草食動物に摂取されたラジノクローバ・サポニンの追跡

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Studies on the Fate of Ingested Ladino Clover Saponin in Herbivorous Animal

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Introduction

Triterpenoidal saponins are special and characteristic constituents of legume plants. Several reviews on their chemical and pharmacological properties have been published in the past[1] and in recent years[4,5]. However, relatively little is known about the metabolic fate of ingested saponins in herbivorous animals. Only four reports, as far as we know, have contributed to give clue to dissolve the problem. GUTIERREZ *et al.* isolated saponin-digesting bacteria from the rumen of steers fed a freshly cut alfalfa diet[6] and of steers on ladino clover pasture[7]. The microorganisms produced a large amount of slime from alfalfa saponins[8]. GESTETNER *et al.* reported that ingested soybean saponins were hydrolyzed into sapogenins and sugars by the caecal microflora of chicks, rats and mice[9].

Ladino clover (*Trifolium repens*) is a most common legume in upland pasture of Japan. The aim of the present study is to follow up quantitatively the fate of ladino clover saponins ingested by animals in which the microbial digestion of saponins in the alimentary tract and the absorption-elimination mechanisms on the digestion products are probably functioning. The present experiment has been carried out using rabbits fed a synthetic diet containing the purified ladino clover saponins prepared in our laboratory.

Materials and Methods

Animals and diets: Two male Japanese white rabbits weighing 3.1 and 3.3 kg were placed individually in metabolic cages in a temperature-regulating room. The metabolic cage was equipped with a stainless steel separator which disposed of feces. For 18 days the animals received control diet of 70 g per day per head and during the last 6 days feces and urine were collected as control specimens, describing below as control feces and as control urine. Following 6 days they were fed on control diet of 35 g plus saponin-treated diet of 35 g and next 6 days on saponin-treated diet of 70 g. In these periods feces and urine were collected daily at 10 a.m. Feces were dried at 50°C in a air-forced oven, weighed, pulverized, transferred into stoppered bottles and stored in refrigerator. Daily collected urine in each period was combined, adjusted to 1500 m*l* and stored in freezer. In text these urines are described as saponin 525 urine and as saponin

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Table 1. Composition of synthetic diets (%)

	control	saponin-treated
saponin	—	1.5
casein	20.0	20.0
corn starch	40.0	38.5
cellulose	15.0	15.0
corn oil	10.0	10.0
liver meal	9.5	9.5
mineral mixture*	4.5	4.5
vitamin mixture**	1.0	1.0

* Composition of mineral mixture as a percentage of total diet: (in %) $CaCO_3$, 0.750; K_2HPO_4, 0.450; Na_2HPO_4, 0.365; $Ca_3(PO_4)_2$, 0.700; NaCl, 0.440; $MgSO_4 \cdot 7H_2O$, 0.250; Fe $(C_6H_{11}O_7)_2 \cdot 2H_2O$, 0.020; $MnSO_4 \cdot 4H_2O$, 0.021; KI, 0.002; $ZnCO_3$, 0.001; $CuSO_4 \cdot 5H_2O$, 0.001; CH_3COOK, 1.250; MgO, 0.250.

** Provided the following per 100 g diet: (in IU) vitamin A palmitate, 2,500; calciferol, 200; (in mg) thiamin·HNO_3, 1; riboflavin, 1.5; niacinamide, 10; pyridoxine·HCl, 1; folic acid, 0.5; calcium pantothenate, 5; ascorbic acid, 37.5; alpha-tocopherol, 3; vitamin K, 2; choline chloride, 100; (in μg) cyanocobalamine, 1.

1050 urine by means of quantities of saponin intake per day. **Table 1** shows composition of diets. The ingredients are based on the purified diet described by HOGAN and HAMILTON[10]. Ladino clover saponins were prepared by the method of WALTER *et al.*[17]. The saponins were furthermore purified by the previously described method[15]. White crystalline saponins were used in this experiment. The mineral mixture was the same as described by BRIGGS *et al.*[3]. Vitamins were supplemented with a purchased vitamin mixture (Takeda Pharm. Ind. Co., Osaka). In the present experiment both diets were pelleted to make the saponin intake possibly valid. Water was given *ad libitum*.

