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Isolation and Characterization of Immunomodulatory Glycoprotein from the Root of *Panax ginseng*

Han Jae Shin, Kyeong Mee Park, Young Sook Kim, Ki Yeul Nam, You Hui Lee and Jong Dae Park#

Korea Ginseng and Tabacco Research Institute, Taejon 305-345, Korea

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Abstract : A high molecular (more than 10 kDa) fraction, showing mitogenic and comitogenic activities in spleen cells of mouse, was isolated from water extract of ginseng. The crude protein substance prepared by 80% $(NH_4)_2SO_4$ precipitation from this fraction was purified and isolated by DEAE Sepharose column chromatography. Among the fractions eluted, it was found that four kinds of fractions eluted with 0 to 1 M NaCl gradient were glycoproteins, which induced proliferation of spleen cells and increased NO production in macrophages. Among them, F-2 fraction, which contained 35.9% protein, 49.4% neutral sugar and 12.5% uronic acid, was found to show mitogenic activity as strong as that of LPS (lipopolysaccharide) at a concentration of 100 μg/m*l* and to remarkably stimulate NO production by murine macrophages at a concentration of 500 μg/m*l*. When F-2 is deproteinized, the mitogenic activity of F-2 was decreased significantly to 70.9% as compared with that of F-2. This results suggests that the protein moiety of F-2 may play an important role in immunomodulating activity of glycoprotein from the root of *Panax ginseng*.

Key words : *Panax ginseng*, glycoprotein, mitogenic activity, macrophage, nitric oxide.

INTRODUCTION

Throughout the years, a number of compounds including ginsenosides, phenolic compounds, alkaloids, fatty acids, esters, polyacetylenes and polysaccharides, have been isolated and elucidated structurally from *Panax ginseng* and found to show various kinds of biological activities. Among them, ginseng saponins and polysaccharides have been long reported to modulate immune responses.[1-7] Recently, it has been reported that nitrogenous compounds have been isolated from non-saponin fraction of ginseng, showing various biological effects. In addition, a number of amino acid derivatives and peptides have been isolated from ginseng roots in China and Japan.[8] Especially an inhibitory neurotransmitter, γ-aminobutyric acid (GABA), a neuroexcitotoxic and hemostatic nonprotein amino acid, β-oxalo-L-α, β-diaminopropionic acid were isolated and their chemical structures identified.[9,10] A recent survey showed that adenosine and pyroglutamic acid from red ginseng, steamed and dried root of fresh ginseng, were known to inhibit epinephrine-induced lipolysis in fat cell and stimulate the insulin-mediated lipogenesis.[11,12] Tetrapeptide from roots of *Panax ginseng* was found to stimulate the proliferation of baby hamster kidney-21 cell[13] and the crude or purified glycoprotein fractions, to exhibit enhanced synthesis of protein and DNA in chicken brain cell as well as mitogenic activity in human peripheral blood lymphocytes and stimulatory activity on cultured brain spinal cord and skeletal muscle cells.[14-16] The protein isolated from white ginseng, peeled roots dried without steaming, showed radio-protective activity and enhanced DNA repair capacity of the UV damaged cells.[17,18] However, very few are known on the immunomodulatory and chemical properties of protein from ginseng. This result has led us to investigate in more detail in this regard.

In a continuation of search for non-saponin bioactive compounds from *Panax ginseng* (ginseng), we fractionated the water-soluble substance of ginseng and demonstrated the presence of a high molecular fraction which had mitogenic and comitogenic effect on mouse spleen lymphocytes. We further attempted to partially purify proteins from this crude protein substance and to characterize its chemical and immunological properties.

