# Isolation and characteristics of trypsin inhibitor from the hepatopancreas of a squid (*Todarodes pacificus*)

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## Abstract

Trypsin inhibitor was purified from the liver of squid *Todarodes pacificus*. The final inhibitor preparation was nearly homogeneous shown by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and its molecular weight was estimated as approximately 6,300. The squid trypsin inhibitor was acid- and heat-stable, and it was active against trypsins from the pyloric ceca of starfish and saury as well as porcine pancreatic trypsin. Amino acid composition of the squid trypsin inhibitor was compared with those of other invertebrate trypsin inhibitors. The squid trypsin inhibitor inhibited the autolysis of walleye pollack myofibrillar proteins.

*Keywords:* Acid- and heat-stability; Amino acid composition; Inhibitory specificity; Isolation; Marine invertebrate; Liver; Squid; *Todarodes pacificus*; Trypsin inhibitor.

## 1. Introduction

Protease inhibitors are widely distributed among animals, plants and bacteria. Especially, trypsin inhibitors from soybean and mammalian pancreas

have been extensively studied for their physiological roles, inhibitory mechanisms and structures. However, few reports exist on molluscan trypsin inhibitors. Tschesche and Dietl (1972a and b) studied the distribution of proteinase inhibitors in eight different organs of Roman snail *Helix pomatia*: the internal organs, such as liver, kidney, stomach, intestines and sex organs, were free from protease inhibitors, but the external organs such as foot, muscle and mucus, contained low molecular weight and acid- and heat-stable inhibitors. Yoshino et al. (1993) isolated a protease inhibitor, tugalistatin, from the muscle of marine gastropod *Tugali gigas*.

Most organs, including external organs such as the mantle and skin of squid *Loligo vulgaris*, contain low molecular weight and acid- and heat-stable inhibitors (Tschesche and Rucker, 1973a). Ishikawa et al. (1966) found acid- and heat-stable protease inhibitory activity in the liver of squid *Ommastrephes sloani pacificus*. Sofina et al. (1988) purified acid- and heat-stable trypsin inhibitor from the liver of squid *Berryteuthis magister*.

We found trypsin inhibitory activity in the visceral tissues of squid *Todarodes pacificus*, but was not detected in mantle muscle or tentacle. In this study, we isolated trypsin inhibitor from the liver of *T. pacificus* and examined its characteristics, such as molecular weight, heat stability, amino acid composition, and inhibitory specificity.

## 2. Materials and methods

### *2.1. Materials*

Squid *T. pacificus* was caught off Hakodate, Hokkaido Prefecture,

Japan. The specimens were stored at −20 ℃ for several months until analysis. Saury *Cololabis saira* and walleye pollack *Theragra chalcogramma* were purchased from a fish market. Diethylaminoethyl (DEAE)-cellulose was purchased from Whatman (Maidston, England). Sephadex G-50 was purchased from Pharmacia Biotech (Uppsala, Sweden). $N^{\alpha}$-*p*-Tosyl-L-arginine methyl ester hydrochloride (TAME) was purchased from Nakalai Tesque, Inc. (Kyoto, Japan).

*2.2. Enzymes*

Porcine pancreatic trypsin was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

Trypsin from the pyloric ceca of starfish *Asterias amurensis* was purified from crude extracts of pyloric ceca acetone powder by the following methods: ammonium sulfate precipitation (40-75 % saturation), gel filtration on Sephacryl S-200 (eluted with 50 mM Tris-HCl buffer (pH 8.0)), and DEAE-cellulose anion exchange chromatography (eluted with a linear gradient of 0-1.2 M NaCl in 10 mM Tris-HCl buffer (pH 8.0)). The final enzyme preparation was nearly homogeneous shown by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and native PAGE.

Crude trypsin from the pyloric ceca of saury was prepared as follows: the pyloric ceca were delipidated with acetone. Trypsin was extracted by stirring the delipidated powder in 5 volumes of 50 mM Tris-HCl buffer (pH 8.0) containing 5 mM $CaCl_2$ at 4 ℃ for 3 h. The extracts were centrifuged at 10,000×g for 10 min, and the supernatant was used as crude trypsin.

