Partial Purification and Characterization of a Glycoprotein Factor from Fresh Ginseng

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Abstract□ The aqueous extract of fresh ginseng (*Panax ginseng* C.A. Meyer) contains a macromolecular fraction that showed mitogenic and co-mitogenic activities in human peripheral blood lymphocytes. Purification of the crude extract by size (ultrafiltration, Sephadex G-200) and charge (DEAE-cellulose, DEAE-Sepharose) yielded a semi-purified fraction (DS-3). This fraction contains at least three subgroups of anionic macromolecules with apparent molecular weight greater than 600 kilodaltons. It is a glycoprotein with a large amount of glucuronic acid. It acts as a mitogen in both T and B cells of human peripheral blood lymphocytes. It could also potentiate the mitogenic action of Concanavalin A in lymphocyte T cells. Such potentiation is not due to increased binding of Concanavalin A to the cell surface. Its mitogenic and co-mitogenic effects do depend on the presence of extracellular Ca^{2+}.

Keywords□ *Panax ginseng* C.A. Meyer, mitogenic/co-mitogenic activity.

Introduction

The medicinal value of ginseng has now been confirmed by laboratory studies showing a wide spectrum of biological effects including sedative and nerve calming effect, tonic effect for visceral and neural functions; it also has a property to ward off diseases. Studies to understand the chemical and biochemical basis of the various beneficial effects of ginseng have largely borne out the medicinal values ascribable to ginseng for a long time. Throughout the years, a number of different molecules, including ginsenosides, phenolic compounds, peptides, alkaloids, fatty acids, esters, polyacetylenes and polysaccharides, have been isolated from ginseng and shown to underlie many important biochemical activities. For example, we have demonstrated the presence of a macromolecular fraction, mainly polysaccharide in nature, in fresh ginseng which has mitogenic and co-mitogenic effect in human peripheral blood lymphocytes (PBL).[1] Further attempts to purify this macromolecular fraction and to characterize its chemical and biological properties are reported in this communication.

Materials and Methods

Purification of the mitogenic factor

Fresh ginseng (4-year old roots from Kum San area) was homogenized in water (1:1 w/v), and filtered through gauze. The aqueous extract was concentrated by ultrafiltration (Amicon), using a YM-100 membrane. The retentate was lyophilized, reconstituted in 20 mM NH_4HCO_3, pH 7.3, and then applied to a Sephadex G-200 column (5.5 × 21 cm) developed with the same buffer. Fractions with mitogenic activity were pooled and lyophilized. It was then reconstituted and applied to a DEAE-cellulose column (3.3 × 10 cm) equilibrated with 10 mM Tris-Cl, pH 8.0. The column was washed with the same buffer, and then eluted with the inclusion of 1 M NaCl in the buffer. Again, the active fractions were pooled, lyophilized and reconstituted.

After removing the salt, the sample was applied to a DEAE-Sepharose column (1.8 × 15 cm), and eluted with a step gradient of NaCl.

FPLC analysis of the preparation

The partially purified mitogenic factor was analyzed by the FPLC system (Pharmacia). Gel filtration was performed on a Superose-12 column (0.75 × 30 cm) equilibrated and eluted with 150 mM NaCl, 50 mM NaPi, pH 7.0 at a flow rate of 0.4 m*l*/min. The column was calibrated with the following molecular weight markers: thyroglobulin (670K), fibrinogen (341K), γ-globulin (160K) and cytochrome C (12.4K). Ion exchange chromatography was performed on Mono-Q (0.5 × 5 cm) and Mono-S (0.5 × 5 cm) columns. Mono-Q was equilibrated with 20 mM Tris-Cl, pH 8.0 and eluted by a linear gradient of 0–1 M NaCl in the same buffer. Mono-S was washed with 50 mM Mes buffer, pH 6.0, followed by a linear gradient of 0–0.5 M NaCl in the same buffer. In both cases, the flow rate was 1 m*l*/min.

