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A Globin Specific Protease in *Schistosoma mansoni* I. Cooperation with a Protease in Mouse Serum

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Introduction

A globin specific protease, first demonstrated in *Schistosoma mansoni* (Times and Bueding, 1959), has been purified and characterized chemically and physically in detail (Grant and Senft, 1971; Sauer and Senft, 1972). Among numerous substrates, this enzyme could only hydrolize globin or hemoglobin into small peptides (Sauer and Senft, 1972; Aoki and Oya, 1976).

In vitro studies on the cultivation of *S. mansoni* demonstrated that host hemoglobin is involved in schistosome nutrition (Cheever and Weller, 1958; Clegg, 1965). Zussman *et al.* (1970) also found that hemoglobin degradation products are incorporated and extensively distributed in the schistosome tissues. These evidences suggest that this parasite actively utilize the hemoglobin degradation products for own protein synthesis. It is, therefore, assumed that the globin specific protease may participate in a fundamental protein metabolism in *S. mansoni*.

We have been interested in studying the physiological effect of the host serum on the schistosome metabolism. Our preliminary investigation on the effect of host sera on the globin specific protease in *S. mansoni* demonstrated that some host sera markedly change the enzyme activity (Asami *et al.*, 1979). The present communication deals with the effect of various sera or serum components on the globin specific protease in *S. mansoni*.

Materials and Methods

Parasite: Adult worms of *Schistosoma mansoni* (Puerto Rican strain) were harvested by perfusion of the liver and portal vein system of ICR strain mice infected 7 to 9 weeks previously with 250 cercariae. They were immediately placed and washed in a chilled physiological saline, and then the worms were lyophilized and stored at −20 C until used.

Chemicals: Crystalline human hemoglobin was purchased from Milus Laboratories Ltd. (Elkart, Ind. USA). Sephadex G-200, DEAE-Sephadex A-25 and Blue dextran 2000 were purchased from Pharmacia Fine Chemicals Japan (Tokyo, Japan). All chemicals were of the highest purity commercially available.

Assays: The activity of the globin specific protease was assayed at 37 C by measuring the absorbance change at 280 nm of the TCA deprotenized supernatant prepared according to the method of Sauer and Senft (1972). The activity of mouse serum protease was assayed in the same manner as above.

The standard assay mixture for the globin specific protease and the mouse serum protease contained 0.1 ml of hemoglobin solution (50 mg/ml) and 200 mM acetate buffer, pH

3.9 in a final volume of 0.5 ml.

One unit of activity is expressed as 1.0 absorbance in the TCA soluble supernatant isolated after incubation for 60 min.

Protein was determined by Folin-phenol procedure (Lowry *et al.*, 1951) using bovine serum albumin as a standard.

Purification of the globin specific protease: Lyophilized worms, usually about 50 to 70 mg, were suspended in about 6 ml of 10 mM citrate buffer, pH 3.0 containing 1 mM 2-mercaptoethanol. The parasite was disrupted by homogenization for 2 min using glass homogenizer and sonicated at 9 KHz for 10 min. The supernatant fluid was isolated by centrifugation at 105,000 g for 60 min and dialyzed against 40 mM acetate buffer, pH 3.9 containing 1 mM 2-mercaptoethanol for 4 hours. The sample was concentrated on a collodion bag and applied on a Sephadex G-200 column (20×600 mm) previously washed and equilibrated with the same buffer. Elution was performed with the same buffer. The elution speed was approximately 10 ml per hour. The active fraction (the second protein peak) was used in all enzyme studies. All procedures were done at 4 C.

