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Presence of ubiquitin in bovine post-mortem cardiac muscle

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Abstract

We prepared sarcoplasmic proteins from bovine cardiac muscle immediately after slaughter (1.0 h; 0 days) and from stored muscle at 1, 2 and 7 days post-mortem for SDS-PAGE and Western blotting analysis. Characterization of the ubiquitin antiserum (Sigma, St Louis) showed clear and strong recognition of the ubiquitin band (8.6 kDa) and another minor band (17 kDa) in the purified ubiquitin sample (Sigma, St Louis). Among the sarcoplasmic proteins prepared from stored muscle at 0 and 7 days, this antiserum also reacted with bands corresponding to purified ubiquitin and small amounts of some other, higher-molecular-mass proteins (about 25 and 30 kDa) which were considered to be ubiquitin–protein conjugates. However, the 25 kDa band was faint in the 7 days sample, suggesting that it had degraded. We compared these results with those from our previous study of bovine skeletal muscles, in which both ubiquitin and the ubiquitin-protein conjugates had almost disappeared in the samples tested at 10 days post-mortem.

1. Introduction

It is well known that low-molecular-mass peptides and free amino acids accumulate in the sarcoplasmic fraction of various meats from skeletal muscles during conditioning (Field, Riley & Chang, 1971; Nishimura, Rhue, Okitani & Kato, 1988; Sekikawa, Seno, Shimada, Fukushima & Mikami, 1999). Although the structures and sources of these peptides and amino acids have not been definitively identified, it is generally considered that these compounds accumulate because of the action of proteases (Dransfield, 1994; Etherington, Taylor, Wakefield, Cousins & Dransfield, 1990;, Koohmarie, 1994; Mikami, Nagao, Sekikawa, Miura & Hongo, 1994). This intracellular protein degradation by different proteolytic mechanisms occurs through either lysosomal or cytoplasmic (non-lysosomal) pathways in living cells (Ciechanover, Gonen, Elias & Mayer, 1990). In the cytoplasmic pathway, there are ATP-dependent and independent mechanisms. Recent studies have suggested that the ubiquitin system, consisting of ATP, proteasomes and ubiquitin, plays an important role in the degradation of muscle proteins under various catabolic conditions (Taillandier et al., 1996). Ubiquitin is conjugated with proteins targetted for degradation. The ubiquitin molecule is a single polypeptide chain containing 76 amino acid residues, and its amino acid sequence has been highly conserved during evolution (Ciechanover et al., 1990). Another common property of this peptide is its extremely high stability over a wide range of pH and temperature. Although the ubiquitin system of cellular protein degradation has been investigated in various fields, such as clinical medicine and cellular biology (Fang, Tiao, James, Olge, Fischer & Hasselgren, 1995), this peptide seems to have received little attention in the field of meat science.

Our previous work on bovine skeletal muscle indicated that ubiquitin was present in muscle immediately after slaughter, but had almost disappeared at 10 days post-mortem, suggesting that it had been degraded by a proteinase (Sekikawa, Seno & Mikami, 1998). The purposes of this study were to demonstrate electrophoretically that ubiquitin is present in post-mortem cardiac muscle and to compare the patterns of degradation of this protein during conditioning of cardiac and skeletal muscles.

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2. Materials and methods

Samples of cardiac muscle (left ventricular wall) were obtained from three Holstein steers (average age 20 months) within 20 min after slaughter, and the excess fat, the fascia and the large blood vessels were removed. Sarcoplasmic protein was prepared from the muscle samples immediately after slaughter and at 1, 2 and 7 days post-mortem from samples stored at $4 \pm 1°C$. The pH was measured by a metal electrode (HM-17MS, Toa, Tokyo) directly inserted into the sample.

The samples were homogenized with twice their weight of distilled water, boiled for 5 min, and then allowed to cool to room temperature. Precipitates were removed by centrifugation (0°C, $8000 \times g$, 30 min) and the supernatant was freeze-dried. The freeze-dried sample obtained was dissolved in distilled water (1:25 w/v) and dialyzed against distilled water at $4 \pm 1°C$ for 24 h. The dialysate obtained was freeze-dried again, and the final lyophilized sample was considered to be the sarcoplasmic fraction for the present study.

The prepared sarcoplasmic fractions were analyzed in a 15% Tris–glycine slab gel with a 6% stacking gel (Laemmli, 1970). The gel was run at 20 mA for 2 h and then stained with 0.1% Coomassie Briliant Blue R-250 in 30% methanol and 10% acetic acid.

