THE PROTEIN OF MEAT TENDERIZED BY PAPAIN-TREATMENT AND ON-CARCASS AGING

— Changes in physico-chemical properties and electrophoretic patterns —

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Studies on meat aging have comprehensively been reviewed[1~3] during the past years. The common belief is that the aging has profound effect on myofibrils. Many past studies were concerned with the interaction of actin and myosin in the region of thick and thin filament overlap. The consensus is that if rigor linkages between these two sets of protein were weakened, meat would become more tender[4~5]. But, an alternative view has also been postulated which suggests that the aging is due to disintegration of the Z lines of sarcomeres leading to the loss in tensile strength of myofibrils and to increased tenderness in cooked meat [7~8]. Despite these intensive studies any direct correlation between structural and tenderness changes is still very much a mystery.

Attempts to make meat artificially tender have consistently progressing. The discoveries that certain plants, fungi and bacteria produce non-toxic proteolytic enzymes was followed by their enlistment as commercial meat tenderizers[10]. Among them papain has helped the muscle biochemists to further understand the myosin molecule by its cleavage to head (heavy meromyosin subfragment-1) and tail (total rod) portions at low ionic strength[11,12]. This tail portion of the myosin molecule is highly helical[11] and easily prepared by ethanol fractionation procedure[11,12].

Although the results[5,6] accumulated so far indicate that post-mortem muscle does not undergo any extensive proteolysis and that proteolysis is probably not an important factor contributing to post-mortem changes in muscle proteins, it is still attractive to imagine that weakening of actin-

myosin interaction could be due to proteolytic cleavage of rigor linkages between the thick and thin filaments. Should that happen, helical fragments of myosin must be present in the myosin fraction of myofibrillar proteins.

No study has yet been undertaken to differentiate between the fragmentation by papain and the aging of myofibrils in muscle. *In situ* rod formation by papain at 2°C was used to invstigate and compare if such fragmentation occurs during on-carcass aging at 2°C, and the changes in protein concentrations of myosin fraction before and after the ethanol fractionation were measured in addition to the measurements of viscosity and optical rotatory dispersion (ORD) and electrophoresis in sodium dodecylsulfate polyacrylamide gel (SDS-PGE) of the ethanol-treated protein fraction. We have directed our efforts toward the clarification of the decisive incongruity between the effects of papain and aging on meat tenderness.

Materials and Methods

A. Protein preparations

Myosin was prepared from rabbit muscle by the method of PERRY[13]. The myofibrils were prepared from 12 months old cock breast muscles according to the procedure described by PERRY and GRAY[14]. Cocks were obtained from Experiment Farm, Faculty of Agriculture, Hokkaido University.

Preparation of standard myosin rod was performed as described by BÁLINT *et al*[12]. The digestion of myosin by papain was carried out as follows. Myosin at a concentration of 9 mg/ml in a medium containing 20 mM KCl, 3 mM EDTA, 5 mM β-mercaptoethanol and 20 mM K-phosphate buffer (pH 6.5) was digested at 25°C for 20 min by the addition of appropriate amount of papain (Sigma, myosin : papain=120 ; 1 (W/W) in a small volume of papain activating solution (50 mM cysteine, 10 mM EDTA, 33 mM phosphate (pH 7.0) and incubated for 3-hr). Reactions were terminated by the addition of 2 mM (final conc.) monoiodoacetic acid and cooled in ice immedeately. Digests were dialyzed against 10 vol. of 10 mM phosphate buffer (pH 7.0) with 1 mM monoiodoacetic acid overnight and pelleted by centrifugation at 22,000$\times g$ for 1 hr to remove soluble peptides. The pellets were fractionated with 75% ehthanol and the precipitate was resuspended in 0.5 M KCl. The suspension was exhaustively dialyzed against 0.5 M KCl (pH 7.0) to remove ethanol. Crude rod preparation was obtained by centrifuging the dialyzate at 10,000$\times g$ for 30 min. The preparations thus obtained were purified further by the Sepharose 2 B gel filtration method developed by Samejima, TAKAHASHI and YASUI (unpublished).

