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# Isolation and Partial Characterization of Cysteine Proteinase from Sparganum

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**Abstract:** A proteolytic enzyme was purified from the tissue extract of spargana (plerocercoids of *Spirometra erinacei*) by DEAE-Trisacryl M ion exchange chromatography and thiopropyl-sepharose affinity chromatography resulted in a 21-fold purification. The proteinase activity was assayed with a synthetic fluorescent substrate, carbobenzoxy-phenylalanyl-7-amino-4-trifluoromethyl-coumarin. SDS-polyacylamide gel electrophoresis of the purified materials revealed a single 28,000 dalton band. Inhibitor profiles of the band indicated that it belonged to cysteine endopeptidases. It exhibited identical pH curves with optimum at pH 5.5, and 50% activity from pH 4.7 to 8. It could completely degrade collagen chains to three identical products. It also showed some activity on hemoglobin. Furthermore, the band on immunoblots was reactive to the sera of sparganosis patients. These results suggest that the proteolytic enzyme belongs to cysteine proteinase which plays a role in the tissue penetration. Also it may be used as the antigen for diagnosis of active sparganosis.

**Key words:** Sparganum, sparganosis, cysteine proteinase, affinity chromatography, SDS-PAGE, immunoblot, human sera, serodiagnosis

## INTRODUCTION

*Spirometra erinacei*, an intestinal tapeworm of wild or domesticated carnivores, is found commonly in the Orient and Southeast Asia, and *S. mansonoides* in the East Africa, North (Sarma and Weilbaecher, 1986) and South America (Kron *et al.*, 1991). Human is usually infected by the plerocercoid of *Spirometra* called sparganum. When the plerocercoid infects a man, it migrates to the tissues such as subcutis, muscle, eye or scrotum where it undergoes no further development (Chi *et al.*, 1980). In rare occasions, the larva invades vital organs or the central nervous system (Fung *et al.*, 1989; Holodniy *et al.*, 1991).

Definite diagnosis of human sparganosis, depends on recovery of the larvae from the lesion. However, it should be invasive, and thus serodiagnosis is required. Okabe and Mutase (1957) found immune responses in sparganosis first. After then, Kim *et al.* (1984) revealed ELISA as a sensitive and specific method for diagnosis of human sparganosis, and Hong *et al.* (1989) detected that sparganum specific IgG antibody began to appear by 4 weeks after infection in experimentally infected mice. Cho *et al.* (1990) found that the whole sparganum antigen con-

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tained two specific antigens reactive to sera of sparganosis patients on immunoblots. Those studies suggested that sparganum-specific antibody is produced at acute phase of infection and the serology is very useful in diagnosis of the disease. However, further analysis on the antigenic components of sparganum is required to minimize the cross reaction.

It was proved that some proteolytic enzymes from parasites played important roles in the infection process including skin penetration (Dresden and Asch, 1972; McKerrow *et al.*, 1985), tissue migration (Rege *et al.*, 1987), and nutrient acquisition (Chappell and Dresden, 1986; Zerda *et al.*, 1987). In *Schistosoma* spp., the proteinases secreted/excreted into the host tissues are known to be immunogenic to the host, and detecting the antibody to the enzymes can be used for specific diagnosis (Ruppel *et al.*, 1985; Chappell and Dresden, 1988).

Ultrastructural studies of the sparganum in hamsters revealed that the secretory granules in the gland cells and the myelin-like bodies in the tegument were produced during migration (Osaki, 1990). The product was suggestedly associated with tissue invasion. As for the enzymatic activity of sparganum, Kwa (1972) demonstrated a caseinolytic enzyme from the scolex, and later it was found that the larval extract contained cysteine proteinase against azocoll, actin and myosin (Nakamura and Yanagisawa, 1982a & b; Nakamura *et al.*, 1984). Fukase *et al.* (1985) isolated an aminolytic enzyme against BZ-arg-PNA of 19-21 KD molecular weight, the activity of which was maximum at pH 7.0 and significantly decreased in the presence of thiol proteinase inhibitors and pepstatin. However, the kind of enzymes, their relationships to each other and their functional roles in the pathogenesis remain unclear.

