

Identification and Characterization of Major Allergens in Excretory/Secretory Products of the Worm *Paragonimus ohirai*

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Key Words

Paragonimus ohirai · Cysteine proteinases · Excretory/secretory products · Paragonimiasis

acidic and neutral cysteine proteinases prepared from the ES products of *P. ohirai* will be useful allergens and antigens for the immunodiagnosis of paragonimiasis.

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Abstract

Allergens present in the excretory/secretory (ES) products of adult *Paragonimus ohirai* were biochemically identified. Immunoblot analysis using sera from *P. ohirai*-infected rats revealed only two allergens to be major proteins in the ES products, with apparent molecular masses (M_r) of 27 and 29 kD. As the ES products contained a high proportion of acidic and neutral cysteine proteinases, we examined whether or not the allergens and the cysteine proteinases were identical. The acidic and neutral cysteine proteinases were biochemically purified from the ES products and showed M_r of 27 and 29 kD, respectively. The two cysteine proteinases had almost identical N-terminal amino acid sequences and were reactive with specific IgE in sera from the infected rats. The allergenicity of the cysteine proteinases was confirmed by 48-hour homologous passive cutaneous anaphylaxis. Immunoblot and immunocapture assays using anti-human IgE monoclonal antibody showed that the proteinase allergens were reactive with specific IgE of patients with paragonimiasis *westermani*. Also, the cysteine proteinases were reactive with specific IgG of both the infected rats and the patients. Therefore the

Introduction

Many attempts have been made to isolate and biochemically characterize helminth allergens [1], since specific IgE production and increase in total IgE are unique features of helminthiasis [2–4]. However, many of these efforts have had limited success and the allergens have not been biochemically characterized or localized in detail. There have been very few reports describing the isolation, biochemical characterization, and localization of allergens of the lung fluke, genus *Paragonimus*. We found that the excretory/secretory (ES) products from excysted juveniles and adult worms of *P. ohirai* contained allergens [5]. In ELISA, the ES antigens of *P. westermani* are more sensitive to IgE of paragonimiasis patients than extracted antigens [6], and the major IgE-reactive antigens were localized in the gut epithelium and luminal contents of *P. westermani* [7]. As ES products contain a large amount of material released by the adult worm gut, we isolated and characterized the major allergens in the ES products of *P. ohirai*.

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Materials and Methods

Antigen Preparation

Adult worms of *P. ohirai* were recovered from the lungs of rats infected for 8–15 weeks. After washing in saline, the worms were incubated in 3 changes of saline for 2 h each at room temperature. The incubation solutions were combined and centrifuged at 20,000 *g* for 30 min at 4°C. The supernatant was concentrated by Amicon ultrafiltration, dialyzed with 10 mM phosphate-buffered saline (PBS, pH 7.2) containing 100 µM *p*-hydroxymercuriphenylsulfonate (HMPS) at 4°C, and then used as ES products. Adult worm extract was prepared by extracting homogenized adult worms with PBS/HMPS. After centrifugation of the homogenate at 20,000 *g* for 30 min at 4°C, the supernatant was dialyzed as described above.

Rat Sera and Patients' Pleural Effusions

Rats producing high levels of *P. ohirai*-specific IgE were obtained as described previously [5]. Briefly, outbred female Wistar rats (160–180 g) were infected intraperitoneally with 10 metacercariae of *P. ohirai* X-irradiated at 2 krad, then challenged 6 weeks later by intraperitoneal infection with 10 normal metacercariae. The rats were bled 1 or 2 weeks after challenge infection and sera were separated. Pleural effusions were obtained from Japanese patients with paragonimiasis westermani or pulmonary tuberculosis.

