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A cysteine protease of *Paragonimus westermani* eggs

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Abstract: Protease activity was identified in crude extracts of *Paragonimus westermani* eggs which were purified from infected dog lungs, isolated on 14 weeks after metacercarial challenge. The eggs were used after removing possibly contaminated host or worm tissues on their shell surfaces. In the crude egg extracts, high proteolytic activities against carboxybenzoyl-phenylalanyl-arginyl-4-methoxy-β-naphthylamide (Cbz-phe-arg-MNA) and Azocoll were detected whereas those against succinyl-alanyl-prolyl-phenylalanyl-*p*-nitroanilide (Suc-ala-pro-phe-*p*NA) were not revealed. The enzyme exhibited the maximal activity at pH 6. Its activity was inhibited by specific cysteine protease inhibitors, 10^{-5} M 1-trans-epoxysuccinylleucylamido (4-guanidino) butane (E-64) and 1 mM iodoacetamide (IAA) while potentiated by 6.5-fold in the presence of 2.5 mM dithiothreitol (DTT). When the enzyme was purified partially by Sephacryl S-300 High Resolution gel filtration, it migrated as a single homogeneous band at 35 kDa. The 35 kDa cysteine protease has been recognized neither in the metacercariae nor in the adult. These findings indicated the presence of at least one protease of cathepsin family in immature eggs of *P. westermani*.
Key words: *Paragonimus westermani*, egg, cysteine protease

INTRODUCTION

In clinical setting, most lesions of extrapulmonary paragonimiasis are chronic egg granuloma. It is mainly because the ectopic lesions are usually, though not always, detected long after egg-producing adults are dead and absorbed while the produced eggs are neither drained out nor absorbed. These egg granuloma have been presenting clinical problems especially in neurological and abdominal surgery (Lee *et al.*, 1993; Cha *et al.*, 1994). Most eggs in pulmonary lesions are, however, expectorated because lung granuloma are ruptured intermittently and their contents are drained through bronchioles as an egg-containing rusty sputum (Shim *et al.*, 1991).

Cysteine proteases of *Paragonimus westermani* have been studied as important pathophysiological factors in paragonimiasis. They were involved either in metacercarial excystment (Chung *et al.*, 1995) or in tissue penetration during its early infection period (Yamakami, 1986; Yamakami and Hamajima, 1989, 1990; Song and Dresden, 1990; Song and Kim, 1994). However, proteases of *P. westermani* eggs have rarely been investigated. In this connection, proteases in schistosome eggs have been purified and characterized including its antigenicity (Ash and Dresden, 1979; Sung and Dresden, 1986), though the relations between schistosome egg proteases

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and granuloma formation are not yet clearly established (McKerrow and Doenhoff, 1988).

In the present study, we described the presence of a cysteine protease activity in egg extracts of *P. westermani*, and partially purified the enzyme.

MATERIALS AND METHODS

Preparation of egg extracts of *P. westermani*

Eggs of *P. westermani* were collected from experimentally infected dog lungs. After squeezing and washing the infected lungs in physiological saline, liberated eggs were cleared by centrifugation at 500 rpm for 10 minutes for 6 times. Subsequently, a pellet, precipitated egg mass of dark brown color, was saved. The partially purified egg mass was cleared further by removing particulated contaminants using a fine needle under a dissecting microscope at room temperature. The grossly purified eggs were then dialyzed against distilled water overnight to eliminate host and adult worm debris or secretions which might be attached on surface of egg shells. They were washed, again by centrifugation at 500 rpm for 10 minutes twice. About 500 mg of eggs of over 99.9% purity were obtained. The eggs were stored at −70°C until used.

The eggs were thawed and homogenized with a teflon-pestle homogenizer and centrifuged at 15,000 g for 30 minutes. The resulting supernatants were regarded as crude egg extracts. Protein content was measured by the method of Lowry *et al.* (1951). All procedures were carried out at 4°C unless otherwise specified.