*2.3. Preparation of myofibrils*

Myofibrils and sarcoplasm were prepared from the dorsal muscle of walleye pollack using the modified method of Ichikawa and Nishita (1987). The dorsal muscle was homogenized with 10 volumes of 20 mM Tris-HCl buffer (pH 7.5) containing 0.1 M NaCl at 4 ℃ for 1 min, and the homogenate was centrifuged at 3,300×g for 5 min. The precipitate was homogenized with 10 volumes of 20 mM Tris-HCl buffer (pH 7.5) containing 0.1 M NaCl at 4 ℃ for 1 min, and the homogenate was centrifuged at 3,300×g for 5 min. The precipitate suspended in 10 volumes of 20 mM Tris-HCl buffer (pH 7.5) containing 0.1 M NaCl was used as myofibrillar fraction and the pooled supernatant was used as sarcoplasmic fraction.

*2.4. Preparation of crude trypsin inhibitor*

The liver of squid was delipidated with acetone. Trypsin inhibitor was extracted by stirring the delipidated powder in 5 volumes of distilled water at 5 ℃ for 3 h. The extracts were centrifuged at 10,000×g for 10 min, and then the supernatant was incubated at 80℃ for 5 min. The heat-treated solution was centrifuged at 10,000×g for 10 min, and the supernatant was concentrated by lyophilization and was used as the crude trypsin inhibitor.

*2.5. Purification of trypsin inhibitor from the liver of squid*

Crude trypsin inhibitor prepared from the squid liver was applied to a Sephadex G-50 column (3.9×64 cm) preequilibrated with 10 mM Tris-HCl buffer (pH 8.0) and the protein was eluted with the same buffer. A single inhibitory peak was pooled and was concentrated by lyophilization. The concentrated solution was mixed with an excess molar quantity of porcine pancreatic trypsin in 20 mM Tris-HCl buffer (pH 8.0) containing 4 mM $CaCl_2$ at 30 ℃ for 5 min to make a porcine trypsin-squid trypsin inhibitor complex. The mixture was then applied to a Sephadex G-50 column (3.9×64 cm) preequilibrated with 10 mM Tris-HCl buffer (pH 8.0) and the protein was eluted with the same buffer. The trypsin-trypsin inhibitor complex fraction was pooled and the porcine trypsin was denatured by adding 2.5 % (final concentration) of perchloric acid. Subsequenty, the acidic solution was centrifuged at 10,000×g for 10 min, and the supernatant was prepared at pH 6.0 by adding 0.5 M NaOH. This acidic solution was applied to a Sephadex G-50 column (3.9×64 cm) preequilibrated with 10 mM Tris-HCl buffer (pH 8.0) and the protein was eluted with the same buffer. The main inhibitory peak was pooled, and was applied to a DEAE-cellulose column (1.1×18 cm) preequilibrated with 10 mM Tris-HCl buffer (pH 9.0). Three inhibitory peaks were eluted with the non-adsorbed fraction and the main inhibitory peak was pooled into the purified trypsin inhibitor (Fig. 1). The final inhibitor was purified 160-fold from the crude inhibitor in a yield of 7 % (Table 1). The squid trypsin inhibitor was found to be nearly homogeneous using SDS-PAGE (Fig. 1).

*2.6. Assay for trypsin inhibitory activity*

Inhibitor solution (10 μl) was mixed with 20 μl of 30 mM Tris-HCl buffer (pH 8.0) containing 6 mM $CaCl_2$ and porcine pancreatic trypsin (8.8 μg, 0.168 units), and the mixture was incubated at 30 ℃ for 5 min. Residual trypsin activity was measured using the method of Hummel (1959) with TAME as the substrate. The assay was carried out at 30 ℃ in 50 mM Tris-HCl buffer (pH 8.0). The change in the absorbance at 247 nm was observed using a spectrophotometer, model 100-60 (Hitachi, Tokyo Japan) One unit (U) of trypsin activity was defined as the amount of enzyme causing an increase of 1.0 in absorbance at 247 nm per minute, one U of trypsin inhibitory activity was defined as the amount of inhibitor that decreased the activity of one milligram of trypsin to 50 %.

