Evaluation of *Clonorchis sinensis* Recombinant 7-Kilodalton Antigen for Serodiagnosis of Clonorchiasis

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The diagnostic applicability of the *Clonorchis sinensis* recombinant 7-kDa protein was evaluated. In enzyme-linked immunosorbent assays and immunoblots, the protein showed high sensitivities (81.3 and 71.9%, respectively) and specificities (92.6 and 89.7%, respectively) for sera obtained from various helminthic infections. Some paragonimiasis sera showed cross-reactions. The antigen might be valuable in the serodiagnosis of human clonorchiasis.

Clonorchis sinensis, a trematode parasite, causes hepatobiliary disease and is prevalent in Asian countries. It has been reported that more than 7 million people are infected with this parasite worldwide (4, 11). Humans are infected with the parasite by eating raw freshwater fishes or by consuming dried, salted, smoked, or pickled freshwater fishes containing infective metacercariae. In the acute stage of heavy infection, patients might have abdominal pain, nausea, diarrhea, eosinophilia, and jaundice. Chronic and heavy infections might be complicated by cholangitis, cholelithiasis, and cholangectasis and possibly by cholangiocarcinoma (11). Clonorchiasis is diagnosed by the detection of the characteristic eggs in patients' stool, bile drainage, or biopsy materials. An intradermal test is also applicable in large-scale epidemiological surveys. However, these classical techniques have several limitations. Stool examination is impossible when patients are not cooperative and might not be sensitive in cases of light infections. An intradermal test also revealed a low specificity, especially in cases in which patients were treated with anthelminthics (11). Various immunodiagnostic tests, including enzyme-linked immunosorbent assay (ELISA) and immunoblotting, have been used to overcome these shortcomings. Searches for sensitive and specific antigens have been undertaken to isolate and characterize the specific antigenic proteins that can be used in the serodiagnosis of clonorchiasis (1, 2, 9, 12). Among antigenic proteins that are reactive with the sera of patients with *C. sinensis* infections, excretory-secretory products (ESP) were shown to be sensitive and specific (1, 8). Cysteine proteases and glutathione *S*-transferase of *C. sinensis* were also reported to be specific antigens for serological diagnosis (5, 6, 7, 10). In addition, the 7-kDa molecule of *C. sinensis* ESP was suggested to be a specific antigen (8). Recently, the 7-kDa protein was purified and partially characterized as a serodiagnostic antigen

and the gene encoding the protein was also identified (9). However, the diagnostic capability of the protein, especially that of the recombinant protein, could not be properly evaluated based on large numbers of samples. In this study, we expressed the 7-kDa protein in a yeast expression system by using *Pichia pastoris* and evaluated its potential as a serodiagnostic antigen for clonorchiasis.

Total RNA was isolated from *C. sinensis* adult worms with a SNAP total RNA isolation kit (Invitrogen, Carlsbad, Calif.) according to the manufacturer's instructions. The cDNA encoding the 7-kDa antigen was amplified by reverse transcription-PCR with ReddyMix reverse transcription-PCR master mix (ABgene, Austin, Tex.) by using *C. sinensis* RNA and specific primers (9). The PCR product was analyzed on 1.2% agarose gel, gel purified, and ligated into the pCR2.1 vector (Invitrogen). After *Escherichia coli* TOP10 cells (Invitrogen) were transformed, the nucleotide sequence of the cloned gene was determined by automatic DNA sequencing. To prepare the expression construct, the flanking region of the mature 7-kDa protein without signal peptide was amplified by using gene-specific primers (5′-CTCGAGAAAAGACGTCCCAGT GAAGAGACC-3′ [forward primer] and 5′-GCGGCCGCTC ACTCCCCAACATAAGT-3′ [reverse primer]). The amplified PCR product was purified and subcloned into the pCR2.1 vector, and *E. coli* TOP10 cells were transformed with it. A positive transformant was screened for the presence of the plasmid with an appropriate insert by PCR and sequencing, after which it was purified and digested with XhoI and NotI. The resulting insert was purified and ligated into the pPICZαA vector (Invitrogen). *E. coli* TOP10 cells were then transformed with the construct. Bacteria were plated on agar plates containing low-salt Luria broth (1% tryptone, 0.5% NaCl, 0.5% yeast extract) and 25 µg of Zeocin/ml. Positive clones were selected by PCR and sequenced to confirm the reading frame of the insert. Recombinant plasmids were linearized by digestion with SacI, and then *P. pastoris* strain KM71H (Mut^S phenotype) was transformed with them by using an EasySelect kit (Invitrogen). Transformed cells were selected on YPDS (1%

