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Electrophoretic Patterns of Proteins from *Paragonimus westermani* in Early Developmental Stages

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INTRODUCTION

For immunological diagnosis of human paragonimiasis various serological techniques have been developed. In evaluating the applicability of such various serological techniques, however, the nature of antigenic preparations should also be considered not only because the crude antigens consisted of many different proteins but also because the specificity and sensitivity of the test could be affected by the variously prepared antigens.

In order to increase the sensitivity and specificity of serodiagnosis of active cases of paragonimiasis, and to minimize the cross reactivity with other parasitic infections, efforts have been made to purify the antigens of *Paragonimus westermani* (Sawada *et al.*, 1964; Imai, 1979; Kim *et al.*, 1983). From these studies it was found that some fractionated antigens by column chromatography through Sephadex G-200 were more sensitive than the crude saline extracts although the specificity was not improved (Kim *et al.*, 1983).

The reports on the purification of antigenic proteins raised the following questions; how many kinds of proteins are present in the crude extracts of *P. westermani;* are the proteins changing through development; among these proteins how many are actually immunogenic in human infection; by what proteins the non-specific cross reactions are elicited; what are the biochemical properties of major immunogens of *P. westermani etc.*

In schistosomiasis model, many studies have revealed that tegumental proteins of *Schistosoma mansoni* played a significant role as stage-specific antigens of which molecular weights were determined (Hayunga *et al.*, 1979; Cordeiro and Gazzinelli, 1979; Simpson *et al.*, 1981). In conjunction with these studies it was also found that not only the protein compositions of *S. mansoni* differed from each developmental stage, but also the antigenic profiles of tegumentum changed during the maturation to adult worm (Ruppel and Cioli, 1977; Simpson *et al.*, 1984; Payares *et al.*, 1985).

The present study was undertaken to define the composition of proteins in the crude extracts of early developmental stages of *P. westermani* by electrophoresis.

MATERIALS AND METHODS

1. Worms used:

The metacercariae of *P. westermani* were obtained from naturally infected crayfish (*Cambaroides similis*) collected in Kiljong-Ri, Yangdo-Myun, Gangwha-Gun, Korea. Dogs, weighing about 10 kg, were fed orally with 100 or 150 metacercariae, and were killed sequentially on 4, 6, 8, 10 and 12 weeks after the experimental infection. The worms were harvested from the organs in abdominal and/or thoracic cavities, and washed with cold 0.85% saline and once with distilled water.

2. Preparation of crude extracts from *P. westermani*:

The numbers of 4, 6, 8, 10 and 12 week-old

P. westermani used for the preparation of crude extracts were 90, 57, 88, 45, and 44 respectively. To remove excess surface water, the worms were rolled on filter paper. Total weights of worms at each age were 122, 141, 1,036, 535 and 890 mg on 4, 6, 8, 10 and 12 week-old worms (1.36, 2.47, 11.76, 11.89 and 20.22 mg per worm respectively). The worms of the same age were subjected to prepare the whole worm extracts. The worms were homogenized in 0.85% saline with Teflon-coated hemogenizer in ice-water bath. Each homogenate was shaked for 2 hours at 25°C and kept at 4°C overnight. After the extraction of soluble proteins, each homogenate was centrifuged at 10,000 *g* for 30 minutes at 4°C. The resulting supernatant was collected, and the protein content was determined by the method of Lowry *et al.* (1951). Each supernatant was freeze-dried to adjust protein content to 1.0 mg/ml, and they were allocated into freezing vials to store at −40°C until used. In case of 12 week-old worm, the extracts were prepared in two different way; *i.e.*, the conventional whole worm extract and somatic extract. The somatic extract was prepared using the worms that eliminated the metabolite from actively moving worms by overnight incubation at 36°C in phosphate buffered saline (pH 7.2).