*2.7. Assay for inhibitory specificity*

Porcine pancreatic trypsin (8.8 μg, 0.168 U), starfish *A. amurensis* trypsin (4.4 μg, 0.104 U), or saury crude trypsin (484 μg, 0.182 U) were incubated with various concentrations of the squid trypsin inhibitor (0-5.0 μg) in 20 mM Tris-HCl buffer (pH 8.0) containing 4 mM $CaCl_2$ at 30 ℃ for 5 min. The residual trypsin activity was determined using TAME as a substrate.

The inhibition by purified squid trypsin inhibitor of the autolysis of walleye pollack myofibrillar proteins was examined as follows: myofibrillar proteins (50 μl) in 20 mM Tris-HCl buffer (pH 7.2) containing 0.1 M NaCl were incubated at 60 ℃ with 20 μl of the squid trypsin inhibitor or sarcoplasmic fraction of walleye pollack. The breakdown of walleye pollack myofibrillar

proteins was analyzed using SDS-PAGE.

*2.8. Electrophoresis*

SDS-PAGE was done using a 0.1 % SDS-20 % polyacrylamide slab-gel using the method of Fling and Gregerson (1977) or using a 0.1 % SDS-10 % polyacrylamide slab-gel using the method of Laemmli (1970). The gel was stained with 0.1 % Coomassie brilliant blue R-250 in 50 % methanol-7 % acetic acid and was destained with 7 % acetic acid.

*2.9. Analysis of amino acid composition*

The squid trypsin inhibitor was hydrolyzed with 6 N HCl at 110 ℃ for 24 h in a vacuum and was subjected to a PICO-TAG work station (Waters, Milford, MA, USA) to analyse the amino acid composition.

The protein concentration was measured using the method of Lowry et al. (1951) with bovine serum albumin fraction V as a standard protein.

## 3. Results and discussion

*3.1. Properties of squid trypsin inhibitor*

The molecular weight of the squid trypsin inhibitor was estimated as approximately 6,300 using SDS-PAGE (Fig. 2), which was similar to trypsin-

kallikrein isoinhibitors of squid *L. vulgaris* (molecular weight: 6,800–7,200) (Tschesche and Rucker, 1973b), to mammalian pancreatic Kuniz-type inhibitor (6,513) (Kassell et al., 1965), and to mammalian pancreatic Kazal-type inhibitor (6,155) (Greene et al., 1966). The molecular weights of three isoinhibitors from Roman snail *H. pomatia*, a trypsin inhibitor from the coelomic fluid of starfish *Asterias forbesi*, Kuniz-type protease inhibitor from Japanese horseshoe crab *Tachypleus tridentatus* hemocytes, and protease inhibitors from sea anemones *Radianthus rhodostoma*, *Stoichactis* sp. and *Actinia equina* were about 6,500, <6,500, 6,824, and 5,500 –7,000, respectively (Tschesche and Dietl, 1972b; Gebauer, 1973; Mebs et al., 1983; Marcum, 1987; Nakamura et al., 1987; Shiomi et al., 1989). These inhibitors are low molecular weight trypsin inhibitors. However, the molecular weight of the serine protease inhibitor, tugalistatin, from marine gastropod *T. gigas* (Yoshino et al., 1993) was estimated as 22,000, which is larger than the molecular weights of the serine protease inhibitors in the animal tissues and organs described above in 3.1.

As the squid trypsin inhibitor in this study was stable aganst the treatment of 2.5 % perchloric acid and/or 80℃ for 5 min, it was acid- and heat-stable trypsin inhibitor, similar to other low molecular weight trypsin inhibitors (Kassell et al., 1965; Greene et al., 1966; Tschesche and Dietl, 1972b; Tschesche and Rucker, 1973b). Fig. 3 shows the stability of the squid trypsin inhibitor at continuous temperature. The residual inhibitory activity of squid trypsin inhibitor after incubation at pH 8.0 and at 80 ℃ for 120 min was about 70 %.

Fig. 4 shows the inhibitory specificity of the squid trypsin inhibitor to

the trypsin of three different animals. The squid trypsin inhibitor was active against trypsins from the pyloric ceca of starfish *A. amurensis* and saury as well as porcine pancreatic trypsin.

