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Influence of Total Saponin from Korean Red Ginseng on Structural Changes in Phospholipid Membranes and Ghost Erythrocytes

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Abstract□Total saponin from Korean red ginseng changed thermodynamic parameters of membranes from dipalmitoylphosphatidylcholine (DPPC) and ghost erythrocytes of human. In liposomes from DPPC, temperature of the main transition (Lb'-La) in liquid-crystalline phase increases by 0.2℃ in average, but enthalpy does not change. Total saponin at a concentration of smaller than 10^{-5}% "stabilizes" the lipid bilayers. At larger than 0.07 of saponin/DPPC ratio, saponin leads to an exclusion of the bound lipid molecules from the main phase transition into lamellar liquid crystalline La-phase. Total saponin influences specifically all erythrocyte membrane transitions in a concentration-dependendent manner, i.e. on the structures of all the main membrane skeleton proteins. A high structural specificity of saponin with membrane proteins, could be a base of specificity of physiological response of not only erythrocytes, but also other cells.

Key words□Korean red ginseng, total saponin, thermodynamic parameter, phase transition, liposome, erythrocytes.

Introduction

Biological membrane plays a functional role in maintaining homeostasis of cells. It acts as not only diffusional barrier but also transportation system for substances. The function of biological membrane is coordinated by the physical state and structural organization of lipid bilayer. It is necessary, in that context, to study the changes of structural organization and physical state of lipid bilayer.

So far there have been many model systems to study the function of biological membranes which show similar characteristics of biological membranes. Among them, liposome is a preferred model membrane system adopted by many researchers. Liposome is also utilized as a vesicle to transport drugs to organisms at present. When a drug is introduced to an organism, the structural change of biological membrane and the mechanism of interaction between drug and membrane is not easy to interpret. Therefore, using liposome in the study of structural and physical changes of membranes is a good tool to elucidate the mechanism of interaction with biological membranes.

Saponins are classically defined as a group of natural products which show a haemolytic activity, induce a lysis of erythrocytes and leukocytes with releases of alkaline phosphatase and esterase and form complexes with cholesterol in artificial and biological membranes[1,2] and transmembrane ion channels.[3] Saponins showed a property of membrane active agents. Saponin from Korean red gin-

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seng, however, modulated various physiological functions of human body, implying that its pharmacological effect is not totally explained by membrane surfactant activity. If taking it into consideration that liposome is used as both a good substitute for biological membrane and a great important means of transportation for drugs,[4] it is necessary to study the effects of saponin on structural state of membranes.

Therefore, we carried out researches how total saponin affects structural states of biological and artificial membranes.

Materials and Methods

1. Materials

The chemicals used in this experiment, NaCl, Na_2HPO_4 and NaH_2PO_4 were obtained from Sigma Chemical Co (St. Louis, MO, U.S.A), and dipalmitoylphosphatidylcholine were from Fluka Chemical Co. (Buchs, Switzerland). Total saponin was offered from the analysis center, Korea Ginseng & Tobacco Research Institute, Taejon, Korea.

2. Preparation of liposome from DPPC and ghost erythrocytes from human

DPPC was dispersed in the solution of 150 mM NaCl in 5 M phosphate buffer (pH 7.4). Multilamellar vesicle (MLV) dispersions were obtained by various methods: ultrasonic and thorough mixing at various temperatures, etc. The total saponin was added directly to a vesicle suspension or during MLV preparation.

The ghost membranes were isolated from human erythrocytes by means of hypotonic hemolysis in 5 mM phosphate buffer (pH 7.4),[5] Before microcalorimetric study, ghost membranes were suspended in a phosphate buffer (310 mosm, pH 7.4). The concentration of ghost membrane was defined by dry weight (mg/m*l*) after drying it up to a constant weight at 110 for 30 min. Saponin was added directly to ghost membrane suspensions and incubated at room temperature for 30 min.

3. Microcalorimetric studies

Calorimetric studies were carried out on a differential adiabatic scanning microcalorimeter, DASM-4 (USSR) with platin cells volume of 0.47 m*l*. Heating rate was 1 K/min in all experiments. We determined enthalpy (ΔH_{cal}) which is defined as the energy needed for destructing intramolecular bonds, cooperativity parameter, $\sigma(\sigma = \Delta H_{cal}/H_{V\text{-}H})^2$ where $\Delta H_{V\text{-}H}$ is the enthalpy of Vant-Hoff, and cooperativity unit, $n(n = \sqrt{\sigma})$.

