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Mechanistic Studies on Biological Effects of Soybean Saponins on Cell Membrane

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ABSTRACT

Saponins are glycosidic compounds present in many plant foods. They are characterized by their ability to lyse cell membranes due to their surface-active properties. Saponins are believed to interact primarily with cholesterol in the cell membrane. In this study, the interaction of soybean saponins(SS) with cell membrane was investigated using erythrocytes as a model. Mechanisms of interaction was also investigated by measuring their binding capacity with different membrane lipid fractions. Throughout the study, gypsophilla saponin(GS) and quillaja saponin(QS) were used to evaluate the membranolytic activity of soybean saponins. All saponins released hemoglobin in a concentration-dependent manner. SS induced 40% hemolysis at the concentration of 400 ppm, however there was no increase in hemoglobin release above 400 ppm concentration. 5 ppm of GS and 8 ppm of QS hemolyzed 100% of erythrocytes. Isolation of SS fractions by thin layer chromatography revealed that only one non-polar saponin possesses strong hemolytic activity. When saponins were incubated with cholesterol in dialysis membrane bags(pore size 500 MW), only GS significantly decreased the release of cholesterol. When the hemolytic activity of saponins was measured in the presence of other major membrane lipid components, sphingomyelin significantly reduced the hemolytic activity of SS, while cholesterol reduced the activity of QS. GS showed high affinity to other component(s) in the incubation media as well as lipids. These results suggest that the membranolytic activity of saponins are related to their specific chemical structure, which determines the interaction behavior between saponins and different membrane components, and thereby influence the biological activity.

KEY WORDS : soybean saponins · phytochemicals · membranolysis · gypsophilla saponin · quillaja saponin.

Introduction

Saponins are surface-active compounds widely distributed in plant foods especially in soybeans. They possess several biological activities including the ability to hemolyze erythrocytes[1], inhibition of nutrient absorption[2], and fungitoxic activity[3]. More recently, some saponins have been shown to possess anticarcinogenic[4-7], hypocholesterolemic[8-10], and im-

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mune-stimulatory effects[11-13]. However, the relationship between chemical structure and mode of action is not well understood, although it is believed that the surface-active property which enables saponin to interact with cell membrane is an important characteristic responsible for above biological activities.

The main binding site for saponins on the cell membrane is thought to be cholesterol[14][15]. There is also evidence suggesting cholesterol may not be the only cell membrane component which saponins bind[16][17]. In addition to cholesterol, alfalfa saponins interact with cell membrane proteins, and phospholipids in a non-specific manner[17]. These differences among saponins may be due to their differences in chemical structure.

Basic chemical structure of saponin consists of a non-polar steroid or triterpenoid ring(sapogenin) attached to one or more polar sugar chain. Dietary saponins differ in the types and numbers of sugars bound to the triterpenoidal sapogenins[18]. The polarity, hydrophobicity, and acidity of saponins depend on the types and numbers of sugars as well as the presence of functional groups in their structures.

At least 10 saponins, differing in their numbers and types of sugar in their structure[19], have been isolated and characterized from soybeans. However, very little information is available about their physiological activities relating to chemical structure. The aim of this study was to investigate the activity of total soysaponin extract and its individual fractions on cell membrane using erythrocytes as a model cell membrane. To compare the activity of SS with other non-dietary saponins of known membranolytic activity, GS and QS were also used. Interaction between these saponins and major cell membrane components was also studied to understand the mechanisms of action by different saponins.

Materials and Methods

1. Preparation of Saponins

Crude soybean saponin extract was prepared based on the method of Wolf and Thomas[20] with minor modifications. Soybean flour was defatted in a Soxhlet apparatus with *n*-hexane. Defatted sample was extracted with absolute methanol and the methanol extract evaporated under vacuum in a rotary evaporator. The remaining syrup-like sample was diluted with water and subjected to ether extraction. The water portion was collected and mixed with 80% butanol. The water and butanol soluble fractions were taken to dryness under vacuum. Residues were redissolved in water and freeze-dried to acquire each fraction. Based on the hemolytic activity, it was shown that only the butanol fraction contained saponins. Therefore, butanol fraction was used as crude saponin extract. Gypsophilla and quillaja saponin were purchased from Sigma Chemical Co. (St. Louis, MO, USA) and Eastman Kodak Company(Rochester, NY, USA) respectively.

