STUDIES ON CONCOMITANT ANTIGENS OF *BITHYNIA FUNICULATA* FOR DETECTION OF ANTIBODY TO *OPISTHORCHIS VIVERRINI*: EFFECT OF DIFFERENT CENTRIFUGAL SPEEDS ON ANTIGEN PREPARATION

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Abstract. Antigens derived from somatic extracts of *Bithynia funiculata*, an intermediate snail host of *O. viverrini*, have been demonstrated to be highly heterogeneous in molecular weight (MW). These antigens have been suggested to be of potential use for serodiagnosis. In this study, *B. funiculata* somatic antigens were extracted using five different centrifugal speeds, namely 10,000 (C1); 20,000 (C2); 30,000 (C3); 40,000 (C4) and 50,000 (C5) rpm, with the aim of removing some non-specific antigens and determining the optimal centrifugal speed to obtain the highest efficiency of the test for which they will be used. The enzyme-linked immunosorbent assay technique was used to compare the reactivity of the five different centrifugal speed-prepared antigens. The sensitivity and specificity of all five antigens were compared by testing against sera from 81 opisthorchiasis patients, 30 parasite-free healthy individuals and 50 individuals infected with other helminthic infections, using $\overline{X}$ + 4SD of all healthy individuals as the cut-off value. The sensitivity of these antigens was 69.1, 84.0, 80.2, 84.0 and 70.4, respectively; while the specificity was 66.2, 76.2, 82.5, 86.2 and 71.2, respectively. Immunoreactive components of each antigen were analyzed by SDS-PAGE and Western blot technique. The assay showed that three pairs of antigens with MW of 29 and 30, 47 and 50, and 86 and 90 kDa of all five antigens, which have previously been shown to be highly immunogenic, still reacted with a pooled serum from patients with opisthorchiasis. However, the C4 antigens gave more distinct components. Our results showed that 40,000 rpm is the optimal speed for antigen preparation for use in the serological diagnosis of opisthorchiasis, as demonstrated by the most satisfactory results of both sensitivity and specificity in the indirect ELISA and Western blot technique.

INTRODUCTION

Results of previous studies, which have highlighted the potential of using antigens shared between *Opisthorchis viverrini* and its snail intermediate host, *Bithynia funiculata*, for the serodiagnosis of opisthorchiasis, have provided the impetus for preliminary serodiagnostic tests giving acceptable results (Watthanakulpanich *et al*, 1997). However, cross-reaction with other parasitic infections and a moderate sensitivity is a puzzle. It is essentially important to improve sensitivity and to reduce cross-reaction of the crude extract antigens for more accurate diagnosis. It has been known that a more refined antigen would provide a more sensitive and sensitive serological test for the diagnosis of opisthorchiasis. Attention has been drawn to the production of sufficient quantities of purified antigens which may contribute significantly to the success of serological procedures (Watthanakulpanich *et al*, 1998; Waikagul *et al*, 2001). To date, there is a dearth of information regarding the optimal method for preparating antigen from *Bithynia* snails. Each snail has such a complex structure, which consists of the whole snail tissue, the hemolymph, the foot muscle, the digestive gland and the visceral structures and has different antigenicity among the snail components (Guha and Sornmani, 1970). Studies on the association between the centrifugal speed used for antigen preparation and composition of *Bithynia* snail antigens extracted therefrom are lacking. The aim of this study is to observe the optimal centrifugal speed to be used in antigen preparation. An attempt to evaluate the serodiagnostic potential of varying centrifugal speed-prepared antigens by the ELISA and Western blot technique is made.

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MATERIALS AND METHODS

Preparation of *B. funiculata* antigens

The shells of the *B. funiculata* snails were gently crushed and the pieces of shell removed. The bodies of the snails were examined for the presence of parasites (sporocysts, rediae or cercariae) under a stereomicroscope. Only uninfected snail bodies were collected and washed with distilled water. They were lyophilized and hand-homogenized using a glass mortar and pestle with a small volume of distilled water, then the homogenate was sonicated in an

ultrasonic disintegrator set (Sonicator Ultrasonic Processor; Model XL 2020-010, Heat System, Inc, USA; Standard probe No. 419) at maximum amplitude for 2-minute intervals for 30 minutes. The homogenate was separated and then centrifuged at different speeds as follows: 10,000; 20,000; 30,000; 40,000 and 50,000 rpm at 4˚C for 1 hour. The supernatant was collected in small aliquots and kept frozen at -20˚C until used.

