[Jap. J. Parasit., Vol. 32, No. 6, 577-586, December, 1983]

Studies on an Acid Protease from *Dirofilaria immitis*

KUMIKO SATO* AND MAMORU SUZUKI†

(Received for publication; October 5, 1983)

Key words: filariae, *Dirofilaria immitis*, acid protease, globin specific protease, enzyme characterization

Introduction

Previous investigators suggested that the growth of some parasitic helminths in the blood vascular system was associated with the degradation of host-cell hemoglobin. The findings attracted attentions of parasitologists as one of the mechanism whereby parasites digest host cell components. An initial approach to this problem was carried out using *Schistosoma mansoni*. Rogers (1940) described that *S. mansoni* might incorporated ingested hemoglobin (Hb) for the bulk of their amino acids because material resembling hematin was found in the guts of adult worms. Timmus and Beuding (1959) demonstrated that these phenomenon was presumably involved in the presence of proteolytic enzyme in ground-up preparations of worms, and the enzyme hydrolyzed specifically human and bovine Hb. Partial purification and characterization of the schistosoma enzyme was reported by Grant and Senft (1971), and Sauer and Senft (1972). On the other hand, Nimmo-Smith and Keeling (1960) extracted an acid protease from *Trichuris muris* and characterized the enzyme. Recently, the existence of the similar enzyme was comfirmed not only in blood helminths such as *Angiostrongylus cantonensis* and *Dirofilaria immitis*, but also in organ living helminths such as *Paragonimus* sp., *Clonorchis sinensis* and also intestinal helminths such as *Ascaris suum*, and *Ancylostoma caninum* (Aoki and Oya, 1977; Sato *et al.*, 1976a, 1976b; Howell, 1973; Maki *et al.*, 1982; Juhasz, 1979; Oya and Noguchi, 1977). Since 1975, we have noted the presence of an acid protease in *D. immitis*. To date, partial purification and characterization of the enzyme were attempted, and intracellular localization of the enzyme in the worm was studied. The series of preliminary studies on the enzyme have been already reported (Sato *et al.*, 1976a, 1976b, 1978, 1979). Using partially purified materials, present paper deals with some physicochemical properties and enzymatic characters of protease of *D. immitis*.

This study was partly supported through funds provided by U.S.-Japan Cooperative Science Program and by Grant No. 237017 of a Cooperative Research (A) from the Ministry of Education, Japan.

* *College of Medical Care and Technology, Gunma University.*

† *Department of Parasitology, Gunma University School of Medicine.*

Materials and Methods

MATERIALS:

Adult *D. immitis* were collected from the pulmonary arteries and hearts of dogs and washed twice with water and finally with distilled water. They were lyophilized and stored at −20 C until use. Human γ-

globulin, egg albumin and azocasein were purchased from INC Pharmaceuticals, USA. Equine myoglobin, edestin, diazoacetyl-DL-norleucine methyl-ester (DAN), α-chymotrypsinogen and human crystallized Hb were Sigma products. Human Hb prepared from blood as described by Drabkin (1949) was used in some part of the experiment. N-α-tosyl-L-lysyl-chloromethane hydrochloride was obtained from Calbio Chem. and 1, 2-epoxy-3-(P-nitrophenoxy) propane (EPOXYD) was obtained from Eastman Company. P-tosyl-L-lysyl-chloromethyl ketone, p-tosylphenylalanine chloromethyl ketone and p-chloromercuribenzoic acid were purchased from Nakarai Co., Japan, monoiodoacetic acid, from Wako Pure Chemicals and diethyl dithiocarbamate (DEDTC), from Kanto Chemicals. Pepstatin, chymostatin, leupeptin, antipain and the other antibiotic inhibitors were kindly donated by Dr. T. Takeuchi, Institute of Microbial Chemistry, Tokyo, Japan. Sephadex G-200, G-100 and Blue dextran 2000 were purchased from Pharmacia. CM-cellulose was a product of Brown Co., USA.