SDS-PAGE and Immunoblot

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting were performed as described previously [8]. Briefly, samples to which *L*-trans-epoxysuccinyl-leucylamide-(4-guanidino)-butane (E-64) was added at a final concentration of 50 µM were heated with 5% β-mercaptoethanol, 2% SDS, and 62.5 mM Tris-HCl buffer (pH 6.8) for 2 min in boiling water, then resolved by electrophoresis in an SDS-PAGE system. The gel was electroblotted onto a membrane, which was washed with PBS containing 0.05% Tween 20 (PBS/T). Strips of the membrane were incubated in blocking solution (2% inactivated normal rabbit serum, 2% BSA in PBS). To detect the allergens, the strips were treated with 1:10 diluted sera of the challenge-infected rats, biotin-conjugated anti-rat IgE monoclonal antibody (mAb) (Zymed Laboratories Inc., San Francisco, Calif., USA) diluted to 1:200, horseradish peroxidase-conjugated avidin D (Vector Laboratories Inc., Burlingame, Calif., USA) diluted to 1:500, and 0.02% 3,3'-diaminobenzidine substrate. Between successive incubations, the strips were washed 5 times in PBS/T. To detect the antigens, the strips were treated with rat sera diluted to 1:200 and peroxidase-conjugated goat anti-rat IgG (Cappel Laboratories, Westchester, Pa., USA) diluted to 1:1,000. When the strips were incubated in pleural effusions (1:40 and 1:400) of patients with paragonimiasis westermani, they were treated with peroxidase-conjugated rat anti-human IgE mAb (Experimental Immunology Unit, Louvain, Belgium) and goat anti-human IgG (Cappel) diluted to 1:1,000, respectively.

Assays of Cysteine Proteinase

The activities of acidic and neutral cysteine proteinases were measured essentially as described by Yamakami [9], and Yamakami and Hamajima [10], respectively. In an assay of the acidic hemoglobinolytic activity, 25 µl of each sample were added to 75 µl of 0.2 M acetate buffer (pH 4.0) containing 0.5 mg of bovine hemoglobin (Sigma Chemical Co., St. Louis, Mo., USA) and 5 mM *L*-cysteine. After the reaction mixture had been incubated at 37°C for 2 h, 25 µl of

12% TCA was added. The TCA soluble products were measured for absorbance at 280 nm. To assay azocollolytic activity, 50 µl of each sample was added to 150 µl of 0.2 M Tris-HCl (pH 8.6) containing 1 mg azocoll and 5 mM *L*-cysteine, then incubated at 37°C for 1–6 h. After the reactive mixture had been centrifuged at 4°C, 100 µl of the supernatant was measured at 550 nm on an ELISA microplate in a SLT EASY READER (SLT Labinstruments GmbH, Grödig, Austria). To assay the proteinases using the synthetic peptide substrates, 10 µl of the sample was added to 100 µl of 5 mM *L*-cysteine and 0.25 M sodium acetate buffer (pH 5.0) containing 20 µM Z-Phe-Arg-MCA, or 0.25 M imidazole-HCl buffer (pH 7.5) containing 20 µM Boc-Val-Leu-Lys-MCA (Peptide Institute, Osaka, Japan). After the reaction mixture had been incubated for 5 min at room temperature, the enzyme activity was measured in a Fluoroskan II (Labsystems, Espoo, Finland) at excitation and emission wavelengths of 355 and 460 nm, respectively. Protein concentration was determined by BCA Protein Assay (Pierce, Rockford, Ill., USA).

Purification of Cysteine Proteinases

The ES products were adjusted to 40% ammonium sulfate saturation and centrifuged at 8,000 *g* for 20 min. The supernatant was 75% saturated with ammonium sulfate, then centrifuged. The precipitate was dissolved in starting buffer (25 mM sodium acetate buffer, pH 6.0, containing 0.2 M ammonium sulfate) and applied to a phenyl-cellulofine column (0.8 × 3.5 cm, Seikagaku Kogyo Co., Tokyo, Japan) equilibrated with the starting buffer. The column was eluted stepwise with starting buffer, buffer alone, then distilled water. The active fractions eluted with distilled water were combined and applied to an arginine-Sepharose 4B column (0.8 × 2 cm, Pharmacia Fine Chemicals, Uppsala, Sweden) equilibrated with 25 mM Tris-HCl buffer (pH 6.8). The sample was eluted stepwise with the above buffer, then with the same buffer containing 0.15 M and 0.5 M NaCl. The active fractions eluted with the buffer containing 0.5 M NaCl were combined and dialyzed against 25 mM Tris-HCl buffer (pH 7.3) or 50 mM Tris-HCl buffer (pH 8.0), then applied to a DEAE-Biogel column (0.8 × 2 cm, Bio-Rad Laboratories, Richmond, Calif., USA) equilibrated with the above Tris-HCl buffers. The proteinases were eluted stepwise with the buffers containing various concentrations of NaCl.

