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Tissue origin of soluble component proteins in saline extract of adult *Paragonimus westermani**

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Abstract: Tissue origin of individual component proteins in crude extract of adult *Paragonimus westermani* was investigated. Major soluble component proteins were separated by disc-PAGE in 8% slab gel. By predefined R_f values, strips of gel containing each band protein was cut out. Each band protein was eluted by electrophoresis. Monospecific antibodies were prepared by immunizing rabbits with each band protein. When peroxidase-antiperoxidase (PAP) staining was done, antiserum to Band 1 reacted to content of eggs both in the worm and in the infected lung tissue. Antiserum to Band 2 reacted to parenchymal tissue of the worm. Antiserum to Band 4 showed the positive reaction at intestinal content while that to Band 5 reacted to the intestinal epithelial border. Antiserum to combined proteins of Bands 6/7 and that to Band 8 reacted to parenchymal tissue of the worm respectively. From the results, the origin of individual proteins in crude extract of adult *P. westermani* could be differentiated.

Key words: *Paragonimus westermani*, antigenic component protein, disc-PAGE, monospecific antibody, PAP staining

INTRODUCTION

Nowadays, the diagnosis of human paragonimiasis depends largely on the specific antibody test such as ELISA (Cho *et al.*, 1981; Yokogawa *et al.*, 1983; Choi *et al.*, 1986; Shim *et al.*, 1991). One of the critical aspects of the antibody test is quality of a diagnostic antigen as well as a sensitivity of the serologic technique itself. In this connection, knowledges on the component proteins in the crude extract are prerequisite to improve the quality.

Considerable informations have been accumulated on the component proteins of *P. westermani.* For example, the Band 1 protein, as seen in the crude extract by non-denaturing disc-PAGE (Huer *et al.*, 1985), is derived from eggs (Kim *et al.*, 1986; Imai and Nawa, 1988; Kang *et al.*, 1991). It is now known to have molecular mass of 440 kDa (Imai and Nawa, 1988; Kong *et al.*, 1991). Its subunits are of 92, 46 and 23 kDa when observed by SDS-PAGE/immunoblot using a monoclonal antibody (Kang *et al.*, 1991), which has already been known as strong antigenic components in human paragonimiasis (Kim *et al.*, 1988). Sugiyama *et al.* (1987 & 1988) reported that 28/26 kDa subunit was derived from intestinal epithelium and 35, 17, 15.5 and 12.5 kDa subunits were produced at parenchymal tissues of the worm by immunohistochemistry utilizing monoclonal antibodies. Kang *et al.* (1991)

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described that Band 4 protein of 17 kDa was localized immunohistochemically at intestine of the worm using a monoclonal antibody. By using gel filtration, Imai and Nawa (1988) recognized a 13 kDa protein which might originated from excretory-secretory product of *Paragonimus*. Its origin in the worm is not determined yet.

To find out the potent antigenic proteins of *P. westermani*, many studies have been made by using different techniques such as column/affinity chromatography, SDS-PAGE and SDS-PAGE/immunoblot using homologous infected human or animal sera (Lee and Chang, 1986; Choi *et al.*, 1986; Imai and Nawa, 1988; Kim *et al.*, 1988; Slemenda *et al.*, 1988; Joo *et al.*, 1989a). The antigenic proteins, thus recognized, were either non-denatured proteins or SDS-subunits according to employed biochemical techniques. Therefore, subunit relations in antigenic proteins could not yet been fully understood. The origins of individual antigenic proteins or their SDS-subunits have been remained largely unknown except the egg and a part of intestinal proteins.

In the previous study for an antigenic proteins in adult *P. westermani*, we had estimated molecular masses of 8 component proteins which are seen in the non-denaturing disc-PAGE of the crude saline extract (Kong *et al.*, 1991). The molecular masses were 440 kDa in Band 1 protein, 386 kDa in Band 2, 17.4 kDa in Band 3, 17 kDa in Band 4, 14.3 kDa in Band 5, 46 kDa in Band 6, 38 kDa in Band 7 and 23 kDa in Band 8. In this study, we verify the origin of the individual band proteins of disc-PAGE by immunohistochemistry.

