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Cytotoxicity of a cysteine proteinase of adult *Clonorchis sinensis*

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Abstract: To clarify the correlation of the proteinase activity with pathogenicity of *Clonorchis sinensis*, the proteinase activity either in excretory-secretory products (ESP) or in crude extracts of adult *C. sinensis* was examined. Substrate gel electrophoresis of the ESP and crude extracts revealed four distinct enzyme bands, which were differently inhibited by the specific proteinase inhibitors. The proteinase of the ESP with molecular mass of 24 kDa, was purified 23-fold with 14.5% yield by spectra gel ACA 44 gel filtration. It exhibited optimal pH at 7.5 in sodium phosphate (0.1 M). Its activity was inhibited specifically by *N*-ethylmaleimide (NEM) and antipain whereas potentiated 1.9 folds in the presence of 5 mM dithiothreitol (DTT). Cytotoxicity of the proteinase increased in a dose-dependent manner up to 120 μg/ml while reduced by NEM and antipain, indicating that cysteine proteinase was responsible for the cytotoxicity. This result shows that the 24 kDa cysteine proteinase is deeply correlated with the pathogenicity of *C. sinensis* infection.
Key words: *Clonorchis sinensis*, cysteine proteinase, cytotoxicity

INTRODUCTION

Clonorchis sinensis, which causes liver and biliary diseases in man, is one of the most important trematode parasite in China, Korea, Japan and south-east Asia (Rim, 1988). *C. sinensis* is orally infected with metarcercariae in intermediate hosts. The parasite excysts in host's digestive tract and migrates up to the bile duct where it grows to adults. During development of *C. sinensis*, biliary epithelial hyperplasia, periductal fibrosis and cystic change of the ducts occurred (Hou, 1955; Lee *et al.*, 1978; Song *et al.*, 1989). However, little is known in regard to the role of proteinase on the pathogenicity of *C. sinensis*.

Proteinases, demonstrated in many parasites, are suggested that they may play a pivotal role either in host-parasite interaction by facilitating the parasite invasion and nutrient uptake (North, 1982; McKerrow, 1989) or in immune evasion via proteolytic cleavage of the immune effector molecules (Kong *et al.*, 1994). Proteinase has also been implicated in intracellular digestion of phagocytes and may have importance in interactions between host cells (Mckerrow, 1989).

In this paper, we screened the proteinase activity either present in worm extracts or in ESP of adult *C. sinensis* and purified the proteinase in ESP of adult *C. sinensis*. Its properties were elucidated to verify the role on the pathogenicity, especially with reference to

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cytotoxic acivity.

MATERIALS AND METHODS

Preparation of the worm extracts and excretory-secretory products (ESP)

The metacercariae of *C. sinensis* were obtained from naturally infected *Pseudorasbora parva* collected in Kimhae-Gun, Korea. Rabbits were infected with oral feeding of 1,000 metacercariae and were sacrificed on the third month of experimental infection. The collected worms were washed three times with physiological saline. The suspensions of *C. sinensis* in sodium phosphate buffer (0.01 M, pH 7.4) was sonicated 4 times with 5-10 pulses at 100 watts for 15 seconds (Blackstone-Sheffield, U.S.A). The homogenates was then centrifuged at 10,000 g for 15 minutes. The supernatant was used as a crude enzyme.

The ESP were collected by incubating the 200 adult worms in 100 ml sodium phosphate buffer (0.01 M, pH 7.4) for 12 hours at 37°C. The incubation medium was dialyzed against Tris-HCl (0.01 M, pH 7.4) overnight at 4°C and centrifuged at 10,000 g for 15 minutes and the supernatant was regarded as a ESP. Protein content was measured (Bradford *et al.*, 1976). They were stored at -70°C until use.

Enzyme assay

Proteinase activity was assayed as described elsewhere (McLaughlin and Baubert, 1977). A total of 200 μl of the reaction mixture, which comprised of 50 μl sodium phosphate (0.1 M, pH 6) and 100 μl of 1% (w/v) azocasein (Sigma, St. Louis, U.S.A.) supplemented with 50 μl of the enzyme (5 μg of protein), was incubated at 37°C for 1 hour. The reaction was terminated by adding final concentrations of 50% (v/v) trichloracetic acid and let it stand for 10 minutes at room temperature. The supernatant was obtained by centrifugation at 10,000 g for 15 minute and the proteinase activity was determined at 420 nm by adding 2.4 ml of 0.5 N NaOH. One unit of the enzyme activity was defined as the amount of enzyme required to causes a unit (0.1) increase in absorbance through a 1 cm light path.

