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Study on the Molecular Weights of Radioprotective Ginseng Proteins by HPLC Method

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Abstract□Partially purified ginseng proteins were either treated with sodium dodecyl sulfate (SDS) and ß-mercaptoethanol to denature the proteins or not, and subjected to Thin Layer Chromatography (TLC) and High Performance Liquid Chromatography (HPLC) to compare the components of each fraction. Standard proteins of known molecular weights (MW) were also either treated with SDS and ß-mercaptoethanol or not, and subjected to HPLC to obtain regression lines for MW determination. From the retention times obtained from samples in either case by HPLC, the MW were estimated as following; In SDS treated condition, GI fraction showed three peaks each with MW of above 100,000, 51,000 and 19,000. GII showed one original peak with MW of 21,000 and GIII, two peaks each with MW of 19,000 and 14,000. On the other hand, in non-SDS treated condition, GI fraction showed two peaks each with MW of above 200,000 and 52,000. GII showed one original peak with MW of 41,000 and GIII, three peaks each with MW of 28,000, 19,000 and 14,000.

Keywords□Ginseng Protein, TLC, HPLC, SDS, ß-mercaptoethanol, Molecular weights

Korean ginseng has been known to contain greater amounts of pharmacologically effective components than ones from other countries.[1]Hence, studies on the effects of these components are being carried out in many different parts of the world.[2] However, most of the works has been centered on saponins and there are not many reports on protein components.

In 1963, Gstirner and Braun[3] found 24 different substances from Korean white ginseng and identified some of them as glutamic acid, cystein, tyrosine, etc. In 1966, Gstirner and Vogt[4] extracted Korean ginseng with methanol-water (v/v) and carried out paper electrophoresis to find out five ninhydrin positive fractions.

Yonezawa, in 1976, reported that ginseng extract showed radioprotective effects in X-ray irradiated NIH-SWISS mouse.[5] And Chang et al.[6] reported clinical data that the ginseng extract administered to cervical cancer patients showed protection against bone marrow death caused by radiation therapy. Moreover, in 1981, Kimura et al.[7] reported that the radioprotective substance was not saponins which were known to be the major components in ginseng. At the same year, Yonezawa et al.[8,9] partially purified the active components and found out that they were protein-like substances which showed positive Biuret test and maximum absorption at UV 280 nm. Later, Takeda et al.[10,11] tested radioprotective activity on X-ray irradiated mouse, rats and guinea pigs with thermostable ginseng proteins and reported the protective activity in all three species. Recently kim and Han[12] published results that showed radioprotective effects of GI fraction from Sephadex G-75 column chromatography on γ-ray irradiated ICR mouse, while GII fraction showed no such effect.

Based on the above works, we set up our project to isolate pure proteins from ginseng to pick up the one that shows the radioprotective activity. In the process of achieving this goal, we determined the MW of proteins obtained from purification procedure by HPLC method.

EXPERIMENTAL METHODS

Materials

CM-Cellulose, Sephadex G-75, blue dextran, bovine serum albumin, and as standard proteins, ferritin, lactate dehydrogenase, phosphorylase b, ovalbumin and cytochrome C were purchased from Sigma Co., U.S.A.. Cellulose membrane for dialysis were the product of VWR Scientific Co., U.S.A., Sodium azide, ß-mercaptoethanol, SDS and other chemicals used were special grade reagents made in Japan.

Instrumentation

HPLC apparatus was consisted of Universal Injector

(Waters Associates Model U6K), Solvent Delivery System (Waters Associates Model 6000A), Absorbance Detector (Waters Associates Model 440UV) and Recorder (Houston Instrument, Omniscribe chart). The column used was a steric exclusion type from Toyo Soda Manufacture (TSK-Gel type SW 3,000 column, 7.5 × 300 mm).[13,14]

Isolation and purification of ginseng proteins

This process was carried out as described earlier.[12,15,16,17]

Thin Layer Chromatography[18,19]

Samples in non-SDS buffer were prepared by dissolving GI, GII and GIII fractions in distilled water to final concentration of 3mg protein/ml. On the other hand, samples in SDS buffer were prepared by dissolving the same fractions in distilled water containing 1% SDS and 5% ß-mercaptoethanol to the same concentration and keeping them in boiling water bath for 5 minutes. All samples were spotted on silica gel plate and developed in a solvent consisting of n-butanol: pyridine: acetic acid: water=30: 20: 6: 24.[20] Spots were detected by short wave UV and ninhydrin spary.