MATERIALS AND METHODS

1. Paraffin sections of *Paragonimus westermani* and cat lung cyst

A total of 10 adult *P. westermani* and a cat lung cyst were secured from a cat on 13 weeks after an experimental infection. Worms were washed 3 times in physiologic saline, fixed in Bouin's solution, dehydrated through graded alcohol and embedded in paraffin. A lung cyst of the experimental cat was fixed in 10% neutral formalin, dehydrated and embedded. The paraffin blocks were cut in 5 μm thickness and mounted on slide glasses.

2. Crude saline extract of *P. westermani*

The extract was prepared as described by Kong *et al.* (1991) using 13-week old adult worms which had been harvested in an experimental dog. Protease inhibitor, 0.006% (W/V) phenylmethylsulfonyl fluoride, was added. All procedures from worm collection to protein purification were done at 4°C, unless otherwise specified. The protein content was 3.98 mg/ml when measured by the method of Lowry *et al.* (1951).

3. Preparation of band proteins in the crude extract

1) **Disc-PAGE:** The methods of Hames (1981) were adopted. A vertical slab gel electrophoresis system of 16×17×0.15 cm size was used for the protein separation in nondenaturing condition. Stacking gel of 2.5% and resolving gel of 8% were used. In a 9.6×2.2×0.15 cm sized well, 200 μl of the crude extract mixed with the same amount of sample buffer, was electrophoresed. Constant current of 15 mA in stacking gel and 30 mA in resolving gel was supplied. The separated proteins were stained with Coomassie Blue for 3 times. As described by Kong *et al.* (1991) the bands were numbered 1~8 in order of appearance from cathodal side. The mean R_f value of each band was calculated. In the following electrophoreses, unstained gel was cut according to predefined R_f value. The disc-PAGE was repeated 80 times at the same condition. Strips of gel containing a band protein were pooled and stored in 1.5 M Tris-glycine buffer (pH 8.3) containing 0.001% (W/V) merthiolate.

After the electrophoretic elution, each band protein was revisualized by Coomassie Blue staining in 8% disc-PAGE.

2) **Electrophoretic elution:** To isolate the separated proteins from the gels, the method of

Waterborg and Matthews (1984) was applied. Chopped gels of a protein band were packed in a dialysis sac in 1.5 M Tris-glycine buffer (pH 8.3). They were electrophoresed at 100V for 5 hours. During the last 3 minutes, the electric current was reversed. After removing the gels from sac, protein containing buffer was dialyzed against PBS (50 mM, pH 7.4) for 72 hours and lyophilized. Rehydrated proteins were centrifuged at 10,000 *g* for an hour. The resulting supernatant was regarded as "Band protein". Protein content was measured as described by Lowry *et al.* (1951).

4. SDS-PAGE

To observe the subunit compositions of each band protein, the method of reducing SDS-PAGE described by Laemmli (1970) was adopted. Stacking gel of 3% and 10~15% gradient separating gel were used. Constant current of 30 mA was supplied. The separated proteins were stained with 0.125% Coomassie Blue.

5. Preparation of monospecific antibodies in rabbits

In order to obtain monospecific antibodies against each band protein, rabbits of 3.0~3.4 Kg were immunized. Two rabbits were injected intradermally at 9~10 places with 50 μg of each protein mixed with Freund's complete adjuvant, respectively. Fifty μg of each band protein mixed with Freund's incomplete adjuvant were boostered twice in 2-week intervals. Finally, 5 μg of each protein was injected through ear vein. Four days later, the rabbits were killed to bleed. The specific IgG antibody levels in serum were measured by ELISA. Rabbit antiserum to the crude extract was also prepared in the same way.

