기 생 충 학 잡 지 제29권 제 2 호
The Korean Journal of Parasitology
Vol. 29, No. 2, 113-120, June 1991

Molecular weight of major component proteins in crude saline extract of adult *Paragonimus westermani**

Yoon Kong, Woong Bong Kim, Shin-Yong Kang and Seung-Yull Cho
Department of Parasitology, College of Medicine, Chung-Ang University, Seoul 156-756, Korea

Abstract: When the component proteins in crude saline extract of 13-week old adult *Paragonimus westermani* were observed by non-denaturing discontinuous-polyacrylamide gel electrophoresis (Disc-PAGE), 8 distinct bands were clearly recognized. Molecular weight (MW) of each band protein, numbered in sequence from cathodal side which appeared in 10% separating gel, was measured first by Ferguson plot utilizing different gel concentrations from 10% to 4.5%. MW of band 1 protein (known as egg protein) was 440 kDa. And MW of other band proteins were: 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. When the proteins in the crude extract were separated into fractions by molecular sieve chromatography through 1.6(ϕ) × 70 cm sized Sephacryl S-300 Superfine column and revisualized by Disc-PAGE in 8% gel, the sequence of eluted proteins was band 1, band 2, band 6, band 7 and bands 3, 4, 5 and 8. This elution profile confirmed MW of each band protein in the crude extract as measured by Ferguson plot.

Key words: *Paragonimus westermani*, component protein, molecular weight, non-denaturing Disc-PAGE, Ferguson plot, molecular sieve chromatography

INTRODUCTION

The main target of serologic diagnosis for paragonimiasis has been a proper detection of *Paragonimus*-specific antibody (IgG) in patients' sera. The antibody test is now increasingly applied because both the sensitivity of egg detection and specificity of intradermal test are low (Cho *et al.*, 1983). One important aspect of the antibody test is its antigen preparation. Since the antibody formations are induced by a variety of antigenic components of the worms, compositions of soluble proteins in an antigen is important to detect the various polyclonal antibodies. Up until now, differently prepared extracts such as acid extract, activated polypeptides, veronal buffered saline extract or saline extract of the worm have been used as diagnostic antigens (Sadun *et al.*, 1959; Biguet *et al.*, 1965). Of them, crude saline extract of adult worm is now widely used in enzyme-linked immunosorbent assay (ELISA) for paragonimiasis (Cho *et al.*, 1981; Knobloch and Leder, 1983; Kojima *et al.*, 1983; Yokogawa *et al.*, 1983; Imai, 1987). Though excretory-secretory antigen of the worm was confirmed to be more sensitive (Kim *et al.*, 1983), its preparation is difficult and yield is limited.

In improving the diagnostic sensitivity and specificity of the antibody test, knowledge on

* This study was supported in part by a Research Grant for Genetic Engineering, Ministry of Education, Republic of Korea (1988).

the protein composition of the crude extracts is a prerequisite as far as quality improvement of antigen is considered. Hitherto, a few studies have been contributed on the nature of native, non-denatured proteins in the crude extract of *Paragonimus*. Yoshimura (1969) was the first in identifying component proteins in saline extract of *P. westermani* by Disc-PAGE. Huer *et al.* (1985) demonstrated by Disc-PAGE that proteins in crude extract of *P. westermani* were changing by developmental stages from 4 to 12 weeks in its final host. Kim *et al.* (1986) proved that first band in Disc-PAGE of the crude extract came from eggs. Imai and Nawa (1988) confirmed the finding of Kim *et al.* (1986) and reported that MW of the protein was 440 kDa when estimated by gel filtration. In addition to the egg protein, a 13 kDa protein was also identified (Imai and Nawa, 1988). Recently, Song and Dresden (1990) identified cysteine protease activity in crude extract of *P. westermani* by column chromatography and MW of the enzyme was estimated to be 20 kDa.