Table 2 compares the amino acid composition of the squid trypsin inhibitor with the those of other invertebrate trypsin inhibitors. The number of Asx, Glx, and Cys/2 of the squid trypsin inhibitor were smaller than those of squid *L. vulgaris* trypsin-kallikrein isoinhibitor, but the number of Arg and Lys of the squid trypsin inhibitor were larger than those of squid *L. vulgaris* isoinhibitor. On the other hand, protease inhibitor from sea anemone *R. rhodostoma*, trypsin-kallikrein isoinhibitor from Roman snail *H. pomatia*, and Kuniz-type protease inhibitor from Japanese horseshoe crab *T. tridentatus* hemocytes have no Met, but the squid trypsin inhibitor has three Met, similar to trypsin-kallikrein inhibitor of squid *L. vulgaris* (Table 2).

### *3.2. Inhibiton of the autolysis of myofibrillar proteins by squid trypsin inhibitor*

The *modori*-phenomenon lowers elasticity, which is sometimes observed at 50 to 70 ℃ in the heating process of fish jelly production, and therefore lowers the commercial value of boiled fish paste products. The *modori*-phenomenon is closely related with the breakdown of the myosin heavy chain, which is induced by a group of heat-activated trypsin-like serine proteinases, so-called *modori*-inducing proteinases (Toyohara et al., 1990a) and also by intracellular myofibril cysteine proteinases (Toyohara et al., 1990b). It was reported that walleye pollack has only sarcoplasmic- 60 ℃-*modori*-inducing proteinase (Kinoshita et al., 1990). In this study, SDS-PAGE

analyses showed that the squid trypsin inhibitor inhibited the breakdown of walleye pollack myofibrillar proteins (Fig. 5), which implies that the squid trypsin inhibitor may inhibit the *modori*-phenomenon.

In conclusion, acid- and heat-stable trypsin inhibitor (about molecular weight 6,300) was isolated from the liver of squid *T. pacificus*. The squid trypsin inhibitor was active against trypsins from porcine, starfish, and saury, and also inhibited the autolysis of myofibrillar proteins. The amino acid composition of the squid trypsin inhibitor, similar to squid *L. vulgaris* inhibitor, differed from the amino acid composition of sea anemone, horseshoe crab, and snail trypsin inhibitors in the number of Met. The physiological roles and amino acid sequence of the squid trypsin inhibitor remain to be clarified.

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## (captions to figures)

Fig. 1. DEAE-cellulose column chromatogram of the active fraction by gel filtration on Sephadex G-50 (second).

The active fraction was applied to a column (1.1×18 cm) preequilibrated with 10 mM Tris-HCl buffer (pH 9.0) and proteins were eluted with the same

buffer containing 0 M and/or 0.3 M NaCl at a flow rate of 18 ml/h. Each 1.8 ml fraction was collected . The solid line, dotted line and closed circle indicate absorbance at 280 nm, NaCl concentration (M) and trypsin inhibitory activity (U/ml), respectively. Inset shows the electrophoretic pattern on a SDS-PAGE of the main active fraction.

Fig. 2. Estimation of molecular weight of squid trypsin inhibitor by SDS-PAGE.

SDS-PAGE was carried out using a 0.1 % SDS-20 % polyacrylamide slab-gel using the method of Fling and Gregerson (1977). 1, lysozyme (molecular weight: 14,300); 2, $\alpha$-bungarotoxin (molecular weight: 7,980); 3, squid trypsin inhibitor; 4, bacitracin (molecular weight: 1,400).

Fig. 3. Effect of continuous temperature on the stability of squid trypsin inhibitor.

Squid trypsin inhibitor was kept at 80 ℃ for 0 min to 120 min and at pH 8.0, and then the residual inhibitory activity was determined.

Fig. 4. Effects of squid trypsin inhibitor on trypsins from three different animals.

Trypsin was incubated with various concentrations of squid trypsin inhibitor (0-5.0 $\mu$g) in 20 mM Tris-HCl buffer (pH8.0) containing 4 mM $CaCl_2$

at 30 ℃ for 5 min. The residual trypsin activity was determined using TAME as a substrate. a: porcine pancreatic trypsin (8.8 μg, 0.168 U); b: starfish *A. amurensis* trypsin (4.4 μg, 0.104 U); c: saury crude trypsin (484 μg, 0.182 U). Each value is the mean of two determinations.

Fig. 5. Inhibitory effect of squid trypsin inhibitor on the autolysis of walleye pollack myofibrillar proteins.

