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Title: **Changes in concentration of sarcoplasmic free calcium during post-mortem ageing of meat**

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Abstract

We studied post-mortem changes in the concentrations of sarcoplasmic free calcium in various meats to clarify the tenderisation mechanism of meat, taking sufficient care to prevent contamination with calcium ions other than those in meat. When minced meats were homogenised and concentrations of soluble calcium ions were determined by atomic absorption spectrophotometry, it was found that the concentration of free calcium increased, the rate of increase varying depending on the species, and reached a narrow range of 210 to 230 μM regardless of the anatomical class of skeletal muscle, chronological ageing or animal and fowl species. The calcium concentration was 100 nM immediately post-mortem when homogenisation was performed in the presence of 2 mM ATP. Phospholipids were liberated from the sarcoplasmic reticulum during ageing of meat. It is likely that calcium ions leak into the sarcoplasm through channels formed by phospholipid liberation.

Keywords: Sarcoplasmic calcium; Post-mortem changes; Meat tenderisation; Post-mortem ageing; Calcium theory

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1. Introduction

The concentration of free calcium in the sarcoplasm is exactly regulated by the sarcoplasmic reticulum to be at 100 nM for relaxation and at 5 μM for contraction of skeletal muscle. However, it increases during post-mortem ageing of meat due to loss of the calcium-accumulating ability of the sarcoplasmic reticulum (Greaser, Cassens,

Hoekstra & Brisky, 1969) accompanied by a gradual leakage of calcium ions into the sarcoplasm. Nakamura (1973) found that the concentration of free calcium in chicken pectoral muscle increases from 60 μM immediately post-mortem to 180 μM within 24 h post-mortem. Jeacocke (1993) reported that free calcium ions increase after the onset of rigor to more than 100 μM in mouse muscle fibres. The increase in calcium ions contributes to meat tenderisation. The mechanism by which calcium ions weaken myofibrillar structures during post-mortem ageing of meat has been suggested to be direct action of calcium ions (Takahashi, 1992, 1996, 1999) or indirect action of calcium ions through activation of μ-calpain (Koohmaraie, 1992; Dransfield, 1993), m-calpain (Boehm, Kendall, Thompson & Goll, 1998), calpain 10 (Ilian, Bekhit & Bickerstaffe, 2004a) and calpain 3 (Ilian,, Bekhit & Bickerstaffe, 2004b).

μ-Calpain and m-capain are calcium-activated neutral proteases in the sarcoplasm, and the optimal pH value and temperature for their activities are around 7.5 and 25 ˚C, respectively. These conditions are markedly different from those of meat during post-mortem ageing, i.e., pH values of 5.5 to 5.7 and temperatures of 3 ˚C to 5 ˚C. Although numerous studies have been performed by many workers to clarify the contributions of μ-calpain and m-calpain to meat tenderisation, the involvement of μ-calpain and m-calpain in meat tenderisation has been questioned. Etherington, Taylor and Dransfield (1987) did not find any definite relationship between ageing rate and protease levels in pork, beef, lamb and chicken, and they concluded that the rapid ageing time for chicken could not be explained by the free or total levels of individual activities of μ-calpain and m-calpain. Ouali and Talmant (1990) also concluded that no relationship seems to exist between meat ageing rate and calpain concentrations. Boehm et al. (1998) reported that it is not clear whether μ-calpain is responsible for any appreciable post-mortem degradation of myofibrellar proteins. Recently, we have

demonstrated that μ-calpain is inactive under conditions similar to those of meat during post-mortem ageing, i.e., pH values below 5.89 and temperatures below 15 ˚C (Kanawa, Ji & Takahashi, 2002). We have some doubt about the possibility of m-calpain contributing to the degradation of myofibrillar proteins during post-mortem ageing of meat, since pH- and temperature-dependencies of m-calpain activity are similar to those of μ-calpain activity and the amount of α-actinin, which is the major protein of Z-discs, remains unchanged even after prolonged ageing of meat (Takahashi, Kim & Yano, 1987; Hwan & Bandman, 1989; Ahn, Shimada & Takahashi, 2003), though one of the characteristics of m-calpain is that it removes α-actinin from Z-discs (Goll, Dayton, Sigh & Robson, 1991). The possibility of contribution of other endogenous proteases, i.e., lysosomal cathepsins, to meat tenderisation has been ruled out (Lacourt, Obled, Christiane, Ouali & Valin, 1986; Whipple, Koohmaraie, Dikeman, Crouse, Hunt & Klemm, 1990; Koohmaraie, 1994, Uytterhaegen, Claeys & Demeyer, 1994; Hopkins and Thompson, 2001). The evidence is in favour of a controversial theory of calcium-ion tenderisation of meat that has been proposed by one of the authors of this article (Takahashi, 1992, 1996, 1999).

