Purification and Characterization of Serine Protease Inhibitor from White Croaker Argyrosomus argentatus Ordinary Muscle

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A serine protease inhibitor was isolated from white croaker Argyrosomus argentatus ordinary muscle by ammonium sulfate fractionation, gel filtration on Sephadex G-100, column chromatographies on CM-Sephadex C-50 and DEAE-Sephadex A-50, and preparative polyacrylamide gel electrophoresis. The inhibitory activity against trypsin increased about 1,500-fold, and the purified inhibitor was homogeneous as judged by polyacrylamide gel electrophoresis. The molecular weight of the inhibitor was estimated to be about 100,000 by gel filtration on Sephadex G-100, but was estimated to be about 55,000 by SDS polyacrylamide gel electrophoresis after reduction with 2-mercaptoethanol. The inhibitor was stable over a range of pH 6.0-9.0, but unstable below pH 5.0, and completely inactivated by heating at 60°C for 30 min at pH 6.0. The isoelectric point was found to be about 4.7, and N-terminal glycine was found by dansylation. Trypsin, α-chymotrypsin and elastase were strongly inhibited with the inhibitor, but pepsin, thermolysin and papain were not inhibited. Each E-I complex of the inhibitor with the proteases, trypsin, α-chymotrypsin and elastase, were stable to the denaturing reagents such as SDS and 2-mercaptoethanol, however each complex was digested in the presence of the excess protease.

Since KUNITZ1,2) isolated the trypsin inhibitors from bovine pancreas and soybean, a large number of protease inhibitors has been found in animals, plants and certain bacteria.3-6) Especially, trypsin inhibitor from soybean and mammalian pancreas have been extensively studied from the stand point of their physiological roles, inhibitory mechanism and structure.4-7) However, very few studies according to the presence of trypsin inhibitor in muscle have been reported.8) Recently, Ca2+-activated neutral protease (CANP) was isolated from various skeletal muscle, chicken,9,10) rabbit10,11) and pig.12) And subsequently, CANP-inhibitor which named as calpastatine was isolated from various animal tissues.13-15) The present paper describes isolation and characterization of serine protease inhibitor from white croaker ordinary muscle.

Materials and Methods

Materials

White croaker Argyrosomus argentatus was obtained from commercial supplier near Nagasaki city, and ordinary muscle was collected. Sephadex G-100, CM-Sephadex C-50 and DEAE-Sephadex A-50 were purchased from Pharmacia Fine Chemicals. Bovine trypsin, bovine α-chymotrypsin, porcine pancreas elastase, thermolysin and subtilisin BPN' (2×crystallized, respectively) were purchased from Sigma Chemical Co., and pronase from Kaken Chemical Co. Casein was Merck product. Congo red elastin was United States Biochemical Co. product. Standard proteins for estimation of molecular weight were purchased from Pharmacia Fine Chemicals. All other reagents were either reagent grade or best grade available.

Assay of Inhibitory Activity

The caseinolytic activity of trypsin was determined at 37°C, pH 8.0, according to the modified method of HAGIHARA.16) 0.25 ml of inhibitor solution (usually, it was adjusted at pH 8.0 with 50 mM H3BO3-KCl-Na2CO3 buffer) was mixed with same volume of an enzyme solution. After preincubation for 5 min at room temperature, 0.25 ml of 2% casein solution, pH 8.0, was added and incubated at 37°C for 25 min. The reaction was stopped by addition of 1 ml of 10% trichloroacetic acid and the reaction mixture was filtered. In the control experiment, 0.25 ml of the buffer solution was added in place of the inhibitor solu-
tion. The amount of proteolytic products in the filtrate was measured according to the method of LOWRY. One unit of inhibitory activity was defined as the inhibition of the activity of 1 μg of trypsin.

**Determination of Protein**

Protein concentrations were determined by the method of LOWRY after every purification steps, using bovine serum albumin as standard protein.

**Electrophoresis**

Polyacrylamide gel electrophoresis in the presence and absence of SDS was carried out as described by WEBER and OSBORN, and DAVIS, respectively. Preparative polyacrylamide gel electrophoresis was carried out by modification of the analytical method of DAVIS, using an apparatus (URECOVERA II) from Fuji-Riken Co., Ltd. 5% and 7.5% polyacrylamide gel (20 × 40 mm) were prepared with 750 mM Tris-250 mM HCl buffer, pH 9.0, as a separating gel and a stopper gel, respectively. 25 mM Tris-200 mM glycine buffer, pH 8.7 was used as electrod buffer, and protein was recovered by 12.5 mM Tris-100 mM glycine buffer, pH 8.7, with a peristatic pump at flow rate of 0.5 ml per min. After preelectrophoresis for 10 h at 10 mA at 4°C, about 5.5 mg of the partially purified inhibitor (the DEAE-Sephadex fraction) was applied to the gel column, and then electrophoresis was carried out by the same condition described above.

