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Characterization of proteases of *Toxoplasma gondii**

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Abstract: The proteases of *Toxoplasma gondii* were purified partially and characterized for some biochemical properties including various chromatographic patterns, major catalytic classes, and conditions to promote the activity of these enzymes. When *Toxoplasma* extract was incubated with ^{3}H-casein at various pH, peak hydrolysis of casein was observed at pH 6.0 and at pH 8.5. Proteases working at pH 6.0 and at pH 8.5 were purified partially by conventional methods of chromatographies of DE52 anion exchange, Sephadex G-200 gel permeation, and hydroxylapatite chromatography. Partially purified enzymes were tested by site-specific inhibitors and promotors. The protease working at pH 6.0 was inactivated by iodoacetamide with LD_{50} of 10^{-3} M and promoted by dithiothreitol, while the protease working at pH 8.5 was inhibited by phenylmethylsulfonyl fluoride with LD_{50} of 10^{-5} M and was promoted by ATP (excess ATP beyond 2 mM inhibited the activity reversely). The protease of pH 8.5 had the activity of ATPase which might exert the energy to its action. Therefore the former was referred to as a cysteinyl acid protease and the latter, ATP-dependent neutral serine protease.

Key words: *Toxoplasma gondii*, chromatography, purification, cysteinyl acid protease, ATP-dependent neutral serine protease

INTRODUCTION

Toxoplasma gondii is an obligatory intracellular protozoan parasite that infects mammals and birds with very low specificity (Levine, 1977; Choi *et al.*, 1987) and can cause abortion, fetal abnormalities or prenatal death(Krick and Remington, 1978). Recently, there have been many reports on this parasite which increased the morbidity and mortality of the immune compromised patients such as acquired immune deficiency syndrome(AIDS) (Anderson *et al.*, 1983; Luft *et al.*, 1984).

In recent years there have been increased interests in the proteinases of protozoan parasites and the parts they might play in the parasitic way of life, the infection of their hosts, the pathology of the diseases they cause, and the nature of protease itself. In respect to the parasitic way of life, cysteinyl protease from amastigote form of *Leishmania mexicana* was suggested to play a crucial role in the survival of the parasite within the host macrophage (Coombs, 1982; Pupkis *et al.*, 1986) and cysteinyl protease of *Trypanosoma cruzi* was also suggested to exert on sustained growth, differentiation, or metabolism of this parasite (Rangel *et al.*, 1981). When the infection of their host cells was concerned, it was suggested that the role of proteases in the entry mechanism, for example, proteolytic step in the course of invasion of *Plasmodium falciparum*(Dluzewski *et al.*, 1986), *P. berghei*(Bernard and Schrevel, 1987) and *L. amazonensis*(Alfieri *et al.*, 1989),

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Some proteases were supposed to be involved in tissue damage of the host. Cysteinyl protease of *Entamoeba histolytica* was correlated with virulence of this parasite to host(Gadasi and Kobiler, 1983; Lushbaugh *et al.*, 1985) and neutral aminoacyl-peptide hydrolase of *Naegleria fowleri* was described in the context of potential cytolytic factors(Marciano-Cabral *et al.*, 1987). Finally, some proteases were characterized in various kinds of parasitic protozoa. Letch and Gibson(1981) reported the peptidase of bloodstream trypanosome of *T. brucei*, Rautenberg *et al.*(1982) characterized the proteolytic enzymes from *T. congolense*, and Coombs and North(1983) described the cysteinyl proteinases of *Trichomonas vaginalis*. Perez-Montfort *et al.*(1987) characterized the catalytic classes of proteinases of *E. histolytica* to be cysteinyl proteases, and Lindmark(1988) found the localization of *Giardia lamblia* in lysosome-like organelles of trophozoites.

Our knowledge about *Toxoplasma* proteases, however, is limited. In this report we purified partially the proteases of *Toxoplasma* and characterized some biochemical properties including various chromatographic patterns, major catalytic classes and conditions promoting the activity of these enzymes. These basic data would offer a way to elucidate the physiological functions of these proteases, locality of these proteases in *Toxoplasma*, and the roles in interactions between host cell and intracellular parasitic protozoa.

