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Pattern of Molecular Aggregation of Ginsenosides in Aqueous Solution

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水溶液에서 人蔘配糖體의 分子結合樣相

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초　록

精製人蔘사포닌의 수용액을 分子透析膜(分子量 12000)을 通하여 透析하거나 Bio-Gel P-2 (分子量 200~2000)gel을 통과시켜 分取하고 HPLC로 ginsenoside를 分析하였다. Ginsenoside는 分子結合樣相과 分子內 親水性基의 空間配列에 따라 3群으로 分類되었다. 第Ⅰ群은 巨大미셀 形成者로 結合分子數가 10이상이며 1面親水基形이고 panaxadiol인 Ginsenoside Rb_1, Rb_2, Rc 및 Rd가 포함된다. 第Ⅱ群은 小미셀形成者로 結合分子數가 10以上에서 1까지이고 不完全 兩面親水基形이며 triol인 Rg_2와 Rf 및 diol인 Rg_3가 포함된다. 第Ⅲ群은 單分子로 存在하며 兩面親水基形이고 triol인 Re와 Rg_1을 포함한다.

Introduction

Large number of papers on *Panax ginseng* are reported in relation to pharmacology and clinics every year. Ginsenosides (ginseng saponin) are considered as one of active agents for medicinal efficacy of of *P. ginseng*. Most chemical studies on ginsenosides were isolation in various plant parts and investigation of molecular structure. Twenty five ginsenosides were found in *P. ginseng* root and 4 of them are specific in red ginseng[1]. Interaction between plant saponin and cell membranes has fairly early investigated[2]. However, such study with ginseng saponin was very limited. In spite of active investigation in chemistry, pharmacology and clinics, molecular status in physiological fluid is rarely investigated[3,4]. Molecular status of ginsenosides in the cell of ginseng plant is also unknown.

Recently a few ginsenosides can be isolated to the amount of laboratory use in Korea and abroad with preparative HPLC in Korea Ginseng and Tobacco Research Institute. Thus the efficacy of ginseng will be elucidated on each ginsenoside level more rapidly than ever before. For the thorough understand of physiological function of ginsenosides in human or ginseng plant itself the information on mole-

cular status in physiological fluid is important as much as that on the action with biological membrane.

Critical micelle concentration (CMC) of ginseng saponin was once reported as about 20 mM (around 2% solution) and CMC was 0.1% with cholesterol[5]. In this case saponin contaiinted at least 6 ginsenosides. We are interested in whether all ginsenosides react for micelle formation in the same way or not. We found that pattern of molecular aggregation depends much on the molecular structure of each ginsenoside.

Materials and Methods

Dialysis

Purified ginsenosides (1g, above 95%) dissolved with 5ml distilled water was dialyzed through dialysis tubing (Sigma, MW 12000) against distilled water(200 ml each, 7 times) at 4°C for 4 days. Nondialyzed and dialyzed fractions were dried under vaccum at 40°C respectively, weighed and investigated forginsenoside composition with HPLC.

Gel filtration

Purified ginsenosides (2g) were dissolved in 3ml of 10% methanol, applied to Bio-Gel P-2 (100-200 mesh for MW200~2000, Bio-Rad, 90g) column(50cm L×2.6cmD) with 10% methanol, eluted with 10% methanol at the rate of 4ml per hour and collected into 1ml fraction. The ginsenoside composition of eluate was detected with HPLC.

High performance liquid chromatography (HPLC)

Waters Associates Model 244, carbohydrate analysis column, solvent system CH_3CN/H_2O (82/18)[6], CH_3CN/H_2O/n-BuOH(82/18/3)[7], flow rate 1.1ml/min., RI detector, attenuation 8×. chart speed 1.0cm/min. Recoder data module.

Impurity test

Protein was detected by folin phenol reagent before and after dialysis. Total nitrogen content of purified saponin was measured by microkjeldahl method. Protein in saponin was detected by precipitation with TCA. Impurity was compared by UV absorbance at the same concentration of saponin before and after dialysis.