**Determination of Isoelectric Point**

The isoelectric focusing was carried out by the method of VESTERBERG, using a column (110 ml) of LKB Biochrom Ltd.

**Determination of N-Terminal Amino Acid**

The N-terminal amino acid of the inhibitor was determined by dansylchloride method according to GRAY, and then analyzed by the thin layer chromatography using polyamide sheet.

**Purification of the Inhibitor**

Five kg of minced ordinary muscle of white croaker was suspended in 30 l of 0.2% KCl solution at 5°C and gently stirred for 5 min. After centrifugation at 7,000 rpm for 20 min, solid (NH₄)₂SO₄ was added in the supernatant to 75% saturation. The resulting precipitate was collected by centrifugation and dissolved in distilled water, and then dialyzed against distilled water for 2 days. After centrifugation, the supernatant was lyophilized. The lyophilizate was dissolved in distilled water and insoluble matter was removed by centrifugation. The supernatant was heated at 45°C for a half min and cooled in an ice bath immediately. Resulting precipitate was removed by centrifugation, and the supernatant (18.8 g of protein per 230 ml) was divided into three portion. Each portion was applied to a Sephadex G-100 column (4.0 × 88 cm) previously equilibrated with 20 mM KH₂PO₄-10 mM Na₂B₄O₇ buffer, pH 6.0, and eluted with the same buffer. The inhibitor activity was revealed at the behind of the void volume. The active fractions in the each gel filtration were combined (5.0 g of protein per 260 ml) and dialyzed against 20 mM KH₂PO₄-10 mM Na₂B₄O₇ buffer, pH 6.0. The solution was applied to a CM-Sephadex C-50 column (2.64 × 88 cm) previously equilibrated with the same buffer. The inhibitor was not adsorbed on the CM-Sephadex C-50 under the condition and major inactive protein was eluted in the second elution buffer containing 500 mM NaCl. The active fractions were pooled (1.5 g of protein per 300 ml) and dialyzed against 20 mM KH₂PO₄-10 mM Na₂B₄O₇ buffer, pH 6.0, and applied to a DEAE-Sephadex A-50 column (1.9 × 40 cm) previously equilibrated with the same buffer. A large amount of contaminated

Table 1. Summary of purification process of the inhibitor

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Volume (ml)</th>
<th>Total protein (mg)</th>
<th>Specific activity (units/mg)</th>
<th>Purification</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>29300</td>
<td>172870</td>
<td>0.34</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>(NH₄)₂SO₄ fraction</td>
<td>3260</td>
<td>44540</td>
<td>0.78</td>
<td>2.3</td>
<td>60</td>
</tr>
<tr>
<td>Heat treatment</td>
<td>230</td>
<td>18860</td>
<td>1.1</td>
<td>3.2</td>
<td>36</td>
</tr>
<tr>
<td>Sephadex G-100</td>
<td>260</td>
<td>5044</td>
<td>2.3</td>
<td>6.8</td>
<td>20</td>
</tr>
<tr>
<td>CM-Sephadex</td>
<td>300</td>
<td>1500</td>
<td>8.6</td>
<td>25.4</td>
<td>22</td>
</tr>
<tr>
<td>DEAE-Sephadex</td>
<td>90</td>
<td>17</td>
<td>515.1</td>
<td>1514.0</td>
<td>15</td>
</tr>
<tr>
<td>Preparative disc-electrophoresis</td>
<td>59</td>
<td>2</td>
<td>530.1</td>
<td>1558.8</td>
<td>2</td>
</tr>
</tbody>
</table>
Fig. 1. Polyacrylamide gel electrophoresis of the inhibitors.  
(A) Analytical electrophoresis. 27 μg of the partially purified inhibitor (DEAE- Sephadex fraction) and 20 μg of the purified inhibitor were applied to disc gel electrophoresis (7.5% gel and Tris-glycine buffer, pH 8.3), respectively. Electrophoresis was carried out at 3 mA per tube. Protein bands were stained with amido black 10B. (B) SDS polyacrylamide gel electrophoresis. 18 μg of the purified inhibitor (20 μl) was mixed with 20 μl of 20 mM sodium phosphate buffer, pH 7.2, containing 2% SDS and 50% glycerol, and whole was heated at 100°C for 5 min in the presence of 5% 2-mercaptoethanol. The sample was then applied to 10% gel column, and electrophoresis was carried out at 8 mA per tube. Protein bands were stained with coomassie brilliant blue.

proteins were eluted in the first and second step (100 mM KH₂PO₄, 50 mM Na₂B₄O₇, pH 7.5), and the inhibitory activity was eluted in the third step (100 mM KH₂PO₄, 50 mM Na₂B₄O₇ buffer, pH 7.5, containing 500 mM NaCl). The active fractions were pooled and dialyzed against distilled water for overnight, and concentrated by Toyo ultrafilter UK-20. The concentrated solution (171.1 mg of protein per 9 ml) was divided into three portions, and each portion (3 ml) was applied to a preparative polyacrylamide gel electrophoresis respectively. The inhibitory activity was separated from some contaminated proteins. The active fractions of the each electrophoresis were combined, and concentrated by ultrafiltration and stored at −70°C.

