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Involvement of cysteine proteinases in excystment of *Paragonimus ohirai* metacercariae induced by sodium cholate and A23187

T. Ikeda*

Department of Medical Zoology, Kanazawa Medical University, Uchinada, Ishikawa, 920-0293, Japan

Abstract

The involvement of intrinsic proteinases in the excystment of *Paragonimus ohirai* metacercariae was studied in *in vitro* excystment induced by sodium (Na) cholate, a bile salt and A23187, a Ca^{2+} ionophore. The effects of various proteinase inhibitors on the *in vitro* excystment were examined and similar inhibitory profiles were obtained. Benzyloxycarbonyl-L-leucyl-L-leucinal (Z-Leu-Leu-H), a cysteine proteinase inhibitor and 4-(2-aminoethyl)-benzenesulfonyl fluoride (Pefabloc SC), a serine proteinase inhibitor completely inhibited excystment, while L-3-carboxy-2,3-trans-epoxypropionyl-leucylamido (4-guanidino)-butane (E-64), a cysteine proteinase inhibitor and leupeptin, a cysteine/serine proteinase inhibitor permitted partial excystment at a lower rate, but inhibited it from proceeding from the partial excystment stage. In secretions released from metacercariae during excystment, proteinase activities detected towards various fluorogenic peptidyl substrates were almost completely inhibited by Z-Leu-Leu-H and E-64, but not by Pefabloc SC. Sodium cholate induced a higher secretion of cysteine proteinases and a higher rate of excystment than A23187. Profiles of cysteine proteinase activities towards five peptidyl substrates detected were markedly different among the two secretions and the lysate of newly excysted juveniles. Newly excysted juveniles released cysteine proteinases with similar activity profiles and levels to metacercariae induced by Na cholate-incubation, whereas the release of cysteine proteinases was reduced compared with metacercariae induced by A23187-incubation. These results provide valuable information about the involvement of intrinsic proteinases in metacercarial excystment.

Introduction

In a number of digenetic trematodes, various factors are involved in the excystment of metacercariae (Lackie, 1975; Sommerville & Rogers, 1987; Fried, 1994). These are extrinsic factors associated with the host's intestine and intrinsic factors from the parasite itself. However, the mechanism of excystment has not yet been fully elucidated. Bile salts are one of the most important extrinsic factors for excystment of metacercariae, because, in a number of digeneans, bile salts are either a prerequisite for *in vitro* excystment or greatly enhance the rate and percentage of excystment, but this mechanism remains poorly understood.

In *Paragonimus* species also, bile salts such as sodium (Na) cholate, Na tauroglycocholate, and Na deoxycholate efficiently induced the excystment of *P. westermani* metacercariae (Oshima *et al.*, 1958). The reducing agent

*Fax: +81 76 218 8336
E-mail: ikeda@kanazawa-med.ac.jp

dithiothreitol (DTT), which is an activator of cysteine proteinases, was also an effective stimulus for *P. westermani* metacercariae (Chung *et al.*, 1995). For *P. heterotremus* metacercariae, however, DTT was not an effective stimulus under the conditions of incubation at 37–38°C (Intapan & Maleewong, 2001). More recently, Ikeda (2001), using *P. ohirai* metacercariae, added Ca^{2+} ionophores, which appear to be unrelated substances with the host's environment, as stimuli for excystment.

In DTT-induced excystment, based on the inhibition of cysteine proteinase inhibitors, cysteine proteinase(s) contributed to the excystment (Chung *et al.*, 1995; Intapan & Maleewong, 2001). It is important to clarify whether these stimuli activated a common mechanism for excystment or different ones. The aim of the present study was to examine the involvement of various proteinases in excystments induced by Na cholate and A23187 (Ca^{2+} ionophore).

