Effects of Saikosaponin Metabolites on the Hemolysis of Red Blood Cells and Their Adsorbability on the Cell Membrane

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The hemolytic properties and the adsorbability on red blood cells of saikosaponin a, saikosaponin d and 13 metabolites formed in the alimentary tract were investigated. Among these compounds, saikosaponin d and its intestinal product, prosaikogenin G, which possess an α-hydroxyl function at C16, showed the strongest hemolytic activity at the dose range of 1.0 to 5.0 μg/ml. Saikosaponin a and its intestinal product, prosaikogenin F, which possess a β-hydroxyl function at C16, showed activity above 10 μg/ml. In this case, the monoglycoside, prosaikogenin F, showed the stronger activity than the diglycoside, saikosaponin a. Among the gastric products whose ether ring was cleaved to produce a carbinol, the monoglycosides, prosaikogenin A and prosaikogenin H, showed a slight activity above 25 μg/ml, and the saikogenins except saikogenin A were inactive. Saikogenin A, however, had hemolytic activity at a dose of 15 μg/ml. The adsorbabilities of these compounds on red blood cell membranes closely paralleled their degrees of hemolytic activity.

Keywords hemolytic activity; adsorbability; cell membrane; erythrocyte; structure–activity relationship; saikosaponin; prosaikogenin; saikogenin; gastric metabolite; intestinal metabolite

The hemolytic activity of saponins is well known, but the structure–activity relationhsip is still unclear. It had been shown that the hemolytic effect could be ascribed to the aglyconic part of the molecule, namely the sapogenin, and all hemolytically active saponins yield active sapogenins,[1] but not quantitative correlation could be found between hemolytic potency and the presence of the sugar moiety in the molecule.[2] In some cases, the sugar moiety enhances the hemolytic capability of the sapogenin, while in others it reduces or even annuls the hemolytic activity. As to saikosaponins, the hemolytic activities of saikosaponins a, b_1, d and b_2 were reported.[3,4] It was stated that high concentrations of saikosaponins (10^{-4}—10^{-3} M) have hemolytic activities, but low concentrations of saikosaponins (10^{-7}—10^{-6} M) protect or stabilize rat erythrocytes against both hypotonic and heat-induced hemolysis. Saikogenins also protected erythrocytes from hypotonic hemolysis, but did not show any prevention of heat-induced hemolysis. Thus, different action mechanisms between saikosaponins and saikogenins were expected.

In general, anti-inflammatory drugs protect or stabilize erythrocytes from hemolysis,[5] and this is an important pharmacological action of the drugs. Saikosaponins are also reported to have anti-inflammatory effects.[6] Furthermore, saikosaponins have been reported to influence biological metabolic reactions.[7,8] These actions may also be influenced by the stabilization of the fluidity of cell membranes by saikosaponins.

In our previous paper, we reported that saikosaponins a, c and d were transformed to 27 metabolites in the alimentary tract when they were administered orally,[9-11] and some of them had remarkable corticosterone secretion-inducing activities.[10,12] This paper reports the structure-hemolytic activity relationship of saikosaponin metabolites with a view to identifying the active metabolites on the cell membrane.

Materials and Methods

Reagents Saikosaponin a and d were isolated from the root of *Bupleurum falcatum* L. according to the reported method.[13,14] Gastric and intestinal metabolites, saikosaponins b_1, g and b_2, prosaikogenins F, A, H and G, and saikogenins F, A, H and G, were derived from saikosaponin a and d by the alcoholic alkali metal degradation and acid treatment as reported in our previous papers.[9,15] Sheep red blood cells (SRBC) were purchased from Nippon Bio-supp. Center (Tokyo, Japan). Other chemicals were of analytical grade.