B. Papain treatment of muscle

Papain used for this purpose was crude preparations from Tokyo Kasei Kogyo Co.. To 20 g of minced cock breast muscle was added 0.46 g of papain in 10 ml of the activating medium described above and thoroughly mixed with 2 mM (final conc.)NaN_3. The mixture was incubated for specified periods of time at 2°C and the reaction was stopped by the addition of 2 mM (final conc.) monoiodoacetic acid. The reaction mixture which contained 10 ml of H_2O instead of papain solution, served as control (0-hr sample). The activity of all papain preparations used in this study was monitored by using rate of splitting 1% casein as a substrate per 1 min[15]. Purified preparations from Sigma and crude preparations from Tokyo Kasei Kogyo had activities of approximately 0.69 mg and 10.75 mg casein units, respectively.

C. Aging of muscle

Breast mucle was allowed to age on carcass and was excised just prior to protein extraction or myofibril preparation. The carcasses were wrapped with cheese cloth which was dipped in 3 mM NaN_3 solution and further covered with polyethylene film. Between the cloth and film were placed filter papers dipped in toluene. The carcasses were stored at 2°C.

D. Measurement of the degree of the shortening of myofibrils

Measurement of the degree of the shortening of myofibrils was carried out as described previously[8]. Myofibril preparations were observed under phase contrast microscope at a magnification of ×1,500. The degree of the shortening of myofibrils was expressed as ratio of the number, [R], of sarcomeres at rest length to total number, [Σ], of sarcomeres. In sarcomeres counted as contracted, a dense band developed in the midregion accompanied with the further contraction. In every case, about 500 myofibrils were counted.

E. Preparation of ethanol-treated protein fraction

Briefly, the procedure involved the incubation of muscle mince with papain for measured times, termination of the digestion with monoiodoacetic acid, removal of the digested peptides and soluble proteins by extraction with buffer solution at low ionic strength, extraction of myosin with modified Hasselbach-Schneider solution (0.6 M NaCl, 0.01 M $Na_4P_2O_7$, 0.1 M Na-phos phate buffer (pH 6.4), and 1 mM $MgCl_2$) and recovery of ethanol fractionated protein by precipitation with 75% ethanol. A flow sheet is given in Fig. 1. The same procedure was applied for muscles aged on carcass also.

