

Tertiary Structure of Ginsenoside Re Studied by NMR Spectroscopy

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Ginseng has long been used as a traditional medicine in Asian countries including Korea and China. In recent years, it has been reported that the biological activities of ginseng are due to its active components, ginsenosides. Ginsenosides are represented by triterpenes of the dammarane type. Ginsenoside Re consists of two glucose rings, one rhamnose ring, and the triterpene ring. In the present study ginsenoside Re has been isolated from the Korean ginseng (*Panax ginseng*) and the tertiary structure has been determined using NMR spectroscopy. Flexibilities around each linkages described by seven torsion angles were considered. The structures of ginsenoside Re obtained by NMR spectroscopy show the rigidity around the glucopyranosyl ring II and alkene side chain. The dihedral angles of ϕ_5 , ϕ_6 , ϕ_7 are about 150° , 50° and 45° , respectively. In addition, flexibility exists around rhamnopyranosyl and glucopyranosyl moiety. The linkage around the rhamnopyranosyl and glucopyranosyl ring I, are divided into three groups. This flexibility seems to play important role in regulation of the hydrophobic surface exposed to the solvent. Because of the growing need for the structural determination of ginsenoside, this result can help to understand their well-accepted pharmacological effects of ginsenoside Re.

Key Words : Ginsenoside Re, Ginseng, Tertiary structure, NMR spectroscopy

Introduction

Ginseng has long been used as a tonic, health promoter in Asian countries including Korea and China.^{1,2} Ginsenoside is one of the derivatives of triterpenoid dammarane and consists of 30 carbon atoms. Each ginsenoside has a common hydrophobic triterpenoid to which the hydrophilic sugar moieties are attached. About 30 different types of ginsenosides have been isolated and identified from the root of *Panax ginseng*. Ginsenosides also have at least three side chains, at carbon-3, -6, and -20, which are free or connected to sugar rings. Ginsenoside Re is a major constituent of *Panax ginseng* and *Panax quinquefolium* (American Ginseng).³ Recent reports showed that the ginsenoside Re especially has anti-diabetic and anti-oxidant effects and prevent PC 12 cells from lesion induced by serum-free medium and b-amyloid peptide.^{4,5}

The three-dimensional (3D) structures of biopolymers are known to play important roles in a variety of biological functions. There are several analytical methods applicable for the structural determination of ginsenosides in ginseng extracts.⁶⁻⁸ Previously, we determined the three dimensional structure of ginsenoside-Rg₃ by NMR spectroscopy and proposed that the hydrophobic packing near the chiral center is important for Na⁺ channel regulation.⁹ At present the best method with which to study solution structure of carbohydrate containing molecule with accuracy is high-resolution NMR spectroscopy.¹⁰ We have completed the assignment of ¹H and ¹³C resonances of ginsenoside Re in DMSO-d₆ and tertiary structure has been investigated through NMR

spectroscopy. Structure of ginsenoside Re will be helpful to understand its biological roles.

Experimental

Naming scheme. Naming scheme of Re (20(S)-protopanaxatriol 6-O- β -D-Glucopyranosyl-20-O- α -L-Rhamnopyranosyl-(1 \rightarrow 2)- β -D-Glucopyranoside) are shown in Figure 1. Re consists of two glucose rings, one rhamnose ring, and the triterpene ring. Flexibilities around each linkage are described by seven torsion angles: ϕ_1 , ϕ_2 , ϕ_3 , ϕ_4 , ϕ_5 , ϕ_6 , and ϕ_7 as shown in Figure 1.

