Abstract. An ELISA was developed using chicken cystatin as a capture agent for the immunodiagnosis of paragonimiasis and fascioliasis. The assay detected specific antibodies to fluke cysteine proteinases without the need for purified proteinases. An ELISA plate was sensitized with chicken egg white cystatin, incubated with excretory-secretory (ES) products of adult flukes, and standard ELISA procedures were then followed. The ELISA plates incubated with the ES products of Paragonimus westermani and Fasciola sp. showed high reactivity to sera from patients with paragonimiasis westermani and fascioliasis, respectively. The capture ELISA showed little cross-reactivity with sera from patients with paragonimiasis and fascioliasis, which showed immunodiagnostic cross-reactivity in a conventional ELISA using crude fluke antigens. Moreover, the capture ELISA showed little reactivity with sera from patients with five other helminth diseases and from healthy volunteers. Omitting either sensitization with cystatin or incubation with fluke ES products abolished high ELISA reactivity, as did a prior exposure of the ES products to cystatin. The addition of papain to an incubation solution of the ES products greatly reduced ELISA reactivity. Incubating the cystatin-sensitized plates with partially purified cysteine proteinases from flukes instead of the ES products also maintained a similar high ELISA reactivity. These results indicate that the cystatin capture ELISA elicits a cystatin and fluke cysteine proteinase antigen-mediated reaction and measures fluke cysteine proteinase-specific antibodies. Prior exposure to low molecular weight inhibitors of cysteine proteinases and other proteinases, such as E-64, leupeptin, aprotinin, and pepstatin, had no effect, suggesting that these inhibitors can be added to cysteine proteinase preparations to prevent autoproteolysis. This assay has good sensitivity and high specificity and is useful for the immunodiagnosis of paragonimiasis and fascioliasis.

Cysteine proteinases of parasitic trematodes not only act as digestive enzymes in the trematode gut, but are also major antigens of their hosts because the enzymes are continuously released outside the flukes. Therefore, fluke cysteine proteinases have been used as antigens for the immunodiagnosis of trematode infections since Senft and Maddison1 used a hemoglobinolytic enzyme of Schistosoma mansoni as an antigen for a skin test. An ELISA has been applied to the seroimmunodiagnosis of many infectious diseases, including those of parasites, because of its high sensitivity, requirement for low amounts of antigens, and simplicity. The ELISAs using cysteine proteinases for immunodiagnosis of parasitic diseases provide good sensitivity and specificity for fascioliasis,2 schistosomiasis,3 and paragonimiasis.4 However, purification of cysteine proteinase is a complex and time-consuming process.2, 5-7 If specific antibodies to fluke cysteine proteinases can be detected by an ELISA that does not require purified proteinases, the assay will be widely applicable to research and routine laboratory identification of all types of trematode infection.

Cystatins are reversible, competitive, and tight binding protein inhibitors of cysteine proteinase.8 Cystatin present in chicken egg white has a molecular mass of 13 kD and very low Ks values for inhibition of cysteine proteinases.8-10 Due to its tight binding to cysteine proteinases, the chicken cystatin has been used as a capture agent in an ELISA to detect antibodies specific for rabbit cathepsins.11 The cystatin capture ELISA could detect anti-cysteine proteinase antibodies without the need for purified cysteine proteinases. Recently, a capture ELISA using recombinant cystatin C was applied to the seroimmunodiagnosis of Chagas’ disease.12 The present study reports that the cystatin capture ELISA is a simple and useful method for detection of antibodies specific for fluke cysteine proteinases in sera of patients with paragonimiasis and fascioliasis.

MATERIALS AND METHODS

Antigens. Excretory-secretory (ES) products of adult Paragonimus westermani were prepared as previously described.13 Adult worms were recovered from dogs infected with 20 metacercariae collected from Eriocheir japonicus. The worms were incubated in three changes of saline (2 hr per change) at room temperature. The incubation solutions were pooled, centrifuged at 20,000 × g for 30 min, concentrated to more than 4 mg of protein/ml by ultrafiltration (PM 10 membrane; Amicon, Danvers, MA), dialyzed against 10 mM phosphate-buffered saline (PBS, pH 7.2) at 4°C, then used as ES products. The ES products of Fasciola sp. were prepared as described above from adult worms recovered from rats infected with five metacercariae. Protein concentration was determined by the bicinchoninic acid protein assay (Pierce, Rockford, IL).

