Changes in the Properties of Porcine Myosin during Postmortem Aging

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Physicochemical and conformational changes, water-holding capacity (WHC) and the structure of heat-induced gel of porcine myosin were investigated to elucidate the relationship between denaturation of myosin and gelation properties during postmortem aging. The turbidity of porcine myosin upon heating increased as the period of postmortem aging increased. During postmortem aging, the increased velocity in values of aliphatic hydrophobicity of porcine myosin by heating was prominent, which suggested that porcine head was susceptible to conformational changes during storage. On the other hand, according to the changes in circular dichroism (CD) spectra of heated porcine myosin, the decrease in α -helix content was almost the same during postmortem aging, indicating no conformational changes in porcine myosin rod during aging. WHC values of porcine myosin gels showed a gradual decrease during storage. This coincided with the progressive loosening in its three dimensional ordering with increased postmortem aging period, as was revealed by SEM studies. In conclusion, conformational changes of myosin head by denaturation during postmortem aging of pork could cause a progressive loosening of heat-induced gel of porcine myosin. This result could be, in part, helpful in the subsequent use of pork in meat processing.

Keywords: myosin (porcine), physicochemical changes, myosin subfragments (head, rod), conformational changes, postmortem aging, scanning electron microscope, gel matrix

The gel forming ability of myofibrillar proteins is important for many processes involved in the production of a variety of products made from meat such as sausages. This and other functional properties determine the textural qualities and physical behavior of meat products during preparation, processing and storage, as well as stabilizing fat and water-holding capacity (Schmidt, 1981; Morrissey *et al.*, 1987; Cofrades *et al.*, 1997). The formation of a protein network in these products contributes not only to the elastic texture, but also to other functional properties that are important in stimulating the appearance, flavor and palatability of the material meats (Park *et al.*, 1997). For example, palatability of pork sausages is known to be better than that of beef ones, which could probably be due to species-specific properties of network or gel formation.

The ability to form gel upon heating has been shown to be brought about mainly by myosin. It was also shown that other myofibrillar proteins such as actin cannot in themselves form a gel upon heating but modify the gelling characteristics of myosin gel (Yasui *et al.*, 1980; Visessanguan *et al.*, 2000). Since the pioneering findings on myosin binding ability to adhere meat pieces together by gelation through thermal processing (Fukazawa *et al.*, 1961), many studies have been performed. These studies investigated the properties of myosin as the main gelling protein of the muscle and characterized the mechanism of gelation over the past 30 years (Samejima *et al.*, 1969; Macferlane *et al.*, 1977;

Ishioroshi *et al.*, 1979; Acton & Dick, 1989; Ziegler & Foegeding, 1990; Liu & Foegeding, 1996; Visessanguan *et al.*, 2000).

Myosin is a multi-domain protein with two large heavy chains and four light chains arranged into an asymmetrical molecule with two globular heads attached to a long α -helical rod-like tail (Privalov, 1982; Lopez-Lacombe *et al.*, 1989; Visessanguan & An, 2000). ATP- and actin-binding sites are located on the heads, whereas filament-forming ability and the property that myosin dissolves in high-salt medium relies on the rod portion (Harrington, 1979; Wang & Smith, 1994; Konno *et al.*, 2000). Both head and rod portions of myosin have been shown to participate in gel formation (Taguchi *et al.*, 1987; Chan *et al.*, 1993).

Heat-induced gelation is a complex physicochemical process involving structural and functional changes of myosin. It is represented by three stages, that is, dissociation, thermal denaturation and aggregation (Roussel & Cheftel, 1990). In the presence of salts, myosin undergoes major structural and conformational changes leading to solubilization of myosin and actin (Parsons & Knight, 1990). Partial unfolding of the protein structure is accelerated by an increase in temperature, which results in the aggregation of the unfolded regions between protein molecules to form a three-dimensional network (Visessanguan *et al.*, 2000). The structural and conformational changes that occur in the head and rod portions as a result of thermal denaturation enable myosin to form gel. It is also well known that the gelling properties of myosin are highly related to species, conditions of myosin preparations and postmortem aging period as well.