4. Freeze-fracture study

Freeze-fracture experiments were performed by the techniques described by Costello.[6] Cell samples without cryoprotectants, were placed between two cooper strips and quenched by plunging them into liquid propane (-196℃) at a cooling rate of greater than 8000 grad/sec. Frozen samples were inserted into a double replica device of freeze-fracture unit (JEOL, Japan). Up to 9 samples were simultaneously fractured at -150℃ under a high vacuum. The fractured samples were replicated with a carbon-platinum of 45 grad angles and with a carbon of 90 grad angles by means of electron guns. The replicas were cleaned in nitric acid, washed in water and picked up on uncoated 400-mesh electron microscope grids. The samples were observed with JEM-100B electron microscope at 80 kV and magnification of 20000.

Results and Discussion

1. Interaction of total saponin with artificial phospholipid membranes

Phase transition of lipid membrane from liquid crystalline state to gel and vice versa is the first range transition. In the phospholipid phase transition region is two border phase, and the whole transformation rate of one phase into the other depends on the structural defects impairing the cooperativity of transition process.[7] Presence of the defects causes pretransitions to proceed to the base phase transition. The DSC method allows to reveal the temperature of phase transition of lipid and calculate the transition enthalpy. The phase transition from gel state to a liquid crystalline phase in lipid bilayers is accompanied by a thinning of bilayers and surface increment fitting each molecule into bilayers. At the phase transition temperature, phospholipids in a gel state form a bilayer structure in which the fatty acid chains are packed in a hi-

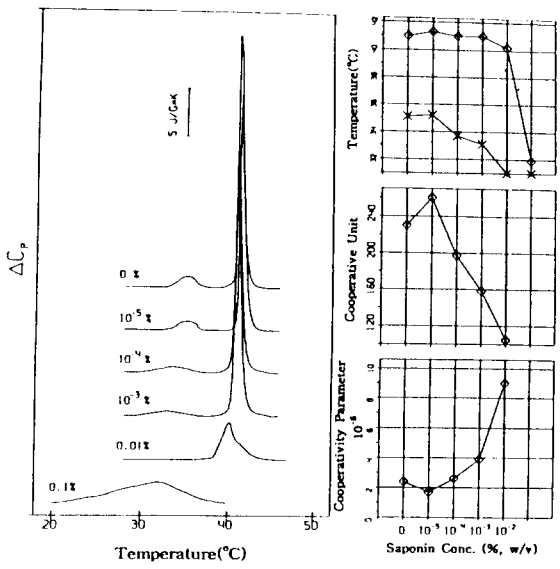

Fig. 1. Temperature dependence of excess specific heat capacity of suspension of DPPC multilamellar vesicles in 5 mM phosphate buffer (pH 7.4) and 150 mM NaCl. In the presence of saponins: 0~0.1%. velocity of scanning: 1 K/min (K : Kelvin).

ghly arranged hexagonal lattice. The phase transition in a bilayer from DPPC occurs at 34± 2C in pretransition and at 42C in main.[8]

The result represented in Fig. 1 is a microcalorimetric study of the interaction of total saponin with the multilamellar vesicle (MLV) dispersions from DPPC.

According to the generally accepted notions in the field of researches of the interaction of organic substances with the lipid bilayers,[9-12] the thermogram appearance and transition parameters are defined by the penetration depth of a substance into a hydrophobic bilayer region or, in other words, by the localization of substance along bilayer thickness (in the region of a lipid polar head, along the glycerol or methylene fatty acid residues selection). Besides, one can value the molecular size and polarity.

The analysis of results that total saponin influences upon thermodynamic parameters of the phase transitions from the lamellar gel phase (Lb') to the ripple (Pb') and liquid crystalline phase (La) of MLV dispersion showes (Fig. 1) that :

a) Saponins are not strong agents to alter bilayer. Its action mechanism is complex and depends on the concentration of saponins used. At low concentrations, incorporation effectiveness and binding quantity to lipid bilayer greatly depend on the preparation way of vesicle mixture with saponins.

b) At a concentration of 10^{-5}% saponin or the saponin/DPPC ratio of 7×10^{-4}, the cooperativity of phase transition and the cooperative unit size are increased. Temperature of the main transition (Lb'-La) in the liquid crystalline phase increases by 0.2C degrees in average, but the enthalpy does not change. Saponins "stabilize" the bilayer membranes (Fig. 1, Right).

c) At concentrations of higher than 10^{-4}% of saponin, the bound lipid molecules are excluded from main phase transition into lamellar liquid crystalline La-phase (Fig. 1, Left). Simultaneously the size of cooperative unit in a lipid domain decreases from 210~260 lipid molecules in control to 70~100 molecules at the saponin/DPPC ratio of 0.7, with an increment of saponins. As the parameter of cooperativity increases, the transition cooperativity lowers (Fig. 1, Right).

d) When its concentration is increased after a simple mixing of saponins at room temperature, saponins induce the second lipid phase formation about at 43C, with saponin placing mostly in a polar bilayer region (Fig. 1, Left). Temperatures of the maximum pretransition and main phase transition decrease as the concentration of saponin is increased. At a concentration of 10^{-2}%, saponin removed pretransition from lamellar gel phase (Lb') to ripple phase (Pb'), and at 0.1%, the main transitions disappeared and only a very new wide transition (20~38C) remained (Fig. 1, Right).

