The Korean Journal of Parasitology
Vol. 36, No. 2, 133-141, June 1998

Isolation and characterization of a 40 kDa cysteine protease from *Gymnophalloides seoi* adult worms

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Abstract: A 40 kDa cysteine protease was purified from the crude extract of adult worms of *Gymnophalloides seoi* by two consecutive steps: Sephacryl S-200 HR and DEAE-Sephacel chromatography. Enzyme activities were completely inhibited by cysteine protease inhibitors, L-trans-epoxysuccinylleucylamido (4-guanidino) butane (E-64) and iodoacetic acid, strongly suggesting that the purified enzyme belongs to the cysteine family of proteases. The enzyme was maximally acive at pH 4.5 in 0.1 M of buffer, and its activity was greatly potentiated in the presence of 5 mM dithiothreitol. The protease degraded macromolecules with differential capabilities; it degraded extracellular matrix proteins, such as collagen and fibronectin, with a stronger activity against collagen than fibronectin. However, the enzyme digested hemoglobin and human immunoglobulins only slightly, leaving them nearly intact after an overnight reaction. Our results suggest that the cysteine protease of *G. seoi* adults is potentially significant in the nutrient uptake from the host intestine.

Key words: *Gymnophalloides seoi*, adult, cysteine protease, nutrition

INTRODUCTION

Among the gymnophallid flukes, which are generally known as parasites of shore birds (Schell, 1985), *Gymnophalloides seoi* is the only known trematode infecting humans (Lee *et al.*, 1993). Human infection with *G. seoi* highly prevails in the southwestern coastal islands of Korea, where many people enjoy consuming raw oysters, *Crassostrea gigas*, the second intermediate host of *G. seoi* (Lee *et al.*, 1994 & 1995). Symptoms in the infected persons include mild to moderate gastrointestinal problems, such as epigastric discomfort, indigestion and diarrhea (Lee *et al.*, 1993 & 1994). For a proper understanding of the pathogenesis of *G. seoi* infection, details of the disease pattern deserve further investigation.

The morphologic characteristics of the adult and metacercaria of *G. seoi* are quite similar except for the body size and presence of intrauterine eggs in the adult flukes (Lee *et al.*, 1995). Other biological properties may, however, be different in the two developmental stages because they have to adapt to different microenvironments in their respective suitable hosts, *i.e.*, metacercariae in oysters and adults

• Received 6 April 1998, accepted after revision 12 May 1998.

• This study was partly supported by the Basic Medical Research Fund, the Ministry of Education, Republic of Korea, 1995-1996.

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in the final host including humans. In order to survive in their appropriate hosts, *G. seoi* may secrete a number of proteases with characteristic functions according to its developmental stages.

Parasites have been shown to secrete several kinds of proteases during their life cycle, which can play important roles in invasion and migration through the host tissue, nutritional uptake for parasite metabolism, and immune evasion from the host by degrading host tissues, cells, and macromolecules (McKerrow, 1989). Maturing and adult schistosomes secrete acid cysteine proteases responsible for the digestion of host hemoglobin to maintain their viability and egg production (McKerrow and Doenhoff, 1988). Neutral proteases of sparganum, 198 and 104 kDa, degrade hemoglobin, gelatin, and collagen, which suggests that the enzymes play major roles in the migration of worms and nutritional uptake (Kong *et al.*, 1994a).

A cysteine protease of 16 kDa was purified from the crude extract of *G. seoi* metacercariae and was suggested to be responsible for the nutrition of worms and evasion from host immune responses (Choi *et al.*, 1998). However, no information is available yet on the proteases of adult flukes. In view of potential importance of proteases in parasite metabolism and host-parasite interactions, the present study was carried out to identify the presence of proteases in the crude extract of *G. seoi* adults.