Results and Discussion

High performance liquid chromatogram of sample before dialysis and non-dialyzed fraction were shown in Fig.1. Peaks of ginsenoside Rg_1 and Re were deleted from non-dialyzed fraction while other peaks are all appeared. Percent composition of ginsenosides in sample saponin and non-dialyzed fraction was shown in Table 1. Thirty eight percent of total saponin was dialyzed. Percentage of non-dialyzed portion was shown in each ginsenosides. Percent dialysis seemed to have no relation to the amount before dialysis among ginsenosides. Percentage of dialyzed and non-dialyzed portion of each ginsenoside shown in Fig. 2.

Ginsenosides could sorted into three groups according to dialysis. Ginsenosides, Re and Rg_1 belong to the extremely well dialyzed group, Rf, Rg_2 and Rg_3 to the well dialyzed and others to the hardly dialyzed one. The hardly dialyzed ginsenosides have all proto panaxadiol aglycone. The other two groups belong to protopanaxatriol except Rg_3 (diol). From this result we can expect that the hardly dialyzed ginsenosides mostly form aggregates with molecular weight above 12000 while the extremely well dialyzed ginsenosides do not aggregate or form small aggregates with molecular weight below 12,000. Joo and Lee[5] reported that ginseng saponin composed of 6 ginsenosides formed micelle and CMC was about 2%.

The aggregates of ginsenosides mentioned above are likely to be micelles and micellar size could be different among ginsenosides. It

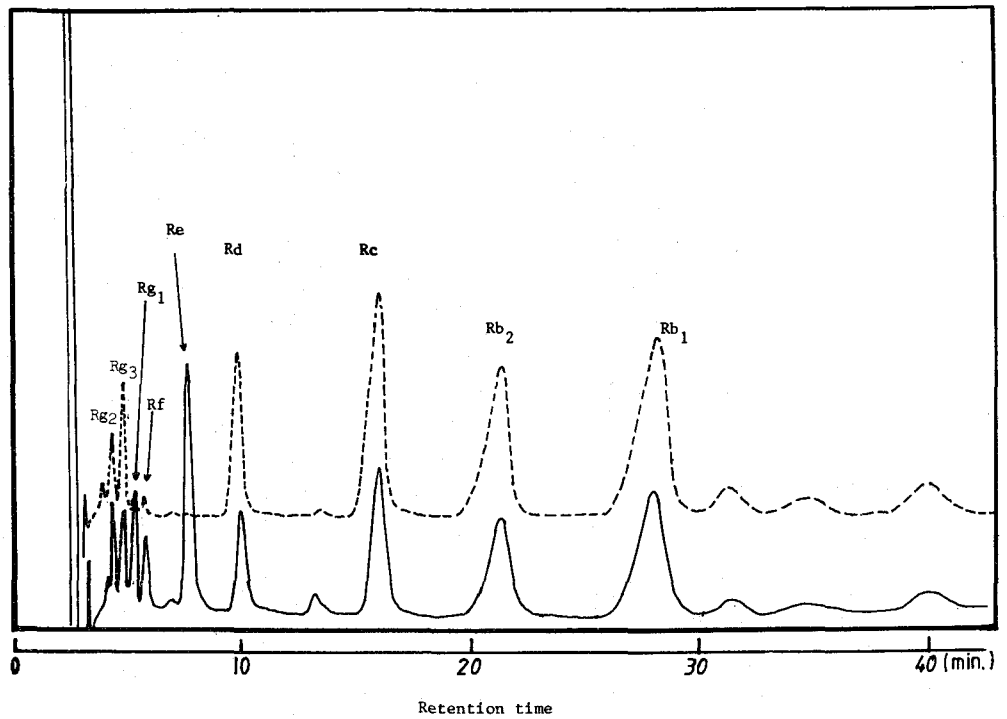

Fig. 1. HPLC chromatogram of purified ginseng saponin before (——) and after (---) dialysis. Column: carbohydrate analysis, eluent: AcCN/H_2O/n-BuoH(82/18/3), flow rate: 1.2ml/min, detector: RI, attenuation: 8X

Table 1. Percent composition of ginsenoside before and after dialysis.