Purification of the mouse serum protease: Pooled sera isolated from normal ICR strain mice was dialyzed against 20 mM sodium phosphate buffer, pH 7.0 (SP buffer) overnight, and brought to 30% saturation by adding solid ammonium sulfate with stirring. After stirred for 60 min, the supernatant fluid was isolated by centrifugation. This fraction was also brought to 50% saturation and the precipitate was isolated by centrifugation in the same manner as above. The final precipitate was dissolved in 10 ml of the SP buffer and dialyzed against 500 ml of the same buffer overnight. The dialysate was concentrated on a collodion bag and applied to a Sephadex G-200 column (20×550 mm) previously washed and equilibrated with the SP buffer. The elution speed was 10 ml per hour. The second protein peak, which contained the most activity, was concentrated in the same manner as above and applied to the column of DEAE-Sephadex A-25 (15×250 mm) previously equilibrated with the SP buffer. The sample was eluted on a linear gradient of sodium chloride (zero to 500 mM in the SP buffer). The elution speed was approximately 20 ml per hour. The active fraction was pooled and used for the enzyme studies. All procedures were done at 4 C.

Results

Purification procedure of the globin specific protease in *Schistosoma mansoni* is summarized in Table 1. The specific activity of the purified enzyme increased approximately 10-fold compared with that of the crude extract.

In Table 2 are shown the effect of various sera and serum proteins on the activity of the purified globin specific protease. The globin specific protease activity was markedly decreased by addition of human serum, and was slightly decreased by rabbit serum. Conversely, addition of mouse serum resulted in marked increase of this enzyme activity. The mouse serum factor inducing the increase of the globin specific protease activity would be refered to as a enhancing factor. This factor could not be removed by dialysis, was

Table 1 Purification of the globin specific protease *Schistosoma mansoni*

Fraction	Total volume (ml)	Total protein (mg)	Total activity (units)	Specific activity (units/mg protein)	Recovery (%)
Whole homogenate	14.0	56.0	18.2	0.33	100
105,000g supernatant	25.8	35.8	18.6	0.52	102
Sephadex G-200	15.4	4.6	14.3	3.14	78

Details of the assay are given in the text.

Table 2 Effect of various sera and serum proteins on the activity of the globin specific protease in *Schistosoma mansoni*

Assay condition	Units/mg protein
Control assay mixture	3.1
+0.05 ml of Human serum	1.2
+0.05 ml of Rabbit serum	2.5
+0.05 ml of Rat serum	3.0
+0.05 ml of Mouse serum	7.6
+Bovine albumin (5 mg)	3.1
+Horse albumin (5 mg)	3.3
+Human γ-globulin (5 mg)	3.0

Details of the assay are given in the text. Sera and serum proteins were present before the reaction was started by addition of substrate. Data are average of at least two independent determinations.

Table 3 Effect of the mouse serum on the activity of the globin specific protease in *Schistosoma mansoni*

Assay condition	Absorbance change at 280 nm/60min
GPE*+Mouse serum	0.023
GPE*+ Human hemoglobin	0.092
Mouse serum+Human hemoglobin	0.145
GPE*+Human hemoglobin+ Mouse serum	0.195†

* The abbriviation of the globin specific protease.

† This activity was obtained by subtracting the mouse serum protease activity from the total activity.

Details of the assay are given in the text and the legend to Table 2.

stable to store at −20 C at least for 60 days, and was completely inactivated by boiling for 20 min. It was therefore, assumed that this factor may be a protein-like substance.

Table 3 clearly shows that the globin specific protease activity increases approximately 2-fold by addition of mouse serum. The activity increased by addition of mouse serum would be refered to as a enhanced activity. As shown in Table 3, it also appeard that mouse serum containes protease(s) which actively split hemoglobin under acidic assay condition. It was therefore, necessary to determine whether this protease take part in the enhancing mechanism of the globin specific protease by mouse serum.