The calcium theory of meat tenderisation (Takahashi 1992, 1996, 1999) is based on evidence that all structural weakenings of myofibrils and disassembly of desmin intermediate filaments, which occur during post-mortem ageing of meat, are fully induced by calcium ions at concentrations above 100 μM in vitro as follows. (1) Calcium ions bind to phospholipids in Z-discs, and liberation of calcium-phospholipid compounds weakens the Z-disc structure, resulting in fragmentation of myofibrils (Shimada & Takahashi, 2003). (2) Calcium ions cause detachment of paratropomyosin from connectin/titin filaments (Fei, Yamanoue & Okayama, 1999). Detached paratropomyosin binds to actin (Nakamura & Takahashi, 1985) and weakens

linkages formed between actin and myosin upon the onset of rigor mortis (Takahashi, Yamanoue, Murakami, Nishimura & Yoshikawa, 1987). (3) Connectin/titin filaments are severed by the binding of calcium ions (Tatsumi and Takahashi, 2003). (4) Nebulin filaments are also severed by the binding of calcium ions (Tatsumi & Takahashi, 2003). (5) Desmin intermediate filaments are rapidly disassembled by the binding of calcium ions (Takahashi, 1999). Although these structural weakenings of myofibrils are also induced by magnesium ions, the action of calcium ions is 100-times more effective than that of magnesium ions. We consider that the structural weakening of myofibrils and the disassembly of desmin intermediate filaments are induced when the concentration of sarcoplasmic free calcium increases to more than 100 μM, causing tenderisation of meat.

Various results have been reported regarding the ultimate concentration of free calcium in the sarcoplasm; reported values vary from 120 μM for the ovine *longissimus* muscle (Geesink, Taylor, Bekhit & Bickerstaffe, 2001) to 970 μM for the bovine *longissimus* muscle (Parrish, Selvig, Culler & Zeece, 1981). The objectives of this study were to determine post-mortem changes in the concentration of free calcium in the sarcoplasm using various skeletal muscles of animals and birds and to clarify the tenderisation mechanism of meat during post-mortem ageing.

2. Materials and methods

2.1. Muscle samples

The following muscle samples were used: Six *psoas major* muscles of three rabbits, three *longissimus thoracis* (dorsi) and three *biceps femoris* muscles of another three

rabbits, three *longissimus thoracis* muscles of three Landrace pigs aged around six months and three *longissimus thoracis* muscles of three Japanese Black steers aged around 30 months. Three *pectoralis superficialis* muscles of three Rhode Island Red chickens were used as samples for 4-month, 6-month and 16-month-old chickens. These animals and birds were slaughtered conventionally and carcasses were refrigerated at 5 ˚C. Unaged samples were dissected from carcasses of rabbits and chickens 20 min post-mortem and from carcasses of pigs and steers 40 min post-mortem. Aged samples were dissected from carcasses of rabbits and chickens 3 h post-mortem, from carcasses of pigs 24 h post-mortem and from carcasses of steers 48 h post-mortem, and they were stored at 5 ˚C.

2.2. Prevention of contamination with calcium

Deionized water was distilled twice with a Pyrex apparatus to remove calcium ions, and this water and Pyrex tools were used in all experiments. The calcium concentration of the water determined by atomic absorption spectroscopy was less than 100 nM. Dissolved reagents were passed through a column of chelex 100 (Bio-Rad Laboratories, USA).

2.3. Determination of calcium

Free calcium was determined according to the method of Parrish et al. (1981) with some modifications. Ten grams of meat, which had been minced with stainless steel scissors, was homogenised with 50 ml of cold water or a cold solution containing 2 mM ATP and 2 mM $MgCl_2$, pH 7.0, for 1 min at 12,000 rpm in a blade-type homogeniser

(Virtis; Research Equipment Gardiner, USA). The homogenate was centrifuged for 2 h at 145,000 × g_{max}. Two milliliters of the supernatant was removed and treated with 4 ml of 5% TCA. Ten minutes later, 0.5% strontium chloride was added to suppress anionic interference. The mixture was centrifuged for 10 min at 1,700 × g_{max}. Calcium was determined with an atomic absorption spectrophotometer (170-10; Hitachi Co., Japan). The concentration of free calcium in the sarcoplasm was calculated assuming the moisture content of skeletal muscle as to be 70% and expressed as molar concentration. The calculated concentrations are considered to be near the true concentration of free calcium because the moisture contents of various meats are around 70%. These procedures are denoted as the homogenisation-atomic absorption method in the text. Three animals or three birds were used for each experiment and values shown in the text and the figures are each the average of three replications. The standard deviation divided by the mean was less than 10% in the case of calcium concentrations measured immediately post-mortem and after reaching the maximum.