6. Enzyme-linked immunosorbent assay (ELISA)

To observe the quality of the antisera to band proteins, antibody test was done. ELISA method described by Cho *et al.* (1981) was modified. Each band protein and the crude extract was diluted in concentration of 1 μg/ml in carbonate buffer (pH 9.6) and coated microtiter plates overnight at 4°C. Anti-*Paragonimus* antisera diluted in PBS containing 0.05% Tween 20 (PBS/T, pH 7.4) were reacted. Peroxidase-conjugated anti-rabbit IgG (Cappel, U.S.A.) was reacted at 1 : 1,000 dilution in PBS/T for 2 hours at 36°C. The reaction was colored by OPD solution. The absorbance was read at 492 nm using Gilford spectrophotometer.

7. Immunoperoxidase-antiperoxidase staining

Immunoperoxidase staining was carried out as described in handbook of immunochemistry laboratory of Dako Co. (Bourne, 1983). Deparaffinized sections of the worm and cat lung cyst were equilibrated with PBS (0.1 M, pH 7.2). Endogenous peroxidase activity was inhibited by treating the sections in 0.05% H_2O_2. After washing, 1 : 20 diluted normal swine serum in PBS (Dako, U.S.A.) was incubated for 30 minutes. Sections were reacted with differently diluted anti-*Paragonimus* antisera overnight each. Swine anti-rabbit IgG (Dako, U.S.A.) diluted at 1 : 200 in PBS was reacted. Then, 1 : 200 diluted peroxidase-antiperoxidase complex (Dako, U.S.A.) was reacted for an hour. The reaction was colored by diaminobenzidine chromogen. The sections were counterstained with Harris hematoxylin. All procedures were done at room temperature.

RESULTS

1. Recovery rate and purity of the proteins from preparative disc-PAGE and electrophoretic elution

The recovery rate was calculated by protein content (Table 1). When a total of 45 mg of protein in the crude extract was subjected to disc-PAGE and electrophoretic elution, the recovered protein was 31.4 mg (69.7%).

Lanes 1~7 of Fig. 1 showed disc-PAGE findings of the separated band proteins at 8% gel. Bands 1, 2 and 3 were purified clearly without visible contamination. Band 6 and Band 7 proteins were not separately collected because of their proximity in the gel. When electrophoretic elution was done, these 2 bands were not

Table 1. Recovery of each Band protein when 45 mg of protein in the crude extract of adult *P. westermani* was purified by disc-PAGE and electrophoretic elution

Protein Band	Recovered amount (mg)*	% to applied crude extract	% to total recovered protein
1	4.2	9.3	13.4
2	5.6	12.4	17.8
3	4.3	9.6	13.7
4	3.7	8.1	11.8
5	3.9	8.7	12.4
6/7	5.4	12.9	17.2
8	4.3	9.6	13.7

* Total recovery rate: 69.7% (31.4 mg of protein)

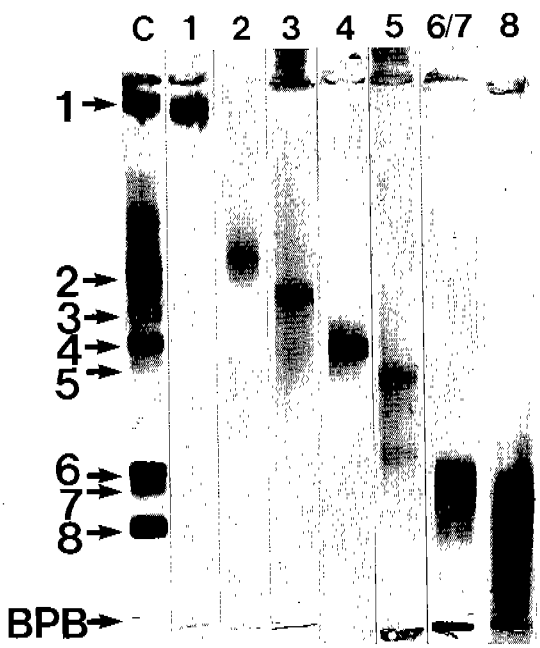


Fig. 1. Purity of the band proteins of adult *P. westermani* after the disc-PAGE and electrophoretic elution. The proteins were visualized in 8% disc-PAGE. Coomassie stained. Numericals stand for an each band protein.
Mr: Molecular mass in kDa,
C: Crude extract.