N-Terminal Sequence Analysis

The active fraction from arginine-Sepharose 4B chromatography was concentrated by using a Centricon-10 centrifugal concentrator (Amicon Inc., Beverly, Mass., USA). The sample was separated by reduced SDS-PAGE and electroblotted to PVDF Transfer Membrane (Millipore Co., Bedford, Mass., USA). The membrane was stained with Coomassie brilliant blue R-250. The stained bands were analyzed for N-terminal amino acid sequence using a Shimadzu protein sequencer.

Homologous Passive Cutaneous Anaphylaxis

Homologous passive cutaneous anaphylaxis (PCA) was performed as described previously [11]. Briefly, 50 µl of 2-fold serially-diluted serum of the challenge-infected rat was injected intradermally into the shaved back of a female Wistar rat (160–200 g). Forty-eight hours later, the recipient rat was challenged with an intravenous injection of 1 ml of 1% Evans blue containing ES products (500 µg protein) or partially purified cysteine proteinases (eluted with 0.5 M NaCl on arginine-Sepharose 4B, 50 µg protein). The skin reactions were examined after 30 min.

Table 1. Proteolytic activities of ES products from adult *Paragonimus ohirai*

Substrate	pH	Enzyme activity		
		with cysteine	without cysteine	with cysteine and E-64
Hemoglobin, A ₂₈₀ /min/mg protein	4.0	3.37	1.78	0.28
Azocoll, A ₅₅₀ /min/mg protein	4.0	3.17	0.40	0
Azocoll, A ₅₅₀ /min/mg protein	8.6	4.20	0.06	0
Z-Phe-Arg-MCA, MCA*/min/mg protein	5.0	552	0	0
Boc-Val-Leu-Lys-MCA, MCA*/min/mg protein	7.5	893	0	0

ES products were assayed for proteolytic activities as described in Materials and Methods. Each value represents the average of triplicate samples incubated separately.

* nmoles MCA released.

Immunocapture Assay

An mAb immunocapture assay was performed essentially as described in the protein A immunocapture assay by Ikeda [12]. Briefly, wells of microtiter plates were sensitized with mouse anti-human IgE mAb (10 µg/ml PBS) (Cosmo Bio, Tokyo, Japan). After blocking with 2% BSA/PBS, the wells were incubated overnight at 4°C with pleural effusions or sera diluted 1:40 with BSA/PBS/T, then incubated for 2 h at 4°C with the fraction containing partially purified cysteine proteinases (0.15 µg). Whenever the incubation solutions were exchanged, the wells were washed 5 times with PBS/T. After the final washing, fluorogenic peptidyl substrates (20 µM) (Peptide Institute) were added to the wells. The fluorescent products released after incubation for 1 h at 30°C were measured. The protein A immunocapture assay was performed as described previously [12], except that the above proteinase fraction was used as the source of proteinase antigens in place of ES products of *P. westermani*.

Results

Allergens in the ES products of adult *P. ohirai* were examined by reduced SDS-PAGE and subsequent immunoblotting. As shown in figure 1, lanes a, b, although several antigens were detected with specific IgG in serum from a challenge-infected rat, only two allergens were detected with specific IgE, and an apparent molecular mass (M_r) of 27 and 29 kD. The profile of the allergen bands was the same in the sera from all of the 5 infected rats examined, whereas no band was detected in normal rat sera (fig. 1, lane c).

The ES products contained a large amount of acidic and neutral cysteine proteinases which hydrolyzed hemoglobin, azocoll, and synthetic peptides. The proteinases were sensitive to the cysteine proteinase inhibitor, E-64, and were markedly activated by, or dependent upon, the addition of cysteine (table 1). The smaller of the two allergens showed an M_r identical to an acidic cysteine proteinase purified from the homogenate of adult *P. ohirai* by

