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STRUCTURAL WEAKENING OF SKELETAL MUSCLE TISSUE DURING POST-MORTEM AGEING OF MEAT: THE NON-ENZYMATIC MECHANISM OF MEAT TENDERIZATION

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Five main factors contribute to the overall eating quality of meat tenderness, taste, juiciness, appearance and aroma. Of these factors 'tenderness' is considered the most important by the average consumer. Tenderness of meat is the sum total of the mechanical strength of skeletal muscle tissue and its weakening during post-mortem ageing of meat. The mechanical strength of skeletal muscle tissue depends on species, breed, age, sex and individual skeletal muscle tissue of animals and fowl.

Slide 1. Meat tenderness stems from structural and biochemical properties of skeletal muscle fibres, especially myofibrils and intermediate filaments, and of the intramuscular connective tissue, the endomysium and perimysium, which are composed of collagen fibrils and fibres. The mechanical stability of collagen fibrils and fibres increases markedly with chronological ageing, and the endomysium and perimysium end up in extremely regular and thickened patterns. Therefore, meat produced from old animals and fowl is tough and of lower eating quality.

Slide 2. To obtain meat of high quality, reasonably fattened animals and fowl should be slaughtered at the proper age and post-mortem ageing of meat at around 3°C for a certain period is required. Ageing periods are usually more than 10 days for beef, 5 to 6 days for pork and half a day to one day for chicken. Both tenderness and flavour improve during this time.

Slide 3. This slide shows changes in the shear-force value of raw beef, pork and chicken during post-mortem ageing at 4°C. Tenderness of beef is improved significantly by ageing over a period of 28 days, but tenderization of pork almost stops within 10 days post-mortem. The tenderization of each meat occurs in two steps, a rapid first phase and a slow phase thereafter. The rapid increase in tenderness is mainly due to the structural weakening of myofibrils, and the slow process is caused chiefly by the structural weakening of the endomysium and perimysium, as shown in the following summary of our recent research.

Slide 4. For what reason does the skeletal muscle tissue weaken during post-mortem ageing of meat? Meat during ageing is under non-physiological conditions; pH decreases ultimately to near 5.5,

ATP disappears completely and temperature is maintained at around 3°C. Furthermore, the sarcoplasmic calcium ion concentration increases from 0.1 μM to 0.2 mM due to the loss of the ability of sarcoplasmic reticulum and mitochondria to accumulate calcium ions.

Slide 5. My research derived its origin in the findings of two kinds of post-mortem changes in myofibril structures in 1967; first, the fragmentation of myofibrils due to the weakening of Z-disks, and second, the restoration of rigor-shortened sarcomeres caused by the weakening of rigor linkages formed between actin and myosin. In 1992, I proposed 'the calcium theory of meat tenderization' on the basis of the following facts: firstly, the weakening of Z-disks and rigor linkages are reactions specific to calcium ions at 0.1 mM regardless of proteolysis, and secondly, the same changes in connectin or titin filaments, nebulin filaments and desmin molecules as those observed on aged meat can be induced by the treatment of isolated myofibrils with a solution containing 0.1 mM calcium ions and 70 μM leupeptin, which is the most effective inhibitor of proteases in myofibril preparation. I would like to talk about the non-enzymatic mechanism of these phenomena, and the structural weakening of the endomysium and perimysium during post-mortem ageing of meat.

Slide 6. My first theme today is the structural weakening of myofibrils by 0.1 mM calcium ions. The Z-disk of vertebrate skeletal muscle connects neighbouring sarcomeres and possesses a structure strong enough to transmit the tension developed by the interaction of thin and thick filaments of individual sarcomeres. In post-mortem muscle, however, Z-disks are characteristically weakened with time, and myofibrils tend to break by homogenization into fragments.

Slide 7. The structure of Z-disks is composed of two phases, Z-filaments and amorphous matrix. This slide shows electron micrographs of the longitudinal section of Z-disks. As shown on the left hand side in A, the intact Z-disk is almost totally masked by an abundance of amorphous matrix materials, and its internal structure can not be discerned. The structural appearance of Z-disks is changed during post-mortem ageng of meat or by the treatment of muscle fibres with a solution containing 0.1 mM calcium ions. As shown in B, the amorphous matrix materials are removed, and the internal Z-filaments composed of α-actinin molecules are clearly observable.