MATERIALS AND METHODS

Preparation of the crude extract of *G. seoi* adults

Metacercariae of *G. seoi* were collected from the oyster, *C. gigas*, naturally produced in the endemic area, and fed orally to six-week-old C3H/HeN mice using a gavage needle. In order to recover as many adult flukes as possible, prednisolone at a daily dose of 10 mg/kg was injected intramuscularly one day before and one day after infection (Lee *et al.*, 1997). Mice were killed by cervical dislocation on day 3 postinfection, and their small intestine was removed and opened in 0.85% saline solution. Adult worms were harvested by Baermann's sieve-in-funnel technique (Beaver *et al.*, 1984), homogenized in sodium acetate buffer (0.1 M, pH 4.5), and the supernatant was used as the crude extract after centrifugation at 20,000 *g* for 1 hr. All procedures for enzyme purification were carried out at 4°C, unless otherwise specified, and the protein content was measured by the method of Lowry *et al.* (1951).

Proteolytic assay

Proteolytic activity was assayed using a synthetic substrate, carboxybenzoyl-phenyl-alanyl-arginyl-4-methoxy-β-naphthylamide (CBZ-phe-arg-MNA; Sigma, USA). Briefly, a 20 μl enzyme solution was incubated with the assay mixture, comprising 0.44 ml sodium acetate (0.1 M, pH 4.5) and 20 μl CBZ-phe-arg-MNA in the presence of 5 mM dithiothreitol (DTT), for 3 hr at 37°C. After stopping the reaction with 10% (v/v) trichloroacetic acid and iodoacetic acid (IAA), color reactions were developed by adding 0.01% (w/v) tetrazotized *o*-dianisidine (Sigma). The released-MNA was measured at 530 nm using a spectrophotometer (UV-160A, Shimadzu, Japan). One unit of enzyme activity was expressed as the amount of enzyme for a one unit (0.1) increase in absorbance through a 1 cm light path corresponding to the enzyme activity that hydrolyses 2.94×10^{-3} pmol/min of MNA under the conditions specified.

Purification of the enzyme

The crude extract was dialysed against sodium acetate (0.1 M, pH 4.5), and then centrifuged at 700 *g* for 5 min. A total of 5.0 ml (16.0 mg/ml) of the supernatant was applied to a Sephacryl S-200 HR column (Pharmacia, Sweden), 1.6 × 70 cm long that was pre-equilibriated with sodium acetate (0.1 M, pH 4.5) containing 0.5 mM EDTA. The column was eluted using the same buffer at a flow rate of 50 ml/cm^2/hr. Eighty 2.3 ml fractions were collected and fractions with high enzyme activities were pooled, dialysed, lyophilized, and reconstituted with 0.5 ml sodium acetate (0.1 M, pH 4.5).

For a final purification, a DEAE-Sephacel column (Pharmacia), 1.6 × 4 cm long and pre-equilibriated with sodium acetate (0.1 M, pH 4.5), was used. A total of 0.45 ml of clarified

enzyme was eluted by sodium acetate (0.1 M, pH 4.5) containing 0, 0.05, 0.1 or 0.2 M NaCl in stepped increments. The highly active fractions were pooled, dialysed, concentrated, and redissolved in 0.2 ml sodium acetate (0.1 M, pH 4.5).

SDS-PAGE

Proteins in each purification step and degradation products of macromolecules by the purified enzyme were analyzed by 7.5-15% separating gels at 20 mA for 8 hr using Laemmli's method (1970). The molecular weight standards were phosphorylase B (94 kDa), albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20.1 kDa), and α-lactalbumin (14.4 kDa) (Pharmacia).

Biochemical properties of the enzyme

Optimal buffer molarity was determined by incubation of 20 μl enzyme in 0.01, 0.05, 0.1, 0.2, 0.3, 0.4, and 0.5 M sodium acetate (420 μl each, pH 4.5) at 37°C for 3 hr. Enzyme activity was also monitored in sodium acetate (0.1 M) at pH 4, 4.5, 5, and 5.5, in sodium phosphate (0.1 M) at pH 6, 6.5, 7, and 7.5, and in Tris-HCl (0.1 M) at pH 8.

