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CLONING AND CHARACTERIZATION OF A NEW CYSTEINE PROTEINASE SECRETED BY *PARAGONIMUS WESTERMANI* ADULT WORMS

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Abstract. The cysteine proteinases of *Paragonimus westermani* are known to play important roles in invasion and pathogenesis to hosts and in immune modulation and nutrient uptake. In this study, we have cloned a new cysteine proteinase of *P. westermani,* PwCP2, from adult worms and tested its diagnostic usefulness. The PwCP2 gene had an open reading frame of 816 base pairs and a conserved catalytic triad of cysteine, histidine, and asparagine residues. The mature form of recombinant PwCP2 (rPwCP2) lacking a proregion was overexpressed in *Escherichia coli* and used to produce antiserum. Western blot and immunohistochemical analyses using this antiserum showed that PwCP2 was expressed as a mature form, 24-kD product in a crude extract and in the excretory-secretory product of *P. westermani,* and was localized mainly in the intestinal epithelium of the adult worm. Western blot analysis using the rPwCP2 showed not only high sensitivity (90%) and specificity (100%) to sera from patients with paragonimiasis westermani, but also no cross-reactivity with sera from patients with clonorchiasis, sparganosis, or cysticercosis. Furthermore, an enzyme-linked immunosorbent assay using rPwCP2 exhibited a sensitivity of 93% and a specificity of 93% with sera of rats infected with *P. westermani* metacercariae. These results suggest that the excretory-secretory PwCP2 can be used for the diagnosis of paragonimiasis.

INTRODUCTION

Paragonimus westermani causes pulmonary, neurologic, and abdominal diseases by infecting the lung, brain, spinal cord, and other organs in humans and animals such as dogs, tigers, cats, pigs, cattle, mink, and feral carnivores.[1–3] Parasite cysteine proteinases are known to play important roles in the pathogenesis of diseases caused by these organisms and invasion of their hosts.[4–8] Parasites can invade their hosts and obtain nutrients using these enzymes to hydrolyze host hemoglobin and collagen.[9,10] In addition to the pathologic roles, proteinases have been implicated in the reproduction of parasites and in the excystment of metacercaria.[11] Previous reports showed that eggs, metacercariae, juveniles, and adults of *P. westermani* produced at least six different types of cysteine proteinase with molecular masses of 53, 34, 28, 27, 17, and 15 kD.[11–15] Among these, the 27-kD and 28-kD proteinases released from the metacercariae were shown to share biochemical features with cathepsin L of *Fasciola hepatica* and cathepsin S of *Sparganum* spp., respectively.[16,17] These proteinases were also believed to play important roles in metacercarial excystment, immune modulation, and tissue penetration.[11–13,15] Previously, we found at least nine proteinases in *P. westermani* by gelatin sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and reported that a cysteine proteinase in *P. westermani*, PwCP1, was localized mainly in the vitelline gland of the adult worms, although its function was not characterized.[18]

Parasitologic diagnosis is conventionally performed by the detection of eggs either in sputum and stool or in tissue. However, it is difficult to detect eggs not only in the stages of early and late infections but also in the stage of infection in extrapulmonary lesions because the eggs rarely exist in such stages. In human paragonimiasis, diagnosis currently depends on serologic assays that detect specific antibodies in serum, cerebrospinal fluid, or pleural effusion.[2,19] Specific antibody tests can discriminate patients with active disease from cured or previously infected patients and from patients with other etiologies. Crude extracts from the adult worms have widely been used as antigens for diagnosis.[19–22] However, these antigens have less sensitivity and specificity in diagnosing a wide clinical spectrum of human paragonimiasis because these crude extracts are composed of many proteins, resulting in serologic cross-reactions with other parasitic antigens. Since the cysteine proteinases are continuously released outside the parasites, the enzymes have recently been studied for the purpose of diagnosis and as a potential target for chemotherapy.[4,23–25] However, a diagnosis using a specific cysteine proteinase from *P. westermani* with sera from patients with paragonimiasis has not been studied in detail.

