기 생 충 학 잡 지 제28권 제3호
The Korean Journal of Parasitology
Vol. 28, No. 3, 135-142, September 1990

Purification of antigenic protein of sparganum by immunoaffinity chromatography using a monoclonal antibody*

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Abstract: The quality improvement of antigen (crude saline extract) of *Spirometra mansoni* plerocercoid (sparganum) was investigated by protein purification. The crude extract was fractionated by gel filtration through Sephacryl S-300 Superfine. Its third fraction was purified by affinity chromatography using a monoclonal antibody as ligand. When observed by SDS-PAGE, the purified protein was composed of 2 bands of 36 kDa and 29 kDa which were found already as the most sensitive components in the crude extract by immunoblots with patients sera. The quality of the purified antigen was evaluated in comparison with the crude extract by enzyme-linked immunosorbent assay (ELISA) for the specific (IgG) antibody in sera of human sparganosis, other parasitic and neurologic diseases, and normal control. When the purified antigen was used, the sensitivity was not altered but remained high (96.4%) while the specificity was increased from 86.8% to 96.9%.

Key words: Human sparganosis, serologic diagnosis, antigen purification, monoclonal antibody, immunoaffinity chromatography, gel filtration

INTRODUCTION

Human sparganosis, a disease caused by tissue invading plerocercoid of *Spirometra mansoni*, is a surgical disease because its final diagnosis depends on the recovery of the larva from lesions. However, by recent application of sensitive serologic test such as ELISA in detecting the specific (IgG) antibody (Kim *et al.*, 1984), it is now nearly possible to determine whether a patient is infected or not. What is impossible by the serologic test is the exact localization of the infected worm in body.

Human sparganosis is a rarely occurring disease even in endemic areas. Probably because of the reason, very few studies have been done on the immunologic/serologic aspects of the disease. Wider application of ELISA, however, in screening neurologic diseases for specific (IgG) antibody revealed that many neurologic diseases in Korea were caused by cerebral infection of sparganum (Chang *et al.*, 1987). Moreover, in other countries as well as in Korea, cerebral sparganosis is now increasingly detected (Anders *et al.*, 1984; Fan and Pezeshkpour, 1986; Chan *et al.*, 1987; Anegawa *et al.*, 1989). The incidence of cerebral sparganosis is not low than previously considered. The major symptoms of neurosparganosis is hemiparesis, seizures, headache and deterioration of intelligence in young adults (Chang *et al.*, 1987). Therefore, more attention should be paid on the clinical implication of human sparganosis in endemic areas such as Japan, China and Korea (Cho, 1987). In applying serologic test for sparganosis, crude saline extract of the larval worm has been used (Kim *et al.*, 1984). Of many different proteins in the crude extract, major antigenic components were 36 and 29 kDa proteins (Choi *et al.*, 1988). Because human cysticercosis, hydatidosis

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

or other parasitic diseases may cause cross-reactions with sparganum antigen (Cho *et al.*, 1986; Kim and Yang, 1988; Choi *et al.*, 1988), the crude extract needs improvement in its quality for the improved serodiagnosis. This study was done to investigate whether the improvement of quality is possible by purifying the component proteins in the crude extract.

MATERIALS AND METHODS

1. Crude saline extract of sparganum

Crude extract was prepared using naturally infected worms in *Natrix tigrina lateralis* as Kim *et al.* (1984) did. Worms were collected from muscle of snakes; host tissue on the worms was removed; worms were washed with cold saline for 3 times. The worms were emulsified by teflon-coated tissue homogenizer with physiologic saline containing 0.006% (W/V) phenylmethylsulfonyl fluoride (PMSF) and 10^{-8} M E-64 in ice bath. The emulsion was shaken for 2 hours and kept for 24 hours at 4°C. After centrifugation at 4°C by 10,000 *g* for 30 minutes, the supernatant was regarded as the crude saline extract. Protein content was 5.0 mg/ml when measured by Lowry *et al.* (1951).