2. Hemolytic activity of total saponins

Finger tip human blood samples were collected into tubes cotaining phosphate buffer solution(pH 7.2). Erythrocytes were recovered by centrifugation at 2500 rpm for 20 minutes, and washed three times with phosphate buffer solution. Erythrocytes were diluted in phosphate buffer solution to a concentration of 1.4×10^{8} cells/ml. Saponin solutions were prepared from stock solution by serial dilution using phosphate buffer. Two ml of cell suspension were incubated with 1 ml of saponin solution for 24 hours. Each vial was centrifuged at 2500 rpm and release of hemoglobin in the supernatant was measured with spectrophotometer(SP8-100UV Spectrophotometer, Pye Unicam Ltd., Cambridge, England) at 540nm.

3. Hemolytic activity of each saponin fraction

Crude saponin extract was subjected to two sets of thin layer chromatography. Saponin extract was dissolved in 80% ethanol(0.04g/ml) and 5ul of the solution applied on precoated silica gel plates(Whatman, England) and chromatographed using chloroform : methanol : water(65 : 25 : 4) as the mobile phase. The plates were then dried. One plate was developed by spraying 50% sulfuric acid and heated at 100℃.

Purple-magenta bands were identified as saponins[20]. The other plate was covered with erythrocyte gel solution to identify the hemolytic saponins[21]. The erythrocyte gel solution was prepared by dissolving gelatin powder in saline solution(4.5g/100ml) and left at room temperature. After 30 minutes, the gel was heated in a water bath at 85℃ . Gelatin solution was then cooled to 45℃ and 6ml of erythrocyte solution added. The gel-covered plate was allowed to set at 6℃ for 20 hours. Hemolytic activity was characterized by a clear band on the gel.

4. Dialysis assay

To assess the amount of cholesterol bound to saponin, the release of free cholesterol through dialysis bag(500 MW, Spectrum, Los Angeles, California) was measured. Cholesterol was dissolved in *n*-propanol (6mg/ml), and saponin solution was prepared by dissolving saponin in ethanol(6mg/ml). An aliquot(0.5ml) of each solution was pipetted into a dialysis bag and both sides were tied. The bag was immersed in the media(*n*-propanl : ethanol, 1 : 1), and incubated at room temperature. Four ml of media were used for each incubation time. The media were changed twice during the incubation. The accumulated release of cholesterol was calculated at each incubation time.

5. Cholesterol assay

Cholesterol was measured according to the method described by Zlatkis et al[22]. Four ml of sample solution containing cholesterol were pipetted into a test tube and evaporated to dryness using nitrogen gas. Three ml glacial acetic acid were added to the botom of the tube containg cholesterol residue and vortexed. Two ml of ferric chloride color reagent(10g $FeCl_3 \cdot 6H_2O$) were added and thoroughly mixed. The absorbance at 560nm were read using spectrophotometer (SP8-100 UV Spectrophotometer, Pye Unicam Ltd., Cambridge, England). The amount of cholesterol was calculated based on a cholesterol standard curve.

6. Saponin-cell membrane component interaction

The albumin-stabilized emulsion containing one of three different types of lipids was prepared as follow. Bovine serum albumin(2% W/V, Sigma Chemical Co., MO). One ml of this lipid emulsion solution was preincubated with 1 ml of each saponin solution (soybean 0.6mg/ml, gypsophilla 2ug/ml, quillaja 2ug/ml) for 1 hour. Two ml of erythrocyte suspension(3×10^7 cells/ml) were added to saponin-lipid emulsion and incubated for 24 hours. Hemoglobin release from erythrocytes was measured spectrophotometically at 540 nm.

7. Statistical analysis

Differences in cholesterol release from dialysis bag treated with different saponins were compared using the Analysis Of Variance. The value used for each saponin was calculated by "area under the curve" where the area between two incubation times were calculated and each area was added. Duncan's Multiple Range Test was used for pairwise comparisons with a family-wise p-value of 0.05.

The hemolytic activities of saponins incubated with different lipids were compared using the Analysis Of Variance. Duncan's Multiple Range Test was used for pairwise comparisons with a family-wise p-value of 0.05.

Results

1. Hemolytic activity of crude soybean saponin extract

Fig. 1a-1c shows the hemolytic activity of saponins. All saponins induced hemolysis in a dose-dependent manner. Gypsophilla saponin(GS) at a concentration of 5 ppm hemolyzed 100% erythrocytes while quillaja saponin(QS) induced near 100% hemolysis at 8 ppm. Soybean saponins(SS) induced only 40% at a concentration of 300 ppm, and there was no more increase in hemoglobin release above 300 ppm.

