Solubilization of Steviolbioside and Steviolmonoside with γ-Cyclodextrin and Its Application to Selective Syntheses of Better Sweet Glycosides from Stevioside and Rubusoside

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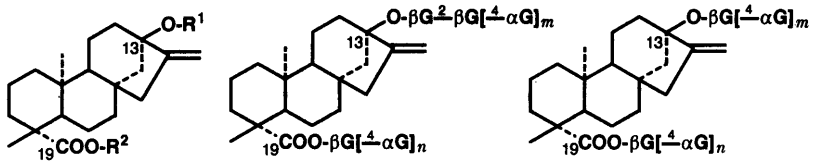
1,4-α-Glucosylation at the 13-*O*-glycosyl moiety of stevioside (S) and rubusoside (RU) results in a significant increase of sweetness. Saponification of the 19-COO-β-glucosyl linkage of S and RU yielded steviolbioside (SB) (=13-*O*-β-sophorosyl-steviol) and steviolmonoside (SM) (=13-*O*-β-glucosyl-steviol), respectively, both of which are poorly soluble in an acetate buffer. It was found that the solubilities of SM and SB in the buffer solution were remarkably increased in the presence of γ-cyclodextrin (γ-CD). SB was solubilized in the buffer solution with the aid of γ-CD, and the solution was subjected to 1,4-α-transglucosylation by using a cyclodextrin glucanotransferase–starch system to give a mixture of products which were glucosylated at the 13-*O*-glycosyl moiety. This mixture was acetylated, and the acetate was subjected to chemical β-glucosylation of 19-COOH followed by deacetylation to afford compounds which have superior sweetness to S. In the same way, derivatives with superior sweetness were selectively prepared from RU through SM.

Keywords sweetener; stevioside; rubusoside; steviolbioside; steviolmonoside; cyclodextrin; cyclodextrin glucanotransferase; solubilization

Stevioside (S),[1] a glycoside of steviol (ST) was isolated from *Stevia rebaudiana* BERTONI (Compositae) as the major sweet principle. Rebaudioside A (RA),[2] which is one of the congeners of S and has a subjectively better sweetness than S, was also isolated from this herb. Both S and RA are currently used as low calorie sweeteners in Japan. Rubusoside (RU), another sweet congener of S was isolated from leaves of a Chinese rosaceous shrub, *Rubus suavissimus* S. LEE in a yield of more than 5%.[3-5]

In order to improve the intensity and character of sweetness, enzymic transglycosylation of these glycosides has been extensively investigated.[6-12] Cyclodextrin glucanotransferase (CGTase) catalyzes regio- and stereo-selective α-D-glucosylation from starch or cyclodextrin (CD) to the 4-hydroxyl group of a glucopyranoside moiety. On treatment of S with this CGTase system, 1,4-α-mono-, di-, tri-, and greater glucosylation occurred at both the 13-*O*-glycosyl and 19-COO-β-glucosyl moieties, affording a complex mixture of glucosylated products (S-1a, -1b, -2a, -2b, -2c, -3a, -3b, -3c, -3d, *etc.*: structures, see Chart 1). This mixture derived from S, commercially named "glucosyl stevioside," is regarded as a better sweetener than S. The transglucosylation of RU under the same condition also yielded a mixture of similar 1,4-α-glucosylated products (RG-1a, -1b, -2a, -2b, -2c, -3a, -3b, -3c, -3d, -4a, -4b, -4c, -4d, -4e, *etc.*: structures, see Chart 1). Recently, isolation and identification of these products (Chart 1) from the complex mixtures of the glucosylated S and RU have been achieved (RG-4c and -4d were obtained as a mixture.[9,12] The sweetness evaluation of each product revealed that in the case of the 1,4-α-transglucosylation of S and RU with the CGTase system, glucosylation at the 13-*O*-glycosyl moiety (totally three or four glucosyl units at 13-OH) resulted in a great improvement of sweetness, while more glucosylation at this glycosyl moiety (totally more than five glucosyl units at 13-OH) or the glucosylation at the 19-COO-glycosyl moiety led to a decrease of the sweetness[7-9,12] (see Chart