The effect of the proteinase inhibitors, such as pepstatin (aspartic acid-), ethylene diamine tetraacetate (EDTA, metallo-), phenyl methyl sulfonyl flouroide (PMSF), N-tosyl-L-lysine chloromethyl ketone (TLCK) and phenylglyoxal (Phgx; serine-), leupeptin, antipain, 5,5-dithiobis 2-nitrobenzoate (DTNB) and N-ethylmaleimide (NEM, cysteine-proteinase inhibitor), was observed. Proteinase inhibitors were purchased from Sigma (St. Louis, U.S.A.). The reaction mixture was pre-incubated with the respective inhibitors for 1 hour at 37°C then incubated with azocasein as described before.

Substrate gel electrophoresis for the proteinase inhibitor treated samples was carried out by pre-incubating the crude extracts or ESP with various proteinase inhibitors, respectively (Laemmli, 1970; Lockwood *et al.*, 1987). Gelatin (0.075%) was used as a substrate. After electrophoresis, the gel was washed with 500 ml of 2.5% (v/v) Triton X-100 at 37°C for 1 hour to reactivate the proteinase activity. The gel was then transferred to sodium phosphate buffer at pH 6 for worm extracts and 7.5 for ESP. They were stained with Coomassie blue, and destained by 10% (v/v) acetic acid and 10% (v/v) methanol.

Cytotoxic activity assay

The Chinese hamster ovary (CHO) cells were used as targets for cytotoxic assay. The cells were grown in Eagle's minimal essential medium (EMEM) supplemented with 10 mM N-2-hydroxy-ethylpiperazine-n-2-ethanesulfonic acid, 100 unit/ml penicillin, 100 mg/ml streptomycin and 10% fetal calf serum. The cells were grown in a 75 cm^2 plastic tissue culture flask at 37°C in 5% CO_2 incubator (NAPCO, Oregon, U.S.A). A total of 10^7 cells were labeled with 100 μCi of ^{51}Cr in 0.2 ml EMEM at 37°C for 1 hour. After washing, the cells were suspended in a complete medium to 1×10^4 cells/0.1 ml. The cells (100 μl) were incubated with the enzyme at 37°C in a 5% CO_2 incubator for 4 hours in wells of 96 microplates (Costar, Cambridge, U.S.A.). The released ^{51}Cr from 100 μl of supernatant fluid was detected using gamma count (Packard, Downers Glove, U.S.A.). As maximal release, all cells were lysed with 1 N NaOH. Incubated cells, without enzyme, were used as a spontaneous release. The cytotoxic activity (=

% specific release) was determined using the following formula (Ulberg and Jondal, 1981)

$$\% \text{ specific release} = \frac{\text{Test release} - \text{spontaneous release}}{\text{Maximal release} - \text{spontaneous release}} \times 100$$

Partial purification of the proteinase from ESP of adult *C. sinensis*

The crude ESP was saturated with 30-65% ammonium sulfate at 4°C. The precipitate was dialyzed against Tris-HCl (0.01 M, pH 7.4) overnight at 4°C and centrifuged at 10,000 g for 15 minutes. It was applied to spectra AcA 44 gel filtration (2.6 × 75 cm) which previously equilibrated with 0.01 M Tris-HCl (pH 7.4). The enzyme was eluted with the same buffer. Flow rate was 9 ml/hour. The enzyme activity in each fraction (3 ml) was assayed as described above. All the procedures were carried out at 4°C.

RESULTS

Identification of the enzyme activities

When the proteinase activities of the worm extracts and ESP were screened at various pHs, the worm extracts showed the highest enzymic activity at pH 6.0 while that of ESP was observed at pH 7.5 (Fig. 1).

Gelatin gel electrophoresis of the worm extracts and ESP revealed enzyme multiplicity. At least four distinct enzyme bands in the ESP and worm extracts were observed, respectively (Fig. 2A & B). The proteinases had a different mobility as well as a sensitivity towards various proteinase inhibitors.

Modulation of the proteinase activity by inhibitors

To determine the active site of the enzyme of the worm extracts and ESP, the effect of various proteinase inhibitors were tested (Table 1). The worm proteinases were sensitive to inhibitors acting on histidine (TPCK) and cysteine (leupeptin and NEM) proteinases while DTT (5 mM) potentiated the activity up to 1.4-fold. Similar result was observed when the proteinase activities in the ESP were examined.

