Transfructosylation of Rebaudioside A (a Sweet Glycoside of Stevia Leaves) with Microbacterium β-Fructofuranosidase

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It was found that a β-fructofuranosidase produced by Microbacterium sp. H-1 has potent trans-β-fructofuranosylation activity from sucrose (donor). By means of this enzyme system, rebaudioside A (RA), the second major sweet steviol glycoside of the leaves of Stevia rebaudiana, was subjected to transfructosylation, affording a mono-β-fructofuranosylated product (RA-F) in a high yield. The structure of RA-F was elucidated as β-D-fructofuranosyl-(2→6)-β-D-glucopranosyl ester of steviol-13-O-[β-D-glucopyranosyl-(1→2)]-β-D-glucopyranosyl-(1→3)]-D-glucopyranoside. Some improvement in the quality of sweetness was observed for RA-F.

Keywords rebaudioside A; Stevia rebaudiana; sweet natural glycoside; trans-β-fructofuranosylation; β-fructofuranosidase; Microbacterium sp. H-1; β-fructofuranosyl-rebaudioside A; steviol glycoside

Introduction

Stevioside (S),1) the major sweet steviol glycoside from the leaves of Stevia rebaudiana BERTONI (Compositae) is commercially used as a low calorie sweetener in Japan. Several related sweet steviol glycosides called rebaudiosides-A (RA),2) C3) (= dulcoside B4), D and E5) and dulcoside A6) were also isolated from the leaves of this plant. Of these, RA which is the second most plentiful sweet glycoside of the leaves, has greater sweetness than S, and is now commercially utilized as a good sweetener in Japan.

To further improve the sweetness, enzymic glycosylation of S and its congeners has been extensively studied by our group. It has been reported that the 1,4-α-transglucosylation of S with soluble starch and cyclomaltoolactone glucanotransferase (CGTase) resulted in improvement of the taste6) and a mixture of the glucosylated products (so-called glucosystevioside) is now commercially used as a better sweetener than S. In this enzymic reaction, 1,4-α-glucosylation occurs to both the 13-O-α-sophorosyl and 19-O-α-glucosyl moieties of S, affording a complex mixture. We recently succeeded in the separation of all of the mono-, di- and tri-glucosylated products from this complex mixture and evaluated the sweetness of each product.7) Rubusoside (desglucostevioside, RU),8) the major sweet component from leaves of Rubus suavissimus S. Lee (Rosaceae) growing in Southern China, was also subjected to transglucosylation by the CGTase system, and separation and sweetness evaluation of the mono-, di-, tri- and tetra-glucosylated products were achieved. Based on these results, the structure-sweetness relationship of these steviol glycosides was investigated, disclosing that the glucosylation of the 13-O-glycosyl chain led to increased sweetness. It has been reported that a galactosyl group is not an efficient acceptor for the 1,4-α-transglucosylation by CGTase.9) Selective elongation of the 13-O-glycosyl moiety of S or RU for production of the superior sweeteners was established by means of the chemical10) or enzymic11,12) protection of 19-O-glycosyl moiety with a β-galactosyl group against CGTase glucosylation.

Fig. 1. High Performance Liquid Chromatogram of Trans-β-fructofuranosylation Products [on ODS-Column with CH3CN-H2O (31:69)]

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TABLE I. Influence of Concentration of RA and Reaction Time on Yield of RA-F

<table>
<thead>
<tr>
<th>Conc. of RA (m)</th>
<th>Yield (%) of RA-F</th>
<th>Reaction time (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0.5</td>
</tr>
<tr>
<td>0.025</td>
<td>79</td>
<td>82</td>
</tr>
<tr>
<td>0.05</td>
<td>55</td>
<td>67</td>
</tr>
<tr>
<td>0.1</td>
<td>45</td>
<td>58</td>
</tr>
<tr>
<td>0.5</td>
<td>11</td>
<td>16</td>
</tr>
</tbody>
</table>

Alkaline saponification of the esteric glycosyl linkage at 19-carboxyl group of RA-F gave rebaudioside B (RB)\(^2\) which had already been obtained from RA by alkaline saponification. This indicated that the fructosylation occurred not at the 13-O-glycosyl moiety but at the 19-O-glycosyl moiety. Methylation analysis of RA-F demonstrated the presence of 6-linked glucopyranosyl and 2,3-linked glucopyranosyl units together with terminal fructofuranosyl and glucopyranosyl units, proving that the fructosylation occurred at 6-hydroxyl group of the 19-O-glycosyl moiety. It follows that RA-F can be formulated as illustrated in Chart 1.

