

# Molecular Expression of a Cysteine Proteinase of *Clonorchis sinensis* and Its Application to an Enzyme-Linked Immunosorbent Assay for Immunodiagnosis of Clonorchiasis

Isao Nagano,<sup>1\*</sup> Fuquan Pei,<sup>2</sup> Zhiliang Wu,<sup>1</sup> Jun Wu,<sup>2</sup> Huier Cui,<sup>2</sup> Thidarut Boonmars,<sup>1</sup> and Yuzo Takahashi<sup>1</sup>

Department of Parasitology, Gifu University School of Medicine, Gifu, 500-8705 Japan,<sup>1</sup> and Institute of Anti-parasitic Diseases of Guangdong Province, Guangzhou, 510300 China<sup>2</sup>

Received 14 October 2003/Returned for modification 17 November 2003/Accepted 5 December 2003

**We produced a recombinant cysteine proteinase of *Clonorchis sinensis* and tested its value as an antigen for serologic diagnosis of *C. sinensis* infections. The predicted amino acid sequence of the cysteine proteinase of *C. sinensis* was 58, 48, and 40% identical to those of cathepsin L cysteine proteinases from *Paragonimus westermani*, *Schistosoma japonicum*, and *Fasciola hepatica*, respectively. Western blotting analysis showed that sera from patients infected with *C. sinensis* strongly reacted with the recombinant protein and that sera from patients infected with *S. japonicum* weakly reacted with the recombinant protein. Antibody against the recombinant protein stained proteins migrating at about 37 and 28 kDa in *C. sinensis* adult worm crude extracts. Immunostaining revealed that the cysteine proteinase of *C. sinensis* was located in the intestinal epithelial cells of the adult parasite and in intrauterine eggs. The specificity and sensitivity of the recombinant antigen or *C. sinensis* adult worm crude extracts were assessed by an enzyme-linked immunosorbent assay (ELISA) using serum samples from humans infected with different parasites, including 50 patients with clonorchiasis, and negative controls. The sensitivities of the ELISA with the recombinant antigen and *C. sinensis* adult worm crude extracts were 96 and 88%, respectively. The specificities of the ELISA with the recombinant antigen and *C. sinensis* adult worm crude extracts were 96.2 and 100%, respectively. The results suggested that the recombinant cysteine proteinase-based ELISA could provide a highly sensitive and specific assay for diagnosis of clonorchiasis.**

Clonorchiasis, which is caused by *Clonorchis sinensis*, is an important food-borne parasitic disease of humans in Eastern Asian countries, including China, Korea, and Vietnam. Reportedly, about 7 million people are infected in these areas by eating raw or undercooked freshwater fish (6). Definitive diagnosis is achieved by finding the eggs in feces, but more cooperative diagnostic methods are necessary for epidemiological surveys because of the difficulties of collecting stool samples from individuals. Additionally, the method is not adequate for early diagnosis, because the flukes start releasing eggs in feces after 4 weeks of infection (16). With the advance of immunological diagnostic methods, the detection of specific antibody has been used as one of the routine diagnostic tests for clonorchiasis, especially for epidemiological surveys.

In conventional serological diagnosis of clonorchiasis, the adult worm crude antigens of *C. sinensis* are used. However, the crude antigens reduce the specificity of the serological test due to cross-reactivity with parasites sharing similar antigens (8). Cathepsin L, one of the cysteine proteinases, has been found in many species of parasites (7, 15, 21, 22). Cathepsin L is secreted by all stages of the developing parasites and is highly antigenic in infected animals. Reportedly, the purified or recombinant cysteine proteinase antigens have been used for diagnosis of human paragonimiasis (9), fascioliasis (1, 4, 5), and schistosomiasis (2, 11).

In this study, we cloned and expressed a *C. sinensis* cysteine proteinase and first evaluated the specificity and sensitivity of the recombinant protein in an enzyme-linked immunosorbent assay (ELISA) by comparing it with adult worm crude antigen.

## MATERIALS AND METHODS

***C. sinensis* adult worms and crude extracts.** *C. sinensis* metacercariae were obtained from naturally infected fish in the city of Shunde in China. Rabbits were infected with 400 metacercariae, and adult worms were collected from bile ducts of the infected rabbits at 12 days postinfection. Adult worms were freeze-dried and crushed to powder. Cold acetone was added to the worm powder, and then the sample was centrifuged at  $2,000 \times g$  for 10 min at 4°C. The precipitate was incubated in physiological saline for 6 days at 4°C and centrifuged to remove the pellets.

**Sera.** A total of 76 human serum samples of parasitologically and clinically proven cases were used. Serum samples referred to herein as clonorchiasis sera ( $n = 50$ ) or schistosomiasis sera ( $n = 5$ ) were collected from the patients infected with *C. sinensis* or *Schistosoma japonicum*, respectively, in the areas where these parasites are endemic in China. The sera from patients infected with other parasites, including *Paragonimus westermani* ( $n = 2$ ), *Fasciola hepatica* ( $n = 3$ ), *Taenia solium* ( $n = 2$ ), *Gnathostoma spinigerum* ( $n = 3$ ), *Dirofilaria immitis* ( $n = 3$ ), *Diphyllobothrium latum* ( $n = 3$ ), *Trichuris trichiura* ( $n = 2$ ), *Anisakis simplex* ( $n = 2$ ), and *Entamoeba histolytica* ( $n = 1$ ), were collected from patients in Japan. Serum samples from 48 healthy Japanese subjects without parasitological infections were used as negative controls.

