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Purification and Some Properties of Cysteine Protease of *Spirometra erinacei* Plerocercoid (Cestoda ; Diphyllobothriidae)

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Key words: *Spirometra erinacei*, plerocercoid, cysteine protease

Introduction

Biochemical studies are necessary to explicate the metabolism in enzymatic systems of parasites. On hydrolyzing enzymes of *Spirometra erinacei*, Kwa (1972) detected proteolytic enzyme(s), hydrolyzing casein, in the scolex of plerocercoids. Nakamura and Yanagisawa (1982 a, b) and Nakamura *et al.* (1984) reported thiol protease in plerocercoids, and Maki *et al.* (1982) carboxyl protease in adults. We described in the previous paper (Fukase *et al.*, 1984) that such hydrolyzing enzymes as *N*-α-benzoyl-DL-arginine-*p*-nitroanilide (Bz-Arg-*p*NA) amidolytic, glutaryl-L-phenylalanine-*p*-nitroanilide amidolytic, L-leucine-*p*-nitroanilide amidolytic, and α-amylase exist in plerocercoids and that the Bz-Arg-*p*NA amidolytic enzyme adsorbed in DEAE-cellulose can be divided into two forms with different molecular weights and isoelectric points. However, it is not known whether the enzymes in our previous report are identical with those of Kwa (1972), Nakamura and Yanagisawa (1982 a, b) and Nakamura *et al.*(1984), and Maki *et al.* (1982) or not. In order to make this problem clear and to elucidate the physiological roles of the enzymes, more detailed examinations must be made on each enzyme.

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In the present paper, we report purification and characterization of a Bz-Arg-*p*NA amidolytic enzyme, adsorbed in DEAE-cellulose, of plerocercoids of *S. erinacei*.

Materials and Methods

Plerocercoids of Spirometra erinacei :

Plerocercoids were collected from the subcutis of *Elaphe quadrivirgata* (Japanese rat snake) captured in Okayama Prefecture, Japan. The plerocercoids were washed in tap water and then freezed until use.

To identify the plerocercoids, three of them were fed to a domestic cat, which was autopsied 20 days after feeding. The adult worms obtained at autopsy were identified as *S. erinacei*.

Chemicals and proteins :

The following chemicals and proteins were all supplied commercially : DEAE-cellulose, *p*-chloromercuribenzoic acid, *p*-chlorophenacyl bromide, *N*-ethylmaleimide, dithiothreitol, and 5, 5′-dithio-bis-(2-nitrobenzoic acid) (Nakarai Chemicals, Ltd., Kyoto, Japan); Sephadex G-50, Sephadex G-100, Sepahdex G-200, QAE-Sephadex A-50, Thiopropyl Sepharose 6B, and Blue Dextran 2000 (Pharmacia Fine Chemicals AB, Sweden); Ampholine carrier ampholyte (LKB Produkter AB, Sweden); *N*-α-benzoyl-DL-arginine-*p*-nitroanilide HCl (Bz-Arg-*p*NA), *N*-α-carbobenzoxy-L-arginine-*p*-nitroanilide HCl (CBZ-Arg-*p*NA), *N*-α-tosyl-L-arginine methyl ester HCl (Tos-Arg-Me), *N*-carbobenzoxy-L-alanyl-L-glycine methyl ester (CBZ-Ala-Gly-Me),

phenylmethylsulfonyl fluoride, human plasma α_1-antitrypsin, bovine serum albumin, equine skeletal muscle myoglobin, and horse heart cytochrome c (Sigma Chemical Co., USA); D-prolyl-L-phenylalanyl-L-arginine-*p*-nitroanilide 2HCl (Pro-Phe-Arg-*p*NA), D-valyl-L-leucyl-L-lysine-*p*-nitroanilide 2HCl (Val-Leu-Lys-*p*NA), *N*-α-benzoyl-L-arginine methyl ester HCl (Bz-Arg-Me), *N*-α-benzoxy-L-lysine methyl ester HCl (α-Z-Lys-Me), *N*-ε-benzoxy-L-lysine methyl ester HCl (ε-Z-Lys-Me), *N*-acetyl-L-phenylalanine methyl ester (Ac-Phe-Me), and *N*-α-benzoyl-L-citrullin methyl ester (Bz-Cit-Me) (Serva Feinbiochemica GmbH, West Germany); *N*-α-tosyl-L-lysine methyl ester HCl (Tos-Lys-Me) and pepstatin A (Protein Research Foundation, Osaka, Japan); *N*-α-tosyl-L-lysylchloromethane hydrochloride (Merck and Co., Inc., USA); *N*, *N'*-*o*-phenylenedimaleimide (Aldrich Chemical Co., Inc., USA); diphenylcarbamoyl chloride (Tokyo Kasei Kogyo Co., Ltd., Tokyo, Japan); bovine plasma α_2-macroglobulin (Boehringer Mannheim GmbH, West Germany); aprotinin (Teikoku Hormone Mfg. Co., Ltd., Tokyo, Japan); and ovo trypsin inhibtor and lima bean trypsin inhibitor (Worthington Biochemical Corp., USA). All the other chemicals used were of analytical reagent grade.

