Enzymes Responsible for the Metabolism of Saikosaponins from *Eubacterium* sp. A-44, a Human Intestinal Anaerobe

Hiroaki KIDA,^a Teruaki AKAO,^b Meselhy R. MESELHY,^a and Masao HATTORI^{*a}

Department of Cell-Resources Engineering, Research Institute for Wakan-Yaku (Traditional Sino-Japanese Medicines),^a and Department of Hygienic and Biochemistry, Faculty of Pharmaceutical Sciences, Toyama Medical and Pharmaceutical University,^b 2630, Sugitani, Toyama 930-0194, Japan. Received May 20; accepted August 26, 1997

From a human intestinal bacterium, *Eubacterium* sp. A-44, which is capable of hydrolyzing saikosaponins to saikogenins, two glycosidases, β -D-glucosidase and a novel type of β -D-fucosidase, were isolated and characterized as saikosaponin-hydrolyzing β -D-glucosidase and prosaikogenin-hydrolyzing β -D-fucosidase.

Relative to the hydrolyzing activities toward saikosaponins a, b₁ and b₂, the β -D-glucosidase showed lower ability to hydrolyze saikosaponin d, but no ability to hydrolyze saikosaponin c or prosaikogenins.

By Sephacryl S-300 column chromatography, the molecular weight of prosaikogenin-hydrolyzing β -D-fucosidase was estimated to be about 130 kDa. The β -D-fucosidase could hydrolyze prosaikogenins A and F, but not prosaikogenins D and G or saikosaponins. Relative to *p*-nitrophenyl β -D-fucoside-hydrolyzing activity, this enzyme had 32.0% and 22.2% of its hydrolyzing ability toward *p*-nitrophenyl β -D-glucoside and *p*-nitrophenyl β -D-galactoside, respectively. *p*-Nitrophenyl β -D-fucoside-hydrolyzing activity was inhibited by D-fucose, and was weakly inhibited by D-glucose, D-glucono δ -lactone, D-galactose and D-galactono δ -lactone.

By combining these two glycosidases, saikosaponins a and b₁ were converted to their saikogenins *via* the corresponding prosaikogenins.

Key words β -D-fucosidase; β -D-glucosidase; intestinal bacteria; prosaikogenin; saikogenin; saikosaponin

Various unique glycosidases from human intestinal bacteria, such as β -glucosidase from *Bifidobacterium* sp. SEN for sennoside¹⁾ or *Eubacterium* sp. A-44 for geniposide,²⁾ and β -glucuronidase from *Eubacterium* sp. GLH for glycyrrhizin,³⁾ have been reported to play an important role in the metabolism of glycosides (aryl- and alkyl-types) which remain unabsorbed from the upper intestine after oral administration.

Saikosaponins (Chart 1), the major triterpene saponins in the radix of *Bupleurum falcatum* L. used widely in traditional Chinese medicine, show anti-inflammatory,⁴⁾ anti-hypercholesteremic⁵⁾ and hepatoprotective action.⁶⁾ It has been reported that intestinal bacteria are essential for the transformation of saikosaponins to their saikogenins,^{7,8)} and that saikogenins F and G show the same activity as potent as their corresponding saikosaponins.⁹⁾

We have previously reported that the human fecal flora are capable of transforming saikosaponins to their corresponding saikogenins *via* their prosaikogenins.¹⁰⁾ Thirty defined bacterial strains tested and various colonized bacteria from human feces were not capable of hydrolyzing saikosaponins. Only three bacterial strains isolated from human feces were responsible for the metabolism of saikosaponins. These three bacterial strains hydrolyzed saikosaponins a, b₁, b₂ and d to the corresponding prosaikogenins F, A, D and G. Of the three strains, *Eubacterium* sp. A-44 could effectively hydrolyze prosaikogenins to the respective saikogenins. Accordingly, these reactions were considered to be catalyzed by unique enzyme(s). Glycosidases responsible for the hydrolysis of saikosaponins a, b₁, b₂ and d have not been isolated so far from human intestinal bacteria.

In the present paper, we report the isolation and characterization of two saikosaponin-hydrolyzing glycosidases, β -D-glucosidase and a novel type of β -D-fucosidase, from *Eubacterium* sp. A-44.