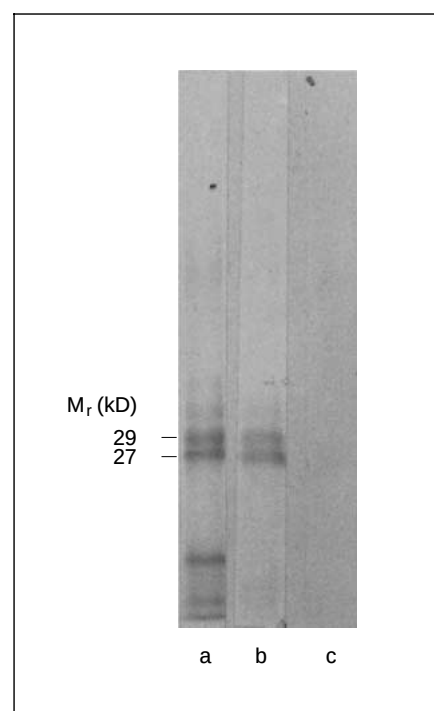


Fig. 1. Immunoblots of antigens recognized by specific IgG and IgE in the ES products of adult *P. ohirai*. Lane a: antigens recognized by specific IgG in serum from a rat challenge-infected with *P. ohirai*; lane b: allergens recognized by specific IgE; lane c: immunostaining with serum from a normal rat.

Yamakami [9]. To examine whether the cysteine proteinases were the allergens, the proteinases were purified from the ES products.

The 40–75% ammonium sulfate fractions of the ES products were separated by means of hydrophobic chromatography on phenyl-cellulofine. As shown in figure 2a,

