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Transglycosylation of Mogroside V, a Triterpene Glycoside in *Siraitia grosvenori*, by Cyclodextrin Glucanotransferase and Improvement of the Qualities of Sweetness*

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Abstract: Mogroside V (MV), a main sweet triterpene glycoside in the extract of the fruit of Luo-han-guo (*Siraitia grosvenori* Swingle), was transglycosylated by cyclodextrin glucanotransferases (CGTases) using starch as a donor substrate. CGTases from *Bacillus macerans*, *B. circulans*, *B. stearothermophilus* and *Thermoanaerobacter* sp. were effective for the transglycosylation of MV. It was appropriate for the production of glycosylated MV to react 9% (w/v) MV with 20% (w/v) tapioca starch and *B. macerans* enzyme (30 U/g-starch) at 50°C and pH 6.5. Under these conditions, the reaction finished within 24 h and more than 90% of a glycosylation yield was attained. Three products that have 1–3 additional glucose residues were identified as transglycosylation products by mass spectrometry. The greater number of glucose residues introduced, the less intensity of sweetness compared to MV was estimated by sensory evaluation. However the qualities of sweetness such as bitterness, after-taste, and peculiarity were improved by transglycosylation.

Key words: mogroside V, transglycosylation, cyclodextrin glucanotransferase, sweetness, quality of sweetness

Luo-han-guo (scientific name, *Siraitia grosvenori* Swingle) is a perennial herb of the gourd family cultured in the cold highlands of Kuangtung and Kuanhsi Provinces in China. The extract of the fruits of Luo-han-guo has been popularly used from ancient time not only as a sweetening ingredient but also as a folk medicine for remediation of fever, for suppression of cough, for removal of phlegm, for promotion of gastro enteric functions, for getting rid of stress, *etc.* In recent years, it has also been reported that the extract of Luo-han-guo possessed free-radical eliminating activity in the peroxide generation process (anti-peroxidation activity) and anti-tumor activity.[1–3]

The extract of the fruit of Luo-han-guo contains several sweet triterpene glycosides of the cucurbitan type (Fig. 1) referred as mogroside V (MV), mogroside IV, siamenoside I, and 11-oxo-mogroside V.[4–6] MV, which exhibits approximately 300-times relative sweetness to sucrose (Suc), amounts to about 75% of these glycosides. In spite of such high sweetness and medical usefulness of the extract, however, the qualities of taste are not necessarily satisfactory, and therefore, their improvement is demanded from the viewpoint of application to foods and beverages.

Improvement of unfavorable tastes of some natural sweet glycosides has been attained by enzymatic glycosylation of sugar moiety in the glycosides[7–11]: For instance, stevioside, which is about 150 times sweeter than Suc, has been transglycosylated by cyclodextrin glucanotransferase (CGTase, EC 2.4.1.19)[7–12] and β-fructofuranosidase[13] to improve the bitter taste and unfavorable after-taste together with the insufficient water-solubility. CGTases are generally recognized as one of the most useful enzymes for glycosylation in view of their high transglycosylation efficiency and broad acceptor specificity as well as industrial availability of the enzymes.[10,11] This is the first report on the transglycosylation of MV, the main sweet substance of Luo-han-guo fruit, by CGTases to improve qualities of sweetness.

MATERIALS AND METHODS

Materials. A crude extract of the fruit of Luo-han-guo was purchased from Guilin Shite New Tech Co. (Guilin, China). The extract contained about 30% (w/w) MV. Glycosides relating to MV were contained in the following ratio in the extract: MV, 84.2% (w/w); mogroside IV, 4.4% (w/w); siamenoside I, 3.8% (w/w); 11-oxo-mogroside V, 5.4% (w/w) and mogroside III, 2.2% (w/w). Tapioca starch was purchased from Matsutani Chemical Industry Co. (Hyogo). The other chemicals used were of the highest grade commercially available.

Enzymes. CGTases from *Thermoanaerobacter* sp. and *Bacillus macerans* were commercial products of Novo Nordisk A/S (Bagsvaerd, Denmark; CAN-0002) and Amano Enzyme Inc. (Nagoya, "Contizyme"), respectively. The enzymes from *B. stearothermophilus* (*Geobacillus stearothermophilus*) and *B. circulans* were supplied by Hayashibara Biochemical Laboratories Inc. (Okayama).

