Journal of Helminthology (2001) **75,** 245–249 DOI: 10.1079/JOH200161

Protein A immunocapture assay detecting antibodies to fluke cysteine proteinases for immunodiagnosis of human paragonimiasis and fascioliasis

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Abstract

Enzyme-linked immunosorbent assays (ELISAs) which detect specific antibodies to fluke cysteine proteinases have provided good sensitivity and specificity for the immunodiagnosis of trematode diseases. To detect specific antibodies without the need for purified proteinase antigens, an immunocapture assay using Protein A was applied for the immunodiagnosis of paragonimiasis and fascioliasis. ELISA plate wells were coated with Protein A, incubated with diluted patient sera, then incubated with a preparation containing fluke cysteine proteinases, excretory–secretory (ES) products of adult *Paragonimus westermani* or *Fasciola* sp. The activity of fluke cysteine proteinases bound on the wells was measured by adding fluorogenic peptidyl substrate, Z-Phe-Arg-MCA or Boc-Val-Leu-Lys-MCA. This assay detected specific immunoglobulin G to cysteine proteinases of *P. westermani* and *Fasciola* sp. by measuring proteinase activity on the plate wells. Patient sera showed significant high values of proteinase activity when the wells were treated with the respective homologous ES products, whereas the sera had low values after treatment with the heterologous ES products. The sera of patients with other parasitoses and uninfected healthy individuals also showed low values after treatment with the above fluke ES products. Thus, Protein A immunocapture assay, which detected IgG specific for fluke cysteine proteinases, provided a high sensitivity and specificity for immunodiagnosis of paragonimiasis and fascioliasis.

Introduction

Cysteine proteinases of parasitic trematodes play important roles in the digestion of proteins in the gut and the histolysis of host tissues during parasite migration. Moreover, proteinases are major antigens for their hosts because they are continuously released by the flukes. Therefore, fluke cysteine proteinases have been used as enzyme-linked immunosorbent assay (ELISA) immunodiagnostic antigens (Yamasaki *et al.*, 1989; Chappell *et al.*, 1990; Ikeda *et al.*, 1996; O'Neill *et al.*, 1998). However, conventional ELISAs require purified cysteine proteinase antigens which are prepared by a complex and time-consuming process, with a large loss of enzyme recovery. In order to measure the level of specific antibodies without the need of purified cysteine proteinase antigens, an ELISA using chicken cystatin, a protein inhibitor of cysteine proteinase, as a capture agent was developed (Ikeda, 1998).

The present study evaluates an alternative method that satisfied the above purpose. Previously, an immunodiagnosis method which used Protein A as a capture agent for immunoglobulin G (IgG) and detected IgG specific for alkaline phosphatase of *Schistosoma mansoni* on

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microtitre plates was reported (Pujol *et al.*, 1989). The Protein A immunocapture assay was applied to detect specific IgG to fluke cysteine proteinases. The proteinases, which were bound by specific IgG captured on Protein A-coated plates, were measured using fluorogenic peptidyl substrates.

Materials and methods

Antigens

The excretory–secretory (ES) products of trematodes, which contain large amounts of fluke cysteine proteinases were used as cysteine proteinase-rich preparations. The ES products of adult *Paragonimus westermani* and *Fasciola* sp. were prepared as previously described (Ikeda *et al.*, 1992; Ikeda, 1998). Adult worms of *P. westermani* and *Fasciola* sp. were recovered from infected dogs and rats, respectively. Washed worms were incubated in three changes of 0.9% saline (2 h per change) at room temperature. The incubation solutions were pooled and centrifuged at 20,000×*g* for 30 min. The supernatants were concentrated by using Amicon ultrafiltration cell, dialysed against 10 mM of phosphate-buffered saline (PBS, pH 7.2) at 4°C and then used as ES products.