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yeast extract, 2% peptone, 2% dextrose, 1 M sorbitol) plates supplemented with 100 μg of Zeocin/ml and grown for several days at 30°C. Positive clones containing the 7-kDa insert were selected and grown at 30°C in 10 ml of BMGY medium (1% yeast extract, 2% peptone, 1.34% yeast nitrogen base, 1% glycerol, 0.00004% biotin, 0.1 M potassium phosphate [pH 6.0]) in 50-ml tubes with vigorous shaking. Cells were harvested by centrifugation, resuspended in 2 ml of BMMY medium (BMGY medium in which 0.5% methanol was substituted for glycerol), and cultured for an additional 6 days. During the induction period, methanol was added every 24 h to maintain the final concentration of 0.5% (vol/vol). Supernatants were screened by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) every 24 h. Large-scale culture was carried out for 5 days after induction. The cells were pelleted by centrifugation, and the resulting supernatants were harvested and concentrated by 75% ammonium sulfate precipitation. The resulting pellet was dissolved in distilled water and equilibrated with phosphate-buffered saline (PBS) (pH 7.4) by using a PD-10 column (Amersham Biosciences, Uppsala, Sweden). Recombinant *C. sinensis* 7-kDa protein was purified by gel filtration chromatography by using Ultrogel AcA202 and Supedex 75 (Amersham Biosciences) and concentrated by lyophilization.

The diagnostic applicability of the recombinant protein was evaluated by ELISA. ELISA was performed in triplicate for each serum sample with 96-well flat-bottom microtiter plates (Costar, Cambridge, Mass). Each well was coated with a total of 50 μl (2.5 μg of antigen/ml) overnight at 4°C in PBS (pH 7.4). Serum samples were diluted at 1:50 in PBS containing 0.05% Tween 20. Peroxidase-conjugated anti-human immunoglobulin G (Sigma, St. Louis, Mo.) was used at a dilution of 1:1,000. Color reaction was developed by using 2,2′-azino-di(3-ethyl-benzothiazoline-6-sulfonate) chromogen. Absorbance at 405 nm was read with a Universal Microplate Recorder EL800 (Bio-Tek, Winooski, Vt.). Serum samples used in this study included those obtained from patients with clonorchiasis (64 samples), paragonimiasis (34 samples), fascioliasis (24 samples), schistosomiasis japonica (20 samples), sparganosis (24 samples), and cysticercosis (13 samples) and from healthy controls (60 samples). Clonorchiasis and schistosomiasis japonica were diagnosed by stool examination, and paragonimiasis, fascioliasis, sparganosis, and cysticercosis were diagnosed by typical imaging findings together with positive antibody reactions in the sera of patients as determined by ELISA with crude antigens, combined with clinical symptoms compatible the respective disease. Control serum samples were obtained from healthy individuals working at the National Institute of Health, Korea. The cutoff was determined to be the mean plus 2 standard deviations of the absorbance of negative control samples. Samples with an absorbance readings of <0.26 were considered negative, and those with absorbance readings of ≥0.26 were considered positive. To confirm diagnostic reliability, all of the serum samples used in ELISA were examined again by immunoblotting by the standard method (3). The recombinant protein was separated by 7-to-18%-gradient SDS-PAGE and transferred to nitrocellulose membrane. After being cut into strips, each strip was incubated overnight with serum samples obtained from patients with different parasitic infections at a dilution of 1:100 and subsequently with peroxidase-conjugated
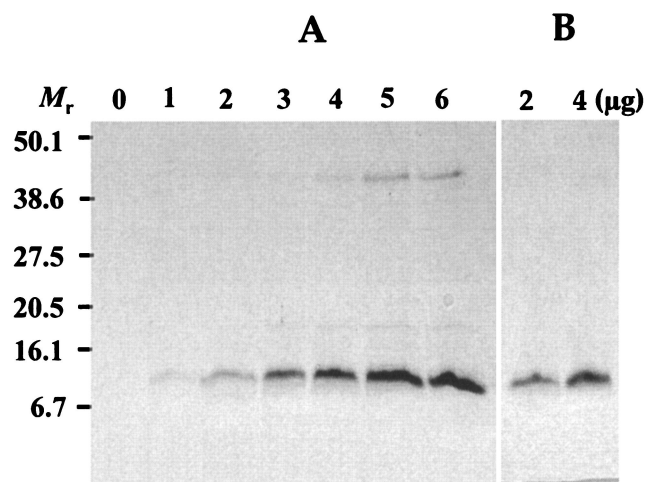