2. Thin layer chromatography and hemolytic activity of each soybean saponin fraction

Fig. 2-a shows a typical chromatogram of crude soybean saponin extract. Among all fractions, there were 10 different purple bands corresponded to saponin

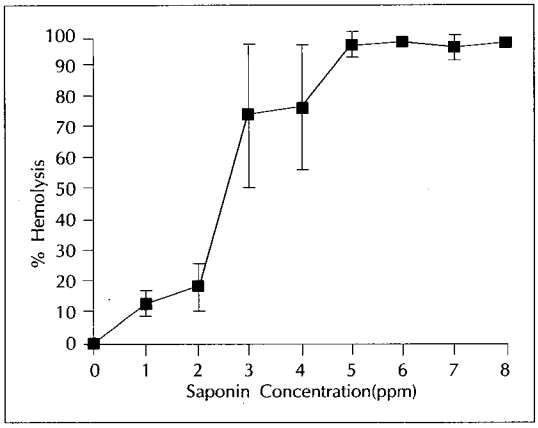

Fig. 1. (a) Hemolytic activity of gypsophilla saponin. Erythrocytes were incubated with saponin for 24 hr and hemolytic activity measured by hemoglobin release. Vertical lines represent standard deviation of the mean.

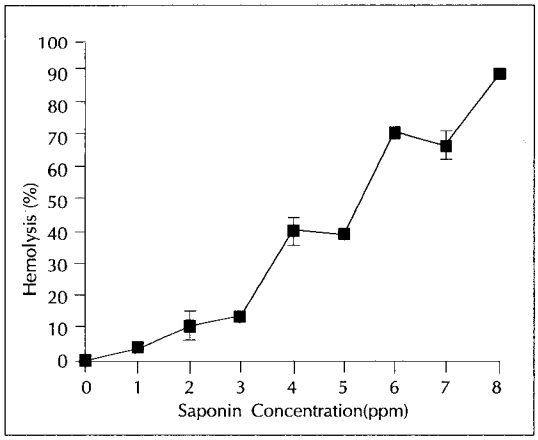

Fig. 1. (b) Hemolytic activity of quillaja saponin. Erythrocytes were incubated with saponin for 24 hr and hemolytic activity measured by hemoglobin release. Vertical lines represent standard deviation of the mean.

fraction. The hemolytic activity of each fraction was shown using chromatogram covered with erythrocyte gel(Fig. 2-b). Only one saponin fraction had strong hemolytic activity while two other fractions revealed minor activity.

3. *Dialysis test*

Fig. 3 shows the ability of different saponins to bind cholesterol. Only GS significantly bound cho-

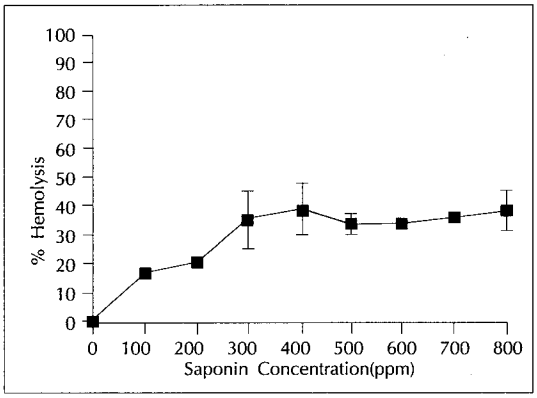

Fig. 1. (c) Hemolytic activity of soybean saponins. Erythrocytes were incubated with saponin for 24 hr and hemolytic activity measured by hemoglobin release. Vertical lines represent standard deviation of the mean.

