Purification and Properties of Bovine Skeletal Muscle Proteasome

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ABSTRACT : This paper describes the purification and properties of a multicatalytic proteinase complex, proteasome, from bovine skeletal muscle, in comparision with proteasome prepared from other species or organs. The purified bovine skeletal muscle proteasome exhibited a single band on polyacrylamide gel electrophoresis under nondenaturing conditions. Bovine skeletal muscle proteasome degraded synthetic peptides maximally at pH 8.0. Relative to pH 8.0, activities were gradually decreased with the lowering pH, but the extent of decrease was substrate-dependent, and the activity at pH 5.5 still retained 78-10% of the activity at pH 8.0, indicating the possibility that the proteasome is active in muscle during aging. When the proteasome was heated at 60°C for 15 or 30 min and treated in the presence of 0.0125% SDS, the activity increased over 1.8 and 3.1 times (LLVY (Suc-Leu-Leu-Val-Tyr-NH-Mec) as a substrate), respectively. These results (activation with heat or SDS) indicate that the hydrolytic activity of proteasome was stimulated under mild denaturing conditions. The characteristics of the bovine skeletal muscle proteasome obtained in our experiment were almost the same as those of the proteasome prepared from other species or organs. ***(Asian-Aust. J. Anim. Sci. 2005. Vol 18, No. 6 : 884-891)***

Key Words : Bovine, Proteasome, SDS Treatment, Heat Treatment, Immunobloting, Meat Aging

INTRODUCTION

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), which has a complex subunit composition and multicatalytic proteolytic activities with different specificities (Orlowski, 1990; Rivett, 1989a,b, 1993).

Proteasomes have been classified into two isoforms with apparent sedimentation coefficients of 20S proteasome and 26S proteasome (1,600 kDa), respectively. The 20S proteasome is considered to be the core unit of proteinase activity of 26S proteasome, and other components are assumed to be the subunits that make the proteinase activity of the 26S proteasome ATP-dependent. It is also shown that ATP is needed to keep the 26S complex and the depletion of ATP cause rapid dissociation of the 26S complex into the 20S proteasome and multiple components (Kanayama et al., 1992). However, postmortem situation in which ATP disappears, it is necessary to investigate the properties of the 20S proteasome that dose not show ATP-dependence rather than the 26S proteasome.

The proteasome has two unique enzymological properties as protease: multiple peptidase activities and a latent form. According to Mykles and Harie (1995), the proteasome has at least five activities-peptidylglutamyl peptide hydrolase, trypsin-like, chymotrypsin-like, branched-chain amino acid-preferring, and small neutral amino acid-preferring activities-within a single proteasome complex. The proteasome 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., 1997) and fatty acids (Wilk and Orlowski, 1983), and heat treatment (Mykles, 1989a,b; Koohmaraie, 1992; Otsuka et al., 1997) and high hydrostatic pressure treatment (Otsuka et al., 1997; Gradrat et al., 1999).

Several researchers have studied the properties of proteasome prepared from various species or organs. Wilk and Orlowski (1983) studied multicatalytic functions on purified bovine pituitary proteasome. Dahlmann et al. (1985) reported that proteasome prepared from rat skeletal muscle was activated by fatty acids or SDS. Tanaka et al. (1986) proposed that the proteasome may play a general role in extralysosomal proteolysis based on its cytocalization. Mykles (1989a,b) reported that the lobster muscle proteasome degraded most myofibrillar proteins. Robert et al. (1999) noticed that when bovine myofibrils were incubated with the liver proteasome, their structure was rapidly damaged with loss of material. However in the report describing the purification and characterization of ovine skeletal muscle proteasome, and the comparision of its effect on ovine myofibrils with μ-calpain, Koomharaie (1992) demonstrated that of all myofibrillar proteins, proteasome degraded only troponin-C, and myosin light chain-2 and -3, and had no detectable effects on the morphological structure of myofibrils. Matsuishi and Okitani (1997) observed that the degradation of myofibrillar proteins by rabbit skeletal muscle proteasome was noticed in the presence of SDS, but not observed in the absence of

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SDS. But Otsuka et al. (1998) concluded that the complete loss of M-line and partial loss of Z-line structure were caused by the rabbit skeletal muscle proteasome in the absence of SDS.

These reports suggest that properties of proteasome are dependent on species or organs.

Incidentally, aging is a very important process for meat industries, especially for beef productions. Now, the availability of intramuscular proteinase for meat aging is well investigated. Especially, cathepsin and calpain are assumed to participate in proteolysis during meat aging (Oulli, 1992; Kim et al., 1995; Sentandreu et al., 2002). However, the role of proteasome on meat aging is not yet fully elucidated.

In addition, lack of research describing bovine skeletal muscle proteasome necessitates studies on bovine skeletal muscle proteasome to compare the properties with other proteasome and to investigate the role of proteasome in aging.

This paper describes the purification and properties of bovine skeletal muscle proteasome in comparison with other species or organs proteasome.

MATERIALS AND METHODS

Purification of proteasome

Proteasome was purified from defrosted bovine shoulder muscle by the method of Otsuka et al. (1997).

Lean meat was excised from the shoulder of a beef carcass two days after slaughter and stored at -20°C. As required, it was tempered overnight in a cold room (4°C).

