Stability of Proteasomes Extracted from Pressurized, Aged Skeletal Muscles

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ABSTRACT : The present paper describes the effects of pressure and post-mortem aging treatments on in situ proteasome activity in rabbit and bovine skeletal muscles. Synthetic peptide hydrolyzing activity of rabbit proteasomes remained in the muscle after exposure to pressures up to 100 MPa. However, when a pressure of 400 MPa or more was applied, proteasomes were markedly inactivated. The extraction of proteasomes from excessively pressurized muscle appeared to be difficult. Proteasomes in aged muscle remained relatively stable throughout the aging process, with activity after 168 h (7 days) being 35%, 48%, 53% and 31% of the 0 h post-mortem LLVY, LSTR, AAF and LLE total hydrolyzing activities, respectively. The synthetic peptide hydrolyzing activities of bovine muscle proteasomes were similar to those of rabbit skeletal muscle proteasomes. The results suggest that synthetic peptide hydrolyzing activity remains in muscle exposed to relatively low pressures. Furthermore, it is known that high-pressure treatment induces fragmentation of myofibrils, modification of actin-myosin interaction and activation of intramuscular proteinases, cathepsins and calpains. Thus, proteasomes are probably involved in the tenderization process in combination with other intramuscular proteinases under high-pressure conditions. Our findings confirmed that proteasomes play a role in meat tenderization induced by high-pressure treatment or aging. (Key Words : High Pressure, Proteasome, Meat Aging, Meat Tenderization, Intramuscular Proteinases)

INTRODUCTION

The proteasome or multicatalytic proteinase complex (MCP) was first isolated from bovine pituitaries (Orlowski and Wilk, 1981). This enzyme is a high-molecular-mass intracellular proteinase (20S proteasome; 700 kDa) that has a complex subunit composition and multicatalytic proteolytic activities with different specificities (Rivett, 1989a, 1989b, 1993; Orlowski, 1990).

Proteasomes have two unique enzymological properties as a protease; multiple peptidase activities, and a latent form. According to Mykles and Harie (1995), proteasomes have at least five activities - peptidylglutamyl peptide hydrolase (PGPH), trypsin-like, chymotrypsin-like, branched-chain amino acid-preferring, and small neutral amino acid-preferring activities - within a single complex. Proteasomes isolated from tissues in a latent form can be activated by various chemicals and treatments, such as polylysine (Tanaka et al., 1986; Mellgren, 1990), SDS (Wilk and Orlowski, 1983; Dahlmann et al., 1985; Tanaka et al., 1986; Otsuka et al., 1998; Yamamoto et al., 2005a), fatty acids (Wilk and Orlowski, 1983), heat treatment (Mykles, 1989a; Mykles, 1989b; Koohmaraie, 1992; Otsuka et al., 1998; Yamamoto et al., 2005a, 2005b), and high hydrostatic pressure treatment (Otsuka et al., 1998; Gardrat et al., 1999; Yamamoto et al., 2005b). These results indicate that proteasome activity is stimulated by mild denaturing treatments.

High hydrostatic pressurization is a new technology for meat tenderization or acceleration of meat aging, which is a very important process for meat industry (Bouton et al., 1977; Macfarlane, 1985; Suzuki et al., 1992; Rusman et al., 2007).

Several researchers have studied the effects of high hydrostatic pressurization treatment on intramuscular proteinases. Homma et al. (1994) reported that the pressure-induced increase in proteolytic levels in the muscle was due to a release of cathepsins from lysosomes. With regard to calpain systems, Suzuki et al. (1993) provided direct...
evidence for pressure-induced Ca$^{2+}$ release from the sarcoplasmic reticulum. Ca$^{2+}$ dispersion into myofibrils may cause an increase in activated calpain. Homma et al. (1994) reported that calpain levels remained in muscle pressurized at up to 200 MPa, whereas calpastatin levels were lowered by pressurization. Thus, the total calpain activity in pressurized muscle appears to have been increased, resulting in meat tenderization (Koomaraie, 1984; Kim et al., 1992).

