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Some Hydrolyzing Enzymes, Especially Arginine Amidase, in Plerocercoids of *Spirometra erinacei* (Cestoda ; Diphyllobothriidae)

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Introduction

A pseudophyllidean tapeworm, *Spirometra erinacei* utilizes many species of vertebrates including man as a second intermediate and/or a paratenic host. It is very interesting from the viewpoint of host specificity that this tapeworm has very wide host range in its plerocercoid stage. On the other hand, when plerocercoids of *S. erinacei* in second intermediate or paratenic hosts were ingested by final hosts such as dogs and domestic cats, they grow rapidly into adults which begin to oviposit about 8 days after infection (Takahashi, 1959). This rapid growth and maturation in final hosts is thought to be associated with drastic changes in metabolic system and rapid synthesis of a large quantity of protein. However, the mechanisms of these phenomena have not yet been made clear.

In order to elucidate rapid growth in the early developmental stage in final hosts, the hydrolyzing enzymatic system relating to the digestion of the cestode, *S. erinacei*, should be studied biochemically. Nakamura and Yanagisawa (1982 a, b) and Maki *et al.* (1982) described acid thiol proteinase in plerocercoids and probably-carboxyl protease in adults of the cestode, respectively. But the detailed properties of these enzymes and the occurrence of other proteolytic enzymes have not been known enough.

In the present paper the authors report some hydrolyzing enzymes, especially arginine amidase(s) in plerocercoids of *S. erinacei*.

Materials and Methods

Plerocercoids

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

In order to identify the plerocercoids, a domestic cat was inoculated with three of them and autopsied 20 days after infection. The adult worms obtained at autopsy were identified as *S. erinacei*.

Chemicals and proteins

The following chemicals and proteins were obtained commercially: DEAE-cellulose, CM-cellulose, glutaryl-L-phenylalanine-*p*-nitroanilide (Glt-Phe-*p*NA), and L-leucine-*p*-nitroanilide (Leu-*p*NA) (Nakarai Chemicals, Ltd., Kyoto, Japan); Sephadex G-150 (Pharmacia Fine Chemicals AB, Sweden); carrier ampholyte (LKB Produkter AB, Sweden); N-α-benzoyl-DL-arginine-*p*-nitroanilide HCl (Bz-Arg-*p*NA), cytochrome c, soybean trypsin inhibitor, bovine serum albumin, and human hemoglobin (Sigma Chemical Co., U.S.A.); and the amylase test kit (Shionogi and Co., Ltd., Osaka, Japan). All the other chemicals

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used were of analytical reagent grade.

Estimation of protein concentration

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

Assay of enzyme activity

Amidolytic activity for Bz-Arg-*p*NA, Glt-Phe-*p*NA, and Leu-*p*NA was measured by the modified method of Amundsen *et al.* (1979) in 5×10^{-4} M substrate concentration and was expressed in terms of n mol of substrate hydrolyzed per minute at 37°C in 0.1 M Tris-HCl buffer, pH 8.0. And for Bz-Arg-*p*NA, activity was also measured in 0.1 M acetate buffer, pH 5.0.

α-Amylase activity was assayed by the amylase test kit, "Amylase Test A Shionogi" (Ceska *et al.*, 1959) and was represented in international units (IU).

Isoelectric focusing

Isoelectric focusing was conducted by the apparatus of LKB Produkter developed by Vesterberg and Svensson (1966) using carrier mpholyte, "Ampholine" in the pH range 3.5 to 5. Electrophoresis was carried out at 10°C for 40 hours at 500 V constant voltage and pH of each fraction (1 ml/tube) was measured at 20°C.

Extraction of crude enzyme preparation from plerocercoids

Plerocercoids (24.6 g in wet weight) of *S. erinacei* were crushed in a porcelain mortar and Triton X-100 was added to the crushed material to get a final concentration of 1 %. This material 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 at room temperature and was centrifuged at 11,000×g for 20 minutes. The supernatant containing crude enzymes of plerocercoids was stored in a refrigerator until use in ion exchange chromatographies.

