Relationship between proteolysis and water-holding of myofibrils

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Abstract

The purpose of this study was to increase the knowledge on the relationship between proteolysis of myofibrillar proteins and the water-holding of meat. Myofibrils isolated from porcine *longissimus thoracis et lumborum* muscle were used as a model system. Myofibrils were incubated with either calpain-2, the proteasome or a lysosomal extract at 25 ºC for 2 h. All three proteolytic systems improved the relative water-holding and generally there was a larger effect with increasing amount of enzymes in the incubation. The improved water-holding occurred in parallel to degradation of myofibrillar proteins. Desmin was degraded by calpain-2 as well as by lysosomal enzymes and α-actinin was released by the proteasome. We here propose a model in which degradation of proteins in and around the Z-disk allows overall swelling of the filament lattice and more specifically in the I-band area. In conclusion, proteolytic degradation of myofibrillar proteins by calpain-2, the proteasome or lysosomal enzymes improves the water-holding of myofibrils.

Keywords: Calpain-2; Proteasome; Lysosomal enzymes; Meat; Porcine muscle; Calcium

1. Introduction

It is estimated that around 85% of the water in the muscle fiber is held in the myofibrils (Huff-Lonergan & Lonergan, 2005), mainly being located within and between the myosin and actin filaments of the myofibrils. After slaughter, both the sarcomere length and myofibril diameter is reduced (Diesbourg, Swatland, & Millman, 1988). The shrinkage of the myofibrillar lattice contributes to a gradual mobilization of water from the intramyofibrillar space to the extramyofibrillar space, which subsequently results in increased drip loss (Hughes, Oiseth, Purslow, & Warner, 2014). During storage of meat an initial decrease in water-holding capacity followed by an improvement after several days of additional storage has been observed in pork (Kristensen & Purslow, 2001; Straadt, Rasmussen, Andersen, & Bertram, 2007; Wang et al., 2016) and beef (Farouk, Mustafa, Wu, & Krsinic, 2012). In addition, both Kristensen & Purslow (2001) and Farouk et al (2012) hypothesized that postmortem proteolysis is associated with the improved water-holding capacity observed at longer storage. In postmortem pork, changes of water-holding capacity during ageing was suggested to be related to the extent of proteolysis of cytoskeletal proteins such as desmin, the major muscle-specific intermediate filament protein (Kristensen & Purslow, 2001). Further evidence that proteolysis is related to water-holding capacity was shown by Melody et al. (2004) who found that different rates of degradation of desmin in *longissimus dorsi*, *semimembranosus*, and *psoas major* muscles were associated to variation in drip loss. Similarly, high levels of desmin degradation has been associated with low drip loss, and limited desmin degradation with increased drip loss during postmortem storage (Zhang, Lonergan, Gardner, & Huff-Lonergan, 2006).). In addition, the degradation of integrin, which has a role in attachment of the cell membrane to the cytoskeleton, has been suggested to correlate to the opening of so-called drip channels in pork (Lawson, 2004).

The calpain system is an endogenous protease system constituting several isoforms of the enzyme. The most studied are the two Ca^{2+}-dependent proteases, calpain-1 and calpain-2, and their specific inhibitor, calpastatin. Even though the requirement of two proteases on Ca^{2+} concentration differ, being 3-50 μM and 400-800 μM for half-maximal proteolytic activity, respectively, calpain-1 and calpain-2 cleave the same substrates (Goll, Thompson, Li, Wei, & Cong, 2003). Therefore, both of the two calcium-dependent cysteine proteases degrade the same specific set of myofibrillar and cytoskeletal proteins (Geesink & Koohmaraie, 1999; Huff-Lonergan et al., 1996). There is evidence that calpain-1 is important for postmortem proteolysis (Koohmaraie et al., 1987; Koohmaraie & Geesink, 2006), and some studies have implied that calpain-2 may also play a role in proteolysis of beef (Camou, Marchello, Thompson, Mares, & Goll, 2007) and pork (Pomponio et al., 2008, Pomponio & Ertbjerg, 2012). The proteasome is another endogenous proteolytic enzyme in muscle. It is composed of multiple subunits and has a high molecular weight of 2000 kDa. The catalytic core, the 20S proteasome, is of the form of a barrel and is assembled of 28 protein subunits, which possesses five peptidase activities (Farout et al., 2000). When incubated with the proteasome, some myofibrillar proteins were reported to be hydrolysed and α-actinin was released but not degraded (Dutaud, Aubry, Guignot, Vignon, Monin, & Ouali, 2006). Another major proteolytic system in skeletal muscle is the lysosomal system. Results from immunochemical studies (Dutson & Lawrie, 1974; Mikami, Whiting, Taylor, Maciewicz, & Etherington, 1987) and studies in which released and bound lysosomal enzymes activities were measured (Ertbjerg, Larsen, & Møller, 1999; Wu, Dutson, & Carpenter, 1981) indicate that cathepsins are effectively released from the lysosomes in postmortem muscle. Incubation of myofibrils with cathepsin B and L resulted in some degradation

of myofibrillar proteins (Baron, Jacobsen, & Purslow, 2004; Mikami, Whiting, Taylor, Maciewicz, & Etherington, 1987). Even though the role in tenderization of the lysosomal system is controversial (Sentandreu, Coulis, & Ouali, 2002), some results have supported that cathepsins may play a role in postmortem proteolysis (Chéret, Delbarre-Ladrat, Lamballerie-Anton, & Verrez-Bagnis, 2007; Lana & Zolla, 2016; Lomiwes, Farouk, Wu, & Young, 2014; Thomas, Gondoza, Hoffman, Oosthuizen, & Naudé, 2004; Wang et al., 2014). Overall, some studies suggest that there is a relationship between postmortem proteolysis and water-holding in meat. However, the mechanisms behind how protein degradation relates to water-holding capacity of meat are not well understood. The aim of this study was to evaluate influence of calpain-2, a lysosomal extract and the proteasome on postmortem water-holding of myofibrils, in order to increase our knowledge on the relationship between proteolysis and water-holding of meat.

2. Materials and Methods

2.1 Raw materials

Porcine *longissimus thoracis et lumborum* muscles were excised from 6 different carcasses the day after slaughter. Pigs belonging to the cross Norwegian Landrace × Swedish Yorkshire × Danish Landrace were slaughtered and chilled at a commercial slaughterhouse in Finland. Muscles were transported refrigerated and arrived in the lab 24 h postmortem. The muscles were assumed not to be PSE meat based on the Minolta L* values being less than 54 as described previously (Liu, Arner, Puolanne, & Ertbjerg, 2016). The ultimate pH was measured by an insertion electrode (Mettler-Toleda Inlab 427) and was for all muscles in the range 5.5 to 5.6. After that, visible connective tissue and external fat were trimmed form muscle and frozen at -80 °C until use. Three independent muscles were used for purification of the calpain-2 (section 2.2), for purification of the lysosomal extract (section 2.3), and for purification of the proteasome (section 2.4). Three other muscles were used for isolation of myofibrils (section 2.5). Myofibril preparations from these three muscles were subsequently randomly incubated with either calpain-2, lysosomal extract or the proteasome to determine the effect on water-holding (section 2.6).

