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## Utility of a Protein Fraction with Cathepsin L-Like Activity Purified from *Cysticercus* Fluid of *Taenia solium* in the Diagnosis of Human Cysticercosis

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### Abstract

Neurocysticercosis, an endemic parasitic disease in most developing countries, is caused by *Taenia solium* and compromises the human central nervous system. Cathepsin L-like proteases are secreted by several parasites including *T. solium* and constitute important antigens for immunodiagnosics. A protein fraction with cathepsin L-like activity was purified from the cysticercus fluid by size exclusion and ion exchange chromatography. Cathepsin L-like activity was measured fluorometrically by detecting the hydrolysis of the fluorogenic substrate Z-Phe-Arg-AMC. The purified protein fraction included antigens of 53 and 25 kD that were tested in a Western immunoblot and in an enzyme-linked immunosorbent assay (ELISA) for detection of human cysticercosis. The sensitivity of the Western immunoblot was 96% for patients infected with multiple cysts and 78%

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for patients with a single cyst. Specificity was 98%. The sensitivity of the ELISA was 98% in patients with multiple cysts and 84% in patients with a single cyst. Specificity was 92.7%.

## INTRODUCTION

Taeniasis/cysticercosis caused by the cestode *Taenia solium* is an important public health problem in developing countries, where spread of the tapeworm persists in communities with poor sanitation.<sup>1</sup> This condition has been recognized as an increasing cause of human disease,<sup>2</sup> where its major complication, neurocysticercosis (NCC), is an important cause of morbidity,<sup>3</sup> accounting for more cases of epilepsy than any other pathology in these areas.<sup>4</sup>

The standard diagnosis of NCC requires expensive imaging methods such as computed-tomography or magnetic resonance, which are difficult to access in disease-endemic areas. The highly sensitive and specific enzyme-linked immunotransfer blot (EITB) is the immunodiagnostic test of choice for confirming a clinical and radiologic presumptive diagnosis of NCC.<sup>5</sup> The EITB is the Centers for Disease Control and Prevention (CDC) Western immunoblot for cysticercosis. This assay is based on detection of antibody to one or more of the seven lentil-lectin glycoproteins (LLGPs) of the *T. solium* cysticercus. This test is nearly 100% specific and is highly sensitive in cases of cysticercosis with more than one cyst.

The enzyme-linked immunosorbent assay (ELISA) format is simpler to perform in most laboratories and is less costly than the EITB. The ELISA is a quantitative test that can be used in high throughput studies and is more objective than the EITB because it does not rely on individual interpretation to determine a positive case of disease.

In recent years, several ELISAs have been evaluated for their efficacy in diagnosing cysticercosis. Over time, the antigenic proteins used in these tests have become more sophisticated; they have evolved from crude cysticercus fluid, to purified fractions, to recombinant proteins and synthetic peptides.<sup>6-15</sup> Most parasites secrete proteases to facilitate tissue penetration and infection. Cathepsin L is such a protease that is secreted by several parasites, and is an important immunodiagnostic antigen for the trematode *Fasciola hepatica*.<sup>16-21</sup> In a previous report, we demonstrated that cathepsin L is secreted by *T. solium* oncospheres and is a relatively abundant antigen among serine and cysteine proteases in the cyst fluid.<sup>22</sup>

In the present study, we purified a 53/25-kD protein fraction with cathepsin L-like activity from cysticercus fluid and then evaluated its utility as an immunodiagnostic antigen for human cysticercosis in Western immunoblot and ELISA.

## MATERIALS AND METHODS

### Cysticercus fluid

Eight pigs naturally infected with cysticercosis were selected from a disease-endemic area in the central highlands of Peru and were confirmed by tongue test.<sup>23</sup> Selected pigs had 6–7 positive bands to the standard CDC Western immunoblot test. The carcasses were dissected and *T. solium* cysts were identified. Without extracting the cysticerci, the cyst fluid content was recovered by aspiration with a syringe, carefully avoiding contamination with the animal blood. Approximately 400 mL of cyst fluid was recovered from 4,000 viable cysts and stored at  $-70^{\circ}\text{C}$ .

## Protein purification

**Ethanol precipitation and concentration**—Particulate material was removed from the cyst fluid by centrifugation ( $10,397 \times g$  for 30 minutes at  $4^{\circ}\text{C}$ ). Cold absolute ethanol ( $-20^{\circ}\text{C}$ ), was added to the supernatant (30% [v/v]), and this was mixed with an inhibitor cocktail (0.01 mM trans-epoxysuccinyl-L-leucylamido-(4-guanidino)butane [E64], 0.1 mM chymostatin, 0.1 mM leupeptin, 1  $\mu\text{M}$  bestatin, 0.1 mM tosylphenylalanylchloromethane, 0.1 mM antipain, 1 mM 4-[2-aminoethyl] benzenesulfonyl fluoride, 1.5 mM pepstatin, 10 mM EDTA) to prevent protein degradation. After incubation for 20 minutes at  $-20^{\circ}\text{C}$ , precipitated proteins were eliminated by centrifugation ( $10,397 \times g$  for 20 minutes at  $4^{\circ}\text{C}$ ). The supernatant was concentrated to one-fifth of its original volume by using an Amicon system with a 10-kD (YM-10) membrane (Millipore, Billerica, MA). The final protein concentration of four different batches was 6–8 mg/mL.