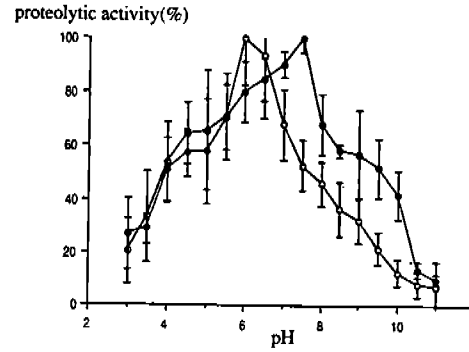

Fig. 1. The pH optimum for proteinase activity in *C. sinensis* worm extracts and ESP. The optimal pHs of the proteinase activities were determined by replacing the buffer used in the respective enzyme assays in 50 mM glycine buffer for pH 3, in 50 mM sodium acetate buffer at pH 4, 4.5, 5 and 5.5, in 50 mM sodium phosphate buffer for pH values between 6.0 and 8.5 and in 50 mM Trisaminomethane buffer for pH values between 9 and 11. ○, worm extract; ●, ESP proteinase.

Cytotoxicity assay

Cytotoxicity of the ESP and worm extracts against CHO cells was assayed. As shown in Fig. 3, cytotoxicity increased in a dose dependent manner up to protein concentration of 120 μg/ml. The activity of the ESP was almost 3 times higher than that of the worm extracts. When the ESP was eluted through gel filtration, the peak of cytotoxic activity was observed at molecular weight around 24 kDa.

Properties of the purified proteinase from ESP of *C. sinensis*

A total of 11,000 units of the enzyme activity was detected in the ESP (specific activity: 37). When the ammonium precipitation was carried out, the cytotoxic activity increased 474 with 34.2% of recovery rate. The clarified proteins were further resolved by spetra AcA 44 gel filtration (Fig. 4). From the step, a total of 1615 units of the enzyme was harvested. Cytotoxic activity increased 850. The protein was purified by 23-fold. The molecular weight of the partially purified enzyme was 24 kDa (Inset of Fig. 4). The overall purification was summarized in Table 2. Employing azocasein as an *in vitro* substrate, the enzyme having optimal pH around 7.5. Table 3 showed the modulation of the partially purified enzyme

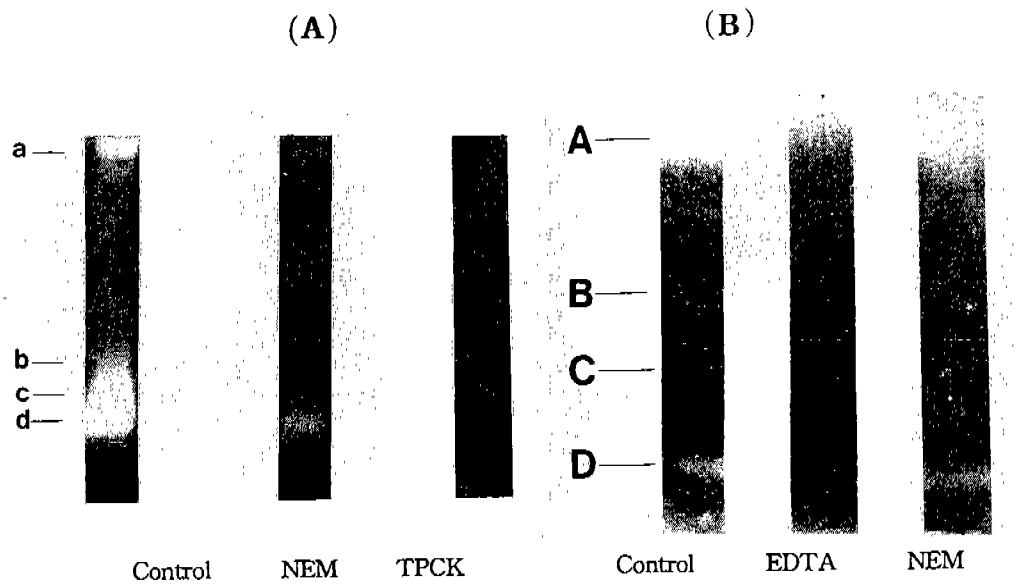

Fig. 2. Identification of proteinase activities of adult *C. sinensis* is shown by substrate gel electrophoresis (each lane contained the 20 μg protein, 5 mM inhibitor). **(A)** Effect of NEM and TPCK on the worm proteinase. Rf values at a, 0.04; b, 0.61; c, 0.71; d, 0.78. **(B)** Effect of EDTA, NEM on ESP enzymes. Rf values are A, 0.11; B, 0.46; C, 0.62; D, 0.92.