2.2 Partly purification of calpain-2

Muscle samples (100 g) were homogenized by an IKA UltraTurrax T25 homogenizer (Labortechnik, Staufen, Germany, 3 × 20 s at 13,500 rpm), 10 s cooling between bursts in 6 vol. (w/v) of cold 100 mM Tris-HCl buffer, pH 8.0, containing 5 mM EDTA and 10 mM monothioglycerol. Then the homogenate was centrifuged (15,000 × *g*, 30 min, 4 °C), and the supernatant was filtered through a 0.45-µm filter. The filtered extract was loaded onto a 26/10 DEAE Sepharose Fast Flow column (GE Healthcare, Uppsala, Sweden) pre-equilibrated in 20 mM Tris-HCl buffer, pH 7.5, containing 5 mM EDTA and 10 mM monothioglycerol (buffer A) using a Pharmacia Biotech system® (AKTA prime) FPLC. Loading and eluting rate was 30 ml/min. The column was eluted using gradient from 0 to 0.6 M NaCl in buffer A. Fractions eluting at 190 to 300 mM NaCl were collected and precipitated with 50% ammonium sulfate. The precipitate was sedimented by centrifugation at 10,000 × *g* for 30 min and the pellet re-suspended in buffer A. This fraction was centrifuged again and filtered through a 0.45-µm membrane before being loaded onto a Sephacryl S-300 HR 26/60 column (GE Healthcare) pre-equilibrated with buffer A. Loading rate was 3 ml/min and eluting rate was 5 ml/min. After that, the fractions containing calpain activity were pooled, concentrated and purified by

chromatography on a 1 ml RESOURCE Q (GE Healthcare) with buffer A. The partly purified calpain-2 preparation was adjusted to contain 30% (v/v) glycerol, and the protein content was determined by the RC DC Protein Assay Kit (Bio-Rad Laboratories, Hercules, CA) before storage at -20 °C until use. Calpain-2 activity was determined using casein as substrate by the method described by Ertbjerg, Henckel, Karlsson, Larsen, & Møller (1999) with some modification. In the assay 100 µl of the calpain fraction was incubated with 300 µl incubation medium (100 mM Tris-HCl, 10 mM monothioglycerol, 5 mg/ml casein and 5.0 mM $CaCl_2$, pH 7.5). After 30 min incubation at 25 °C, the reaction was stopped by addition of 400 µl of 10% trichloroacetic acid, and tubes were centrifuged at 20,000 × *g* for 5 min. One unit of calpain activity was defined as an increase in absorbance at 280 nm of 1.0 per hour at 25 °C. Results were corrected by blanks with the same content incubated 0 min.

2.3 Purification of the proteasome

Finely chopped meat cubes (100 g) were homogenized by an IKA UltraTurrax T25 homogenizer (Labortechnik, 3 × 20 s at 13,500 rpm), 10 s cooling between bursts in 6 vol. (w/v) of cold 100 mM Tris-HCl buffer, pH 8.3, containing 5 mM EDTA and 10 mM monothioglycerol. Then the homogenate was centrifuged (15,000 × *g*, 30 min, 4 °C), and the supernatant was filtered through a 0.45-µm filter. The filtered extract was loaded onto a 26/10 DEAE Sepharose Fast Flow column (GE Healthcare) pre-equilibrated in 20 mM Tris-HCl buffer, pH 7.7, containing 5 mM EDTA and 10 mM monothioglycerol (buffer B) using a Pharmacia Biotech system® (AKTA prime) FPLC. Loading and eluting rate was 30 ml/min. The column was eluted using gradient from 0 to 0.6 M sodium chloride in buffer B. Fraction eluting at 170 to 280 mM NaCl were collected and adjusted to 65% ammonium sulfate. The ammonium sulfate precipitate was sedimented by centrifugation at 10,000 × *g* for 30 min and the pellet re-suspended in buffer B. The fraction was further centrifuged and filtered through a 0.45-µm membrane and loaded onto a sephacryl S-300HR 26/60 column (GE Healthcare) pre-equilibrated in buffer B. Elution was done by the same buffer and the fractions containing proteasome activity were pooled, concentrated and purified by chromatography on a 1 ml RESOURCE Q column (GE Healthcare) eluting with a gradient of 60% NaCl in buffer B. The purified proteasome preparation was adjusted to contain 30% (v/v) glycerol and the protein content was determined by RC DC Protein Assay Kit (Bio-Rad Laboratories) before storage at -20 °C until use. The proteasome activity was determined using carbobenzoxy-Gly-Gly-Leu-7-amido-4-methylcoumarin (Z-GGL-AMC) (Sigma, Saint Louis, MO) as substrate for chymotrypsin-like activity. Ten µl of the proteasome fraction was incubated with 20 µM Z-GGL-AMC at 25 °C in 250 µl of buffer B. The amount of fluorescent product (AMC) released was determined fluorometrically using a spectrofluorometer Infinite M200 scalable microplate reader (Tecan, Mannedorf, Germany). Excitation and emission wavelength were 360 and 450 nm for AMC.

2.4 Preparation of lysosomal extract

Lysosomal enzymes were prepared as described by Ertbjerg et al. (1999) with slight modification. All procedures were carried out at 0 - 4 °C. Coarsely chopped meat cubes (15 g) were added to 30 ml of buffer C (100 mM sucrose, 100 mM KCl, 50 mM Tris-HCl, 10 mM sodium pyrophosphate, 1 mM Na_2EDTA, pH 7.2), containing 50 µg/ml nagarse (Sigma, protease Type XXIV) dissolved immediately before use, and were finely minced with scissors and incubated for 5 min. Excess buffer was decanted off and buffer C (20 ml/g meat) without nagarse was added.

Homogenisation was performed using an IKA Ultra-Turrax T25 homogenizer (Labortechnik) at 8,000 rpm for 5 s. The homogenate was filtered through cheesecloth. The filtrate was centrifuged at 1,100 × *g* for 10 min and the supernatant was then centrifuged at 3,000 × *g* for 10 min. Thereafter, the supernatant was centrifuged at 18,000 × *g* for 30 min to obtain a lysosomal fraction. The pellets were suspended in 2 ml buffer (85 mM Na acetate, 15 mM acetic acid, 1 mM Na_2EDTA, pH 5.5) and rapidly frozen at -80 °C until use. This preparation was after thawing taken as the lysosomal extract. The protein content was determined by the RC DC Protein Assay Kit (Bio-Rad Laboratories).

2.5 Preparation of myofibrils

Myofibrils were prepared as described by Liu, Puolanne & Ertbjerg (2014) with slight modifications. Ten g of frozen meat was homogenized by IKA Ultra-Turrax T25 homogenizer (Labortechnik, Staufen, Germany) at 13,500 rpm for 20 s in 100 ml cold rigor buffer (75 mM KCl, 20 mM Tris-HCl, 2 mM $MgCl_2$, 2 mM EGTA, pH 7.0). The supernatant was discarded after centrifugation at 10,000 × *g* at 4 °C for 10 min. The pellet was then washed twice more by the same process in buffer D (75 mM KCl, 20 mM MES, 2 mM $MgCl_2$, 2 mM EGTA, pH 5.5). The pellet after the final wash was taken as the myofibril preparation.

