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Purification and Inhibition of Acid Hemoglobin Protease of a Lung Fluke by Antiproteases from Human Plasma

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P. westermani is highly important as a lung fluke causing disease in some countries of the Orient (Yokogawa *et al.*, 1960; Miyazaki, 1978). In recent years, an acid hemoglobin protease which hydrolyzes hemoglobin has been found in some parasitic helminths (Timms and Bueding, 1959; Grant and Senft, 1971; Zussman and Bauman, 1971; Sauer and Senft, 1972; Aoki and Oya, 1976; Sato *et al.*, 1976; Oya and Noguchi, 1977; Asami *et al.*, 1979; Dresden and Deelder, 1979; Hamajima *et al.*, 1979; Bolla and Weinstein, 1980; Maki *et al.*, 1980). Thus, we have been interested in studying the inhibitory effect of mammalian host serums on the hemoglobin protease activity of *P. westermani* relative to the host-parasite relationship. Hamajima *et al.* (1979) found that the enzyme activity was inhibited by human plasma. These findings suggest that inhibition of the enzyme by serum may probably be brought about by some antiproteases of plasma (Schwick *et al.* 1966; Heimburger, 1975). It was, therefore, thought worthwhile to elucidate the inhibitors which interact with the enzyme with the aim of investigating the parasitism of *Paragonimus*. However, to accomplish this, it is necessary to purify the enzyme. This paper reports the purification and inhibition of the enzyme by the antiproteases from human plasma.

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Materials and Methods

The adult worms of *Paragonimus westermani* (triploid type) used in the experiments were removed from worm cysts in the lungs of dogs 10 months after inoculation with metacercariae isolated from *Eriocheir japonicus* collected on Tsushima Is., Japan. α_1-Antitrypsin for affinity chromatography was purchased from Sigma Chemical Co. Antiproteases used in the inhibitory experiments were kindly supplied by Dr. N. Heimburger, Dr. H. Karges, Dr. E. Dietzel and Dr. H. H. Weimar of the Behring Institute. These substances were of high purity (>90%) except inter-α-trypsin inhibitor. The latter consisted of two active components of inter-α-trypsin inhibitor which comprised 80 to 90% of the total protein. The main impurity was prealbumin. Sepharose 4B, DEAE-cellulose and CM-cellulose were obtained from Pharmacia Fine Chemicals, Whatman Biochemicals and Serva Feinbiochemica GmbH, respectively. All chemicals employed were of the highest purity.

For affinity column chromatography, α_1-antitrypsin (13 mg/ml of packed wet beads) was coupled to Sepharose 4B activated with cyanogen bromide according to the procedure of Cuatrecasas *et al.* (1968). Columns of DEAE-cellulose and CM-cellulose were

used as recommended by the manufacturer's instructions. All columns were run at 4 C. Fractions were monitored for protein content at 280 nm with an LKB Instrument. Protein concentration was determined by the method of Lowry *et al.* (1951) with bovine serum albumin as a standard. Assay of proteolytic activity was basically performed by the method reported by Anson (1939). Reaction mixtures containing 50 μl enzyme, 750 μg human hemoglobin and 0.1 M citrate-phosphate buffer, pH 3.9 in a final volume of 150 μl were incubated for 40 minutes at 37 C. Then trichloroacetic acid was added to a final concentration of 2%. The mixtures were allowed to stand for 60 minutes at 0 C, and then centrifuged. The liberated peptides in the supernatant were measured by the methods of Lowry *et al.* (1951). The absorbance was determined at 660 nm with a Beckman spectrophotometer. One unit of the enzyme activity was defined as the activity producing an increase of 1.0 in absorbance at 660 nm per minute per ml.

The reaction system for the inhibitory experiments contained 10 μg of inhibitor in the standard assay mixture (the amount corresponding in 150 μg protein of human plasma to concentration of antiproteases inhibiting the hemoglobin protease according to Hamajima *et al.*, 1979). Purified enzyme (9.0 μg protein/ml) was used in the experiment. The enzyme was treated with each inhibitor for 5 minutes at 4 C, and then the substrate was added to the reaction mixture. Evaluations of inhibition of the protease by antiproteases were based on reduction in formation of the hydrolysates of substrate. The activity of the enzyme was expressed as ΔOD at 660 nm.