Chemical determination of the preparation

Protein concentration was determined by the method of Lowry *et al.*[2], using bovine serum albumin as the standard. Carbohydrate was determined by the anthrone-sulfuric acid method,[3] using glucose as the standard. Glucuronic acid was determined according to the method of Bitter and Muir.[4]

Mitogenic/co-mitogenic activity assay

Human PBL was obtained from heparinized umbilical cord blood.[5] 1×10^5 PBL in 0.1 m*l* culture medium [RPMI 1640 (Gibco)] supplemented with 10% heat-inactivated fetal calf serum (Gibco) was added to each well of a flat-bottom 96-wells culture plate. The ginseng extract was dissolved in 50 μ*l* phosphate buffer saline and added to the plate. For the study of co-mitogenic activity, Concanavalin A (Con A) dissolved in 50 μ*l* culture medium was also added. The cultures were incubated in 5% CO_2 at 37 °C for 3 days. They were then labelled for 6 h with [methyl-^{3}H]thymidine (Amersham, 2 Ci/mmol) at 0.5 μCi/well. The cells were harvested with a cell harvester (Dynatech, Automash 2000) and the radioactivity was counted in a liquid scintillation counter (Beckman). Untreated cells were also cultured to compare the activity under the same condition.

Elimination of specific cell type

To obtain the T cell-depleted lymphocytes, human PBL were treated with 203T monoclonal antibody (Serotec) at predetermined optimal dilution (1:100) at room temperature for 30 min. The cells were washed to remove excess antibody and then resuspended in diluted rabbit complement and incubated at 37 °C for 40 min. The cells were washed for two times with plain RPMI medium before being used in the culture experiment. The macrophage-depleted lymphocytes were prepared by preincubating the PBL in culture dish for 3 h. Non-adherence cells were collected by washing with plain medium and resuspended in culture medium.

Binding of DC-2/Con A to cell surface

The binding of ginseng extract to the cell surface was examined by preincubating human PBL with DC-2 in the presence and absence of Con A for different periods of time. The lymphocytes were washed for two times before being cultured. The binding of Con A to the cell surface was determined by incubating the PBL with different concentrations of ^{3}H-Con A for 4 h. The lymphocytes were washed for two times before counting.

Effect of extracellular Ca^{2+}

The effect of extracellular Ca^{2+} was examined by incubating human PBL for 3 days with DS-3 in (a) normal RPMI medium, (b) Ca^{2+}-free medium, and (c) Ca^{2+}-free medium supplemented with 2 mM $CaCl_2$, before thymidine incorporation was determined.

Results and Discussion

Purification

A glycoprotein mitogenic factor was partially purified from fresh ginseng by ultrafiltration, gel

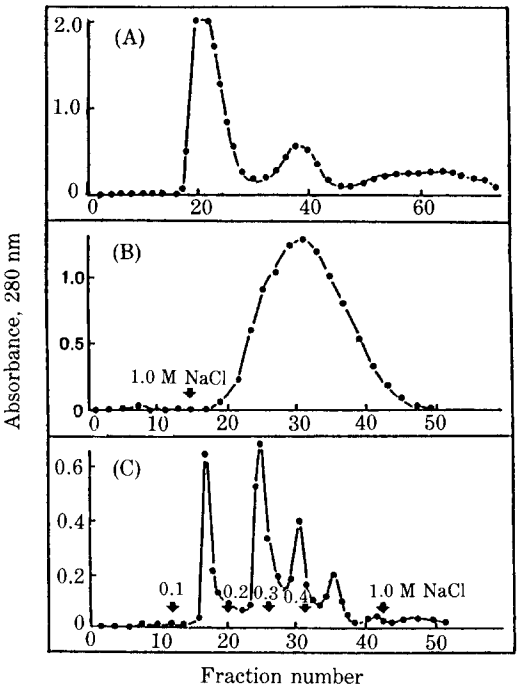

Fig. 1. Purification of the mitogenic factor from fresh ginseng. (A) The elution profile of 100 K-In on Sephadex G-200. Fractions 17-30 were pooled as SG-1. (B) The elution profile of SG-1 on DEAE-cellulose. Fractions 20-48 were pooled as DC-2. (C) The elution profile of DC-2 on DEAE-Sepharose. Fractions 23-28 were pooled as DS-3.