**Size exclusion chromatography**—The concentrated batches of supernatant from the ethanol precipitation (500  $\mu\text{L}$ ) were passed through a column packed with G75 Sephadex (120 mL, 75 cm length), equilibrated with citrate buffer (0.1 M, pH 4, 0.1 mM  $\text{HgCl}_2$ ) at  $4^{\circ}\text{C}$ . Buffer at  $4^{\circ}\text{C}$  (300 mL) was passed at a flow rate of 0.2 mL/minute and fractions of 1 mL were collected. Fractions were analyzed for cathepsin L-like activity using the fluorometric assay described below. The fraction with the highest activity was selected for ion exchange chromatography.

**Ion exchange chromatography**—The selected fraction (1 mL) was loaded into a 15-mL column packed with SP Sephadex, equilibrated with citrate buffer (50 mM, pH 4, 0.1 mM  $\text{HgCl}_2$ ) at  $4^{\circ}\text{C}$ . After washing (30 mL, 50 mM citrate pH 4, 0.1 mM  $\text{HgCl}_2$ ), protein fractions were eluted with 20 mL of a NaCl gradient (0–2 M) in the same citrate buffer at a flow rate of 0.2 mL/minute. The fraction showing the highest cathepsin L-like activity was selected for being tested as an immunodiagnostic antigen for cysticercosis.

## Fluorometric assay for cathepsin L-like activity

Cathepsin L-like activity of cyst supernatant protein fractions was detected by the hydrolysis of the peptide substrate coupled to the fluorogen 7-amino-4-methylcoumarin (AMC), Z-Phe-Arg-AMC (MP Biomedicals, Aurora, OH).<sup>24</sup> The hydrolysis reaction was performed in 100 mM citrate buffer, pH 5, with 5 mM dithiothreitol (DTT) to reduce trypsin-like interference. Urea at a final concentration of 4 M was included in the reaction buffer to reduce cathepsin B-like interference.<sup>22,25</sup> Reaction buffer (92 mL), purified *T. solium* cyst protein (4  $\mu\text{L}$ ), and the fluorogenic substrate Z-Phe-Arg-AMC (4  $\mu\text{L}$ , 5 mM), were incubated at  $37^{\circ}\text{C}$  in a 0.2-mL tube and followed for 4 hours and 24 hours.<sup>22,25</sup> The AMC fluorogen released after the cleavage of the peptide substrate was measured by fluorescence in a TD-700 fluorometer (Turner Designs, Sunnyvale, CA) as the excess of absolute fluorescence units above the blank reaction with buffer replacing the purified protein. Excitation was performed at 360 nm and emission was detected at 460 nm using a 70- $\mu\text{L}$  microcuvette. The supernatant protein fraction with the highest cathepsin L-like activity was selected for testing as an immunodiagnostic antigen for cysticercosis.

## Cathepsin L-like protein identification

**Protein sizing**—An alternate purification was as described above but in the absence of protease inhibitors except  $\text{HgCl}_2$ . Fractions with cathepsin L-like activity purified from 10 runs were pooled and concentrated to approximately 1 mg/mL, and analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) (12% acrylamide gel, 1.2  $\mu\text{g}$  protein/5-mm well). To determine the location of the proteins on the gel, one lane was excised and stained with silver nitrate. Resolved proteins in the matrix of the unstained gel that co-migrated

with the stained proteins were excised according to molecular weight, minced, and tested for cathepsin L-like activity in the fluorometric assay. The control reaction for the cathepsin L-like activity was performed in the fluorometric assay using a similar amount of minced gel corresponding to one lane free of common-stainable protein.

**Gelatin zymography**—To confirm the proteolytic activity and to estimate the molecular weight of the protease associated, the ion exchange chromatography cathepsin L-like purified antigen was tested in a gelatin zymography.<sup>26</sup> The purified antigen was resolved by electrophoresis on an 11% polyacrylamide gel co-polymerized with 0.12 mg/mL of gelatin (buffer 25 mM, Tris, pH 7.2, 190 mM glycine, 0.1% SDS). After electrophoresis, the gel was washed in 2.5% Triton X for 1 hour, rinsed twice in 100 mM citrate buffer, pH 5.0, and incubated at 37°C for approximately 48 hours in 100 mM citrate buffer, pH 5, 5 mM DTT. The gelatinolytic activity was revealed as clear bands against a blue background by a single step stain-destain method using 0.02% Coomassie blue at an acetic acid: methanol: water ratio of 1:3:6.

### Inhibition assays

To confirm the specific cathepsin L-like activity, inhibition with 10  $\mu$ M E64 and 100  $\mu$ M chymostatin was tested. Cathepsin L-like activity is inhibited by E64 but not by chymostatin.<sup>27,28</sup>

The total purified protein and the minced gels corresponding to each band revealed by SDS-PAGE were pre-incubated with 0.01 mM E-64 or 0.1 mM chymostatin for 10 minutes at room temperature. After pre-incubation with inhibitors, each sample was tested for proteolytic inhibition in the fluorometric hydrolysis reaction and in the gelatin-zymography described above. In gelatin zymography, proteolytic inhibition was detected by a lack of gelatin degradation shown by the absence of a revealed band in the gel.

