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Purification and Characterization of Two Novel β-D-Glucuronidases Converting Glycyrrhizin to 18β-Glycyrrhetinic Acid-3-O-β-D-Glucuronide from *Streptococcus* LJ-22

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Abstract Two novel β-glucuronidases, which metabolize glycyrrhizin (GL) to 18β-glycyrrhetinic acid-3-O-β-D-glucuronide (GAMG), were purified from *Streptococcus LJ-22* isolated from human intestinal microflora. β-Glucuronidases I and II were purified to apparent homogeneity, using a combination of ammonium sulfate fractionation, butyl toyopearl, Q-Sepharose, hydroxyapatite Ultrogel, and GL-attached Sepharose column chromatographies, with the final specific activities of 137 and 190 nmole/min/mg, respectively. The molecular sizes of both β-glucuronidases were found to be 140 kDa by gel filtration, and they consisted of two identical subunits (M.W. 67 kDa by SDS-PAGE). β-Glucuronidases I and II showed optimal activity at pH 7.0 and pH 6.5, respectively. Both purified enzymes were potently inhibited by Cu^{2+} and PCMS, and had maximum activity on glycyrrhizin, but did not hydrolyze p-nitrophenyl-β-glucuronides, baicalin, or GAMG. These findings suggest that the biochemical properties and substrate specificities of these enzymes are different from those of the previously purified β-glucuronidases. This is the first reported purification of sugar (not aglycone)-recognizing β-glucuronidases from intestinal bacteria.

Key words β-Glucuronidase, *Streptococcus* LJ-22, glycyrrhizin, 18β-glycyrrhetinic acid-3-O-β-D-glucuronide, purification

Glycyrrhizin [18β-glycyrrhetinic acid-3-O-β-D-glucuronopyranosyl-2-β-D-glucuronide, GL], which is a main component of licorice extract (*Glycyrrhiza glabra*), is ingested orally as a sweetener as well as a component in Oriental medicine. GL has a steroid-like action, antiviral and anti-inflammatory activities [1, 20, 26]. On oral and administration of GL to humans, 18β-glycyrrhetinic acid (GA) was detected in serum, but GL was not [24]. Hattori *et al.* [9] reported that GL was transformed to GA by human intestinal bacteria. Akao *et al.* [2] reported that 18β-glycyrrhetinic acid-3-O-β-D-glucuronide (GAMG) and GL are metabolized by the β-glucuronidase(s) of human intestinal bacteria. We previously reported that GAMG and GA are minor and main metabolites, respectively, when GL was incubated with human intestinal microflora [14, 16]. Therefore, it seems that there are two pathways concerning the metabolism of GL to GA by human intestinal bacteria: one is the main pathway, directly metabolizing GL to GA, and the other one is the minor pathway, metabolizing GL to GA via GAMG. *Streptococcus* LJ-22, which metabolizes GL to GAMG, is from human intestinal microflora [16]. However, the purification and characterization of this bacteria's β-glucuronidase(s), metabolizing GL to GAMG, have not been attempted.

In the present study, therefore, the purification and characterization of β-glucuronidases from *Streptococcus* LJ-22 were performed.

Materials and Methods

Materials

Glycyrrhizin, baicalin, p-nitrophenyl-β-D-glucuronide (PNGu), p-nitrophenyl-β-D-glucopyranoside (PNG), sodium thioglycolate, tosyl-L-lysine chlormethyl ketone (TLCK), iodoacetic acid (IAA), N-ethylmaleneimide (NEM), p-chlormercuryphenylsulfonic acid (PCMS), carbodiimide, paraoxon, dithiothreitol (DTT), mercaptoethanol, ascorbic acid, DEAE-cellulose, hydroxyapatite ultrogel, and butyl-toyopearl were purchased from Sigma Chem. Co. (St. Louis, MO, U.S.A.). A general anaerobic medium (GAM) was purchased from Nissui Pharmaceutical Co., Ltd., (Tokyo, Japan). Tryptic soy broth (TS) and other media were purchased from Difco Co. (Sparks, MD, U.S.A.). EAH-Sepharose 4B, Sephacryl S-300, molecular weight

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markers for gel filtration and the protein electrophoresis markers were purchased from Amersham Pharmacia Biotech. (Buckinghamshire, England, U.K.). The protein assay reagent was purchased from Bio-Rad Laboratories (Hercules, CA, U.S.A.). All other chemicals were of analytical reagent grade.

