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Effect of proteasome inhibitor on sarcoplasmic protein of bovine skeletal muscle during storage

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Abstract

Bovine skeletal muscle, immediately after slaughter (1.5 h; 0 d), was buffered, homogenized treated with proteasome inhibitor, and stored (2 d) for SDS-PAGE and Western blotting analysis. Ubiquitin antiserum (Sigma, St Louis) reacted with bands corresponding to purified ubiquitin and small amounts of other higher-molecular-mass proteins (about 30 and 40 kDa) which were considered to be ubiquitin-protein conjugates. These bands were faint in the control 2 d sample, suggesting that they had degraded. However, these tendencies of the ubiquitin positive bands to decrease were not clearly observed in the sample treated with proteasome inhibitors (MG132 and Lactacystin). These results suggest that both ubiquitin and the ubiquitin-protein conjugates were present in the skeletal muscle immediately after slaughter and they were then degraded during storage. This degradation was partially due to the action of proteasome.

1. Introduction

Although various types of meat from skeletal muscles are obtainable, the intracellular proteolytic system of muscle cells is considered to play an important role in improving the texture and taste of all meat. It is well known that low-molecular-mass peptides and free amino acids accumulate in the sarcoplasmic fraction of various meats during conditioning (Field, Riley, & Chang, 1971; Nishimura, Rhue, Okitani, & Kato, 1988; Sekikawa, Seno, Shimada, Fukushima, & Mikami, 1999). The structures and sources of these peptides and amino acids have not been definitively identified, but it is generally considered that they accumulate because of the action of proteases (Dransfield, 1994; Etherington, Taylor, Wakefield, Cousins, & Dransfield, 1990; Koohmariae, 1994; Mikami, Nagao, Sekikawa, Miura, & Hongo, 1994). This intracellular protein degradation, by different proteolytic mechanisms occurs, by either lysosomal or cytoplasmic 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. This system is involved in various cellular functions: the regulation of intracellular protein degradation, cell cycle regulation, and the stress response (Fang, Tiao, James, Olge, Fischer, & Hasselgren, 1995; Sharma, Malukik, Gho, Das, & Verdouw, 1996). The ubiquitin, a 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. Taylor, Tassy, Briand, Robert, Briand, and Ouali, (1995), Matsuishi and Okitani (1997) and Robert, Briand, Taylor, and Briand (1999) reported that purified 20S proteasome degraded myofibrils and/or myofibrillar proteins. The function of the 20S proteasome in the living body is not yet clear, but ATP depletion, which is a condition of postmortem muscle cells, results in reversible dissociation of the 26S into the 20S proteasome

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(Robert et al. 1999). However, it is uncertain whether the ubiquitin-proteasome system functions in the muscle cell after slaughter. There is a small amount of ATP in the muscle cell until rigor mortis occurs, e.g. 24 h after slaughter in beef. During this period, immediately after slaughter until rigor mortis, there is a possibility that the ubiquitin system could be acting in the bovine muscle cell. Moreover, our previous work on bovine skeletal and cardiac muscles indicated that ubiquitin and ubiquitin linked-proteins were present in muscle immediately after slaughter, but almost disappeared during storage, suggesting that the ubiquitin and linked proteins had been degraded by proteinases (Sekikawa, Seno, & Mikami, 1998, Sekikawa, Shimada, Fukushima, Ishikawa, Wakamatsu, & Mikami, 2000). The purpose of this study was to demonstrate, electrophoretically, the effects of proteasome inhibitor on sarcoplasmic protein of bovine skeletal muscle during storage.

2. Materials and methods

Samples of *quadriceps femoris* muscle were obtained from three Holstein cows (average age 38 months) within 90 min of slaughter, and the excess fat, the fascia and the large blood vessels were removed. The samples were homogenized in 5-fold (w/v) of 0.2 M Tris–HCl buffer containing 0.05 % NaN3, pH 7.4, with or without proteasome inhibitor and then stored at 4±1°C for 48 h (2 d). The inhibitors used were 10μM MG132 (Calbiochem, USA), 10 μM lactacystin (Kyowa Medics, Japan) and a complete™ (Boehringer Mannheim, Germany; protease inhibitor cocktail, one tablet was dissoluble in 50 ml Tris–HCl buffer). Each inhibitor was individually added.

Sarcoplasmic proteins were prepared from aliquot volumes of the homogenized samples at 0 h and 48 h after storage. These samples were boiled for 5 min, and then allowed to cool to room temperature. Precipitates were removed by centrifugation (0°C, 8000×***g***, 30 min) and the supernatant was dialyzed against distilled water at 4±1°C for 24 h. The dialysate obtained was freeze-dried, and the lyophilized sample was considered to be the sarcoplasmic fraction for the present study.

Muscle pH was measured 90 min after slaughter by a metal electrode (HM-17MS, Toa, Tokyo) directly inserted into the sample, and the pH of the homogenized samples was measured two days after slaughter using a pH meter with a glass electrode (Toa, HM-5s).

