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In vitro study to evaluate the degradation of bovine muscle proteins *post-mortem* by proteasome and μ-calpain

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Abstract

The degradation of bovine muscle proteins by proteasome and ubiquitous calpains was explored via 2D gel proteome analysis by inhibition of the physiological level of the proteases by specific inhibitors.

The inhibition of the proteasome chymotrypsin- and trypsin-like activity results in the lack of degradation of several fragments of structural proteins such as actin, troponin T, myosin light chain and nebulin. In addition the degradation of several sarcoplasmatic proteins was eliminated when proteasome was inhibited. The inhibition of the ubiquitous calpain only resulted in minor changes in the degradation pattern, which might indicate that p94, which is not inhibited by calpastatin, is involved in the degradation *post-mortem*. The results of the present study indicate a sequential degradation of the structural proteins *post-mortem*, where calpain initiates the disruption and destabilisation of the myofibrillar structure, and thereby allows the proteasome to act.

Keywords: μ-Calpain; Proteasome; Proteome analysis; Myofibrillar proteins; Bovine

1. Introduction

Tenderness is an important quality trait in meat, especially in beef, and it depends on several factors, such as the amount of connective tissue and the myofibrillar protein degradation. The contribution from connective tissue to the toughness of the meat does not decrease considerably during storage, while the toughness related to myofibrillar proteins decreases during storage resulting in tenderisation of the meat (Harper, 1999). The proteases responsible for protein degradation in muscle tissue during the tenderisation process have been debated for decades (Koohmaraie, 1996; Sentandreu, Coulis, & Ouali, 2002). The three main proteolytic systems mentioned in connection with tenderisation of meat are the lysosomal proteolysis by cathepsins, Ca^{2+}-dependent proteolysis by μ-calpain and p94 and finally proteolysis by proteasome.

Several studies have proposed that the *post-mortem* degradation is a result of the sequential action of the proteolytic enzymes, suggesting that the early *post-mortem* changes are due to calpain that initiates disruption and destabilisation of the myofibrillar structures, allowing the proteasome and cathepsines to act on the partially degraded proteins (Calkins & Seideman, 1988; Price & Stevens, 1999; Robert, Briand, Taylor, & Briand, 1999; Taylor, Goll, & Ouali, 1995b).

Proteasome (26S) is a high molecular weight, multicatalytic protease complex involved in a variety of basic cellular processes, e.g., in control of cell cycle, transcription, signalling and metabolism, antigen processing and apoptosis (Coux, Tanaka, & Goldberg, 1996). The proteasome (26S) consists of a 19S regulatory cap structure attached to either end of a barrel-shaped 20S catalytic structure. The 20S complex has six different subtypes in the muscle tissue (Dahlmann, Ruppert, Kloetzel, & Kuehn, 2001). Within these subunits there are five different proteolytic activities including trypsin-like and chymotrypsin-like activities. Only

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proteins ligated to multiple units of ubiquitin are degraded by the 26S proteasome, and the tagging of proteins to ubiquitin requires ATP (Taillandier et al., 2004).

It is not clear how important proteasome is for the muscle proteolysis *post-mortem*, but a study by Sekikawa et al. (2001) examined the effect of proteasome during storage (48 h) of beef, and found that ubiquitin and ubiquitin-protein conjugates were present in the skeletal muscle immediately after slaughter and were degraded during storage partly due to the action of proteasome. When ATP is depleted, it results in a reversible dissociation of 26S proteasome into 20S proteasome and 19S domains (Peters, Franke, & Kleinschmidt, 1994). The 20S proteasome does not require ATP and ubiquitin, and its proteolytic actions can therefore contribute to meat conditioning after rigor mortis has occurred. Studies on beef have shown that the activity of proteasome is high 7 days *post-mortem* (Lamare, Taylor, Farout, Briand, & Briand, 2002), whereas Jia, Hollung, Therkildsen, Hildrum, and Bendixen (2006) found that degradation of several proteasome subunits had already occurred one day *post-mortem*.

Several studies have examined the effect of purified 20S proteasome on degradation of myofibrils and myofibrillar proteins (Eble, Spragia, & Ferguson, 1999; Robert et al., 1999; Taylor et al., 1995c). They found that despite its large size (20S), proteasome is able to degrade structural myofibrillar proteins. The degradation showed some specificity, because actin, myosin and desmin were degraded faster than alpha-actinin, troponin T and tropomyosin. Changes in the ultra structure of the myofibril were slow but included a general loss of structure where Z and I bands were affected before the M band and the costameres.