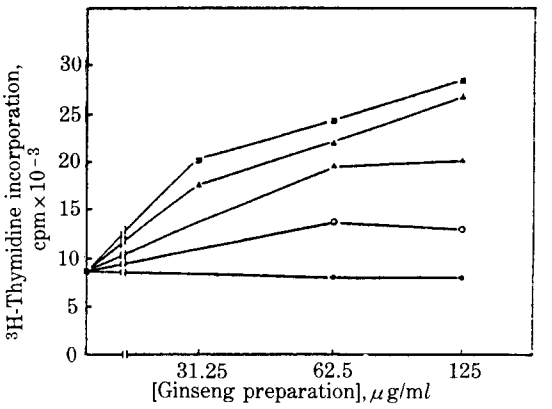

Fig. 2. The co-mitogenic effect of the ginseng extract at different stages of purification with Con A (0.5μg/ml) on human PBL. The various samples are aqueous extract (●), 100 K-In (○), SG-1 (▲), DC-2 (△) and DS-3 (■).

Table 1. Yield and chemcial composition of the ginseng extract at different stages of purification

Purification step	Yield	Carbohydrate (%)	Protein (%)	Glucronic acid (%)
Fresh ginseng	1 kg			
Aqueous extract	50.53g	60	4	9
Ultrafiltration, 100 k-In	1.49g	28	23	8
Sephadex G-200, SG-1	1.17g	27	7	11
DEAE-cellulose, DC-2	243 mg	40	14	19
DEAE-sepharose, DC-3	103 mg	40	18	23

filtration and ion exchange chromatography. The mitogenic factor was retained by the YM-100 membrane, suggesting that it is a macromolecule with molecular weight greater than 100K. When the retentate (100K-In) was applied to the Sephadex G-200 column, it was eluted at the void volume (Fig. 1A). This preparation (SG-1) has been shown to possess mitogenic and co-mitogenic effects on human PBL.[1] Further purification of this mitogenic factor was achieved by ion exchange chromatography. The mitogenic factor bound to the anion exchanger, DEAE-cellulose, and was eluted by using 1 M NaCl (DC-2) (Fig. 1B). In an attempt to have better resolution, DC-2 was rechromatographed on a DEAE-Sepharose column, and eluted with a step gradient of NaCl (Fig. 1C). Several peaks were obtained and all of them showed considerable mitogenic/co-mitogenic activity. The major peak was DS-3, which was eluted at a NaCl concentration of 0.2 M. The co-mitogenic effect of the ginseng preparation at different stages of purification is shown in Fig. 2. From 1 kg of fresh ginseng, 103 mg of DS-3 can be obtained (Table 1).

Chromatographic profiles

DS-3 was analyzed on the FPLC system. The elution profile on the Superose-12 column indicates that it consists mainly of macromolecules with molecular weight greater than 600K (Fig. 3A). DS-3

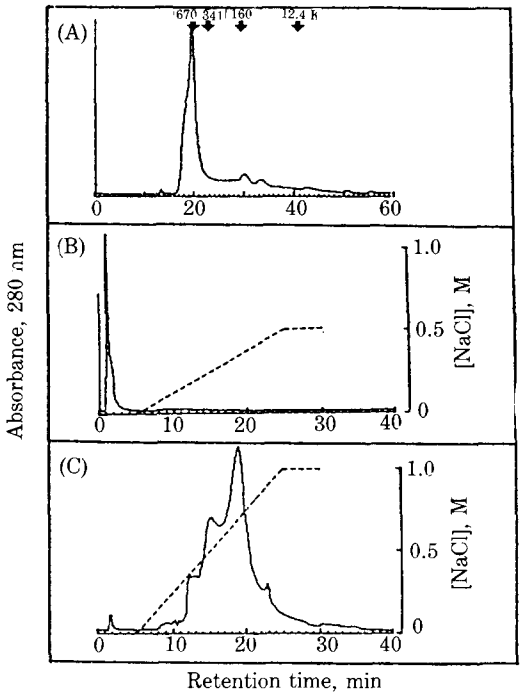

Fig. 3. FPLC analysis of DS-3 on (A) Superose-12 (B) Mono-S and (C) Mono-Q columns. See materials and methods for conditions.

failed to bind to the Mono-S cation exchange column and gave a single breakthrough peak, revealing the anionic nature of the macromolecules (Fig. 3B). As expected, DS-3 was retained by the Mono-Q anion exchange column, and can be eluted by a linear gradient of 0–1 M NaCl. The elution profile indicates the presence of a heterogeneous population consisting of at least three subgroups of anionic macro-molecules (Fig. 3C).