**Preparation of *C. sinensis* cDNA.** Total RNA was isolated from adult worms using TRIZOL (Invitrogen, Carlsbad, Calif.) according to the manufacturer's instructions and treated with DNase (Promega, Madison, Wis.). Reverse transcription was performed using Ready-To-Go You-Prime First-Strand beads (Amersham Biosciences Co., Piscataway, N.J.) according to the manufacturer's instructions. Briefly, 3  $\mu$ g of the total RNA and 1  $\mu$ g of oligo<sub>12-18</sub> (dT) (0.5  $\mu$ g/ $\mu$ l; Amersham Biosciences Co.) were added to the Ready-To-Go tube, and this was followed by the addition of RNase-free water to produce a final volume of 33  $\mu$ l. The tube was incubated at 37°C for 60 min and then at 90°C for 5 min.

\* Corresponding author. Mailing address: Department of Parasitology, Gifu University School of Medicine, Tsukasa 40, Gifu 500-8705 Japan. Phone: 81 58 267 2253. Fax: 81 58 267 2960. E-mail: isao@cc.gifu-u.ac.jp.

**Sequencing of cysteine proteinase of *C. sinensis*.** The gene encoding full-length cysteine proteinase of *C. sinensis* was amplified by PCR from *C. sinensis* cDNA using oligonucleotide primers with *Bam*HI and *Eco*RI restriction enzyme sites added (underlined). The primers were designed from the reported nucleotide sequences of *C. sinensis* cysteine proteinase (GenBank accession number AF093242) as follows: 5'-CGG GAT CCC GAT GCG ACT TTT CGT GTG TTG-3' and 5'-CGG AAT TCC GCT ATT TGA TAA TCG CTG TAG TA-3'. The PCR mixture (total volume of 100  $\mu$ l) consisted of 10  $\mu$ l of 100 ng of *C. sinensis* cDNA, 10  $\mu$ l of 10 $\times$  PCR buffer, 4  $\mu$ l of each deoxynucleoside triphosphate (2.5 mM), 1.5 U of *Taq* polymerase (Takara Shuzo Co., Kyoto, Japan), and 10  $\mu$ l of 10  $\mu$ M concentrations of each primer. DNA was amplified for 35 cycles (each cycle consisted of 30 s of denaturation at 94°C, 30 s of annealing at 52°C, and 60 s of extension at 72°C). The purified PCR products were digested with *Bam*HI and *Eco*RI (New England BioLabs, Beverly, Mass.), and the resulting fragments were cloned into pGEM-3Zf (+) vector (Promega). The nucleotide sequences were determined using Dye Primer Cycle Sequencing kits (Applied Biosystems, Foster City, Calif.) and an ABI DNA automatic sequencer (model 373S). The DNA sequences were assembled and analyzed using the DNASIS software (Hitachi Software Engineering, Tokyo, Japan). The BLAST network service was used to search the DNA and protein database at the National Center for Biotechnology Information.

**Expression and purification of recombinant protein.** The gene encoding pro-cysteine proteinase of *C. sinensis* was amplified by PCR from *C. sinensis* cDNA using oligonucleotide primers with *Bam*HI and *Eco*RI restriction enzyme sites added (underlined). The primers were designed from the nucleotide sequences which were determined in "Sequencing of cysteine proteinase of *C. sinensis*" as follows: 5'-CGG GAT CCC GGC TTT GGC CAG AAC TAC T-3' and 5'-CGG AAT TCC GCT ATT TGA TAA TCG CTG TAG TA-3'. The conditions for PCR were the same as those in "Sequencing of cysteine proteinase of *C. sinensis*." The purified PCR products were digested with *Bam*HI and *Eco*RI and were cloned into the pTrcHis expression vector (Invitrogen). The recombinant plasmid was transformed into an *Escherichia coli* DH5 $\alpha$  strain, and the expression of polyhistidine-containing recombinant proteins was induced by adding isopropyl- $\beta$ -D-thiogalactopyranoside (IPTG) at a final concentration of 1 mM at 37°C for 3 h. The induced cells were harvested and disrupted by sonication in 20 mM Tris-HCl buffer (pH 8.0). The recombinant protein expressed as inclusion bodies was solubilized completely with 6 M urea in 20 mM Tris-HCl buffer (pH 8.0) and then subjected to a His Trap kit (Amersham Biosciences Co.) for affinity purification of histidine-tagged proteins according to the manufacturer's instructions. Urea was removed from the sample protein solution by stepwise dilution of urea with 20 mM Tris-HCl buffer (pH 8.0) in a His Trap chelating column according to the method of Colangeli et al. (3). The recombinant protein was eluted with 500 mM imidazole, and then the imidazole was removed from samples with a PD-10 column (Amersham Biosciences Co.) and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (11% gel) and stained with Coomassie brilliant blue to assess their purity.

**Preparation of antiserum.** Antiserum against the recombinant protein was collected from BALB/c mice that received intradermal injections with approximately 100  $\mu$ g of recombinant protein mixed with complete Freund's adjuvant followed by four booster injections of 50  $\mu$ g of protein mixed with incomplete Freund's adjuvant at 2-week intervals.

