Journal of Traditional Medicines **14**, 34－40, 1997

Isolation and identification of human intestinal bacteria capable of hydrolyzing saikosaponins

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(*Received November 25, 1996. Accepted December 20, 1996.*)

Abstract

For studying biotransformation of saikosaponins by human intestinal bacteria, thin-layer chromatography-densitometric analysis was performed on the metabolites obtained by anaerobic incubation of saikosaponins with a human fecal suspension. It revealed that saikosaponins a, b_1, b_2 and d were converted to the corresponding prosaikogenins and saikogenins in order. In the case of saikosaponin c, saikogenin E was obtained as a sole product. Of 31 defined human intestinal bacterial strains, only *Eubacterium* sp. A-44 could metabolize saikosaponins a, and b_1 to the corresponding prosaikogenins and saikogenins. Saikosaponins d and b_2 were hydrolyzed to the respective prosaikogenins, but no saikosaponin c by this strain.

After screening bacterial colonies from fresh human feces for metabolizing activity of saikosaponins, two of 60 isolates showed appreciable ability of hydrolyzing saikosaponins a, b_1, b_2 and d, except for c, to the corresponding prosaikogenins. However, both strains did not further hydrolyze the prosaikogenins to saikogenins. They were identified as *Bifidobacterium* spp., named *Bifidobacterium* sp. Saiko-1 and Saiko-2, close species to *Bifidobacterium breve* ss *breve* and *Bifidobacterium adolescentis*, respectively.

Key words *Bifidobacterium* sp. Saiko, intestinal bacteria, metabolism, saikosaponin.

Introduction

In traditional Chinese medicine, the radix of *Bupleurum falcatum* L. has been used as antipyretic, detoxicating, analgesic and anti-inflammatory agents for treatment of feeling of fullness and oppression in the chest and hypochondria, alternating episodes of chills and fever, jaundice, chest and abdominal pain and pain in the side including menstrual pain. Saikosaponins, the main constituents in this radix, have been extensively investigated for their pharmacological actions, such as anti-inflammatory,[1)] anti-hypercholesteremic[2)] and hepatoprotective actions.[3)] As regards the metabolism of saikosaponins, Shimizu *et al.* and Fujiwara *et al.* reported that saikosaponins a, b_1, b_2, c, and d were metabolized by mouse intestinal bacteria *in vitro*,[4)] and that saikosaponin a and its metabolites were detected in the blood stream[5)] and excreted in feces[6)] after oral administration to rats. However, there has been no report on the metabolism of saikosaponins by human intestinal bacteria and the isolation and identification of bacterial species capable of metabolizing saikosaponins.

In this paper, we examined the *in vitro* transformation of saikosaponins by a bacterial mixture from fresh human feces and the characterization of bacterial strains capable of hydrolyzing saikosaponins.

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Materials and Methods

Apparatus : Saikosaponins and their metabolites on TLC plates were determined using a dual wavelength chromatoscanner CS-9300 (Shimadzu Co., Kyoto, Japan). For anaerobic cultivation, an anaerobic incubator EAN-140 (Tabai Co. Osaka, Japan) was used. A gas chromatograph GC-8A (Shimadzu) was used for determination of fatty acids under the following conditions : column, packed chromosorb (Reoporex 400, 10 %, Shimadzu) ; detector, FID ; carrier gas, N_2 ; temperature, 140°C.