* This paper is dedicated to our mentors, the late Professors Dr. Michinori Nakamura, Dr. Susumu Hizukuri and Dr. Toshiaki Komaki, in memory of their numerous pioneering works and leadership in the field of starch and its related science.

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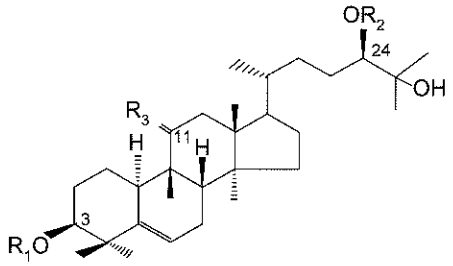

	R_1	R_2	R_3
mogroside V	–Glc^{6}–Glc	–Glc^{2}(–Glc^{6}–Glc)–Glc	OH / H
mogroside IV	–Glc^{6}–Glc	–Glc^{2}–Glc	OH / H
11-oxo-mogroside V	–Glc^{6}–Glc	–Glc^{2}(–Glc^{6}–Glc)–Glc	= O
siamenoside I	–Glc	–Glc^{2}(–Glc^{6}–Glc)–Glc	OH / H

Glc;β-D-glucopyranosyl

Fig. 1. Structures of MV and related glycosides found in the Luo-han-guo fruit.

Anomeric type of all glucosidic linkages is β-type.

These CGTases were used without purification. The enzyme activity was measured by dextrogenic activity and one unit was defined as the amount of enzyme that decreased 1% of absorbance of the iodine-starch complex at 660 nm.[11] α-Glucosidase from *Aspergillus niger* was purified according to the method of Kita *et al.*[14] using a commercial product, "Transglucosidase Amano L", of Amano Enzyme Inc., as a starting material. Glucoamylase from *Rhizopus niveus* was purchased from Seikagaku Kogyo (Tokyo).

HPLC. High-performance liquid chromatography (HPLC) for the measurement of MV and its transglycosylation products was performed using a Shimadzu LC-AD (Shimadzu Co., Kyoto) and a Shimadzu RID-6A detector under following conditions: Column, YMC-pack ODS-AQ (4.6×250 mm, YMC Co., Kyoto); solvent, 25% (v/v) acetonitrile; flow rate, 0.8 mL/min; temperature, 40°C.

Transglycosylation ratio. MV was separated from other minor glycosides and from other transglycosylation products by HPLC under the conditions as described above. Then, transglycosylation ratios (%) were calculated from the decrease of peak area of MV that corresponded to the molar reduction of the acceptor.

Transglycosylation of MV by CGTases from four origins. The reaction mixtures (1.0 mL) containing 6% (w/v) MV, 10% (w/v) tapioca starch, 25 mM acetate buffer (pH 6.5), and CGTases from different origins (2 U/mL, 20 U/g-starch) were incubated at 50°C for 24 h and 48 h. After stopping the reaction by boiling for 15 min, the samples were diluted 10–20 times with water, and the remaining MV was measured with HPLC.

Effect of acceptor and donor concentrations on the transglycosylation of MV. Reaction mixtures (1.0 mL) containing 1.5–12% (w/v) MV (5–40%, w/v, as crude extract), 2.5–25% (w/v) tapioca starch in ratios indicated in Fig. 3, and the *B. macerans* enzyme (7.8 U/g-starch) in 50 mM acetate buffer (pH 6.5) were incubated at 40°C. After incubation for 24 and 48 h, the samples were boiled for 5 min, appropriately diluted (1–20 times) with water, and subjected to HPLC analysis to determine the remaining MV.

Time course of transglycosylation of MV. A reaction mixture (10 mL) containing 9% (w/v) MV, 20% (w/v) starch, 50 mM acetate buffer (pH 6.5) and *B. macerans* CGTase (30 U/g-starch) was incubated at 50°C. At intervals, samples (1.0 mL) were withdrawn and boiled for 5 min to stop the reaction. Samples diluted 10 times with water were subjected to HPLC analysis to measure the remaining MV.

LC-MS. HPLC was performed under the following conditions: Column, Asahipak NH2P50-4D (4.6×150 mm, Showa Denko Co., Tokyo); solvent, 75% (v/v) acetonitrile; flow rate, 1.0 mL/min; temperature, 40°C. Mass spectrometry (MS) was carried out under the following conditions: spray Voltage, 10 kV; spray current, 10 μA; sheeth gas (N_2) flow rate, 20 mL/min; aux gas (N_2) flow rate, 20 mL/min; capillary voltage, 10.24 V; capillary temperature, 350°C; ion gauge, 0.72×10^{5} torr.