Results

Hydrolyzing enzymes adsorbed in DEAE-cellulose

A half of the crude enzyme preparation was

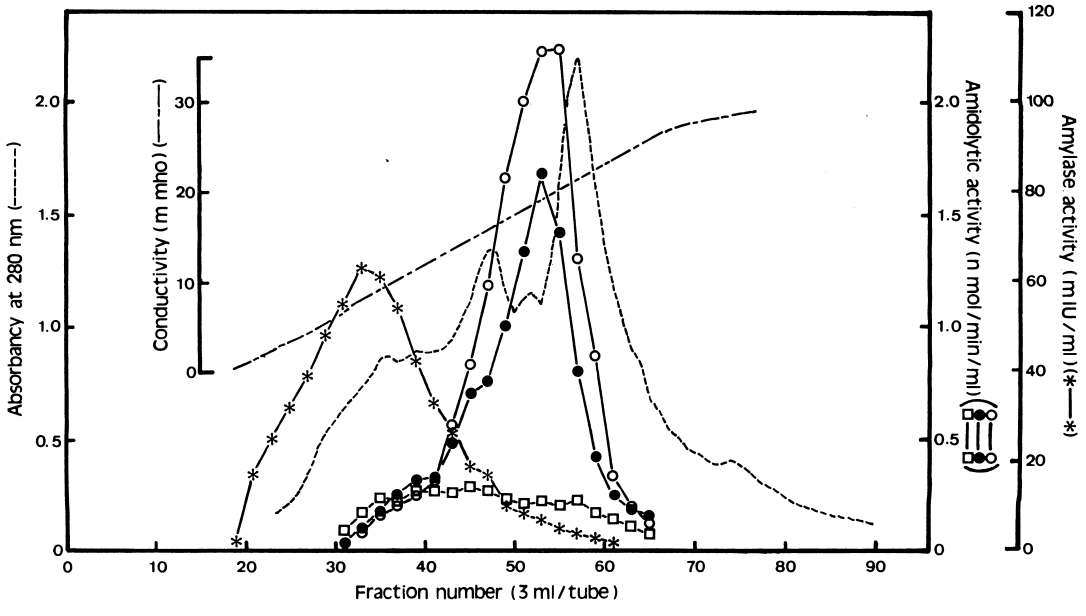

Fig. 1 DEAE-cellulose chromatography of crude enzyme preparation from plerocercoids of *Spirometra erinacei*.

····: absorbancy at 280 nm, ——: conductivity concentration, O—O: Bz-Arg-*p*NA amidolytic activity at pH 8.0, ●—●: Bz-Arg-*p*NA amidolytic activity at pH 5.0, □—□: Leu-*p*NA amidolytic activity, ✳—✳: α-amylase activity