Proteins were transferred from the slab gel to a nitrocellulose membrane (Advantec, Japan) by a buffer-transfer method (Negishi, Yamamoto & Kuwata, 1996; Towbin, Staehelin & Gordon, 1979). After transfer, the membrane was incubated in phosphate-buffered saline (PBS, pH 7.4) containing 5.0% skim milk overnight at $4 \pm 1°C$, and then washed three times in PBS for 5 min at room temperature. The transferred membrane was incubated with ubiquitin antiserum (Sigma, USA) for 1 h at 37°C, washed three times in PBS, and then incubated with peroxidase-labelled goat anti-rabbit secondary antibody (Bio-Rad, USA) for 1 h at 37°C, followed by two washes in PBS. The peroxidase was detected with 0.6% (w/v) 4-chloro-1-naphthol, 20% ethanol and 0.02% H_2O_2.

Each analysis was done at least in duplicate. The significance of differences among storage day groups was determined by analysis of variance with Duncan's multiple-range test (SAS Institute, Cary, NC). Significance of differences was defined at $P < 0.05$.

3. Results and discussion

In 1998, approximately 1.3 million cows were slaughtered for meat in Japan. Many of the hearts obtained are thought to be consumed as human food. We use bovine heart as a table meat, and it is frequently sliced and grilled, sauteed or skewered and barbequed. However, it is not known whether cardiac muscle intended for these uses should be conditioned in the same way as skeletal muscle.

In the current study, the average (S.E., standard error) pH of the cardiac muscle samples was 6.19 (0.03) at 2.0 h post-mortem and 6.03 (0.06) at 7 days post-mortem. The lowest pH — 5.97 (0.07) — was observed 6.0 h post-mortem (Table 1). This ultimate pH value was higher than that of bovine skeletal muscle (5.5), and the rate of pH decline was faster than in bovine skeletal muscle (Lawrie, 1998). We also observed that the mean yield value of the sarcoplasmic protein tended to decrease (Table 1). We noted that although the sample size (12) was not large, the correlation coefficient between the pH and the yield value was 0.86. This might have reflected the finding that the isoelectric point of the principal proteins in muscle is close to the ultimate pH (Lawrie).

It is well known that low-molecular-mass peptides and free amino acids accumulate in the sarcoplasmic fraction of various skeletal muscles during conditioning (Field et al., 1971; Nishimura et al., 1988; Sekikawa et al., 1999). This tendency was observed in the SDS-PAGE profiles of the sarcoplasmic proteins, especially the 8.6-kDa band (Fig. 1, lanes b and c). However, when these results were compared with our previous results in bovine skeletal muscle (Sekikawa et al., 1998), there was a smaller increase in the intensity of CBB staining, and new bands did not appear in the heart. This suggests that, in cardiac muscle, the production of low-molecular-mass proteins is lower than that seen in skeletal muscle. Accumulation of peptides in the skeletal muscles is considered to be caused by certain proteases: calpain, lysosomal cathepsins, or multicatalytic proteinase complex (Dransfield, 1994; Etherington et al. 1990; Koohmaraie, 1994; Mikami et al., 1994).

Recent studies have suggested that the ubiquitin system, consisting of ATP, proteasomes and ubiquitin, plays an important role in the degradation of muscle proteins under various catabolic conditions (Taillandier et al., 1996). This protein is involved in various cellular functions: regulation of intracellular protein degradation, cell cycle regulation and the stress response (Fang et al., 1995; Taillandier et al. 1996). The ubiquitin, a

Table 1
Means of pH and the yield of sarcoplasmic protein in bovine cardiac muscles during storage[a]

Time after slaughter	pH		Yield[b]	
	Mean	S.E.	Mean	S.E.
2 h	6.19	0.03a	29.3	2.2a
6 h	5.97	0.06b	–	–
1 day	6.01	0.08b	21.7	4.8ab
2 days	5.99	0.08b	20.0	4.0b
7 days	6.03	0.05b	14.7	1.7b

[a] Means with the same letter are not significantly different ($P < 0.05$) within the same rows.

