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MECHANISM OF MEAT TENDERIZATION DURING POST-MORTEM AGEING: CALCIUM THEORY

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Key words: calcium ions, Z discs, rigor linkages, connectin/titin filaments, nebulin filaments, intermediate filaments, μ-calpain

Abstract

Tenderization of meat during post-mortem ageing is caused by the structural weakening of myofibrils, desmin intermediate-filaments and the intramuscular connective tissue. The weakening of myofibrils, including Z-discs, rigor linkages formed between actin and myosin, connectin (titin) filaments, and nebulin filaments, is induced specifically by 0.1 mM of calcium ions, which is similar to the ultimate concentration in sarcoplasm of meat during ageing. Desmin intermediate-filaments are also disassembled non-enzymatically by 0.1 mM calcium ions. These facts are the basis of 'the calcium theory of meat tenderization,' which I have proposed. Furthermore, μ-calpain, which is the only possible candidate protease for the hydrolysis of myofibrillar proteins, is completely inactive under conditions similar to those of meat during post-mortem ageing, *i.e.* below pH 5.85 and below 15℃. Since it has been proven that there is no relationship between proteolysis and tenderization of meat, the calcium theory seems to have a great deal of validity.

Introduction

Most consumers consider tenderness to be the most important quality of meat. To improve meat tenderness, post-mortem ageing at 3 ~ 5℃ is required. Ageing periods are 2 ~ 4 weeks, 6 ~10 days and 0.5 ~ 1 day for beef, pork and chicken, respectively. Figure 1 shows changes in the shear-force values of raw beef, pork and chicken during post-mortem ageing. The toughness is reduced to 50 ~ 60% of the initial value in all meats. The tenderness of beef is improved significantly by ageing for 4 weeks, but the tenderization of pork almost stops within 10 days post-mortem. The rapid increase in tenderness is mainly due to the structural weakening of myofibrils and desmin intermediate-filaments: and structural weakening of the endomysium and perimysium occurs after 10 days, 5 days and 12 hr post-mortem in beef, pork and chicken, respectively.

Meat during ageing is under non-physiological conditions; pH decreases ultimately to near 5.5, ATP disappears completely, and the temperature is maintained at 3 ~ 5℃. Moreover, the sarcoplasmic calcium-ion concentration increases ultimately to 0.2 mM (Fig. 2), which is about 2,000-times higher than the initial value. The concentration increases gradually at 4℃ and reaches 0.2 mM within 4 days, 3 days and 20 hr in beef, pork and chicken, respectively. I have proposed 'the calcium theory of meat tenderization' on the basis of the following facts (Takahashi, 1992; Takahashi, 1996; Takahashi, 1999): Weakening of Z-discs and rigor linkages are reactions specific to calcium ions at 0.1 mM regardless of proteolysis, the same changes in connectin (titin) and nebulin filaments as those observed in aged meat can be induced non-enzymatically by 0.1 mM calcium ions *in vitro*, and desmin intermediate-filaments are disassembled non-enzymatically by 0.1 mM calcium ions . I report here recent studies in my laboratory on the molecular mechanisms of these phenomena.

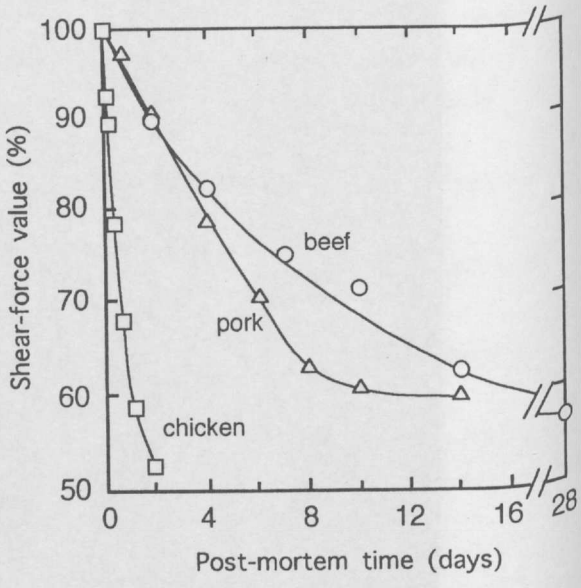

Fig. 1. Tenderization of raw meat during post-mortem ageing. Bovine, porcine and chicken *semitendinosus* muscles were aged at 4℃.