In this study, we cloned a new cysteine proteinase (PwCP2) that is secreted as a 24-kD protein and is localized in the intestinal epithelium of the adult worm. In addition, we tested the diagnostic potential of recombinant PwCP2 (rPwCP2) with sera from patients with paragonimiasis or with sera from rats infected with *P. westermani* metacercariae.

MATERIALS AND METHODS

Parasites and isolation of RNA. The metacercariae of *P. westermani* were obtained from naturally infected crayfish (*Cambaroides similis*) collected in Wando-gun, Korea. Dogs were orally infected with 150 metacercariae and killed on day 90 after infection. The worms were harvested from cysts in their lungs. The dead worms were homogenized in 0.01 M Tris buffer (pH 7.2) at 4°C for 30 minutes. The supernatant was obtained by centrifugation at 15,000 rpm for one hour at 4°C and used as crude extract. The live worms were cultured in Krebs-Ringer solution at 37°C. After several days of cultivation, the supernatant was obtained by centrifugation at 15,000 rpm for 30 minutes at 4°C and used as an excretory-secretory (ES) product. The mRNA was purified from the adult worms and reverse-transcribed into single-stranded cDNA with an oligo$(dT)_{17}$ primer as previously described.[26]

Cloning of PwCP2. A 500-base pair cDNA fragment was amplified by a polymerase chain reaction (PCR) from single-stranded cDNA using two degenerate primers (dP5: 5′-CARGGICARTGYGGIWSITGYTGG-3′ and dP3: 5′- CCAISWRTTYTTIACRATC-3′ (where I = inosine, R = A or G, Y = T or C, W = A or T, and S = C or G) that correspond to highly conserved regions of eukaryotic cysteine proteinases. The following PCR conditions were used: 35 cycles at 95°C for 50 seconds, 50°C for two minutes, and 72°C for three minutes, followed by an extension at 72°C for 10 minutes. The amplified fragments were cloned into a T-vector (Novagen, Darmstadt, Germany) and sequenced. To characterize the full-length PwCP2 cDNA, 5′ and 3′ rapid amplification of cDNA ends (RACE) were used according to the manufacturer's instructions (Invitrogen, Carlsbad, CA) with mRNA isolated from *P. westermani* adult worms. The following primers were used for the 5′ RACE: GSP1 primer: 5′-AACGCCCAACACGAACCA-3′ and GSP2 primer: 5′- GCACGCGTTTCACCTGGT-3′. In addition, the GSP1 primer 5′-CCAAAATCGATGATTCAAT-3′ was also used for the 3′ RACE. The PCR was carried out for 30 cycles with denaturation at 94°C for 30 seconds, annealing at 68°C for four minutes, and extension at 68°C for four minutes, and the amplified fragments were cloned into a T-vector (Novagen) and sequenced.

Expression and purification of PwCP2. The mature form of PwCP2 cDNA was obtained by a PCR using the upstream primer 5′-GAA TTC GCT CCC GAA CGT ATT GAC-3′ and the downstream primer 5′-GTC GAC CAT ATC ACC TTC CTT ATC-3′ from mRNA of *P. westermani* adult worms. The underlined sequences are *Eco* RI and *Sal* I restriction sites, respectively, which were included for cloning. The following conditions for the PCR were used: 30 cycles at 95°C for 50 seconds, 50°C for 30 seconds, and 72°C for 30 seconds. A 657-base pair band was subjected to agarose gel electrophoresis, eluted from the gel, cloned into an *E. coli* expression vector pET21a(+) (Novagen), and sequenced. *E. coli* BL21 (DE3) cells (Novagen) transformed with the recombinant plasmid were grown to an absorbance value of 600 (A_{600}) of 0.6 in Luria-Bertania broth and induced by the addition of isopropylthio-β-D-galactopyranoside to a final concentration of 0.8 mM. Incubation was continued at 37°C for an additional four hours, at which time the cells were harvested by centrifugation at 3,000 × *g* for 10 minutes, resuspended in buffer A (6 M guanidine-HCl, 0.1 M sodium phosphate, 0.01 M Tris-HCl, pH 8.0), and lysed by rocking for four hours. After centrifugation, the supernatant was incubated with 1 mL of Ni^{2+}-NTA resin (Peptron, Daejeon, Korea) and equilibrated with buffer A for three hours. The lysate-resin mixture was loaded on a mini-column (Sigma, St. Louis, MO) and sequentially washed with 10 bed volumes of buffer A, buffer B (8 M urea, 0.1 M sodium phosphate, 0.01 M Tris-HCl, pH 8.0), and buffer C (buffer B, pH 6.3) at a flow rate of 10–15 mL/hour. Bound proteins were eluted with buffer F (buffer B, pH 4.5, supplemented with 200 mM EDTA), dialyzed in phosphate-buffered saline (PBS), and quantified with Bradford solution (Bio-Rad, Hercules, CA).