Assay of protease activity

Endopeptidolytic activity: Protease activity was assayed by endopeptidolysis employing synthetic substrates as described by McKerrow *et al.* (1985). The reaction mixture, consisted of 0.44 ml sodium acetate buffer (0.1 M, pH 6) and 20 μl of carboxybenzoyl-phenylalanyl-arginyl-4-methoxy-β-naphthylamide (Cbz-phe-arg-MNA), carboxybenzoyl-alanyl-arginyl-arginyl-4-methoxy-β-naphthylamide (Cbz-ala-arg-arg-MNA), carboxybenzoyl-arginyl-arginyl-4-methoxy-β-naphthylamide (Cbz-arg-arg-MNA) and succinyl-alanyl-prolyl-phenylalanyl-*p*-nitroanilide (Suc-ala-pro-phe-*p*NA) (each in 0.1 mM of final concentrations) supplemented with 2.5 mM DTT, was incubated with 10-20 μl enzyme solution at 37°C for 1-3 hours. The reaction was stopped by adding 0.5 ml 10% (v/v) trichloroacetic acid (final 5% concentration). The liberated MNA or *p*NA was detected by adding 0.01% (w/v) tetrazotized *o*-dianisidine (Fast blue N salt) at 530 nm or at 410 nm using a spectrophotometer (Smart 190DU, Korea). One unit of the enzyme activity was defined as the amount of enzyme required to cause a unit (0.1) increase in absorbance through 1 cm light path. It corresponded to the enzyme activity required to hydrolyzing 2.94×10^{-3} picomole/minute of MNA or *p*NA under the conditions stated.

Azocoll hydrolyzing activity was also determined (McKerrow *et al.*, 1985). The reaction mixture, comprised of 0.465 ml of sodium acetate (0.1 M, pH 6), 5 mg Azocoll and 10-20 μl enzyme solution, was incubated and the reaction was terminated as decribed above. Enzyme activity in the supernatant fraction, which was obtained by centrifugation at 12,000 rpm for 10 minutes, was measured at 540 nm. One unit of the activity was expressed as described before which corresponded to enzyme activity that hydrolyzed 8.32×10^{-2} mg/minute of Azocoll under the conditions specified. Specific activity was defined as the units of enzyme activity per milligram protein. All chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All determination was triplicated and standardized using an appropriate blank.

Determination of optimal pH: The optimal pH of the enzyme activity was monitored by incubating the enzyme with Cbz-phe-arg-MNA in sodium acetate buffer (0.1 M) at pH 5.5 and 6, in sodium phosphate buffer (0.1 M) at pH 6, 6.5, 7 and 7.5 (Chappell and Dresden, 1986). Procedures for enzyme assay were performed as described above.

Active site titration: The effect of 2.5 mM DTT, a disulfide group protector, was observed. The cysteine protease inhibitors, 10^{-5} M E-64 and 1 mM IAA as well as serine protease inhibitors including 0.1 mM 3,4-

dichloroisocoumarine (3,4-DCI) and 2 mM diisopropyl fluorophosphate (DFP) were examined for their effects on the proteolytic activity. The effects of 1,10-phenanthroline (2 mM) and ethylenediamine tetraacetic acid (EDTA, 0.1 mM) were also tested.

Polyacrylamide and activity gel electrophoresis: Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out on 12.5% separating gel in reducing or non-reducing conditions or on 7.5-15% gel in reducing condition (Laemmli, 1970). The gel was stained with 0.125% (w/v) Coomassie blue.

To observe the direct proteolytic activity of the egg proteases, activity gel, which was copolymerized with a final concentration of 0.2% (w/v) gelatin and 12% acrylamide, was performed using 10 μg egg protein with non-reducing sample buffer (McKerrow *et al.*, 1985). After electrophoresis, the gel was washed with 2.5% (v/v) Triton X-100 solution for 1 hour to remove SDS and to reactivate the enzyme. The gel was further incubated with sodium phosphate buffer (0.1 M, pH 6) containing 2.5 mM DTT overnight at 37°C to allow proteolysis. The gel was finally visualized with Coomassie blue staining.

Partial purification of egg protease by gel filtration

The crude egg extracts (1 ml) were resolved by 1.6 × 70 cm sized Sephacryl S-300 HR gel permeation chromatography (Pharmacia-LKB, Piscataway, NJ, USA), which previously equilibrated with sodium acetate (0.05 M, pH 5.7) containing 0.15 M NaCl. Protein was eluted using the same buffer at a flow rate of 40 ml/hour · cm^2. Eighty 2.3 ml fractions were collected. Fractions with high enzyme activity were pooled, dialyzed against sodium acetate (0.01 M, pH 5.7), lyophilized and reconstituted with 0.1 ml of sodium acetate (0.01 M, pH 5.7).

