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Characterization of immunogenic *Clonorchis sinensis* protein fractions by gel filtration chromatographyDuan Pham Ngoc<sup>1,2\*</sup>, Yuji Arimatsu<sup>1</sup>, Sasithorn Kaewkes<sup>3,4</sup>, Sung-Jong Hong<sup>5</sup>, Banchob Sripa<sup>1,4\*</sup><sup>1</sup>WHO Collaborating Centre for Research and Control of Opisthorchiasis (Southeast Asian Liver Fluke Disease), Tropical Disease Research Laboratory, Department of Pathology, Faculty of Medicine, Khon Kaen University, Khon Kaen 40002, Thailand<sup>2</sup>Department of Parasitology, Ha Noi Medical University, Ha Noi 10000, Viet Nam<sup>3</sup>Department of Parasitology, Faculty of Medicine, Khon Kaen University, Khon Kaen 40002, Thailand<sup>4</sup>Liver Fluke and Cholangiocarcinoma Research Center, Khon Kaen University, Khon Kaen 40002, Thailand<sup>5</sup>Department of Medical Environmental Biology, Chung-Ang University College of Medicine, Dongjak-gu, Seoul 156-756, Republic of Korea

## PEER REVIEW

## Peer reviewer

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## Comments

For immunodiagnosis of clonorchiasis, such as ELISA, better sensitivity and specificity are required. In this study, a low molecular protein fraction, No. 20 (53 kDa), was found to be a unique antigen of *C. sinensis*. The result will be helpful for future immunodiagnosis of clonorchiasis patients in endemic areas.

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## ABSTRACT

**Objective:** To characterize immunogenic protein fraction of *Clonorchis sinensis* (*C. sinensis*) by partial purification.

**Methods:** A total of 30 hamsters were infected with 50 *C. sinensis* metacercariae, and then *C. sinensis* protein was purified by gel filtration chromatography. Indirect ELISA and immunoblot were used to detect the antibody in sera of hamsters infected with *C. sinensis*.

**Results:** The gel filtration showed 2 peaks at high (fraction No. 10 to 14) and low (fraction No. 21 to 26) molecular weight proteins. Indirect ELISA showed that both antibodies of clonorchiasis and opisthorchiasis reacted strongly with early fractions (6 to 14) and the reaction was gradually reduced at middle and late fractions (15 to 50). Both antibodies showed different individual fraction of *C. sinensis* by immunoblot. It showed several protein bands that the 34 and 37 kDa were major proteins. The 53 kDa protein which was only found in the clonorchiasis reacted with fraction 20.

**Conclusions:** The purified antigen of *C. sinensis* reacted similarly with both antibodies of clonorchiasis and opisthorchiasis where strong reaction was seen with early fractions. The *C. sinensis* protein fraction No. 20 may be useful for immunodiagnosis of clonorchiasis.

## KEYWORDS

*Clonorchis sinensis*, Gel filtration chromatography, Indirect ELISA, Immunoblot

## 1. Introduction

*Clonorchis sinensis* (*C. sinensis*) is a major food-borne trematode in Southeast and East Asia, principally endemic in China, Northern Vietnam, Southern Korea and the eastern part of Russia[1]. The human infection occurs by eating raw freshwater fish containing

*C. sinensis* metacercariae[2]. More than 200 million people are at risk of infection in the endemic areas; around 15–20 million people are infected but only 1.5–2 million are found with the complications or symptoms[1,3,4]. The *C. sinensis* adults inhabit the bile ducts, mainly inducing clinical biliary and hepatic diseases, with cholangiocarcinoma that is the most serious consequence