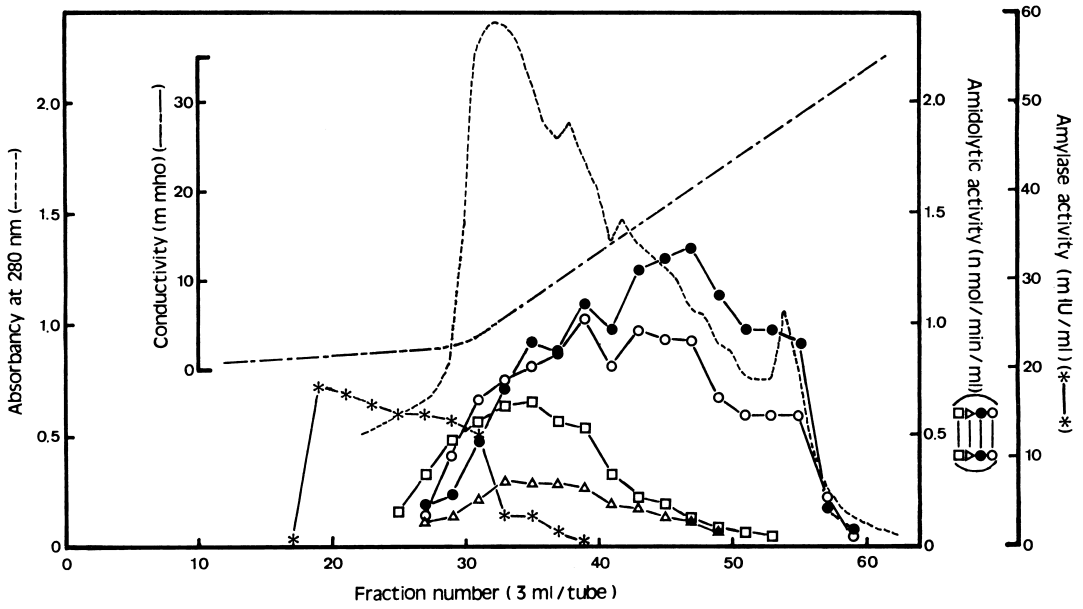

Fig. 2 CM-cellulose chromatography of crude enzyme preparation from plerocercoids of *Spirometra erinacei*.
····: absorbancy at 280 nm, ——: conductivity concentration, O—O: Bz-Arg-*p*NA amidolytic activity at pH 8.0, ●—●: Bz-Arg-*p*NA amidolytic activity at pH 5.0, △—△: Glt-Phe-*p*NA amidolytic activity, □—□: Leu-*p*NA amidolytic activity, *—*: α-amylase activity

adjusted to a conductivity concentration under 1 m mho and to pH 7.5. Then the resulting solution was applied to a DEAE-cellulose column (1.6×90 cm) pre-equilibrated with 0.02M Tris-HCl buffer, pH 7.5. The enzymes were eluted in a gradient elution system using the buffer containing 0 to 0.6 M NaCl, and fractions, each of 3 ml were collected.

The results obtained by the chromatography are shown in Fig. 1. Activity of Bz-Arg-*p*NA amidolytic enzyme at pH 8.0 and 5.0, Leu-*p*NA amidolytic enzyme, and α-amylase was detected in some of the fractions, but Glt-Phe-*p*NA amidolytic activity was not distributed in all the fractions.

Hydrolyzing enzymes adsorbed in CM-cellulose

The other half of the crude enzyme preparation was adjusted to a conductivity concentration under 1 m mho and to pH 7.0. Then the resulting solution was applied to a CM-cellulose column (1.6×90 cm) pre-equilibrated with 0.025 M phosphate buffer, pH 7.0. The

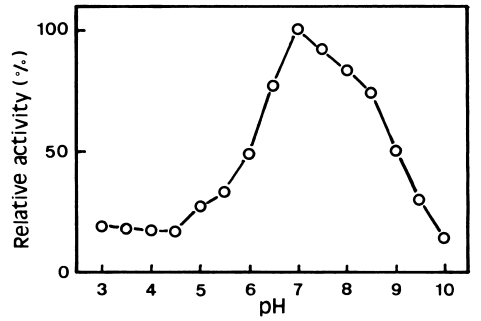

Fig. 3 Effect of pH on the activity of Bz-Arg-*p*NA amidolytic enzyme obtained by DEAE-cellulose chromatography.
The activity was shown in percentage to that at the optimum pH.

enzymes were eluted in a gradient elution system using the buffer containing 0 to 0.6 M NaCl, and fractions, each of 3 ml were collected.

The results obtained are shown in Fig. 2. Activity of Bz-Arg-*p*NA amidolytic enzyme at pH 8.0 and 5.0, Glt-Phe-*p*NA amidolytic