Ginsenoside	Rg_2	Rg_3	Rg_1	Rf	Re	Rd	Rc	Rb_2	Rb_1	Total
Sample (A)	1.06	2.83	5.01	1.98	13.3	9.15	20.1	18.6	28.1	100.0
Non-dialyzed (B)	0.37	1.66	T	0.43	0.11	7.57	16.0	13.1	22.6	61.8
B/A × 100	34.9	58.7		21.7	0.83	82.7	79.6	70.4	80.4	61.8

seems to be questionable that the composed saponin in which the content of each ginsenoside was different showed relatively sharp one CMC[5], although changes in the hydrophilic part of the amphiphile generally have insignificant effects on CMC[9].

Ultraviolet absorption of saponin solution before and after dialysis was shown in Fig. 3. The UV absorption material (impurity) decreased considerably after dialysis indicating that non-dialyzed fraction is related to impurity no more than dialyzed fraction is if the impurity might be concerned in aggregation.

Folin-phenol test showed 1.9% as bovine serum albumin in dialyzed fraction and 0.83% in non-dialyzed fraction. Microkjeldahl method showed 0.66% crude protein in sample saponin but no precipitation was appeared with 10% trichloroacetic acid. It seemed to be difficult for protein to remaine during purification process of saponin. Above results do not rule out probable concern of phenolic compound.

Gel filtration profile of saponin through Bio-Gel P-2 column was shown in Fig. 4. Ginsenosides Rd, Rc, Rb_2 and Rb_1 were eluted first all together. They are the hardly dialyzed ginsenosides. The HPLC chromatogram of the eluate from 120 to 140ml (Fig 5) also showed

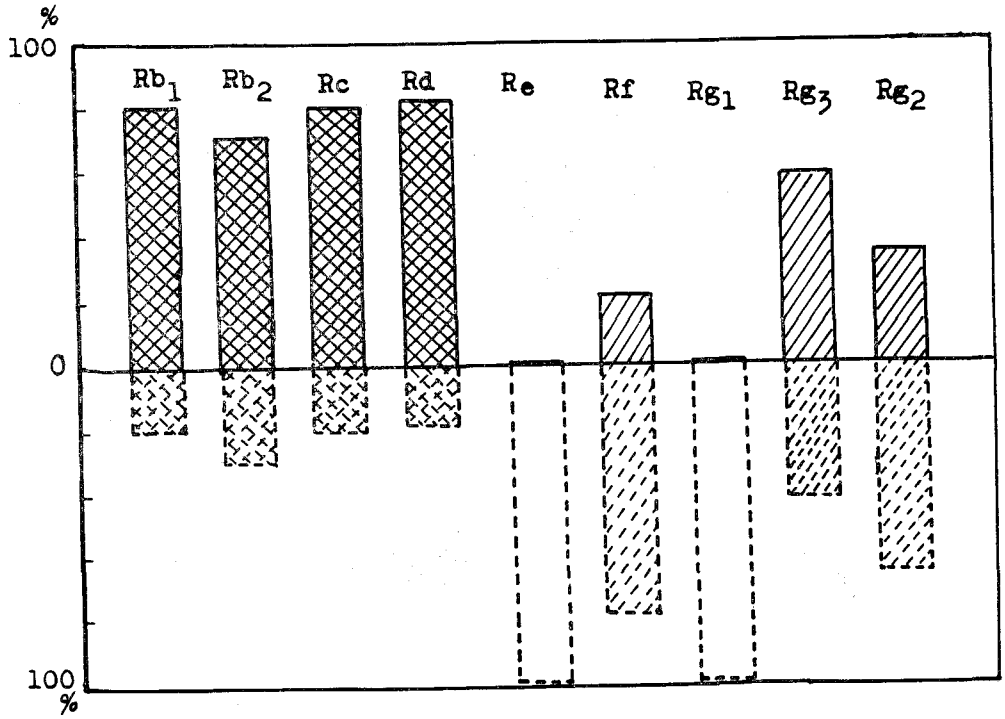

Fig. 2. Percentage of dialyzed and non-dialyzed ginsenoside portion of each ginsenoside.