METHODS:

Enzyme assay: The assay routinly used was modification of the method of Sauer and Senft (1972). Each 5 mg of various substrates was dissolved into 1 ml of 0.1 M citrate-0.2 M Na_2HPO_4 buffer, pH 3.2, and incubated with 0.5 ml of enzyme solution dissolved into the same buffer at 37 C for one hour if not otherwise indicated. The reaction was stopped by adding 1.5 ml of cold 10% trichloroacetic acid solution, to give final 5% concentration. After standing in an ice bath for 30 min, precipitated protein was removed by centrifugation at 3,000 rpm for 15 min. Enzyme activity was determined spectrophotometorically by measuring the absorption at 280 nm on the supernatant. One unit of proteolytic activity was defined as the amount of the absorption rate through a 1 cm light path at 280 nm which 5 mg of human Hb was hydrolyzed for 1 hour under the indicated conditions. Specific activity refered to units of proteolytic activity per mg protein in the enzyme fraction. Occasionally, 0.5 ml of the supernatant was measured by the method of Lowry *et al.* (1951) as mentioned below.

Preparation of crude enzyme: Twelve g of lyophilized *D. immitis* worms were cut finely with scissors, suspended into 500 ml of 0.1 M acetate buffer, pH 4.0 and kept at 4 C overnight. The prepared material was homogenized with Cell Dispertor 200, Branson for 15 min. The sonicated specimen was filtered through gauze to eliminate large scum and then centrifuged at 13,000 g at 4 C for 30 min. The resulting supernatant used as a crude enzyme fraction. Acetate buffer was selected according to preliminary test on the efficiencies to extract protein from the worm.

Partial purification procedure of acid protease: The purification procedure are illustrated in Fig. 1. The crude enzyme

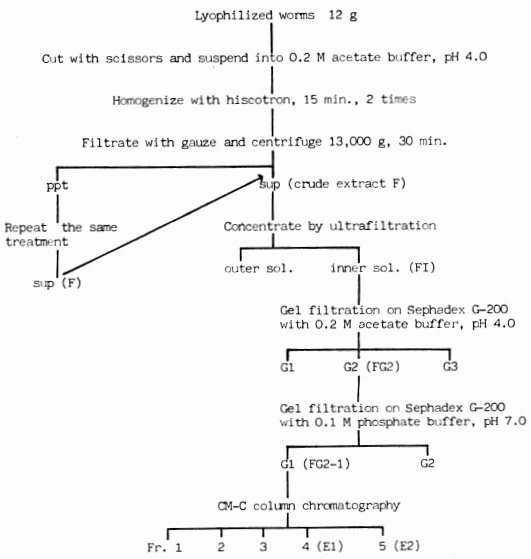

Fig. 1 Purification steps of acid proteases from *Dirofilaria immitis*.

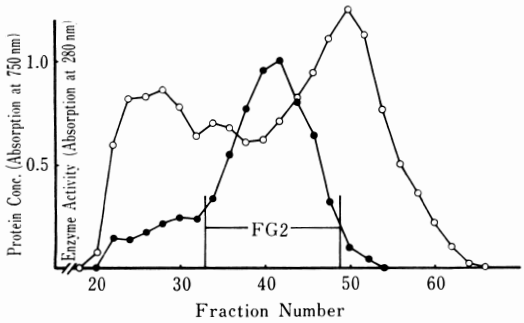

Fig. 2 Gel exclusion chromatography of the crude inner solution FI. The sample reduced in volume to 18 ml, containing approximately 741.8 mg of protein and 1,617.1 units of enzyme activity was applied to a column (2.5×55 cm) of Sephadex G-200 which was equilibrated with 0.2 M acetate buffer, pH 4.0, at a rate of 12 ml per hour. After the sample passed into the gel, elution was continued with the same buffer at a flow rate of 20 ml/hour. The effluent was divided into fractions about 7 ml each. Thirty μl of each fraction was tested for protein concentration and 50 μl of the same fraction was assayed for enzyme activity.
Protein conc. —○— Enzyme activity —●—