Determination of saponin in feces: Three grams of pulverized feces were extracted with ether for 24 hours in a conventional Soxhlet apparatus. Then the thimble containing the residual feces was air-dried, transferred into a refluxing apparatus and again extracted 3 times with 50% hot ethanol. The ethanol solutions were combined and evaporated to dryness by a rotary evaporator. The residue was dissolved in 1 m*l* of warmed butanol-ethanol-water (2:4:4) and the solution was used for saponin determination by the densitometric thin-layer chromatography described previously[15].

Determination of sapogenin in feces: Etheric solution in Soxhlet's flask was evaporated to dryness and the residue was dissolved in 1 m*l* of chloroform-methanol (6:4). The solution was used for sapogenin determination by tne densitometric method similar with saponin determination. Standard solution of sapogenin was prepared with purified ladino clover sapogenin and solvent system for development was isopropyl ether-acetone as that described previously[12].

Fractionation and determination of materials appeared in urine: The procedure involving all of the fractionation for materials appeared in urine is illustrated schematically in **Fig. 1**. Three fractions were obtained by the procedure. Each fraction was

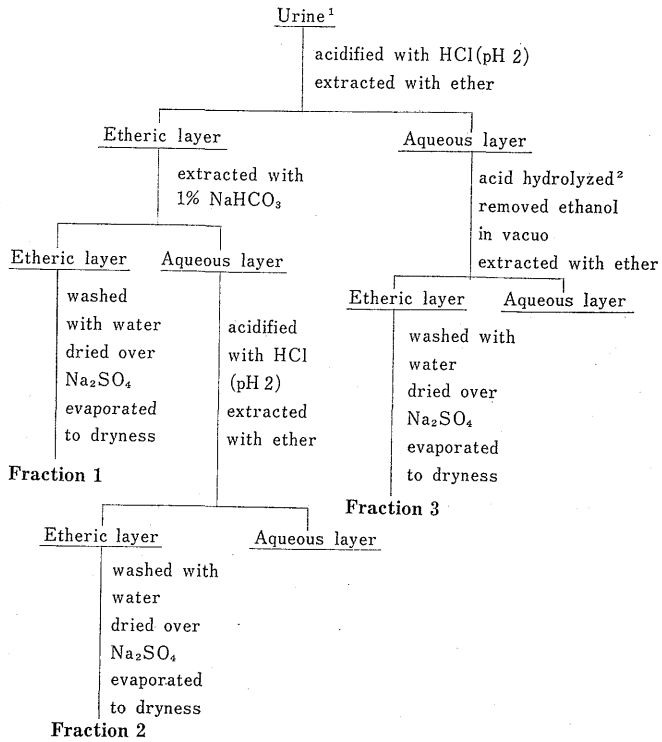

Fig. 1. Fractionation procedure for materials appeared in urine
1. Three hundred m*l* of urines were used.
2. To the aliquot, added 300 m*l* of ethanol and 20 m*l* of sulfuric acid.

dissolved in 3 m*l* of chloroform-methanol (6:4) and stored in a refrigerator until analysis. Qualitative analyses were carried out mainly by thin-layer chromatography using silica gel plate. Solvent systems, colour reactions and other details were cited in the footnote of each figure. Of three fractions, fraction 2 was also analysed by gas-chromatography. The method was similar with that of the sapogenin determination previously reported[12)]

Results and Discussion

Animals fed synthetic diet: Altering ration to a synthetic diet from a commercial feed (Nihon Clea Ltd., Osaka) did not cause any avoidance in feed intake of animals. Even when received saponin-treated diet, the animals fed the ration completely. No body loss occured in the experimental period and there were nothing indicating any physiological disturbances. As described previously[15)], it was recognized that the determination of saponin in feces, in the case of ladino clover feeding, led to an erroneous result by interfering effects of phenolic substances co-existing with saponins in ladino clover. To eliminate those obstacles, the present work was designed with a synthetic diet. By the alteration of feed, feces became grayish one and urine lost the brownish colour.