Determination of Hemolytic Activity SRBC suspension in Alsever's solution was washed with phosphate buffered saline (PBS) including 0.2% glucose until the supernatant was colorless. Then the erythrocytes were diluted with the same 0.2% glucose–PBS solution to give 4% SRBC suspension. The test solutions consisted of 0.3 ml of erythrocyte suspension, 0.3 ml of various concentrations of saikosaponin, prosaikogenin or saikogenin in methanol (10—500 μg/ml) and 2.4 ml of 0.2% glucose–PBS solution. The components were prepared as follows; first the erythrocyte suspension, then the buffer was added. The mixture was preincubated for 30 min at 37 °C. After that, saikosaponin or its metabolite was added. The mixture was incubated for 30 min at 37 °C, then centrifuged at 700 × *g* for 10 min and the optical density of the supernatant was determined at 540 nm. The percentage of hemolysis was determined by comparison with a sample in which 100% hemolysis was obtained by treatment with distilled water.

Adsorption of Saikosaponin, Prosaikogenin or Saikogenin on Erythrocyte Membrane The percent SRBC solution, 0.6 ml, was diluted with 2.1 ml of 0.2% glucose–PBS solution, then preincubated for 30 min at 37 °C. Next, 0.3 ml of saikosaponin or its derivative in methanol (100—400 μg/ml) was added to the preincubated solution and incubation was continued for 30 min at 37 °C. Then the incubation mixture was centrifuged at 700 × *g* for 10 min to give the supernatant (saponin-hemolyzed blood cells) and the precipitate (saponin-non-hemolyzed blood cells).

a) Isolation of Ghost Cells Ghost cells were obtained from the hemolytic supernatant and the non-hemolytic precipitate, respectively, as follows. The supernatant was centrifuged at 25000 × *g* for 20 min and the pellet was washed twice with distilled water to give ghost cells(I). The precipitate was hemolyzed by distilled water and treated as described above to give ghost cells(II).

b) Extraction of Saikosaponins, Prosaikogenins and Saikogenins from Ghost Cells Ghost cells obtained from saponin-hemolyzed blood cells and non-hemolyzed blood cells were collected and extracted with 3.0 ml of methanol under ultrasonic treatment for 30 min. Each extract was filtered through a 0.45 μm membrane filter and concentrated to dryness *in vacuo*. The residue was dissolved in 100 μl of methanol, then a 20 μl aliquot was analyzed by high performance liquid chromatography (HPLC). The adsorbability of saikosaponin or its metabolites was calculated by means of the following equation:

$$\text{adsorbability (\%)} = \frac{\text{(adsorbed amount in the ghost cells)}}{\text{(initial amount)}} \times 100$$

HPLC Conditions[10] A Shimadzu LC-4A chromatograph with a Shimadzu SPD-2A ultraviolet (UV) detector was used. A stainless steel column (250 × 4 mm i.d.) packed with reversed-phase Hypersil ODS (5 μm, Eruma, Tokyo, Japan) was used. The mobile phase was acetonitrile–water (46 : 54). The column temperature was 50 °C, the flow rate was 1.0 ml/min, the detection wavelength was 210, 254 or 280 nm and the sensitivity was

0.04 or 0.08 a.u.f.s. Peak area was measured using a Shimadzu C-R3A computing integrator.

Results

Hemolytic Activity The structures of saikosaponin a, saikosaponin d, and their metabolites formed in the alimentary tract are shown in Charts 1 and 2. Saikosaponin a, prosaikogenin F and saikogenin F showed hemolytic activities (Fig. 1). Among them, prosaikogenin F was the most potent and the preferred structures for hemolysis were monoglycoside > diglycoside > aglycone. Figure 2 shows the results for saikosaponin b_1, prosaikogenin A and saikogenin A, and Fig. 3 shows the results for saikosaponi g, prosaikogenin H and saikogenin H. The hemolytic activities of saikosaponins and prosaikogenins, which possess carbinol functions in the aglycones were reduced relative to those of both saikosaponin a and prosaikogenin F. The hemolytic action of saikogenin A was stronger than that of other aglycones, but its dose-response curve was different from the curves of other active compounds.