Structural Weakening of Myofibrils

Weakening of Z-discs

The Z-discs of vertebrate skeletal muscle connect neighbouring sarcomeres and possess a structure strong enough to transmit the tension developed by the interaction of thin and thick filaments of individual sarcomeres. Since we found that skeletal muscle myofibrils tend to break into small fragments composed of 1 ~ 4 sarcomeres with post-mortem time (Takahashi *et al.*, 1967), much evidences have demonstrated a close relationship between the fragmentation of myofibrils and tenderization of meat. The fragmentation of myofibrils is caused by structural weakening of Z-discs. The structure of Z-discs is composed of Z-filaments, whose key component is α-actinin, and an amorphous matrix (Takahashi & Hattori, 1989).

Z-Discs split into halves after removal of amorphous matrix materials by 0.1 mM calcium ions (Takahashi *et al.*, 1987), indicating that these materials cement neighbouring Z-filaments; once the cementing materials are solubilized by 0.1 mM of calcium ions, Z-discs are weakened and easily split by homogenization.

We have recently identified the main components of the amorphous matrix-materials as lipids. The content of lipids in bovine *semitendinosus* muscle Z-discs is 1.8 g per 100 g of myofibrillar proteins. They are composed of phospholipids, triacylglycerols, cholesterol and free fatty acids; the proportions are 65.8, 23.2, 8.6 and 2.4%, respectively. The weakening of Z-discs is induced by liberation of phospholipids during post-mortem ageing of meat, whereas the amount of α-actinin remains constant in these processes (Hattori & Takahashi, 1982; Takahashi *et al.*, 1987; Hwan and Bandman, 1989). Since calcium ions bind to phospholipids, it is very probable that phospholipids bind electrostatically to α-actinin under physiological conditions, and that this interaction is broken by the binding of calcium ions at 0.1 mM to phospholipids, resulting in the liberation of phospholipids from Z-discs. Figure 3 shows the molecular mechanism underlying the weakening of Z-discs during post-mortem ageing of meat.

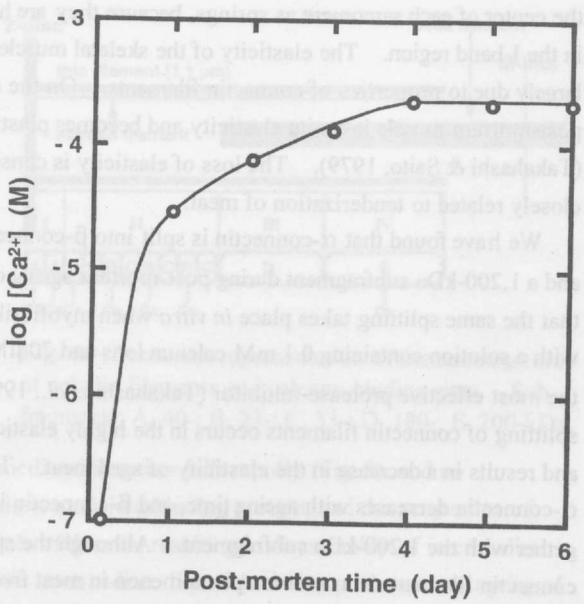

Fig. 2. Increase in sarcoplasmic calcium-ion concentrations during post-mortem ageing of beef. Bovine *semitendinosus* muscle was aged at 4℃, and samples were homogenized with deionized water containing 2 mM ATP. Calcium-ion concentrations were determined by atomic absorption analysis.

