Production of Rubusoside Derivatives by Transgalactosylation of Various α -Galactosidases

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Incubation of rubusoside with raffinose in the presence of various α -galactosidases yielded three galactosylated rubusosides. *Mortierella vinacea* α -galactosidase transferred a galactosyl residue preferentially to the C6-hydroxyl group of the glucosyl residue at the 13-hydroxyl group of rubusoside to produce mainly 13-*O*- α -D-galactosyl-(1 $\rightarrow$ 6)- β -D-glucosyl-19-*O*- β -D-glucosyl-steviol. α -Galactosidases from *Absidia reflexa*, *Escherichia coli*, and coffee beans transferred a galactosyl residue to the C6-hydroxyl group of both glucosyl residues at the 13-hydroxyl and 19-carboxyl groups of rubusoside, yielding the above galactosylated rubusoside and 13-*O*- β -D-glucosyl-19-*O*- α -D-galactosyl-(1 $\rightarrow$ 6)- β -D-glucosyl-steviol.

Rubusoside (β -D-glucosyl ester of 13-*O*- β -D-glucosyl-steviol) was isolated in a high yield (5.4%) from the leaves of a *Rubus suavissimus* S. Lee (Rosaceae) collected in Kwangchow, China.^{1,2)} The relative sweetness of rubusoside to sucrose at a concentration of 0.025% is 114-fold. Rubusoside, however, has a slightly bitter taste like stevioside.

Recently, the modification of rubusoside has been studied to improve the quality of its taste and to increase its sweetness. Darise *et al.*³⁾ synthesized various rubusoside derivatives by transglycosylation of cyclomaltodextrin glucanotransferase (CGT-ase) and studied the relationship between the chemical structures and their tastes. It was revealed that the glucosylation of 13-*O*-glucosyl moiety of rubusoside significantly improved the sweetness, while the elongation of a glucosyl unit at the 19-position changed both the intensity and quality of sweetness for the worse. We have intended the improvement of sweetness of rubusoside by selective elongation of the 13-*O*-glucosyl moiety of rubusoside.

It is already known that glucose is an efficient acceptor for the intermolecular transglycosylation of CGT-ase, but galactose is not an efficient acceptor.⁴⁾ Based on this, we planned to block the C4-hydroxyl group of the glucosyl moiety at the 19-position of rubusoside with galactose using transgalactosylation of α -galactosidase.

In this work, we have tested whether α -galactosidases produce galactosylated rubusosides using a mixture of rubusoside and raffinose. In this paper, we report the production of three transfer products and investigation of their structures.

Materials and Methods

Materials. Rubusoside was isolated from the leaves of *Rubus suavissimus* (Rosaceae) collected in Kwangchow, China, by the method of Tanaka *et al.*¹⁾ Melibiose and raffinose were purchased from Wako Pure Chemical Industries. α -Galactosidases from *Mortierella vinacea* and *Absidia reflexa* were prepared by Hokkaido Sugar Co., Ltd. α -Galactosidases from *Escherichia coli* and green coffee beans were purchased from the Sigma Chemical

Company.

Assay of α -galactosidase activity. α -Galactosidase activity was assayed spectrophotometrically with *p*-nitrophenyl α -galactoside as the substrate. The reaction mixture contained 200 μ l of 10 mM *p*-nitrophenyl α -galactoside, 200 μ l of 40 mM acetate buffer (pH 6.0), and 50 μ l of an enzyme solution. After incubation for 10 min, the reaction was stopped by adding 500 μ l of 200 mM sodium carbonate. One unit of α -galactosidase activity was defined as the amount releasing 1 μ mol of *p*-nitrophenol per min.

High performance liquid chromatography. A high performance liquid chromatography (HPLC) for the measurement of the transgalactosylation products was done under the following conditions: column, TSK gel ODS 120T (4.6 $\times$ 250 mm); detector, Shimadzu SPD-6A (213 nm); solvent, acetonitrile–water (25 : 30 : 75 : 70, v/v); flow rate, 0.5 ml/min. Preparative HPLC was done under the following conditions: column, TSK gel ODS-120T (21.5 $\times$ 300 mm); detector, Shodex RE-11 refractometer; solvent, acetonitrile–water (30 : 70 v/v); flow rate, 6 ml/min.