2. Gel filtration

Crude saline extract of sparganum was fractionated by gel filtration through 1.6(ϕ)×40 cm Sephacryl S-300 Superfine at flow rate of 30 ml•cm^{-2}•hour. Eluents were divided into peaks by absorbance at 280 nm. Protein contents in each fraction were measured. Each fraction was dialysed separately in 0.05 M phosphate buffered saline(PBS, pH 7.4) containing 0.01% merthiolate (W/V) for 24 hours and lyophilized to concentrate protein contents to over 1.0 mg/ml. Standard proteins of blue dextran (M.W. 2,000,000), thyroglobulin (M.W. 669,000), alcohol dehydrogenase (M.W. 150,000), bovine serum albumin (M.W. 66,000) and cytochrome C (M.W. 12,400) (Sigma, USA) were also filtrated through the gel to estimate the molecular weight of the fractions.

3. Observation of protein composition by disc-PAGE and SDS-PAGE

Non-denaturing discontinuous polyacrylamide gel electrophoresis (disc-PAGE) was done as described by Davis (1964) to observe the protein compositions in the crude saline extract and its fractions. Disc-PAGE was done using 10% separating gel and 3% stacking gel. SDS-PAGE of Laemmli(1970) was done in reducing condition to observe subunits of the proteins in the crude extract and its fractions. SDS-PAGE was done using 3% stacking gel and 10~15% linear gradient separating gel. Samples were heated at 95°C for 5 minutes with sample buffer containing 0.4% SDS, 10% 2-mercaptoethanol and 0.05% bromophenol blue.

4. Preparation of monoclonal antibody

BALB/c mice were immunized with the crude saline extract as Kim *et al.*(1986) did. Spleen cells were hybridized with plasmacytoma cells of SP 2/0 according to Cha *et al.*(1984). Dispensed cells of hybridization were incubated at 5~10% CO_2 incubator in HAT medium. Antibody secreting colonies were selected by testing culture media for sparganum-specific (IgG) antibody by ELISA (Kim *et al.*, 1984). Antibody-secreting colonies in HT media were dispensed again by limiting dilution. Monoclonal antibody-secreting wells were screened again by ELISA. Monoclonal antibody secreting cells were expanded in BALB/c mice peritoneum. IgG fraction in mice ascites was isolated by filtering through Protein A-Sepharose CL-4B column(Sigma, USA).

5. Immunoblot

Western blot was undertaken according to the technique of Tsang *et al.*(1983). Separated proteins in the gel were transferred to nitrocellulose paper by electrophoresis at 100 V for 1 hour at 4°C. Monoclonal antibodies were reacted for 1 hour on a shaker. After washing the papers, peroxidase-conjugated anti-mouse IgG (Cappel, USA) was reacted in 1 : 1,000 dilution for 1 hour. After washing, reacting bands were stained with 3,3′-diaminobenzidine.

6. Immunoaffinity chromatography

Affinity chromatography was carried out as de-

scriptions in Pharmacia Fine Co(1983). A total of 50 mg of monoclonal antibody was coupled with 10 ml of CNBr-activated Sepharose 4B(Pharmacia). Uncoupled sites were blocked by 1 M ethanolamine. Crude saline extract and Fraction 3 of gel filtration were charged to the column at flow rate of 10 ml/hour, respectively. Each elution was monitored by UV-monitor until absorbance at 280 nm was lowered below 0.002. After washing, desorption buffer (glycine-HCl, pH 2.5) was eluted to separate the bound protein.