MATERIALS AND METHODS

Apparatus Saikosaponins and their metabolites were quantitatively determined on thin-layer chromatography (TLC) plates using a dual wavelength chromatoscanner CS-9300 (Shimadzu Co., Kyoto, Japan). A sonicator, Sonifier 250 (Branson Co., Danbury, CT, U.S.A.) was used to disrupt bacterial cells.

Chemicals and Media Saikosaponins a, b₁, b₂, c and d were isolated from the dried roots of *Bupleurum falcatum*

Chart 1. Structures of Saikosaponins

* To whom correspondence should be addressed.

L. as reported by Shimaoka *et al.*¹¹⁾ Prosaikogenins and saikogenins were obtained as described previously.¹⁰⁾ General anaerobic medium (GAM) was purchased from Nissui Seiyaku Co. (Tokyo, Japan). Silica gel plates 60 F₂₅₄ (Merck Co., Darmstadt, Germany) were used for TLC. *p*-Nitrophenyl β -D-glucoside, L-glucose and D-galactose were purchased from Nacalai Tesque Co. (Kyoto, Japan). *p*-Nitrophenyl β -D-fucoside, *p*-nitrophenyl β -L-fucoside, *p*-nitrophenyl α -L-fucoside, D-glucono δ -lactone, D-galactono δ -lactone, bovine serum albumin (BSA), bovine liver catalase and human transferrin were products of Sigma Chemical Co. (St. Louis, U.S.A.). D-Glucose was purchased from Wako Pure Chemical Industries Co. (Osaka, Japan). Butyl-Toyopearl 650M was purchased from Tosoh Co. (Tokyo, Japan). Sephacryl S-300 gel was obtained from Pharmacia Fine Chemical Co. (Uppsala, Sweden). Rabbit muscle aldolase A was a kind gift from Dr. N. Nakai, Department of Chemistry, Fukui Medical School.

Bacterial Strains *Eubacterium* sp. A-44,¹²⁾ *Bifidobacterium* sp. Saiko-1 and Saiko-2¹⁰⁾ had been previously isolated from human feces.

Enzyme Purification Bacterial cells from 6 l of the culture medium were harvested and suspended in 45 ml of 50 mM potassium phosphate buffer (pH 7.2). The bacterial cells were disrupted by sonication (two times). The supernatants obtained by centrifugation at $22500 \times g$ for 30 min were combined and designated as crude enzyme (90 ml). The crude enzyme (15 ml $\times$ 6) was applied to a column (30 $\times$ 150 mm) of Butyl-Toyopearl 650M equilibrated with 50 mM potassium phosphate buffer (pH 7.2) containing 1.3 M $(\text{NH}_4)_2\text{SO}_4$ (pH 7.2). The column was eluted with a linear gradient of $(\text{NH}_4)_2\text{SO}_4$ (1.3–0 M) in the same buffer, and 2 ml fractions were collected. Enzymatic activity of the fractions was monitored using *p*-nitrophenyl β -D-glucoside, *p*-nitrophenyl β -D-fucoside, saikosaponin a and prosaikogenin F as substrates. Fraction A (fr. 43–45) containing saikosaponin a-hydrolyzing activity and fraction B (fr. 49–51) containing prosaikogenin F-hydrolyzing activity were separately concentrated to small volumes with Centriprep-10 (Amicon Co., Tokyo, Japan), which were subsequently subjected to column chromatography of Sephacryl S-300 (30 $\times$ 900 mm). The column was eluted with 50 mM potassium phosphate buffer (pH 6.5), and fractions of 1.2 ml per tube were collected.

Molecular Weight Determination The molecular mass of native enzymes was determined by Sephacryl S-300 column chromatography using a series of standard proteins: BSA (M_r = 67000), bovine liver catalase (M_r = 232000), rabbit muscle aldolase A (M_r = 158000), human transferrin (M_r = 81000), and arylsulfotransferase (M_r = 320000) isolated from *Eubacterium* sp. A-44.¹²⁾

Assay Saikosaponin-hydrolyzing activity and prosaikogenin-hydrolyzing activity were assayed as follows. The reaction mixture (0.1 ml) containing 50 μ l of enzyme sample, 50 mM potassium phosphate buffer (pH 6.5) and 1 mM saikosaponins or prosaikogenins was incubated at 37°C for 30 min. Amounts of saikosaponins and their metabolites were determined as reported previously.¹⁰⁾ The hydrolyzing activity toward saikosaponins was also assayed by determining the amount of glucose released.¹³⁾