Determination of Molecular Weights by HPLC[21,22]

In the case of non-SDS buffer, samples were prepared by dissolving GI, GII and GIII fractions in 0.1M sodium phosphate buffer (pH 7.0) to a final concentration of 5mg protein/ml and filtering through sample clarification kit (Millipore Co.). As standard proteins, ferritin (MW 440,000), lactate dehydrogenase (MW 132,000), phosphorylase b (MW 97,400), ovalbumin (MW 45,000) and cytochrome C (MW 12,384) were prepared by the same method as samples. After the base line was stabilized in HPLC apparatus, 20 l each of samples and standard proteins was injected and the absorbance was recorded at 280 nm against retention time. All procedures were carried out at room temperature. From the known MW of standard proteins and their retention times obtained, a regression line was drawn by the least square method. According to this regression line, MW of sample proteins were estimated by their retention times obtained.

In the case of SDS buffer, samples were prepared by dissolving GI, GII and GIII fractions in 0.1M sodium phosphate buffer (pH 7.0) containing 1% SDS and 5% ß-mercaptoethanol to a final concentration of 5mg protein/ml and kept in boiling water for 5 minutes to filter prior to column injection. As standard proteins, ferritin (Subunit MW 18,500), lactate dehydrogenase (SMW 36,000), phosphorylase b (SMW 97,400), ovalbumin (SMW 45,000) and cytochrome C (SMW 12,384) were prepared by the same method as samples. Elution was carried out with 0.1M sodium phosphate buffer (pH 7.0) containing 0.1% SDS. Column was kept at 65.°C to prevent SDS from forming turbidity at room temperature. All other procedures were the same as those in the case of non-SDS buffer. From the known subunit MW of standard proteins and their retention times obtained, a regression line was drawn by the least square method. Using this regrression line, MW of subunit polypeptides of sample proteins were determined from the retention times recorded.

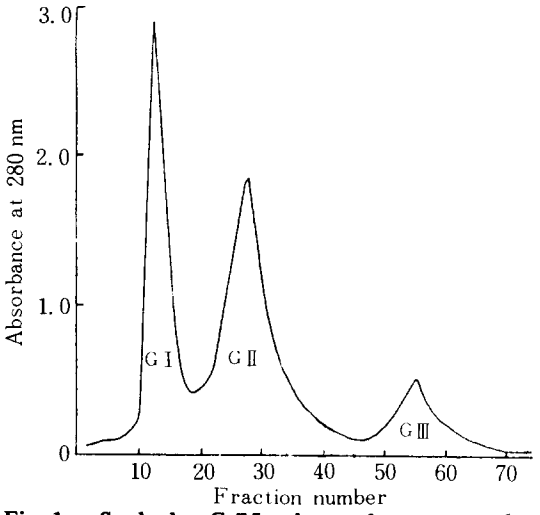

Fig. 1. Sephadex G-75 column chromatography of the thermostable fraction of CM-B.

The column (2.5 x 34.5cm) was pre-equilibrated and eluted with 50 mM potassium phosphate buffer (pH 7.2). Each fraction of 5 ml was collected at a flow rate of 15 ml/hr.

RESULTS

Chromatogram of Sephadex G-75 column chromatography is shown in Fig 1. Three fractions, GI, GII and GIII were obtained and relative yields of proteins in each fraction were 3.2%, 0.85% and 0.06% respectively when compared with the amount of proteins in crude extract. These fractions were subjected to TLC and HPLC.

Results of TLC are given in Fig. 2. In non-SDS buffer, GI showed three strong spots (a, b and c), GII, one weak (d) and one strong (e) spots and GIII, one long spot (f). In SDS buffer, GI showed three strong sports (g,h and i), GII, one weak (j) and one strong (k) spots and GIII, two very close spots (l and m).