RESULTS

Proteolytic activity of the crude egg extracts was monitored using synthetic molecular and macromolecular substrates. Table 1 showed the specific activity against each substrate. Out of five substrates examined, the extracts exhibited the strong activity against Cbz-phe-arg-MNA and Azocoll. It also degraded Cbz-ala-arg-arg-MNA and Cbz-arg-arg-MNA, though the activity was weak. However, it could not hydrolyse Suc-ala-pro-phe-*p*NA.

Table 1. Substrate specificity of *Paragonimus westermani* egg extracts against synthetic and macromolecular substrates

Substrate[a)]	Specific activity (units/mg protein)
Cbz-phe-arg-MNA	30.4
Cbz-ala-arg-arg-MNA	2.4
Cbz-arg-arg-MNA	1.5
Suc-ala-pro-phe-pNA	0
Azocoll	19.2

[a)]Cbz-phe-arg-MNA, carboxybenzoyl-phenyl-alanyl-arginyl-4-methoxy-β-naphthylamide; Cbz-ala-arg-arg-MNA, carboxybenzoyl-alanyl-arginyl-arginyl-4-methoxy-β-naphthylamide; Cbz-arg-arg-MNA, carboxybenzoyl-arginyl-arginyl-4-methoxy-β-naphthylamide; Suc-ala-pro-phe-*p*NA, succinyl-alanyl-prolyl-phenylalanyl-*p*-nitroanilide.

Fig. 1 demonstrated the optimal pH for the enzymic activity. The protease revealed the maximal activity at pH 6. Between pH 5.5-7, it retained about 50% of the specific activity while it showed highly decreased activity at pH 7.5 (23-38% of the peak activity).

Modulation of enzyme activity by different inhibitors was shown in Table 2. DTT potentiated the proteolytic activity up to 6.5-fold whereas E-64 (10^{-5} M) and IAA (1 mM) inhibited over 90% of the activity, but not completely. Neither inhibitors acting on serine protease such as DFP (2 mM) and 3,4-DCI (0.1 mM) nor metalloprotease inhibitors including 1,10-phenanthroline (2 mM) and EDTA (2 mM) affected the activity. About 90% of the control activity was observed.

In Fig. 2, comparative protein composition of the crude egg extracts and adult worm extracts was analysed by reducing and non-reducing conditions. In reduced gel (lanes 1 & 2), the egg extracts (lane 2) exhibited 47, 46, 28 and 27 kDa bands as major components, while they showed two distinct bands larger than 200 kDa and ca. 150, 120, 47, 46, 28 and 27 kDa in non-reducing condition (lane 4).

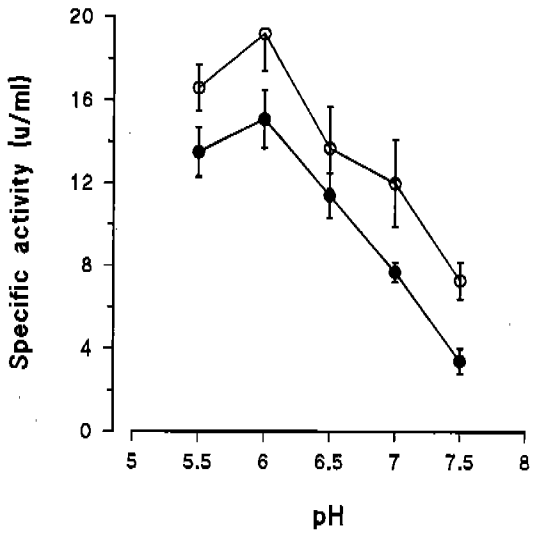

Fig. 1. Determination of optimal pH for proteolytic activity of the partially purified enzyme of *P. westermani* egg. The enzyme activity was detected in 0.1 M sodium acetate and 0.1 M sodium phosphate buffer. ●-●, activity against Cbz-phe-arg-MNA; ○-○, activity against Azocoll. See also the "Materials and Methods" for detailed description.