Assay of enzyme activity:

Amidolytic activity and esterolytic one were measured by a modified method of Amundsen *et al.* 's (1979) in 4×10^{-4} M substarte concentration and by a modified method of Moriwaki *et al.* 's (1971) in 2×10^{-2} M substrate concentration, respectively. The activity was expressed in terms of nmol of substrate hydrolyzed per minute at 37°C in 0.1 M phosphate buffer, pH 7.0, containing dithiothreitol to get a final concentration of 10^{-3} M, because the enzyme was activated by dithiothreitol in preliminary experiments. Bz-Arg-*p*NA was used as a substrate in all the purification procedures.

To know the effect of inhibitors and chemical modifiers against the enzymes, the purified enzyme (0.074 A_{280}) was incubated with different amounts of them for 20 minutes, and then remaining amidolytic activity was measured, in 0.1 M phosphate buffer, pH 7.0, without dithiothreitol.

Estimation of protein concentration:

Protein concentration was estimated by measuring absorbance at 280 nm in a cuvette of 1 cm light path.

Determination of dithiothreitol concentration:

Dithiothreitol concentration was determined by the method of Ellman (1958) in 5, 5'-dithio-bis-(2-nitrobenzoic acid).

SDS-polyacrylamide gel electrophoresis:

Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis was done by the method of Laemmli (1970) in 16 % gel. After electrophoresis, the proteins in the gel were stained with Coomassiee Brilliant Blue R-250, and densitometry was done using the Jookoo densitometer, "Densitoron model-PAN" (Jookoo Co., Ltd., Tokyo, Japan).

Isoelectric focusing:

The apparatus of LKB Produkter was used for isoelectric focusing with Ampholine carrier ampholite at the pH range 3.5–5, by the method of Vesterberg and Svensson (1966). Electrophoresis was done at 10°C for 40 hours at 500 V constant voltage. Then 1 ml fractions of the column were collected and pH of them was measured at 20°C.

Results

Purification of the enzyme

All the processes were done at room temperature unless otherwise indicated.

Step 1. Extraction of crude enzyme preparation from plerocercoids:

Frozen plerocercoids (14.7 g in wet weight) were crushed in a porcelain mortar and Triton X-100 was added to the resulting preparation to get a final concentration of 1 %. This preparation was then homogenized and deionized water was added to it in a ratio of 3 ml per gram of plerocercoids. Then the diluted homogenate was shaken vigorously for 1 hour and was centrifuged at $11\,000\times g$ for 20 minutes at 4°C. The supernatant (crude enzyme extract) showed the total A_{280} and Bz-Arg-*p*NA amidolytic activity to be 1508 and 7731 nmol per minute, respectively.

Step 2. DEAE-cellulose adsorption and elution:

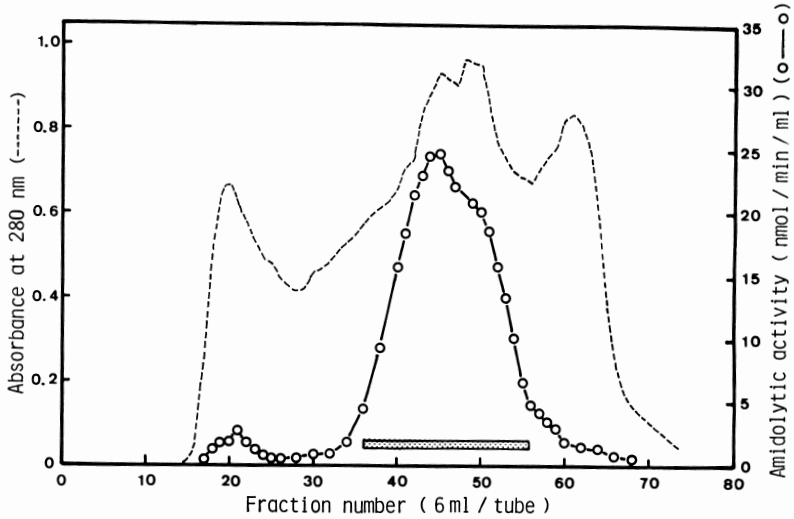

Fig. 1 Elution profile in Sephadex G-200 gel filtration
·····: absorbance at 280 nm ; o——o: Bz-Arg-*p*NA amidolytic activity ;
▒▒: pooled fractions

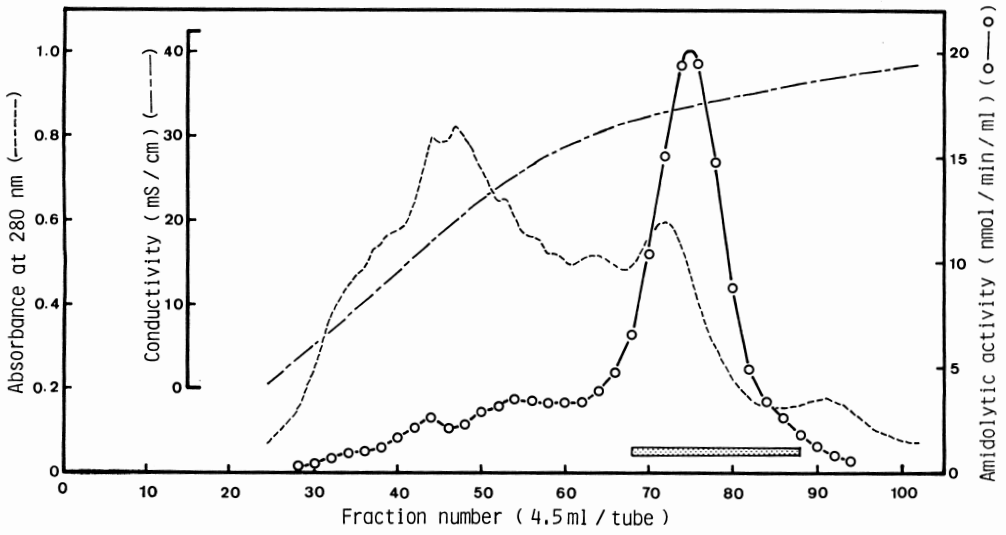

Fig. 2 Elution profile in QAE-Sephadex A-50 ion exchange chromatography
·····: absorbance at 280 nm ; — — —: conductivity concentration ; o——o: Bz-Arg-*p*NA amidolytic activity ; ▒▒ : pooled fractions

The crude extract obtained in Step 1 was adjusted to 1 mS/cm in conductivity concentration and to 7.5 in pH, and then was applied to a DEAE-cellulose column (1.2×12 cm) equilibrated with 0.02 M Tris-HCl buffer, pH 7.5. After rinsed with the same buffer, the enzyme was eluted with 1 M NaCl solution. Five ml fractions were collected and their amidolytic activity was measured toward Bz-Arg-*p*NA. The active fractions pooled showed the total A_{280} and Bz-Arg-*p*NA amidolytic activity to be 365 and 4504 nmol per minute, respectively.

Step 3. Sephadex G-200 gel filtration :

The pooled solution in Step 2 was applied to a Sephadex G-200 column (2.2×97 cm) equilibrated with 0.01 M phosphate buffer at pH 7.0. Elution was done in the same buffer and

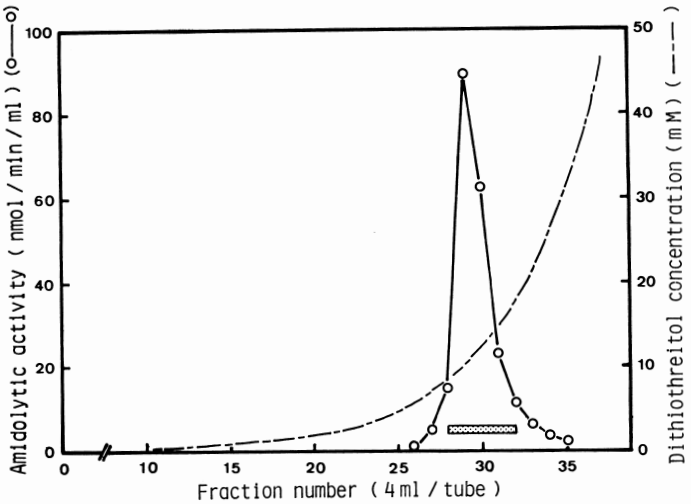

Fig. 3 Elution profile in Thiopropyl Sepharose 6B covalent chromatography
o——o : Bz-Arg-*p*NA amidolytic activity ; — — — : dithiothreitol concentration ;
▒ : pooled fractions