Chemicals and media : Saikosaponins a, b_1, b_2, c, and d were isolated from dried roots of *Bupleurum falcatum* L. (Alps Pharmaceutical Ind. Co., Gifu, Japan) as reported by Shimaoka *et al.*[7] Saikosaponins a, d, b_1 and b_2 were treated to obtain their corresponding prosaikogenins and saikogenins with naringinase from *Penicillium decumbens* (Sigma Chemical Co., St. Louis, USA). Saikogenin E was obtained by alcoholic alkali metal degradation of saikosaponin c as reported by Nose *et al.*[8] Isolated compounds were identified by comparing their ^{13}C-NMR spectra with those reported.[4] General anaerobic medium (GAM) and BL agar broth were purchased from Nissui Seiyaku Co.(Tokyo). Peptone yeast extract Fildes solution (PYF) broth for fermentation tests consisted of 10 g tripticase (BBL, Cockeysville, USA), 5 g yeast extract (Difco, Michigan, USA), 40 ml of Fildes solution (peptic digest of sheep blood), 40 ml of salts solution (8 mg $CaCl_2$, 8 mg $MgSO_4$, 40 mg KH_2PO_4, 40 mg K_2HPO_4, 400 mg $NaHCO_3$ and 80 mg NaCl) and 0.5 g L-cysteine-HCl · H_2O in liter.

Chromatography : Silica gel plates 60 F_{254} (Merck, Darmstadt, Germany) were used for TLC using solvent system A ($CHCl_3$-MeOH-H_2O, 30 : 10 : 1), For determination of saikosaponins a, c and d and their metabolites, spots were detected after spraying with phosphomolybdic acid reagent (phosphomolybdic acid 1 g, AcOH 20 ml and H_2SO_4 5 ml) followed by heating and analyzed by chromatoscanner at a wavelength of 600 nm (sample) relative to a reference wavelength of 700 nm. On the other hand, saikosaponins b_1, b_2 and their metabolites were determined at a wavelength of 262 nm. The calibration lines for saikosaponins and their metabolites were linear in a range of 0.2-10 nmol/spot.

Preparation of a bacterial suspension of human feces : Fresh feces obtained from a healthy young man (age : 25, male) were suspended in five volumes of phosphate buffer (pH 7.2). The fecal suspension thus obtained was used in the following experiments.

Bacterial strains : *Bifidobacterium* sp. strain SEN,[9] *Clostridium innocuum* ES24-06,[10] *Eubacterium* sp. A-44,[11] *Eubacterium* sp. strain BAR[12] and *Ruminococcus* sp. PO1-3[10] had been isolated from human feces previously. Other defined strains of human intestinal bacteria were provided by Dr. T. Mitsuoka, The University of Tokyo.

Time course of the metabolism of saikosaponins by an intestinal bacterial suspension : GAM broth (9 ml) containing saikosaponin a (**1**), b_1 (**3**), b_2 (**4**), c (**5**) or d (**2**) (a final concentration, 1 mM) was incubated with an intestinal bacterial suspension (1 ml) in an anaerobic incubator at 37°C. A 100 μl portion was taken out at intervals (4, 10, 24 and 48 hours) and extracted with BuOH (100 μl). Five microliters of the BuOH layer were applied to a TLC plate, which was developed with solvent system A. Saikosaponins and their metabolites were determined as described above.

Screening of defined bacterial strains for their ability to metabolize saikosaponins : Each precultured bacterium (10 μl) was added to GAM broth (10 ml) and cultured in an anaerobic incubator at 37°C for 24 hours. Individual cultures were centrifuged at 1500×g for 15 min. The pellets were washed with 0.9 % NaCl solution (10 ml), and suspended in 1.5 ml of phosphate buffer (pH 7.2). Individual saikosaponins were separately added to 100 μl of each bacterial suspension to give 1 mM of the compound, and incubated in the anaerobic incubator at 37°C for 24 hours. The reaction mixture was extracted with BuOH (100 μl) and products were analyzed by TLC-densitometry as described above.

Isolation of bacteria capable of metabolizing saikosaponins from human feces : A 10 μl portion of 250-fold diluted intestinal bacterial suspension was seeded on GAM agar plates and cultured in an anaerobic incubator at 37°C for 24 hours. Colonies were picked up and re-cultured in GAM broth as mentioned above.

After centrifugation, the isolated bacteria were screened for their ability to metabolize saikosaponins as described above.

