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Single step purification of potent antigenic protein from sparganum by gelatin-affinity chromatography

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Abstract: Out of many component proteins in crude saline extract of *Spirometra mansoni* plerocercoid (sparganum), 36 kDa and 29 kDa proteins were found to be the most antigenic and were already purified by immunoaffinity chromatography using monoclonal antibody as a ligand. In this study, a single step purification of these potent antigenic proteins of sparganum extract was investigated. When the crude saline extract was charged to gelatin-Sepharose 4B affinity column, 36 kDa and 29 kDa protein fractions were bound. SDS-polyacrylamide gel electrophoresis(PAGE) and SDS-PAGE/immunoblot confirmed that the bound protein to gelatin was serologically pure. When evaluated by ELISA with patients sera, the purified protein of 36 and 29 kDa also showed improved antigenicity.

Key words: *Spirometra mansoni* plerocercoid (sparganum), human sparganosis, serodiagnosis, protein-antigen, protein purification, gelatin-Sepharose 4B affinity chromatography

INTRODUCTION

Human sparganosis, caused by tissue invading plerocercoid larva of *Spirometra mansoni* is manifested usually by migrating granulomatous lesions(Cho *et al.*, 1975; Cho, 1987). Though frequently found in subcutaneous tissue, it is a potential systemic infection. Recently, sparganosis of the central nervous system attracted much attentions world-widely because of its scattered discoveries (Mineura and Mori, 1980; Anders *et al.*, 1984; Chan *et al.*, 1987; Fan and Pezeshkpour, 1986; Chang *et al.*, 1987; Anegawa *et al.*, 1989).

For correct diagnosis of cerebral sparganosis, antibody test by ELISA (Kim *et al.*, 1984), characteristic findings of brain CT (Chang *et al.*, 1987) together with clinical history and manifestations are available. Of them, serologic diagnosis of sparganosis is directed to detect the sparganum-specific(IgG) antibody. However, in spite of its high sensitivity, non-specific cross reactions with other diseases caused by taxonomically related and/or unrelated parasites often confuse the correct diagnosis. For example, non-specific positive reactions with paragonimiasis, cysticercosis and hydatidosis (Choi *et al.*, 1988; Kim and Yang, 1988) are observed. In this respect, antibody test for sparganosis needs the improved antigen.

Recently, Choi *et al.* (1988) identified the 36 and 29 kDa proteins in crude saline extract of sparganum were the most sensitive antigenic component when observed by SDS-PAGE/immunoblot. Eventually, by immunoaffinity chromatography using a monoclonal antibody, these proteins were purified(Cho *et al.*, 1990). And improved quality of the purified antigen

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was achieved especially in aspect of specificity.

Though immunoaffinity chromatography using a monoclonal antibody is powerful to purification, it is expensive, low-yield, time-consuming and complicated processes in itself. In the present study, therefore, we report a simple and novelette affinity chromatography using gelatin as a ligand in purifying the 36 and 29 kDa proteins from crude saline extract of sparganum.

MATERIALS AND METHODS

1. Crude saline extract of sparganum

The crude extract was prepared as described by Kim *et al.* (1984). Worms were collected from muscle of naturally infected *Elaphe rufodorsata*; they were washed 3 times in saline to remove host tissue debris. The worms were emulsified by teflon-pestle tissue homogenizer in physiologic saline containing 0.006% (W/V) phenylmethylsulfonyl fluoride (PMSF) and 0.01 % (W/V) merthiolate. The homogenate was shaken for 2 hours and kept overnight. Centrifuged by 45,000 *g* for 1 hour followed by 3,500 *g* for 15 minutes, the resulting supernatant was used as crude saline extract of sparganum (de Duve, 1975). All procedures were done at 4°C. Protein content of the crude extract was 6.6 mg/ml by Lowry *et al.* (1951).

2. Gelatin-affinity chromatography

(1) Coupling of gelatin to CNBr-activated Sepharose 4B

The methods described in handbook of Pharmacia Fine Co. (1983) was adopted. After swelling and washing in 1 mM HCl, a total of 5 g of CNBr-activated Sepharose 4B was equilibriated with coupling buffer (0.1 M $NaHCO_3$, 0.5 M NaCl, pH 8.3). After 50 mg of gelatin (Difco, USA, Lot No. 745361) was dissolved in 20 ml of coupling buffer at 50°C, the activated Sepharose 4B was added. The mixture was incubated for 2 hours at 25°C on shaker. Then, uncoupled sites of CNBr-activated Sepharose 4B were blocked by 0.1 M Tris-HCl (pH 8.0). Gelatin coupled Sepharose 4B was washed 3 times in coupling buffer (pH 8.3) and sodium acetate buffer (0.1 M Na•acetate, 0.5 M NaCl, pH 4.0), alternatively. Finally the media was equilibriated with PBS (0.15 M, pH 7.4).