	R^1	R^2
ST	H	H
S	βG²-βG	βG
RA	βG²-βG (3-βG)	βG
RU	βG	βG
SM	βG	H
SB	βG²-βG	H

	m	*n*
S-1a	1	0
S-1b	0	1
S-2a	2	0
S-2b	1	1
S-2c	0	2
S-3a	3	0
S-3b	2	1
S-3c	1	2
S-3d	0	3

	m	*n*
RG-1a	1	0
RG-1b	0	1
RG-2a	2	0
RG-2b	0	2
RG-2c	1	1
RG-3a	3	0
RG-3b	2	1
RG-3c	1	2
RG-3d	0	3
RG-4a	4	0
RG-4b	3	1
RG-4c	2	2
RG-4d	1	3
RG-4e	0	4

G; D-glucopyranosyl

Chart 1

TABLE I. Relative Sweetness of Glucosylated Steviosides with Respect to Sucrose

Compound	$m^{a)}$	$n^{b)}$	RS[c)]
S	2	1	160
S-1a	3	1	180
S-1b	2	2	133
S-2a	4	1	205
S-2b	3	2	136
S-2c	2	3	136
S-3a	5	1	117
S-3b	4	2	146
S-3c	3	3	150
S-3d	2	4	121

a) Number of glucose residues at 13-*O*-position. *b*) Number of glucose residues at 19-*O*-position. *c*) Relative sweetness. B/A; A, concentration (w/v%) of aqueous solution of sample (0.025%); B, concentration (w/v%) of aqueous solution of sucrose with the same sweetness as the sample solution.

TABLE II. Relative Sweetness of Glucosylated Rubusosides with Respect to Sucrose

Compound	$m^{a)}$	$n^{b)}$	RS[c)]
R	1	1	114
RG-1a	2	1	132
RG-1b	1	2	102
RG-2a	3	1	278
RG-2b	1	3	99
RG-2c	2	2	95
RG-3a	4	1	214
RG-3b	3	2	182
RG-3c	2	3	110
RG-3d	1	4	58
RG-4a	5	1	115
RG-4b	4	2	202
RG-4c	3	3	184
RG-4d	2	4	
RG-4e	1	5	49

a) Number of glucose residues at 13-*O*-position. *b*) Number of glucose residues at 19-*O*-position. *c*) Relative sweetness. B/A; A, concentration (w/v%) of aqueous solution of sample (0.025%); B, concentration (w/v%) of aqueous solution of sucrose with the same sweetness as the sample solution.

1 and Tables I and II). The products S-1a and -2a from S as well as RG-1a, -2a and -3a from RU, all of which are 1,4-α-glucosylated exclusively at the 13-*O*-glycosyl moiety, have intense sweetness, and mixtures which are mainly composed of these glycosides, have attracted much attention as more desirable sweeteners than RA, S, RU and "glucosyl stevioside." Recently, we reported the regioselective syntheses of RG-1a, -2a and -3a from RU by means of enzymic protection of the 19-COO-glucosyl group against the CGTase glucosylation.[11,12] The present study deals with another route for selective syntheses of the better sweeteners such as S-1a and -2a or RG-1a, -2a and -3a.

On saponification of the 19-COO-glucosyl linkage with alkali[6] or with an LiI-2,6-lutidine–methanol system,[13] S and RU yielded steviolbioside (SB) and steviolmonoside (SM), respectively. It seems to be possible that transglucosylation of SB or SM by using the CGTase system followed by chemical β-glucosylation of the 19-COOH group affords better sweeteners which are glucosylated exclusively at the 13-*O*-glycosyl moiety. However, as shown in Fig. 1, the yield of this *trans*-glucosylation is inadequate because of the low solubility of SB and SM in

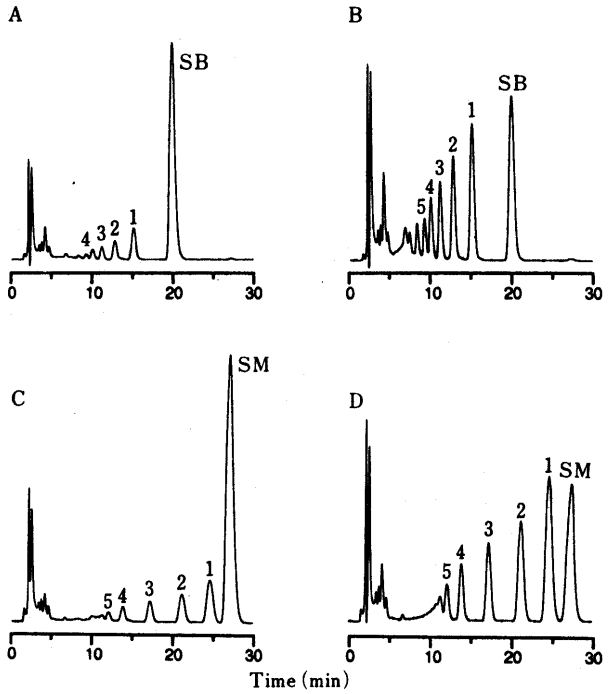


Fig. 1. High-Performance Liquid Chromatograms of Transglucosylated Products from SB and SM