MATERIALS AND METHODS

Parasites: Virulent tachyzoites of RH strain of *Toxoplasma* were maintained and propagated by continuous passages in the peritoneal cavity of ICR mice. Tachyzoites were purified from mouse peritoneal exudate by the method of Choi *et al.*(1988). Briefly, peritoneal exudate of mouse infected with tachyzoites four days earlier was saved and incubated with 1×10^{-5} M formyl-methionyl-leucyl-phenylalanine (FMLP) for 15 min at 37°C, and centrifuged over 40% Percoll(Pharmacia Fine Chemicals) density gradient at 2,000 rpm(Beckman, TJ-6) for 30 min. The precipitates were saved, washed with phosphate buffered saline(PBS) for 3 times, and frozen to −70°C until used.

Preparation of ^{3}H-labelled substrate: ^{3}H-formalin (5 mCi) was purchased from New England Nuclear Co. Reductive methylation of casein was performed according to the method of McKeen *et al.*(1979) with some modifications. Five mCi of ^{3}H-formalin was added to 250 μl of 1 mg/ml solution of casein in 40 mM potassium phosphate buffer, pH 7.0, followed by the addition of 1 ml of freshly prepared solution of $NaBH_3CN$ (6 mg/ml in 40 mM phosphate buffer, pH 7.0). The reaction mixture was incubated at 25°C for 1 hr with shaking at 15 min interval. The volume of the reaction mixture was increased to 10 ml by adding 40 mM phosphate buffer, pH 7.0 and the low molecular weight components were removed by dialysis against distilled water for 16 hr at 4°C.

Assay of protease activity: Protease activity was measured according to the method of Barrett(1977). Reaction mixture consisted of 10 μl of ^{3}H-casein(1 mg/ml), 50 μl of acetate (100 mM, pH 6.0) or Tris-HCl(100 mM, pH 8.5), 10 μl of enzyme, 10 μl of ATP(20 mM) in the case of Tris buffer, and was adjusted to 100 μl with distilled water. The reaction mixture was incubated for 90 min at 37°C. After cooling to 0°C, 50 μl of 10 mg/ml bovine serum albumin (BSA) and 50 μl of 40% trichloroacetic acid (TCA) were added. It was incubated for 10 min at 0°C, then centrifuged at 13,000 rpm for 5 min. One hundred μl of supernatant was saved, and the radioactivity was calculated as the percentage conversion of the radiolabelled casein to the 10% TCA acid-soluble products. Assays were run in duplicate and the data were expressed as the mean percentage in cpm. Background cpm was obtained from incubation of casein alone.

Protein concentration: Concentration of protein was estimated by the absorbance at 280 nm(Packard Spectrometer), using BSA as a

standard.

Purification of proteases: Frozen *Toxoplasma* tachyzoites were thawed and homogenized with a Dismembrator(Fisher Co.) in 10 mM Tris-HCl, pH 7.4. Extracts were centrifuged for 1 hr at 15,000 g. First, the supernatant was absorbed to DE52(Whattman Chem. Co.)(2.5 $\phi \times 40$ cm), washed extensively with the above buffer, and then eluted with 1,200 ml of linear salt gradient made by a gradient mixer(Hoefer Sci. Ins.) from 0 to 0.4 M NaCl. Two peak fractions which showed high caseinolytic activity were rescued, and then treated separately in the next fractionation steps. Fractions obtained from DE52 chromatography were dialyzed overnight against 10 mM Tris-HCl, pH 7.4, and subjected to gel permeation with Sephadex G-200(Pharmacia Fine Chemicals) ($2.5\phi \times 90$ cm). Then the fractions of proteolytic activity were pooled, dialyzed against 10 mM phosphate buffer, pH 7.4, and absorbed to hydroxylapatite column (Whattman Chem. Co.) ($2.5\phi \times 10$ cm for pH 6.0 peak and $2.5\phi \times 20$ cm for pH 8.5 fractions). Proteins were eluted with 200 ml for pH 6.0 and 400 ml for pH 8.5 by phosphate gradient of 0.01 M to 0.2 M, respectively.