About 400 g of muscle was homogenized twice for 45 s in 2.5 volumes of buffer A (50 mM Tris-HCl, containing 10 mM EDTA and 10 mM 2-mercaptoethanol, pH 8.3) using a Waring Blender. After centrifugation at 8,000×g for 30 min, the supernatant was filtered through two layers of gauze. The filtrate was salted out between 0 and 45% ammonium sulfate saturation, left at 0°C for 30 min, and then centrifuged at 8,000×g for 30 min. The 0-45% supernatant was subsequently salted out between 45 and 65% ammonium sulfate saturation, left at 0°C for 30 min, and then centrifuged at 8,000×g for 30 min. The precipitate was dissolved in buffer B (40 mM Tris-HCl, containing 10 mM EDTA, 10 mM 2-mercaptoethanol and 100 mM NaCl, pH 7.5). After dialysis against buffer B at 2°C for 48 h, the solution was clarified at 8,000×g for 30 min. The supernatant from this clarification is designated crude proteasome.

The crude proteasome solution was loaded onto DEAE-Sephacel column (2.5 cm×50 cm) equilibrated with the same buffer at flow rate of 0.7 ml min^{-1} and collected in 7.0 ml fractions. Unbound proteins were removed by washing the column with the same buffer B. The bound proteins were eluted with a linear gradient of NaCl from 100 to 400 mM. The fractions with proteasome activity were pooled, concentrated with polyethylene glycol 500,000, adjusted to 500 mM NaCl and loaded onto phenyl Sepharose HP column (1.0 cm×80 cm) equilibarated with 40 mM Tris-HCl buffer (pH 7.5) containing 10 mM EDTA, 10 mM 2-mercaptoethanol and 500 mM NaCl. Under these conditions, the proteasome did not bind to the column, although most of the impurities were retained. The fractions with activity were concentrated and rechromatographed on the phenyl Sepharose HP column under the same conditions as mentioned above. The active fraction from the second chromatograph on the phenyl Sepharose HP column is designated purified proteasome.

Determination of proteasome activities

Proteasome activities were 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), Ala-Ala-Phe-NH-Mec (AAF), and Z-Leu-Leu-Glu-NH-Mec (LLE) purchased from SIGMA (USA) and Boc-Leu-Ser-Thr-Arg-NH- Mec (LSTR) purchased from the Peptide Institute (Japan), were chosen to assay distinct proteolytic activities of the proteasome.

All methylcoumarylamide substrates were stored as 1 mM solutions in N, N- dimethylformamide at -20°C and diluted with water to the 20 μl solution prior to use.

Enzyme solution (50 μl) was mixed with 150 μl of 100 mM Tris-HCl (pH 8.0) and 50 μl of 6 mM DTT. After preincubation at 37°C for 5 min, 50 μl of substrate was added to the reaction mixture. After 60 min, the reaction was stopped by addition of 2.0 ml of 100 mM Tris-HCl and 5% SDS (pH 9.0).

Fluorescence was measured by a HITACHI F 2000 spectrofluorimeter with an excitation wavelength set to 370 nm and emission wavelength set to 480 nm.

Electrophoresis

Polyacrylamide gel electrophoresis (PAGE) under non-denaturing conditions was performed according to the method of Davis (1964) using 5% gel. SDS-PAGE was conducted according to the procedure described by Laemmli (1970) with a slight modification. The electrophoresis was carried out for 1.5 h on slab gels (70 mm×90 mm×1 mm) containing 12.5% polyacrylamide [bisacrylamide/acrylamide, 1:20 (w/w)]. Staining of the gel was carried out by the method of Trinick et al. (1984).

pH dependence of activity

Activity was determined over a pH range of 5.0 to 9.0. For pH 5.0 to 6.5, the buffer used was 100 mM NaH_2PO_4-NaOH and for pH 6.5 to 9.0 was 100 mM Tris-HCl.

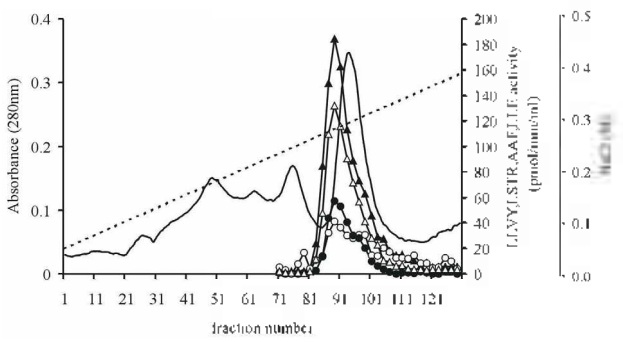

Figure 1. Elution profile resulting after application of the crude proteasome extract to DEAE-Sepharose column.The column (2.5 cm×50 cm) was loaded with crude proteasome, washed with buffer B (40 mM Tris-HCl, 10 mM 2-mercaptoethanol, 100 mM NaCl, pH 7.5), and bound protein was eluted at a flow rate of 42 ml h^{-1} with a linear gradient of NaCl from 100 to 400 mM in buffer B. Fractions of 7 ml were collected. LLVY, LSTR, AAF and LLE hydrolyzing activities were expressed as pmol AMC min^{-1} ml^{-1} released from the substrate. The result shown in the figure is representative of those obtained by repeated experiments for different muscle sample. —, absorbance at 280 nm; •••, NaCl concentration; ●, LLVY hydrolyzing activity; ○, LSTR hydrolyzing activity; ▲, AAF hydrolyzing activity; △, LLE hydrolyzing activity.

