Purification of a Pepstatin Insensitive Protease From Mackerel White Muscle*1

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A pepstatin insensitive protease was purified from mackerel white muscle by a combination of acid treatment, ammonium sulfate fraction, Q Sepharose Fast Flow ion exchange chromatography, Cellulofine GC-200 gel filtration, hydrophobic chromatography on Phenyl-Superose column, chromatofocusing on Mono P column, re-chromatofocusing on the column, and ion exchange chromatography on Mono Q column. The specific activity of the enzyme increased about 7,750-fold in 1.6% yield, and the final preparation proved to be homogeneous on polyacrylamide slab gel electrophoresis.

Proteases, present in lysosomes, play an important role in the normal turnover of tissue protein. Of lysosomal proteases, cysteine proteinases such as cathepsins B, H, S, and L, which have a broad activity on protein and are involved in many physiological processes of protein turnover, have become of major interest lately.1-3)

In fish muscle, few work has been done about lysosomal cysteine proteinases in detail although the study of the proteinase is considered to be important physiologically and pathologically, and also from the standpoint of preservation or utilization of fish as food. Recently, attention has been paid to the existence of cysteine proteinase in several fish muscles such as clun salmon4) and mackerel.5) Hara et al.6) reported that there existed a cysteine proteinase like activity differing from cathepsin B or L in muscle and liver of many fish species. During tracing the behavior of lysosome particles of mackerel muscle by using cathepsin D as a marker enzyme,7,8) we found the existence of two different types of proteases which hydrolyze hemoglobin at pH 4.0, one being strongly inhibited by pepstatin, and the other not.9) The former might correspond to cathepsin D, and the latter, pepstatin insensitive protease, was strongly inhibited by leupeptin which is one of the cysteine proteinase inhibitors.9) Furthermore, the ratio of the pepstatin insensitive activity to cathepsin D activity in mackerel muscle homogenate was 1:1 for white muscle and 1:9 for red muscle.9) Therefore, it seemed necessary to investigate the characteristics of the protease in order to understand the role and the mechanism of the enzyme action in mackerel muscle, especially white muscle. The present paper describes various chromatographic procedures for completely purifying the mackerel white muscle pepstatin insensitive protease.

Materials and Methods

Fresh mackerel Scomber Japonicus with average body weight about 400 g, was obtained from Tsu Fish Market (Mie, Japan). Bovine hemoglobin was from Cooper-Biochemical Co., U.S.A. Fluorescamine was from Japan Roche Co. L-Leucyl-L-Leucine and Cellulofine-GC-200 for gel chromatography were purchased from Seikagaku Kyogyo Co., LTD, Japan. Pepstatin and leupeptin were from Peptide Institute, Inc., Japan. PD-10 Sephadex G-25 column for desalting, Q Sepharose Fast Flow Gel and Mono Q column for anion exchanger, Phenyl-Superose column for hydrophobic chromatography and Mono P column and Polybuffer 74 for chromatofocusing were obtained Pharmacia Fine Chemicals Co., Sweden. Silver stain reagent kit for slab polyacrylamide gel stain and bovine plasma albumin as protein assay standard were purchased from Bio-Rad Laboratories, U.S.A. Centrifo CF25 for ultrafiltration was from Amicon Corp, U.S.A. All other reagents used were of analytical grade or of the best grade available.

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Enzyme Assay

For proteolytic activity assay, to 0.25 ml of enzyme solution were added 0.25 ml of 1 mM protease inhibitor (pepstatin for pepstatin insensitive protease assay and leupeptin for cathepsin D assay) and 1.5 ml of 2.4% urea denatured hemoglobin, pH 4.0. The reaction mixture was incubated at 37°C for 60 min, and then reaction was stopped by addition of 2.0 ml of 0.6 M trichloroacetic acid. After incubation for further 30 min, the mixture was filtered through a Toyo No. 131 filter paper. The filtrate was used for the fluorometric determination according to the method of Ueno et al.10) To 0.1 ml of the filtrate was added 3.5 ml of 0.5 M borate buffer, pH 8.5. As soon as 0.25 ml of a fluorescamine solution (0.6 mg/ml of acetone) was added to the solution, the mixture was shaken vigorously for 5 s on a test tube mixer (NS-8, luchiseieido Co., LTD., Japan). The resulting fluorescence was measured at Ex 390 nm and Em 475 nm by a spectrofluorometer (Type FP-4, Japan Spectroscopic Co., LTD., Japan). One unit of the enzyme activity was expressed as nM of L-Leucyl-L-Leucine per min,11) and the specific activity as units per mg protein. The method of Lowry et al.12) was also used for the assay when necessary.