These specimens were preferable for chromatographic determination of triterpenoidal substances.

Quantities of saponin and sapogenin appeared in feces: As shown in **Table 2**, when the animals received 525 mg of saponin daily, the average daily excretion of saponin in feces was merely 53 mg. The quantity was about 10 per cent of ingested saponin. Even with twice dose of saponin (1050 mg) the excretion was 143 mg per day and it was about 14 per cent of dose. When the rabbits fed control diets, the 50% ethanol extract of feces did not contain any materials positive with the antimony trichloride reagent. There seemed to be no evidence about the quantitative feature of saponin digestion by the intestinal microorganisms. The quantitative determination of saponin in feces has shown that most of ingested saponin, in degrees of such 86 to 90 per cent, disappeared while the passing through the digestive tract. The rabbits excreted sapogenin of 6 mg in feces per day when received saponin of 525 mg and excreted 8 mg when received twice dose of saponin. The quantity of sapogenin appeared in feces was very small compared with that of saponin disappeared.

Two assumptions would be led from these results. It is assumed firstly that one part of disappearing saponin is absorbed directly through the intestinal mucosa and another part is hydrolyzed to sapogenin by intestinal microflora as GESTETNER *et al.* mentioned[9]. Consecutively it is supposed that one part of sapogenin derived from the microbial digestion of saponin is absorbed through the intestinal mucosa and most of the remainder is further metabolized to unknown materials by intestinal microflora. To investigate the absorption and the following renal excretion of saponin and its microbial digestion products in animal body, the urines collected were analysed as follows.

Chromatographic analyses of materials involved in three urine fractions: Saponin 1050 urine was used for chromatographic analyses of materials appearing in urine when the rabbits fed saponin-treated diet. Control urine was always used as non-treated specimen.

Fraction 1. Materials transfering into the fraction are substantially ether-soluble and non-acidic substances. Ladino clover sapogenins also belong to the category by means of their chemical structure of polyhydroxy oleanan. As shown in **Fig. 2**, the chromatogram indicated that this fraction did not contain any sapogenins and other

Table 2. Fecal excretion of ladino clover saponin and sapogenin in experimental animals

saponin intake* mg/day	saponin excreted in feces** mg/day	sapogenin excreted in feces** mg/day
525	53	6
1050	143	8

* Calculated from diet composition
** Mean value of daily excretion of two rabbits

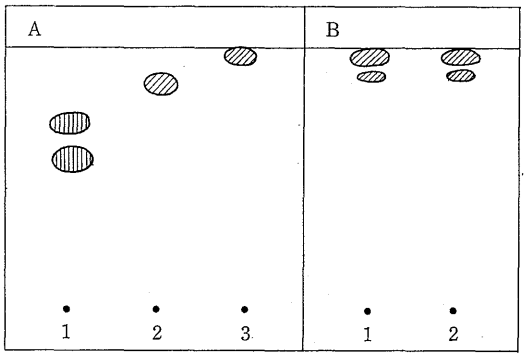

Fig. 2. Schematic diagram of silica gel thin-layer chromatogram of fraction 1 of saponin 1050 urine and control urine

A1: Ladino clover sapogenin, A2: Cholesterin, A3: Cholesterin acetate, B1: Fraction 1 of saponin 1050 urine, B2: Fraction 1 of control urine. Solvent system is isopropyl ether-acetone (5 : 2). Spots were visualized with Liedermann-Burchard reagent; ▨ red ▨ violet