(2) Protein elution on gelatin-Sepharose 4B column

In affinity chromatography, general procedures described by Rouslahti *et al.* (1983) were followed at room temperature. A total of 50 mg of protein in sparganum extract was charged on the column, and the unbound protein was filtrated. After washing the column with 20 bed volumes of PBS, the bound protein was eluted with the same buffer containing 4 M urea and 0.1 M NaCl. Immediately after the elution, the protein was dialyzed against PBS (0.15 M, pH 7.4) for 72 hours at 4°C.

3. Visualization of protein by SDS-PAGE

Reducing SDS-PAGE was done according to Laemmli (1970). Stacking gel was 3% and separating gel was 10~15% linear gradient gel. Samples were treated at 95°C for 5 minutes with the same amount of sample buffer containing 0.4% SDS, 10% 2-mercaptoethanol and 0.05% bromophenol blue. Electrophoresis was carried out at 30 mA constant current. The gel was stained and destained with 0.125% Coomassie brilliant blue R-250 and methanol/acetic acid solution.

4. Western blot analysis

The method of Tsang *et al.* (1983) was adopted. After SDS-PAGE of the crude extract, unbound and bound proteins in gelatin affinity chromatography, the separated protein bands were transferred onto nitrocellulose paper by electrophoresis at 100 V for 2 hours at 4°C. After washing in saline/Tween 20 solution, a 1:100 diluted confirmed sparganosis serum was reacted for 1 hour. Peroxidase conjugated anti-human IgG (heavy- and light-chain specific, Cappel, USA) was reacted for 1 hour in 1:1,000 dilution. Finally, the color was developed by chromogen (50 mg of 3,3′-diaminobenzidine, 10 μl of 30% H_2O_2 dissolved in 99 ml of distilled water). A piece of nitrocellulose paper containing the crude extract was stained with amido

black B (0.1% in 45% methanol/10% acetic acid) and destained with 90% methanol/2% acetic acid according to Towbin *et al.* (1979).

5. Antigenicity evaluation

The antigenicities of the purified protein, unbound protein and the crude extract were evaluated for sparganum specific (IgG) antibody by serologic test of ELISA (McLaren *et al.*, 1978; Kim *et al.*, 1984). Subjected sera were 27 sparganosis, 21 cysticercosis, 3 diphyllobothriasis, 10 *T. saginata* infections, 16 paragonimiasis, 10 clonorchiasis, 4 fascioliasis and 12 neurologic diseases of other causes. Fifteen normal human sera were also used in the study. In determining the sensitivity and specificity of the respective antigens, a cut-off absorbance of 0.22 was used as in Kim *et al.* (1984).

RESULTS

1. Protein composition of the purified protein

Banding patterns of proteins on reducing SDS-PAGE were shown in Fig. 1 using the crude extract (lane C), the unbound (lane U) and the purified proteins (lane B). The crude extract revealed at least 30 bands while the purified protein exhibited only 2 bands of 36 and 29 kDa. Unbound protein fraction from gelatin-affinity chromatography showed decreased densities at location of 36 and 29 kDa bands.

2. Findings in western blot

As shown in Fig. 2, a sparganosis patient serum reacted multiple bands to the crude extract (lane C) and the unbound protein fraction (lane U) whereas only 2 bands of 36 and 29 kDa were reacted to the purified protein (lane B). Decreased reactions at 36 kDa and 29 kDa bands were also observed in lane U.