Determination of Protein

Protein was determined by the method of Lowry et al.12) or the absorbance at 280 nm with bovine plasma albumin as the standard.

Slab Polyacrylamide Gel Electrophoresis

Electrophoresis was carried out on 7.5% (w/v) polyacrylamide slab gel (8.5 × 5 × 0.08 cm) using the discontinuous buffer system (Tris-glycine buffer, pH 8.3).13) A constant current of 12 mA per slab was applied until the bromophenol blue tracking dye front was about 1.0 cm from the bottom of the gel. After electrophoresis, the gel was stained by using the Silver stain kit from Bio-Rad.

Results and Discussion

Preparation of Crude Enzyme

White muscle of mackerel (3.5 kg) separated from red muscle and skeleton was rinsed with 1% NaCl and cut into small pieces with scissors. A portion was suspended in two volumes of 1% NaCl and disrupted with a Waring Blender (Sakuma Seisakusho Co., LTD., Japan) for 5 min. to the homogenate was added 0.1% Triton X-100 and stood at 4°C for 30 min. Then the homogenate was centrifuged at 15,000 × g for 45 min and the supernatant was filtered through two layers of gauze. The filtrate (7.1 l) thus obtained was used to purity pepstatin insensitive protease.

All the purification steps were performed at 0-4°C unless otherwise stated. Desalting and ultrafiltration of an enzyme solution were performed by using a PD-10 column of Sephadex G-25 equilibrated with the buffer indicated and by using Centriflo CF25, respectively.

Step 1. Acid Treatment

To the crude enzyme, 1 N HCl was added to adjust pH to 4.0, and the solution was kept at 4°C for 30 min. The resulting precipitate was removed by centrifugation, and then the pH of the supernatant was readjusted to the original pH with 1 N NaOH. The solution was centrifuged again to remove the precipitate and referred as acid treated fraction (6.6 l). At this step, the bulk of inert proteins were removed from the crude enzyme, the specific activity of the enzyme being raise from 0.4 in the crude enzyme solution to 3.0 in the acid treated fraction.

Step 2. Ammonium Sulfate Fractionation

The acid treated fraction was brought to 40% saturation by addition of solid ammonium sulfate. After standing for 2 h or more, the resulting precipitate was discarded by centrifugation, and then the pH of the supernatant was then brought to 70% saturation. The precipitate was collected and suspended in 0.01 M phosphate buffer, pH 6.2. The suspension was dialyzed overnight against the same buffer as above and centrifuged to remove the precipitate. The supernatant (400 ml) obtained was submitted to the following procedure.

Step 3. Q Sepharose Fast Flow Ion Exchange Chromatography

Four hundred ml of the prepared enzyme solution was applied to a column of Q Sepharose Fast Flow (2.6 × 10 cm, a strong anion exchanger), which had been connected to a PFLC system (Fast Protein Liquid Chromatography, made by Pharmacia Fine Chemicals Co., LTD.) and equilibrated with 0.01 M phosphate buffer, pH 6.2. After washing the column with 1.5 bed-volume of the buffer, adsorbed proteins were eluted with a linear gradient of NaCl (0-1.0 M) in the buffer. The Cl− concentration in the elution buffer increased at a rate of 0.8 mM/ml. Fractions of 20
Fig. 1. Q Sepharose Fast Flow ion exchange chromatography of mackerel pepstatin insensitive protease after ammonium sulfate fractionation. The enzyme solution (400 ml) was applied to a column (2.6 × 10 cm) of Q Sepharose Fast Flow connected with a FPLC system and eluted in a linear gradient of NaCl in 0.01 M phosphate buffer, pH 6.2, at a flow rate of 5 ml/min. Fractions of 10 ml were collected. • — Pepstatin insensitive protease activity, ○ — cathepsin D activity, —— protein.

Fig. 2. Cellulofine GC-200 gel filtration of mackerel pepstatin insensitive protease after Q Sepharose Fast Flow ion exchange chromatography. The enzyme solution (30 ml) was loaded to a column (2.6 × 100 cm) of Cellulofine GC-200 equilibrated with 0.01 M phosphate buffer containing 0.1 M NaCl, pH 6.0, and 10 ml fractions were collected at a flow rate of 30 ml/h. • — Enzyme activity, ○ — protein.

ml were collected at a flow rate of 5 ml/min. The active fractions were pooled and to it was added solid ammonium sulfate. The resulting precipitate was collected by centrifugation and suspended in a small volume of 0.01 M phosphate buffer containing 0.1 M NaCl, pH 6.0. As shown in Fig. 1, the pepstatin insensitive protease which required a higher salt concentration (0.25 M) for
**Fig. 3.** Hydrophobic chromatography of mackerel pepstatin insensitive protease on Phenyl-Superose column. The enzyme solution (5 ml) was applied to a Phenyl-Superose column (0.5 × 5 cm) connected with a FPLC system and eluted by a linear gradient of ammonium sulfate from 1.7 to 0 M in 0.05 M phosphate buffer, pH 7.0, at a flow rate of 0.4 ml/min. Fractions of 1 ml were collected. ●—● Enzyme activity, ------ protein.