To whom correspondence should be addressed.
(Tel) 042-866-5534; (Fax) 042-861-1949
(E-mail) jdpark@gtr.kgtri.re.kr

MATERIALS AND METHODS

1. Reagents

Ginseng roots, *Panax ginseng* C.A. Meyer, were kindly given from Korea Ginseng Corporation (Taejon, Korea). DEAE Sepharose CL-6B was obtained from Pharmacia Fine Chemical (Uppsala, Sweden). Lipopolysaccharide (LPS), concanavalin A (Con A), carbazole, glucose, glucuronic acid, bovine serum albumin (BSA), 2-Mercaptoethanol (2-ME), 3-(4,5-Dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), protease and dialysis membranes were purchased from Sigma Chemical Co. (St. Louis, MO). Absolute alcohol and sulfuric acid were purchased from Fluka (Buchs, Switzerland). Earle's balanced salt solution (EBSS), HEPES, fetal bovine serum(FBS), L-glutamine, penicillin/streptomycine solution, RPMI 1640 medium were purchased from GIBCO (Grand Island, NY). All other chemicals and reagents used were of the best grade available.

2. Mice

Specific pathogen-free female BALB/c mice were purchased from Daehan Laboratory Animal Research Center Co. (Eumsong, Korea), weighing 25~30 g were used as the experimental animals. The animals were housed and maintained at 22±2°C with constant humidity (55±5%). They had free access to food and water until the beginning of the experiments.

3. General procedures

Total neutral cabohydrate, uronic acid and protein contents were determined by the phenol-sulfuric acid, carbazole, and Bradford, methods using glucose, glucuronic acid and bovine serum albumin as the respective standard.[19-21]

4. Fractionation of ginseng

The powder (100 g) of white ginseng was extracted with 3 volumes of water at 20 for 3 hours with 3 times. The water extract was filtered through gauze and centrifuged at 10,000×g for 20 minutes at 4°C. This procedure was twice repeated to eliminate the water insoluble precipitates. The supernatant was applied to Amicon stirred concentrator (Amicon, U.S.A.) using a YM-10 membrane and divided into two fractions, one fraction showing more than 10 kDa of molecular weight and the other fraction, less than 10 kDa.

5. Ion exchange chromatography of crude protein fraction

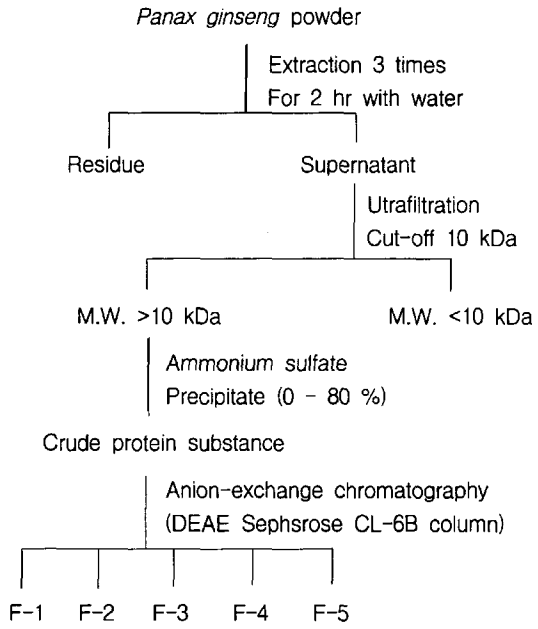
80% $(NH_4)_2SO_4$ saturation was done with the fraction having more than 10 kDa molecular weight at 4°C. The precipitate was collected by centrifugation (12000×g, 20 min) and the pellet was dissolved with minimum volume of distilled water. This solution was dialyzed against deionized water for four days by changing the water several times. The crude protein solution was obtained by centrifugation (12000×g, 20 min) from the dialysates. This crude protein substance prepared from more than 10 kDa fractions was applied onto a DEAE Sepharose column (2.5×25 cm, Pharmacia, Sweden), which had been equilibrated with 20 mM Tris-HCl buffer (pH 8.8). The non-adsorbed materials were obtained by washing the column with 5 volume of bed volume containing this buffer (F-1). Four fractions (F-2, F-3, F-4, F-5) were eluted with NaCl linear gradient (0 to 1 M) in the above described buffer, at a rate of 1 ml/min.