3. Antigenicity of the purified protein

The antigenicity of the purified protein was evaluated for its sparganum specific (IgG) antibody levels by ELISA. Patients' sera of sparganosis, other cestodiases, trematodiases

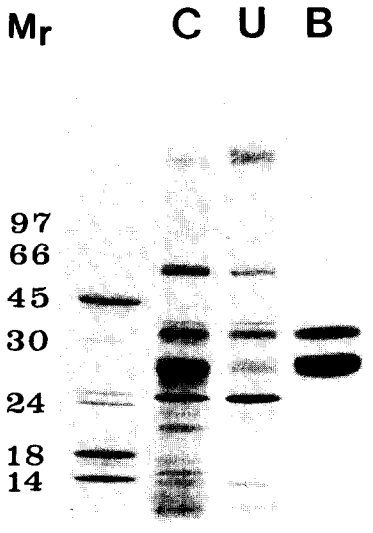

Fig. 1. Findings on reducing SDS-PAGE of sparganum extract. For protein separation, 10~15% linear gradient gel was used.
M_r: Molecular weight in kDa, C: Crude extract U: Unbound protein, B: Bound protein

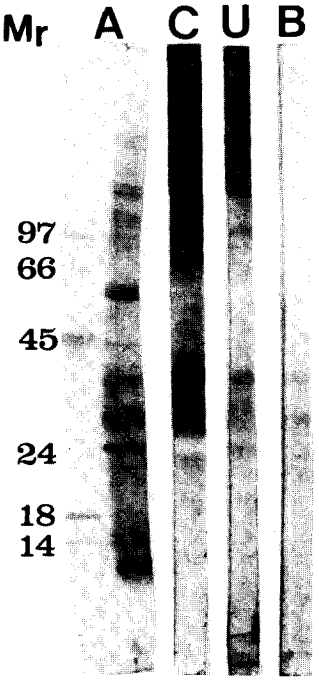

Fig. 2. Western blot analysis of antigenicity of the crude extract (C), unbound protein(U) and bound protein(B) using a patient serum of surgery confirmed sparganosis. M_r: Molecular weight in kDa. A: Amido black B stained nitrocellulose strip of crude extract.

Table 1. Mean absorbance and standard deviations of sparganum-specific IgG antibody by ELISA in human sparganosis and other conditions against crude and purified sparganum antigens

Patient category	No. of cases	Mean absorbance & standard deviations for antigen		
		Crude	Unbound	Bound
Sparganosis	27	0.72±0.29	0.74±0.30	0.64±0.25
Cysticercosis	21	0.15±0.09	0.14±0.09	0.09±0.06
Diphyllobothriasis	3	0.14±0.05	0.15±0.08	0.10±0.05
T. saginata infection	10	0.11±0.05	0.14±0.08	0.08±0.03
Paragonimiasis	16	0.11±0.05	0.13±0.05	0.09±0.03
Clonorchiasis	10	0.09±0.06	0.13±0.06	0.06±0.03
Fascioliasis	4	0.23±0.13	0.30±0.17	0.15±0.06
Neurologic diseases	12	0.10±0.02	0.13±0.03	0.08±0.01
Normal control	15	0.03±0.01	0.04±0.01	0.03±0.01

and neurologic diseases of other causes were used. As presented in Table 1, the mean absorbance of the bound protein was lower than those of both the crude extract and unbound fraction in sparganosis patients. The mean absorbance of the bound protein for other cestodiases, trematodiases, other neurologic diseases and normal control were also lower than those of the crude extract and the unbound protein. The mean absorbance were al most invariably same with or slightly higher in the unbound protein than in the crude extract.

Sensitivity in sparganosis and specificity in other conditions were summarized in Table 2 when 3 different antigens were used. The sensitivity of the purified protein was same with that of the crude extract while the specificity was increased from 89.0% to 95.6%.

Table 2. Comparison of sensitivity and specificity of the purified antigen in 27 sparganosis and 91 other conditions

Sensitivity/ Specificity	Percent sensitivity and specificity for antigen of		
	Crude	Unbound	Bound
Sensitivity	92.6	96.3	92.6
Specificity	89.0	82.4	95.6

DISCUSSION

In this study, we could isolate the 36 and 29 kDa protein from crude saline extract of sparganum by gelatin affinity chromatography instead of immunoaffinity chromatography using a monoclonal antibody (Cho *et al.*, 1990). When the purified proteins were evaluated by SDS-PAGE/immunoblot, no other antigenic protein bands were reacted. Antibody test by ELISA using the purified protein showed improved antigenicity.