Results of HPLC in non-SDS buffer are given in Fig. 3. GI showed two peaks (GI-A and B) at the retention time of 10.0 min. and 17.3 min., GII showed two peaks (GII-A and B) at 10.0 min. and 18.6 min. and G III showed three peaks (GIII-A,B and C) at 20.8 min., 23.0 min. and 24.5 min.

Fig. 4 shows the elution peaks of five standard proteins in non-SDS buffer. Since only three peaks were ap-

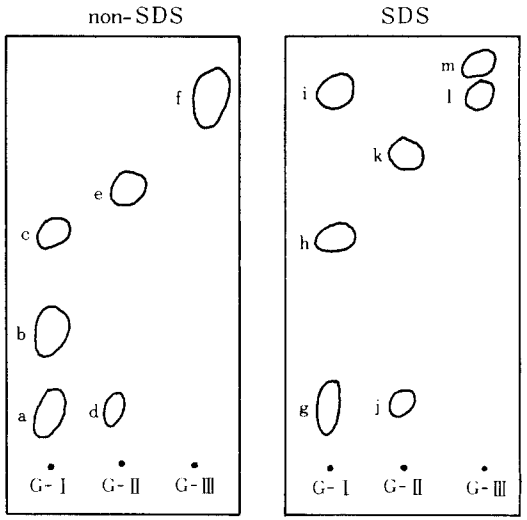

Fig. 2. Thin Layer chromatography of Sephadex G-75 fractions.

Stationary phase; Silica gel 60, 0.25 mm thickness Solvent; n-BuOH:Pyridine: Acetic acid: H_2O = 30:20:6:24
Detection; UV and Ninhydrin spray
Rf values of each spot; a(0.20), b(0.41), c(0.57), d(0.20), e(0.67), f(0.79), g(0.22), h(0.56), i(0.78), j(0.22), k(0.68), l(0.78), m(0.79).

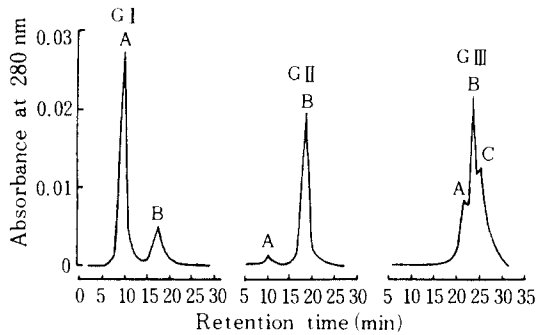

Fig. 3. HPLC of Sephadex G-75 fractions in non-SDS buffer.

Solvent and Eluent; 0.1M Sodium. phosphate buffer (pH 7.0)
Temperature; Room Temperature
Flow rate; 0.5 ml/min.
Column; TSK Gel type SW 3,000, 7.5 x 300mm

peared when five proteins were mixed and run together, each protein was run separately at the exactly same conditions and peaks were assembled together according to their retention time. Ferritin peak was appeared at the retention time of 10.1 min., lactate dehydrogenase at 13.5 min., phosphorylase b at 15.4 min., ovalbumin at

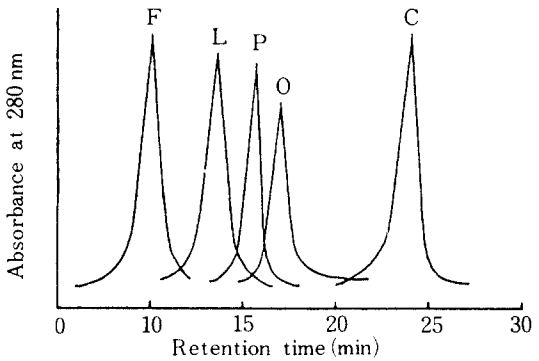

Fig. 4. HPLC of standard proteins in non-SDS buffer.

