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Relationship between proteolysis and water-holding of myofibrils

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A B S T R A C T

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 °C 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.

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 and 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 were 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 have 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 differs, 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, Seideman, Schollmeyer, Dutson, & Crouse, 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 & Ertbjerg, 2012; Pomponio et al., 2008). 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

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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; Li et al., 2012; 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 et al., 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, de 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 < 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 microliters 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, Henckel, 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 8000 rpm for 5 s.

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