### Sample sera

Serum samples were selected from the repository of the Cysticercosis Working Group in Peru. All patient and healthy control sera were collected under appropriate informed consent processes including permission for future use and approved by the Institutional Review Board of the Universidad Peruana Cayetano Heredia (FWA 00000525). These included 100 samples from clinically defined NCC cases (50 from patients with a single viable cyst and 50 from patients with more than one viable cyst, range = 2–53; 20 patients had more than 10 cysts), 48 NCC-negative control sera, and 109 sera from patients with different helminth infections to determine cross-reactions.

Cases were defined by the identification of active lesions in the brain using computed tomography and/or magnetic resonance imaging. Cases could have additional calcified or inactive lesions and although they were selected on the basis of imaging characteristics alone, all had positive LLGP-EITB results. Negative control sera were collected from volunteers in Iquitos, a city in the Amazon basin in northern Peru where NCC is not endemic and other helminth infections are common. All negative control sera were tested by LLGP-EITB to confirm that they were not infected with cysticerci, but we did not rule out other helminth infections by using other serologic assays.

To determine cross-reactions, LLGP-EITB-negative sera (n = 109) from patients who were infected with a single helminth were tested. These sera included samples from patients proven by positive serologic results to have infections with *Ancylostoma duodenale* (n = 9), *Ascaris lumbricoides* (n = 3), *Enterobius vermicularis* (n = 14), *Hymenolepis nana* (n = 34), *Strongyloides stercoralis* (n = 13), *Terranova trichiuris* (n = 6), *Taenia saginata* (n = 10), and

*Echinococcus granulosus* (n = 10). Additionally, sera from patients who had negative serologic results for *E. granulosus*, but had hydatid cysts recovered from surgery (n = 10) were also used as NCC-negative controls. Five sera positive for *T. saginata* were also positive in the LLGP-EITB.

### Western immunoblot assay

The ion exchange chromatography-purified antigen with cathepsin L-like activity was resolved by SDS-PAGE in gradient polyacrylamide gels (5–22.5%) at a concentration of 0.024 µg/mm of gel width. The fraction was heated at 65°C for 15 minutes in sample buffer (0.1% SDS, 0.025% [w/v] bromophenol blue, 1% glycerol, 0.0025 M Tris-glycine-HCl, pH 8, and 5 mM DTT). Electrophoresis was performed at 5 mA for stacking and at 25 mA for resolving. Proteins were electrically transferred onto a nitrocellulose membrane at 2 A for one hour in a Transfer-Blot Cell (Bio-Rad, Hercules CA) in buffer (20% methanol, 0.212 M Tris glycine-HCl). Nitrocellulose membranes were cut into small strips to be tested using individual serum samples. A blocking solution of phosphate-buffered saline (PBS), pH 7.4, 0.3% Tween 20, 5% nonfat dry milk was prepared as a diluent for the serum samples. Each sera at a final dilution 1:300 was incubated with a nitrocellulose strip overnight at 4°C.<sup>5</sup> The proteins were visualized using a peroxidase-labeled anti-human IgG, followed by the addition of 3,3'-diaminobenzadine plus hydrogen peroxide as the substrate. When a positive reaction was present, it was further classified as weak positive, positive, or strong positive on the basis of intensity of the band measured by simple observation.

### ELISA

The ion exchange chromatography-purified antigen with cathepsin L-like activity was diluted in PBS (0.8 µg/mL) and was used to coat the wells (100 µL each) of Immulon 1 microtiter plates (Dynatech Laboratories, Chantilly, VA). After incubation for two hours with shaking, the 96-well plates were washed five times with PBS, 0.1% Tween using a manual washer. The wells were blocked with 100 µL of PBS, 0.1% Tween at room temperature for 30 minutes with shaking. After washing, the wells were incubated in duplicate with 25 µL of serum diluted in 75 µL of inactivated fetal bovine serum (Invitrogen, Carlsbad, CA) at room temperature for one hour with shaking. The plates were washed and antibodies were detected with anti-human IgG labeled with peroxidase (Kirkegaard and Perry, Gaithersburg, MD) diluted 1:20,000 in a 5% milk solution of PBS, 0.3% Tween and incubated at room temperature for one hour with shaking. The reaction was developed after incubation with tetramethyl benzidine SureBlue substrate (Kirkegaard and Perry) (100 µL/well) at room temperature for 30 minutes with shaking. The optical density (OD) was determined at 650 nm using a spectrophotometer (Molecular Devices, Toronto, Ontario, Canada).

To ensure ELISA validity, each plate included two strong positive control wells, two weak positive wells, and six negative control wells. The positive controls were prepared from a cysticercosis-positive serum pool diluted in a negative serum pool at 1:8 (strong positive) and 1:128 (weak positive). The six negative control wells corresponded to six individual serum samples from persons who were negative for cysticercosis. If any of the two weak positive wells did not give a positive reaction, results from the entire plate were discarded.

Every test sample was run in duplicate. An ELISA result was rejected if the difference of the ODs in the duplicate wells was greater than 15% or if the positive/negative reactivity in the duplicate wells were different, in which case the sample was tested again. For testing the ELISA, 21 additional NCC-negative control serum samples became available and were included. A total of 69 NCC-negative control serum samples were used to test the specificity.

