[Jpn. J. Parasitol., Vol. 35, No. 4, 287–293, August, 1986]

Some Hydrolyzing Enzymes, Especially Leucine Amidases, in Adult *Spirometra erinacei* (Cestoda; Diphyllobothriidae)

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(Received for publication; December 5, 1985)

Key words: *Spirometra erinacei,* hydrolyzing enzyme

Introduction

Parasites have specific digestive and absorptive mechanisms to obtain all nutrients from their hosts although the biochemical aspects of the mechanisms such as enzymes are not well known.

In *Spirometra erinacei* (=*Diphyllobothrium erinacei*) plerocercoids, Kwa (1972) detected a casein hydrolyzing enzyme(s) in the protoscolex. Nakamura and Yanagisawa (1982a, b) and Nakamura *et al.* (1984, 1985) reported a cysteine protease which hydrolyzes azocoll, myosin, actin, and others. In addition, we detected some hydrolyzing enzymes (Fukase *et al.*, 1984) and reported the purification and some properties of a cysteine protease, one of the enzymes (Fukase *et al.*, 1985, 1986).

The plerocercoids of *S. erinacei* entirely differ from the adults in morphology, host specificity, and habitat. Therefore, the adult cestodes will possess different hydrolyzing enzymes from those of the larvae. Maki *et al.* (1982) described a hemoglobin hydrolyzing enzyme, presumably a carboxyl protease in adult *S. erinacei,* but the detailed properties of the enzyme and the presence of other enzymes are not known. In the present experiments, we examined some hydrolyzing enzymes in adult *S. erinacei.*

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Materials and Methods

Parasites

Five adult cestodes (33.7 g in wet weight) were obtained from the small intestine of a naturally infected dog, and they were washed in tap water and stored frozen until use.

Chemicals

The following chemicals were supplied commercially: diethylaminoethyl(DEAE)-cellulose, carboxymethyl(CM)-cellulose, L-leucine-*p*-nitroanilide (Leu-*p*NA), ethylenediaminetetraacetic acid 2Na-salt (EDTA), and *p*-chloromercuribenzoic acid (PCMB) (Nakarai Chemicals, Ltd., Kyoto, Japan); Sephadex G-200 and Blue Dextran 2000 (Pharmacia Fine Chemicals AB, Uppsala, Sweden); N-α-benzoyl-DL-arginine-*p*-nitroanilide HCl (Bz-Arg-*p*NA) and glutaryl-L-phenylalanine-*p*-nitroanilide (Glt-Phe-*p*NA) (Sigma Chemical Co., St. Louis, U.S.A.); Amylase Test A Shionogi (Shionogi & Co., Ltd., Osaka, Japan); *N*-α-tosyl-L-lysylchloromethane hydrochloride (TLCK) (E. Merck AG, Darmstadt, West Germany); and pepstatin A (Protein Research Foundation, Osaka, Japan). All the other chemicals used were of analytical reagent grade.

Assay of enzyme activity

Amidolytic activity to Leu-*p*NA, Bz-Arg-*p*NA and Glt-Phe-*p*NA was measured by the modification of the method of Amundsen *et al.* (1979) in 0.1 M Tris-HCl buffer (pH 8.0) with 4×10^{-4} M substrate concentration at 37°C. The activity was expressed in nmol of substrate hydrolyzed per minutes.

α-Amylase activity was assayed by the blue starch method (Ceska *et al.*, 1969) using an amylase test kit, "Amylase Test A Shionogi" and was expressed in international unit (IU).

Estimation of protein concentration

Protein concentration was estimated by measuring absorbance at 280 nm (A_{280}) in a cuvette of 1 cm light path.

Extraction of crude enzyme preparation

Worms were crushed in a porcelain mortar and Triton X-100 was added to the crushed material 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 initial material. The diluted homogenate was shaken vigorously for 1 hour and was centrifuged at 11 000 × *g* for 20 minutes at 4°C. The supernatant was used as the crude enzyme preparation for ion exchange chromatographies.

Results

Hydrolyzing enzymes in the crude enzyme preparation

The crude worm extract showed high Leu-*p*NA amidolytic and α-amylase-like activities, but amidolytic activity to Bz-Arg-*p*NA and Glt-Phe-*p*NA was hardly detected (Table 1).

