Structural and Immunological Characteristics of a 28-Kilodalton Cruzipain-Like Cysteine Protease of *Paragonimus westermani* Expressed in the Definitive Host Stage

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A complete cDNA sequence encoding a 28-kDa cruzipain-like cysteine protease of adult *Paragonimus westermani*, termed Pw28CCP, was isolated from an adult cDNA library. The cDNA contained a single open reading frame of 975 bp encoding 325 amino acids, which exhibited the structural motif and domain organization characteristic of cysteine proteases of non-cathepsin B5 including a hydrophobic signal sequence, an ERFNIN motif, and essential cysteine residues as well as active sites in the mature catalytic region. Analysis of its phylogenetic position revealed that this novel enzyme belonged to the cruzipain-like cysteine proteases. The sequence of the first 13 amino acids predicted from the mature domain of Pw28CCP was in accord with that determined from the native 28-kDa enzyme purified from the adult worm. Expression of Pw28CCP was observed specifically in juvenile and adult worms, with a location in the intestinal epithelium, suggesting that this enzyme could be secreted and involved in nutrient uptake and immune modulation. The recombinant protein expressed in *Escherichia coli* was used to assess antigenicity by immunoblotting with sera from patients with active paragonimiasis and from those with other parasitic infections. The resulting sensitivity of 86.2% (56 of 65 samples) and specificity of 98% (147 of 150 samples) suggest its potential as an antigen for use in immunodiagnosis.

*Paragonimus westermani* is a trematode parasite that causes chronic inflammatory lung disease as well as systemic infection including cerebral invasion in humans and carnivorous mammals. Human infection occurs by ingesting undercooked freshwater crayfish or crabs containing metacercaria or eating raw boar meat. The metacercariae excyst in the duodenum, penetrate the peritoneal cavity, and finally, migrate to the lung, in which they become adults and are surrounded by a thick granulomatous wall (1, 18). The adult worms can survive for approximately 5 years.

Parasite cysteine proteases are known to play critical roles in parasitic infections. They participate in a broad range of biological processes including egg hatching and subsequent stage transitions, invasion and migration through host tissues, and immune modulation and nutrient uptake (4, 25, 28, 29, 39, 43). Recent studies have shown that these enzymes might be good targets for the development of vaccines (20, 33) and antiparasitic drugs (7, 9).

In *P. westermani*, at least six different species of cysteine protease with molecular sizes of 53, 34, 28, 27, 17, and 15 kDa, respectively, have been characterized in eggs, metacercariae, juveniles, and adults (4, 5, 14, 22, 45). The 28- and 27-kDa proteases released by the metacercariae have been shown to share biochemical features with cathepsin L of *F. hepatica* (8) or with cathepsin S of sparganum (25) and are believed to play important roles in metacercarial excystment, tissue migration, and immune evasion (4, 6, 14, 45).

It is of particular interest that *P. westermani* possesses multiple cysteine proteases of similar sizes ranging from 29 to 27 kDa (4, 6, 14, 45). To date, however, only one gene encoding a 28-kDa neutral thiol protease (NTP) of *P. westermani* has been well characterized in terms of its molecular and biological properties (45, 46). Recently, a gene encoding a putative cysteine protease (PwCP) was reported (32), but its protein and enzymatic identities have not been described.

In the present study, we elucidate the molecular properties of a new 28-kDa cysteine protease (Pw28CCP) that is expressed specifically in juvenile and adult stages of *P. westermani*. We characterize it as a 28-kDa cruzipain-like cysteine protease on the basis of its molecular structure. The potential usefulness of a recombinant protein as an immunodiagnostic antigen has also been investigated.

**MATERIALS AND METHODS**

*Parasite materials from different developmental stages of *P. westermani*. Cats and dogs were fed metacercariae isolated from crayfish (*Cambareolus similis*) and were killed 1, 2, 3, 4, 7, and 16 weeks after infection. The 1- and 2-week-old juveniles were harvested from the peritoneal cavity, and 3- and 4-week-old juveniles were harvested from the peritoneal, thoracic, and pleural cavities of infected cats. The 7-week-old juvenile and 16-week-old adult worms were collected from the lungs of infected dogs (5). Eggs were collected by incubating the adult worms in physiological saline overnight at 37°C (22). Eggs, metacercariae, 1-, 2-, 3-, 4-, and 7-week-old juveniles, and adult worms were either used immediately for RNA preparation or stored at −70°C prior to protein extraction.