Results

Purification of Acid Hemoglobin Protease

Step 1: Extraction

The lyophilized adult worms (750 mg) were homogenized in 10 ml of 0.01 M citrate-phosphate buffer, pH 3.8 (buffer 1), for 4 minutes with a motor driven glass homogenizer fitted loosely with a teflon pestle kept in an ice bath. The homogenate was centrifuged at 25,000$\times g$ for 10 minutes. The supernatant as a crude extract was dialyzed for 16 hr against 0.02 M acetate buffer, pH 4.5 (buffer 2).

Step 2: Affinity Chromatography on α_1-Antitrypsin-Sepharose 4B

The extract was applied to a column (0.9×6.0 cm) of α_1-antitrypsin-Sepharose 4B equilibrated with buffer 2. The column was washed with starting buffer until the absorbance at 280 nm of the eluate returned to base line, and the adsorbed enzymes were eluted with the same buffer containing 0.1 M sodium chloride. The fractions (1.5 ml) were collected at a flow rate of 6.0 ml/hr. The pooled enzyme fraction was dialyzed for 16 hr against 0.01 M phosphate buffer, pH 6.0 (buffer 3). The elution profile by affinity chromatography on α_1-antitrypsin-Sepharose 4B column is illustrated in Fig. 1 a. An activity peak of the enzyme was found in the fraction 90 to 100 eluted with buffer 2 containing sodium chloride. Chromatography in this step gave a 73-fold increase in specific activity over that of the crude extract with a 67% yield.

Step 3: DEAE-cellulose Column Chromatography

For further purification, the dialyzed enzyme fraction obtained in the previous step was placed on a column (1.5×6.0 cm) of DEAE-cellulose equilibrated with buffer 3. The column was washed with the same buffer until the absorbance at 280 nm of eluate reached the base line. The adsorbed enzymes were eluted with a linear sodium chloride gradient to 0.5 M in the same buffer. The fractions (1.5 ml) were collected at a flow rate of 5.0 ml/hr. The