Myofibrillar proteins (50 μl) in 20 mM Tris-HCl buffer (pH 7.2) containing 0.1 M NaCl were incubated with 20 μl of squid trypsin inhibitor (a), or sarcoplasmic fraction of walleye pollack (b), at 60 ℃ for 0 min to 120 min. Then, the breakdown of walleye pollack myofibrillar proteins was analyzed using SDS-PAGE using a 0.1 % SDS-10 % polyacrylamide slab-gel using the method of Laemmli (1970). MHC: myosin heavy chain; A: actin; MLC: myosin light chain.

However, inhibitory specificity of the squid trypsin inhibitor is slightly different between porcine and starfish trypsin. It had been found that

starfish trypsins were unstable at acidic pH and were not activated or stabilized by calcium ions unlike mammalian pancreatic trypsin (Winter and Neurath, 1970; Bundy and Gustafson, 1973). These findings were considered that there may be notable structural differences between mammalian pancreatic and starfish trypsins (Bundy and Gustafson, 1973). Therefore, the difference of inhibitory specificity may be caused by these structural differences.

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Table 1
Purification of trypsin inhibitor from the liver of squid *T. pacificus*

| Purification step | Protein (mg) | Total activity (U)* | Specific activity (U/mg) | Purity (fold) | Yield (%) |
|---|---|---|---|---|---|
| Crude inhibitor | 5,067 | 43.2 | 0.009 | 1 | 100 |
| Sephadex G-50 (first) | 1,247 | 33.9 | 0.027 | 3 | 78 |
| Sephadex G-50 (second) | 42 | 12.8 | 0.30 | 33 | 30 |
| DEAE-cellulose | 2.1 | 3.0 | 1.44 | 160 | 7 |

* One unit (U) of inhibitory activity was defined as the amount of inhibitor that decreased the activity of one milligram of porcine pancreatic trypsin to 50 %.
DEAE: diethylaminoethyl.

Table 2
Amino acid composition of trypsin inhibitor from the liver of squid *T. pacificus* compared with other invertebrates

| Amino acid | Mol % | | | | |
|---|---|---|---|---|---|
| | squid *T. pacificus* | squid *L. vulgaris*[*1] | sea anemone *R. rhodostoma*[*2] | snail *H. pomatia*[*3] | horseshoe crab *T. tridentatus*[*4] |
| Asx | 5.9 (6)[*5] | 11.1 (11) | 5.1 (5) | (5) | (7) |
| Glx | 2.6 (3) | 6.0 ( 6) | 4.0 (4) | (9) | (5) |
| Cys/2 | 3.8 (4) | 7.2 ( 8) | 4.2 (4) | (6) | (6) |
| Ser | 0.8 (1) | 3.1 ( 3) | 1.8 (2) | (4) | (3) |
| Gly | 7.8 (8) | 6.7 ( 7) | 5.6 (6) | (8) | (8) |
| His | 1.0 (1) | 0.3 ( 0) | 0.9 (1) | (0) | (0) |
| Arg | 2.8 (3) | 1.1 ( 1) | 5.1 (5) | (4) | (5) |
| Thr | 3.9 (4) | 4.8 ( 5) | 0.8 (1) | (3) | (5) |
| Ala | 2.3 (2) | 3.1 ( 3) | 1.7 (2) | (2) | (2) |
| Pro | 2.2 (2) | 2.0 ( 2) | 2.1 (2) | (3) | (4) |
| Tyr | 2.3 (2) | 1.0 ( 1) | 3.1 (3) | (4) | (4) |
| Val | 3.0 (3) | 1.9 ( 2) | 2.9 (3) | (2) | (1) |
| Met | 3.0 (3) | 1.6 ( 2) | 0 (0) | (0) | (0) |
| Ile | 1.8 (2) | 2.0 ( 2) | 1.6 (2) | (1) | (0) |
| Leu | 1.3 (1) | 2.9 ( 3) | 2.0 (2) | (1) | (2) |
| Phe | 2.5 (3) | 2.9 ( 3) | 2.7 (3) | (4) | (4) |
| Trp | – (–)[*6] | – ( –)[*6] | 0 (0) | (0) | (0) |
| Lys | 5.7 (6) | 3.3 ( 3) | 3.1 (3) | (2) | (5) |
| Total | (54) | (62) | (48) | (58) | (61) |

[*1] Tschesche and Rucker (1973b).
[*2] Mebs et al. (1983).
[*3] Dietl and Tschesche (1976).
[*4] Nakamura et al. (1987).
[*5] Values in parentheses are to the nearest integer.
[*6] Decomposed during hydrolysis with 6 N HCl.