As for the component proteins of *P. westermani*, many studies were done by sodium dodecyl sulfate polyacrylamide electrophoresis (SDS-PAGE) or SDS-PAGE/immunoblot. For example, 30 kDa (Choi *et al.*, 1986), 27 kDa protein (Sugiyama *et al.*, 1987), 23, 30, 46, and 92 kDa proteins (Kim *et al.*, 1988), 8 and 62 kDa proteins (Slemenda *et al.*, 1989), 92 and 223 kDa proteins (Joo *et al.*, 1989) were shown to be antigenic component proteins in the crude extract when observed by SDS-PAGE / immunoblot. Yamakami and Hamajima (1987) estimated the purified enzyme of neutral thiol protease by SDS-PAGE as 27 kDa. When the component proteins in the crude extract is observed by SDS-PAGE, however, proteins were denatured into its subunits by SDS, 2-mercaptoethanol, dithiothreitol, urea or boiling. Therefore, the relation between these subunit components recognized in SDS-PAGE and non-denatured, native proteins in the crude extract is not verified yet.

In the process of the preparing better diagnostic antigen, handling of the native proteins in the crude extract is unescapable especially when the chromatography should be included. This study was conducted, therefore, to estimate MW of major component proteins in crude saline extract of *P. westermani* by Ferguson plot and molecular sieve chromatography.

MATERIALS AND METHODS

1. Crude saline extract of *P. westermani*

Saline extract of 13-week old adult *P. westermani* was prepared as described by Cho *et al.* (1981). Briefly, a total of 50 adult worms which were collected from an experimentally infected dog was emulsified with teflon-pestle tissue homogenizer in physiologic saline containing 0.006% (W/V) phenylmethylenesulfonyl fluoride (PMSF). After shaking at 4°C for 2 hours, the homogenate was kept in refrigerator overnight. It was centrifuged at 4°C by 30,000*g* for 30 minutes. Resulting supernatant was regarded as a crude saline extract of adult *P. westermani*. The protein content was 3.92 mg/ml when measured by Lowry *et al.* (1951).

2. Estimation of molecular weight of component proteins

1) Ferguson plot

Disc-PAGE : Disc-PAGE of Davis (1964) was adopted. Stacking gel was 2.5%. A pair of 4.5%, 5%, 5.5%, 6%, 7%, 8%, 9% and 10% separating tube gels was prepared. In each pair of different separating gel concentration, the crude extract in amount of 75 μg of protein and standard proteins of α-lactalbumin (M.W.: 14.2 kDa), carbonic anhydrase (29 kDa), chicken egg albumin (45 kDa), bovine serum albumin (monomer 66 kDa, dimer 132 kDa), urease (trimer 272 kDa, hexamer 545 kDa) (Sigma, USA) together with the same amount of sample buffer were loaded. Samples were electrophoresed by direct current supply of 1.5 mA per tube for stacking gel and 3 mA per tube for separating gel. After the electrophoresis, gels were stained with 0.125% Coomassie brilliant blue R-250.

Proteins, which were fractionated through gel filtration, were also observed for their composition by Disc-PAGE of 8% separating gel concentration.

Molecular weight estimation by Ferguson plot : R_f value of each band of the standard proteins was measured in each % gel concentration. Then the minus slopes of $100 \cdot \log(R_f \cdot 100)$ of the standard proteins against % gel concentrations were plotted against molecular weights in log-log paper to make a standard straight line. In the same way, the minus slope of each component protein in crude extract of *P. westermani* was calculated. Using the value of the minus slope, molecular weight of each protein was calculated in the standard line (Hedrick and Smith, 1968).

2. Molecular sieve chromatography

To confirm the molecular weight of each component proteins estimated by Ferguson plot, proteins in the crude extract was separated into fractions by gel filtration. A total of 40 mg of protein was filtrated through 1.6 (ϕ) × 70cm sized column of Sephacryl S-300 Superfine (Pharmacia) at flow rate of 15 ml/cm²·hour. A total of 80 tubes which allocated 35 drops of eluent was collected using Multirec fraction collector (LKB, Sweden). Eluents were divided into 7 peaks by absorbance at 280 nm. Protein content of each fraction was measured by Lowry *et al.* (1951) and dialyzed in 0.15 M phosphate buffered saline (PBS) (pH 7.4) for 24 hours at 4°C. Proteins were concentrated by lyophilization. Molecular weight marker proteins of blue dextran (MW: 2,000 kDa), thyroglobulin (669 kDa), alcohol dehydrogenase (150 kDa), bovine serum albumin (66 kDa) and cytochrome C (12.4 kDa) (Sigma, USA) were also filtrated to make a standard line. Molecular weight of each fraction was estimated by Ve/Vo value of each peak.