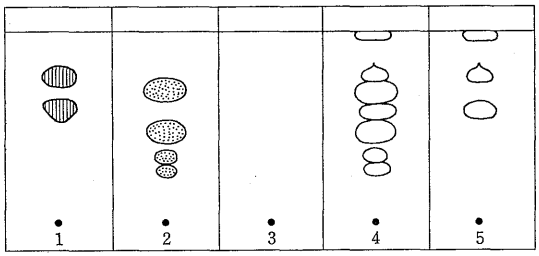

Fig. 3. Schematic diagram of silica gel thin-layer chromatogrem of fraction 2 of saponin 1500 urine and control urine

Solvent system is isopropyl ether-acetone (5 : 2).
1: Ladino clover sapogenins, 2: Fraction 2 of saponin 1050 urine, 3: Fraction 2 of control urine. Spots were visualized with Liebermann-Burchard reagent. ▨ red, ▨ slightly reddish brown 4: Fraction 2 of saponin 1050 urine, 5: Fraction 2 of control urine, Spots were blackened with 50% sulfuric acid and heat.

substances positive with Liebermann-Burchard reagent excepting cholesterides. It means that thers is scarce of renal excretion of sapogenins at least as those free forms or non-conjugated ones.

Fraction 2. This fraction includes acidic substances soluble in ether. Thin-layer chromatography developed four spots slightly positive with Liebermann-Burchard reagent as shown in **Fig. 3**, Plates were also splayed with 50% sulfuric acid and heated in 150°C. Blackening indicated that 4 spots were sole target-materials appeared in the fraction. The appearance of acidic substances suggested that sapogenins might be carboxylated by some intestinal microorganisms or done so in certain animal tissues after the absorption of sapogenins. For identifying the materials, fraction 2 was silylated with silylation mixture in addition of or in absence of purified ladino clover sapogenins and then

determined gaschromatographically. It was, however, obvious that no apparent peak was found in the neighborhood of two peaks of the purified sapogenins. The authors concluded that there were no carboxylation products, acidic sapogenins, in the urine. Although there remains to be controversial, it is assumed that four materials are partial degradation products of sapogenins, being characterized by weak reactivity with Liebermann-Burchard reagent and receiving carboxylation reaction through the degradation processes.

Fraction 3. The fraction included no sapogenins as shown in **Fig. 4.** It meant that no saponin might be excreted. Provided that the adsorption and the renal excretion occurs, the saponin has to be transferred into aqueous layer of primary ether extraction of the systemic fractionation in the present work and its hydrolysate, sapogenin moiety, must be transferred into the fraction 3. There is no evidence at present that saponins and the other glycosides are hydrolyzed in tissues of animal body. In the present experiment, there also seemed to be little possibility of the hydrolysis since sapogenins did not appear in fraction 1 when fed the saponins. Even if the hydrolysis of saponins occurs in animal body, it would be supposed that hydrolysis products, sapogenins, had to be dissimilated towards further degradation. The materials of fraction 3 were supposed to be degradation products of sapogenins. It was not investigated any further whether such a dissimilation of sapogenins occurred in the intestinal tract by microorganisms or in animal body. We supposed the action of microorganisms in possibility of sapogenin dissimilation. By this consideration the possibility of saponin absorption and its consecutive excretion has been abandoned. The plate developed fraction 3 was heated with 50% sulfuric acid. Compared with the plate of control urine, as shown in

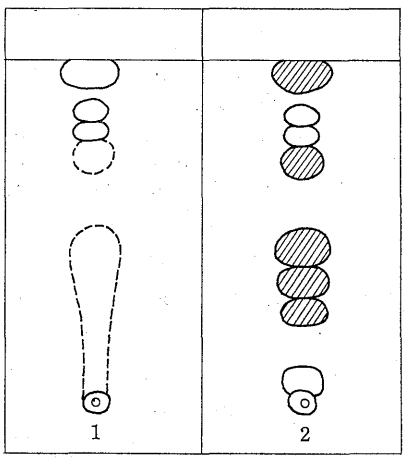

Fig. 4. Schematic diagram of silica gel thin-layer chromatogram of fraction 3 of control urine and saponin 1050 urine