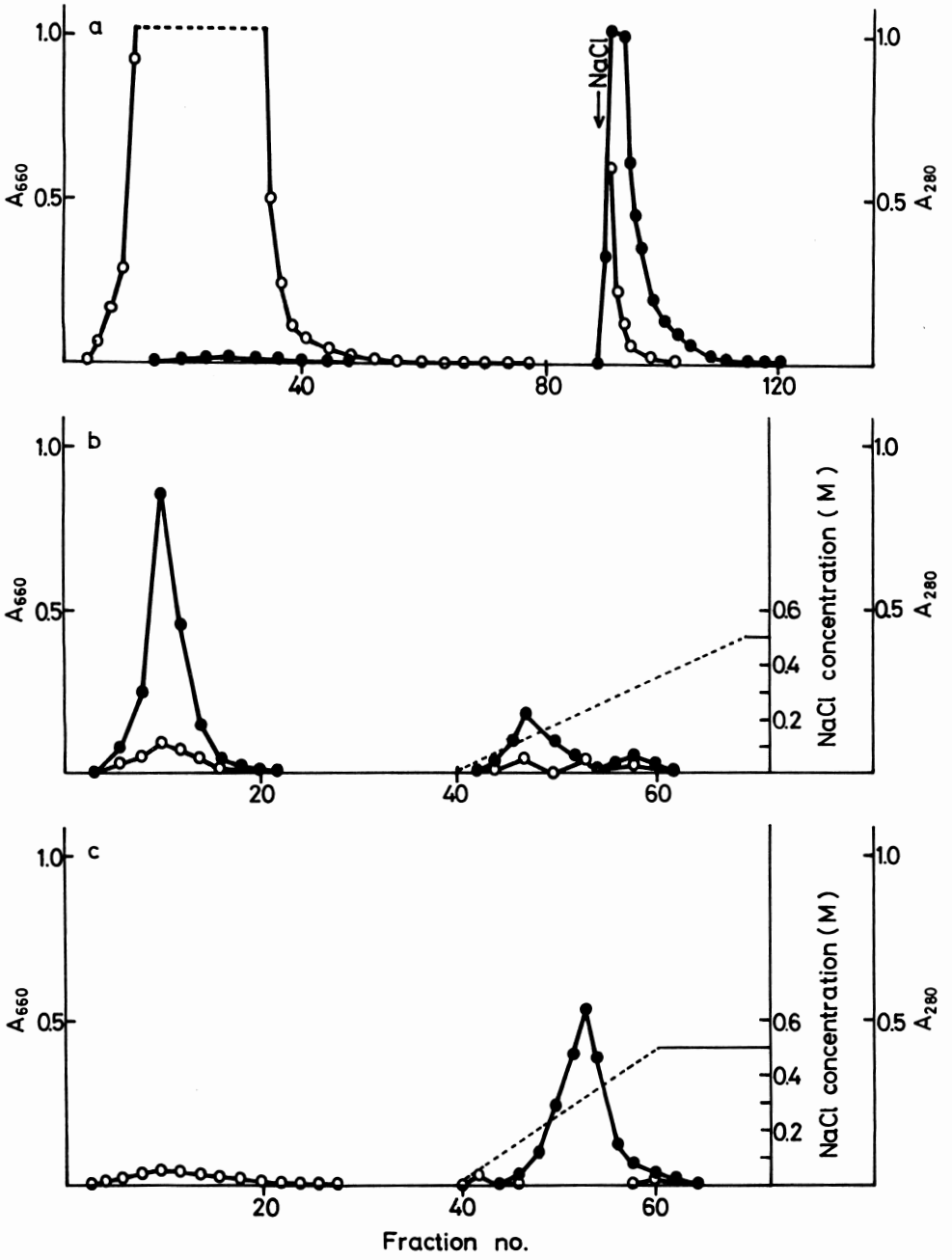

Fig. 1 a, α_1-Antitrypsin-Sepharose 4B affinity chromatography of acid hemoglobin protease from the crude extract of *P. westermani* adult worms. b, DEAE-cellulose column chromatography of the protease fraction purified with α_1-antitrypsin-Sepharose 4B affinity chromatography. c, CM-cellulose column chromatography of the enzyme fraction purified by DEAE-cellulose column chromatography. Open and closed circles represent absorbances at 280 nm (A_{280}) of protein concentration and proteolytic activity at 660 nm (A_{660}) of liberated hydrolysate.

typical elution profile in DEAE-cellulose column chromatography is shown in Fig. 1 b. The column yielded three activity peaks of the enzyme. A major peak was found in the washing effluent fraction of the fraction 6 to 15, and was used for the purification. The other two chromatographic activity peaks were eluted with gradients of sodium chloride. The yield of the enzyme from the major peak was 39% of the crude extract, and the specific activity was increased 124-fold in this step.

Step 4: CM-cellulose Column Chromatography

The major enzyme fraction from Step 3 was applied to a column (0.9×9.0 cm) of CM-cellulose equilibrated with buffer 3 for the repeated purification. The column was washed with the same buffer until the absorbance at 280 nm of the eluate approached zero, and the adsorbed enzyme was eluted with a sodium chloride gradient to 0.5 M in the same buffer. The fractions (1.2 ml) were collected at a flow rate of 5.0 ml/hr. The pooled enzyme was dialyzed for 16 hr against buffer 1, and concentrated by ultrafiltration. The elution profile in CM-cellulose column chromatography is shown in Fig. 1 c. Active material was eluted with a sodium chloride gradient, and it was found in the fractions 48 to 56. The chromatography in this step gave a 1,888-fold increase in specific activity with a 34% yield of the crude extract. The sample was used as the purified enzyme preparation. The summary of a representative purification is shown in Table 1.

Inhibition of the Protease by Antiproteases

Table 2 indicates inhibitions of the purified enzyme by 6 known antiproteases from human plasma. The extent of hydrolysis of hemoglobin by the enzyme was decreased below the level of the control without inhibitor when α_1-antitrypsin, α_1-antichymotrypsin or Cl-inactivator were added as an inhibitor. The differences are statistically significant. On the contrary, inter-α-trypsin inhibitor, antithrombin III and α_2-macroglobulin did not significantly inhibit formation of the hydrolysates.