Figures 4 and 5 summarize the hemolytic activity of saikosaponin d and 5 of its metabolites. Saikosaponin d and prosaikogenin G showed marked activities. These acions were stronger than those of saikosaponin a and prosaikogenin F, judging from the percent hemolysis curve. When the actions of saikosaponin d and prosaikogenin G were examined at concentrations below 5 μg/ml, saikosaponin d showed the activity at 1 μg/ml and prosaikogenin G showed it at 2 μg/ml. Maximal activity was seen at 4 μg/ml. Saikosaponin b_2, prosaikogenin D and saikogenin D which possess carbinol functions in the aglycone also showed no activity. On the other hand, saikogenin G showed a 50% hemolysis above 10 μg/ml.

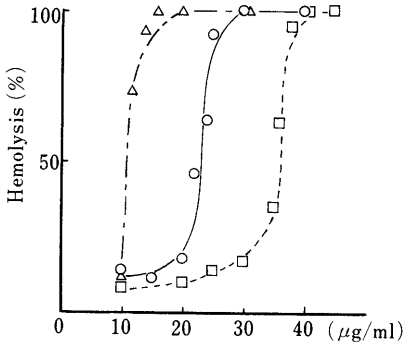

Fig. 1. Hemolytic Activities of Saikosaponin a and Its Intestinal Products (Prosaikogenin F and Saikogenin F)

○—○, saikosaponin a; △—△, prosaikogenin F; □—□, saikogenin F. Data are means of 6 samples.