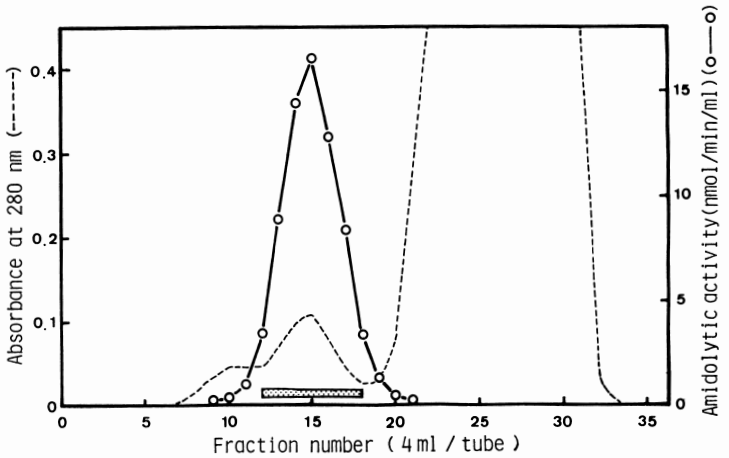

Fig. 4 Elution profile in Sephadex G-50 gel filtration
····· : absorbance at 280nm ; o——o : Bz-Arg-*p*NA amidolytic activity ;
▒ : pooled fractions

6 ml fractions were collected. Fractions obtained were divided into two: Those first eluted with the weaker activity and those secondly eluted with the stronger activity; and the latter were collected (Fig. 1). The total A_{280} and Bz-Arg-*p*NA amidolytic activity of the solution were 200 and 3853 nmol per minute, respectively.

Step 4. QAE-Sephadex A-50 ion exchange chromatography :

The solution obtained in Step 3 was adjusted to 2 mS/cm in conductivity concentration and to 7.5 in pH, and then was applied to a QAE-Sephadex A-50 column (1.6×74 cm) equilibrated with Tris-HCl buffer of the same conductivity concentration and pH as the sample. After rinsed with the buffer, the enzyme was eluted with a gradient elution system in the buffer containing 0 to 0.6 M NaCl, and 4.5 ml fractions were collected. Then the fractions with the strong amidolytic activity (fraction number 68-88 in Fig. 2) were col-

Table 1 Purification of cysteine protease of *Spirometra erinacei* plerocercoid

Step	Procedure	Total A_{280}	Total activity (nmol/min)	Specific activity (nmol/min/A_{280})	Purification factor
1	Extraction	1508	7731	5.1	1
2	DEAE-cellulose adsorption & elution	365.4	4504	12.3	2.4
3	Sephadex G-200 gel filtration	199.8	3853	19.3	3.8
4	QAE-Sephadex A-50 chromatography	33.17	989.4	29.8	5.8
5	Thiopropyl Sepharose 6 B Chromatography and Sephadex G-50 gel filtration	2.546	394.4	154.9	30.4

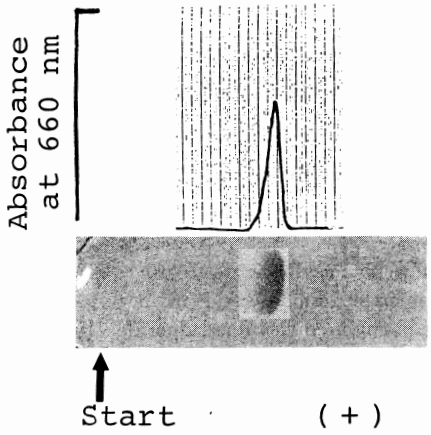

Fig. 5 Homogeneity of the purified enzyme in SDS-polyacrylamide gel electrophoresis