## Statistical analysis

To control for inter-plate variability of the OD absolute values, the percentage of positivity (PP), was calculated for each tested sera by dividing its mean OD by the mean OD of the strong positive controls of the corresponding plate. A simple logistic regression to model the disease condition (positive/negative) using the PP of all samples as the predictor covariate was performed. A linear score to estimate the probability of the positive-disease condition was developed based on the PP and the regression coefficient. A sample was considered positive if the probability was greater than the best cutoff (Pr-cutoff/PP). A receiver operating characteristic curve (ROC) was calculated and the Pr-cutoff/PP was estimated to maximize the Youden's J-index (sensitivity + specificity - 1).<sup>29</sup> The same Pr-cutoff/PP was used to test every sample in all plates.

As an alternative method of calculating a positive result, a sample was considered positive if the mean OD of the tested serum sample was greater than the mean OD plus three standard deviations of the six negative controls, which defines the classification cutoff (NC-cutoff) of a plate.

The 95% confidence intervals (CIs) for estimated sensitivities and specificities were calculated for proportions following a binomial distribution. All analysis was performed using Stata 10 statistical software (Stata Corp., College Station, TX).

## RESULTS

### Protein purification

The size exclusion and the ion exchange chromatography patterns were similar and showed two major peaks of protein (Figure 1). The specific cathepsin L-like activity after each purification step is shown in Table 1. After the ion exchange chromatography, the final purification factor was 42.5. The silver-stained SDS-PAGE of the ion exchange chromatography-purified antigen processed without inhibitors showed a partially purified complex with four intense bands of 53, 25, 10, and 7.5 kD plus a weak and smeared band of 21 kD (Figure 2). In contrast, the SDS-PAGE pattern of the purified antigens processed with inhibitors showed the only three intense bands (53, 25, and 21 kD).

### Cathepsin L-like activity in purified antigens and inhibition assays

The fluorometric assay from the slices of gel showed that the protein fraction of 53 kD is associated with a cathepsin L-like activity; the 25-kD band was only associated to a marginal activity. The 21-, 10-, and 7.5-kD bands were not associated with cathepsin L-like activity. These reactions were inhibited by E64 but not by chymostatin, which confirms the cathepsin L-like nature of the proteolytic activity. We observed systematically that pre-incubation with chymostatin enhanced the cathepsin L-like activity.

The gelatin zymography showed a strong gelatinolytic activity at 53 kD; no gelatinolytic activity was observed in the 25-kD band. The gelatinolytic activity of the 53-kD protein fraction was partially inhibited by E64, but not by chymostatin (Figure 3). Similarly, as in the fluorometric assay, pre-incubation with chymostatin enhanced the gelatinolytic activity.

### Western immunoblot assay

The ion exchange chromatography-purified antigen showed a Western immunoblot pattern similar to the silver-stained SDS-PAGE. Among the 50 positive samples with multiple viable cysts, 48 reacted positively against the 53-kD and 25-kD bands, giving a sensitivity of 96% (95% CI = 86.3–99.5%). Serum samples from patients with a single cyst showed a sensitivity of 78% (95% CI = 64.0–88.5%) against the 53-kD band and a sensitivity of 76% (95% CI =

61.8–86.9%) against the 25-kD band. Negative controls (47/48) had no detectable reactivity with the 53-kD band, giving a specificity of 97.9% (95% CI = 88.9–99.9%). Negative controls (45/48) did not show reactivity with the 25-kD band, giving a specificity of 93.8% (95% CI = 82.8–98.7%). Positive bands were classified as weak positive, positive, and strong positive depending on the visual intensity of the staining. Serum samples from patients with many cysts had visibly stronger reactions than patients with fewer numbers of cysts (Table 2). The sensitivity of the 21-, 10.5-, and 7.5-kD bands were lower than 70% with either the 53-kD or the 25-kD bands.

**Cross-reactions**—In the Western immunoblot, 11 of 109 serum samples from persons with other helminthic infections had a cross-reaction. However all were weak reactions and not easily detected visually: 1 of 14 serum samples with *E. vermicularis* cross-reacted with the 53-kD band, 1 of 13 serum samples with *S. stercoralis* cross-reacted with the 53-kD and 25-kD bands, 3 of 33 serum samples with *H. nana* cross-reacted with the 53-kD band; 1 of 10 serum samples with *T. saginata* cross-reacted with the 53-kD band (however, this serum sample was also LLGP-EITB positive), and 5 of 20 serum samples with *E. granulosus* cross-reacted with the 53-kD and 25-kD bands. None of the serum samples with *A. duodenale*, *A. lumbricoides*, or *T. trichiura* cross-reacted with the 53-kD or 25-kD bands.

## ELISA

The average OD of the positive sera samples was approximately 15-fold greater than the OD of the negative controls. On the basis of the Pr-cutoff/PP (18.5%), the sensitivity of the cathepsin L ELISA was 84% (42 of 50, 95% CI = 70.9–92.8%) in patients with one cyst, and 98% (49 of 50, 95% CI = 89.4–99.9%) in patients with more than one cyst. The specificity of the cathepsin L ELISA was 92.7% (64 of 69, 95% CI = 86.3–95.7%). The assay efficacy, measured by Youden's J index, was 0.87, and the area under the ROC curve was 0.975. As shown in Figure 4, the PP values for the group of patients with multiple cysts are higher than those with a single cyst. The PP values of the cross-reactions against other helminths are slightly higher than PP values of the NCC-negative controls. When we excluded serum samples from NCC-positive patients with a single cyst and used only serum samples with high antibody responses from patients with multiple cysts, a new Pr-cutoff/PP was calculated (34.5%). The estimated sensitivity of the cathepsin L ELISA was 98% and the estimated specificity was 98.5%, with an assay efficacy of 0.965 and an area under the ROC curve of 0.9903.