**Purification and N-terminal amino acid sequencing of a 28-kDa native cysteine protease of adult *P. westermani*.** The 28-kDa cysteine protease of adult *P. westermani* was purified by Sephacryl S-300 gel filtration (1.6 by 70 cm long), followed by DEAE anion-exchange chromatography (1.6 by 2 cm long), and enzyme activity was monitored with a synthetic substrate, carboxybenzoyl-phenylalanyl-arginyl-7-aminomethylcoumarin (Cbz-phe-arg-AMC) (5). The purified protein was resolved by 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and transblotted onto a polyvinylidene difluoride (PVDF) micro porous membrane (Millipore, Bedford, Mass.). The enzyme band excised from the membrane was used for protein sequencing. N-terminal sequence determination was carried out on an ABI model 477A sequencer and an

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Results

Purification and N-terminal amino acid sequencing of the native 28-kDa protease of P. westermani. The use of gel filtration and DEAE ion-exchange chromatography allowed effective purification of the native 28-kDa protease from adult P. westermani. The purified enzyme showed maximal activity against Cbz-phe-arg-AMC at pH 6.0 in the presence of 2 mM diithiothreitol, with a molecular mass of 28 kDa by SDS-PAGE, as described previously (5). The N-terminal sequence, (3)PA (3)VDWERKGA, showed significant homology with the cathepsin family of cysteine proteases from parasites to mammals. The five residues including Pro, Asp, Trp, Arg, and Val were conserved among members of this enzyme family (25, 27, 28, 44).
The Ala at position 214 of 63.4%.

Significant homology was also observed with the 3

acids with an estimated molecular mass of 36,105 Da. The 3

was used as a probe to screen the adult worm cDNA library.

known cysteine proteases of the cathepsin family available in

zyme.

Papain-like, papain (M15263) ................................................. 37.5

Cruzipain-like

P. westermanni NTP (D21124) ................................................. 63.4
P. sinensis cysteine protease 1 (AF093243) .................................. 59.1
P. westermanni cysteine protease (U36861) .................................. 50.5
S. mansoni Puerto Rican cathepsin L1 (U07345) .......................... 50.5
T. congolense cysteine protease (L25101) .................................. 38.2
L. major cathepsin L (U43706) .................................................. 37.8
T. cruzi cruzipain (M84342) .................................................... 37.5

Cysteine proteases (12, 23, 41), was found; of seven residues, it conserved

constitute the core globular portion of the

be involved in the formation of catalytic triad, as all other

cysteine residues (at positions

22, 56, 63, 96, 154, and 202, respectively) proposed to be

essential for the composition of the tertiary structure by disul- 

dide bridges were also recognized in the same domain. The

potuitive S1 subsites were located at Try, Pro, Ala, and Ala at

positions 67, 68, 136, and 162, respectively, and those corre-

sponding to S2 subsites were Pro, Ser, and Ile (at positions 69, 210,

and 212, respectively). The potential S1` sites were iden-

tified at Gln 19 and Trp 177. A single site for substrate binding

was present at Gly 65. All these sites might be involved in the

formation of the active-site cleft that determines the substrate

specificity and active catalysis (2).

The phylogenetic trees constructed to examine the evolu-

tory relationship of Pw28CCP to other cysteine proteases

revealed that Pw28CCP belonged to the cruzipain-like cysteine

protease of branch B (Fig. 1A) (41). It formed a strong clade

with the cysteine proteases of P. westermanni, C. sinensis, and S.

mansonii Puerto Rican preprocathepsin L1 with a 100% boot-

strap value (Fig. 1B).