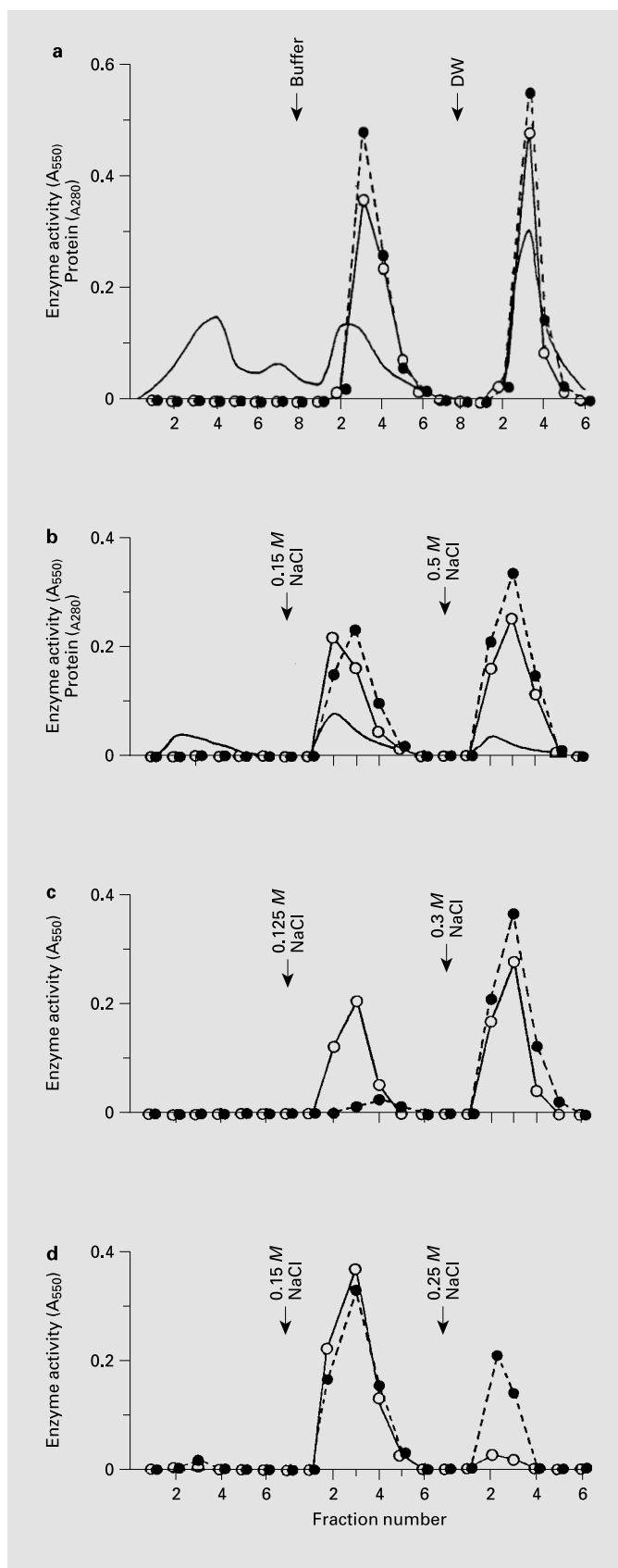


Fig. 2. Chromatography of azocolytic cysteine proteinases from the ES products of adult *P. ohirai*. a Phenyl-cellulofine chromatography of the 40–75% ammonium sulfate fraction. b Arginine-Sepharose 4B chromatography of the active fraction eluted with distilled water (DW) shown in a. c, d DEAE-Biogel chromatography of the active fraction eluted with 0.5 M NaCl shown in b, under pH 7.4 and pH 8.0 conditions, respectively. Fractions of 0.7 ml were monitored by their absorbance at 280 nm (—○—) and assayed for azocolytic cysteine proteinases under acidic (—○—) or neutral (---●---) conditions. Enzyme activity was measured by incubation at 37°C for 1 (a), 2 (b), or 6 h (c, d).

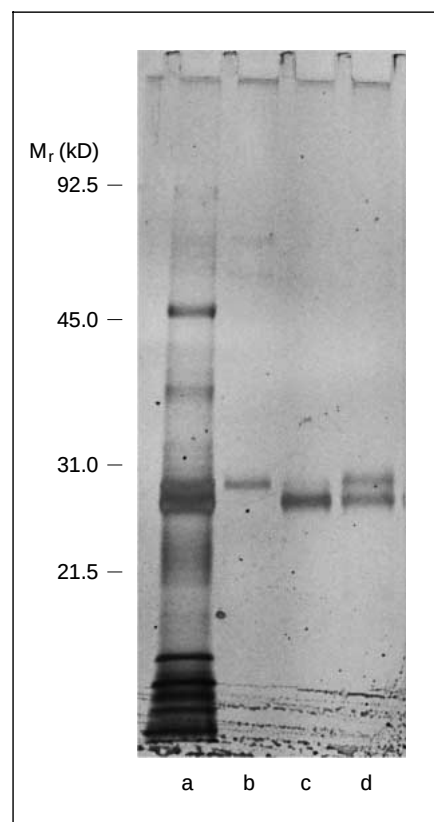


Fig. 3. Silver-stained SDS-PAGE gel of cysteine proteinases purified from the ES products of adult *P. ohirai*. Lane a: the ES products; lane b: eluted with 0.25 M NaCl from DEAE-Biogel (pH 8.0); lane c: eluted with 0.125 M NaCl from DEAE-Biogel (pH 7.4) chromatography; lane d: eluted with 0.5 M NaCl from arginine-Sepharose 4B chromatography.

Table 2. N-terminal amino acid sequences of cysteine proteinases of *Paragonimus ohirai* adult worm

Species	Stage	M _r	N-terminal amino acid sequences																				
<i>P. ohirai</i>	adult	27 kD	A	A	P	E	S	I ¹	D	W	R	E	K	G	A	V	T	P	V	E	X	Q	G
	adult	29 kD	A	A	P	E	R	I ¹	D	W	R	E	K	G	A	V	T	P	V	E	N	Q	G
<i>P. westermani</i>	metacercaria	22 kD		A	P	E	S	I	D	W	R	E	K	G	A	V	A	P	V	E	D	Q	G
	adult			A	P	E	R	M	D	W	R	E	W	G	A	V	G	P	V	E	N	Q	G

The 27- and 29-kD bands shown in figure 3 were analyzed. Boxed amino acid residues were the unanimously homologous ones among the acid and neutral cysteine proteinases of the *P. ohirai* adult worm, the neutral cysteine proteinase of *P. westermani* metacercariae [12] and the cysteine proteinase of *P. westermani* adult worm (amino acid sequence estimated from nucleotide sequence of cDNA) [13].