The purpose of the present study was to confirm the activity of cysteine proteinase from spargana, and to characterize the enzyme including its antigenicity.

## MATERIALS AND METHODS

**Collection of parasites:** The spargana were obtained from naturally infected snakes, *Rhabdophis tigrina*, collected in Korea. The parasites were carefully dissected from the snake tissue. The collected spargana were washed several times with sterile physiological saline and once with distilled water prior to lyophilization. All experimental samples were stored at −20°C.

**Extraction of proteinase:** All procedures were carried out at 4°C unless otherwise stated. The lyophilized spargana (10 mg dry weight/ml) were homogenized with a tissue grinder in 0.1 M sodium acetate buffer (pH 5.5) and the homogenate was centrifuged at 40,000 g for 15 minutes. The supernatant was used for test of proteinase activity.

**Proteinase purification:** The crude supernatant was subjected to purification steps as described by Fukase *et al.* (1985). Briefly, the preparation was fractionated in a DEAE-Trisacryl M column (2.6×14 cm) equilibrated and washed with 20 mM tris, pH 7.5 and eluted with 0∼0.5 M NaCl in the same buffer. Fractions (3 ml) were collected at flow rate of 60.0 ml/hr, and assayed for the enzyme activity. The eluted active fractions were pooled, and low molecular weight components were removed on a sephadex G-25 column (1.5×13 cm). The pool was then applied to an activated thiolpropyl sepharose column (bed volume 10 ml), and eluted with 25 mM cysteine. Cysteine and 2-thiopyridone were removed and the buffer was exchanged by a sephadex G-25 column equilibrated and eluted with 0.1 M sodium acetate, pH 5.5. The fractions (2.0 ml) were collected at flow rate of 10 ml/hr, and were assayed for enzyme activity and protein content by spectrophotometry at 280 nm. The eluted active fractions were pooled, and column molecular weight determination was removed on an AcA54 gel filtration column (1.6×60 cm) equilibrated with 0.1 M sodium acetate, pH 5.5. Fractions (1.5 ml) were collected, assayed, and stored at −20°C.

**Synthetic substrate:** Proteinase activity in the acid-soluble sparganum extract was assayed on a synthetic substrate of low molecular weight with fluorescent carboxy terminal group, carboxybenzoyl-phenylalanyl-arginyl-7-amino-4-trifluoromethyl-coumarin (CBZ-phe-arg-AFC; Enzymes Systems Products, Livermore, CA). The assay buffer, 0.1 M sodium acetate pH 5.5, contained 5 mM dithiothreitol(DTT) and 10 $\mu$g of substrate in a total volume of 0.5 ml. After incubation for 3 hours at 37°C, the released AFC was measured in a Turner fluorometer(Model 111, Sequoia Turner Corp., Mountain View, CA) at excitation and emission wavelengths of 400 and 505 nm, respectively. A standard curve was constructed with various concentrations of AFC; activity is expressed as nM AFC $hr^{-1}$ $ml^{-1}$.

**Inhibitor studies:** Purified proteinase was preincubated with inhibitor for 30 minutes at 37°C. The substrate was added afterthen, and the tubes were further incubated for 3 hours at 37°C. Inhibitors included iodoacetic acid(1 mM; Wha Kwang Laboratories, Inc., Japan), L-trans-epoxysuccinyl-leucylamide(4-guanidino) butane (E-64, 5 $\mu$M; Enzymes Systems Products, Livermore, CA), leupeptin(100 mM) and pepstatin (10 mM, Peninsula Laboratories, Inc., Belmont, CA), N-tosyl-L-lysine chloromethyl ketone (TICK, 100 $\mu$M), N-a-p-tosyl-L-lysine chloromethyl ketone(TLCK, 100 $\mu$M), diisopropyl fluoro-phosphate(DEP, 1 mM), phenylmethylsulfonyl fluoride(PMSF, 1 mM), and 1, 10 phenanthroline (1 mM; all from Sigma Chemical Co., St. Louis, MO), and ethylene diamine tetraacetate(EDTA, 10 mM; Yakuri Co., Japan).