Further comparison of the ERFNIN motif demonstrated that

the cruzipain-like cysteine proteases, including Pw28CCP,

tended to share higher levels of sequence homology in the first

half of the motif than the posterior half. In cathepsin L-like

proteases, the first half of the motif appeared to be less con-

served than the posterior half (Fig. 2). In addition, the pro-

line at position –60 and Ala at position –50 were highly conserved in

cruzipain-like cysteine proteases but absent from cathepsin

L-like enzymes. The Phe at position –57, which was highly

conserved in papain- and cruzipain-like proteases, was re-

placed by Trp in five of seven cathepsin L-like proteases ex-

amined. The His residue at position –47 was well conserved in

all cathepsin L-like proteases examined. Notably, NTP con-

tained only one residue of the ERFNIN motif on the basis of

this alignment. It is likely that NTP did not contain the motif,

which further supported the possibility that, in fact, it might be

another member of cathepsin family.

Southern blotting with the full-length cDNA probe showed that

the size of Pw28CCP genomic DNA was approximately 2.5

kb on the basis of the BamHI digestion pattern (data not shown).

Northern blot analysis with the same probe revealed a single

band at ca. 1.0 kb (data not shown), consistent with the

length (1.052 bp) of the Pw28CCP cDNA sequence.

Comparison of expression levels of Pw28CCP, NTP, and

PwCP among different developmental stages of P. westermanni.

Semiquantitative RT-PCR was performed with primers spe-

cific for Pw28CCP, NTP, and PwCP to examine the levels of

expression of the mRNAs of these three genes among different

life cycles including eggs, metacercariae, juveniles 1, 2, 3, 4, and

7 weeks old, and adults. As shown in Fig. 3A, the expression

patterns of the three enzymes appeared to be different from

each other. The mRNA of Pw28CCP was absent from eggs and

metacercariae but was present in 1- to 7-week-old juveniles and

adults. Expression of NTP and PwCP mRNAs was de-

tected in all stages except the egg stage. The levels of expres-

sion of Pw28CCP mRNA in juveniles of different ages and

adults appeared to be similar. In contrast, the levels of expres-

sion of NTP and PwCP mRNAs were shown to be age de-

pendent, with the expression of both mRNAs being down-regu-

lated in the adult. The results of Northern blot analysis of

Pw28CCP and NTP mRNA expression were similar to those

obtained by RT-PCR, although the expression levels could not

be directly compared due to the low sensitivity of the test (Fig. 3B).

The expression of NTP declined dramatically in the adult

stage as determined by both Northern blot and RT-PCR anal-

yses. Figure 3C shows the immunoreactivity of Pw28CCP with

pooled serum collected sequentially from four cats experiment-

ally infected with 50 metacercariae. Specific antibody reac-

TABLE 1. Comparison of homologies between Pw28CCP and other cysteine proteases members

| Description (GenBank accession no.) | Homology |
|------------------------------------|----------|
| Papain-like, papain (M15263)       | 37.5     |
| Cruzipain-like                     |          |
| P. westermanni NTP (D21124)        | 63.4     |
| P. sinensis cysteine protease 1    | 59.1     |
| P. westermanni cysteine protease   | 50.5     |
| S. mansoni Puerto Rican cathepsin L1 | 50.5   |
| T. congolense cysteine protease    | 38.2     |
| L. major cathepsin L               | 37.8     |
| T. cruzi cruzipain (M84342)        | 37.5     |
| Cathepsin L-like                   |          |
| F. hepatica secreted cathepsin L2  | 47.4     |
| F. hepatica cathepsin L (AB009306) | 38.1    |
| Fasciola sp. cysteine protease (S70380) | 38.1  |
| Mouse cathepsin L (M20495)         | 36.0     |
| Human cathepsin L (M20496)         | 29.8     |

*The sequence used for comparison included the whole region of propro-

ten-zyme.

partial fragment clearly encoded a unique cysteine protease

which shared a significant homology to, but was different from,

known cysteine proteases of the cathepsin family available in

the EMBL, GenBank, and DDBI databases. This fragment

was used as a probe to screen the adult worm cDNA library.