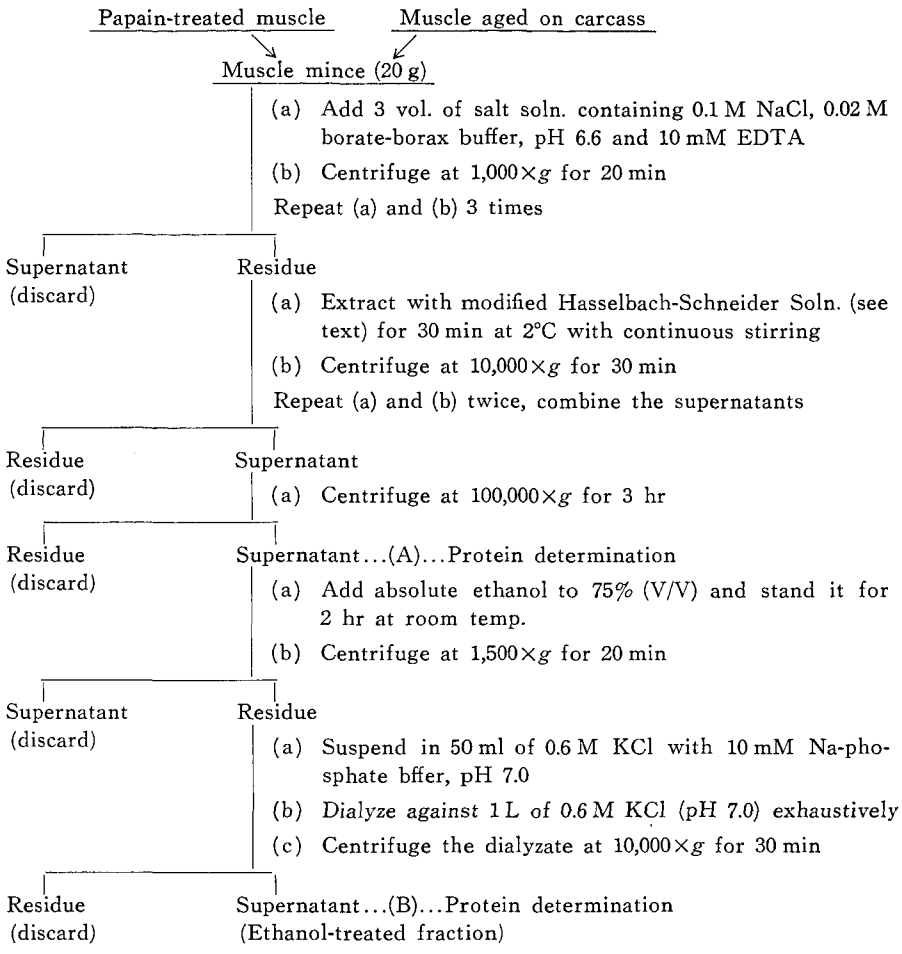

Fig. 1. Preparation of ethanol-treated protein fraction.

F. Viscosity measurement

The viscosity of myosin rod and the protein ethanol-treated fraction was measured at 20°C using Ostwald-type viscometer with an outflow time for buffer of 140–180 sec. Protein concentration was kept 1 mg/ml.

G. Optical rotatory dispersion

The measurement of optical rotatory dispersion (ORD) was carried out with a JASCO ORD/UV-5 at 25°C. Helix content was calculated from the following formula using the $(m')_{233}$ value:

$$\text{Helix}\,(\%) = -[(m')_{233}+2000]\times 100/13{,}000\,.$$

H. Sodium dodecylsulfate polyacrylamide gel electrophoresis

The procedure adopted for sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PGE) was essentially the same as that of WEBER and OSBORN[16], using ethanol recrystallyzed SDS. Samples in 0.01 M Na-phosphate buffer (pH 7.0), 1% β-mercaptoethanol solution were incubated 5 min at 100°C. Samples of 10-20 μg were applied to the 6% gel which were run at 8 mA per tube for 4-5 hr.

I. Measurement of ATPase activity

The ATPase measurements were performed at 25°C in the presence of 0.03 M KCl, 5 mM $MgCl_2$, 20 mM Tris-maleate buffer (pH 7.6), 0.5 mg protein/ml and 1 mM ATP with or without 1 mM EGTA. After 5 min of incubation, the reaction was stopped by the addition of 15% trichloroacetic acid. The liberated phosphate was measured by the method of Fiske and SubbaRow. The ratio of Mg^{2+}-ATPase activity/Mg^{2+}-EGTA-ATPase activity was used to express the calcium-sensitivity of the contractile system.

J. Protein determination

Protein concentration was determined by LOWRY's phenol reagent method[17] or by the biuret reaction, standardized by determining the nitrogen by the micro-Kjeldahl method.

K. Bacterial counts

Bacterial counts were obtained periodically from oncarcass aged muscle by plate method. A sample was asceptically cellected and minced with a sterile meat grinder. With the addition of the equal quantity of dilutent (phosphate buffered 0.85% saline solution pH 7.2), the sample was homogenized by means of a Universal Homogenizer (Tokyo Nihon Seiki). Out of each dilution, duplicate standard agar plates (5 g of meat extract, 10 g of polypeptone, 1 g of glucose and 15 g of agar dissolved in 1 L of deionized water, pH 7.0) were prepared and incubated at 25°C for 3 days.

Results

A. Formation of Myosin Rod from Myosin by Papain Digestion

Fig. 2 represents a typical Sepharose 2 B column chromatogram of ethanol fractionated myosin subfragments as well as some electrophoretograms of certain chromatographed fractions. Molecular weight determinations were made on the basis of the distance *vs* molecular weight relationship on SDS-PGE indicated in Fig. 3.