Gelatin affinity chromatography has been widely used for fibronectin purification (Engvall and Ruoslahti, 1977; Ruoslahti *et al.*, 1982; Gelder and Brown, 1987). As a dimer glycoprotein of molecular weight of 450 kDa (subunit 220 kDa), fibronectin distributed ubiquitously in mammalian cell surface (cellular fibronectin) and plasma (plasma fibronectin) (Yamada and Olden, 1978; Yamada, 1980; Engvall, 1985). Because of its adhering capacity, cellular fibronectin plays a critical role for collagen organization (McDonald *et al.*, 1982) and chemotaxis (Engvall, 1985). It can also interact with proteoglycan, fibrin and bacterial cell wall (Engvall, 1985). On the other hand, circulating plasma fibronectin serves as a non-specific opsonin that promote the removal of collagen-containing cells and plays a role in cellular cytotoxicity of natural killer cells (Katzman *et al.*, 1987). The possible purification of the 36 and 29 kDa protein from sparganum extract by gelatin ligand did not directly refer that the protein was a fibronectin in nature. Our purified protein had different molecular weight from the

classical mammalian fibronectin. Therefore, the protein was not the same with mammalian fibronectin although it seemed to possess the adhering property to collagen/gelatin as in other fibronectinoid proteins. There have been many reports which showed the presence of fibronectin-like proteins in parasites. For example, *Taenia solium* metacestodes has fibrinectin-like proteins of 95 and 85 kDa which are bound to collagen (Plancarte *et al.*, 1982; Laclette *et al.*, 1990) while *Trypanosoma cruzi* has 68 and 58 kDa proteins which are assumed to be important in entering host cells in the initial phase of infection(Velge *et al.*, 1988). The exact role and function of 36 and 29 kDa proteins produced by sparganum are not known yet especially in its host-parasite relationship.

It is interesting that cysteine protease of sparganum has a comparable molecular weight with the purified protein in this study. Fukase *et al.*(1986) identified the 38~19 kDa cysteine protease from sparganum. In addition, protease of 28 kDa was found in excretory secretory product of sparganum (Song and Choi, 1990). Though the molecular weight of the proteases are not exactly same with the present protein, it is quite possible that these proteins share fibronectin-like properties. The roles of this protease are well known in aspect of degradation of collagen and hemoglobin, and provoking inflammatory processes in pathogenesis of *Schistosoma monsoni* infections (Mckerrow *et al.*, 1985; Dresden *et al.*, 1985; Cappell and Dresden, 1987; Mckerrow, 1989). Further studies are needed to elucidate the relationships between the fibronectinoid property and protease activity in this sparganum protein.

This study made it easier to investigate the physiologic, pathogenetic and immunologic roles of the highly antigenic protein in sparganum in the future by opening a simple, single step purification process.

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=국문초록=

젤라틴 친화성 크로마토그래피를 이용한 스파르가눔 성분단백질의 순수분리

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스파르가눔 생리식염수 추출액 내에 포함되어 있는 성분단백질 중 스파르가눔증 환자 혈청내 특이 IgG 항체와 민감하고 특이하게 반응하는 항원단백질인 36, 29 kDa 단백질을 단세포군 항체를 이용한 면역친화성 크로마토그래피로 순수분리할 수 있음은 이미 보고하였다. 이 연구에서는 스파르가눔 추출액 내에 포함된 이 36, 29 kDa 단백질이 젤라틴을 고리로 한 친화성 크로마토그래피로 훨씬 쉽게 순수분리할 수 있음을 증명하고자 하였다. 젤라틴을 고리로 부착시킨 Sepharose 4B column에 스파르가눔 추출액을 통과시키고 젤라틴에 부착한 단백질은 4 M urea/0.1 M NaCl 용액을 분리완충액으로 분리하였다. 이렇게 분리한 단백질은 SDS-PAGE에서 36, 29 kDa band로 구성되어 있었고, SDS-PAGE/immunoblot 결과 환자의 polyclonal 항체는 이들 band에만 반응하였다. 스파르가눔증, 기타 기생충증 환자 및 건강대조군 혈청내 스파르가눔 특이항체가(IgG)를 면역효소측정법으로 측정한 결과 순수분리한 이 단백질은 특히 특이도가 95.8%로 생리식염수 추출액의 89%보다 우수하였고 민감도는 차이가 없었다.

이상의 결과는 젤라틴을 고리로 이용한 친화성 크로마토그래피는 스파르가눔 생리식염수 추출액 내의 36 및 29 kDa 단백질을 간편하게 순수분리할 수 있고 단백질의 항원성도 유지할 수 있음을 보이고 있었다.

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