GL-attached EAH-Sepharose 4B was prepared according to the modified method of Kim and Kobashi [12]. Swollen EAH Sepharose 4B (3.5 g) was washed with 100 ml of 1 M KCl and then with 100 ml of 0.05 M boric acid, pH 4.0. Ethylcarbodiimide (30 mg) and glycyrrhizin (10 mg) were added to the beads and gently stirred overnight at 4°C, and successively washed with 100 ml of 0.1 M Tris-HCl buffer, pH 8.3, 100 ml of 0.1 M Tris-HCl buffer (pH 8.3) containing 0.5 M NaCl, 100 ml of 0.1 M acetate buffer (pH 4.5), distilled water, and finally with 300 ml of 10 mM phosphate buffer (pH 7.0). The final beads were used as a matrix for the GL-attached Sepharose.

Isolation of Bacteria, Metabolizing GL to GAMG, from Human Intestinal Microflora

Bacterial strains, previously isolated from the fresh feces of a healthy Korean man, were cultured in GAM broth, and assayed for the activity to transform GL to GAMG. The positive bacterium LJ-22, which is a Gram-positive *Streptococcus*, was identified by means of Bergey's manual [23] and analysis of the elongation factor EF-Tu DNA sequence.

To analyze the elongation factor EF-Tu DNA sequence of LJ-22, the chromosomal DNA was isolated using a Wizard® Genomic DNA purification kit (Promega, Madison, WI, U.S.A.). The amplification of the EF-2 DNA was conducted using two primers (Ent1, 5'-TACTGACAAAC-CATTCATGATG-3'; Ent2, 5'-AACTTCGTCACCAACG-CGAAC-3') according to the method of Ke *et al.* [11, 17]. PCR amplification was performed with 35 cycles in a DNA thermocycler (CoreBio 25 PCR Thermocycler, Germany). The purified PCR product was cloned into the pGEM® T-easy vector (Promega, Madison, WI, U.S.A.) and sequenced with a MegaBASE 1000 sequencer (PE biosystems, Foster City, CA, U.S.A.). The newly obtained sequence was aligned with the previously published sequences, which were retrieved from the GenBank database, using the alignment algorithm Blast 2 sequences.

Biotransformation of GL and Isolation of GAMG

Streptococcus LJ-22, which was isolated from human intestinal bacteria, was cultured in 1 l of tryptic soy (TS) broth with 1 g of GL. After cultivation at 37°C for 24 h, the pH was adjusted to 2 with 1 M HCl, and the culture extracted twice with ethyl acetate. After evaporating the ethyl acetate fraction to dryness, the resulting powder was applied to a silica gel chromatography column (2.5×40 cm) using $CHCl_3$/methanol (10:15:1) as the eluent. The isolated GAMG (90 mg) was crystallized from $CHCl_3$/MeOH (5:1).

The ^{13}C-NMR (125 MHz, $CDCl_3$) of GAMG: 40.21 (C-1), 27.61 (C-2), 90.74 (C-3), 40.53 (C-4), 56.43 (C-5), 18.44 (C-6), 33.82 (C-7), 44.64 (C-8), 63.13 (C-9), 38.08 (C-10), 202.65 (C-11), 128.96 (C-12), 172.77 (C-13), 46.76 (C-14), 26.97 (C-15), 27.41 (C-16), 32.98 (C-17), 49.93 (C-18), 42.43 (C-19), 44.90 (C-20), 32.02 (C-21), 39.02 (C-22), 28.41 (C-23), 16.94 (C-24), 16.96 (C-25), 19.31 (C-26), 23.81 (C-27), 28.75 (C-28), 29.19 (C-29), 180.38 (C-30), 106.95 (C-1'), 77.71 (C-2"), 76.54 (C-3'), 75.31 (C-4'), 73.18 (C-5'), 172.7 (C-6').