Protein determination

The protein content of the respective antigens prepared at different centrifugal speeds was determined by Lowry's method (1951) using serum albumin as the standard.

Collection of sera

Serum samples were obtained from three groups of individuals.

Group 1: sera obtained from 81 patients living in the endemic area in Prachin Buri Province. They were positive for *O. viverrini* infection by fecal examination.

Group 2: sera obtained from 30 apparently healthy adults residing in non-endemic areas. All were negative for *O. viverrini* eggs and other parasites in the feces at the time of blood collection.

Group 3: sera obtained from 50 patients with helminthic infections other than *O. viverrini*, as described by fecal examination. Their distribution, by helminthic infection, was as follows: paragonimiasis 23, taeniasis 6, sparganosis 1, strongyloidiasis 7, hookworm infection 5, gnathostomiasis 4, capillariasis 2 and toxocariasis 2.

Antibody determination

The serodiagnostic potential of the different centrifugal speed-prepared antigens was evaluated by indirect ELISA. Each antigen was tested for antigenic activity against sera from patients infected with *O. viverrini* (Group 1), normal healthy controls (Group 2) and other parasites (Group 3). The solid phase ELISA was performed in a microtiter plate (Nunc, Denmark) as described elsewhere (Dharmkrong-at *et al*, 1988). The optimal conditions of the assay were determined by checkerboard titration. Each well of the microtiter plate was coated with 100 µl of an appropriate concentration of antigen (20 µg/ml each for the respectively prepared antigen in carbonate-bicarbonate buffer pH 9.6). The plate was incubated in a humidified chamber at 37˚C for 1 hour and kept overnight at 4˚C. The unbound antigens were eliminated by washing 5 times with PBS-T (Tween-20 in PBS, pH 7.4). After blocking with 1% BSA in PBS-T pH 7.4, the plate was incubated at 37˚C for 1 hour. After washing, the sera, diluted to 1:400 for each antigen, were added to each well and allowed to react at 37˚C for 1 hour, then washed as above. The peroxidase-conjugated rabbit immunoglobulins to human IgG, diluted to 1:1,000, were applied to each well and incubated at 37˚C for 1 hour. The enzymatic reaction was developed by the addition of 100 µl of para-phenylenediamine (PPD, Sigma) substrate in citrate buffer containing hydrogen peroxide (H_2O_2) to the wells and the plate was kept at room temperature in the dark for 30 minutes. The reaction was stopped by the addition of 50 µl of 1 N NaOH. The intensity of color reaction was read by an ELISA reader (Titertek Multiskan Plus, MKII) at 492 nm using the diluent as a blank.

Data analysis

The sensitivity, specificity and positive and negative predictive values of the ELISA were determined by the method of Garen (1980).

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis

The SDS-PAGE was carried out in a vertical slab gel apparatus (AE-6200, ATTO Corporation, Japan). The 13% separating gel was prepared, poured onto the slab gel apparatus and allowed to polymerize at room temperature. The 4% stacking gel was then prepared and poured on top of the separating gel. Each antigen, with a concentration of 40 µg/ml, was mixed with an equal volume of sample (0.5 M Tris-HCl pH6.8, 7.5%2-mercaptoethanol, 3.5%SDS). The protein contents were completely dissociated by boiling at 100˚C for 3 minutes, then loaded onto the gel. Electrophoresis was carried out with a current of 20 mA for the stacking gel, then the current was increased to 30 mA for the separating gel. The resolved polypeptide bands on the gel were either revealed by staining with Coomassie brilliant blue or electrophoretically transferred onto a 0.45 µm nitrocellulose membrane by semi-dry transfer cell "Horis Blot" (ATTO Corporation, Japan) with a constant current at 200 mA for 4 hours. After blotting, non-specific binding was blocked by immersing the membrane into 3% gelatin in 0.04% NaN_3-PBS pH 7.4 for 1 hour on a rocking platform, washed in PBS-T, and the membrane was then cut into strips. The strips were reacted with a pooled positive serum at a dilution of 1:50 for 4 hours on a rocking platform, washed in PBS-T 3 times and subsequently incubated with peroxidase conjugated rabbit anti-human IgG diluted

to 1:1,000 for 2 hours. The 2,6-dichlorophenol-indophenol substrate, containing hydrogen peroxide (H_2O_2), was added and the enzyme activity was allowed to develop. The strips were rinsed in distilled water, air-dried and photographed.