In Japan meats, stored at 4°C for several days and imported under vacuum-packing from foreign countries, are used for making meat products such as sausages (Okumura *et al.*, 1996). The

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Abbreviations: CD: circular dichroism, cPA: *cis*-parinaric acid, DW: distilled water, DTT: dithiothreitol, EDTA: ethylenediamine tetracetate, SDS-PAGE: sodium dodecyl sulphate-polyacrylamide gel, SEM: scanning electron microscopy, WHC: water-holding capacity.

aging period of meat differs from one type of meat to another; while beef is stored from 2–4 weeks, pork is stored for only about 6–10 days (Takahashi, 1999). During this postmortem aging, various changes occur in muscle proteins even at low temperature. Many studies investigated the physiological and biochemical changes during postmortem aging (Alarcon-Rojas & Dransfield, 1995; Steen *et al.*, 1997; Nishimura, 1998). On the other hand, few studies referred to the effect of postmortem storage on the physicochemical changes of porcine myosin, its gelling process or its water binding properties. In this study, we examined the physicochemical and conformational changes, water-holding capacity of porcine myosin and observed the structure of its heat-induced gel to elucidate the relationship between denaturation of myosin and gelation properties during postmortem aging.

Materials and Methods

Meat samples Porcine muscles (*M. longissimus dorsi*) were supplied by Nippon Meat Packers Company, and transferred to our laboratory two days after slaughter. Meat was then stored at 4°C. Myosin was successively extracted from porcine meat 3, 5, 7, and 9 days after slaughter.

Myosin extraction Myosin was extracted from porcine meat by “Hasselbach-Schneider” buffer (0.6 M KCl/10 mM MgCl₂/200 mM K-phosphate/20 mM pyrophosphoric acid) (Hasselbach & Schneider, 1951). In each extraction, 200 g of minced meat was used, to which 600 ml of the extraction buffer was added. Extraction was done within 15 min by slow and continuous stirring with occasional pauses and was then stopped by the addition of 800 ml of cold distilled water (DW). The mixture was centrifuged at 3000×*g* for 10 min, and the muscle residue was separated by filtration through four layers of gauze. To get myosin as a precipitate, the filtrate was diluted with an equal volume of cold DW, and then centrifuged at 10,000×*g* for 15 min. Myosin pellets were suspended in 0.12 M KCl/20 mM K-phosphate (pH 7.2)/1 mM ethylenediamine tetracetate (EDTA)/1 mM dithiothreitol (DTT) and then washed twice with the same buffer. During the whole period of experiments, myosin solution was kept at 4°C.

Protein concentration Myosin concentration was measured by the method mentioned by Bradford (1976) using a commercial kit (Bio-Rad, Japan). Colored products were measured at 595 nm on a spectrophotometer (Jasco V-530 UV/Vis, Japan).

Sodium dodecyl sulphate-polyacrylamide gel (SDS-PAGE) measurements Electrophoresis on polyacrylamide gels (7.5%) was carried out according to the method mentioned by Laemmli (1970). Porcine myosin was dissolved with 10 mM Tris-HCl buffer (pH 6.8), containing 1% SDS, 1% 2-mercaptoethanol and 20% glycerol. The solution was then boiled for 2 min before being applied to the gel. Electrophoresis was carried out and the resulting gel was stained with Coomassie brilliant blue. The density of each band was measured by a densitometer (Toyo, DMU-33C, Japan).

Turbidity Myosin (1 mg/ml) was dissolved in 0.6 M KCl/50 mM K-phosphate buffer (pH 6.5). The absorbance was monitored during heating of the porcine myosin solution from 30 to 80°C at an increasing rate of 3°C/min using a spectrophotometer (Shimadzu UV-250, Japan) at 340 nm.