Hydrolyzing enzymes detected by DEAE-cellulose chromatography

The crude enzyme preparation was adjusted to 1 mS/cm in conductivity and 7.5 in pH. The resulting solution was then applied to a DEAE-cellulose column (1.5 × 87 cm) equilibrated with 0.02 M Tris-HCl buffer at pH 7.5. The enzymes were eluted by an increasing gradient elution system with 0 to 0.6 M NaCl in the buffer, and 5 ml fractions were collected.

The results are shown in Fig. 1. High Leu-*p*NA amidolytic and α-amylase-like activities were detected with the increase in conductivity, but Bz-Arg-*p*NA amidolytic and Glt-Phe-*p*NA amidolytic activities were hardly detected. The fractions with high Leu-*p*NA amidolytic activity (fraction numbers 34–48 in Fig. 1), which seemed to be composed of two parts, were collected into the same lot.

Hydrolyzing enzymes detected by CM-cellulose chromatography

The crude enzyme preparation was adjusted to 0.7 mS/cm in conductivity and 7.0 in pH. The resulting solution was then applied to a CM-cellulose column (1.5 × 87 cm) equilibrated with 0.02 M phosphate buffer at pH 7.0. The enzymes were eluted by an increasing gradient elution system with 0 to 0.6 M NaCl in the buffer, and 5 ml fractions were collected.

Table 1 Activity of hydrolyzing enzymes in the crude extract of adult *S. erinacei*

Enzyme	Unit	Activity	
		Total	Per gram of parasites
Bz-Arg-*p*NA amidolytic	nmol/min	7.75	0.23
Glt-Phe-*p*NA amidolytic	nmol/min	10.45	0.31
Leu-*p*NA amidolytic	nmol/min	4101.63	121.71
α-Amylase-like	mIU/min	1864.62	55.33

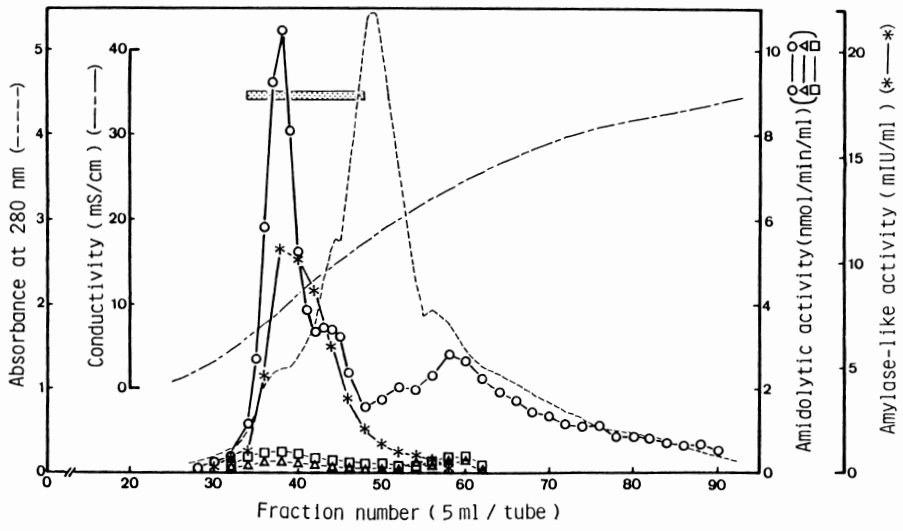

Fig. 1 Elution profile in DEAE-cellulose chromatography of crude enzyme preparation of adult *S. erinacei.*
- - - - -: absorbance at 280 nm; – – –: conductivity; ○—○: Leu-*p*NA amidolytic activity; △—△: Bz-Arg-*p*NA amidolytic activity; □—□: Glt-Phe-*p*NA amidolytic activity; *—*: α-amylase-like activity; ▨ : pooled fractions