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of *C. sinensis* infection[5]. For control and elimination in endemic areas, clonorchiasis is mainly diagnosed by conventional egg examinations in feces. A diagnostic method is able to cover a large area, easy, rapid, cheap, and can be applied to estimate the intensiveness of infection[6]. However, the sensitivity and specificity of the conventional egg examination is low, especially in low levels of infection intensity[7]. Molecular techniques can be applied for diagnosis of clonorchiasis with high sensitivity and specificity. However, these methods are expensive and require high technical skill levels and are not feasible on a large-scale[8-12]. Several other methods have been developed including immunodiagnosis. Crude worm extract and recombinant proteins have been used as an antigen for indirect ELISA of *C. sinensis* infection. However, the disadvantage is cross-reaction with other helminths infection[13-17]. Better sensitivity and specificity of these immunodiagnostic tests are still required for development of the diagnostic test. A previous study used purified protein and detected immunoglobulin E in the serum of clonorchiasis patients[18]. However, the study only focused on immunoglobulin E and the character of the protein fraction was poorly understood. In another study on liver fluke, the purified protein also detected antibody of opisthorchiasis[19].

In this study, we therefore aimed to characterize the immunogenic protein fractions of *C. sinensis* using partially purified antigens fractionated by gel filtration.

## 2. Materials and methods

### 2.1. Parasites and experimental infections

The *C. sinensis* metacercariae was recovered from naturally infected freshwater fish (*Pungtungia herzi*) collected from Jinju, Gyeongsangnam-do, Republic of Korea. The collected metacercariae was kept in 0.1 mol/L phosphate-buffered saline (pH 7.4) with antibiotics at 4 °C until used[20]. According to the study design, 30 adult Syrian golden hamsters, obtained from the Animal Unit, Faculty of Medicine, Khon Kaen University, were used in the experiments. The experimental animals were housed under controlled temperature (24±2) °C and they were fed with commercial feed pellets and water *ad libitum*. Animals were orally infected with 50 metacercariae of *C. sinensis* using gastric intubation.

### 2.2. Sephacryl S-200 gel filtration column chromatography

Adult *C. sinensis* worms were homogenized using extraction buffer and the supernatants were collected after centrifugation (13000 r/min, 10 min at 4 °C). Somatic protein extracted from one hundred adult worms was subjected to sephacryl S-200 gel filtration column (1.0 cm×16.3 cm, i.d.) equilibrated with phosphate-buffered saline buffer (pH 7.2) using an AKTAprime machine (GE HealthCare, USA). Fifty fractions were collected (1 mL each) at a flow rate of 1 mL/min. Each fraction was measured for protein concentration at optical density (OD) 280 and analyzed by indirect ELISA, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot.

### 2.3. Indirect ELISA of fractions

Fractions from gel filtration column were diluted 1:10 using carbonate buffer (pH 9.6) and coated to Nunc MaxiSorp plate overnight at 4 °C. The plates were washed three times with washing buffer (0.15 mol/L NaCl, 0.05% Tween 20). Each well was blocked with 200 µL of 5% skim milk in coating buffer for 2 h at 37 °C.

After washing, the wells were incubated with 100 µL of diluted *C. sinensis* infected hamster serum (1:500) and *Opisthorchis viverrini* (*O. viverrini*) infected hamster serum in incubation buffer containing 2% skim milk for 1 h at 37 °C. After washing, 100 µL of goat polyclonal secondary antibody to anti-hamster immunoglobulin G conjugated horseradish peroxidase (Invitrogen, USA) in incubation buffer (1:2000) was applied to each well and incubated for 1 h at 37 °C. After extensive washing, 50 µL of 3,3',5,5'-Tetramethylbenzidine substrate solution (Thermo Fisher Scientific Inc., USA) was added and the reaction was stopped with 50 µL of stop solution (0.5 mol/L H<sub>2</sub>SO<sub>4</sub>). OD were read at 450 nm (VasaMax, USA).

### 2.4. SDS-PAGE and immunoblot of fractions assay

*C. sinensis* antigen in each fraction was treated with 30% trichloroacetic acid to desalt the fractions. About 15 µL of each fraction was applied to 12.5% SDS-PAGE. Prestained protein ladder of 5 µL (Fermentas, USA) was used for markers. The gels were stained with Coomassie brilliant blue. The resolution proteins by SDS-PAGE were transferred onto nitrocellulose membrane (GE Healthcare, UK), reacted with sera of clonorchiasis, opisthorchiasis in 1:200 dilution and then incubated with horseradish peroxidase conjugated anti-hamster immunoglobulin G (dilution 1:2000). Then finally, the colors of membrane were developed with the diaminobenzidine solution (Boster). The strip images were scanned by Amersham Image Scanner (GE HealthCare, USA).