7. Evaluation of the antigenicity

By serologic test of ELISA(Kim *et al.*, 1984), sparganum-specific (IgG) antibody levels were measured in human sera to evaluate the antigenicity of the different antigenic preparations. Tested human sera were 28 sparganosis, 27 cysticercosis, 3 diphyllobothriasis, 8 *Taenia saginata* infections, 16 paragonimiasis, 15 clonorchiasis, 4 fascioliasis, 12 neurologic diseases other than parasitic diseases and 14 normal control. Antigens were coated to polystyrene plate wells in protein concentration of 2.5 μg/ml. Serum was diluted in 1 : 100 with PBS. Peroxidase-conjugated anti-human IgG(heavy- and light-chain specific) (Cappel, USA) was diluted in 1 : 1,000. Absorbance of 0.22 was used as cut-off value of positive reaction.

RESULTS

1. Protein composition in crude saline extract of sparganum

Proteins in non-denaturing disc-PAGE were shown in Fig. 2(Lane C). The exact number of protein bands were hardly countable but at least 15 bands were identified. In SDS-PAGE of the crude extract, 30 stained bands were represented. The bands were 158, 130, 107, 87, 78, 72, 67, 60, 58, 55, 53, 52, 43, 41, 39, 36, 32, 29, 26, 23, 22, 21.5, 21, 18, 17, 15, 14, 13.5, 9 and 6 kDa respectively. Of them, 87, 78, 52, 36, 29, 23, 21, 15, 14, and 6 kDa bands were major bands.

2. Fractionation of the proteins in the crude extract

By gel filtration, 5 fractions were obtained (Fig. 1). Calculated mean molecular weight of each fraction was: 270 kDa in Fraction 1, 90 kDa in Fraction 2, 36 kDa in Fraction 3, lower than 10 kDa in Fractions 4 and 5 (Fig. 1).

Fig. 2 shows the findings of non-denaturing disc-PAGE in 5 fractions. Each fraction did not consist of a single protein band but of multiple. Fig. 3 shows the findings of reducing SDS-PAGE in 5 fractions. Fraction 1 consisted of subunits of higher molecular weight over 60 kDa. Fraction 2 had subunits of Fractions 1 and 3; but main bands were 87, 78, 52, 21 kDa bands and bands of lower than 14 kDa. Main bands in Fraction 3 were 36, 29 and 23 kDa. Fraction 4 and 5 shows only faintly stained bands of molecular weight lower than 14 kDa.

3. Preparation of monoclonal antibody

Out of 192 wells of dispensed hybrid cells, 71 wells showed cell growth in 10 days. Of them, culture media in 20 wells showed significant activity of sparganum-specific (IgG) antibody. Of them, hybrids in 6 wells were selected and dispensed by limiting dilution. By this way, 14 strains of monoclonal cell lines were kept. Of them, 7 strains secrete significant amount

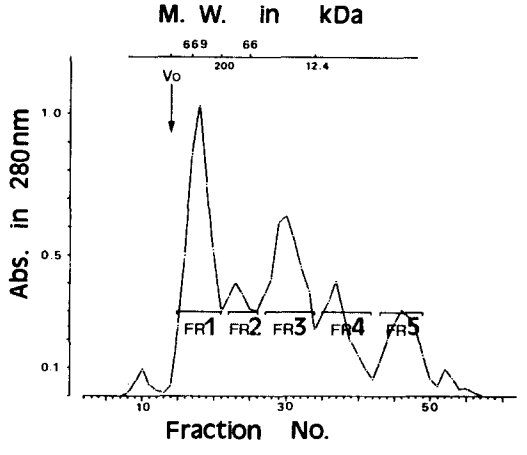

Fig. 1. Fractionation of crude saline extract of sparganum through Sephacryl S-300 Superfine. 1.6(φ)×40 cm sized column was used. Eluent: 0.15 M PBS containing 0.01% merthiolate. Flow rate: 30 ml·cm^{-2}·hour. Collection volume: 35 drops/tube.