Classification of the serum samples with the NC-cutoff (mean = 0.16, SD = 0.05) had a sensitivity of 94% (95% CI = 87.4–97.8%) for patients with one cyst and a sensitivity of 100% (95% CI = 96.4–100%) for patients with more than one cyst, but the specificity was only 88.8% (95% CI = 83.0–93.7%).

**Cross-reactions**—On the basis of the PP test, 8 of 109 serum samples with other helminth infections showed a cross-reaction: 1 of 14 serum samples with *E. vermicularis*, 3 of 34 serum samples with *H. nana*, 2 of 10 serum samples with *T. saginata* (however, these were also LLGP-EITB positive), and 2 of 20 serum samples with *E. granulosus* showed cross-reactions. The PP values of the cross-reactions were lower than those of the NCC-positive samples and slightly higher than those of the NCC-negative controls (Figure 4). Serum samples that showed cross-reactions in the Western immunoblot and the ELISA were not the same, but approximately 50% of them overlapped.

## DISCUSSION

This study demonstrates that a partially purified antigen from *T. solium* cysticercus fluid that contains two protein fractions of 53 kD and 25 kD associated with cathepsin L-like activity

performed well in the immunodiagnosics of human cysticercosis in an ELISA and a Western immunoblot assay.

It has been demonstrated that the *T. crassiceps* metacestode secretes a number of proteolytic enzymes including metallo-aminopeptidases and cysteine proteases that degrade human IgG,<sup>30</sup> suggesting that this parasite may be using these proteases to modulate the host immune response. Cathepsin L proteases that degrade human IgG have been isolated from *T. solium* cysticerci in two prior studies; a 48-kD protein thought to be involved in immune response evasion<sup>31</sup> and a 29-kD protein that also degraded collagen and bovine serum albumin.<sup>32</sup> The 29-kD cathepsin L protease was shown to be reactive against sera from cysticercosis patients in an immunoblot format, but neither of these proteases were fully evaluated for their immunogenicity or diagnostic potential.

In this study, the 53-kD, and to a lesser extent, the 25-kD partially purified protein fractions were able to hydrolyze the fluorogenic substrate Z-Phe-Arg-AMC. This activity was inhibited by E64 but not by chymostatin, confirming that the protein fraction includes at least one cathepsin L-like peptidase. The relatively low total purification factor of 42.4 suggests that the 53/25-kD cathepsin L-like fraction is highly abundant in the cyst fluid or that other proteins are present in this fraction.

The 53-kD and 25-kD protein fractions performed well in the Western immunoblot and in the ELISA, with a sensitivity and specificity similar to that of the LLGP-EITB. The sensitivities of the cathepsin L Western immunoblot and the cathepsin L ELISA were higher in NCC patients with multiple cysts compared with patients with a single cyst, which suggests that patients with multiple cysts have a higher antibody response than patients with a single cyst infection. Remarkably, the sensitivities of these two tests for patients with one cyst were similar to the sensitivity of the LLGP-EITB in the same group of patients.

It is possible that the immunoreactive antigen recognized in the ELISA is the cathepsin L-like peptidase. However, further studies are required to confirm this hypothesis. Further analyses are needed to identify the cathepsin L-like protease in the 53/25-kD purified fraction and its relationship with the 48-kD and 29-kD cathepsin L-like proteases previously identified.

Currently, on the basis of *T. solium* metacestode lentil-lectin structural glycoproteins,<sup>5</sup> the best immunologic test to diagnose human cysticercosis is the LLGP-EITB. However, this assay is expensive, requires specialized trained personnel, and is not quantitative. An ELISA is a simpler assay and can be performed in most laboratories at a lower cost than a Western immunoblot. The ELISA is also quantitative and lends itself to high throughput studies, and its results are not subject to individual interpretation as is the case with blot analyses. We have not tested the seven LLGPs used in the CDC Western immunoblot for cathepsin L-like activity. However, neither of the amino acid sequences of the LLGP (GenBank accession nos. AAF25005.1, AAM00206.1, AAM00207.1, AAM00208.1, AAD51763.2, AAD51767.2, AAD51768.1, AAD51769.1, and AAF60975.1) showed a protease motif in a protein BLAST against the entire GenBank within an e value from 1e-42 to 0.020).<sup>33</sup>

The major limitation of our cathepsin L ELISA is the difficulty in obtaining and processing the cyst fluid. Despite its high sensitivity and specificity, our cathepsin L ELISA requires purification of antigens from cyst fluid, which is time-consuming and requires infected pigs. As disease control measures increase, the ability to obtain large amounts of antigen may become more difficult. To make this test practical, it will be necessary to produce reliable recombinant antigens.

Another limitation of the cathepsin L ELISA is its cross-reactivity with *E. granulosus* (5 of 20, 25%), *T. saginata* (1 of 10, 10%), and *H. nana* (3 of 33, 9%), which are common parasites



in developing countries, and to a lesser extent its cross-reactivity with *E. vermicularis* (1 of 14, 7%). However *E. granulosis* and *T. saginata* are close related to *T. solium* and have always shown high cross-reactivity in immunodiagnostic tests for cysticercosis.<sup>34</sup>

The cathepsin L-based ELISA for the diagnosis of human cysticercosis may have a significant clinical impact. The high sensitivity of the assay implies that it can be used for detection of NCC in patients, even those with one cyst who make up a large proportion of the infected population. The ELISAs relatively low cost and ease of use suggests that this assay can be used as a bedside diagnostic test. The use of the antigen has the potential to be further expanded for its use in a bedside diagnostic test and as a vaccine candidate. Further studies are required to produce these antigens as recombinant proteins.

