

Protease activity of 80 kDa protein secreted from the apicomplexan parasite *Toxoplasma gondii*

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Abstract: This study describes the characterization of 80 kDa protease showing gelatinolytic property among three proteases in the excretory/secretory proteins (ESP) from *Toxoplasma gondii*. The protease activity was detected in the ESP but not in the somatic extract of RH tachyzoites. This protease was active only in the presence of calcium ion but not other divalent cationic ions such as Cu²⁺, Zn²⁺, Mg²⁺, and Mn²⁺, implying that Ca²⁺ is critical factor for the activation of the protease. The 80 kDa protease was optimally active at pH 7.5. Its gelatinolytic activity was maximal at 37°C, and significant level of enzyme activity of the protease remained after heat treatment at 56°C for 30 min or 100°C for 10 min. This thermostable enzyme was strongly inhibited by metal chelators, i.e., EDTA, EGTA, and 1,10-phenanthroline. Thus, the 80 kDa protease in the ESP secreted by *T. gondii* was classified as a calcium dependent neutral metalloprotease.

Key words: *Toxoplasma gondii*, excretory/secretory proteins, gelatinolytic activity, thermostable, metalloprotease

INTRODUCTION

The apicomplexan parasite, *Toxoplasma gondii*, is an obligatory Protozoa with a worldwide distribution, which infects the central nervous system of warm blooded animals, including humans. Although toxoplasmosis is clinically asymptomatic in healthy individuals, there are many reports which indicate that this parasite increases the rate of severe complications in fetuses and in immunocompromised patients, such as AIDS patients (Luft et al., 1984;

Wanke et al., 1987).

There are many evidence for the involvement of protozoan proteases in host-parasite interactions, such as in the digestion of exogenous proteins for nutrition, in the invasion into host cells and tissues, and in the inactivation of host proteins detrimental to parasite survival (Santana et al., 1997; Eggleston et al., 1999; Spinella et al., 1999). Several studies have pointed out that the processes associated with invasion into host cells by protozoan zoites involve proteases in the breakdown of the host cell membrane and the shedding of zoite surface antigens (Carruthers et al., 2000; Huynh et al., 2003). As suggested in apicomplexan invasion, such process requires a contractile system (Russell and Sinden, 1981; Ghazali et al., 1995) and parasitic proteases (Schrevel et al., 1990). Degradation of the host's skeletal proteins during invasion or the degradation of connective

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tissues could facilitate the parasitic invasion into host cells.

Under the presumption that parasite proteases should be secreted to modify other secreted functional proteins, we attempted to identify proteases in the excretory/secretory proteins (ESP) from *T. gondii* in a continuation of the work on the ESP by Son and Nam (2001). We previously reported a 42 kDa rhoptry protein, which has protease activity and a host cell binding property, in the *T. gondii*-derived ESP (Ahn et al., 2001). Here we describe the identification of 80 kDa protease released from *T. gondii* and describe its proteolytic properties.

MATERIALS AND METHODS

Parasite

The RH strain of *T. gondii* was maintained by peritoneal passages in Balb/c mice. Tachyzoites were purified by centrifugation over 40% Percoll (Amersham Pharmacia Biotech, Uppsala, Sweden) in PBS solution as the method previously described (Sohn and Nam, 1999).

Preparation of ESP

Purified tachyzoites (3×10^8) were incubated at 37°C for 1 hr under mild agitation in 1.0 ml Hank's balanced salt solution (Gibco BRL, Rockville, MD, USA). After centrifugation for 5 min at 6,000 rpm, the supernatant was saved as ESP. The protein concentration of ESP ranged from 0.8 to 1.0 mg/ml by the batches prepared.

Gelatin acrylamide gel electrophoresis (GAGE)

Protease activity was assayed by gelatin acrylamide gel electrophoresis as the method described by Heussen and Dowdle (1980). SDS-PAGE was performed on 8% gels containing 0.1% gelatin as a copolymerized substrate under non-reducing conditions (i.e., without 2-mercaptoethanol). After electrophoresis, the gels were shaken gently at room temperature for 1 hr in 2.5% Triton X-100 solution to renature the proteins, and then incubated in appropriate buffers containing 5 mM CaCl_2 at 37°C

for 5-8 hrs. The gels were fixed and stained with 0.15% Coomassie brilliant blue R-250 for 1 hr and destained in 40% methanol/10% acetic acid solution. Relative enzymatic activity was determined from band densities using an Image Gauge program (Fujifilm, Tokyo, Japan).