Hydrophobicity Hydrophobicity was measured according

Fig. 1. SDS-PAGE of myosin isolated from porcine muscles. Myosin was isolated with Hasselbach-Schneider buffer from porcine muscles, stored at 4°C, and was extracted on the 3, 5, 7 and 9 days after slaughter. Acrylamide concentration of the separating gel was 7.5%.

to the method mentioned by Boyer *et al.* (1996). Three milliliters of porcine myosin (0.04 mg/ml) in 0.6 M KCl/50 mM K-phosphate buffer (pH 6.5) was heated for 5 min at each temperature from 30 to 80°C and, after heating, cooled on ice. Ten microliters of 1 mM *cis*-parinaric acid solution (cPA) was then added to each solution. The fluorescence of cPA protein complex was measured with a spectrofluorometer (Jasco FP-750, Japan). Hydrophobicity was expressed as μM^{-1} of myosin· μM^{-1} of cPA.

ATPase activity ATPase activity measurements were carried out at 25°C by the method of Maruyama and Ishikawa (1963). Myosin solution (0.25 mg/ml) was incubated with 1 mM ATP, 2 mM MgCl₂, 50 mM KCl, 2 mM NaN₃ in 20 mM Tris-HCl buffer (pH 8) for 10 min. ATPase activity was expressed as μg phosphorus liberated for 1 min by 1 mg of protein.

α -Helix measurements Myosin (0.05 mg/ml) was dissolved in 0.6 M KCl/50 mM K-phosphate buffer (pH 6.5). The solution was then heated gradually from 15 to 75°C. The α -helix content of myosin was calculated based on a CD spectrum (Jasco J-720 W). The molar ellipticity [θ] at 222 nm was monitored during heating.

Water-holding capacity (WHC) measurements WHC was performed according to the method mentioned by Smyth *et al.* (1998). Briefly, porcine myosin (10 mg/ml) in 0.6 M KCl buffer (pH 6.5) was heated from 20 to 90°C at a rate of 1.75°C/min. The gels were transferred to a cold room (4°C) and held for 12 h. Gels of known weight (W_1) were centrifuged at 800×*g* for 15 min at 4°C. The weight of the resulting supernatant (W_2) was recorded. WHC was calculated as follows:

$$\text{WHC} = (W_1 - W_2)/W_1 \times 100.$$

Scanning electron microscope (SEM) Porcine myosin gel samples were prepared for SEM observation adopting the method mentioned by Yasui *et al.* (1979). In this method, protein solutions (10 mg/ml) of myosin in 0.6 M KCl and 20 mM K-phosphate buffer (pH 6.0) were incubated at 60°C for 30 min. Gel pieces less than 1 mm³ were immersed in 2.5% glutaraldehyde in 0.1 M K-phosphate buffer (pH 7.0) for 2 h at 4°C. All fixed specimens were dipped in distilled water and dehydrated in graded ethanol of 30, 50, 70, 90, 95, and 100% for at least 15 min per station. After dehydration, they were transferred to 50 and 100% isoamylacetate for 30 min each. Samples were dried by a carbon

Fig. 2. Changes in turbidity at 340 nm of porcine myosin during storage. Myosin (1 mg/ml) was dissolved with 0.6 M KCl/50 mM K-phosphate buffer (pH 6.5) and then heated from 30 to 80°C at a rate of 3°C/min. The arrow in the Figure indicates that the temperature, at which the absorbance of porcine myosin solution started to increase steeply, became lower as the aging period increased.

dioxide critical point dryer (Hitachi HCP-2, Japan). After the dried specimens were mounted on brass studs and coated with a thin layer of platinum in an Ion Sputter (Hitachi E-1010), they were observed with a scanning electron microscope (Hitachi S-2380N) at an accelerating voltage of 15 KV.