Sample purification. Ginsenosides were extracted from

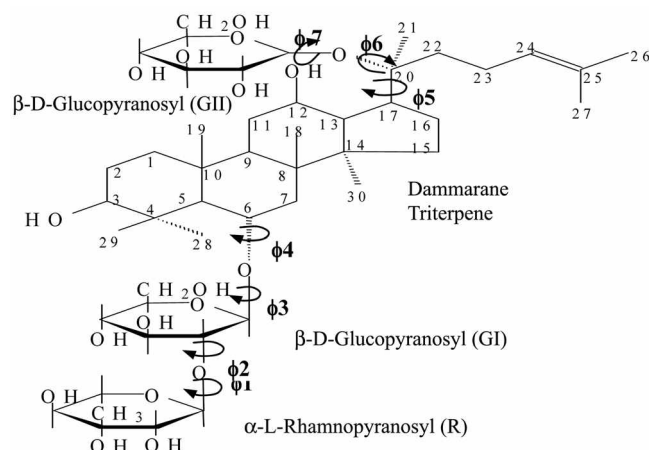


Figure 1. The structure and naming scheme of Ginsenoside Re.

commercially available Korean Ginseng by the Folch-Suzuki partition methods.^{11,12} Glycolipids purified by preparative thin layer chromatography (PREP-TLC) were separated into seven bands. The components of fifth were further purified several times by silica gel column chromatography with various eluents. By using ESI-MS and FAB-MS, fifth band was analyzed as Ginsenoside Re.¹³ The compositions of ginsenosides Re have been investigated by means of high performance thin layer chromatography (HPTLC), high performance liquid chromatography (HPLC), and mass spectrometry (MS), and NMR spectroscopy.

NMR spectroscopy. All the phase sensitive two-dimensional experiments such as DQF-COSY, TOCSY, and NOESY experiments were performed using TPPI method, *i.e.*, time-proportional phase incrementation.¹⁴⁻¹⁶ For these experiments, 256-400 transients with 2048 complex data points were collected for each of the increments with a relaxation delay of 1.5 sec between successive transients and the data along the t_1 dimension were zero-filled to 1024 before 2D-fourier transformation. Data were recorded with 48 scans for each t_1 increments with spectral width of 4504.5 Hz. Prior to Fourier transformation, shifted skewed sine bell weighting function and zero filling were applied to give a final data matrix of 1024 × 1024 real points. HMQC (heteronuclear multiple-quantum coherence spectroscopy) experiments were performed in phase sensitive mode using TPPI method, 256 FID of 2048 complex points were acquired.^{17,18} HMBC (heteronuclear multiple-bond correlation spectroscopy) experiments were performed in magnitude mode and a total of 256 FIDs were acquired.¹⁹ Both of HMQC and HMBC experiments were conducted in the inverse mode, with the ^1H spectral width set to 3521.1 Hz and ^{13}C spectral width set to 23770.9 Hz. TOCSY pulse program used an 80 msec, MLEV-17 spin-lock mixing pulse. Mixing time of 250 and 600 msec was used for NOESY experiments. All spectra were recorded at 303 K on a Bruker AMX-500 spectrometer in Inter-University Center for Natural Science Research Facilities at Seoul National University and in Korea Basic Science Institute. All NMR spectra were processed off-line using the FELIX software package on SGI workstation in our laboratory.²⁰

For the assignment and NOE integration of nonexchangeable protons, Ginsenoside Re was deuterium-exchanged by repeated lyophilization from DMSO- d_6 -D₂O mixture and finally dissolved in 0.4 mL of 98:2 DMSO- d_6 -D₂O solvent for NMR spectroscopy that made a 5 mM sample. For the spectral assignments and NOE integrations of all the protons, including exchangeable protons, Ginsenoside Re was dissolved in 100% DMSO- d_6 . Chemical shifts of ^1H and ^{13}C resonances are expressed relative to an internal tetramethylsilane signal at 0 ppm.

Structure Calculation. Structure calculations were carried out using X-PLOR, version 3.851.²¹ All of the NOE intensities were divided into three classes (strong, medium, and weak) with distance ranges of 1.8-2.8, 1.8-3.5, and 1.8-5.0 Å, respectively. A hybrid distance geometry-dynamical simulated annealing protocol was employed to generate the