Production of antibodies. Purified rPwCP2 (60 μg/400 μL) was subcutaneously injected into six-week-old (BALB/C mice) with Freund's complete adjuvant (Difco, Detroit, MI). A booster immunization with rPwCP2 was performed with the same amount two weeks after the first immunization. Blood was obtained from the heart two weeks after the second immunization. A rPwCP2 antiserum was obtained from the blood by centrifugation at 13,000 rpm for 10 minutes at 4°C.

Collection of rat sera. To produce a *P. westermani* antiserum, each of 30 rats were orally infected 10 metacercariae and killed on day 24 after infection. Sera were obtained from the heart. Sera were also obtained from the tails of 30 rats before infection.

Western blotting. The crude extract and the ES product of *P. westermani* adult worms and rPwCP2 protein were separated by SDS-PAGE on a 12% polyacrylamide gel and transferred to a nitrocellulose membrane. The membrane was blocked overnight with 5% non-fat milk in PBS at 4°C, washed with PBS containing 0.05% Tween 20 (PBST), incubated with the rPwCP2 antiserum (1:2,000 dilution), washed, incubated with goat anti-mouse IgG conjugated with horseradish peroxidase (1:5,000 dilution), and subjected to an enhanced chemiluminescence detection system (Amersham Pharmacia Biotech, Uppsala, Sweden). For Western blot analysis of sera from patients with paragonimiasis, clonorchiasis, sparganosis, or cysticercosis, rPwCP2 was separated by SDS-PAGE on a 12% polyacrylamide gel and transferred to a membrane. The membrane was incubated with serum from a patient with paragonimiasis (1:100 dilution), clonorchiasis (1:100), sparganosis (1:100), cysticercosis (1:100), or serum from a healthy person (1:100) as a negative control and then with goat anti-human IgG conjugated with horseradish peroxidase (1:5,000 dilution). Color reactions were observed by the addition of diaminobenzidine (DAB; Sigma). Serum from healthy individuals was confirmed to be free of other parasites. Paragonimiasis (*P. westermani*) and clonorchiasis (*Clonorchis sinensis*) were confirmed by the microscopic detection of eggs in the sputum (*P. westermani*) and feces (*C. sinensis*). Sparganosis *(Sparganium* spp.) and cysticercosis (*Taenia solium*) were confirmed by the histologic detection of worms. Sera from healthy individuals were obtained from the Wonkwang Medical Center of Clinical Pathology Laboratory (Iksan, Korea). All patients and healthy individuals agreed to provide serum samples after filling out and signing standard informed consent forms. The study was reviewed and approved by University Faculty of Medicine Local Ethics Council of Wonkwang University.

Immunohistochemical staining. Paraffin-embedded sections of tissues infected with *P. westermani* were stained with rPwCP2 antiserum (1:1,000 dilution) or uninfected mouse serum (1:1,000 dilution) and incubated with goat anti-mouse IgG conjugated with horseradish peroxidase. Color reactions were observed by the addition of DAB. The slides were counterstained with hematoxylin.