3. Electrophoresis:

Disc polyacrylamide gel electrophoresis (=disc-PAGE) was performed by the method of Davis (1964) with 7.5% acrylamide running gels using tris-glycine buffer (pH 8.3). Protein amount of 80∼100 μg in each sample were placed in gel tubes (5×100 mm), and DC power supply was adjusted to 1.5mA per tube for half an hour, afterwards to 3mA for 2 hours. After the electrophoreses, the gels were stained with 0.25% Coomassie brilliant blue R-250 for 2∼4 hours and destained by repeated washings in 5% methanol and 7.5% acetic acid. Densitometric profiles of each gel were recorded using automatic computing densitometer (Gelman, U.S.A.) at 565 nm.

RESULTS

1. Electrophoretic patterns of proteins of each developmental stage:

Throughout the electrophoregram of 5 developmental stages, a total of 15 distinct bands of different *Rf* values was identified (Table 1). The

Table 1. Mean *Rf* values of protein bands identified in disc-PAGE of 5 developmental stages of *P. westermani*

Band No.	Mean *Rf* values in					
	Pw4WWE	Pw6WWE	Pw8WWE	Pw10WWE	Pw12WWE	Pw12SME
1	—	—	—	—	0.064	0.061
2	0.090	0.092	0.090+NUB**	0.092+NUB	0.097	0.091
3	0.194	0.198	—	NUB	NUB	NUB
4	0.262	0.265	NUB	NUB	NUB	NUB
5	0.369	0.374	0.370	0.368	0.376	0.383
6	0.445	0.454	0.454	0.454	0.468	0.467
7	0.508	0.514	0.512	0.511	0.524	0.515
8	0.555	0.565	0.558	0.556	—	—
9	0.597	0.607	—	—	—	—
10	0.638	0.643	—	NUB	—	—
11	0.673	—	—	—	—	—
12	0.751	0.758+NUB	0.758	0.744	0.757	0.760
13	—	—	—	—	0.786	0.791
14	0.832	0.840	0.840+NUB	0.837	0.839	0.841
15	0.928	0.933	0.934	0.929	—	—
BPB*	1.00	1.00	1.00	1.00	1.00	1.00

*BPB: front dye of Bromophenol blue **NUB: nearby unidentified band

Table 2. Relative composition of protein bands in disc-PAGE as measured by densitometric tracing

Band No.	Relative composition(%) in					
	Pw4WWE	Pw6WWE	Pw8WWE	Pw10WWE	Pw12WWE	Pw12SME
1	—	—	—	—	17	5
2	3	4	1+2*	4+3*		
3	8	10	—	7*	5*	4*
4	15	19	8*	8*	12*	6*
5	6	9	10	51	18	60
6	15	17	36		10	
7	11	14	7		18	
8	4		13		—	—
9	5	3	—	—	—	—
10	6	5	—	9*	—	—
11	11	—	—	—	—	—
12	5	7+*	3	8	8	11
13	—	—	—	—	6	
14	3	6	15+*	6	6	11
15	2	2	<1	3	—	—
BPB	6	5	7	1	1	3

*Nearby unidentified bands.

bands were numbered according to *Rf* from cathodal side. The bands which were not consistently observed at every runs or which were not comparable with distinct bands were not numbered, and regarded as unidentified bands.

The relative compositions of protein amount in each band were presented in Table 2, as read by densitometry. The term "major band" should represent the prominent, conspicuous bands of electrophoregram, however, this term was applied to rather subjectively selected bands, considering the clear separation from nearby bands and relatively dense stainability. Therefore, the "major band" may not be compatible with densitometric reading.

(1) Whole worm extract of 4 week-old (= Pw4WWE)

As shown in Fig. 1(1), 13 bands were observed from Band 2 to Band 15. Of 13 bands, Bands 4, 6, 7, 11, 12, 14 and 15 represented as major bands (marked with arrow head).

(2) Whole worm extract of 6 week-old (= Pw6WWE)

A total of 12 different bands was identified (Fig. 1(2)). The major bands were Bands 4, 6, 7, 12, 14 and 15. One unidentified band was recognized next to Band 12.