The modulating effects on the enzyme activity were examined using various effectors, such as DTT (5 mM), L-trans-epoxysuccinyl-leucylamido (4-guanidino) butane (E-64, 10^{-5} M), IAA (1 mM), leupeptin (0.1 mM), 4-(amidinophenyl)methanesulfonyl fluoride (APMSF, 0.1 mM), aprotinin (10 μg/ml), ethylene diamine tetraacetate (EDTA, 2 mM), and 1,10-phenanthroline (5 mM). Twenty μl of the purified enzyme in 0.44 ml sodium acetate (0.1 M, pH 4.5) were pre-incubated with the respective inhibitors for 10 min at room temperature. After adding 20 μl CBZ-phe-arg-MNA and 25 μl 0.1 M DTT to the reaction mixtures, they were incubated at 37°C for 1 hr. All chemicals were purchased from Sigma except for E-64 (Enzyme System Products, USA).

Degradation of macromolecules and human immunoglobulins

Cleaving activities of the enzyme were determined using macromolecular substrates such as acid soluble calf skin collagen (type I, Boehringer Mannheim Biochemicals, Germany), hemoglobin (Sigma), and fibronectin isolated from human plasma. Five duplicate reaction mixtures comprised 20 μl diluted enzyme (6 μg of protein), 5 μl 0.1 M sodium acetate (pH 4.5), making a total volume for proteolysis of 100 μl, including macromolecules. The amounts of collagen, fibronectin, and hemoglobin added to the mixtures were 10 μl (50 μg), 35 μl (70 μg), and 15 μl (75 μg), respectively. After incubation of the mixtures for 1, 3, 5 hr, and overnight at 37°C, degradation products were visualized by SDS-PAGE.

Degradation of human immunoglobulins was observed using IgG2a isolated from human sera and sIgA (Chemicon, USA). Reaction mixtures comprised 20 μl enzyme, 25 μl IgG2a (47 μg) or 20 μl sIgA (40 μg) in the presence of 5 μM DTT. After adding 0.1 M sodium acetate (pH 5.0) to make a total volume of 100 μl, these mixtures were incubated for 1, 3, 5 hr, and overnight, at 37°C. SDS-PAGE was undertaken for analyses of degradation products.

RESULTS

Protein peaks were obtained by applying the crude extract of *G. seoi* adults to a Sephacryl S-200 HR column; they were sharp and narrow peak with high absorbances and a broad one showing absorbances lower than 0.1 (Fig. 1A). Fractions with high proteolytic activities were further purified by DEAE-sephacel chromatography. The unbound and the 0.2 M NaCl fraction exhibited enzyme activites, that were higher in the former than in the latter (Fig. 1B). SDS-PAGE analysis for proteins in each purification step revealed a single protein band with a molecular weight of 40 kDa in the unbound fraction after DEAE-Sephacel chromatography (Fig. 2). The activities and yields of clarified enzymes during each step are shown in Table 1. The peak activity of the purified enzyme was observed at pH 4.5 and in 0.1 M sodium acetate (Fig. 3).

Proteolytic activities were greatly enhanced by the addition of 5 mM DTT into the reaction