**Western blotting analysis.** Test samples included crude extracts from adult worms and the recombinant protein. Aliquots of crude extracts (20  $\mu$ g) or recombinant protein (0.4  $\mu$ g) were subjected to SDS-PAGE (11% gel), electrotransferred to nitrocellulose sheets, and immunostained with sera from patients infected with *C. sinensis*, sera from patients infected with *S. japonicum*, sera from healthy adults, or sera from mice immunized against recombinant protein (1:100 dilution). Goat anti-human or mouse immunoglobulin G (Fab specific) alkaline phosphatase conjugate (Sigma Chemical Co., St. Louis, Mo.) was used as the second antibody, and the alkaline phosphatase was developed in 5-bromo-4-chloro-3-indolyl-*p*-toluidine salt and nitroblue tetrazolium.

**Immunocytochemical localization.** *C. sinensis* adult worms were processed for light microscopic observation according to the established method (10% formalin fixation and hematoxylin and eosin staining). For immunohistochemical staining, formalin-fixed sections 4  $\mu$ m thick were incubated with the sera from mice immunized against recombinant protein or normal mouse serum (1:100 dilution) for 1 h, washed, and further processed using the HistoStain SP kit (ZYMED Laboratories, South San Francisco, Calif.) according to the manufacturer's instructions.

**ELISA.** The antigens were assayed at several concentrations to determine the sensitivity of the ELISA, and the optimal concentration of the antigens was determined to be 5  $\mu$ g/ml (data not shown). ELISA was performed as reported by Matsuda et al. (12) with slight modifications. Briefly, 96-well microtiter plates

(MaxiSorp; Nalge Nunc International, Tokyo, Japan) were sensitized with either recombinant protein or adult worm extracts at a concentration of 5  $\mu$ g/ml in 0.05 M bicarbonate buffer, pH 9.6 (100  $\mu$ l/well), for 3 h at 37°C and overnight at 4°C. After the microplates were washed three times with 0.15 M phosphate-buffered saline (PBS)-0.05% Tween 20, they were blocked with 150  $\mu$ l of PBS containing 2% bovine serum albumin for 1 h at 37°C. After washing as described above, the microplates were probed with a 1:200-diluted human serum sample (100  $\mu$ l/well), in PBS containing 1% bovine serum albumin for 1 h at 37°C. After washing, 100  $\mu$ l of 1:10,000-diluted goat anti-human immunoglobulin G (Fab specific) peroxidase conjugate (Sigma Chemical Co.) was incubated for 45 min at 37°C. For color development, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (Sigma Chemical Co.) was added to each well as substrate (0.3 mg/ml; 100  $\mu$ l/well), and the reaction was terminated after 60 min by adding 50  $\mu$ l of 1.25% sodium fluoride per well. Absorbance at 414 nm was monitored with a Multiskan JX plate reader (Labsystems, Helsinki, Finland). All samples were carried out in duplicate. The cutoff point was set at three times the mean values of the  $A_{414}$  for the negative samples from 48 healthy persons.

**Nucleotide sequence accession number.** Nucleotide sequence data reported in this article are available in the GenBank, EMBL, and DDBJ databases under accession number AY273802.

## RESULTS

**Molecular characterization of cysteine proteinases of *C. sinensis*.** The gene encoding the cysteine proteinase was successfully amplified by PCR from cDNA of *C. sinensis* collected from China using the primers designed from the reported nucleotide sequences of *C. sinensis* cysteine proteinase. The amplicon from the cysteine proteinase, designated Cs-cp, was sequenced, and its amino acid sequence was deduced. The Cs-cp clone was 981 bp, which encoded a protein of 326 amino acid residues, with a molecular mass of 36,541 Da (Fig. 1). Weight matrix calculations based on the algorithm determined by von Heijne (19) allows predictions of the most likely cleavage site for the N-terminal signal sequence. In the case of Cs-cp, possible signal sequence cleavage sites of Cs-cp were predicted after Ser 15 (Fig. 1), which after cleavage results in a proprotein of 311 residues with a predicted molecular mass of 34,788 Da. The amino acid sequence of Cs-cp had a similarity of 99% to that of the reported sequence of *C. sinensis* cysteine proteinase (GenBank accession number AAF40479). The Cs-cp protein was 58% identical to the cathepsin L from *P. westermani* (GenBank accession number AAB93494), 48% identical to the cathepsin L from *S. japonicum* (GenBank accession number AAM44058), and 40% identical to the cathepsin L from *F. hepatica* (GenBank accession number BAA23743) (Fig. 1).

**Expression of Cs-cp recombinant protein.** The Cs-cp recombinant protein was much more highly expressed in inclusion bodies (Fig. 2, lane 5) than in the supernatant of the induced cells (Fig. 2, lane 4). Protein synthesis was induced more efficiently in the sample with 1 mM IPTG treatment (Fig. 2, lane 3) than in the sample without treatment (Fig. 2, lane 2). The recombinant protein could be purified at the single-band level using a His Trap kit and eluted with 500 mM imidazole (Fig. 2, lane 6).

**Western blotting analysis of Cs-cp recombinant protein.** The pooled sera from patients infected with *C. sinensis* positively immunostained adult worm crude extracts and the Cs-cp recombinant protein (Fig. 3A, lanes 2 and 4), and the pooled sera from patients infected with *S. japonicum* weakly stained adult worm extracts and the Cs-cp recombinant protein (Fig. 3A, lanes 6 and 8).