6. Preparation and incubation of splenocytes

Mice were sacrificed by cervical amputation and their spleens were removed using aseptic techniques. The spleens were minced and the cells were released from the spleen fragments by tapping with a flat end of sterile syringe plunger on a stainless mesh sheet. Cell suspension was stood for 10 minutes to precipitate the unbroken cell debris. Upper part of cell suspension was carefully transferred to a new centrifugal tube and centrifuged at 400×g for 10 minutes. The cell pellet was suspended in RPMI 1640 culture medium containing 10% FBS, 15 mM HEPES, 5×10^{-2} mM 2-ME, 2 mM glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. The cells were treated with 4 volumes of SRBC lysis buffer and incubated at 37°C. After 10 minutes, the total number of viable splenocytes were counted by the method of dye-exclusion test using 0.4% trypan blue solution. Spleen cells at a concentration of 2×10^5 cell/well were cultured in 96 well plastic culture plate with this medium containing various concentrations of test compounds with or without 1 μg/ml of concanavalin A (Con A) or 10 μg/ml lipopolysaccharide (LPS) at 37°C in a humidified 5% CO_2 -95% air incubator.

7. Determination of lymphocyte proliferation[22]

MTT was dissolved in PBS at 5 mg/ml, filtered to sterilize and remove a small amount of insoluble residue and



Scheme 1. Extraction and isolated of active protein fraction form the root of *Panax ginseng*.

stored at -20°C until required. Four hours prior to culture termination, the MTT solution (10 μl) was added to each well of the culture plate after removing 100 μl of each supernatant. The cells were then continuously cultured until culture termination. The culture was stopped by adding 15% SDS containing 0.01 N HCl solution to each well, and the optical density was then measured in an ELISA plate reader using a measure of 570 nm and a reference wavelength of 690 nm.

7. Measurement of NO production in murine macrophages

Murine macrophage cell line, RAW264.7, was obtained from Korean Cell Line Bank (KCLB, Seoul, Korea). RAW264.7 cells were maintained in 75 cm^3 plastic culture flasks in RPMI-1640 medium supplemented with 10 % FBS and then these cells were seeded in 96-well plastic culture plate at a concentration of 2×10^5 cell/well. After pre-incubation for 24 hours, the medium was replaced with fresh medium containing various concentrations of test compounds. Cells were incubated at 37°C in a 5% CO_2 incubator for 48 hours, and then supernatant medium was collected for measurement of nitric oxide (NO). Supernatants (70 μl) of culture media were incubated with an equal volume of Griess reagent at room temperature for 10 minutes in 96-well microplate.[23] Absorbances at 540 nm were read using an ELISA plate reader. Standard calibration curves were prepared using $NaNO_2$ as a standard.

8. Protease digestion

Based on the method of Yamada *et al.*,[24] F-2 was incubated with protease at 37 for 24 hours in 50 mM Tris-HCl (pH 7.9).

9. Statistical analysis of data

Data obtained from the pharmacological experiments were expressed as mean±S.D. Differences between the control and test groups in this experiments were measured for statistical significance by Student's t-test.

RESULTS AND DISCUSSION

1. Fractionation of water soluble fraction

For the purpose of search for immunomodulatory protein from the root of *panax ginseng*, water soluble fraction was divided into two fractions by ultrafiltration system, one fraction showing more than 10 kDa of molecular weight, and the other fraction, less than 10 kDa. We found that the high molecular fraction stimulated the proliferation of mouse spleen cells as shown in Table 1. In addition, this fraction also significantly augmented both B cell-specific (LPS-stimulated) and T cell-specific (Con A-stimulated) lymphocytes proliferation in the presence of LPS or Con A. In contrast, the low molecular fraction was not found to show mitogenic and comitogenic effect on spleen lymphocytes, indicating the high molecular fraction to be immunomodulatory active fraction.