β-D-Glucosidase activity : β-Glucosidase activity was determined using *p*-nitrophenyl β-D-glucoside (pNPG) as a substrate as described.[13]

Fructose 6-phosphate phosphoketolase activity : Fructose 6-phosphate phosphoketolase activity was determined as described by Schraman *et al.*[14]

Fermentation test : Sugar fermentation test was carried out according to the method of Sneath *et al.*[15] Fatty acids obtained in PYF broth containing D-glucose were determined using gas chromatography.

Results

Time course of the metabolism of saikosaponins by human feces

Saikosaponins a (**1**), b_1 (**3**), b_2 (**4**) and d (**2**) were converted to the corresponding prosaikogenins F (**6**), A (**8**), D (**9**) and G (**7**) and saikogenins F (**10**), A (**12**), D (**13**) and G (**11**) in order by incubation with a human fecal suspension (Fig. 1). Saikosaponins a (**1**), b_1 (**3**) and b_2 (**4**) were completely hydrolyzed after 24 hours, but complete hydrolysis of saikosaponin d (**2**) was achieved after 48 hours. In the case of saikosaponin c (**5**), only saikogenin E (**14**) was detected without formation of intermediate prosaikogenins.

Screening of defined bacterial strains for their ability to metabolize saikosaponins

Defined bacterial strains isolated from human feces were screened for their ability to metabolize saikosaponins (Table I). Of 31 strains tested, only *Eubacterium* sp. A-44 could metabolize saikosaponins a (**1**), b_1 (**3**), b_2 (**4**) and d (**2**) to the corresponding prosaikogenins, and the produced prosaikogenins F (**6**) and A (**8**) were further transformed to the corresponding saikogenins. However, saikosaponin c (**5**) was not metabolized by this strain.