Materials and methods

Proteinase inhibitors, chymostatin, L-3-carboxy-2,3-trans-epoxypropionyl-leucylamido (4-guanidino)-butane (E-64), leupeptin, pepstatin A, phosphoramidon and benzyloxycarbonyl-L-leucyl-L-leucinal (Z-Leu-Leu-H), were all purchased from Peptide Institute (Osaka, Japan). Three fluorogenic peptidyl substrates, benzoyl-L-phenylalanyl-L-valyl-L-arginine-4-methylcoumaryl-7-amide (Bz-Phe-Val-Arg-MCA), Suc-Ala-Phe-Lys-MCA (Suc, succinyl) and Tos-Gly-Pro-Arg-MCA (Tos, tosyl), and 17 fluorogenic peptidyl substrates other than the above three were purchased from Sigma (St Louis, Missouri, USA) and Peptide Institute, respectively. Proteinase inhibitors bestatin and 4-(2-aminoethyl)-benzenesulfonyl fluoride (Pefabloc SC) were purchased from Roche (Mannheim, Germany). The A23187 and 3-[4-(2-hydroxyethyl)-1-piperazinyl] ethanesulfonic acid (HEPES) were purchased from Sigma and Dojin Laboratories (Kumamoto, Japan), respectively. All other reagents were from Wako Pure Chemicals (Osaka, Japan).

Metacercariae of *P. ohirai* were obtained as previously described (Ikeda, 2001a). Briefly, the livers of naturally infected freshwater crabs, *Sesarma dehaani*, were stirred with a whisk in a beaker with cold 0.2% NaCl solution. After filtration, the liquid was left to settle to allow the metacercariae to sink. Intact metacercariae were collected by pipette under a binocular dissecting microscope, stored at 4°C in salt solution to preserve the *Paragonimus* metacercariae (1.2 g NaCl, 0.04 g KCl, 0.04 g $CaCl_2$ per 100 ml distilled water), and then used for excystment studies within one week. Newly excysted juveniles (NEJs) were obtained by overnight incubation in Tyrode's medium (pH 8.0) containing 1000 U ml^{-1} penicillin and 100 μg ml^{-1} streptomycin, as previously described (Ikeda & Oikawa, 1991).

Excystment of metacercariae was carried out in 0.5 ml of 25 mM HEPES-buffered 0.85% NaCl solution (pH 7.6) containing 4.6 mM Na cholate or 0.2 μM A23187 in a 24-well cell culture multidish. Ten metacercariae per well were incubated at 39°C and observed under the microscope. Secretions during the excystment were obtained from 25 metacercariae.

Proteinase activities were measured with fluorogenic peptidyl substrates in a 96-well microtitre plate as previously described (Ikeda, 2001b). The assay solution consisted of 10 μl of sample solution and 150 μl of 0.25 M Tris-HCl buffer (pH 7.5) containing 5 mM L-cysteine and 40 μM fluorogenic substrate. Duplicate wells per sample were incubated at 37°C for 30 min, and then measured in a Flow Fluoroscan II plate reader (Labsystems, Helsinki, Finland). The NEJ lysate was prepared as follows: four NEJs were suspended in 2 ml of 0.25 M Tris-HCl buffer (pH 7.5) containing 0.1% Triton X-100, frozen and thawed. After centrifugation at 10,000 g for 30 min at 4°C, the supernatant was used as the NEJ lysate.

Results

The effect of various proteinase inhibitors (100 μM) on the *in vitro* excystment of *P. ohirai* metacercariae induced by 4.6 mM Na cholate and 0.2 μM A23187 was examined (fig. 1). Sodium cholate induced 90% complete