2. Purification of active protein fraction

The crude protein substance was obtained by 80% ammonium sulfate precipitation from the high molecular

Table 1. Mitogenic and co-mitogenic effect of each water soluble fraction of ginseng on lymphocytes from normal BALB/c mice

Sample	Absorbance at 570 nm		
	No mitogen	+LPS	+Con A
Control (PBS)	0.19±0.11	1.01±0.09	1.06±0.05
High molecular fraction	0.35±0.03*	1.68±0.20**	1.58±0.10**
Low molecular fraction	0.22±0.01	1.14±0.05	1.28±0.13

Each substance (100 μg/ml) was added to spleen cell culture medium with or without either LPS (10 μg/ml) or Con A (1 μg/ml). BALB/c spleen cells (2×10^5 cells/well) were cultured for 72 hours at 37°C under 5% CO_2 incubator and then cell proliferations were measured by MTT method as described in materials and methods. Results are expressed as meanSD, n=10 Significant difference from each control; *P<0.05, **P<0.01

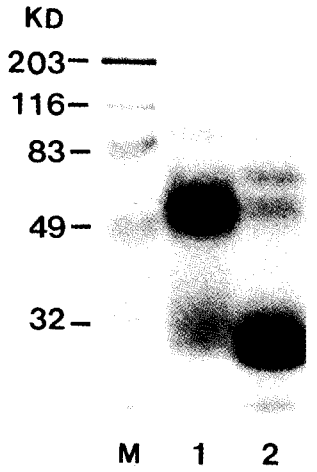


Fig. 1. Alteration of molecular size of crude protein substance by -mercaptoethanol. 10% acrylamide gel was used. Lane M, molecular weight markers; Lane 1, SDS-PAGE pattern of protein substance under nonreducing condition; Lane 2, SDS-PAGE pattern of protein substance under reducing condition.

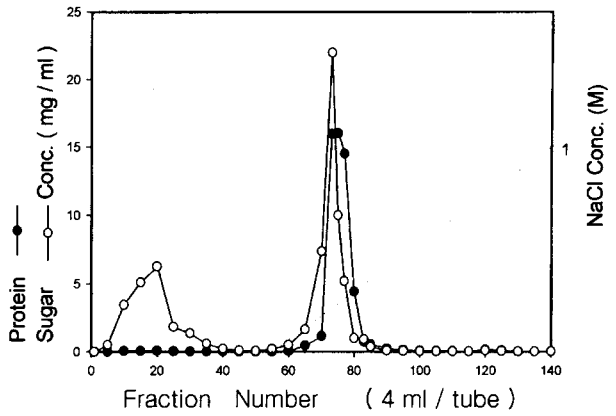


Fig. 2. DEAE Sepharose CL-6B chromatography of crude protein substance. Crude protein substance of ginseng water extract was purified with DEAE Sepharose CL-6B column (2.5× 25 cm) that had been equilibrated with 20 mM Tris-HCl buffe (pH 8.8). The unbound substance was washed with the same buffer and bound materials were eluted with linear gradient of 0 to 1 M NaCl at a flow rate of 1 ml/min

Table 2. Yield and chemical composition of ginseng extract at different stages of separation

Step	Yield[a]	Protein[b] (%)	Neutral[c] sugar (%)	Glucronic[d] acid (%)
Water extract	29.5 g	2.6	89.1	
Ultrafiltration (>10 kDa)	9.5 g	7.4	93.7	
$(NH_4)_2SO_4$ precipitation	1.7 g	39.9	43.5	11.8
DEAE-Sepharose unbound fraction (F-1)	177.6 mg	n.d.[e]	44.0	10.6
0.0~0.3% NaCl (F-2)	194.8 mg	45.9	59.4	12.5
0.3~0.5% NaCl (F-3)	813.5 mg	48.3	36.1	12.6
0.5~0.8% NaCl (F-4)	335.8 mg	41.1	50.2	9.2
0.8~1.0% NaCl (F-5)	38.1 mg	66.7	24.4	5.5

[a]dry weigh (each fraction was isolated from 100 g of ginseng powders)
[b]Assayed by Bradford method
[c]Assayed by penol-sulfuric acid method
[d]Assayed by carbazole method
[e]Not determined

active fraction. As shown in Table 2, this crude protein substance contained 39.9% of protein, 43.5% of neutral sugar and 11.8% of uronic acid indicating that it is acidic glycoprotein. To determine the protein pattern, this crude protein substance was analyzed by SDS-PAGE. As shown in Fig. 1, this substance exhibited a major band corresponding to 60 kDa of molecular weight, and a second one of about 29 kDa under nonreducing condition. However, 60 kDa molecule band was mostly shifted to 29 kDa band in the reducing SDS-PAGE condition, suggesting that protein of ginseng is formed a dimer of 60 kDa having disulfide bonds with 29 KDa subunits.