The results clearly showed that 120,000 dalton fragment known to be

a total myosin rod[11,12] was effectively produced by limitted proteolysis under our experimental conditions. It is, however, to be emphasized that simple ethanol fractionation procedure yields a mixture of several subfragments

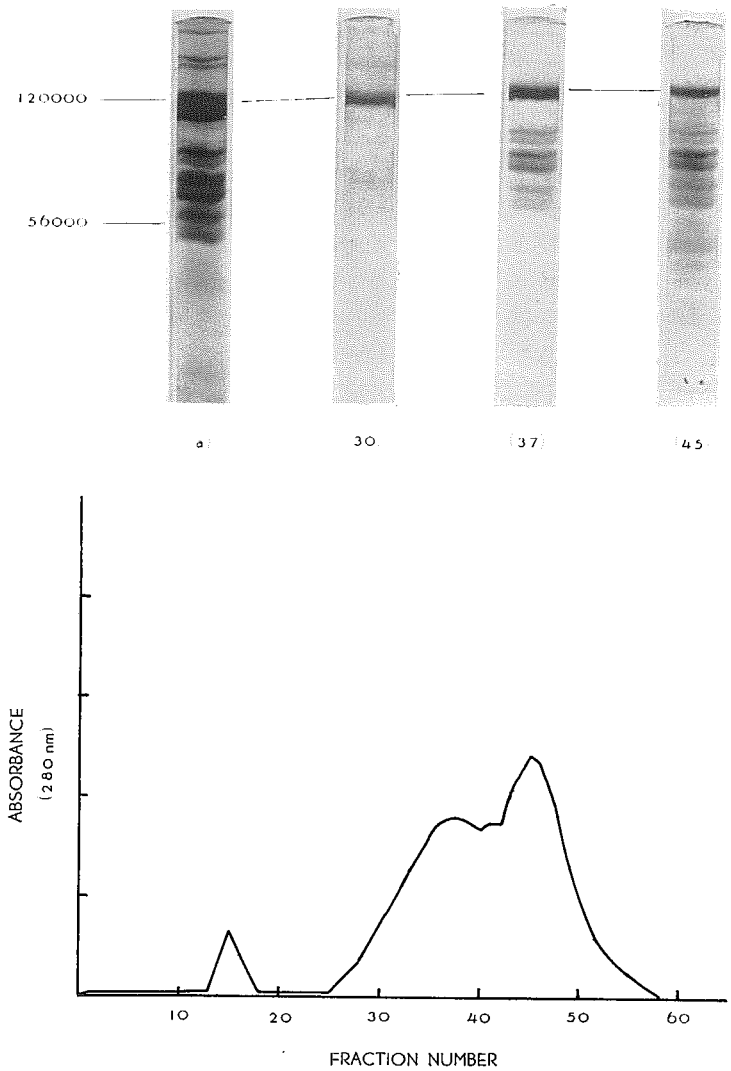

Fig. 2. Elution patterns of Sepharose 2B column chromatography of ethanol fractionated myosin rod fron papain treated myosin at $I'/_2=0.2$, and SDS-PGE patterns of some chromatographed fractions: (a) unchromatographed control; (30) fraction 30; (37) fraction 37; (45) fraction 45.

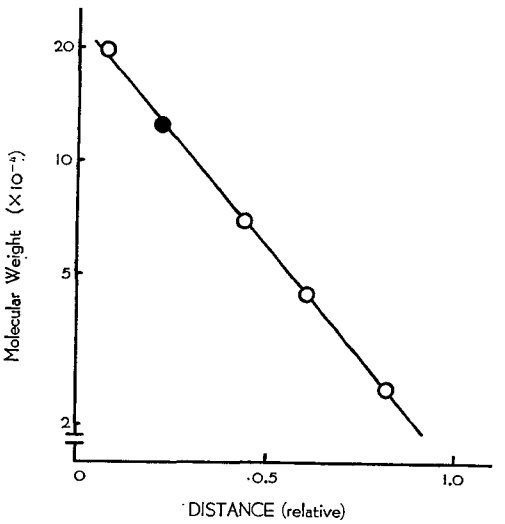

Fig. 3. Determination of the molecular weight of myosin rod on SDS-PGE. (○); the 4 marker proteins used were myosin heavy chain (200,000 dalton), bovine serum albumin (69,000 dalton), ovalbumin (45,000 dalton) and chymotrypsinogen A (25,000 dalton) from the top. (●); myosin total rod from fraction 30 in Fig. 2.

over 56,000 to 120,000 dalton (Fig. 2). The results obtained by SAMEJIMA *et al.* (unpublished data), show that the most homogeneous myosin rod can be obtainable by their further purification using Sepharose 2 B column chromatography (Fig. 2). The ethanol fractionated subfragments conssit of several smaller subfragments with the molecular weight of 120,000 daltons. The fractions were highly helical and exhibited no ATPase activity. Therefore they were thought to be the parts of helical tail of myosin.