2. 6 Incubation of myofibrils with calpain-2, proteasome and lysosomal enzymes

For calpain-2 incubation, 0.5 g myofibrils were washed in buffer E (75 mM KCl, 50 mM Tris-HCl, 2 mM $MgCl_2$, 2 mM EGTA) adjusted to pH 8.0 and then washed in buffer F (75 mM KCl, 20 mM Tris-HCl, 2 mM $MgCl_2$, 2 mM EGTA) adjusted to pH 7.0. After addition of 500 µl incubation buffer (75 mM KCl, 20 mM Tris-HCl, 2 mM $MgCl_2$, pH 7.0), myofibrils were incubated with different amounts of $CaCl_2$ (0, 0.04, 0.4, 4 mM) and calpain-2 (0, 0.4, 1.2, 2.8 µg) at 25 °C for 2 h.

For lysosomal extract incubation, 0.5 g myofibrils were washed twice in buffer D. After addition of buffer D, myofibrils were incubated with different amount of lysosomal extract (0, 20, 100, 200, 300, 400 µg of protein) at 25 °C for 2 h; the total volume of each tube was 500 µl.

For the proteasome incubation, 0.5 g myofibrils were washed in buffer E adjusted to pH 8.3 and then washed in buffer F adjusted to pH 7.6. After that, myofibrils in 500 µl buffer F adjusted to pH 7.6, were incubated in different amount of proteasome (0, 1, 5, 10, 15 µg) at 25 °C for 2 h.

2.7 Water-holding capacity and protein content of supernatant

All enzyme incubations were repeated 5 times on a myofibril preparation. Each incubation used around 0.5 g of myofibrils in an Eppendorf tube, however, the exact weight of the used myofibrils (*Wm*) in each incubation was recorded. After incubation, each tube with myofibrils was mixed with 500 µl buffer G (75 mM KCl, 100 mM MES, 2 mM $MgCl_2$, 2 mM EGTA, pH 5.5) and centrifuged at 10,000 × *g* at 4 °C for 10 min, and the supernatant was collected for protein content determination. The myofibrils were washed two additional time in buffer G as above. Buffer G was added again and then the tubes were centrifuged at 2,400 × *g* for 8 min followed by decanting of

supernatant and weighing of the myofibrillar pellet including the tube (*W*1). The relative water-holding of myofibrils was calculated as below:

$$Relative\ water-holding\ (\%)\ = \frac{W1 - Wt - Wm}{Wm} \times 100$$

*W*1: Weight of tube + pellet after centrifugation and decanting of supernatant; *Wt*: Weight of tube; *Wm*: Weight of myofibrils.

The protein content of the supernatant after the first wash was determined by measuring the absorbance at 280 nm and taking a reading of 1 as 1.00 mg /ml.

2.8 SDS-PAGE

SDS-PAGE was run according to Liu, Puolanne, & Ertbjerg (2014) with slight modification. A total of 32 ml of sample was mixed with 12.5 ml NuPAGE® LDS Sample Buffer (4X) and 5 ml NuPAGE® Sample Reducing Agent (10X) (Invitrogen, Carlsbad, CA). Then the mixture was heat treated at 70 ºC for 10 minutes and applied onto NuPAGE (Invitrogen, Carlsbad, CA) Novex 12% Bis-Tris gels. Each sample was loaded 3 times on the gel. Gels were settled in XCell SureLock® Mini-Cell electrophoresis chamber and the electrophoresis was run at 200 V for approximately 50 min. Gels were after staining (400 ml/l ethanol, 100 ml/l acetic acid and one g/l Coomassie Brilliant Blue R-250) and distaining (100 ml/l ethanol and 75 ml/l acetic acid) placed on a light board. The gels were scanned and analyzed by AlphaImager® HP Versatile gel imaging system (ProteinSimple, Santa Clara, CA)

2. 9 Western blot

Western blot method was according to Bao & Ertbjerg (2015) with slight modification. The amount of protein loaded into each lane was 15 µg. After electrophoresis, proteins in gels were transferred to Immobilon-FL Transfer Membrane (Millipore, Bedford, MA) in XCell II® Blot Module with NuPAGE_ Transfer Buffer (20X) from Invitrogen. The blotting process was performed for 1 h at 30 V. After blotting, membranes were blocked for 1 h in 20 ml of TBS (50 mM Tris-HCl, 150 mM NaCl, and pH 7.5) with 50 g/l skim milk powder at room temperature. Then membranes were washed in TBS-T (50 mM Tris-HCl, 150 mM NaCl, 0.5 g/L Tween-20, pH 7.5) for 10 min. Membranes were then incubated with 2 µl mouse monoclonal anti-desmin antibody clone DE-R-11 (LifeSpan Biosciences, Santa Cruz, CA) in 10 ml TBS-T with 20 g/l skim milk powder for 1 h. Afterwards, membranes were washed three times in TBS-T for 5 to 10 min and incubated with secondary antibodies, which were 3.0 µl IRDye® 800 CW Donkey anti-mouse IgG (H+L) in 15 ml TBS-T with 20 g/l skim milk powder and 0.1 g/l SDS for 1 h. After incubation was completed, membranes were washed twice in TBS-T and once in TBS for 5 min. After washing, membranes were scanned by Odyssey Infrared Imaging System-CLx (LI-CoR Cop, Lincoln, NE) with the 800 nm channel. Relative intensity of each individual band was calculated by the LI-COR Odyssey program. The background was calculated using the median method. The fluorescence intensity of each band was calculated based on the integrated intensity divided by the mean value of the control (no enzyme addition).

2.10 Data analysis

Data were analyzed by the IBM SPSS Statistics 21 software using general linear model. Tukey HSD test was used to find significant differences between means at a level of $P < 0.05$.

3. Results and discussion

3.1 Incubation of myofibrils with calpain-2

Incubation of myofibrils with Ca^{2+} alone improved the relative water-holding of the myofibrils and a further increase was observed when calpain-2 was added in the presence of Ca^{2+} (Fig 1). In parallel, the protein content of the supernatant increased with Ca^{2+} and calpain-2, indicating that an increasing amount of peptides/proteins were released from the myofibril protein network. Desmin is a known substrate of calpain (Huff-Lonergan et al., 1996), and incubation of myofibrils with greater amounts of calpain-2 resulted in increased degradation of desmin (Fig. 2). Degradation products of desmin appeared with increasing degradation in a pattern similar to what have previously been reported for calpain-induced desmin degradation (Baron et al., 2004; Huff-Lonergan et al., 1996). One important observation from the representative Western blot against desmin is that calcium alone without calpain addition could induce some desmin degradation. Calpains are located exclusively intracellularly and seem to be partly associated with subcellular organelles such as myofibrils in skeletal muscle (Goll et al., 2003), and some calpain-1 has been reported to be bound to myofibrils (Delgado, Geesink, Marchello, Goll, & Koohmaraie, 2001). In present study, 0.04 mM Ca^{2+} was assumed to be enough to fully active calpain-1 but not calpain-2 considering the known calcium sensitivity of the calpains (Goll et al., 2003). Increasing the amount of Ca^{2+} to 0.4 mM would be enough to start to active calpain-2, and 4 mM Ca^{2+} would fully activate calpain-2. Therefore, adding more Ca^{2+} to the incubation buffer would be expected to increase the degradation of desmin by any endogenous calpain bound to myofibrils. The degradation of desmin in myofibrils incubated by different amounts of Ca^{2+} (Fig. 2) suggests that not only calpain-1 but also a significant amount of calpain-2 remained associated to the myofibrils during their isolation. Calpain bound to myofibrils activated by added Ca^{2+} thus likely account for the increased relative water-holding by Ca^{2+} alone (Fig. 1).