FIG. 1. (A) Time-dependent expression of *C. sinensis* recombinant 7-kDa protein. Aliquots taken from the yeast culture medium at the indicated time intervals (in days) were analyzed by SDS-PAGE. M_r, molecular size in kilodaltons; lane 0, uninduced yeast supernatant. Lanes 1 to 6, yeast supernatant 1 to 6 days after induction with methanol. (B) Purification of recombinant protein by molecular sieve chromatography. Lanes 2 and 4, 2 and 4 μg of the purified proteins, respectively.

anti-human immunoglobulin G (Sigma). The color reaction was developed with 4-chloro-1-naphthol chromogen (Sigma).

SDS-PAGE analysis of the yeast culture supernatants shows that the recombinant protein is a major component protein (Fig. 1A). The protein, with an approximate molecular mass of 7 kDa, which coincides well with the predicted molecular mass, was observed after 1 day of induction. The expression levels increased with induction time and reached a plateau 5 days after induction. After purification, the purity of the recombinant protein increased to more than 95% (Fig. 1B), as confirmed by spray mass spectrum chromatography (data not shown).

The diagnostic applicability of the recombinant protein as a serodiagnostic antigen was evaluated. As shown in Table 1, significant statistical differences in specific antibody levels were observed between clonorchiasis samples and other parasitic infection samples ($P < 0.01$) and between clonorchiasis samples and healthy control samples ($P < 0.01$). Although 35.3% of serum samples from patients with paragonimiasis showed

TABLE 1. Levels of antibodies specific for different parasitic infections as determined by ELISA using serum samples

Disease or group	No. of samples tested	Specific antibody level in sera[a]	No. (%) of positive reactions
Clonorchiasis	64	0.83 ± 0.69	52 (81.3)
Paragonimiasis	34	0.30 ± 0.21	12 (35.3)
Fascioliasis	24	0.10 ± 0.05	0 (0)
Schistosomiasis japonica	20	0.13 ± 0.05	0 (0)
Sparganosis	24	0.14 ± 0.04	1 (4.2)
Cysticercosis	13	0.12 ± 0.04	0 (0)
Healthy controls	60	0.11 ± 0.05	0 (0)

[a] Values are means ± standard deviations and were determined by measuring absorbance at 405 nm.
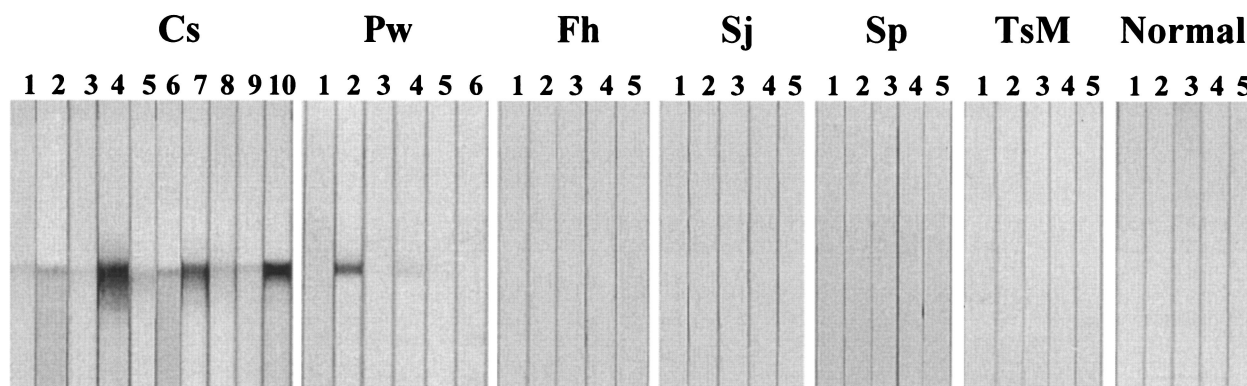
FIG. 2. Immunoreactivity of *C. sinensis* recombinant 7-kDa protein against sera from patients with different parasitic infections. Each strip was incubated with individual serum samples from patients with clonorchiasis (Cs), paragonimiasis (Pw), fascioliasis (Fh), schistosomiasis japonica (Sj), sparganosis (Sp), or cysticercosis (TsM) or healthy controls (Normal). Representative reactions are shown. Sera from patients with clonorchiasis demonstrated positive reactions, and some paragonimiasis sera (16 of 34 samples) exhibited cross-reactions. Sera from patients with other parasitic infections and from healthy controls showed no reactions. Different lanes show sera from different patients.

