Some Properties and Postmortem Changes of Cysteine Protease Inhibitors from Rabbit Skeletal Muscles

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Rabbit skeletal muscles were demonstrated to contain several inhibitors of cysteine proteases, cathepsins B and L, and papain.

Sephadex G-100 column chromatography exhibited two inhibitor peaks at the positions corresponding to 9,500 and 52,000 daltons, which commonly inhibited the above three cysteine proteases. There were two other peaks (corresponding to 21,000 and 28,000 daltons) inhibiting papain, one peak (25,000 daltons) inhibiting cathepsin B and one peak (36,000 daltons) inhibiting cathepsin L.

Muscle extracts contain an unknown factor which can degrade the inhibitors during incubation at pH 4.0 and 37°C. This factor is not cathepsin D.

Total inhibitor activities slightly changed on 9-day storage of rabbit skeletal muscle at 4°C, while the extractable activity of cathepsin L increased and that of cathepsin B slightly decreased.

In a previous work¹⁾ we demonstrated the occurrence of a cysteine protease in rabbit skeletal muscles which is capable of degrading myofibrillar proteins and identified it as cathepsin L. Cathepsin L seems to have an important role in myofibrillar protein turnover, since it is able to hydrolyze myosin, actin, tropomyosin, and α -actinin.²⁾ Cathepsin L also seems to make more of a contribution to postmortem proteolysis of muscle compared with cathepsin D³⁾ and calpain,⁴⁾ since the optimum pH of its activity (pH 4~5) is closer to the pH of the postmortem muscle (pH 5.5) than the optimum pH of the other two proteases are.

There are many papers describing the purification of cysteine protease inhibitors from various animal tissues including skin,^{5~8,11)} liver,^{9,10,12,13)} spleen,¹⁴⁾ kidney,¹²⁾ and lung.^{11,12)} Järvinen¹¹⁾ demonstrated the occurrence of two papain inhibitors showing Mw of 13,400 and 74,000 respectively, in rat skeletal muscles. Schwartz and Bird¹⁵⁾ also demonstrated the occurrence of two cathepsin B inhibitors showing Mw of 12,500 and

62,000 respectively, in rat skeletal muscles. In the present study, we surveyed cathepsin L inhibitors in rabbit skeletal muscles and examined whether or not they were identical with the reported inhibitors against papain and cathepsin B. We also examined the change in the inhibitor activity during storage of the rabbit skeletal muscles and discussed the extent of contribution of cathepsin L to postmortem proteolysis of the muscles.

MATERIALS AND METHODS

Muscles. Rabbit muscles (longissimus dorsi) were removed from carcasses immediately after slaughter. Some blocks were used as 0-day muscles and the other blocks were stored for up to 9 days at 4°C in polyethylene bags after being sprayed with 1 mM NaN₃. The muscle blocks were used after mincing with a meat grinder.

Chemicals. Sephadex G-100 was purchased from Pharmacia Fine Chemicals, Sweden. Bovin serum albumin, chymotrypsinogen, and cytochrome *c* were purchased from Boehringer Mannheim GmbH, FRG.

BANA,* papain, and bovine trypsin were obtained from Sigma Chemical Co., U.S.A. Leupeptin and pepstatin A were purchased from the Peptide Institute, Osaka. TLCK and PMSF were obtained from Nakarai Chemicals Ltd., Kyoto. TPCK was obtained from Seikagaku Kogyo Co., Tokyo.

Preparation of cathepsin B. The enzyme was prepared by the method shown in Fig. 1 from both fresh and stored muscles. The precipitates between 25 and 65% ammonium sulfate saturation (P25~65) were dissolved in 10 mM sodium acetate buffer (pH 5.0)/0.1 M NaCl/0.1% MCE and dialyzed against the same solution. The dialysate was used as the cathepsin B preparation after it had been centrifuged at $7000 \times g$ for 20 min to remove insoluble materials.