Protease inhibitors and promotors: Phenylmethylsulfonyl fluoride (PMSF), iodoacetamide (IAA), Pepstatin A(Pep A), ethylenediaminetetraacetic acid(EDTA), dithiothreitol(DTT), and ATP were purchased from Sigma Chem. Co. Inhibition or promotion of caseinolytic activity was determined with various concentrations of the above mentioned reagents. Assays were run in duplicate and the data were expressed as the mean percentage of decrease or increase in cpm compared to an inhibitor-free or promotor-free control.

RESULTS

To detect the presence of a variety of proteolytic enzymes, *Toxoplasma* was homogenized in 10 mM Tris-HCl, pH 7.4. Extracts were assayed on the general proteolytic enzyme substrate, casein.

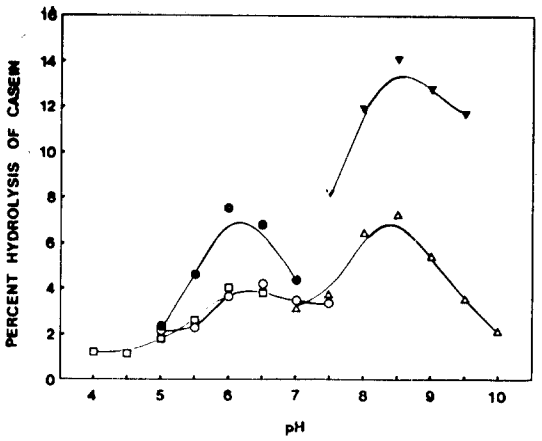

Fig. 1. Effects of pH on the caseinolytic activity of *Toxoplasma* extract. Proteolytic activity was tested at various pH with casein in 50 mM citrate buffer from pH 4.0 to 6.5 (—□—), acetate buffer from pH 5.0 to 7.5 (—○—), and Tris buffer from pH 7.0 to 10.0(—△—). The effects of DTT(—■—) and ATP (—▼—) was also tested with the above buffers.

The effect of pH on *Toxoplasma* proteolytic activity was shown in Fig. 1. Peak hydrolysis of casein was observed at pH 6.0(acetate buffer) and at pH 8.5(Tris buffer) with significant levels of proteolysis between pH 5.0 and pH 9.5. Therefore, proteolytic peak which formed at pH 6.0 was referred to as an acid protease and that at pH 8.5 was referred to as a neutral protease. The acid protease activity was elevated by the addition of DTT and the neutral pro-

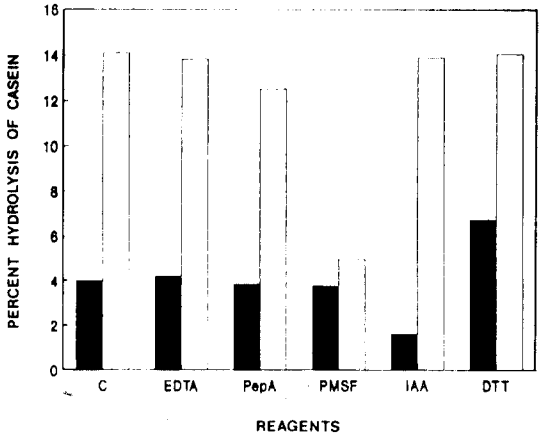

Fig. 2. Effects of protease inhibitors on *Toxoplasma* extract at pH 6.0(■) and pH 8.5(□).

