IDENTIFICATION OF ANTIGENIC COMPONENTS OF *GNATHOSTOMA SPINIGERUM* ADVANCED-THIRD STAGE LARVAE BY TWO-DIMENSIONAL GEL ELECTROPHORESIS AND MASS SPECTROMETRY

Porntip Laummaunwai[1,4,5], Pewpan M Intapan[1,4],
Chaisiri Wongkham[2], Viraphong Lulitanond[3,4] and Wanchai Maleewong[1,4]

[1] Department of Parasitology, [2] Department of Biochemistry, [3] Department of Microbiology, Faculty of Medicine; [4]Research and Diagnostic Center for Emerging Infectious Diseases; [5]Faculty of Graduate School, Khon Kaen University, Khon Kaen, Thailand

Abstract. The reactive spots of crude somatic antigen of *Gnathostoma spinigerum* third-stage larvae were verified and identified by two-dimensional gel electrophoresis (2DE) and liquid chromatography/tandem mass spectrometry (LC/MS-MS). Specific proteins of an approximate molecular weight of 23 to 24 kDa with a pI of 8.1 to 9.3 that were recognized by human gnathostomiasis serum could be demonstrated in Coomassie blue stained-2DE gel and were then identified by LC/MS-MS. The derived peptide sequences matched the sequence regions of cyclophilin, hypothetical protein, actin, matrix metalloproteinase-like protein and intermediate filament protein B in the nr.fasta database. These peptide sequences can be useful for the strategic planning of *G. spinigerum* recombinant antigen production.

INTRODUCTION

Gnathostoma spinigerum, a spirurid nematode, is widely distributed in Asia, especially Japan, China, Burma, Vietnam, Philippines, Malaysia and Thailand (Miyazaki, 1960; Daengsvang, 1981). Humans acquire gnathostomiasis mainly by consuming raw or semi-cooked meat contaminated with infective larvae (Daengsvang, 1981). Cases occur continuously despite public health campaigns. The worm usually migrates into the subcutaneous tissue causing an intermittent migratory swelling; sometimes it reaches the central nervous system producing various signs and symptoms, at which point the disease can become life threatening (Boongird *et al*, 1977; Jaroonvesama, 1988; Schmutzhard *et al*, 1988). Protein analysis of this parasite using one-dimensional polyacrylamide gel electrophoresis under reducing conditions can identify a specific polypeptide for use as an antigen in the immunodiagnosis of this disease (Nopparatana *et al*, 1988, 1991; Tapchaisri *et al*, 1991). A specific *G. spinigerum* antigen with an approximate molecular mass of 24 kDa was determined using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting (Tapchaisri *et al*, 1991). Antigenic spots with an approximate molecular mass of 23-25 kDa and a pI of 8.3-8.5 revealed by two-dimensional gel electrophoresis (2DE) and immunoblotting have been reported and are used as diagnostic spots for human gnathostomiasis (Wongkham *et al*, 2000). However, there are no reports regarding the biochemical properties of these protein spots by amino acid sequencing. Therefore this study was initiated to evaluate specific antigenic spots of *G. spinigerum* using 2-DE-based-liquid chromatography/tandem mass spectrometry (2DE-LC/MS-MS), and a database search. Knowledge regarding these specific spots gained by this study is expected to further the designation of degenerate primers used in PCR reactions, followed by the full-length cDNAs synthesis of these proteins. The results should be applicable for the molecular cloning of gnathostomiasis antigens for diagnostic purposes.

Correspondence: Wanchai Maleewong, Department of Parasitology, Faculty of Medicine, Khon Kaen University, Khon Kaen 40002, Thailand.
Tel : 66 (043) 348387; Fax : 66 (043) 202475
E-mail: wanch_ma@kku.ac.th

MATERIALS AND METHODS

G. spinigerum antigen

Advanced third-stage larvae of *G. spinigerum* (al3) were obtained from mice which were orally inoculated with early third-stage larvae from infected copepods (Maleewong *et al*, 1988). Al3 somatic extract was prepared as previously described (Maleewong *et al*, 1997). Briefly, the worms were homogenized with a tissue grinder in a small volume of 0.1 M phosphate buffered saline at pH 7.4 containing 0.1 mM of phenylmethylsulfonylfluoride, 0.1 mM of tosylamide-2-phenyl-ethyl-chloromethylketone, and 1 μM of L- trans-3-carboxyoxiran-2-carbonyl-6-leucylagmatine. The preparation was then sonicated with an ultrasonic disintegrator and centrifuged at 10,000*g* for 30 minutes at 4˚C. The supernatant was dialyzed against distilled water containing the same proteinase inhibitors and kept at -20˚C until further analysis. The protein content of the antigen was determined by the Folin phenol method (Lowry *et al*, 1951).