¹ Phenylalanine was detected at a low level.

the acidic and neutral cysteine proteinases were eluted with the buffer alone and with distilled water. Active fractions eluted with distilled water were fractionated by affinity chromatography on arginine-Sepharose 4B. This chromatography also provided a mixed fraction of the two cysteine proteinases (fig. 2b). On reduced SDS-PAGE, the ES products migrated as numerous protein bands (fig. 3, lane a), although there were much fewer bands than in adult worm extract, whereas the active fractions eluted with 0.5 M NaCl resolved into 2 protein bands with M_r of 27 and 29 kD (fig. 3, lane d). The 0.5 M NaCl fractions from arginine-Sepharose 4B were dialyzed against 25 mM Tris buffer (pH 7.4) or 50 mM Tris buffer (pH 8.0), then purified by ion exchange chromatography on DEAE-Biogel. As shown in figure 2c, fractions containing only acidic cysteine proteinase activity were obtained by elution with 0.125 M NaCl on DEAE-Biogel under pH 7.4 conditions, although much of the proteinase was eluted together with neutral cysteine proteinase with 0.3 M NaCl. Similarly, fractions containing only neutral cysteine proteinase activity were obtained by elution with 0.25 M NaCl after large amounts of the two cysteine proteinases had been eluted with 0.15 M NaCl under pH 8.0 conditions (fig. 2d). Reduced SDS-PAGE analysis of these purified acidic cysteine proteinase and the neutral cysteine proteinase revealed a single protein band with M_r 27 and 29 kD, respectively (fig. 3, lanes b, c).

For the analysis of the N-terminal amino acid sequence of the two cysteine proteinases, the 27- and 29-kD protein bands in the 0.5 M NaCl fraction from arginine-Sepharose 4B chromatography were analyzed due to insufficient amounts of purified proteinases. As can be seen in table 2, the two protein bands had an almost identical N-terminal sequence (19 of 20 amino acid residues; 95%). Phenylalanine was detected at a low level at the sixth amino acid

residue from the N-terminal end. When this amino acid sequence was compared with those of a neutral cysteine proteinase with M_r 22 kD purified from *P. westermani* metacercariae [13] and a cysteine proteinase cDNA cloned from *P. westermani* adults [14], absolutely identical residues of the 4 cysteine proteinases were found in 15 of 20 residues (75%). The cysteine proteinases of *P. ohirai* had an additional alanine residue at the N-terminal end compared with the cysteine proteinase of *P. westermani* metacercariae.

When the partially purified cysteine proteinases (eluted with 0.5 M NaCl on arginine-Sepharose 4B) and the purified acidic and neutral cysteine proteinases were immunoblotted using anti-human IgE mAb and pleural effusion from a paragonimiasis westermani patient with a high level of specific IgE for *P. westermani*, the two cysteine proteinases were reactive with human specific IgE (fig. 4). The 27- and 29-kD bands of the proteinases were reactive with both specific IgE and IgG in the pleural effusions from the other two patients with paragonimiasis westermani and in the serum from the challenge-infected rat (data not shown). Control pleural effusions from three patients with pulmonary tuberculosis did not yield any positive bands. The allergenicity of the partially purified proteinases was confirmed by rat 48-hour homologous PCA using serum of the challenge-infected rat. The injection of the partially purified cysteine proteinases (50 µg) elicited a PCA titer (1:500) that was identical to that of the whole ES products (500 µg protein).

To confirm specific human IgE reactivity to the acidic and neutral cysteine proteinases, an immunocapture assay using anti-human IgE mAb was applied to pleural effusions from three patients with paragonimiasis westermani. As shown in figure 5a, all pleural effusions from paragonimiasis patients showed obviously higher hydro-