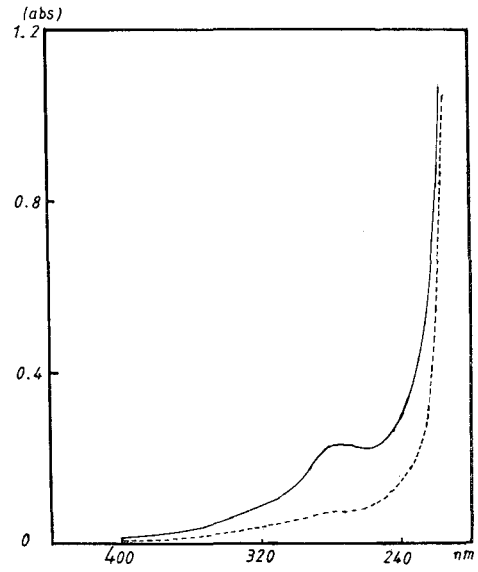

Fig. 3. Ultraviolight absorption spectra of total (——) and non-dialyzed (---) fraction after 5 days dialysis. (0.1% aqueous soln.)

Rg_2, Rg_3 and Rf (tha well dialyzed group) but Re and Rg_1 (the extremely well dialyzed group). Re was eluted before Rg_1 due to greater molecular weight (947) than Rg_1 (MW 801). Since this gel is for the separation of MW 200 to 2000 it strongly indicates that Re and Rg_1 exist as dimer or single molecule. Some of Rg_2, Rf and Rg_3 were eluted with Rg_1 and Re. Bio-Gel P-2 was reported being successful for the fractionation of TGF of ginseng[10]. TGF seems to be triol ginsenosides.

Separation with gel filtration was well in accordance with that of dialysis. Ginsenosides in the same dialysis group have the simillar spatial arrangement of hydrophilic moiety, sugars, in their molecules. Two hydrophilic moieties are located in one side of hydrophobic part of aglycone in the hardly dialyzed group while two hydrophilic parts are located symetrically in both sides of hydrophobic part in the extremely well dialyzed group as schematically shown in Fig 6. The well dialyzed group has hydrophilic part in two sides but size of

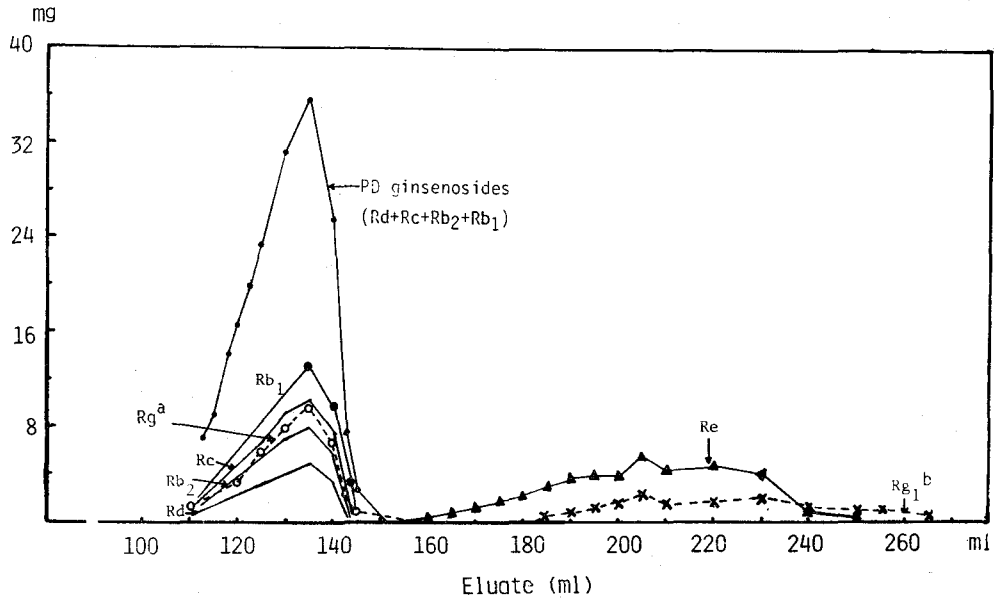

Fig. 4. Separation of ginsenosides on Bio- Gel P-2 column. Demension: 2.6cm×5cm, eluent: 10% methanol, flow rate: 4ml/hour, sample: 2g crude saponin, Rg^a: some of Rg_2, Rg_3 and Rf, $Rg_1{}^b$: Rg_1 and some of Rg_2, Rg_3 and Rf.