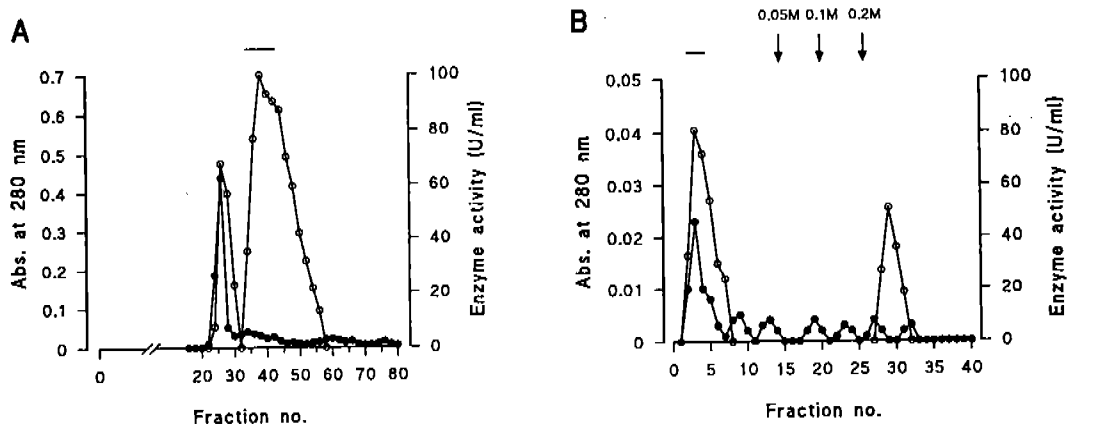

Fig. 1. Elution profile of protease during purification by Sephacryl S-200 HR (A) and DEAE-Sephacel chromatography (B). Fractions were assayed for activity on CBZ-phe-arg-MNA (○) and monitored for protein content (●) at 280 nm. Fractions with high enzyme activities were pooled, as shown by the bar (—). Vertical arrows in Fig. 1B indicate stepped salt gradients.

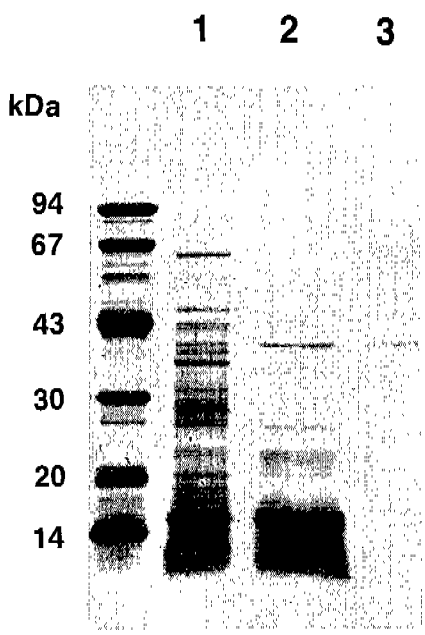

Fig. 2. SDS-PAGE analysis of proteins purified from crude extract to cysteine protease by sequential chromatographic steps. 1, crude extract; 2, active fractions containing enzyme activity from Sephacryl S-200 HR; 3, the purified enzyme from DEAE-Sephacel.

mixtures, and therefore the modulation effect of various protease inhibitors was determined in the presence of 5 mM DTT (Table 2). Cysteine protease inhibitors, E-64 and IAA, and a common inhibitor of both cysteine and serine proteases, leupeptin, completely inhibited the proteolytic activity of the purified enzyme (Table 2). On the other hand, serine protease inhibitors, APMSF and aprotinin, and metallo-protease inhibitors, EDTA and 1,10-phenanthroline, had no influence on the enzyme activity.

Degradation of macromolecules and immunoglobulines by the cysteine protease of *G. seoi* adults was observed by incubation of reaction mixtures for 1, 3, 5 hr, and overnight. The enzyme began to digest β-chains of collagen after 1 hr of incubation, producing degradation products. When incubated overnight, α- and β-chains of collagen were completely hydrolysed, yet showing several degradation products (Fig. 4A). The protease also degraded fibronectin with less proteolytic activity compared to that against collagen, and digestive products were still remained after overnight reaction (Fig. 4B). By contrast, the cleaving activities of the cysteine protease were negligible against hemoglobin (Fig. 4C) and human immunoglobulins, IgG2a or sIgA (Fig. 5).

DISCUSSION

The purification of a 40 kDa protease from the crude extract of *G. seoi* adults involved 2 separation methods; gel filtration and ion-exchange chromatography. The purified

Table 1. Summary of the purification of a cysteine protease from the crude extract of *Gymnophalloides seoi* adults

Step	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Purification (fold)	Recovery (%)
Crude extract	16.0	3288.9	205.6	1.0	100
Sephacryl S-200 HR	1.8	721.5	400.8	1.9	21.9
DEAE-Sephacel	0.2	592.6	2963.0	14.4	18.0