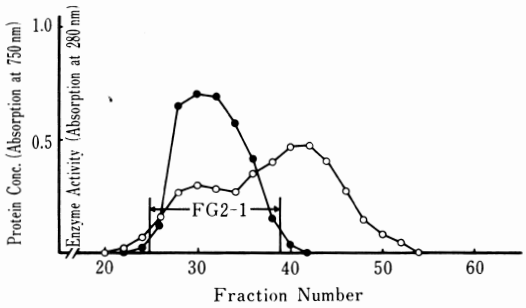

Fig. 3 Rechromatography of the enzyme fraction FG2 on Sephadex G-200. The concentrated and dialyzed enzyme fraction FG2 which contained 131.1 mg of protein and 1,200 unit of enzyme activity in 12.5 ml was applied to a column (2.5×55 cm) of Sephadex G-200 equilibrating with 0.1 M phosphate buffer, pH 7.0, at a rate of 12 ml per hour. After the sample passed into the gel, elution was continued with the same buffer at a flow rate of 20 ml per hour. The effluent was divided into fractions about 7 ml each. Twenty μl of each fraction was tested for protein concentration, and 50 μl of the same fraction was assayed for enzyme activity.
Protein conc. —○— Enzyme activity —●—

fraction was first passed through a Sephadex G-200 column in 0.2 M acetate buffer, pH 4.0 as shown in Fig. 2. Fraction FG2, having enzyme activity was collected and concentrated by ultrafiltration with Visking cellulose tubing 8/32. The fraction FG2 was dialyzed against 0.1 M phosphate buffer, pH 7.0 stirring at 4 C overnight and rechromatographed on a Sephadex G-200 column in 0.1 M phosphate buffer, pH 7.0. Effluent diagram was shown in Fig. 3. The fraction, FG2–1, having enzyme activity was collected again and dialyzed against 0.01 M acetate buffer, pH 4.0 at 4 C for 15 hours, and then the fraction FG2–1 was charged onto CM-cellulose column, equilibrated with 0.01 M acetate buffer, pH 4.0. As shown in Fig. 4, two peaks, E1 and E2, showing enzyme activity were obtained by eluting from the column

Table 1 Summary of purification of the enzyme from 12 g of lyophilized *Dirofilaria immitis* worms

Fraction	Total Protein (mg)	Total Activity (unit)	Specific Activity (unit/mg pro.)	Yield (%)	Purification (fold)
Crude extract F	1,444.8	1,820	1.26	100	1.0
Inner solution FI	741.8	1,617	2.18	88.8	1.7
FG2	131.1	1,200	9.20	65.9	7.3
FG2-1	92.5	1,110	12.00	61.0	9.5
CM-C, E1	6.1	488	80.00	26.8	63.5
CM-C, E2	5.5	358	64.00	19.7	50.8

Unit was calculated as described under "Methods".

with a slight step-wise increase of sodium ion. Details of purification data is shown in the results.

Analytical methods: Protein content was measured according to the method described by Lowry *et al.* (1951). The standard curve was prepared using BSA. Hb content was measured by cyanmethohemoglobin method described by Drabkin (1936, 1949). Extinction of cyanmethohemoglobin at 540 nm was measured and the concentration of Hb was calculated by the following formula; $\Delta E_{540} \times 0.146 \times$ dilution = Hb concentration (g/dl).

Estimation of molecular weight: Molecular weight of enzyme was determined in the chromatography on Sephadex G-200 column (column size : 1.5 × 60 cm) in 0.2 M acetate buffer, pH 4.0 and 0.1 M phosphate buffer, pH 7.0 following the method described by Andrews (1964).