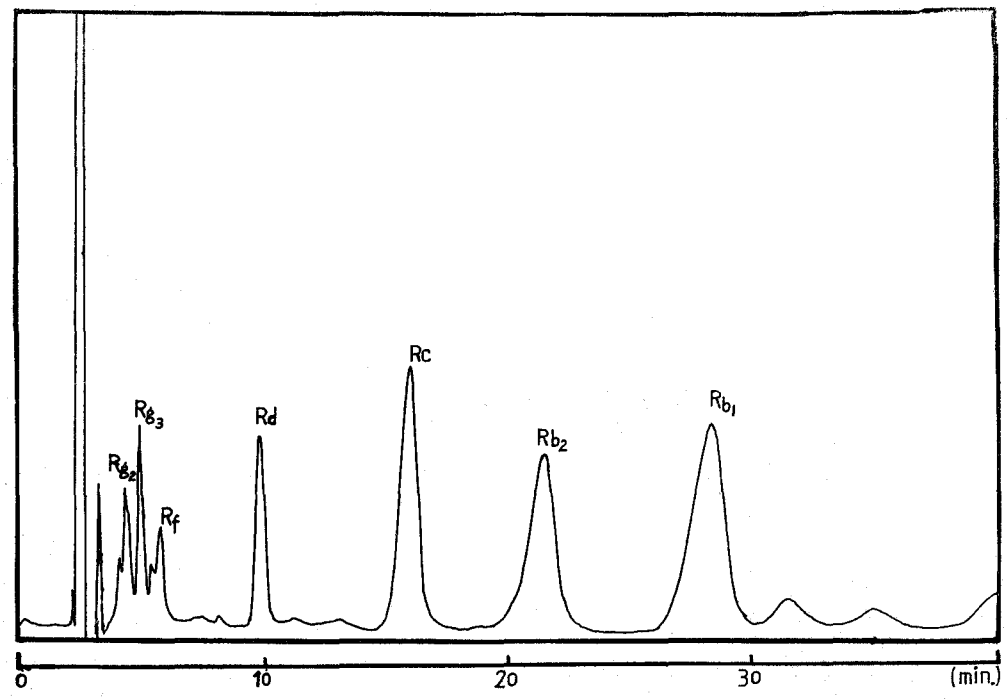

Fig. 5. HPLC chromatogram of ginsenosides in 120 to 140ml fraction of Bio-Gel P-2 column eluate.

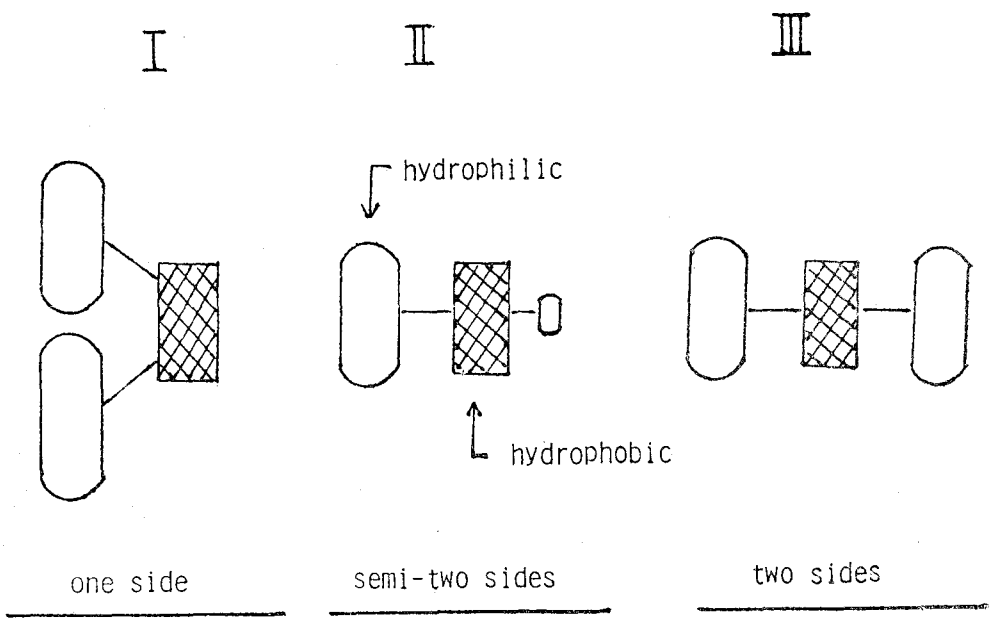

Fig. 6. Schematic diagram of amphiphatic form of ginsenosides

Table 2. Classification of molecular aggregation for ginsenosides in aqueous solution