Slide 8. These electron micrographs show the splitting of Z-disks after the removal of matrix materials. The structure of intact Z-disks is most resistant to alkali. When amorphous matrix materials are removed, Z-disks split into halves after treatment with NaOH, as shown in this slide. It seems likely that these materials cement neighbouring Z-filaments; and once the cementing materials are

solubilized by 0.1 mM calcium ions, Z-disks are weakened, and easily split by alkali or homogenization. A new fact which no one has ever imagined before has been discovered.

Slide 9. We have recently identified the chief components of the amorphous matrix materials as lipids. The content of lipids in bovine *semitendinosus* muscle Z-disks is 1.8 g per 100 g of myofibrillar proteins. They are composed of phospholipids, triacylglycerols, cholesterol and free fatty acids. Phospholipids occupy about 66% of total lipids. Because the amount of α-actinin is 2.9% and the sum of many minor proteins known to be present in the Z-disk is less than 1% of the total myofibrillar protein, proteins occupy at most about 4%. The total amount of lipids and proteins coincides well with the estimation from the results obtained by interference microscopic observations that the amount of Z-substance is 6% of the total myofibrillar material.

Slide 10. The weakening of Z-disks is induced by the liberation of phospholipids. This slide shows that the Z-disk weakening agrees well with the liberation of phospholipids during post-mortem ageing of beef, pork and chicken at 4°C. The amount of α-actinin remains constant in these processes. This fact denies any participation of μ-calpain in the post-mortem weakening of Z-disks, because its specificity is the removal of α-actinin from Z-disks. It is the only possible candidate protease for the hydorolysis of myofibrillar proteins.

Slide 11. One aspect of the calcium-specific weakening of myofibrillar structures is the restoration of rigor-shortened sarcomeres. The restoration of the sarcomere length indicates that rigor linkages formed between actin and myosin have been weakened. Our discovery of a new myofibrillar protein in 1985, which we named paratropomyosin, has clarified the non-enzymatic mechanism of the weakening of rigor linkages and of the concomitant lengthening of rigor-shortened sarcomeres.

Slide 12. This slide shows the translocation of paratropomyosin during post-mortem ageing of pork. The upper panels are phase-contrast images and the lower panels are immunofluorescence images. As shown in B, paratropomyosin is exclusively located at the A-band and I-band junction region of sarcomeres in myofibrils prepared immediately post-mortem. Striking changes in the distribution are observable in myofibrils prepared from aged pork. At the final stage of post-mortem ageing, as shown in F, the I-band becomes strongly fluorescent, and the A-band becomes distinctly more fluorescent. Paratropomyosin is released from its original position by 0.1 mM calcium ions non-enzymatically and translocated onto the thin filaments in the A-band, where rigor linkages have been formed.

Slide 13. The rate of the post-mortem translocation of paratropomyosin, PTM, from the A-I junction region of sarcomeres onto the thin filaments in the A-band agrees well with the rate of increase in the length of rigor-shortened sarcomeres, as shown in this slide. Thus, translocated paratropomyosin weakens rigor linkages and brings about the recovery of the length of rigor-shortened sarcomeres.

Slide 14. Purified paratropomyosin binds to F-actin and strongly inhibits the Mg-ATPase activity of actomyosin and myofibrils. It relieves the rigor tension generated in glycerinated muscle fibres, and restores rigor-shortened sarcomeres. This slide shows the effect of paratropomyosin on the rigor tension developed. The rigor contraction of muscle fibres is elicited at arrow A. The broken line shows the tension record in the absence of paratropomyosin. The rigor tension remains constant for at least 3 hours in this case. When paratropomyosin is added one minute after the full development of rigor tension, at arrow B, the rigor tension is reduced to about 65% of the initial value within 10 minutes. This result directly demonstrates that paratropomyosin weakens rigor linkages. Thus, paratropomyosin dominates the competition for myosin binding sites on actin due to its greater affinity for them, and speeds up the dissociation of actin and myosin. Paratropomyosin stimulates the resolution of *rigor mortis*, and is a key factor in meat tenderization during post-rigor ageing.