Thus, with an increase of its concentrations, saponin removes the lamellar Lb' phase at room temperature firstly, and then completely removes gel formation so that all lipids are in a liquid crystalline state at the physiological temperatures.

Fig. 2 represents the fractured hydrophobic surfaces of DPPC multilamellar vesicles which were frozen before (A) and after (B) the addition of 10^{-3}% saponin. The cryofixation was performed after

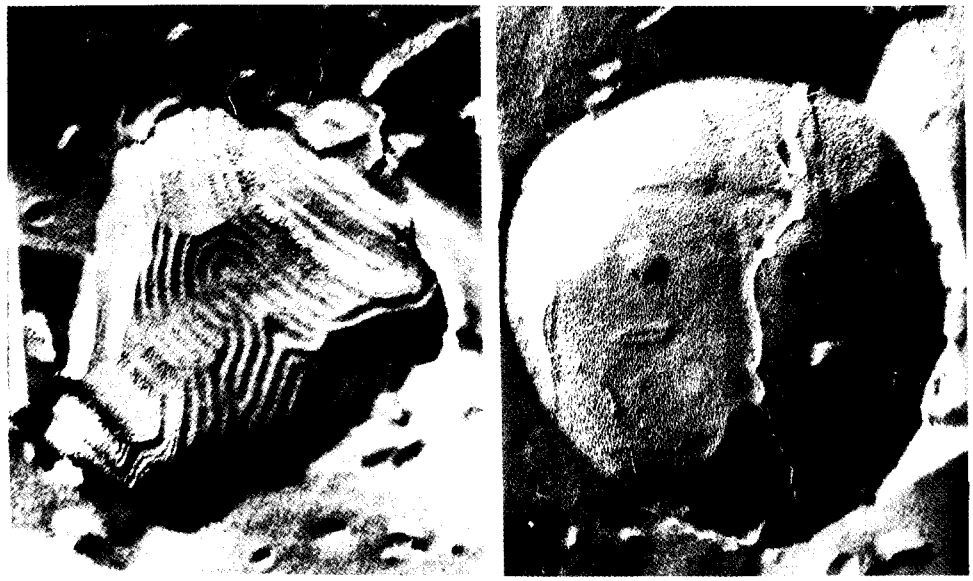

Fig. 2. Electron microscope images of hydrophobic surface of membranes formed by DPPC multilamellar vesicles in control (A) and in the presence of saponin, 0.001% (B). It can be noticed that "ripple" phase is absent in the presence of saponin.

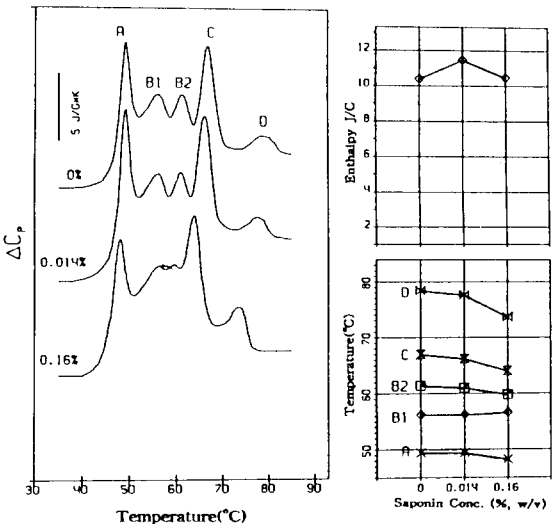

Fig. 3. Temperature dependence of excess specific heat capacity of suspension of human erythrocyte membranes in 310 mosm phosphate buffer (pH 7.4): 1; control. In the presence of saponins: 2; 0.014%, 3; 0.16%. Transitions: A; spectrin and actin proteins, B1; ankyrin and protein band-4.1, -4.2 and -4.9, B2; protein band 3 (forms a cytoplasmic domain), C; protein band 3 (membrane fragments).

the incubation of samples at 39℃ for 3 min. As demonstrated in Fig. 2A, the pure DPPC forms the ripple phase which is typical of that lipid. The addition of saponin effectively eliminated the observed ripple phase and smoothed lipid bilayer (Fig. 2B).

It is well known[11-14] that various additives such as membrane proteins or cholesterol into hydrophobic bilayer region can stabilize the structure of ripple phase by increasing the temperature interval which promote the exhibition of that structure.