In Fig. 1 are shown the results of the determination of the relationship between the enhanced activity and the mouse serum protease activity under the constant activity of the globin specific protease. The enhanced activity increased proportionally to increase of the activity of mouse serum protease (Fig. 1-A). When the enhanced activity was plotted against the mouse serum protease activity, it was clearly demonstrated that the enhanced activity positively correlates

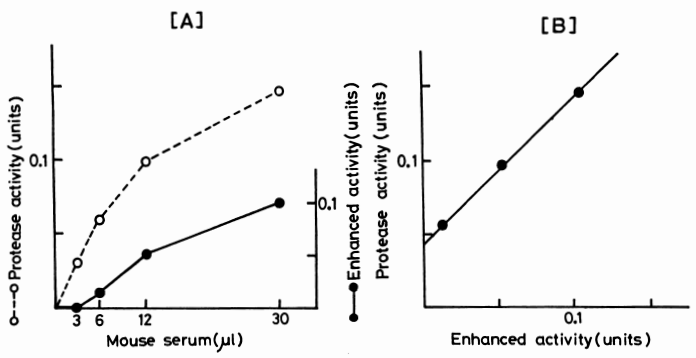

Fig. 1 Correlation between the enhanced activity and the mouse serum protease activity under the constant activity of the globin specific protease.

Details of the assay are given in the legend to Table 2. Dialyzed mouse serum was used as the enhancing factor.

Table 4 Purification of the mouse serum protease

Fraction	Total protein (mg)	Total activity (units)	Specific activity (units/mg protein)	Recovery (%)
Mouse serum (Dialyzed)	543	18.5	0.03	100
Ammonium sulfate (30–50%)	223	15.6	0.07	84
Sephadex G-200	43	13.1	0.31	71
DEAE-Sephadex A-25	1.3	6.4	4.79	35

Details of the assay are given in the text.

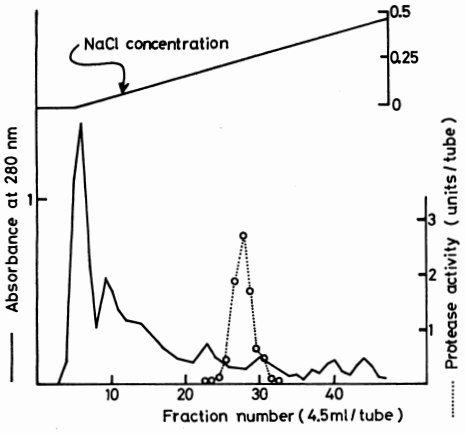

Fig. 2 Elution profile of the mouse serum protease from DEAE-Sephadex A-25 chromatography.

with the protease activity in mouse serum (Fig. 1-B). This indicated that there may be close correlation between the enhancing factor and mouse serum protease. Accordingly, we tried to purify the mouse serum protease in order to further confirmation of this supposition.

Purification procedure of the mouse serum protease is summarized in Table 4. The activity of this enzyme was concentrated in the fractions eluted with about 250 mM sodium chloride from DEAE-Sephadex A-25 chromatography (Fig. 2). The specific activity of the mouse serum protease in this fraction increased approximately 160-fold compared with that of the starting material.

As shown in Fig. 3, the activity of the purified mouse serum protease also exhibited the positive correlation with the enhanced

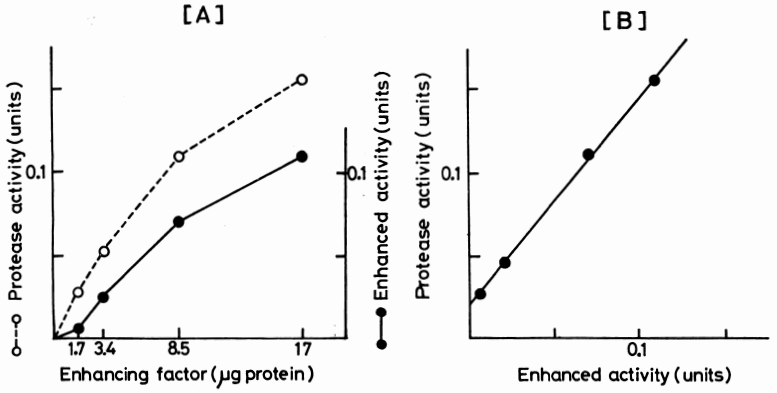

Fig. 3 Correlation between the enhanced activity and the purified mouse serum protease activity under the constant activity of the globin specific protease.