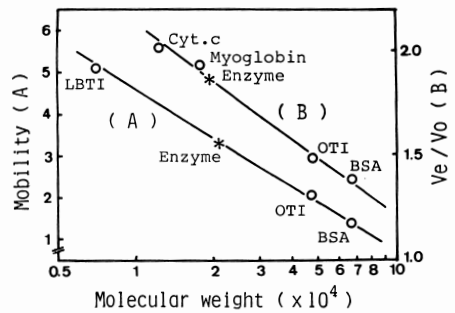

Fig. 6 Molecular weight of cysteine protease of *Spirometra erinacei* plerocercoid estimated by SDS-polyacrylamide gel electrophoresis (A) and Sephadex G-100 gel filtration (B)

Reference proteins used were authentic lima bean trypsin inhibitor (LBTI), cytochrome c (Cyt. c), myoglobin, ovo trypsin inhibitor (OTI), and bovine serum albumin (BSA). And blue dextran was used for determination of void volume in the gel filtration.

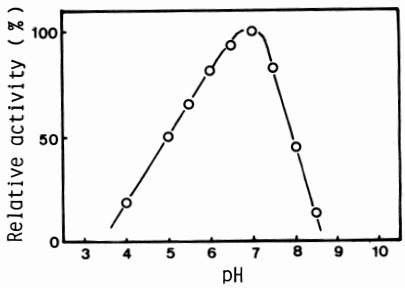

Fig. 7 Effect of pH on the amidolytic activity of cysteine protease of *Spirometra erinacei* plerocercoid

Activity is expressed as a percentage of that at optimum pH.

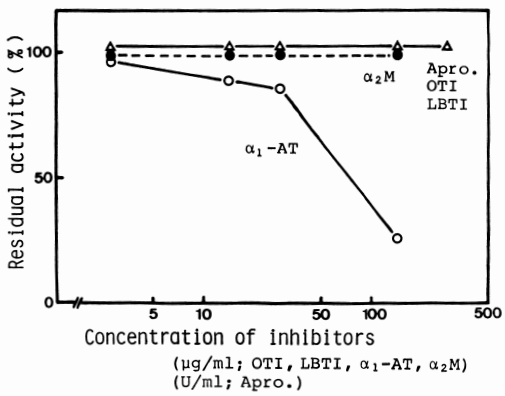

Fig. 8 Effect of protease inhibitors on the amidolytic activity of cysteine protease of *Spirometra erinacei* plerocercoid

Activity is expressed as a percentage of that of control (intact).

○——○ : α_1-antitrypsin (α_1-AT); ●——● : α_2-macroglobulin (α_2M); △——△: aprotinin (Apro.), ovo trypsin inhibitor (OTI), and lima bean trypsin inhibitor (LBTI)

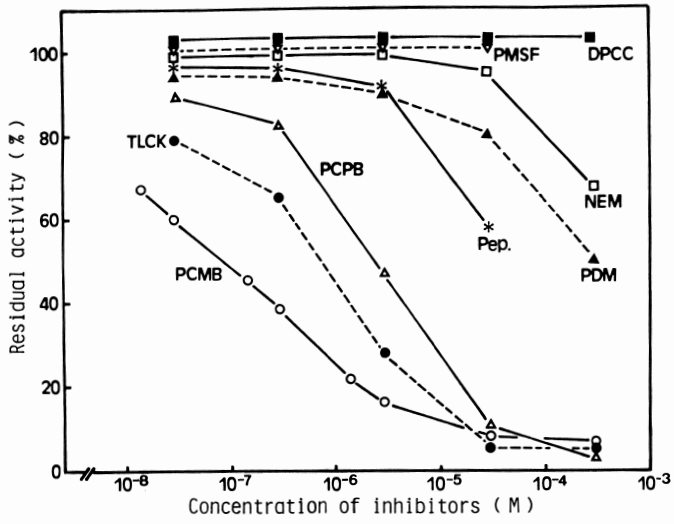

Fig. 9 Effect of chemically synthesized modifiers on the amidolytic activity of cysteine protease of *Spirometra erinacei* plerocercoid

Activity is expressed as a percentage of that of control (intact).
○——○ : *p*-chloromercuribenzoic acid (PCMB); ●····● : *N*-α-tosyl-L-lysylchloromethane (TLCK); △——△: *p*-chlorophenacylbromide (PCPB); ✳——✳: pepstatin (Pep.); ▲····▲: *N*, *N'*-*o*-phenylenedimaleimide (PDM); □——□: *N*-ethylmaleimide (NEM); ▽····▽: phenylmethylsulfonyl fluoride (PMSF); ■——■: diphenylcalbamoyl chloride (DPCC)

lected. The total A_{280} and Bz-Arg-*p*NA amidolytic activity of the solution were 33.17 and 989.4 nmol per minute, respectively.