Patient sera. Patient sera were prepared as described.4 Briefly, sera were obtained from Japanese patients confirmed parasitologically or suspected by clinical signs and medical history of having paragonimiasis westermani and fascioliasis. Sera were also obtained from parasitologically confirmed Japanese patients with clonorchiasis and anisakiasis, Japanese and Chinese patients with cysticercosis, Philippine and Kenyan patients with schistosomiasis, and Guatemalan patients with onchocerciasis. Serum samples of Japanese patients were sent by referring physicians to the Medical Zoology Department of Kanazawa Medical University, the Parasitology Department of the Nara Medical University and the Miyazaki Medical College for immunodiagnosis of parasitic diseases. Sera from patients with onchocerciasis were obtained from the Onchocerciasis Research and Control Project in Guatemala (1975–1980). Other sera were provided by various investigators.

Cystatin capture ELISA. Wells of microtiter plates were sensitized overnight at 4°C with 1 µg of chicken egg white
cystatin (Sigma, St. Louis, MO) in 0.1 ml of 0.1 M NaHCO₃ buffer (pH 9.6). After the wells were treated with 2% bovine serum albumin (BSA) in 10 mM PBS containing 0.05% Tween 20 (PBS/T) for 1 hr at room temperature, they were incubated for 4 hr at 4°C with ES products of either adult *P. westermani* or *Fasciola* sp. (1.5 µg of protein/0.1 ml) in BSA/PBS/T. The wells were incubated at room temperature for 1 hr with human sera diluted 1:2,000 with BSA/PBS/T and peroxidase-conjugated anti-human IgG (Cappel Laboratories, Westchester, PA) diluted 1:1,000 with BSA/PBS/T. The wells were then washed five times with PBS/T, and incubated for 30 min with the substrate 2,2’-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) diammonium salt. The absorbance was measured at 405 nm (A₄₀₅).

**Other ELISAs.** The ELISA plates were sensitized overnight at 4°C with crude extracts of antigens (1 µg of protein/0.1 ml) prepared from adult *P. westermani* or *Fasciola* sp. The crude antigens were prepared by homogenizing adult worms with PBS containing 100 µM p-hydroxymercuriphenylsulfonate (HMPS; Sigma), centrifugation at 20,000 ×g for 30 min, and dialysis against PBS. The cysteine proteinases of *P. westermani* were partially purified from ES products of adult worms as previously described.²⁴ Briefly, the ES products were fractionated by 40–75% ammonium sulfate saturation and phenyl-cellulose and arginine-Sepharose 4B column chromatography. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis under reducing conditions showed that the fraction of the partially purified proteinases contained two major bands of cysteine proteinases and a faint band of another substance.

**Exposure to proteinase inhibitors and papain.** The ES products of adult *P. westermani* (15 µg of protein/ml) in BSA/PBS/T were incubated with the cysteine proteinase inhibitors of cystatin, E-64, leupeptin, and HMPS, as well as the aspartic, serine, and metalloproteinase inhibitors of pepstatin A, aprotinin, and EDTA, respectively, for 4 hr at 4°C. Papain (Worthington Biochemical Corp., Freehold, NJ) was added to the ES products in BSA/PBS/T. These mixtures were then tested by the cystatin capture ELISA.

**RESULTS**

The optimal conditions of the cystatin capture ELISA were determined using sera pooled from three patients with paragonimiasis westermani, as shown in Figure 1. Figure 1A shows the effect of the cystatin concentration used in the plate sensitization on the ELISA after a 4-hr incubation with ES products of *P. westermani* at a protein concentration of 15 µg/ml. The ELISA values reached a plateau at a cystatin concentration of 6.25 µg/ml. Figure 1B shows the effect of the concentration of *P. westermani* ES products on the ELISA in a plate sensitized with 10 µg of cystatin/ml. Although there was a rapid increase in the ELISA result at protein concentrations less than 10 µg/ml, there was only a slight increase in the ELISA result at protein concentrations greater than 10 µg/ml. Figure 1C shows the time course of binding of fluke cysteine proteinases to a cystatin-sensitized plate at 4°C at an ES product concentration of 15 µg of protein/ml. The degree of binding increased with incubation periods up to 4 hr. Replicates of the these three experiments showed identical results. Therefore, the capture ELISAs using cystatin capture ELISA for two trematode diseases
tatin were performed after overnight sensitization of the plates with 10 μg of cystatin/ml and a 4 hr-incubation with 15 μg of fluke ES products/ml at 4°C.