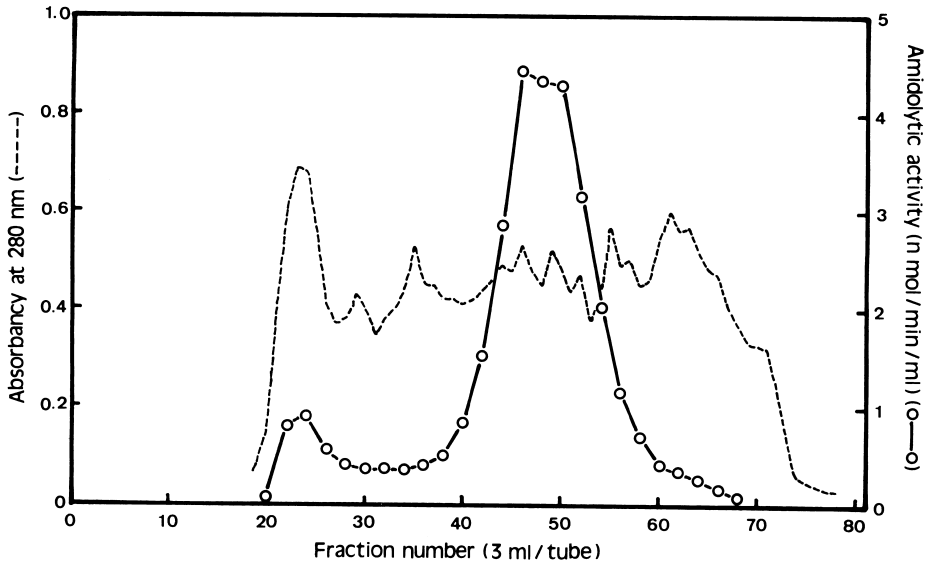

Fig. 4 Sephadex G-150 gel filtration of Bz-Arg-*p*NA amidolytic enzyme following DEAE-cellulose chromatography.
···· : absorbancy at 280 nm, O—O : Bz-Arg-*p*NA amidolytic activity

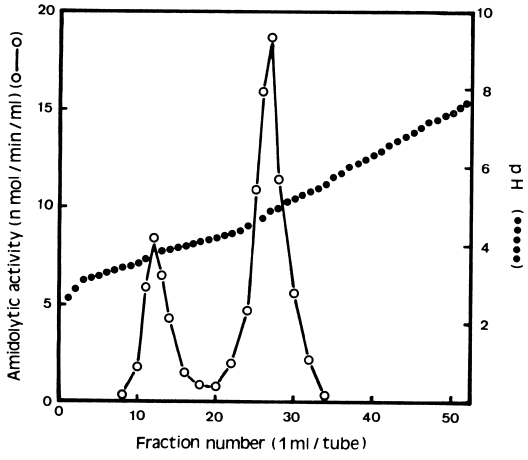

Fig. 5 Isoelectric focusing of Bz-Arg-*p*NA amidolytic enzyme following DEAE-cellulose chromatography.
O—O : Bz-Arg-*p*NA amidolytic activity,
●●● : pH

enzyme, Leu-*p*NA amidolytic enzyme, and α-amylase was distributed in the fractions.

Optimum pH of Bz-Arg-pNA amidolytic enzyme(s) obtained by DEAE-cellulose chromatography

Optimum pH of Bz-Arg-*p*NA amidolytic enzyme(s) obtained by DEAE-cellulose chromatography was determined with 0.08 M modified Britton and Robinson's universal buffer consisted of H_3PO_4, CH_3COOH, H_3BO_3, and NaOH (Britton and Robinson, 1931) at pH 3.0 to 10.0. The maximum relative activity was detected at pH 7.0, as shown in Fig. 3.

Two forms of Bz-Arg-pNA amidolytic enzyme obtained by Sephadex G-150 gel filtration

The fractions with Bz-Arg-*p*NA amidolytic activity obtained by DEAE-cellulose chromatography were collected in a Visking tube and were concentrated by polyethylene glycol. The concentrate showing Bz-Arg-*p*NA amidolytic activity was applied to a Sephadex G-150 column (2.2×60 cm) pre-equilibrated with 0.05 M Tris-HCl buffer, pH 7.5. Elution was made with the same buffer, and fractions, each of 3 ml were collected. And amidolytic activity for Bz-Arg-*p*NA was measured at pH 8.0 in each faction. As shown in Fig. 4, two peaks of Bz-Arg-*p*NA hydrolyzing activity were noticed. The first and the secondly eluted peaks were tentatively called Bz-Arg-*p*NA amidolytic enzyme-1 and -2, respectively.