Isolation of human intestinal bacteria capable of metabolizing saikosaponins

Of 60 colonies obtained on agar plates, two col-

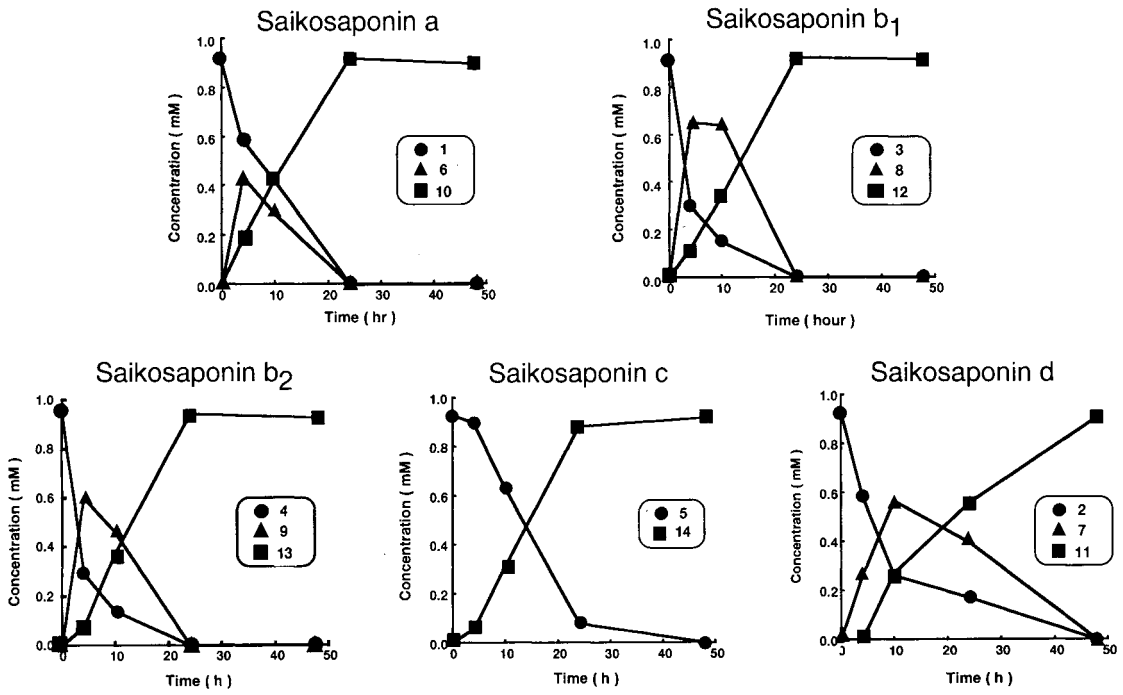

Fig.1 Time course of metabolism of saikosaponins a (**1**), b_1 (**3**), b_2 (**4**), c (**5**) and d (**2**) by a human fecal suspension.
Symbols : **1**, saikosaponin a ; **2**, saikosaponin d ; **3**, saikosaponin b_1 ; **4**, saikosaponin b_2 ; **5**, saikosaponin c ; **6**, prosaikogenin F ; **7**, prosaikogenin G ; **8**, prosaikogenin A ; **9**, prosaikogenin D ; **10**, saikogenin F ; **11**, saikogenin G ; **12**, saikogenin A ; **13**, saikogenin D ; **14**, saikogenin E

Table I Saikosaponin-metabolizing activity of human intestinal bacterial strains

Intestinal bacteria	Substrate / Products (%)	Saikosaponin a			Saikosaponin d			Saikosaponin b_1			Saikosaponin b_2			Saikosaponin c	
		1	6	10	2	7	11	3	8	12	4	9	13	5	14
Bacteroides fragilis ss *thetaotus*		91.6	–	–	89.9	–	–	90.6	–	–	84.0	–	–	86.8	–
B. fragilis ss *vulgatus*		94.4	–	–	88.2	–	–	82.3	–	–	90.5	–	–	88.6	–
Bifidobacterium adolescentis		93.7	–	–	91.9	–	–	86.5	–	–	90.0	–	–	83.1	–
B. angulatum		92.7	–	–	82.7	–	–	84.7	–	–	84.0	–	–	82.3	–
B. bifidum a E 319		98.0	–	–	94.3	–	–	83.0	–	–	86.2	–	–	91.6	–
B. breve S-2 kz 1287		91.6	–	–	93.9	–	–	85.0	–	–	85.7	–	–	84.8	–
B. longum IV-55		97.3	–	–	91.1	–	–	93.0	–	–	84.0	–	–	93.1	–
B. pseudolongum PNC-2-9-G		94.7	–	–	93.4	–	–	85.0	–	–	91.8	–	–	91.7	–
B. sp. SEN		90.7	–	–	87.9	–	–	83.0	–	–	88.3	–	–	90.6	–
Clostridium butyricum		88.6	–	–	89.1	–	–	84.2	–	–	94.0	–	–	90.0	–
C. innocuum ES 24-06		89.3	–	–	91.6	–	–	80.6	–	–	86.2	–	–	90.7	–
C. innocuum KZ-633		94.2	–	–	91.3	–	–	83.0	–	–	85.7	–	–	82.2	–
C. perfringens TO-23		89.7	–	–	89.1	–	–	89.7	–	–	89.4	–	–	83.5	–
Escherichia coli O 127		94.2	–	–	93.8	–	–	95.5	–	–	93.5	–	–	91.2	–
Eubacterium. aerofaciens		90.3	–	–	87.6	–	–	94.5	–	–	90.5	–	–	89.0	–
E. sp. A-44		38.6	37.8	22.9	70.0	24.5	–	15.6	53.6	23.4	65.2	35.1	–	89.4	–
E. sp. BAR		95.1	–	–	87.6	–	–	86.5	–	–	86.9	–	–	89.3	–
Fusobacterium nucleatum		88.9	–	–	86.6	–	–	89.0	–	–	86.4	–	–	91.1	–
Gaffkya anaerobia G-0608		91.2	–	–	89.4	–	–	82.3	–	–	84.7	–	–	82.5	–
Klebsiella pneumoniae ATCC 13883		97.3	–	–	93.1	–	–	84.2	–	–	87.5	–	–	82.8	–
Lactobacillus acidophilus ATCC 4356		96.1	–	–	94.1	–	–	88.7	–	–	84.6	–	–	89.8	–
L. brevis II 46		89.3	–	–	93.1	–	–	85.1	–	–	89.4	–	–	83.6	–
L. fermentum ATCC 9338		89.3	–	–	91.4	–	–	90.7	–	–	84.7	–	–	82.3	–
L. plantarum ATCC 14927		91.3	–	–	89.4	–	–	92.0	–	–	85.8	–	–	83.8	–
L. lactis ss *lactis*		97.3	–	–	91.1	–	–	86.3	–	–	86.9	–	–	91.1	–
Peptostreptococcus intermedius EBF77/25		86.8	–	–	90.4	–	–	87.5	–	–	84.4	–	–	89.9	–
P. anaerobius 0240		97.9	–	–	90.3	–	–	86.1	–	–	87.1	–	–	91.9	–
Proteus mirabilis S2		94.1	–	–	91.4	–	–	89.0	–	–	88.2	–	–	83.5	–
Enterococcus faecalis II-136		93.2	–	–	89.4	–	–	83.8	–	–	88.6	–	–	89.3	–
Ruminococcus sp. PO1-3		93.8	–	–	88.4	–	–	86.7	–	–	85.0	–	–	82.9	–
Veillonella parvula ss *parvula* ATCC 10790		93.1	–	–	90.6	–	–	84.7	–	–	85.6	–	–	83.1	–
Strain X		42.0	65.6	–	65.6	30.8	–	45.4	45.2	–	30.6	60.9	–	87.8	–
Strain Z		24.7	71.1	–	45.3	48.9	–	39.4	58.9	–	19.3	67.1	–	82.2	–