Preparation of cathepsin L. The enzyme was prepared by the method reported previously.¹¹ Minced muscle was homogenized with two volumes of 15 mM HCl containing 3% NaCl. The pH of the homogenate was adjusted to 3.7 with 1 N HCl and stirred for 2 hr. The homogenate was then centrifuged at $6,500 \times g$ for 15 min. The supernatant obtained was fractionated with ammonium sulfate. The precipitate between 25 and 65% saturation was collected and dissolved in 10 mM sodium acetate buffer (pH 5.0)/0.1 M NaCl/0.1% MCE and then dialyzed against the same solution. The dialysate was used as the cathepsin L preparation after it had been centrifuged at $7,000 \times g$ for 20 min to remove insoluble materials.

Preparation of myofibrils. Myofibrils were prepared as described by Yang *et al.*¹⁶ from fresh muscle.

Assay of cathepsin B and its inhibitors. The enzyme was incubated with 0.5 mM BANA at 37°C in 50 mM potassium phosphate buffer (pH 6.2)/0.4 mM EDTA/0.3 mM DTT. After 1 hr a half volume of 0.23 N HCl in ethanol and a half volume of 0.06% *p*-dimethylaminocinnamaldehyde in ethanol were added to the incubated mixture. The developed red color was measured at 540 nm.¹⁷ One unit of cathepsin B activity was defined as the amount that hydrolyzed 1 μ mol of substrate/min. Assay of the inhibitors was done under the same conditions as above in the presence of about 10 mU/ml cathepsin B prepared from fresh muscles. One unit of cathepsin B inhibitor was defined as the amount which depressed the enzyme activity by 1 unit.

Assay of cathepsin L and its inhibitors. Cathepsin L activity was assayed by the measurement of its Ca-sensitivity-removing activity towards myofibrils, essen-

tially according to the method described in the previous paper.¹¹ Myofibrils (1 mg/ml) was incubated with cathepsin L in 10 mM potassium citrate buffer (pH 5.0)/0.3 mM DTT/1 mM EDTA/0.1 mM pepstatin at 25°C for 2 hr. The reaction was stopped by the addition with 6.5 volumes of 30 mM Tris-HCl buffer (pH 8.0)/77 mM KCl/3 mM MgCl₂. The ATPase activity of cathepsin L-treated myofibrils was assayed by incubation with 1 mM ATP in 20 mM Tris-HCl buffer (pH 7.8)/2 mM MgCl₂/50 mM KCl at 25°C for 15 min in the presence of 1 mM GEDTA or 0.1 mM CaCl₂. The reaction was stopped by the addition of trichloroacetic acid (final concentration, 4%). Liberated phosphorus was determined by the method of Nakamura.¹⁸ The amount which reduced the Ca-sensitivity of 0.4 mg of myofibrils by 1% per 1 hr was expressed as one unit of cathepsin L activity. Assay of the inhibitors was done under the same conditions as above in the presence of about 100 U/ml of cathepsin L prepared from fresh muscles. One unit of cathepsin L inhibitor was defined as the amount inhibiting 1 unit of cathepsin L activity.

Assay of papain and its inhibitors. Papain (1.5 μ g/ml) was pre-incubated with 50 mM Tris-HCl buffer (pH 7.5)/2 mM EDTA/1 mM DTT at 37°C for 30 min and then assayed with 0.5 mM BANA in the same solution as above at 37°C for 15 min in the presence or absence of the inhibitors. Liberated 2-naphthylamine was determined by the same method used for the cathepsin B assay.¹⁷ One unit of papain activity was defined as the amount that hydrolyzed 1 μ mol of substrate/min. One unit of the inhibitor was defined as the amount inhibiting 1 unit of papain activity.