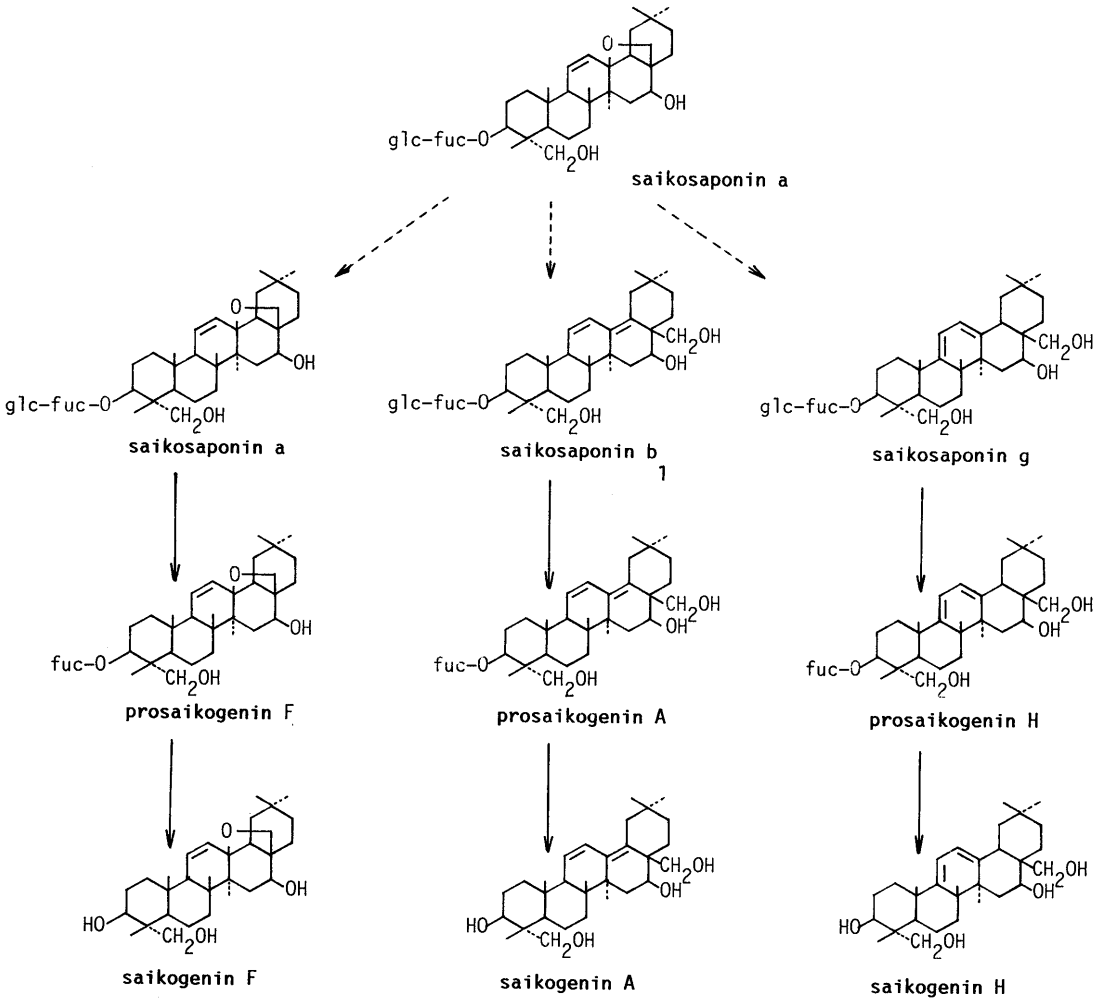

Chart 1. Metabolism of Saikosaponin a in the Alimentary Tract

----→, structural transformation in gastric juice; ——→, structural transformation in intestinal contents.

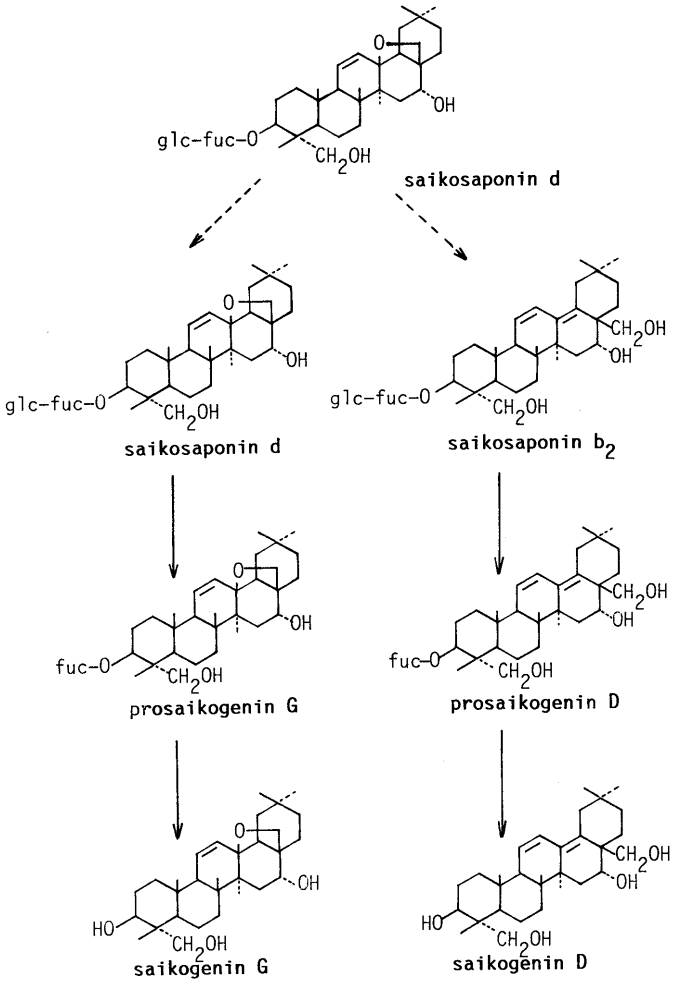

Chart 2. Metabolites of Saikosaponin d in the Alimentary Tract

----→, structural transformation in gastric juice; ——→, structural transformation in intestinal contents.