Further purification of the crude protein substance was undertaken by anion exchange chromatography. As shown in chromatogram of Fig. 2, most proteins were bound to the DEAE Sepharose resin, and these proteins were eluted with linear gradient of 1 M NaCl to give unbound fraction (F-1), and four eluted fractions (F-2, F-3, F-4, F-5), respectively. The chemical properties of these fractions are summarized in Table 2. The unbound fraction (F-1) did not contained any of protein but 44.0% of neutral sugar and 10.6% of uronic acid. The other four fractions obtained by 1 M NaCl linear gradient contained a large amount (35.9~66.7%) of protein, 24.4~50.2% of neutral sugar and 5.5~12.6% of uronic acid. For further study, we concentrated each fraction with amicon concentrator and dialyzed against the deionized water, followed by lyophilization to evaluate the immunological activity.

3. Immunomodulatory activity of protein fractions

To evaluate the immunological activity of protein fractions from ginseng, we investigated the effect of each protein fractions on the proliferation of spleen cells and NO production by murine macrophages, RAW 264.7 cells. Polyclonal cell proliferation of mouse cell has been widely used as basic experimental model to evaluate T and B lymphocytes functions *in vitro*.[25] And also, NO

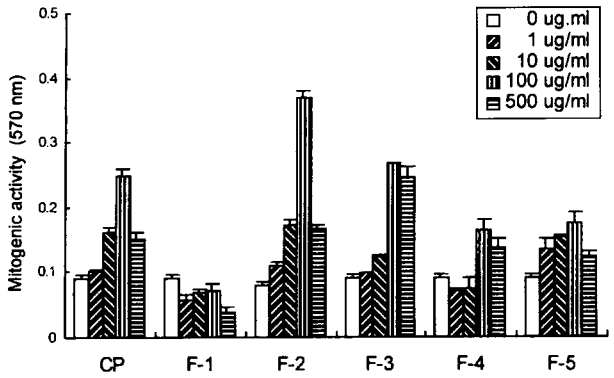


Fig. 3. Mitogenic activities of various protein fractions from *Panax ginseng*. BALB/c Spleen cells (2×10^5 cells/well) were cultured with different concentrations of crude protein substance (CP), unbound fraction (F-1), F-2, F-3, F-4 and F-5 for 72 hours at 37°C under 5% CO_2 incubator. Cell proliferations were measured by MTT method as described in materials and methods.

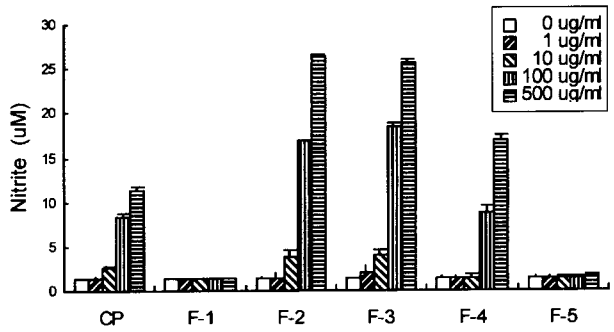


Fig. 4. The effect of protein fractions from *Panax ginseng* on the NO production by macrophages. RAW 264.7 cells (2×10^5 cells/well) were cultured with different concentrations of crude protein substance (CP), unbound fraction (F-1), F-2, F-3, F-4 and F-5 for 48 hours at 37°C under 5% CO_2 incubator. NO contents in supernatant of macrophage cultures were determined using the Griess reaction as described in materials and methods.

released from macrophage has become a new target for pharmacological research to find new substances for the treatment of cancer because NO, TNF- and IL-1 produced by macrophage exhibit cytotoxic activities mediated by modulation of the immune system against tumors.[26] As shown in Fig. 3 and 4, F-1, protein-free fraction, did not affect both lymphocytes proliferation and NO production by RAW 264.7 cells, while large protein-containing fractions, F-2, F-3, F-4 and F-5, induced proliferation of spleen cells and increased NO production by macrophages. Of these protein fractions, F-2 remarkably induced proliferation of mouse spleen cells in a concentration-