Proteasome activation induced by high pressure may be involved in the pressure-induced modification or breakdown of muscle proteins (Otsuka et al., 1998; Gardrat et al., 1999; Yamamoto et al., 2005b). However, the effects of pressure and postmortem aging treatments on proteasome in in situ muscle are not yet clear. In addition, Farout et al. (2000) and Lamare et al. (2002) reported that proteasome purification was likely to alter the activity, which mimics intercellular conditions.

In this paper, we describe the effects of pressure and postmortem aging treatments on in situ proteasome activity of rabbit and bovine skeletal muscles. The reason why the rabbit and bovine muscles used in present experiment were as follows; the rabbit muscle was widely used as a model to investigate the mechanism of postmortem aging and the bovine muscle was generally considered as a muscle requiring long aging period for the improvement of meat quality in comparison with the other meats.

**MATERIALS AND METHODS**

**Muscle**

A rabbit (6 week-old) was anaesthetized with sodium pentobarbital (2.5 ml) and d-tubocurarine chloride pentahydrate (0.5 ml) just prior to exsanguination. The carotid artery was stuck for exsanguinations. The back and leg muscles were immediately excised and subjected to extraction and aging. Lean meat was excised from the shoulder of a beef carcass (76 to 86 week-old Holstein cows) two days after slaughter, and was stored at -20°C. As required, it was tempered overnight in a cold room (4°C) and then cut into small pieces (about 5×5×3 cm) for aging. The experimental protocol was carried out in accordance with the Guide for the Care and Use of Laboratory Animals prepared by Niigata University.

**High pressure treatment**

Pressurization was performed using the method described by Homma et al. (1995). Briefly, each skeletal muscle was vacuum-sealed in a polyethylene bag, and this bag was then transferred to a larger polyethylene bag. The space between the two bags was filled with water. Each double-bag was then placed in a pressure vessel containing water, and was pressurized at 0.1-400 MPa at about 10°C for 10 min using a Cold Isostatic Press (CIP) apparatus from Nikkiso Co., Ltd., Tokyo, Japan.

**Preparation of proteasome**

Crude proteasomes were extracted from pressurized (0.1 to 400 MPa at 10°C for 10 min) or aged rabbit and bovine muscle (0 to 168 h for rabbit muscle or 0 to 21 days for bovine muscle at 4°C) in accordance with the method of Farout et al. (2000). Muscle was suspended (1:10 w:v) in 50 mM Tris-HCl buffer, pH 8.0, containing 10% glycerol, 1 mM EDTA, 1 mM EGTA, 50 mM E64, 2.5 μM peptatin A, and was homogenized. Crude extracts were prepared by centrifuging the homogenates at 100,000 g for 1 h and were studied immediately. Purified rabbit or bovine proteasomes were prepared by the method of Otsuka et al. (1998).

Protein concentration was determined using the method of Bradford et al. (1976).

**Measurement of proteasome activities**

The activity of proteasomes extracted from aged or pressurized muscle was measured using the method described by Lamare et al. (2002). Proteasome activity was assayed by fluorometric measurement of the release of 7-amino-4-methylcoumarin (-NH-Mec; AMC) after incubation with synthetic substrates. The substrates Suc-Leu-Leu-Val-Tyr-NH-Mec (LLVY; chymotrypsin-like activity), Ala-Ala-Phe-NH-Mec (AAF; chymotrypsin-like activity), and Z-Leu-Leu-Arg-NH-Mec (LLE; PGPH activity), purchased from Sigma (St. Louis, MO, U.S.A.), and Boc-Leu-Ser-Thr-Arg-NH-Mec (LSTR; trypsin-like activity), purchased from the Peptide Institute (Osaka, Japan), were chosen to assay the distinct proteolytic activities of proteasome.