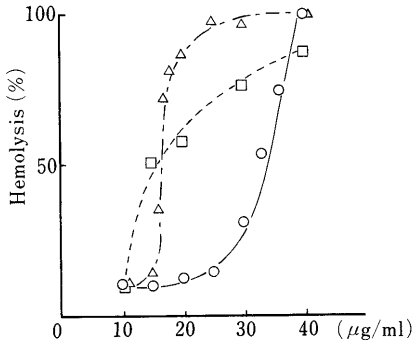

Fig. 2. Hemolytic Activities of Saikosaponin b_1 and Its Intestinal Products (Prosaikogenin A and Saikogenin A)

○—○, saikosaponin b_1; △—△, prosaikogenin A; □—□, saikogenin A. Data are means of 6 samples.

Adsorbability on Cell Membranes The adsorbability of saikosaponins and their metabolites on red blood cell membranes was studied. Figure 6 shows the amounts of saikosaponin d and its metabolites adsorbed on the cell membranes. When saikosaponin d, prosaikogenin G and saikogenin G at a concentration of 10 μg/ml were added, they were adsorbed on the cell membranes and the preferred structures for the adsorbability were monoglycoside = diglycoside > aglycone. Saikosaponin b_2, pro-

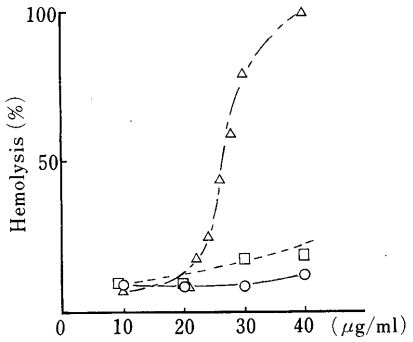

Fig. 3. Hemolytic Activities of Saikosaponin g and Its Intestinal Products (Prosaikogenin H and Saikogenin H)

○—○, saikosaponin g; △—△, prosaikogenin H; □—□, saikogenin H. Data are means of 6 samples.

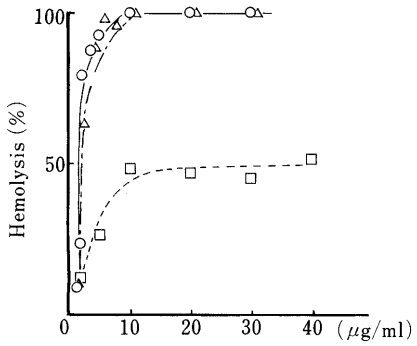

Fig. 4. Hemolytic Activities of Saikosaponin d and Its Intestinal Products (Prosaikogenin G and Saikogenin G)

○—○, saikosaponin d; △—△, prosaikogenin G; □—□, saikogenin G. Data are means of 6 samples.

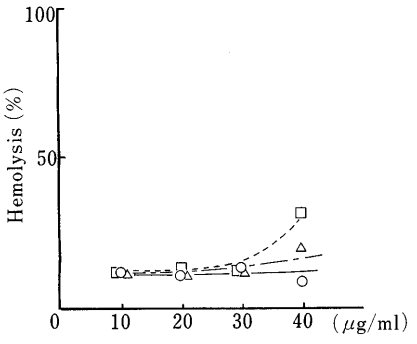

Fig. 5. Hemolytic Activities of Saikosaponin b_2 and Its Intestinal Products (Prosaikogenin D and Saikogenin D)

○—○, saikosaponin b_2; △—△, prosaikogenin D; □—□, saikogenin D. Data are means of 6 samples.

saikogenin D and saikogenin D were not adsorbed at the same concentration. Compounds derived from saikosaponin a were not adsorbed at a concentration of 10 μg/ml (data not shown). When the concentration of compounds was increased, saikosaponin d and prosaikogenin G showed remarkable hemolytic activities and adsorbabilities on cell membranes, as shown in Fig. 7. Although saikosaponin a and prosaikogenin F were also adsorbed on cell membranes when the concentration was increased, their adsorbabilities were lower than those of saikosaponin d and prosaikogenin G. The aglycones, saikogenin G and saikogenin F, were not adsorbed on cell membranes.