Free calcium was also determined by a mild extraction from fibre bundles of rabbit *psoas* muscle to examine whether calcium ions bound with proteins were released by homogenisation and TCA treatment described above. Fibre bundles of 200 to 300 μm in diameter and about 50 mm in length were cut off from rabbit *psoas* muscle at 20 min post-mortem and stored in a petri dish at 5 ˚C. Calcium ions were extracted from 0.5 g of the fibre bundles over 10 min at 5 ˚C with 6 ml of a modified Ringer's solution containing 119 mM NaCl, 5 mM KCl, 2 mM $MgCl_2$ and 20 mM Tris-maleate buffer, pH 7.0, with gentle stirring. Proteins in the extract were coagulated by heating for 15 min at 90 ˚C and spun down for 10 min at 1,700 × g_{max}. Calcium in the supernatant was determined using quin2 (8-amino-2-[(2-amino-5-methylphenoxy)-methyl]-6-methoxy-quinoline-*N*, *N*, *N'*,

N'-tetraacetic acid, tetrapotassium salt; Dojindo Laboratories, Japan), a fluorescent quinoline calcium-indicator, with a spectrofluorometer (FP-770; Jasco, Japan). The excitation and emission wavelengths were set at 339 nm and 500 nm, respectively (Tatsumi, Shimada & Hattori, 1997). The calcium concentration was calculated and expressed in the same way as that in the case of the homogenisation-atomic absorption method. These procedures are denoted as the mild extraction-quin2 method in the text.

2.4. Determination of phospholipids

The sarcoplasmic reticulum was prepared from unaged and aged pork and beef according to the method of Ogawa (1970). Lipids were extracted from the sarcoplasmic reticulum with chloroform : methanol at a ratio of 2 : 1 (vol/vol) using the method of Folch, Lees and Stanley (1957); the protein concentration was adjusted to 5 mg/ml by adding a solvent mixture, and the extraction was continued for 24 h at room temperature with gentle stirring. The suspension was then centrifuged for 10 min at 1,000 × g_{max}. Twenty microliters of the lipid extract was pipetted from the lower chloroform layer and dried by heating. To the dried lipids, 0.5 ml of water was added, and the amount of phospholipids was determined by measuring the amount of inorganic phosphate according to the method of Bartlett (1959). The amount of phospholipids was expressed as mg per g of sarcoplasmic reticular proteins.

2.5. SDS-PAGE

Preparations of the sarcoplasmic reticulum were washed with a solution containing 5 mM EDTA and 5 mM Tris-HCl buffer, pH 8.0, and dissolved by heating in 1% SDS

solution containing 1% β-mercaptoethanol, 10% glycerol, 5 mM EDTA and 5 mM Tris-HCl buffer, pH 8.0, for 2 min at 100 ˚C. The 15 μg-proteins was subjected to SDS-PAGE by the method of Laemmli (1970) employing a two-phase resolving gel of 4.5% (upper) polyacrylamide and 10% to 20% polyacrylamide-gradient gels. After each run, the gels were stained with Coomassie Brilliant Blue R-250 and destained.

2.6. Protein determination

Concentrations of proteins were measured by the biuret reaction (Gornall, Bardawill & David, 1949) standardised by the micro-Kjeldahl process.

3. Results and discussion

3.1. General tendencies

Figure 1 shows post-mortem changes in concentrations of calcium in rabbit *psoas* muscle measured by the homogenisation-atomic absorption method and by the mild extraction-quin2 method. In the former case, the concentration of calcium was 33 μM at 20 min post-mortem, increased with the passage of time, and reached an ultimate level of 230 μM at 30 h post-mortem. The calcium concentration measured by the mild extraction-quin2 method was 11 μM at 20 min post-mortem and increased to a peak of 230 μM at 30 h post-mortem. The calcium concentrations between 20 min and 24 h post-mortem measured by the mild extraction-quin2 method were less than those during the same period measured by the homogenisation-atomic absorption method, showing that lesser calcium was extracted by the mild extraction-quin2 method at

earlier times post-mortem. The agreement of ultimate concentrations of calcium indicated that bound calcium was not released by homogenisation of minced meat and that the calcium concentration measured by the atomic absorption method is equal to that measured using fluorescent quin2. We, therefore, measured the concentration of free calcium by the homogenisation-atomic absorption method in the following experiments. Post-mortem changes in the concentrations of free calcium in rabbit *longissimus thoracis* and *biceps femoris* muscles were very similar to those in the rabbit *psoas* muscle; concentrations of free calcium at 20 min post-mortem were 26 μM and 24 μM, respectively, and reached the ultimate concentration of 230 μM at 30 h post-mortem in both muscles. Thus, the ultimate concentration of free calcium was the same regardless of the class of skeletal muscle.

In chicken pectoral muscle, concentrations of free calcium were around 70 μM at 20 min post-mortem and reached a maximum of 220 μM at 18 h post-mortem regardless of chronological ageing of birds (Fig. 2). The ultimate calcium concentration of 220 μM was comparable to that of 180 μM reported by Nakamura (1973) in the same skeletal muscle of chicken, and it remained constant after prolonged ageing of chicken for 5 days post-mortem, indicating that bound calcium was not released during ageing, and that the ultimate concentration of free calcium remains constant even after prolonged ageing of meat. The initial concentration of free calcium is higher and the post-mortem time required to reach the ultimate concentration is faster in chicken than those in rabbit meat (Fig. 1), pork and beef (Fig. 3). Calcium ions probably leaked rapidly from the chicken sarcoplasmic reticulum because of the high rates of various chemical reactions that take place in chicken muscle fibres post-mortem (De Fremery & Pool, 1960).