Table 1. ^{13}C and ^1H chemical shifts of Ginsenoside Re in DMSO at 303 K

	Aglycone		Glycone				
	Re		Re				
	^1H	^{13}C	^1H	^{13}C	^1H	^{13}C	
1	1.55	0.93	38.44	Glucopyranosyl (G I)			
2	1.44		26.59	GI-1	4.47	99.74	
3	2.90		77.08	GI-2	3.26	77.06	
4	–		38.70	GI-3	3.14	77.06	
5	0.91		59.89	GI-4	3.15	70.70	
6	3.99		72.56	GI-5	3.28	76.46	
7	1.64	1.46	44.57	GI-6	3.29	3.34	62.96
8	–		39.66	GI-HO3	5.16		
9	1.30		48.45	GI-HO4	5.11		
10	–		38.60	GI-HO6	4.45		
11	1.60	0.99	29.86				
12	3.53		68.88	Glucopyranosyl (G II)			
13	1.50		48.04	GII-1	4.41	96.47	
14	–		50.60	GII-2	2.86	73.79	
15	1.43	0.93	30.06	GII-3	3.18	77.06	
16	1.74	1.25	25.77	GII-4	3.05	69.90	
17	2.15		50.29	GII-5	3.42	77.83	
18	0.94		16.57	GII-6	3.45	61.69	
19	0.97		15.35	GII-HO2	4.89		
20	–		81.90	GII-HO3	5.00		
21	1.23		21.48	GII-HO4	5.00		
22	1.69	1.39	35.17	GII-HO6	4.38		
23	2.03	1.91	22.09				
24	5.06		125.28	Rhamnopyranosyl (R)			
25	–		130.10	R-1	5.11	99.74	
26	1.62		25.36	R-2	3.65	70.31	
27	1.55		17.60	R-3	3.40	70.11	
28	1.21		30.88	R-4	3.16	71.95	
29	0.74		15.96	R-5	3.88	67.66	
30	0.81		16.57	R-6	1.06	17.59	
HO3	4.26			R-HO2	4.59		
HO12	4.62			R-HO3	4.50		
				R-HO4	4.65		

structures. The distance restraints used for the structure calculations are listed in Table 1. A total of 100 structures were generated, and the 10 structures having lowest energies were selected for the further analysis.

Results and Discussion

Assignment of NMR spectra. Even though the assignment was not completed by proton-proton through-bond and through-space correlation spectra (Figure 2), proton resonances show well resolved correlations to the ^{13}C resonances in HMQC and HMBC spectra (Figure 3). They offer a variety of ways to identify all directly bonded and long-range carbon-proton pairs in Ginsenoside Re and allowed to complete the assignment of ^1H and ^{13}C resonances. As shown in ^{13}C NMR spectrum of Ginsenoside Re (Fig. 3), the

