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Clonorchis sinensis: Analysis Characterization of Somatic and Metabolic Antigen (II) Profile of the Worm, Excretory-secretory and Billis Antigen in *C. sinensis* Infected Rabbit

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Abstract: The authors characterized the proteins of the crude antigen obtained from *Clonorchis sinensis* worm and excretory-secretory and billis from rabbits, experimentally infected for 3 months. Protein composition was observed after adding a cysteine proteinase inhibitor E-64 and a serine proteinase inhibitor PMSF, respectively. SDS-PAGE of the crude antigen from *C. sinensis* recovered from the infected rabbits, the crude antigen from the adult worm excretory-secretory, and the crude antigen from billis of the rabbits resolved 26, 27 and 19 profiles between 200-9 kDa, respectively. When E-64 supplemented 29, and 22 bands, respectively. More study should be carried out in the future on the immunological characteristics and the monoclonal antibody of the each antigen.

Key Words: *Clonorchis sinensis,* Antigen, Worm, Excretory-secretory, Billis, E-64, PMSF

INTRODUCTION

Clonorchiasis is widely distributed throughout Southeast Asia. In Korea, the infected are estimated to be about one million at present. Egg detection through stool examination and intradermal reaction test developed in 1950s have been used for diagnosis. But more improved methods are required because egg detection has shortcomings that the estimated worm burden has no relation to the infected amount and the symptom and/or nonsymptom, and intradermal test is suffered from cross reaction problem and lacking in sensitivity and specificity.

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A lot of researches have been conducted on the crude antigen protein from *Clonorchis sinensis.* Lee (1988) reported that the crude antigen of adult *C. sinensis* consisted of about 35 proteins in the range of 11-80 kDa. Kim (1994) reported that SDS-PAGE of the worm excretory fluid resolved 30 protein bands between 11.5-114 kDa. Yong (1994) reported that SDS-PAGE/immunoblot of the worm crude antigen showed 34 and 10 kDa proteins to be specific antigens for monoclonal antibody. Still, the adult *C. sinensis* and the worm excretory-secretory proteins should be characterized more definitely, and the immunoreactions for the each antigen, e.g. serum reaction, should be investigated.

Proteinases are classified into four groups, according to the active site residues of the enzyme; cysteine proteinase, serine proteinase, aspartic proteinase and metallo proteinase. Parasi-

tic proteinase is known to help the parasite invade into the host by hydrolyzing the host tissue protein and to play an important role in the immunoreaction of the host[8]. *C. sinensis* proteinases are also reported to have critical effects on both parasite and host[3], and more precise examination is needed.

In this study, the authors showed the SDS-PAGE profiles of the *C. sinensis* worm, the worm excretory-secretory and the host billis proteins, particularly when a proteinase inhibitor was supplemented, and intended to prepare the groundwork for the molecular biological and immunological studies.

MATERIALS AND METHODS

1. Collection of metacercariae of *C. sinensis*

Metacercariae of *C. sinensis* were collected from *Pseudorasbora parva*, the second intermediate host, obtained at the lower Nakdong River, Pusan, Korea. *P. parva* were ground by mortar and pestle, and treated with artificial juice to collect the metacercariae from.

2. Infection of animals with *C. sinensis*

Rabbits were purchased from Korea Experimental Animal Center. Each rabbit was infected with 500 metacercariae of *C. sinensis* and sacrificed after 90 days for the worm to be collected.

3. Preparation of the antigens of *C. sinensis*, the worm excretory-secretory and the rabbit billis

C. sinensis collected from rabbits 3 months after the infection were washed by water repeatedly at PBS pH 7.4, homogenized in some PBS, ultrasonicated for 5-10 seconds several times, and centrifuged for 30 minutes at 4℃, 10000 rpm. The supernatant was dialyzed in 4℃ distilled water for 24 hours, thoroughly freeze-dried and kept at -70℃.

The worm excretory-secretory was obtained as follows. After washing the collected worms by saline repeatedly, about 100-200 active worms were transferred to 20 ml saline and incubated for 18 hours at 36℃. Collected *C. sinensis* excretory-secretory was centrifuged at 3000g for 30 minutes. The supernatant was freeze-dried, kept at -70℃ and used as excretory-secretory antigen.

Billis were collected from control rabbits and infected ones. After adding PBS pH 7.4, they were centrifuged at 3000g for 30 minutes. The supernatant was freeze-dried, kept at -70℃ and loaded on SDS-PAGE.