Figure 3 shows changes in concentrations of free calcium during post-mortem ageing

of pork and beef. The calcium concentrations at 40 min post-mortem were 28 μM for pork and 16 μM for beef and reached a maximum of 210 μM at 3 days post-mortem in pork and 4 days post-mortem in beef, differing from appropriate ageing periods to improve tenderness, i.e., beef ages twice as slow as pork. Although Parrish et al. (1981) reported that concentrations of free calcium vary from 640 to 970 μM depending on maturity of beef aged for 10 to 14 days post-mortem, the ultimate concentration of free calcium was in the narrow range of 210 to 230 μM in all meats used in this study. It is likely that the ultimate concentration of free calcium is almost the same in mammalian and poultry meats and that a large amount of calcium in muscle fibres binds with proteins, such as calsequestrin in the sarcoplasmic reticulum, parbalbumin in the sarcoplasm, and troponin C, connectin/titin, nebulin and other proteins in myofibrils. The high and large range of concentrations of free calcium reported by Parrish et al. (1981) are probably due to release of bound calcium or contamination with calcium ions other than those in meat during preparation of their samples. Based on the results of Parrish et al. (1981), Boehm et al. (1998) suggested that m-calpain contributes to degradation of myofibrillar proteins during post-mortem ageing of meat. Kendall, Koohmaraie, Arbona, Williams and Young (1993) determined m-calpain activities in the presence of 5 mM $CaCl_2$ and at 25 °C and reported that m-calpain activities at pH 5.7 ranged from 10 to 17% of the activities at pH 7.0. Based on their results, they speculated that m-calpain is responsible for the changes in myofibrillar proteins observed during post-mortem ageing of meat. However, the ultimate concentration of free calcium in the range of 210 to 230 μM is insufficient to activate m-calpain in porcine skeletal muscle, because it requires 740 μM of calcium ions for half maximal rate of proteolysis at pH 7.5 and 25 °C (Dayton, Schollmeyer, Lepley & Cortes, 1981) and shows no activity at a calcium ion concentration of 200 μM even under optimal

conditions, i.e., at pH 7.5 and at 25 ˚C (Cong, Goll, Peterson & Kapprel, 1989). It is clear that m-calpain is inactive throughout the period of post-mortem ageing of meat, because of low concentrations of free calcium. On the other hand, Geesink et al. (2001) reported that free calcium concentrations determied by atomic absorption spectroscopy were in the range of 120 to 230 μM in lambs at 7 days post-mortem. The concentrations lower than 200 μM are probably caused by insufficient extraction of free calcium, because they extracted calcium from minced meat without homogenisation.

3.2. Effect of ATP on extractability of free calcium

We considered that high concentrations of free calcium at 40 min post-mortem, such as 28 μM for pork and 16 μM for beef, were caused by decrease in the concentration of ATP. The ATP concentration in the sarcoplasm had been decreased by adding 5 vol of water to homogenise minced meat and to extract free calcium, so that the Ca-ATPase pump could no longer maintain high concentrations of calcium ions inside the sarcoplasmic reticulum, accompanying leakage of free calcium into the sarcoplasm. We, therefore, homogenised minced meat in a solution containing 2 mM ATP. Figure 4 shows that the free calcium concentration measured in the presence of ATP was 100 nM for pork and beef at 40 min post-mortem, being equivalent to the free calcium concentration in the sarcoplasm of living skeletal muscle at rest. The results indicate that the amount of blood calcium remaining is small and that the calcium concentration measured by the homogenisation-atomic absorption method precisely reflects the free calcium concentration in the sarcoplasm. Figure 4 also shows that the free calcium concentration increased with the passage of time and reached a maximum of 210 μM at 3 days post-mortem in pork and at 4 days post-mortem in beef. The post-mortem

times required for free calcium to reach its ultimate concentration coincided with those determined in the absence of ATP (Fig. 3).