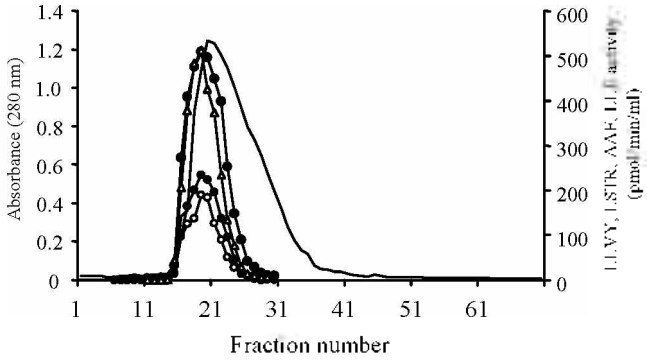

Figure 2. Elution profile resulting from application of the proteasome fraction eluted from DEAE-Sepharose to phenyl Sepharose HP column. The proteasome fraction from the DEAE-Sepharose loaded on the phenyl Sepharose HP column (1.0 cm×80 cm) was eluted with 40 mM Tris-HCl buffer (pH 7.5) containing 10 mM EDTA, 10 mM 2-mercaptoethanol, 500 mM NaCl at a flow rate of 12 ml h^{-1} and collected 2.0 ml fractions. Refer to the legend to Figure 1 for details.

Activation with SDS

The activity of the proteasome in the presence of SDS was determined using 150 µl Tris-HCl buffer (pH 8.0) containing 0 to 0.5% SDS. Otherwise, the assay was conducted using the method outlined in above.

Temperature dependence of activity

The purified proteasome was heated from 37 to 70°C at 30 min. After chilling on ice for 10 min, the remaining activity was measured. And the remaining activity heated at 60°C from 0 to 60 min was also measured after chilling on ice for 10 min.

Effect of bovine proteasome on bovine myofibrils

Myofibrils were made from bovine thigh muscle according to the procedure described by Kimura et al. (1989), and were suspended in buffer (50 mM NaCl, 5 mM EGTA, 1 mM $NaHCO_3$, 1 mM NaN_3, pH 8.2).

Myofibrils were incubated with the purified proteasome (proteasome/myofibrils 1/10, w/w) in 100 mM Tris-HCl pH 8.0, 5 mM EDTA, 100 mM NaCl, 1 mM DTT for 0, 3, 6, and 24 h at 37°C.

The reaction was stopped by addition of equal volume 0.6 M TCA, allowed to stand at room temperature for overnight with shading, and then filterate using TOYO-No.5 filter paper.

The tyrosine contents in the filtrates were measured fluorometrically by the method of Ambrose et al. (1974). Fluorescence was measured by a HITACHI F 2000 spectrofluorimeter with an excitation wavelength set to 460 nm and emission wavelength set to 530 nm.

Electrophoretic pattern and immunoblotting of the materials released from myofibrils

The incubation sample of the myofibrils with proteasome was same to 2.7. The reaction was stopped by addition of 0.1 mM $ZnCl_2$. After centrifugation at 10.000×g for 15 min, supernatant was treated for SDS-PAGE samples (refer to Electrophoresis).

Immunoblotting was performed according to Towbin et al. (1979). After SDS-PAGE, the proteins were transferred from the gel on to a PVDF (polyvinyliden difluoride) membrane (Bio-Rad., USA) with a 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 2% gelatin-PBST (Tween20-phosphate-bufferd saline) solution. The blocked membrane was rinsed with PBS (phosphate-bufferd saline) and then incubated overnight in PBST containing the first antibody (Monoclonal anti-troponin T or Monoclonal anti-tropomyosin (SIGMA)) diluted 1:50 vol/vol (anti-troponin T) or 1:250 vol/vol (anti-tropomyosin) at 25°C. The membrane was then washed three times with PBST. Subsequently, the membrane was incubated in PBST containing a 1:100 (anti-troponin T) or 1:250 (anti-tropomyosin) dilution of Peroxidase Labeled Anti-mouse IgG Goat IgG (Bio-Rad) as second antibody for 90 min at 25°C. After washing three times with PBST and once with PBS, the proteins on the PVDF membranes were stained with 4-chloro-1-Naphthol·H_2O_2 solution (60 mg 4-chloro-1-Naphthol solved 20 ml methanol and added 100 ml PBS and 60 µl 31% H_2O_2).

Table 1. Purification of proteasome

Purification step	Total volume (ml)	Total protein (mg)	Total activity (pmol min)				Specific activity (pmol min mg)				Yield (%)				Purity (-fold)			
			LLVY	LSTR	AAF	LLE	LLVY	LSTR	AAF	LLE	LLVY	LSTR	AAF	LLE	LLVY	LSTR	AAF	LLE
Crude extract	70	2,611.0	4,956.03	4,247.25	9,062.93	6,975.68	1.90	1.63	3.47	2.67	100	100	100	100	1.00	1.00	1.00	1.00
DEAE-sephacel	200	25.6	3,401.10	3,209.75	6,876.35	5,533.30	132.86	125.38	268.61	216.14	68.63	75.57	75.87	75.57	69.99	77.08	77.38	80.90
1st phenyl-sepharose HP	18	12.6	2,318.11	1,632.53	4,941.58	4,805.62	183.98	129.55	392.19	381.40	46.77	38.45	54.53	38.43	96.93	79.64	112.99	142.76
2nd phenyl-sepharose HP	11	6.116	1,524.38	1,021.86	2,747.41	3,351.68	249.24	167.08	449.22	548.02	30.76	24.06	30.32	24.06	131.31	102.71	129.42	205.12