Enzyme-linked immunosorbent assay. The purified rPwCP2 (50 ng/well) was coated onto 96-well microtiter plates (Corning, Corning, NY) for two hours at 37°C. The plates were blocked with 3% casein (Sigma) in PBS for one hour at 37°C, incubated with sera (1:800 dilution) from rats infected with *P. westermani* metacercariae or with sera of rats before infection for one hour at 37°C, washed with PBST, incubated with goat anti-human IgG conjugated with horseradish peroxidase (1:15,000 dilution), and washed with PBST. The color reaction was detected by incubating samples with 100 μL of the 3, 3′5, 5′-tetramethylbenzidine peroxidase substrate (Kirkegaard and Perry Laboratories, Gaithersburg, MD) for 10 min-

utes. The absorbance was measured at 450 nm with a 650-nm reference filter. Results are shown as the mean ± SD of triplicate experiments.

RESULTS

We cloned a new cysteine proteinase from *P. westermani* adult worms using a degenerate PCR with primers that correspond to highly conserved regions of eukaryotic cysteine proteinases and RACE. The new enzyme has been designated PwCP2. The gene has an open reading frame of 816 base pairs encoding a 272-amino acid polypeptide with a calculated molecular mass of 30 kD and a theoretical pI of 5.71. Consistent with the putative function as a cysteine proteinase, PwCP2 has a conserved catalytic triad of cysteine, histidine, and asparagine residues. The amino acid sequences of the gene showed moderate similarity with that of other cysteine proteinases (Figure 1). The similarity with the cysteine proteinases of *P. westermani* (PwCP1) and *Schistosoma mansoni* and with human cathepsin F was 42%. A fragment lacking the amino terminal 53 amino acids of PwCP2 cDNA, designated here as the mature form of PwCP2, was amplified, cloned into the expression vector pET21a(+), and used to transform *E. coli* BL21 (DE3). The PwCP2 enzyme was abundantly expressed in an insoluble form, purified by metal chelate affinity chromatography and refolded by dialysis in PBS. The molecular mass of the purified rPwCP2 was approximately 24 kD, which is consistent with the deduced molecular mass (Figure 2A, lane 3). Interestingly, staining of the crude extract and ES product of *P. westermani* adult worms with Coomassie blue showed an intense 24-kD band in each sample that corresponded to the deduced molecular size of the mature form and rPwCP2 (Figure 2A). To confirm whether the bands are PwCP2, six-week-old mice were subcutaneously injected with rPwCP2, and antiserum was pooled. Western blotting of the crude extract and ES product of adult *P. westermani* with the rPwCP2 antiserum indicated that the 24-kD band in each sample was PwCP2 (Figure 2B). This result suggests that PwCP2 is secreted as a mature form, and is present in the ES product of *P. westermani* adult worms. To examine the localization of PwCp2 in the adult worm, immunohistochemical

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P2   ------------------------------------------------------------
P1   ---CG-LKEHPPRRLSVEFFYRPWMDAFQRLAIEQLRISRRSIELVSLPSNIELLGFRLP  120
SM   ------------------------------------------MPVNLEYLGFKLP   13
HF   RKDCGPVDTKVPGAGEPKSAFTQGSAMISSLSQNHPDNRNETFSSVISLLNEDPLSQDLP  180

P2   ----------------------------------MRAQKLQLKDQGTARYGVTQFSDLT   25
P1   QNTSRLFEEFQRKFRKSYSS-DTAKR-YALFKYNLLKMQLIQRLEKGTANYGITKFSDLS  178
SM   GNVDEKYVQFKLKYRKQYHETEDEIR-FNIFKSNILKAQLYQVFVRGSAIYGVTPYSDLT   72
HF   VKMASIFKNFVITYNRTYESKEEARWRLSVFVNNMVRAQKIQALDRGTAQYGVTKFSDLT  240