Protein A immunocapture assay

Wells of microtitre plates (Nunc, Roskilde, Denmark) were sensitized overnight at 4°C with 1 μg of Protein A (Sigma, St Louis, Missouri) in 0.1 ml of PBS. After the wells were washed with PBS containing 0.05% Tween 20 (PBS/T), they were treated with 0.1 ml of 2% bovine serum albumin (BSA) (Sigma) in 10 mM of PBS for 1 h at room temperature, washed with PBS/T and then stored at −20°C until use. The wells were incubated for 2 h at room temperature with diluted human sera. After washing, the wells were incubated for 1 h at 4°C with ES products of either adult *P. westermani* or *Fasciola* sp. (1.5 μg of protein per 0.1 ml) in BSA/PBS/T. The wells were washed five times with PBS/T and once with distilled water, then fluorogenic peptidyl substrates (40 μM) (Peptide Institute, Osaka, Japan) were added to the wells. Fluorescent products, 7-amino-4-methyl coumarin (AMC), released after incubation at 30°C were measured in a Flow Fluoroscan II plate reader (Labsystems, Helsinki, Finland) at excitation and emission wavelengths of 355 and 460 nm, respectively. Background fluorescence obtained when treatment with diluted human sera was omitted from a series of the above procedures showed a negligible level and was subtracted from the fluorescence values of serum-treated wells.

Serum samples

Patient sera were prepared as described by Ikeda *et al.* (1996) and Ikeda (1998). Paragonimiasis and fascioliasis sera were obtained from Japanese patients confirmed parasitologically or who showed clinical signs, dietary history, and/or a positive immunodiagnosis. Clonorchiasis and anisakiasis sera were obtained from parasitologically confirmed Japanese patients. Patient sera were also obtained from Philippine patients with schistosomiasis japonicum and Japanese and Chinese patients with cysticerciasis. Control sera were obtained from uninfected healthy Japanese volunteers.

Results

Little is known about the hydrolytic activity of ES products of adult *Paragonimus* and *Fasciola* flukes towards various peptidyl fluorogenic substrates. Excretory–secretory products of adult *P. westermani* and *Fasciola* sp. were tested for hydrolytic activity for various fluorogenic peptidyl substrates at pH 7.5 and 5.5. Both ES products highly hydrolysed Boc-Val-Leu-Lys-MCA and Z-Phe-Arg-MCA but hydrolysed Tosy-Gly-Pro-Arg-MCA and Boc-Val-Pro-Arg-MCA much less (table 1). Suc-Leu-Leu-Val-Tyr-MCA was also slightly hydrolysed,

Table 1. Hydrolytic activity of ES products of adult *Paragonimus westermani* and *Fasciola* sp. towards various peptidyl fluorogenic substrates.

Substrate	*P. westermani* ES		*Fasciola* sp. ES	
	pH 7.5	pH 5.5	pH 7.5	pH 5.5
Boc-Val-Leu-Lys-MCA	1082 (131)	905 (66)	1767 (202)	1351 (63)
Boc-Val-Pro-Arg-MCA	167 (153)	72 (20)	153 (0)	97 (4)
Suc-Ala-Ala-Ala-MCA	8 (0)	0	0	0
Suc-Gly-Pro-Leu-Gly-Pro-MCA	0	0	0	0
Suc-Leu-Leu-Val-Tyr-MCA	104 (10)	68 (4)	22 (0)	9 (0)
Tosy-Gly-Pro-Arg-MCA	360 (22)	274 (15)	138 (8)	184 (11)
Z-Arg-Arg-MCA	88 (3)	18 (10)	3 (0)	0
Z-Phe-Arg-MCA	1046 (109)	912 (55)	1517 (154)	1385 (121)
Ala-MCA	0	0	0	0
Arg-MCA	0	0	0	0
Leu-MCA	5 (0)	0	0	0
Phe-MCA	0	0	0	0

Each value is the mean fluorescence intensity of triplicate wells.
Activities were measured in 0.25 M Tris-HCl buffer (pH 7.5) and 0.25 M acetate-NaOH buffer (pH 5.5) with or without (in parentheses) 5 mM of cysteine for 30 min at 30°C.

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and Z-Arg-Arg-MCA was hydrolysed only by *Paragonimus* ES products. These hydrolytic activities markedly decreased in the absence of 5 mM of cysteine. E-64, a cysteine proteinase inhibitor, completely inhibited these hydrolytic activities (data not shown).