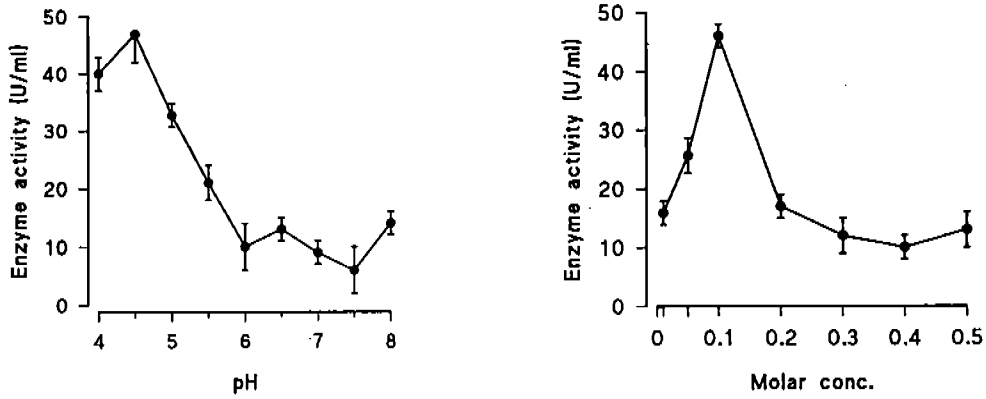

Fig. 3. Effects of pH (A) and molar concentration (B) on proteolytic activity of the cysteine protease. Mean ± SD, n = 3.

enzyme showed maximal activity at pH 4.5 with 0.1 M sodium acetate, which was greatly potentiated in the presence of 5 mM DTT. The enzyme was found to be an acidic cysteine protease as analyzed by cathepsin L-like activity of degrading CBZ-phe-arg-MNA, and acid pH optimum, and specific inhibition by cysteine protease inhibitors, E-64 and IAA.

It has been suggested that cysteine proteases of parasites play an extracorporeal function in the digestion of host tissues (Rhoads and Fetterer, 1997). The fourth-stage larvae and adults of *Haemonchus contortus* have been shown to release cathepsin L-like cysteine proteases, and degradation and uptake of extracellular matrix components by live parasites were specifically inhibited by Z-phe-arg-FMK, a cysteine protease inhibitor (Rhoads and Fetterer, 1997). The cysteine protease of *G seoi* adults degraded extracellular matrix proteins such as type I collagen and fibronectin with different cleaving activities, while it digested hemoglobin only slightly even after overnight incubation. Metacercarial cysteine protease of 16 kDa also showed a similar pattern of proteolysis as observed in an adult cysteine protease of *G. seoi* (Choi *et al.*, 1998). Therefore, cysteine proteases of *G. seoi* may play a significant role in nutrient uptake by degrading host tissue proteins. Immunohistochemical localization of the protease secretion in *G. seoi* needs to be elucidated further.

Several proteases with differing functions specific to different developmental stages have been characterized from a number of parasites. Egg proteases of schistosomes help eggs pass through the wall of the intestine or bladder, and cysteine proteases of maturing schistosomula and adults actively degrade host hemoglobin to obtain nutrition (McKerrow and Deonhoff, 1988). Two metacercarial cysteine proteases of *Paragonimus westermani*, 27 and 28 kDa, have been proven to be responsible for excystment of metacercariae (Chung *et al.*, 1995). Their specific enzyme activities decline as the worm matures after excystation, implying their possible roles as a driving force for migration of the juvenile worms in the final host (Chung *et al.*, 1997).

Table 2. Modulatory effects of various effectors on the enzyme activity

Inhibitors	Final concentration	Relative activity[a]
Control (DTT-activated)	5 mM	100.0 ± 7.5
without DTT		7.4 ± 5.6
E-64	10 μM	0.4 ± 0.2
IAA	1 mM	0.5 ± 0.3
Leupeptin	0.1 mM	9.1 ± 3.8
APMSF	0.1 mM	99.9 ± 3.8
Aprotinin	10 μg/ml	107.0 ± 3.7
EDTA	2 mM	103.4 ± 2.5
1,10-phenanthroline	5 mM	97.9 ± 7.1

[a]mean ± SD, n = 3.