**Protein substrates:** Degradation of protein substrates was carried out as previously described by Rege and Dresden(1987) using acidic soluble collagen, type 1 of calf skin and bovine hemoglobin(Sigma Chemical Co., St. Louis, MO). Briefly, 40 $\mu$g of substrate were incubated at 37°C alone(control) or with proteinase in 0.1 M sodium acetate, pH 5.5 containing 5 mM DTT. The reaction was stopped by the addition of an equal volume of SDS sample buffer containing $\beta$-mercaptoethanol followed by boiling for 2~5 minutes. Samples were then electrophoretically separated on SDS gels and visualized by Coomassie Blue R-250 staining. Degradation was judged as the disappearance of uncleaved substrate and/or appearance of product. Protein concentration was determined by the method of Lowry *et al.*(1951). Bovine serum albumin(10~70 $\mu$m; Sigma Chemical Co., St. Louis, MO) was used to construct a standard curve. All assays were duplicated.

**Estimation of native molecular weight:** Purified proteinase was applied to an ultragel AcA54 gel filtration column(1.6×60 cm; IBF Biotechnics, Villenueve-la-Garenne, France) equilibrated and eluted with 0.1 M sodium acetate buffer, pH 5.5. Fractions(1.5 ml) were collected and assayed for activity with CBZ-phe-arg-AFC. The column was precalibrated with proteins of known molecular weight, including bovine serum albumin(67 kDa), ovalbumin(43 kDa), chymotrypsinogen(25 kDa) and ribonuclease(13.7 kDa).

**SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblot analysis:** Aliquots from various stages of purification were analyzed on 7.5~12.5% a polyacryamide gradient slab gel 4°C as described by Laemmli (1970). Molecular weight markers included the following proteins(Pharmacia LKB Biotechnology, Piscataway, NJ); phosphorylase B(94 kDa), albumin(67 kDa), ovalbumin(43 kDa), carbonic anhydrase(30 kDa), trypsin inhibitor(20 kDa) and $\alpha$-lactalbumin(14.4 kDa). After electrophoresis, the gel was stained with Coomassie Blue R-250 as described(Rege *et al.*, 1987).

The purified proteinase by electrophoresis under denaturing conditions was transferred to nitrocellulose membrane according to the method of Towbin *et al.*(1979). The nitrocellulose strips were incubated for 2 hours at 37°C with sera (1 : 100) collected from sparganosis patients which were stocked at Department of Parasitology, Seoul National University. Control sera were collected from uninfected volunteers. The immunoreactive bands were visualized by incubation for 2 hours at room temperature with

horseradish peroxidase-conjugated anti-human IgG(1 : 1000) followed by a further incubation for 5~15 minutes and chrongen reaction.

## RESULTS

Proteinase was extracted under acidic conditions to select for and to stabilize acidic proteinases. From 10 mg dry weight per ml of extraction buffer, 4.76 mg of protein was recovered on the soluble fraction(Table 1). To optimize extraction of proteinase activity in soluble extracts, various ionic strengths of the buffer ranging from 0.01~0.05 M were tested(data not shown). All concentrations had supported proteinase activity by 2-fold increase over that in 0.01 M solution.

The proteinase was purified from acidic extracts of spargana. Chromatographic separations, based on the purification scheme by Fukase *et al.*(1985), revealed in one major activity peak at 0.2 M NaCl by DEAE-Trisacryl M column. The active fractions were pooled, concentrated and applied to activated thiolpropylsepharose 4B affinity column. The column revealed two peaks of activity (Fig. 1). The active fractions were pooled, and applied to on AcA54 molecular sieve column. The AcA54 column revealed the presence of one activity peak at an approximate molecular weight of 23 kDa.