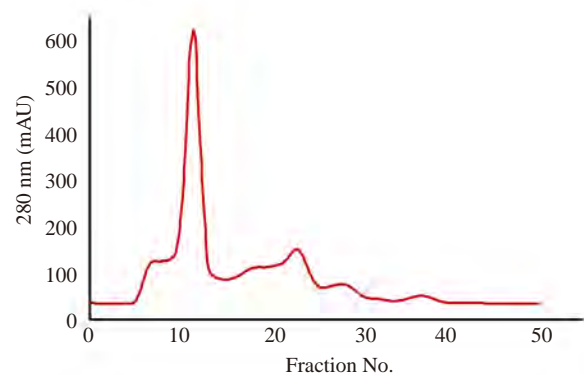
### 2.5. Data analysis

Statistical analysis was performed as follows: a Student's *t*-test was used to compare between antibodies in *C. sinensis* and *O. viverrini* infection (using *P* value less than 0.05 for statistical significance). The protein weight was determined by Image J software and the equation of  $y=aln x+b$ .

## 3. Results

### 3.1. Purification of *C. sinensis* crude antigen by gel filtration chromatography

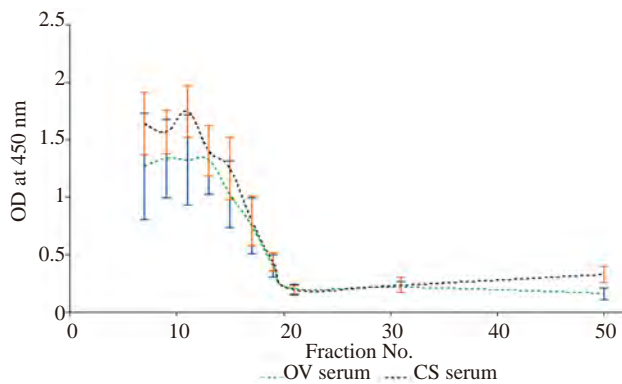
A total of 50 protein fractions were collected from sephacryl S-200 gel filtration column chromatography of *C. sinensis* crude antigen. The result showed 2 peaks at high molecular weight protein (fraction 10 to 14) and low molecular weight protein (fraction 21 to 26) in Figure 1.



**Figure 1.** Purification of *C. sinensis* crude antigen by gel filtration chromatography.

### 3.2. Indirect ELISA of *C. sinensis* protein fractions reacted with both antibodies against *C. sinensis* and *O. viverrini*

The serum antibody of *C. sinensis* infected hamster responded to *C. sinensis* protein fractions as shown in Figure 2. The antibody reacted strongly with early protein fraction (fraction 6 to 14), with reduced reaction at middle and late protein fractions. The antibody from serum of *O. viverrini* infected hamster also reacted similarly with *C. sinensis* protein fractions. However, the *C. sinensis* protein fractions reacted with *C. sinensis* antibody more strongly than it reacted with *O. viverrini* antibody at early fraction (6 to 14) though the difference was not statistically significant. The reaction of *C. sinensis* protein fractions (No. 50) with *C. sinensis* antibody was statistically significantly stronger than that with *O. viverrini* antibody ( $P < 0.05$ ). However, the absorbance values are too low. This result is also similar with the first peak of gel filtration chromatography.



**Figure 2.** Indirect ELISA of *C. sinensis* fractions reacted with *O. viverrini* antibody (green line) and *C. sinensis* antibody (black line) from sera of infected hamsters.