**Fig. 4.** Chromatofocusing of mackerel pepstatin insensitive protease on Mono P column. The enzyme solution (5 ml) was applied to a Mono P column (0.2 × 20 cm) connected with a FPLC system. A pH gradient from 7.1–4.0 was formed by two kinds of buffer; 0.025 M Bis-Tris buffer containing 0.025 M NaCl and 20% glycerol, pH 7.1, and Polybuffer 74 diluted with 0.025 M NaCl containing 20% glycerol, pH 4.0. The enzyme was eluted by 0.2 M NaCl in above Polybuffer at a flow rate of 0.4 ml/min. Fractions of 1 ml were collected. ●—● Enzyme activity, ------ protein.
elution was completely separated from cathepsin D which was not adsorbed onto the column. The use of Q Sepharose Fast Flow column not only improved effectively the enzyme purity and the elimination of cathepsin D but also made a large enzyme preparation possible.

**Step 4. Cellulofine GC-200 Gel Filtration**

The above concentrated enzyme solution (30 ml) was loaded to a column of Cellulofine GC-200 (2.6×100 cm) equilibrated with 0.01 M phosphate buffer containing 0.1 M NaCl, pH 6.0. The elution was carried out at a flow rate of 30 ml/h, and fractions of 10 ml were collected. The fractions containing the enzyme activity were pooled and to it was added solid ammonium sulfate. The resulting precipitate was collected by centrifugation and dissolved with 5 ml of 0.05 M phosphate buffer containing 1.7 M ammonium sulfate, pH 7.0 (Fig. 2).

**Step 5. Hydrophobic Chromatography on Phenyl-Superose Column**

A Phenyl-Superose column (0.5×5 cm) was connected to a FPLC system and equilibrated with the buffer mentioned above. Five ml of enzyme solution from gel filtration was applied to the column and eluted with a linear concentration gradient of ammonium sulfate (1.7→0 M) in the buffer. Fractions of 1 ml were collected at a flow rate of 0.4 ml/min. The active fractions were pooled and concentrated by ultrafiltration through Centrificlo CF25. The buffer was changed over to 0.025 M Bis-Tris buffer containing 0.025 M NaCl and 20% glycerol, pH 7.1, using a PD-10 Sephadex G-25 column. At this step, most of inert proteins were separated from the enzyme preparation, and the purification factor was raised from 330 in the elute from the Cellulofine GC-200 column to 1,630 (Fig. 3).

**Step 6. Chromatofocusing on Mono P Column (pH 7.1→4.0)**

The column (0.5×20 cm) connected with a FPLC system was equilibrated with 0.025 M Bis-Tris buffer containing 0.025 M NaCl and 20% glycerol, pH 7.1. The eluent buffer was prepared by diluting Polybuffer 74 with 0.025 M NaCl and 20% glycerol in a ratio of 1:9. The pH of the eluent buffer was adjusted to 4.0 with 1 N HCl. By running 3 ml of the eluent buffer, a pre-gradient of pH (7.1→4.0) was formed in the column, and then 5.0 ml of the enzyme solution was applied to the column. After eluted with 30 ml of the

![Fig. 5. Re-chromatofocusing of mackerel pepstatin insensitive protease on Mono P column. The enzyme solution (5 ml) was applied to a Mono P column (0.5×20 cm) connected with a FPLC system. A pH gradient from 7.1→4.0 was formed by two kinds of buffer; 0.025 M Bis-Tris buffer containing 0.025 M NaCl and 20% glycerol, pH 5.0, and Polybuffer 74 diluted with 0.025 M NaCl containing 20% glycerol, pH 3.5. The flow rate was 0.5 ml/min, and fractions of 0.5 ml were collected. Enzyme activity, protein.](image-url)
eluent buffer, the same buffer containing 0.2 M NaCl was introduced to the column. The flow rate was 0.4 ml/min, and fractions of 1 ml were collected. The active fractions were pooled and concentrated by ultrafiltration through Centriflo CF25. The buffer was changed over to 0.025 M Bis-Tris buffer containing 0.025 M NaCl and 20% glycerol, pH 5.0, using a PD-10 Sephadex G-25 column. As seen in Fig. 4, about 83% of inert proteins having their pH 7.1-4.0 were eluted at the first elution condition. The enzyme was eluted at 0.2 M NaCl in a sharp peak.