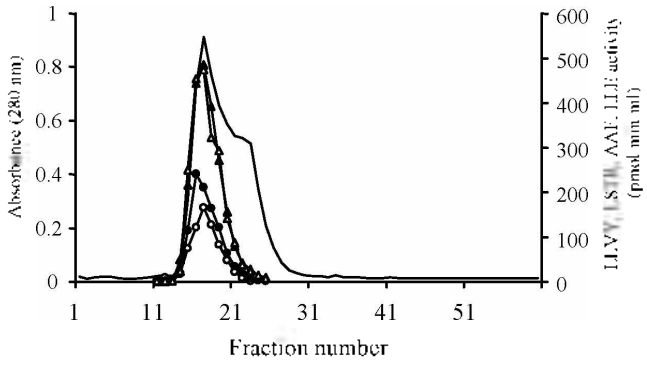

Figure 3. Elution profile resulting from application of the proteasome fraction eluted from the first phenyl Sepharose HP column to the second phenyl Sepharose HP column. The proteasome fraction from the first phenyl Sepharose HP column loaded on the second phenyl Sepharose column was eluted and collected under the same conditions as shown in the Figure 2. Refer to the legend to Figure 1 for details.

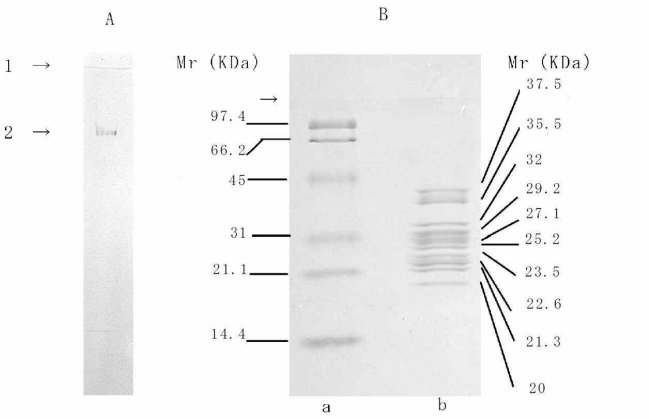

Figure 4. Polyacrylamide gel electrophoresis of purified proteasome. A purified proteasome (3.6 μg) was applied to a 5.0% gel under non-denaturing conditions. Arrow 1 and 2 indicates the origin of separation gel and purified proteasome, respectively. B The molecular mass standards (lane a) and purified proteasome (0.88 μg, lane b) applied to a 12.5% gel under denaturing conditions. The molecular mass standards consist of phosphorylase b (97,400), bovine serum albumin (66,200), ovalbumin (45,000), carbonic anhydrase (31,000), soybean trypsin inhibitor (21,500), lysozyme (14,400). Arrow indicates the origin of separation gel.

RESULTS

Purification and characterization of proteasome

At the first step, crude proteasome extracted from bovine skeletal muscle was purified by chromatography on DEAE-Sephacel column, and LLVY, LSTR, AAF and LLE hydrolyzing activities were measured (Figure 1). Proteasome degrading activities were confirmed from 0.23 to 0.25 M NaCl concentration. These fractions were applied to further purification on phenyl Sepharose HP column. The elution profile and enzyme activities are shown in Figure 2. Proteasome activities to the three substrates emerged in a sharp and almost single peak at the same place. The fractions with activities were concentrated and rechromatographed on phenyl Sepharose HP column. As shown in Figure 3, a single peak with activities to the three substrates was obtained. The proteasome obtained in the present experiment exhibited a single band on PAGE (Figure 4 (a)) and 10 bands ranging from 20,000 to 37,500 Da on SDS-PAGE (Figure 4 (b)). So, this active fraction is designated purified proteasome and used for the characterization of the proteasome.

The date of a typical purification process of proteasome is summarized in Table 1. Finally, hydrolyzing activities representing a characteristic of proteasome were purified about 131 fold (LLVY), 103 fold (LSTR), 129 fold (AAF), and 205 fold (LLE) over the crude extract.

pH dependence of activity

The effects of pH on the proteasome activities are shown in Figure 5. The relative activity is expressed as a percentage of the activity at pH 8.0. All synthetic peptides used in the present experiment were degraded maximally at pH 8.0. However, proteasome activities gradually decreased with the lowering of pH. The degrees of the decrease were substrate-dependent, and activities at pH 5.5 still retained about 78 to 10% of activity at pH 8.0. The decrease in LLVY and LLE degrading activity were slower than LSTR and AAF degrading activities.

Activation with SDS

The effect of SDS on proteasome activity is shown in Figure 6. The relative activity is expressed as a percentage of the activity of control (0% SDS). LLVY and LSTR hydrolyzing activities remarkably increased with increasing SDS concentration up to 0.0125%, then rapidly decreased over 0.0125%. At 0.0125% of SDS, LLVY, LSTR and LLE hydrolyzing activity reached 3.1, 4.7 and 3.2 times of control, respectively. The activation of AAF hydrolyzing activity was small as compared with them (1.2 times of control).

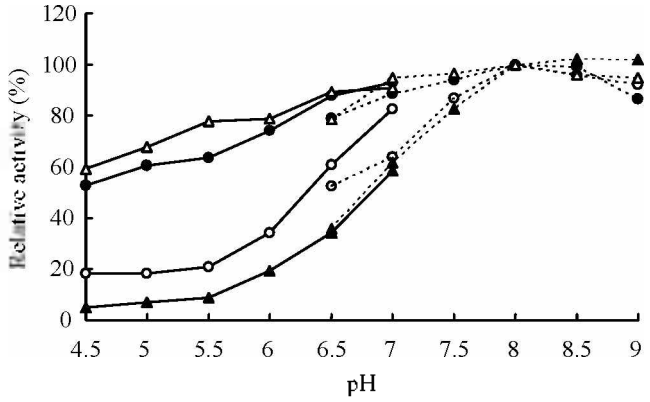

Figure 5. Effect of pH on the activity of purified proteasome. Relative activity was expressed as a percentage of the activity at pH 8.0. The result shown in the figure is representative of those obtained by repeated experiments for different muscle samples. –, 100 mM NaH_2PO_4-NaOH buffer; •••, 100 mM Tris-HCl buffer; ●, LLVY hydrolyzing activity; ○, LSTR hydrolyzing activity; ▲, AAF hydrolyzing activity; Δ, LLE hydrolyzing activity.