Enzyme Activity Assay

The GL-hydrolyzing activity was measured as follows: The assay mixture, containing 0.2 ml of 2 mM GL (or other substrates) and 0.2 ml of the enzyme, was made up with 0.1 M phosphate buffer (pH 7.0) to a final volume of 1 ml. The mixture was incubated at 37°C for 2 h, and was then extracted twice with 5 ml of ethyl acetate. The ethyl acetate fraction was analyzed by TLC or HPLC. The quantity of these compounds was determined with a TLC scanner (Shimadzu CS-9301, Tokyo, Japan).

The TLC for GL, GAMG, and GA and for baicalin and baicalein were performed on silica-gel plates (Merck, silica gel 60F-254, Aston, PA, U.S.A.). The developing solvents were $CHCl_3$/petroleum ether/AcOH (6:6:1) for GA, BuOH/$CHCl_3$/AcOH/H_2O (4:1:4:1) for GAMG and GL, and $CHCl_3$/MeOH (3:1) for baicalin and baicalein.

HPLCs for GL, GAMG, GA, baicalin, and baicalein were performed as follows: HPLC system (Hitachi, Tokyo, Japan) - column, μ-Bondapak C_{18} (3.9×300 mm); elution solvent, methanol/water/glacial acetic acid (55:42.5:2.5); elution rate, 1.0 ml/min; detection wavelength, 254 nm.

Protein Measurement

Protein was measured using the Bradford method with bovine serum albumin as the standard [6, 10].

Purification of β-Glucuronidases from *Streptococcus* LJ-22

The *Streptococcus* LJ-22 was cultured at 37°C for 20 h in 50 l of tryptic soy broth, containing 0.1% ascorbic acid, 0.01% sodium thioglycolate, and 0.02% GL, under anaerobic conditions, and harvested by centrifugation for 30 min at 5,000 ×*g*. The pellets were washed twice with cold 50 mM sodium phosphate buffer (pH 7.0) (Buffer A), and suspended in 200 ml of the same buffer, and the suspended cells were ultrasonicated on ice for 15 min (100 watt, 60% pulsed mode). The disrupted cells were centrifuged at 15,000 ×*g* for 60 min, and the supernatant was used as a crude enzyme suspension. The crude enzyme suspension was treated with 35% saturated ammonium sulfate and centrifuged at 10,000 ×*g* for 60 min. The resulting supernatant was precipitated with 70% saturated ammonium sulfate and centrifuged again at 10,000 ×*g*

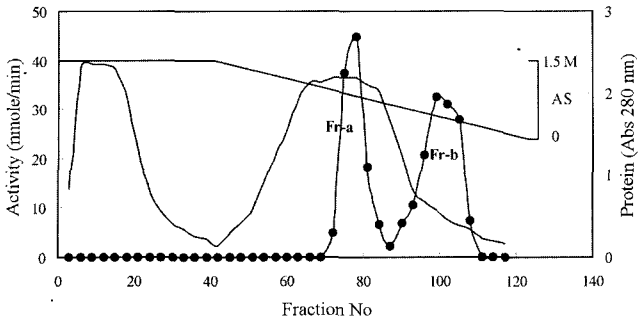

Fig. 1. Elution profile of the activities of β-glucuronidases I and II, from the butyl-toyopearl column chromatography.
Solid circle, β-glucuronidase activity; simple line, absorbance at 280 nm; Fr-a, β-glucuronidase I; Fr-b, β-glucuronidase II; AS, ammonium sulfate. Fraction volume, 20 ml.

for 60 min. The pellets were resuspended in 70 ml of Buffer A containing 1.5 M ammonium sulfate (Buffer B). All purification procedures were performed at 4°C.

The suspended solution was applied to a butyl toyopearl column (3.5×10 cm), which had previously been equilibrated with Buffer B, and the column was washed with 200 ml of the same buffer (Fraction volume, 20 ml). A linear gradient elution was undertaken with 600 ml of Buffer B and 600 ml of Buffer A (Fig. 1). All the fractions obtained were assayed for β-glucuronidase activity.

Two peaks (**Fr-a,** β-glucuronidase I; **Fr-b,** β-glucuronidase II), containing the enzyme activity were separately collected, and assayed for enzyme activity levels.