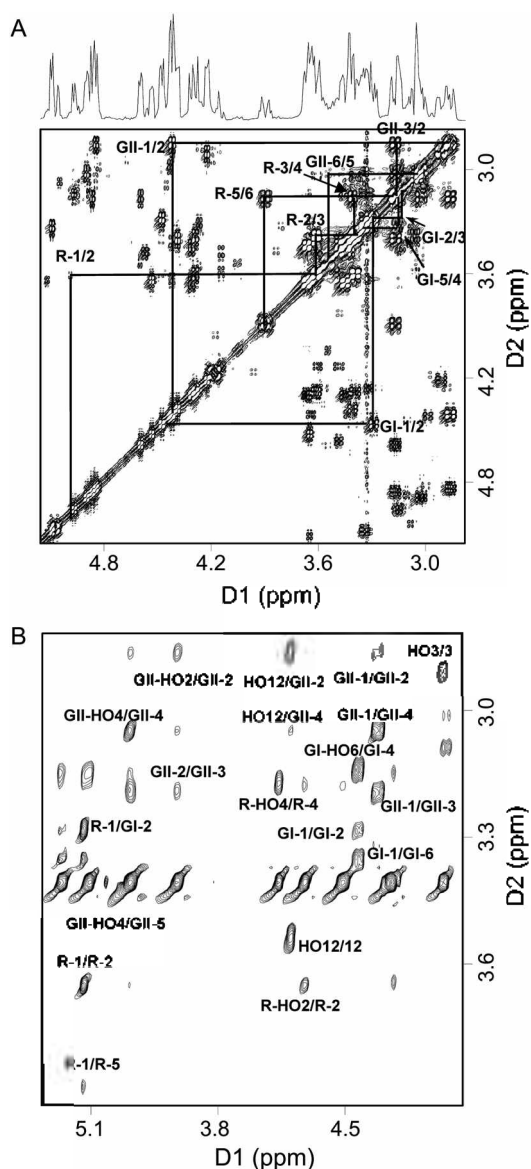


Figure 2. 500 MHz ^1H NMR spectrum of ginsenoside Re in DMSO-d_6 at 303 K. A) DQF-COSY B) NOESY (mixing time = 250 ms)

signals attributed to the aglycon range from 10 to 50 ppm. The part of the spectrum between 60 and 100 ppm contains resonances for sugar carbons, except R-6 (17.7 ppm). Table 1 summarizes the assignment of ^1H and ^{13}C resonances of ginsenoside Re in DMSO-d_6 at 303 K. If ^{13}C chemical shifts of aglycon moiety are compared with previously reported chemical shifts of 20(S)-protopanaxatriol, only the chemical shifts of C-6, 17, and 20, 21 were much different from the reported data because of the different linkages in each ginsenosides.²² ^1H NMR spectrum and HMQC spectrum of ginsenoside Re (Fig. 3) shows the ring proton signals are exist between 2.8 and 5.1 ppm, except R-6. As shown in Figure 2B, there are many intra-ring NOEs in NOESY spectrum. Table 2 lists the long range NOEs including the inter-ring NOEs obtained from the NOESY cross peaks of ginsenoside Re and these NOEs were important to determine

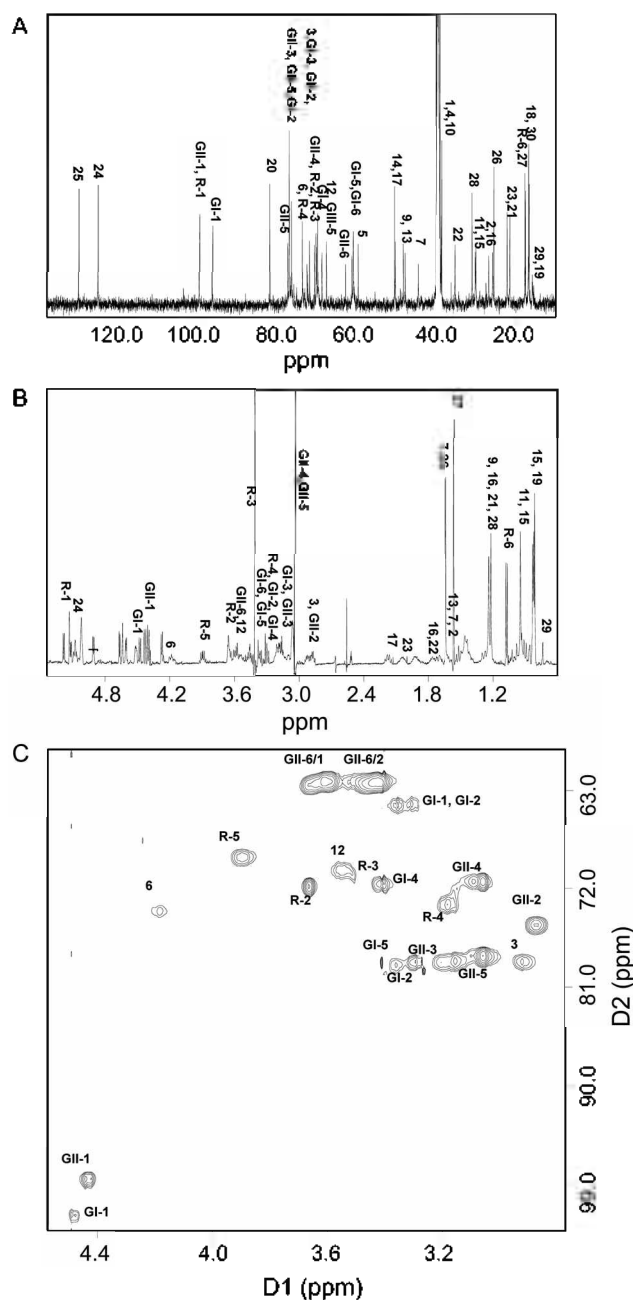


Figure 3. 500 MHz spectra and the assignments of ginsenoside Re in DMSO-d_6 at 303 K. A) Proton 1D, B) Carbon 1D, C) HMQC

the tertiary structures of ginsenoside Re.