All conditions were the same as in Fig.3.
Here, F: Ferritin, L: Lactate Dehydrogenase, P: Phosphorylase b, O: Ovalbumin, C: Cytochrome c

16.7 min. and cytochrome C at 23.9 min., Equation for regression line was calculated by the least square method and obtained as log y = −0.07907x + 6.0829. Here, Ferritin data was not included in the calculation since the data was too much off line. MW of proteins in sample peaks were determined according to this equation. Results are shown in Table I.

MW of GI-A was above 200,000 GI-B was 52,000 GII-A was above 200,000, GII-B was 41,000. GIII-A was 28,000, GIII-B was 19,000 and GIII-C was 14,000. Here,

Table I. Chromatographic behavior and apparent molecular weights of proteins in non-SDS buffer.

Protein	Retention time (min)	Molecular weight (dalton)
Ferritin	10.1	440,000
Lactate dehydrogenase	13.5	132,000
Phosphorylase b	15.4	97,400
Ovalbumin	16.7	45,000
Cytochrome C	23.9	12,384
Sephadex G-75 fractions		
GI-A	10.0	>200,000
B	17.3	52,000
GII-A	10.0	>200,000
B	18.6	41,000
GIII-A	20.8	28,000
B	23.0	19,000
C	24.5	14,000

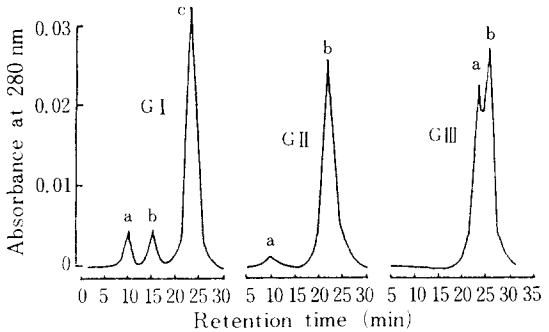

Fig. 5. HPLC of Sephadex G-75 fractions in SDS buffer.

Solvent; 0.1M Sodium phosphate buffer (pH 7.0) containing 1% SDS and 5% β-mercaptoethanol.
Eluent; 0.1M Sodium phosphate buffer (pH 7.0) containing 0.1% SDS.
Temperature; 65°C
Flow rate; 0.5 ml/min.
Column; TSK Gel type SW 3,000, 7.5 x 300mm

GI-A and GII-A seem to be the same protein which was not completely separated between two fractions.

When SDS was added as shown in Fig. 5, GI showed three peaks (GI-a,b and c) at the retention time of 10.2 min., 14.8 min. and 23.2min., GII showed two peaks (GII-a and b) at 10.2min. and 22.4 min. and GIII showed two peaks (GIII-a and b) at 23.2 min and 25.8 min..

Fig. 6 shows elution behavior of five standard proteins in SDS buffer. Each protein was also run separately and peaks were assembled together according to their retention time. Phosphorylase b peak was appeared at the retention time of 10.2 min., ovalbumin at 14.6 min.,

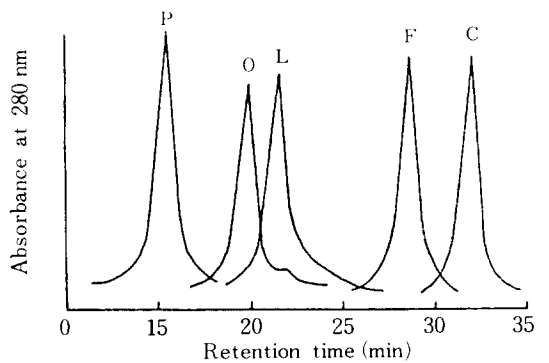

Fig. 6. HPLC of standard proteins in SDS buffer.

All conditions were the same as in Fig.5.
Here, P: Phosphory lase b O: Ovalbumin, L: Lactate Dehydrogenase, F: Ferritin. C: Cytochrome C.

Table II. Chromatographic behavior and apparent subunit molecular weights of proteins in SDS buffer.