The prepared sarcoplasmic fractions were analyzed in a 15% Tris–HCl slab gel with a 6% stacking gel (Laemmli, 1970). The gel (85×90×1.5 mm) was run at 20 mA for ca. 2 h and then stained with 0.1% Coomassie Brilliant 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°C, and then washed three times in PBS for 5 min at room temperature. The transferred membrane was incubated with rabbit ubiquitin antiserum (Sigma, USA) for 1 h at 37°C, washed three times in PBS, and then incubated with peroxidase-labeled 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% H2O2. Each analysis was done at least in duplicate.

3. Results and discussion

In the current study, the average pH (S.E.; standard error) of a sample of *quadriceps femoris* muscle 90 min after slaughter was 7.07 (±0.03) and that of the homogenized muscle samples, after storage for 48 h, was 6.79 (±0.04) in the control sample, and the range was from 6.60 to 6.76 (±0.04) in the samples with added inhibitor. There were no significant mean differences among groups at the 5% level using the Student *t* test.

The color of the homogenized muscle in the control became darker due to met-myoglobin formation, whereas the MG132-treated sample showed a bright red color after storage for 48 h. Although the reason for this is unclear, MG132 is the peptide aldehyde inhibitor N-carbobenzoxyl-Leu-Leu-leucinal; in general, aldehyde is considered a reducing agent.

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 under 15-kDa band (Fig. 1: lanes 1 and 2) in the control. However, in comparison with our previous results for bovine skeletal muscle (Sekikawa et al., 1998), there was a smaller increase in the intensity of CBB staining, and few new bands appeared in the present study, indicating that the production of low-molecular-mass proteins was lower than in the previous study. This difference of SDS-PAGE profiles might be related to the age of the animals and/or storage conditions. In the present study, muscle samples were obtained from old milking cows and were homogenized with buffer and then stored. In our previous study, muscle samples were taken from young steers and these were stored intact. Distilled water extraction was also used.

There were no apparent differences of the SDS-PAGE profiles between the control and MG132 or lactacystin treatments (Fig.1: lanes 4–5), indicating that proteasome

inhibitors, MG132 and lactacystin, had no effect on the SDS-PAGE profiles stained with CBB. The peptide aldehyde inhibitor MG132 can also inhibit calpains and certain lysosomal cysteine proteases (Lee & Goldberg, 1998; Tawa, Odessey, & Goldberg, 1997), although there was no apparent difference of the SDS-PAGE profiles between the control and this inhibitor treatment (Fig 1: lanes 2 and 4). However, lactacystin, originally isolated from actinomycetes, is considered to be a specific inhibitor of proteasomes. This reagent is a natural product, and not considered to affect other proteases (Tawa et al.; Lee & Goldberg). The SDS-PAGE profile after lactacystin treatment was similar to that of the control (Fig 1: lanes 2 and 5). This present result suggests that neither MG132 nor lactacystin inhibit the low-molecular-mass peptide production of the sarcoplasmic fraction caused by proteases in bovine skeletal muscles after slaughter. However, the SDS-PAGE profile after treatment with Complete™ for 48 h was similar to that of the control at 0 h (Fig 1: lanes 1 and 3). The Complete™ inhibited the production of low-molecular-mass peptide, indicating that the latter was produced by proteases, which were inhibited by this inhibitor cocktail.

In our previous study (Sekikawa et al., 1998), the characterization of ubiquitin antiserum (Sigma, USA) showed that it clearly and strongly recognized the ubiquitin band (8.7 kDa) in the purified ubiquitin sample (Sigma, USA). We have also reported that when the 8-kDa band purified by preparative SDS-PAGE (Prep cell, BioRAD), was subjected to amino acid sequence analysis by Edman degradation (model 470, ABI), the sequence of the five N-terminal residues (MQIFV) was the same as that of ubiquitin (Schlesinger, Goldstein, & Niall, 1975). These results suggest that the 8-kDa band, among the sarcoplasmic proteins, includes ubiquitin as a major component.

The ubiquitin antiserum reacted with bands corresponding to ubiquitin (about 8 kDa) and small amounts of other higher-molecular-mass proteins (about 30 and 40 kDa), which were considered to be ubiquitin-protein conjugates (Fig. 2: lane 1). In a preliminary experiment, the 30-kDa band was purified by two-dimensional electrophoresis and its amino acid sequence was analyzed. However, at this stage the N-terminal amino acid was not detected. After pyroglutamyl peptidase digestion, the amino acid sequence of six residues was determined as RQTAAG. When this sequence was compared with the ubiquitin reported previously (Schlesinger et al., 1975), there was no correlation. This suggests that the 30-kDa protein is a ubiquitin-protein conjugate, and not poly-ubiquitin.