Purification of β-Glucuronidase I. The active Fr-a (Fr. Nos. 54-63) was pooled and dialyzed three times against 3 l of Buffer A for 18 h. The dialysate was applied to a Q-Sepharose column (3.5×20 cm), previously equilibrated with Buffer A, and the column was washed with 150 ml of the same buffer. A linear gradient elution was undertaken with 500 ml of Buffer A and 500 ml of Buffer A containing 0.5 M KCl (Fraction volume, 20 ml). All the fractions obtained were assayed for β-glucuronidase activity. The active fractions (Fr. Nos. 110–120) were pooled and dialyzed twice against 3 l of 10 mM phosphate buffer (Buffer C). The dialysate was applied to a hydroxyapatite ultrogel column (3.5×5.0 cm) previously equilibrated with Buffer C. The β-glucuronidase was eluted from the column with 600 ml of a linear gradient formed by 10 to 150 mM sodium phosphate buffer (fraction volume, 7.5 ml), the enzyme-active fractions (Fr. Nos. 36–53, 131 ml) were pooled and dialyzed twice against 3 l of Buffer C for 18 h. The dialysate was applied to a GL-attached sepharose column (1.0×3.0 cm), previously equilibrated with Buffer C. The column was washed with 50 ml of Buffer C containing 0.15 M KCl. A linear gradient elution was undertaken with 100 ml of Buffer A containing 0.15 M KCl and 100 ml of Buffer A containing 0.5 M of KCl. All the fractions obtained were assayed for β-glucuronidase activity (fraction volume, 2 ml). The active fractions (28 ml in Fr. Nos. 68–82) were found to be homogeneous β-glucuronidase, evidenced by native and denatured PAGE.

Purification of β-Glucuronidase II. The active Fr-a (Fr. Nos. 75–86), collected from the butyl-toyopearl column chromatography in the above purification procedure, was further purified according to the above purification (Q-Sepharose, hydroxyapatite ultrogel, and GL-attached sepharose column chromatographies) methods. The active fractions (5.9 ml in Fr. Nos. 77–79) were found to be homogeneous β-glucuronidase, determined by native and denatured PAGE.

Characterization of β-Glucuronidases

Electrophoresis was performed by a discontinuous polyacrylamide gel (10% separating gel and 4% stacking gel, 1 mm thickness) under native or denatured conditions using the procedure described by Laemmli [21, 22]. The gel was treated with Coomassie brilliant blue R250, and further stained with silver stain. The molecular weights of the purified enzymes were estimated by comparison with molecular weight markers. The enzyme activity staining was performed as follows; the electrophoretic gel was cut at 5 mm intervals, immersed in the enzyme reaction mixture, instead of the enzyme, and incubated at 37°C for 12 h. The reaction mixtures were extracted with 1 ml of EtOAC, and the reaction products were assayed by TLC. The molecular weight of the native enzyme was estimated by gel filtration using a Sephacryl S-300 column (1.6×70 cm), previously calibrated with a gel filtration low and high molecular weight calibration kit (from Sigma and Amersham Pharmacia Biotech).

The optimum pHs for the purified enzymes were examined using the following buffers: 50 mM acetate (pH 3.5–6.5), 50 mM phosphate (6.0–7.5), and 50 mM NaOH glycine (8.0–9.0).

To investigate the effects of salt concentrations on the enzyme activity, the enzymes were incubated with a substrate in reaction mixtures containing various concentrations of NaCl, KCl, or ammonium sulfate, for 30 min at 37°C, and their activities were assayed.

The kinetic constants of β-glucuronidases were determined by measuring the initial rates at various substrate concentrations of each substrate under standard reaction conditions.

To investigate the effect of metals and chemical modifying agents on the enzyme activity, the enzymes were incubated with various concentrations of metals and chemical modifying agents for 30 min at 37°C, and their activities were measured.

RESULTS

Screening of GL-Hydrolyzing Human Intestinal Bacteria

When GL-hydrolyzing activities were preliminarily assayed in five samples of human feces, enzyme activity was detected in four of the specimens, but the degrees of the

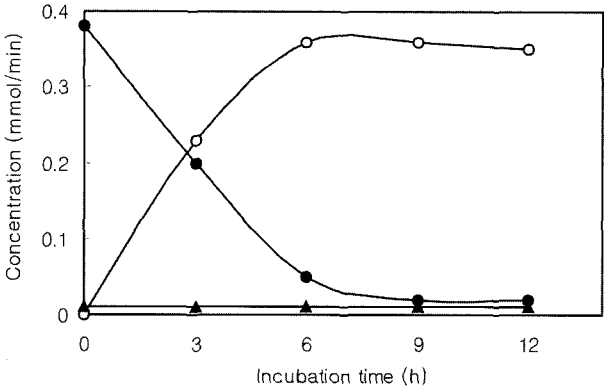

Fig. 2. Time course of reaction products during incubation of GL with LJ-22 β-glucuronidase.
1.5 nmol/min of the β-glucuronidase partially purified by ammonium sulfate fractionation was used. Closed circle, GL; open circle, GAMG; closed triangle, GA.