Slide 15. I would like to move on to the next item, which is the calcium-specific splitting of connectin or titin filaments. Firstly, I'd like to give a brief explanation about connectin. Connectin is a huge molecule over 3,000 kDa of chain weight, and is an elastic protein of vertebrate striated muscle. It was discovered by Dr. Maruyama of Japan in 1976, and named titin by Dr. Wang of the United States in 1979. It exists as a very thin filament connecting the thick filament with the Z-disk in a sarcomere. A single connectin filament equals one molecule of α-connectin. Because connectin filaments are highly elastic in the I band region, they help position the thick filaments at the center of each sarcomere by acting as 'springs' attached to both ends of the thick filaments. The elasticity of the skeletal muscle tissue is largely due to the property of connectin filaments.

Slide 16. On the other hand, post-mortem muscle loses its elasticity and becomes plastic with time. The loss of elasticity is considered to be closely related to the tenderization of meat. We have found that connectin filaments are split into β-connectin and a 1,200-kDa subfragment during post-mortem ageing of meat at 4°C. The splitting of connectin filaments occurs in the highly elastic part and results in the decrease in the elasticity of aged meat.

Slide 17. This slide shows post-mortem changes in SDS-polyacrylamide gel electrophoretic patterns. The amount of α-connectin decreases with ageing time and β-connectin is formed together with a 1,200-kDa subfragment. There is no sign of further degradation of either β-connectin or the subfragment. The same splitting takes place, when isolated myofibrils are treated with a solution containing 0.1 mM calcium ions and 70 μM leupeptin.

Slide 18. The rate of decrease in the amount of α-connectin varies with the origin of meat; α-connectin disappears in beef, after 10 days storage, and in chicken, within 1 day. This slide shows the relationship between the decrease in the elasticity of post-mortem muscle and in the amount of α-connectin. The initial rate of these two changes is characteristic of beef, pork and chicken and agrees well in each meat. It is clear that the elasticity of post-mortem muscle is lost by the splitting of connectin filaments into β-connectin and the 1,200-kDa subfragment.

Slide 19. We have proposed that non-enzymatic cleavage of some peptide bonds between β-connectin and 1,200-kDa subfragment is induced specifically by the binding of calcium ions at 0.1 mM, because calcium ions bind to the β-connectin portion of connectin filaments. The calcium-binding site on β-connectin is restricted to a 400-kDa fragment which is produced by tryptic digestion of β-connectin. There is a possibility that the N-terminal of the 400-kDa fragment is located at the junction between β-connectin and the 1,200 kDa subfragment, and its N-terminal region has a high affinity for calcium ions.

Slide 20. Nebulin is another giant filamentous protein with a chain weight of about 800 kDa and constitutes a set of long inextensible longitudinal filaments. One molecule of nebulin spans the space between the Z-disk and the free end of a thin filament. Nebulin adheres to the thin filament, thereby stabilizing its organization.

Slide 21. We have found that nebulin filaments are fragmented into five subfragments during post-mortem ageing of meat.

Slide 22. Nebulin disappears during post-mortem ageing of meat, though the initial lag time and the rate of decrease vary with the species, as shown in this slide. The disappearance is caused by the fragmentation of nebulin filaments.

Slide 23. This slide shows the results of an immunoblot analysis. Nebulin filaments break up into five subfragments. All the subfragments other than the 180 kDa one are released from myofibrils, as shown on the right hand side. The same fragmentation occurs when isolated myofibrils are treated with a solution containing 0.1 mM calcium ions and 70 μM leupeptin. Nebulin is

proved to be a calcium-binding protein. Based on these findings, we have proposed a non-enzymatic fragmentation of nebulin filaments induced by 0.1 mM calcium ions. The Ca-specific fragmentation of nebulin filaments must destabilize the organization of thin filaments, and thus contribute to the tenderization of meat during post-mortem ageing.

Slide 24. My second theme today is the weakening of intermediate filaments, in other words, the fragmentation of desmin molecules and depolymerization of the filament.

Slide 25. Desmin was discovered in 1976 and its chain weight is about 50 kDa. It polymerizes to form intermediate filaments of 10 nm in diameter *in situ*. As shown in this slide, desmin intermediate filaments surround each Z-disk and function in muscle fibres as a three dimensional matrix which interconnects individual myofibrils to one another and to the cell membrane at the level of their Z-disk. In this manner desmin intermediate filaments provide a framework that mechanically integrates myofibrils during the contraction and relaxation of skeletal muscle.

Slide 26. We have studied the fragmentation mechanism of desmin molecules using anti-desmin antiserum to detect all fragments by immunoblotting. When beef, pork and chicken are aged at 4°C, the amounts of desmin decrease to 70 to 80% of the initial values within 14, 8 and 2 days, respectively.