The largest insert identified (1,052 bp in length) had a 975-bp

complete open reading frame encoding a protein of 325 amino

acids with an estimated molecular mass of 36,105 Da. The 3’

untranslated region contained a putative polyadenylation

signal (AATAACA), followed by a short poly(A) + tail of 17

nucleotides. A sequence similarity search of the GenBank data-

base demonstrated that Pw28CCP was most closely related to

the NTP of P. westermanni metacercariae (46) with an identity

of 63.4%. Significant homology was also observed with the
different cysteine proteases from other parasites as well as

mammals, with identities ranging from 39.1% with Clonorchis

sinensis cysteine protease 1 to 29.8% with human cathepsin L

(Table 1).

The deduced amino acid sequence comprised an 18-residue

hydrophobic region followed by a 93-residue propeptide region

and a 214-residue mature protein domain. The putative rec-

ognition site for a signal peptidase (42) was identified between

and a 214-residue mature protein domain. The putative rec-

(43).

The potential ERFNIN motif, which may constitute the core globular portion of the α-helix and which is

characteristic of the non-cathepsin B family of cysteine protease cysteine proteases (12, 23, 41), was found; of seven residues, it conserved four amino acids of Arg, Ile, Phe, and Asn at positions –61, –58, –57, and –54, respectively. The mature peptide started from Ala at position 1 since it immediately preceded Pro 2, which is highly conserved in all family members. It was con-

firmed by N-terminal amino acid sequencing. The sequence of the first 13 amino acids predicted from the mature domain showed a perfect match with the N-terminal sequence deter-

mined from the native enzyme from adult worm except for the

first and the fourth residues, which could not be clearly deter-

mined with the model 120A PTH-analyzer (Perkin-Elmer Ap-

plied Biosystems) due to a high background. The mature do-

main contained three conserved residues of Cys, His, and Asn

at positions 25, 161, and 181, respectively, which are likely to

be involved in the formation of catalytic triad, as all other

cysteine proteases are. The six cysteine residues (at positions

53-258]
tions to rPw28CCP could be detected from 6 weeks after infection.

Localization of Pw28CCP and PwCP by in situ hybridization. To identify the locations of Pw28CCP and PwCP gene expressions in the cells and tissue of the worm, in situ hybridization was performed. As shown in Fig. 4A and B, the hybridization signals with Pw28CCP were detected in the intestinal epithelium of the adult worm but not in the metacercariae.

FIG. 1. Phylogenetic analysis of P. westermani 28-kDa cysteine protease by unrooted neighbor-joining tree (A) and phylogram (B). Analysis shows the position of Pw28CCP as a sister group of the S. mansoni Puerto Rican cathepsin L (branch B), which is one of the ancestral forms of the cruzipain-like cysteine proteases. The alignment set used in the analysis was 115 sites in length in the mature protein domain. The number of each node indicates the bootstrapping value obtained with 100 replicates (GenBank accession nos. are given in parentheses). 1, P. westermani 28-kDa cruzipain-like cysteine protease (Pw28CCP) (U70537); 2, P. westermani NTP (D21124); 3, P. westermani cysteine protease (U56865); 4, Clonorchis sinensis cysteine protease (AB020306); 5, S. mansoni cysteine protease 1 (AF093243); 6, Fasciola hepatica cathepsin L (AB009306); 7, F. hepatica secreted cathepsin L2 (U62289); 8, Fasciola sp. cysteine protease (U57030); 9, Schistosoma mansoni Puerto Rican preprocathepsin L1 (U37045); 10, S. mansoni Liberian cathepsin L (Z35239); 11, S. mansoni Liberian cathepsin C (Z35231); 12, S. mansoni cathepsin B (Sm6) (M213809); 13, Schistosoma japonicum preprocathepsin L (U38476); 14, S. japonicum preprocathepsin C (U77932); 15, S. japonicum cathepsin B (X70968); 16, Striopetricolus erinacei cysteine protease (D63670); 17, Ancylostoma caninum cysteine protease (AcCP1) (U183911); 18, Caenorhabditis elegans (Bristol N2) cathepsin B (L93927); 19, Haemococcus contortus CP1 (HMCP1; Z69342); 20, HMCP2 (Z69343); 21, HMCP4 (Z69345); 22, HMCP5 (Z69346); 23, H. contortus cysteine protease (Z61377); 24, Giardia muri (CP1; AP606198); 25, G. intestinalis cysteine protease (U38327); 26, Giardia intestinalis cysteine protease (U38325); 27, Toxocara canis cathepsin Z1 (AF013817); 28, T. canis cathepsin L (U53712); 29, Trypanosoma cruzi cathepsin B (AF013426); 30, trypanosome cysteine protease (X54155); 31, T. cruzi (M21380); 32, Leishmania donovani cysteine protease (U54155); 33, Leishmania major cathepsin L (U43706); 35, L. major cathepsin B (U43705); 36, mouse cathepsin L (M20495); 37, human cathepsin L (M20496); 38, human cathepsin B (gastric cancer, L6510); 39, human cathepsin B (kidney, M14221); 40, human cathepsin S (M40696); 41, human cathepsin S (alveolar macrophage, M86553); 42, human cathepsin X precursor (ovary) (AF0733890); 43, human cathepsin X (osteoclastoma) (U20280); 44, human cathepsin C (X70968); 45, Carica papaya cathepsin (M15203); 46, Cathepsin Z1 (AF043817); 47, T. canis cathepsin L (U53172); 48, C. papaya chymopapain (X97899); 49, Ananas comosus bromelain (L41407).