4. Supplementation of proteinase inhibitors and SDS-PAGE

A serine proteinase inhibitor PMSF (phenylmethanesulphonyl fluoride) and a cysteine proteinase inhibitor E-64 (c-trans-epoxysuccinyl-leucylamide-(4-guanidino)-butane) were supplemented to the *C. sinensis* crude antigens, respectively. PMSF was adjusted to 1 mM and E-64 to 1 μM. The crude antigens with/without the proteinase inhibitors were applied to SDS-PAGE.

RESULTS

1. SDS-PAGE of the adult *C. sinensis* crude antigen

SDS-PAGE of the adult worm crude antigen showed 26 polypeptide profiles. Molecular weights of each band was 174.0, 125.6, 112.0, 104.6, 85.3, 80.0, 77.0, 74.5, 62.8, 46.3, 43.3, 37.8, 36.5, 35.3, 33.0, 30.0, 25.1, 23.5, 22.0, 19.2, 15.6, 12.7, 12.0, 10.0, 9.4 and 8.5 kDa. Among these, profiles of 15.6 and 12.7 kDa were especially strongly visualized (Fig. 1).

2. SDS-PAGE of the adult *C. sinensis* crude antigen with supplemented E-64

Thirty profiles were observed. 15.6 and 12.7 kDa profiles were the strongest, and 43.0, 37.0 and 26.0 were strong (Fig. 1).

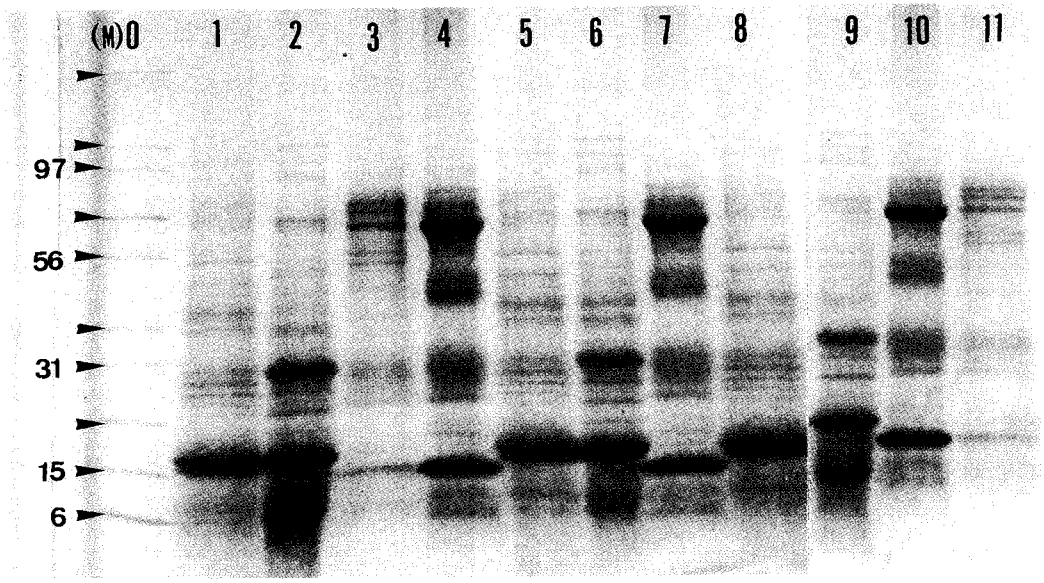

Fig. 1. Electrophoretic pattern of component proteins of the crude antigen obtained from *Clonorchis sinenesis* worm and excretory-secretory and billis from rabbits, experimentally infected for three months. Protein composition was observed after adding a cysteine protainase inhibitor E-64 and a serine proteinase inhibitor PMSF, respectively. Lane (M)0, this lane was contaminated with molecular weight marker. Lanes 1-4, inhibitors were not supplemented before homogenizing: lane 1, crude antigen of *C. sinenesis*. lane 2, crude antigen of excretory-secretory. lane 3, crude antigen of control billis. lane 4, crude antigen of infected billis. Lane 5-7, E-64 were treated before homogenizing; lane 5, worm of *C. sinenesis*. lane 6, excretory-secretoty of *C. sinensis*. lane 7, billis from infected rabbits. Lane 8-11, PMSF were treated before homogenizing; lane 8, worm of *C. sinenesis*. lane 9, excretory-secretory of *C. sinenesis*. lane 10, billis from infected rabbits. lane 11, control billis from normal rabbits.