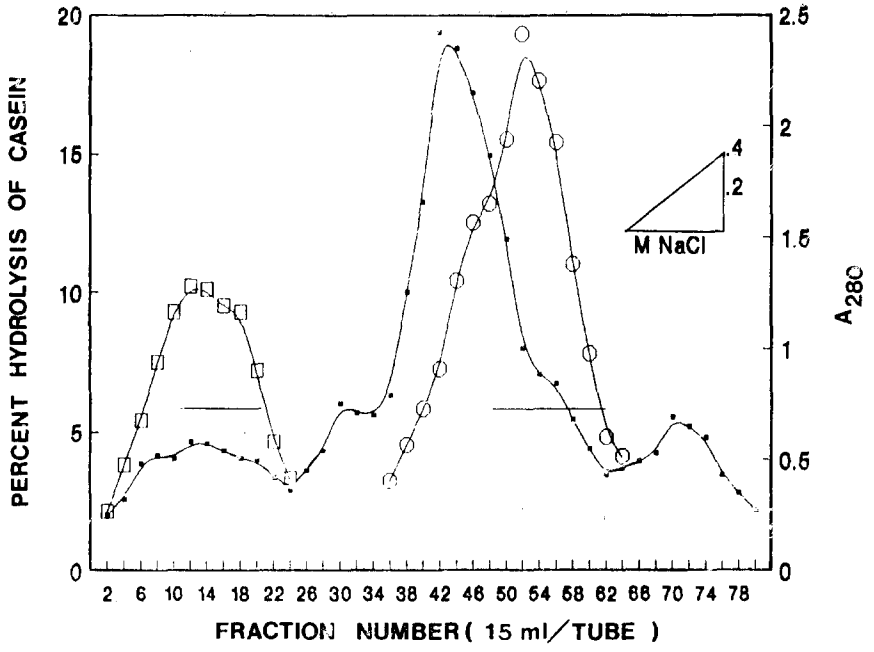

Fig. 3. DE52 chromatography of *Toxoplasma* extract. *Toxoplasma* crude extract was loaded on a DE52 column and eluted with salt gradient of 0 to 0.4 M NaCl in 10 mM Tris buffer, pH 7.4. Salt concentration gradient was indicated by a triangle. (—□—, at pH 6.0; —○—, at pH 8.5; —■—, A_{280}; and —, saved fractions)

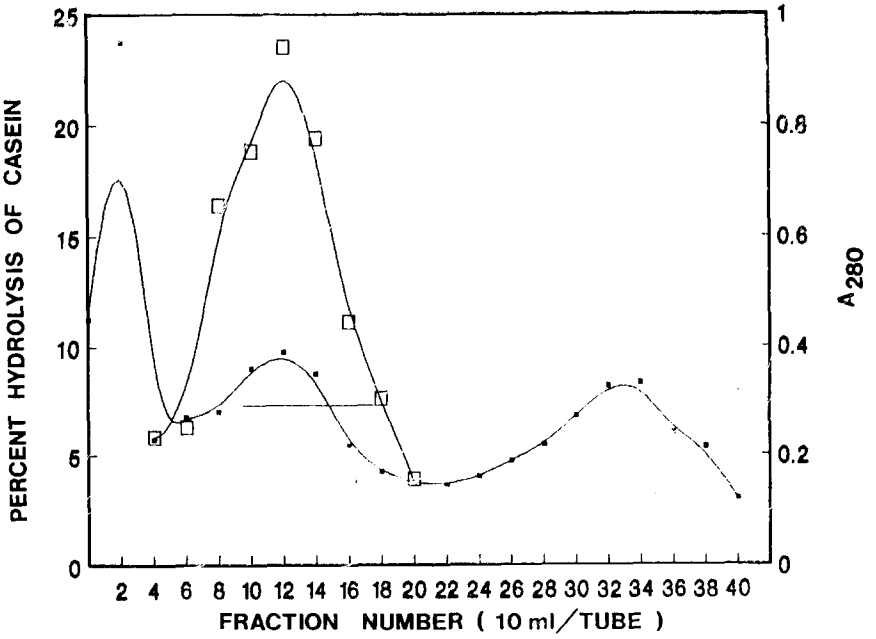

Fig. 4. Sephadex G-200 gel permeation chromatography of fractions of high caseinolytic activity at pH 6.0 from DE52 fractionation. (—□—, % hydrolysis of casein; —■—, A_{280}; and —, saved fractions)