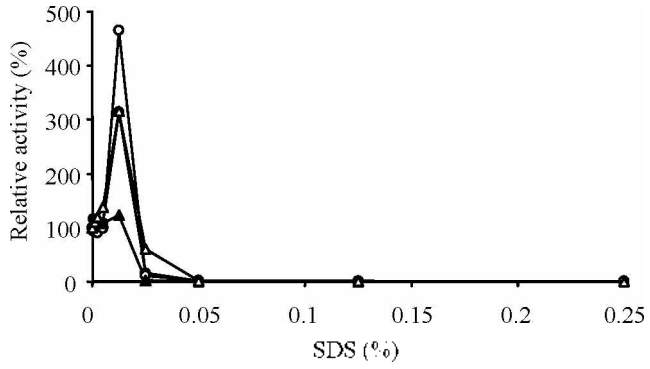

Figure 6. Effect of SDS on the activity of purified proteasome. Relative activity was expressed as a percentage of the activity of the untreated ●, LLVY hydrolyzing activity; ○, LSTR hydrolyzing activity; ▲, AAF hydrolyzing activity; △, LLE hydrolyzing activity.

Temperature dependence of activity

The changes in the proteasome activities heated from 37°C to 70°C are shown in Figure 7. The relative activity is expressed as a percentage to that of the untreated (37°C). LLVY, LSTR and LLE hydrolyzing activities increased with heating up to 60°C, and reached 1.8, 1.6 and 1.3 times of the control, respectively at 60°C. However, change in AAF hydrolyzing activity by heat was not observed.

The changes in the proteasomoe activities heated at 60°C from 0 to 60 min are shown in Figure 8. Relative activities are expressed as a percentage to that of the untreated (0 min). LLVY, LLE hydrolyzing activities gradually increased with increasing heat treatment up to 30 or 15 min, and reached 1.8, 1.4 and 1.6 times of control, respectively, but AAF hydrolyzing activity was not activated for heat treatment at all.

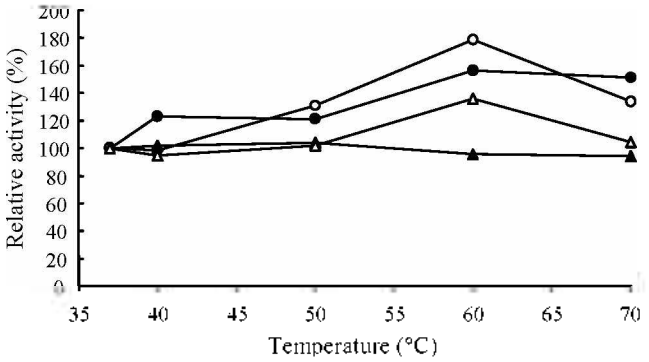

Figure 7. Effect of heating treatment on the activity of purified proteasome. Relative activity was expressed as a percentage of the activity of the untreated. ●, LLVY hydrolyzing activity; ○, LSTR hydrolyzing activity; ▲, AAF hydrolyzing activity; △, LLE hydrolyzing activity.

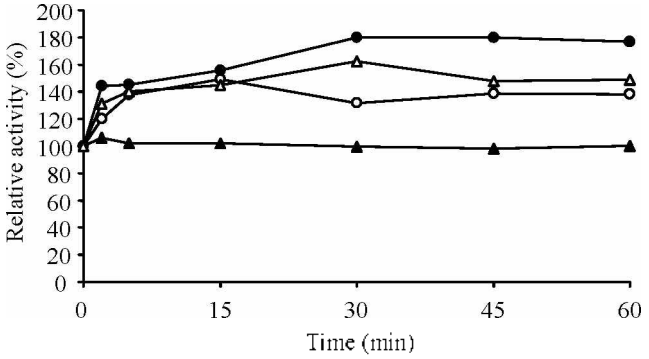

Figure 8. Changes in the proteasome activity heated at 60°C. Relative activity was expressed as a percentage of the activity of the untreated. ●, LLVY hydrolyzing activity; ○, LSTR hydrolyzing activity; ▲, AAF hydrolyzing activity; △, LLE hydrolyzing activity.

Effect of proteasome on myofibrillar structure

The flourometric measurement of tyrosine contents in the filtrates from incubated myofibrils with proteasome is shown in Figure 9. The amount of tyrosine contents in the filtrates almost linearly increased with increasing time of incubation.

The SDS-PAGE pattern and immunoblotting of the materials released from myofibrils are shown in Figure 10 (a), (b), (c). In the Figure 10 (a), it was difficult to see the changes in muscle proteins resulting from incubation with proteasome compared to incubation without proteasome. However, from the immunoblotting, the released troponin-T seemed to be further degraded with the increase of incubation time (Figure 10 (b)), while the released tropomyosin seemed not to be degraded (Figure 10 (c)).