(3) Whole worm extract of 8 week-old (= Pw8WWE)

Eleven bands, composed of 4 major bands (Bands 6, 7, 8 and 14), 4 minor bands (Bands 2, 5, 12 and 15) and 3 unidentified ones were observed (Fig.1 (3)). Band 3, 4, 9 and 10 of Pw4WWE and Pw6WWE were not detected.

(4) Whole worm extract of 10 week-old (= Pw10WWE)

Twelve bands were identified (Fig. 1(4)). Bands 6, 12 and 14 were major bands. Four unidentified bands were recognized between Band 2 and Band 5, and near to Band 10. Bands 9, 10 and 11 of Pw4WWE and Pw6WWE were not detected.

(5) Whole worm extract of 12 week-old (= Pw12WWE)

As shown in Fig. 1(5), 10 different bands were observed. Band 1 was newly observed near the cathode, and Band 13 was recognized adjacent to Band 12. Bands 1, 5, 12, 13, and 14

represented as major bands. Minor bands were Bands 2, 6, and 7. Two unidentified bands were observed near to Band 4. Bands 8 and 15 were not detected.

(6) Somatic extract of 12 week-old (=Pw12 SME)

A total of 10 bands was identified (Fig. 1 (6)). Bands 5, 12 and 14 were major bands, and Bands 2, 6, 7 and 13 were minor bands. Two bands on cathodal side were unidentified ones.