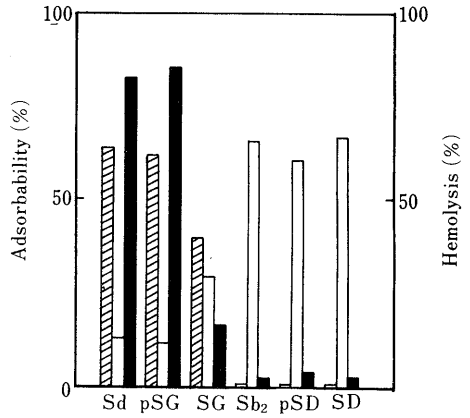

Fig. 6. Adsorbability of Saikosaponin d and Its Alimentary Products at the Concentration of 10 μg/ml

▨, ghost cells (I+II) (adsorbed compounds); □, supernatant (non-adsorbed compounds); ■, hemolytic activity.

Sd, saikosaponin d; pSG, prosaikogenin G; SG, saikogenin G; Sb_2, saikosaponin b_2; pSD, prosaikogenin D; SD, saikogenin D. Each column indicates the mean of 6 samples.

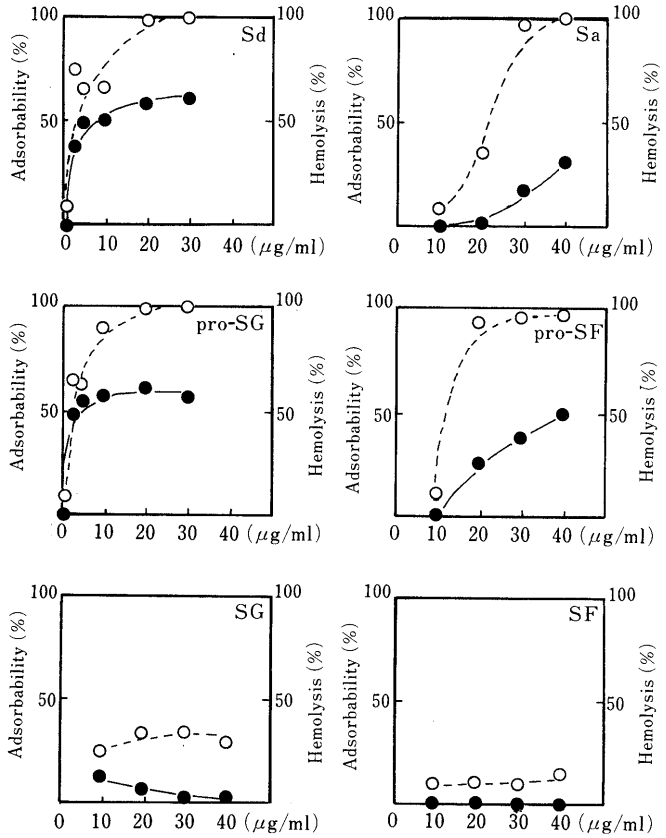

Fig. 7. Adsorbability of Saikosaponin a, Saikosaponin d and Their Intestinal Products at the Concentration Range of 10—40 g/ml

●—●, ghost cells(I) (adsorbed compounds); ○—○, hemolytic activity.

Sd, saikosaponin d; pro-SG, prosaikogenin G; SG, saikogenin G; Sa, saikosaponin a; pro-SF, prosaikogenin F; SF, saikogenin F. Each value indicates the mean of 6 samples.

