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Isolation and identification of proteolytic fragments from TCA soluble extracts of bovine *M. longissimus dorsi*

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Abstract

Hereford cross heifers ($n=3$) were slaughtered and hung conventionally. At 1 h post-mortem the *M. longissimus dorsi* (LD) was excised, vacuum packaged in plastic bags and stored at 4°C. Samples were taken from each of eight different locations along the length of the LD muscle at 1 h, 1, 3 and 15 days post-mortem for extraction using 5% TCA. Supernatants were stored at −20°C until analysis by HPLC. Peptides produced during the storage of beef were isolated by high performance liquid chromatography (HPLC). Results show that these components increase in quantity from 1 h to 15 days post-mortem. Five fractions were collected which correspond to various peaks of interest. These fractions were subsequently analysed by mass spectrometry and amino acid sequencing to reveal their identity. Fractions 1 and 2 were found to be mixtures of low molecular components. Fractions 3, 4 and 5 were found to be proteolytic fragmentation products of glyceraldehyde-3-phosphate dehydrogenase, troponin T and creatine kinase, respectively. Enhanced appearance of proteolytic fragments from these parent proteins with muscle ageing suggest that they may be useful indicators of meat quality. Further work incorporating sensory and/or texture evaluation of muscle tissue is required to investigate this issue

1. Introduction

A large variation exists in the rate and extent of tenderisation which is responsible for variation in meat tenderness at the consumer level (Koohmaraie, 1996). As consumers consider tenderness to be the single most important component of beef quality (Koohmaraie, 1992), solving the problem of inconsistent meat tenderness is of high priority to the meat industry. In order to approach this problem it is important to have a greater understanding of the processes that affect meat tenderness during post-mortem ageing. Although improvement in meat tenderness with post-mortem ageing is measurable both subjectively and objectively, the mechanism(s) of tenderisation has not been conclusively established. However, many researchers agree that biochemical processes during the post-mortem ageing period have a huge influence on the ultimate tenderness of meat (Dutson, 1983; Goll, Otsuka, Nagainis, Shannon, Sathe, & Muguruma, 1983; Koohmaraie, 1996). It has been suggested by many authors that proteolysis of key myofibrillar and associated proteins plays an important role in meat tenderisation. According to Koohmaraie (1996), current data indicates that calpains (and more specifically μ-calpain) are the only proteases that are directly involved in the events leading to meat tenderisation. In spite of this evidence in support of the calpain proteolytic system as the underlying mechanism of post-mortem proteolysis, there have been some conflicting reports. Other proteolytic enzymes such as amino- and peptidyl-peptidases are active in post-mortem muscle and may contain information regarding meat quality. Free amino acids have been shown to be released from peptides by the action of neutral aminopeptidases during meat ageing (Nishimura, Rhyu, Tajima & Kato, 1996; Okitani, Otsula, Sugitani, & Fujimaki, 1974) and even in processed meats like dry-cured ham (Toldra et al., 1992). Such accumulation of free amino acids is potentially undesirable for meat flavour, water holding and drip but may be partially responsible for natural flavour development (Spanier, McMillin & Miller, 1990) and may be associated with tenderness (Feidt, Brun-Bellut & Dransfield, 1998). Muscle cathepsins B, H, D and L, proteinases located in the

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lysosomes (Goll et al., 1983) are another group of enzymes which have been shown to participate in post-mortem proteolysis which results in textural changes in meat (Zeece & Katoh, 1989). Such proteinases have also been shown to be active during the processing of dry-cured ham (Toldra, Rico & Flores, 1993) and fermented sausage (Molly, Deymeyer, Johansson, Raemaekers, Ghistelinck & Geenen, 1997).