Although Koohmaraie, Schollmeyer and Dutson (1986) reported that under conditions similar to those of post-mortem ageing, such as pH 5.5 to 5.8 and 5 °C, μ-calpain retains 24 to 28% of its maximum activity at pH 7.5 and 25 °C, we demonstrated by accurately measuring pH values of reaction mixtures that *μ*-calpain is inactive under the same conditions as those for meat during post-mortem ageing, i.e., at pH values below 5.89 and at temperatures below 15 ˚C (Kanawa et al., 2002). The pH values of reaction mixtures used by Koohmaraie et al. (1986) to determine μ-calpain activities were probably shifted to neutral pH by the substrate, because the buffering action of the substrate, 5 mg of casein or myofibrils per ml, is stronger than that of the 50 mM buffer (Kanawa et al., 2002). Huff-Lonergan, Mitsuhashi, Beekman, Parrish, Olson and Robson (1996) monitored degradation of proteins by SDS-PAGE and Western blotting, and they showed that purified μ-calpain can degrade bovine myofibrillar proteins at pH 5.6, 4 °C and 100 μM $CaCl_2$ in a manner similar to that observed during post-mortem ageing of beef, suggesting that the calpain system plays a key role in post-mortem protein degradation. They used μ-calpain at a ratio of 1 : 800 (wt/wt; μ-calpain : myofibrillar proteins), and the concentration of μ-calpain in their reaction mixtures is far higher than those in muscle fibres. Skeletal muscle contains approximately 3.4 μg of calpains per g of muscle fresh weight (Dayton, Goll, Zeece, Robson & Reville, 1976), and myofibrillar proteins are assumed to account for around 120 mg per g of muscle fresh weight; the weight ratio of calpains to myofibrillar proteins in muscle fibres is calculated to be 1 : 35,000. Therefore, the results obtained by Huff-Lonergan et al. (1996) do not support their hypothesis of contribution of μ-calpain to changes in myofibrillar proteins during post-mortem ageing of meat.

It is possible that μ-calpain is active in the early post-mortem period, when the pH of muscle is above 5.9 and muscle temperature is above 15 ˚C, and that it contributes to the tenderisation of meat. However, the following evidence suggests that μ-calpain is inactive in the early post-mortem period. The pH value of muscle decreases to 5.9 within 5.4 h post-mortem in pork and within 14.6 h post-mortem in beef (Kanawa et al., 2002), and concentrations of free calcium in the sarcoplasm at the post-mortem times stated above are estimated from Fig. 4 to be below 1 μM for pork and about 10 μM for beef. These calcium concentrations are too low to activate μ-calpain, since μ-calpain in porcine skeletal muscle requires 45 μM of calcium ions for half maximal rate of proteolysis at pH 7.5 and 25 ˚C (Dayton et al., 1981). Therefore, the possibility that μ-calpain contributes to degradation of myofibrillar proteins is extremely low throughout the periods of post-mortem ageing of pork and beef, although an appreciable fraction of μ-calpain in beef is autolyzed during post-mortem ageing, indicating that it is proteolytically active (Boehm et al., 1998). Boehm et al. (1998), however, concluded that it is not clear whether μ-calpain is responsible for any appreciable post-mortem myofibrillar proteolysis, unless post-mortem muscle contains some factor that enables μ-calpain in this muscle to be proteolytically active.

Uytterhaegen et al. (1994) reported that the injection of an inhibitor of calpains, i.e., leupeptin at a concentration of 400 μM or calpain inhibitor I at 2 mM, into beef at 24 h post-mortem causes an increase in toughness during ageing for 8 days post-mortem, and they suggested that calpains are the main proteases involved in beef tenderisation, while they reported in the same article that the salt solubilities of titin 2, nebulin, myosin, α-actinin and actin are markedly decreased in beef into which 2 mM of calpain inhibitor I has been injected. We have demonstrated that leupeptin at 19 μM causes aggregation of isolated myofibrils and that the dense aggregates are never dissociated into individual

myofibrils by homogenisation (Hattori & Takahashi, 1982). These results indicate that some myofibrillar proteins are very susceptible to leupeptin. We have also shown that calpastatin domain I, an inhibitor of calpains, at 100 μM binds randomly to conectin/titin, nebulin, myosin, α-actinin, actin and troponin C and induces aggregation of isolated myofibrils (Tatsumi, Hattori & Takahashi, 1998). Moreover, leupeptin at concentrations above 100 μM or calpastatin domain I at concentrations above 30 μM causes deterioration of titin and nebulin in isolated myofibrils, thus interfering with the binding of calcium ions (Tatsumi & Takahashi 2003). Evidence indicates that the results of Uytterhaegen et al. (1994) show not only inhibition of calpain activity but also deterioration of myofibrillar proteins by the protease inhibitors used by them. Hopkins and Thompson (2001) reported that any improvement in tenderness with ageing is prevented by the injection of E-64, an inhibitor of calpains, at 1.4 mM into lamb, and they concluded that calpains have a pivotal role in the proteolysis and tenderisation of meat post-mortem. However, they reported in the same article a significant reduction in protein solubility in muscle injected with E-64. Myofibrillar proteins are probably deteriorated by E-64 and their solubilities are reduced in the same manner as leupeptin and calpastatin domain I stated above, because isolated myofibrils form dense and irreversible aggregates in the presence of E-64 at 23 μM (Hattori & Takahashi, 1982). Doses of protease inhibitors that do not induce any decrease in protein solubility and are necessary for inhibition of calpain activity should be used for such an in situ approach as was done by Uytterhaegen et al. (1994) and by Hopkins and Thompson (2001). Koohmaraie, Crouse and Mersmann (1989) found that infusion of a solution containing $CaCl_2$ into ovine carcasses accelerates tenderisation of lamb during ageing. Although they (1989) suggested that activation of μ-calpain and m-calpain by infused calcium is responsible for the tenderisation, the acceleration of meat tenderisation by infused

calcium can be explained by the direct action of calcium ions at concentrations above 100 μM. Kent, Spencer and Koohmaraie (2004) reported that degradation of desmin and troponin T is reduced during post-mortem storage of skeletal muscle of transgenic mice over-expressing calpastatin, and they concluded that a high level of expression of calpastatin is responsible for a decrease in post-mortem proteolysis. The relationship between the decrease in proteolysis and tenderisation of meat has not been reported.