The yield of transfructosylation with glycosidases essentially depends upon the concentrations of acceptor, donor and enzyme as well as the reaction time. By means of HPLC analysis (Fig. 1), the influence of the reaction time and the concentration of the acceptor (RA) on the yield of RA-F was investigated. As summarized in Table I and Fig. 2, RA-F was more efficiently produced at relatively lower concentration of RA and the yield was decreased with increased RA concentration. It was also found that the maximum yield was obtained at the early stage of the reaction and prolonged reaction resulted in lowered yield of RA-F owing to enzymic hydrolysis of the fructosyl linkage of RA-F. The maximum yield, 82%, was obtained by the reaction at a concentration of 0.025 m RA for 1 h. The yield of RA-F at the concentration of 0.5 m RA for 21 h was only 19%.

The intensity and quality of sweetness of RA-F were compared with those of RA and a commercial glucosyl-stevioside (vide supra) as summarized in Table II (see Experimental). In this fructosylation, some decrease of bitterness and an increase in tastiness over RA were observed, although the aftertaste of RA-F was somewhat stronger than RA. The intensity of sweetness of RA-F was found to be similar to that of RA. As mentioned, remarkable improvement in the quality of sweetness was observed for S-F, which is thus promising as a good commercial sweetener. The present results are significant for the industrial scale production of S-F from crude S which contains some RA.

Results and Discussion
It was found that \(\beta\)-fructofuranosidase (FFase) produced by a bacterium isolated from soil exhibited potent trans-\(\beta\)-fructofuranosylation activity from sucrose (donor); this is described more fully in Experimental. A solution of RA, sucrose and the FFase in phosphate buffer (pH 6.5) was incubated at 40 °C for 2 h. The products were chromatographed on a Diaion HP-20 column with water and then methanol. The methanolic eluate was subjected to high performance liquid chromatography (HPLC) on a reverse phase (ODS) column to give a fructosylated product (RA-F).

Compound RA-F, C\(_{84}H\(_{152}O\(_{153}\) (from elemental analysis and fast atom bombardment mass spectrum (FAB-MS)), afforded fructose and glucose on acid hydrolysis. Comparison of the \(^{13}\)C-nuclear magnetic resonance (\(^{13}\)C-NMR) spectrum of RA-F with that of RA indicated that one \(\beta\)-fructofuranoside unit was introduced to RA.

Experimental
Materials and Method Rebaudioside A (RA) was purchased from Morita Kagaku Kogyo Co., Ltd., Osaka. NMR and FAB-MS spectra were recorded with a JEOL JNM GX-400 spectrometer and with a JEOL JMS SX-102 spectrometer, respectively.

Preparation of Crude FFase A bacterium, strain H-1, which was one of those isolated from soil as a fructose-transferring enzyme producer, was used in the present study. This bacterium is classified as a member of the genus Microbacterium by the National Collection of Industrial and Marine Bacteria, Ltd. in Scotland and tentatively called Microbacterium sp. H-1 by the present authors. The bacterium was cultivated in a 500 ml shaking flask containing the following medium at 28 °C for 48 h on an reciprocating shaker (110 rpm): 1% sucrose, 0.3% NaNO\(_3\), 0.1% KH\(_2\)PO\(_4\), 0.05% MgSO\(_4\)•7H\(_2\)O, 0.02% MnCl\(_2\), and 0.05% yeast extract (pH 7.0). The supernatant obtained by centrifuging the culture broth was dialyzed with 60% (NH\(_4\))\(_2\)SO\(_4\). After standing overnight at 10 °C, the resulting precipitate was collected by centrifugation and dissolved in 20 mm phosphate buffer (pH 7.0), and then the solution was dialyzed against the same buffer overnight. The non-dialyzed solution was used as FFase solution.

Assay of FFase Activity A mixture of 5% sucrose solution in 50 mm phosphate buffer (pH 6.5, 200 \(\mu\)l) and the enzyme solution (50 \(\mu\)l) was incubated at 40 °C for 10 min. The released glucose was measured by an F-kit reagent (Boehringer Mannheim GBM Biochemica). One unit of FFase activity was defined as the amount of enzyme releasing 1 \(\mu\)mol of
glucose per minute under this condition.