Partial purification of transfer products for LC-MS analysis. A reaction mixture (30 mL) containing 9% (w/v) MV (about 30% of the extract), 30% (w/v) tapioca starch, 50 mM acetate buffer (pH 6.5), and *B. macerans* CGTase (360 U) was incubated at 50°C for 48 h. After transglycosylation, ethanol (60 mL) was added and the mixture was centrifuged at 10,000 rpm for 20 min to remove precipitates. The supernatant was evaporated, dissolved in water (about 50 mL) and put onto a column (30×450 mm) of ODS-AQ (Organo Co., Tokyo). The column was washed well with water and eluted with a linear gradient of ethanol (0 to 60%, v/v, 1 L each). Fractions (15 mL) were collected at a flow rate of 30 mL/h. The components in each fraction were analyzed by thin-layer chromatography (TLC) using ethyl acetate–acetic acid–water (3:1:1) as a developing solvent. Fractions containing transfer products (tube number, 70–132) were collected, concentrated by evaporation, and subjected to LC-MS analysis and to further fractionation.

Fractionation of transfer products used for sensory evaluation. The partially purified preparation, which was obtained by the first ODS chromatography performed as above, was further fractionated by re-chromatography on ODS resin. Re-chromatography was performed under the same conditions except that a linear gradient was made from 30 to 60% (v/v) ethanol (1 L each). The preparation was separated into four fractions, A (tube number, 20–23), B (24–27), C (28–34) and D (35–43). Fraction D contained MV. Fractions A-C were found to contain glycosylation products with additional 3-1 Glc residues (abbreviated as 3Glc-MV, 2Glc-MV and 1Glc-MV, see Fig. 5), respectively. Each (fractions A–C) was collected, evaporated, and subjected to preparative HPLC performed under the following conditions: column, SP-120-5-ODS-BP (20×250 mm, DAISO Co., Osaka); solvent, 25% (v/v) acetonitrile; flow rate, 5.0 mL/min; temperature, room temperature.

Sensory evaluation.

(1) Relative sweetness to Suc. Fifteen human panelists performed the sensory evaluation of the fractionated products having different numbers of Glc residues. All samples were dissolved in water to make appropriate con-

centrations that corresponded to 2–10% (w/v) Suc solutions with respect to the intensity of sweetness. The panelists were asked to taste the Suc solutions and estimate their maximum intensity relative to that of the sample solutions. The estimation was conducted several times for each sample. The relative sweetness to Suc was calculated according to the following formula; $\{(\Sigma A/B)/(\text{the number of tests})\} \times (\text{the number of panelists})$, where A is the concentration (%) of the Suc solution with the same intensity of sweetness as the sample solution, and B is the concentration (%) of the sample solution.

(2) Quality of sweetness. Qualities of sweetness, bitterness, after-taste and peculiarity, were evaluated for each sample solution corresponding to 8% (w/v) Suc solution in the intensity of sweetness and were calculated according to the following formula; $\{(\Sigma T)/(\text{the number of tests})\} \times (\text{the number of panelists})$, where T is the following evaluation points; 4, much worse than Suc; 3, somewhat worse than Suc; 2, slightly worse than Suc; 1, same as Suc.

RESULTS AND DISCUSSION

Transglycosylation of MV with CGTases.

CGTase[10,11] is a transglycosylating enzyme that synthesizes α-1,4-linked cyclic maltooligosaccharides, which are generally called cyclodextrins (CDs), by intramolecular transglycosylation. The enzyme also catalyzes another industrially important reaction, intermolecular transglycosylation, where maltooligosyl residues of starch and CDs are transferred to appropriate acceptor molecules. D-Glucose (Glc) and Suc, for instance, are the most preferable group of acceptors.[10–12] Several natural sweet glycosides are also known to act as good acceptors for the intermolecular transglycosylation of CGTases: The glycosylation of stevioside, rebaudioside A, and rubusoside have been undertaken[7–9] to improve the qualities of taste or the water solubility.