We confirmed that the cystatin capture ELISA detected fluke cysteine proteinase–specific antibodies in the paragonimiasis serum. Incubation with partially purified cysteine proteinases of adult *P. westermani* instead of its ES products showed a similar ELISA value (mean \(A_{405} = 1.697\)) in the cystatin capture ELISA compared with that of the ES products (mean \(A_{405} = 1.705\)). When papain, a typical cysteine proteinase, was added to an incubation solution of the ES products at final concentrations of 3.75, 7.5 and 15 μg/ml, the ELISA reactivity was greatly reduced (87%, 94%, and 96%, respectively). A similar reduction was observed in the incubation solution containing papain and E-64 (0.1 mM). Prior exposure of the ES products to cystatin markedly reduced the high ELISA reactivity by 94%, as shown in Table 1.

The effect of exposing the ES products to various proteinase inhibitors was also examined (Table 1). Exposing the ES products to the cysteine proteinase inhibitors of E-64, leupeptin, and HMPS did not affect the high ELISA reactivity, nor did exposure to pepstatin A, aprotinin, and EDTA, respectively. We confirmed that the cystatin capture ELISA detected fluke cysteine proteinase–specific antibodies in the paragonimiasis serum. Incubation with partially purified cysteine proteinases of adult *P. westermani* instead of its ES products showed a similar ELISA value (mean \(A_{405} = 1.697\)) in the cystatin capture ELISA compared with that of the ES products (mean \(A_{405} = 1.705\)). When papain, a typical cysteine proteinase, was added to an incubation solution of the ES products at final concentrations of 3.75, 7.5 and 15 μg/ml, the ELISA reactivity was greatly reduced (87%, 94%, and 96%, respectively). A similar reduction was observed in the incubation solution containing papain and E-64 (0.1 mM). Prior exposure of the ES products to cystatin markedly reduced the high ELISA reactivity by 94%, as shown in Table 1.

The effect of exposing the ES products to various proteinase inhibitors was also examined (Table 1). Exposing the ES products to the cysteine proteinase inhibitors of E-64, leupeptin, and HMPS did not affect the high ELISA reactivity, nor did exposure to pepstatin A, aprotinin, and EDTA, which inhibit aspartic, serine, and metalloproteinases, respectively.

Sera from 12 patients with paragonimiasis westermani and nine with fascioliasis were tested on ELISA plates sensitized with crude antigens of either adult *P. westermani* or *Fasciola* sp. and on plates sensitized with cystatin and incubated with ES products of the two parasites. The results of the ELISA using the crude antigens are shown in Figure 2. The patient sera showed high ELISA reactivity to the respective homologous antigens and also marked cross-reactivity to the heterologous antigens. Control sera from 10 healthy volunteers showed little ELISA reactivity to either antigen. On the other hand, the results of the cystatin capture ELISA shown in Figure 3 indicate that patient sera showed high ELISA reactivity with the plates treated with the respective homologous ES products, but showed little reactivity with the plates treated with heterologous ES products. The control sera showed little reactivity with both types of treated ELISA plates.

All sera from 12 schistosomiasis, nine clonorchiasis, 10 anisakiasis, 10 onchocerciasis, and 11 cysticercosis patients showed little ELISA reactivity (Table 2). Although only one paragonimiasis serum among those from patients with paragonimiasis and fascioliasis showed a relatively low value

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**Table 1**

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Final concentration (mM)</th>
<th>ELISA value (A_{405})</th>
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<tr>
<td>Control</td>
<td>–</td>
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<tr>
<td>Cystatin</td>
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<td>EDTA</td>
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† HMPS = p-hydroxymercuriphenylsulfonate.

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**DISCUSSION**

Cross-reactive antigens among the parasitic trematodes *P. westermani*, *F. hepatica*, and *S. mansoni* have been described. In ELISAs using crude antigens of *Paragonimus* species, some investigators have observed false-positive results with sera from patients with fascioliasis, schistosomiasis, and clonorchiasis. Conversely, an ELISA using partially purified cysteine proteinases of *P. westermani* showed markedly high specificity even for these trematode diseases. In ELISAs for fascioliasis immunodiagnosis, although fluke crude antigens have low specificity, a cysteine proteinase antigen provided similarly good sensitivity and specificity. Also, cysteine proteinases of *S. mansoni* are reportedly valuable ELISA antigens. However, not all laboratories can use the aforementioned ELISAs because purification of the proteinases requires specialized equipment and...
Fasciola sp., which has a very low Ki value and high specificity for several purification procedures. This study used chicken cysteine proteinases by means of several procedures. Furthermore, even when possible, it is troublesome to prepare fluke cysteine proteinases by means of several purification procedures. This study used chicken cystatin, which has a very low Kᵢ value and high specificity for cysteine proteinases, as a capture reagent in ELISA to detect fluke cysteine proteinase-specific antibodies without the need for purified enzymes. This assay can be used even by laboratories that are not familiar with biochemical techniques to analyze most trematode diseases.