Table 1. Purification of proteinase from *C. sinensis* cultured media

Purification step	Total activities (units/ml)	Total protein (mg)	Cytotoxic activity[a)]	Purification fold	Yield (%)
Culture supernatant	11000	300	37	1	100
Ammonium sulfate	3792	8	474	12.8	34.2
Spectra gel AcA 44	1615	1.9	850	23.0	14.5

[a)]Cytotoxic activity = % specific release of ^{51}Cr/mg protein

activity by proteinase inhibitors. NEM and leupeptin inhibited the activity up to 58 and 63%, respectively. However, the enzyme was also susceptible to EDTA.

DISCUSSION

Parasite proteinases facilitate the invasion of host tissues, allowing parasites to digest host proteins, evade the host immune responses, and prevent blood coagulation (Chapman and Michell, 1982; Mckerrow, 1989; Maizels *et al.*, 1993). In the present study, at least four distinct proteinase activities of adult *C. sinensis* either in worm extracts or in ESP were identified by gelatin substrate gel electrophoresis employing proteinase inhibitors, suggesting that several different proteinases were synthesized and released outside the worm. Of these, the cysteine proteinase, which was responsible for the cytotoxicity against CHO cells, was purified partially and characterized. The purified cysteine proteinase from the ESP had optimal pH at 7.5 in 0.1 M sodium phosphate.

In order to identify the proteinase activity and to determine the active site residues, the effects of various proteinase inhibitors on the enzyme activities were examined. The proteinase in the adult worm extracts showed an optimal pH at 6.0 and were sensitive to inhibitors affecting histidine type (TPCK) and cysteine type (leupeptin and NEM) while the 3rd and 4th bands in ESP were susceptible to

inhibitors acting on cysteine type (NEM and leupeptin) and metallo type (EDTA), respectively (Table 1 and Fig. 2). This result demonstrated clearly that *C. sinensis* had different proteinases which were incorporated in performing diverse biological reactivities. This result was also coincided well with the findings of Song *et al.*, (1990) which showed that adult worms of *C. sinensis* contained cysteine proteinase activity, although the ESP contained metallo and cysteine type proteinase.

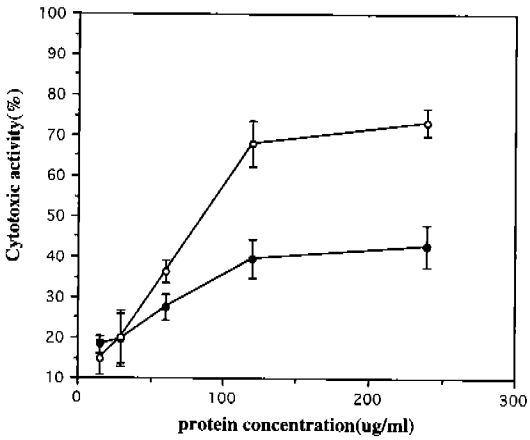

Fig. 3. Cytotoxic activity of *C. sinensis* on CHO cells. Error bars represent the mean ± standard error, n=6. —•—, worm extract; —○—, ESP.

Cultivation of mammalian cells is a useful tool in examining pathogenicity of the invasive parasites (McCaul *et al.*, 1977). When the ESP was added in the culture media of CHO cells, the cytotoxic activity increased dose-dependently up to protein concentration of 120

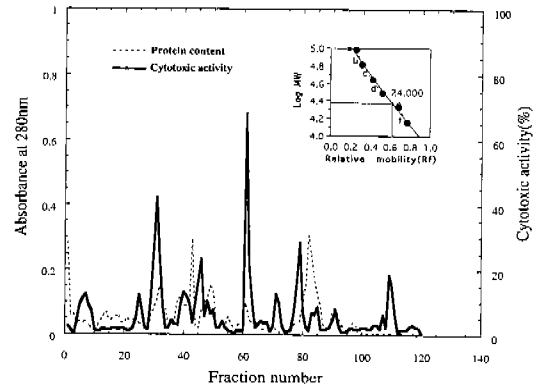

Fig. 4. Elution profile and cytotoxic activity of partially purified enzyme by spectra/Gel AcA 44 gel filtration. Each fractions are assayed for cytotoxicity (——) and protein content (·····). Inset also shows the determination of molecular weight in kDa. a, phosphorylase b (97.4); b, bovine albumin (66); c, egg albumin (45); d, carbonic anhydrase (31); e, trysin inhibitor (21.5); f, lysozyme (14.5).