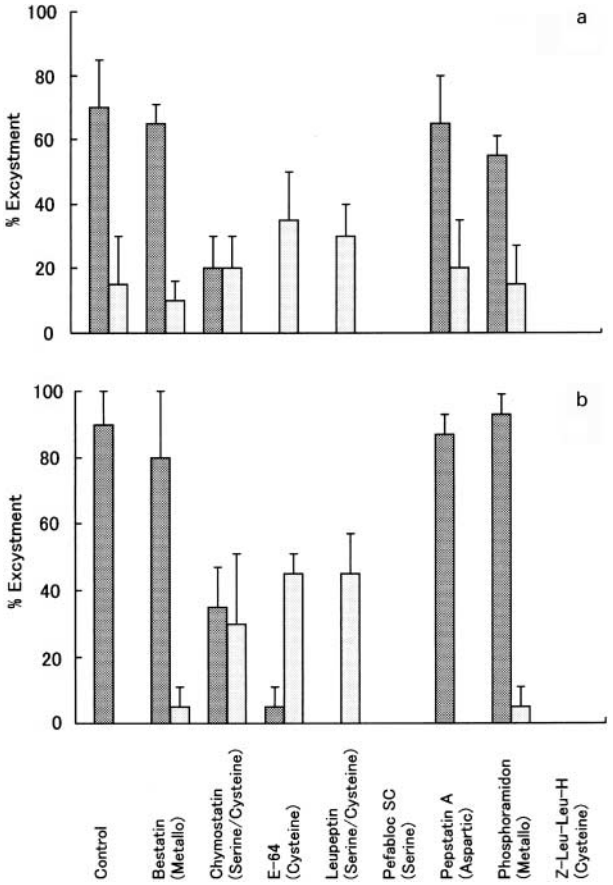

Fig. 1. The effect of various proteinase inhibitors on the *in vitro* excystment of *Paragonimus ohirai* metacercariae induced by (a) A23187 and (b) sodium cholate. Ten metacercariae per well were observed 3 h and 2 h after incubation in 25 mM HEPES-buffered saline (pH 7.6) with 0.2 μM A23187 and 4.6 mM sodium cholate, respectively. Each bar represents the mean percentage +SD of complete excystment (dark column) and partial excystment (light column) from three experiments.

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excystment 2 h after incubation. Bestatin, pepstatin A and phosphoramidon had little effect on excystment, while chymostatin markedly reduced complete excystment and E-64 and leupeptin permitted partial excystment at a reduced rate, but almost completely inhibited emergence from the metacercarial cyst, and Pefabloc SC and Z-Leu-Leu-H completely inhibited excystment. Even after 5 h incubation, the inhibitory effects of the four cysteine proteinase inhibitors continued (data not shown). A23187 induced 85% excystment (complete excystment 70%, partial 15%) 3 h after incubation. The inhibitory pattern of proteinase inhibitors was similar to that of Na cholate-induced excystment. In both Na cholate-and A23187-induced excystments, metacercariae and NEJs were alive during the incubation in the proteinase inhibitors, bestatin, chymostatin, E-64, leupeptin, Pefabloc SC, pepstatin, phosphoramidon and Z-Leu-Leu-H, for 5 h at 39°C (fig. 1), while the metacercariae died a few hours after incubation with 50 μM 3,4-dichloroisocoumarin (3,4-DIC) or N-tosyl-L-phenylalanine chloromethyl ketone (TPCK) proteinase inhibitor (data not shown).

The incubation solution during metacercarial excystment induced by Na cholate was examined for proteolytic activities towards various fluorogenic synthetic peptides (table 1). A high hydrolytic activity was detected consistently with Boc-Val-Leu-Lys-MCA (Boc, butyloxycarbonyl) and Tos-Gly-Pro-Arg-MCA. At a later stage, high activity was detected with Bz-Phe-Val-Arg-MCA and Z-Phe-Arg-MCA. Low but significant activity was detected with Boc-Val-Pro-Arg-MCA, Suc-Ala-Phe-Lys-MCA, Boc-Gln-Ala-Arg-MCA, Bz-Arg-MCA and Boc-Phe-Ser-Arg-MCA. The sensitivity of the detected proteinase activities to the proteinase inhibitors which completely and partially inhibited the excystments was examined. Cysteine proteinase inhibitors E-64 and Z-Leu-Leu-H almost completely inhibited the proteolytic activities, whereas a serine proteinase inhibitor Pefabloc SC had little inhibitory effect.