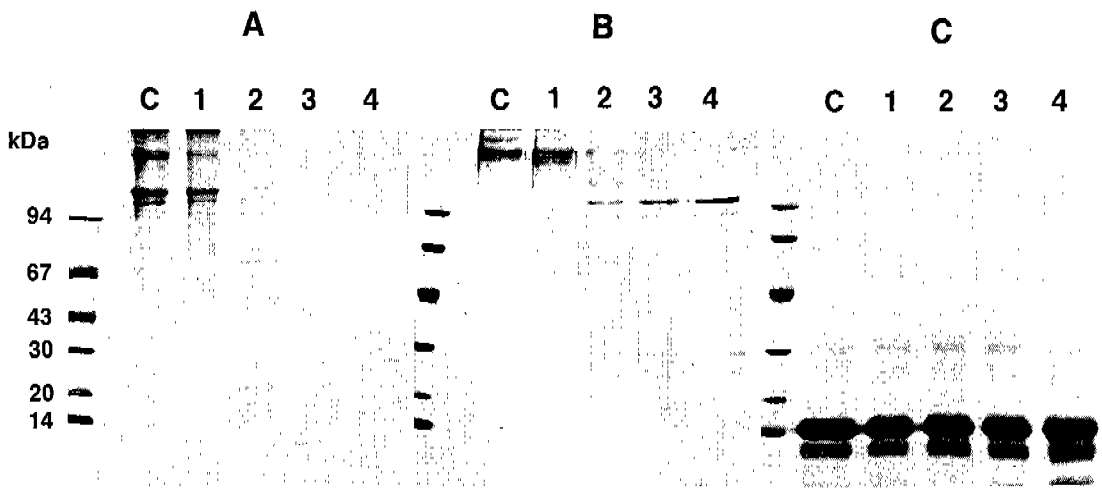

Fig. 4. Cleaving activity of the cysteine protease against collagen (A), fibronectin (B), and hemoglobin (C). C, macromolecules only ; 1-4, macromolecules with the enzyme for 1, 3, and 5 hr, and overnight at 37°C.

We tried to purify a 16 kDa protease from the adult crude extract of *G. seoi* for comparison of its proteolytic activity in the two developmental stages, metacercariae and adults. Unfortunately, the purification of a 16 kDa enzyme to homogeneity was not successful.

Parasites secrete a number of enzymes of different families or having different molecular weights. Several cysteine proteases with relative molecular weights ranging from 96 to 16 kDa were purified from various extracts of *Entamoeba histolytica* (Montfort *et al.*, 1994). *Fasciola hepatica* secretes a number of proteases, the most prominent of which are two cysteine proteases, cathepsins L1 and L2 (Berasain *et al.*, 1997). During the final purification of the adult cysteine protease of *G. seoi* by DEAE-Sephacel chromatography, the 0.2 M NaCl fraction showed a lesser degree of enzyme activity against CBZ-phe-arg-MNA than the purified one in the unbound fraction. The former revealed several protein bands, including a 40 kDa protein, by SDS-PAGE analysis (data not shown). Therefore, *G. seoi* adults may have several cysteine proteases, probably having different molecular weights, the purification and characterization of which will provide more information for the understanding of host-parasite interactions.

Parasites can modify host immune responses by secreting proteases capable of degrading host immunoglobulins for their