Slide 27. The desmin molecules which have disappeared have been fragmented to 43 to 46, 40 and 34 to 36 kDa polypeptides. Desmin purified from porcine skeletal muscle is similarly fragmented by the treatment with a solution containing 0.1 mM calcium ions and 70 μM leupeptin.

Slide 28. The fragmentation depends on calcium ion concentrations; it begins above 10 μM in the same manner as the structural weakening of myofibrils, and reaches a maximum at 10 mM, differing from the weakening of myofibrils which reaches a maximum at 0.1 mM as shown by the broken line. Surprisingly, the fragmentation of desmin molecules does not depend on temperature in the range from 5°C to 40°C. This fact denies any participation of proteases.

Slide 29. Moreover, the viscosity of intermediate filaments prepared from purified desmin drops immediately after the addition of 0.1 mM calcium chloride, indicating that the filaments are depolymerized. This depolymerization occurs prior to the fragmentation of desmin molecules. These findings demonstrate that the depolymerization of desmin intermediate filaments and the fragmentation of desmin molecules during post-mortem ageing of meat are induced by a direct action of calcium ions. The

disintegration of the three dimensional matrix of desmin intermediate filaments must result in the tenderization of meat.

Slide 30. My third theme today is the structural weakening of the intramuscular connective tissue, especially the endomysium and the perimysium. The endomysium resolves into individual collagen fibrils, and the thick sheets of the perimysium separate into collagen fibres during post-mortem ageing of meat.

Slide 31. The left hand side A shows a scanning electron micrograph of the endomysium and perimysium of bovine *semitendinosus* muscle immediately post-mortem. The honeycomb structure of the endomysium for housing individual muscle fibres is clearly observable. The perimysium is composed of several layers of sheets surrounding the endomysia. These structures remain unchanged for up to 10 days post-mortem, but a progression of structural alterations is clearly observable. after 14 days post-mortem. As shown on the right hand side in B, in the muscle aged for 28 days at 4°C, the honeycomb structure of the endomysium markedly deforms, and the perimysial sheets disintegrate into ribbon-like structures.

Slide 32. At a higher magnification, as shown on the right hand side in C and D, the endomysium becomes lacy and resolves into individual collagen fibrils.

Slide 33. A closer view of the perimysium shows that the thick sheets of the perimysium separate into collagen fibres, as displayed on the right hand side in B. These results indicate that the structural changes are induced by the dissociation of collagen fibrils and fibres from the endomysium and the perimysium, respectively. These structural changes in, or in other words, the weakening of the endomysium and perimysium show the effect on tenderization of extended ageing of beef. Similar structural weakening of the endomysium and perimysium occurs at 4°C after 5 days and 12 hours post-mortem in pork and chicken, respectively. It is evident that an extended post-mortem ageing is necessary to obtain satisfactorily tenderized meat.

Slide 34. Collagen fibrils and fibres of the endomysium and perimysium are embedded in ground substances, proteoglycans and glycoproteins. The left hand side A shows the arrangement of proteoglycans in bovine *semitendinosus* muscle immediately post-mortem observed under a transmission electron microscope. In the perimysium, proteoglycans are associated with collagen fibrils at regular intervals of 65 nm, as indicated by the arrows. Proteoglycans remain unchanged up to 14 days post-mortem. As shown on the right hand side in B, in the muscle aged for 28 days at 4°C, the greater part of the proteoglycans disappears. These results indicate that one, proteoglycans associated with collagen fibrils are

degraded with time post-mortem, two, the linkage between collagen fibrils is weakened, and three, collagen fibrils and fibres separate from the endomysium and the perimysium, respectively. The mechanism responsible for the degradation of proteoglycans during post-mortem ageing of meat is unknown. It seems reasonable to assume that the structural weakening of the endomysium and perimysium is induced by 0.1 mM calcium ions under non-physiological conditions, as occurs in the myofibrillar structures.

Slide 35. The direct action of calcium ions which I have just mentioned clears up many questions which could not be well understood in the past. Calcium ions have a dual function in post-mortem muscle; the rise of sarcoplasmic calcium ion concentrations to 5 μM induces rigor-contraction, and the further rise to 0.1 mM weakens structures of myofibrils, desmin intermediate filaments and probably the endomysium and perimysium, thereby bringing about the tenderization of meat.