To visualize the proteolytic activity of the egg protease, gelatin substrate gel electrophoresis was carried out with non-reducing sample buffer. The enzyme activity was observed at pH 6. As shown in Fig. 3, at least four zones of proteolysis were recognized in the adult worm extracts (lane C) whereas two proteolytic zones were faintly observed in the crude egg extracts (lane Egg). The proteolytic zone of lower mobility (cathodic side) protein was more distinct when compared to that of higher mobility (anodic side).

Fig. 4 exhibited the elution profile of the crude egg extracts through Sephacryl S-300 HR gel permeation. When the proteolytic activity was measured along with each fraction, high enzymic activity against Cbz-phe-arg-MNA was observed between 40th and 48th fractions. These fractions were pooled, concentrated by lyophilization and analysed by SDS-PAGE. As shown in Fig. 5, a protease migrated as a single homogeneous band at 35 kDa which was not recognized in the crude egg extracts (lane 1 of Fig. 2 & lane C of Fig. 5).

Table 2. Modulation of inhibitors on the proteolytic activity of the crude egg extracts of *P. westermani*

Effectors[a]	Final concentration (mM)	Relative activity[b]
Control (DTT-activated)	2.5	100
Without DTT		15.4
E-64	0.01	9.5
IAA	1	9.4
DFP	2	114.5
3,4-DCI	0.1	88.8
1,10-phenanthroline	2	95.4
EDTA	2	90.8

[a]DTT, dithiothreitol; E-64, 1-trans-epoxysuccinylleucylamido (4-guanidino) butane; IAA, iodoacetamide; DFP, diisopropyl fluorophosphate; DCI, 3,4-dichloroisocoumarine; EDTA,ethylenediamine tetraacetic acid. [b]Residual activity of the enzyme is expressed as percent activity in comparison to an initial activity of the crude enzyme without inihibitor in sodium acetate (0.1 M, pH 6) using Cbz-phe-arg-MNA at 530 nm as described in the "Materials and Methods".

DISCUSSION

This study demonstrated clearly that egg extracts of *P. westermani* contained proteolytic enzymes which degraded synthetic and macromolecular substrates. It showed strong activity against Cbz-phe-arg-MNA which is a known protease substrate for cathepsin B and L. The enzymic activity was inhibited by specific cysteine protease inhibitors, E-64 and IAA while potentiated by 6.5-fold in the presence of DTT. Inhibitors acting on serine- (DFP and 3,4-DCI) or metallo-protease (1,10-phenanthtoline and EDTA) did not modulate the activity. These findings indicated that crude egg extracts of *P. westermani* had at least a kind of cysteine protease belonging to cathepsin family. The finding that maximal activity of the protease at pH 6 also supported the conclusion. When the protease was purified partially by single step chromatography, it exhibited a single band at 35 kDa which was a little larger than

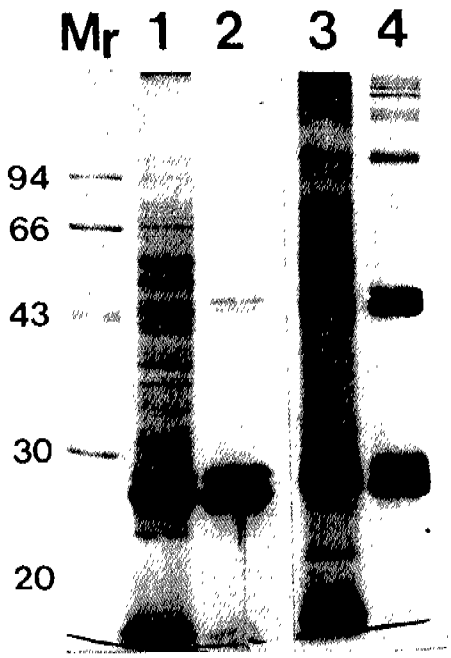

Fig. 2. SDS-PAGE findings of the crude adult and egg extracts of *P. westermani* on 12.5% separating gel. 1 & 3, crude adult extracts in reducing (1) and non-reducing conditions (3); 2 & 4, crude egg extracts in reducing (2) and non-reducing (4) conditions. Coomassie blue stained. Mr, molecular mass in kDa.