The glucose oxidase/oxidase method of Dahlqvist¹⁴⁾ was followed with minor modifications. In the same manner, geniposide-hydrolyzing activity was measured. β -D-Glucosidase and β -D-fucosidase activities were assayed routinely using *p*-nitrophenyl β -D-glucoside and *p*-nitrophenyl β -D-fucoside as substrates, respectively, according to the method described previously by Akao *et al.*¹³⁾ Similarly, hydrolyzing activities for *p*-nitrophenyl β -L-fucoside, *p*-nitrophenyl α -L-fucoside and *p*-nitrophenyl β -D-galactoside were measured.

RESULTS

β -D-Glucosidase and β -D-Fucosidase Activities of Isolated Bacterial Strains Three bacterial strains isolated from human feces, *Bifidobacterium* sp. Saiko-1, Saiko-2 and *Eubacterium* sp. A-44, which were responsible for the metabolism of saikosaponins,¹⁰⁾ had potent hydrolyzing activities for saikosaponin a and *p*-nitrophenyl β -D-glucoside (Table 1), possibly due to β -D-glucosidase. On the other hand, only *Eubacterium* sp. A-44 showed appreciable hydrolyzing activities for prosaikogenin F and *p*-nitrophenyl β -D-fucoside, possibly due to β -D-fucosidase.

Bifidobacterium sp. Saiko-1 had weak hydrolyzing activity for *p*-nitrophenyl β -D-fucoside, and no hydrolyzing activity for prosaikogenin F, while *Bifidobacterium* sp. Saiko-2 had no activity for either substrate.

Isolation of Saikosaponin-Hydrolyzing β -D-Glucosidase and Prosaikogenin-Hydrolyzing β -D-Fucosidase from *Eubacterium* sp. A-44 A supernatant obtained by centrifugation of the disrupted cells of *Eubacterium* sp. A-44 showed appreciable hydrolyzing activities toward saikosaponins a, b₁, b₂, d and prosaikogenins A and F, but not prosaikogenins D and G (data not shown), which was the same as that seen for the native cells.¹⁰⁾

Through Butyl-Toyopearl 650M column chromatography (Fig. 1), two *p*-nitrophenyl β -D-glucoside-hydrolyzing activities and two *p*-nitrophenyl β -D-fucoside-hydrolyzing activities were separated into four different fractions, indicating the presence of two kinds of both β -D-glucosidase and β -D-fucosidase. The first eluted fraction of the two β -D-glucosidase fractions was capable of hydrolyzing saikosaponin a but not prosaikogenin F, while the first eluted one of the two β -D-fucosidase fractions had prosaikogenin F-hydrolyzing activity but no

Table 1. Enzymatic Activities of Saikosaponin-Hydrolyzing Bacterial Strains

Substrate	Enzymatic activity (nmol/min/mg)		
	<i>Bifidobacterium</i> sp. Saiko-1	<i>Bifidobacterium</i> sp. Saiko-2	<i>Eubacterium</i> sp. A-44
<i>p</i> -Nitrophenyl β -D-glucoside	349 $\pm$ 13.7	245 $\pm$ 0.667	92.0 $\pm$ 0.05
<i>p</i> -Nitrophenyl β -D-fucoside	0.99 $\pm$ 0.0128	ND	29.6 $\pm$ 0.623
Saikosaponin a	4.04 $\pm$ 0.086	4.08 $\pm$ 0.047	5.26 $\pm$ 0.211
Prosaikogenin F	ND	ND	1.34 $\pm$ 0.097

Enzymatic activities were determined using the disrupted cell of each bacterium. Quantity of protein was determined by Lowry's method. ND: not detected. Means $\pm$ S.D. are given (n = 3).