Results and Discussion

Myosin preparation Figure 1 shows the SDS-PAGE profiles of porcine myosin, which was extracted on days 3, 5, 7, and 9 of storage, respectively. The myosin obtained was almost pure except for minor contamination with actin, which increased successively during the postmortem aging period. Based on the densities of their bands, the myosin : actin ratio was estimated to be 70 : 1.

Myosin molecule is composed of a coiled coil rod and two globular heads. The heads are responsible for the hydrolysis of ATP into ADP and phosphorus with the liberation of energy necessary for muscle contraction (Goody *et al.*, 1977). During postmortem aging it is stated that, as long as the endogenous ATP is present, actin and myosin filaments are separated and the muscle remains extensible. However, once ATP has been entirely consumed after death, actin and myosin filaments combine with each other to form actomyosin and the muscle becomes rigid. This rigor mortis represents an irreversible contraction and usually occurs within the first 24 h after death (Yang *et al.*, 1970). This seems to be why myosin solution, on different days of storage, contained different amounts of actin contamination.

The effect of postmortem aging on the increase of turbidity of porcine myosin upon heating An increase in absorbance (or optical density) of heated myosin solution, results from the formation of myosin aggregates or associations (Morita *et al.*, 1987). In this study, the absorbance of myosin solution was measured at 340 nm to detect small or fine protein aggregations. The patterns of turbidity changes upon heating, in all myosin solutions from pork stored for 3–9 days after slaughter, showed a gradual increase in values as the period of postmortem aging increased (Fig. 2). Of interest was that the temperature, at which the absorbance of porcine myosin solution started to increase

steeply, became lower as the aging period increased as shown by an arrow in Fig. 2. This might indicate that during storage porcine myosin gradually lost its stability. Our results are in agreement with Egeland and Martinsen (1994) who reported an increase in turbidity values of red and white bovine muscles during storage.

The effect of postmortem aging on hydrophobicity of porcine myosin upon heating Hydrophobicity measurements of porcine myosin solution were used in this study to signify the conformational changes which occurred in the myosin globular heads, since most of the hydrophobic bonds are known to be buried in the head subfragment (Boyer *et al.*, 1996). According to Sklar *et al.* (1977), fluorescent *cis*-parinaric acid (cPA) probes bind to the aliphatic hydrophobic residues of protein.

Among different days of postmortem aging of porcine myosin, it was shown that, as the period of postmortem aging increased, the aliphatic hydrophobicity displayed by porcine myosin increased upon heating (Fig. 3).

Hydrophobicity is known to contribute largely to the stability and structural conformation of proteins, *i.e.*, the greater surface hydrophobicity results in reduced protein stability (Niwa *et al.*, 1989). By applying this to the present study it could be suggested that porcine myosin head gradually lost its stability as the number of exposed hydrophobic bonds increased during aging.

It is well known that, at high temperatures, the unfolding of myosin molecules leads to the exposure at their surfaces of hydrophobic residues that have been initially buried inside in an intact form. In the present study, there was a gradual increase in the hydrophobicity values between 40 and 65°C upon heating the porcine myosin solution followed by a constancy in the values. This increase in hydrophobicity could be attributed to the conformational changes that took place in the head subfragment during heating, and to the release of myosin light chains, preferentially LC1 and LC3 (Sharp & Offer, 1992; Boyer *et al.*, 1996).

The effect of postmortem aging on ATPase activity of porcine myosin The changes in ATPase activity during postmortem aging of porcine myosin were measured as shown in Fig. 4,

Fig. 3. Changes in aliphatic hydrophobicity of porcine myosin during storage. Myosin (0.04 mg/ml) was dissolved with 0.6 M KCl/50 mM K-phosphate buffer (pH 6.5) and then heated from 30 to 80°C for 5 min at each temperature. Hydrophobicity was expressed as μM^{-1} of myosin $\cdot \mu\text{M}^{-1}$ of cPA.

for the purpose of determining the extent of denaturation of head subfragment, since it contains the sites for the hydrolysis of ATP. ATPase activity of porcine myosin showed a slight gradual increase in values during the successive days of storage, which agreed with the results reported by Yang *et al.* (1970) who observed an increase in ATPase activity of myofibrils from rabbit muscle with the progress of aging.