Protein	Retention time (min)	Subunit molecular weight (dalton)
Phosphorylase b	10.2	97,400
Ovalbumin	14.6	45,000
Lactate dehydrogenase	16.3	36,000
Ferritin	23.4	18,500
Cytochrome C	26.8	12,384
Sephadex G-75 fractions		
GI-a	10.2	>100,000
b	14.8	53,000
GII-a	10.2	>100,000
b	22.4	21,000
GIII-a	23.2	19,000
b	25.8	14,000

lactate dehydrogenase at 16.3 min., ferritin at 23.4 min. and cytochrome C at 26.8 min.. Equation for regression line was calculated and obtained as $\log y = -0.05259x + 5.5021$. Subunit MW of proteins in sample peaks were determined. Results are presented in Table II. MW of GI-a was above 100,000, GI-b was 53,000, GI-C was 19,000 GII-a was above 100,000, GII-b was 21,000, GIII-a was 19,000 and GIII-b was 14,000. Here also, GI-a and GII-a seem to be the same protein.

DISCUSSION

SDS, a denaturant, is known to form micellar complex with hydrophobic portion of proteins and denatures them to their subunits.[23] In this study, samples and standard proteins were treated with SDS to obtain subunit polypeptides, and compare their MW with the MW of original molecules.

To measure MW of these proteins, HPLC method was used which had been applied by others successfully.[24-28] However, the problem was the deformation of soft gels by the pressure exerted by repeated passage of mobile phase. To improve this problem, Hefti[21] made use of rigid porous silica gel matrix of 20 μm or below packed in a very narrow column (I.D. below 10mm) and sucessfully separated samples under high pressure. Montelaro et al.,[22] in 1981, examined possibility of accurate MW measurements by HPLC method within an average error of 5-7% even when using denaturants such as SDS and guanidine HCl. In this experiment,

TSK-Gel SW type column was used which was made of silica gel to exert excellent resolution and minimize adsorption of samples to matrix.

In the case of GI fraction, when run in non-SDS buffer, two peaks with peak ratio of 5:1 were appeared (Fig. 3). When this sample was run in SDS buffer, three peaks with ratio of 1:1:12 were shown (Fig. 5). Here, GI-B has similar MW with GI-b, therefore, it seems as if GI-A was divided into GI-a and GI-c. This data agrees with TLC data if it is assumed that GI-A contains both a and b spots in TLC (Fig. 2). Protein in b spot seems to be broken down to one in i spot, which has the same Rf value as in 1 spot. Note here that GI and GIII fractions were reported to have antiradiation effects on mice.[9]

In the case of GII fraction, peak area ratio of GII-B: GII-b was 1:2, indicating the splitting of a dimer molecule in SDS buffer giving about half of the original MW. (Table I and II). TLC spots show similar mobilities in both buffers probably due to the similar migrating properties of the dimer and the two subunits.

In the case of GIII fraction, three peaks with ratio of 3:7:6 were appeared in non-SDS buffer which became two peaks with ratio of 7:11 in SDS buffer. Here, GIII-B shows the same MW and ratio with GIII-a, hence considered as a monomer present in both buffers. From the peak area ratio and MW estimated, it is likely that GIII-A was broken down to appear in GIII-b on top of already present GIII-C. Here, the original GIII-C and the added protein to make GIII-b could be the same molecule or the different melecule with only similar MW.. TLC shows one broad spot which is likely to correspond to all three peaks in non-SDS buffer, however, in SDS buffer, only two spots were appeared which agreed with HPLC data.

In our previous paper of this series,[17] only SDS treated samples were analyzed giving incomplete interpretation. But the peak pattern was very similar with the results obtained in this experiment. Estimated MW were a little higher in the previous experiment due to the difference in the standard proteins employed. However, the absolute MW are not so important at this stage of work.

Clear view came out of all the data obtained up to now that GI-c and GIII-a proteins in SDS buffer by HPLC are the candidates for having radioprotective activity. The reason is because GI and GIII fractions were reported to have the activity and the protein with the same MW of 19,000 was presented by these two peaks, while it did not exist in GII fraction which was reported to have no effect.

Our future work will be concentrated to purify this protein in pure form and test its activity possibly on cellular level since the amount of the protein that can be recovered is extremely small.

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