A, SB without γ-CD; B, SB with γ-CD; C, SM without γ-CD; D, SM with γ-CD. Peak Nos. 1—5 in each chromatogram indicate the number of transferred glucosyl units. Conditions: column, YMC pack ODS-AM302 (4.6 mm × 150 mm); mobile phase, MeOH–0.05% TFA (60 : 40); flow rate, 0.8 ml/min; column temperature, 60 °C; detection, UV 210 nm.

TABLE III. Solubilizing Effect of CDs on SM or SB in 50 mM Acetate Buffer (pH 5.4)

	CD	Concentration of CD (mg/ml)	Solubility of SM or SB (mg/ml)
SM	None		0.69
	α-CD	15.1	1.26
	β-CD	17.7	2.48
	γ-CD	20.2	7.01
SB	None		0.21
	α-CD	20.3	0.28
	β-CD	23.6	0.90
	γ-CD	27.0	>10.0

The amount of SM or SB added was 10 mg/ml.

an acetate buffer. We have looked for a solubilizing agent which would increase the solubilities of SB and SM in 50 mM acetate buffer solution (pH 5.4) and which can be used safely in food-additive production. Investigation of the well-known inclusion agents, cyclodextrins (CDs) disclosed that γ-CD greatly increased the solubility of both SB and SM. As shown in Table III, the solubility of SB in the buffer solution is 0.21 mg/ml at 37 °C, while equimolar addition of γ-CD to a suspension of SB [10 mg ($=1.5\times10^{-5}$ mol)/ml] led to an increase of the solubility to more than 10 mg/ml. In a similar manner, the solubility of SM was remarkably increased with γ-CD, as shown in Table III. This effect is presumably due to the formation of an inclusion complex with γ-CD. Such a remarkable solubilizing effect was not observed with α- and β-CDs, which have a smaller hole size than γ-CD and can not form an inclusion complex with SB or SM.

Subsequently, the 1,4-α-transglucosylation of SB and SM by the CGTase system in the presence of γ-CD was investigated. High performance liquid chromatography (HPLC) of both the reaction mixtures (Fig. 1) demonstrated the evident promotion of the transglucosylation by the addition of γ-CD.

Each mixture, after removal of the enzyme and saccharides, was acetylated and subjected to β-glucosylation of 19-COOH with acetobromoglucose in the presence of silver carbonate-Celite[6] followed by deacetylation with mild alkali. The reaction mixtures from SB and SM were compared by HPLC with the direct 1,4-α-transglucosylation mixtures of S[9] and RU[12] by the same enzyme system. It was demonstrated that the mixture from SB in the present experiment mainly consisted of the superior sweeteners, S-1a and -2a, together with S and more glucosylated products such as S-3a. Similarly, the mixture from SM mainly consisted of good sweeteners, RG-1a, -2a and -3a, along with RU and a small amount of more glucosylated products such as RG-4a. The products, S-1a, -2a and -3a from SB and RG-1a, -2a, -3a and -4a from SM were respectively isolated and identified by comparison of the ^{1}H- and ^{13}C-nuclear magnetic resonance (^{1}H- and ^{13}C-NMR) spectra with those of corresponding authentic samples.

Experimental

Solubilization of SB and SM A suspension of SB [10 mg (= 1.5×10^{-5} mol)/ml] and (or without) equimolar CD [α-CD: 20.3 mg, β-CD: 23.6 mg or γ-CD: 27.0 mg] in 50 mM acetate buffer (pH 5.4, 1 ml) was heated at 100 °C for 10 min and then incubated at 37 °C for 1 h. The mixture was filtered through an HPLC disposable filter (Ekikurodisk 25, 0.25 μm for aqueous solution, Gelman Science Japan Ltd., Tokyo). The filtrate (500 μl) was mixed with 500 μl of a 0.5% aqueous solution of RU (internal standard), and 10 μl of the solution was subjected to HPLC analysis on a ODS column, YMC pack ODS AM302 (4.6 mm × 150 mm, YMC Co., Ltd., Kyoto); mobile phase, MeOH–0.05% aqueous trifluoroacetic acid (TFA) (60 : 40); flow rate, 0.8 ml/min; detection, UV 210 nm. Concentration of SB was determined from the peak area ratio to the internal standard.