P2   PEEFAAKYLSAP----VNNDQVKRVRPTGLKAAPERIDWRAKGAVTAVENQGSCGSCWAF   81
P1   AEEFRHSLANMKRRKSKGSQMETAIFPTTIQSLPPSFDWRANGAVTEVKDQGMCGSCWAF  238
SM   TDEFARTHLTASWVVPSSRSNTPTSLGKEVNNIPKNFDWREKGAVTEVKNQGMCGSCWAF  132
HF   EEEFRTIYLNTLLRKEPGNKMKQAKSVGDL--APPEWDWRSKGAVTKVKDQGMCGSCWAF  298

P2   STAGNVEGQWFIKTGQLVSLSKQQLVDCDRAADGCNGGWPASSYLEIMHMGGLESQDDYP  141
P1   ATTGNIEGQWFRKTNKLISLSEQQLLDCDTKDEACNGGLPEWAYDEIVKMGGLMSEKDYP  298
SM   STTGNVESQWFRKTGKLLSLSEQQLVDCDGLDDGCNGGLPSNAYESIIKMGGLMLEDNYP  192
HF   SVTGNVEGQWFLNQGTLLSLSEQELLDCDKMDKACMGGLPSNAYSAIKNLGGLETEDDYS  358

P2   YAGVKEQ-CFMEKERLLAKIDDSIALGPSEDDNAAYLAEHGPLSTLLNAITLQYYQSGII  199
P1   YEAMKEQSCHLRRPNISAYINGSATLPSDEAKLAAWLVQNGPISVGVNANFLQFYLGGIS  358
SM   YDAKNEK-CHLKTDGVAVYINSSVNLTQDETELAAWLYHNSTISVGMNALLLQFYQHGIS  251
HF   YQG-HMQSCNFSAEKAKVYINDSVELSQNEQKLAAWLAKRGPISVAINAFGMQFYRHGIS  418

P2   HPSYEECSPVDLNHAVLTVGYDKEG--DMPYWIIKNQLECGVGRERLLPTLPGRWNMWNQ  257
P1   HPPHMLCSEAGLDHAVLLVGYGVSTFLRRPYWIVKNSWGGGWGEK-------GYFRMYRG  411
SM   HPWWIFCSKYLLDHAVLLVGYGVSE-KNEPFWIVKNSWGVEWGEN-------GYFRMYRG  303
HF   RPLRPLCSPWLIDHAVLLVGYGNRS--DVPFWAIKNSWGTDWGEK-------GYYYLHRG  469

P2   SHAYIRDHPLMKTM--   271
P1   DGTCGINADPTTSIIQ   427
SM   DGSCGINTVATSAMIY   319
HF   SGACGVNTMASSAVVD   485
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FIGURE 1. Alignment of the deduced amino acid sequence encoding the cysteine proteinase of *Paragonimus westermani* (PwCP2) (P2, GenBank accession number AY527384) with those of other related enzymes. The amino acid sequences of PwCP1 (P1, U69120[18]), cysteine proteinase from *Schistosoma mansoni* (SM, U07345[27]), and human cathepsin F (HF, NM_003793[28]) are shown for comparision. Identical amino acid residues are shaded in gray and gaps (-) are introduced to maximize alignment. The cysteine proteinase catalytic triad residues (C, H, and N) are shown in the box. The N-terminal amino acid sequences of P1 and HF do not have sequence similarity with PwCP2 and are not shown. The **arrow** indicates the potential cleavage site between the proregion and the mature form of PwCP2.