DISCUSSION

The proteasome obtained in the present experiment exhibited a single band on PAGE (Figure 4 (A)) and 10

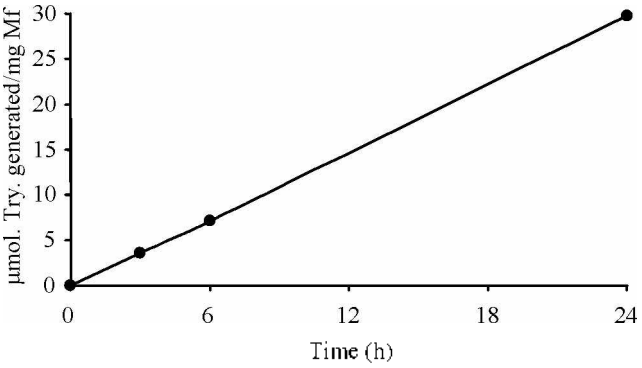

Figure 9. The flourometric measurement of tyrosine contents in the filtrates from incubated myofibrils with proteasome. •, Tyrosine contents in the filtrates from incubated myofibrils with proteasome.

bands ranging from 20,000 to 37,500 Da on SDS-PAGE (Figure 4 (B)-b), indicating that the enzyme comprises multiple hetero subunits with low molecular mass. This result was corresponded with Matsuishi and Okitani (1997; rabbit skeletal muscle) and Rivett (1993). However, 40,000 and 42,000 Da bands that reported by Otsuka (1997; rabbit skeletal muscle) were not observed in our experiment. Koohmaraie (1992; ovine skeletal muscle) claimed that subunit with molecular mass of about 42,000 Da was not the constituent of proteasome on the basis of the immunoblot analysis. Therefore, the 40,000 and 42,000 Da bands may be an ancillary protein, suggesting incomplete purification of the enzyme.

In the present experiment, the proteasome was purified from postmortem muscle in which ATP was not retained. And the proteasome obtained in the present experiment did not exhibit any bands ranging over 40 kDa subunit on SDS-PAGE. If the proteasome was 26S, subunit bands of over 40 kDa should be observed (Akashi et al., 1996; Hoffman et al., 1999). These results indicate that the proteasome shown in this paper is not 26S but 20S.

The effect of pH on the activity obtained in this experiment (Figure 5) corresponded with the previous reports (Tanaka et al., 1986; Koomaraie, 1992 for ovine skeletal muscle; Ugai et al., 1993 for rat liver; Matsuishi and Okitani, 1997; Otsuka et al., 1998 for rabbit skeletal muscle). The LLVY, LSTR, AAF and LLE degrading activities gradually decreased with the lowering of pH, but the extent of decrease was substrate-dependent, and activities at pH 5.5 still retained about 78 to 10% of the activity at pH 8.0. Matsuishi and Okitani (1997) reported that the rabbit skeletal muscle proteasome was stable at pH 5.0 to 9.0 and retained 80% of the optimal activity in postmortem pH range (pH 5.5 to 5.7). The proteolytic activity in the postmortem pH range of the proteasome obtained in the present report is lower than that of the proteasome reported by Matusishi and Okitani (1997). As compared with their proteasome, the activity of the proteasome obtained in this report is lower in the postmortem pH range, but still retained 78 to 10% of the activity at optimal pH. This result was in agreement with the report by Otsuka et al. (1998), describing that rabbit skeletal proteasome retained 60 to 30% of the maximum activity at postmortem pH range. It was well known that the pH of muscle tissue dropped from 7.0 to the ultimate pH of around 5.5 in postmortem. Therefore, the results obtained indicate the possibility that the bovine muscle proteasome is active in the muscle during aging.

Concerning the effect of SDS on the activity of the proteasome obtained in this experiment, LLVY, LSTR and LLE hydrolyzing activities remarkably increased with increasing SDS concentration up to 0.0125%, then rapidly decreased over 0.0125%. Other studies reported that the activities of proteasome were maximum at 0.03% on rat skeletal muscle (Dahlmann et al., 1985), at 0.02% on bovine pituitary (Wilk and Orlowski, 1983) and at 0.05% on rat liver (Tanaka et al., 1986). On the other hand, Otsuka et al. (1998) reported that the activity of rabbit skeletal muscle proteasome increased with SDS concentration up to 0.005%, then rapidly decreased over 0.005%. These differences in optimal SDS concentration may be caused by the differences of the proteasome preparations, depending on species, organs and purification method. Anyway, all reports exhibited that the proteasome activities were increased by mild SDS treatment.

In the present experiment, LLVY, LSTR and LLE hydrolyzing activities increased with heating up to 60°C, and reached 1.8, 1.6, 1.3 times of the control, respectively at 60°C. And LLVY, LSTR and LLE hydrolyzing activities gradually increased with increasing heat treatment up to 30 or 15 min, and reached 1.8, 1.4 and 1.6 times of control, respectively. Wagner and Margolis (1993) reported that the bovine lens proteasome lost 60% of the activity, when it was pre-incubated at 53°C for 10 min. Mykles (1989 a,b) reported that maximum stimulation occurred within 1 to 2 min at 60°C and heating beyond 2 min resulted in rapid loss of the activity of lobster muscle proteasome. Koohmaraie (1992) obtained the same results with Mykles's using ovine skeletal muscle proteasome. Our results are not the same as the results of those reports. The discrepancy in the results was probably caused by the differences in the substrates, enzyme preparations, species, organs and purification methods. However, the present result is in agreement with the idea that the proteasome is activated by heat treatment, because the proteasome was extracted from muscle in a latent state (Tanaka et al., 1986; Mykles, 1989a,b; Matsuishi and Okitani, 1997).

As shown in Figure 9, the tyrosine contents in the filtrates from incubated myofibrils with the bovine muscle proteasome, increased with the increase in the incubation time.