Fig. 1

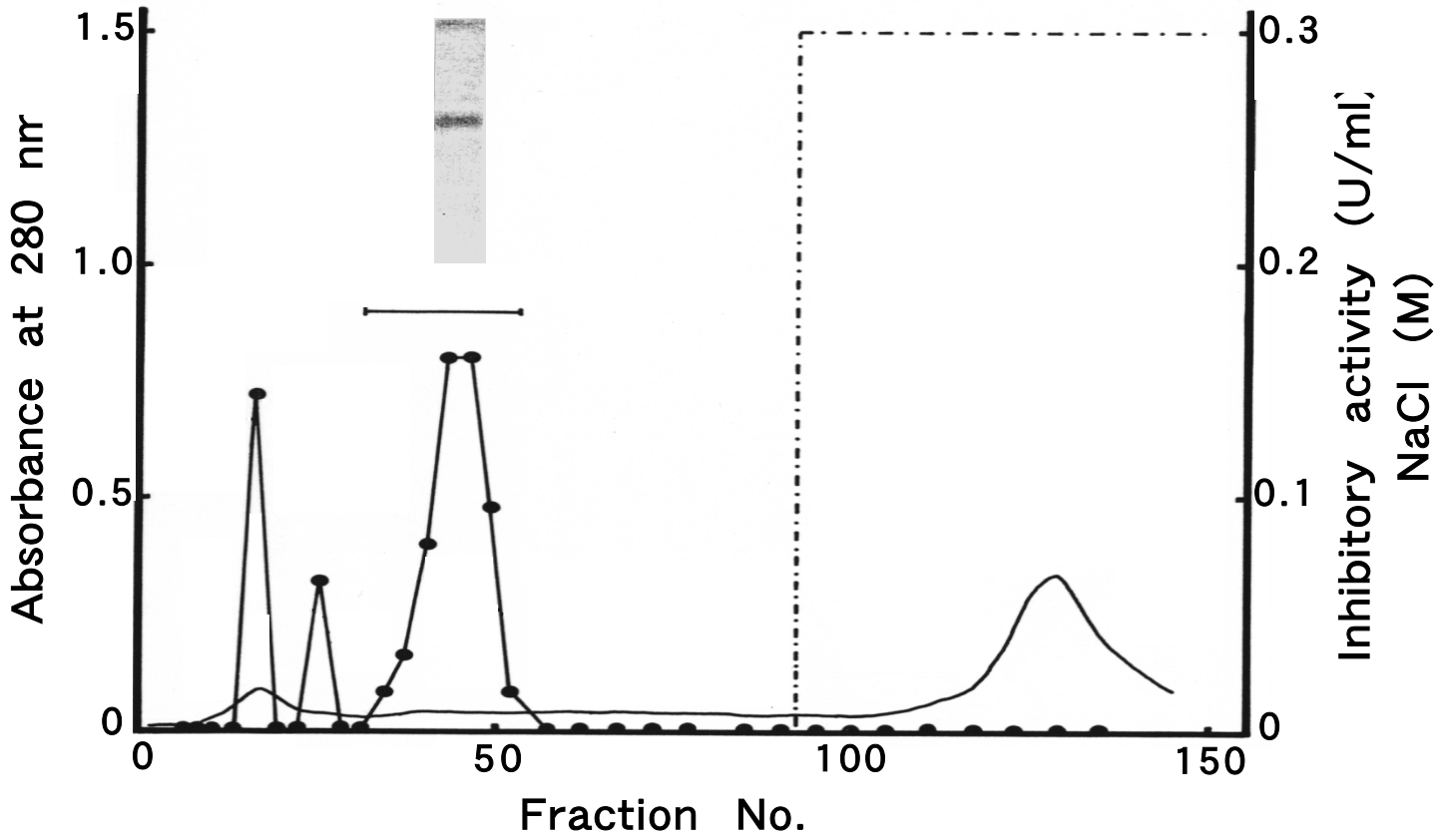
Absorbance at 280 nm
1.5
1.0
0.5
0
0
50
100
150
Fraction No.
0.3
0.2
0.1
0
Inhibitory activity (U/ml)
NaCl (M)