The results of western blotting suggested that both the 8-kDa ubiquitin and the ubiquitin-protein conjugates existed and/or were released in the sarcoplasmic fraction of skeletal muscle cells immediately after circulatory arrest, and that they then degraded during storage

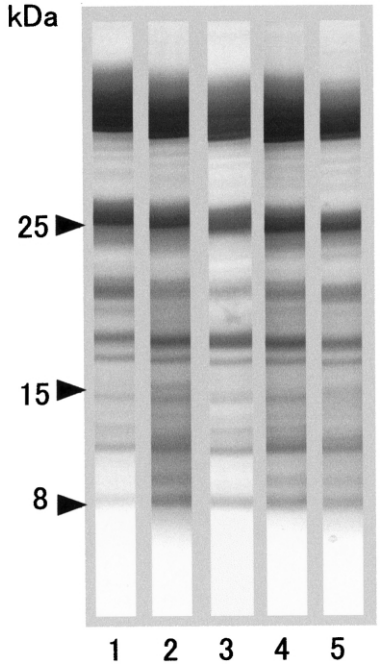

Fig. 1. SDS-PAGE profiles of bovine skeletal sarcoplasmic proteins incubated with or without protease inhibitors. Each lane was loaded with 10 μg of sample proteins and stained with CBB. Lane 1 and 2: incubated for 0 h and 48 h without (control) inhibitor, respectively, lane 3: incubated for 48 h with Complete™, lane 4: incubated for 48 h with MG132, and lane 5: incubated for 48 h with lactacystin.

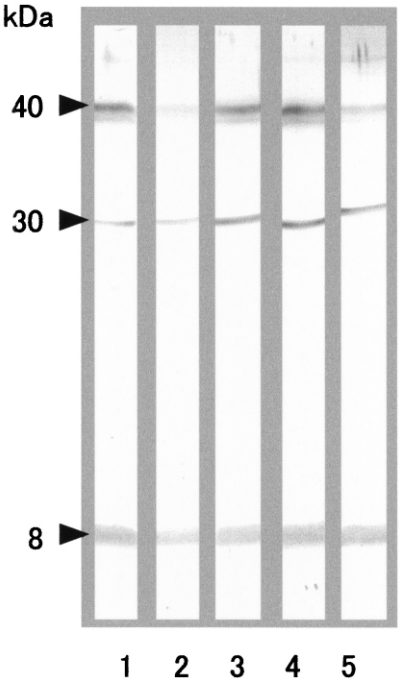

Fig. 2. Western blot analysis of sarcoplasmic proteins obtained from bovine skeletal muscle. Each lane was loaded with 15 μg of sample proteins and stained with rabbit anti-ubiquitin antiserum. Lane 1 and 2: incubated for 0 h and 48 h without (control) inhibitor, respectively, lane 3: incubated for 48 h with Complete™, lane 4: incubated for 48 h with MG132, and lane 5: incubated for 48 h with lactacystin.

(Fig. 2: lane 2). This was similar to the trend observed in our previous study (Sekikawa et al., 1998). However, when using inhibitor treatments (Complete™, MG132 and lactacystin, Fig. 2: lanes 3–5, respectively) the positive bands, stained with ubiquitin untiserum corresponding to ubiquitin and ubiquitin-conjugated proteins, were not affected by storage for 48 h, especially after Complete™ and MG132 treatments. The patterns of Western blotting after lactacystin treatment were similar to, but weaker in intensity than those after Complete™ and MG132 (Fig.2: lanes 3–5).

In summary, Complete™ almost completely inhibited the production of low-molecular-mass protein observed in SDS-PAGE profiles and, in addition, no change was observed in the ubiquitin-positive band upon western blotting. However, neither MG132 nor lactacystin had a large influence on low-molecular-mass protein production, or even changed the ubiquitin-positive band upon Western blotting. These results suggest that the ubiquitin and ubiquitin-conjugated proteins were degraded by proteases, including proteasomes, as well as calpain and cathepsins. The results also suggest the possibility that the ubiquitin-proteasome system functioned from immediately after slaughter until ATP depletion. In beef, this was generally when maximum rigor mortis had occurred.

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 the 20S proteasome (Peters, Franke, & Kleinschmidt, 1994). Recently, Robert et al. (1999) reported that the 20S proteasome degraded myofibrillar proteins and that, after the ATP level had decreased, the 26S proteasome appeared to dissociate into the 20S proteasome and PA700, thus increasing the 20S concentration in muscle cells. As this 20S proteasome does not require ATP and ubiquitin, its proteolytic actions contribute to meat conditioning after maximum rigor mortis has occurred.

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 because of ATP depletion. 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, enhancing the ubiquitin-mediated pathway of protein turnover and causing degradation of striated muscle.

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 the ischemia conditions of muscle cells (Sharma et al., 1996). It is also apparent that the mechanism of action of ubiquitin is important, not only in meat science, but also in general biochemical studies. Therefore, further experiments are needed to demonstrate the contribution of the ubiquitin system to meat conditioning.

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