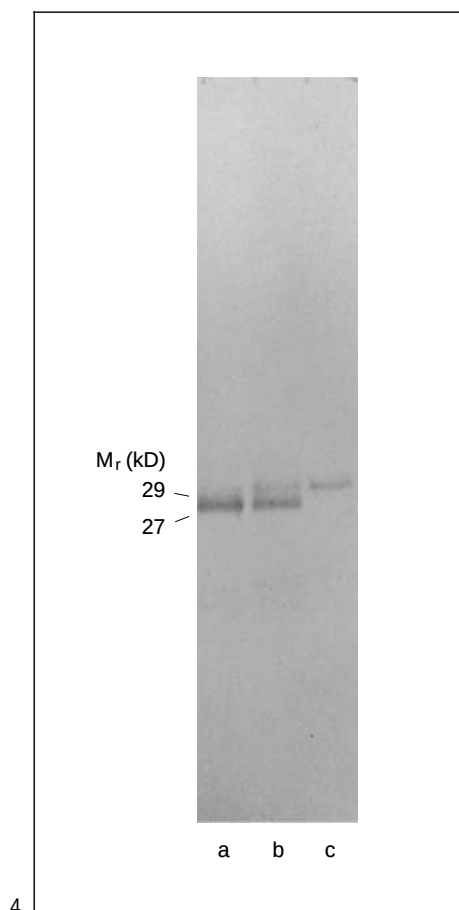
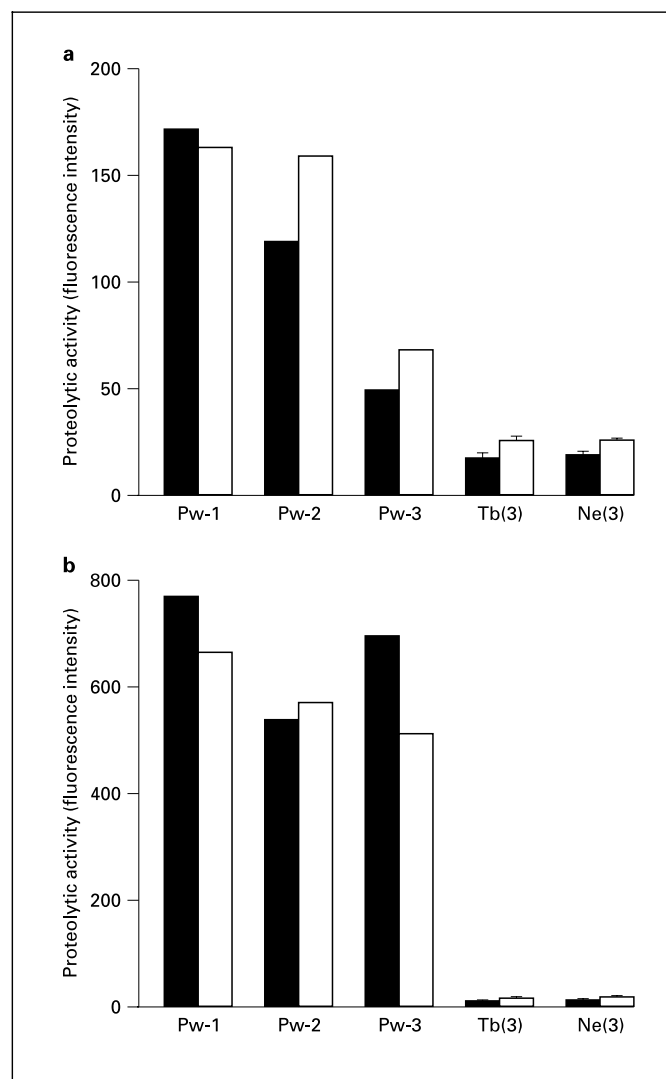


Fig. 4. Immunoblots of purified cysteine proteinases with specific IgE in the pleural effusion from a paragonimiasis westermani patient. Lane a: eluted with 0.125 M NaCl from DEAE-Biogel (pH 7.4) chromatography; lane b: eluted with 0.5 M NaCl from arginine-Sepharose 4B chromatography; lane c: eluted with 0.25 M NaCl from DEAE-Biogel (pH 8.0).

Fig. 5. Reactivity of specific human IgE and IgG to acidic and neutral cysteine proteinases of *P. ohirai* in immunocapture assays. Pleural effusions and sera diluted 1:40 and 1:2,000 were incubated overnight at 4°C in plate wells sensitized with anti-human IgE mAb and protein A, respectively. The acidic and neutral cysteine proteinases captured on the wells were measured in 0.25 M sodium acetate buffer (pH 5.0) containing 5 mM cysteine and 20 µM Z-Phe-Arg-MCA (■), and 0.25 M imidazole-HCl buffer (pH 7.5) containing cysteine and



Boc-Val-Leu-Lys-MCA (□) for 1 h (specific IgE) or 30 min (specific IgG) at 30°C. Pw-1–3 = Pleural effusion from each patient with paragonimiasis westermani. Tb(3) = Mean value + SD of the pleural effusions from three patients with pulmonary tuberculosis. Ne(3) = Mean value + SD of the sera from three patients with nematode diseases. Each individual patient value is the mean fluorescence intensity of triplicate wells.

lytic activities for Z-Phe-Arg-MCA at pH 5.0 and Boc-Val-Leu-Lys-MCA at pH 7.5, compared with those of pleural effusions from three patients with pulmonary tuberculosis and to sera with high total IgE levels from three patients with anisakiasis or toxocariasis. Moreover, an immunocapture assay using protein A confirmed specific human IgG reactivity to the two proteinases (fig. 5b).