Assay of trypsin. Trypsin (7.5 μ g/ml) was incubated with 0.5 mM BANA in 0.1 M Tris-HCl buffer (pH 7.2) at 37°C for 15 min. After incubation, the liberated 2-naphthylamine was determined by the same method used for the cathepsin B assay.¹⁷

Molecular weight determination. Molecular weights of the inhibitors were estimated by gel permeation chromatography using a Sephadex G-100 column (2 $\times$ 44 cm) equilibrated with 10 mM Tris-HCl buffer (pH 7.2)/0.1 M NaCl/1 mM NaN₃. Bovine serum albumin (Mw=67,000), ovalbumin (Mw=45,000), α -chymotrypsinogen (Mw=25,000), and cytochrome *c* (Mw=12,500) were used as marker proteins.

Protein concentration. Protein concentration were determined by the biuret method¹⁹ using bovine serum albumin as a standard.

* The following abbreviations are used: BANA, α -N-benzoyl-DL-arginine-2-naphthylamide; TLCK, *N*- α -*p*-tosyl-L-lysine chloromethylketone HCl; PMSF, phenylmethane sulfonyl fluoride; TPCK, L-1-tosylamide-2-phenylethylchloromethylketone; MCE, 2-mercaptoethanol; DTT, dithiothreitol; EDTA, ethylenediamine tetraacetic acid; GEDTA, glycoetherdiamine tetraacetic acid.

RESULTS AND DISCUSSION

Preparation of the inhibitors

Our previous study¹⁾ showed that muscle cathepsins B and L were obtained from the supernatant when muscle homogenates were centrifuged at pH 3.7. When the resultant residue was extracted with Tris-HCl buffer of pH 7.2, the extract showed inhibitor activity toward cathepsins B and L. It was thus concluded that muscle cysteine protease inhibitors were soluble at pH 7.2 and precipitable at pH 3.7.

To avoid a probable denaturation in acid regions we tried to extract the inhibitors directly from muscles with a neutral extractant. As shown in Fig. 1, muscle was extracted with 40 mM Tris-HCl buffer (pH 7.2) and the extract obtained was centrifuged after its pH was adjusted to 3.7. The resultant supernatant was shown to contain cathepsins B and L by the assay of the fraction (P_{25-65}) salted out between 25 and 65% ammonium sulfate satura-

tion.

On the other hand, the resultant precipitate was mixed with 10 mM Tris-HCl buffer (pH 7.2) using a Waring blender. The mixture was centrifuged after its pH was adjusted to 7.2 with 1 N NaOH.

The supernatant obtained showed inhibitory activity towards cathepsins B and L. Thus, it was indicated that the method shown in Fig. 1 was effective in preparing muscle cysteine protease inhibitors. That crude preparation of the inhibitors was used for further studies after dialysis against 10 mM Tris-HCl buffer (pH 7.2) and then clarification by centrifuge. Representative data for the yields of proteins and activities from 100 g muscles are also shown in Fig. 1.

Distribution of molecular weights of the inhibitors

The crude preparation of the inhibitors was subjected to Sephadex G-100 column chromatography to examine how many inhibitors of

FIG. 1. Preparation Scheme for Cysteine Proteases and Their Inhibitors from Rabbit Skeletal Muscles.

The protein and activity yields of the crude cathepsins B and L and their inhibitors are also presented. A_B , total activity of cathepsin B; A_L , total activity of cathepsin L; I_B , total inhibitor activity against cathepsin B; I_L , total inhibitor activity against cathepsin L; P_{25-65} , the precipitate between 25 and 65% ammonium sulfate saturation.

FIG. 2. Sephadex G-100 Column Chromatography of Cysteine Protease Inhibitors from Rabbit Skeletal Muscles.

A crude preparation of the inhibitors (16.8 mg protein, 6 ml) was applied to a column (2 × 46.5 cm) equilibrated with 10 mM Tris-HCl buffer/0.1 M NaCl (pH 7.2). Fractions of 3 ml were collected. The inhibitors were assayed as described in MATERIALS AND METHODS. -----, absorbance at 280 nm; Δ, inhibitor activity against papain; □, inhibitor activity against cathepsin L; ○, inhibitor activity against cathepsin B.

different molecular weights could be found. Inhibitory activities towards cathepsins B and L and papain were located in the eluted fractions (Fig. 2).