The increase in ATPase activity values during storage could be accounted for by the increase in actin contamination, which coincidentally increased during storage of porcine myosin (Fig. 1). Fujimaki *et al.* (1965) examined the effect of actin addition on the ATPase activity of myosin. It was shown that ATPase activity of myosin increased as the binding ratio of F-actin to myosin became higher. The myosin showed the highest activity when the binding ratio of F-actin to myosin was 0.26 : 1. The binding ratio of F-actin to myosin prepared from muscle 9 days after slaughter in this study was 0.14 : 1. This value was located between the minimum and the maximum ratio of F-actin to myosin in the report by Fujimaki *et al.* (1965). Taken together, the increase in ATPase activity of myosin from stored muscle in the present study seems to have been caused by the increase in actin contamination.

It is worth noting that, contrary to our prior prediction, ATPase activity showed an increase during postmortem aging, while turbidity and hydrophobicity values increased as an indication of protein denaturation. This apparent discrepancy could be explained by the fact that changes in turbidity and hydrophobicity values were measured upon heating porcine myosin, thus causing direct and rapid denaturation of protein. Conversely, ATPase activity was measured at a fixed temperature without applying heat. In other words, ATP hydrolyzing sites located on the head were apparently kept intact during the measuring process.

The effect of postmortem aging on α -helix content of porcine myosin upon heating It is well known that the α -helix content of myosin rod is over 90% (Lowey *et al.*, 1969) and that changes in α -helix content of porcine myosin by heating were, therefore, mainly due to the conformational changes occurring in the rod during storage.

Fig. 4. Changes in ATPase activities of porcine myosin during storage. Myosin solution (0.25 mg/ml) was incubated with 1 mM ATP in 20 mM Tris-HCl buffer (pH 8) at 25°C for 10 min. ATPase activity was expressed as μ g phosphorus (Pi) liberated in 1 min by 1 mg of protein.

Fig. 5. Changes in $[\theta]_{222}$ of porcine myosin during storage. Myosin (0.05 mg/ml) was dissolved with 0.6 M KCl/50 mM K-phosphate buffer (pH 6.5) and then heated from 15 to 75°C. The molar ellipticity $[\theta]$ at 222 nm was monitored during heating.

CD analysis is generally performed to detect the changes in α -helical content of a protein. A decrease of the magnitude of $[\theta]_{222}$ corresponds to the decrease of the α -helical content in protein. Changes in the α -helix content of porcine myosin are shown in Fig. 5. In the present study, denaturation of α -helix of porcine myosin during postmortem aging was almost the same regardless of the aging period. Therefore, it is suggested that no conformational changes took place in porcine myosin rod during postmortem aging.

By heating myosin solution linearly from 15 to 75°C, porcine myosin showed a decrease in the α -helix content derived from myosin rod. This decrease in α -helix content was shown between 40 and 55°C until it reached a plateau at 60°C. Harrington (1979) reported that, by heating myosin rod above 37°C, the coiled-coil α -helix structure of the rod progressively melted to a highly flexible coil, which was almost the same temperature at which porcine myosin rods started to melt.

Fig. 6. Changes in water-holding capacity (WHC) values of porcine myosin during storage. Myosin solution (10 mg/ml) in 0.6 M KCl buffer (pH 6.5) was heated from 20 to 90°C at a rate of 1.75°C/min. Gels of known weight (W_1) were centrifuged at 800×g for 15 min at 4°C, and the weight of the resulting supernatant (W_2) was recorded. WHC was calculated as $(W_1 - W_2)/W_1 \times 100$.