cross-reactions against the protein (12 of 34 cases), those from patients with other parasitic infections such as fascioliasis, schistosomiasis japonica, sparganosis, and cysticercosis showed no or minimal cross-reactions. Healthy control samples also did not show cross-reactivity. Overall sensitivity and specificity as determined by ELISA were 81.2% (52 of 64 cases) and 92.6% (162 of 175 cases), respectively. Representative findings obtained by immunoblotting are shown in Fig. 2. Sera from the patients with clonorchiasis showed strong to moderate reactions against the recombinant 7-kDa protein. Of the 64 serum samples tested, 46 exhibited positive reactions, with a sensitivity of 71.9%. Sera from patients with paragonimiasis exhibited cross-reactions in some cases (16 of 34 cases [47.1%]). In contrast, serum samples from the patients with other parasitic diseases and those from healthy controls did not react, except for two serum samples obtained from patients with sparganosis, which showed weak cross-reactions. The overall sensitivity of the test was determined to be 71.9% (46 of 64 cases), and the specificity was determined to be 89.7% (157 of 175 cases).

Several studies have focused on the detection of specific antigens to improve the diagnostic sensitivity of clonorchiasis. A number of antigens of molecular masses ranging 8 to 90 kDa (1, 12) prepared differently from either crude worm extracts or ESP have been reported to react strongly to human clonorchiasis. However, some cross-reactions against serum samples obtained from patients with other trematode infections, such as paragonimiasis, fascioliasis, metagonimiasis and schistosomiasis japonica, were observed (1, 7, 8, 13). It seemed difficult to minimize serological cross-reactivity between some trematode infections in previous studies. The 7-kDa protein characterized in this study was also cross-reactive with sera from patients with paragonimiasis. In addition, a previous study showed that approximately 43% of serum samples from patients with metagonimiasis, which is caused by a minute intestinal trematode, *Metagonimus yokogawai*, cross-reacted with the natural 7-kDa antigen in ESP of *C. sinensis* (8). It is not clear whether the 7-kDa protein in that study was identical to that used in this study. However, it is obvious that serological cross-reactions, especially between clonorchiasis and paragonimiasis, often cause serious problems with regard to the spec-

ificity of serological tests. The underlying mechanisms of cross-reactivity due either to common antigenic determinants shared by these two trematode parasites or to common recognition by the host immune surveillance system should be elucidated in the future. However, as confirmed in this study, the 7-kDa protein might be applicable to serodiagnosis and thus might strengthen the diagnostic reliability of the tests. This protein might be useful as a component protein in the preparation of an antigenic cocktail or chimeric antigen for the serodiagnosis of clonorchiasis. In a previous study, the 7-kDa protein was localized in the basal layer of the syncytial membrane, in the interstitial matrix, and in uterine content. Furthermore, a typical signal peptide sequence before the N terminus of the native protein was conserved (9). These results suggest that the protein not only might be present in a soluble form in the worm parasite but also may be secreted outside and act as a potent antigen.

In conclusion, we have evaluated the diagnostic applicability of the excretory-secretory 7-kDa recombinant protein of *C. sinensis*. In both ELISA and immunoblots, the recombinant protein showed relatively high sensitivities (71.9 and 81.3%, respectively) and specificities (89.7 and 92.6%, respectively) for sera from clonorchiasis and other parasitic infections. Although serum samples from patients with paragonimiasis showed cross-reactivity (35.3 and 47.1% as determined by ELISA and immunoblotting, respectively), those from other parasitic diseases exhibited no or minimal cross-reactivity. The results presented here suggest that the 7-kDa antigen of *C. sinensis* might be valuable in the serodiagnosis of human clonorchiasis.

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