Cs-cp 1: MRLFVCCVLVTTIWSALARTTQVEPDNARALYEEFTLKYKKTYSNDDDELRFEFKDNLLRAKRLQEMEQG---TAQYGVTFSDLTSEE  
Pw 1: MRLFTVSCLVWVGCAFAVNTVRVPDNARELYEQFKRDYGKAYANEDDQKRFADFKNLVRAGQYQTEQGG---TAKYGVTFSDLTNEE  
Sj 1: -----MPQNLLEYLGFELPENVGEMYAQFKLTYRKQYHETDNEKRFSIFKSNLLKAQLYQVLERG---SAVYGVTPYSDLTDEE  
Fh 1: -----MRLFILAVLTVGVLGSNDDLWHQWKRMYNKEYNGAVDEHRRNIWEENVKHIGEHLNRHDLGLVYTLGLNQLTDMTFEE  
\*\*\* \* \* \* \*

Cs-cp 91: FKTRYLRMRFDGPVISEDLTPEED-VTMDNEKFDWREHGAVGVPVLDQGGKCGSCWAFSVIGNVVGQWFRKTGHLLALSEQQLVDCD---YLD  
Pw 91: FAAMYLGSRID-ERVDR-VQLND-LQTPASVDWREKGAVGVPVEHQSGCGSCWAFSVTANVEGQWFLKTGRLVSLKQQLVDCD---RLD  
Sj 91: FSRTHLTAPWRASSKRNTIPPRRE-VGDI PNFDWREKGAVTEVKNQGMCGSCWAFSTTGNIESQWFRKTGKLLSLSLSEQQLVDCD---SLD  
Fh 91: FKAKYLTEMPRASDILSHGIPYEANNRAVPDKIDWRESGYVTEVKDQGGKCGSCWAFSTTGTMEGQYMKNERTSISFSEQQLVDCSGPWGN  
\* \* \*\*\*\* \* \* \* \* \* \* \*

Cs-cp 181: DGCDDGGYPPTTYTAIQKMGLELASDYPTGVGGICHMDKSKFVAVVNGSTI LPLSEKVQAQKLRAIGPLSSALNADTLQLYKGGIMR---  
Pw 181: HGCSGGYPPTYKEIKRMGGLLEQSAIYPTGWGQACRLDRSKLFAKIDDSIVLEKNEEQAAWLAEHGPMSTCLNAGPLQFYRYGILHPS  
Sj 181: DGCNGGLPSNAYESI IRMGGLLEDNYPYDAKNECHLKVGNVAAVINSSVNLTDQSELAIWLYHSAISVGMNALLQFYRHGISHPW  
Fh 181: YGCMMGLMENAYEYLKQFG-LETESYPTAVEGQCRYNRQLGVAKVTDYIYTVHSGSEVELKNLVGAEGPAAVAVDVEDFMMYSGGIYQ  
\*\* \*\* \* \*\* \*\* \* \* \* \*

Cs-cp 271: PKWCDPAGVNHAVLTVGYGV-QNGKPYWIVKNSWGEDFGKGYFRIYRGDG-TCGINSIVTIAIK--- Identity  
Pw 271: EYACSGPEGLNHAVLTVGYDT-ERGVPYWTVRNSWGTWNGENGYFRIYRGDG-TCGIDRLTTSALIH--- 58%  
Sj 271: WIFCSKYLDDHAVLLVGYGVSEKNEPFIWVKNWGVVEWGEKGYFRMYRGDG-TCGINTGATSALIY--- 48%  
Fh 271: SRTCSSLRVNHAVLAVGYGT-QGGTDYIIVKNSWGSWGER-YIRMVRNRGNMCGIASLASLPMVARFP 40%  
\* \*\*\*\* \*\* \* \* \*\* \* \* \* \*

FIG. 1. Alignment of the deduced amino acid sequence of *C. sinensis* cysteine proteinase (Cs-cp) open reading frame, with that of *P. westermani* cathepsin L (Pw) (GenBank accession number AAB93494), *S. japonicum* cathepsin L (Sj) (GenBank accession number AAM44058), and *F. hepatica* cathepsin L (Fh) (GenBank accession number BAA23743). The putative signal peptide of Cs-cp is shaded. Amino acid residues conserved in all four sequences are indicated by asterisks. The identities with Cs-cp are represented along the margins. Gaps are represented by dashed lines. Amino acid positions are shown to the left of the sequences.

The sera from mice immunized against the Cs-cp recombinant protein stained 2 bands migrating at about 37 and 28 kDa in adult worm extracts (Fig. 3B, lane 2) and stained the Cs-cp recombinant protein (Fig. 3B, lane 4). The positive staining of the 37-kDa protein was expected because this is the predicted size of the Cs-cp protein from the amino acid sequence, and the 28-kDa protein may have been the Cs-cp mature protein.

**Cytolocalization of Cs-cp protein in *C. sinensis* adult worms.**

Intense staining with the sera from mice immunized against the Cs-cp recombinant protein was found within the intestinal epithelial cells of the adult parasite and in the intrauterine eggs (Fig. 4). The tegumental surface and parenchymal tissues had no reaction. Normal mouse sera did not result in any positive reaction (data not shown).