Weakening of rigor linkages

One aspect of the weakening of myofibril structures is the restoration of rigor-shortened sarcomeres (Stromer *et al.*, 1967; Takahashi *et al.*, 1967). The restoration of the sarcomere length indicates that rigor linkages formed between actin and myosin have been weakened. The state of contraction measured by sarcomere length is associated with tenderness of the meat (Herring *et al.*, 1965).

We have found a novel myofibrillar protein, paratropomyosin, which weakens rigor linkages (Takahashi *et al.*, 1985). Although paratropomyosin is exclusively located at the A-band and I-band junction region of sarcomeres *in situ* (Fig. 4A), it is released from its original position by 0.1 mM calcium ions and binds to actin in thin filaments during post-mortem ageing of meat (Hattori & Takahashi, 1988). The post-mortem translocation of paratropomyosin was confirmed by immunoelectron microscopy; this protein binds to actin along the entire length of thin filaments in aged meat (Fig. 4C). As paratropomyosin dominates the competition for myosin binding sites on actin due to its greater affinity for them, it weakens rigor linkages formed between actin and myosin.

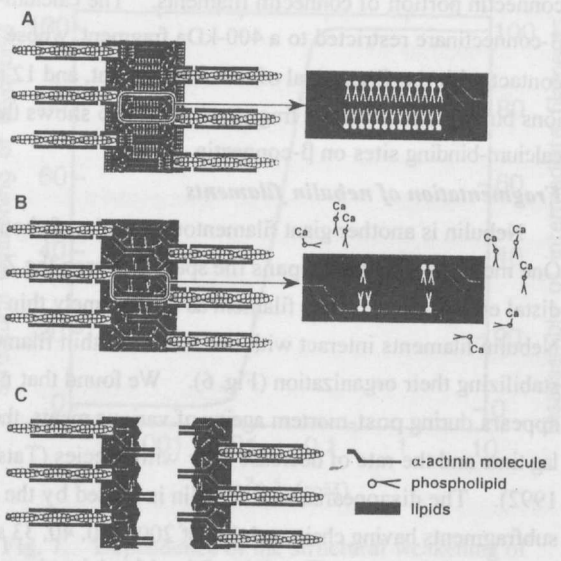

Fig. 3. Schematic representation of Z-disc weakening induced by 0.1 mM calcium ions. A, Intact Z-disc; B, liberation of phospholipids by binding of calcium ions, *i.e.*, Z-disc weakening; C, splitting of the weakened Z-disc into halves.

The rate of the post-mortem translocation of paratropomyosin from the A-I junction region of sarcomeres onto the thin filaments in the A-band, where rigor linkages have been formed, agrees well with the rate of increase in length of rigor-shortened sarcomeres (Takahashi *et al.*, 1995). The sarcomere lengths were found to be maximum at 10, 7 and 1 day post-mortem in beef, pork and chicken, respectively. It is clear that translocated paratrpomyosin weakens rigor linkages and brings about the recovery in the length of rigor-shortened sarcomeres. Thus, paratropomyosin stimulates the resolution of *rigor mortis* and is a key factor in meat tenderization during post-rigor ageing.

Splitting of connectin (titin) filaments

Connectin is an elastic protein of vertebrate striated muscle, and it exists as a very thin filament connecting the thick (myosin) filaments with the Z-disc in a sarcomere (Fig. 5). A single connectin filament of about 1.25 μm in length equals one molecule of α-connectin (titin 1), whose molecular weight is about 3 million. Connectin filaments play a role in positioning the thick filaments at

the center of each sarcomere as springs, because they are highly elastic in the I-band region. The elasticity of the skeletal muscle tissue is largely due to properties of connectin filaments. On the other hand, post-mortem muscle loses its elasticity and becomes plastic with time (Takahashi & Saito, 1979). The loss of elasticity is considered to be closely related to tenderization of meat.