Structures of Ginsenoside Re. Among 100 structures, ten lowest energy structures were analyzed for ginsenoside Re. They were superimposed on the average structure as shown in Figure 4A. When the structures were superimposed on the terpenoid ring of restrained-minimized average structures for Ginsenoside Re, alkene side chain and glucopyranosyl II structures are converged well. As reported in our previous study on ginsenoside Rg3, this rigid structure near the alkene side chain and glucopyranosyl ring II may play important roles in recognition of target protein near the membrane surface.

The linkages between the rhamnopyranosyl and gluco-

Table 2. Long Range NOEs obtained from the NOESY cross peaks of Ginsenoside Re

	Proton1	Proton2	20(S)-Rg ₃
1	H6	GI H1	m
2	H6	GI H5	s
3	H9	GII H6a	w
4	H11	GII H1	m
5	H12	GII H2	m
6	H12	GII HO2	m
7	H22	H13	s
8	H22	GII H2	w
9	H28	R H1	w
10	H28	R H2	w
11	H29	R H1	w
12	GI HO6	R H2	m
13	GII H6a	R H1	s

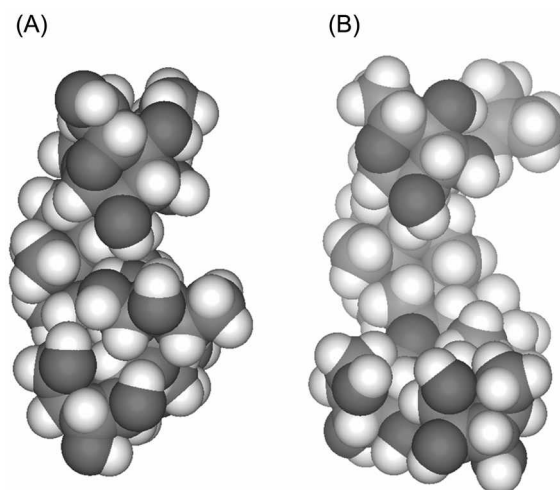
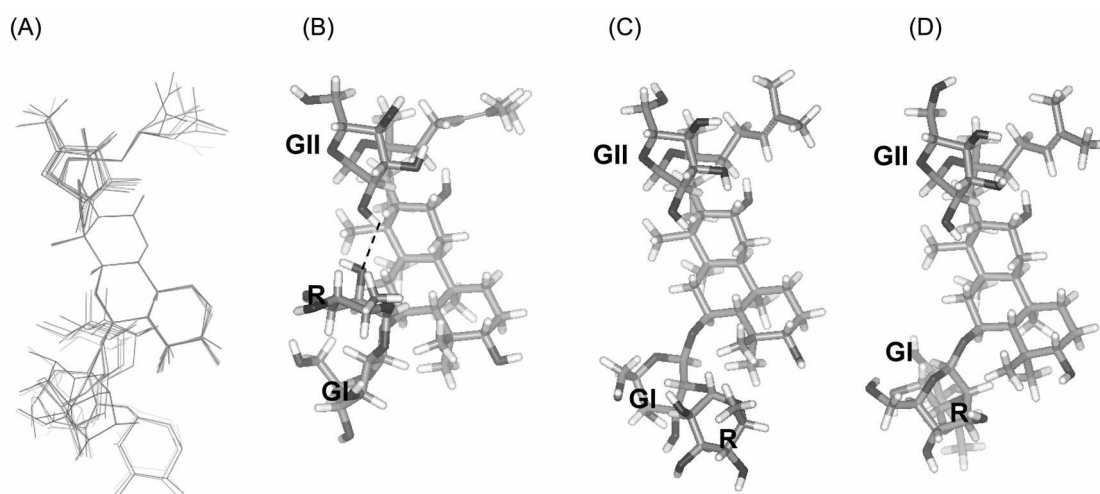
pyranosyl ring I are divided into three groups. The dihedral angles in these three groups are summarized in Table 3 and there is flexibility of around the linkages between rhamnopyranosyl and glucopyranosyl II moieties. The relative energies of group A, B and C are 301.3, 317.9, 350.0 kcal/mol, respectively. Group A has lowest energy and highest population (50%). The rhamnopyranosyl ring in group A orients to the terpenoid and those of group B and C have extended structure. Interestingly, in group A, distance between O2 of rhamnopyranosyl ring and HO2 of glucopyranosyl ring I is very close (2.30 Å), resulting in a stable hydrogen bond between these two atoms as shown in Figure 4B. As shown in Figure 5A, this hydrogen bond shields the hydrophobic terpenoid from the solvent exposure and provides the hydrophilic phase to the ginsenoside Re. However, in group B as shown in Figure 5B, hydrophobic terpenoid is exposed to the surface.