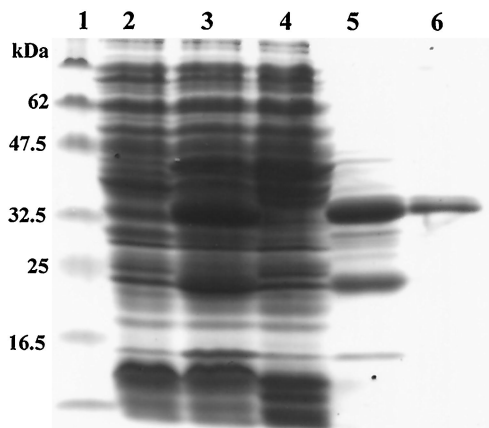


FIG. 2. SDS-PAGE (11% gel) analysis of the Cs-cp recombinant protein. Lane 1, molecular mass standards (masses shown on the left side); lane 2, lysate of *E. coli* with Cs-cp before induction; lane 3, lysate of *E. coli* with Cs-cp after induction; lane 4, supernatants of lysate of *E. coli* with Cs-cp after induction; lane 5, precipitants of lysate of *E. coli* with Cs-cp after induction; lane 6, Cs-cp recombinant proteins purified by the affinity purification method.

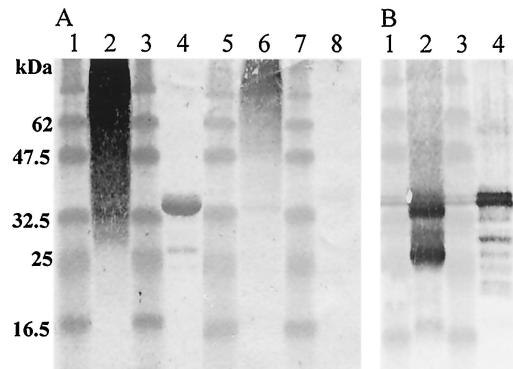


FIG. 3. (A) Western blotting analysis of *C. sinensis* adult worm crude extracts (lanes 2 and 6) and Cs-cp recombinant protein (lanes 4 and 8) stained with the pooled sera from patients infected with *C. sinensis* (lanes 2 and 4) and with the pooled sera from patients infected with *S. japonicum* (lanes 6 and 8). Lanes 1, 3, 5, and 7, molecular mass standards (masses shown on the left side). (B) Western blotting analysis of reactivity of sera from mice immunized against the Cs-cp recombinant protein with *C. sinensis* adult worm crude extracts (lane 2) and Cs-cp recombinant protein (lane 4). Lanes 1 and 3, molecular mass standards (masses shown on the left side).

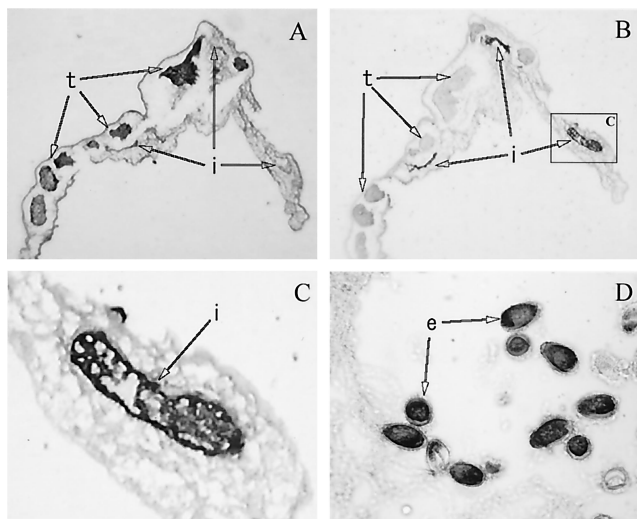


FIG. 4. (B to D) Immunocytochemical staining of *C. sinensis* adult worms with sera from mice immunized against the Cs-cp recombinant protein. (A) Hematoxylin and eosin staining of *C. sinensis* adult worms. Abbreviations: i, intestine; e, intrauterine egg; t, testis.

**Cross-reactivity analysis of Cs-cp recombinant proteins by Western blotting.** The sera from patients infected with *C. sinensis* strongly reacted with the Cs-cp recombinant protein (Fig. 5, lanes 2 to 11), and the sera from patients infected with *S. japonicum* weakly reacted with the Cs-cp recombinant protein (Fig. 5, lanes 12 to 16), but sera from healthy persons did not react with the Cs-cp recombinant protein (Fig. 5, lanes 17 to 21).

**ELISA with human sera.** ELISA plates were sensitized by the Cs-cp recombinant protein and adult worm crude extracts, respectively, and then probed with human sera. The frequency distribution of positive and negative control sera was plotted as a histogram (Fig. 6). The  $A_{414}$  mean value of the negative control group for the Cs-cp antigen was 0.042 with a standard deviation of 0.007, while the  $A_{414}$  mean for adult worm extracts

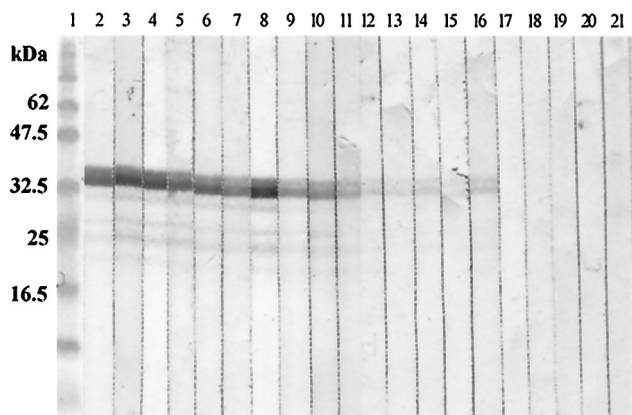


FIG. 5. Western blotting analysis of Cs-cp recombinant protein stained with sera from patients infected with *C. sinensis* (lanes 2 to 11), with sera from patients infected with *S. japonicum* (lanes 12 to 16), and with sera from healthy persons (lanes 17 to 21). Lane 1, molecular mass standards (masses shown on the left side).