The calpains consist of enzymes expressed ubiquitously and tissue-specific calpains. The expression of calpains in the muscle tissue consists primarily of the ubiquitous calpains (μ- and m-calpain) and the muscle-specific calpain (p94). μ- and m-calpains are heterodimers composed of two subunits. The enzymes consist of an 80 kDa catalytic subunit that is responsible for the peptidase activity and is unique to each enzyme, while the 30 kDa regulatory subunit is similar in μ-calpain and m-calpain. p94 consists only of a catalytic subunit that is greater in size than the catalytic subunit of the ubiquitious calpains (Ono, Sorimachi, & Suzuki, 1998; Sentandreu et al., 2002).

The catalytic subunit consists of four domains, domain I (autolytic activation), II (cysteine catalytic site), III (switch domain) and IV (calmodulin-like domain). All members of the calpain superfamily contain homologous catalytic domain II, but some of them lack one or more of the other domains. The activity of the ubiquitous calpains is dependent on the concentration of calcium ions and phospholipids and the amount of their specific inhibitor calpastatin (Sentandreu et al., 2002), whereas p94, lacking the regulatory subunit, is resistent to the inhibition of calpastatin (Ono et al., 2004).

The effect of the calpain system on tenderness development has been explored in several studies (Dransfield, 1992; Ilian et al., 2001; McDonagh, Fernandez, & Oddy, 1999; Therkildsen, Larsen, Bang, & Vestergaard, 2002), and they have found correlations between calpain activity and tenderness development in meat, especially in the initial part of the storage period. Some studies indicate that *post-mortem* tenderisation is primarily a result of μ-calpain-mediated degradation of myofibrillar proteins (Koohmaraie, 1992; Koohmaraie, 1996). This is consistent with the strong correlations between the myofibrillar fragmentation index (MFI), Warner Bratzler shear force and μ-calpain activity in lamb (McDonagh et al., 1999) and beef (Dransfield, 1992; Dransfield, Etherington, & Taylor, 1992), and an *in vitro* study on pork by Lametsch, Roepstorff, Moller, and Bendixen (2004), who found that the proteins degraded by μ-calpain *in vitro* are identical to proteins degraded during *post-mortem* tenderisation.

Altogether these results indicate that μ-calpain is active during *post-mortem* proteolysis, and that the activity has a significant influence on meat tenderness.

The purpose of this study was to examine the effect of inhibition of the proteasome and μ-calpain activity on the degradation pattern of bovine muscle tissue via 2DE proteome analysis.

Previous studies have investigated substrates for the calpain (Lametsch et al., 2004) and proteasomes (Robert et al., 1999; Taylor et al., 1995c) by adding extra enzymes to the meat. However, these results can be questioned as the enzymes have been used in non-physiological concentrations. In the present study we address the question of whether and how enzymes are involved in *post-mortem* proteolysis from another angle, by investigating inhibition of the enzyme already present in the meat.

2. Material and methods

The experiment was done on muscle tissue (*Longissismus dorsi*) from several young bull calves fed a high energy diet from weaning until slaughter at the age of 16 months.

The meat samples were collected within 15 min *post-mortem*, frozen in liquid nitrogen immediately and stored at −80 °C.

Pools of muscle tissue were generated by mixing samples from six young bulls, followed by mincing in a blender and portioned out in vials containing 250 mg. The vials were incubated with inhibitor/enzyme dissolved in 100 μl 10 mM $CaCl_2$ at 24 °C for 5 h on a minishaker. Different treatments were used.

1. Control was incubated with 10 mM $CaCl_2$.
2. Calpastatin (#c9857, Merck Biosciences) in either 9 or 75 μM dissolved in 10 mM $CaCl_2$.
3. μ-Calpain (#2078129, Calbiochem®), 1 unit dissolved in 10 mM $CaCl_2$.
4. Lactacystin (#426100, Calbiochem®) in either 10 μM or 100 μM dissolved in 10 mM $CaCl_2$. Lactacystin is a specific proteasome inhibitor, inhibiting the chymotrypsin and trypsin-like activity of proteasome.