We have found that α-connectin is split into β-connectin (titin 2) and a 1,200-kDa subfragment during post-mortem ageing of meat, and that the same splitting takes place *in vitro* when myofibrils are treated with a solution containing 0.1 mM calcium ions and 70 μM leupeptin, the most effective protease-inhibitor (Takahashi *et al.*, 1992). The splitting of connectin filaments occurs in the highly elastic I-band region and results in a decrease in the elasticity of aged meat. The amount of α-connectin decreases with ageing time, and β-connectin is formed together with the 1,200-kDa subfragment. Although the splitting of connectin filaments is a common phenomenon in meat from different species, α-connectin almost disappears 10 days, 5 days and 1 day post-mortem in beef, pork and chicken, respectively. The splitting of connectin filaments depends on the calcium ion concentration *in vitro*; it begins above 0.01 mM and reaches a maximum at 0.1 mM. The calcium-specific splitting of connectin filaments takes place in all myofibrils prepared from various skeletal muscles of cattle, swine, chicken and rabbit in the presence of 0.1 mM calcium ions and 70 μM leupeptin.

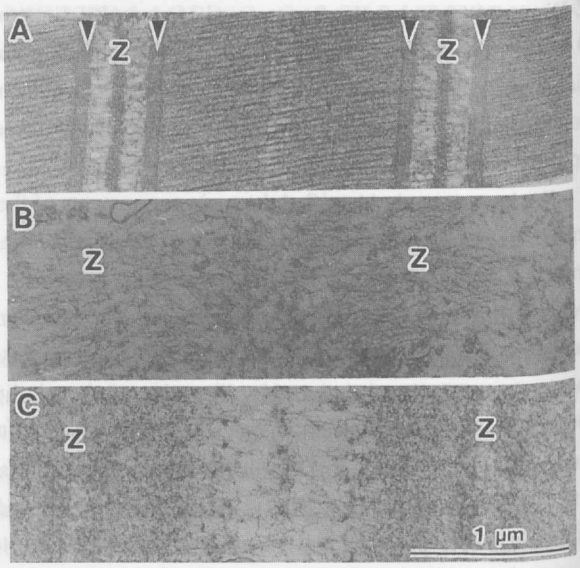

Fig. 4. Immunoelectron micrographs displaying post-mortem translocation of paratropomyosin in chicken. A, A fresh myofibril reacted with antiparatropomyosin antiserum. Arrowheads show the localization of the protein; B, a myofibril of chicken aged for 3 days at 4℃, after removal of the A-band; C, as in B but reacted with the antiserum.

We have proposed that non-enzymatic cleavage of some peptide bonds between β-connectin and the 1,200-kDa subfragment is induced specifically by the binding of calcium ions at non-physiological concentrations (0.1 mM), because calcium ions bind to the β-connectin portion of connectin filaments. The calcium-binding sites on β-connectinare restricted to a 400-kDa fragment, whose N-terminal is in contact with the C-terminal of the subfragment, and 12 moles of calcium ions bind to 1 mole of the fragment. Figure 5 shows the location of calcium-binding sites on β-connectin.

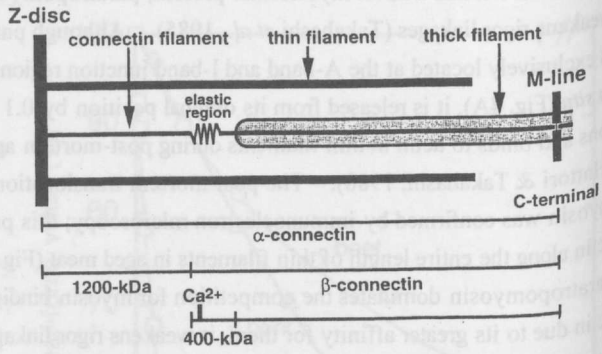

Fig. 5. Schematic representation of calcium-binding sties on β-connectin.