3.2 Incubation of myofibrils with lysosomal enzymes

Other enzymes than calpain-2 were investigated to obtain a more general view on the effect of proteolysis on water-holding. The relative water-holding was also improved when myofibrils were incubated with up to 100 µg protein of lysosomal extract (Fig. 3), and with no further increase with higher amounts. With increasing amount of lysosomal enzymes (up to around 200 µg protein) also the protein content of the supernatant augmented, indicating that an increasing amount of peptides/proteins were released from the myofibril protein network. Desmin is degraded e.g. by the lysosomal enzyme cathepsin B (Baron et al., 2004), and incubation of myofibrils with an increasing amount of lysosomal extract resulted in an increasing amount of desmin being degraded (Fig. 4).

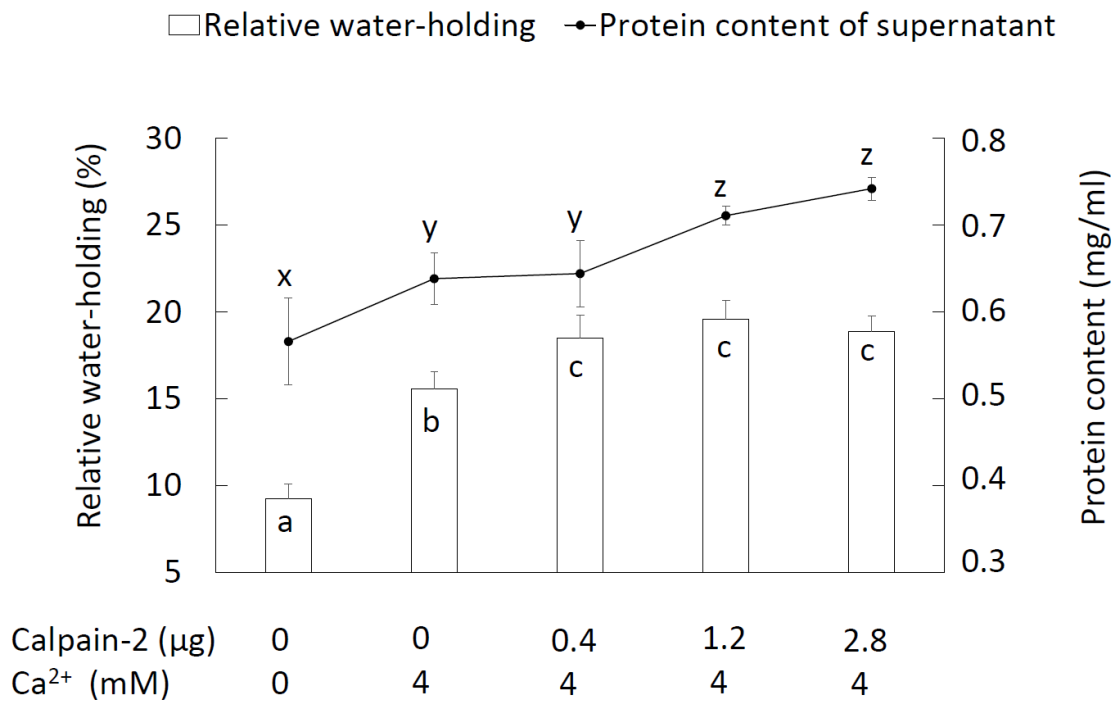

Fig. 1. The relative water-holding of myofibrils and protein content of supernatant. Myofibrils were incubated with $CaCl_2$ alone (0 and 4 mM) or calpain-2 (0.4, 1.2 and 2.8 µg) together with 4 mM $CaCl_2$ at 25 °C for 2 h. Means with standard deviation (n = 5) are shown. $^{a–c, x-z}$ Within traits, means with different letters differ ($P < 0.05$).