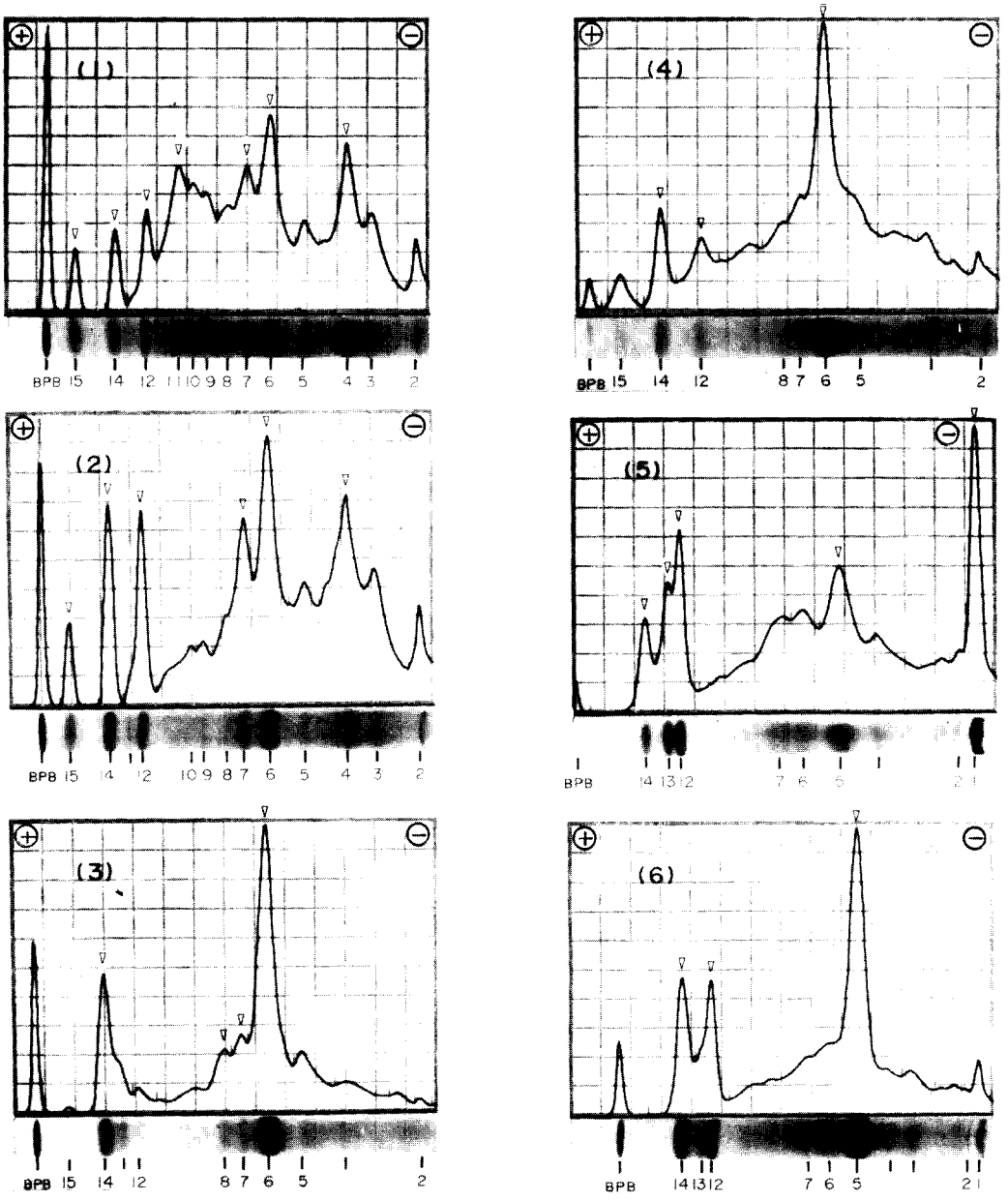

Fig. 1. Electrophoretic patterns and their densitometric profile of saline extracts from *P. westermani*. All identified bands are numbered, and those marked with open arrow heads are the major bands. (1) Whole worm extract of 4 week-old *Paragonimus westermani* (=Pw4WWE) (2) Pw6WWE (3) Pw8WWE (4) Pw10WWE (5) Pw12WWE (6) Somatic extract of 12 week-old *P. westermani* (=Pw12SME)

2. Comparison of electrophoretic patterns of protein:

The banding patterns of Pw4WWE and Pw6 WWE were similar each other, and the bands of Pw8WWE was similar with that of Pw10 WWE, while the bands of Pw12WWE were different from those of younger worms.

As shown in Fig. 2, Band 2 at cathodal zone, Bands 5, 6 and 7 at mid-zone and Bands 12 and 14 at anodal zone were always observed as common bands throughout all of developmental stages, though the stainabilities were variable.

When compared by densitograms (Figs. 1(1) ~1(6)), Band 5 was a minor band until 10 week-old, but became a major band in 12week-old worms (marked with dashed arrow head in Fig. 2). On the other hand, Band 6, which was a major band until 10 week-old, turned to be minor band in 12 week-old worms.

Bands 3 and 4 at cathodal zone, Bands 8, 9, 10 and 11 at mid-zone and Band 15 at anodal zone, which were recognized in Pw4WWE and Pw6WWE, progressively lowered their stainabilities in Pw8WWE to be unrecognized in Pw12 WWE (marked with open arrow head).

As shown in Figs. 1(5) and 2, a prominently peaked and intensely stained Band 1 appeared firstly in 12 week-old worms(marked with closed arrow head), and Band 13 became distinctly recognized only at 12 weeks.

Between the banding patterns of Pw12WWE and Pw12SME (columns 5 and 6 in Fig. 2 in respect), there existed only quantitative differences; Band 5 in Pw12SME was much more prominent than in Pw12WWE while Band 1 in PwSME was stained less prominently than in Pw12WWE.