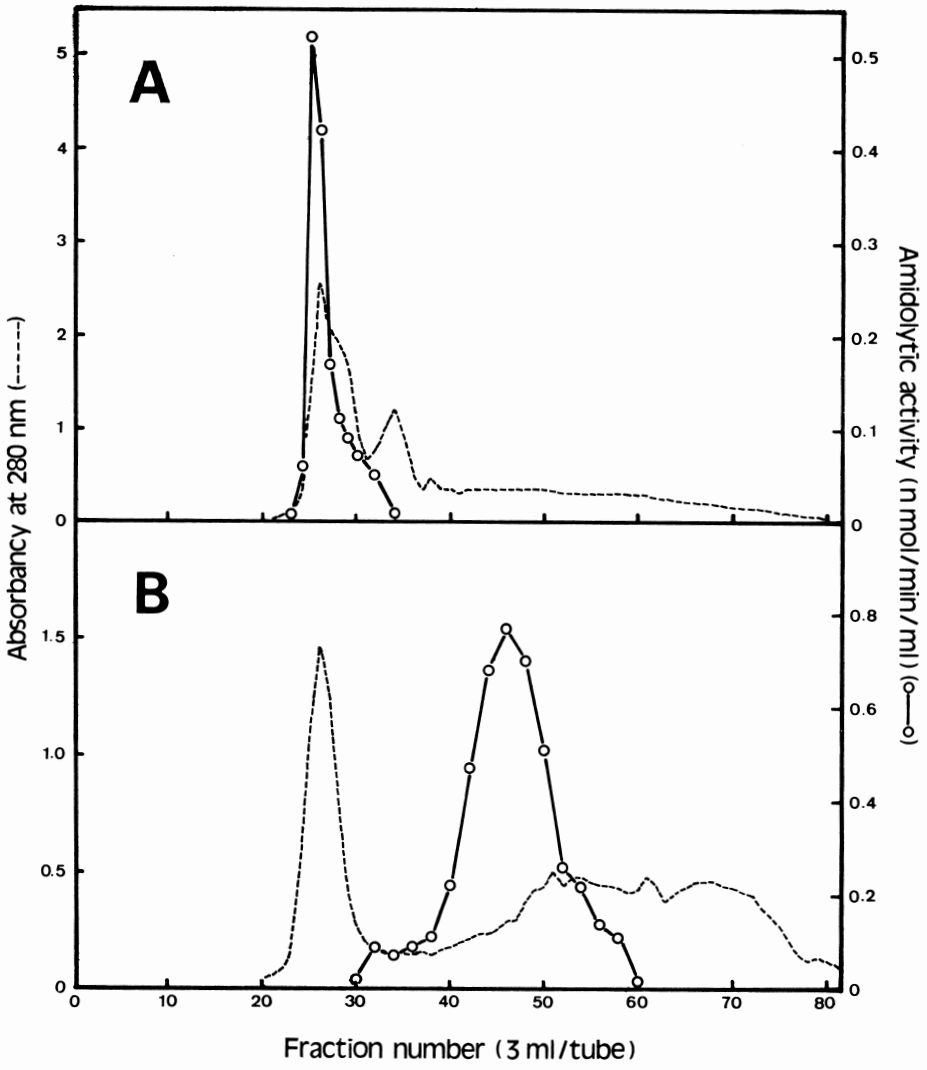

Fig. 6 Sephadex G-150 gel filtration of Bz-Arg-*p*NA amidolytic enzymes following isoelectric focusing.

A: Bz-Arg-*p*NA amidolytic enzyme-1 with a pI value of 3.8

B: Bz-Arg-*p*NA amidolytic enzyme-2 with a pI value of 4.9

···· : absorbancy at 280 nm, O—O : Bz-Arg-*p*NA aimdolytic activity

Two forms of Bz-Arg-pNA amidolytic enzyme obtained by isoelectric focusing

The results of isoelectric focusing of the fractions with Bz-Arg-*p*NA amidolytic activity obtained by DEAE-cellulose chromatography are shown in Fig. 5. Bz-Arg-*p*NA amidolytic activity at pH 8.0 was clearly separated into two peaks, one having an isoelectric point (pI) of 3.8 and the other, 4.9.