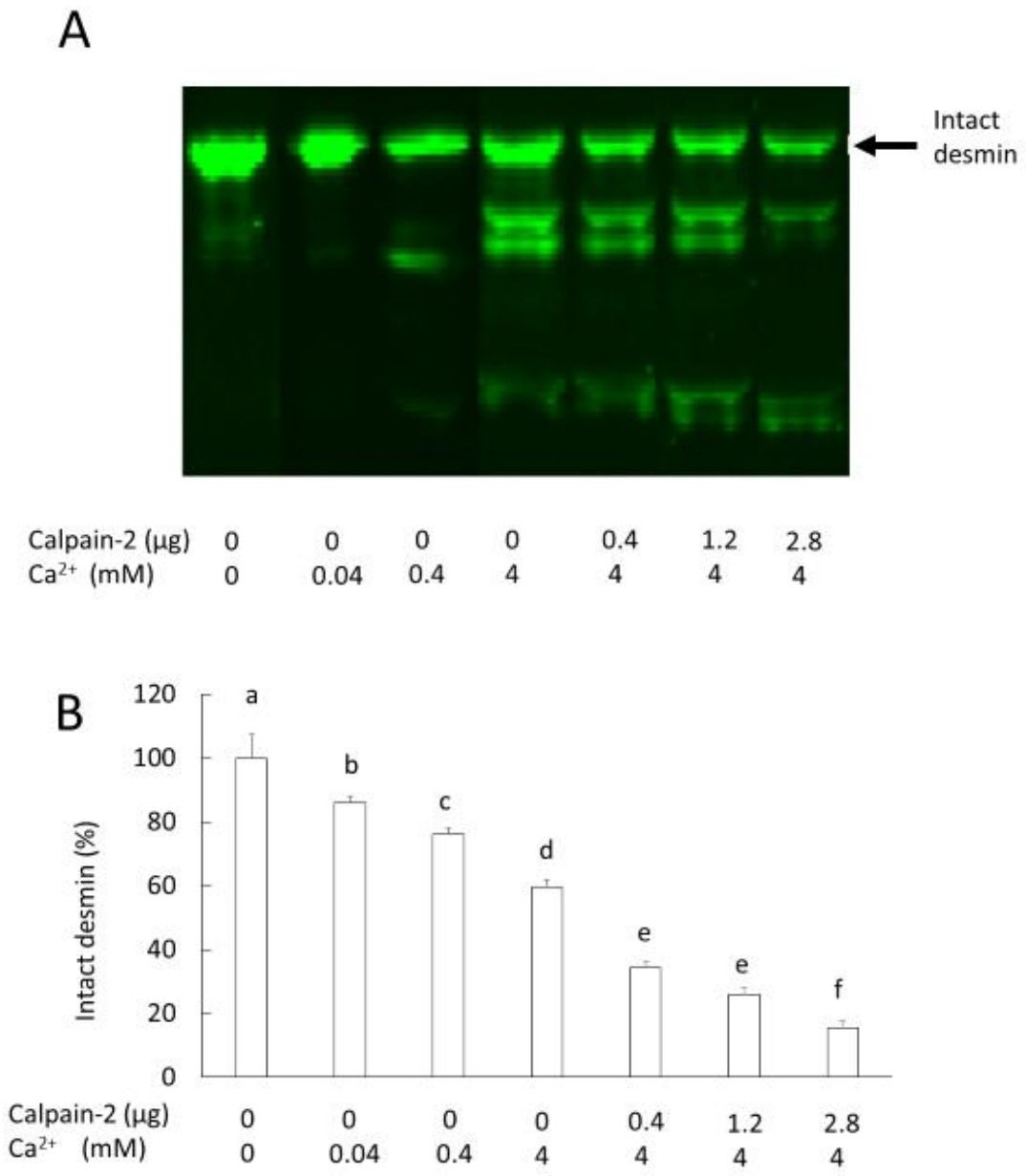

Fig. 2. Effect of incubating myofibrils with $CaCl_2$ and calpain-2. Myofibrils were incubated with $CaCl_2$ alone (0, 0.04, 0.4 and 4 mM) or calpain-2 (0.4, 1.2 and 2.8 μg) together with 4 mM $CaCl_2$ at 25 °C for 2 h. (A) Representative Western blot of desmin. (B) Band intensity of intact desmin relative to control (no Ca^{2+} and calpain-2). Means with standard deviation (n = 3) are shown. $^{a–f}$ Means with different letters differ ($P < 0.05$).

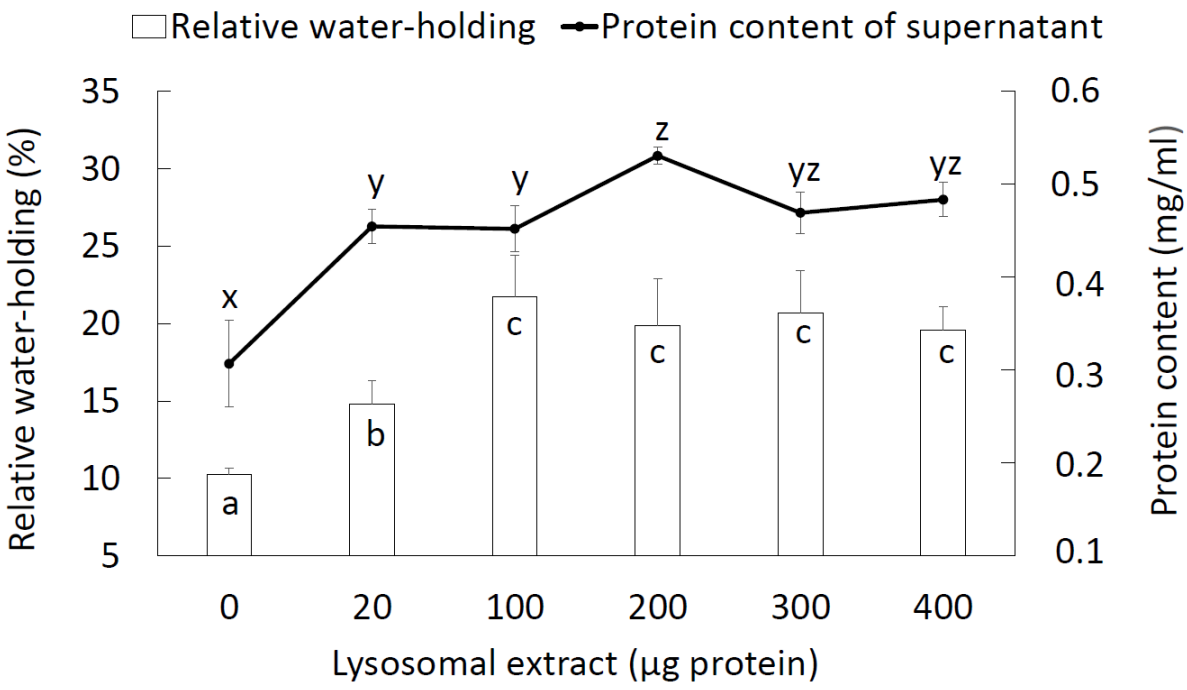

Fig. 3. The relative water-holding and protein content of supernatant. Myofibrils were incubated with lysosomal extract (0, 20, 100, 200, 300 and 400 µg protein) at 25 °C for 2 h. Means with standard deviation (n = 5) are shown. $^{a–c}$ Means with different letters differ ($P < 0.05$). $^{x–z}$ Means with the same letter do not differ ($P > 0.05$).