The action of proteolytic enzymes such as calpains and cathepsins, on the myofibrillar fraction results in the production of protein fragments (Feidt et al., 1998). It is extensively recognised that several new bands appear in the 25–34 kDa region on the SDS-PAGE separation patterns of myofibrils during the post-mortem storage of muscles (Ouali, 1990). In these bands, the appearance of the 30 kDa band, which has been shown to originate from troponin T (Negishi, Yamamoto & Kuwata, 1996), is very predominant and has been reported by many researchers. It has also been demonstrated that the 30 kDa component is related to post-mortem muscle tenderisation (Olson & Parrish, 1977) and can be related to shear force measurements (Buts, Claeys & Demeyer 1986). A second fragment, the 110 kDa fragment has also been shown to appear during ageing of beef on SDS-PAGE of myofibrils (O'Halloran, 1996). Later work by Troy et al. (1997) has shown a band at 110 kDa to be 80% homologous with human C-protein for the first 17 amino acids. Such fragmentation products whose appearance are enhanced with ageing and whose parent compounds are structurally important, are potentially useful in the prediction of meat quality. However, isolation and identification of fragmentation products of myofibrillar proteins is a tedious and time consuming process. A more rapid method of analysing proteolytic degradation of structurally important myofibrillar fragments could potentially be of more interest to the industry. Degradation of myofibrillar proteins can also lead to soluble proteolytic fragments which may provide a clearer understanding of the tenderisation process. Therefore, the objective of this research is to isolate and characterise soluble proteolytic fragments produced during post-mortem ageing in bovine *M. longissimus dorsi* (LD).

2. Materials and methodology

2.1. Preparation of samples

Hereford cross heifers ($n = 3$) of similar age, size and grade, were selected. Slaughtering was effected by stunning using a captive-bolt pistol and exsanguination, after which each carcass was conventionally dressed and split into two sides. The LD muscles were excised from the carcass at 1 hour *post-mortem*, vacuum packaged in plastic bags and stored at 4°C for 15 days. At 1 h, 1, 3 and 15 days post-mortem. Samples of approximately 40 g weight were excised from each of eight locations (A–H) along the LD, location A and H being the cranial and caudal ends of the LD muscle, respectively and locations B–G taken at regular intervals between the two Extractions were carried out on these samples using a procedure adapted from Nakai, Nishimura, Shimizu and Arai (1995). This involved homogenisation of muscle tissue in a two-fold volume of deionised water, after which tri-chloroacetic acid (TCA) was added to a final concentration of 5%. Supernatants were collected after centrifugation at 3600 rpm for 30 min, filtered through cheesecloth, divided into aliquots and stored at −20°C until analysis.

2.2. Isolation of fragmentation products by HPLC

Free amino acids and peptides, produced during the storage of beef, were analysed by reverse phase high performance liquid chromatography (RPHPLC). The HPLC system (Eppendorf, Hamburg, Germany) consisted of two Model BT 810 pumps with a controller for gradient programming and a Rheodyne injection valve BT 8121 with a 500 µl loop. The separations were carried out on LiChrospher 100 RP-18 (5 µm; 250 × 4 mm) and Nucleosil 100 RP-18 (7 µm; 250 × 10 mm) columns using a TFA/CH_3CN gradient system: reservoir A contained 0.05% TFA in water, reservoir B 80% CH3CN and 0.05% TFA in water. Linear gradients from 0 to 70% B and 10 to 100% B in 60 min were used, respectively, at a flow rate of 1.0 ml/min. The column eluate was monitored at 214 nm using a UV detection system (Eppendorf, Hamburg, Germany). After collection, the peptide fractions corresponding to the relevant peaks were lyophilised.

2.3. Analysis of fragmentation products by mass spectrometry and amino acid sequencing

The purity was controlled and the molecular mass of the compounds analysed by laser desorption mass spectrometry (MALDI MS; Kratos, MALDI I equipment, Shimadzu, Europe) and the structure elucidated by automatic Edman degradation using a pulsed liquid amino acid sequencer (ABI 473A). The search for protein homologies was carried out by comparisons of the sequences obtained with protein and DNA-derived protein sequence databases, carried out with the aid of the European Molecular Biology Laboratory (EMBL) via an Internet connection.