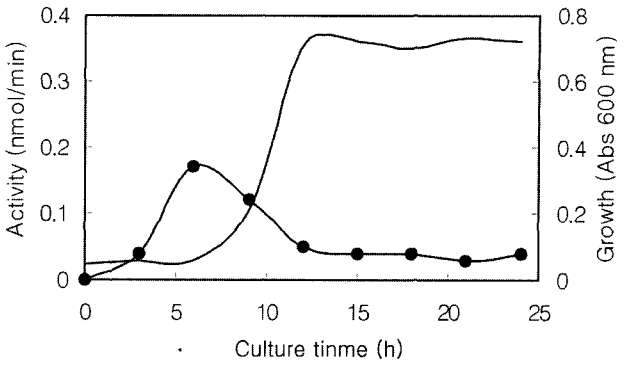

Fig. 3. Growth and β-glucuronidase productivity of *Streptococcus* LJ-22.
Closed circle, β-glucuronidase activity; simple line, growth (Abs 550 nm).

activity varied with individual samples (data not shown). Among 200 intestinal bacteria collected from human feces, three potently hydrolyzed GL to GA, and one (LJ-22) converted GL to GAMG. Only LJ-22 alone transformed GL to GAMG (not GA) (Fig. 2) and was identified as anaerobic *Streptococcus* sp. by means of Bergey's manual [23]. The sequence of the LJ-22 elongation factor EF-Tu showed 99% homology to that of the previously reported *Streptococcus casseliflavus* (data not shown).

The β-glucuronidase (the enzyme hydrolyzing GL to GAMG) activity was observed to increase with the growth of *Streptococcus* LJ-22, reaching a plateau 9 h after cultivation, and then constitutively decreased (Fig. 3). The GL-hydrolyzing-β-glucuronidase activity was strongly induced by GL at 0.1% concentration, but high concentrations (more than 0.2%) of GL inhibited its inducibility (data not shown). To purify the β-glucuronidase produced by the *Streptococcus* LJ-22, it was anaerobically cultured in tryptic soy broth, containing 0.01% sodium thioglycolate, 0.1% ascorbic acid, and 0.2% GL, for 20 h at 37°C.

Purification of β-Glucuronidases

The β-glucuronidases I and II were purified 587- and 950-fold from the crude extract, with a yields of 13.5% and 1.9%, respectively, using the procedures shown in Table 1. The specific activity of the homogeneously purified β-glucuronidases I and II were 137 and 190 nmole/min/mg, respectively. Only a single band was observed in PAGE of each purified enzyme with SDS (Fig. 4). The silver stained band of each purified enzyme on the gels of the non-SDS-PAGE was identical to each enzyme activity peak assayed with fragments of each enzyme electrophoresis gel (data not shown).

Characterization of β-Glucuronidases

The molecular masses of both β-glucuronidases I and II were 140 kDa by Sephacryl S-300 HR, and 67 kDa by the SDS-PAGE methods (Fig. 4).

Table 1. Summary of the purification of β-glucuronidase from *Streptococcus* LJ-22.