Chemical constituents

DS-3 was also analyzed for its chemical composition. It was found to be a glycoprotein consisting of 40% carbohydrate and 18% protein (Table 1). The presence of large amount of carbohydrate suggests that the affinity gels Con A-Sepharose and wheat germ lectin-Sepharose might be useful for further purification of the mitogenic factor. However, both affinity gels failed to retain the mitogenic factor (data not shown), indicating the absence of terminal α-D-glucopyranosyl, α-D-mannopyranosyl, N-acetyl-D-glucosamine or sterically similar residues in the mitogenic factor. The mitogenic/co-mitogenic activity of the preparation after passing through these columns was virtually identical to that of DS-3. Glucuronic acid was also present in large amount, accounting for 23% of the weight of DS-3 (Table 1). Such high glucuronic acid content accounts for the anionic nature of the preparation.

Absence of saponins

Our previous report[1] demonstrated the presence of a large amount (22%) of saponin in the void volume fraction of Sephadex G-200 (SG-1). Ion exchange chromatography on DEAE-cellulose and DEAE-Sepharose was quite successful in removing the saponin while retaining the mitogenic/co-mitogenic activity of the preparation. The preparation, DS-3, contained less than 5% of saponin, as determined by the vanillin-sulfuric acid method. To eliminate any saponin that may be present, organic extraction was employed. DS-3 was dissolved in water and the same amount of saturated n-butanol was added and vigorously shaken to promote the dissociation of bound saponin, if there was any. After 4 h of standing at room temperature, the two layeres were separated. The aqueous layer was concentrated by ultrafiltration, lyophilized and then dissolved in culture medium. Its mitogenic/co-mitogenic activity was similar to that of the untreated one. On the other hand, the n-butanol layer, after removal of the organic solvent and subsequent lyophilization, failed to show any mitogenic/co-mitogenic effect (data not shown). Such observation suggests that saponin does not contribute to the mitogenic/co-mitogenic effect of the preparation.

Enzyme digestion

The susceptibility of DS-3 towards enzymatic degradation was also examined. DS-3 was incubated with different enzymes, including α-amylase, pronase, trypsin, arg-C endopeptidase, and neuraminidase, at 37 °C for 6 h. The enzymes were used at a maximally tolerated dose and the total enzymatic digests were incubated with human PBL. None of these enzymatic treatments showed any apparent

digestion of the mitogenic factor. After various treatments, the elution profile of DS-3 on the Superose-12 FPLC column remained virtually identical to that of the untreated sample (data not shown). Such treatment also failed to abolish the mitogenic/co-mitogenic activity of the preparation.

Monomeric structure

Preincubation with 0.02 M dithiothreitol and subsequent reaction with 0.04 M iodoacetamide also failed to affect the biological activity and the apparent molecular weight of DS-3. Even 6 M guanidine hydrochloride failed to cleave the macromolecule into smaller fragments, as indicated by the elution profile on the Superose-12 FPLC column using a buffer system containing 6 M guanidine hydrochloride (data not shown). These observations seem to suggest the monomeric nature of the macromolecule. However, it should be cautioned that minor changes of molecular weight might not be apparent, as the mitogenic factor was eluted near the "void" volume of the column.

Negative charges

The presence of negative charges on the mitogenic factor may make it more resistant to various enzymatic digestions. In an attempt to remove the negative charge, β-glucuronidase was used. Preliminary result showed that the mitogenic factor became less anionic after treating with β-glucuronidase (data not shown). Whether such treatment will affect the mitogenic/co-mitogenic activity, or alter the susceptibility of the preparation to enzymatic digestion, remains to be seen.

Mitogenic/co-mitogenic effect

To study the biological activity of the mitogenic factor, either the preparation DC-2 or DS-3 was used. They differ only slightly in the potency of mitogenic/co-mitogenic activity (Fig. 2). The mitogenic and co-mitogenic effects of DS-3 is shown in Fig. 4. In the absence of Con A, DS-3, at a concentration of 100 μg/ml, stimulated thymidine incorporation. It also enhanced the effect of Con A throughout the concentrations of Con A examined.