RESULTS

1. Molecular weight estimated by Ferguson plot

As illustrated in Fig. 1, crude saline extract

Fig. 1. Separation of component proteins in crude saline extract of adult *P. westermani* at different gel concentrations in Disc-PAGE. A: 10% separating gel, B: 9% gel, C: 8% gel, D: 7% gel, E: 6% gel, F: 5.5% gel, G: 5% gel and H: 4.5% gel. Protein bands were numbered as appeared from cathodal side in 10% separating gel.

of *P. westermani* was separated into 9 bands including bromophenol blue (BPB) dye front in 10%, 9%, 8% and 7% separating gels. In the separating gels of lower concentrations, several protein bands were superimposed on BPB (lanes E-H in Fig. 1). Each band was numbered from 1 to 8 according to their appearance from cathodal side in 10% gel except BPB. Of them, band 1, band 2, band 4, band 6 and band 8 were stained darkly. Between bands 1 and 2, faintly stained bands were observed at higher gel concentration from 10% to 7%. As the gel concentration lowered, the bands in 10% gel moved at the same sequence from cathodal to anodal side. The only exception was band 2 protein of 10% gel which migrated below band 3 in gel concentration of 7% or lower.

R_f value of each band as numbered in 10% gel was measured in the different gel concentrations. Based on that, $100 \cdot \log(R_f \cdot 100)$ was

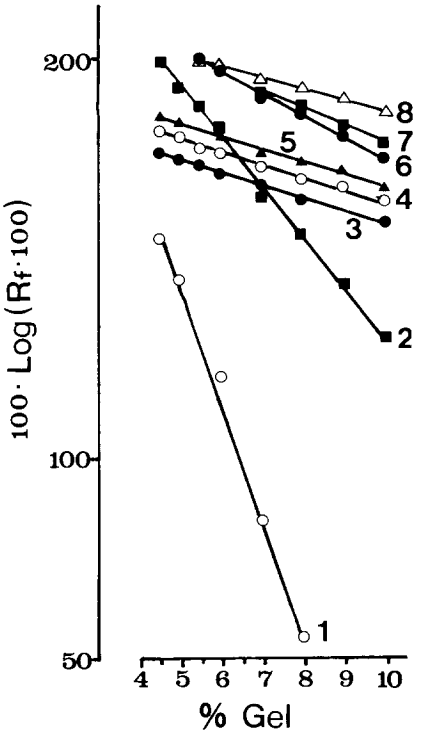

Fig. 2. Calculation of minus slope of $100 \cdot \log(R_f \cdot 100)$ against % gel concentration of each protein band in the saline extract of *P. westermani*. Numericals stand for the band number in Fig. 1.

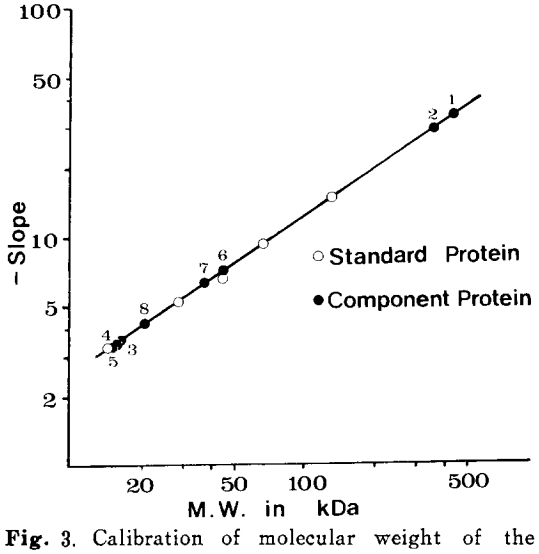

Fig. 3. Calibration of molecular weight of the component proteins in the crude saline extract from the minus slopes. Numericals mean the band number in Fig. 1.