Stage	Total activity (nmol/min)	Total protein (mg)	Specific activity (nmol/min/mg)
β-Glucuronidase I			
Crude extract	306.3	1,301.0	0.2
Ammonium sulfate precipitation	202.6	984.4	0.2
Butyl-toyopearl column chromatography	143.0	190.6	0.8
Q-Sepharose column chromatography	130.4	9.4	14
Hydroxyapatite ultrogel column chromatography	79.3	0.7	137
GL-attached Sepharose column chromatography	41.2	0.3	137
β-Glucuronidase II			
Crude extract	306.3	1,301.0	0.2
Ammonium sulfate precipitation	202.6	984.4	0.2
Butyl-toyopearl column chromatography	44.6	89.7	0.5
Q-Sepharose column chromatography	36.2	9.6	3.8
Hydroxyapatite ultrogel column chromatography	10.0	0.06	167
GL-attached Sepharose column chromatography	5.7	0.03	190

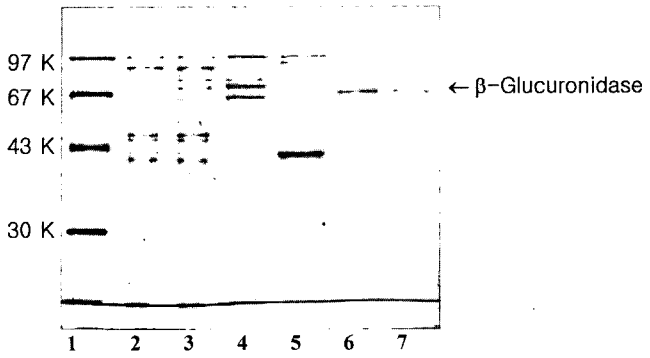

Fig. 4. SDS-PAGE of the β-glucuronidases during the purification stages.
Lane 1, molecular markers; Lane 2, crude extract; Lane 3, Ammonium sulfate precipitation; Lane 4, Fr-a of butyl toyopearl (β-glucuronidase I); Lane 5, Fr-b of butyl toyopearl (β-glucuronidase II); Lane 6, GL-attached sepharose (β-glucuronidase I); and Lane 7, GL-attached sepharose (β-glucuronidase II). Molecular markers contain phosphorylase (97.4 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45 kDa), and carbonic anhydrase (29 kDa).

When the activities of the purified β-glucuronidases I and II were assayed at 37°C, the optimal pH of these enzymes were found to be 7.0 and 6.5, respectively (Fig. 5). The activities of both purified enzymes were unaffected by NaCl or KCl, but the β-glucuronidases were inhibited by ammonium sulfate with an IC_{50} value of 0.7 M. When these enzymes were incubated at 37°C for 1 h, the residual enzyme activities were more than 90%.

The effects of the chemical modifying agents and metal ions on the purified α-L-β-glucuronidases I and II were investigated (Tables 2 and 3). PCMS alone potently inhibited both enzymes. Both enzymes were inhibited by Cu^{++} and β-glucuronidase II was inhibited also by Ni^{++}. However, most metal ions had no inhibitory effect on the enzymes.

The pI values of the purified β-glucuronidases I and II were 4.0 and 4.1, respectively, as determined by chromatofocusing column chromatography (Sigma Co., U.S.A.).

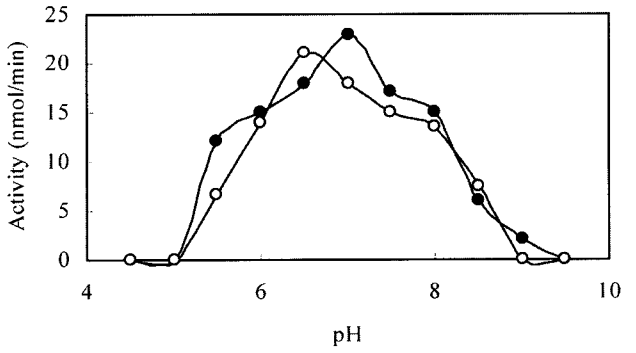

Fig. 5. pH profiles of the activities of β-glucuronidases I and II.
The enzyme activities were assayed under standard conditions. Solid circle, β-glucuronidase I activity; open circle, β-glucuronidase II activity.

Table 2. Effects of chemical modifying agents on β-glucuronidase activities.

Sugar[a]	IC_{50} (mM)[b]	
	β-Glucuronidase I	β-Glucuronidase II
PMSF	88	102
TLCK	101	78
IAA	105	92
Paraoxon	92	98
PCMS	16	7
DTT	92	99
Mercaptoethanol	98	102
2,3-Butanediol	93	91
NEM	96	95
Carbodiimide	105	91

[a]Final concentration, 0.1 mM.
[b]0.05 nmol/min of the homogeneously purified enzyme activity was taken as 100%.