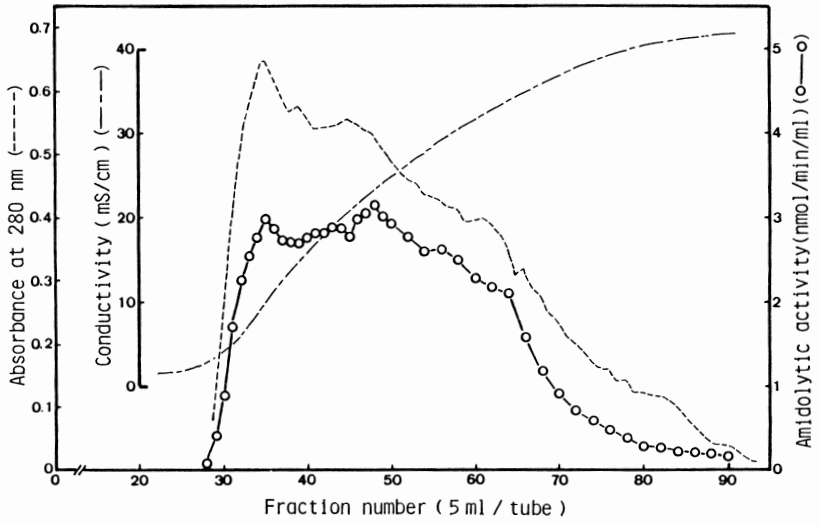

Fig. 2 Elution profile in CM-cellulose chromatography of crude enzyme preparation of adult *S. erinacei.*
- - - - -: absorbance at 280 nm; – – –: conductivity; ○—○: Leu-*p*NA amidolytic activity

The results are shown in Fig. 2. Leu-*p*NA amidolytic activity was detected in most of the fractions, but Bz-Arg-*p*NA amidolytic, Glt-Phe-*p*NA amidolytic, and α-amylase-like activities were not detected.

Leu-pNA amidolytic enzymes in Sephadex G-200 gel filtration

The enzyme preparation obtained by DEAE-cellulose chromatography (fraction numbers 34–48 in Fig. 1) was applied to a Sephadex G-

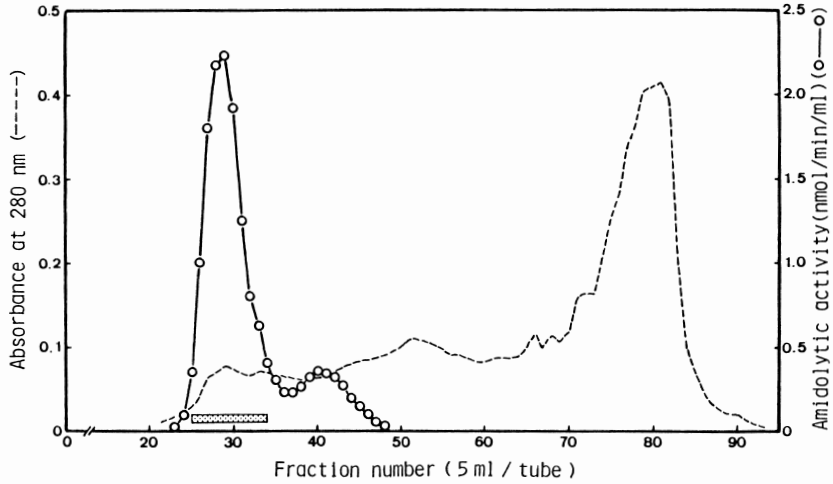

Fig. 3 Elution profile in Sephadex G-200 gel filtration of Leu-*p*NA amidolytic enzyme(s) obtained by DEAE-cellulose chromatography.
- - - - -: absorbance at 280 nm; o—o: Leu-*p*NA amidolytic activity; ▨: pooled fractions

200 column (2.2 x 98 cm) equilibrated with 0.05 M Tris-HCl buffer at pH 7.5. Elution was done with the same buffer and 5 ml fractions were collected.

Leu-*p*NA amidolytic enzyme(s) was clearly divided into two: the first enzyme with higher activity eluted just following the void volume which was determined to be about 225 ml by the use of blue dextran, and the secondly eluted one with lower activity. The fractions containing the former enzyme were collected and pooled (Fig. 3).

Effects of pH and some reagents on the activity of the first eluted Leu-pNA amidolytic enzyme

The activity of the Leu-*p*NA amidolytic enzyme obtained by Sephadex G-200 gel filtration (fraction numbers 25–34 in Fig. 3) was examined in different pH's with GTA buffer (Kohda, 1981) and glycine-NaOH buffer in a range of 5.00–9.00 and of 8.75–11.00 respectively. The optimum pH was observed to be 8.50–8.75 (Fig. 4).