The proteinase activity in the final preparation was purified approximately 21-fold compared to the extracts (Table 1) recovered activity in the purified proteinase was substantially higher than the extracts(42%), and the cysteine proteinase was more stable in the acidic homogenization buffer(pH 5.5) than in the saline(pH 7.2) incubation buffer. Purification from prepa-

**Table 1.** Purification of cysteine proteinase from soluble extracts of sparganum

| Purification steps | Total activity* | Yield (%) | Total protein (mg) | Specific activity* | Purification (fold) |
|---|---|---|---|---|---|
| Soluble extract | 2,904 | 100 | 4.76 | 610.1 | 1.0 |
| DEAE-Trisacryl M | 1,655 | 57 | 1.82 | 909.3 | 1.5 |
| Thiolpropyl Sepharose | 1,224 | 42 | 0.10 | 12,750.0 | 21.0 |

* n mole AFC/hr/ml

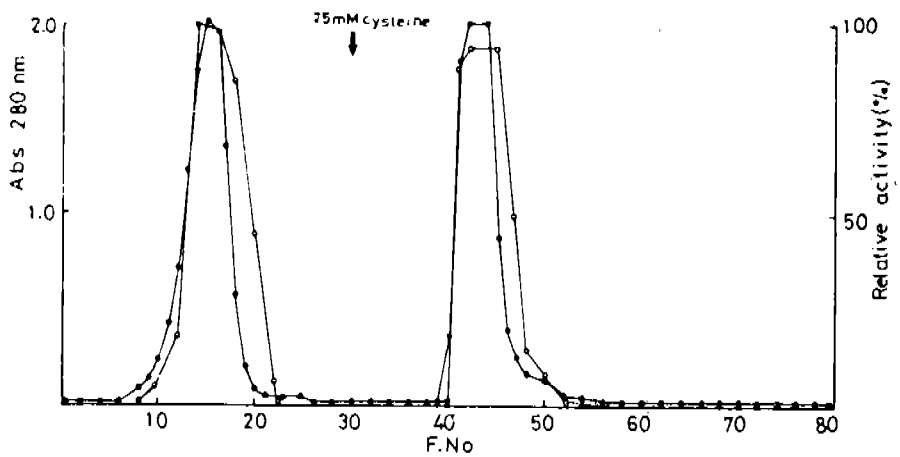


**Fig. 1.** Affinity chromatography with thiolpropyl-sepharose 4B. The supernatants were loaded onto DEAE-Trisacryl M chromatography, pooled, dialyzed with Sephadex G-25 and concentrated. The active peaks were loaded onto thiolpropyl-sepharose 4B, 0.1 M Tris-HCl containing 0.3 NaCl, 1 mM EDTA buffer(pH 7.5) and 2.0 ml fractions(Fno) were assayed for activity on CBZ-phe-arg-AFC (○). Fractions were monitored for protein content(●) at 280 nm.

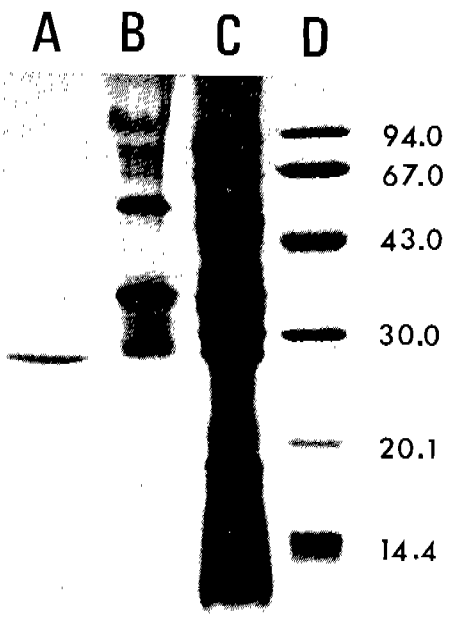


**Fig. 2.** 10~20% gradient SDS-PAGE of purification steps of plerocercoid extracts. Lane A, active peak from thiolpropyl-sepharose 4B; Lane B, active peak from DEAE-Trisacryl M; Lane C, extracts; Lane D, molecular weight protein markers; phosphorylase B(94 kDa), albumin(67 kDa), ovalbumin(43 kDa), carbonic anhydrase(30 kDa), trypsin inhibitor (20 kDa), α-lactalbumin(14 kDa).

ration, however, resulted in an active fraction containing a single 28 kDa band on SDS-PAGE (Fig. 2).