contaminated with nearby bands. Each of Bands 4, 5 and 8 was contaminated with neighbouring bands. But contaminations were not significant. Band 8 was contaminated with Band 6/7 as much as 34% when observed by densitometry (Lane 7).

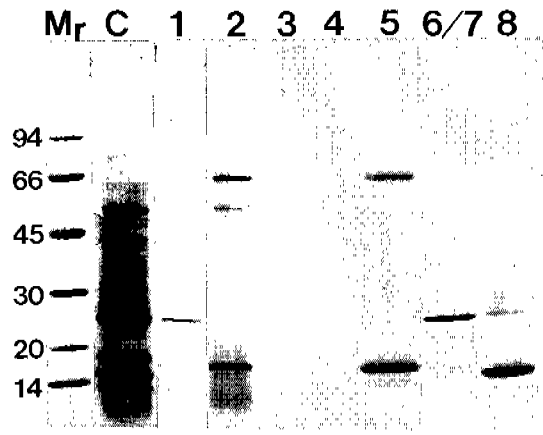


Fig. 2. SDS-PAGE findings of the band protein. A gradient separating gel of 10~15% was used. Markings and numericals are the same as described in Fig. 1.

2. Subunits composition of the band proteins

As illustrated in Fig. 2, the crude extract was resolved into at least 27 bands. Of them, 84, 64, 51, 46, 45, 35, 30/28, 23, 17, 15, 12.5, 10 and 8 kDa were the major subunits. Band 1 protein was composed of 46 and 23 kDa subunits. Those of band 2 were 62, 52, 26, 17, 12.5 and 8 kDa. Bands 3, 4, 5 revealed a common subunits of 17 kDa. In Band 5, 62 and 10 kDa were found in addition to 17 kDa. Bands 6/7 were subdivided into 23, 17 and 16.5 kDa. The subunits of Band 8 were 23 and 15 kDa.

3. Specific IgG antibody levels in immunized rabbits antisera

The rabbit sera collected before immunization showed negligible absorbances both to the band proteins and to the crude extract (mean±S.D.: 0.07±0.043).

After immunization, antibody levels (IgG) in rabbit antisera were elevated. Even when 1 : 6,400 diluted serum was tested by ELISA, absorbance were near to or above 1.0 (Table 2). Rabbit antiserum to the crude extract exhibited the highest absorbance to the extract while it showed different absorbances (from 0.29 to 1.00) to each band protein. Antisera to proteins of Bands 1~8 showed the highest absorbance against the homologous band proteins.

Table 2. Specific IgG antibody levels (abs. by ELISA) in differently diluted rabbit antisera against respective antigens

Rabbit antisera against	Absorbance to antigen of							
	Crude extract	Band						
		1	2	3	4	5	6/7	8
Crude*	1.58	0.74	0.93	1.00	0.67	0.57	0.48	0.29
Band 1*	0.41	0.95	0.11	0.32	0.28	0.36	0.39	0.20
Band 2*	0.48	0.27	0.83	0.39	0.38	0.66	0.20	0.31
Band 3**	0.79	0.56	1.90	1.21	0.66	0.48	0.35	0.15
Band 4***	0.38	0.09	0.81	0.29	1.76	0.85	0.19	0.68
Band 5**	0.32	0.37	0.32	0.60	0.28	1.45	0.39	0.34
Band 6/7*	0.31	0.39	0.53	0.51	0.32	0.55	1.17	0.56
Band 8**	0.31	0.30	0.31	0.44	0.30	0.28	0.56	1.98

* Rabbit antisera diluted at 1:1,600
** Rabbit antisera diluted at 1:3,200
*** Rabbit antiserum diluted at 1:6,400

The only exception was the antisera to Band 3 which showed high absorbance also to the Band 2 protein. We referred each antiserum, except for that to Band 3 protein, as a monospecific antiserum.