Fragmentation of nebulin filaments

Nebulin is another giant filamentous protein of about 800 kDa. One molecule of nebulin spans the space between the Z-disc and the distal end of a thin (actin) filament as an extremely thin filament. Nebulin filaments interact with and adhere to thin filaments, thereby stabilizing their organization (Fig. 6). We found that nebulin disappears during post-mortem ageing of various meats, though the initial lag time and the rate of decrease vary with species (Tatsumi & Takahashi, 1992). The disappearance of nebulin is caused by the fragmentation of nebulin filaments. Nebulin filaments break up into five subfragments having chain weights of 200, 180, 40, 33 and 23 kDa. The same fragmentation takes place *in vitro* when myofibrils are treated with a solution containing 0.1 mM calcium ions and 70 μM leupeptin. It has been proved that calcium ions bind to the 200-, 40- and 23-kDa subfragments (Tatsumi *et al.*, 1992). The fragmentation of nebulin filaments is probably induced by the binding of calcium ions to these subfragments, and this binding destabilizes the organization of thin filaments. This must be a factor in meat tenderization during post-mortem ageing.

Based on the results obtained by immunoelectron microscopy, we have proposed a model for the substructure of nebulin filaments (Tatsumi *et al.*, 1993), as shown in Fig. 6. There are four domains in a nebulin filament. Domain I is composed of 2 mol of the 40-kDa subfragment; domain II is composed of 4 mol of the 33-kDa subfragment alternating with 4 mol of the 23-kDa subfragment; domain III is composed of 1 mol of the 180-kDa subfragment, and domain IV composed of 1 mol of the 200-kDa subfragment.

Weakening of Intermediate Filaments

Disassembly of desmin intermediate-filaments

Desmin molecules, whose molecular weight is about 50,000, assemble to form intermediate filaments of 10 nm in diameter *in situ*. Desmin intermediate-filaments surround each Z-disc, extend between Z-discs of adjacent myofibrils, and interconnect individual myofibrils to the cell membrane. In this manner, desmin intermediate-filaments provide a framework that mechanically integrates myofibrils during the contraction and relaxation of skeletal muscle.

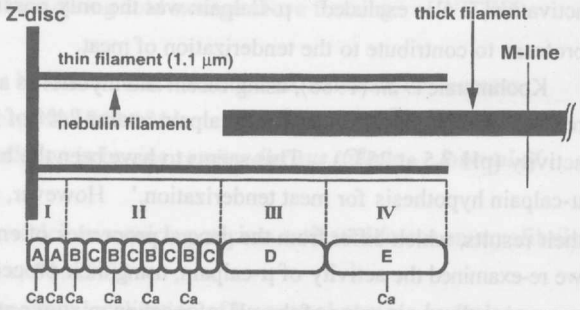

Fig. 6. Schematic representation of the substructure of nebulin filaments and calcium-binding sites. Sub-fragments: A, 40-; B, 23-; C, 33-; D, 180-; E, 200-kDa.

Recently, we have found that desmin intermediate-filaments disappear during post-mortem ageing of meat, and that intermediate filaments, which have been assembled from purified desmin, are disassembled by 0.1 mM calcium ions. After addition of the ions, the viscosity of desmin intermediate-filaments falls rapidly (Fig. 8), and no intermediate filament can be observed under an electron microscope. Desmin is a calcium-binding protein, and the properties of desmin molecules are changed by the binding of 1 mole of calcium ion to 1 mole of desmin, resulting in disassembly of intermediate filaments. These findings clearly demonstrate that the disassembly of desmin intermediate-filaments during post-mortem ageing of meat is caused by a direct action of calcium ions, and the findings strongly support 'the calcium theory of meat tenderization.' The dependence of the structural weakening of myofibrils described above and the disassembly of desmin intermediate-filaments on calcium-ion concentrations is summarized in Fig. 7. All structural weakenings, which contribute to tenderization of meat, take place at calcium ion concentrations above 0.01 mM and reach a maximum at 0.1 mM.