Discussion

Although there are several reports describing parasite allergens, few allergens have been shown to possess biological and biochemical activity other than their allergenicity. This study clarified that the two allergens present in the ES products of adult *P. ohirai* are cysteine proteinases, one is acidic and the other is neutral. In schistosomi-

asis mansonii, although the major allergens of the parasite are antigens derived from eggs [15], an acidic proteinase present in the adult worm is also reportedly an allergen with high specificity, but low sensitivity in an immediate skin reaction test [16]. The acidic proteinase was shown to be a cysteine proteinase [17, 18]. The findings from *Schistosoma mansoni* and *P. ohirai* indicate that the acidic cysteine proteinases of other parasitic trematodes might also be allergenic. Moreover, in house dust mite allergens causing atopic asthma and allergenic rhinitis, one of the major allergens is a cysteine proteinase [19, 20].

In contrast with studies of acidic cysteine proteinases in helminth parasites, there have been few investigations into neutral cysteine proteinases. This study showed that the neutral cysteine proteinase of *P. ohirai* is also allergenic. We did not determine whether the two cysteine proteinases possess similar or different allergen epitopes. The two cysteine proteinases have markedly similar biochemical characteristics, as it was difficult to separate the two proteinases efficiently by various means. Analysis of the N-terminal amino acid sequences confirmed the markedly similar biochemical characteristics of the two proteinases. We prepared an mAb reactive to the 29- and 27-kD cysteine proteinases of *P. ohirai* [21], thinking that the two cysteine proteinases might possess cross-reactive allergenic epitopes. Although many kinds of cysteine proteinases have been reported in *S. mansoni* [22, 23], their allergenicity remains unknown.

The acidic and neutral cysteine proteinases of adult *P. ohirai* were reactive on immunoblots against specific IgE of infected rat sera, and their allergenicity was confirmed by 48-hour homologous PCA. Since the two cysteine proteinases constitute a high proportion of the ES products, they are probably released continuously from the parasite into the host. Thus, continuous antigen stimulation may be an important factor for inducing IgE antibody formation, since efforts to induce IgE antibody formation by injecting parasite-derived allergens without adjuvant into experimental animals have rarely succeeded, even though most helminth parasites can elicit an IgE antibody response in their hosts.

Allergen cross-reactivity among adult antigens extracted from *P. westermani*, *P. miyazakii*, and *P. ohirai* was shown in the immediate skin reaction of paragonimiasis patients [24]. We found that the specific IgE of patients with paragonimiasis westermani intensely reacted with the gut epithelium and luminal contents of adult *P. ohirai* and *P. miyazakii*, as well as those of adult *P. westermani* [7]. An immunoblot and mAb immunocapture assay in the present study showed that specific IgE of

patients with paragonimiasis westermani recognized the two cysteine proteinases of *P. ohirai*. Moreover, an immune serum against a neutral cysteine proteinase of *P. westermani* metacercariae reacted with the gut epithelium of the juvenile [25], and an mAb recognizing the acidic and neutral cysteine proteinases of adult *P. ohirai* also reacted with the gut epithelium and luminal contents of adult worms of these three *Paragonimus* species [21]. These results confirm that the cross-reactive allergens among the above *Paragonimus* species are the acidic and neutral cysteine proteinases formed in the gut epithelium, secreted into the gut lumen, and finally released from the fluke.

The acid and neutral cysteine proteinases of *P. ohirai* were also recognized by specific IgG of the patients with paragonimiasis westermani as well as that of the infected rats. The N-terminal amino acid sequences of the *P. ohirai* cysteine proteinases were highly homologous with the neutral cysteine proteinase from *P. westermani* metacercariae [12] and the cysteine proteinase from *P. westermani* adult [13]. Antigenic and allergenic cross-reactivity among cysteine proteinases from different *Paragonimus* species can be explained by the high homology of the N-terminal amino acid sequence. Therefore, the two cysteine proteinases of adult *P. ohirai*, which can be obtained more easily in larger quantities than cysteine proteinases of other human-related *Paragonimus* species, will provide useful allergens and antigens for the immunodiagnosis of human paragonimiasis.

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