4. Reactions with monospecific rabbit antisera by immunoperoxidase-anti-peroxidase staining

Using rabbit sera collected before immunization and antiserum against the crude extract and each band protein, PAP staining was carried out on the worm sections or on cat lung cysts. Each antiserum was tested on the tissue sections which had all organ/tissues. When a section had not certain tissues, it tested repeatedly using 2~3 different kinds of sections. Antiserum to Band 3 did not reacted due to its cross reaction.

(1) **Negative controls:** When the worm sections were reacted with PBS and normal rabbit serum as primary antibodies, no organs showed the positive reactions (Figs. 3 & 4).

(2) **Positive control:** When worm sections were reacted with antiserum against the crude extract, positive reactions were observed at intestinal epithelial border, intestinal content, eggs and parenchymal tissue(Fig. 5). Reactions to vitellarian follicles and ovary were equivocal. Tegument, testes, basal layer of intestine and suckers were not stained positively with PAP.

(3) **Reactions with monospecific antibodies:** As exhibited in Fig. 6, the rabbit antiserum to Band 1 protein reacted to the contents within egg shell. The eggs which were scattered in a cat lung tissue also showed the positive reaction (Fig. 7).

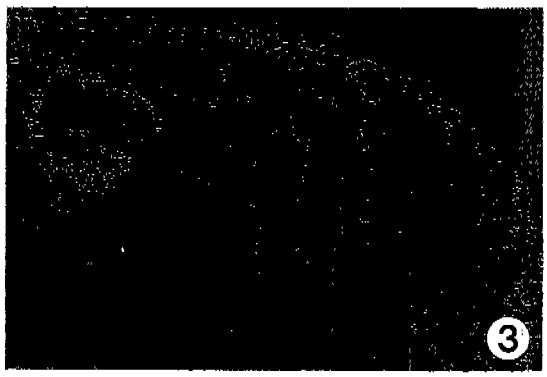
When the antiserum to Band 2 protein was reacted, positive reaction was observed in the parenchymal tissue(Fig. 8). No other tissues reacted positively.

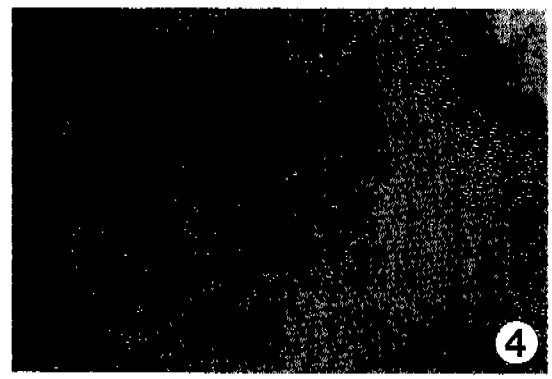
Fig. 9 showed the positive reaction at intestinal content of the worm, when antiserum to Band 4 protein was reacted. Intestinal epithelia did not reacted to this antiserum. Unlike this, antiserum to Band 5 protein reacted to the luminal side of intestinal epithelium. Basal part of epithelium was not stained with chromogen (Fig. 10).