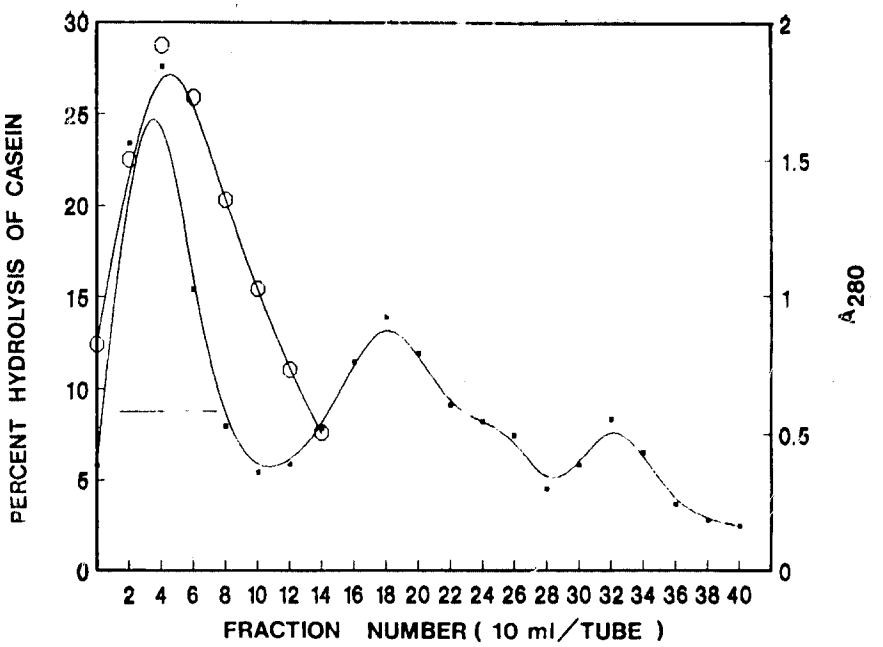

Fig. 5. Sephadex G-200 gel permeation chromatography of fractions of high caseinolytic activity at pH 8.5 from DE52 fractionation. Refer to the legend of Fig. 4.

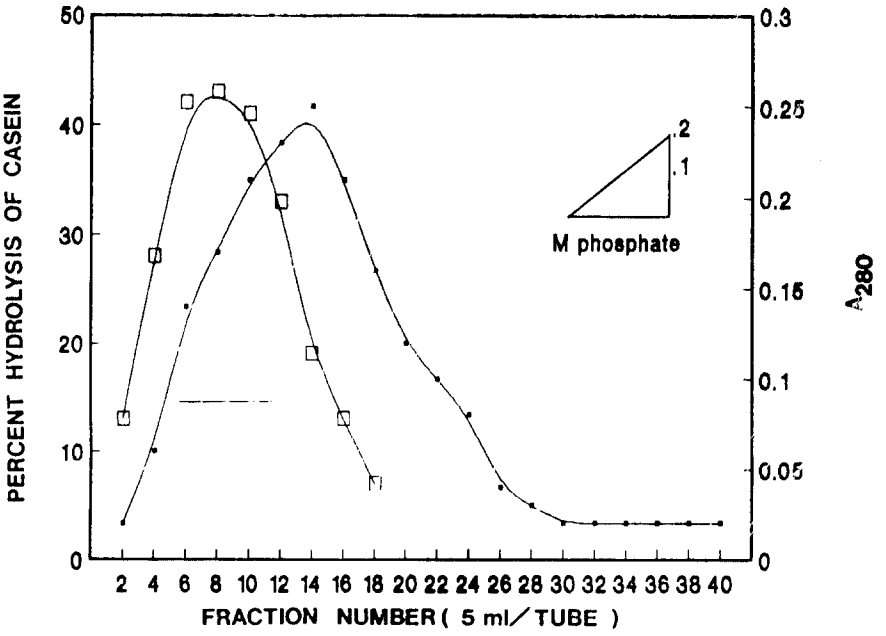

Fig. 6. Hydroxylapatite chromatography of pH 6.0 proteolytic fractions. Highly active fractions from Sephadex G-200 column were pooled, dialyzed against 10 mM phosphate buffer, pH 7.4, and loaded on a hydroxylapatite column as described in the text. Phosphate concentration gradient was indicated by a triangle. (—□—, % hydrolysis of casein; —■—, A_{280}; and ——, saved fractions).

tease activity was promoted when ATP was added.

When treated with various catalytic site-specific protease inhibitors, extract enzymes active at pH 6.0 were inactivated by IAA and those active at pH 8.5 were inactivated by PMSF as shown in Fig. 2.