Crude proteasome extract (50 μl) was mixed with 210 μl of 50 mM Tris-HCl (pH 7.5) and 100 μl of 6 mM DTT. After preincubation at 37°C for 5 min, 240 μl of a 20 μM fluorometric substrate was added to the reaction mixture. After 60 min, the reaction was stopped by addition of 2.4 ml of 100 mM monochloroacetate/30 mM sodium acetate.

Fluorescence was measured using a Hitachi F 2000 spectrofluorimeter with an excitation wavelength of 370 nm and an emission wavelength of 460 nm. The results are given as averages±SE of 6 (LLVY, LSTR, AAF for rabbit proteasome) or 3 (LLE for rabbit proteasome and synthetic substrates for bovine proteasome) different experiments. The Student’s t-test was used for statistical analysis.

**Electrophoresis and immunological analysis**

SDS-PAGE was conducted in accordance with the procedure described by Laemmli (1970), with slight modification. Electrophoresis was carried out for 1.5 h using slab gels (70 mm×90 mm×1 mm) containing 12.5% polyacrylamide (bisacrylamide/acrylamide, 1:20 (w/w)).

Gels were stained using the method of Trinick et al. (1984). Immunoblotting was performed in accordance with the method of Towbin et al. (1979). After SDS-PAGE, proteins were transferred from the gel to a polyvinylidene difluoride (PVDF) membrane (Bio-Rad., Oakland, CA, USA) using transfer buffer consisting of 25 mM Tris, 192 mM glycine and 5% methanol. The PVDF membrane was blocked for 2 h at 25°C in a buffer containing 5% skim milk and 0.1% Tween in phosphate-buffered saline (PBS). The blocked membrane was rinsed with PBS, and was then incubated overnight in Tween/phosphate-buffered saline (PBST) containing the first antibody (20S proteasome subunit β7 (NH3), mouse monoclonal antibody (Biomol, San Diego, CA, USA) diluted 1:200 vol/vol at 25°C. The membrane was then washed three times with PBST and once with PBS. Subsequently, the membrane was incubated in PBST containing the second antibody, a 1:200 dilution of goat anti-mouse IgG (H+L)-HRP conjugate (Bio-Rad), for 90 min at 25°C. After being washed three times with PBST and once with PBS, the proteins on PVDF membranes were visualized by chemiluminescence using the ECL Plus kit (GE Healthcare, Little Chalfont, Buckinghamshire, UK).

RESULTS AND DISCUSSION

Proteasomes from rabbit muscle

The total activities of proteasomes extracted from pressurized rabbit muscle are shown in Table 1. The ability of proteasomes to all hydrolyze synthetic peptides was retained in muscle exposed to pressures of up to 100 MPa, being about 96 to 56% of the activities extracted from unpressurized muscle. However, when a pressure of 400 MPa or more was applied, proteasomes were markedly inactivated with regard to all hydrolyzing activity. This finding was confirmed by SDS-PAGE and immunoblotting analysis (Figure 1A and B). The results of our experiment show that when pressures of 400 MPa or more are applied, bands equivalent to proteasomes decrease markedly.

Table 1. Total activities of the proteasome extracted from pressurized rabbit muscle

<table>
<thead>
<tr>
<th>Pressure (MPa)</th>
<th>LLVY hydrolyzing activity</th>
<th>LSTR hydrolyzing activity</th>
<th>AAF hydrolyzing activity</th>
<th>LLE hydrolyzing activity</th>
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<td>1.18±1.03*</td>
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Relative activity was expressed as a percentage of the activity of the 0.1 MPa. Each value is expressed as the mean±standard deviation (LLVY, LSTR, AAF; n = 6; LLE, n = 3). * p<0.01.