Fragmentation of desmin molecules

Desmin molecules disappear during post-mortem ageing of beef at 15℃ (Young *et al*., 1980), and Hwan and Bandman (1989) showed, using anti-desmin monoclonal antibodies, that fragments of desmin are present in aged beef. We have studied the fragmentation mechanism of desmin using anti-desmin antiserum to detect all fragments produced. The amounts of desmin decreased to 70 ~ 80% of the initial values within 14, 8 and 2 days post-mortem in beef, pork and chicken, respectively, and disintegrated desmin-molecules appeared as 43 ~ 46, 40 and 34 ~ 36-kDa polypeptides. A similar fragmentation was observed in isolated myofibrils treated with a solution containing 0.1 mM calcium ions and 70 μM leupeptin. The fragmentation depends on calcium ion concentrations; it begins above 0.01 mM, in the same manner as the disassembly of intermediate filaments, and reaches a maximum at 10 mM. Surprisingly, the fragmentation does not depend on temperature in the range from 5℃ to 40℃. This fact denies any participation of proteases. Moreover, purified desmin is fragmented into 43 ~ 46, 42 and 34 ~ 36-kDa polypepetides by 0.1 mM calcium ions, as in the cases of aged meat and isolated myofibrils. Because the disassembly of desmin intermediate-filaments takes place prior to the fragmentation of molecules (Fig. 8), the collapse of mechanical integration of myofibrils must result in tenderization of meat.

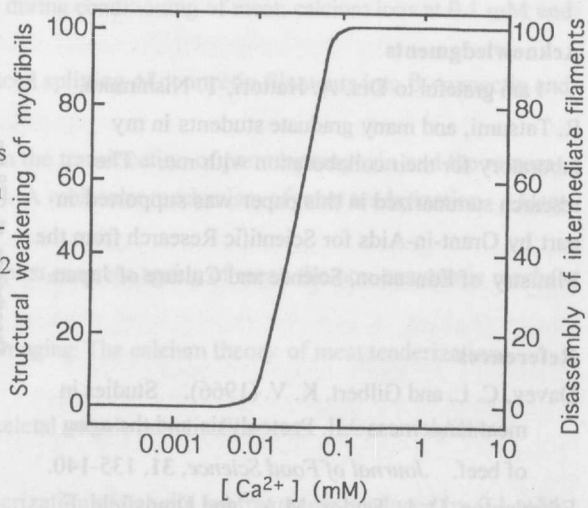

Fig. 7. Dependence of the structural weakening of myofibrils and the disassembly of desmin intermediate-filaments on calcium-ion concentrations. The structural weakening of myofibrils includes four classes of weakening described in the text.

Activity of μ-Calpain under Non-physiological Conditions

Since Hoagland *et al*. (1917) suggested the possibility of proteolysis during post-mortem ageing of meat, numerous investigations have been carried out to demonstrate the role of proteolysis in tenderizing meat. Endogenous proteases, *i.e.*, lysosomal cathepsins and sarcoplasmic calpains, were believed to attack myofibrils, whereas this process was found to occur to a very limited extent in post-mortem muscle (Davey & Gilbert,1966; Hamm, 1969). It has been proven that cathepsins do not take part in meat tenderization: 1) Cathepsin B remains in lysosome even after one-month ageing of beef at 4℃, and it does not diffuse into sarcoplasm (Lacourt *et al*., 1986). 2) Etherington *et al*. (1987) did not find any clear relationship between the rate of tenderizing meat and levels of cathepsins B and L in meat from different species. 3) Although cathepsins degrade many myofibrillar proteins, such as actin, myosin and α-actinin, these proteins remain unchanged during post-mortem ageing of meat. The possibility of m-calpain, which requires 1 ~ 5 mM calcium ions for

activation, is also excluded. μ-Calpain was the only possible candidate protease to contribute to the tenderization of meat.