The conditions of Protein A immunocapture assay were determined using sera from fascioliasis patients with a relatively high ($A_{405} = 1.83$), medium (1.47), or low (0.81) value of cystatin capture ELISA (Ikeda, 1998) detecting antibodies against fluke cysteine proteinases and from an uninfected healthy volunteer. The effect of incubation time for patient sera in Protein A-coated wells on Protein A immunocapture assay values was examined (fig. 1). The serum with the higher level almost reached a plateau value of hydrolytic activity for Boc-Val-Leu-Lys-MCA at 1 h after incubation. The serum with the lower level showed 70, 86, and 94% of the 8-h value at 1, 2, and 4 h after incubation, respectively, compared with the value after 8 h of incubation. A 2-h incubation period was selected. The appropriate dilution of sera and reaction time of substrates were determined. In serum with a high ELISA level, the hydrolytic activity for Boc-Val-Leu-Lys-MCA was linear with time during 30 min at all serum dilutions from 1:500 to 1:16,000 (fig. 2A). Sera diluted from 1:500 to 1:2000 showed almost identical curves. In serum with a medium ELISA level, the hydrolytic activity was linear with time during 45 min at all serum dilutions (fig. 2B). At serum dilutions of 1:4000 and 1:8000, linear reactions were observed for 60 min. A control serum showed a negligible value even at a serum dilution of 1:500. Therefore, a serum dilution of 1:2000 and a substrate reaction time of 30 min were selected for immunodiagnosis of fascioliasis in this assay. The linear reaction in hydrolytic activity for Z-Phe-Arg-MCA was also confirmed at the above conditions in a serum of a paragonimiasis westermani patient with a high ELISA level against fluke cysteine proteinases (data not shown).

A Protein A immunocapture assay using sera from a paragonimiasis and a fascioliasis patient with a medium ELISA level was used to determine the pH profiles of hydrolytic activity for Boc-Val-Leu-Lys-MCA and Z-Phe-Arg-MCA (fig. 3). The pH profile for Boc-Val-Leu-Lys-MCA was similar to the result obtained from fluke ES products, but that for Z-Phe-Arg-MCA was different at an acidic pH. The assay values for Z-Phe-Arg-MCA at pH 4 were similar to those at pH 7.5 showing peak activity, whereas the proteinase activities of ES products at pH 4.0 were much lower than those at pH 7.5 and 5.5 (data not shown). Z-Phe-Arg-MCA for a paragonimiasis

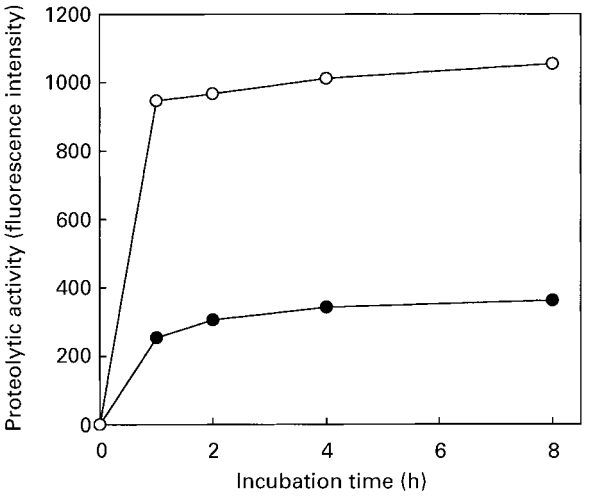

Fig. 1. Effect of incubation time of patient sera on Protein A immunocapture assay. Two fascioliasis sera with high (○) and low (●) ELISA levels against fluke cysteine proteinases were examined using Boc-Val-Leu-Lys-MCA at pH 7.5. Each point is the mean of triplicate wells.