calculated and plotted against gel concentration as shown in Fig. 2. Then, minus slope of each band was plotted in the standard line (Fig. 3). MW was calculated as 440kDa in band 1 protein, 386kDa in band 2 protein, 17.4kDa in band 3, 17kDa in band 4, 14.3kDa in band 5, 46kDa in band 6, 38kDa in band 7 and 23kDa in band 8, respectively.

2. Molecular weight estimated by molecular sieve chromatography

Elution profile of proteins in the crude extract which filtrated through Sephacryl S-300 Superfine was shown by absorbance at 280nm (Fig. 4). At the top of figure, tube number of peak absorbance of molecular weight marker proteins were shown. Using a standard line of Ve/Vo from marker proteins, molecular weight of each fraction was calculated. Peak of fraction 1 corresponded to 560 kDa, mid-point of fraction 2 did to 385 kDa, peak of fraction 3 to 135 kDa, mid-point of fraction 4 to 42 kDa, peak point of fraction 5 to 12.9 kDa and peak point of fraction 6 to 10 kDa. Molecular weight in fraction 7 was hardly calculable.

Protein composition of each fraction was observed by Disc-PAGE using 8% gel (Fig. 5). Fraction 1 was composed only of band 1 protein; fraction 2 of band 1 and band 2. Faint bands

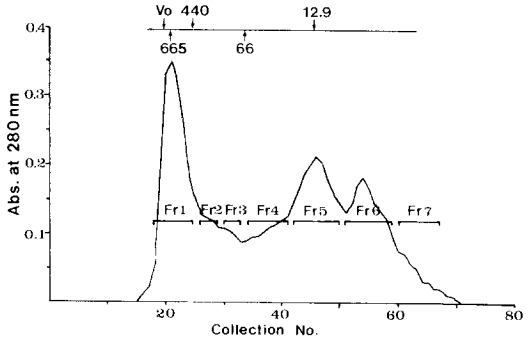

Fig. 4. Elution profile of crude extract of *P. westermani* filtrated through Sephacryl S-300 Superfine. Eluent: 0.15 M PBS containing 0.01% merthiolate and 0.006% PMSF. Flow rate: 15 ml·cm^{-2}·hr. Collection volume: 35 drops/tube.

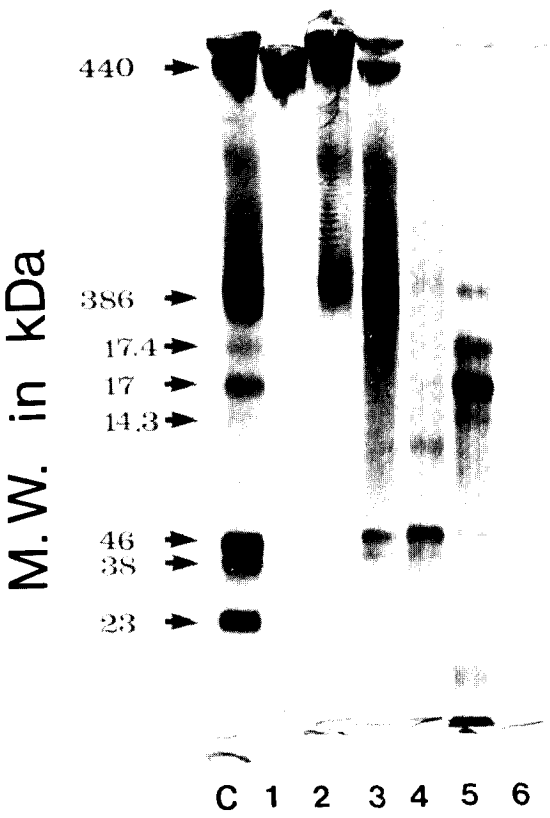

Fig. 5. Banding patterns of each fraction collected by Sephacryl S-300 Superfine. Proteins in each fraction were revisualized in 8% Disc-PAGE. C: Crude saline extract, 1: Fraction 1, 2: Fraction 2, 3: Fraction 3, 4: Fraction 4, 5: Fraction 5, 6: Fraction 6.