The crude extract of *Toxoplasma* soluble at 10 mM Tris-HCl, pH 7.4 was loaded onto a DE52 column equilibrated with the same buffer. Unbound proteins were washed with the buffer, eluted with salt gradient of 0 to 0.4 M NaCl, and collected at a flow rate of 90 ml/hr. Fractions eluted with 0.05 M to 0.1 M NaCl showed high caseinolytic activity at pH 6.0 and those eluted with 0.25 M to 0.3 M NaCl appeared to have high casein degrading activity as shown in Fig. 3. Pooled fractions of anterior and posterior peak were concentrated with a freeze drier(Labconco Co.), and dialyzed against 10 mM Tris-HCl, pH 7.4, and then loaded on a Sephadex G-200 column separately. Fig. 4 shows the profile of Sephadex G-200 chromatography of anterior peak which expressed high proteolytic activity at pH 6.0. Fractions next to the exclusion limit showed high casein degrading activity. The posterior peak which showed high caseinolytic activity at pH 8.5 was also fractionated by a Sephadex G-200 chromatography. Fractions out of exclusion limit had the high proteolytic activity as shown in Fig. 5.

The active fractions were pooled, dialyzed against 10 mM phosphate buffer, pH 7.4, and then loaded on a hydroxylapatite column. Columns were washed with the above buffer extensively and eluted with 10 mM to 0.2 M phosphate gradient as shown in Fig. 6 and Fig. 7. Fractions eluted with 0.05 M phosphate buffer in both chromatographies expressed high caseinolytic activity.

Through the purifying procedures performed, specific activity of protease that worked at pH 6.0 was concentrated about 8 times and that wcrkedat pH 8.5 was concentrated about 4 times.

The effects of various protease inhibitors and site-specific reagents were tested by incubating them with the partially purified proteases. As shown in Fig. 8 and Fig. 9, acid protease of pH 6.0 was inhibited by the addition of IAA

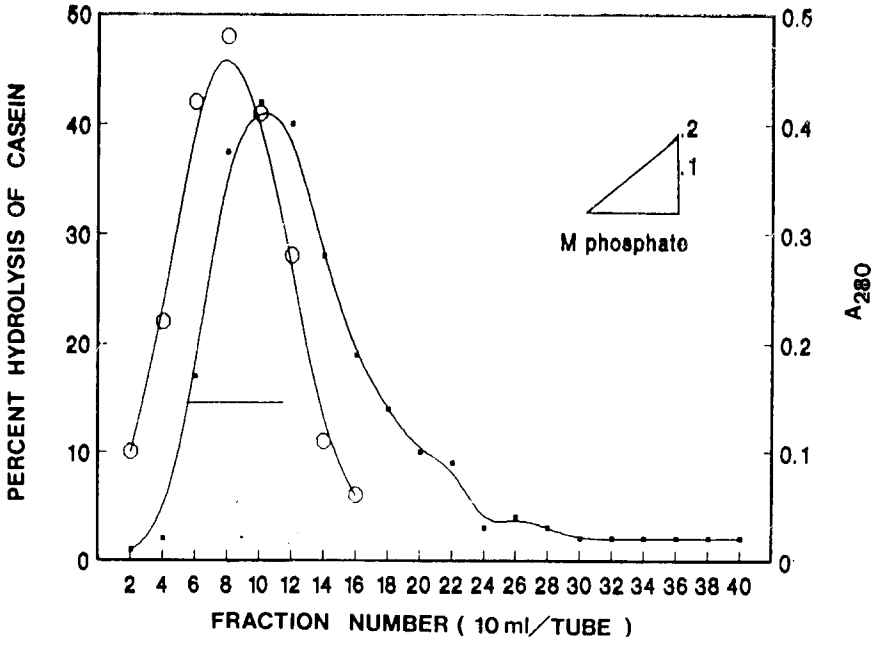

Fig. 7. Hydroxylapatite chromatography of pH 8.5 proteolytic fractions. Refer to the legend of Fig. 6.