Dialysis	Group I Hardly	Group II Well	Group III Extremely well
Agrycone	Protopanaxadiol	Protopanaxadiol Protopanaxatriol	Protopanaxatriol
Ginsenosides	Rd, Rc, Rb_2, Rb_1	Rg_2, Rf, Rg_3	Re, Rg_1
Molecular weight	947—1211	639—801	801—947
Hydrophilic part	One side	Semi-two sides	Two sides
Micellization	Large micelle former	Small micelle former	No micelle former
Aggregation number	>10	>10~1	1

each part is unequal. One is sugar residue and the other is hydroxyl group.

Thus we can classify ginsenosides into three groups according to spatial arrangement and its size of hydrophilic part, that is, the one side, the two-side and the semi-two side group, and they are well matched to the groups of dialysis. The one side ginsenosides can form large micelles easily while the two side ginsenosides will be in difficulty for micelle formation and exist as single molecule. The semi-two side group will partly form small micelles and partly exist as single molecule.

The relationship between molecular structure, and the characteristics of micelle formation may be summerized as Table 2. In the semi-two side group Rg_3 is only panaxadiol but molecular structure is much simillar to Rg_2 and Rf as shown in Fig. 7.

In Fig. 2 ginsenosides are in order of approximate polarity (inverse order of HPLC). Dialysis characteristics has no consistent relation to polarity. But generally one side type will have higher polarity than two side type. It can be said in general that the greater the polarity, the more the dialysis hindered.

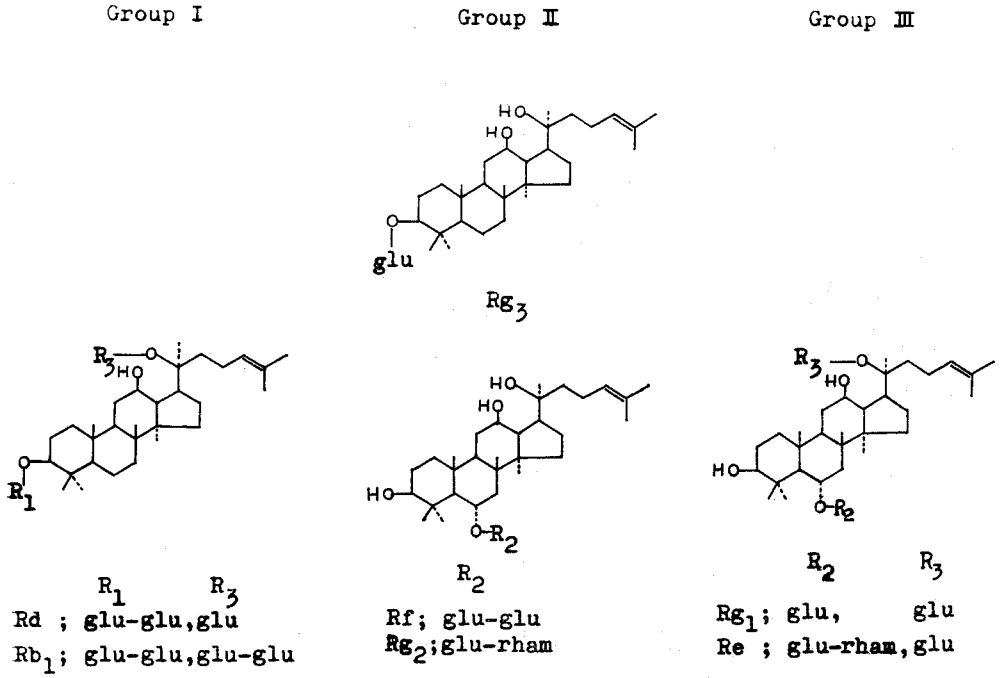

Fig. 7. Representative molecular structure of molecular aggregation groups.