B. Effects of Papain and Post-mortem Aging on Ethanol-treated Fraction of Muscle

As described earlier in this paper, if the cleavage of rigor linkage has occured, highly helical rod fragments would certainly appear in the ethanol-treated fraction (Fig. 1). In addition, the amount of proteins extracted with Hasselbach-Schneider solution showed almost no time-dependence during post-mortem storage of muscle (MORITA, TAKAHASHI and YASUI, unpublished data). Therefore, extraction and fractionation of myofibrillar proteins according to Fig. 1 will enable us to learn if there is any proteolytic degradation of cross linkages. The ratio of B/A (Fig. 1, protein concentration of ethanol-treated fraction/protein concentration of Hasselbach-Schneider fraction, most-

ly myosin) was then plotted against digestion or aging time (Fig. 4). As expected, papain digestion did show a remarkable increase in the concentration of ethanol-treated fraction, indicating molecular fragmentation of myosin in myofibrils. At 2°C the reaction reached maximum after 8 hr-digestion and leveled off thereafter. Aging of muscle on carcass at 2°C, on the other

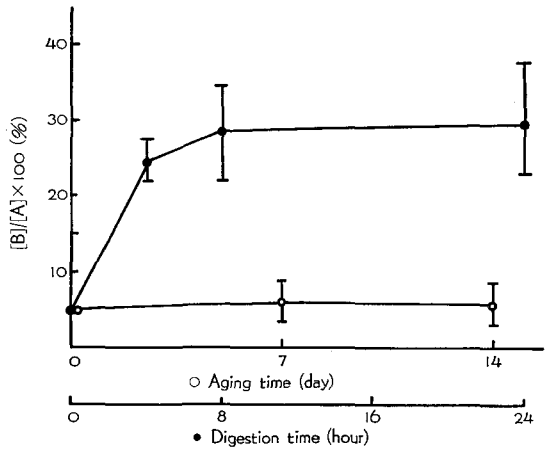

Fig. 4. Liberation of ethanol fractionated protein during papain-treatment (●) or on-carcass aging (○) at 2°C. (A); protein concentration of Hasselbach-Schneider fraction in Fig. 1, B; protein concentration of ethanol-treated fraction in Fig. 1. Bars in the figure indicate standard deviations of 4 xperiments.

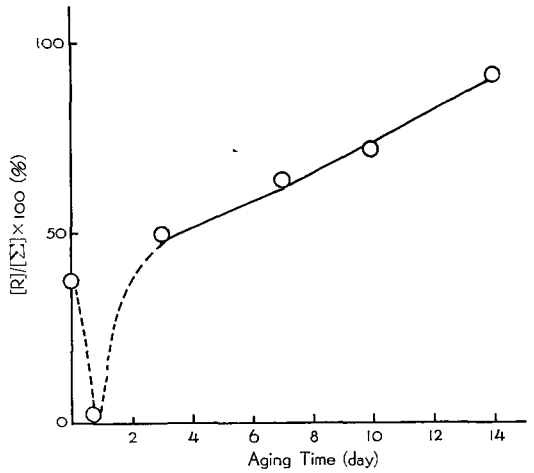

Fig. 5. The reversibility of post-mortem contraction.

hand, revealed no increase in B/A ratio even after 14-day storage (Fig. 4), though the resolution of rigor was morphologically well established (Fig. 5). Changes in pH values and bacterial counts of breast muscle during on-carcass aging are illustrated in Table I. The decrease in the bacterial counts with the progress of aging would imply that under our experimental conditions the changes induced in myofibrillar proteins by proteolysis during aging, if any, may not be of bacterial origin.

TABLE I. Changes in pH and bacterial number during on-carcass aging of cock breast muscle

Storage time (days)	pH	Bacterial number/g muscle
0	6.38 ± 0.12*	102,000
7	5.76 ± 0.06*	28,000
14	5.85 ± 0.22*	7,000

* Standard deviations from 6 experiments.