The solubility of SM was determined for a suspension of SM [10 mg (= 2.1×10^{-5} mol)/ml] and (or without) equimolar CD (α-CD: 15.1 mg, β-CD: 17.7 mg or γ-CD: 20.2 mg) in the same way as above.

Transglucosylation of SB and SM without γ-CD A suspension of SB (10 mg) and soluble starch (30 mg) in 50 mM acetate buffer (pH 5.4, 1 ml) was heated at 100 °C for 10 min. After cooling of the mixture, CGTase solution (125 μl, from *Bacillus circulans*, 2000 unit/ml,[14] supplied by Dr. S. Kitahata, Osaka Municipal Technical Research Institute) was added and the mixture was incubated at 40 °C for 8 h. The reaction mixture was heated at 100 °C, and after cooling, was extracted with 1-BuOH saturated with H_2O. The BuOH layer was concentrated to dryness and a methanolic solution of the residue was subjected to HPLC analysis on a ODS column, YMC pack ODS-AM302 (4.6 mm × 150 mm); mobile phase, MeOH–0.05% aqueous TFA (60 : 40); flow rate, 0.8 ml/min; detection, UV 210 nm. The transglucosylation of SM and analysis of the products were also carried out in the same way as above.

Transglucosylation of SB and SM in the Presence of γ-CD and Subsequent Chemical Glucosylation of the 19-COOH A suspension of SB (1.0 g), soluble starch (2.7 g) and γ-CD (2.7 g) in 50 mM acetate buffer (pH 5.4, 20 ml) was heated at 100 °C for 10 min to give a clear solution. After cooling and subsequent addition of CGTase (1.25 ml, from *B. circulans*, 2000 unit/ml), the solution was incubated at 40 °C for 8 h. After heating at 100 °C for 30 min and dilution with H_2O, the solution was extracted with 1-BuOH saturated with H_2O. The BuOH layer was concentrated to dryness. [An aliquot of the residue was subjected to HPLC analysis on an ODS column, YMC-pack ODS AM302 (4.6 mm × 150 mm, YMC Co., Ltd., Kyoto); mobile phase, MeOH–0.05% aqueous TFA (60 : 40); flow rate, 0.8 ml/min; detection, UV 210 nm.] The above residue was acetylated by refluxing with anhydrous Ac_2O–C_5H_5N (1 : 1, 20 ml) for 3 h and the solution was concentrated to dryness. A mixture of the residue and Ag_2CO_3–Celite (6 g) in anhydrous dichloromethane was partly distilled to remove moisture. Then, acetobromoglucose (0.5 g) was added and the mixture was refluxed for 1.5 h. Further acetobromoglucose (0.5 g) was added and the mixture was further refluxed for 1.5 h. The mixture was filtered through a column of silica gel and the column was washed with $CHCl_3$. The filtrate and washing were concentrated to dryness. The residue was deacetylated by treatment with 0.2 M BaO in anhydrous MeOH at room temperature for 30 min under stirring. The reaction mixture, after neutralization with Amberlite MB-3, was concentrated to dryness. [An aliquot of the residue was analyzed by HPLC on an ODS column of YMC-pack ODS AM302 (4.6 mm × 150 mm); mobile phase, MeOH–0.05% aqueous TFA (60 : 40); flow rate, 0.8 ml/min; detection, UV 210 nm.] The remaining residue was separated by HPLC on a column of YMC-pack D-ODS-5 (20 mm × 250 mm); mobile phase, 65% MeOH; flow rate, 6.0 ml/min; detection, UV 210 nm, to give S (78 mg), S-1a (62 mg), S-2a (56 mg) and S-3a (42 mg).

A mixture of SM (950 mg), γ-CD (1.92 g) and soluble starch (3.0 g) in 50 mM acetate buffer (pH 5.4, 100 ml) was heated at 100 °C for 10 min and then cooled to room temperature, affording a clear solution. CGTase (1.19 ml, 2000 U/ml) was added to the solution and the mixture was incubated at 40 °C for 8 h. After work up in the same manner as for SB, an aliquot of the reaction mixture was analyzed by HPLC under the same conditions as for SB. The remaining reaction mixture was glucosylated as in the case of SB and an aliquot of the products was analyzed by HPLC under the same conditions as for SB. The remaining products were separated by preparative HPLC in the same manner as the products from SB to give RU (136 mg), RG-1a (90 mg), RG-2a (71 mg), RG-3a (43 mg) and RG-4a (24 mg).

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