The following results verified that the cystatin capture ELISA elicited cystatin- and fluke cysteine proteinase-mediated reactions. 1) Omitting cystatin sensitization or the prior incubation of fluke ES products with cystatin abolished the high ELISA reactivity. 2) Omitting the incubation with the ES products abolished the high ELISA reactivity, but this was reversed by incubation with partially purified fluke cysteine proteinases. 3) Papain, which has a lower Kᵢ value than cathepsin, largely reduced the ELISA reactivity even in the presence of E-64. Thus, the cystatin capture ELISA elicited cystatin- and fluke cysteine proteinase-specific antibodies. Exposing the ES products to cysteine proteinase inhibitors other than cystatin did not affect the cystatin capture ELISA. This result agrees with the finding that tight complexes between cystatin and cysteine proteinases were formed even after cysteine proteinases had been allowed to react with active-site blocking agents. The cystatin molecule forms a wedge-shaped edge complementary to an active-site cleft of a cysteine proteinase (papain), and the edge penetrates into the cleft. This binding appeared to be independent of the blocking of the active-site of cysteine proteinases. Also, exposure to aspartic, serine, and metalloproteinase inhibitors had no effect. These results indicate that the inhibitors can be added to the ES products to prevent autoproteolysis of the proteinases during the preparation and storage of the products. By adding E-64, a strong irreversible inhibitor of cysteine proteinases, fluke ES products containing a large amount of cysteine proteinases can be handled more easily without the need for precautions against proteolysis.

The cystatin capture ELISA showed high reactivity to all paragonimiasis and fascioliasis sera tested except for one of the former. Thus, parasitic trematodes such as Paragonimus, Fasciola, and Schistosoma invading host tissues must induce high antibody responses to fluke cysteine proteinases. This high antibody response appears to be due to the continuous stimulation of the host immune mechanism by cysteine proteinase antigens periodically released by the flukes. The cystatin capture ELISA showed little cross-reactivity with sera from patients with other parasitic diseases, even between paragonimiasis and fascioliasis sera, which showed considerable cross-reactivity on ELISA plates sensitized with the crude antigens. Good sensitivity and markedly high specificity in the cystatin capture ELISA were predicted by the results on ELISA plates sensitized with fluke cysteine proteinases. Consequently, the cystatin capture ELISA is a simple assay with good sensitivity and high specificity for use in the immunodiagnosis of paragonimiasis and fascioliasis.

Financial support: This work was supported in part by a Grant for Project Research from Kanazawa Medical University (P95-17) and by a Grant-in-Aid for Scientific Research (06670276) from the Ministry of Education, Science and Culture, Japan.

Author’s address: Teruaki Ikeda, Department of Medical Zoology, Kanazawa Medical University, Uchinada, Ishikawa 920 0293, Japan.

REFERENCES

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<th>Schistosomiasis</th>
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<th>Anisakiasis</th>
<th>Onchocerciasis</th>
<th>Cysticercosis</th>
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<td>12</td>
<td>9</td>
<td>10</td>
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<td>0.031 ± 0.010</td>
<td>0.031 ± 0.008</td>
<td>0.037 ± 0.014</td>
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<td></td>
<td></td>
<td></td>
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<tr>
<td>Mean ± SD</td>
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<td>0.032 ± 0.012</td>
<td>0.031 ± 0.009</td>
<td>0.042 ± 0.016</td>
<td>0.028 ± 0.017</td>
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<tr>
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<td>0.019–0.049</td>
<td>0.029–0.074</td>
<td>0.015–0.075</td>
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* The cystatin capture ELISA using ES products of P. westermani and Fasciola sp. was performed in sera (1 : 2,000) of the patients. The ELISA values are as described in the legend to Figure 1. ES = excrery-secretory.