The effect of postmortem aging on water-holding capacity (WHC) of porcine myosin gels WHC is the ability of meat or gel to hold its own or added water during the application of any force such as pressing, grinding, heating and so on (Grau & Hamm, 1956; Zamorano & Gambaruto, 1997). In the present study, WHC values of porcine myosin gels extracted on different days of storage were measured. It was found that during storage, WHC values of porcine gels decreased (Fig. 6).

Heat-induced gelation is represented by three stages, that is, dissociation, thermal denaturation and aggregation (Roussel & Cheftel, 1990). Partial unfolding of the protein structure is accelerated by an increase in temperature resulting in the aggregation of the unfolded regions between protein molecules to form a three-dimensional network matrix (Visessanguan *et al.*, 2000).

The structural and conformational changes that occur in the head and rod portions as a result of thermal denaturation enable myosin to form gel. During postmortem aging, denaturation of myosin took place and the shrinkage of myosin heads occurred, which in consequence resulted in more water being expelled outside the gel matrix by centrifugation. To study the changes in porcine myosin gels during storage, observations by SEM were performed.

Effect of postmortem aging on porcine myosin gel structure

Figure 7 illustrates the changes in structure of the heat-induced porcine myosin gels on different days of storage. On the third day of storage, the gels appeared to be dense and of a well-structured matrix with highly interconnected and ordered network strands of uniform thickness (Fig. 7A). Distributed evenly

Fig. 7. Scanning electron micrographs of heat-induced gels of porcine myosin (bar length is 50 μm): (A–D) gel of extracted myosin on the third day (A), fifth (B), seventh (C) and ninth (D) day of storage. Myosin (10 mg/ml) in 0.6 M KCl and 20 mM phosphate buffer (pH 6.0) was incubated at 60°C for 30 min. Gel pieces less than 1 mm³ were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.0) for 2 h at 4°C. The specimens were then dehydrated using carbon dioxide critical point dryer. Finally, the dehydrated specimens were observed with SEM, at an accelerating voltage of 15 KV.

throughout the matrix were numerous small-sized pores that may cause resistance to applied stress and greater water-holding capacity. On the fifth day of storage, the gel was still dense but was less uniform and not as highly aggregated or interconnected (Fig. 7B). It contained many larger-sized pores not as evenly distributed. On the seventh day of storage, gels became a loose lacy network with obvious decrease in gel strands thickness and the degree of inter-linking between strands (Fig. 7C). The pores became of increased sizes and more irregular. As aging period increased, gel structure seemed to become somewhat discontinuous with less linkage between protein strands and large-sized pores could be observed, as was the case on the ninth day of storage (Fig. 7D).

These obvious physical changes in gel structure during post-mortem aging help to explain the functionality differences among the gels, which were expressed by the gradual decrease in their water-holding capacity. It could be concluded that a fine uniform structure with numerous pores would probably result in more absorptive capacity and better retention of water compared to coarse structures with large pores (Hermansson, 1985; Defreitas *et al.*, 1997). Protein denaturation during postmortem aging could account for those physical changes in gels, which disabled them so that they could not firmly retain water. Samejima *et al.* (1969) pointed out that intact myosin molecule, extracted from rabbit muscle, had been shown to be required for the development of good protein binding properties of heated gels.

In conclusion, porcine myosin is suggested to be unstable during postmortem storage. Taking into account the fact that pork sausages are of high palatability, it could be assumed that denaturation of head during postmortem aging is an important aspect in making sausages. However, prolonged aging may lead to a decrease in the capacity of gels to retain water and consequently affect the quality of the sausages produced. Other than this, there are many factors which are known to be responsible for the physicochemical properties of the heat-induced gel, such as pH, ionic strength, and specific differences between species (Ishioroshi *et al.*, 1983; Lefevre *et al.*, 1998; Wang & Xiong, 1998). The results revealed in this study would be, in part, informative for the elucidation of properties of the heat-induced gels from pork.

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