RESULTS

Detection of antibody to *O. viverrini* by different centrifugal speed-prepared antigens

The *Bithynia* snail proteins prepared at different centrifugal speeds (10,000; 20,000; 30,000; 40,000 and 50,000 rpm), namely C1, C2, C3, C4 and C5, respectively, were used as antigens for indirect ELISA. Checkerboard titration determined the optimal concentration to be 20 μg/ml for all antigens. All serum samples were tested at a dilution of 1:400 and the conjugate was diluted to 1:1,000. The means of optical densities (OD) of the sera from group 1 were significantly higher than those from group 2 and 3 ($p<0.05$), for all antigens used. When each mean of OD+4SD from a healthy control group was used as a cut-off value belonging to each antigen for comparison, any reading with an OD higher than those cut-off values would be considered ELISA-positive for *O. viverrini* infection (Fig 1). The sensitivities, specificities and positive and negative predictive values of the test for all antigens used are summarized in Table 1. The C2 and C4 antigens gave sensitivities as high as 84.0%, which were better than the other antigens. However, the specificity of the C4 antigens (86.2%) was rather higher than the C2 antigens (76.2%), while those of other antigens were also poor when C1, C3 and C5 antigens were used in the assay.

Analysis by SDS-PAGE and Western blotting

The SDS-PAGE analysis and Coomassie brilliant blue staining of each prepared antigen are shown in Fig 2. The results showed that there were some differences in protein patterns among the antigens used. Besides the intensity of each band not being equal, there were some deteriorations in some bands. C1 antigens seemed to give more prominent bands, with high MW (over 94 kDa), observable where some macromolecules of complex proteins had broadly diffuse stains at the top. The predominant protein bands of the C1 antigens were revealed, with MW of 50, 47, 42, 37, 32, 27, 26.5, 22 and 18 kDa, respectively. For the 42 kDa band, it was clearly shown to be more prominent than that of 42 kDa belonging to the other antigens. The other minor bands faintly stained were 90, 86, 58, 30 and 25 kDa, respectively. Similarly, the protein patterns observed from C2 antigens were thickly stained at the top region but fewer; and the 42 kDa protein band was rather faintly stained compared with the former antigens. However, the low MW of protein bands seemed to be more prominent, particularly the 37, 32, 27, 26.5 kDa ones. The C3 and C4 antigens were nearly identical, as they produced protein bands with similar mobility. However, the C4 antigens appeared to give more prominence in the low MW of 50, 47, 37, 32, 27, 26.5 than the C3 antigens and the other antigens. The 42 kDa protein band was also weakly stained, like the other antigens, by comparison with the C1 antigens, whereas none of the C5 antigens produced any more prominent bands than the other antigens after staining with Coomassie brilliant blue. The Western blot technique was utilized for studying the antigenic communities of the antigens. The reactive bands of each antigen, when reacted with a pooled positive serum, revealed approximately 13 identical bands. Furthermore, the C4 antigens appeared to be composed of more prominent and intensely reactive bands of 90, 86, 50, 47, 32, 30, 29, 27 and 26.5 kDa, respectively. The three pairs of 29-30, 47-50 and 86-90 kDa bands were of special interest, in view of the fact that they still appeared as potent immunogen.

DISCUSSION

The phenomenon of antigen-sharing between a parasite and its snail intermediate host is currently receiving considerable attention. Several investigators have studied the possibility of using antigens shared by trematodes and their intermediate snail hosts for immunodiagnosis. This finding leads to the interesting possibility of using *Bithynia* snails as alternative diagnostic antigens for opisthorchiasis. For the preparation of *Bithynia* antigens, some of the body regions that appear not to be antigenic should be removed. *Bithynia* snails have a complex structure, being composed of a head, a long foot, a smooth mantle region (pallial region) and a visceral mass. The head and foot are united to form a head-foot region, which bears long tentacles that are thin and round, and eyes. *Bithynia* snails also possess a mantle that covers the pallial region (mantle edge, pseudobranch and kidney) and that is continuous with a tunica propria covering the visceral mass (Chitramvong, 1992). Of course, some contaminating and nonspecific components occur, and they have to be removed. A previous study showed that the two sources of antigens, the head-foot and the visceral mass, both gave a lower specificity, of about 70.0% (Watthanakulpanich *et al*, 1997). This implies that some components and body fluids of snails may have influenced that unsatisfactory result, such as the snail's blood sugars or stored glycogen and fatty