The pattern of proteolytic activity towards the five substrates with a relatively higher sensitivity was compared among the incubation solutions either during excystment induced by Na cholate, A23187, or overnight incubation in Tyrode's medium (pH 8.0) and the NEJ lysate with 0.1% Triton X-100 (fig. 2). In the Na cholate-induced excystment, the proteolytic activity towards Tos-Gly-Pro-Arg-MCA and Boc-Val-Leu-Lys-MCA was about two times or more higher than the other three. In the A23187-induced excystment, Tos-Gly-Pro-Arg-MCA alone showed a high hydrolytic activity, although about one half of that of the Na cholate-induced excystment. Similar substrate specificities and levels were observed in slow excystment, which occurred by overnight incubation in Tyrode's medium. The NEJ lysate showed the highest activity with Boc-Val-Leu-Lys-MCA and a later high activity with Z-Phe-Arg-MCA. Thus, the activity profiles towards the five substrates were markedly different among the four samples.

The NEJs obtained by overnight incubation in Tyrode's

Table 1. Substrate specificity of proteinases released by *Paragonimus ohirai* metacercariae and the effect of three proteinase inhibitors on the proteinase activity.

		Proteinase inhibitor (50 μM)		
Substrate	Proteolytic activity	E-64	Z-Leu-Leu-H	Pefabloc SC
Boc-Gln-Ala-Arg-MCA	122	0	0	104
Boc-Phe-Ser-Arg-MCA	40	0	0	30
Boc-Val-Leu-Lys-MCA	1053	3	2	1035
Boc-Val-Pro-Arg-MCA	259	0	0	249
Bz-Arg-MCA	59	0	0	37
Bz-Phe-Val-Arg-MCA	557	7	7	579
Suc-Ala-Ala-Ala-MCA	0	0	0	0
Suc(OMe)-Ala-Ala-Pro-Val-MCA	0	0	0	0
Suc-Ala-Ala-Pro-Phe-MCA	0	0	0	0
Suc-Ala-Phe-Lys-MCA	146	0	0	125
Suc-Ala-Pro-Ala-MCA	3	0	0	3
Suc-Gly-Pro-Leu-Gly-Pro-MCA	0	0	0	0
Suc-Leu-Leu-Val-Tyr-MCA	7	0	0	5
Tos-Gly-Pro-Arg-MCA	995	3	9	941
Z-Arg-Arg-MCA	2	0	0	3
Z-Phe-Arg-MCA	572	3	5	543
Ala-MCA	0	0	0	0
Arg-MCA	0	0	0	0
Leu-MCA	0	0	0	0
Phe-MCA	0	0	0	0

Secretions from 25 metacercariae were obtained by 2 h incubation in 1 ml of excystment solution containing 4.6 mM Na cholate.
Ten μl of secretions was assayed for 30 min in a microtitre well with 150 μl substrate solution containing 5 mM L-cysteine.
Proteolytic activity is expressed as the average pmoles of AMC produced in duplicate wells.
A similar result was obtained from another experiment using a different sample of metacercarial secretions.

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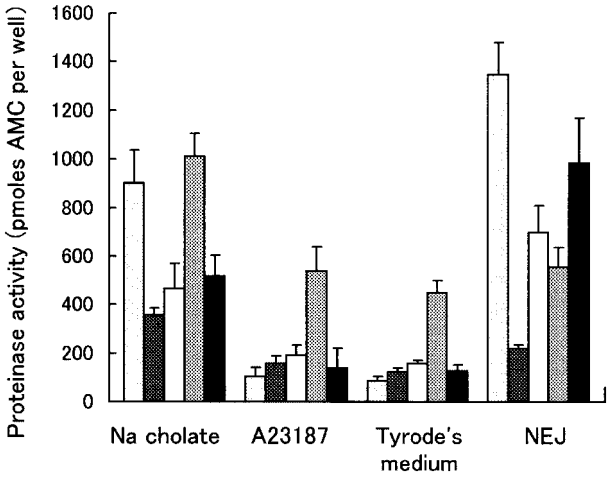