C. Physico-chemical Properties of Ethanol-treated Protein Fractions from Papain-treated and Aged Muscles

In Table II, are shown the changes in helical contents and viscosity of ethanol-treated fractions obtained from the papain digested and aged breast muscles during the course of reaction at 2°C. At death muscle, helical content of the protein fraction was about 60%. In the case of papain digestion, values of both parameters increased by more than 20% as the reaction proceeded, but in the case of aging on carcass, the increase in helical contents was less than 10% whereas viscosity decreased conversely after 14 days' of storage. The initial helical contents of this protein fraction are unexpectedly high and are almost equivalent to the helical contents of native myosin[18]. To investigate the cause of this higher helicity and the apparent

TABLE II. Changes in α-helix content and reduced viscosity of etoanol-treated protein fractions from papain-treated and aged on carcass muscles.

	Papain-treatment at 2°C (hr)				On-carcass aging at 2°C (day)		
Storage time	0	0	8	24	0	7	14
Helical content (%)	58.7	76.3	77.7	82.3	58.7	58.2	64.6
Reduced viscosity (dl/g)	1.2	1.8	2.0	2.1	1.2	0.8	0.8

* Values were the average of two experiments.

increase in either values during the reaction, ethanol-treated protein fractions from variously treated muscles were subjected to SDS-PGE. At the same time myofibrils prepared from at-death and 7 day-aged muscles were also examined as references.

Fig. 6 depicts the results of the above experiments. On gels (1) and (2) (myofibrils from at-death and 7 day-aged muscles), myosin heavy chains, α-actinin, actin, tropomyosin were apparent, while the lower gel region probably included myosin light chains and some troponin components. These results are consistent with the recent report by SAMEJIMA and WOLFE[19]. Gel (2) clearly shows a 29,000 dalton protein which can not be seen in gel (1). No 120,000 dalton band is visible either in gel (1) or gel (2).

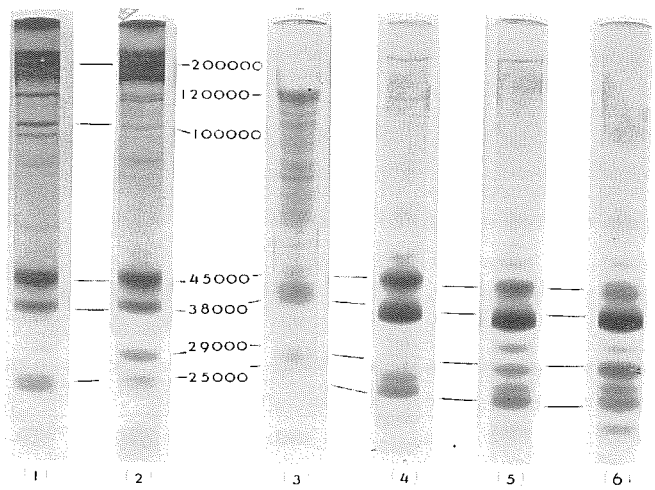

Fig. 6. SDS-PGE patterns of myofibrils, and ethanol-treated protein fractions from papain-treated and aged on carcass muscle. (1) and (2); myofibrils from at death muscle and 7 day-aged muscle, respectively. (3); ethanol-treated protein fraction from 4 hr-papain-treated muscle. (4), (5) and (6); ethanol-treated protein fractions from at death muscle, 7 day-aged muscle and 14 day-aged muscle, respectively.

Fig. 6, (3) shows the SDS-PGE patterns of ethanol-treated fraction from 4 hr-papain digested muscle. The patterns resemble the crude preparation shown in Fig. 2 except for the existence of 38,000 dalton band which presumably corresponds to tropomyosin. In the gel patterns of both fresh (Fig. 6, (4)) and aged samples (Fig. 6, (5) and (6)), no 120,000 dalton protein is apparent, but instead the middle and lower regions include actin-like protein

(45,000), tropomyosin and some troponin components. The absence of protein bands in the upper region suggests that myosin and α-actinin have been removed by ethanol-treatment. SDS-PGE patterns on gels (4)-(6) in Fig. 6 suggest that ethanol-treated protein fractions from aged muscle consist of actin, tropomyosin and troponin and that the former two proteins are the main components. Tropomyosin is a rod shaped and highly helical protein[20], and that is why its presence results in higher value of helical contents of the ethanol-treated protein fractions. Since the density of actin band decreased with the time of aging (Fig. 6, (4)-(6)), while no such effect was noticed for tropomyosin, relative amount of tropomyosin appears to increase as aging proceeds. Obviously the increase in its amount causes the increase in hilical contents of the protein fraction.