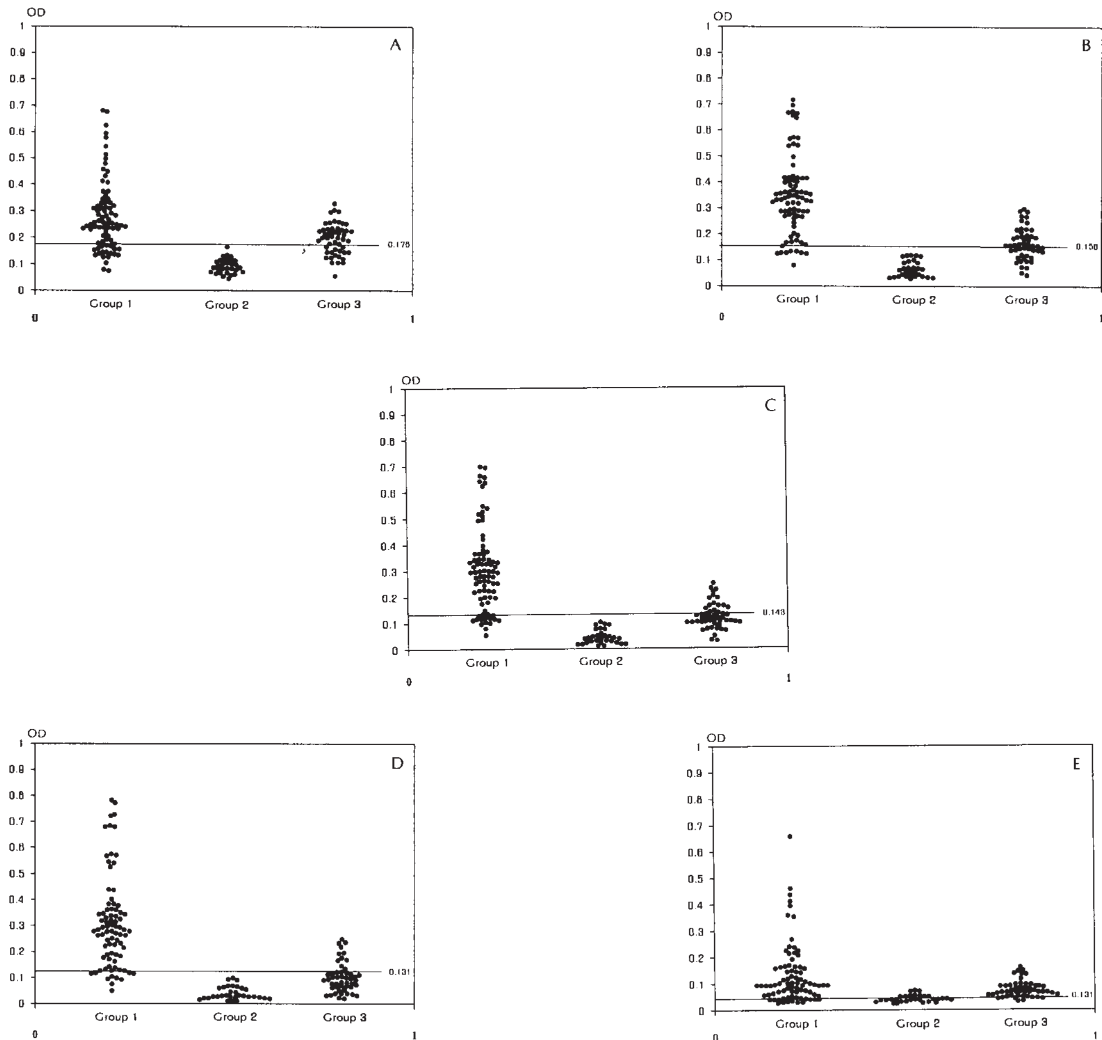

Fig 1- Optical densities of indirect ELISA using various-prepared antigens from different centrifugal speeds. 10,000(A); 20,000(B); 30,000(C); 40,000(D); 50,000(E); against serum samples of opisthorchiasis (Group1), normal controls (Group2) and other parasitic infections (Group3).