Figure 1. The SDS-PAGE pattern and immunoblotting of the proteasome extracted from pressurized rabbit muscle. (A) SDS-PAGE of the proteasome extracted from pressurized rabbit muscle. A purified proteasome (Lane 1, 1.2 μg) and crude proteasome (Lane 2 to 9, 18.8 μg) applied to a 12.5% gel under denaturing conditions. Lane 1: purified rabbit muscle proteasome. Lanes 2 to 9: crude rabbit muscle proteasome extracted from pressurized rabbit muscle for 0.1 (control), 50, 100, 200, 300, 400, 500 and 600 MPa, respectively. (B) The immunoblotting of the pressurized proteasome using anti 20S proteasome subunit β7 (HN3), mouse monoclonal antibody. The lane conditions are the same as in (A).
also found that extractability of proteasomes from excessively pressurized meat was difficult. Based on activity measurement, and SDS-PAGE and immunoblotting analysis, when pressures of 400 MPa or more were applied, proteasome activity decreased markedly, depending on both the denaturation of proteasomes by high pressure treatment and the amount of proteasome extracted from pressurized meat. However, proteasome activity was largely retained at an applied pressure of 100 MPa. It has been demonstrated that activation of cathepsins (Ohmori et al., 1991; Homma et al., 1994) and calpain (Kooohmaraie et al., 1984; Suzuki et al., 1993; Homma et al., 1995; Gerel et al., 2006), and the disintegration of myofibrillar structure, are induced by high pressure treatment (Macfarlane, 1973; Suzuki et al., 1990). In addition, it is known that proteasomes more easily hydrolyze denatured proteins than non-denatured proteins. Considering these findings, our data show that proteasomes are able to tenderize meat if low pressures are applied.

The total activities of proteasomes extracted from aged rabbit muscle are shown in Table 2. Proteasomes from aged muscle remained relatively stable throughout the aging process, with total hydrolyzing activity after 168 h of aging (7 days) being 35%, 48%, 53% and 31% of the 0 h postmortem hydrolyzing activities against LLVY, LSTR, AAF and LLE, respectively. The results of SDS-PAGE and immunoblotting for proteasomes extracted from aged skeletal muscles, Lamare et al. (2002) reported that bovine skeletal muscle proteasome activity was stable until beyond 24 h postmortem and was 54 to 28% of at-death values after 168 h. Thomas et al. (2004) reported that ostrich proteasome activity remained at about 82 to 74% of at-death values after 288 h. Our investigation supported these results. Furthermore, other investigators have reported

Table 2. Total activities of the proteasome extracted from aged rabbit muscle

<table>
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<tr>
<th>Time (h)</th>
<th>LLVY hydrolyzing activity</th>
<th>LSTR hydrolyzing activity</th>
<th>AAF hydrolyzing activity</th>
<th>LLE hydrolyzing activity</th>
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<tr>
<td>120</td>
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<td>168</td>
<td>35.31±9.73*</td>
<td>47.78±16.19*</td>
<td>52.70±9.23*</td>
<td>30.71±6.28*</td>
</tr>
</tbody>
</table>

Relative activity was expressed as a percentage of the activity of the 0.1 MPa. Each value is expressed as the mean±standard deviation (LLVY, LSTR, AAF; n = 6; LLE, n = 3). * p<0.01.

Figure 2. The SDS-PAGE pattern and immunoblotting of the proteasome extracted from aged rabbit muscle. (A) SDS-PAGE of the proteasome. A purified proteasome (Lane 1, 1.2 μg) and crude proteasome (Lane 2 to 10, 18.8 μg) applied to a 12.5% gel under denaturing conditions. Lane 1: purified rabbit muscle proteasome. Lanes 2 to 10: the proteasome during aging 0 (control), 1, 2, 4, 8, 24, 72, 120, 168 h, respectively. (B) The immunoblotting of the pressurized proteasome using anti 20S proteasome subunit β7 (HN3), mouse monoclonal antibody. The lane conditions are the same as in (A).
that proteasome activity was stable at pH 5.0 to 9.0 (Matsuishi and Okitani, 1997; Otsuka et al., 1998; Yamamoto et al., 2005a) and was retained in the postmortem pH range. Therefore, proteasomes may play a role in meat tenderization during postmortem aging.