3.3. Post-mortem changes in the sarcoplasmic reticulum

It seemed likely that apparent concentrations of free calcium, which were measured after inactivation of the Ca-ATPase pump in the pressence of 2 mM ATP (Fig. 4), included calcium ions that were released from the sarcoplasmic reticulum artifactually during homogenisation of minced meat and extraction of calcium. To know the real post-mortem time required for free calcium to reach its ultimate concentration in the sarcoplasm, we studied post-mortem changes in components of the sarcoplasmic reticulum, proteins and phospholipids. Figure 5 shows the results obtained by SDS-PAGE of proteins of the sarcoplasmic reticulum isolated from unaged and aged beef. There were 30 protein bands in the unaged sample. Based on SDS-PAGE alone, it seemed likely that the major proteins, Ca-ATPase and calsequestrin, and almost all minor proteins remained unchanged until 8 days post-mortem. The same results were obtained for sarcoplasmic reticular proteins of pork aged for 8 days post-mortem.

Figure 6 shows post-mortem changes in the amounts of sarcoplasmic reticular phospholipids. The amounts of phospholipids were 423 mg per g of proteins for unaged pork and 455 mg for unaged beef, and they decreased gradually up to 3 days post-mortem in pork and up to 6 days post-mortem in beef. Thus, phospholipids were

liberated from the sarcoplasmic reticulum during ageing of pork and beef. The ratios of liberated phospholipids to initial amounts in the sarcoplasmic reticulum were 16.5% for pork aged more than 3 days and 23.3% for beef aged more than 6 days. The rate of the phospholipid liberation corresponded well to the fact that pork ages twice as fast as beef. It seems likely that the electrostatic interaction between phospholipids and proteins has been disrupted by the decrease in pH values to 5.5 – 5.7 and that calcium ions accumulated in the sarcoplasmic reticulum leak into the sarcoplasm through channels formed by the liberation of phospholipids. In addition to the liberation of phospholipids due to decrease in pH values, leakage of calcium ions from the sarcoplasmic reticulum may be related to loss of ATP. The results shown in Figs. 1 – 4 support the importance of ATP, which functions to maintain the activity of the Ca-ATPase pump. If the liberation of phospholipids is a cause of leakage of calcium and reflects changes in the true concentration of sarcoplasmic free calcium, time courses of the increase in the calcium concentration shown in Figs. 3 and 4 are too fast because of an artifactual leakage of calcium from the sarcoplasmic reticulum and fragmented sarcoplasmic reticula during the homogenisation and extraction processes. The leaked calcium ions seem to diffuse slowly in the sarcoplasm at meat temperatures of 3 to 5 ˚C, since the sarcoplasm is viscous due to a high concentration of proteins. Supposing these presumptions are valid, free calcium in the sarcoplasm reaches the ultimate concentration of 210 μM after 3 days post-mortem in pork and after 6 days post-mortem in beef. This presumption is supported by evidence that myofibrillar fragmentation due to Z-disc weakening, which is fully induced by calcium ions at concentrations above 100 μM (Shimada & Takahashi, 2003), reaches a maximum at 6.5 days post-mortem in pork and at 3 weeks post-mortem in beef (Ahn et al., 2003). In this connection, appropriate periods of post-morten ageing to improve tenderness, which is

due to weakening of myofibrillar structures, disassembly of desmin intermediate filaments and structural weakening of the intarmuscular connective tissue (Takahashi 1996, 1999), are 6 to 10 days for pork (Takahashi, 1999) and 2 to 4 weeks for beef (Nishimura, Hattori & Takahashi, 1995) at 4 ˚C. For clarification of the exact post-mortem time required for sarcoplasmic free calcium to reach its ultimate concentration, it is necessary to measure free calcium in muscle fibres without homogenisation and extraction, i.e., directly by a non-destructive method. For example, Jeacocke (1993) injected the calcium- and magnesium-binding dye arsenazo III into single muscle fibres of the mouse and observed that a large increase in intracellular free calcium to more than 100 μM occurred after the onset of rigor at 35 °C.

4. Conclusions

The concentration of free calcium in the sarcoplasm increases from 100 nM to a narrow range of 210 to 230 μM during post-mortem ageing of meat, and the ultimate concentration remains constant even after prolonged ageing of meat. The increase in the concentration of sarcoplasmic free calcium is thought to be caused by liberation of phospholipids from sarcoplasmic reticular membranes and by loss of ATP. The possibility of contributions of μ-calpain and m-calpain to meat tenderisation seems to be very low. In this paper, evidence in favour of the controversial theory of calcium ion tenderisation of meat is presented.