acids, including the body-surface mucus of the snails (Wright, 1959; Cheng, 1968). That is why, when the snail bodies were prepared as antigens, these contaminating components should be removed using appropriate techniques, such as incubating in ethyl carbamate (Capron *et al*, 1965), homogenizing in 1% Triton X-100 (Jackson and Moor, 1976), washing in cold buffer (PBS containing 0.1% sodium azide and 2mM methylene diamine tetraacetic acid) (Devine and Kemp, 1984), swabbing with 70% alcohol (Harris *et al*, 1993), filtering through glass wool (Waikagul *et al*, 2002). In addition, centrifugal force is a powerful separation technique for separating the available supernatant, which is comprised of rich antigenic proteins from the protein pellet, on the basis that a larger body will sediment faster than a smaller one and denser particles will sediment faster than lighter ones (Stryer, 1995). The speed required for the separation of those available antigens into the supernatant was assessed by spinning for equal times at variable speeds. As the *Bithynia* snails have complex structures that are composed of many components, little is known about how to prepare the antigens from the *Bithynia* snails themselves. There should be an optimal

Table 1
Summary of sensitivity, specificity and predictive values of indirect ELISA using different centrifugal speed prepared-antigens.

Antigens	Cut-off value at $\overline{X}$+4SD	Sensitivity (%)	Specificity (%)	Predictive values	
				Positive (%)	Negative (%)
C1	0.176	69.1	66.2	67.5	67.9
C2	0.158	84.0	76.2	78.2	82.4
C3	0.143	80.2	82.5	82.3	80.5
C4	0.131	84.0	86.2	86.1	84.1
C5	0.059	70.4	71.2	71.2	70.4

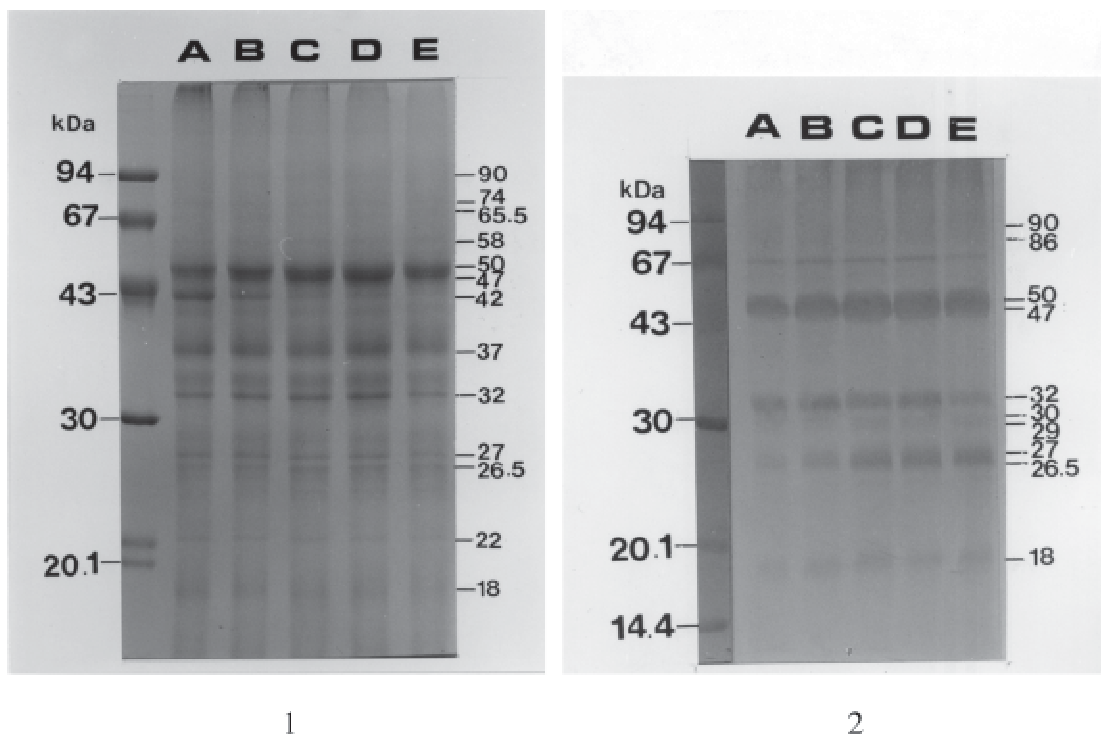

Fig 2- SDS-PAGE and Western blot patterns of various-prepared antigens from different centrifugal speeds; 10,000 (A); 20,000 (B); 30,000 (C); 40,000 (D); 50,000 (E).
1: Stained with Coomassie brilliant blue; 2: Reacted with a confirmed opisthorchiasis pooled serum.