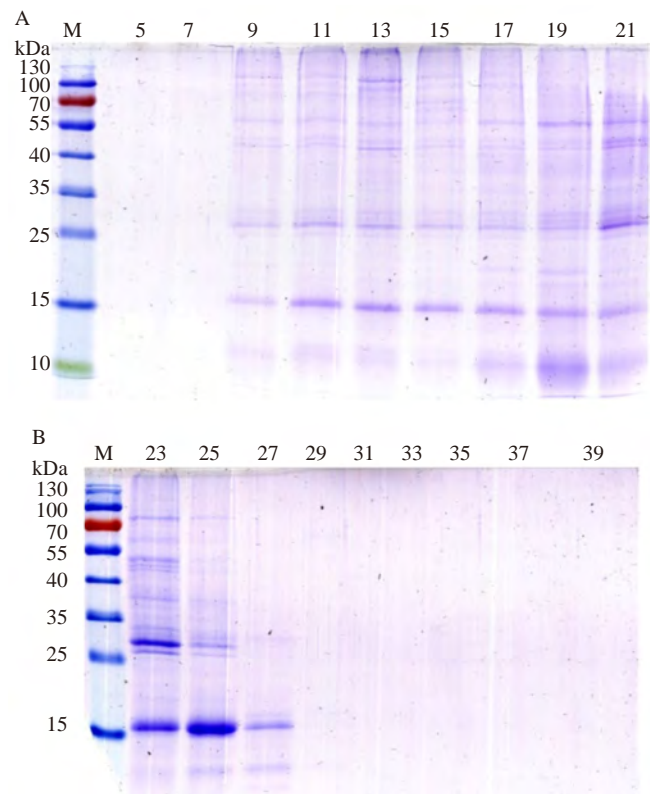
OV: *O. viverrini*; CS: *C. sinensis*.

### 3.3. The SDS-PAGE of *C. sinensis* protein fractions

About 12.5% gel of SDS-PAGE was run with individual fraction and stained with Coomassie brilliant blue. The result showed several protein bands at early and middle fraction (odd fractions from 9 to 25). The 11, 17, 26, 28, 49, 52, 62 and 89 kDa were major protein bands as showed in Figure 3A and 3B.

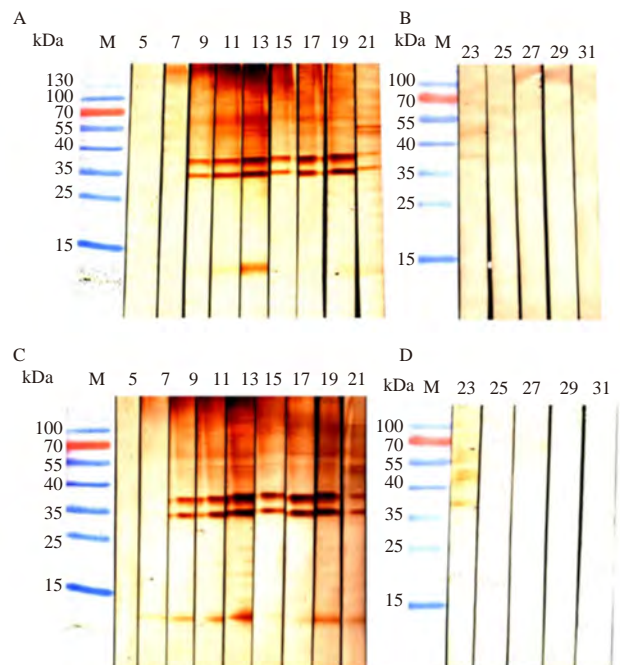
### 3.4. The characterization of *C. sinensis* protein fractions by immunoblotting

The individual fraction of *C. sinensis* antigen samples was loaded to 12.5% gel of SDS-PAGE and transferred to nitrocellulose membrane. Hamster antibodies infected with *C. sinensis* reacted with several protein fractions of *C. sinensis* (fraction No. 7 to 29), and identified over 20 protein bands. The antibody reacted very strongly with high molecular weight protein. The 34 and 37 kDa were detected as common protein bands of fraction 9 to 21. The 10, 50, 53, 59-68, and 123-177 kDa protein bands were detected in the serum of *C. sinensis* infected hamster. The fraction number 11-13 gave the strongest reaction with antibody. The *O. viverrini* antibody also reacted similar as *C. sinensis* antibody did. However, the *C. sinensis* antibody only reacted with the fraction 25 to 29 (Figure 4).