Product (%) represents a relative concentration of recovered substrate and its metabolite. Symbols : **1**, saikosaponin a ; **2**, saikosaponin d ; **3**, saikosaponin b_1 ; **4**, saikosaponin b_2 ; **5**, saikosaponin c ; **6**, prosaikogenin F ; **7**, prosaikogenin G ; **8**, prosaikogenin A ; **9**, prosaikogenin D ; **10**, saikogenin F ; **11**, saikogenin G ; **12**, saikogenin A ; **13**, saikogenin D ; **14**, saikogenin E ; –, not detected.

onies (strains X and Z) showed ability to hydrolyze saikosaponin a (**1**). These bacteria also hydrolyzed saikosaponins b_1 (**3**), b_2 (**4**) and d (**2**) to the corresponding prosaikogenins (Table I). However, neither strain X nor Z could hydrolyze the produced prosaikogenins and saikosaponin c (**5**).

Characterization of the isolated strains

The biochemical characteristics of the isolated strains (X and Z) are as shown in Table II. Both were gram-positive bud-forming rods (Table II) and strict anaerobes, forming brown colonies when cultured on BL agar plates. Acetic acid and lactic acid were produced when D-glucose was fermented by both bacterial strains. In addition, both strains had fructose 6-phosphate phosphoketolase activity. These findings led us to confirm that both strains belong to the genus *Bifidobacterium*. Accordingly, we named them *Bifidobacterium* sp. Saiko-1 and Saiko-2, respectively. In the former strain, the ability of sugar fermentation was quite similar to that of *Bifidobacterium breve* ss *breve* except for the cases of D-xylose, D-mannose and amygdalin (Table III). On the other hand, a pattern of sugar fermentation by the latter strain was close to that shown by *B. adolescentis* except for L-

Table II Characteristics of bacterial strains (X and Z)

	Strain X	Strain Z	A-44
1) Gram stain	+	+	+
2) BL agar broth	Brown colony	Brown colony	Milky-white colony
3) Aerobic growth	—	—	—
4) Major fatty acids	Lactic acid Acetic acid	Lactic acid Acetic acid	Lactic acid Acetic acid
5) Fructose-6-phosphate phosphoketolase	+	+	—
6) β-glucosidase	+	+	+
Genus	*Bifidobacterium*	*Bifidobacterium*	*Eubacterium*

Symbols : +, positive ; —, negative.