The two forms of active preparation were each applied to the same Sephadex G-150 column as that used in the above experiment. As shown in Fig. 6A, Bz-Arg-*p*NA amidolytic enzyme with low pI value corresponded to Bz-Arg-*p*NA amidolytic enzyme-1. On the other hand, Bz-Arg-*p*NA amidolytic enzyme

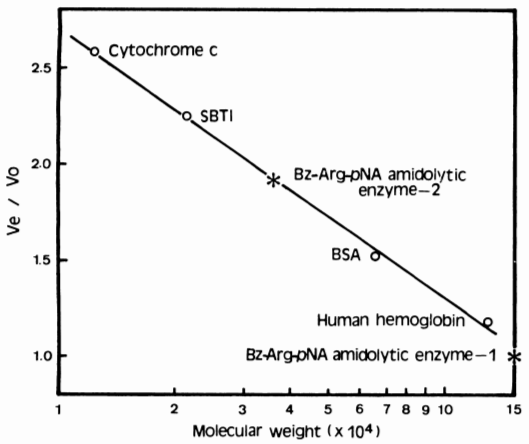

Fig. 7 Estimation of molecular weights of Bz-Arg-*p*NA amidolytic enzymes by gel filtration on a Sephadex G-150 column.

Authentic cytochrome c, soybean trypsin inhibitor (SBTI), bovine serum albumin (BSA), and human hemoglobin were used as reference proteins, and blue dextran was used for determination of void volume.

with high pI value, which was eluted following that with low pI value, corresponded to Bz-Arg-*p*NA amidolytic enzyme-2 (Fig. 6B).

Molecular weights of Bz-Arg-pNA amidolytic enzymes

The molecular weights of Bz-Arg-*p*NA amidolytic enzyme-1 and -2 were estimated by gel filtration on a Sephadex G-150 column (2.2×60 cm) according to the method of Andrews (1964). Approximate molecular weight of Bz-Arg-*p*NA amidolytic enzyme-2 was 3.6×10^4 daltons as shown in Fig. 7. In the case of Bz-Arg-*p*NA amidolytic enzyme-1, it was 1.5×10^5 daltons or more because the enzyme was eluted following void volume of the Sephadex G-150 column.

Discussion

In the present investigation, some hydrolyzing enzymes such as Bz-Arg-*p*NA amidolytic, Glt-Phe-*p*NA amidolytic, Leu-*p*NA amidolytic enzymes and α-amylase were detected in plerocercoids of *S. erinacei*.

Strong Bz-Arg-*p*NA amidolytic activity was observed at pH 8.0 in Tris-HCl buffer and at pH 5.0 in acetate buffer in each of DEAE- and CM-cellulose chromatographies, and the elution patterns at both the pH's were almost parallel to each other in the two chromatographies. Moreover, looking over the effect of pH on the activity of Bz-Arg-*p*NA amidolytic enzyme obtained by DEAE-cellulose chromatography, optimum pH was 7.0 in Britton and Robinson's universal buffer, and only slight activity was observed at pH 5.0. Thus, the difference in the buffers appears to affect activity of the enzyme. In order to make clear whether the Bz-Arg-*p*NA amidolytic enzyme detected at pH 8.0 is identical with that at pH 5.0 or not, purification and characterization of the two enzymes are necessary at each condition of pH and buffer for enzyme assay.

Glt-Phe-*p*NA amidolytic enzyme, which seems to be chymotrypsin-like enzyme because it hydrolyzed Glt-Phe-*p*NA, a specific synthetic substrate for chymotrypsin, was detected not by DEAE-cellulose chromatography, but by CM-cellulose chromatography. In fact, chymotrypsin having a low pI value adsorbed in DEAE-cellulose has not ever been detected.

α-Amylase activity was detected by both DEAE- and CM-cellulose chromatographies. The enzyme eluted in CM-cellulose chromatography, however, does not seem to be adsorbent to the cellulose because its activity began to be detected prior to rise in NaCl concentration. Therefore, α-amylase eluted by CM-cellulose chromatography appears to correspond to that by DEAE-cellulose chromatography.

The occurrence of several hydrolyzing enzymes, at any rate, suggests that a digestive enzymatic system exists in the cestode larva.