Discussion

Some investigators have attempted to achieve purification of acid hemoglobin protease of some parasitic helminths with affinity chromatography. However, little is known about high affinity purification of the protease by antiprotease from plasma. Timms and Bueding (1959) reported that ultracentrifugation at pH 3.0 gave a 20-fold purification in specific activity compared with the crude preparation of the enzyme of adult *Schistosoma mansoni*. Also, the specific activity was increased 7- to 9-fold over that of the supernatant by the use of ethyl alcohol precipitation between 50 and 75% for the adult worms (Grant and Senft, 1971). On the other hand, Sauer and Senft (1972) found that affinity chromatography of the crude extract on agarose-L-phenylalanine gave a 15.15-fold increase in specific activity with a 52% yield. Similarly, the specific activity was increased 9-fold over that of the crude extract of adult *Schistosoma japonicum* with a 40% yield by affinity chromatography on the same agarose (Aoki and Oya, 1976). Furthermore, Dresden and Deelder (1979) reported that the specific activity of the enzyme was increased 7-fold over that of the crude extract of adult *S. mansoni* with a 60 to 70% yield by affinity chromatography using an aminophenylmercuric acetate-Sepharose 4B column. From these results, the preparations reported so far seem not to have attained a highly purified state. In the present study, we were able, however, to obtain a highly purified enzyme from adult *P. westermani* worms using a column of α_1-antitrypsin-Sepharose 4B and two columns of DEAE-cellulose and CM-cellulose. The purifica-

Table 1 Purification of acid hemoglobin protease from adult worms of *P. westermani*

	Step	Protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Purification (-fold)
1	Crude extract	111.5	40.25	0.36	100	1
2	α_1-Antitrypsin-Sepharose 4B	1.025	26.93	26.27	67	73
3	DEAE-cellulose	0.349	15.57	44.61	39	124
4	CM-cellulose	0.02	13.59	679.5	34	1,888

Table 2 Inhibition of purified acid hemoglobin protease from adult worms of *P. westermani* by six antiproteases from human plasma

Inhibitor	ΔOD ($\times 10^{-3}$) Mean±SD	Inhibition (%)	P
Control	185± 0.8	0	—
α_1-Antitrypsin	140± 0.5	24	<0.001
α_1-Antichymotrypsin	153± 0.9	17	<0.001
Cl-Inactivator	146± 1.7	21	<0.001
Inter-α-Trypsin Inhibitor	182± 5.2	2	(NS)
Antithrombin III	182± 4.9	2	(NS)
α_2-Macroglobulin	175±14.4	5	(NS)

The concentrations of the inhibitors in each reaction system were: α_1-antitrypsin, 185.2 pmoles; α_1-antichymotrypsin, 145.0 pmoles; Cl-inactivator, 96.2 pmoles; inter-α-trypsin inhibitor, 62.5 pmoles; antithrombin III, 153.8 pmoles; and α_2-macroglobulin, 13.8 pmoles. Each value represents the mean of triplicate determinations with the standard deviations (SD). Probability (P) is expressed in P-value. The abbreviation in parenthesis is not significant (NS) by t-test.

tion ratio of the enzyme from the crude extract achieved a higher level than that reported by the above investigators for *S. mansoni* and *S. japonicum*. Affinity chromatography of α_1-antitrypsin-Sepharose 4B was the important step for facilitating the further purifications. It seems that the reason is due to the high affinity of α_1-antitrypsin for the enzyme.

According to Yamagami and Hamajima (1980), the pH optimum for proteolytic action of the purified enzyme on human hemoglobin was around pH 3.9. The enzyme appeared to be homogeneous on polyacrylamide gel electrophoresis, and the molecular weight was estimated to be about 30,000 by gel filtration. Both hemoglobin and globin were hydrolyzed by the enzyme. The enzyme was inhibited by soy bean trypsin inhibitor, chymostatin, leupeptin and antipain, but pepstatin showed no effect. Similar results were obtained by Yamamoto *et al.* (1979) for the pH optimum and substrate specificity of cathepsin D. On the other hand, our enzyme was most closely similar to cathepsin B rather than cathepsin D in sensitivity to trypsin inhibitor, chymostatin, leupeptin and antipain (Barret, 1973; Yamamoto *et al.*, 1979; Towatari *et al.*, 1979).