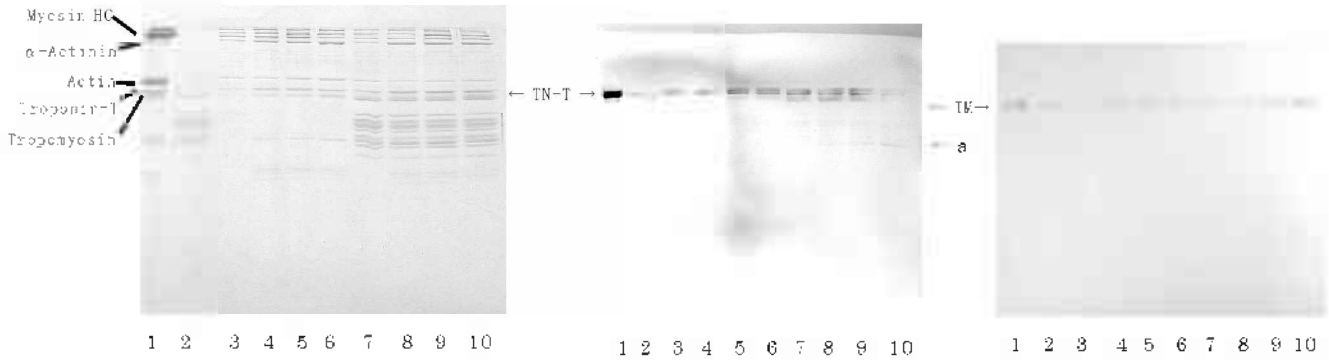

Figure 10. The SDS-PAGE pattern and immunoblotting of the materials released from myofibrils (a) SDS-PAGE of the materials released from myofibrils incubated with proteasome applied to a 12.5% gel under denaturing conditions. Lane 1: standard myofibrils. Lanes 2: purified proteasome. Lanes 3 to 6: myofibrils incubated for 0, 3, 6 and 24 h respectively. Lanes 7 to 10: myofibrils incubated with purified proteasome for 0, 3, 6 and 24 h respectively (b) The immunoblotting of the materials released from myofibrils incubated with proteasome using anti troponin-T antibody. Lane 1: standard myofibrils. Lanes 2. purified proteasome. Lanes 3 to 6: myofibrils incubated for 0, 3, 6 and 24 h respectively Lanes 7 to 10: myofibrils incubated with purified proteasome for 0, 3, 6 and 24 h respectively (c) The immunoblotting of the materials released from myofibrils incubated with proteasome using anti tropomyosina antibody Lane 1: standard myofibrils. Lanes 2: purified proteasome. Lanes 3 to 6. myofibrils incubated for 0, 3, 6 and 24 h respectively. Lanes 7 to 10: myofibrils incubated with purified proteasome for 0, 3, 6 and 24 h respectively.

From the immunoblotting analysis (Figure 10 (b), (c)), the release of the troponin-T and tropomyosin from the myofibrils seems to be accelerated with the progress of incubation.

Mykles (1989a.b) reported that proteasome prepared from lobster muscle degraded most myofibrillar proteins, especially paramyosin, troponin-I and -C, and myosin α light chain on the basis of the SDS-PAGE analysis. On the other hand, Koohmaraie (1992) reported that myofibrils were a very poor substrate for proteasome and of all myofibrillar proteins, only troponin-C and myosin light chain-2 and -3 were degraded by proteasome from the results of the SDS-PAGE analysis. No difference in the ultrastructure between proteasome-treated and -untreated myofibrils was observed in the electron micrograph in the paper by Koomaraie (1992). Taylor et al. (1995) claimed that bovine liver proteasome caused significant changes in bovine myofibrillar structure incubated at 37°C for 24 h in 50 mM Tris buffer (pH 7.4), 100 mM KCl and 0.1% 2-mercaptoethanol. They reported that actin, myosin and desmine were degraded faster than troponin-T, tropomyosin and α-actinin, and changes in the ultrastructure were slow and included a general loss of structure with Z and I bands effected before the M band and costameres. However, it is uncertain whether the changes in myofibrillar structure were induced by proteasome or not, because the condition used in their experiment could not inhibit the calpain activity possibly bound to the myofibrils. Matsuishi and Okitani (1997) reported that the proteasome prepared from rabbit skeletal muscle caused the degradation of myosin heavy chain, α-actinin, actin, tropomyosin, troponins and myosin light chain in the presence of SDS. However, in the absence of SDS, no changes in myofibrillar proteins were obserbed in SDS-PAGE profile. On the other hand, Otsuka et al. (1998) revealed that obvious gaps between filamentous structures, the complete loss of M-line and partial loss of Z-line structure were caused by rabbit skeletal muscle proteasome. Robert et al. (1998) reported that when bovine myofibrils were incubated with bovine liver proteasome, their structure was rapidly damaged with loss of material, particularly from the Z discs and I bands. After 24 h of incubation, the myofibrils rupture and scraps appeared. Certain myofibrillar proteins, including nebline, myosin, actin and tropomyosin, were hydrolyzed during the incubation. And α-actinin was solubilized.

Our results were not necessarily in complete agreement with their results. The release and further degradation of troponin-T shown in our results were not mentioned in their report. However, from the results obtained in the present experiment and in other experiments (Taylor et al., 1995; Otsuka et al., 1998; Robert et al., 1999), it is presumed that proteasome is able to cause the degradation of myofibrils in the absence of SDS and may play a role on the postmortem change of muscle in meat during aging.