3. Results and discussion

Figs. 1 and 2 represent typical profiles obtained following HPLC analysis of the supernatant from beef

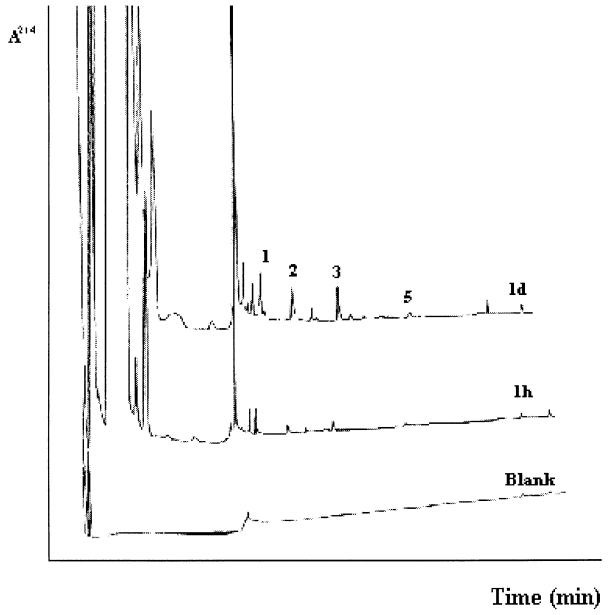

Fig. 1. HPLC profiles of extracts of beef *M. longissimus dorsi* after storage for 1 h and 1 day.

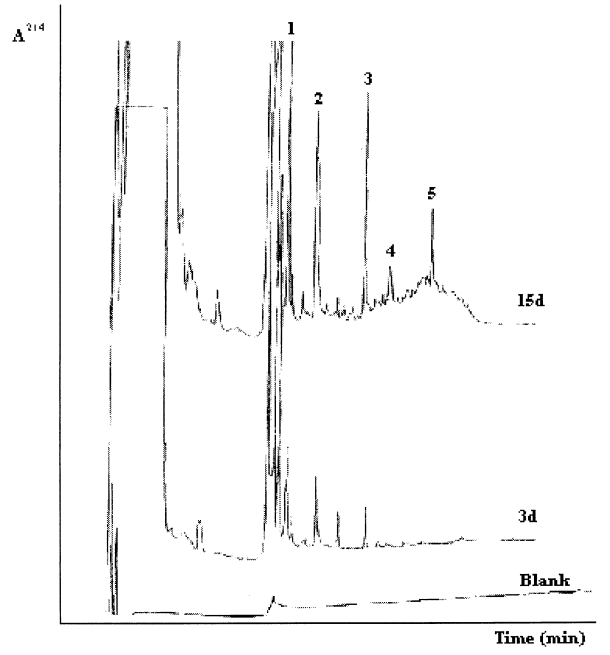

Fig. 2. HPLC profiles of extracts of beef *M. longissimus dorsi* after storage for 3 and 15 days.