Table 3. Dialysis characteristics of ginsenosides

Investigator and year	Plant part	Ginsenosides		Dialysis condition
		Dialyzed	Non-dialyzed	
Yahara et al[11] 1976	Leaves	Re, Rg_1 F_1, F_2, F_3	Rd	10g/50ml H_2O Cellophane film
Yahara et al[12] 1976	Buds and flower	Re, Rg_1	Rd	Aqueous soln.
Yahara et al[13] 1979	Root	Re, Rg_1, Rh_1 (small)	Rh_1 (large)	Aqueous soln. Gradient dialysis
	Leaves	Re, Rg_1	Rb_1, Rb_2, Rc	
	Flower bud	Re, Rg_1, F_3 M_7(a-d)	Rb_1, Rb_2, Rc, Rd, M_{3-6}, M_7(a-d) M_7cd (from M_7(a-d))	
Zhou et al[14] 1981	Root (P. notoginseng)	Re, Rg_1, NR_1	Rb_1, Rd	42g/100ml H_2O Cellulose film (MW 8000)

Dialysis of saponin has recently been used especially for the saponin investigation in above ground parts[11~13]. Dialysis results of other inve stigators[11~14] were shown in Table 3. Ginsenosides are clearly into three groups namely dialyzed, partially dialyzed and nondialyzed group. These groups are well in accordance with the present study. The great dif-

ference is that the hardly dialyzed group in the present study appeared as the non-dialyzed group in their studies. It might be due to smaller pore size(MW 8,000) they used than that (MW 12,000) in the present study.

According to Table 3 ginsenoside Rh, M_7(a-d) and M_7cd are the well dialyzed ones. Molecular structure of M_7 (a-d) seemed to be unknown yet. Rh is a semi-two side triol and M_7cd is a semi-two side pentaol. Thus they obeyed to the molecular stucture in dialysis pattern. F_1, F_2, F_3 and NR_1 belong to the extremely well dialyzed group. F_1, F_3 and NR_1 were the two side triol[1] and thus well in accordance with molecular structure. F_2 is a one side diol[1] and a unique exception. According to dialysis characteristics the spatial arrangement of two glucoses should be symetrical as triol.

Its exceptional phenomenon can not be explained at present in relation to molecular stucture. The difference in molecular structure of F_2 from other diols is that it has only one glucose on 3rd carbon of aglycone while others have two. It is remained unsolved how the size of hydrophilic part affects micelle formation in one side ginsenosides.

According to this study and others (Table 3) aggregation number of micelle formed by the hardly dialyzed group is postulated as greater than 10. Aggregation number of surfactants in aqueous solution generally ranges between 10 and 100[9]. Aggregation number of two sides ginsenosides could be 2 or 1 as mentioned above. But formation of dimer seems to be difficult in two side shape. Thus the chance for single molecule will be high. The semi-two side, group may range about 20 to 1. It is uncertain whether ginsenosides in the same group form a micelle of one kind ginsenoside or a micelle of many kind ginsenosides. Pattern of molecular assembly in ginsenoside micelles is also one of interesting point to be elucidated.

Summary

For the information on micellization at each ginsenoside level aqueous solution of purified saponin of *Panax ginseng* root was dialyzed through dialysis tubing (MW 12,000) or eluted through Bio-Gel P-2 (MW 200-2,000) and analysed for ginsenosides by high performance liquid chromatography. Ginsenosides can be classified into three groups depending upon molecular aggregation pattern and spatial arrangement of hydrophilic parts in molecule. Group I that is large micelle former(aggregation number: above 10) and one side hydrophilic part (HP) includes ginsenosides Rb_1, Rb_2, Rc and Rd (diols). Group Ⅱ that is small micelle former (aggregation number:>10-1) and semi-two sides HP includes Rg_2, Rf (triol) and Rg_3 (diol). Group Ⅲ that is no micelle former (aggregation number: 1) and two sides HP includes Re and Rg_1 (triol).

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