**Chromatography**  Conditions of thin layer chromatography (TLC) on Kiesel gel 60 plate (Merck Co., Ltd.); solvent system, CHCl₃-MeOH-H₂O (10:5:1 or 6.4:1); detection, spraying methanolic H₂SO₄ followed by heating at 110°C. HPLC conditions for analysis of glycosides: on a column of TSK gel ODS 120T (4.6 mm i.d. x 250 mm), Tosoh Co. Ltd.; detection, ultraviolet (UV) 213 nm; mobile phase, CH₃CN-H₂O (25:30 to 75:25); flow rate 0.5 ml/min (see Fig. 1). HPLC condition for analysis of saccharides: on a column of Shimpack CLC-NH₂ (4.6 mm i.d. x 250 mm), Shimadzu Co., Ltd.; detection, refractive index; mobile phase, CH₃CN-H₂O (75:25); flow rate 0.8 ml/min.

**Rotation and Properties of RA-F**  A solution of RA (0.05 m), sucrose (2.0 m), FFase (50 units) in 50 mM phosphate buffer (pH 6.5, final volume 31 ml) was incubated at 40°C. The reaction process was followed by HPLC analysis of aliquots of the mixture. After 2 h, the mixture was heated at 100°C for 15 min and chromatographed on Diaion HP-20 with H₂O and then MeOH. The MeOH eluate was separated by HPLC on TSKgel ODS 120T (2.5 mm i.d. x 30 cm) with 2% CH₃CN (flow rate: 6 ml/min) to give RA-F (800 mg) together with recovered RA.

RA-F: a white powder, [α]D²¹ + 68.5° (c = 0.97, MeOH). Anomeric carbon signals in C₂D₂N: 13-O-glycosyl moiety δ: 79.9 (13-O-Glc), 164.5, 164.6; 19-O-glycosyl moiety δ: 95.8 (β-Glc), 105.7 (β-Fru). Negative FAB-MS: 1127. [M-H]⁻. Anal. Calcd. for C₃ₓHₓₓOₓₓH₂O C: C52.35; H: 7.20. Found: C: 52.30; H: 7.09. A solution of RA-F in 8% aqueous KOH was heated at 80°C for 1 h. The reaction mixture was acidified with 2N HCl and extracted with 1-BuOH (saturated with H₂O). The BuOH layer was washed with H₂O and concentrated to dryness, affording RA which was identified by comparison of TLC and the 1H-NMR spectrum with those of an authentic sample. Methylation analysis of RA-F was conducted by the procedure reported previously.

**Effect of Acceptor Concentration and Reaction Time on Formation of RA-F**  A solution of the FFase (0.6 units) and 2.0 m sucrose in 50 mM phosphate buffer (pH 6.5, 400 µl) was incubated in the presence of various concentrations of RA at 40°C. After 0.5, 1.0, 3.0 and 21 h, the amounts of RA-F in the reaction mixture were determined by HPLC. The results are summarized in Table I and Fig. 2.

**Sensory Evaluation**  Sweetness of RA-F was evaluated and compared with RA and commercial glycosyl-stevioside (vide supra) by human sensory panels. All samples were dissolved in water to make appropriate concentrations which corresponded to 3–5% (w/v) sucrose solutions in intensity of sweetness, and standard sucrose solutions were prepared at graduated concentrations from 3 to 6% (w/v) with intervals of 0.5%. The panels were asked to taste a sucrose solution and estimate its total taste intensity relative to that of the sample solutions. Panelists tested each sample several times. The relative sweetness to sucrose was calculated according to the following formula:

\[
\frac{\sum A \cdot B}{\text{number of tests} \times \text{number of panelists}}
\]

A, concentration (w/v, %) of sucrose solution with the same intensity of sweetness as the sample solution; B, concentration (w/v, %) of the sample solution.

The qualities of taste, aftertaste, bitterness and deliciousness were evaluated for each sample solution at a concentration corresponding to 5% (w/v) sucrose solution in intensity of sweetness and were calculated according to the following formula:

\[
\frac{\sum T}{\text{number of tests} \times \text{number of panelists}}
\]

**Table II. Sensory Evaluation**

<table>
<thead>
<tr>
<th>Compd.</th>
<th>Bitterness</th>
<th>Aftertaste</th>
<th>Deliciousness</th>
<th>Sweetness²</th>
</tr>
</thead>
<tbody>
<tr>
<td>RA</td>
<td>4.0</td>
<td>4.0</td>
<td>2.0</td>
<td>198</td>
</tr>
<tr>
<td>RA-F</td>
<td>4.3</td>
<td>3.7</td>
<td>2.1</td>
<td>200</td>
</tr>
<tr>
<td>Glucosyl-stevioside</td>
<td>3.8</td>
<td>3.6</td>
<td>1.6</td>
<td>53</td>
</tr>
</tbody>
</table>

²a Relative sweetness to sucrose. b Commercial sample.

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**References and Notes**