Fig. 2. Substrate specificity of cysteine proteinases of secretions during *in vitro* excystments induced by sodium cholate, A23187, and Tyrode's medium (pH 8.0) and the lysate of newly excysted juveniles (NEJs) of *Paragonimus ohirai*. Secretions were obtained 1 h after Na cholate-incubation, 2 h after A23187-incubation, and 14–16 h after Tyrode's medium-incubation. Each bar represents the mean proteinase activity + SD from three experiments. Cysteine proteinases towards Boc-Val-Leu-Lys-MCA (▒), Boc-Val-Pro-Arg-MCA (■), Bz-Phe-Val-Arg-MCA (□), Tos-Gly-Pro-Arg-MCA (▒), and Z-Phe-Arg-MCA (■).

medium were incubated in excystment solutions containing Na cholate or A23187 and saline alone. The pattern of cysteine proteinase activities towards the above five substrates was examined in the incubation solutions for 1 h (fig. 3). Sodium cholate induced similar patterns and levels of cysteine proteinase activities to the metacercarial excystment. A23187 did not stimulate cysteine proteinase secretion more than the control incubation in saline.

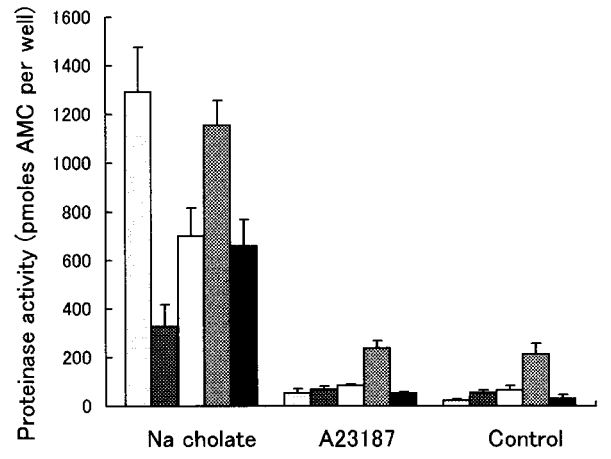

Fig. 3. Substrate specificity of cysteine proteinases in secretions from newly excysted juveniles of *Paragonimus ohirai* during incubation with sodium cholate, A23187, and saline (control). Secretions were obtained 1 h after incubation. Each bar represents the mean proteinase activity + SD from three experiments. Cysteine proteinases towards Boc-Val-Leu-Lys-MCA (▒), Boc-Val-Pro-Arg-MCA (■), Bz-Phe-Val-Arg-MCA (□), Tos-Gly-Pro-Arg-MCA (▒), and Z-Phe-Arg-MCA (■).

Discussion

In addition to the observation that bile salts enhanced the movement of metacercarial juveniles (Dixon, 1965), bile salts were observed to induce metacercarial juveniles to regurgitate caecal fluid (Sukhdeo & Mettrick, 1986; Bock, 1989). Secretions released from metacercariae and excysted juveniles showed a high proteinase activity (Yamakami & Hamajima, 1988; Bock, 1989; Carmona *et al.*, 1993). However, there have been many reports suggesting but very few reports supporting the involvement of secreted proteinases in bile salt-induced excystment.

In the present study using *P. ohirai* metacercariae, *in vitro* excystment induced by Na cholate was completely inhibited by Z-Leu-Leu-H, a cysteine proteinase inhibitor and Pefabloc SC, a serine proteinase inhibitor. The cysteine proteinase inhibitor E-64 and the cysteine/serine proteinase inhibitor leupeptin, markedly inhibited excystment but allowed partial excystment of about 40% of the metacercariae. These four proteinase inhibitors did not influence the active movement of excysted juveniles to the same degree as cyst-enclosed ones during at least a 5 h incubation period, suggesting that unlike DIC and TPCK, which kill cyst-enclosed juveniles, they exerted little harmful effect except for the inhibition of proteolytic activity. Thus, both cysteine and serine proteinases are likely to be involved in the Na cholate-induced excystment. Both Z-Leu-Leu-H and E-64, cysteine proteinase inhibitors, almost completely inhibited proteinase activities in secretions from the metacercariae during the excystment process. However, Z-Leu-Leu-H completely inhibited *in vitro* excystment while E-64 allowed some metacercariae to partially excyst. This discrepancy in the inhibitory effect on excystment may be due to differences in the permeability of the cyst wall.