Thin layer chromatography. Thin layer chromatography (TLC) was done on a plate of Kiesel gel 60 TLC plate (Merck Co., Ltd.) with a solvent system of chloroform–methanol–water (10 : 5 : 1 or 6 : 4 : 1, v/v). The sugars on the TLC plate were visualized by heating at 110–120°C after spraying with 50% sulfuric acid–methanol solution.

Measurement of nuclear magnetic resonance (NMR) spectra. NMR spectra were recorded with a JEOL GX-400 spectrometer at 28°C for pyridine-*d*₅ solutions at 400 MHz for protons and at 100 MHz for carbon-13 with tetramethylsilane as an internal standard.

Optical rotation. $[\alpha]_D$ was measured on a Union automatic digital polarimeter.

Selective cleavage of ester type glycoside linkage.⁵⁾ A mixture of a glycoside (5 mg), anhydrous LiI (5 mg), and anhydrous 2,6-lutidine (3 ml) in anhydrous methanol (3 ml) was refluxed for 24 hr. The mixture was diluted with 50% methanol and then deionized with Amberlite MB-3. In TLC, the *R*_f value of the resulting 13-*O*-glycosyl-steviol decreased in the order of 13-*O*-mono-, di-, and tri-glycosides, which made distinction of these compounds easy.

Results and Discussion

Transgalactosylation by *M. vinacea* α -galactosidase

When α -galactosidase from *M. vinacea* (1.3 units) was incubated with raffinose and

Fig. 1. Thin Layer Chromatogram of *M. vinacea* α -Galactosidase Action Products on the Mixtures of Raffinose and Rubusoside and Melibiose and Rubusoside.

1, rubusoside (Ru); 2, glucose (Gl) and melibiose (Me); 3 and 4, reaction products on melibiose and rubusoside; 5, glucose and raffinose (Ra); 6 and 7, reaction products on raffinose and rubusoside.

melibiose (1 M each) as donors in the presence of rubusoside (0.1 M) in 50 mM acetate buffer (pH 6.0, final volume, 200 μ l), separately, two new spots besides the spots of raffinose, melibiose, and rubusoside, were detected on the TLC plate after TLC (Fig. 1). Their *R*_f values were between those of rubusoside and glucose. We suppose from their *R*_f values that these new spots may be transgalactosylated derivatives of rubusoside.

Isolation of transgalactosylated products

To isolate the rubusoside derivatives described above, the reaction was run on a large scale with 3 g of rubusoside, 20 g of raffinose, and 1340 units of the enzyme in a total volume of 47 ml at 50°C for 12 hr. After being heated at 100°C for 10 min, the reaction mixture was centrifuged to remove insoluble materials and the supernatant solution was analyzed by HPLC (Fig. 2). Two peaks, RGal-1a and

Fig. 2. High Performance Liquid Chromatogram of *M. vinacea* α -Galactosidase Action Products on the Mixture of Raffinose and Rubusoside.

Compound	R ₁	R ₂
Rubusoside	β -Glc	β -Glc
RGal-1a	β -Glc ⁶⁻¹ α -Gal	β -Glc
RGal-1b	β -Glc	β -Glc ⁶⁻¹ α -Gal
RGal-2	β -Glc ⁶⁻¹ α -Gal ⁶⁻¹ α -Gal	β -Glc

RGal-2, were detected as reaction products. The supernatant solution was put on a column (30 $\times$ 210 mm) of Diaion HP-20 (Nippon Rensui Co.). After washing the column with distilled water to remove galactose and olig-

osaccharides, a mixture of rubusoside and its derivatives were eluted with 70% methanol. The eluate was chromatographed on silica gel with a linear gradient of chloroform-methanol-water (13:7:1-10:5:1, homogeneous) to separate two products. We obtained 150 mg of RGal-1a and 70 mg of RGal-2 by the above procedures.