Results

The yield and specific activity of the fraction obtained in each purification steps are shown in Table 1. The two enzyme fractions, E1 and E2 purified to 64-fold and 51-fold respectively were obtained from the final purification step with CM-cellulose column chromatography. The recovery rate of these fractions was 46.5% of the

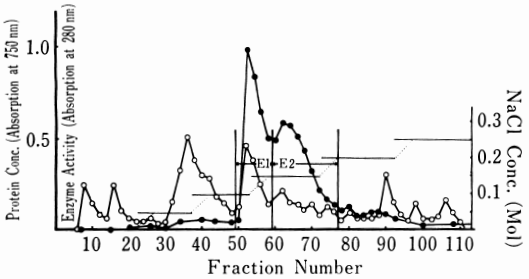

Fig. 4 Ion exchange chromatography on CM-cellulose column of the gel-chromatographed enzyme fraction FG2-1. The column (1.5×45 cm) was equilibrated with 0.01 M acetate buffer, pH 4.0. The sample FG2-1 was dialyzed against the same buffer and applied to the column. The column was washed with 100 ml of equilibrating buffer and then eluted with a 400 ml step-wise increase of NaCl (0 to 0.25 M) in the same buffer. Each 5 ml of effluent was collected. Fifty μl of each fraction was tested for protein concentration and 100 μl of the same fraction was assayed for enzyme activity.
Protein conc. —○— Enzyme activity —●—

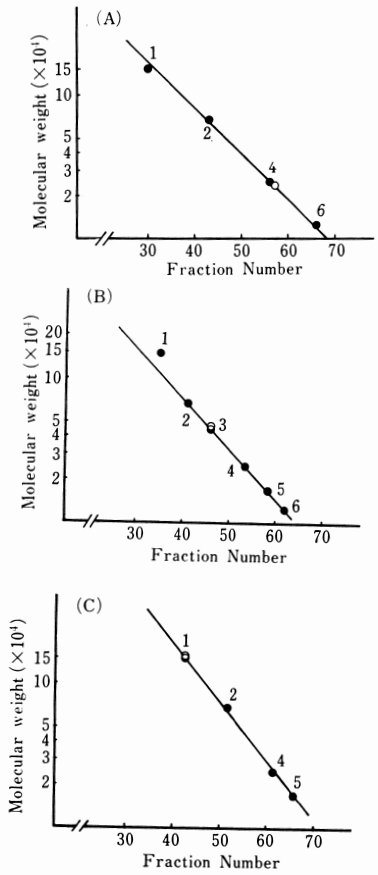

Fig. 5 Estimation of molecular weight of the enzyme E1 and E2 by gel chromatography. Proteins used as references were 1; γ-globulin, 2; bovine serum albumin (monomer), 3; egg albumin, 4; α-chymotrypsinogen A, 5; myoglobin, 6; cytochrome C. The open circle represents the E1 and E2. (A) Sephadex G-100 column, 0.2 M acetate buffer, pH 4.0. (B) Sephadex G-100 column, 0.1 M phosphate buffer; pH 7.0. (C) Sephadex G-200 column, 0.1 M phosphate buffer; pH 7.0 the enzyme fractions were dialyzed against the buffer for 7 days before gel chromatography.

Table 2 Hydrolysis rate of various substrates purified enzymes E1 and E2

Substrate	Hydrolysis rate by E1		Hydrolysis rate by E2	
	280 nm	Lowry	280 nm	Lowry
Human crys. hemoglobin	0. 495	0. 280	0. 425	0. 245
Human hemoglobin	0. 460	0. 256	0. 398	0. 230
Myoglobin	0. 780	0. 340	0. 634	0. 290
γ-globurin	0. 026	0. 020	0. 002	0. 000
Human serum protein	0. 080	0. 155	0. 068	0. 134
Bovine serum albumin	0. 060	0. 120	0. 044	0. 115
Ovalbumin (pH 3. 8)	0. 000	0. 024	0. 000	0. 006
Edestin (pH 3. 8)	0. 004	0. 014	0. 012	0. 010

The reaction mixture consisted of 1. 4 ml of 0. 1 M citrate—0. 2 M Na_2HPO_4 buffer containing 5 mg of the substrate, pH 3. 2 and 0. 1 ml of enzyme. Enzyme assay was carried out as described under "Methods". The hydrolysis rate was measured by extinction at 280 nm compared with results from Lowry's method.