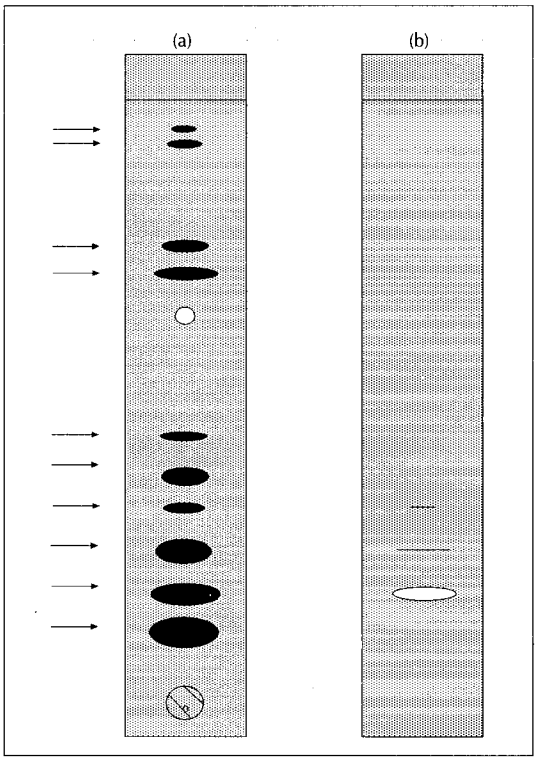

Fig. 2. (a) Thin layer chromatogram of soybean saponin extract. Arrows indicate saponin fractions.
(b) Thin layer chromatogram of soybean saponin extract covered with blood gelatin film. Only one fraction of saponin caused strong hemolysis which was shown as a clear band. Two other bands showed only minor hemolytic activity.

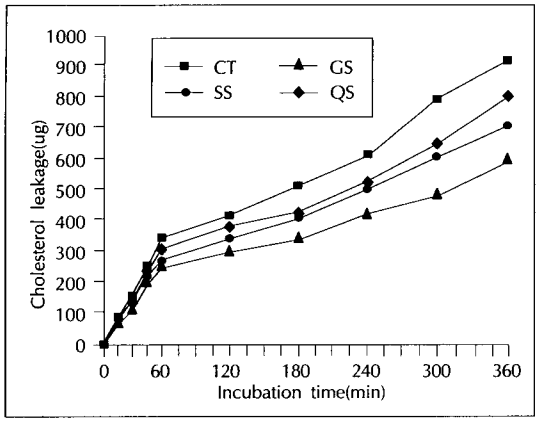

Fig. 3. The amount of cholesterol diffused into the media from the diaysis bag at each incubation time. Each dialysis bag(pore size, 500 MW) contained cholesterol(MW 386) and saponin(MW > 700) at a ratio of 1 : 1. Analysis of variance test was performed using AUC(area under the curve) values to compare the effect of different saponins. Pairwise comparisons were mande with Duncan's multiple range test. Each saponin which does not share same letter is significantly different. CT(control), SS (soybean saponin) GS(gypsophilla saponin), QS (quillaja saponin).

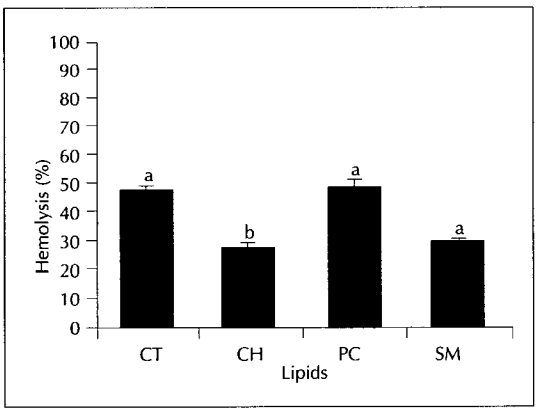

Fig. 4. (a) The effect of preincubation of quillaja saponin with different lipid components on hemolytic activity. Vertical lines represent standard deviation of the mean. Different lipids were compared using analysis of variance test. Paiwise comparisons were made with Duncan's multiple range test. Each lipid which does not share a same letter is significantly different. CT(control), CH(cholesterol), PC (phosphatidylcholine), SM(sphingomyelin).

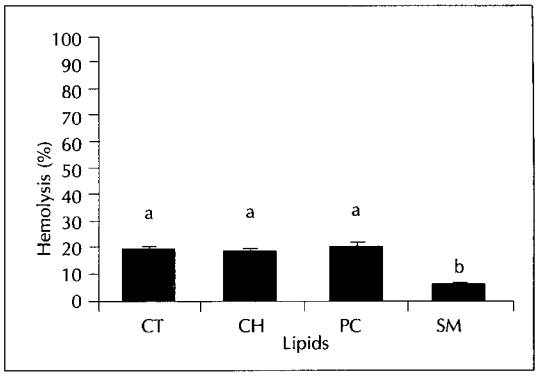

Fig. 4. (b) The effect of preincubation of soybean saponins with different lipid components on hemolytic activity. Vertical lines represent standard deviation of the mean. Different lipids were compared using analysis of variance test. Pairwise comparisons were made with Duncan's multiple range test. Each lipid which does not share a same letter is significantly different. CT(control), CH(cholesterol), PC (phosphatidylcholine), SM(sphingomyelin).

lesterol, thus decreasing its release. QS and SS reduced the release of cholesterol, however, the quantity was not significantly different from the control.