It is known that acceptor specificity in the intermolecular transglycosylation depends on the source of CGTase.[10–12] For example, CGTase from *B. stearothermophilus* showed wider acceptor specificity than *B. macerans CGTase* and the former enzyme produced glycosylation product from lactose, which was an inefficient acceptor for the latter enzyme.[12] Therefore, efficiencies in transglycosylation of MV were compared among CGTases from four different origins indicated in Fig. 2, which were commercially available enzymes. Six percent (w/v) of MV (about 20% (w/v) as a crude extract of the Luo-han-guo fruit) and 10% (w/v) tapioca starch were used as an acceptor and a donor, respectively. After incubation at pH 6.5 and 50°C for 24 and 48 h, MV was measured by HPLC.

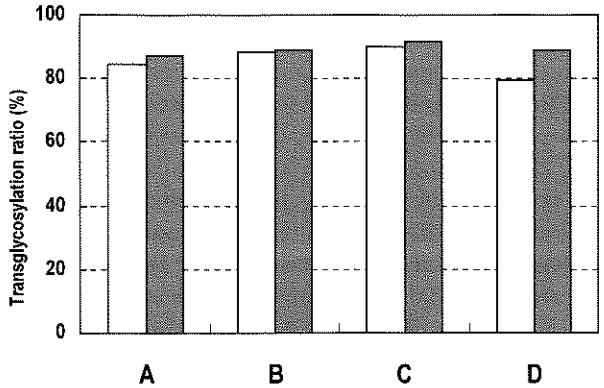

Fig. 2. Transglycosylation of MV by CGTases from four origins.

The reaction and analytical conditions are described in MATERIALS AND METHODS. A, *B. macerans*; B, *B. circulans*; C, *B. stearothermophilus*; D, *Thermoanaerobacter* sp. Reaction time: 24 h (open bars) and 48 h (shaded bars).

The CGTases caused no decrease of MV in the absence of starch. Other glycosides in the extract (*e.g.* mogroside IV) were not considered to effect the transglycosylation of MV so significantly because of their low contents compared to MV (see "MATERIALS AND METHODS"). In this study, therefore, the transglycosylation ratio (%) was tentatively calculated from the molar reduction of MV. As shown in Fig. 2, MV was found to be a considerably preferred acceptor for all CGTases tested: More than 80% of MV was transglycosylated within 24 h and no significant differences were observed in the ratios among the enzymes after the prolonged reactions (48 h). Such extremely high efficiencies may be attributable to the several Glc residues to be glycosylated in the MV molecule (Fig. 1). No considerable differences were also observed in the profiles for the transglycosylation products obtained by the CGTases under the reaction and analytical conditions. In the following experiments, therefore, the enzyme from *B. macerans* was adopted because of its moderate heat stability that enables the reaction to be stopped easily and precisely.

Reaction conditions for effective transglycosylaion.

Reaction conditions for effective transglycosylation with *B. macerans* CGTase were determined. Figure 3 shows the effects of the acceptor/donor concentration ratios on the transglycosylation of MV at 40°C and pH 6.5. The transglycosylation tended to be suppressed at higher MV concentrations, which was more evident at the shorter incubation time (24 h): For example, in the reactions of 9 and 12% (w/v) of MV (30 and 40%, w/v, as the crude extract) with 20% starch, the ratios were 83 and 64% at 24 h, respectively. Such decreases may be due to the inhi-

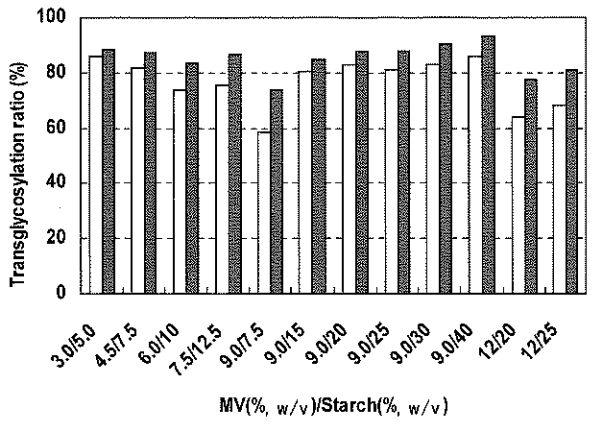

Fig. 3. Effect of acceptor concentrations on the transglycosylation of MV.

Ratios of MV (%, w/v) to starch (%, w/v) used for the reactions are indicated in the figure. The reaction and analytical conditions are described in MATERIALS AND METHODS. Reaction time: 24 h (open bars) and 48 h (shaded bars).