speed that can separate those contaminating and nonspecific proteins from the desired ones. For the C1 antigens, the results showed that the sensitivity and specificity were 69.1% and 66.2%, respectively. The sensitivity and specificity of the test were rather lower than those of the C2, C3 and C4 antigens. An explanation may be that the centrifugation speed was not high enough to cause the contaminating proteins to form a pellet. It was virtually impossible to reduce the level of contaminating proteins below detection limits with a single spin, particularly with such low speed that diffusion counterbalanced the centrifugal force (Stryer, 1995). This made the macromolecules and contaminating proteins distribute throughout the supernatant, which could be seen clearly from the turbidity of the supernatant. SDS-PAGE supported the

supposition that the macromolecules of the proteins were mostly still retained in the supernatant and were stained as complex protein patterns at the top, while the reactive proteins were not prominent and intense due to the contaminating proteins. A similar approach was attempted with C2-C5 antigens to improve both the sensitivity and specificity of the assay. The results showed that the C5 antigens gave similar results to the C1 antigens but might be explained contrarily, as the speed of the centrifugation used was probably excessive. Therefore, little of the contaminating proteins, or the reactive proteins, were left in the supernatant, which led to low sensitivity, while the disproportion of the antigen-antibody reaction would lead to low specificity, despite there being less antigen in the supernatant due to false positives as no specific antigens against *O. viverrini* antibodies were retained. The results showed that, among the three antigens left, C4 antigens were the most reactive ones in the indirect ELISA against sera from patients with opisthorchiasis and gave the highest sensitivity (84.0%) and highest specificity (86.2%). Serum IgG from patients with paragonimiasis (10 out of 23 cases) remained cross-reactive, but with fewer cases than the previous study (Watthanakulpanich *et al*, 1997). *Paragonimus* was one of the parasites that usually gave cross-reaction with *O. viverrini*, which might be due to the fact that there are common antigens between these two parasites. Surprisingly, there was a case infected with taeniasis (1 out of 7 cases), which was also cross-reactive. This case was used to give a negative result in the previous study. However, compared with the background exhibited by the OD, the OD in both situations showed a marked elevation of IgG antibodies to both antigens tested, for which the latter one, unfortunately, gave a slightly higher OD than the cut-off value. It may be expected that the C4 antigens containing complex mixtures of proteins had a higher content of functional antigens that cross-reacted with taeniasis, while the patients with other parasitic infections had no detectable cross-reaction. The speed of 40,000 rpm for antigen preparation was appropriate to cause more specific antigens to float to the top, and more contaminating and nonspecific antigens to sediment to the bottom. Both processes must occur in the equilibrium density gradient to accomplish a separation (Schumaker and Puppione, 1986). Eventually an exact balance is reached between flotation and sedimentation, and the concentration distribution reaches an equilibrium. When C2 and C3 antigens were used, they appeared to be less sensitive and specific than C4 antigens, as shown in Table 1. C3 antigens that were prepared at 30,000 rpm gave sensitivity and specificity equal to those used in a previous study, which were also prepared at a speed of 30,000 rpm (Watthanakulpanich *et al*, 1997). As in previous explanation, the centrifugation speeds for C2 and C3 antigen preparations were not rapid enough to reach equilibrium and yield the more specific antigens and eliminate the contaminating and nonspecific antigens.

Characterization of the *Bithynia* snail antigens by SDS-PAGE stained with Coomassie brilliant blue revealed at least 30 peptides. The reactive band patterns showed that each antigen gave identical antigenic components. The result of Western blot analysis was in agreement with that obtained from ELISA, as C4 antigens that revealed more prominent reactive bands than the other antigens gave the highest sensitivity. This indicated that centrifugal force could reduce some contaminating proteins from crude extract antigens but should be used at an optimal speed. Protein components with MW of 29-30, 47-50 and 86-90 kDa were potent immunogens and had potential in the immunodiagnosis of opisthorchiasis, as it reacted with the sera of all patients with opisthorchiasis (Watthanakulpanich *et al*, 2002). These three pairs were still found in the C4 antigens; however, the protein pair of 86-90 kDa might be glycoprotein, because it could react with a pooled positive reference serum and gave reactive bands in Western blotting, but were weakly stained and inconspicuous in SDS-PAGE analysis. Furthermore, the specificity of the C4 antigens was improved, so that there was less cross-reactivity with the sera of patients infected with other parasites. Further attempts should be made to prepare more specific antigens from C4 antigens to improve the specificity of the assay.

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