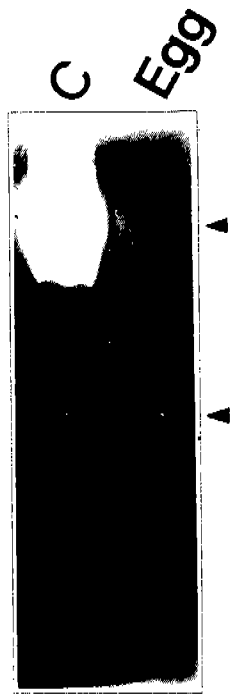

Fig. 3. Activity gel electrophoresis of the crude egg extracts of *P. westermani* showing two proteolytic zones. The 12.5% separating gel, copolymerized with 0.2% (w/v) gelatin was used for protein resolution. C, adult worm extracts of *P. westermani*; Egg, egg extracts. Proteolytic zones by the egg extracts are indicated (◀).

approximate size of 20-30 kDa of cysteine proteases (Barrett and Kirschke, 1981). Gelatin substrate gel electrophoresis of the crude egg extracts showed two zones of proteolysis indicating that at least two proteolytic activities are working at pH 6 in the presence of DTT. At this moment, however, identification of the partially purified 35 kDa protease was not determined between the two gelatinolytic bands of the eggs.

In regards with proteolytic enzymes of *P. westermani*, some cysteine proteases have already been described in its developmental stages. Of these, 28 and 27 kDa enzymes of adult *P. ohirai* (Yamakami, 1986) and that of 17.5 kDa of *P. westermani* (Song and Kim, 1994) which shared the biochemical properties with cathepsin B have been characterized. In addition, Yamakami and Hamajima (1989, 1990), Song and Dresden (1990) reported the 20 kDa cysteine protease of *P. westermani* from adult or from various develpmental

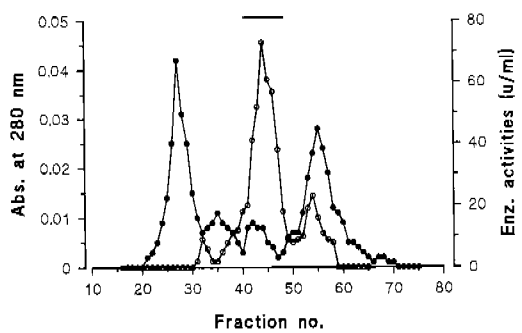

Fig. 4. Partial purification of the cysteine protease from the egg extracts. The crude extracts were eluted through Sephacryl S-300 HR gel permeation column (1.6 × 70 cm). Aliquots of each fraction were assayed for Cbz-phe-arg-MNA degrading activity (○-○) and for protein (●-●). Pooled fractions with high enzyme activity are shown by a bar (–).

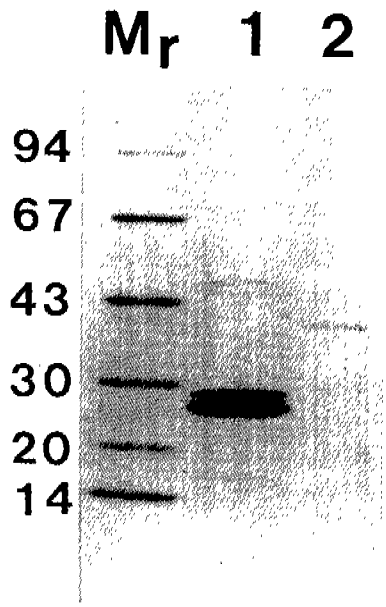

Fig. 5. Electrophoretic analysis of crude egg extracts (1) and partially purified cysteine protease of *P. westermani* eggs (2) shown in 7.5-15% SDS-PAGE. The partially purified egg protease migrates as a single band at 35 kDa (Coomassie blue stained). Mr, molecular mass in kDa.

stages. Chung and coworkers (1995) also described recently the cysteine proteases at 28 and 27 kDa in the metacercaria which might play a pivotal role in its excystment. In the present study, we purified the 35 kDa cysteine protease from the eggs of *P. westermani* which might be different from those of other stages. The egg protease seems to be incorporated with vitelline cells because an immature egg of *P. westermani* has abundant vitelline cells. This presumption deserves further investigation.