was 0.078 with a standard deviation of 0.017. The  $A_{414}$  means plus 3 standard deviations were 0.063 for the Cs-cp antigen and 0.129 for adult worm extracts, and three times the  $A_{414}$  means were 0.126 for the Cs-cp antigen and 0.234 for adult worm extracts. Since three times the  $A_{414}$  means were larger than the means plus 3 standard deviations, three times the  $A_{414}$  mean of the negative control group was determined as the cutoff value in further experiments, resulting in 0.126 for the Cs-cp antigen and 0.234 for adult worm extracts. The  $A_{414}$  mean and standard deviation of sera from patients infected with *C. sinensis* were  $0.331 \pm 0.160$  for the Cs-cp antigen and  $0.386 \pm 0.147$  for adult worm extracts. The sensitivities of the test using Cs-cp antigen and adult worm extracts were 96% (48 of 50) and 88% (44 of 50), respectively (Fig. 7). The specificity of this test was analyzed by measuring the reactivity of sera from 26 patients infected with other parasites. Only one sample from a patient with *G. spinigerum* infection produced a positive reaction when the Cs-cp antigen was used, while adult worm extracts showed no cross-reaction.

## DISCUSSION

There is a need for a reliable diagnostic tool to solve the clinical and parasitological diagnosis problems in clonorchiasis. An immunodiagnostic method is a highly sensitive and early method to diagnose *C. sinensis* infections.

Cysteine proteinases are highly antigenic because parasitic trematodes continually release them into the host. Therefore, cysteine proteinases are possible diagnostic antigens. Reportedly, the acidic extracts of various developmental stages of *C. sinensis* (metacercariae and adult worms) have cysteine proteinase activity (17). The present experiment showed that a recombinant cysteine proteinase of *C. sinensis* (Cs-cp) ensured the sensitivity and specificity of ELISA for serological diagnosis of clonorchiasis.

The amino acid sequence of the Cs-cp cysteine proteinase was less similar to the cathepsin L cysteine proteinases from *P. westermani*, *S. japonicum*, and *F. hepatica*. These data suggest that for serological diagnosis, the Cs-cp recombinant protein probably does not cross-react with sera from patients with *P. westermani*, *S. japonicum*, and *F. hepatica* infections. Indeed, in an ELISA, the Cs-cp recombinant protein did not cross-react with sera from patients with *S. japonicum* infection, and cross-reaction was observed in only one patient with *G. spinigerum* infection. On the contrary, adult worm extracts did not cross-react with all sera from patients with parasitic infections, but the sensitivity of the test using adult worm extracts was lower than that of Cs-cp antigen.

Cysteine proteinases from some trematodes were reported to localize in the intestine or in cercarial extracts or immature eggs (10, 13, 20), but immunocytochemical analysis of *C. sinensis* cysteine proteinases has not been reported. In this study, *C. sinensis* cysteine proteinases are localized in the intestinal epithelial cells of the adult parasite and in the intrauterine eggs. These cysteine proteinases are parts of the excretory-secretory products of *C. sinensis* and are mainly synthesized in the intestinal epithelia, secreted into the lumen, and have been shown to be highly antigenic in infected animals.

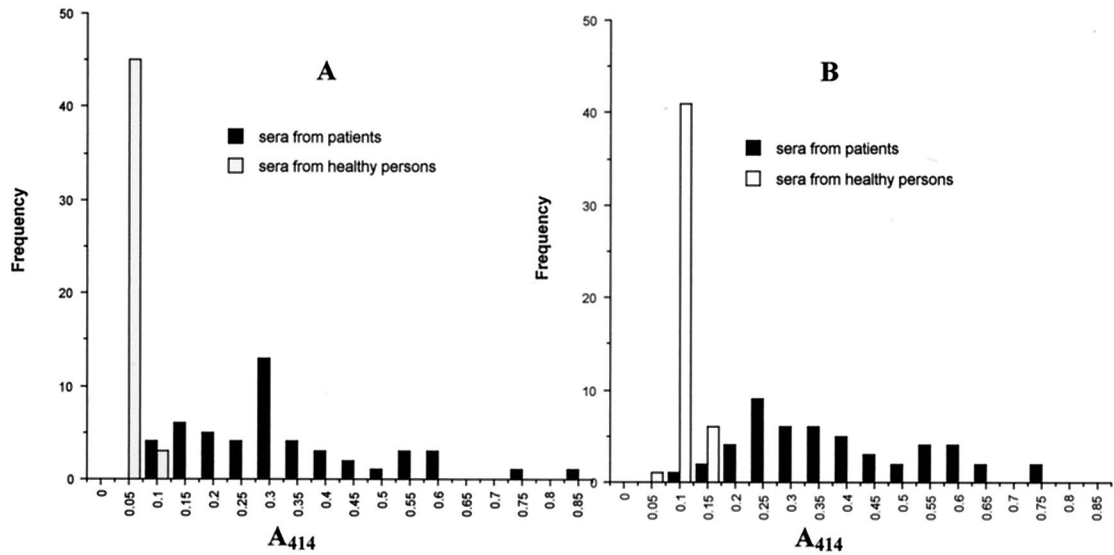


FIG. 6. Immunoreactivity of the Cs-cp recombinant protein (A) and *C. sinensis* adult worm extracts (B). Analysis by ELISA of 50 sera from patients infected with *C. sinensis* and 48 sera from healthy persons. The y axis shows the frequency of absorbance measurements.