which were not designated between band 1 and band 2 were also found in fraction 2. In fraction 3, bands of 1, 2, 6 and 7 were observed. In fraction 4, band 6 and band 7 were the major components; and other faintly stained bands were also observed. In fraction 5, band 3, band 4, band 5 together with a portion of band 6 and band 8 were revealed. In fraction 6, faint band of band 4 was a major component. Fraction 7 did not show any band (data not shown).

When the Disc-PAGE findings of fractions were analysed by each band, band 1 of estimated MW 440 kDa was found in fractions 1 (560 kDa), 2 (385 kDa) and 3 (135 kDa). The minor band between band 1 and band 2 were found in fractions 2 and 3 suggesting its molecular weight being over 100 kDa. Band 2 protein (370 kDa) was observed in fractions 2-5, but the majority was in fraction 2 (385 kDa) and fraction 3 (135 kDa). Band 3 (17.4 kDa), band 4 (17 kDa) and band 5 (14.3 kDa) were found in fractions 4 and 5. But majority of their proteins were eluted in fraction 5 (12.9 kDa). Band 6 (46 kDa) and band 7 (38 kDa) appeared in fractions 3 (135 kDa) and 4 (42 kDa). Band 8 (23 kDa) appeared in fraction 5 (12.9 kDa).

DISCUSSION

In this study, crude extract of adult *P. westermani* revealed at least 8 distinct protein bands when observed by Disc-PAGE. Although some minor bands were interspersed between them, their presence was not always consistent as the 8 major bands. Same findings were described by Yoshimura (1969) and Huer *et al.* (1985). To minimize the autodigestion of native proteins, we mixed PMSF to the extract and kept 4°C in all processings except for an electrophoresis. Therefore, these 8 bands can be regarded as major soluble proteins in crude extract of adult *P. westermani*. However, it was not completely ruled out that there were some degradation products because the extract contained possibly different kinds of proteases which were not inhibited by PMSF.

When MW of these proteins was measured by two different methods of Ferguson plot using Disc-PAGE and column chromatography, the results were comparable each other. Because protein of a certain MW dispersed considerably in gel filtration, contamination of a protein into nearby fractions was inevitable. Therefore, we thought the findings of Fig. 5 in this study was confirming evidence of MW measurement by Ferguson plot.

In the present study, we did not undertake SDS-PAGE to observe the subunit compositions of component proteins of *P. westermani*. In

interpreting the findings by either SDS-PAGE or SDS-PAGE/immunoblot, the relation between the native proteins and their subunits were important. But the fractionated proteins in this study were contaminated with neighbouring proteins of similar MW. The correct relations between the native component proteins and their subunits could not, therefore, properly recognized when the fractionated proteins were subjected. For that purpose, each protein should be purified first, then their subunits should be observed next.

The band 1 protein in this study was already known to be derived from eggs (Kim *et al.*, 1986; Imai and Nawa, 1988). MW of this protein was 440 kDa, which was the same with that reported by Imai and Nawa (1988). Proteins of bands 3, 4, 5 had similar ionic charges and MW each other as revealed by pararell migration patterns throughout the different gel concentrations. Of them, band 4 protein was found in excretory-secretory product (ESP) of *P. westermani* (Imai and Nawa, 1988) and its MW was 13 kDa (Imai and Nawa, 1988) or 17 kDa as shown in this study. In addition, characterization of other component proteins in crude saline extract of *P. westermani* should be studied in the future as a native proteins especially in their origin in the worm and their biological roles.

REFERENCES

Biguet, J., Rose, F., Capron, A. and Tran Van Ky, P. (1965) Contribution de l'analyse immunoelectrophoretique a la connaissance des antigens verminеux. Incidences pratiques sur leur standardisation, leur purification et le diagnostic des helminthiases par immuno-electrophorese. *Rev. Immunol.*, **29**:5-30.