Details of the assay are given in the legend to Table 2. Purified mouse serum protease was used as the enhancing factor.

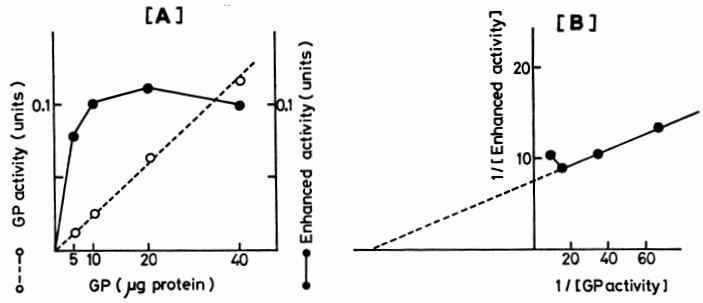

Fig. 4 Relationship between the enhanced activity and the globin specific protease activity under the constant activity of the mouse serum protease.

Details of the assay are given in the legend to Table 2. Purified mouse serum protease was used as the enhancing factor.

activity. This evidence, therefore, indicates that the increase of the globin specific protease activity by mouse serum probably depend on direct or indirect action of mouse serum protease.

In the next place, we also investigated on the determination of the relationship between the globin specific protease and the enhancing factor under the constant activity of the purified mouse serum protease. As shown in Fig. 4-A, the activity of the globin specific protease linearly increased in proportion to its volume. At a low globin specific proteasc activity, the enhanced activity was nearly proportional to the globin specific protease activity. However, as the globin specific protease activity was increased, the enhanced activity increased less, so that it was no longer nearly proportional to the globin specific protease activity. With a further increase in the globin specific protease activity, the enhanced activity became essentially independent of the globin specific protease activity, asymptotically approached a constant rate and gradually decreased at last. When a reciprocal value of the enhanced activity was plotted against that of the globin specific protease activity (Fig. 4-B), a straight line was obtained. This indicates that the enhanced activity is undoubtedly dependent on the globin specific protease activity and that Michaelis-Menten relationship establishes between the enhanced activity and the globin specific protease activity.

Discussion

Present studies clearly demonstrated that addition of mouse serum results in marked increase of the globin specific protease activity in *Schistosoma mansoni*. The enhanced activity positively correlated with the activity of the purified mouse serum protease. This suggests that the mouse serum protease may be the enhancing factor in the mouse serum. Moreover, under the constant activity of the mouse serum protease, Michaelis-Menten relationship established between the enhanced activity and the globin specific protease activity. This relationship may be explained by the idea that the mouse serum protease further hydrolyzes hemoglobin degradation products produced by the globin specific protease (Sauer and Senft, 1972). As shown in Fig. 4-A, the activity of the globin specific protease increased in proportion to its volume, so that hemoglobin degradation product was also produced in proportion to the enzyme volume. If the enhancing factor could hydrolyze the hemoglobin degradation products, the enhanced activity probably depends on the amount of this product, so that Michaelis-Menten relationship may eatablish between the enhanced activity and globin specific protease activity. On the other hand, this relationship suggests that the globin specific protease may cooperate with the mouse serum protease, and that the enhanc-

ing of the globin specific protease activity by mouse serum may be caused by the co-operation of both enzymes. However, it is still unknown why the cooperative reaction of both enzymes results in marked increase in the production of acid-soluble peptides. Details of this mechanism remains to be elucidated.