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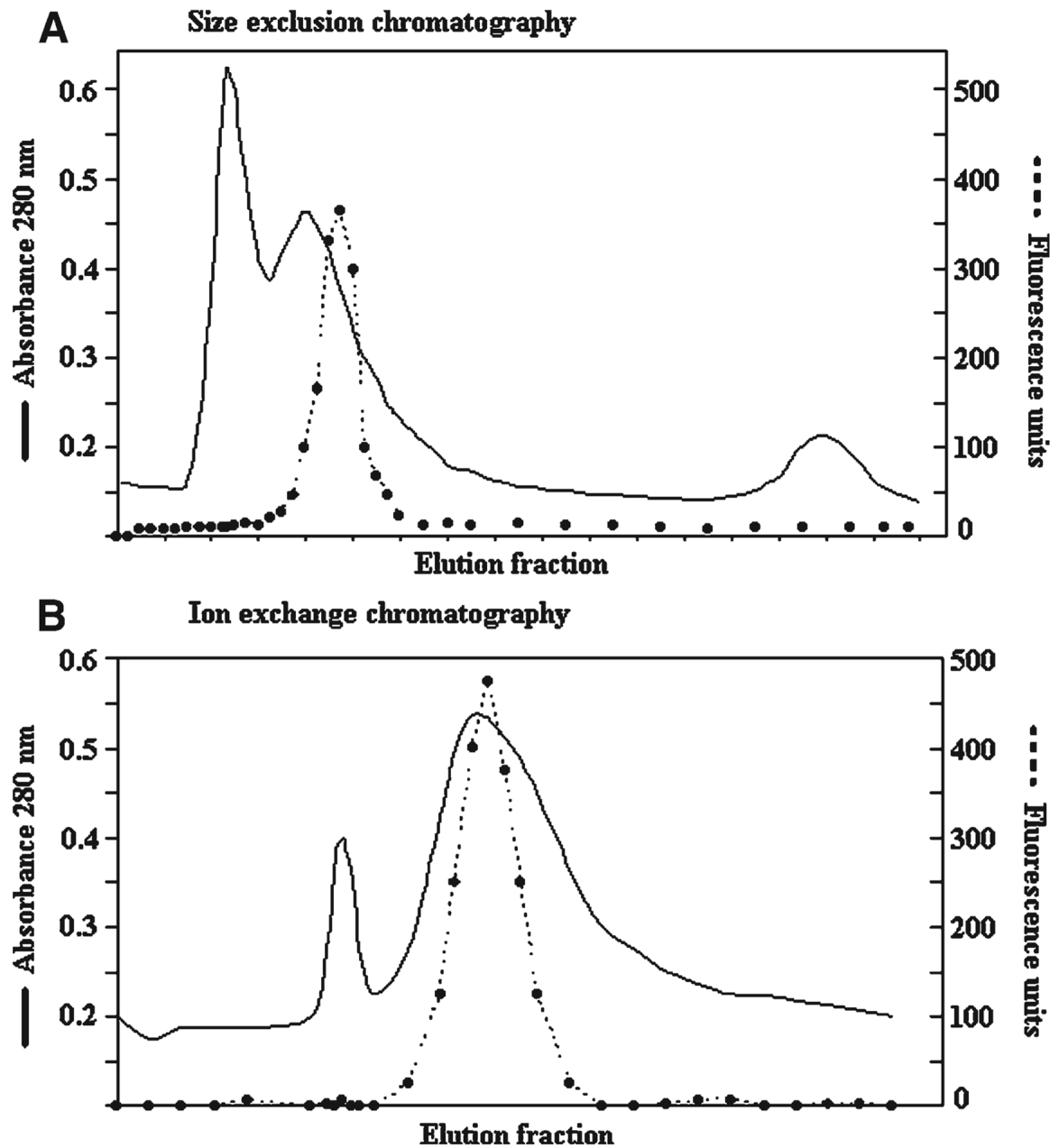
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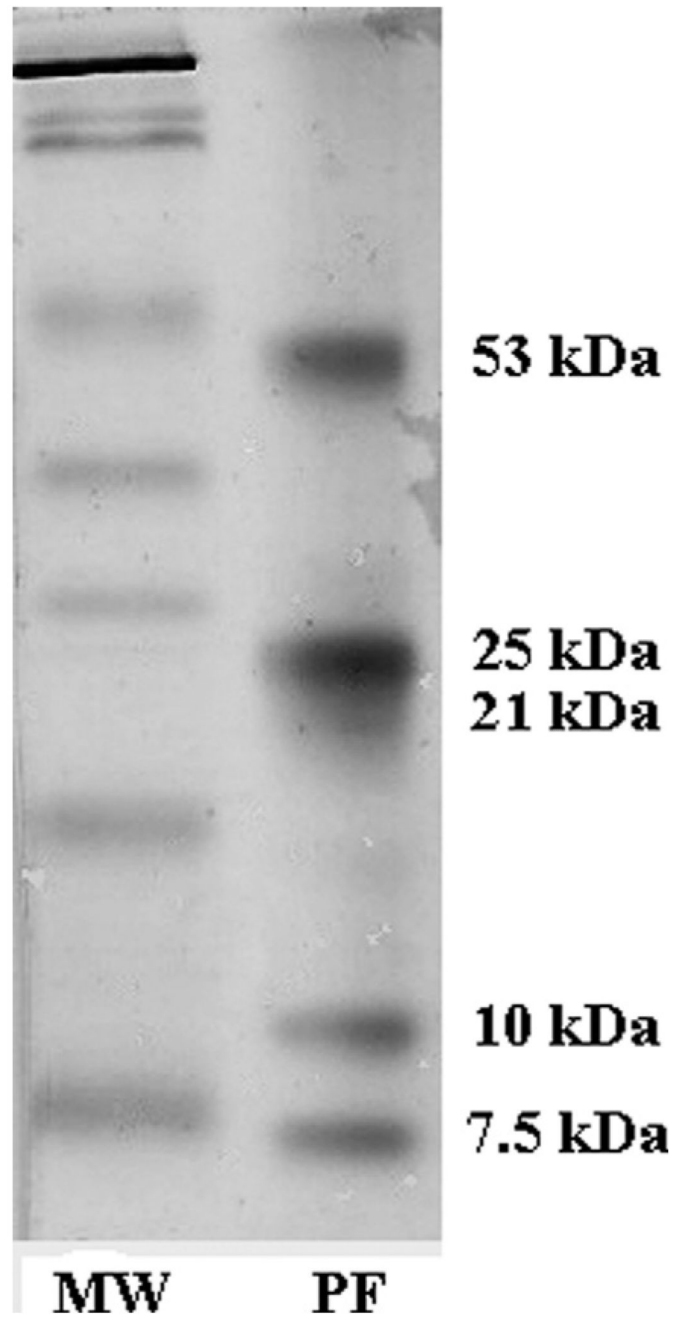
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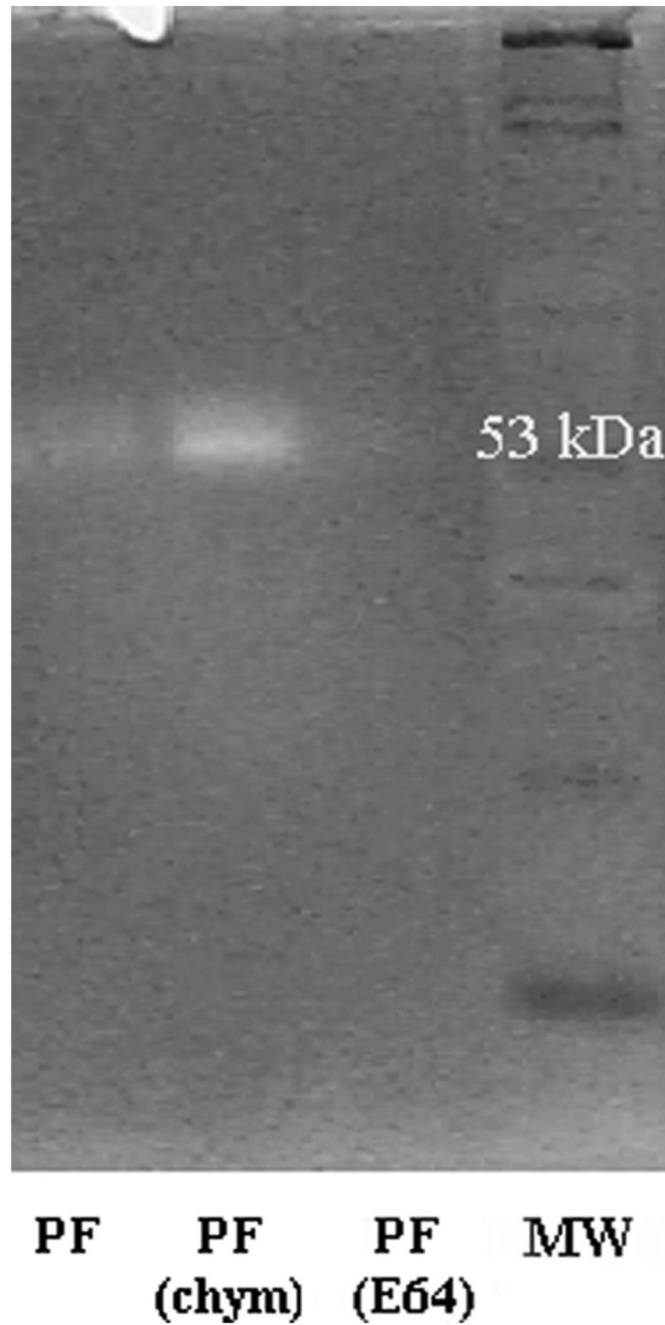
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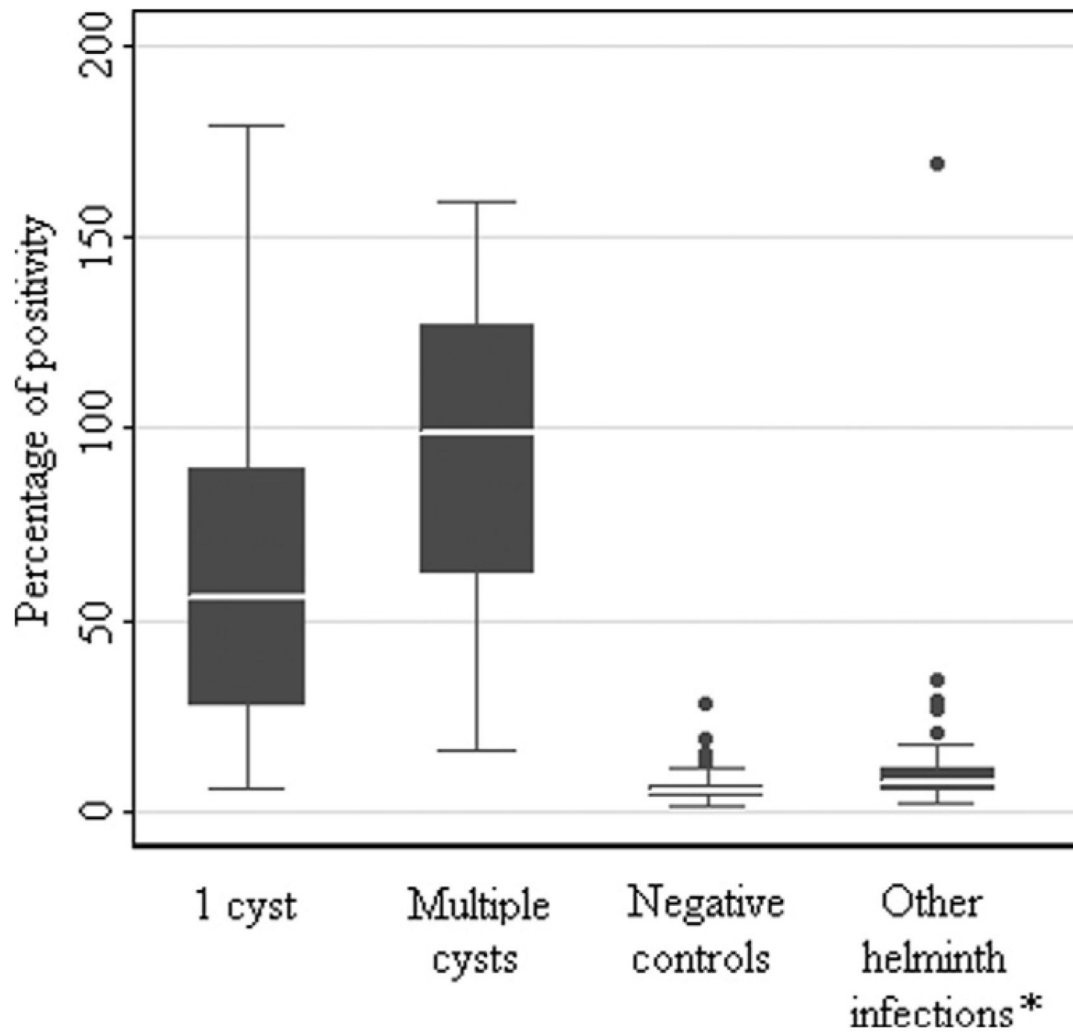
**Figure 1.** Chromatographic and Z-Phe-Arg-AMC fluorometric patterns during **A**, Size exclusion chromatography, followed by **B**, ion exchange chromatography. The continuous line represents the total protein concentration, and the dotted line represents the cathepsin L-like activity.