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Figure captions

Fig. 1. Postmortem changes in calcium concentrations measured by the homogenisation-atomic absorption method (○) and by the mild extraction-quin2 method (●) using rabbit psoas muscle.

Fig. 2. Post-mortem changes in free calcium concentrations in pectoral muscle of chicken at 3 different ages. ○, Two-month-old; ●, 4-month-old; △, 16-month-old chickens.

Fig. 3. Changes in free calcium concentrations during post-mortem ageing of pork (○) and beef (●).

Fig. 4. Changes in concentrations of sarcoplasmic free calcium extracted with a solution containing 2 mM ATP during post-mortem ageing of pork (○) and beef (●).

Fig. 5. Post-mortem changes in proteins of the bovine sarcoplasmic reticulum.

Fig. 6. Changes in amounts of sarcoplasmic reticular phospholipids during post-mortem ageing of pork (○) and beef (●).

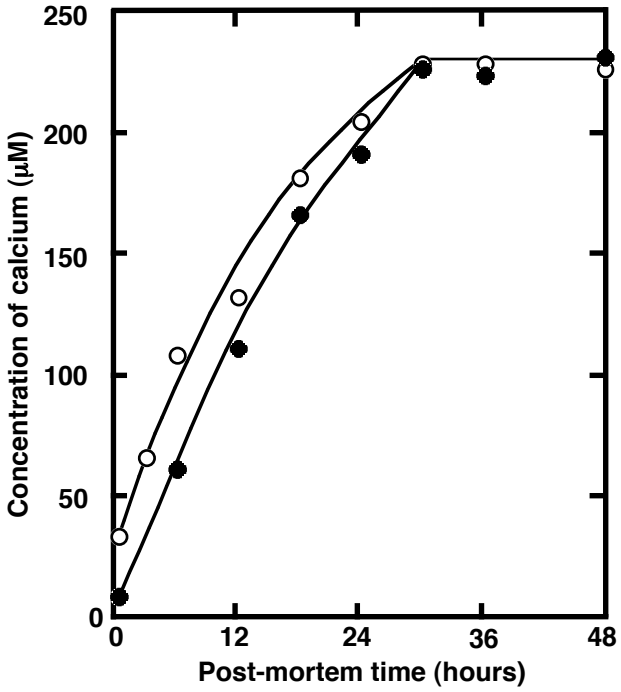

Fig. 1.
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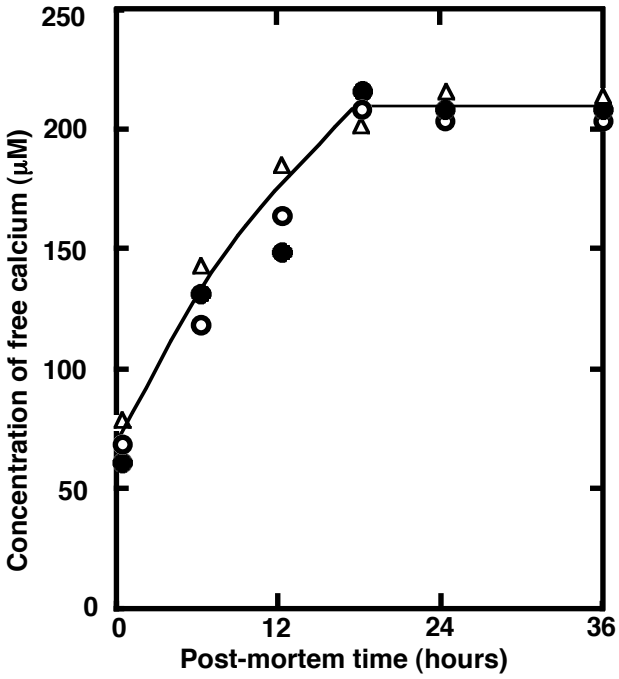

Fig. 2.
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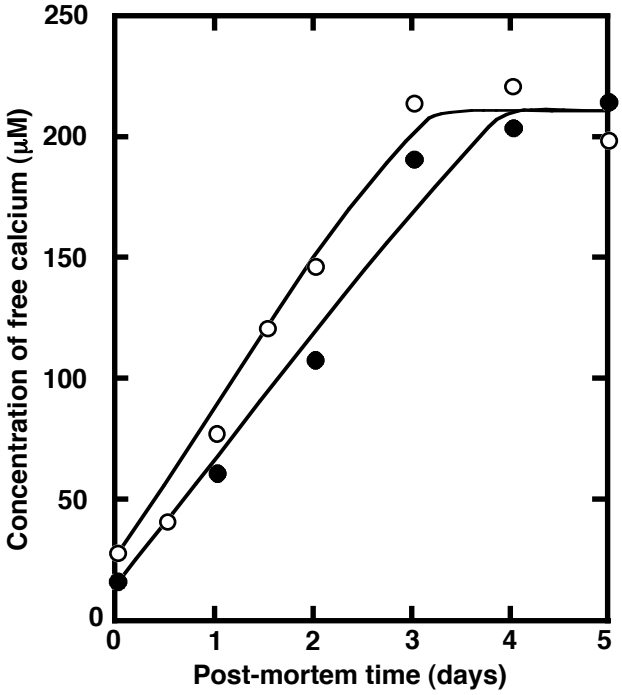