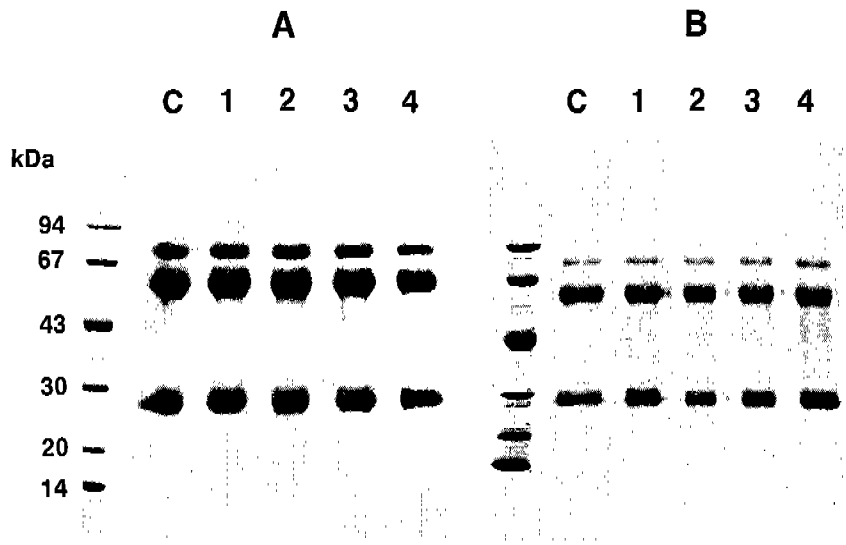

Fig. 5. Degradation of IgG2a (A), and sIgA (B) by cysteine protease. No digestive products are shown in comparison with the control. C, immunoglobulins only; 1-4, immunoglobulins with the enzyme for 1, 3, and 5 hr, and overnight at 37°C.

survival (Maizels *et al.*, 1993). Cleaving activities against IgG molecules by parasite proteases have been demonstrated in *Schistosoma mansoni* schistosomula and sparganum (Auriault *et al.*, 1981; Kong *et al.*, 1994b), and cysteine protease of *E. histolytica* is capable of degrading human sIgA (Kelsall and Ravdin, 1993). A metacercarial cysteine protease of *G. seoi* cleaves heavy chains of human IgG and sIgA; the latter is more susceptible than the former to digestive activity (Choi *et al.*, 1998). However, the 40 kDa protease purified in the present study could not degrade human immunoglobulins, indicating that the enzyme is not associated with immunological modulation strategies of *G. seoi*.

The exact roles and biochemical properties of the protease of *G. seoi* are not fully understood. To elucidate the biological functions and reactivity of the protease, and its primary structure including N-terminal amino acid sequence, cellular and molecular biological investigations are needed.

ACKNOWLEDGEMENTS

We sincerely thank Dr. Young-Bae Chung, Department of Parasitology, Seoul National University College of Medicine, for his advice in enzyme characterization, and also express our gratitude to Miss Yeon Hee Lim, Department of Parasitology, Seoul National University College of Medicine, for her technical assistance in the enzyme purification.

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=초록=

참굴큰입흡충 (*Gymnophalloides seoi*) 성충에서 정제한 40 kDa 시스테인계열 단백분해효소의 특성

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참굴큰입흡충 (*Gymnophalloides seoi*)의 병원성을 규명하기 위한 연구의 일환으로 성충의 조효소에서 단백분해효소를 분리 정제한 후 생화학적 특성을 관찰하였다. 조효소를 0.1 M sodium acetate (pH 4.5)로 투석한 후 원심분리하여 얻은 상층액을 Sephacryl S-200 HR column chromatography로 부분 정제한 후 DEAE-Sephacel column chromatography를 실시하여 순수 정제하였다. SDS-PAGE를 실시하여 각 정제 단계별 시료의 정제도를 확인한 결과 분자량이 40 kDa인 단일 분획이 관찰되었다. 정제된 효소는 시스테인 단백분해효소의 특이억제제인 L-trans-epoxysuccinylleucylamido (4-guanidino) butane (E-64)와 iodoacetic acid, 세린 및 시스테인 단백분해효소의 일반 억제제인 leupeptin에 의해 활성이 억제되어 시스테인 단백분해효소임을 확인하였다. 정제된 효소는 콜라겐, 파이브로넥틴과 같은 세포외 기질을 분해하였으나 헤모글로빈은 분해정도가 낮아 반응 12시간 후에도 단량체 (menomer)와 이합체 (dimer)의 양이 대조군과 큰 차이가 없었으며, IgG2a와 sIgA도 거의 분해하지 못하였다. 따라서, 참굴큰입흡충 성충의 40 kDa 시스테인 단백분해효소는 충체가 숙주 체내에서 기생생활을 하는데 필요한 영양분 섭취에 주로 관여할 것으로 생각되었다.

〔기생충학잡지 36(2): 133-141, 1998년 6월〕