Table III Comparative biochemical characteristics of the bacterial strains

Sugar	*B.* sp. Saiko-1 (Strain X)	*B. breve* ss *breve**	*B.* sp. Saiko-2 (Strain Z)	*B. adolescentis**
D-Arabinose	—	—	++	++
D-Xylose	+	—	++	++
L-Rhamnose	—	—	+	—
D-Ribose	++	++	++	++
D-Glucose	++	++	++	++
D-Mannose	—	++	+	d
D-Fructose	++	++	++	++
Maltose	++	++	++	++
Cellobiose	++	++	++	++
Lactose	++	++	++	++
Trehalose	—	—	++	d
Melibiose	++	++	++	++
Raffinose	++	++	++	++
Melezitose	—	—	++	++
Starch	++	d	++	++
Inulin	++	d	—	d
D-Mannitol	++	++	++	d
D-Sorbitol	++	++	++	d
Inositol	—	—	—	—
Esculin	++	++	++	++
Salicin	++	++	++	++
Amygdalin	—	++	—	++

Symbols : —, >pH 6.0 ; +, pH 5.5~6.0 ; ++, <pH 5.5 ; d, 11~89 % positive. *Mitsuoka, T. (see reference 16)

rhamnose and amygdalin. Both strains showed appreciable β-D-glucosidase activity when assayed with *p*-nitrophenyl β-D-glucoside as a substrate.

Discussion

In the course of our studies on the metabolism of crude drug components by intestinal bacteria from human feces, we tried to isolate human intestinal bacteria capable of metabolizing saikosaponins. All saikosaponins examined were effectively and completely metabolized to the corresponding prosai-

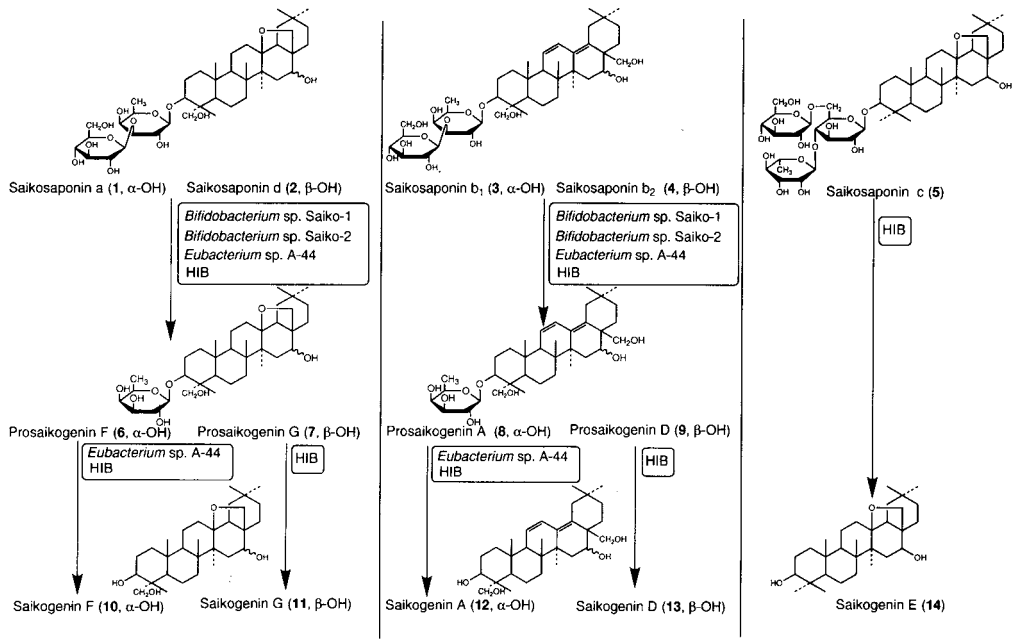

Fig. 2 Metabolic pathway of saikosaponins by human intestinal bacteria (HIB)

kogenins and saikogenins by a fecal suspension of humans (Fig. 2). The bacterial mixture from human feces metabolized saikosaponins to the corresponding saikogenins, similar to the case of metabolism of saikosaponins a and d by animal intestinal flora as reported by Shimizu *et al.*[6] However, the transformation of saikosaponins by a mixture of bacteria from human feces was much faster than by that from rat feces under the same conditions (data not shown).