### Proteasomes from bovine muscle

The total activities of proteasome extracted from pressurized bovine muscle are shown in Table 3. The ability of bovine proteasomes to hydrolyze synthetic peptides was retained in muscle exposed to pressures of up to 200 MPa, being about 72 to 56% of the activities extracted from unpressurized muscle. However, when pressures of 500 MPa or more were applied, proteasomes were notably inactivated, although activities against LLVY, LSTR and AAF were slightly retained at 600 MPa. Based on these results, bovine proteasomes are more resistant to pressure treatment when compared with rabbit proteasomes. The results of SDS-PAGE and immunoblotting for proteasomes extracted from pressurized bovine muscle are shown in Figure 3A and B. Similarly to proteasomes extracted from pressurized rabbit skeletal muscle, the results of our experiment show that when a pressure of 400 MPa or more was applied, bands equivalent to proteasomes decreased markedly.

### Table 3. Total activities of the proteasome extracted from pressurized bovine muscle

<table>
<thead>
<tr>
<th>Pressure (MPa)</th>
<th>LLVY hydrolyzing activity</th>
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<th>LLE hydrolyzing activity</th>
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<tr>
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<td>50</td>
<td>83.71±13.23</td>
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<td>200</td>
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Relative activity was expressed as a percentage of the activity of the 0.1 MPa. Each value is expressed as the mean±standard deviation (n = 3). * p<0.01.

The total activities of proteasomes extracted from aged bovine muscle are shown in Table 4. Proteasomes in aged muscle remained relatively stable throughout the aging process, with total hydrolyzing activity after 21 days of aging being 17%, 50%, 58% and 21% of the 0 day postmortem LLVY, LSTR, AAF and LLE values, respectively. The results of SDS-PAGE and immunoblotting for proteasomes extracted from aged bovine muscle are shown in Figure 4A and B. Similarly to aged rabbit muscle proteasomes, marked changes were not seen with age treatment, as compared with bovine skeletal muscle extracted from pressurized bovine skeletal muscle. Table 4 and Figure 4 show that the activity levels of proteasomes

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**Figure 3.** The SDS-PAGE pattern and immunoblotting of the proteasome extracted from pressurized bovine muscle. (A) SDS-PAGE of the proteasome. A purified proteasome (Lane 1, 0.6 μg) and crude proteasome (Lane 2 to 9, 30 μg) applied to a 12.5% gel under denaturing conditions. Lane 1: purified rabbit muscle proteasome. Lanes 2 to 9: crude rabbit muscle proteasome extracted from pressurized rabbit muscle for 0.1 (control), 50, 100, 200, 300, 400, 500 and 600 MPa, respectively. (B) The immunoblotting of the pressurized proteasome using anti 20S proteasome subunit β7 (HN3), mouse monoclonal antibody. The lane conditions are the same as in (A).
remained constant during the aging period. Thus, bovine skeletal muscle is more stable during the aging process than rabbit skeletal muscle.

Our results suggest that bovine proteasomes treated by high-pressure and aging are more stable than rabbit proteasomes. Therefore, it is thought that bovine proteasome can affect more effectively on meat tenderization induced by both high-pressure and aging treatments in comparison with rabbit proteasome.

CONCLUSION

In this experiment we found that the ability to hydrolyze synthetic peptides was retained in muscle exposed to low-pressure pressurization. It is known that high pressure treatment induces fragmentation of myofibrils (Macfarlane, 1973; Suzuki et al., 1990), modification of actin-myosin interactions (Nishiwaki et al., 1996) and activation of intramuscular proteinase, cathepsins (Ohmori et al., 1991; Homma et al., 1994) and calpain (Koohmaraie et al., 1984; Suzuki et al., 1993; Homma et al., 1995; Gerelt et al., 2006). Thus, the meat tenderization process under high-pressure conditions probably involves proteasomes, in combination with other intramuscular proteinases.

Given the results obtained in the present study, it is very likely that proteasomes play a role in meat tenderization induced by both high-pressure and aging treatments.

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