Structure elucidation of transgalactosylated products

RGal-1a was obtained as a white powder: $[\alpha]_D^{19}$, -3.07° ($c=1.01$, methanol); *Anal.* Found: C, 53.31; H, 7.83%. Calcd. for $C_{38}H_{60}O_{18} \cdot 3H_2O$: C, 53.13; H, 7.75%. The 1H -NMR spectrum of RGal-1a exhibited three anomeric proton signals at δ 6.16 (1H, d, $J=7.7$ Hz, 19-COO- β -Glc), 5.54 (1H, d, $J=3.4$ Hz, α -Gal) and 5.03 (1H, d, $J=7.7$ Hz, 13-O- β -Glc) and the ^{13}C -NMR spectrum had three anomeric carbon signals at δ 95.9 (19-COO- β -Glc), 99.5 (13-O- β -Glc), and 100.0 (α -Gal), indicating that RGal-1a must be a mono- α -galactosylated product. Rubusoside had two carbon signals due to C-6 of non-linked glucosyl units at δ 62.5 and 63.3, while RGal-1a had a carbon signal due to C-6 of a 6-linked glucosyl unit at δ 68.8 together with signals due to C-6 of non-linked glucosyl and galactosyl units at δ 62.5 and 62.1, indicating that 6-hydroxyl group of one of two β -glucosyl units was galactosylated. RGal-1a was selectively cleaved at the 19-COO- β -Glc linkage with LiI-reagent⁵⁾ to give an acidic glycoside (RGal-1a-G). The TLC-*R_f* value of RGal-1a-G was evidently lower than that of 13-O-glucosyl-steviol (steviolmonoside), and rather similar to that of 13-O- β -sophorosyl-steviol (steviolbioside). This shows that RGal-1a-G is 13-O-galactosyl-glucosyl-steviol. Consequently, we concluded that RGal-1a was 13-O- α -D-galactosyl-(1 $\rightarrow$ 6)- β -D-glucosyl-19-O- β -D-glucosyl-steviol.

RGal-2 was obtained as a white powder: $[\alpha]_D^{19}$, $+29.2^\circ$ ($c=0.79$, methanol); *Anal.* Found: C, 52.94; H, 7.22%. Calcd. for $C_{44}H_{70}O_{23} \cdot 2H_2O$: C, 52.68; H, 7.44%. The four anomeric proton signals of RGal-2 ap-

Table I. EFFECTS OF RAFFINOSE CONCENTRATION ON THE PRODUCTION OF RGal-1a AND RGal-2 BY *M. vinacea* α -GALACTOSIDASE

Raffinose (M) Rubusoside (M)	Product	Reaction time (hr)		
		1.0	3.5	15
0.1	RGal-1a	4.8%	6.2%	6.0% ^a
0.1	RGal-2	0.5	0.5	<0.1
0.4	RGal-1a	8.1	10.8	11.7
0.1	RGal-2	1.3	2.1	1.9
1.0	RGal-1a	8.6	11.3	12.6
0.1	RGal-2	1.6	3.0	3.3
0.4	RGal-1a	6.4	7.1	8.8
0.2	RGal-2	1.4	1.0	1.7
1.0	RGal-1a	5.1	7.0	7.9
0.2	RGal-2	0.6	1.4	1.4

^a [Amount of transfer product (M)/Amount of rubusoside used (M)] $\times$ 100.