4. Saponin-membrane lipids interaction

Fig. 4a and 4b show the effect of preincubation of saponins with different lipids on their hemolytic activity. The hemolytic activity of QS was significantly decreased when it was preincubated with cholesterol (57.4% of control), indicating significant binding of QS to cholesterol. For SS, hemolysis was significantly decreased only when preincubated with sphingomyelin(32.2% of control). When GS was preincubated in media with or without lipids(control), no hemolysis occured even in the control samples indicating possible binding of GS to components of the incubation media. Therefore, no spectrophotometrical observation could be made.

Discussion

Biological activity of plant saponins, including hypocholesterolemic effect[8-10], immune stimulatory ef-

fect[11-13], and anticarcinogenic effect[4-7] are well documented. Although these effects are thought to be related to their surface-active property, not much information is available on their mode of interaction. This may be due to the chemical diversity of saponins in different plant sources and difficulties associated with their extraction and purification. However, as the nutritional and physiological importance of saponins are recognized, investigations on the biological activity of each saponin and mechanisms of action become necessary.

One of the most important sources of saponin in the diet is soybean. There are at least ten saponins seperated from soybeans[19]. But their biological activity is not well established and not consisitant. Birk et al[23]. found that soybean saponins are thermostable and highly hemolytic, while other investigators reported no homolytic activity[24][25]. The result from this study shows that soybean saponin mixture is hemolytic, although the activity is much weaker when compared with saponins from non-dietary sources. Also there was no sharp increase in hemoglobin release in erythrocytes treated with SS while GS and QS induced 100% hemolysis in a narrow concentration range. These observations suggest that soybean saponins may interact with cell membranes in a manner which is different from other saponins.

Soysaponin Ⅰ, Ⅱ, and Ⅲ containing soysapogenol B as their aglycon are classified as monodesmosides[26]. On the other hand, soysaponin A_1 and A_2 contain soysapogenol A possessing one more hydroxyl group and are classified as bisdesmosides[26]. Soysaponin A_1 and A_2 contain more sugars and therefore, more polar compared to others. In this study, 10 saponin bands were isolated by thin layer chromatogram of the crude soybean saponin extract. Among these fractions, only one showed strong hemolytic activity, while two others possessed weak hemolytic activity. These three saponins were less polar compared to the other soybean saponins. This result suggests that the biological activity of soybean saponin fractions measured by their hemolytic activity depends on their chemical characteristics including their polarity. Gestetner et al[27]. showed that lucerne saponin containing five sugars attached to soysapogenol B did not have hemolytic activity while the saponin, which had a ratio of soysapogenol B : sugar of 1 : 1, possessed hemolytic activity. Our result also suggests that the polarity of saponin is an important determinant of its biological activity.

To further investigate differences between SS and the other two saponins with respect to the effect on erythrocytes, the ability of saponins to bind the main lipid components of cell membrane were tested. It is generally recognized that saponin-induced erythrocyte hemolysis is related to its ability to bind cell membrane cholesterol. Several studies have shown cholesterol to be the binding site for saponins in the cell membrane[14][15], However, the results are not consistant[16][17][25]. In our experiment, to test the affinity of saponins for cholesterol, saponins were incubated with cholesterol in a dialysis bag with pore size of 500 MW, and the release of unbound free cholesterol (MW 386.67) through the membrane to the media was measured. Although, the amount of unbound cholesterol decreased in the order of GS$>$ QS$>$ SS, only GS significantly reduced the release of cholesterol compared to the control. The observed differences between saponins with regard to erythrocyte hemolysis may therefore be due to the affinity of saponins for membrane lipids. This implies that soybean saponins interact with cell membrane components other than cholesterol inducing hemoglobin release and may explain the delayed hemoglobin release in erythrocytes treated with soybean saponins.