In *C. sinensis*, two kinds of cysteine proteinases have been reported (14, 15, 18). We preliminarily produced these two kinds of cysteine proteinases in the *E. coli* expression system and analyzed these recombinant cysteine proteinases by Western blotting. The sera from patients infected with *C. sinensis* strongly reacted with the Cs-cp recombinant protein but did not react with other kinds of recombinant cysteine proteinase.

These data suggested that all of the cysteine proteinases of *C. sinensis* were not immunodominant and did not induce immunological reaction during infection.

In conclusion, it has been demonstrated that the recombinant cysteine proteinase is useful for immunodiagnosis of clonorchiasis by ELISA and will provide more reliable results not only for routine diagnosis but also for epidemiological

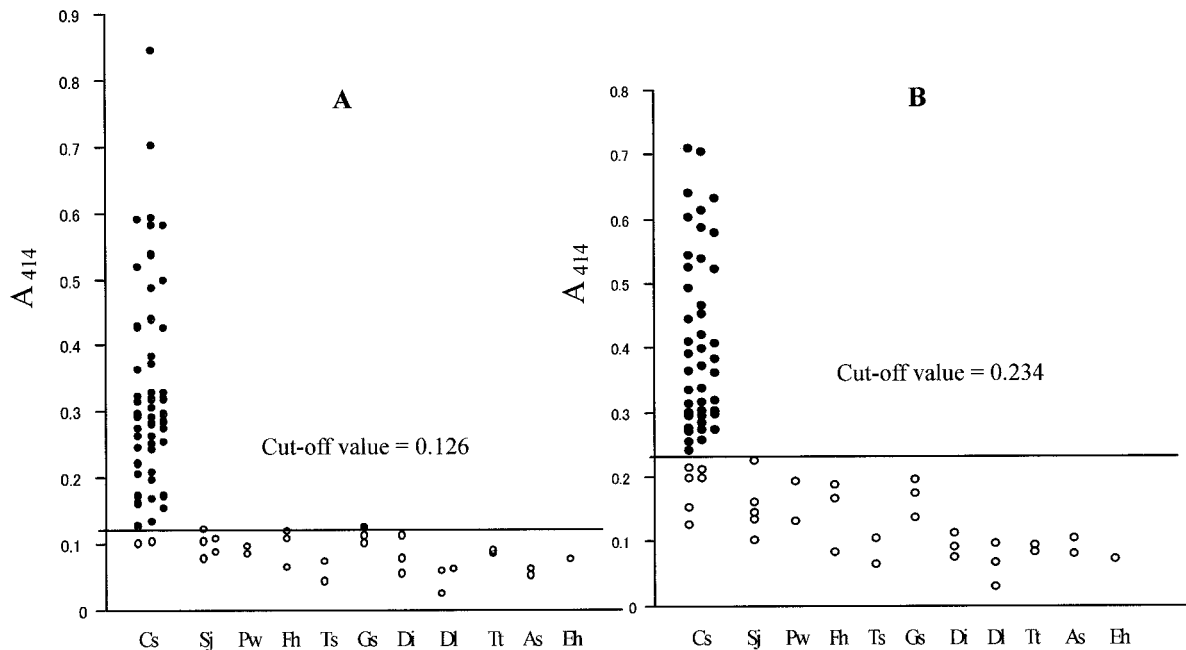


FIG. 7. Comparison of reactivity in enzyme-linked immunosorbent assay of the Cs-cp recombinant protein (A) and *C. sinensis* adult worm extracts (B) against those of sera from patients infected with *C. sinensis* (Cs), *S. japonicum* (Sj), *P. westermani* (Pw), *F. hepatica* (Fh), *T. solium* (Ts), *G. spinigerum* (Gs), *D. immitis* (Di), *D. latum* (Dl), *T. trichiura* (Tt), *A. simplex* (As), and *E. histolytica* (Eh). The cutoff values are indicated by the long horizontal lines. Open circles indicate values below the cutoff, and solid circles indicate values above the cutoff.

surveys of clonorchiasis. Thus, the use of recombinant antigen may provide a new source of diagnostic reagent and more reliable results for diagnosis of clonorchiasis.

#### ACKNOWLEDGMENT

This study was partially supported by a grant-in-aid from the Japan-China Medical Association.