Table 3 Effect of various compounds on the activity of purified enzymes E1 and E2

Compound	Concentration	Relative activity	
		E1	E2
None	(10^{-3}M)	100. 0%	100. 0%
Diazoacetyl-DL-Norleucine Methylester	1	100. 0	100. 0
1, 2-Epoxy-3-(p-nitro-phenoxy) Propane	1	102. 0	95. 5
P-Tosyl-L-Lysine Chloromehtyl Ketone Hydrochloride	1	100. 0	105. 0
P-Tosylphenylalanine Chloromethyl Ketone	1	98. 5	105. 0
N-α-Tosyl-L-Lysyl-Chloromethane Hydrochloride	1	100. 0	103. 5
P-Chloromercurobenzoate	1	98. 0	95. 0
Monoiodoacetic Acid	1	100. 0	103. 5
Diethyl Dithiocarbamate	1	105. 0	103. 0
Pepstatin	10 ng/ml	0. 0	0. 0
Chymostatin	20 ng/ml	100. 0	101. 0
Antipain	20 ng/ml	101. 5	100. 0
Leupeptin	20 ng/ml	100. 0	101. 0
Elastatinal	20 ng/ml	100. 0	98. 0
Phosphoramidon	20 ng/ml	102. 0	98. 5

enzyme activity of the starting material. The obtained E1 and E2 enzyme fractions were concurrently studied as follows.

Molecular weights of E1 and E2 were determined on Sephadex G-200 column as described in Materials and Methods. Fig. 5 shows the elution position during gel chromatography of the two enzyme fractions relative to several protein standards when the chromatography was carried out using 0.1 M acetate buffer, pH 4.0. Both E1 and E2 enzymes eluted at the same position around standard chymotrypsinogen (Mr. 25,000) (Fig. 5-A). Assuming that these enzymes formed globular protein, a molecular weight of about 23,000 was estimated from the elution pattern. When the gel chromatography of E1 and E2

enzymes were carried out in 0.1 M phosphate buffer, pH 7.0 after being dialyzed against the same buffer for 15 hours, the enzyme activities were found in the eluate in the same region as egg albumin (Mr. 45,000) (Fig. 5-B). Occasionally, enzymes were eluted at the region representing molecular weight of 68,000 shown by BSA. The same phosphate buffer at pH 7.0 was used as the solvent of the enzyme after being stored in a refrigerator for 7 days, the specimens were subjected to the determination of molecular weight by gel chromatography using the same column with γ-globulin reference (Fig. 5-C). As large as 150,000 molecular weight was estimated under the condition. However the difference in molecular weight between E1 and E2 was not remarked if they were kept under the same condition.

The optimal pH of purified E1 and E2 enzymes were examined using 0.1 M citrate-0.2 M Na_2HPO_4 buffer at various pH. Both of the pH profiles showed sluggish curve; Optimal pH was ranged 2.5–4.0, hence no difinite difference between optimal pH of E1 and E2 was noted (Fig. 6).

Kinetic studies of E1 and E2 enzymes were carried out and Km value were calculated from Lineweaver-Burk plot. The apparent Km value for human Hb hydrolysis was about 0.02 mM with the enzyme E1 and about 0.15 mM with the enzyme E2. The Km values of E1 and E2 were close each other, indicating high affinities to the substrate of both enzymes.

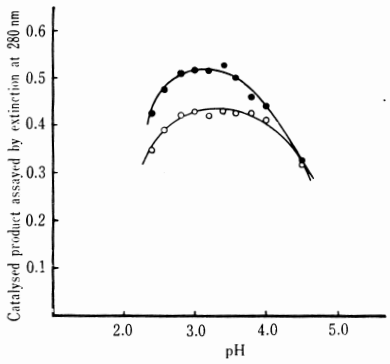

Fig. 6 Effect of pH on activity of purified enzymes E1 (—●—) and E2 (—○—) for human crystallized hemoglobin. The buffer was 0.1 M citrate–0.2 M disodium hydrogenphosphate to the indicate pH. The pH of the reaction mixture after incubation was measured with a glass electrode. The other conditions were as described for the standard assay.