Inhibitory effect of some reagents on the activity of the Leu-*p*NA amidolytic enzyme was also examined. The activity was inhibited by about 30% by EDTA at a concentration of 10^{-3} M, but it hardly inhibited by PCMB,

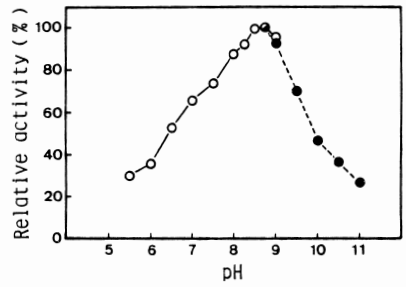

Fig. 4 Activity of a Leu-*p*NA amidolytic enzyme at different pH's.
Relative activity is represented in per cent to that at the optimum pH.
o—o: in GTA buffer; •—•: in glycine-HaOH buffer

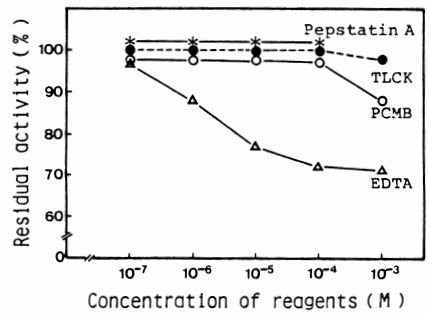

Fig. 5 Inhibitory effect of some reagents on a Leu-*p*NA amidolytic enzyme.
Residual activity is represented in per cent to that of control (intact).

TLCK, and pepstatin A (Fig. 5).

Discussion

In *S. erinacei* plerocercoids, high Leu-*p*NA amidolytic activity and α-amylase-like activity were present, but Glt-Phe-*p*NA amidolytic and Leu-*p*NA amidolytic activities were not so high (Fukase *et al.*, 1984), and the purified Bz-Arg-*p*NA amidolytic enzyme seemed to be a cysteine protease (Fukase *et al.*, 1985). In the present examination on adult *S. erinacei*, on the other hand, high Leu-*p*NA amidolytic and α-amylase-like activities were detected, whereas amidolytic activity to Bz-Arg-*p*NA and Glt-Phe-*p*NA was hardly detected. Since the Bz-Arg-*p*NA amidolytic cysteine protease characteristic of plerocercoids is present densely in the body part other than the protoscolex (Fukase *et al.*, 1986), the enzyme will be lost when the plerocercoid is transformed to an adult cestode in final hosts. Or again, it may be one possibility that the inhibitor is formed against the Bz-Arg-*p*NA amidolytic enzyme with the transformation to an adult. At any rate, from these results of our previous (Fukase *et al.*, 1984, 1985, 1986) and present studies, it seems that the hydrolyzing enzyme system differs between plerocercoids and adults of *S. erinacei*.

Adult *S. erinacei* live in the small intestine of final hosts, whereas the plerocercoids chiefly in the muscle and subcutis of second intermediate and paratenic hosts. The mechanism of nutrient intake seems to differ between the larval and the adult stages. Accordingly, it will be reasonable that hydrolyzing enzyme system is different between the larval and the adult stages.

In the present examinations, some peaks were recognized in Leu-*p*NA amidolytic activity in DEAE- and CM-cellulose chromatograms, and two clear peaks in the elution profile in Sephadex G-200 gel filtration of the preparation obtained by DEAE-cellulose chromatography. This indicates that there are a few kinds of Leu-*p*NA amidolytic enzymes which are different in the isoelectric point and the molecular weight.

One Leu-*p*NA amidolytic enzyme obtained by Sephadex G-200 gel filtration showed an optimum pH of 8.50–8.75, and the activity was slightly inhibited by EDTA but hardly inhibited by PCMB, TLCK, and pepstatin A. The molecular weight of this enzyme will be about 2×10^5 or more, because the enzyme was eluted just following the void volume in Sephadex G-200 gel filtration. Considering the substrate specificity (i.e., Leu-*p*NA amidolytic) and the optimum pH, it seems that this enzyme is similar to leucine aminopeptidase, a typical metal protease. But the present enzyme was not determined to be a metal protease because its activity was not strongly inhibited by EDTA.

It is well known that the body surface of adult cestodes resembles the mucosal epithelium of the small intestine of host animals in structure and function (Lumsden, 1975a, b; Chappel, 1980; Lumsden and Murphy, 1980). Membrane-bound enzymes such as aminopeptidases are known to exist in the brush border of the small intestine of mammals (Kim and Brophy, 1976; Vannier *et al.*, 1976; Gray and Santiago, 1977). Therefore, the present Leu-*p*NA amidolytic enzyme may be located in the tegument and play a part in membrane (contact) digestion. And so, it is possible that the molecular weight of this enzyme was estimated to be large because extracted with the small pieces of tegument membrane.