#### REFERENCES

- Carnevale, S., M. I. Rodriguez, E. A. Guarnera, C. Carmona, T. Tanos, and S. O. Angel. 2001. Immunodiagnosis of fasciolosis using recombinant cathepsin L cysteine proteinase. *Diagn. Microbiol. Infect. Dis.* **41**:43–49.
- Chappell, C. L., M. H. Dresden, B. Gryseels, and A. M. Deelder. 1990. Antibody response to *Schistosoma mansoni* adult worm cysteine proteinases in infected individuals. *Am. J. Trop. Med. Hyg.* **42**:335–341.
- Colangeli, R., A. Heijbel, A. M. Williams, C. Manca, J. Chan, K. Lyashchenko, and M. L. Gennaro. 1998. Three-step purification of lipopolysaccharide-free, polyhistidine-tagged recombinant antigens of *Mycobacterium tuberculosis*. *J. Chromatogr. B Biomed. Sci. Appl.* **714**:223–235.
- Cordova, M., L. Reategui, and J. R. Espinoza. 1999. Immunodiagnosis of human fascioliasis with *Fasciola hepatica* cysteine proteinases. *Trans. R. Soc. Trop. Med. Hyg.* **93**:54–57.
- Cornelissen, J. B., C. P. Gaasenbeek, F. H. Borgsteede, W. G. Holland, M. M. Harmsen, and W. J. Boersma. 2001. Early immunodiagnosis of fasciolosis in ruminants using recombinant *Fasciola hepatica* cathepsin L-like protease. *Int. J. Parasitol.* **31**:728–737.
- Crompton, D. W. 1999. How much human helminthiasis is there in the world? *J. Parasitol.* **85**:397–403.
- Dalton, J. P., K. A. Clough, M. K. Jones, and P. J. Brindley. 1996. Characterization of the cathepsin-like cysteine proteinases of *Schistosoma mansoni*. *Infect. Immun.* **64**:1328–1334.
- Hillyer, G. V., and E. A. Serrano. 1983. The antigens of *Paragonimus westermani*, *Schistosoma mansoni*, and *Fasciola hepatica* adult worms. Evidence for the presence of cross-reactive antigens and for cross-protection to *Schistosoma mansoni* infection using antigens of *Paragonimus westermani*. *Am. J. Trop. Med. Hyg.* **32**:350–358.
- Ikeda, T., Y. Oikawa, and T. Nishiyama. 1996. Enzyme-linked immunosorbent assay using cysteine proteinase antigens for immunodiagnosis of human paragonimiasis. *Am. J. Trop. Med. Hyg.* **55**:435–437.
- Kang, S. Y., M. S. Cho, Y. B. Chung, Y. Kong, and S. Y. Cho. 1995. A cysteine protease of *Paragonimus westermani* eggs. *Korean J. Parasitol.* **33**:323–330.
- Klinkert, M. Q., K. Bommert, D. Moser, R. Felleisen, G. Link, O. Doumbo, and E. Beck. 1991. Immunological analysis of cloned *Schistosoma mansoni* antigens Sm31 and Sm32 with sera of schistosomiasis patients. *Trop. Med. Parasitol.* **42**:319–324.
- Matsuda, H., H. Tanaka, B. L. Bayani, N. S. Julian, T. Tokawa, and S. Ohsawa. 1984. Evaluation of ELISA with ABTS, 2-2'-azino-di-(3-ethylbenzothiazoline sulfonic acid), as the substrate of peroxidase and its application to the diagnosis of schistosomiasis. *Jpn. J. Exp. Med.* **54**:131–138.
- Michel, A., H. Ghoneim, M. Resto, M. Q. Klinkert, and W. Kunz. 1995. Sequence, characterization and localization of a cysteine proteinase cathepsin L in *Schistosoma mansoni*. *Mol. Biochem. Parasitol.* **73**:7–18.
- Na, B. K., H. J. Lee, S. H. Cho, H. W. Lee, J. H. Cho, W. G. Kho, J. S. Lee, J. S. Lee, K. J. Song, P. H. Park, C. Y. Song, and T. S. Kim. 2002. Expression of cysteine proteinase of *Clonorchis sinensis* and its use in serodiagnosis of clonorchiasis. *J. Parasitol.* **88**:1000–1006.
- Park, S. Y., K. H. Lee, Y. B. Hwang, K. Y. Kim, S. K. Park, H. A. Hwang, J. A. Sakanari, K. M. Hong, S. I. Kim, and H. Park. 2001. Characterization and large-scale expression of the recombinant cysteine proteinase from adult *Clonorchis sinensis*. *J. Parasitol.* **87**:1454–1458.
- Rim, H. J. 1986. The current pathology and chemotherapy of clonorchiasis. *Korean J. Parasitol.* **24**(Suppl.):78–85.
- Song, C. Y., and A. A. Rege. 1991. Cysteine proteinase activity in various developmental stages of *Clonorchis sinensis*: a comparative analysis. *Comp. Biochem. Physiol. B* **99**:137–140.
- Song, C. Y., M. H. Dresden, and A. A. Rege. 1990. *Clonorchis sinensis*: purification and characterization of a cysteine proteinase from adult worms. *Comp. Biochem. Physiol. B* **97**:825–829.
- von Heijne, G. 1986. A new method for predicting signal sequence cleavage sites. *Nucleic Acids Res.* **14**:4683–4690.
- Yamasaki, H., E. Kominami, and T. Aoki. 1992. Immunocytochemical localization of a cysteine protease in adult worms of the liver fluke *Fasciola* sp. *Parasitol. Res.* **78**:574–580.
- Yamasaki, H., R. Mineki, K. Murayama, A. Ito, and T. Aoki. 2002. Characterisation and expression of the *Fasciola gigantica* cathepsin L gene. *Int. J. Parasitol.* **32**:1031–1042.
- Yun, D. H., J. Y. Chung, Y. B. Chung, Y. Y. Bahk, S. Y. Kang, Y. Kong, and S. Y. Cho. 2000. Structural and immunological characteristics of a 28-kilodalton cruzipain-like cysteine protease of *Paragonimus westermani* expressed in the definitive host stage. *Clin. Diagn. Lab. Immunol.* **7**:932–939.