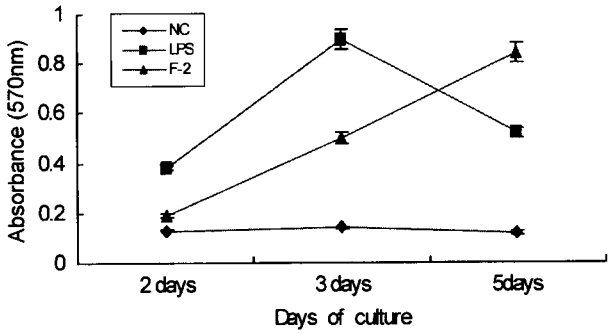


Fig. 5. The effect of F-2 isolated from *Panax ginseng* on the proliferation of spleen cells. BALB/c spleen cells were cultured with F-2 (100 μg/m*l*), LPS (10 μg/m*l*) and PBS buffer alone (NC) for the indicated number of days at 37°C under 5% CO_2 incubator. Cell proliferations were measured by MTT method.

Table 3. The effect of protease treatment on the mitogenic activity of F-2

Treatment	Relative activity
Control	100.0±3.0%
Protease	70.9±5.9%

BALB/c spleen cells were cultured with F-2 for 72 hours at 37°C under 5% CO_2 incubator. Cell proliferations were measured by MTT method. Each value was expressed as the mean SD, n=3.

dependent manner (1-100 μg/m*l*) and significantly stimulated NO production by macrophages at a concentration of 500 μg/m*l*. These results indicated that F-2 have a potent mitogenic activity of splenocytes and stimulating activity of macrophages.

To compare the mitogenic effect of F-2 with LPS, which have been widely known as mitogen, both F-2 and LPS were added to the culture medium at a concentration of 100 μg/m*l* or 10 μg/m*l*, respectively, and spleen cells were cultured for 5 days. The proliferation of the spleen cells cultured with LPS was peaked on day 3 and gradually decreased with culture time. In contrast, the proliferation level of the spleen cells cultured with F-2 were increased continuously as high as that of LPS on day 5.

Besides, to evaluate whether the protein moiety is involved in spleen cell proliferation or not, F-2 was treated with protease to provide deproteinized F-2 whose mitogenic activity was compared with F-2 untreated with protease. The mitogenic activity of the deproteinized F-2 significantly decreased to 70.9% compared with that of F-2. From this result, it is suggested that the protein moiety

of F-2 may contribute to mitogenic activity. Finally, to clarify the mechanism of the immunostimulating activities of this glycoprotein, F-2, further studies on the purification are in progress.

요 약

인삼(*Panax ginseng*)의 물추출물로부터 분리한 분자량 10 kDa 이상의 분획에서 강한 비장세포 분열증식 효과를 볼 수 있었다. 또한 이 분획은 LPS와 Con A 에 의해서 유도된 B-cell과 T-cell의 증식효과를 66%와 49%씩 더 증가시켰다. 이 분획을 80% ammonium sulfate 침전법을 실시해서 조 단백질 분획을 얻었으며 이를 다시 DEAE Sepharose CL-6B column chromatography를 수행해서 단백질 분획들을 분리 하였다. 대부분의 단백질은 NaCl gradient에 의해서 용출 되었고, 0~1 M NaCl gradient에 의해서 분리된 4종류의 당단백질 분획에서 비장세포 증식효과와 대식세포의 NO 생산촉진 효과가 나타났다. 특히 단백질 35.9 %, 중성당 49.4%, 산성당 12.5%로 구성되어있는 F-2 분획에서는 LPS 수준의 높은 면역세포 증식효과를 보였다. 그러나 F-2를 protease로 처리한 후의 비장세포 증식 효과는 대조군과 대비해서 29.1%가 낮아졌다. 이상의 결과로부터 인삼의 물추출물에서 분리된 F-2 분획의 면역세포 활성효과에는 단백질 부분이 중요한 역할을 한다고 생각된다.

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