Table 2. Modulation of inhibitors on proteinases of *C. sinensis* worm extract and ESP

	Remaining activity[a] of proteinase			
	Worm extract		ESP	
	Final concentration (mM) of inhibitor			
Inhibitors	2	5	2	5
PMSF	103 ± 19	114 ± 7	112 ± 9	112 ± 9
Pepstatin	104 ± 27	119 ± 15	85 ± 12	88 ± 12
Phgx	106 ± 22	98 ± 16	98 ± 8	124 ± 17
EDTA	89 ± 7	80 ± 8	23 ± 6	15 ± 4
TPCK	71 ± 8	59 ± 7	39 ± 8	33 ± 5
TLCK	72 ± 6	62 ± 15	37 ± 10	31 ± 19
Leupeptin	62 ± 16	52 ± 12	42 ± 3	35 ± 7
Antipain	77 ± 17	72 ± 26	25 ± 3	21 ± 3
NEM	66 ± 7	49 ± 7	34 ± 1	28 ± 2
DTT	132 ± 28	135 ± 29	179 ± 10	189 ± 17
DACM	82 ± 36	72 ± 22	45 ± 29	42 ± 31

[a]Activity = $\frac{\text{Experimental absorbance}}{\text{Control absorbance}} \times 1$, the activity was a mean ± standard deviations, n = 5.

Table 3. The effect of various inhibitors on partially purified proteinase from ESP against CHO cell

Inhibitors[a]	Cytotoxic activity[b]
Control[c]	68.5 ± 5.7
PMSF	92.5 ± 14.3
Pepstatin	75.3 ± 22.0
NEM	32.0 ± 9.7
TLCK	39.0 ± 13.4
Leupeptin	27.0 ± 7.2
Antipain	35.0 ± 9.0
EDTA	47.0 ± 4.3

[a]Final concentration of the respective inhibitors are 2 mM. [b]Cytotoxic activity = $\frac{\text{test release} - \text{spontaneous release}}{\text{maximal release} - \text{spontaneous release}} \times 100$

[c]Protein concentration of control ESP proteinase is 120 μg/ml.

μg/ml. However, relatively little cytotoxic activities were observed when the worm extracts were added in the culture media, which suggested strongly that the pathogenicity of the parasite might mainly be due to a excretory-secretory cysteine proteinase. The secretory proteinase might affect the biliary epithelium which finally resulted in epithelial metaplasia and cellular hyperplasia; the main pathological changes in clonorochiasis (Lee *et al.*, 1978; Hong *et al.*, 1993, 1994). More studies deserve to elucidate the roles of the released cysteine proteinase especially on its roles in invoking pathophysiological and morphological changes in clonorchiasis.

The purification of proteinase in the ESP was performed by 30-65% ammonium sulfate precipitation and gel filtration through a spectra/Gel AcA 44 column. The molecular weight of the purified enzyme was estimated to be 24 kDa. The partially purified proteinase exhibited also significant cytotoxic activity and proteinase activity which was modulated by cysteine proteinase inhibitors. This result confirmed that cysteine proteinase was responsible for the cytotoxic activity. Nevertheless, extensive studies on the role of cysteine proteinase of *C. sinensis* in the pathogenicity are not yet clarified. Further studies are needed to define the molecular mechanism for secretion, subcellular localization of the proteinase.

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=초록=

간흡충의 cysteine 단백분해효소의 세포독성

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간흡충의 병원성과 단백분해효소 활성도의 상관성을 밝히기 위하여 간흡충 추출물과 분비배설물의 단백분해효소 활성도와 세포독성을 평가하였고 분비배설물에서 단백분해효소를 부분정제하고 생화학적 성질을 규명하였다. 여러 가지 단백분해효소 억제제를 사용하여 단백분해효소의 활성을 측정한 결과 간흡충에는 Rf값을 서로 달리하는 효소분획으로 되어있음이 관찰되었으며, 이러한 효소분획은 azocasein을 기질로 한 활성부위 잔기 억제 실험에서 서로 상이한 활성부위잔기를 갖고 있음을 알 수 있었다. 간흡충 분비배설물의 세포독성은 단백질 농도를 120 μg/ml까지 증가시키자 세포독성이 3배 증가했고, 이 효과는 NEM과 antipain에 의해 억제되었다. 이 사실은 cysteine 단백분해효소가 세포독성에 관여하는 것을 보여주고 있었다. 이 단백분해효소는 최적활성치가 pH 7.5이었다. 이 효소를 분비배설물로부터 23배 정제하였고, 이때 회수율은 14.5%이었다. 부분정제한 단백분해효소의 분자량은 24 kDa이었다. 이 효소는 NEM, antipain에 의해 효소활성이 억제되었고, 동시에 세포독성도 억제되었다. 이 사실로부터, 부분정제한 효소의 활성부위잔기는 cysteine이고 이 효소가 또한 분비배설되어 세포독성을 나타낸다는 것을 알 수 있었다.

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