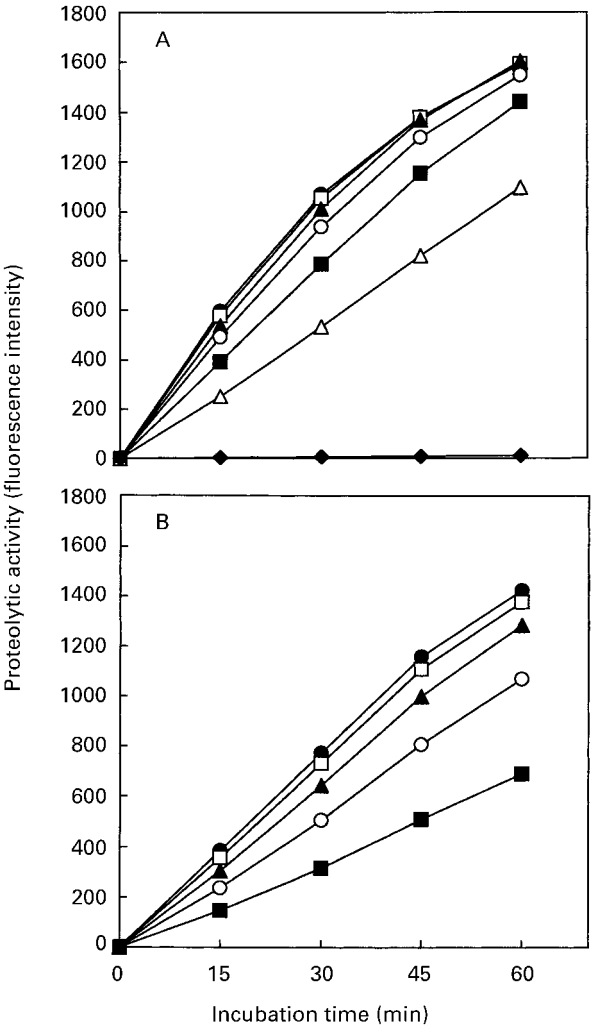

Fig. 2. Effect of serum dilution and incubation time for Boc-Val-Leu-Lys-MCA reaction solution on Protein A immunocapture assay. Diluted fascioliasis sera with high (A) and medium (B) ELISA levels were added into Protein A-coated wells, then incubated for 2 h at room temperature. 1:500 (●), 1:1000 (□), 1:2000 (▲), 1:4000 (O), 1:8000 (■), 1:16,000 (△). A healthy control serum diluted to 1:500(◆) was also examined. Each point is the mean of triplicate wells.

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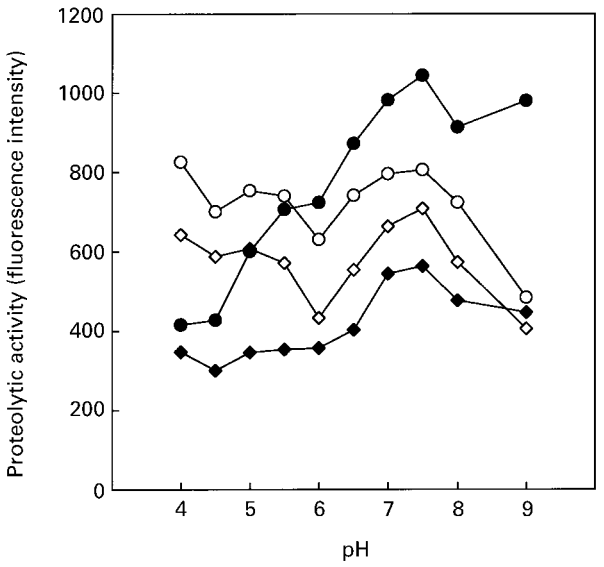

Fig. 3. Effect of pH of substrate solution on Protein A immunocapture assay. Diluted sera (1:2000) with paragonimiasis (square) and fascioliasis (circle) and with a medium ELISA level were incubated in Protein A-coated wells for 2 h, in fluke ES products for 1 h and then in various pH buffers containing Boc-Val-Leu-Lys-MCA (closed) or Z-Phe-Arg-MCA (open) for 30 min.

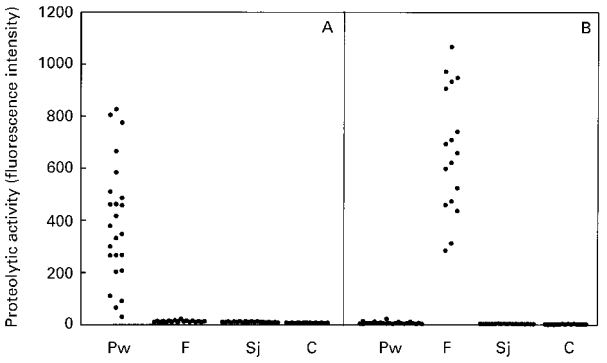

Fig. 4. Protein A immunocapture assay using fluke ES products in sera from patients with paragonimiasis westermani, fascioliasis, and schistosomiasis japonicum and from uninfected healthy individuals. The assay was determined using *Paragonimus* ES products and Z-Phe-Arg-MCA substrate (A) and using *Fasciola* ES products and Boc-Val-Leu-Lys-MCA (B). Each point is the mean value of triplicate wells. Pw, paragonimiasis westermani sera ($n = 24$); F, fascioliasis sera ($n = 17$); Sj, schistosomiasis japonicum sera ($n = 15$), C, healthy control sera ($n = 10$).

serum and Boc-Val-Leu-Lys-MCA for a fascioliasis serum were higher than the reverse combination. Therefore, assays for paragonimiasis and fascioliasis used Z-Phe-Arg-MCA and Boc-Val-Leu-Lys-MCA substrates, respectively.