Fig. 2

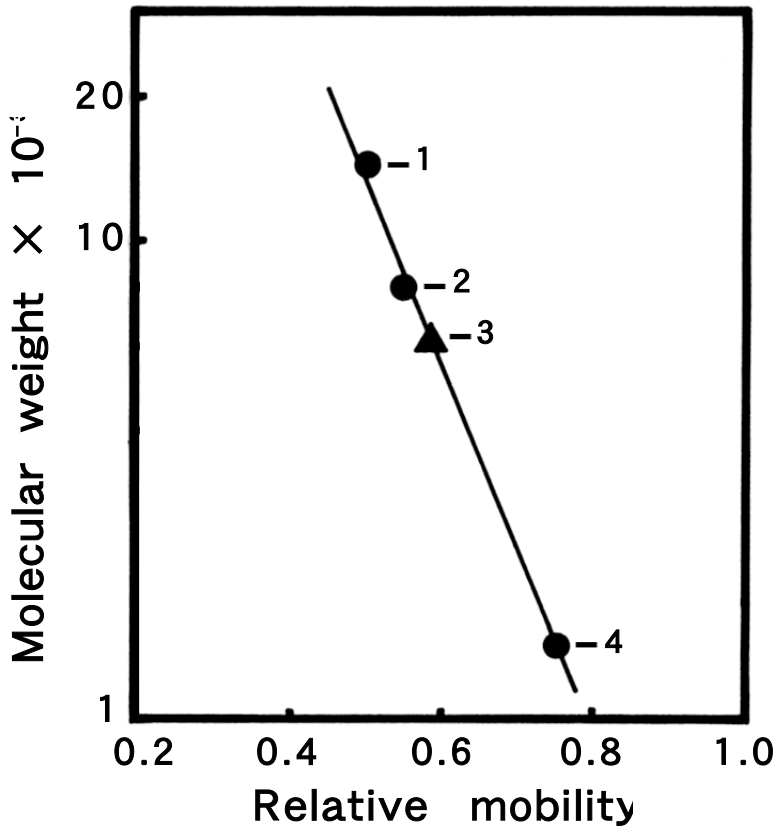
Molecular weight × 10⁻⁴
20
10
1
0.2
0.4
0.6
0.8
1.0
Relative mobility
1
2
3
4