Step 5. Thiopropyl Sepharose 6B covalent chromatography and Sephadex G-50 gel filtration :

The solution obtained in Step 4 was adjusted to pH 7.0 and then mixed with Thiopropyl Sepharose 6B pre-equilibrated with 0.01 M phosphate buffer, pH 7.0, which contained 0.1 M NaCl and 10^{-3} M ethylenediaminetetraacetic acid (EDTA). The mixture was stirred overnight. Then the Thiopropyl Sepharose 6B was packed in a column (2.25×13.5 cm). After rinsed with the buffer containing 1 M NaCl and 10^{-3} M EDTA, the enzyme was eluted with a gradient elution system in the buffer containing 0 to 0.05 M dithiothreitol, and 4 ml fractions were collected. The fractions with strong Bz-Arg-*p*NA amidolytic activity were collected (Fig. 3), and they showed the total Bz-Arg-*p*NA amidolytic activity of 806.0 nmol per minute.

The pooled active fractions in the covalent chromatography were applied to a Sephadex G-50 column (2×27 cm) equilibrated with 0.01 M phosphate buffer at pH 7.0, and 4 ml fractions were collected (Fig. 4). The total A_{280} and Bz-Arg-*p*NA amidolytic activity were 2.546 and 394.4 nmol per minute, respectively. The specific activity was 154.9 nmol per minute per A_{280}, namely, being about 30-times higher than that of the extract in Step 1.

Confirmation of the homogeneity :

The final preparation obtained in Step 5 showed a single band in SDS-polyacrylamide gel electrophoresis, and a single peak was detected by densitometry (Fig. 5).

Properties of the enzyme

Molecular weight :

Approximate molecular weight of the purified enzyme was 2.1×10^4 daltons by SDS-polyacrylamide gel electrophoresis and 1.9×10^4 daltons by the gel filtration method in a Sephadex G-100 column (2×90 cm) of Andrews (1964) as shown in Fig. 6.

Effect of pH on enzyme activity :

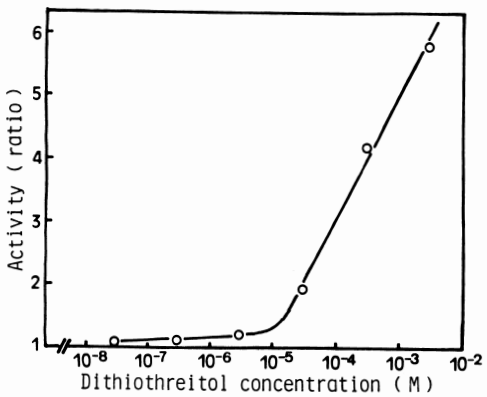

Fig. 10 Effect of dithiothreitol on the amidolytic activity of cysteine protease of *Spirometra erinacei* plerocercoid

Activity is expressed as a ratio to that of control (intact).

The Bz-Arg-*p*NA amidolytic activity of the purified enzyme varied with pH from 3.0 to 10.0 in 0.1 M modified Britton and Robinson's universal buffer which contains H_3PO_4, CH_3COOH, H_3BO_4, and NaOH (Britton and Robinson, 1931), and optimum pH was 7.0, as shown in Fig. 7.

Effect of various reagents on enzyme activity :

Effect of several organism-originated protease inhibitors and chemically synthesized modifiers was examined on the amidolytic activity of the purified enzyme. Of the organism-originated protease inhibitors, human plasma α_1-antitrypsin inhibited the enzyme activity, but bovine plasma α_2-macroglobulin, aprotinin, ovo trypsin inhibitor, and lima bean trypsin inhibitor did not (Fig. 8). Of the chemically synthesized modifiers, on the other hand, *p*-chloromercuribenzoic acid, *N*-α-tosyl-L-lysylchloromethane, and *p*-chlorophenacylbromide strongly inhibited the activity. And the activity was inhibited also by pepstatin, *N*, *N'*-*o*-phenylenedimaleimide, and

Table 2 Substrate specificity of cysteine protease of *Spirometra erinacei* plerocercoid