Effect of pH on proteolytic activity

Gelatinolytic activity was assayed over a range of pH values in 10 mM MES buffer (2-[N-morpholino] ethanesulfonic acid) for pH 5.5, PIPES buffer (piperazine-*N,N*-bis[ethanesulfonic acid]) for pH 6.5, HEPES buffer (*N*-[2-hydroxyethyl] piperazine-*N*-[2-ethanesulfonic acid]) for pH 7.5, Tris-HCl (tris [hydroxymethyl] aminomethan) for pH 8.5, CHES buffer (2-[*N*-cyclohexylamino]-1-propanesulfonic acid) for pH 9.5, or in CAPS buffer (3-[cyclohexylamino]-1-propanesulfonyl fluoride) for pH 10.5, respectively. GAGE was performed after incubating at 37°C for 1 hr.

Effects of temperature on proteolytic activity

The thermostability of the enzyme was assayed by measuring the activity remained after incubating the enzyme in 40 mM Tris-HCl buffer, pH 7.5 containing 5 mM CaCl_2 at 36°C for 30 min, 56°C for 1, 5, 10, and 30 min, and 100°C for 1, 5, 10, and 30 min, respectively.

RESULTS

Three proteases were detected in the ESP derived from *T. gondii* by gelatin activity gel electrophoresis (GAGE), but not in the somatic extract of RH tachyzoites (Fig. 1A). Of these, we characterized the biochemical properties of the 80 kDa protease. Its gelatinolytic activity was found to be dependent on the concentration of calcium ions in the reaction mixture. Moreover, no enzyme activity was detected in the presence of micromolar concentrations of calcium ion (10-100 μM), but this was recovered when the concentration of calcium ion reached the millimolar range (over 1mM) (Fig. 1B). In contrast, other divalent cations such as copper, zinc,

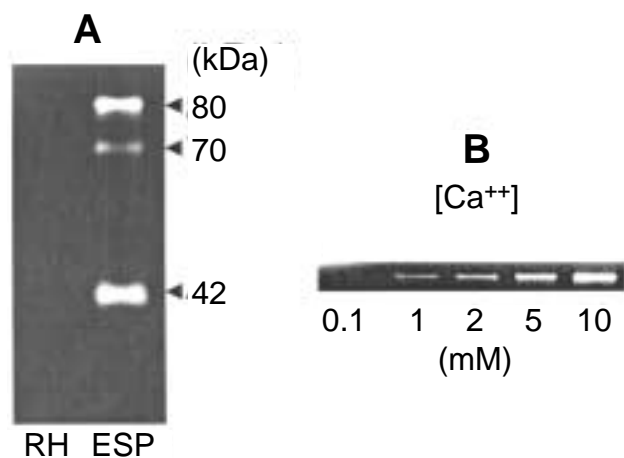


Fig. 1. Gelatin acrylamide gel electrophoresis (A) of RH tachyzoites (RH) and the ESP of *Toxoplasma gondii* (ESP) and the effect of calcium ion on the gelatin degrading activity of the 80 kDa protease in ESP (B).

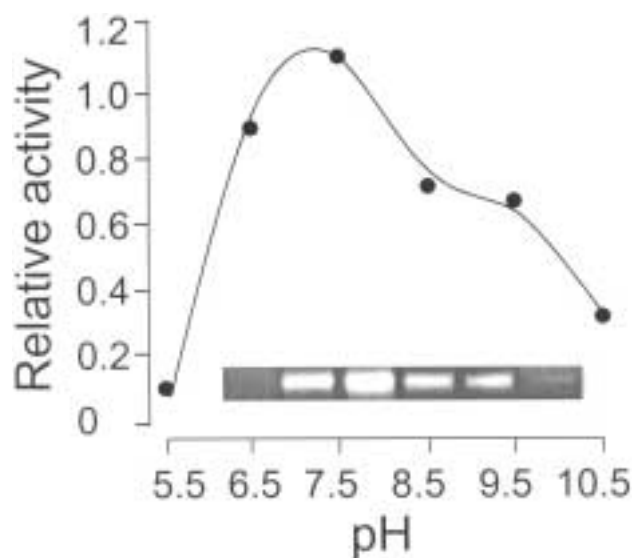


Fig. 2. Effect of pH on the gelatin-degrading activity of the 80 kDa protease in ESP from *Toxoplasma gondii*. The buffers (10 mM) used were MES for pH 5.5, PIPES for pH 6.5, HEPES for pH 7.5, Tris-HCl for pH 8.5, CHES for pH 9.5, and CAPS for pH 10.5.

magnesium, or manganese, did not support its enzyme activity (data not shown). Maximal enzymatic activity was obtained at pH 7.5, and little or no activity was observed at pH over 10.5 or below 5.5 (Fig. 2).