**Figure 3.** SDS-PAGE of *C. sinensis* protein fractions.

M: Protein marker.



**Figure 4.** *C. sinensis* protein fractions reacted with both antibodies against *C. sinensis* and *O. viverrini* by immunoblot.

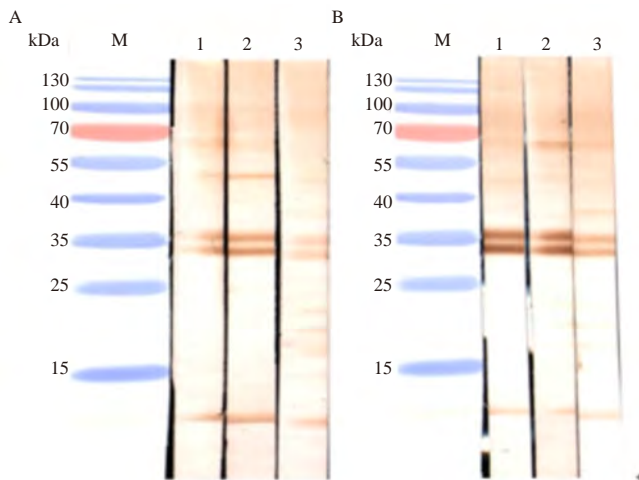
A and B: *C. sinensis* antibody reacted with 5 to 31 of *C. sinensis* protein fractions; C and D: *O. viverrini* antibody reacted with 5 to 31 of *C. sinensis* protein fractions; M: Protein marker.

### 3.5. The characterization of *C. sinensis* protein fraction No. 20

The *C. sinensis* fraction No. 20 was detected in sera of *C. sinensis* and *O. viverrini* infected hamsters. The 10, 34, and 37 kDa were



common protein bands of serum of *C. sinensis* and *O. viverrini* infected hamster. However, the 53 kDa protein band was only found in the serum of *C. sinensis*-infected hamster. Moreover, the 10 kDa protein band reacted with *C. sinensis* antibody more strongly than that with *O. viverrini* antibody (Figure 5).



**Figure 5.** Characterization of *C. sinensis* protein fractionation No. 20.

A: Protein fraction No. 20 reacted with sera of *C. sinensis* infected hamster;

B: Protein fraction No. 20 reacted with sera of *O. viverrini* infected hamster.

#### 4. Discussion

Different fractions of *C. sinensis* worm extract possess different immunogenicity. The present study focused on the characterization of immunogenic fractions of *C. sinensis* crude extraction using partially purified antigen fraction by automatic gel filtration chromatography system. The result showed 2 peaks of protein fraction at high molecular weight (fraction 10 to 14) and low molecular weight (fraction 21 to 26). The antibodies reacted differently with individual protein fractions. Immunoblotting was observed over 20 protein bands while the 34 and 37 kDa were major protein bands of fractions 9 to 21 which reacted with antibodies of *C. sinensis* and *O. viverrini* infected hamsters. Moreover, the *C. sinensis* fraction 25–29 only reacted with *C. sinensis* antibody. The 53 kDa was only found in serum of *C. sinensis* infected hamster reacted with *C. sinensis* fraction 20. In comparison with previous studies, the *C. sinensis* antibody reacted differently with individual fraction. The 15.5, 23.5, 28.5, 37, 45, 61.5 and 66 kDa proteins showed reaction of immunoglobulin E with protein fraction[18]. The different molecular sizes may be due to difference in the type of antibody and in the preparation of fractions. The study was compared to previous studies that the 34, 37, 43 and 50 kDa were major proteins[21,22]. However, in the present study, we did not observe the 17, 26–28, 43, 70 and 100 kDa protein bands[21–23]. The different molecular sizes may be due to the difference in the preparation of protein antigen and the running condition of SDS-PAGE and protein marker. On the other hand, it is impossible to verify possible factors of size discrepancy because there was little information to compare with each other. Especially in fraction No. 20, only the 53 kDa protein reacted with *C. sinensis* antibody. It was also found that the 10 kDa protein bands reacted with both *C. sinensis* and *O. viverrini* antibodies. However, the circulating antibody of *C. sinensis* reacted with *C. sinensis* protein fraction with more reactive bands than *O. viverrini* antibody

reacted with protein antigen. The character of 53 kDa protein is still poorly understood. The result also suggested that the fraction No. 20 may be the antigenic protein fraction for clonorchiasis immunodiagnosis. A limitation of this method is that it uses a lot of fraction volume when compared with ELISA technique.