When saponins were preincubated with each of the major membrane lipid components, SS showed significant binding activity with sphingomyelin, while QS had a strong affinity for cholesterol. The latter result contradicts the result from the dialysis test where QS did not show significant affinity towards cholesterol, although the release of cholesterol was decreased compared to control. This may be due to the additional binding of QS to albumin in the incubation mixture,

which further reduced the available QS to hemolyze erythrocytes. GS also bound to compound(s), possibly albumin, in the incubation mixture, such that even GS in the incubation mixture without the presence of any lipid did not have any hemolytic activity. These results suggest that each saponin possesses a specific affinity for different membrane lipid components.

In this study, SS are shown to be biologically active although the activity is moderate compared to the other two saponins used in this study. Cell membrane is an important site to control cell growth and function. Also, cancer cell membrane is known to contain numerous growth factors receptors directly related to the cancer cell growth[28]. Therefore, the negative relationship between legume consumption and the occurence of cancer may be attributed to these biologically active saponins. Future research should be directed towards studying the role of these less toxic dietary saponins in disease management.

References

1) Birk Y, and Peri I. Saponins. In : Toxic constituents of plant foodstuffs(Liener IE ed.), pp. 161-182. Academic Press, New York, 1980
2) West LG, and Greger JL. In vitro studies on saponin-vitamin complexation. *J Food Sci* 43 : 1340-1341, 1978
3) Leath KT, Davis KH Jr, Wall ME, and Hanson CH. Vegetative growth responses of alfalfa pathogens to saponin and other extract from alfalfa (Medicago sativa L.), *Crop Sci* 12 : 851, 1972
4) Yu L, Ma R, Wang Y, Nishino N, Takayasu J, He W, Chang M, Zhen J, Liu W, and Fan S. Potent antitumorigenic effect of tubeimoside I isolated from the bulb of bolbostemma paniculatum (maxim) franquet. *Int J Cancer* 50 : 635-638, 1992
5) Tokuda H, Konoshima T, Kozuka M, and Kimura T. Inhibition of 12-o-tetradecanoylphorbol-13-acetate-promoted mouse skin papillama by saponins. *Oncology* 48 : 77-80, 1991
6) Wu R-T, Chiang H-C, Fu W-C, Chein K-Y, Chumg Y-M, and Horng L-Y. Formosanin-C, an immunomodulator with antitumor activity. *Int J Immunopharmacol* 12 : 777-786, 1990
7) Ota T, Fujikawa-yamamoto K, Zong Z-P, Yamazaki M, Odashima S, Kitakawa I, Hiroko A, and Arichi S. Plant-glycoside modulation of cell surface related to control of differentiation in cultured B16 melanoma cells. *Cancer Res* 47 : 3863-3867, 1987
8) Malinow MR, McLaughlin P, Stafford C, Livingstone AL, Cohler CO, and Cheek PR. Comparative effects of alfalfa saponins and alfalfa fiber on cholesterol absorption in rats. *Am J Clin Nutr* 30 : 2061-2067, 1979
9) Oakenfull DG, Topping DL, Illman RJ, and Fenwick DE. Prevention of dietary hypercholesterolemia in the rat by soya bean and quillaja saponins. *Nutr Rep Int* 29 : 1039-1046, 1984
10) Rao AV, and Kendall CW. Dietary saponins and serum lipids. *Fd Chem Toxicol* 24 : 441, 1988
11) Chavali SR, Barton LD, and Campbell JB. Immunopotentiation by orally-administered quillaja saponins : effects in mice vaccinated intraperitoneally against rabies. *Clin Exp Immunol* 74 : 339-343, 1988
12) Chavali SR, and Campbell JB. Adjuvant effects of orally administered saponins on humoral and cellular immune responses in mice. *Immunobiol* 174 : 347-359, 1987
13) Kenarova B, Neychev H, Hadjiivanova C, and Petkov VD. Immunomodulating activity of ginsenoside Rg_1 from panax ginseng. *Jpn J Pharmacol* 54 : 447-454, 1990
14) Gogelein H, and Huby A. Interaction of saponin and digitonin with black lipid membranes and lipid monolayers. *Biochem Biophy Acta* 773 : 32-38, 1984
15) Shany S, Bernheimer W, Grushoff PS, and Kim Y-S. Evidence for membrane cholesterol as the common binding site for cereolysin, streptolysin O, and saponin. *Mol Cell Biochem* 3 : 179-186, 1974
16) Segal R, and Milo-Goldzweig I. The susceptability of cholesterol-depleted erythrocytes to saponin and sapogenin hemolysis. *Biochem Biophy Acta* 512 : 223-226, 1978