Tropomyosin has been known to polymerize in the absence of neutral salt and to depolymerize into monomers at high ionic strength[20]. The dependence of viscosities on salt concentrations of ethanol-treated protein fractions from aged muscles was, therefore, examined. The results in Fig. 7 are indicative of the characteristic salt-dependence of the viscosity of tropomyosin. Therefore, it may safely be concluded that relatively higher values of initial helical contents of the ethanol-treated protein fraction are due to the presence of tropomyosin in this fraction and that the apparent slight but significant, increase in helical content during aging (Table II) is originated

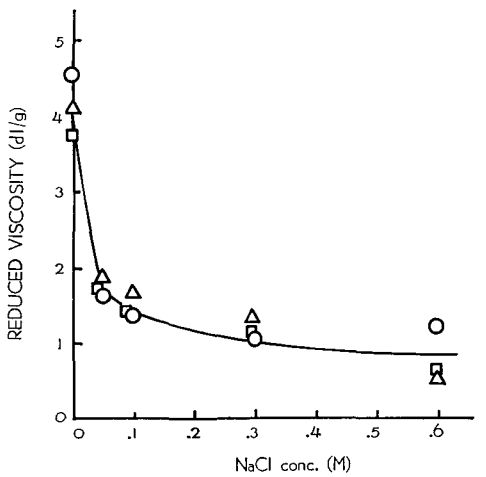

Fig. 7. Ionic strength dependence of reduced viscosity of the ethanol-treated protein fractions from aged muscle. (○); at death muscle, (△); 7 day-aged muscle, (□); 14 day-aged muscle.

from the increase in the relative amount of tropomyosin as judged by SDS-PGE patterns (Fig. 6., (4)-(6)).

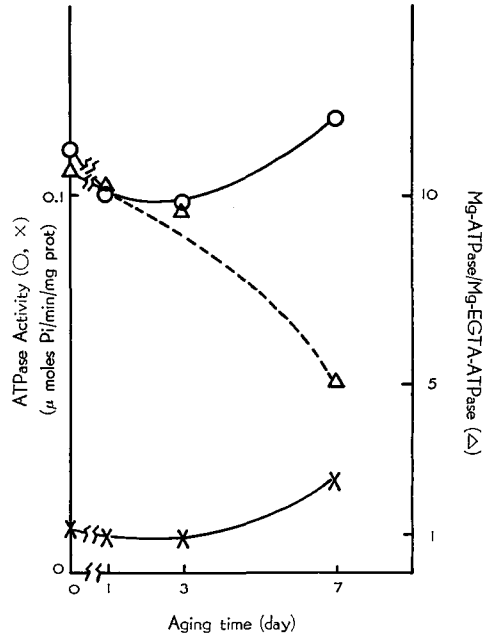

Fig. 8. Changes in ATPase activities and calcium sensitivity of myofibrils during the course of aging on carcass.
(○); Mg^{2+}-ATPase activity, (×); Mg^{2+}-EGTA-ATPase activity, △---△; Ca^{2}-sensitivity. Myofibril preparatsons were treated with Triton X-100 as described by YASUI *et al.*[27]

Discussion

Results in the present study never showed that incubation of muscle homogenate with papain at 2°C mimics the effect of on-carcass aging at 2°C for 14 days. The ethanol-treated protein fraction from the papain-treated muscle exhibited the appearence of rod fragments of myosin in all aspects (Figs. 2, 4 and 6 and Table II), whereas existance of such fragments in the fractions of muscle aged on carcass could never be observed throughout the reaction (Figs. 4 and 5). Nevertheless, rigor mortis of myofibrils was clearly resolved, as shown in Fig. 5. These results strongly suggest that the resolution of rigor mortis *i. e.*, lengthening of supercontracted sarcomeres occurring without scission of myosin molecules within thick filaments (Figs. 5 and 6), should result from a completely different mechanism rather than proteolysis

such as papain digestion. On the other hand, addition of proteolytic enzymes as artificial tenderizers, indeed, fragments the myosin molecules (Figs. 2, 4 and 6 and Table II).