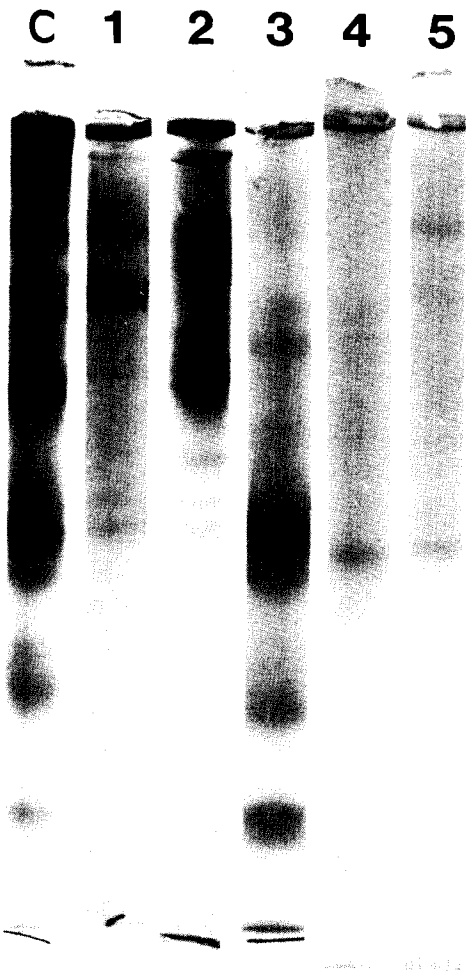

Fig. 2. Disc-PAGE findings of the crude and fractions at 10% gel concentration. C: Crude extract,
1: Fraction 1, 2: Fraction 2,
3: Fraction 3, 4: Fraction 4,
5: Fraction 5.

of antibody(SP-3-1, SP-37-1, SP-41-3, SP-53-2, SP-53-6, SP-53-118, SP-53-23, SP-56-13). The monoclonal antibodies were harvested in mice ascites.

When SDS-PAGE/immunoblot was done, monoclonal antibody of SP-3-1 reacted to 2 bands of 36 and 29 kDa weakly, SP-41-3 reacted to 72 kDa, SP-37-1 to bands of higher molecular weight over 100 kDa. Monoclonal antibody of SP-53-2, SP-53-18, SP-53-6 reacted to 29 and 36 kDa bands strongly (Fig. 4).

4. Purification of antigenic protein by immunoaffinity chromatography

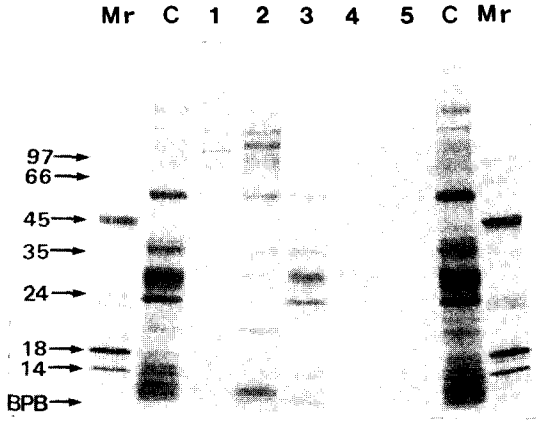

Fig. 3. SDS-PAGE findings of the crude and fractions. Ten-15% linear gradient gel was used for protein separation. M_r: Molecular weight in kDa, C, 1-5: Same as described in Fig. 2.

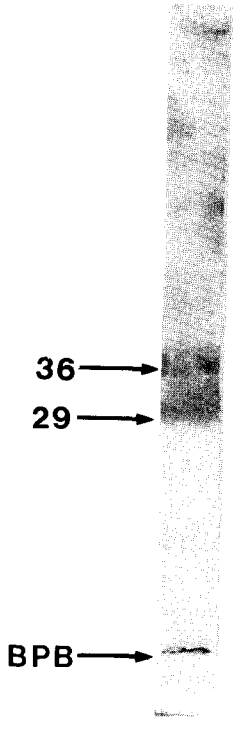

Fig. 4. SDS-PAGE/immunoblot finding of SP-53-2 monoclonal antibody to sparganum antigen.