L. as reported by Shimaoka *et al.*¹¹⁾ Prosaikogenins and saikogenins were obtained as described previously.¹⁰⁾ General anaerobic medium (GAM) was purchased from Nissui Seiyaku Co. (Tokyo, Japan). Silica gel plates 60 F₂₅₄ (Merck Co., Darmstadt, Germany) were used for TLC. *p*-Nitrophenyl β -D-glucoside, L-glucose and D-galactose were purchased from Nacalai Tesque Co. (Kyoto, Japan). *p*-Nitrophenyl β -D-fucoside, *p*-nitrophenyl β -L-fucoside, *p*-nitrophenyl α -L-fucoside, D-glucono δ -lactone, D-galactono δ -lactone, bovine serum albumin (BSA), bovine liver catalase and human transferrin were products of Sigma Chemical Co. (St. Louis, U.S.A.). D-Glucose was purchased from Wako Pure Chemical Industries Co. (Osaka, Japan). Butyl-Toyopearl 650M was purchased from Tosoh Co. (Tokyo, Japan). Sephacryl S-300 gel was obtained from Pharmacia Fine Chemical Co. (Uppsala, Sweden). Rabbit muscle aldolase A was a kind gift from Dr. N. Nakai, Department of Chemistry, Fukui Medical School.

Bacterial Strains *Eubacterium* sp. A-44,¹²⁾ *Bifidobacterium* sp. Saiko-1 and Saiko-2¹⁰⁾ had been previously isolated from human feces.

Enzyme Purification Bacterial cells from 6 l of the culture medium were harvested and suspended in 45 ml of 50 mM potassium phosphate buffer (pH 7.2). The bacterial cells were disrupted by sonication (two times). The supernatants obtained by centrifugation at 22500 $\times g$ for 30 min were combined and designated as crude enzyme (90 ml). The crude enzyme (15 ml $\times$ 6) was applied to a column (30 $\times$ 150 mm) of Butyl-Toyopearl 650M equilibrated with 50 mM potassium phosphate buffer (pH 7.2) containing 1.3 M (NH₄)₂SO₄ (pH 7.2). The column was eluted with a linear gradient of (NH₄)₂SO₄ (1.3—0 M) in the same buffer, and 2 ml fractions were collected. Enzymatic activity of the fractions was monitored using *p*-nitrophenyl β -D-glucoside, *p*-nitrophenyl β -D-fucoside, saikosaponin a and prosaikogenin F as substrates. Fraction A (fr. 43—45) containing saikosaponin a-hydrolyzing activity and fraction B (fr. 49—51) containing prosaikogenin F-hydrolyzing activity were separately concentrated to small volumes with Centriprep-10 (Amicon Co., Tokyo, Japan), which were subsequently subjected to column chromatography of Sephacryl S-300 (30 $\times$ 900 mm). The column was eluted with 50 mM potassium phosphate buffer (pH 6.5), and fractions of 1.2 ml per tube were collected.

Molecular Weight Determination The molecular mass of native enzymes was determined by Sephacryl S-300 column chromatography using a series of standard proteins: BSA (*Mr*=67000), bovine liver catalase (*Mr*=232000), rabbit muscle aldolase A (*Mr*=158000), human transferrin (*Mr*=81000), and arylsulfotransferase (*Mr*=320000) isolated from *Eubacterium* sp. A-44.¹²⁾

Assay Saikosaponin-hydrolyzing activity and prosaikogenin-hydrolyzing activity were assayed as follows. The reaction mixture (0.1 ml) containing 50 μ l of enzyme sample, 50 mM potassium phosphate buffer (pH 6.5) and 1 mM saikosaponins or prosaikogenins was incubated at 37 °C for 30 min. Amounts of saikosaponins and their metabolites were determined as reported previously.¹⁰⁾ The hydrolyzing activity toward saikosaponins was also assayed by determining the amount of glucose released.¹³⁾

The glucose oxidase/peroxidase method of Dahlqvist¹⁴⁾ was followed with minor modifications. In the same manner, geniposide-hydrolyzing activity was measured. β -D-Glucosidase and β -D-fucosidase activities were assayed routinely using *p*-nitrophenyl β -D-glucoside and *p*-nitrophenyl β -D-fucoside as substrates, respectively, according to the method described previously by Akao *et al.*¹³⁾ Similarly, hydrolyzing activities for *p*-nitrophenyl β -L-fucoside, *p*-nitrophenyl α -L-fucoside and *p*-nitrophenyl β -D-galactoside were measured.