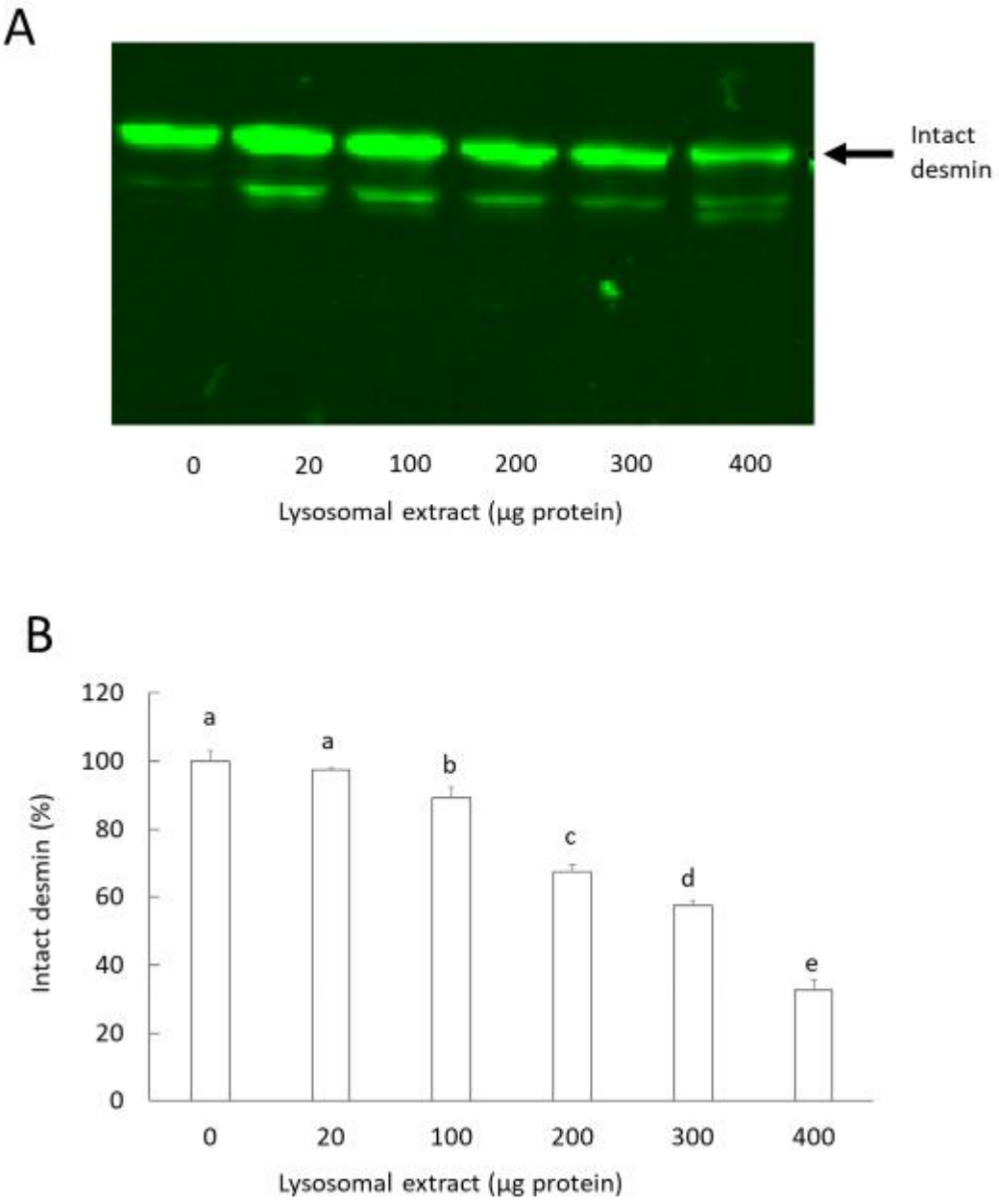

Fig. 4. Effect of incubating myofibrils with lysosomal extract. (A) Representative Western blot of desmin. (B) Band intensity of intact desmin relative to control (no lysosomal extract). Means with standard deviation (n = 3) are shown. $^{a–e}$ Means with different letters differ ($P < 0.05$).

3.3 Incubation of myofibrils with the proteasome

The progress of the purification and of the proteasome preparation is shown in Fig. 5. As the proteasome is composed of multiple subunits, the purity was illustrated by native gel electrophoresis. The preparation used for the myofibril incubations was mainly containing the 20S

proteasome migrating at a molecular weight of around 700 kDa as also reported for the 20S proteasome from bovine (Dutaud, Aubry, Sentrandreu, & Ouali, 2006). Similar to the effect of calpain-2 and the lysosomal extract, the relative water-holding of myofibrils was improved after incubation with proteasome (Fig. 6). Incubating myofibrils with an increasing amount of proteasome up to 10 µg caused greater relative water-holding. Also the protein content of the supernatant was increased up to 1 µg but without further increase at greater amounts, revealing a general release of peptides/proteins into the supernatant after incubating myofibrils with a rather low amount of proteasome. SDS-PAGE of the supernatant showed that α-actinin was released from myofibrils following incubation with the proteasome (Fig. 7). The release of α-actinin reflected the increase in water-holding and reached a maximum with 10 – 15 µg of proteasome.

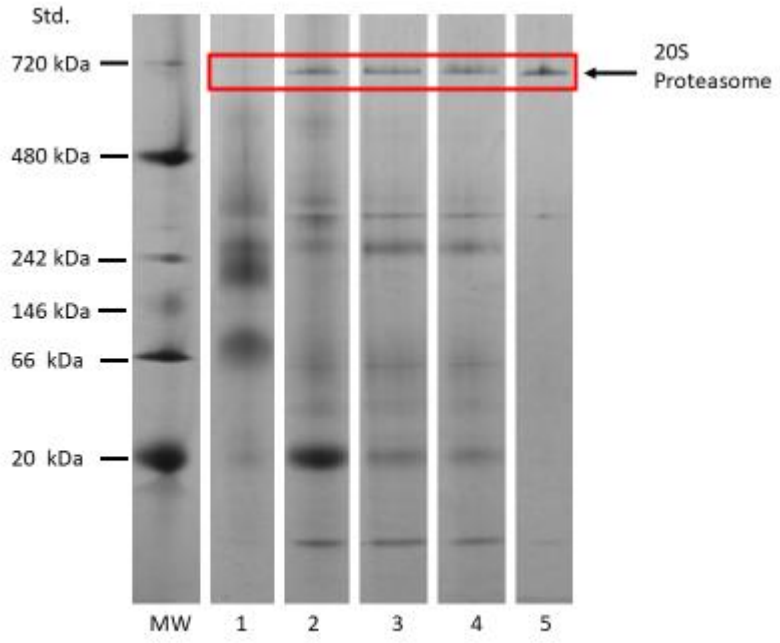

Fig. 5. Native PAGE illustrating the purification of the proteasome. Lane 1: Homogenate, 2: DEAE Sepharose Fast Flow, 3: Ammonium sulfate precipitate, 4: Sephacryl S-300HR, 5: RESOURCE Q.

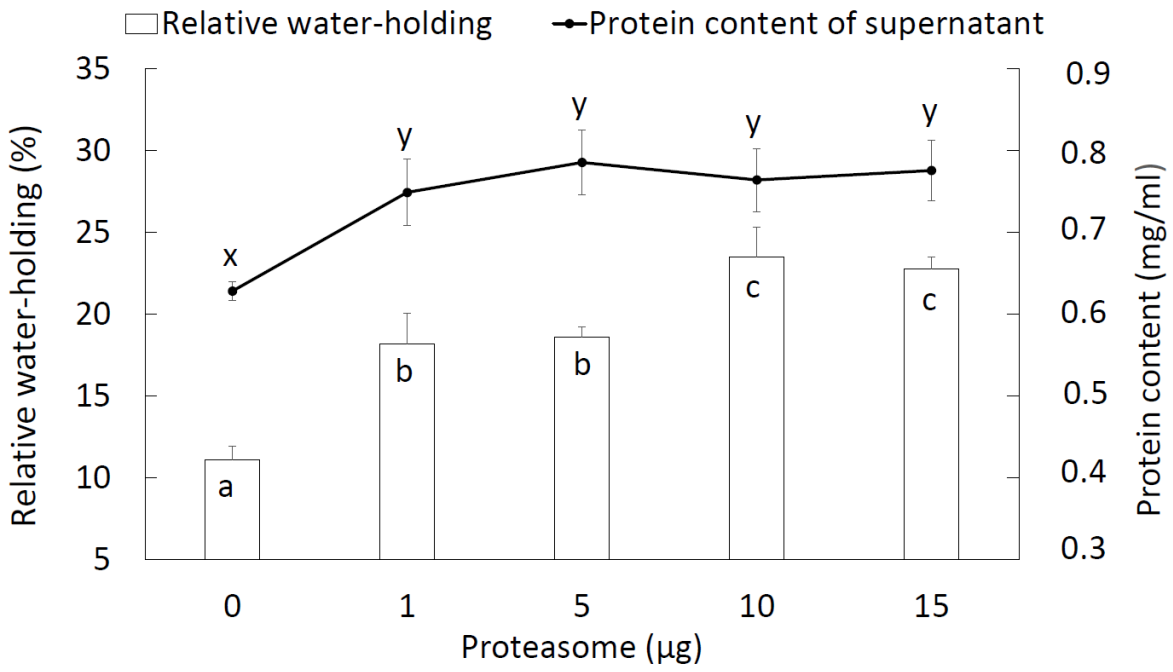

Fig. 6. The relative water-holding and protein content of supernatant. Myofibrils were incubated with proteasome (0, 1, 5, 10 and 15 µg) at 25 °C for 2 h. Means with standard deviation (n =5) are shown. [a–c, x-y] Within traits, means with different letters differ ($P < 0.05$).