Based on the above results, we selected the monoclonal antibody SP-53-2 as a ligand antibody in immunoaffinity chromatography. Fig. 5 shows the elution profile of affinity chromato-

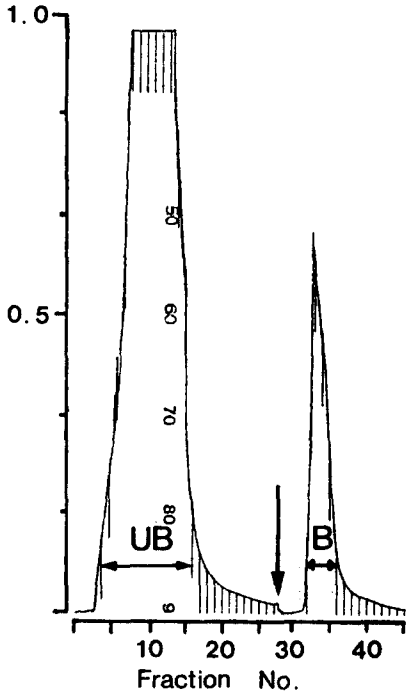

Fig. 5. Elution profile of immunoaffinity chromatography.
UB: Unbound protein, B: Bound protein.

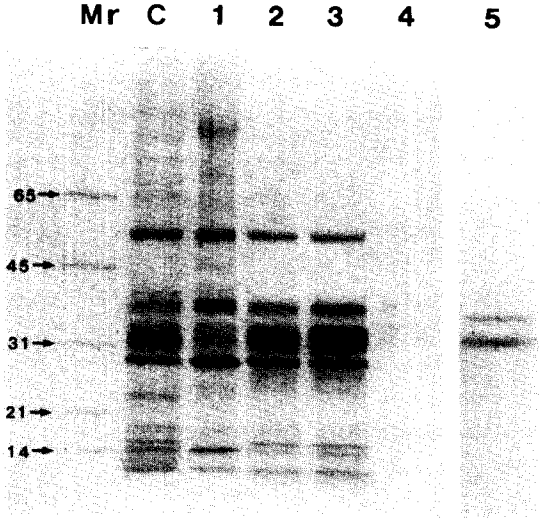

Fig. 6. Finding of SDS-PAGE of the crude and purified proteins.
M_r: Molecular weight in kDa,
C: Crude extract, 1: UB of crude extract, 2: Fraction 3, 3: UB of Fraction 3, 4: B of crude extract, 5: B of Fraction 3.

Table 1. Mean absorbance for sparganum-specific IgG antibody in human sparganosis and other conditions against the crude and processed antigens of sparganum

Category	No. of cases	Mean absorbance and standard deviations for antigen of								
		crude	UB* crude	Fr. 1	Fr. 2	Fr. 3	UB Fr. 3	BP** Fr. 3	Fr. 4	Fr. 5
Sparganosis	28	0.75 ±0.29	0.84 ±0.41	0.61 ±0.27	0.68 ±0.26	0.64 ±0.35	0.62 ±0.33	0.53 ±0.26	0.45 ±0.18	0.25 ±0.13
Cysticercosis	27	0.20 ±0.09	0.25 ±0.12	0.19 ±0.08	0.16 ±0.08	0.16 ±0.09	0.16 ±0.10	0.14 ±0.07	0.11 ±0.05	0.08 ±0.04
Diphyllobothriasis	3	0.12 ±0.05	0.18 ±0.09	0.17 ±0.07	0.11 ±0.05	0.11 ±0.03	0.10 ±0.03	0.09 ±0.02	0.08 ±0.01	0.06 ±0.01
T. saginata infection	8	0.09 ±0.03	0.13 ±0.04	0.11 ±0.04	0.08 ±0.03	0.06 ±0.02	0.07 ±0.03	0.06 ±0.01	0.06 ±0.01	0.07 ±0.02
Paragonimiasis	16	0.10 ±0.04	0.13 ±0.07	0.14 ±0.04	0.08 ±0.03	0.07 ±0.03	0.06 ±0.03	0.05 ±0.03	0.08 ±0.03	0.09 ±0.04
Clonorchiasis	15	0.14 ±0.10	0.19 ±0.12	0.17 ±0.09	0.12 ±0.04	0.09 ±0.04	0.07 ±0.04	0.06 ±0.02	0.10 ±0.04	0.10 ±0.04
Fascioliasis	4	0.18 ±0.12	0.23 ±0.13	0.38 ±0.23	0.16 ±0.08	0.09 ±0.03	0.05 ±0.03	0.06 ±0.03	0.10 ±0.03	0.14 ±0.07
Neurological diseases	12	0.09 ±0.02	0.10 ±0.03	0.11 ±0.02	0.09 ±0.02	0.09 ±0.02	0.08 ±0.02	0.07 ±0.02	0.10 ±0.03	0.14 ±0.03
Normal	14	0.04 ±0.01	0.05 ±0.02	0.03 ±0.01	0.03 ±0.01	0.03 ±0.01	0.04 ±0.01	0.04 ±0.01	0.04 ±0.01	0.04 ±0.01