FIG. 2. Alignment of the ERFNIN motif of Pw28CCP with those of other related enzymes. Cruzipain-like cysteine proteases display a high degree of homology in the first half, while cathepsin L-like proteases do so in the posterior half. Notably, NTP is not likely to have an ERFNIN motif. The percent conservation is indicated at the end of each sequence. Numbering starts from the N terminus of Pw28CCP. The sequence specific to the ERFNIN motif is set in reverse type. Highly conserved sequences among cruzipain-like cysteine proteases of Phe and Ala are indicated in boxes. His, which is well conserved in cathepsin L-like proteases, is also highlighted. Circled letters show possible replacement of Phe by Trp, which share a high degree of biochemical similarity in cathepsin L-like proteases.
suggesting that this enzyme may be synthesized and secreted outside only when the worm is grown in the definitive hosts. Positive signals with PwCP occurred in the parenchymal cells of the adult worm in an ill-defined fashion, suggesting that PwCP is a lysosomal enzyme, as reported previously (28). In the metacercarial section, positive signals with PwCP were also seen in the parenchymal cells.

In vitro expression and immunological evaluation of rPw28CCP. We expressed the 642-bp mature protein domain in the pET-28a (+) expression vector, which adds an N-terminal leader sequence with a 6× His tag to aid the purification. Recombinant protein, expressed as inclusion bodies, was solubilized by denaturing in 6 M urea and was purified by Ni-NTA affinity chromatography. The protein migrated as a single band at about 27 kDa, in good accord with the molecular mass calculated from the cDNA sequence by SDS-PAGE analysis (data not shown). However, the refolded rPw28CCP did not exhibit any enzyme activity either by substrate gel electrophoresis or in the fluorogenic molecular substrate (data not shown).

Table 2 summarizes the results of the evaluation of rPw28CCP as a diagnostic antigen by immunoblotting with sera from patients with different parasitic infections. Representative findings are also shown in Fig. 5. Strong antibody reactions against rPw28CCP were observed in 56 of 65 serum samples from patients with active pulmonary paragonimiasis (Fig. 5a), whereas 2 of 20 samples from patients with chronic inactive neuroparagonimiasis showed weak positive reactions (Fig. 5b). Two of five serum samples from patients with schistosomiasis japonicum (Fig. 5d) and 1 of 20 serum samples from patients with fascioliasis (Fig. 5e) exhibited positive reactions. None of the serum samples from patients with clonorchiasis, cystic and alveolar echinococcoses, sparganosis, and cysticercosis or sera from healthy controls reacted positively. The sensitivity of rPw28CCP as a diagnostic antigen was determined to be 86.2% (56 of 65 samples) for active paragonimiasis, and the specificity was 98% (147 of 150 samples).