In our previous study concerning the three dimensional structure of ginsenoside Rg₃, we reported that the O12-

Table 3. The dihedral angles in three groups of lowest energy structures of ginsenoside Re

Dihedral Angles	Group A	Group B	Group C
ϕ_1	28 ± 2.6	43 ± 1.3	100 ± 0.2
ϕ_2	71 ± 1.9	131 ± 1.1	-67 ± 2.4
ϕ_3	151 ± 3.4	139 ± 4.0	55 ± 0.7
ϕ_4	172 ± 1.5	123 ± 5.3	123 ± 0.1
ϕ_5	149 ± 0.8	148 ± 1.1	148 ± 0.3
ϕ_6	52 ± 2.4	47 ± 1.7	46 ± 1.6
ϕ_7	42 ± 2.4	46 ± 1.6	47 ± 1.6

HO20 hydrogen bond is a critical factor for the overall structure and proposed that the accessibility of solvents to these hydroxyl group would be crucial for the activity of Ginsenoside Rg₃.⁹ S form of ginsenoside Rg₃ has tight

**Figure 5.** (A) Space filling representation of the lowest energy structure of group A of ginsenoside Re. (B) Space filling representation of the lowest energy structure of group B of ginsenoside Re.**Figure 4.** (A) Superimposition of the 10 lowest energy structures of ginsenoside Re. (B) The lowest energy structure in group A. Hydrogen bond between O2 of rhamnopyranosyl and HO2 of glucopyranosyl ring II is dictated by dashed line. (C) The lowest energy structure in group B. (D) The lowest energy structure in group C.

hydrophobic packing near the chiral center and this factor may provide strong interactions with the receptor region in ion channels. Similarly, the flexibility in the linkages between rhamnopyranosyl and glucopyranosyl II moieties and the hydrogen bond between O2 of rhamnopyranosyl ring and HO2 of glucopyranosyl ring I of ginsenoside Re may be the critical factor for the controlling the hydrophobic environment, and its action eventually.

Hydrogen bond is an important factor in the structures of carbohydrates. Structures of carbohydrates such as gangliosides and many different kinds of disaccharides or trisaccharides have been determined in water or DMSO by NMR spectroscopy.^{9-10,23-27} In ginsenosides, because they have several carbohydrates, it can be proposed that the hydrogen bond between the sugar rings can be an important factor in structure and function of ginsenoside. Here, we studied the structure of ginsenoside Re and revealed that the inter-sugar moiety hydrogen bond between glucopyranoside II and rhamnopyranoside exists. We suggested here that this hydrogen bond and flexibility in the linkage between rhamnopyranoside and glucopyranoside I would be the crucial factor for regulation of its function by controlling of the hydrophobicity of terpenoid ring of ginsenoside Re. Structure of Ginsenoside Re as well as our previously reported ginsenoside Rg₃ will be helpful to understand biological roles of ginsenosides.

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