Cho, S.Y., Hong, S.T., Rho, Y.H., Choi, S.Y. and Han, Y.C. (1981) Application of micro-ELISA in serodiagnosis of human paragonimiasis. *Korean J. Parasit.*, **19**:151-156.

Cho, S.Y., Lee, D.K., Kang, S.Y. and Kim, S.I. (1983) An epidemiological study of human paragonimiasis by means of micro-ELISA. *Korean J. Parasit.*, **21**:246-256.

Choi, W.Y., Yoo, J.E., Nam, H.W. and Choi, H.R. (1986) Purification of antigenic proteins of *Paragonimus westermani* and their applicability to experimental paragonimiasis. *Korean J. Parasit.*, **24**:177-186.

Davis, B.J. (1964) Disc-electrophoresis. II. Method and application to human serum proteins. *Ann. N.Y. Acad. Sci.*, **121**:404-427.

Hedrick, J.L. and Smith, A.J. (1968) Size and charge isomer separation and estimation of molecular weights of protein by Disc gel electrophoresis. *Arch. Biochem. Biophys.*, **126**:155-164.

Huer, B., Kim, S.I., Kang, S.Y. and Cho, S.Y. (1985) Electrophoretic patterns of proteins from *Paragonimus westermani* in early developmental stages. *Korean J. Parasit.*, **23**:189-196.

Imai, J. (1987) Evaluation of ELISA for the diagnosis of paragonimiasis westermani. *Trans. Roy. Soc. Trop. Med. Hyg.*, **81**:3-6.

Imai, J. and Nawa, Y. (1988) Immunochemical charaterization of major antigenic components of *Paragonimus westermani* (Triploid type) adult worms. *Jpn. J. Parasitol.*, **37**:398-404.

Joo, K.H., Ahn, H., Chung, M.S. and Rim, H.J. (1989) Demonstration of species-specific and cross-reactive components of *Paragonimus westermani* crude worm antigen by EITB. *Korean J. Parasit.*, **27**:9-14.

Kim, D.C., Lee, O.W., Lee, J.S. and Ahn, S.A. (1983) Immunodiagnosis of human paragonimiasis by ELISA using secretory-excretory antigen. *Rep. NIH Korea*, **19**:109-114.

Kim, S.H., Kong, Y., Kim, S.I., Kang, S.Y. and Cho, S.Y. (1988) Immunoblot observation of antigenic protein fractions in *Paragonimus westermani* reacting with human patients sera. *Korean J. Parasit.*, **26**:239-243.

Kim, S.I., Ko, E.K., Kang, S.Y. and Cho, S.Y. (1986) Antigenicity of the soluble egg antigen of *Paragonimus westermani*. *Korean J. Parasit.*, **24**: 49-54.

Knobloch, J. and Leeder, I. (1983) Immunodiagnosis of human paragonimiasis by an enzyme immunoassay. *Tropenmed. Parasit.*, **34**:21-23.

Kojima, S., Kobayashi, M. and Yokogawa, M. (1983) Demonstration of cross-reactivity among antigens extracted from four species of *Paragonimus* and its utilization for the enzyme-linked immunosorbent assay (ELISA). *Jpn. J. Parasitol.*, **32**:413-418.

Lowry, O.H., Rosenbrough, N.J., Lewis-Farr, A. and Randall, R.J. (1951) Protein measurement with Folin-phenol reagent. *J. Biol. Chem.*, **193**: 365-375.

Sadun, E.H., Walton, B.C., Buck, A.A. and Lee, B.K. (1959) The use of purified antigens in the diagnosis of clonorchiasis sinensis by means of intradermal and complement fixation test. *J. Parasit.*, **45**:129-134.

Slemenda, S.B., Maddison, S.E., Jung, E.C. and Moore, D.D. (1988) Diagnosis of paragonimiasis by immunoblot. *Am. J. Trop. Med. Hyg.*, **39**: 469-471.