Serum samples

Positive control serum was obtained from six parasitologically confirmed cases of adult human gnathostomiasis. Negative control serum was obtained from the pooled sera of 30 healthy Thai adults who had no history of intermittent cutaneous migratory swellings and were negative for intestinal parasitic infection at the time of blood collection. The serum collections were started several years before the present investigation began though a study was envisioned and therefore informed consent was obtained from the study subjects using an approved standard procedure. The study protocol was approved by the Khon Kaen University Ethics Committee of Human Research.

2DE and immunoblotting

2DE was performed using the IPGphor system (GE Healthcare Bio-Sciences AB, San Francisco). For the first dimension electrophoresis, IEF was performed using ready-made 7 cm Immobiline DryStrip gels with a non-linear pH gradient of 3-11. Briefly, a strip was rehydrated with rehydration solution [8 M urea, 2% (w/v) CHAPS, 0.5% IPG buffer, 0.28% dithiothreitol (DTT)] and crude extract of *G. spinigerum* advanced third-stage larvae (200 μg protein in 50 μl distilled water). Then the IPG strip was placed in the gel upside down in the holder and covered with 1 ml of IPG Cover Fluid. The holder was closed with a lid and the IPG strip was incubated in the holder for 13 hours at 20˚C followed by focusing at 8,000 Vhr at 20˚C. After focusing, the strip was equilibrated twice for 15 minutes each, with the first equilibration solution (6 M urea, 30% glycerol, 2% SDS, 1% DTT, supplemented with trace amounts of bromophenol blue in 50 mM Tris-HCl, pH 8.8) and with the second equilibration solution (6 M urea, 30% glycerol, 2% SDS, 2.5% iodoacetamide, supplemented with trace amounts of bromophenol blue in 50 mM Tris-HCl, pH 8.8), respectively. Then, each of the focused gel strips was further subjected to SDS-PAGE (12% gel) (Laemmli, 1970). After electrophoresis, each gel was stained with Coomassie blue; the interesting spots were excised or the separated proteins were electro-transferred onto a nitrocellulose membrane for immunoblotting.

The membrane was immersed in a blocking solution containing 1% skim milk in a 100 mM phosphate buffered saline (PBS) solution containing 0.1% Tween 20 for 30 minutes at room temperature and was then incubated with pooled positive or negative reference serum samples (diluted 1:100 in blocking solution) for 2 hours at room temperature. Thereafter, it was washed 5 times with PBS at pH 7.5 containing 0.05% Tween-20, followed by incubation for 2 hours with goat anti-human IgG (Fc) labeled horseradish peroxidase conjugate (Zymed laboratories Inc, San Francisco, CA) (diluted 1: 2,500 in blocking solution). For visualization of the antibody reaction 3,3´-diaminobenzidine

tetrahydrochloride (DAB) and hydrogen peroxide were used as chromogenic substrates. To ensure reproducibility of the tests, each experiment was performed in triplicate; the subsequent tests produced uniform results.

2DE-based-liquid chromatography/tandem mass spectrometry (LC/MS-MS)

Coomassie-stained individual protein spots of interest were excised and sent for analysis to the Proteomics service center of the Bioservice Unit, Biotech Thailand, National Science and Technology Development Agency, Thailand. Briefly, the gel plugs were destained, air-dried and digested with trypsin. The tryptic peptides were acidified and analyzed by liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS). A LC-MS/MS model Finnigan LTQ Linear Ion Trap Mass Spectrometer was used. The HPLC system was a Finnigan Surveyor™ MS pump with a flow splitter. The trap column (ZORBAX 300SB-C18, 5 µm, 5×0.3 mm) was from Agilent. The eluates were analyzed by mass spectrometer (Finnigan LTQ). For data analysis, the ion spectra of the peptides generated by mass spectrometry were interpreted using the Turbo SEQUEST algorithm of the BioWorks™3.1SR1 software package (Thermo Electron) and the nr.fasta database. The identified peptides were further evaluated using charge state versus cross-correlation numbers (Xcorr). The criteria for positive identification of peptides were Xcorr > 1.5 for singly charged ions, Xcorr > 2.0 for doubly charged ions, and Xcorr > 2.5 for triply charged ions. A delta correlation (ΔC_n) > 0.08 was used as a cutoff for peptide acceptance.