Proteinase purified from extracts was tested with CBZ-phe-arg-AFC substrate for inhibition by various endopeptidase class-specific compounds (Table 2). Significant inhibition was seen when the known inhibitory compounds on cysteine proteinase such as E-64, iodoacetate, NEM and leupeptin were present. TPCK and TLCL, which inhibited cysteine as well as serine proteinases, also decreased proteinase activity. In additione the absence of thiol resulted in 95% decrease of its activity.

Molecular weight of the purified proteinase

**Table 2.** Effect of endopeptidase inhibitors on proteinase purified from sparganum extracts

| Inhibitors | % Activity* soluble extract |
|---|---|
| None | 100 |
| Serine class: | |
| PMSF (1 mM) | 94 |
| DFP (1 mM) | 102 |
| TPCK (100 μg) | 85 |
| TLCK (100 μg) | 96 |
| Metallo class: | |
| 1, 10 phenanthroline (1 mM) | 0 |
| EDTA (10 mM) | 108 |
| Cysteine class: | |
| Without DTT | 4 |
| E-64 (10 μM) | 0 |
| Iodoacetate (1 mM) | 0 |
| NEM (10 mM) | 1 |
| Leupeptin (100 μM) | 0 |
| Aspartic class: | |
| Pepstatin (10 μM) | 112 |

* Percentages based on activity in the presence of 5 mM DTT without inhibitors

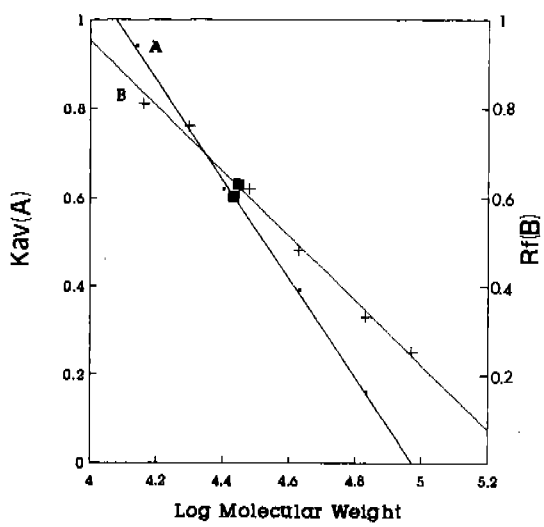


**Fig. 3.** Estimation of molecular weight of proteinases from *Spirometra* plerocercoid by AcA54 gel filtration(A) and SDS-PAGE(B).

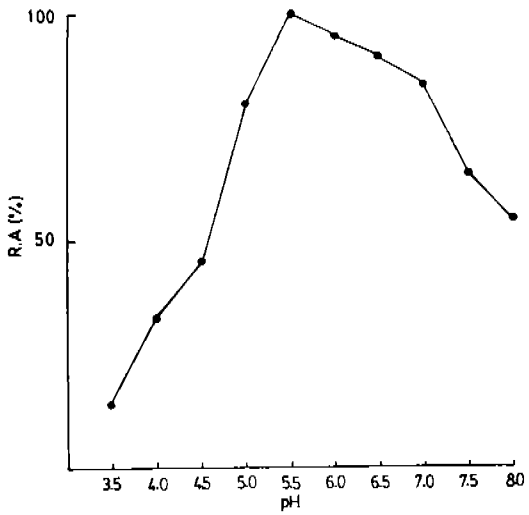


**Fig. 4.** Effect of pH on cysteine proteinase activity from plerocercoid extracts.

was estimated under native and denaturing conditions(Fig. 3). Native proteinase eluted from the gel filtration column in a single peak of activity was of approximately 28 kDa. On reducing or denaturing PAGE, the molecular weight of the purified protein was approximately 28 kDa either. The proteinase activity was shown in the range of pH 4.7~8.0 with a peak of 50% more at pH 5.5, and at least partial stability in neutral pH.

The proteins tested as protential substrates for the sparganum cysteine proteinase was shown in Fig. 5. Alpha and beta chains of collagen (upper panel) were completely degraded to three distinct products (Ⅰ-Ⅲ). Long incubation times did not, however, result in further degradation of these products to peptides. In contrast, only a limited degradation of hemoglobin was noted (lower panel). A slight decrease in the mobility of the dimer band and a marginal decrease in the density of the monomer band were noted. The results of this experiment were similar when the purified proteinase was applied. The purified proteinase was applied to SDS-PAGE and transferred to the nitrocellulose membrane.