Song, C.Y. and Dresden, M.H. (1990) Partial purification and characterization of cysteine proteinases from various developmental stages of *Paragonimus westermani*. *Comp. Biochem. Physiol.*, **95B**:473-476.

Sugiyama, H., Sugimoto, M., Akasaka, K., Horiuchi, T., Tomimura, T. and Kazaki, S. (1987) Characterization and localization of *Paragonimus westermani* antigens stimulating antibody formation in both the infected cat and rat. *J. Parasit.*, **73**:363-367.

Yamakami, K. and Hamajima, F. (1989) Purification of neutral thiol protease from *Paragonimus westermani* metacercariae by single-step chromatography and preparation of antibodies. *Int. J. Parasit.*, **19**:9-12.

Yokogawa, M., Kojima, S., Kobayashi, M., Hata, H., Ito, J., Tsuji, M., Miranda, H., Ibanz, N., Fernandez, E. and Guerra, A. (1983) Peruvian paragonimiasis: Diagnostic value of enzyme-linked immunosorbent assay (ELISA). *Jpn. J. Parasitol.*, **32**:317-322.

Yoshimura, K. (1969) *Paragonimus westermani*, *P. ohirai* and *P. miyajakii*: Electrophoretic comparison of whole body proteins. *Exp. Parasit.*, **25**: 118-130.

=국문요약=

폐흡충 성충 생리식염수 추출액의 성분 단백질의 분자량

중앙대학교 의과대학 기생충학교실
공 윤 • 김웅봉 • 강신영 • 조승열

혈청내 특이 항체가를 측정하여 폐흡충증을 면역학적으로 진단할 경우 그 민감도와 특이도가 뛰어난 것은 이미 잘 알려져 있다. 이때에 항원으로 사용하는 폐흡충 추출액의 구성 단백질 중 항원성과 특이성이 높은 단백질을 알면 항원을 질적으로 향상시킬 수 있을 것으로 기대하고 있다. 최근 SDS-PAGE/immuoblot을 이용하여 환자 혈청내의 특이 항체(IgG)와 민감하게 반응하는 폐흡충의 수용성(水溶性) 항원 단백질 분획이 보고되고 있다. 그러나 이 단백질 분획은 SDS-PAGE 후 특이항체와 반응한 polypeptide 수준의 subunit이어서 이들이 분해되기 전 성분 단백질 어느 것에서 유래한 것인지를 전혀 알 수 없는 단점이 있다. 따라서, 이와 같은 문제를 해결하고 나아가 종특이(種特異) 항원 단백질을 찾기 위해서는 분해, 변성되기 전의 성분 단백질의 성질을 파악할 필요가 있다. 이 실험에서는 폐흡충 성충 생리식염수 추출액내의 구성 단백질을 파악하고 그 분자량을 측정하기 위하여 Disc-PAGE를 이용한 Ferguson plot과 column chromatography를 실시하였다.

우선 폐흡충 추출액을 4.5~10%까지의 각기 다른 gel 농도에서 전기영동을 실시한 결과 추출액은 최소 8개 이상의 주요 성분 단백질로 구성되어 있었고 각각의 분자량은 band 1은 440 kDa, band 2는 386 kDa, band 3은 17.4 kDa, band 4는 17 kDa, band 5는 14.3 kDa, band 6은 46 kDa, band 7은 38 kDa 그리고 band 8은 23 kDa 이었다. 이어 추출액을 1.6(ϕ)×70 cm의 Sephacryl S-300 Superfine column을 통과시켜 7분획을 얻은 후 각 분획을 다시 8% gel에서 전기영동으로 확인한 결과 band 1은 1~3분획, band 2는 2,3분획, band 3은 5분획, band 4는 5,6분획, band 5는 5분획, band 6,7은 3,4분획, band 8은 5분획에서 각각 관찰되어 Ferguson plot에 의해 계산한 분자량과 유사한 결과를 보이고 있었다. 〔기생충학잡지, 29(2):113-120, 1991년 6월〕