Substrate Specificity

The substrate specificities of the β-glucuronidases I and II with synthetic substrates and natural glycosides were investigated (Table 4). The best substrate for both enzymes was GL. Neither of the enzymes hydrolyzed p-nitrophenyl-β-glucuronide and p-nitrophenyl-β-D-glucopyranoside. Also, neither of the enzymes hydrolyzed natural β-glucuronic acid conjugates, baicalin, and GAMG.

Commercial *E. coli* β-glucuronidase and *E. coli* HGU-3 β-glucuronidase weakly hydrolyzed GAMG to GA, but did not hydrolyze GL. The β-glucuronidase of commercial bovine and that partially purified from rat livers hydrolyzed both GL and GAMG to GA.

Kinetic Constants of β-Glucuronidases

Using GL as the substrate for both β-glucuronidases I and II, the K_m and V_{max} values were estimated to be 0.27 mM

Table 3. Effects of metals on β-glucuronidase activities.

Metal[a]	Residual activity (%)[b]	
	β-Glucuronidase I	β-Glucuronidase II
Control	100	100
Mg^{++}	78	79
Ca^{++}	94	96
Ni^{++}	93	52
Ba^{++}	115	122
Co^{++}	92	79
Cu^{++}	9	1
Pb^{++}	97	76
Fe^{++}	103	72
Li^{++}	105	87
Zn^{++}	91	93
EDTA	102	101

[a]Final concentration, 1 mM.
[b]0.05 nmol/min of the homogeneously purified enzyme activity was taken as 100%.

Table 4. Substrate specificity of LJ-22 β-glucuronidases.

Substrate	Activity (%)					
	β-Gase I	β-Gase II	*E. coli*	HGU-3	Bovine	Rat
GL	100 (137)[a]	100 (190)	0	0	3	81
GAMG	0	0	2	<1	1	3
Baicalin	0	0	4	1	2	5
Bilirubin DiG	0	0	–[b]	–	–	–
PNGu	0	0	100 (1840)	100 (48)	100 (62)	100 (67)
PNG	0	0	–	0	–	–
Maltose	0	0	–	0	–	–
Cellobiose	0	0	–	0	–	–

β-Gase I, LJ-22 β-glucuronidase I; β-Gase II, β-glucuronidase II; *E. coli*, commercial *E. coli* β-glucuronidase; HGU-3, *E. coli* HGU-3 β-glucuronidase; bovine, bovine β-glucuronidase (Sigma Co.); Rat, β-glucuronidase partially purified from livers of male SD rats (180–200 g) according to Akao *et al.* [3].
[a]The enzyme substrate specificities for GL (β-Gase I or II) and for PNGu (the other enzymes) were taken as 100%. Number in parenthesis indicates substrate specificity of each enzyme.
[b]Not determined.

and 0.77 μmol/min/mg, and 0.98 mM and 0.98 μmol/min/mg, respectively (Fig. 6).

DISCUSSION

Intestinal microflora play significant roles in the metabolism of unabsorbable natural herbal components, such as GL and ginsenosides, and in the enterohepatic circulation of endogenous and exogenous substances [4, 18]. In order to investigate the biological action of the components of licorice, the metabolism of GL from licorice by human intestinal bacteria has been studied [2, 9, 14, 16]. GL, a major component of licorice, is a glycoside that contains diglucuronic acid with 18β-glycyrrhetinic acid [7]. To absorb and have the pharmacological actions of GL, GL must be metabolized, by human intestinal bacteria, after oral ingestion [18, 27]. Related to biotransformation of GL, Akao *et al.* [2] and Kim *et al.* [15, 16] isolated *Eubacterium* sp., *Bacteroides* sp., and *Streptococcus* sp. from human intestinal feces. However, purification and characterization of the β-glucuronidases of intestinal bacteria, related to the metabolism of GL, have not been studied. Therefore, we isolated *Streptococcus* LJ-22 from human feces, and its GL-hydrolyzing β-glucuronidases, which hydrolyzed GL to GAMG but not GA, were purified.

