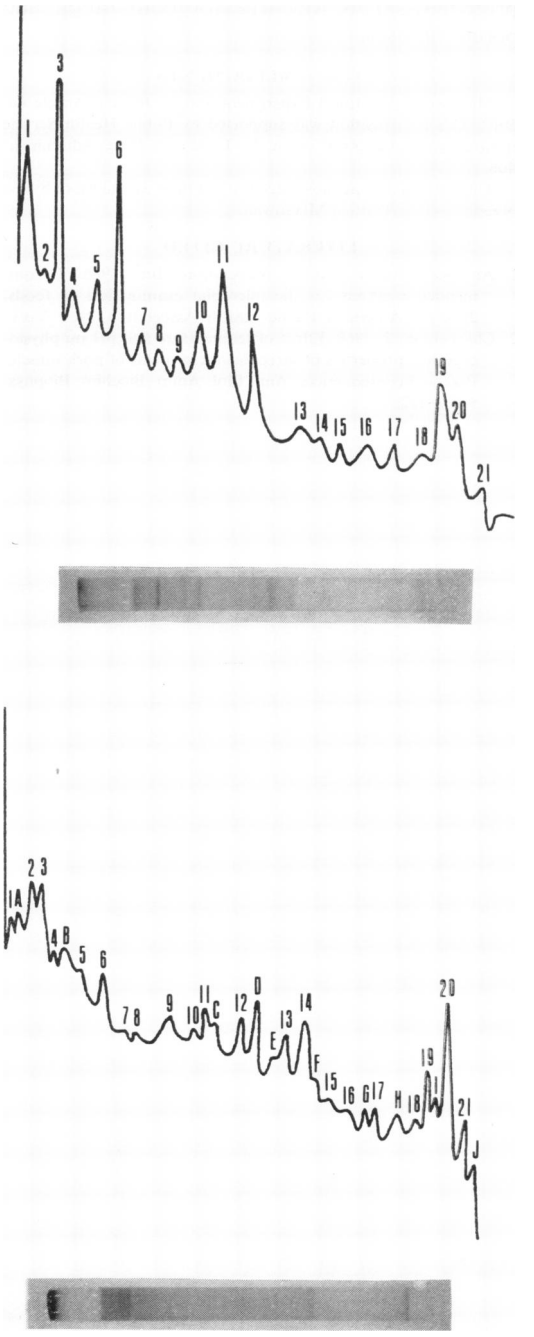


FIG. 2. *Disc-gel patterns and densitometer tracings for 8 M urea-soluble proteins from porcine muscle after incubation at 30 C for 8 days. Top, uninoculated control. Bottom, inoculated with C. perfringens. Numbers for all peaks correspond for control and inoculated samples. The absence of any number indicates that the peak disappeared from the disc-gel pattern, whereas a letter indicates the presence of a new peak.*

proteolysis. This was true for both rabbit and porcine muscle.

ACKNOWLEDGMENTS

Michigan Agricultural Experiment Station Journal Article no. 5017. This investigation was supported by Public Health Service research grant FD-00097 from the Food and Drug Administration.

The authors also acknowledge the technical assistance of Sylvia Ngoddy and of Patricia McGowan.

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