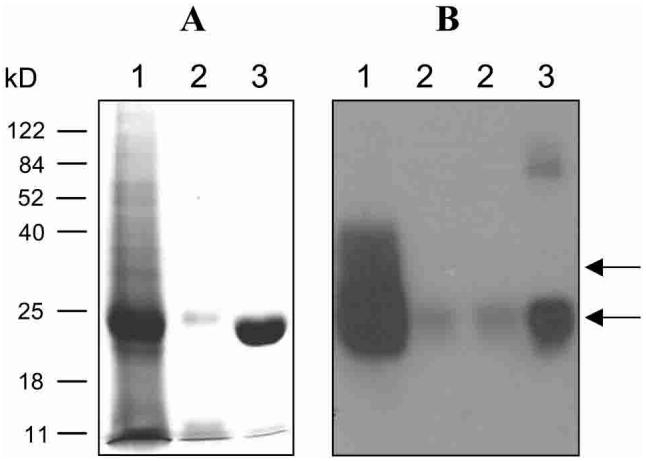

FIGURE 2. Western blot analysis of the cysteine proteinase of *Paragonimus westermani* (PwCP2) by **A**, staining with Coomassie blue and **B**, Western blotting. Lane 1, crude extract; lane 2, excretory-secretory product of *P. westermani*; lane 3, recombinant PwCP2 (rPwCP2). The proteins were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis on a 12% polyacrylamide gel and stained with 0.25% (w/v) Coomassie blue staining solution (**A**) or transferred to a nitrocellulose membrane for Western blotting (**B**) with rPwCP2 antiserum described in the Materials and Methods. The **upper and lower arrows** indicate the immature and mature form of PwCP2, respectively.

staining was performed with paraffin-embedded sections of tissues infected with *P. westermani* using the rPwCP2 antiserum. Staining with this antiserum demonstrated that PwCP2 was found mainly in the intestinal epithelium of *P. westermani* adult worm (Figure 3B and D), whereas staining with uninfected serum showed no reaction (Figure 3A and C).

We next examined whether PwCP2 could be a diagnostic antigen for the detection of infection with *P. westermani*. Western blotting of the sera of 10 patients with paragonimiasis using rPwCP2 antigen showed that 90% (9 of 10, sensitivity = 90%) of the sera reacted with rPwCP2 antigen (Figure 4A). However, no reaction was shown with 11 sera from healthy individuals without infection (0 of 11, specificity = 100%) (Figure 4B). When sera from patients with clonorchiasis, sparganosis, or cysticercosis as well as paragonimiasis were analyzed, only serum from patient with paragonimiasis reacted with rPwCP2 (Figure 4C), suggesting that PwCP2 is a specific antigen for the detection of infection with *P. westermani*. To further confirm the diagnostic potential of PwCP2 by increasing sample numbers, we infected 30 rats each with 10 metacercaria, and the antisera were obtained on day 24 after infection. An ELISA using these antisera and rPwCP2 antigen showed a reactivity with sera (28 of 30, sensitivity = 93%) from 28 rats infected with *P. westermani* (Figure 5). However, when 30 sera from healthy rats were tested, only two sera reacted with rPwCP2 (2 of 30, specificity = 93%) with a low ELISA value. Incubation with crude extracts of adult *P. westermani* instead of the rPwCP2 showed a similar result.

DISCUSSION

In this study, we have cloned a cDNA encoding a new cysteine proteinase (PwCP2) from the adult *P. westermani* through a degenerate PCR with primers that correspond to highly conserved regions of eukaryotic cysteine proteinases. The PwCP2 enzyme has a short proregion of 53 amino acids compared with the other cysteine preteinases (Figure 1), and the deduced molecular mass of the mature form was 24 kD.