Step 7. Re-chromatofocusing on Mono P Column (pH 5.0-3.5)

The column connected with a FPLC system was equilibrated with 0.025 M Bis-Tris buffer containing 0.025 M NaCl and 20% glycerol, pH 5.0. The eluent buffer was prepared as described above. The pH was adjusted to 3.5 with 1 N HCl. An aliquot of the above concentrated enzyme solution (5.0 ml) was applied to the column. The column was washed with 30 ml of the eluent buffer, pH 5.1 in order to attain better concentration and separation of enzyme protein,14) and then 25 ml

Table 1. Purification of pepstatin insensitive protease from mackerel white muscle

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Total volume (ml)</th>
<th>Total protein (mg)</th>
<th>Total Activity (Units)</th>
<th>Specific Activity (Units/mg)</th>
<th>Purification factor (fold)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude enzyme</td>
<td>7,700</td>
<td>102,000</td>
<td>40,600</td>
<td>0.4</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>Acid treatment (pH 4.0)</td>
<td>6,600</td>
<td>8,680</td>
<td>26,000</td>
<td>3.0</td>
<td>7.5</td>
<td>64</td>
</tr>
<tr>
<td>Ammonium sulfate (40-70%)</td>
<td>400</td>
<td>3,180</td>
<td>24,300</td>
<td>7.6</td>
<td>19.0</td>
<td>60</td>
</tr>
<tr>
<td>Q Sepharose Fast Flow</td>
<td>30</td>
<td>248</td>
<td>19,200</td>
<td>77.4</td>
<td>194</td>
<td>47</td>
</tr>
<tr>
<td>Cellulofine GC-200</td>
<td>10</td>
<td>131</td>
<td>17,300</td>
<td>132</td>
<td>330</td>
<td>42.6</td>
</tr>
<tr>
<td>Phenyl-Superose</td>
<td>10</td>
<td>8.3</td>
<td>5,400</td>
<td>651</td>
<td>1,630</td>
<td>13.3</td>
</tr>
<tr>
<td>Mono P (pH 7.1-4.0)</td>
<td>5</td>
<td>1.23</td>
<td>3,300</td>
<td>2,680</td>
<td>6,710</td>
<td>8.1</td>
</tr>
<tr>
<td>Mono P (pH 5.0-3.5)</td>
<td>5</td>
<td>0.39</td>
<td>1,200</td>
<td>3,080</td>
<td>7,690</td>
<td>3.0</td>
</tr>
<tr>
<td>Mono Q</td>
<td>2</td>
<td>0.21</td>
<td>650</td>
<td>3,100</td>
<td>7,750</td>
<td>1.6</td>
</tr>
</tbody>
</table>

* Activity (nm tyrosine/min per mg protein) as measured by Lowry's method.12)
of the eluent buffer was introduced to the column. The flow rate was 0.5 ml/min, and fractions of 0.5 ml were collected. A peak containing the enzyme activity was eluted nearly at pH 3.8. The fractions consisting of the peak were pooled and concentrated by ultrafiltration through Centriflo CF25 (Fig. 5).

In a preliminary experiment, chromatofocusing on Mono P column could not achieve a good separation of the enzyme from a lot of inert proteins, by using a pH gradient from 7.1 to 3.5. Therefore, chromatofocusing was carried out two times at different elution conditions, as in steps 6 and 7. However, the enzyme from re-chromatofocusing was still mingled with at least three kinds of inert proteins when examined by slab polyacrylamide gel electrophoresis.

**Step 8. Ion Exchange Chromatography on Mono Q Column**

A Mono Q column (0.5 × 5 cm, a strong anion exchanger) was connected to a FPLC system and equilibrated with 0.01 M phosphate buffer, pH 6.2. The enzyme preparation (5 ml) from re-chromatofocusing was applied to the column and eluted with a linear gradient of NaCl (0–1.0 M) in the phosphate buffer. The Cl⁻ concentration in the elution buffer increased in a rate of 33.3 mM/ml. Fractions of 0.5 ml were collected at a flow rate of 1.0 ml/min. As shown in Fig. 6, the enzyme was eluted at 0.28 M NaCl in a sharp peak. The active fractions pooled in this step gave the final purified pepstatin insensitive protease.

Details of a typical purification are summarized in Table 1. A 7,750-fold purification was achieved with a yield of 1.6%.

**Homogeneity**

The final enzyme preparation showed a single protein band on slab polyacrylamide gel electrophoresis (Fig. 7), when stained with a high sensitive silver stain method, which has been reported to be 10–50 fold more sensitive than the conventional Coomassie brilliant blue stain for protein.¹⁵

**References**