DISCUSSION

Despite the significant advances in purification and enzymological characterization of several cysteine proteases of *P. westermani* in recent years (4–6, 11, 16, 17, 22, 45), the genetic information about these enzymes is poorly understood (32, 46). In this study, we have cloned a cDNA encoding a 28-kDa cysteine protease of adult *P. westermani* (Pw28CCP) that exhibited a phylogenetic relationship to the cruzipain-like cysteine protease. This enzyme was expressed specifically in the developmental stages of juveniles and adults, when the parasite resides in the definitive hosts. Immunoblot analysis with the recombinant protein showed that it has a strong potential to be an antigen for the serological diagnosis of active paragonimiasis.

To examine whether the isolated cDNA encoded a functional cysteine protease and to provide an abundant source for further biological study of the enzyme, we produced the recombinant enzyme in a bacterial expression system. The mature domain of Pw28CCP cDNA was expressed at a high level as inclusion bodies in *E. coli*. We solubilized the inclusion bodies using either urea or guanidine and reconstructed the enzyme in soluble form. However, the recombinant enzyme refolded under various conditions did not show any proteolytic activity, which suggested that the native Pw28CCP is synthesized as a precursor molecule and that posttranslational modification is important and is required for conversion of the...
enzyme to its active form. It has been proposed that many helminth and protozoan parasites, including *P. westermani*, as demonstrated in the present study, synthesize the cruzipain-and cathepsin-like proteases as an inactive prepro form to protect themselves from autodigestion (23, 27, 37, 41, 44). The lack of catalytic activity of the bacterially expressed recombinant enzyme may be attributable to the lack of the appropriate posttranslational process. On the other hand, the deduced amino acid sequence of the Pw28CCP did not contain any putative N- or O-linked glycosylation sites. Instead, there was Pro at position 2, which is highly conserved in several cysteine proteases and which is believed to be a potential site for posttranslational modification. In *F. hepatica* cathepsin L, it has been shown that the replacement of Pro at this position with hydroxyproline is essential for enzymatic maturation and excretion (44). It has also been demonstrated in *S. mansoni* cathepsin B that the conservation of Cys at position 25 is crucial for enzyme processing and catalysis (10). Testing of these hypotheses awaits analysis of site-directed mutagenesis as well as a reliable expression system that yields enzymatically active protein, such as baculovirus vector and insect cells.

It is interesting that NTP had a unique primary structure; it showed a high degree of sequence homology with Pw28CCP and other cruzipain-like cysteine proteases, by which it could be classified as a member of the cruzipain family (Fig. 1). In contrast, it did not contain the ERFNN motif in the propeptide region (Fig. 2), which hardly allowed it to be categorized as a cruzipain-like protease (23, 41). Enzymologically, it also has a neutral pH optimum, which is an unusual finding for the parasite cysteine proteases. From this point of view, further studies will be necessary to clearly evaluate the characteristics of the enzyme.

Studies of the patterns of expression of Pw28CCP in different developmental stages and in the worm compartment would provide some information about the potential functional significance of this enzyme. We have previously demonstrated that the changes in the activities of five different cysteine proteases during the maturation stages of *P. westermani* may be associated with the successful adaptation of parasites to the changing environment of the host (5). In this study, semiquantitative RT-PCR and Northern blot analysis revealed that the expression levels of Pw28CCP varied in different stages and appeared to be different from those of other two cysteine proteases, NTP and PwCP. These results suggest strongly that cysteine proteases of *P. westermani* may consist of very similar but different entities, further supporting the notion that the different species of cysteine protease play different roles in specific stages. Immunoblotting with sera from *P. westermani*-infected cats showed that specific antibody reactions against rPw28CCP could be detected from 6 weeks after infection, suggesting that Pw28CCP might be expressed from the early juvenile stage shortly after infection. This appeared to coincide

**FIG. 4.** Localization of Pw28CCP and PwCP by in situ hybridization. (A) Hybridization signals with cDNAs of Pw28CCP are observed in the intestinal epithelium of the adult worm (arrow), while PwCP shows a positive reaction in the parenchymal cells (arrowhead). (B) In the metacercarial section, Pw28CCP exhibits no reaction, whereas PwCP reveals a positive signal in the parenchymal cells (arrowhead). pa, parenchyma; ic, intestinal contents; ie, intestinal epithelium; tg, tegument; eb, excretory bladder.