At a position corresponding to a molecular weight of 9,500, we detected a large peak which showed inhibitory activities towards all of the three enzymes. At a position corresponding to a molecular weight of 52,000 occurred another peak which showed inhibitory activity towards papain and cathepsin L. The fractions at that position also exhibited inhibitory activity towards cathepsin B, although the peak of the activity was not clearly

separated from the other peak eluting later. Between the two peaks corresponding to 9,500 and 52,000 daltons there were two peaks (21,000 and 28,000 daltons) of inhibitory activity towards papain, one peak (25,000 daltons) towards cathepsin B, and one peak (36,000 daltons) towards cathepsin L.

The results suggest that rabbit skeletal muscles contain at least two inhibitors of 9,500 and 52,000 daltons, respectively, which commonly inhibit three cysteine proteases, cathepsins B and L and papain, and some other inhibitors each of which is specific to the individual enzyme. The inhibitors of 9,500 and

52,000 daltons might correspond to the two cathepsin B inhibitors ($M_w=12,500$ and $62,000$) of rat skeletal muscle reported by Schwartz and Bird.¹⁵⁾

Some properties of the inhibitors

It is known that spleen and leucocytes contain an inhibitor, sericystatin,^{14,20)} which inhibits both cysteine proteases and serine proteases.

On the other hand, it was found that the crude inhibitor preparation from skeletal muscles did not inhibit a serine protease, trypsin, but enhanced it by about 60% (Fig. 3). Therefore, it could be concluded that muscle cysteine protease inhibitors did not fall in the category of sericystatin.

The thermostability of the crude cysteine protease inhibitors was examined. In 10 mM Tris-HCl buffer of pH 7.2, the inhibitory activity towards both cathepsins B and L was not changed by a 10-min incubation at 50°C. A 10-min incubation at 60°C destroyed about

50% of the activity and a 10-min incubation at 80°C caused a complete loss of the activity. Lenney *et al.*¹²⁾ reported that rat lung cathepsin B inhibitor retained 40% of its activity on a 10-min incubation at 80°C (pH 7.0). Thus, the muscle cathepsin B inhibitors seemed to be less heat-stable than the corresponding inhibitor in rat lung.

The pH-stability of the crude cysteine protease inhibitors was examined by measurement of their activity towards papain (Fig. 4). A 60°C-treatment for 10 min caused a greater inactivation in neutral regions than in acid regions. However, a 50°C-treatment for 10 min caused less inactivation in neutral regions than in acid regions. The inactivation in acid regions was magnified by 37°C-treatment for 1 hr. This treatment brought about a 70% loss of activity at pH 4.0 but nothing at pH 7.0. These results strongly suggested the presence of a protease in the crude inhibitor preparation capable of degrading the cysteine protease inhibitors in acidic conditions. Schwartz and Bird¹⁵⁾ reported that when a rat skeletal muscle extract were kept at 25°C and pH 4.5 for

FIG. 3. Effect of a Crude Preparation of Cysteine Protease Inhibitors from Rabbit Skeletal Muscles on the Activity of Trypsin.

Trypsin (7.5 μg/ml) was assayed using BANA as substrate in the presence of varying amounts of an inhibitor preparation. The inhibitor preparation used showed no hydrolytic activity towards BANA.

FIG. 4. pH-Stability of Crude Cysteine Protease Inhibitors from Rabbit Skeletal Muscles at Three Different Temperatures.

Inhibitor preparations (3 mU activity against papain/ml) were kept at 50°C for 10 min (○), at 60°C for 10 min (△), or at 37°C for 1 hr (□) in 50 mM sodium acetate buffer of various pH values/0.1 M NaCl. After treatment the remaining activity against papain was measured.