3. SDS-PAGE of the adult *C. sinensis* crude antigen with supplemented PMSF

Twenty-eight bands were observed. Strongest were 25.1, 15.6 and 12.3 kDa (Fig. 1).

4. SDS-PAGE of the *C. sinensis* excretory-secretory crude antigen

SDS-PAGE of the worm excretory-secretory crude antigen resolved 26 profiles. Molecular weights of each band was 193.0, 174.0, 137.2, 128.2, 112.0, 105.0, 98.0, 82.5, 74.5, 67.2, 63.0, 61.0, 55.0, 51.2, 50.0, 45.0, 39.0, 34.1, 25.1, 21.2, 19.8, 18.5, 15.6, 14.0, 10.3 and 9.0 kDa. Among these, characteristic main proteins were of 128.2, 74.5, 34.1, 25.1, 15.6 and 9.0 kDa (Fig. 1).

5. SDS-PAGE of the *C. sinensis* excretory-secretory crude antigen with supplemented E-64

About 30 profiles were observed. 25.1, 19.0, 15.6 and 10.0 kDa bands were the strongest (Fig. 1).

6. SDS-PAGE of the *C. sinensis* excretory-secretory crude antigen with supplemented PMSF

Twenty-nine profiles were observed. Strongest were 25.1, 19.8, 15.6, 12.3 and 10.4 kDa bands (Fig. 1).

7. SDS-PAGE of the control rabbit billis protein

SDS-PAGE of billis from the control rabbit showed 22 profiles. Molecular weight of each band was 105.0, 88.4, 82.4, 77.0, 69.5, 57.0,

53.0, 46.2, 45.0, 37.0, 35.2, 32.0, 28.0, 26.0, 25.1, 23.4, 20.0, 15.1, 14.0, 12.0, 10.3 and 9.4 kDa. Generally, the protein profile of the billis was weak. Strongest were 82.4, 77.0 and 69.5 kDa bands. E-64 supplemented billis was resolved into 17 profiles, and 77.0, 69.5 and 65.7 were the strongest. Billis treated with PMSF showed 23 bands and 77.0, 73.0 and 69.5 kDa were the strongest (Fig. 1).

8. SDS-PAGE of the infected rabbit billis protein

The infected rabbit billis protein was resolved into 19 profiles on SDS-PAGE. Molecular weight of each profiles was 157.2, 105.0, 91.3, 85.3, 69.5, 61.0, 55.0, 53.0, 45.0, 41.8, 25.1, 20.0, 17.0, 16.1, 13.0, 12.0, 10.3, 10.0 and 9.0 kDa. Strongest were 69.5, 45.0, 41.8, 25.1, 16.1 and 13.0 kDa. E-64 supplemented billis showed 18 bands, and the strongest were 74.5, 41.8, 25.2, 20.5, 15.6 and 12.0 kDa. Billis treated with PMSF showed 22 profiles, and the strongest were 69.5, 41.8, 25.1, 18.0, 15.6 and 12.3 kDa (Fig. 1).

DISCUSSION

Lee (1988) reported that the crude antigen of adult *C. sinensis* consisted of about 35 proteins in the range of 11-80 kDa and Yong (1991) reported that 34 and 10 kDa proteins were specific antigens for monoclonal antibody. It was reported that the egg protein protein consisted of 33-35 proteins among which 38.5 kDa protein was the main antigen of egg antigen.

Proteinases are classified into four groups, according to the active site residues of the enzyme; cysteine proteinase, serine proteinase, aspartic proteinase and metallo proteinase. Some authors reported that the cysteine proteinase isolated from *Schistosoma mansoni* was a proteinase that hydrolyzed hemoglobin, the *Schistosoma* nutrient, and could be an antigen available for diagnosis[1,2]. For *Paragonimus westermani* and *C. sinensis*, cysteine proteinases were purified following the growth stage and observed comparatively[11]. For *sparganum*, cysteine proteinases having lowest activity at pH 5.7 and 7.0, respectively, were isolated and purified[2,11]. and serine proteinases of 36, 104 and 198 kDa were reported to induce the strong antibody reaction in the infected individual[5]. *C. sinensis* worm crude antigen was reported to show comparingly strong profiles more than 20 in the range of 200-14 kDa when a cysteine proteinase inhibitor E-64 was supplemented, and 43, 34 and 28-25 kDa antigens were shown to be specific antigens by immunoblotting with the infected serum and ELISA[3]. Park (1995) observed that *C. sinensis* cysteine proteinases are concerned in cytotoxicity and the specific proteinase was of 24 kDa.