muscle samples for location A (cranial end of the LD muscle) during storage for 1 h, 1, 3 and 15 days post-mortem. Similar patterns were observed for locations B–H, with no significant differences between locations. Results show an increase in the appearance of five peaks with ageing from 1 h to 15 days post-mortem for all three samples. These peaks were labelled 1 to 5 (Figs. 1 and 2). HPLC fractions corresponding to peaks 1 to 5 were collected and lyophilised. These components were assessed by mass spectrometry and amino acid sequencing to reveal their identity. LDMS analysis of fractions 1 and 2 did not yield any signals above 500 Da, indicating that the fractions contain low molecular components (free amino acids, di-, tri-, tetra- and/or pentapeptides). Amino acid analysis in the presence of internal standards showed that these two fractions contain free amino acids, tyrosine and phenylalanine, respectively [retention times (RT) 27 and 32 min]. The LDMS spectra of fractions 3–5 are shown in Figs 3–5, respectively, demonstrating that the isolated peptides 4 and 5 are homogeneous and peptide 3 is heterogeneous. The observed molecular masses were 1282.8 (main component), 1807.5 and 3607.3 (fraction 3), 1734.8 (fraction 4) and 5712.9 Da (fraction 5). These fractions seem to be both sarcoplasmic and myofibrillar in origin (Table 1). The primary structure of the higher molecular weight HPLC fraction, 5712.9 Da (fraction 5, RT 54 min) was elucidated by Edman degradation and demonstrated 100% homology with 39 overlapping amino acid residues of creatine kinase of rat muscle (positions 4–42), rat skeletal muscle, rabbit M chain (positions 90–128) and 97% identical with 39 overlapping amino acid residues of creatine kinase of human M chain, chicken muscle, *T. californica*, *T. marmorata*, trout, *G. gallus*, dog brain, rabbit brain and mouse (positions 90–128). Creatine kinase is a sarcoplasmic protein as is glyceraldehyde-3-phosphate dehydrogenase (G-3-PD). The 1282.8 Da fragment eluting at 39 min (fraction 3) revealed an 87.5% homology with this latter protein in eight overlapping amino acid sequences. G-3-PD is an enzyme which catalyses the oxidative phosphorylation of its aldehyde substrate, glyceraldehyde-3-phosphate into 1,3-bisphosphoglycerate (1,3-BPG) during post-mortem glycolysis. The reported molecular weight of this protein in mammals is about 35 kDa. On examination by SDS-PAGE of myofibrillar proteins, Troy et al. (1997) reported that a 16 kDa myofibrillar fragment is a N-terminal fragmentation product of G-3-PD, having an identical sequence to the first 15 residues of G-3-PD. Other authors have also shown that this protein decreases during storage of beef at 4°C (Nakai et al., 1995; Okayama, Fukumoto, Nakagawa, Yamonoue & Nishikawa, 1992;).

Comparison of the 1734.8 Da component (fraction 4, RT 46 min) with the known troponin T structures revealed an average identity score of 50% including isofunctional amino acid residues. This is in agreement with results obtained by other authors (Nakai et al., 1995). However, following our analysis the presence of one additional histidine residue at the C-terminus was detected. During the conditioning of beef, the loss of troponin T with time post-mortem is paralleled by a decrease in meat toughness (Penny & Dransfield, 1979). Concomitant with this loss, the gradual appearance of the 30 and 32 kDa components, resulting from the