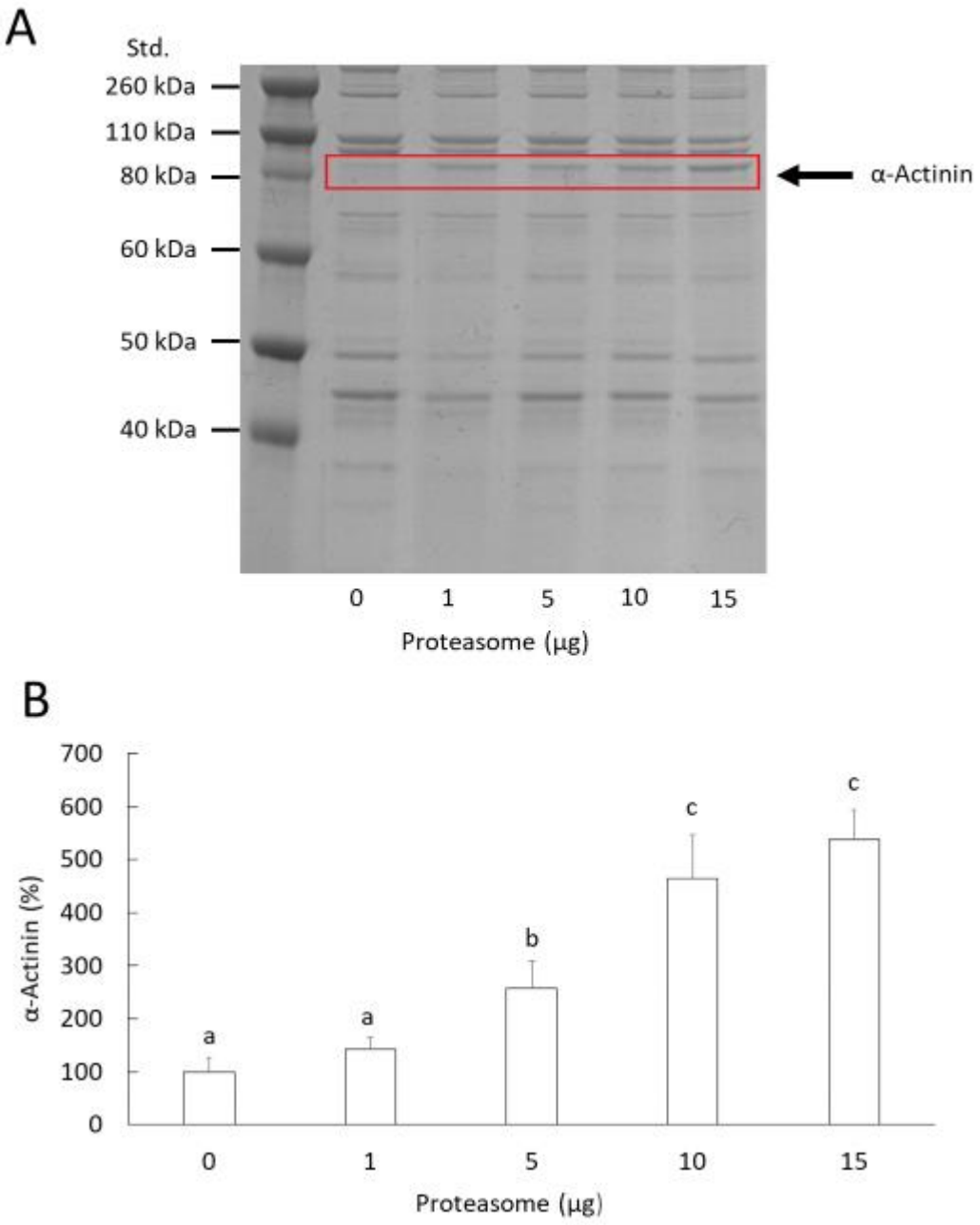

Fig. 7. Effect of incubating myofibrils with proteasome. (A). SDS-PAGE of supernatant after incubation. (B) Band intensity of α-actinin in the supernatant after incubation relative to control (no proteasome). Std.: molecular weight of standard proteins. Means with standard deviation (n = 3) are shown. $^{a–c}$ Means with different letters differ ($P < 0.05$).

3.4. Hypothesis for the effect of proteolysis on water-holding

Structural proteins within the myofibrils form a flexible framework inside the muscle fiber. Some intermediate filament proteins such as desmin and Z-disk proteins like α-actinin play a role in organizing the structure of the myofibrils. The intermediate filaments comprised by desmin run between myofibrils thereby linking myofibrils together and the filaments also extends and encircle myofibrils constituting a three-dimensional scaffold around the myofibrillar Z-disk, and in addition connect the entire contractile apparatus to the subsarcolemmal cytoskeleton, the nuclei, and other organelles (Capetanaki, Milner, & Weitzer, 1997; Paulin & Li, 2004). α-Actinin is a major component of the Z-disk and associates with actin filaments and structural proteins to stabilize the cytoskeleton (Berman & North, 2010). In the present study, desmin was degraded (calpain-2 and lysosomal extract) and α-actinin was released (the proteasome) by incubation of myofibrils with proteolytic enzymes. The change in these two proteins likely reflected that the Z-disk and intermediate filaments had been damaged, i.e. structural elements highly important for the integrity of the myofibrils. Weakening of the Z-disk structure and degradation of desmin filaments has been observed after incubation of myofibrils with calpains (Taylor, Geesink, Thompson, Koohmaraie, & Goll, 1995). Similarly, electron micrographs showed damage of the Z-disk after myofibrils were incubated with either the proteasome (Robert, Briand, Taylor, & Briand, 1999) or cathepsin L (Mikami et al., 1987). The density of the Z-line was decreased by treatment of myofibrils with either calpain, a lysosomal extact or the proteasome (Sentandreu et al., 2002). Some studies have suggested a sequential degradation of structural proteins postmortem. Calpain is believed to initiate the disruption by attacking the Z–disk and by degrading desmin and initiating the release α-actinin, then further proteolysis has been proposed to be carried by the proteasome and lysosomal enzymes like the cathepsins (Goll, Thompson, Taylor, & Christiansen, 1992; Houbak, Ertbjerg, & Therkildsen, 2008, Sentandreu et al., 2002). Apart from desmin and α-actinin, many other myofibrillar proteins are substrates of calpains, the proteasome and lysosomal enzymes, such as titin, nebulin, fliamin, myosin heavy chain, myosin light chain I, troponin T, tropomyosin (Lametsch, Roepstorff, Møller, & Bendixen, 2004; Robert et al., 1999; Ladrat, Verrez-Bagnis, Noël, & Fleurence, 2003; Mikami et al., 1987). Several of these proteins are common substrates for the proteases used in the present study. When incubating myofibrils with these enzymes separately, myofibrils were generally partly degraded as the protein content of the supernatant increased as shown in Fig 1, 3 and 5. The affected proteins likely contribute to some important structures in myofiribrils, such as costameres, I band organization, N_2-line and Z –disk (Bloch et al., 2002; Labeit & Kolmerer, 1995; O'Neill et al., 2002; Wang & Williamson, 1980). Once proteolysis progressed and proteins were degraded or released, the original structure may have become unstable resulting in an ultrastructural change. For example, after addition of proteasome Dutaud et al. (2006a) observed that the I-band became overlapped by an amorphous structure and the M-lines disappeared and after addition of lysosomal extract the myofibrils were degraded near N_2 lines and at the A/I junction area (Sentandreu et al., 2002).