20 hr, cathepsin B activity in it was increased by 6 times. Our present result suggests that such a phenomenon is also due to the decomposition of inhibitors by a co-existing protease. The data for pH-stability obtained by the 60°C-treatment for 10 min seemed to be less influenced by the inhibitor-degrading protease, because such treatment might inactivate it. Hence, the inhibitors themselves are assumed to be more stable in acid regions than in neutral regions, if they are isolated from contaminants. However, the crude inhibitor preparation should be kept at a neutral pH, as indicated by the present result. Thus, it is elucidated that on preparation of muscle cysteine protease inhibitors, it is better to extract them directly from muscles with a neutral solution, as shown in Fig. 1, than to extract them from the residue which is obtained after cathepsins B and L were removed by a long (2 hr) extraction at pH 3.7.

Characterization of a factor degrading the inhibitors

We examined effects of various protease inhibitors on the inactivation of the muscle cysteine protease inhibitors during the incubation of the crude inhibitor preparation at pH 4.0 and 37°C. Under the same condition as in Fig. 4, the addition of PMSF (final concentration; 1 mM), EDTA (1 mM), TLCK (0.1 mM), TPCK (0.1 mM) or pepstatin (0.1 mM) showed no effect. Thus, the enzyme supposed to decompose the muscle cysteine protease inhibitors was indicated not to be cathepsin D. The result also indicated a low possibility for it being a metallo- or serine-protease. It may be a cysteine protease which is not inhibited by the cysteine protease inhibitors dealt with at the present study. We are now making an effort to purify the inhibitor-degrading enzyme.

Postmortem changes of the inhibitors

We examined whether the cysteine protease inhibitors were inactivated in postmortem muscles.

Crude inhibitor preparations were obtained

FIG. 5. Changes in Total Activities of Cysteine Protease Inhibitors during Storage of Rabbit Skeletal Muscles at 4°C.

Inhibitor activities against cathepsin B (Δ), cathepsin L (●), and papain (○) were assayed as described in MATERIALS AND METHODS.

by the method shown in Fig. 1 from muscles immediately after slaughter and from muscles stored at 4°C for 3 and 9 days, and their total inhibitory activities towards cathepsins B and L and papain were measured. As shown in Fig. 5, yields of inhibitory activities towards the three enzymes changed slightly during the storage up to 9 days. The pH values of the examined muscles were around 5.5 on the third day after slaughter. These results indicated that at such a pH an inhibitor-degrading enzyme is latent or far away from its substrate *in situ* muscles.

On the other hand, the total cathepsin B activity extracted from 9-day stored muscles decreased to 93.4% of that from fresh muscles. The total cathepsin L activity extracted from 9-day stored muscles increased to 150% of the value from the fresh muscles. These results show that cathepsin L becomes more extractable during the storage of muscle.

It is well known that decomposition of myofibrillar proteins by proteases happens in postmortem muscles. We suggested in previous papers^{1,2)} that cathepsin L contributed to the postmortem proteolysis more than cathepsin D and calpain on the basis of

their optimum pH. However, the contribution of cathepsin L should be re-examined because the co-existence of cathepsin L inhibitors was revealed by the present study.

Previously²¹⁾ we examined the autolysis of rabbit skeletal muscle homogenates at various pH values and found a peak of proteolysis around pH 4.5 which was ascribable to the action of cathepsin L. Penny *et al.*²²⁾ also studied the autolysis of bovine skeletal muscle homogenates at various pH values and showed that the decomposition of troponin in the pH range from 5 to 6 was mostly attributable to the action of cysteine proteases including cathepsin L. In both cases, the hydrolysis were observed with the co-existence of cathepsin L inhibitors. Therefore, cathepsin L appears to have an opportunity to decompose myofibrillar proteins during the storage of muscles even in the presence of the inhibitors. It is still to be clarified in what circumstances the event occurs.

In order to understand the physiological role of the inhibitors of cathepsins B and L, it is necessary to purify them and investigate their properties in detail. The information obtained by the present work should be of great help in the purification of them.

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