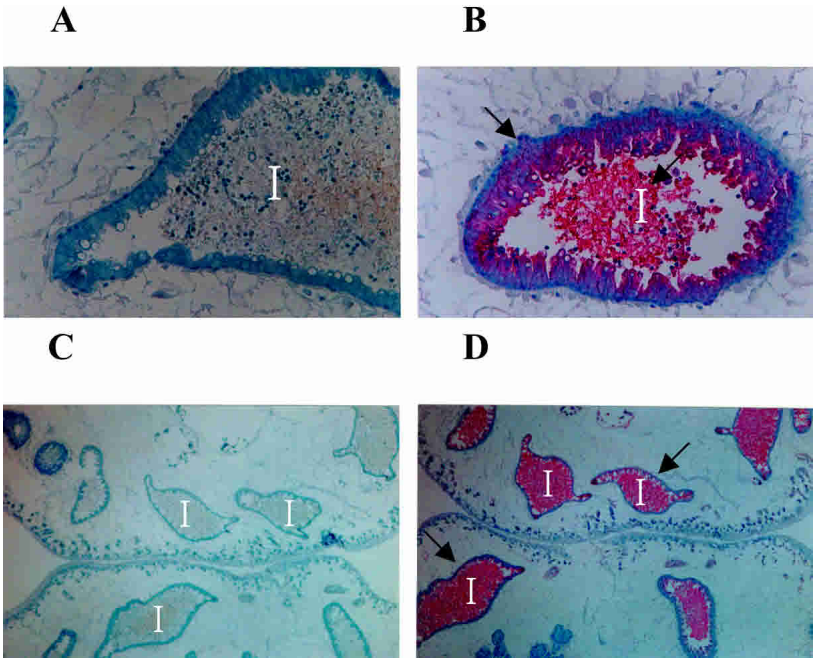

FIGURE 3. Localization of the cysteine proteinase of *Paragonimus westermani* (PwCP2) in the adult worm. Immunohistochemical staining of PwCP2 was performed with uninfected mouse serum (**A** and **C**) and recombinant PwCP2 antiserum (**B** and **D**) described in the Materials and Methods. Note that PwCP2 was expressed mainly in the intestinal epithelium of the *P. westermani* adult worm (indicated by the **arrows** in **B** and **D**). **I** = intestine. (Magnifications × 200 in **A** and **B** and × 40 in **C** and **D**.)

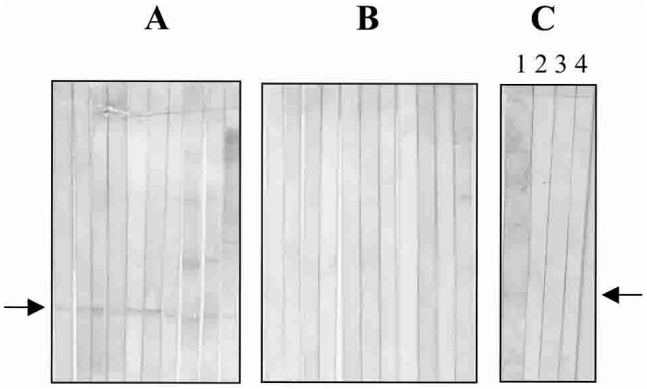

FIGURE 4. Western blotting of the sera of patients with paragonimiasis using recombinant cysteine proteinase of *Paragonimus westermani* (rPwCP2). One hundred nanograms of rPwCP2 was separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis on a 12% polyacrylamide gel, transferred to a nitrocellulose membrane, and probed with **A**, the sera of 10 patients with paragonimiasis (1:100 dilution) and **B**, uninfected serum (1:100 dilution). **C**, Western blotting of the sera of patients with paragonimiasis (lane 1, 1:100 dilution), clonorchiasis (lane 2, 1:100 dilution), sparganosis (lane 3, 1:100 dilution), or cysticercosis (lane 4, 1:100 dilution) using rPwCP2. **Arrows** indicate PwCP2 bands recognized by the sera of patients with paragonimiasis.

To examine its expression and localization in adult *P. westermani*, we produced an antiserum against rPwCP2 that lacks a proregion and is expressed in *E. coli*. Interestingly, Western blot analysis using this antiserum showed that PwCP2 exists in an ES product as a 24-kD protein that corresponds to the mature form size. Also, in crude extract of adult *P. westermani*, the majority of PwCP2 was detected as a mature form, although there was some immature form (30 kD). In addition, immunohistochemical staining showed that PwCP2 was localized in intestinal epithelium of adult *P. westermani*.