Discussion

Saikosaponin a and saikosaponin d extracted from *Bupleurum falcatum* L. differ in the configuration of the hydroxyl function at C16. Saikosaponin a has a β-hydroxyl function and saikosaponin d has an α-hydroxyl function, and a β-hydroxyl function is rather more polar than the α-hydroxyl function.[10] Under gastric conditions, saikosaponin a and saikosaponin d, extracted from the root of *Bupleurum falcatum* L., are transformed to saikosaponin b_1 and saikosaponin b_2, respectively. They possess heteroannular diene moieties at C11, 13(18) and a polar carbinol function at C28 due to cleavage of the ether ring. Furthermore, saikosaponin a yeilds saikosaponin g, which possesses a homoannular diene moiety at C9(11), 12 under the same acidic conditions. In the intestinal contents, saikosaponins passed through the stomach are transformed into each prosaikogenin by cleavage of the terminal glucose and into saikogenin by cleavage of fucose.[10] Therefore, we examined the structure–hemolytic activity relationship and membrane affinities of saikosaponin a, saikosaponin d and their metabolites to identify the active forms of saikosaponin a and saikosaponin d, using erythrocytes as a model biomembrane.

In the hemolysis test, saikosaponin d and prosaikogenin G, which possess the ether ring and α-hydroxyl function at C16, showed remarkable hemolytic activities. Saikosaponin a and prosaikogenin F, which possess the ether ring and β-hydroxyl function at C16, also showed hemolytic activities, although their activities were lower than those of saikosaponin d and prosaikogenin G. These differences of hemolytic activities between the saikosaponin d group and the saikosaponin a group was due to the configuration of hydroxyl function at C16. When the ether ring was cleaved to give a carbinol function at C28, the hemolytic activities were reduced in both the saikosaponin a and the saikosaponin d groups. Namely, saikosaponin b_2, prosaikogenin D and saikogenin D, the metabolites obtained from saikosaponin d, showed slight hemolytic activities. Among the metabolites obtained from saikosaponin a, however, prosaikogenin A and prosaikogenin H, showed hemolytic activities. From these results, it seems likely that the existence of the ether ring is essential for the hemolytic activities of saikosaponins. The configuration of the hydroxyl function at C16 and a sugar moiety were also clarified to be the important for the hemolytic activities of saikosaponins. The structure–hemolytic activity relationship of saikosaponin metabolites, however, can not be explained fully in terms of these factors. Our results suggest that the proper polar balance between a sugar moiety (polar position) and an aglycone moiety (non-polar position) is one of the important factors in the hemolytic activity of saikosaponins. The ether ring and α-hydroxyl function at C16 reduce the polarity of the aglycone part and may induce the proper polar balance in the molecule for hemolytic activity. These structure–activity relationships were also recognized for corticosterone-secreting activity,[12] and the corticosterone-secreting activities were correlated to a certain extent to the hemolytic activities and the adsorbability on the cell membrane, but it is unknown whether the biological actions of saponins on the cell membrane participate in the corticosterone-secreting activity.

When the adsorption of saikosaponin derivatives on cell membrane was investigated to confirm the hemolytic activity, the adsorbability of saikosaponin a, saikosaponin d and their metabolites was found to be correlated with their hemolytic activities. When the concentrations of saikosaponins and metabolites were increased, the adsorbabilities increased in parallel with the hemolytic activities. These data suggest that the adsorption of saikosaponins and

metabolites on erythrocyte membrane may result in the hemolysis, as reported by Abe *et al.*[16] Saikosaponins were reported to possess affinity for the cell membrane based on changes of the membrane fluidity of erythrocytes monitored by electron spin resonance (ESR) spectroscopy[16] and of the charges on the surface of cell membrane using the histopathological method.[17] How these compounds cause hemolysis after adsorption on the membrane is unknown and further studies on the changes of the membrane functions—the membrane fluidity, the membrane composition, adenylcyclase activity, cellular Ca^{2+} concentration *etc.*—will be required.

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