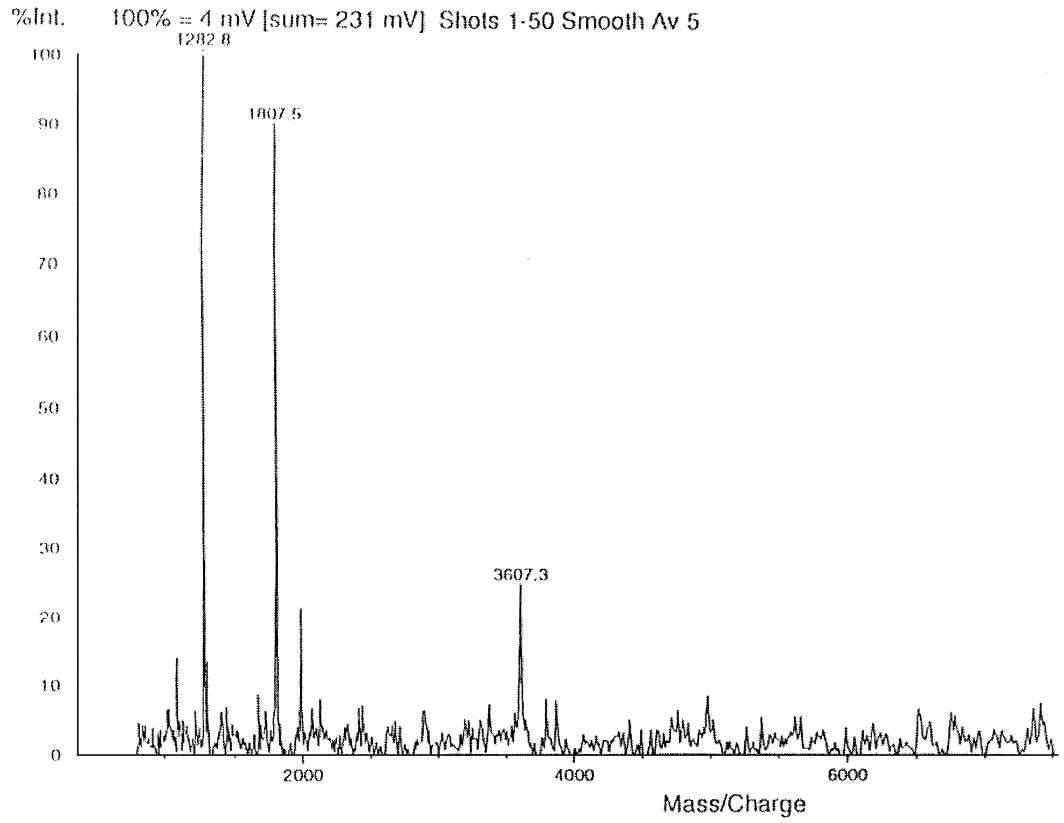

Fig. 3. LDMS spectrum of HPLC fraction 3; matrix: α-cyano-4-hydroxycinnamic acid; number of shots: 50; laser power: 109.

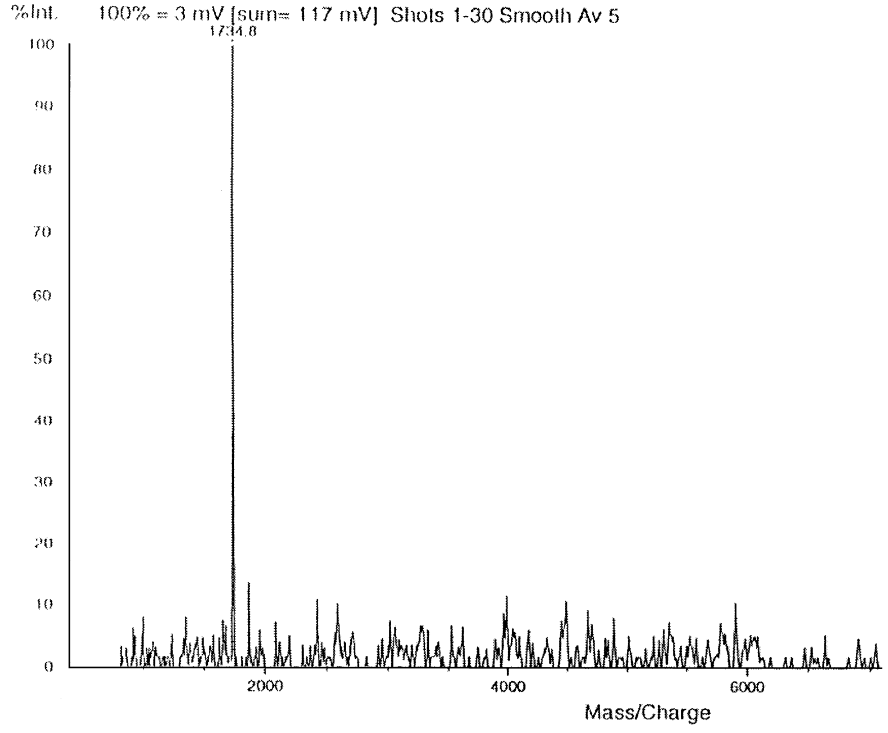

Fig. 4. LDMS spectrum of HPLC fraction 4; matrix: α-cyano-4-hydroxycinnamic acid; number of shots: 30; laser power: 98.