In contrast, Pefabloc SC, which showed little inhibitory effect on proteinase activities detected in metacercarial secretions, completely inhibited Na cholate-induced excystment. In the present experiment, the activity of serine proteinases was not detected in secretions even by using various fluorogenic synthetic substrates for serine proteinases. These results raise speculation that Pefabloc SC may inhibit a process other than proteolysis of the cyst wall.

A23187, a Ca^{2+} ionophore, also induced *in vitro* excystment of *P. ohirai* although at a slower rate than Na cholate-induced excystment. A23187-induced excystment showed a similar inhibitory effect of proteinase inhibitors on excystment to the Na cholate-induced excystment. Chung *et al.* (1995) showed that DDT, a reducing agent, efficiently induced the *in vitro* excystment of *P. westermani* and the DTT-induced excystment was completely inhibited by E-64 and leupeptin. Thus, whatever stimulus was used for the *in vitro* excystment, cysteine proteinase inhibitors largely inhibited the *in vitro* excystment of *Paragonimus* species. These results confirm the involvement of cysteine proteinases in excystment. In other digeneans, cysteine proteinases seem to play a crucial role in excystment, as cysteine proteinases are major proteinases in secretions from metacercariae or excysted juveniles (Yamakami & Hamajima, 1988; Bock, 1989; Carmona *et al.*, 1993).

Between Na cholate-and A23187-induced excystment,

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the patterns of substrate specificity of cysteine proteinases present in the excystment secretions were markedly different. Moreover, the pattern of NEJ lysate clearly differed from the Na cholate and A23187. The results indicate that metacercarial juveniles of *P. ohirai* possess several cysteine proteinases with different substrate specificities. The presence of some distinct cysteine proteinases has been reported in digenetic trematodes, *Paragonimus* species (Yamakami, 1986; Yamakami & Hamajima, 1987; Yamakami *et al.*, 1995; Chung *et al.*, 1997; Yun *et al.*, 2000), *Fasciola hepatica* (Dalton & Heffernan, 1989; Smith *et al.*, 1993; Carmona *et al.*, 1993; Dowd *et al.*, 1994; Heussler & Dobbelaere, 1994), and *Schistosoma* species (Klinkert *et al.*, 1989; Merckelbach *et al.*, 1994; Smith *et al.*, 1994; Butler *et al.*, 1995). The difference in the substrate specificity patterns of Na cholate-and A23187-induced excystments suggests that the source of the secreted cysteine proteinases is not only one organ. In addition to the gut caeca, the anterodorsal glands opening at the anterior extremity of the head were proposed as sources of proteolytic enzymes for excystment (El-Mayas & Kearn, 1995).

Secretions during Na cholate-induced excystment showed higher proteinase activity towards Tos-Gly-Pro-Arg-MCA and Boc-Val-Leu-Lys-MCA amongst the five fluorogenic substrates. In contrast, secretions during A23187-induced excystment showed the highest activity for Tos-Gly-Pro-Arg-MCA, but a marked low activity for Boc-Val-Leu-Lys-MCA. In *P. westermani* metacercariae, neutral cysteine proteinase with a high activity towards Boc-Val-Leu-Lys-MCA was localized in the gut caeca (Hamajima *et al.*, 1985). The NEJ lysate containing the gut caeca with a relatively large portion of the body also showed the highest activity towards Boc-Val-Leu-Lys-MCA. These results suggest that Na cholate induces cysteine proteinase secretions mainly from the gut caeca, whereas A23187 induces cysteine proteinase secretions from organs other than the gut caeca, due to the low activity of the secretions for Boc-Val-Leu-Lys-MCA. Overnight incubation in Tyrode's medium induced A23187-type secretions. The NEJ lysate obtained by overnight incubation in Tyrode's medium stimulated similar proteinase secretions to metacercariae by Na cholate, but no longer stimulated proteinase secretions by A23187. The secretory bodies in the anterodorsal glands were much less distinct after excystment (El-Mays & Kearn, 1995). These results suggest that A23187 may stimulate proteinase secretions from the anterodorsal glands, but further investigations are necessary to support the above hypothesis.

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