The strips were then reacted to sera from patients with confirmed sparganosis or from normal individuals(Fig. 6). The IgG antibodies from infected patients bound to the 28 kDa protein in purified proteinase preparations; whereas, IgG from normal sera showed no reactivity to it. These findings indicate that the proteinase may be a useful indicator for serologic evaluation of patients with sparganosis.

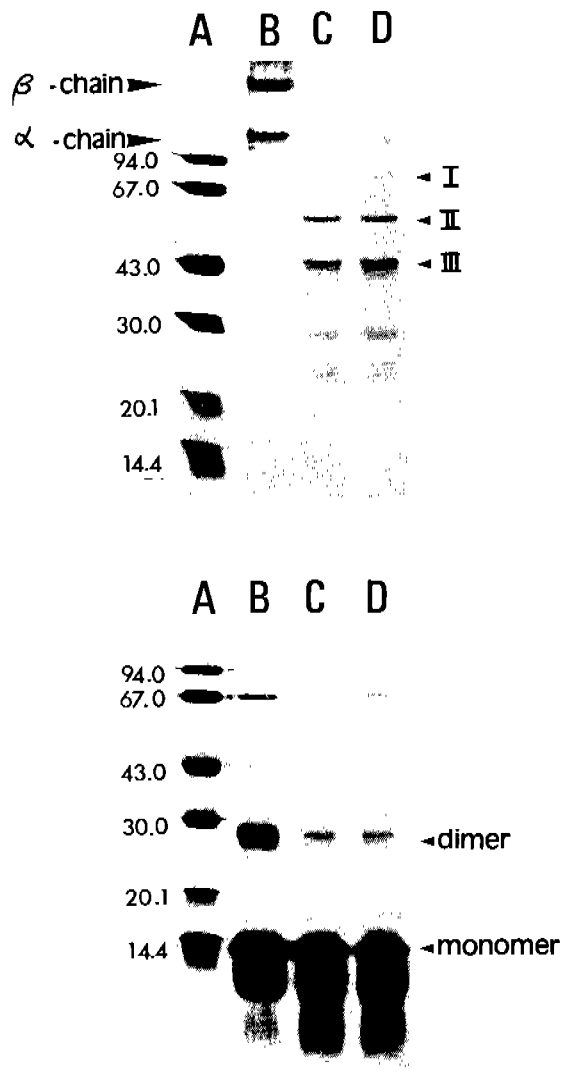


**Fig. 5.** Degradation of collagen(upper) and hemoglobin(lower) by proteinases from plerocercoid extracts. Upper; Lane A, molecular weight protein marker; Lane B, collagen control; Lane C, 30 min; Lane D, 1 hr. Lower; Lane A, molecular weight protein markers; Lane B, hemoglobin control; Lane C, 2 hr; Lane D, 4 hr.

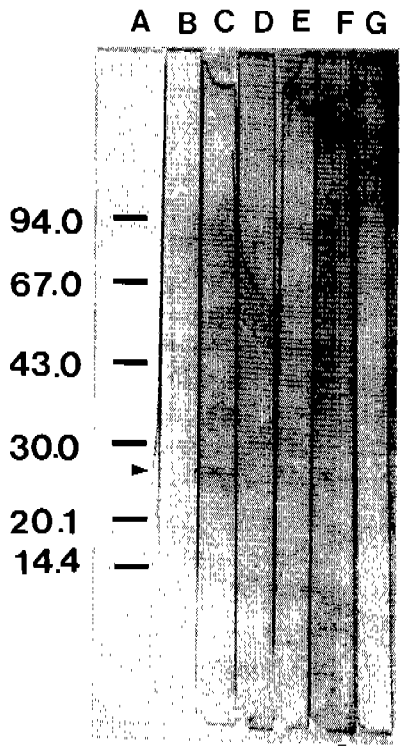


**Fig. 6.** IgG reactivity to purified plerocercoid cysteine proteinase(▲) from patients with sparganosis. Lane A, molecular weight protein marker; Lane B, C, D, E and F, human sera of sparganosis: Lane G, normal human serum.