1: Fraction 3 of control urine, 2: Fraction 3 of saponin 1050 urine. Solvent system is chloroform-methanol-water (87 : 10 : 4). Spots were visualized with 50% surfuric acid and heat. Intensity of black colour is marked as following: ▨ > ⬭ > ⬭

Fig. 4, apparently large amounts of organic materials were detected on the plate. As the materials did not react with Liebermann-Burchard reagent, almost all of the materials seemed to be derived from the degradation of triterpenoidal nucleus. Absorbed degradation products may be converted into highly polar conjugates in animal body and excreted in urine as water-soluble materials. These degradation of sapogenins may be supposed from the fact that quantity of sapogenins appeared in feces is unsuspectingly small. These findings suggest that such a dissimilation of sapogenin probably occurs in the intestinal tract by the activities of some saponin-decomposing bacteria although the bacteria and the mechanism of breakdown remains to be undetermined.

General consideration on the fate of ingested ladino clover saponin: Our previous work[11] has demonstrated that the ladino clover saponin comprises large amounts of poorly water-soluble and easily crystallizable saponins and comparatively very small amounts of moderately water-soluble and amorphous saponins. The former saponins are harmless but the latters are slightly harmful as they possess hemolytic activity and weak fish toxicity. Biological properties of the latter saponins resemble to those of alfalfa saponins[16]. Several investigators[2,13,14] show that alfalfa saponins depress the growth of young chicks, and the antinutritional property has been discussed. No one demonstrates the growth-depressing effect of ladino clover saponins. As the present work using the poorly water-soluble and easily crystallizable saponins, the saponin administration would be accepted to be scarcely antinutritional but at least to be unnutritional to the animals. According to GESTETNER *et al.*[9], caecal microflora of chicks, rats and mice hydrolyze soybean saponins into sapogenins and sugars. Sugar moiety of the saponin glycoside seems to be metabolized to volatile fatty acids in the alimentary tract. On the other hand sapogenin moiety, if it penetrates into blood vessel through the intestinal mucosa, may be entirely a foreign substance owing to its impossibility of entry into the animal nutrients. The present investigation has revealed the quantitative, but partially qualitative feature of metabolic fate of ladino clover saponins ingested by a herbivorous animal. Most of ingested saponins are undoubtedly dissimilated to various decomposition products in the intestinal tract and the materials are easily absorbed from the intestinal mucosa. The animals eliminate the materials mainly by means of functioning some conjugation mechanisms which convert them to water-soluble substances.

It would be assumed that when young animals received legume saponins, if the intestinal microflora already developed to be able to metabolize the saponins but the elimination mechanisms did not develope completely, many physiological functions, namely liver function, renal function or circulation system might be affected by the absorbed dissimilation products. Generally, herbage contains various specific substances which convert to some absorbable forms in the intestine and transfer to the blood vessel but not contribute to the animal nutrition. Except saponins, flavonoids and other phenolic substances should be additionally pointed out.

Herbivorous animals intake from herbage not only many nutrients but also various antinutritional or unnutritional materials. Completeness of the elimination mechanisms would be important in the animals.

Summary

To follow up quantitatively the metabolic fate of labino clover saponins ingested by herbivorous animals, the present experiment has been carried out using rabbits fed a synthetic diet containing the purified ladino clover saponins. Two male Japanese white rabbits weighing 3.1 and 3.3 kg were placed individually in metabolic cages. For 18 days the rabbits received saponin-free diet (control diet) of 70 g per day per head and during the last 6 days feces and urine were collected as control specimens. Following 6 days they were fed on control diet of 35 g plus saponin-treated diet of 35 g (saponin intake 525 mg per day per head) and next 6 days on saponin-treated diet of 70 g (saponin 1050 mg per day per head). Through the period feces and urine were collected daily. Saponins and sapogenins excreted in feces were determined by densitometric thin-layer chromatography. Materials derived in urine were divided into three fractions, 1) ether-soluble and neutral fraction, 2) ether-soluble and acidic fraction, and 3) water-soluble fraction. Each fraction was determined by thin-layer chromatography and/or gas-liquid chromatography, and compared with each of control urine. The results obtained were as follows.