It is noteworthy that the proteins originated from thin filaments, actin, tropomyosin and troponins are found in the ethanol-treated protein fractions. There seems to be no protein belonging to thick filaments appearing on the SDS-PGE patterns of the ethanol-treated protein fractions from muscles aged on carcass (Fig. 6, (4)-(6)). What is more significant is the absence of α-actinin band.

It is of interest that distinct alterations in the bands are observable on these SDS-PGE patterns. While tropomyosin and troponin-T (38,000 dalton) band remains almost constant, 45,000 band becomes less dense as aging proceeds and instead a band develops at 29,000 daltons (Fig. 6). That the 29,000 dalton protein band is clearly evident on SDS-PGE pattern of myofibrils from aged muscle (Fig. 6, (2)) is comparable to the data of YAMAMOTO *et al.*[24]. and SAMEJIMA and WOLFE[19]. The latter authors presumed on the basis of their finding on the natural F-actin that the 30,000 dalton protein band was intimately associated with the thin filament. DABROWSKA *et al.*[21,24], and TAKAHASHI[23] gave evidence that certain proteolytic enzymes were responsible for the appearence of a 30,000 dalton protein in their troponin preparations. They further suggested that the proteolytic activity was tightly bound to the myofibrils. It is, therefore, reasonable to conclude that the 29,000 dalton protein developed in the SDS-PGE patterns of both myofibrils and ethanol-treated protein fractions from aged muscles is derived from the proteolytic degradation of troponin in the thin filaments.

It is, however, rather puzzling that in the SDS-PGE patterns of the ethanol-treated protein fractions from aged muscle, the 29,000 band appears to increase in density accompanying the decrease in density of the 45,000 dalton protein (Fig. 6, (4)-(6)). Since such a phenomenon is not recognized in the case of myofibrils (Fig. 6, (1) and (2)) and actin is the most resistant against proteolysis[20], one can hardly offer any satisfactory explanation for this experimental fact. According to Fig. 1, preparation of the ethanol-treated protein involves high speed centrifuge and ethanol fractionation procedures. There might be a possibility that aging causes changes in properties of F-actin, thus resulting in the apparent loss of its concentration in the final preparations. Alternatively, 45,000 dalton protein band in the SDS-PGE patterns of this protein fraction might not be composed of mainly F-action, but also include 10 s-actinin which can not be differentiated from actin on SDS-PGE gels[20]. Anyhow, a reasonable interpretation can not be

offered at the moment, and hence it merits futher study.

In conclusion, although both papain (as the representative of artificial tenderizers)-treatment and on-carcass aging induce post-mortem fragmentation of myofibrils[6,25] as well as resolution of rigor-mortis[6], events occurring at the molecular level are quite different from each other. The effect of the papain-treatment on myofibrillar proteins is characterized by the formation of myosin rods in the ethanol-treated protein fraction, indicating the cleavage of lateral projection of thick filaments in myofibrils. On the other hand, on-carcass aging at 2°C for 14 days exerts no influence on the thick filament, but causes the formation of 30,000 dalton protein which is originated from thin filament component, presumably troponin. A strong support for this presumption comes from the fact shown in Fig. 8 in which the post-mortem changes in Mg^{2+}-ATPase as well as Mg^{2+}-EGTA-ATPase have been illustrated. Despite the apparent changes in these ATPase activities, the ratio of Mg^{2+}-ATPase/Mg^{2+}-EGTA-ATPase tended to decrease with storage period of time. This may reflect the degradation of troponin subunits, especially troponin T and troponin I, for among troponin subunits, troponin T and I are the most susceptibile to proteolytic enzymes[21~24], and the Ca^{2+}-sensitivity of the contractile system can not be restored, unless the both subunits are added to the desensitized system[23,26] in the presence of tropomyosin.

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Summary

A 120,000 dalton protein is produced from rabbit myosin as a result of limited papain digestion. The fresh cock muscle homogenates incubated at a temperature of 2°C with papain also develop this band. Myofibrils from the pectoral muscle during the course of on-carcass aging show no sign of such a band. Electrophoresis and viscosity and ORD measurements indicate that 120,000 may correspond to myosin rod and that tenderizing mechanism of on-carcass aging of meat is quite different from that of papain-treatment.

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