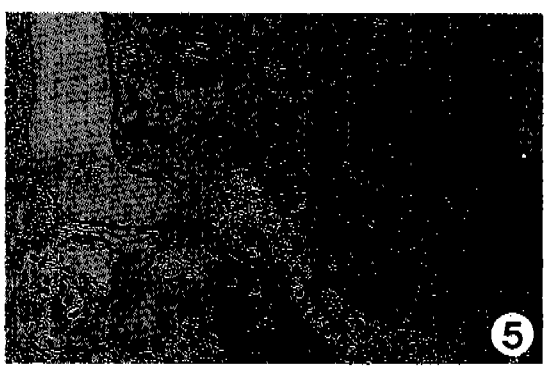
As presented in Fig. 11, rabbit antiserum to Bands 6/7 proteins showed the diffuse positive reaction to parenchymal tissue of the worm. Antiserum to Band 8 protein also reacted to the parenchymal tissue(Fig. 12).

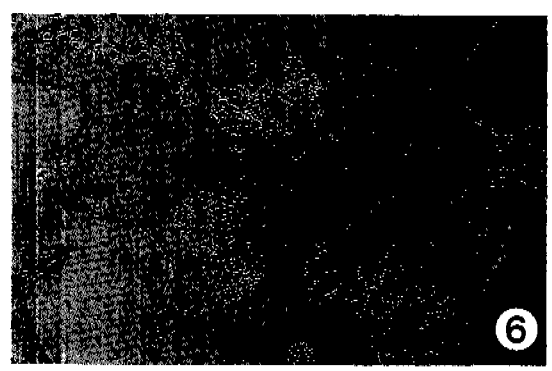
DISCUSSION

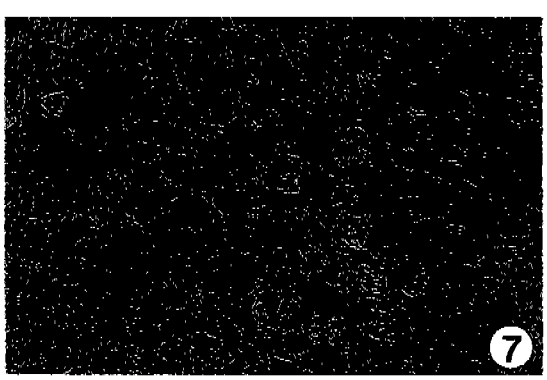
This study showed clearly that major antigenic proteins of adult *P. westermani* originated from the eggs, intestinal content, intestinal epithelial

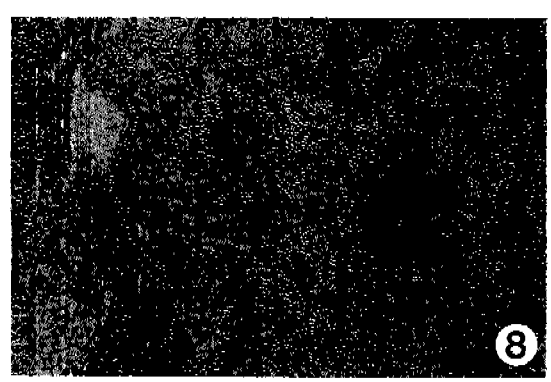












Figs. 3 & 4. Negative control I & II (×100). The worm sections were treated with PBS (I) and rabbit serum collected before immunization (II) as primary antibodies. No structure showed the positive reactions.

Fig. 5. Positive control(×100). Antiserum against the crude extract was reacted at intestinal epithelial border, intestinal content, eggs and parenchymal tissue. Cvary and vitellarian follicles showed equivocal reaction. Tegument, testes, basal layer of intestine and suckers were not stained positively with PAP.

Fig. 6. The rabbit antiserum to Band 1 reacted to the cells within egg shells in uterus (×100).

Fig. 7. Another PAP staining of antiserum to Band 1 (×100). The eggs which were scattered in cat lung tissue showed the stronger positive reaction.

Fig. 8. When the antiserum to Band 2 was reacted, positive reaction was observed at the parenchymal tissue of the worm (×100).