1) Quantitative determination of saponin in feces indicated that 86-90% of ingested ladino clover saponin disappered while passing through the digestive tract.

2) Sapogenins appeared 6-8 mg in feces per day when received saponin 525 mg and even its twice dose.

3) In the ether-soluble and neutral fraction of urine, ladino clover sapogenins were not detected.

4) In the ether-soluble and acidic fraction of urine of saponin-fed rabbits, four materials positive with Liebermann-Burchard reagent were detected. These materials, however, could not be detected by gas-chromatography.

5) The water-soluble fraction of urine was hydrolyzed by sulfuric acid. In the hydrolyzate, ladino clover sapogenins were not determined, but large amounts of unknown materials blackened by 50% sulfuric acid and heat were obviously detected.

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草食動物に摂取されたラジノクローバ・サポニンの追跡

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要 約

草食動物に摂取されたラジノクローバ・サポニンがいかなる推移をたどるかを明らかにするため，ラジノクローバより分離，精製したサポニンを合成飼料に混じて，うさぎに投与し，そのサポニンの糞と体内への移行を定量的に測定する試験を設定した。2頭の雄うさぎをそれぞれ糞尿分離式の代謝ケージに収容し，サポニンを含まない合成飼料（対照飼料）で18日間飼育し，その末期6日間の糞と尿を集めてこれらを対照とした。続いて6日間対照飼料とサポニン含有飼料の等量混合物（1日1頭当りサポニン 525 mg）を与え，更に6日間サポニン含有飼料のみ（1日1頭あたりサポニン 1050 mg）を与え，糞と尿をそれぞれ6日間採取した。

糞は乾燥粉砕し，エチルエーテルによってサポゲニンを抽出し，次に 50%熱エタノールによってサポニンを抽出した。これらのシリカゲルの薄層によるクロマトグラムをデンシトメトリーによって定量した。尿についてはエーテル抽出，1%$NaHCO_3$ 溶液洗滌によってエーテル可溶の中性物質群，エーテル可溶の酸性物質群および水溶性物質群の3分画に分け，これらについて更に酸加水分解処理をおこない，薄層クロマトグラフィーおよびガスクロマトグラフィーによって比較した。えられた結果は次の通りである。。

1) うさぎのラジノクローバ・サポニンを1日1頭当り 525 mg と 1050 mg を摂取させた場合，それらの 86～90%が消化管内を通過するうちに消失することが判明した。

2) 消失したサポニンのうち，サポゲニンとして糞に現われる部分は極めて少なく，1日当り 6～8 mg であって，残りは更に消化管内で分解されるか体内に移行することを示唆した。

3) サポニン摂取動物の尿のエーテル可溶の中性分画にはラジノクローバ由来のサポゲニンを検出できなかった。

4) 尿のエーテル可溶の酸性分画の薄層クロマトグラムにおいては，対照尿には見られない Liebermann-Burchard 反応陽性の4つのスポットが出現した。したがって，これら4物質はラジノクロバ・サポニン由来の代謝物質と考えられた。しかし，この分画のガスクロマトグラムにおいて，index として加えたサポゲニン TMS エーテルのピークの周辺に，これら物質に由来するピークを見出すことができなかった。

5) 尿の水溶性部分を加水分解してサポゲニンの存在をしらべたが，サポゲニンは検出されなかった。これはサポニンそのものの吸収はおきないことを示唆した。更にこの加水分解物中には，対照尿のこの分画にくらべると，薄層クロマトグラム上に，50%硫酸加熱によって黒化するスポットが多量に検出された。

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