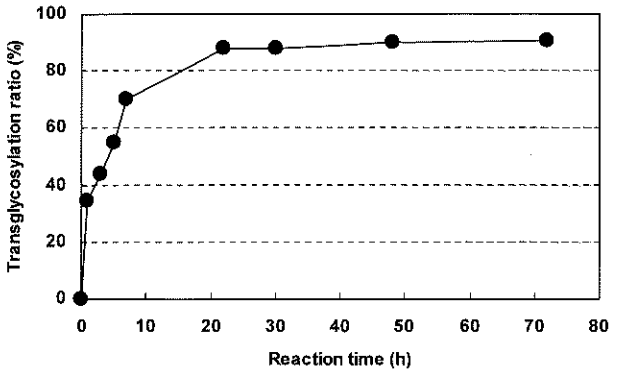

Fig. 4. Time course of transglycosylation of MV.

The reaction and analytical conditions are described in MATERIALS AND METHODS.

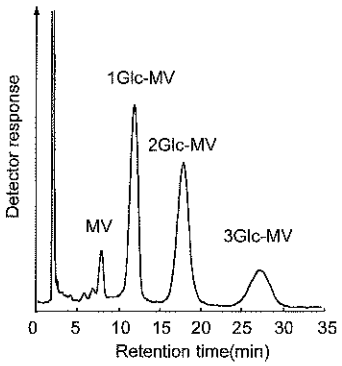

Fig. 5. HPLC analysis of transglycosylation products of MV.

Partially purified products were prepared as described in MATERIALS AND METHODS. HPLC conditions were same as those for LC-MS.

bition of the CGTase by MV or by other substances in the crude extract. The suppression was quite diminished by the extension of incubation or the increase of starch concentration: For example, when 9% (w/v) MV was reacted with increasing concentrations (7.5–40%, w/v) of starch, the ratios increased gradually from 59 to 86% at 24 h, and from 74 to 93% at 48 h, respectively.

The incubation temperature and pH were found to be appropriate at 50–55°C and pH 6–7.3, respectively (data not shown): When 9% (w/v) MV and 8% (w/v) tapioca starch were incubated with the enzyme (49 U/g-starch) at pH 6.5 for 48 h at 40 and 50°C, the transglycosylation ratios were about 55 and 75%, respectively. At 60°C, the ratio decreased to 48% due to inactivation of the enzyme. At pH 6–7.3, the ratios appeared to be almost constant (75%) in the reaction at 50°C for 48 h.

As practical reaction conditions, eventually, we concluded the reaction of 9% (w/v) of MV (about 30%, w/v, as the extract of the Luo-han-guo fruit), 20% (w/v) tapioca starch, and the *B. macerans* CGTase (30 U/g-starch) at 50°C and pH 6.5 were appropriate for the glycosylation of MV. Under these conditions, the reaction was completed within 24 h and a maximum yield of approximately 90% was attained as shown in Fig. 4. Such a high transglycosylation ratio seems to be advantageous from the industrial viewpoint. Although the yield was maintained at high level afterwards, a gradual decrease of the products with larger numbers of glycosyl residues together with an increase of those with smaller numbers of glycosyl residues was observed in HPLC or TLC, which was attributable to the disproportionation activity and weak hydrolytic activity of the CGTase.[10–12]

Fractionation and structure elucidation of the transfer products.

Figure 5 shows a typical HPLC chromatogram of transglycosylation products which were partially purified by ODS chromatography. In addition to the small peak of MV, there were detected at least three peaks of products, 1Glc-MV, 2Glc-MV and 3Glc-MV. Each product was separated by re-chromatography on ODS resin and preparative HPLC. In LC-MS analysis, 1Glc-MV, 2Glc-MV and 3Glc-MV gave molecular peaks of m/z 1451, 1612 and 1774, respectively, suggesting they were glycosylated MV (s) having 1–3 additional Glc residues in addition to 5 intrinsic Glc residues, respectively. These products were digestible either by *A. niger* α-glucosidase or by *R. niveus* glucoamylase to produce MV and Glc (data not shown), obviously indicating the additional Glc residues had been bound with α-glucosidic linkages.