Fig. 9. Intestinal content was stained positively with antiserum to Band 4(×100).
Fig. 10. Antiserum to Band 5 reacted to the intestinal epithelial border. Basal part of epithelium was not stained with chromogen (×100).
Fig. 11. Rabbit antiserum to Bands 6/7 showed the diffuse positive reaction to parenchymal tissue of the worm (×200).
Fig. 12. Parenchymal tissue of the worm was stained with antiserum to Band 8 (×100).

border and parenchymal tissue. The tegument, tegumental cells, testes and suckers were not found to be sources of the antigenic materials.

The present results agreed with those reported by Lee *et al.* (1989) in which major antigenic sources of adult *P. westermani* were evaluated by indirect immunoperoxidase staining using infected cat sera. They reported that main antigens were excretions from the intestine and eggs of the worm.

Band 1 protein(440 kDa) was reacted to eggs as described previously(Kim *et al.*, 1986; Imai and Nawa, 1988; Kang *et al.*, 1991). Its subunits were reported to be composed of 92, 46 and 23 kDa when observed by SDS-PAGE/immunoblot using a monoclonal antibody or 23 kDa by reducing SDS-PAGE(Kang *et al.*, 1991). In this study, subunits of the Band 1 protein were 46 and 23 kDa in reducing SDS-PAGE. Kim *et al.*(1986) reported that the soluble egg protein was less antigenic than the crude extract in diagnosing early cat paragonimiasis. On the point, Kim *et al.* (1986) assumed that the weaker antigenicity of the eggs might be due to immaturity of cells while schistosome eggs had fully matured miracidia within the egg shell. Unlike this speculation, egg antigen was looked just to be weaker, because it reacted only with monospecific antibody out of polyclonal antibodies in the infected serum. Imai and Nawa (1988) and Lee *et al.* (1989) showed that the egg protein was one of major antigens in paragonimiasis. The present results also agreed that the egg protein seemed to be a relatively strong antigen out of component proteins (Table 3).

In schistosomiasis, egg proteins has long been known as a major antigen source. Several aspects of egg proteins such as molecular mass, subunits compositions, physicochemical characteristics and protease activities have been well studied (Carter and Harrison, 1983; Damonneville *et al.*, 1984; Mckerrow and Doenhoff, 1988; Deelder *et al.*, 1989). Because schistosomiasis and paragonimiasis share a common pathologic character of egg granuloma, *Paragonimus* egg protein deserves further studies especially on its biological activities.

When PAP staining was done, Band 4 and 5 proteins were localized at the intestinal content and intestinal epithelial border of the worm respectively. Molecular masses were reported as 17 kDa (Band 4) and 14.3 kDa (Band 5) (Kong *et al.*, 1991). In this study, main subunits of Band 4 and Band 5 proteins were all 17 kDa. In reducing SDS-PAGE of Band 5 protein, however, additional subunits of 62 and 10 kDa were found. We thought these 2 subunits may be either contaminants of neighbouring band or aggregate or degradation product of the subunit.

Using a monoclonal antibody, Kang *et al.* (1991) showed that the Band 4 (monomer of 17 kDa) was produced at the intestine of the worm. Unlike their report, Sugiyama *et al.* (1987) described that antigenic protein of 28/26 kDa reacted to the intestine of the worm. Of these 2 different reports, the present study agreed with that of Kang *et al.* (1991). The 28/26 kDa subunits of Sugiyama *et al.* (1987) were hardly identified even in SDS-PAGE of the crude saline extract. In this connection, Kim *et al.* (1988) and Cho *et al.* (1989) reported that patient sera of paragonimiasis reacted strongly to 32/30 kDa bands in SDS-PAGE/immunoblot even though the bands were not stained in SDS-PAGE of the crude extract. The 28/26 kDa bands of Sugiyama *et al.* (1987) and the 32/30 kDa bands of Kim *et al.* (1988) may be identical. The molecular masses were differently calculated by using different molecular mass markers.