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REFERENCES

Akashi, T., T. Shiomi, H. Sawada and H. Yokosawa. 1996. Purification and properties of the 26S proteasome from the rat

brain: Evidence for its degradation of myelin basic protein in a ubiquitin-dependent manner. Brain Research 722:139-144.

Dahlmann, B., M. Rutschmann, L. Kuehn and H. Reinauer. 1985. Activation of the multicatalytic proteinase from rat skeletal muscle by fatty acid or sodium dodecyl sulphate. Biochem. J. 228:171-177.

Davis, B. J. 1964. Disk electrophoresis-II. Method and application to human serum protein. Annals of the New York Academy of Sci. 121:404-427.

Gardrat, F., B. Fraigneau, V. Montel, J. Raymond and J. L. Azanza. 1999. Effect of high hydrostatic pressure on 20S proteasome activity. Eur. J. Biochem. 262:900-906.

Hoffman, L., C. Gorbea and M. Rechsteiner. 1999. Identification, molecular cloning, and characterization of subunit 11 of the human 26S proteasome. FEBS Lett. 449:88-92.

Kanayama, H., T. Tamura, S. Ugai, S. Kagawa, N. Tanahashi, T. Yoshimura, K. Tanaka and A. Ichihara. 1992. Demonstration that a human 26S proteolytic complex consists of a proteasome and multiple associated protein components and hydrolyzes ATP and ubiquitin-ligated proteins by closely linked mechanisms. Eur. J. Biochem. 206:567-578.

Kim, K., Y. Ikeuchi and A. Suzuki. 1995. Cleavage of connectin by calpain and cathepsin D. Biosci. Biotech. Biochem. 59:896-899.

Kimura, S. and K. Maruyama. 1989. Isolation of α-connectin, an elastic protein, from rabbit skeletal muscle. J. Biochem. 106:952-954.

Koohmaraie, M. 1992. Ovine skeletal muscle multicatalytic proteinase complex (proteasome): purification, characterization and comparision of its effects on myofibrils with μ-calpains. J. Anim. Sci. 70:3697-3708.

Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage. Nature 227:680-685.

Matsuishi, M. and A. Okitani. 1997. Proteasome from rabbit skeletal muscle: Some properties and effect on muscle proteins. Meat Sci. 45:451-462.

Mellgren, R. L. 1990. Interaction of human erythrocyte multicatalytic proteinase with polycations. Biochimica et Biophysica Acta. 995:181-186.

Mykles, D. L. 1989a. High-molecular-weight serine proteinase from lobster muscle that degrades myofibrillar proteins. J. Exp. Zool. 250:244-252.

Mykles, D. L. 1989b. Purification and characterization of multicatalytic proteinase from crustacean muscle: comparison of latent and heat-activated forms. Arch. Biochem. Biophys. 274:216-228.

Mykles, D. L. and M. F. Haire. 1995. Branched-chain-amino-acid-preferring peptidase activity of the lobster multicatalytic proteinase (proteasome) and the degradation of myofibrillar proteins. Biochem. J. 306:285-291.

Orlowski, M. and S. Wilk. 1981. A multicatalytic proteinase complex from pituitary that froms enkephalin and enkephalin containing peptides. Biochem. Biophys. Res. Comm. 101:814-822.

Orlowski, M. 1990. The multicatalytic proteinase complex, a major extralysosomal proteolytic system. Biochemistry. 29:10289-10297.

Otsuka, Y., N. Homma, K. Shiga, J. Ushiki, Y. Ikeuchi and A. Suzuki. 1998. Purification and properties of rabbit muscle proteasome, and its effect on myofibrillar structure. Meat Sci. 49:365-378.

Ouali, A. 1992. Proteolytic and physicochemical mechanisms involved in meat texture development. Biochinie. 74:251-265.

Rivett, A. J. 1989a. The multicatalytic proteinase of mammalian cells. Arch. Biochem. Biophys. 268:1-8.

Rivett, A. J. 1989b. The multicatalytic proteinase. J. Biolog. Chem. 264:12215-12219.

Rivett, A. J. 1993. Proteasome: multicatalytic proteinase complexs. Biochem. J. 291:1-10.

Robert, N., M. Briand, R. Taylor and Y. Briand. 1999. The effect of proteasome on myofibrillar structures in bovine skeletal muscle. Meat Sci. 51:149-153.

Sentandreu, M. A., G. Coulis and A. Ouali. 2002. Role of muscle endopeptidases and their inhibitors in meat tenderness. Trends Food Sci. Tech. 13:398-419.

Tanaka, K., K. Ii, A. Ichihara, L. Waxman and A. L. Goldberg. 1986. A high molecular weight proteinase in the cytosol of rat liver. J. Biol. Chem. 261:15197-15203.

Taylor, R. G., C. Tassy, M. Briand, N. Robert, Y. Briand and A. Ouali. 1995. Proteolytic activity of proteinase on myofibrillar structure. Mol. Biol. Rep. 21:71-73.

Towbin, H., T. Staehelin and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheet: Procedure and some applications. Proc. Natl. Acad. Sci. USA. 76:4350-4354.

Trinick, J., P. Knight and A. Whiting. 1984. Purification and properties of native titin. J. Mol. Biol. 180:331-356.

Wagner, B. J. and J. W. Margolis. 1993. Thermal stability and activation of bovine lens multicatalytic proteinase complex (proteasome). Arch. Biochem. Biophys. 307:146-152.

Wilk, S. and M. Orlowski. 1983. Evidence that pituitary cation-sensitive neutral endopeptidase is a multicatalytic proteinase complex. J. Neurochem. 40:842-849.