## DISCUSSION

The methodological difference in the enzyme purification resulted in disparate results. For example, when the extraction buffer, used in the earlier study, consisted of deionized water with a detergent Triton-100, a single band of 19~21 kDa molecular weight was made up of two components with different isoelectric points. However, the present study employed acetate buffer of pH 5.5 following Fukase *et al.* (1985), and purified a single 28 kDa band on SDS-PAGE and a single peak of activity at 28 kDa on gel filtration. The protein purified from the plerocercoid extracts, native or denaturing condition yielded the band of same molecular weight. Therefore, the starting materials may well be composed of a mix of different components since acidic enzymes in other systems have enhanced solubility in low pH and high ionic concentrations (Chappell and Dresden, 1987). Furthermore, although similar synthetic substrates were used, minor differences in substrate composition can sometimes result in major differences in the activity. Therefore it is difficult to make direct comparisons in the proteinases described in these two studies. Their molecular weight differences, however, suggest that they are distinct proteinases.

The present experiment on the sparganum revealed an active fraction containing a single proteinase was same as the purified protein from whole sparganum extracts. The stability and activity of 28 kDa cysteine proteinase in neutral conditions further suggested that the proteinase would be active in extracellular host tissues against collagen or other protiens. The finding was consistent with the cysteine proteinase activity described by Nakamura *et al.* (1982a & b; 1984), which showed degradation of azocoll and the muscle proteins, actin and myosin. The capacity of the cysteine proteinase(s) from spargana to digest these tissue proteins, is certainly suggestive for its role in tissue migration.

Furthermore, the extracted proteinase was recognized on immunoblots by IgG antibodies in all of the sparganosis patients tested. Although still the present results should be confirmed and evaluated further, it strongly raises the applicability of the 28 kDa cysteine proteinase as an indicator of active sparganosis.

As for the antigenic proteins of sparganum, several papers have recorded various bands. Choi *et al.*(1988) found 30 bands on the gel and suggested that 29 and 36 kDa bands were prominent. The two proteins were observed to share common antigenic determinants and the serology with the antigens showed improved specificity (Cho *et al.*, 1990). They were produced at the tegumental cells, and concentrated on the tegument (Kim *et al.*, 1992). Kim and Choi (1991) also described 23 antigenic bands to IgG and 15 bands to IgM in the sparganum extract. They

also found the bands of 29 kDa on immunoblots. Whether the cysteine proteinase of the present study is the same molecule with previously described protein of 29 kDa or not should be a subject for further study.

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=국문초록=

## 스파르가눔 충체에서 분리한 cysteine proteinase의 정제 및 부분 특성

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한국산 유혈목이에서 스파르가눔 충체를 수집하고, 이들 충체의 추출액에서 ion-exchange chromatography와 affinity chromatography를 실시하여 cysteine proteinase를 순수 정제하였다. 정제된 효소의 최적 pH는 5.5이었고, 최적 mole 농도는 0.1 M (0.1 M sodium acetate, pH 5.5) 이었다. 정제된 효소는 thiol-dependent이고, 4°C에서 pH 5.0일 때 24시간 동안 안전성을 보였다. 효소의 활성도는 저분자 합성기질인 CBZ-phe-arg-AFC에 대해 활성이 높았다. 정제된 효소는 척추동물의 산성 cysteine proteinase의 억제인자에 감수성을 보였다. Ultrogel AcA54 column chromatography로 정제된 cysteine proteinase의 분자량을 측정한 결과 28,000 dalton이었다. 정제된 효소는 collagen type I과 hemoglobin을 분해하였다. Immunoblot한 결과 정제된 효소는 스파르가눔증 환자의 혈청과 반응하였다. 이상의 결과에서 스파르가눔의 cysteine proteinase는 숙주 체내이동, 조직침수성 및 영양소 섭취에 관여할 것이라 추정되며, 정제된 효소는 스파르가눔 현증의 혈청학적 진단에 이용될 수 있을 것으로 생각된다.

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