CGTases are known to transglycosylate specifically the C4-OH group of non-reducing end Glc residues of various glycosides.[10,11] In the glycosylation of stevioside, for instance, α-1,4-glucosylation occurred at the C4-OH groups both of 13-*O*-β-sophorosyl and 19-*O*-β-glucosyl residues.[8] MV has three Glc residues with free C4-OH groups[4,5]; two Glc residues linking at C24 through β-1,6- and β-1,2-linkages, and β-1,6-linked Glc residue at C3 (Fig. 1). When all these possible residues were assumed to be glycosylated, then the preparation of 1Glc-MV should contain 3 species with different glycosylation sites. Because of the difficulty in the separation of each product species, however, we have not determined so far whether the glycosylation occurred equally to these three sites or preferentially to one or two certain sites. Furthermore, CGTases generally produce a mixture of glycosides having Glc and a series of α-1,4-linked maltooligosyl residues due to disproportionation action. This action makes the structures of 2Glc-MV and 3Glc-MV more complex, because 6 and 10 species were conceivable, respectively, depending on the allocation of the glycosylated residues and on the number of Glc residues added. Detailed structures, especially the glycosylation sites, remain a topic to be studied.

Sweetness of the transfer products.

Most natural glycosides with high sweetness such as stevioside, rubusoside and glycyrrhezin are known to have bitterness, after-taste, and peculiarity compared to the familiar taste of Suc. Such unfavorable qualities of sweetness could be improved by the transglycosylation with CGTases.[7–10] In this study, quality of sweetness together with relative intensity of sweetness of 1Glc-MV, 2Glc-MV, and 3Glc-MV were compared with MV through the sensory evaluation performed by 15 panelists using Suc as a standard sweetener. As summarized in Table 1, the glycosylated MV(s) were judged to have improved qualities of sweetness, especially, with respect to the after-taste. The improvements in bitterness and peculiarity were smaller and the evaluation scores did not necessarily increase with the number of Glc residues introduced.

Table 1. Sensory evaluation of the transglycosylation products with different numbers of Glc residues.

Product	Sweetness	Quality of sweetness		
		Bitterness	After-taste	Peculiarity
Suc	1	1.00	1.00	1.00
MV	378	1.50	2.50	1.81
1Glc-MV	191	1.36	1.91	1.36
2Glc-MV	97	1.23	1.69	1.46
3Glc-MV	53	1.30	1.40	1.40

Sensory evaluation was performed by 15 panelists as described in MATERIALS AND METHODS. The qualities of sweetness for Suc were expressed as an evaluation point of 1.0. Note that the points close to 1.0 implied the samples have similar (and thus more preferred) qualities of sweetness to those of Suc.

On the contrary, the intensity of sweetness considerably diminished with the increasing number of Glc residues: The addition of one Glc residue resulted in the reduction in sweetness almost to half. A relatively large decrease in sweetness had been also observed in the glycosylation of glycyrrhezin,[15] a triterpenoide glycoside with 170 times the sweetness of Suc. The transglycosylation products with shorter Glc chains, especially 1Glc-MV, still retained sufficient sweetness, which was almost comparable to that of stevioside (150-fold). Furthermore, the studies on the transglycosylation of rubusoside[7,9] and stevioside[8] with CGTases indicated that the intensity and the quality of sweetness depended not only on the number of Glc residues introduced but also on the glycosylation positions. Therefore, one possible strategy for accumulation of the glycosylated MV with shorter Glc chains which have both sufficient intensity and satisfactory qualities of sweetness may be partial hydrolysis of the transglycosylation products with, for example, appropriate amylases or glycosidases. The partial hydrolysis of transglycosylation products will be reported elsewhere.

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Siraitia grosvenori 中のトリテルペンであるモグロシドVのシクロデキストリン合成酵素の転移反応による配糖化と甘味質の改善

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羅漢果 (*Siraitia grosvenori* Swingle) エキス中に存在する甘味トリテルペノイド配糖体の主成分であるモグロシドV (MV) を，デンプンを供与体基質としてシクロデキストリングルカノトランスフェラーゼ (CGTase) の転移反応で配糖化した．*Bacillus macerans*, *B. circulans*, *B. stearothermophilus* および *Thermoanaerobacter* 属由来のCGTaseはMVを転移反応で効率的にグリコシル化した．グリコシル化には9% (w/v) MV，20%のタピオカデンプンに*B. macerans*のCGTase (30単位/gデンプン) を50°C，pH 6.5で24時間作用させた場合が適当であり，この条件下では24時間以内に最大約90%という配糖化率が達成できた．マススペクトル分析で1~3残基のグルコースが付加した3種類の転移生成物が同定された．官能検査で評価した結果，導入したグルコース残基数の増加とともに，甘味強度はMVと比べて低下したが，苦み，後引き，くせなどの甘味質は十分改善できることがわかった．