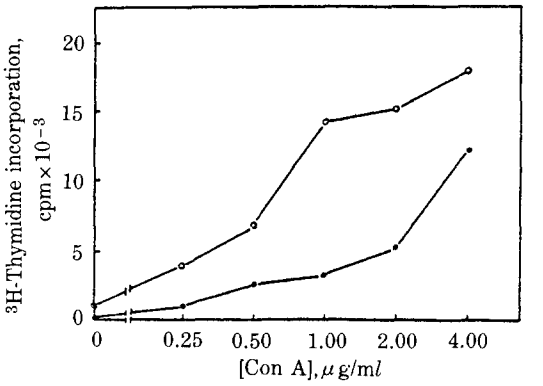

Fig. 4. The mitogenic/co-mitogenic activity of DS-3 on human PBL. ^{3}H-ehymidine incorporation was determined at various concentrations of Con A in the in the absence (●) and presence (○) of 100μg/ml DS-3.

Target cells

DC-2 in the presence of Con A, a T cell mitogen, enhanced thymidine incorporation in total lymphocytes beyond their additive value, especially at higher concentration. This potentiation was not observed with SAC (*Staphylococcus aureus* Cowan), a B cell mitogen (Table 2). Such difference in the co-mitogenic effect of DC-2 on different cell types was confirmed by the results obtained with the T cell-depleted lymphocytes where most of the Con A activity was abolished. Thus, it is fair to conclude that DC-2 was active as a mitogen in both T and B cells, and it could potentiate the activity of lectin mitogens in human lymphocyte T cells. The results obtained with macrophage-depleted lymphocytes were similar to those obtained with total lymphocytes, indicating that the mitogenic and co-mitogenic activities of DC-2 on human PBL is macrophage-independent.

Cell surface binding

Previous studies have shown that the transformation of lymphocytes by antigen, lectin, and anti-Ig sera is initiated by the cross-linkage of specific receptors on the cell surface. Such ligand-receptor interaction is believed to induce a conformational change of the receptor; and subsequently, a second messenger is released to transfer the information

Table 2. Stimalation index[a] of the mitogenic and co-mitogenic activities of DC-2 on different cell types

Cell type	w/o Mitogen	[DC-2], μg/ml 0	1	10	100
Total lymphocytes	DC-2 only	1.0	1.5	2.4	6.3
	+Con A (0.5 μg/ml)	8.2	9.7	14.1	31.5
	+SAC (25 μ g/ml)	3.1	2.9	3.2	6.3
T cell-depleted lymphocytes[b]	DC-2 only	1.0	1.2	1.7	4.2
	+Con A (0.5μ g/ml)	1.1	1.4	2.1	4.9
	+SAC (25 μ g/ml)	1.6	1.7	1.9	3.8
Mφ-depleted lymphocytes[b]	DC-2 only	1.0	1.4	1.7	4.8
	+Con A (0.5μ g/ml)	9.4	9.4	12.8	30.2
	+SAC (25 μ g/ml)	2.6	2.2	3.0	5.2

[a] The stimulation index is the ratio of thymidine incorporation of the stimulated cells to that of the unstimulated cells.

[b] The T cell-depleted lymphocytes and the macrophage-depleted lymphocytes were prepared as described in materials and methods.

from the cell surface to the interior of the cell. However, the mitogenic/co-mitogenic effect of the ginseng preparation did not appear to act at the receptor level (Table 3). Unlike Con A, DC-2 itself had negligible mitogenic activity when the cells were washed before being cultured; suggesting that (a) it did not bind to the cell surface receptor, and (b) within 6 h of preincubation, it failed to generate any signal for cell proliferation. However, it could potentiate the mitogenic effect of Con A (Table 3). Such potentiation was not due to an increase in the binding of Con A to the cell surface. The binding curve for Con A is virtually identical in the presence and absence of DS-3 (Fig. 5). On the other hand, α-methylmannopyranose decreased while fibrinogen increased the binding of Con A to the cell surface.[6] Thus the co-mitogenic activity of DS-3 is probably exerted directly on the cell rather than indirectly by an interaction with the lectin.