2. Influence of total saponin on structural transitions of human erythrocyte membranes

The results of microcalorimetric study are presented in Fig. 3, concerning the influence of total saponin on the human erythrocyte membranes. Curve 1 represents the membranes in the absence of saponins, the control.

Previously it was shown that thermoabsorbance peaks are due to a denaturation of the highly cooperative membrane skeleton domain proteins.[11,12] They exhibit 4~6 thermoabsorbance peaks with a dependence on ionic power and pH at intervals of 30~90℃ (Fig. 3, Left). In each domain that is called

as A-, B1-, C-transition, the specific membrane skeleton proteins denaturalize. It was shown that in A-transition with an average maximum temperature about 50℃, α- and β-spectrin and actin chains denaturalize. In B1-transition with a maximum about 54℃, ankyrin and protein bands-4.1, -4.2 and -4.9 denaturalize. B2-transition with a maximum near 62℃ triggers the denaturation of a cytoplasmic domain of the erythrocyte anion transport protein, protein band 3 (Fig. 3, Left). The transmembrane domain of protein band 3 and perhaps some other membrane lipid domain denaturalize in the C-transition. A thermoabsorbance nature of D-transition region is not known so far, but it is assumed that an unfolding of some protein structures and the decay, the vesiculation of membrane are the primary cause.[12]

As shown in Fig. 3, total saponins in a concentration-dependent manner, are able to influence most of erythrocyte membrane transitions specifically; i.e. on all the structure of main membrane skeleton proteins.

At 0.014% of saponin concentration, the thermograms differ from the control: a) the temperature of C- and D-transitions was lowered by 1℃ and 3℃, respectively, but the denaturation enthalpy was heightened by 2 J/g (Fig. 3, Right). It is obvious that saponins slightly affect the structural proteins of anion transport, but not influence the state of proteins which determine the mechanic and characteristic properties of erythrocytes such as spectrin, ankyrin, actin, band proteins-4.1 and -4.2. b) at concentrations of increase up to 0.16%, saponins affect all the structure of erythrocyte membrane domains that is expressed by a lowering of enthalpy value, decrease of maximum temperature of transition except B1-transition and change of thermocapacity value ratios in a transition maximum.

The presented results demonstrate (Fig. 1, 3) the capability of saponin to influence very important physical properties of lipids such as phase transitions and structural organizations. Liposomes have been used effectively to introduce various drugs, radiotherapeutic agents, enzymes and transcription factors into the cytoplasm of living cells.[9]

It is necessary for us to make clear the problems caused by bringing saponins into liposomes from phospholipids.

As a result of microcalorimetric and electron microscopic studies(Fig. 1, 2), it was shown that total saponin from Korean red ginseng was able to incorporate into the gel phase of MLV membrane from DPPC and to alter the lipid packing characteristic and phase transformations of DPPC.

An ability of total saponin to interact with DPPC bilayers reflects a fundamental property of saponins, its lipophilia which evidently enables total saponin to influence a wide spectrum of membrane such as the process related to lipids and signal transductions in intracellular compartments of all living cells.

Results obtained from human ghosts erythrocytes are difficult to interpret so far. Among all changes of ghost erythrocyte membrane, however, it is inferred that saponin exerts on the state of membrane domain of band protein-3 (C-transition) and its cytoplasmic fragment (B2-transition). A high structural sensitivity of protein band 3 to the action of saponin in vitro with other membrane proteins, could be a base of the specificity of physiological response in not only erythrocytes, but also other cells the membranes of which contain protein band 3.

Further studies in ghosts and whole erythrocytes *in vitro* with ginseng fractions could allow to obtain a detail information about the behavior of structural domains of erythrocyte membranes in the presence of saponins.

요 약

고려홍삼의 총사포닌은 사람의 적혈구막과 dipalmitoylphosphatidyl choline(DPPC)로 제조한 리포좀에서의 열역학적 매개변수를 변화시켰다. DPPC로 제조한 리포좀에서 총사포닌은 액체-결정상의 주전이 온도를 평균 0.2℃올렸으나, 엔탈피에는 아무 영향을 주지 않았다. 10 %보다 낮은 농도에서 총사포닌은 막이중층을 안정화시켰으며, DPPC에 대한 사포닌의 비율이 0.07보다 높을 때에는 막에 결합된 지질의 주전이를 막았다. 총사포닌은 주요한 막골격 단백질의 구조를 비롯하여 적혈구막의 막전이를 농도에 비례

하여 특이적으로 변화시켰다. 시험관 내에서의 이와 같은 사포닌의 구조적 특이성은 적혈구막 뿐만이 아니라 다른 세포계에서의 생리적 특이성의 근거가 될 것으로 사료된다.

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