In this study, proteins of Bands 2 (386 kDa), 6/7 (46/38 kDa) and 8 (23 kDa) were localized in parenchymal tissue of the worm. As shown in lane 2 of Fig. 2, the Band 2 protein was composed of 62, 52, 26, 17, 12.5 and 8 kDa subunits. According to Sugiyama *et al.* (1988), 35, 17, 15.5 and 12.5 kDa subunits were antigens originated from parenchymal tissue of adult *P. westermani* when observed by SDS-PAGE/immunoblot using a monoclonal antibody. We think the 35/17/15.5/12.5 kDa subunits of Sugiyama *et al.* (1987) corresponded partly to the subunits of the Band 2 protein in this study.

The antiserum to the proteins of Band 6/7 reacted no other tissues of the worm than the parenchyme. Therefore, the origins of these 2 band proteins should be all the parenchymal tissue. Another protein of Band 8 also localized at the parenchyme. Unless Bands 6/7/8 are degradation products of Band 2 protein, the parenchymal tissue of adult *P. westermani* is a source of several component proteins.

In this study we have concerned only about the soluble component proteins in crude extract of adult *P. westermani*. Early stage infection with invading stages of the juvenile *Paragonimus* should also induce immune responses to the host. The early immune responses seem to depend on the nature of the proteins of the juvenile worms. In this respect, the protein composition in crude extract of juvenile *P. westermani* showed differences from that of adult (Huer *et al.*, 1985; Joo *et al.*, 1989b). Recently Kwon *et al.* (1991) reported that the tegument and intestine were origins of antigen in 4-week old worms by using an immunogold labelling. Hitherto, the tegument was not recognized as the source of antigen in adult *Paragonimus*. Further studies are needed to elucidate the immune reactions in early paragonimiasis.

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=국문초록=

폐흡충 성충 수용성 단백질의 성분별 생성위치

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폐흡충의 생리식염수 추출액 내에 있는 여러 가지 성분 단백질의 생화학적 성상, 면역학적 특징 그리고 충체 내에서의 생성위치 등이 단세포군 항체나 전기영동을 이용한 연구에서 일부 밝혀졌다. 이 실험은 폐흡충 성충의 생리식염수 추출액 내에 있는 각 성분단백질이 폐흡충의 어느 부위에서 유래한 것인지를 알기 위하여 실시하였다. 먼저 생리식염수 추출액을 8% disc-PAGE로 전기영동하여 성분단백질을 분리하고 각 단백질 대(帶)를 포함하는 젤을 잘라 내었다. 이어 전기영동으로 젤에서 성분단백질을 용출(溶出)하였다. 각각의 단백질을 토끼에 면역시켜 성분 단백질별 항폐흡충 면역혈청을 만들고 이 항혈청으로 폐흡충 및 감염 고양이 폐의 절편에 면역효소염색법을 시행하였다. 그 결과 성충추출액으로 면역한 항폐흡충 면역혈청은 폐흡충 성충의 충란, 장관상피세포, 장관 내용물에 강한 반응을 보였고 실질조직에도 염색이 되었다. 그러나 표피, 표피하세포, 고환, 흡판등은 반응이 없었다. 1번 Band 단백질의 항혈청은 충란내 세포에 염색이 되었고 충체에서 배출되어 폐실질 조직에 들어있는 충란에 더욱 강하게 반응하였다. 2번 Band 단백질의 항혈청은 폐흡충의 실질조직에 반응하였다. 3번 Band 단백질의 항혈청은 2번 Band 단백질과 교차반응을 일으켜 염색 반응을 관찰하지 않았다. 4번 Band 단백질의 항혈청은 폐흡충의 장관 내용물과 강한 반응을 보였고 5번 Band 단백질의 항혈청은 장관상피세포에 반응하였다. 6/7번 Band 단백질과 8번 Band 단백질의 항혈청은 각각 실질조직에 반응하였다.

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