Koohmaraie *et al.* (1986), using casein and myofibrils as substrates, reported that at pH 5.5 and 5℃, μ-calpain retains 24% of its maximum activity (pH 7.5 at 25℃). This seems to have been the basis of 'the μ-calpain hypothesis for meat tenderization.' However, we doubted their results, which differ from the general properties of enzymes, and we re-examined the activity of μ-calpain, using their procedure but measuring the exact value of the pH of reaction mixtures at each temperature. μ-Calpain purified from porcine *biceps femoris* and *semitendinosus* muscles is completely inactive under non-physiological conditions similar to those of meat during post-mortem ageing, *i.e.* below pH 5.85 and below 15℃ (Fig. 9). The discrepancy between Koohmaraie's and our results can be explained by the pH of reaction mixtures; the buffering action of 5 mg of casein or myofibrils per ml is stronger than that of 50 mM Tris-acetate buffer. Commercial casein-powder should be solubilized by adjusting pH above 7.0 to use as a substrate, and pH of myofibrils is around 7.0. If casein at pH 8.0 is used, the pH of the reaction mixture changes to 5.91 even in the presence of 50 mM buffer at pH 5.5. It is reasonable to conclude that Koohmaraie *et al.*(1986) used alkaline casein and obtained incorrect results.

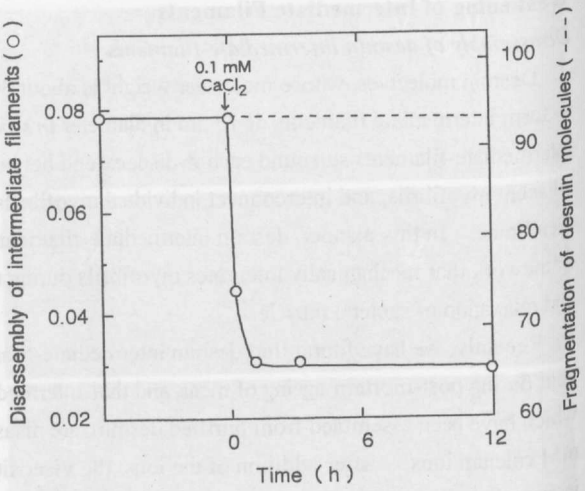

Fig. 8. Difference between the disassembly rate of desmin intermediate-filaments and the fragmentation rate of desmin molecules. Desmin was purified from porcine *semitendinosus* muscle.

Conclusions

Proteolysis does not contribute to the tenderization of meat during post-mortem ageing. Rather, the post-mortem increase in the sarcoplasmic calcium-ion concentration to 0.1 mM weakens the structures of myofibrils and desmin intermediate-filaments, resulting in tenderization of meat, *i.e.* the calcium theory.

Acknowledgments

I am grateful to Drs. A. Hattori, T. Nishimura, R. Tatsumi, and many graduate students in my laboratory for their collaboration with me. The research summarized in this paper was supported in part by Grant-in-Aids for Scientific Research from the Ministry of Education, Science and Culture of Japan.

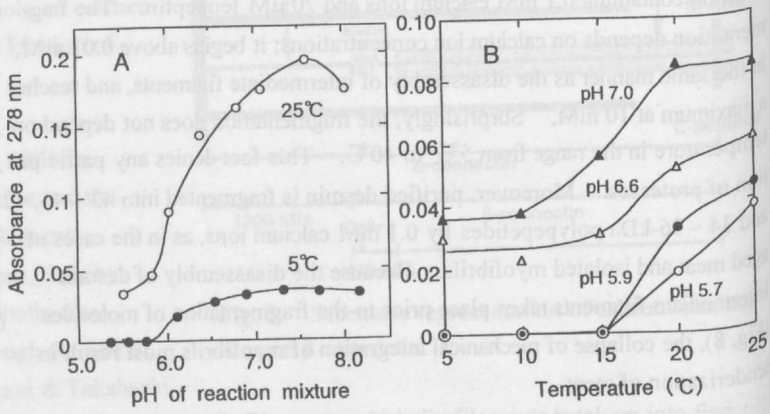

Fig. 9. Activity of μ-calpain. A, Dependence on pH; B, dependence on temperature. Reaction mixture: 5 mg/ml of casein (A) or myofibrils (B), 25 μg/ml μ-calpain, 0.1 M KCl, 0.1 mM $CaCl_2$, 10 mM 2-MeOH and 50 mM Tris-maleate buffer.

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