*UB: Unbound protein in immunoaffinity chromatography
**BP: Bound protein in immunoaffinity chromatography (the purified antigen)

Table 2. Comparison of sensitivity and specificity in 28 sparganosis and 99 other conditions in different antigens

	Percent sensitivity and specificity of								
	Crude	UB crude	Fr. 1	Fr. 2	Fr. 3	UB Fr. 3	BP Fr. 3	Fr. 4	Fr. 5
Sensitivity	96.4	100	96.4	96.4	96.4	96.4	96.4	89.3	35.7
Specificity	86.8	75.4	77.7	84.8	91.9	92.9	96.9	92.3	94.3

graphy when the crude extract was charged. Fraction 3 was also used in the purification. The quality of purified protein was analysed by SDS-PAGE as shown in Fig. 6. Bound protein to the monoclonal antibody was stained weakly to Coomassie brilliant blue R-250 but evidently composed of 2 bands of 29 and 36 kDa.

5. Evaluation of antigenicity

By ELISA, sera from human sparganosis, other parasitic/neurologic patients and normal control were tested for their sparganum-specific (IgG) antibody levels. Antibody levels as shown by mean absorbance was the highest in unbound protein of the crude extract not only in human sparganosis but also in other conditions. The bound protein of crude extract and Fraction 3 showed comparable absorbance with that of Fraction 3. Fraction 4 and 5 showed far lower mean absorbance(Table 1).

High antibody levels to sparganum antigen in patients sera other than sparganosis were observed in cysticercosis, diphyllobothriasis and fascioliasis. Antigens of the crude extract, unbound crude extract and Fraction 1 showed higher antibody levels in patients sera of other conditions(Table 1).

Table 2 summarized the results of sensitivity and specificity evaluation of each antigenic preparation. Except for Fraction 4 and 5, sensitivity was satisfactory in all antigenic preparations. In view of specificity, purified protein by immunoaffinity chromatography showed the highest value(96.9%) while the lowest was observed in unbound fraction of the crude extract(75.4%).

DISCUSSION

From this study it is clear that protein of 36 and 29 kDa were the most sensitive and specific components as antigen out of many proteins in the crude saline extract of *Spirometra mansoni* plerocercoid. In the previous study of immunoblot of the crude extract of sparganum with patients sera (Choi *et al.*, 1988), the two protein bands of 36 and 29 kDa were the most sensitive and strong antigen. Though strong antigen they are, there were many other strong antigenic proteins in the crude extract. Accordingly antibodies to 36 and 29 kDa protein were only parts of different polyclonal antibodies in patients sera. Therefore, the antibody levels as measured by ELISA was not high enough when compared with that by the crude extract. Also decreased levels of antibody were observed in fractionated antigens probably because of the same reason.