In present study, characterization of the *C. sinensis* crude antigen protein showed 28 profiles of which 15.6 and 12.7 kDa bands were the strongest (Fig. 1). This result is similar to those obtained by Lee (1988), Yong (1991) and Kim (1994). Comparing a cysteine proteinase inhibitor E-64 supplementation and a serine proteinase inhibitor PMSF supplementation, large profile was best obtained with E-64. This is a similar result to that of Hong (1997). Therefore, it is believed that using the cysteine proteinases is advantageous for immunology and molecular biology study in the future.

C. sinensis excretory-secretory were reported to have more than 30 protein profiles, the main being 11.5, 12.5, 17.5, 16, 16.3 and 15.1 kDa.[4]. In present study, 28 profiles were observed, 25.1, 20.0, 15.6 and 12.7 kDa being the strongest. This is a similar result to those by Choi and Kim. Comparing E-64 supplementation and PMSF supplementation, largest profile was obtained with E-64, having 32 bands. Strong profiles were 25.1, 19.0, 15.6 and 10.0 kDa. 25.1, 19.8, 15.6 and 10.4 kDa were strong in both PMSF and E-64 supplementation and seemed to be principal (Fig. 1).

It was reported that *C. sinensis* infection had

histological and immunological effects on the epithelium of bile duct tissue[7]. Lee (1994) reported that the infection leaded to bile duct dilation, bile duct wall hypertrophy, adenoma hyperplasia and goblet cell metaplasia in the epithelium of bile duct and a few serotonin-excreting cell in the epithelium presumed to be mast cell. In this study, 17-22 protein profiles were observed from billis from control and infected rabbits. Strong and principal profiles from the infected ones were 70.0, 42.0 and 25.1 kDa. Comparing E-64 supplementation and PMSF supplementation, more profiles were observed with PMSF. 70.0, 42.0, 25.1 and 15.6 kDa were the strongest, estimated as main proteins (Fig. 1).

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=국문초록=

간흡충 : 충체 및 대사성 항원의 특성분석 (II) 간흡충 감염 가토에서 간흡충, 분비배설액 및 담즙 항원의 분획 양상

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가토에게 간흡충을 감염시키고 3개월 후 거살한 다음 간흡충의 충체, 간흡충의 분비배설액 그리고 간토의 담즙을 조항원으로 제조한 후 이에 대한 항원단백질의 구성물질을 분석하였다. 그리고 cysteine계 면역억제제인 E-64와 serine계 면역억제제인 PMSF를 첨가하였을 때 단백질 구성물질의 발현 양상을 관찰하였다. 실험적으로 가토에서 얻은 간흡충 성충의 조항원은 200-9 kDa의 범위에서 26개의 분획을 관찰하였으며, 간흡충 성충 조항원에 cysteine계 단백질분해효소 억제제인 E-64를 첨거하였을 때 잘 보존하여 200-9 kDa의 범위에서 29개의 분획을 관찰하였다. 간흡충 성충의 분비배설액 조항원은 200-9 kDa 범위에서 27개의 분획이 관찰되었으며, cysteine계 단백질분해효소 억제제인 E-64를 첨거하였을 때 잘 보존하여 200-9 kDa의 범위에서 29개의 분획이 관찰되었다. 그리고 간흡충을 감염시킨 가토의 담즙 조항원은 200-9 kDa의 범위에서 19개의 분획이 관찰되었으며, serine계 단백질분해효소인 PMSF를 첨거하였을 때 200-10 kDa의 범위에서 22개의 분획이 관찰되었으나, cysteine계 단백질분해효소인 E-64에서도 비슷한 양상을 보였다. 대조군인 건강 가토의 담즙 조항원은 200-10 kDa의 범위에서 22개의 분획이 관찰되었으며, serine계 단백질분해효소 억제제인 PMSF를 첨가하였을 때 잘 보존하여 200-12 kDa의 범위에서 23개의 분획이 관찰되었다. 앞으로 각 항원에 대한 면역학적 특징 및 단세포군 항체에 대한 구체적인 규명이 계속되어야 겠다.

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