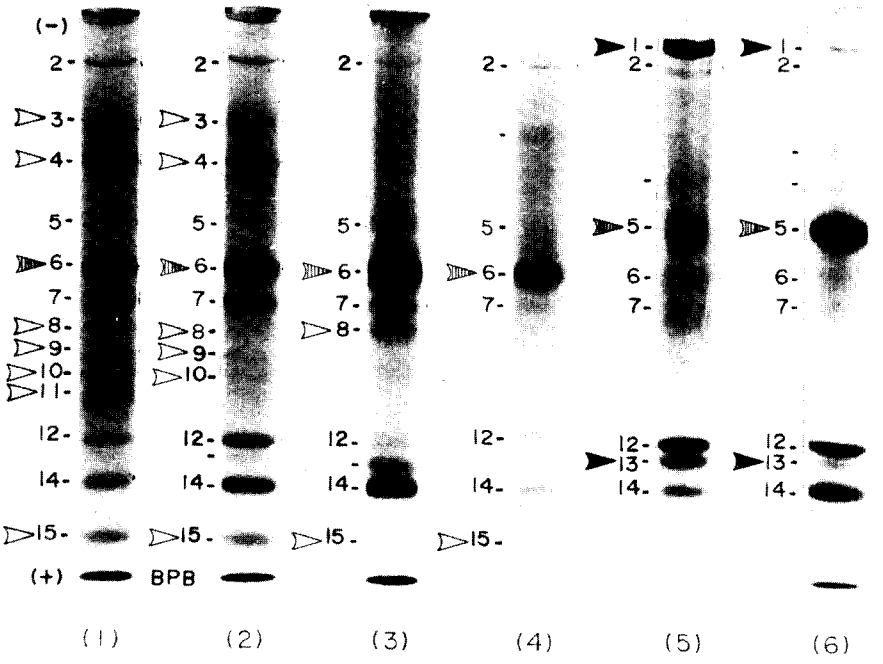

Fig. 2. Comparison of electrophoretic patterns of saline extracts from early developmental stages of *P. westermani*. All identified bands were numbered; and changing bands were marked with 3 kinds of arrow heads. Open arrow head: Progressively decreasing stainability with development
Closed arrow head: Newly appeared in 12 week-old
Dashed arrow head: Those turned from major band to minor, or *vise versa*
(1) Pw4WWE (2) Pw6WWE (3) Pw8WWE (4) Pw10WWE (5) Pw12WWE (6) Pw12SME

DISCUSSION

Yoshimura (1969 a, b) made pioneering studies on the protein compositions of *Paragonimus westermani* by electrophoresis on polyacrylamide gel. He actually observed the protein profiles only of 80~90 day-old worms. One densitometric profile in his study matched very well with the pattern of Pw12WWE of this study. However, he also recognized three other variations in electrophoregrams that were not observed in this study. These variations in protein bands with more numerous bands could have been derived from the usage of the crude worm extract prepared by centrifuging the worm homogenate at 1,500*g*.

Imai (1979) also made an observation on the electrophoretic pattern of 20 week-old *P. westermani* in which the pattern of protein bands was far different from that of Pw12WWE of this study.

The data available at the moment are not conclusive to define a typical electrophoretic pattern of adult *P. westermani* proteins. The electrophoretic pattern of proteins seemed to be hardly acceptable as a taxonomic criterion among various species that belonged to genus *Paragonimus* (Yoshimura, 1969 a, b; Yoshimura *et al.*, 1969), because there observed so many variations of banding pattern, even in a species, *P. westermani*. In addition proteins from different developmental stages provided many different profiles as observed in this study.

The results of disc-PAGE showed that the protein patterns were very similar between Pw4 WWE, and Pw6WWE, and between Pw8WWE and Pw10WWE respectively. Incidentally the light microscopic findings of worm development at those relevant ages were very similar each other as far as the development of sexual organs were concerned.

It was evident that the electrophoretic patterns changed according to the worm growth and development up to 12 weeks. Band 1 was not recognized in proteins from 4 week-old to 10 week-old worms, but firstly sppeared in 12 week-old worms. This suggested that Band 1 may be derived from the proteins of eggs that deposited in significant amount only in 12 week-old worms. To confirm this, electrophoregram of soluble proteins from eggs of *P. westermani* should be made to compare with that of Pw12WWE. Another possible source of Band 1 was metabolite of 12 week-old worms since Pw12SME showed significantly smaller amount of Band 1 than in Pw12WWE

Band 5, which became a major band in 12 week-old, may be derived from parenchymatous organs of 12 week-old worms, because it was more prominent in Pw12SME than in Pw12 WWE.