RESULTS

The separation patterns of *G. spinigerum* larval extract using 2-DE were highly complex, consisting of more than 75 visible protein spots (Fig 1) which were mostly located in the acidic and neutral areas. Immunoblots of the pooled

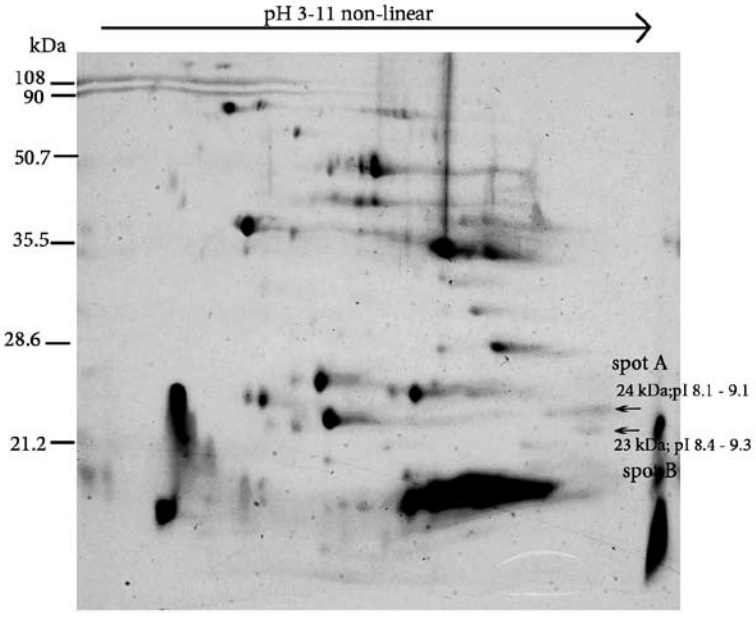

Fig 1- Coomassie blue staining of the 2DE protein spot pattern of *Gnathostoma spinigerum* advanced third-stage larval extract. The pH markers are indicated on the top. The molecular weight markers are indicated on the left in kilodaltons (kDa). The arrowhead indicates the interesting spots. Spot A has an approximate molecular mass of 24 kDa with a pI between 8.1 and 9.1. Spot B has an approximate molecular mass of 23 kDa with a pI between 8.4 and 9.3.

proven gnathostomiasis cases are shown in Fig 2. An analysis of the blots showed various numbers of prominent antigenic peptides, with at least 70 spots and an approximate molecular mass of less than 21.2 to more than 108 kDa with pIs between 5 and 10. Two antigenic spots (A and B) were specifically recognized by human gnathostomiasis serum. Spot A was located at an approximate molecular mass of 24 kDa with a pI of 8.1-9.1 and spot B was located at an approximate molecular mass of 23 with a pI of 8.4-9.3. These spots did not react with pooled healthy control sera.

The orthologous proteins in the database matching spots A and B are shown in Table 1. A total of 8 orthologous proteins belonging to 4 types of proteins (cyclophilin, matrix metalloproteinase-like protein, actin and hypothetical protein) matched the peptide sequences derived from spot A. While 4 orthologous proteins belonging to 3 types of protein (cyclophilin, intermediate filament protein B and hypothetical protein) matched with the peptide sequences derived from spot B.

DISCUSSION

LC/MS-MS is widely used to determine the internal protein sequences in parasites, such as *Taenia solium* (Levine *et al*, 2004), *Trichinella spiralis* (Robinson and Connolly, 2005), *Leishmania donovani* (Forgber *et al*, 2006) and *Entamoeba histolytica* (Tolstrup *et al*, 2007). In the present study, a protein extract from *G. spinigerum* al3 was separated by 2DE and subsequent immunoblotting. The results showed that proteins at an approximate molecular weight of 23 to 24 kDa with a pI of 8.1 to 9.3 were specifically recognized by human gnathostomiasis sera. The data correlate with a previous report by