The results of substrate specificity studies using E1 and E2 were summarized in Table 2. Both enzymes hydrolyzed these substrates at the similar rate. They showed a marked activity to hydrolyze crystallized human Hb, human Hb and myoglobin. The enzymes displayed very limited hydrolysing activity to serum proteins, so far as hydrosis rate was measured by the absorption at 280 nm. When Lowry's method was employed for the measurement of enzyme products, however, the enzymes hydrolyzed 40–50% BSA and human serum protein against 100% decomposition for reference human Hb. Both E1 and E2 were inactive to egg albumin and edestin when assayed by spectrophotometic measurements and by Lowry's method. Thus, difference in the substrate specificities of E1 and E2 were not observed.

Inhibition experiments are shown in Table 3. Both E1 and E2 enzymes were completely inhibited by pepstatin at 10^{-6}M. The other peptide aldehyde protease inhibitors, chymostatin, antipain, leupeptin, elastatinal and phosphoramidon were rather ineffective comparing to the effects by pepstatin. On the other hand, the enzymes were not largely affected by various organic compounds, p-chrolomercuribenzoate, p-tosyl-L-lysine chloromethyl ketone hydrochloride, p-tosylphenylalanine chloromethyl ketone, N-α-tosyl-L-lysyl-chrolomethan hydrochloride, monoiodoacetic acid and diethyl dithiocarbamate at the concentration of 10^{-4}M and 10^{-3}M. Diazoacetyl-DL-norleucine methylester and 1, 2-epoxy-3-(p-nitrophenoxy) propane which were potent inhibitors of pepsin showed

no remarkable effect to the reacting enzymes, nor did p-chloromerculibenzoate to E2 at 10^{-3}M.

Discussion

In the present results two enzyme fractions, E1 and E2 were resulted from the final purification steps by CM-cellulose column chromatography. Distinctive elution of E1 and E2 was achieved by a slight stepwise increases of sodium ion in the buffer. Two enzymes manifested features similar to each other in terms of molecular weight, substrate specificity and response to inhibitors, still showing different elution peaks. The results suggested that the enzyme built up a multiple forms in the worm. Aoki and Oya (1978) carried out disc electrophoresis of the partially purified enzymes from *Fasciola hepatica* and *A. cantonensis* using layered Hb as an indicator substrate. The results strongly suggested the presence of multiple forms of the enzyme sharing common function and forms. Introduction of such kind of enzyme technology will clarify difference of two types of the resulted enzymes in the present study.

Physico-chemical investigation on the acid protease suggested that the enzyme unit easily polymerized. The findings would allow a speculation that the enzyme units polymerized each other under respective conditions. If the speculation is true with other helminth enzymes, it would be well explained that different molecular weights of helminth acid protease were proposed by different workers.

The enzyme activity of the present acid protease was remarkably affected by pepstatin as reported by us previously (Sato *et al.*, 1978). Recent publication by Sreedhara Swamy and Jaffe (1983) has reported the presence of two acid proteases from *D. immitis* adult worms. The paper also supports our finding that one of the enzyme is comparable to cathepsin D regarding the sensitivity to the inhibiting activity by pepstatin. While the enzyme was not affected by other inhibitors from actinomycetes, nor by several organic compounds. Generally, proteases are classified into 4 categories according to the difference of catalytic residues of the active site, namely thiol, carboxyl, metallo and serine proteases. It will be soundly justified to categorize *D. immitis* protease in carboxyl protease from the results of inhibition experiments. Maki *et al.* (1982) presented that the acid proteases found in *A. cantonensis, D. immitis, T. muris, A. suum, Paramphistomum* sp., *Diphyllobothrium erinacei* and *Hymenolepis nana* belonged to the carboxyl proteases. Levy *et al.* (1974) also reported the exsistence of the enzyme similar to cathepsin D in *Plasmodium falciparum, P. kowlesi* and *P. berghei* affected strongly by pepstatin. Rūpova and Keilova (1979) reported that the acid protease in *F. hepatica* might be inclusive in the group of carboxyl proteases. On the other hand, enzymes of thiol protease group was found in *S. mansoni* and *Trichomonas foetus.* (McLaughlin and Müller, 1979; Dresden and Deelder, 1979). We also found that the protease purified from *C. sinensis* was grouped in the thiol protease being sensitive to sulfhydryl reagent and insensitive to pepstatin (Sato *et al.*, 1980). Such informations on parasite proteases will provide baseline data for the new approach to helminth evolution in terms of comparative biochemistry.