Results
Purification of the Inhibitor
A summary of the each purification process is shown in Table 1. The specific activity was increased about 1,500-fold from the crude extract. The homogeneity of the purified inhibitor was investigated by polyacrylamide gel electrophoresis. As shown in Fig. 1A, the purified inhibitor migrated as a single protein band in the polyacrylamide gel.

Molecular Weight of the Inhibitor
The molecular weight of the inhibitor was estimated by gel filtration using Sephadex G-100 and SDS polyacrylamide gel electrophoresis. By the gel filtration, the molecular weight of the inhibitor was estimated to be approximately 100,000 as shown in Fig. 2. On the other hand, as shown in Fig. 1B, the inhibitor appeared as a single protein band of molecular weight about 55,000 by the SDS polyacrylamide gel electrophoresis. These results suggested that the inhibitor is dimer protein consist of the same subunits.

Thermo-Stability and pH-Stability
As shown in Fig. 3, the inhibitor was stable over the pH range 6.0 to 9.0 for 15 h at 5°C and 3 h at 37°C. As shown in Fig. 4, when the inhibitor solution were heated at 60°C for 30 min, the loss of activity at pH 6.0 and 8.5 were 98% and 15%, respectively. These results suggested that the inhibitor was unstable in acidic range.

Effects of the Inhibitor on Various Proteases
Effects of the inhibitor on various proteases were investigated. As shown in Fig. 5, trypsin and α-chymotrypsin were remarkably inhibited, and it
was found that 1 µg of the inhibitor could inhibit about 0.4 µg of trypsin. This result suggests that one molecule of the inhibitor may react with two molecules of trypsin, assuming their molecular weight to be 100,000 and 23,000, respectively. For other protease, thermolysin pepsin and papain were not inhibited, and subtilisin BPN' was slightly inhibited in the high concentration of the inhibitor. As shown in Fig. 6, elastase was completely inhibited.

**Isoelectric Point**

When the inhibitor was subjected to isoelectric focusing electrophoresis using Ampholine (pH 3-10, LKB Biochrom Ltd.), isoelectric point of the inhibitor was estimated to be about pH 4.7.

**N-Terminal Amino Acid**

When the inhibitor (about 0.3 mg) was subjected to N-terminal amino acid analysis by the dansyl-chloride method, dansyl-glycine was detected on the thin layer chromatography sheet as a single spot.

**Gel Electrophoretic Analysis of the Reaction of the Inhibitor with Trypsin and α-Chymotrypsin**

The reaction of the inhibitor with trypsin and α-chymotrypsin were analyzed by SDS polyacrylamide gel electrophoresis. As shown in Fig. 7, each of the inhibitor and trypsin migrated as a single protein band. In the mixture of trypsin and the inhibitor (10 µg: 15 µg), five kinds of the protein bands (M. W. 68,000, 63,000, 53,000, 45,000 and trypsin) were represented, but the band of the inhibitor did not detected. As shown in...
0.1 ml of the purified inhibitor (8 μg) was mixed with the same volume of elastase (20 μg) and preincubated for 20 min at room temperature, and then the mixture was added in 3 ml of 0.1% congo red elastin, which was prepared with 20 mM sodium borate buffer, pH 8.5. After incubation at 37°C at various intervals, an absorbance at 495 nm of the supernatant of the reaction mixture was measured. (○), With inhibitor; (○), Without inhibitor.

Fig. 8, α-chymotrypsin migrated as two peptide bands, which were B-chain (M.W. 15,000) and C-chain (M.W. 10,000) of the enzyme. In the mixture of α-chymotrypsin and the inhibitor (15 μg: 25 μg), five kinds of protein bands (M. W. 62,000, 56,000, 49,000, 45,000 and B-chain) were represented, but the band of C-chain (contains 195-Ser which is essential amino acid for activity of the enzyme), was not detected. This results suggested that the protein band of molecular weight 62,000 may be a complex between the inhibitor and C-chain of the enzyme.