Protein A immunocapture assay was examined in sera from patients with paragonimiasis westermani, fascioliasis, or schistosomiasis japonicum and from uninfected healthy individuals. In paragonimiasis and fascioliasis sera, Protein A-coated plate wells, which were treated with the patient sera and then with the respective homologous fluke ES products, showed significant hydrolytic activity for Z-Phe-Arg-MCA and Boc-Val-Leu-Lys-MCA (fig. 4). Treatment with the heterologous ES products resulted in low activity. Sera from patients with schistosomiasis japonicum and from healthy individuals also showed a low activity in wells treated with *P. westermani* or *Fasciola* ES products. Also, sera from patients with clonorchiasis, anisakiasis, and cysticerciasis showed low activity after treatment with the above two ES products (data not shown). All experiments were performed at least twice and the same results were obtained.

Discussion

Excretory–secretory products obtained from adult flukes of *P. westermani* and *Fasciola* sp. showed high hydrolytic activity for Boc-Val-Leu-Lys-MCA and Z-Phe-Arg-MCA substrates. The hydrolytic activities were due to cysteine proteinases due to the dependency on cysteine and the susceptibility to E-64. It has also been shown that cysteine proteinases purified from *P. westermani* and *F. hepatica* had high hydrolytic activity for the above fluorogenic substrates (Yamakami & Hamajima, 1987; Dowd *et al.*, 1994). In newly excysted juveniles, the presence of neutral and acidic cysteine proteinases in ES products of *P. westermani* and *F. hepatica* flukes, respectively, has been previously reported (Yamakami & Hamajima, 1988; Carmona *et al.*, 1993).

For Boc-Val-Leu-Lys-MCA and Z-Phe-Arg-MCA substrates, the pH-activity profiles of the two ES products were much broader than those reported for purified cysteine proteinases (Yamakami & Hamajima, 1987; Rege *et al.*, 1989; Yamakami *et al.*, 1995) (data not shown). The pH profiles of hydrolytic activity for the above substrates in the Protein A immunocapture assay using sera from paragonimiasis and fascioliasis patients were also broad. It has previously been shown that various cysteine proteinases purified from *Paragonimus* and *Fasciola* flukes have different pH optima (Chapman & Mitchell, 1982; Yamakami, 1986; Fagbemi & Hillyer, 1992). These results suggested that specific IgG antibodies in patient sera recognized several cysteine proteinases with different pH optima, because each purified cysteine proteinase has a significant hydrolytic activity only at a narrow pH range (Yamakami, 1986; Yamakami & Hamajima, 1987).

Protein A immunocapture assay in paragonimiasis and fascioliasis sera has proved to be highly sensitive and specific for immunodiagnosis. The homologous combination of patient sera and ES products added to plate wells yielded significant fluorogenic assay values, while the heterologous combination showed low values. Moreover, wells treated with *Paragonimus* and *Fasciola* ES products yielded low fluorogenic assay values in sera from patients with schistosomiasis japonicum, clonorchiasis, anisakiasis and cysticercosis and from healthy individuals. These results are in agreement with those

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of conventional ELISAs using purified cysteine proteinases as antigens (Yamasaki *et al.*, 1989; Ikeda *et al.*, 1996; O'Neill *et al.*, 1998) and cystatin capture ELISA using chicken cystatin as a capture agent for cysteine proteinases (Ikeda, 1998). However, the Protein A immunocapture assay showed much lower nonspecific reactivity and crossreactivity. Therefore, even patient sera with much lower levels of specific IgG become clearly positive by increasing the reaction time of the substrate.

The Protein A immunocapture assay detecting specific IgG to fluke alkaline phosphatase was species-specific for schistosomiasis (Cesari *et al.*, 1998), whereas ELISAs detecting specific IgG to fluke cysteine proteinases were not species-specific, but genus-specific (Chappell *et al.*, 1990; Ikeda *et al.*, 1996) By seeking appropriate antigenic enzymes for which very sensitive substrates are available, the Protein A immunocapture assay might be applied to species-specific immunodiagnosis for trematode diseases and may also provide useful immunodiagnosis for parasitic diseases other than trematode diseases.

Acknowledgements

This work was supported in part by a grant for Project Research from Kanazawa Medical University (P99-1) and by a grant-in-aid for Scientific Research (11670255) from the Ministry of Education, Science and Culture, Japan.

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(Accepted 25 November 2000)