The lung flukes injure various tissues and organs when penetrating and feeding during their migrations to the lungs (Yokogawa *et al.*, 1960). Human plasma contains several inhibitors against trypsin, chymotrypsin, elastase and plasmin as proteolytic enzymes (Schwick *et al.*, 1966; Heimburger, 1975). On the basis of these facts, inhibition of the proteases in the worms by antiproteases from human plasma is of interest

from the point of view of the susceptibility of host to the worms and pathology produced. In the present study, the purified enzyme was appreciably inhibited by α_1-antitrypsin, α_1-antichymotrypsin and Cl-inactivator. Similar results were obtained for the inhibitory effects of these antiproteases on cercarial "penetration" protease of *S. mansoni* (Asch and Dresden, 1977). From these results, it would appear that these antiproteases inhibited the enzyme which hydrolyzes hemoglobin. On the contrary, inter-α-trypsin inhibitor (the amount corresponding to 10 μg as inter-α-trypsin inhibitor), antithrombin III and α_2-macroglobulin did not inhibit the enzyme activity in a statistically significant manner. It seems that these antiproteases belong to inhibitors associated with limitation or prevention of blood coagulation and fibrinolysis according to Heimburger (1975). Also this result is different from those reported by Starkey and Barrett (1973) studying the inhibitory effect of α_2-macroglobulin on the proteolytic activity of human cathepsin B and cercarial protease (Asch and Dresden, 1977). The reason is not yet clear. It may be due to the concentration effect; i. e., 10 μg of α_2-macroglobulin contains relatively few molecules compared to the other inhibitors tested. Human plasma contains α_1-antitrypsin, α_1-antichymotrypsin and Cl-inactivator as inhibitors for the enzyme. Especially, α_1-antitrypsin is found in human plasma at high concentration (Schwick *et al.*, 1966; Heimburger, 1975). It seems probable, therefore, that hydrolysis of ingested hemoglobin by the worms in some hosts may be inhibited by α_1-antitrypsin and other protease inhibitors present in the plasma.

Summary

Purification of the hemoglobin protease of adult *P. westermani* worms has been achieved with affinity chromatography on α_1-antitrypsin-Sepharose 4B, and chromatographic methods using DEAE-cellulose and CM-cellulose. The purified protease showed a 1,888-fold increase in specific activity with a 34% yield of the crude extract in the final step. Six known antiproteases from human plasma were tested for inhibitory activity against the hemoglobin protease of *P. westermani* adults. α_1-Antitrypsin, α_1-antichymotrypsin and Cl-inactivator inhibited the purified enzyme activity with regard to the hydrolysis of human hemoglobin, but the three other antiproteases did not.

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人血漿のプロテイナーゼインヒビターによる肺吸虫ヘモグロビン分解酵素の精製およびその阻害作用

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ウェステルマン肺吸虫 (3n 型) は固有宿主と非固有宿主をもっており，この寄生現象を生化学面から解明することは寄生虫学にとってきわめて枢要なことである．すでに著者らは本肺吸虫成虫におけるヘモグロビン分解酵素および人血漿による本酵素の阻害作用を報告し，その血漿中に本酵素に対するインヒビターが存在するであろうことを示唆した．そこで本酵素に対する人血漿中のインヒビターを明らかにするため，まず本酵素の精製をおこなった．その結果，α_1-antitrypsin-Sepharose 4B アフィニティクロマトグラフィー，DEAE-cellulose および CM-cellulose カラムクロマトグラフィーによって，本酵素を粗抽出液の 1,888 倍 (収率 34%) にまで精製することができた．ついで，この精製酵素に対する人血漿中の α_1-antitrypsin, α_1-antichymotrypsin, Cl-inactivator, inter-α-trypsin inhibitor, antithrombin III および α_2-macroglobulin の阻害効果を検討した．その結果，本酵素活性は α_1-antitrypsin, α_1-antichymotrypsin および Cl-inactivator によって阻害されることが明らかになった．このようなことから，これらのプロテイナーゼインヒビターは肺吸虫の宿主での寄生にとってきわめて重要な関係をもっているものと考えられる．