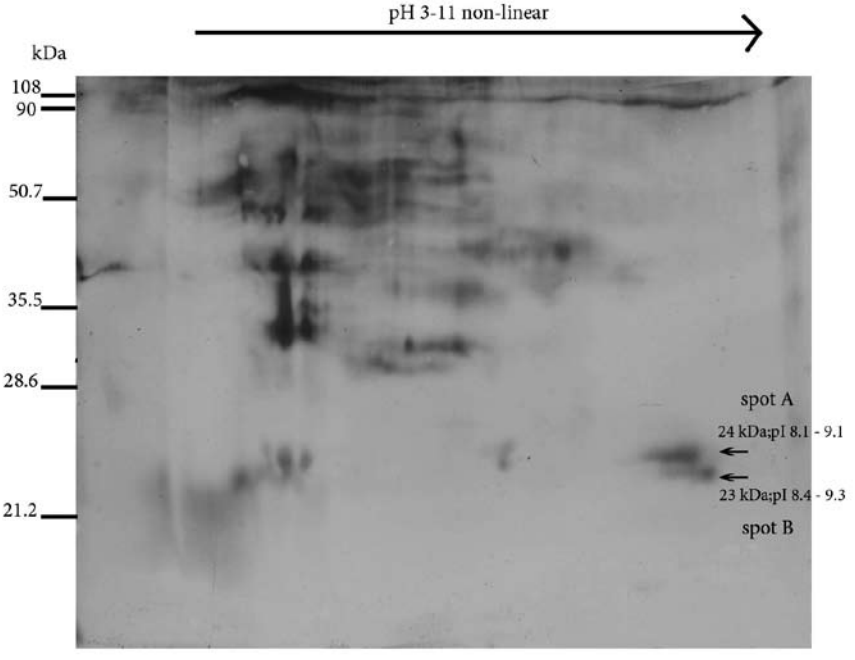

Fig 2- The 2DE-immunoblot pattern of *Gnathostoma spinigerum* advanced third-stage larval extract reacted with pooled proven gnathostomiasis serum. The pH markers are indicated on the top. The molecular mass markers are indicated on the left in kilodaltons (kDa). The arrowhead indicates the interesting spots. Spot A has an approximate molecular mass of 24 kDa with a pI between 8.1 and 9.1. Spot B has an approximate molecular mass of 23 kDa with a pI between 8.4 and 9.3.

Table 1
Orthologous proteins of the database matching the peptide sequences for diagnostic proteins of *Gnathostoma spinigerum* advanced third-stage larvae components identified by 2DE-LC/MS-MS and a database search.

Spot	Orthologous proteins	Accession no.
A	Cyclophilin Bmcyp-2 of *Brugia malayi*	AAC47231
	IVMELFADIVPK	
	VIPNFMLQGGDFTR	
	GNGTGGQSIYGEK	
	GNGTGGQSIYGEKFPDENFQEK	
	Cyclophilin of *Zea mays*	CAA48638
	IVMELYANEVPK	
	VIPEFMCQGGDFTR	
	Cyclophilin of *Caenorhabditis elegans*	1H0P
	IVIGLFGKTVPK	
	Cyclophilin-A	AAB01531
	VIPNFMCQGGDFTR	
	Peptidylprolyl isomerase	S71547
	VLEGMDVVR	
	Matrix metalloproteinase-like protein of *G. spinigerum*	AAF82802
	VLPLTFEFTTGK	
	YGAFTHYDDDELFGEWTQQYIDNGR	
	Actin of *C. elegans*	CAA34719
	SYELPDGQVITVGNER	
	Hypothetical protein of *C. briggsae*	CAE 62837
	GPGTAFEFALK	
B	Cyclophilin Bmcyp-2 of *B. malayi*	AAC47231
	IVMELFADIVPK	
	GNGTGGQSIYGEK	
	FPDENFQEK	
	Cyclophilin-A	AAB01531
	VIPNFMCQGGDFTR	
	Intermediate filament protein B (Common roundworm)	S06954
	EIVYTIPPNTVLK	
	Hypothetical protein of *C. briggsae*	CAE 62852
	IVMELYTDVVPK	
	TEWLDGK	

Spot A: antigenic spot at an approximate molecular mass of 24 kDa, pI 8.1-9.1
Spot B: antigenic spot at an approximate molecular mass of 23 kDa, pI 8.4-9.3

Wongkham *et al*, (2000). Cross-analyses were performed of amino acid sequences for the tryptic peptides derived from the 2DE-LC/MS-MS combination with known databases. The peptides matched sequence regions of cyclophilin, hypothetical protein, actin, matrix metalloproteinase-like protein and intermediate filament protein B. These results provide new data regarding the internal protein sequences of the two diagnostic spots for human gnathostomiasis.

The information gained regarding the two antigenic spots of *G. spinigerum* larval extract by this study should help to further designate degenerated primers which can be used for the 5′ and 3′ rapid amplification of the cDNA end synthesis of these genes. The results can be applied to molecular cloning and to express diagnostic proteins for human gnathostomiasis. Further cloning and expression of *G. spinigerum* antigenic proteins is now in progress. This will help us to develop highly sensitive and specific rapid tests that are simple and low-cost, designed for field use in developing countries.

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