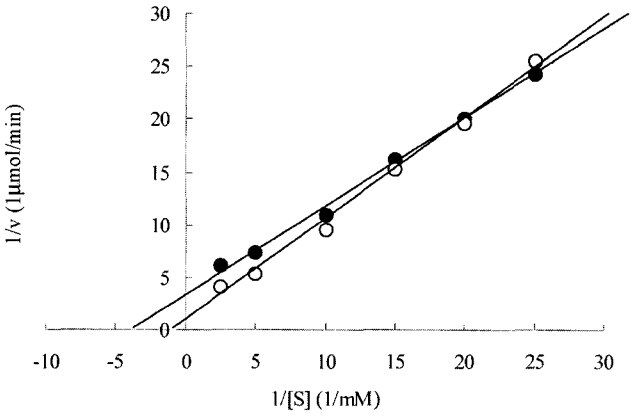

Fig. 6. Lineweaver-Burk plot of β-glucuronidases I (closed circle) and II (open circle) for glycyrrhizin.

The molecular masses of the purified β-glucuronidases were found to be 140 and 67 kDa by a gel filtration method using Sephacryl S-300 HR and by the SDS-PAGE method, respectively. These results suggest that the β-glucuronidases I and II consist of two identical subunits, although the *N*-terminal sequences of the purified enzymes could not be analyzed due to their blockages. The optimal pHs of these enzymes were found to be 7.0 and 6.5, respectively. These enzymes were thermostable during incubation at 37°C. Both enzymes were inhibited by Cu^{++}, and PCMS alone potently inhibited both enzymes. These results suggest that cysteine may be important in the catalysis by these enzymes. The general properties, molecular masses, and optimal pHs of the present purified enzymes are different from those of the β-glucuronidases previously purified from *E. coli*, and rat and human livers [2, 3, 5, 8, 13, 25, 28].

To understand the substrate specificity of β-glucuronidases, the GL-hydrolyzing activities of many β-glucuronidases originating from some intestinal bacteria and animals were investigated. Commercial *E. coli* β-glucuronidase and *E. coli* HGU-3 β-glucuronidase weakly hydrolyzed GAMG to GA, but did not hydrolyze GL. Akao *et al.* [2] also reported that *Eubacterium* sp. GLH β-glucuronidase hydrolyzed GL and synthesized β-GAMG. Kim *et al.* [15] also reported that β-glucuronidase isolated from *Bacteroides* J-37 hydrolyzed both GAMG and GL to GA. The GL-hydrolyzing activities of the putative β-glucuronidase isolated from *Bacteroides* J-37 and *Eubacterium* A-44 were different from those of the above *E. coli* β-glucuronidases [2, 13, 15].

Rat and bovine liver β-glucuronidases hydrolyzed GL to GAMG and then GAMG to GA, but seem not to directly hydrolyze GL to GA.

When the specificities of the present β-glucuronidases I and II toward synthetic substrates and natural glycosides were investigated, the present β-glucuronidases I and II hydrolyzed GL, but did not hydrolyze p-nitrophenyl-β-glucuronide, baicalin, and GAMG. In addition, Kuramoto *et al.* [19] reported that *Cryptococcus magnus* MG-27 produced the β-glucuronidase that hydrolyzed GL to GAMG, but, they did not purify the enzyme. Therefore, its substrate specificity is not known. Nevertheless, the MG-27 is a eukaryotic microbe, in which β-glucuronidase seems to hydrolyze GL to GAMG and GAMG to GA. Based on these findings, the present β-glucuronidases appear to recognize and hydrolyze diglucuronic acids, but did not recognize alycone (not sugar)-conjugated β-monoglucuronic acid. The general properties, molecular masses, and inhibitions of chemical modifying agents were not significantly different between β-glucuronidases I and II. Nevertheless, their affinities for butyl toyopearl matrix and substrate GL were significantly different. These results suggest that the present enzymes may recognize the sugar moiety of substrates, but only some amino acids related to the active site of these enzymes may be different.

In conclusion, this is the first report on the purification and characterization of β-glucuronidases I and II, which hydrolyze GL to GAMG (not GA), a more potent sweetener than GL [7]. The substrate specificity and the characteristics of the present β-glucuronidases I and II are different from those previously reported for β-glucuronidases of *E. coli*, and rat and bovine livers. The present β-glucuronidases I and II produced from human intestinal bacteria can transform GL to GAMG in the human intestines. Finally, it is suggested that the present β-glucuronidases I and II should be classified separately from those previously reported β-glucuronidases.

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