Next, we screened various defined bacteria isolated from human feces for their metabolizing activities of saikosaponins, but only *Eubacterium* sp. A-44 could metabolize saikosaponins a (**1**), b_1 (**3**), b_2 (**4**), d (**2**). Although most of the defined strains used in this experiment had β-D-glucosidase activity which hydrolyzes various glucosides, such as *p*-nitrophenyl β-D-glucoside, paeoniflorin,[17] sweroside[18] and geniposide,[19] they did not hydrolyze saikosaponins except for one strain mentioned above. Moreover, of 60 colonies from human feces of a healthy man, only two bacteria, *Bifidobacterium* sp. Saiko-1 and Saiko-2, could hydrolyze saikosaponin a to prosaikogenin F. These findings indicated that a population of bacterial species capable of hydrolyzing saikosaponins may be low and the three strains (*Eubacterium* sp. A-44, *Bifidobacterium* sp. Saiko-1 and Saiko-2) may produce a new type of glucosidase to hydrolyze saikosaponins.

Although we found that three species of bacterial strains had ability of hydrolyzing saikosaponins a (**1**), b_1 (**3**), b_2 (**4**) and d (**2**) in this study, we did not succeed to isolate any bacterial species capable of hydrolyzing saikosaponin c (**5**) in a direct or stepwise manner. However, the possibility of isolating other bacterial species capable of hydrolyzing saikosaponin c (**5**) and its prosaikogenins from human feces still remains.

Fujiwara *et al.* reported that saikosaponin a and its metabolites were detected in the blood stream after oral administration to rats.[5] Furthermore, Yamamoto *et al.*[2] reported that the radioactivity was detected mainly in the feces, but not in urine after intramuscular injection of both ^{14}C-labeled saikosaponins a and d to rats, and that saikosaponins are expected to contact repeatedly with the intestinal bacteria through the enterohepatic circulation. Namely, saikosaponins are readily metabolized by the intestinal flora and excreted in the feces as its metabolites. In this context, various pharmacological effects of metabolites from saikosaponins by intestinal bacteria

have been reported.[2, 20] In the present study, we showed it is possible for saikosaponins to be metabolized by human intestinal flora after oral administration of a variety of prescriptions containing the radix of *Bupleurum falcatum* as a component. However, further studies on pharmacokinetics of saikosaponins is necessary to clarify ultimate substances which endure pharmacological effects in the human blood stream.

Acknowledgment

This work was financially supported by the Proposal-Based Advanced Industrial Technology R & D Program from the New Energy and Industrial Technology Development Organization (NEDO) of Japan.

和文抄録

嫌気的培養条件下においてヒト腸内細菌はサイコサポニン a, b_1, b_2, c, d を経時的にプロサイコゲニンおよびサイコゲニンに代謝したことから，我々はその代謝に関与する菌株を探索した。31種の既知のヒト腸内細菌株をスクリーニングしたところ，*Eubacterium* sp. A-44 のみがサイコサポニン a, b_1 をプロサイコゲニンを経てサイコゲニンにまで代謝し，またサイコサポニン b_2, d をプロサイコゲニンにまで代謝した。さらにヒト糞便より代謝活性を有する菌種の単離を試み，60のコロニーから2種のサイコサポニン a 代謝能を有する菌株を得た。両菌はサイコサポニン a, b_1, b_2, d をプロサイコゲニンに水解する *Bifidobacterium* 属であることが明らかになり，両菌を *Bifidobacterium* sp. Saiko-1, Saiko-2 と仮名した。これらの結果からヒト腸内細菌叢中に存在する比較的少ない菌種がサイコサポニンの代謝に関わっていることが示唆された。

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