Gel Electrophoretic Analysis of the Reaction of the Inhibitor with Elastase

The inhibitor and elastase were mixed in the same buffer described in Fig. 7, and incubated at room temperature. And the hydrolysis of the E-I complex was observed by SDS polyacrylamide gel electrophoresis. As shown in Fig. 9 and Fig. 10, elastase migrated as two protein bands (M.W 35,000 and 25,000). The former was regarded as a contaminant in the elastase, because it has been known that the molecular weight of porcine pancreas elastase was about 26,000.23) In Fig. 9, where the weight ratio of the inhibitor to elastase 1: 1.25, faint protein bands of molecular weight 68,000 and 49,000, except the obvious bands of the inhibitor and elastase, were observed in the gel at 0 min incubation. On the longer incubation than that of 10 min, the former (M.W 68,000) disappeared and the later (M.W 49,000) distinctly appeared in the gel. As shown in Fig. 10, where the weight ratio of the inhibitor to elastase was 1: 6, a obvious protein band of molecular weight
Fig. 9. SDS polyacrylamide gel electrophoretic analysis of interaction of the purified inhibitor and elastase.

15 μg of the inhibitor and 12 μg of elastase were mixed in the same buffer described in Fig. 7. After incubation at room temperature at various intervals, the mixtures were reduced by 2-mercaptoethanol as described in the legend to Fig. 1, and applied to electrophoresis. Electrophoresis was carried out using 12% polyacrylamide gel, and the other condition were the same as described in Fig. 1.

68,000 and a faint protein band of 49,000 were observed in the gel at the 0 min incubation. On the longer incubation than that of 10 min, the protein band of molecular weight 58,000 faintly appeared, however the gel patterns did not change at the various incubation times.

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Discussion

We had been found that the trypsin inhibitory activity was existent in muscle of various fish unpublished results). This report described a procedure for purification and some characterizations of the serine protease inhibitor from white croaker ordinary muscle.

The specific activity of the purified inhibitor increased about 1,500-fold than that of the crude extract. Molecular weight of this inhibitor was estimated to be about 100,000 by the gel filtration, and that was estimated to be about 55,000 by the SDS polyacrylamide gel electrophoresis. These values are higher than that of Kunitz trypsin inhibitor24,25 (M.W 21,000) and Bowman-Birk inhibitor26,27 (M.W 8,000) from leguminous plant and basic pancreatic trypsin inhibitor28 (M.W 6,500), but lower than that of α1-macroglobulin29 (M.W 725,000) from human serum. Molecular weight of the subunit of this inhibitor is fairly approximation that of α1-proteinase inhibitor (synonymously α1-antitrypsin) from human serum. Molecular weight of the human α1-proteinase inhibitor, it has been known as monomer protein, has been estimated to be from 54,000 to 59,000 by several workers30-34. And recently, amino acid sequence of α1-proteinase inhibitor was determined35.

From Fig. 7, 8, 9 and 10, it is suggested that the bands of the highest molecular weight in the each gel are initial complexes of the inhibitor and the each enzyme (trypsin, α-chymotrypsin and elastase). And the molecular weight of these initial complexes were estimated by SDS polyacrylamide gel electrophoresis to be 68,000, 62,000 and 68,000, which are close to the sum of each molecular weight of these enzymes and the inhibitor, respectively. It was elucidated that these initial complexes were degradated rapidly and limitedly by the each excess protease. Because, when in the presence of large amount of the inhibitor to the enzyme (Fig. 10), the E-I complex band of the molecular weight 68,000 did not disappeared for a long incubation time. On the other hand, these complexes cannot be dissociated with denaturing and reducing reagent such as SDS and 2-mercaptoethanol. This results suggested that the interaction of the inhibitor and the protease was formed with a kind of covalent bond.

The pH-stability and the thermo-stability of this
inhibitor are distinctly different from the protease inhibitors of leguminous plant,\textsuperscript{1,4,7} potato inhibitors\textsuperscript{36} and basic pancreatic trypsin inhibitor.\textsuperscript{37} On the other hand, some properties of $\alpha_1$-proteinase inhibitor, for example pH-stability,\textsuperscript{33} thermo-stability,\textsuperscript{30} isoelectric point,\textsuperscript{38} and stability of the E-I complexes against denaturing and reducing reagents such as SDS and 2-mercaptoethanol,\textsuperscript{31,32,34,40} were very similar to that of this inhibitor. From these similarity with $\alpha_1$-proteinase inhibitor, it is supposed that this inhibitor is originated in serum. However, trypsin and $\alpha$-chymotrypsin inhibitory activities were considerably detected in commercial frozen fish paste “Surimi” of alaska pollack and other fish (unpublished results). Therefore, the investigation with regard to $\alpha_1$-proteinase inhibitor of fish serum should be pursued for compare with this inhibitor.

The presence of serine protease inhibitor in fish muscle is of interest in connection with a autolysis of fish muscle protein. Therefore, it is necessary to clarify the physical and chemical properties of this inhibitor and the role of the inhibitor take part in the degradation of fish muscle protein by endogeneous proteases.

References

34) J. TRAVIS, D. A. JOHNSON and R. PANBELL: in