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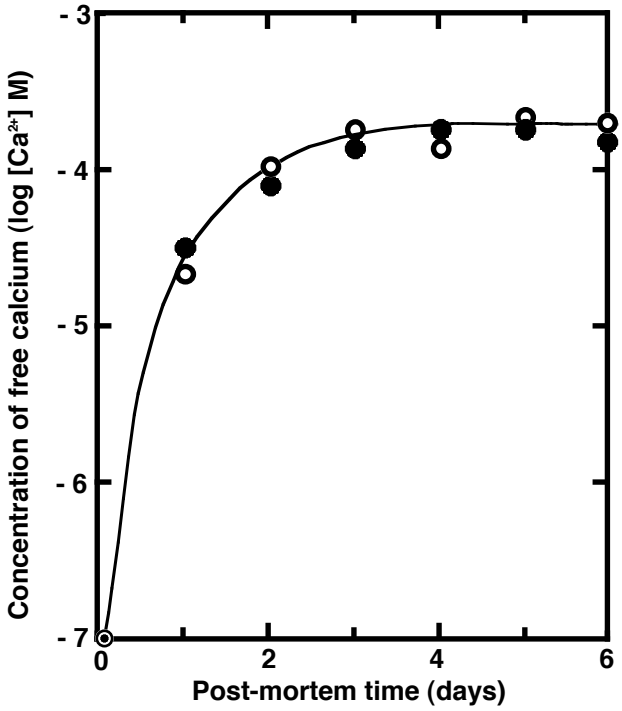

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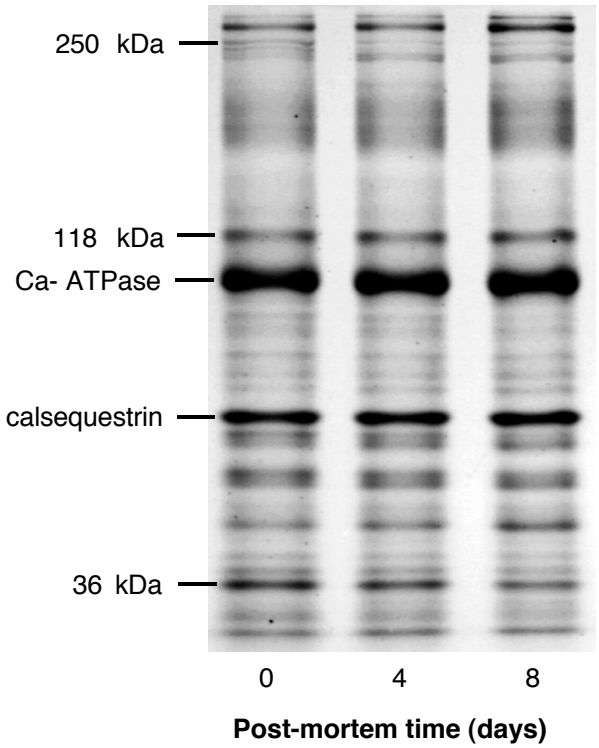

Fig. 5.
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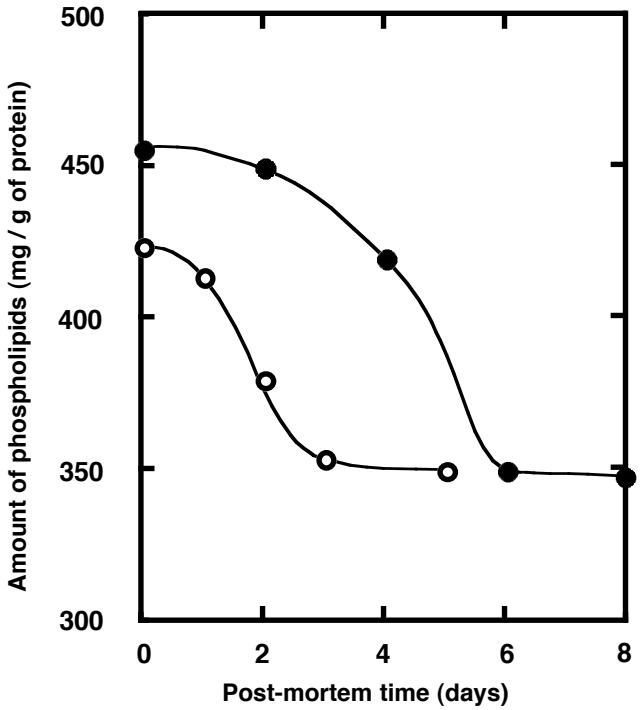

Fig. 6.
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