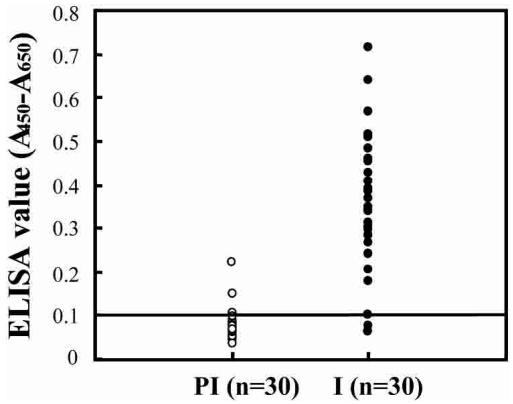

FIGURE 5. Enzyme-linked immunosorbent assay (ELISA) using the recombinant cysteine proteinase of *Paragonimus westermani* (rPwCP2) in sera from rats infected with metacercariae of *P. westermani*. After an ELISA plate was coated with rPwCP2 (50 ng/well) at 37°C for two hours, it was incubated with rat sera (1:800 dilution) infected with *P. westermani* metacercariae (**I**, n = 30) or preimmune sera (1:800 dilution) before infection (**PI**, n = 30) and then with peroxidase-conjugated anti-rat IgG antibody (1:15,000 dilution). The absorbance was measured at 450 nm with a 650-nm reference filter. Each point represents the mean ± SD of triplicate experiments. The cut-off value (**horizontal line**) was determined as the mean + 2 SD (0.10) in uninfected rat sera.

Since there was no cross-reactivity when antiserum was incubated with rPwCP1, which has the highest amino acid sequence homology with PwCP2 among the cysteine proteinases of *P. westermani*, we can exclude the possibility that the specific localization and expression of PwCP2 resulted from the non-specific reaction of antiserum with other cysteine proteinases. The presence of PwCP2 in the ES products as a mature form suggests that the enzyme may be processed as it is trafficked through the cellular secretory pathway before being released outside the parasite.

Since fluke cysteine proteinases become antigenic to the host by being continuously released outside the fluke, these enzymes have been investigated for use in the immunodiagnosis of fluke infections. Using the ES product of *Paragonimus* spp. has improved the sensitivity and specificity of immunodiagnosis to some extent.[29,30] When partially purified cysteine proteinases from the ES products of *P. westermani* were used, increased sensitivity to sera from patients with paragonimiasis westermani and reduced cross-reactivity with the sera of patients with fascioliasis, onchocerciasis, and clonorchiasis were obtained.[31] Also, the cysteine proteinases from *Facciola* spp.[32] and *S. mansoni*[33] for immunodiagnosis have shown good sensitivity and specificity to sera from patients with fluke infections. Since PwCP2 was also detected in the ES product in a mature form, we speculated that this enzyme could become antigenic to the host by inducing a high antibody response. To assess the usefulness of PwCP2 for the diagnosis of paragonimiasis, we analyzed the sera of 10 patients with paragonimiasis and sera from healthy individuals by Western blotting using rPwCP2. The results showed that the sensitivity and specificity of this test were 90% and 100%, respectively. Furthermore, PwCP2 did not cross-react with sera from patients with clonorchiasis, sparganosis, or cysticercosis. Amino acid sequence comparison of PwCP2 with those of the cysteine proteinases of *C. sinensis* was ranged from 13% to 45%, suggesting that the regions showing sequence similarities are not overlapping epitopes. An ELISA using rat sera infected with *P. westermani* metacercariae showed that the sensitivity and specificity were greater than 90%, further confirming the diagnostic potential of PwCP2.

At the present time, we do not know the exact function of PwCP2 in the adult *P. westermani*. It was reported that *P. westermani* produces at least six different species of the cysteine proteinases in its developmental stages.[11,12,14,34] The proteinases have diverse biologic functions, including metacercarial excystment, nutrient uptake, worm migration, and host immune evasion.[11,35] Because PwCP2 was not only excreted and secreted from the adult worm but also localized mainly in the intestinal epithelium, we speculated that PwCP2 may have a diverse functions such as nutrient uptake, worm migration, or immune evasion. The substrate specificity of PwCP2 and its putative function in the adult *P. westermani* are now under investigation.

In conclusion, we report that PwCP2 cloned from *P. westermani* adult worms is an suitable antigen for the serodiagnosis of patients with paragonimiasis since a sufficient amount of antibody is induced in almost all patients, and it can also be a potential target in the treatment of infection.

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