Substrate	Activity	
	nmol/min/A_{280}	Ratio
Bz-Arg-*p*NA	153.9	1
CBZ-Arg-*p*NA	18.5	0.12
Pro-Phe-Arg-*p*NA	307.8	2.00
Val-Leu-Lys-*p*NA	8125.9	52.8
Bz-Arg-Me	10511.4	68.3
Tos-Arg-Me	5417.3	35.2
Tos-Lys-Me	4555.4	29.6
α-Z-Lys-Me	1569.8	10.2
ε-Z-Lys-Me	695.6	4.52
Ac-Phe-Me	449.4	2.92
Bz-Cit-Me	4524.7	29.4
CBZ-Ala-Gly-Me	720.3	4.68

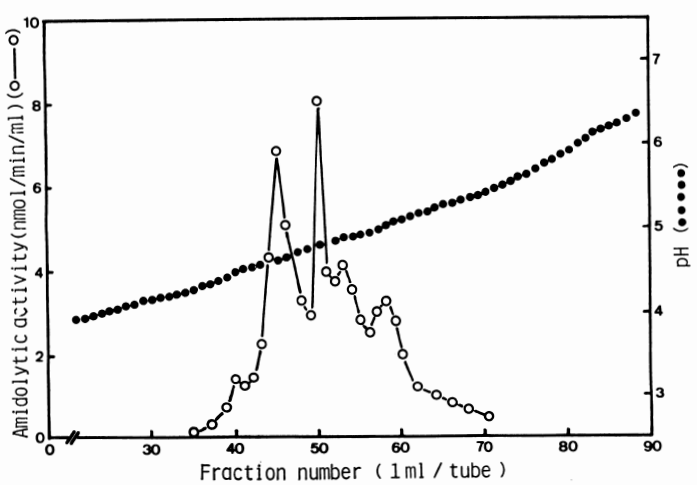

Fig. 11 Multiple forms of cysteine protease of *Spirometra erinacei* plerocercoid detected by isoelectric focusing
amidolytic activity ; ●●●●● : pH o——o : Bz-Arg-*p*NA

N-ethylmaleimide, but not by phenylmethylsulfonyl fluoride and diphenylcarbamoyl chloride (Fig. 9). The enzyme was strongly activated by dithiothreitol (Fig. 10).

Substrate specificity :

Amidolytic and esterolytic activity of the purified enzyme was examined to such synthetic substrates as arginine, lysine, and other amino acid derivatives, among which arginine derivatives and lysine derivatives were sufficiently hydrolyzed by the enzyme. And other amino acid derivatives such as Bz-Cit-Me were also hydrolyzed. The esterolytic activity was generally stronger than the amidolytic one except in the case of Val-Leu-Lys-*p*NA hydrolysis (Table 2).

Multiple forms of the enzyme :

The enzyme had some multiple forms detected by isoelectric focusing. They contained two stronger activities, whose isoelectric points (pI) were 4.7 and 4.9 (Fig. 11).

Discussion

Proteases are classified into four groups by catalytic site : serine, cysteine (or thiol), metal, and aspartic (or carboxyl) proteases. The present protease seems to belong to cysteine proteases by its behaviour to some effectors, especially inhibition by SH-blocking agents such as *p*-chloromercuribenzoic acid and activation by dithiothreitol or a SH-containing compound, although the enzyme slightly inhibited by pepstatin. And further, the success of purification by covalent chromatography using Thiopropyl Sepharose 6B supports that the enzyme is a cysteine protease.

In the present investigation, estimated molecular weight of the enzyme was 2.1×10^4 daltons by SDS-polyacrylamide gel electrophoresis and 1.9×10^4 daltons by Sephadex G-100 gel filtration in phosphate buffer. In our previous examination (Fukase *et al.*, 1984), however, it was 3.8×10^4 daltons by Sephadex G-150 gel filtration in Tris-HCl buffer. This discordance in molecular weight between the present and previous examinations will be caused by that, in the previous examination the enzyme interacted with other substances in the gel filtration because of the imcompletely purified enzyme, or the enzyme formed dimer. On polymerization in gel filtration, Sato and Suzuki (1983) also described that acid protease of canine heartworm, *Dirofilaria immitis* easily polymerized in gel filtration with different buffers.

pI value of the enzyme was 4.9 in the previous paper (Fukase *et al.*, 1984). But in the present examination with purified enzyme, some multiple forms were detected, two of them had strong activity and were 4.7 and 4.9 in pI. Multiple forms exist in many enzymes, e.g., kallikreins (Hojima *et al.*, 1975), and were suggested to exist in such proteases of parasitic helminths as hemoglobin-specific proteases of *Schistosoma mansoni*, *Angiostrongylus cantonensis*, and *Fasciola hepatica* (Aoki and Oya, 1978) and acid protease of *Dirofilaria immitis* (Sato and Suzuki, 1983). But significance of multiple forms is not known.