peared at δ 6.12 (1H, d, $J=7.7$ Hz, 19-*COO*- β -Glc), 5.57 (1H, d, $J=3.7$ Hz, α -Gal), 5.46 (1H, d, $J=3.7$ Hz, α -Gal), and 5.03 (1H, d, $J=7.7$ Hz, 13-*O*- β -Glc). It had four anomeric carbon signals at δ 95.9 (19-*COO*- β -Glc), 99.5 (13-*O*- β -Glc), 100.1 (α -Gal), and 100.3 (α -Gal). On the selective cleavage of an ester type glycoside linkage (see above), RGal-2 gave an acidic product (RGal-2-G). The R_f value of RGal-2-G in the TLC on silica gel was evidently lower than that of RGal-1a-G and similar to that of 13-*O*-tri-glucosyl-steviol.⁶⁾ The ¹³C-NMR spectrum of RGal-2 had two signals due to C-6 of 6-linked galactosyl and glucosyl units at δ 68.1 and 68.2 together with two signals attributable to C-6 of non-substituted galactosyl and glucosyl units at 62.1 and 62.6. Based on these results, RGal-2 can be formulated as 13-*O*- α -D-galactosyl-(1 $\rightarrow$ 6)- α -D-galactosyl-(1 $\rightarrow$ 6)- β -D-glucosyl-19-*O*- β -D-glucosyl-steviol.

Effects of raffinose concentration on the formation of RGal-1a and RGal-2

For the purpose of investigation of the effects of raffinose and rubusoside concentrations on RGal-1a and RGal-2 formation, α -galactosidase from *M. vinacea* (150 units/g

raffinose) was incubated with various concentrations of raffinose in the presence of 0.1 M and 0.2 M rubusoside at 52°C. After 1, 3.5 and 15 hr, the amounts of RGal-1a and RGal-2 produced in the reaction mixture were measured by HPLC. As shown in Table I, *M. vinacea* α -galactosidase produced mainly RGal-1a from the early stage of reaction. The amounts of RGal-1a and RGal-2 increased with increase of raffinose concentration in the case of 0.1 M rubusoside. The ratio of conversion (the amounts of RGal-1a and RGal-2 produced to that of raffinose used) decreased, however, with increase of raffinose concentration. In the case of 0.2 M rubusoside, the amounts of RGal-1a and RGal-2 produced from 0.4 M raffinose were larger than those from 1.0 M raffinose.

Preparation and isolation of transgalactosylated products by *A. reflexa* α -galactosidase

The reaction mixture containing 3 g of rubusoside, 33.5 g of raffinose, and 12.7 ml of *A. reflexa* α -galactosidase (1240 units) was incubated at 50°C for 4 hr. The reaction mixture was heated at 100°C for 10 min and centrifuged to remove insoluble materials. The supernatant solution was analyzed by HPLC (Fig. 3). Two peaks, RGal-1a and RGal-1b, were detected as reaction products. The supernatant solution was chromatographed on Diaion HP-20 by eluting with water and 75% methanol. The 75% methanol eluate was chromatographed on silica gel with chloroform-methanol-water (10:5:1, homogeneous), followed by separation with preparative HPLC. Two galactosylated products, RGal-1a (278 mg) and RGal-1b (261 mg) were obtained by the above procedures.

Structural elucidation of transgalactosylated products

Identification of RGal-1a was confirmed by comparison of ¹H- and ¹³C-NMR spectra with those of a specimen obtained by the transgalactosylation with α -galactosidase from *M. vinacea* (see above).

Another product, RGal-1b, was obtained as

Fig. 3. High Performance Liquid Chromatogram of *A. reflexa* α -Galactosidase Action Products on the Mixture of Raffinose and Rubusoside.

a white powder: $[\alpha]_D^{19}$, $+9.4^\circ$ ($c=0.57$, methanol); Anal. Found: C, 53.38; H, 7.74%. Calcd for $C_{38}H_{60}O_{18} \cdot 3H_2O$: C, 53.13; H, 7.75%. The 1H -NMR spectrum of RGal-1b had three anomeric proton signals at δ 6.07 (1H, d, $J=7.7$ Hz, 19-*O*- β -Glc), 5.42 (1H, d, $J=3.4$ Hz, α -Gal) and 5.15 (1H, d, $J=7.7$ Hz, β -Glc). The ^{13}C -NMR spectrum of RGal-1b showed three anomeric carbon signals at δ 95.7 (19-*O*- β -Glc), 99.8 (13-*O*- β -Glc), and 100.6 (α -Gal) and a signal assignable to C-6 of a 6-linked glucosyl unit as δ 67.9 together with two signals due to C-6 of non-substituted glucosyl and galactosyl units at δ 63.1 and 62.8. The selective cleavage of an ester type glycoside linkage (see above) of RGal-1b afforded an