RESULTS

β -D-Glucosidase and β -D-Fucosidase Activities of Isolated Bacterial Strains Three bacterial strains isolated from human feces, *Bifidobacterium* sp. Saiko-1, Saiko-2 and *Eubacterium* sp. A-44, which were responsible for the metabolism of saikosaponins,¹⁰⁾ had potent hydrolyzing activities for saikosaponin a and *p*-nitrophenyl β -D-glucoside (Table 1), possibly due to β -D-glucosidase. On the other hand, only *Eubacterium* sp. A-44 showed appreciable hydrolyzing activities for prosaikogenin F and *p*-nitrophenyl β -D-fucoside, possibly due to β -D-fucosidase.

Bifidobacterium sp. Saiko-1 had weak hydrolyzing activity for *p*-nitrophenyl β -D-fucoside, and no hydrolyzing activity for prosaikogenin F, while *Bifidobacterium* sp. Saiko-2 had no activity for either substrate.

Isolation of Saikosaponin-Hydrolyzing β -D-Glucosidase and Prosaikogenin-Hydrolyzing β -D-Fucosidase from *Eubacterium* sp. A-44 A supernatant obtained by centrifugation of the disrupted cells of *Eubacterium* sp. A-44 showed appreciable hydrolyzing activities toward saikosaponins a, b₁, b₂, d and prosaikogenins A and F, but not prosaikogenins D and G (data not shown), which was the same as that seen for the native cells.¹⁰⁾

Through Butyl-Toyopearl 650M column chromatography (Fig. 1), two *p*-nitrophenyl β -D-glucoside-hydrolyzing activities and two *p*-nitrophenyl β -D-fucoside-hydrolyzing activities were separated into four different fractions, indicating the presence of two kinds of both β -D-glucosidase and β -D-fucosidase. The first eluted fraction of the two β -D-glucosidase fractions was capable of hydrolyzing saikosaponin a but not prosaikogenin F, while the first eluted one of the two β -D-fucosidase fractions had prosaikogenin F-hydrolyzing activity but no

Table 1. Enzymatic Activities of Saikosaponin-Hydrolyzing Bacterial Strains

Substrate	Enzymatic activity (nmol/min/mg)		
	<i>Bifidobacterium</i> sp. Saiko-1	<i>Bifidobacterium</i> sp. Saiko-2	<i>Eubacterium</i> sp. A-44
<i>p</i> -Nitrophenyl β -D-glucoside	349 $\pm$ 13.7	245 $\pm$ 0.667	92.0 $\pm$ 0.05
<i>p</i> -Nitrophenyl β -D-fucoside	0.99 $\pm$ 0.0128	ND	29.6 $\pm$ 0.623
Saikosaponin a	4.04 $\pm$ 0.086	4.08 $\pm$ 0.047	5.26 $\pm$ 0.211
Prosaikogenin F	ND	ND	1.34 $\pm$ 0.097

Enzymatic activities were determined using the disrupted cell of each bacterium. Quantity of protein was determined by Lowry's method. ND: not detected. Means $\pm$ S.D. are given (*n*=3).

Fig. 1. Butyl-Toyopearl 650M Column Chromatography of a Crude Enzyme Preparation

●, *p*-nitrophenyl β -D-glucoside-hydrolyzing activity; ○, *p*-nitrophenyl β -D-fucoside-hydrolyzing activity.

Table 2. Substrate Specificity of Saikosaponin-Hydrolyzing β -D-Glucosidase and Prosaikogenin-Hydrolyzing β -D-Fucosidase

Substrate	Glucosidase (%) ^{a)}	Fucosidase (%) ^{b)}
<i>p</i> -Nitrophenyl β -D-fucoside	ND	100
<i>p</i> -Nitrophenyl β -L-fucoside	ND	ND
<i>p</i> -Nitrophenyl α -L-fucoside	ND	ND
<i>p</i> -Nitrophenyl β -D-glucoside	100	32.0
<i>p</i> -Nitrophenyl β -D-galactoside	ND	22.2
Saikosaponin a	17.4	ND
Saikosaponin b ₁	14.8	ND
Saikosaponin b ₂	14.8	ND
Saikosaponin c	ND	ND
Saikosaponin d	4.8	ND
Prosaikogenin F	ND	8.6
Prosaikogenin A	ND	8.2
Prosaikogenin D	ND	ND
Prosaikogenin G	ND	ND
Geniposide	41.4	ND

a, b) Hydrolyzing activities of β -D-glucosidase and β -D-fucosidase for all substrates were expressed relative to that shown for *p*-nitrophenyl β -D-glucoside-hydrolyzing activity (100% = 4.90 μ mol/min/mg) and *p*-nitrophenyl β -D-fucoside-hydrolyzing activity (100% = 1.08 μ mol/min/mg), respectively. ND: not detected.