Maki *et al.* (1982) reported on a hemoglobin hydrolyzing enzyme in adult *S. erinacei*. This enzyme has an acidic optimum pH and is strongly inhibited by pepstatin, so that it clearly differs from the present Leu-*p*NA amidolytic enzyme.

Douch (1978) described L-leucyl-β-naphtylamidases in a cyclophyllidean cestode, *Moniezia expansa*, and a nematode, *Ascaris suum*, with molecular weights of about 200 000 and about 60 000 estimated by Sephadex G-200 gel filtration and with optimum pH's of 7.2 and 6.9, respectively. And further, Cesari *et al.*

(1983) detected Leu-*p*NA hydrolyzing activity in *Schistosoma mansoni*. The enzymes described by Douch (1978) and Cesari *et al.* (1983) resemble the present Leu-*p*NA amidolytic enzyme in substrate specificity, but further precise examinations are necessary to define these enzymes.

Many proteases have been reported in many species of parasitic helminths, but the identification of them is generally difficult because experimental methods are different in different reports. The purification and detailed characterization of each enzyme will accelerate the elucidation of enzyme systems in parasitic helminths.

Summary

Adult *S. erinacei* was examined for hydrolyzing enzymes. The crude extract of the cestodes showed high Leu-*p*NA amidolytic activity and α-amylase-like activity, but amidolytic activity toward Bz-Arg-*p*NA and Glt-Phe-*p*NA was hardly detected. In the eluate obtained by DEAE-cellulose chromatography of the crude extract, high Leu-*p*NA amidolytic and α-amylase-like activities were detected, but Bz-Arg-*p*NA amidolytic and Glt-Phe-*p*NA amidolytic activities were hardly detected, as well as in the crude extract. In the eluate obtained by CM-cellulose chromatography of the crude extract, on the other hand, only Leu-*p*NA amidolytic activity was observed. The major fractions with Leu-*p*NA amidolytic activity obtained by DEAE-cellulose chromatography were clearly divided into two active peaks by Sephadex G-200 gel filtration: the first eluted with higher activity and the secondly eluted weaker activity. The first eluted Leu-*p*NA amidolytic enzyme had an optimum pH of 8.50–8.75, and slightly inhibited by EDTA and hardly by PCMB, TLCK, and pepstatin A. This enzyme seems to have a molecular weight of about 2×10^5 or more because it was eluted just following the void volume in Sepadex G-200 gel filtration. From these results, enzyme system of adult *S. erinacei* considered to differ, as far as we examined, from that of plerocercoids in which an arginine amidase(s) is characteristic and Leu-*p*NA amidolytic activity is not detected so high.

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マンソン裂頭条虫の成虫に存在する数種の加水分解酵素，とくにロイシンアミダーゼ

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マンソン裂頭条虫 *Spirometra erinacei* の成虫について，数種の加水分解酵素の存在を検索した．虫体からの粗抽出液では，強い Leu-*p*NA 水解活性と α-アミラーゼ様活性が認められたが，Bz-Arg-*p*NA 水解活性ならびに Glt-Phe-*p*NA 水解活性はごくわずか認められたにすぎなかった．この粗抽出液を用いて DEAE- セルロースクロマトグラフィーを行ったところ，粗抽出液の場合と同様に，Leu-*p*NA 水解活性と α-アミラーゼ様活性が明瞭に認められたが，Bz-Arg-*p*NA 水解活性と Glt-Phe-*p*NA 水解活性はほとんど検出されなかった．一方，CM-セルロースクロマトグラフィーでは，Leu-*p*NA 水解活性のみが認められた．

DEAE- セルロースクロマトグラフィーにより得た Leu-*p*NA 水解活性の主要な画分は，さらにセファデックス G-200 ゲル濾過を行うことにより明確に2つの画分に分離することができた．このうち最初に溶出された酵素について性質を検討したところ，至適 pH は8.50～8.75であり，活性は EDTA によってわずかに阻害されたが，PCMB, TLCK, ペプスタチン A ではほとんど阻害されなかった．なお，この酵素の分子量は，セファデックス G-200 ゲル濾過における溶出位置から，約 2×10^5 あるいはそれ以上と推察された．

以上の成績から，マンソン裂頭条虫の成虫では，われわれが検索した限りにおいては，ロイシンアミダーゼが特徴的であり，アルギニンアミダーゼが特徴的であるプレロセルコイドとはその酵素系が大きく異なっている可能性があると考えられる．