Table II. EFFECTS OF RAFFINOSE CONCENTRATION ON THE PRODUCTION OF RGal-1a AND RGal-1b BY *A. reflexa* α -GALACTOSIDASE

Raffinose (M) Rubusoside (M)	Product	Reaction time (hr)		
		0.5	4.0	15
0.1	RGal-1a	<0.1%	0.8%	2.3% ^a
0.1	RGal-1b	0.6	2.5	3.0
0.8	RGal-1a	0.9	7.2	9.5
0.1	RGal-1b	4.1	10.7	9.4
1.6	RGal-1a	2.8	9.9	10.5
0.1	RGal-1b	4.1	11.7	11.4
0.2	RGal-1a	0.2	1.2	3.5
0.2	RGal-1b	1.1	4.3	6.6
1.6	RGal-1a	1.6	5.3	6.8
0.2	RGal-1b	2.9	7.2	8.0

^a [Amount of transfer product (M)/Amount of rubusoside used (M)] $\times$ 100.

acidic compound (RGal-1b-G) which had the same *Rf* value as that of steviol-monoside in the TLC on silica gel. It follows that RGal-1b can be assigned as 13-*O*- β -D-glucosyl-19-*O*- α -D-galactosyl-(1 $\rightarrow$ 6)- β -D-glucosyl-steviol.

Effects of raffinose concentration on the formation of RGal-1a and RGal-1b

To investigate the effects of raffinose and rubusoside concentrations on RGal-1a and RGal-1b formation, *A. reflexa* α -galactosidase (14.4 units/g raffinose) was incubated with various concentrations of raffinose in the presence of 0.1 M and 0.2 M rubusoside at 52°C. After 0.5, 4 and 15 hr, 10 μ l samples of the reaction mixture were removed to analyze by HPLC (Table II). *A. reflexa* α -galactosidase produced mainly RGal-1b in the early stage of reaction, then RGal-1a. The amounts of RGal-1a and RGal-1b increased with increase of raffinose concentration.

Comparison of transgalactosylated products by α -galactosidases from various origins

α -Galactosidases from *M. vinacea*, *A. reflexa*, *E. coli*, and coffee beans (5~20 units) were incubated with the mixture of 0.5 M raffinose and 0.1 M rubusoside (500 μ l) at 40°C.

Table III. TRANSGALACTOSYLATED PRODUCTS BY VARIOUS α -GALACTOSIDASES^a

	<i>M. vinacea</i>	<i>A. reflexa</i>	<i>E. coli</i>	Coffee beans
RGal-1a	12.0%	8.0%	4.6%	0.6% ^b
RGal-1b	—	8.6	1.1	<0.1
RGal-2	2.7	—	<0.1	—

^a Amounts of transfer products after 15 hr reaction.^b [Amount of transfer product (m)/Amount of rubusoside used (M)] $\times$ 100.

After 1, 2, 3, 5, 7, and 15 hr, 10 μ l samples of the reaction mixture were removed to analyze by HPLC. The amounts of each transgalactosylated product increased with the elapse of the reaction time. As shown in Table III, α -galactosidases from *E. coli* and coffee beans produced RGal-1a and RGal-1b like *A. reflexa* α -galactosidase, though the amount of transfer products was small.

The transgalactosylated products synthesized by α -galactosidases, RGal-1a, RGal-1b, and

RGal-2, have a free C4-hydroxyl group of the glucosyl moiety at 19-position of rubusoside. Consequently, we could not attain the initial purpose. But we found that *M. vinacea* α -galactosidase transferred a galactosyl residue selectively to the 13-O-glucosyl moiety of rubusoside. The sweetness and the quality of its taste is under investigation.

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