Fig. 3

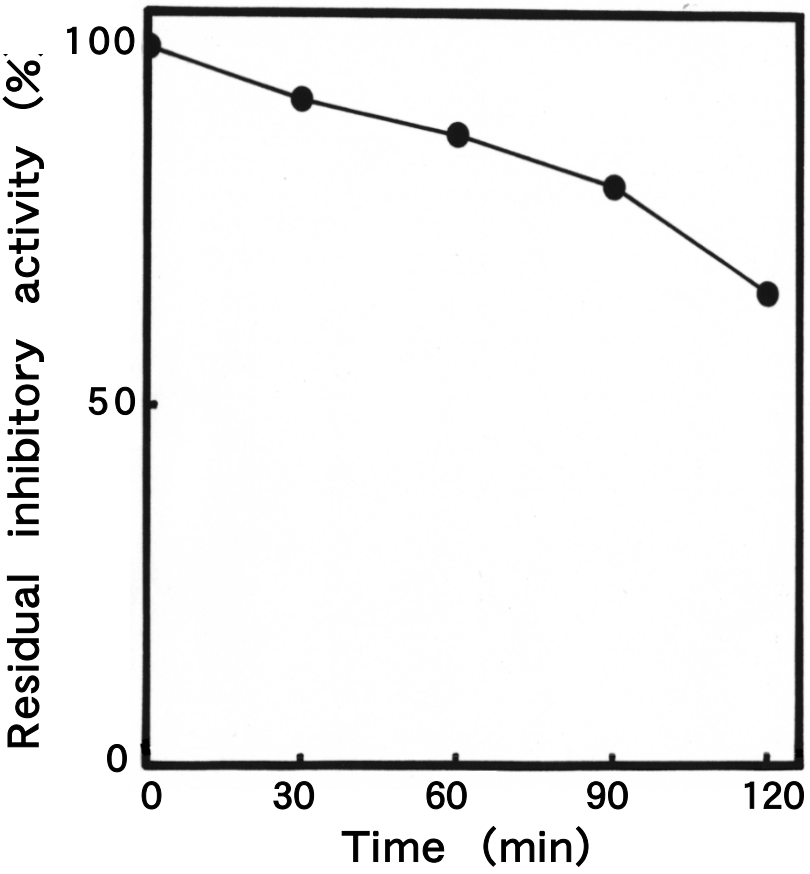

Fig. 4

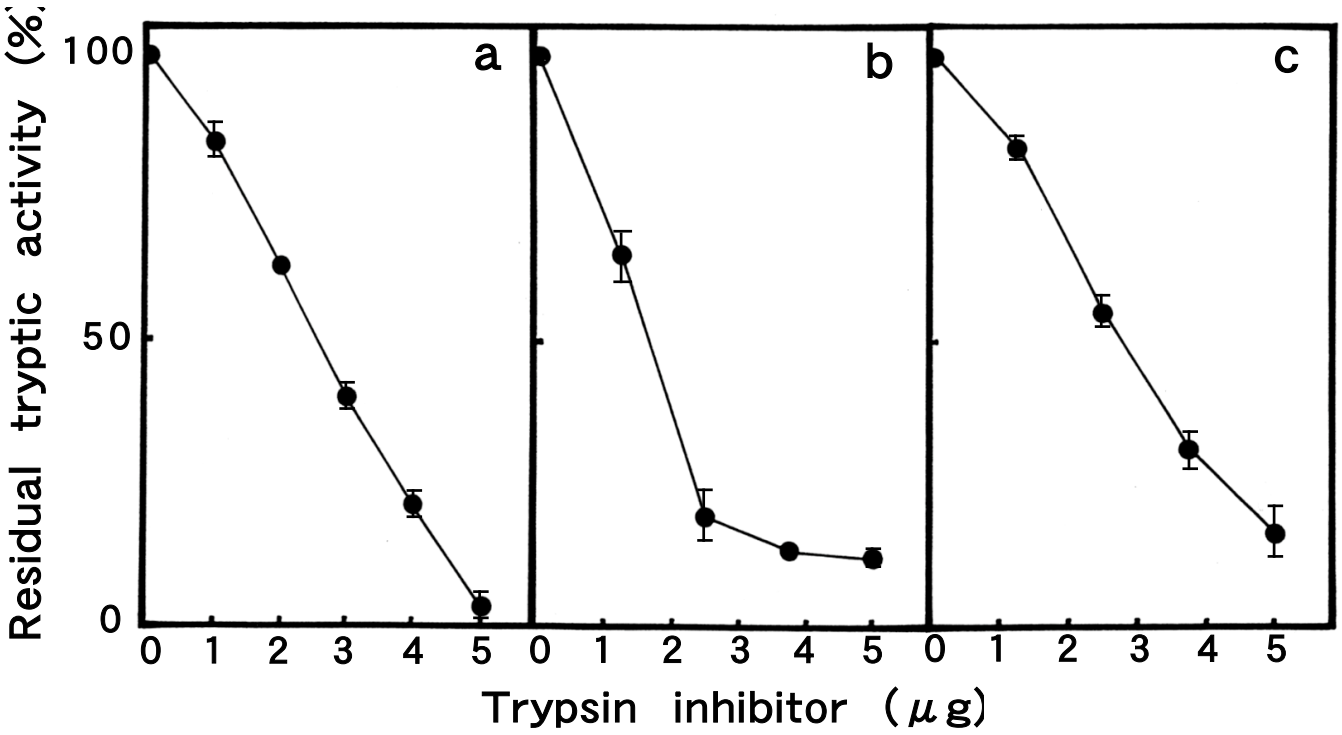
Residual tryptic activity (%)
100
50
0
a
b
c
0 1 2 3 4 5
0 1 2 3 4 5
0 1 2 3 4 5
Trypsin inhibitor (μg)

Fig. 5