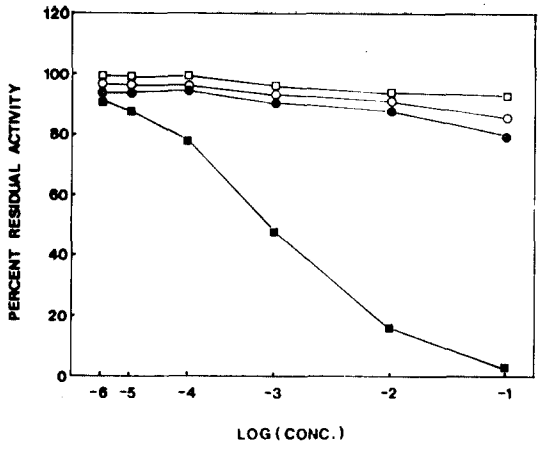

Fig. 8. Effects of inhibitors on the caseinolytic activity of pH 6.0 active fractions. (—□—, Pep A; —○—, EDTA; —■—, IAA; and —●—, PMSF more diluted by 10^2)

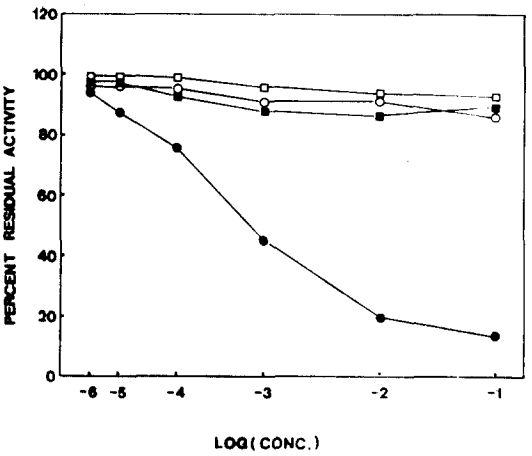

Fig. 9. Effects of inhibitors on the caseinolytic activity of pH 8.5 active fractions. Refer to the legend of Fig. 8.

with the LD_{50} of 1×10^{-3} M and was not affected by Pep A, EDTA, or PMSF, while neutral protease of pH 8.5 was inactivated by PMSF with LD_{50} of 1×10^{-5} M and was not inhibited by Pep A, EDTA, or IAA, respectively.

The effects of various concentrations of ATP on the neutral protease were also tested. ATP promoted the caseinolytic activity until the concentration of ATP added was 2.0 mM, but additional ATP inhibited the caseinolytic activity of the neutral protease as described in Fig. 10.

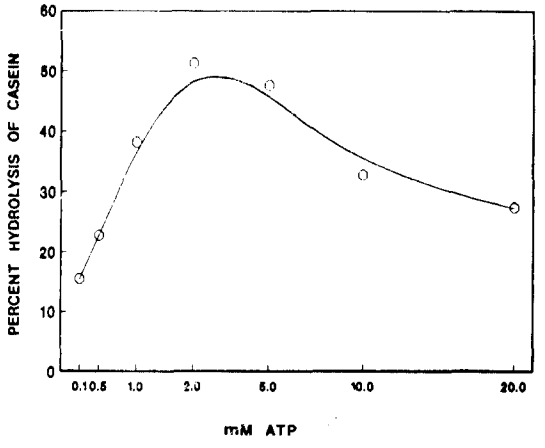

Fig. 10. Effects of various concentrations of ATP on the caseinolytic activity of neutral protease of pH 8.5. (—○—, % hydrolysis of casein)

DISCUSSION

Proteolytic activity was detected in the extract of *Toxoplasma* with significant caseinolytic activity at pH 6.0 and at pH 8.5. Because this assay measured net proteolytic activity of the extract, the range of pH's might indicate the presence of multiple proteases, hence two major proteases working at pH 6.0 and at pH 8.5 were separated during this purifying procedure. Assuming a biological role for such pH optimum, two possibilities might be given. The enzyme could only hydrolyze proteins under very special conditions, *e.g.*, in compartments of low pH such as lysosomes, and neutral pH, on the other hand, could restrict the protease activity at a level required for its role inside the cell.

The properties of the proteases had generally been studied with special reference to inhibitors as well as optimal pH and substrate specificity. Proteases were classified into four groups with respect to the catalytic residues of the active site which specific inhibitors were found to affect; *i.e.*, thiol-, carboxyl-, metallo-, and serine proteases(Asch and Dresden, 1979; Maki *et al.*, 1982; McDonald, 1985). Proteolytic enzyme working at pH 6.0 was inhibited by IAA and

that working at pH 8.5 was inactivated by PMSF and was promoted when ATP was added, therefore, the former was referred to as a cysteinyl acid protease and the latter, an ATP-dependent neutral serine protease, respectively. Thus, it could be suggested that the proteases of *Toxoplasma* may have one active site which become fully active when the environmental pH conditions is pH 6.0 or pH 8.5. It also could be suggested that the acid protease may have sulfhydryl group and the neutral protease may have hydroxyl group which associated with their active site. It was also suggested that the neutral protease need ATP essentially to perform its action, that is, the neutral protease also have a ATPase activity in its substructure to offer some energy to proteolytic potential. It was not explained that excess ATP inhibited the activity of neutral protease, but it might be the role of excess ATP to stabilize the conformation of substrate, casein.