saikosaponin a-hydrolyzing activity. Further purification of these enzymes was carried out through a column of Sephacryl S-300. Using a series of standard proteins, the apparent molecular weight of saikosaponin-hydrolyzing β -D-glucosidase was estimated to be about 100 kDa, and that of prosaikogenin-hydrolyzing β -D-fucosidase was shown to be about 130 kDa. By two steps of purification, the β -D-glucosidase and the β -D-fucosidase were purified 50 and 40-fold, respectively, from the supernatant of disrupted cells, and these specific activities were 4.9 and 1.1 μ mol/min/mg, respectively. By incubation with both glycosidases, saikosaponins a and b₁ were transformed to their corresponding saikogenins *via* prosaikogenins (data not shown).

Properties of Saikosaponin-Hydrolyzing β -D-Glucosidase

Table 3. Inhibitors of β -D-Glucosidase and β -D-Fucosidase

Inhibitors (10 mM)	Glucosidase (%) ^{a)}	Fucosidase (%) ^{b)}
None	100	100
D-Fucose	89.5	33.3
L-Fucose	95.1	82.8
D-Glucose	40.0	69.1
L-Glucose	102	99.7
D-Glucono δ -lactone	12.7	60.7
D-Galactose	99.5	69.8
D-Galactono δ -lactone	72.6	59.0

a) Relative to *p*-nitrophenyl β -D-glucoside-hydrolyzing activity without additive (100% = 4.95 μ mol/min/mg). b) Relative to *p*-nitrophenyl β -D-fucoside-hydrolyzing activity without additive (100% = 0.916 μ mol/min/mg).

and Prosaikogenin-Hydrolyzing β -D-Fucosidase The optimum pH of the saikosaponin-hydrolyzing activity of β -D-glucosidase was found to be pH 6.0 when *p*-nitrophenyl β -D-glucoside and saikosaponin a were used as substrates (data not shown). The substrate specificity of the two glycosidases toward various glycosides is shown in Table 2. Relative to *p*-nitrophenyl β -D-glucoside-hydrolyzing activity (100%), the hydrolyzing activities of saikosaponin-hydrolyzing β -D-glucosidase were 41.4% for geniposide, 17.4% for saikosaponin a, and about 15% for both saikosaponins b₁ and b₂, whereas a weaker activity was observed for saikosaponin d. However, no fucosidase, galactosidase, or prosaikogenin-hydrolyzing activity was detected. These findings, in addition to its molecular weight, indicate that the β -D-glucosidase isolated in this experiment was identical to the geniposide-hydrolyzing β -D-glucosidase purified previously from the same bacterium.¹⁰⁾ The *p*-nitrophenyl β -D-glucoside-hydrolyzing activity of the β -D-glucosidase was effectively inhibited by D-glucose and D-glucono δ -lactone, but not by L-glucose, D-fucose, L-fucose or D-galactose (Table 3). These findings indicate that the β -D-glucosidase has a broad substrate-specificity toward β -D-glucosides but not β -D-

- 21) Walker D. E., Axelrod B., *Arch. Biochem. Biophys.*, **187**, 102—107 (1978).
- 22) Heyworth R., Walker P. G., *Biochem. J.*, **83**, 331—335 (1962).
- 23) Levvy G. A., *Nature* (London), **187**, 1027 (1960).
- 24) Arai K., Kanaseki T., Ohkuma S., *J. Biochem.* (Tokyo), **110**, 541—547 (1991).
- 25) Tochikura T., Sakai K., Fujiyoshi T., Tachiki T., Kumagai H., *Agric. Biol. Chem.*, **50**, 2279—2286 (1986).
- 26) Nunoura N., Ohdan K., Yano T., Yamamoto K., Kumagai H., *Biosci. Biotech. Biochem.*, **60**, 188—193 (1996).