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**TABLE 2. Sensitivity and specificity of rPw28CCP for immunodiagnosis of active paragonimiasis**

| Disease or infection | No. of serum samples tested | No. (%) of positive samples |
|---------------------|-----------------------------|-----------------------------|
| Paragonimiasis      | 65                          | 56 (86.2)                   |
| Active cases        | 20                          | 2 (10)                      |
| Chronic cases       | 32                          | 0 (0)                       |
| Clonorchiasis       | 5                           | 2 (40)                      |
| Schistosomiasis japonicum | 5          | 2 (40)                      |
| Fascioliasis        | 20                          | 1 (5)                       |
| Cystic echinococcosis | 5                        | 0 (0)                       |
| Alveolar echinococcosis | 8                     | 0 (0)                       |
| Sparganosis         | 15                          | 0 (0)                       |
| Cysticercosis       | 15                          | 0 (0)                       |
| Healthy control     | 50                          | 0 (0)                       |
well with the observation that antibody-producing B cells directed to a specific antigen could be generated differentially 4 to 6 weeks after priming during parasite infection (13). Histochemochemical localization of the Pw28CCP in the intestinal epithelium, indicative of excretion-secretion, was consistent with its plausible roles in modulating the host immune responses and nutrient uptake (6).

Although more precise quantitative tests are required to clearly define the changes in enzyme activity, the various levels of expression of each cysteine protease at different stages obtained both by semiquantitative RT-PCR and by Northern blot analysis might reflect the physiological significance of each enzyme during the maturation and migration of *P. westermani*. Alternatively, it might represent a strategy that has evolved with genetic design for the biological adaptation in a given host (38). Many cysteine protease genes of free-living nematodes and other parasites have also been shown to be developmentally regulated (29, 30, 34, 36).

The successful use of *P. westermani* cysteine proteases as diagnostic antigens has been documented with the use of partially purified enzymes or the application of cystatin-capture ELISA (15–17). However, its application is limited not only by the availability of parasite materials but also by the difficulties with the purification of the specific antigens. In the present study, the use of rPw28CCP alone in immunoblotting exhibited a sensitivity and a specificity of 86.2 and 98%, respectively, for the detection of the anti-*P. westermani* antibodies in the patients’ sera. The sera from patients with parasitic infections other than schistosomiasis japonicum and fascioliasis used in this study did not cross-react with rPw28CCP. The mechanism of cross-reactivity due either to common epitopes shared by different trematode parasites or to other host factors possibly associated with major histocompatibility complex type II molecules awaits further elucidation to strengthen the diagnostic sensitivity for paragonimiasis. However, Pw28CCP seems to be an important antigen since its diagnostic value was comparable to those of mixed or partially purified cysteine protease or other protein antigens that have been used in antibody assays (15–17, 26). This study confirmed the usefulness of *P. westermani* cysteine protease as a potent diagnostic antigen. The availability of rPw28CCP in large quantities may facilitate the development of more effective tests for the diagnosis of human paragonimiasis, which is still endemic in several parts of the world (1).

In conclusion, we have characterized a cDNA encoding the 28-kDa cysteine protease of *P. westermani* that has structural motifs and a domain organization specific to the cruzipain-like cysteine protease. Our results indicate that cysteine protease species of similar molecular sizes are differentially expressed during the developmental stages of *P. westermani*. While the metacercarial NTP played critical roles in the early infection stage by suppressing the host immune system (14), Pw28CCP, as a gut-associated enzyme in the juvenile and adult stages, seems to play a major role in nutrient uptake and immune evasion in the definitive host (6). The specific immunoreactivity of rPw28CCP suggests that it could probably be useful for immunodiagnostic applications as well as in the development of a vaccine.

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ADDENDUM

During the revision process, another gene that putatively encodes a cysteine protease of adult *P. westermani* and that had an open reading frame of 687 bp and a deduced amino acid sequence of 229 (estimated molecular mass of approximately 28.5 kDa) was partially characterized. It shared a significant degree of sequence homology (range, 32 to 74%) with other members of the cathepsin family of cysteine proteases of helminth parasites (24).

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