17) Assa Y, Shany S., Gestetner B, Tencer Y, Birk Y, and Bondi A. Interaction of alfalfa saponins with components of the erythrocyte membrane in hemolysis. *Biochim Biophy Acta* 307 : 83-91, 1973
18) Oakenfull D. Saponins in food – A review. *Fd Chem* 6 : 19-40, 1981
19) Fenwick DE, and Oakenfull D. Saponin content of soya beans and some commercial soya bean products. *Sci Food Agric* 32 : 273-278, 1981
20) Wolf WJ, and Thomas BW. Thin layer and anion exchange chromatography of soybean saponin. *J Am Oil Chem Soc* 47 : 86-90, 1970
21) Stahl E. Thin-layer chromatography – A laboratory handbook, Academic Press Publishers, 1965
22) Zlatikis A, Zak B, and Boyle AJ. A new method for direct determination of serum cholesterol. *J Lab Clin Med* 41 : 486-492, 1953
23) Birk Y, Bondi A, Gestetner B, and Ishaaya I. A thermostable haemolytic factor in soybeans. *Nature* 197 : 1089-1990, 1963
24) Elkowicz K, and Sosulski FW. Antinutritive factor in eleven legumes and their air-classified protein and starch fractions. *J Food Sci* 47 : 1301-1304, 1982
25) Curl CL, Price KR, and Fenwick, GR. The quantitative estimation of saponin in pea(Pisum sativum L.) and soya(Glycine max). *Food Chem* 18 : 241-250, 1985
26) Kitagawa I, Saito M, Taniyama T., and Yoshikawa M. Saponin and sapogenol XXXVIII. Structure of soyasaponin A_2, a bisdesmoside of soyasapogenol A, from soybean, the seeds of Glycine max MERRIL. *Chem Pharm Bull* 33 : 598-608, 1985
27) Gestetner B, Assa Y, Henis Y, Birk Y, and Bondi A. Lucern saponins IV. – Relationship between their chemical constitution, and haemolytic and antifungal activities. *J Sci Food Agric* 22 : 168-172, 1970
28) Petty HR. Molecular biology of membranes. Structure and function. pp7-83, Plenum Press, 1993

Effects of Soybean Saponins on Cell Membrane

= 국 문 초 록 =

대두 사포닌의 세포막 활성에 관한 기전 연구

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사포닌은 식물계 특히 두류에 널리 존재하는 배당체 화합물로 계면활성이 있어서 체내의 세포막과 결합하여 막을 파열시키는 기능을 가지며 이는 사포닌과 세포막내의 콜레스테롤의 결합에 의한 것으로 보고되어졌다. 본 연구에서는 적혈구 세포를 model로 이용하여 대두 사포닌의 세포막 활성을 알아보고 사포닌과 주요 세포막 성분과의 결합 여부를 판정하였다. 대두 사포닌의 활성은 세포막 활성이 잘 알려진 gypsophilla saponin(GS)과 quillaja saponin(QS)을 실험 대조군으로 사용하여 평가 하였다. SS는 400 ppm의 농도에서 약 40%의 적혈구를 파열시켰으나 그 이상의 농도증가에서는 변화가 없었다. GS와 QS는 각각 5 ppm 및 8 ppm에서 100%의 용혈을 일으켰다. SS를 박층 크로마토그라피로 분리시켰을 때 10개의 사포닌이 회복되었는데 한 개의 비극성 사포닌만이 강한 용혈작용을 나타내었다. 사포닌의 세포막 활성 기전을 알아보기 위하여 사포닌을 투석막 내에서 콜레스테롤과 배양시킨 결과 GS 만이 유의적으로 콜레스테롤과 결합하여 분자량이 커지게되어 투석막 외로의 방출이 저해되었다. 각 사포닌의 세포막 지질에 대한 친화력을 albumin 으로 안정시킨 배양액 중에서 측정해보았을때 SS는 sphingomyelin에 유의 적으로 결합 하였고 QS 및 GS는 cholesterol, sphingomyelin 및 albumin에 대한 친화력이 유의하였다. 이 결과로 사포닌의 세포막활성은 사포닌의 화학구조에 의해 결정되며 그로 인한 세포막 성분에 대한 친화력의 차이가 사포닌의 생물활성에 영향을 주게 되는 것으로 사료 된다.