Bz-Arg-*p*NA amidolytic enzyme eluted by DEAE-cellulose chromatography was separated into two forms by both Sephadex G-150 gel filtration and isoelectric focusing. Judging from the molecular weights of the two forms, one form with an estimated molecular weight of 1.5×10^5 or more and a pI value of 3.8 seems to be a biomembrane-bound enzyme, because proteases with a large molecular weight such as this form have not been de-

tected except for membrane-bound enzymes. And the other form with an estimated molecular weight of 3.6×10^4 and a pI value of 4.9 seems to be a lysosomal or an externally-secreted enzyme. It is interesting from the physiological meanings of the enzymes that the larval cestode possesses different forms of arginine amidase.

Nakamura and Yanagisawa (1982 a, b) found a thiol proteinase in plerocercoids of *S. erinacei*, which hydrolyzes myoglobin and other substrates. In addition, Maki *et al.* (1982) reported that there was a hemoglobin hydrolyzing enzyme in adults of this tapeworm and that it would be a carboxyl protease. These two enzymes were also reported to be acidic in optimum pH by those authors. In the present investigation, on the other hand, the optimum pH of Bz-Arg-*p*NA amidolytic enzyme adsorbed in DEAE-cellulose was about 7.0. Accordingly the Bz-Arg-*p*NA amidolytic enzyme was not identical, in optimum pH, with the enzymes reported by Nakamura and Yanagisawa (1982 a, b) and Maki *et al.* (1982). However, the difference in optimum pH will be caused by that of the substrates and the buffers used. And all of these enzymes are crude in purity. So, it is unknown whether the Bz-Arg-*p*NA amidolytic enzyme detected in the present study is identical with the enzymes of Nakamura and Yanagisawa (1982 a, b) and Maki *et al.* (1982) or not. Consequently, in order to elucidate the enzymatic system in the larval cestode, purification and characterization of each of the enzymes detected are the most important of all.

Summary

Some hydrolyzing enzymes were detected in plerocercoids of *Spirometra erinacei*. Activity of Bz-Arg-*p*NA amidolytic enzyme at pH 8.0 and 5.0, Leu-*p*NA amidolytic enzyme, and α-amylase was detected by DEAE-cellulose chromatography, but Glt-Phe-*p*NA amidolytic activity was not shown by this method. By cationic ion exchange chromatography using CM-cellulose, activity of Bz-Arg-*p*NA amidolytic enzyme at pH 8.0 and 5.0, Glt-Phe-*p*NA amidolytic enzyme, Leu-*p*NA amidolytic enzyme, and α-amylase was detected.

Bz-Arg-*p*NA amidolytic enzyme obtained by DEAE-cellulose chromatography was separated into two forms by Sephadex G-150 gel filtration, and the molecular weights of these two forms were estimated to be 3.6×10^4 and 1.5×10^5 or more. By isoelectric focusing also, the Bz-Arg-*p*NA amidolytic enzyme was separated into two forms with isoelectric points (pI) of 3.8 and 4.9, and one with a pI value of 3.8 corresponded to the enzyme with the larger molecular weight and the other with a pI value of 4.9 to that with the smaller molecular weight.

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マンソン裂頭条虫プレロセルコイド中に含まれる数種の加水分解酵素，とくにアルギニンアミダーゼ

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マンソン裂頭条虫 *Spirometra erinacei* のプレロセルコイド中に含まれる数種の加水分解酵素について，その存在を検討した．DEAE-セルロースクロマトグラフィーでは，Bz-Arg-*p*NA 水解酵素，Leu-*p*NA 水解酵素および α-アミラーゼの活性を認めたが，Glt-Phe-*p*NA 水解酵素は確認できなかつた．一方，CM-セルロースクロマトグラフィーでは，Bz-Arg-*p*NA水解酵素，Glt-Phe-*p*NA 水解酵素，Leu-*p*NA 水解酵素および α-アミラーゼの活性を認めた．

DEAE-セルロースクロマトグラフィーにより得られた Bz-Arg-*p*NA 水解酵素は，さらにセファデックス G-150 ゲル濾過を行うことによつて2種の活性画分に分かれ，それぞれの分子量は 3.6×10^4 および 1.5×10^5 以上と推定された．また，等電点電気泳動によっても等電点3.8 と 4.9 の2種の活性画分に分かれたが，各々についてさらにゲル濾過を行つて，等電点 3.8 の酵素は高分子量のものに，等電点 4.9 のものは低分子量のものに一致することを確認した．