Some of protein bands such as Bands 3, 4, 8, 9, 10, 11 and 15 were observed in 4~6 week-old juvenile worms, but most of them disappeared in worms older than 8 weeks. Payares *et al.* (1985) also observed that some proteins in tegumentum of schistosomula of *S. mansoni* were replaced by a single protein with maturation to adult worms. Choi *et al.* (1981) also demonstrated that some bands of juvenile worms disappeared in adult stage of *Clonorchis sinensis*. Those proteins, which appeared only in juvenile stages of *P. westermani*, might be related with enzymes such as protease which facilitate the migration of worms through host tissues.

It is now well known that the tegumental proteins of *S. mansoni* changed their surface glycoproteins which sensitized the host as immunogens (Capron *et al.*, 1980; Brink *et al.*, 1980). In that connection, it appeared that the changing pattern of protein composition in electrophoregram of *P. westermani* reflected many changes of morphogenesis as well as changes in proteins from tegumentum and other sources.

This study should be extended to identify and characterize the potent immunogens of *P. westermani*. By applying disc-PAGE to fractionated extracts, and differently prepared extracts, and by concomitant serologic examinations, we could understand more specifically the natures of protein antigens of *P. westermani*.

SUMMARY

In order to observe the protein compositions of soluble extracts of *P. westermani*, and their changes during early developmental stages, the crude saline extracts of 4, 6, 8, 10 and 12 week-old worms which were harvested from experimentally infected dogs were analysed by disc-PAGE. The results were as follows:

1. A total of 15 bands were identified from electrophoregrams of respective developmental stages. Of them, 5 bands were recognized throughout the developmental stages.

2. The number and protein amount of identified bands changed according to the worm development from 4 weeks to 12 weeks. However, the banding patterns of 4 and 6 week-old worms and 8 and 10 week-old worms were similar each other.

3. Of 15 identified bands, Band 1 was recognized only in 12 week-old worms whereas Bands 3, 4, 8, 9, 10, 11 and 15 gradually lowered their amount according to development to disappear in 12 week-old. In addition, Band 5 became a major band in 12 week-old while Band 6 turned to a minor band at the same age.

The possible relations of changing patterns of protein bands with worm development were discussed.

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=국문초록=

초기발육단계 폐흡충에서 추출한 단백질의 전기영동상

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폐흡충 발육에 따른 형태학적 변화와 함께 구성단백질의 전기영동상의 차이점을 관찰할 목적으로 실험 감염 후 4, 6, 8, 10 및 12주 경과한 초기 발육단계의 폐흡충으로부터 각각의 생리 식염수 추출액을 만들고 이들 추출액 내의 단백질 구성을 디스크 폴리아크릴아마이드젤 전기영동(disc-PAGE)양상으로 비교 관찰한 결과는 다음과 같았다.

1. 감염후 4주부터 12주에 이르는 충체들의 생리식염수 추출액 전기영동상에서 관찰된, 서로 다른 *Rf*치를 보인 단백질 band수를 모두 합하면 15개이었다. 그중 5개는 관찰한 전발육단계를 통하여 관찰할 수 있었다.

2. 분리된 구성 단백질 band수와 그 양상은 각 발육단계마다 서로 다르게 관찰되었지만, 4주와 6주의 전기영동상이 비슷하였고, 8주와 10주의 전기영동상 또한 비슷하게 나타났다.

3. 12주 성충에서의 전기영동상은 그 이전의 영동상과 크게 달라졌던 바 가장 낮은 전기이동도 영역에서 새로운 단백질 band가 주(主, major) band로 관찰되었고, 12주 이전의 충체에서 미약하던 단백질 band가 주 band로 전환되었으며 4주와 6주의 미성숙 충체에서 관찰되었던 band들이 소멸되었다.

이상의 결과에서 폐흡충은 성충으로 발육하는 초기 발육단계에서 구성단백질의 전기영동상이 단계별로 다르게 나타나는 바, 특히 8주와 12주에 각각 크게 변화하고 있음을 알 수 있었다.