The ELISA using various different *C. sinensis* protein fraction showed that *C. sinensis* antibody reacted differently with individual fraction. The high molecular weight protein reacted strongly with antibody and with descending antigenicity. The present study demonstrated that the high molecular weight contained more antigenic protein. The *O. viverrini* antibody cross-reacted with the *C. sinensis* protein fractions as *C. sinensis* antibody did. The taxonomy of both trematodes may be very similar. Our method did not find different reactions between the two liver flukes. The sharing antigenic proteins of both liver flukes were demonstrated in previous studies[22,24].

In conclusion, the partial purified crude protein of *C. sinensis* showed 2 peaks at high and low molecular weight protein. The fractionated *C. sinensis* proteins reacted similarly with both clonorchiasis and opisthorchiasis antibodies. The *C. sinensis* protein fraction No. 20 could be useful for immunodiagnosis of clonorchiasis but the character of 53 kDa requires further study.

#### Conflict of interest statement

We declare that we have no conflict of interest.

#### Acknowledgements

This work was partially supported by the Thailand Research Fund under the Thailand Research Fund Senior Scholar (BS); the Higher Education Research Promotion and National Research University Project of Thailand, Office of the Higher Education Commission, through the Health Cluster (SHeP-GMS), Khon Kaen University; the National Institute of Allergy and Infectious Diseases with award number P50AI098639; and the United States Army Medical Research and Materiel Command with contract number W81XWH-12-C-0267. DPN is a postgraduate scholar supported by Khon Kaen University. The content is solely the responsibility of the authors and does not necessarily represent the official views of the United States Army Medical Research and Materiel Command, National Institute of Allergy and Infectious Diseases or the NIH or the funders.

#### Comments

##### Background

*C. sinensis* is a major food-borne trematode in Southeast and East Asia. More than 200 million people are at risk of infection in the endemic areas, around 15–20 million people are infected. For control and elimination in endemic areas, clonorchiasis is mainly diagnosed by immunodiagnosis. However, the disadvantage is cross-reaction with other helminth infection. Better sensitivity and specificity of these immunodiagnostic tests are still required for development of the diagnostic test.

##### Research frontiers

The gel filtration showed 2 peaks at high (fraction No. 10 to

14) and low (fraction No. 21 to 26) molecular weight proteins. Indirect ELISA showed that both antibodies of clonorchiasis and opisthorchiasis reacted strongly with early fractions (6 to 14) and the reaction was gradually reduced at middle and late fractions (15 to 50). Both antibodies showed different individual fraction of *C. sinensis* by immunoblot. The 53 kDa protein which was only found in the clonorchiasis reacted with fraction 20.

### Related reports

The data about the immunoblot of purified *C. sinensis* antigen are in agreement with Li *et al.* (2004) and Choi *et al.* (2003). However, it is not in agreement with Yong *et al.* (1993). The different molecular sizes may be due to the difference in the preparation of protein antigen, the running condition of SDS-PAGE and protein marker. On the other hand, it is impossible to verify possible factors of size discrepancy because there was little information to compare with each other.

### Innovations & breakthroughs

The present study purified *C. sinensis* protein antigen by the gel filtration chromatography. This system can purify small protein in the short time. This study has showed the characterization of antigenic *C. sinensis* protein fraction.

### Applications

The 53 kDa protein and fraction No. 20 may be useful for immunodiagnosis of clonorchiasis.

### Peer review

For immunodiagnosis of clonorchiasis, such as ELISA, better sensitivity and specificity are required. In this study, a low molecular protein fraction, No. 20 (53 kDa), was found to be a unique antigen of *C. sinensis*. The result will be helpful for future immunodiagnosis of clonorchiasis patients in endemic areas.

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