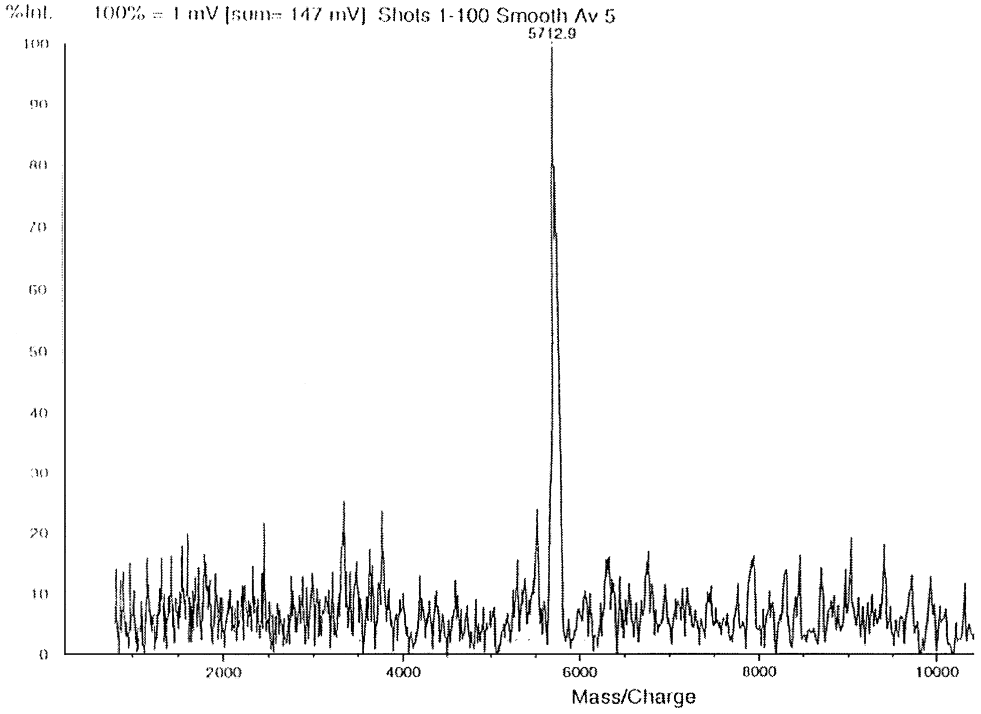

Fig. 5. LDMS spectrum of HPLC fraction 5; matrix: α-cyano-4-hydroxycinnamic acid; number of shots: 100; laser power: 110.

Table 1
Comparison of sequenced primary structure segments of bovine proteins with known proteins

Molecular weight (soluble fragment)	Identified sequence	Parent protein	% homology
Fraction 3 1282.8 Da	KVVKQASEGPLK	Glyceraldehyde-3-phosphate dehydrogenase	
		(258–265) rabbit, mouse, pig	87.5
		(260–267) human	87.5
Fraction 4 1734.8 Da	APPPPAEVPEVHEEVH	Troponin T	
		(62–75) chicken	56.6
		(29–42) quail	37.5
		(39–54) rat	
Fraction 5 5712.9 Da	DPIIQDRHGGFKPTKK KTDLNHENLKGGDD LDPNYVLS	Creatine kinase	
		(4–42) rat muscle	100
		(90–128) rat skeletal muscle, rabbit M chain	100
		(90–128) human M chain, chicken muscle, *T. calfornica*, *T. marmorata*, trout, *G. gallus*, dog brain, rabbit brain, mouse.	97

degradation of troponin T, has been suggested to be a useful indicator of meat tenderness since their concentration is higher in tender meat (Buts et al., 1986; McBride & Parrish, 1977; Troy et al., 1997). Similarly, the 1734.8 Da fraction identified in this study may be useful as an index of beef tenderness.

It is interesting to note that the parent compounds (troponin T, glyceraldehyde-3-phosphate dehydrogenase and creatine kinase) of three of our soluble fragments have previously been identified as the parent compounds of myofibrillar fragments which increase over the ageing process on SDS-PAGE gels (Troy et al., 1997). While the precise role of these three proteins in the ageing process is not clear, they may be indicative of the conditioning process of ageing muscle. Based on these results we propose that HPLC analysis of soluble proteolytic fragments from bovine muscle, may provide a rapid method for indicating and monitoring the ageing process in the muscle.

4. Conclusions

Soluble fragments of myofibrillar proteins produced during the ageing process can be isolated and identified using HPLC analysis. Three of these fragments originate

from a similar parent compound as previously identified fragments on SDS-PAGE gels from aged muscle. The enhanced appearance of such proteolytic fragments with ageing suggests that they may be useful indicators of meat quality.

Acknowledgements

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