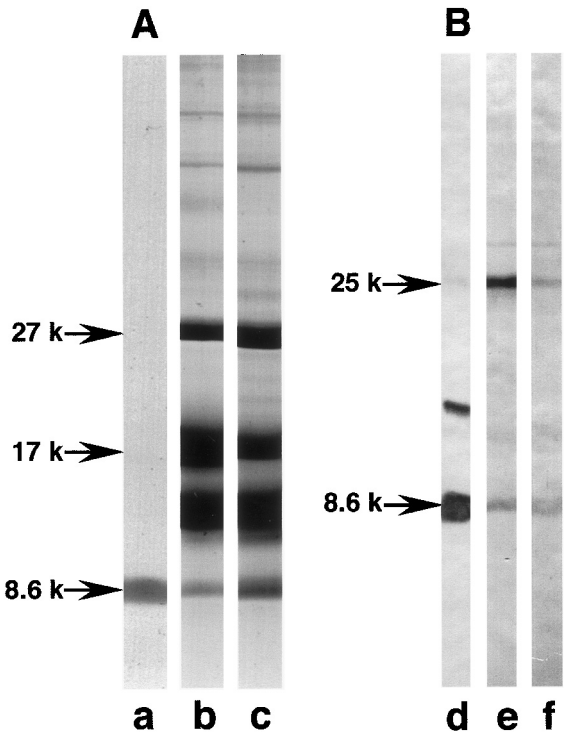

Fig. 1. Western blot analysis of sarcoplasmic proteins obtained from bovine cardiac muscle. A: SDS-PAGE, CBB-stained. B: Western blot with anti-ubiquitin antiserum. Lanes b and e: 0 days, and lanes c and f: 7 days post-mortem samples. Lanes a and d: purified ubiquitin.

highly conserved 76-residue protein found in all eukaryotic cells, is covalently ligated to the target protein. Protein ligated to multiple units of ubiquitin is degraded by the 26S proteasome. The 26S proteasome is involved in ATP-ubiquitin-dependent proteolysis, and the 20S proteasome is the catalytic core of the 26S proteasome. The function of the 20S proteasome in the living body is not yet clear, but ATP depletion results in reversible dissociation of 26S into 20S proteasome (Robert, Briand, Taylor & Briand, 1998). Recently, Matsuishi and Okitani (1997) and Robert et al. reported that the 20S proteasome degraded myofibrillar proteins incubated for about 24 h.

Although the ubiquitin system of cellular protein degradation has been investigated in various fields, such as clinical medicine and cellular biology (Fang et al., 1995), the state of this peptide in muscle cells post-mortem seems to have received little attention. Riley, Bain, Ellis and Haas (1988) and Hilenski, Terracio, Haas and Borg (1992) reported that ubiquitin was conjugated to the Z-bands of normal skeletal muscle and enhanced a ubiquitin-mediated pathway of protein turnover and degradation in striated muscle.

Characterization of ubiquitin antiserum showed that it clearly and strongly recognized the ubiquitin band (8.6 kDa) and another minor band (about 17 kDa) in the purified ubiquitin sample (Sigma, USA: Fig. 1, lane d). This antiserum also reacted with bands corresponding to purified ubiquitin (8.6 and 17 kDa) and small amounts of some other, higher-molecular-mass proteins (about 25 and 30 kDa), which were considered to be the ubiquitin-protein conjugate (Fig. 1, lanes e and f).

In our previous study (Sekikawa et al., 1998), when the 8.6 kDa band purified by preparative SDS-PAGE (Prep cell, Bio-Rad) was subjected to amino acid sequence analysis by Edman degradation (model 470, ABI), the sequence of the N-terminal five residues (MQIFV) was the same as that of ubiquitin (Schlesinger, Goldstein & Niall, 1975). These results suggest that the 8.6 kDa band in the sarcoplasmic proteins includes ubiquitin as a major component.

The results of Western blotting suggested that both the 8.6 kDa ubiquitin and the ubiquitin-protein conjugates existed and/or were released in the sarcoplasmic fraction of cardiac muscle cells immediately after circulatory arrest, and that they were then degraded during storage (Fig. 1, B).

It is generally accepted that accumulation of low-molecular-mass peptides in the sarcoplasmic fraction during conditioning is evident in SDS-PAGE profiles using CBB staining. However, in the sarcoplasmic fraction during conditioning, there might be a decrease in low-molecular-mass components, such as the 8.6 kDa ubiquitin band shown in the present study.

It is considered that protein degradation with the ubiquitin system, comprising ubiquitin, ATP and proteasomes, which act in living muscle cells, is one of the primary factors affecting meat conditioning in the immediate post-mortem period, since this system requires ATP. It is also apparent that the mechanism of action of ubiquitin is important not only in meat science, but also in general biochemical studies, such as those investigating ischemia of muscle cells (Sharma, Malulik, Gho, Das & Verdouw, 1996). Therefore, further experiments are needed to demonstrate the contribution of the ubiquitin system to meat conditioning.

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