Ca^{2+} dependence

The presence of extracellular Ca^{2+} has been demonstrated to be essential for Phytohemagglutinin-induced transformation of human lymphocytes.[7-9] The mitogenic effect has been attributed to an influx of Ca^{2+}.[10-12] The presence of extracellular Ca^{2+} is also essential in the expression of mitogenic activity of the ginseng extract (Fig. 6A). In the presence of DS-3, the stimulation index was decreased for the lymphocytes in the Ca^{2+}-free medium as compared to that in the normal medium; becoming more significant as DS-3 dose increased. Such decrease in stimulation index could be restored when Ca^{2+} was supplemented. This decrease of mitogenic effect was confirmed and magnified many times when Con A and DS-3 were used together. While Con A blastogenesis did not require Ca^{2+}, a significant co-mitogenic effect was observed only when extracellular Ca^{2+} was present in the medium

Table 3. Thymidine incorporation (cpm) of human PBL preincubated with DC-2 and/or Con A[a]

Sample	Preincubation time, h 0	2	4	6
Control (cell only)	886			
DC-2 (500 μg/ml)	624	1100	561	542
Con A (10 μg/ml)	766	2470	5036	2219
DC-2 (500μg/ml) +Con A (10μg/ml)	981	6441	6486	4006

[a] Human PBL were preincubated with DC-2 and/or Con A for different periods of time and then washed for two times before suspending in the culture medium.

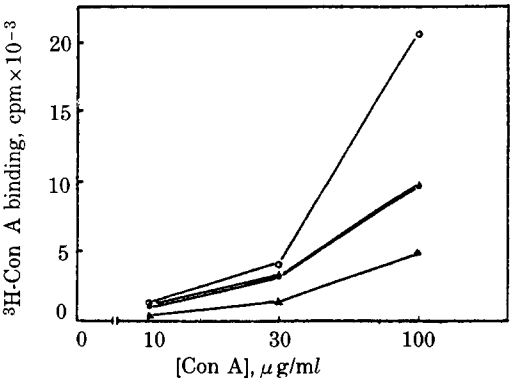

Fig. 5. The binding of ^{3}H-Con A to cell surface in the absence (●) and presence of 500μg/ml fibrinogen (○), 500μg/ml DS-3 (△) or 20 mg/ml α-methyl mannopyrannopyranose (▲).

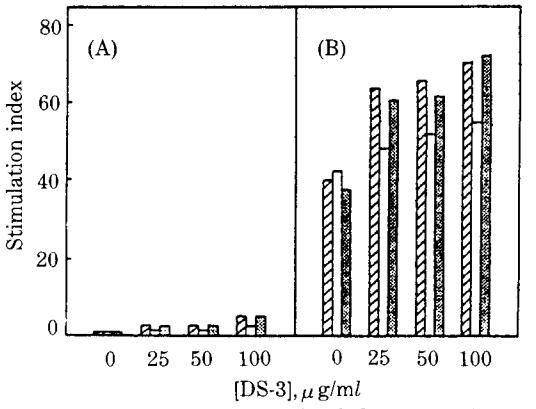

Fig. 6. The effect of extracellular Ca^{2+} on (A) mitogenic and (B) co-mitogenic effect of DS-3. Cells were incubated in the absence and presence of 0.5μg/ml Con A in normal RPMI medium (▨), Ca^{2+}-free medium (□) of Ca^{2+}-free medium + 2 mM $CaCl_2$ (▩).

(Fig. 6B). This observation suggests that DS-3 is partly dependent on the presence of extracellular Ca^{2+} for the expression of its mitogenic/co-mitogenic activity.

To conclude, a macromolecular fraction with strong mitogenic/co-mitogenic activity from fresh ginseng has been partially purified by size selection and anion exchange chromatography. Significant increase in unit weight potency of the mitogenic/co-mitogenic activity was accompanied by increasing protein, carbohydrate and glucuronic acid content. DS-3, the most purified fraction, contains a population of at least three subgroups of macromolecules with molecular weights greater than 600K. The mitogenic/co-mitogenic activity was protease- and glycosidase-resistant, but treatment with β-glucuronidase could remove the negative charges. Extracellular Ca^{2+}, but not macrophage, was required for the expression of the mitogenic/co-mitogenic activity. While mitogenic activity was shown in both T and B cells, the co-mitogenic activity was T-cell specific and was not due to increased binding of the mitogen or co-mitogen.

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