The *D. immitis* enzyme hydrolyzed BSA and human serum proteins although hydrolyzing rates of these proteins were about 50% of that given by human Hb. The role of this enzyme in the protein metablism of *D. immitis* has not yet been defined. Our previous study showed the highest level of enzyme activity in the soluble fraction of the whole worm homogenate by percoll density gradient centrifu-

gation (Sato *et al.*, 1979). Usually, black pigment can be seen in the upper part of worm's intestine. The findings might suggest that the protease secreted into parasites intestine hydrolyzing host's materials in the gut.

Summary

An acid protease in *Dirofilaria immitis* worm was studied. Two purified fractions from worm extract were obtained as a result of gel filtration and CM-cellulose ion exchange chromatography. The eluates of the two distinct fractions showed features similar to each other in terms of molecular weight, substrate specificity and response to inhibitors. The fact suggested that the enzyme built up a multiple form. Details of the studies were as follows.

(1) Molecular weights ranging 23,000–150,000 were calculated by the results from gel filtration according to different kinds of used buffers. The results suggested that the molecular weight of essential unit of enzyme was 23,000 and the unit enzyme tended to be polymerized in a reversible way in different buffer conditions.

(2) The enzyme showed specifically high hydrolysing activity to human Hb and myoglobin. The enzyme hydrolysed 40–50% BSA and human serum protein against 100% hydrolysis for reference human Hb.

(3) The enzyme activity was completely inhibited by pepstatin at 10^{-6} M, which is the potent inhibitor against mammalian cathepsin D. Therefore, the *D. immitis* protease seemed to be grouped in carboxyl protease.

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犬糸状虫の酸性プロテアーゼの研究

佐藤久美子
(群馬大学医療技術短期大学部)
鈴木　守
(群馬大学医学部寄生虫学教室)

種々の寄生虫虫体において酸性域で蛋白質特にヘモグロビンをよく分解する酵素の存在が知られているが，著者らは住血性の線虫，犬糸状虫の酸性プロテアーゼについて部分精製を行い，2，3 の物理化学的および酵素学的性状について検討した．まず虫体粗抽出液からセファデックス G-200 による二度のゲル沪過および CM-セルローズカラムクロマトグラフィーによって酵素を精製し，E1，E2 の二酵素分画を得た．酵素学的性状の検討では両分画の間に顕著な差が認められなかったが，CM-セルローゼカラムクロマトグラフィーの結果から multiple form である可能性が考えられた．以下に検討結果をまとめた．

1)　本酵素の分子量をゲル沪過法によって検討したところ，緩衝液の種類によって変化することがわかった．すなわち酢酸バッファー，pH 4.0 中では分子量，約23,000であり，燐酸バッファー，pH 7.0 中では貯蔵や塩濃度などの条件によって，約45,000，68,000，150,000 と変化した．これらの結果は酵素分子が条件によって容易に解離あるいは重合する可能性を示唆するものと考える．

2)　本酵素の基質特異性を検討したところ，ヒトヘモグロビンやミオグロビンを特異的に分解したが，BSA やヒト血清蛋白もヘモグロビン分解量の約50%の割合で分解した．

3)　本酵素の活性は微量のペプスタチンによって阻害された．ペプスタチンは哺乳類の代表的なカルボキシルプロテアーゼであるカテプシンDの典型的な阻害剤であり，本酵素もカテプシンDのタイプに属すると考えられた．