Egg cysteine protease of *P. westermani* was not a major protein constituting the crude egg extracts. As reported earlier, a major egg protein, 440 kDa (Imai and Nawa, 1988) was highly antigenic (Kang *et al.*, 1991; Kong *et al.*, 1992) and was the first major cathodic protein in non-denaturing PAGE of adult or egg extracts (Huer *et al.*, 1985; Kim *et al.*, 1986). In SDS-PAGE, the subunits of the egg protein was 44 and 23 kDa (Kong *et al.*, 1992). A cysteine protease of *P. westermani* egg at 35 kDa has not been shown in any previous electrophoretic analysis of the crude egg extracts probably due to its relative small amount.

Roles of *Paragonimus* egg cysteine protease are not yet studied especially in aspects of miracidial development and egg hatching. In addition, roles of the eggs in forming a granuloma have not actually been defined in chronic paragonimiasis. Eggs may be existing within the granuloma just as innocuous objects. However, patient sera of paragonimiasis reacted strongly with the egg protein (Kong *et al.*, 1992) which suggested that *Paragonimus* eggs may not be only innocuous materials. In this regards, schistosome eggs are major pathogenic units in the lesion and they cause strong immune reactions to the patient sera (Dunne *et al.*, 1991). If the *Paragonimus* egg is a pathogenetic unit such as depot of granuloma forming factors, the first candidate is a protease. But it is not known whether it is secreted outside to induce a cytokine releases because the cysteine protease usually has an intracelluar confinement. Further studies are necessary in this aspect.

In schistosomiasis, eggs are main pathologic units of granuloma. The initial pathologic finding of the egg granuloma is a focal degeneration of hepatocytes around the eggs which are followed by infiltration of inflammatory cells including macrophage, eosinophil and lymphocyte. A role of egg cysteine protease has long been speculated in this process (McKerrow and Doenhoff, 1988). The cysteine protease of schistosome egg have actually been identified and purified (Ash and Dresden, 1979; Sung and Dresden, 1986). The roles of cysteine protease as a granuloma forming factor, however, has not yet been definitely proved but other cationic glycoproteins have been characterized to be important (Dunne *et al.*, 1991).

In conclusion, this study demonstrated the presence of a 35 kDa cysteine protease which was a minor protein constituting crude egg extracts of *P. westermani*. Its role as a pathogenic factor should be studied in the future especially in relation to granuloma formation.

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=초록=

폐흡충 충란에 존재하는 시스테인 계열 단백질 분해효소

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폐흡충의 여러 발육 단계, 즉 피낭유충, 종숙주 내 성숙단계 충체 및 성충에서 분자량이 각각 28, 27, 22 및 17.5 kDa인 시스테인 계열 단백질 분해효소가 분리 정제되었다. 이 연구는 만성 폐흡충증의 육아종에서 발견되는 충란이 육아종 형성에 관여하는 물질을 분비한다면 그 분비액 중에서는 단백질 분해효소가 중요할 것이라고 가정하고 우선 그 존재를 조사하였으며 이를 부분정제하고 생화학적 특성을 관찰하였다. 실험적으로 개에 폐흡충 피낭유충을 감염시키고, 14주일 후에 개를 도살하여 폐를 분리하였다. 폐를 생리식염수로 씻어 그 세척액에서 충란을 모았다. 그후 해부현미경하에서 이물질(異物質)을 제거하고 증류수에서 하룻밤 투석하여 충란외부에 묻어 있을 수 있는 숙주, 또는 성충의 조직을 제거하였다. 충란을 생리식염수에서 마쇄한 후 원심분리에 의해 충란 조효소(粗酵素)를 제작하였다. 조효소에는 Cbz-phe-arg-MNA와 Azocoll을 분해하는 단백질 분해효소가 존재하였으며 이 활성은 pH 6에서 가장 높았다. 이 활성은 DTT에 의해 6.5배 증강되었고, 시스테인계 단백질 분해효소의 특이 억제제인 E-64나 IAA에 의해서는 90% 억제되었다. 조효소를 Sephacryl S-300 HR column을 통과시켜 효소를 부분정제한 결과 분자량이 35 kDa인 시스테인 계열의 단백질 분해효소을 정제할 수 있었다.

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