**Figure 2.** Silver-stained sodium dodecyl sulfate–polyacrylamide gel electrophoresis of the concentrated purified fraction (PF) with cathepsin ion exchange chromatography–purified cysticercus fraction with cathepsin L-like activity (no protease inhibitor added). MW = molecular weight marker.



**Figure 3.** Gelatinolytic zymography of cathepsin L-like fraction purified without using protease inhibitors (PF). To confirm the cathepsin L-like activity, the purified fraction was incubated with E64 (PF(E64)) and chymostatin (PF(chym)) before electrophoresis. MW = molecular weight marker.



**Figure 4.** Box plot distribution of the percentage of positivity (PP) of the cathepsin L enzyme-linked immunosorbent assay in patients with a single cyst infection, patients with more than 1 cyst, negative controls, and patients with other helminth infections.

**Table 1**

Purification efficiency during the steps used to recover the 53/25-kD cathepsin L-like fraction from *Taenia solium* cyst fluid

Purification step	Total protein (µg/mL)	Specific activity (µmol substrate/minute/mg of protein) × 10 <sup>-3</sup>	Purification factor
Crude cyst fluid	4,933	1.32	1
Ethanol precipitate	3,033	1.32	1.00
Size exclusion G75	278	6.44	4.88
Ion exchange	70	56.00	42.46



**Table 2**Distribution of the *Taenia solium* cathepsin L Western immunoblot band intensity according to sera tested

Protein	Result	Negative controls (%) (n = 48)	Patients with a single viable cyst (%) (n = 50)	Patients with multiple viable cysts (%) (n = 50)
25 kD	Negative	45 (94)	12 (24)	2 (4)
	Weak positive	2	11	6
	Positive	1	21	18
	Strong positive	0	6	24
	% positive	6	76	96
53 kD	Negative	47 (98)	11 (22)	2 (4)
	Weak positive	0	13	6
	Positive	1	21	18
	Strong positive	0	5	24
	% positive	2	78	96