The quality improvement achieved by purification processes was recognized in aspect of specificity rather than in sensitivity. As shown in the Tables, cross-reactive antigens in the crude extract were the proteins of higher molecular weight because the cross-reactions were most frequent in Fraction 1 and 2. By removing these proteins of high molecular weights, non-specific positive reactions can be reduced.

Most of false positive reactions in cysticercosis patients became negative when the purified antigen was used as antigen, because cross-reactions with cysticercosis were more frequent with the higher molecular weight proteins in the crude extract such as 158, 143, 87, 78 and 52 kDa than with those of low molecular weights (Choi *et al.*, 1988). However, some of cysticercosis patients sera reacted to 36 and 29 kDa proteins as shown by Choi *et al.*(1988). Therefore, the cross-reactions in cysticercosis

do not totally disappear even when the purified antigen is used. In this respect, the clinical nature of cysticercosis patients whose serum reacted crossly with 36 and 29 kDa of sparganum antigen should be studied in the future. The possibility of racemose form of cysticercus can not be completely ruled out yet. Actually, histopathologic findings of racemose cysticercosis are often confused with that of sparganosis (Beaver and Rolon, 1981).

Another problem in this study is that monoclonal antibody react two bands in SDS-PAGE rather than one band. Accordingly, in affinity chromatography, two bands of 36 and 29 kDa are purified. This consistent finding suggest the presence of same antigenic determinant in the different proteins. This should be proved by repeated limiting dilution of the antibody secreting hybridoma cells. However, up to present, our data show that the two bands in SDS-PAGE share common antigenic determinant.

The present result made a study of histologic/cytologic localization of antigenic protein in sparganum possible. And improved quality of the purified antigen also made it possible to differentiate sparganosis from other parasitic infections more definitely by means of antibody test.

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=국문초록=

단세포군항체를 이용한 친화성 크로마토그래피에 의한 스파르가눔 항원의 순수분리

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조직기생충증인 스파르가눔증은 우리 나라를 비롯하여 동양 여러 나라에서 드물지 않게 발생하고 있으므로 보조진단법으로서 혈청학적 진단이 필요하다. 그러나, 현재 진단에 사용하는 항원인 스파르가눔의 생리식염수 추출액은 그 단백질 구성이 복잡하고, 낭미충증, 포충증, 폐흡충증 등과 혈청학적 교차반응을 일으키는 경우가 있어 개선의 여지가 많다. 이 실험은 스파르가눔 생리 식염수 추출액의 구성단백질 중 항원성이 높고, 교차반응이 적은 구성단백질 분획만을 단세포군항체를 이용하여 친화성 크로마토그래피로 분리하고 그 항원성을 평가하고자 하였다. 먼저 Sephacryl S-300 Superfine을 통과시켜 스파르가눔 생리 식염수 추출액을 5 분획으로 나눈 결과, 분자량이 90 kDa 이상으로 구성된 제1, 2 분획은 항원성은 높았으나 교차반응을 많이 일으키고 있었다. 제 3 분획의 주요 구성성분은 스파르가눔증 환자 혈청과의 SDS-PAGE/EITB를 통해 가장 민감한 반응을 보였던 36, 29 kDa 단백질이었다. 이어 스파르가눔 추출액으로 면역시킨 BALB/c mice 비장세포에서 기원한 단세포군항체 중 36, 29 kDa에 반응하는 단세포군항체를 리간드로 친화성 크로마토그래피를 실시하여 36, 29 kDa 단백질을 순수하게 분리하였다. 이 두 단백질은 같은 항원결정기를 지니고 있는 것으로 판단하였다. 순수분리한 항원의 항원성을 스파르가눔 감염자 28명과 기타 질환자 및 건강 대조군 99명의 혈청으로 평가한 결과, 이 항원은 조항원(스파르가눔 추출액)에 비해 민감도는 같았으나(96.4%) 특이도는 86.8%에서 96.9%로 개선되었다.

〔기생충학잡지, 28(3):135-142, 1990년 9월〕