The degradation of myofibrillar proteins following the enzyme incubations may thus have caused a structural change that subsequently allowed increased swelling of the filament lattice and the cytoskeletal structures especially around the Z-disk. Such a breakage of the structure will allow water to flow from the space outside myofibrils to inside. Therefore, the relative water-holding increased when myofibrils were incubated with the three different proteases (Fig. 1, 3 and 6). This

hypothesis is outlined in Fig. 8. The degradation of desmin has previously been related to formation of drip loss during postmortem storage (Zhang et al., 2006). In our study it can be noted that the water-holding did not continue to increase at higher levels of calpain-2 and lysosomal extract in spite of increasing desmin degradation (Fig. 1 - 4). This may suggest that only a part of desmin is involved in restraining swelling of myofibrils. We speculate that degradation of desmin in and around the Z-disc is more related to water-holding than degradation of desmin in the intermediate filaments connecting the myofibrils. Straadt et al., (2007) combined confocal laser scanning microscopy and low-field nuclear magnetic resonance measurements and observed that the number of swollen myofibrils increased during fresh meat storage up to two weeks and in parallel also water-holding capacity increased. Furthermore, there were characteristic changes in the intramyofibrillar water, as the distribution of this water population became more homogeneous. This development was suggested to be ascribed to increased proteolytic degradation of the myofibrillar structure (Pearce, Rosenvold, Andersen, & Hopkins, 2011).

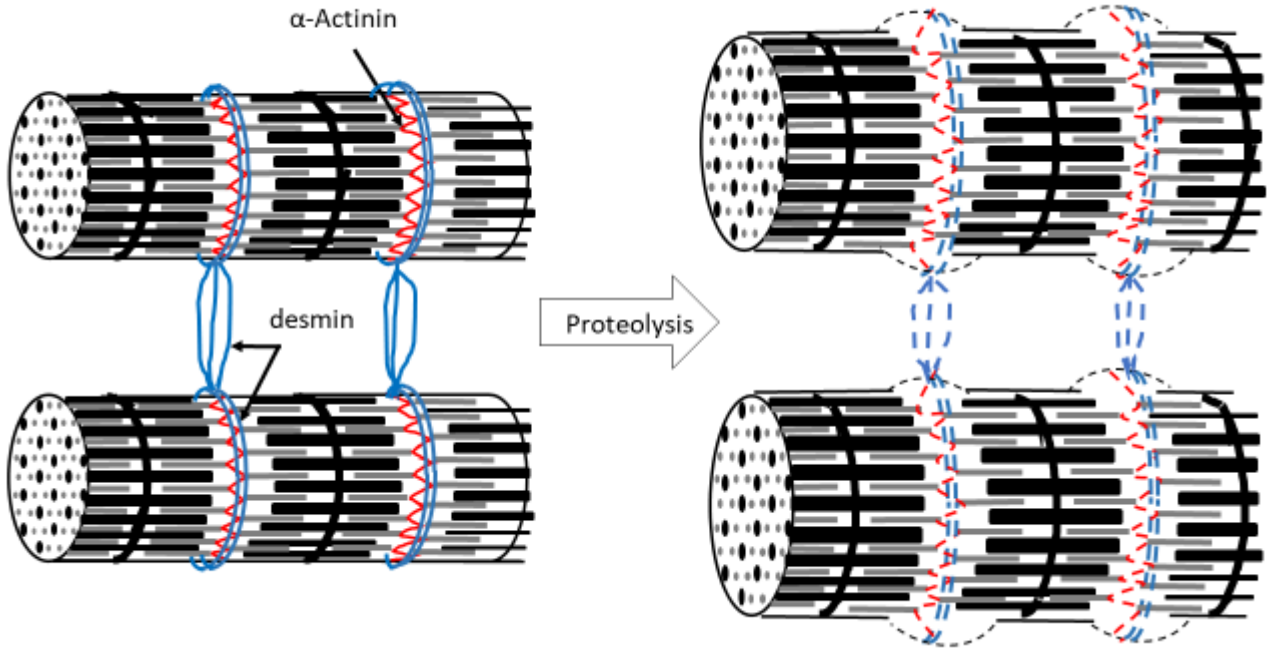

Fig. 8. Hypothesis showing structural changes of myofibrils as influenced by proteolytic degradation of cytoskeletal proteins.

We have here provided direct evidence of improved water-holding following proteolytic degradation of myofibrillar proteins. Previous studies have also attempted to link proteolytic degradation of the meat structure with increased water-holding during prolonged storage. Kristensen & Purslow (2001) observed improved water-holding capacity during ageing of pork and hypothesized that degradation of cytoskeletal proteins may remove the structural linkages between myofibrils and the sarcolemma thereby slowly equalizing the pressure forcing water from the intracellular to the extracellular space. During later postmortem storage, the reduced pressure was

suggested to be the driving force causing inflow of extracellular water to muscle fibers with a following increase in water-holding capacity of the meat. Later, Farouk et al (2012) hypothesized that the improvement in meat water-holding capacity with long term ageing could be due to formation of a “sponge effect” in the drip channels which develop postmortem between muscle fibers and muscle fiber bundles. This “sponge effect” traps the water and prevents it from getting lost. Our present hypothesis (Fig. 8) does not contradict these previous ideas to explain the improved water-holding during prolonged storage. However, they focused on the proteolytic induced structural changes occurring outside the myofibrils, such as the structural linkages between myofibrils and the sarcolemma or the drip channels between the muscle fibers, whereas the present study put more emphasis on the structural change inside myofibrils involving specific degradation of proteins in and around the Z-disk which allows overall swelling of the filament lattice and more specifically in the I-band area. The understanding of how proteolysis is affecting the water-holding of meat is thus expanded and we here provide a new view on the changes occurring in meat and especially in the later times of postmortem storage.

5. Conclusion

Incubation myofibrils with three different endogenous proteolytic enzyme systems, calpain-2, a lysosmal extract and the proteasome, all resulted in increased water-holding of the myofibrils. During the enzyme incubations the intermediate filament desmin was degraded by calpain-2 as well as by a lysosomal extract and the Z-disk protein α-actinin was released by the proteasome. A model is presented in which the degradation of myofibrillar proteins causes a structural change that subsequently allows increased swelling of myofibrils as water flows from the space outside to inside. Hence, the improved water-holding of myofibrils may explain that the rate of drip loss from meat decreases at later times of postmortem storage.

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