The biological role of proteases of *Toxoplasma* was speculative at this time. It was possible that the enzyme was involved in the digestion of exogenous peptides or that it played a role in intracellular protein metabolism. When the digestion of exogenous peptides was concerned, the protease might play in the parasitic way of life, the infection of their hosts, or the pathology of the disease they cause. There were many studies which pointed out the cysteinyl or neutral proteases of various kinds of parasitic protozoa that function in these categories(Dluzewski *et al.*, 1983; Pupkis and Coombs, 1984; Lushbaugh *et al.*, 1984; Munoz *et al.*, 1984; Schrevel *et al.*, 1984; Avila *et al.*, 1985; Friedman *et al.*, 1985; Keene *et al.*, 1986; Rabinovitch *et al.*, 1987). When the regulation of intracellular protein metabolism was concerned, proteases and peptidases might play a role in the degradation of proteins yielding amino acids after complete hydrolysis. Another important function of protein breakdown in cells was to protect the organism against the intracellular accumulation of polypeptides whose conformation became highly abnormal or to regulate the enzyme activity by degrading the target enzyme. Various lysosomal proteases had specific actions on substrate enzymes and might play an important role in the intracellular protein degradation and enzyme regulation.

With the procedure described here we had been able to confirm the existence of two proteases from *Toxoplasma*. This approach allowed the identification of proteases and, in some cases, their classification in the corresponding catalytic class. Such information could be useful for further purification of these molecules to elucidate the role and action mechanism of proteases with host cells.

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=국문초록=

*Toxoplasma gondii*에서 단백질 분해 효소의 특징

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*Toxoplasma*의 추출액을 ^{3}H-casein을 기질로 반응시켰을 때, pH 6.0과 pH 8.5에서 casein을 분해하였으며, pH 6.0에서는 cysteinyl protease의 억제제인 iodoacetamide(IAA)에 의해 억제되었고, 활성제인 dithiothreitol (DTT)에 의해 활성이 증가하였다. 또 pH 8.5에서는 serine protease의 억제제인 phenylmethylsulfonil fluoride (PMSF)에 의해 활성이 억제되었으며, ATP를 첨가할 때 그 활성이 증가하여 ATP 의존성 효소임을 알 수 있었다.

위의 단백질 분해 효소를 부분 정제하기 위해 여러 chromatography를 실시하였는데, 먼저 DE52 (2.5ϕ×40 cm)에 통과시켰을 때, 0.05 M-0.1 M NaCl에 의해 유출되는 분획이 pH 6.0에서 활성을 나타내었으며, 0.25 M-0.3 M에서 유출되는 분획이 pH 8.5에서 활성을 나타내었다. 이 분획들을 각각 Sephadex G-200 (2.5ϕ×90 cm)에 통과시켜 pH 6.0에서 활성을 나타내는 분획은 exclusion limit 내에서, pH 8.5의 분획은 exclusion limit 외에서 분획을 얻었다. 이들을 각각 hydroxylapatite (2.5ϕ×10 cm과 2.5ϕ×20 cm)를 통과시켜 각각을 0.05 M phosphate로 유출되는 분획에서 높은 활성을 얻었다.

부분 정제된 분획들의 특성을 검토하기 위하여 억제제를 농도별로 처리하였을 때, pH 6.0에서의 분해 효소는 10^{-3} M IAA에 의해 활성이 반감되어 cysteinyl acid protease임을 알 수 있었다. pH 8.5에서의 분해 효소는 10^{-5} M PMSF에 의해 활성이 반감되었고, ATP에 의해 활성이 증가(ATP의 농도가 2.0 mM 이상에서는 억제)하여 ATP-dependent neutral serine protease임을 알 수 있었다.