Schistosome actively ingests red blood cells (Cheever and Weller, 1958; Clegg, 1965; Lawrence, 1973) and utilize the globin moiety of hemoglobin to its protein metabolism (Zussman, 1970), particularly to synthesis of yolk granules within the vitelline gland of the worms (Senft, 1969; Sauer and Senft, 1972). These evidences suggest that the globin specific protease may play an important physiological role for schistosome nutrition. As mentioned above, the present results suggest that mouse serum protease may play a helper effect on the splitting the hemoglobin into acid-soluble peptides by the globin specific protease. To our knowledge, no such a cooperation system has yet been reported for parasitic helminths. However, recent findings indicated that the globin specific protease widely distributed in various parasitic helminths (Aoki and Oya, 1977; Oya and Noguchi, 1977; Sato *et al.*, 1979). Therefore, it is expected that such a co-operation system will be also discovered in other parasitic helminths.

Sauer and Senft (1972) suggested that the globin specific protease probably located in schistosome intestine. Although there was few evidence that schistosome can ingest serum as well as blood cell components into its alimentary canal, it is possible that serum components may be also ingested by this parasite. If it was done, such a cooperation system may be functional *in vivo* as *in vitro*. In general, *S. mansoni* is thought to be a well adapted parasite for human. On the other hand, according to the present experiment addition of human serum markedly decreased the globin specific protease activity (Table 2). This evidence, therefore, probably suggests that such a cooperation system may not be directly associated with parasitic adaptation.

Summary

The activity of the globin specific protease, partially purified from *Schistosoma mansoni*, was markedly increased by addition of mouse serum. The enhancing factor consisted in the mouse serum could not be removed by dialysis and was inactivated with boiling for 20 min. It is, therefore, assumed that the enhancing factor may be a protein-like substance. Mouse serum contained a protease which actively split the hemoglobin under the acidic assay condition, and the protease activity was closely associated with the enhanced activity. In order to determine the relationship between the enhancing factor and the mouse serum protease, mouse serum protease was purified by ammonium sulfate fractionation, Sephadex G-200 gel filtration and DEAE-Sephadex A-25 chromatography. The specific activity of the purified mouse serum protease increased 160-fold compared with the starting material. Under the constant activity of the globin specific protease, the enhanced activity positively correlated with the activity of the purified mouse serum protease. This indicates that mouse serum protease may be the enhancing factor consisted in the mouse serum. Moreover, when under the constant activity of the mouse serum protease the reciprocal value of the enhanced activity was plotted against that of the globin specific protease activity, Michaelis-Menten relationship established between both activities. This probably indicates that the increase of the globin specific protease activity by mouse serum may be caused by the cooperation of the globin specific protease and the mouse serum protease.

Acknowledgements

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マンソン住血吸虫のグロビン特異的蛋白分解酵素について
I. マウス血清プロテアーゼとの協同作用

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マンソン住血吸虫から抽出精製したグロビン特異的蛋白分解酵素にマウス血清を添加すると著明な活性の上昇（増加した活性を enhanced activity とする）が観察された．マウス血清中のこの enhancing factor は，透析で除くことができず，しかも沸騰水中 20 分の処理で完全に失活することから，蛋白質様の物質と推測された．一方，マウス血清中にはヘモグロビンを活発に分解するプロテアーゼが含まれており，この enhancing factor とマウス血清プロテアーゼの関係が注目された．

グロビン特異的蛋白分解酵素活性を一定にした条件下において，血清プロテアーゼ活性は enhanced activity に正の相関を示した．この関係をよりはつきりするために，マウス血清プロテアーゼの精製をこころみた．硫安塩析，Sephadex G-200 ゲル濾過，DEAE-Sephadex A-25 クロマト法により，約 160 倍精製した標品が得られた．この精製したマウス血清プロテアーゼの活性が再度 enhanced activity と正の相関を示したことから，マウス血清プロテアーゼがマウス血清中の enhancing factor であることが強く示唆された．

一方，マウス血清プロテアーゼ活性を一定にした条件下で，グロビン特異的蛋白分解酵素と enhancing factor の関係を調べたところ，両者の活性には Michaelis-Menten の関係が成立することが明らかとなつた．このことは，マウス血清によるグロビン特異的蛋白分解酵素の活性化現象が，本酵素とマウス血清プロテアーゼの協同作用に由来するものであることを示唆しているものと考えられた．