The effects of various inhibitors on the activity of

Table 1. Effect of protease inhibitors on the proteolytic activity of the 80 kDa protease of ESP by *Toxoplasma gondii*

Inhibitors	Concentration (mM)	Relative activity (%)
Control ^{a)}		100
Serine class		
AEBSF	1	82.9
PMSF	1	88.3
Serine & cysteine class		
Leupeptine	0.1	77.9
TLCK	0.1	70.1
TPCK	0.1	77.3
Cysteine class		
E-64	1	95.7
Iodoacetamide	0.1	77.7
Aspartic class		
Pepstatin A	0.1	79.8
Metallo class		
EDTA	0.1	51.8
	1	40.6
EGTA	0.1	65.4
	1	8.9
1,10-Phenanthroline	0.1	30.7
	1	15.6

^{a)}Control represents the activity tested without any inhibitors.

AEBSF: 4-(2-aminoethyl)benzenesulfonyl fluoride

PMSF: phenylmethanesulfonyl fluoride

TPCK: N-tosyl-L-phenyl-alanine chloromethyl ketone

TLCK: N-a-p-tosyl-L-lysine chloromethyl ketone

E-64: trans-epoxysuccinyl-L-leucylamino-(4-guanidino) butane

EDTA: Ethylene diamine tetraacetic acid

EGTA: Ethylene glycol-bis-(2-aminoethyl)-N,N,N',N' tetraacetic acid

the protease were determined by preincubating it with various classes of protease inhibitors (Table 1). The metalloprotease inhibitors, EDTA, EGTA, and 1,10-phenanthroline, significantly inhibited its protease activity, and the inhibitory effect of EGTA was stronger than that of EDTA. However, other inhibitors, such as AEBSF and PMSF (inhibitors of serine protease), leupeptine, TLCK, and TPCK (serine and cysteine protease inhibitors), E-64 (inhibitor of cysteine protease), and pepstatin A (inhibitor of aspartic protease) had little or no inhibitory effect.

The enzyme exhibited a broad temperature

Table 2. Thermostability of the 80 kDa protease in ESP from *Toxoplasma gondii*

Temperature (°C)	Incubation time (min)	Relative enzyme activity (%)
37	30	100 (control)
56	1	95
	5	94
	10	90
	30	89
100	1	85
	5	70
	10	47
	30	6

optimum, 37-100°C (5 min), with maximum activity at 37°C. However, it was stable after heat treatment at 56°C for 30 min, with activity of over 89% to the control and even after treatment at 100°C for 5 min, retaining an activity of over 70%. However, this enzyme activity was decreased sharply after 10 min incubation at 100°C (Table 2).

DISCUSSION

In the present study, the 80 kDa band of the ESP from *T. gondii* in the gelatin zymogram was found to be a calcium dependent neutral metalloprotease. Protease activity was observed only in the ESP not in the somatic extracts of tachyzoites. This suggests that excretion/secretion is a critical event for the activation of enzymes. The activity of the secreted protease was dependent upon the concentration of calcium ion. For example, the protease activity was only present at higher (almost equal to the concentration of extracellular milieu), but not lower, concentrations of the Ca²⁺. However, protease activity in the tachyzoite extracts was not detected even in the presence of millimolar concentrations of the calcium ion (data not shown), implying that additional factors were required for the activity. Matrix metalloproteases (MMPs) are produced by cells as pro-enzymes, and then require further processing to generate the enzyme activities (Mazzieri et al., 1997; Le et al., 2003).

Apicomplexa undoubtedly possesses many proteases, as is thought to be the case of all eukaryotic

cells, however, only a few have been reported to date. This may be due to the relatively low abundances of proteases in Apicomplexa (Fuller and McDogald, 1990; Michalski et al, 1994), a higher susceptibility to denaturation during electrophoresis, or a narrower substrate specificity. Although limitations are imposed by the fact that large amount of parasites are required to obtain a reasonable ESP sample, ESP assays are essential in the study of proteases in this phylum. The enzyme activity and the molecular mass of this protease were determined by zymography (Mook et al., 2003). Of these three proteases present, the subject of this study had the highest molecular mass (about 80 kDa) by GAGE. The biochemical characterization of this protease revealed that its gelatinolytic activity was optimal at pH 7.5, and that its gelatinolytic activity was inhibited significantly by EDTA, EGTA, and 1,10-phenanthroline, suggesting that it belongs to the neutral metalloproteases. In addition, it possessed some special characteristics, such as its thermostability and its ability to function in a wide pH range. This result is coincident with a report by Sookkheo et al. (2000), which described highly thermostable extracellular proteases (36, 53, and 71 kDa) in *Bacillus stearothermophilus* TLS33.

The molecular characterization of this protease will enable us to understand its transition from the inactive to the active, and its secretory pathway across the cell membrane, and furthermore, to characterize its substrate(s), in the hope that this will clarify both the function of this protein and the mode of entry of *T. gondii* into host cells.

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