Kwa (1972) detected casein hydrolyzing enzyme(s) in the scolex of plerocercoids of *S. erinacei*. And Nakamura and Yanagisawa (1982 a, b) and Nakamura *et al.* (1984) reported thiol protease in plerocercoids, which hydrolyzes azocoll (a synthetic substrate of collagen combined with azo dye), actin, and myosin. The present enzyme may be identical with that of Nakamura and Yanagisawa (1982 a, b) and Nakamura *et al.* (1984) because of resemblance in some properties. But these two enzymes cannot be directly compared with each other, because our methods of extraction and enzyme assay differ from those used by the other authors. And further, the behaviours to pepstatin and *N*-ethylmaleimide were different between the two enzymes : the enzyme of Nakamura's group was not inhibited by them (Nakamura and Yanagisawa, 1982 b), but the present enzyme was inhibited. Accordingly, it is impossible to discuss fully on the identity of the two enzymes.

Physiological roles of the present cysteine protease are not known. But if the enzyme is identical with that of Nakamura and Yanagisawa (1982 a, b) and Nakamura *et al.* (1984) which hydrolyzes muscle structural proteins, it may be concerned with digestion of host muscles.

Many reports have been recently published on the hydrolyzing enzymes of parasitic helminths. Cystenie proteases were also detected from such helminths as *Schistosoma mansoni* (Dresden and Deelder, 1979), *Clonorchis sinensis* (Sato *et al.*, 1980), *Fasciola* sp. (Yamasaki *et al.*, 1982), and *Paragonimus miyazakii* (Yamakami and Hamajima, 1982) in addition to *S. erinacei*, but their physiological meanings have not been made clear. So, their enzymological characteristics and physiological functions must be studied in near future.

Summary

A protease, which hydrolyzes *N*-α-benzoyl-DL-arginine-*p*-nitroanilide, of plerocercoids of *Spirometra erinacei* was purified by the techniques of DEAE-cellulose adsorption and elution, Sephadex G-200 gel filtration, QAE-Sephadex A-50 ion exchange chromatography, Thiopropyl Sepharose 6B covalent chromatography, and Sephadex G-50 gel filtration. The final preparation was homogeneous in SDS-polyacrylamide gel electrophoresis.

Estimated molecular weight of the enzyme was 2.1×10^4 daltons by SDS-polyacrylamide gel electrophoresis and 1.9×10^4 daltons by Sephadex G-100 gel filtration. Optimum pH of the amidolytic activity was 7.0.

The enzyme activity was strongly inhibited by SH-blocking agents such as *p*-chloromercuribenzoic acid, while accelerated by dithiothreitol. So, the enzyme seems to be a cysteine protease.

The multiple forms were detected by isoelectric focusing.

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マンソン裂頭条虫プレロセルコイドのシステインプロテアーゼの精製とその性質

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マンソン裂頭条虫のプレロセルコイドに含まれる *N*-α-benzoyl-DL-arginine-*p*-nitroanilide を水解するプロテアーゼの精製を行い，その性質について検討した．

精製は，虫体からの抽出液の DEAE-セルロース吸着・溶出，セファデックス G-200 ゲル濾過，QAE-セファデックス A-50 イオン交換クロマトグラフィー，チオプロピールセルファロース 6 B コバレントクロマトグラフィー，およびセファデックス G-50 ゲル濾過によって行い，その結果得られた標品は，SDS-ポリアクリルアミドゲル電気泳動において均一であった．

本酵素の分子量は，SDS-ポリアクリルアミドゲル電気泳動により 2.1×10^4，ゲル濾過により 1.9×10^4 と推定された．また，アミダーゼ活性の至適 pH は 7.0 であった．本酵素の活性は，*p*-chloromercuribenzoic acid などにより阻害を受け，また，dithiothreitol により増強された．したがって，本酵素はシステインプロテアーゼに属するものと考えられる．さらに，等電点電気泳動によって，本酵素には多様成分が存在することが認められた．