Constituents and Their Sweetness of Food Additive Enzymatically Modified Licorice Extract

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Enzymatically modified licorice extract (EMLE) is a natural sweetener, which is prepared with cyclodextrin glucanotransferase. It is used because of its unique properties such as higher solubility and better taste than those of licorice extract. In the present paper, the structures of six major constituents isolated from EMLE were determined, and their sweetness was studied. The isolated compounds were glycyrrhizin (**1**), 3-*O*-[*β*-D-glucuronopyranosyl-(1→2)-*β*-D-glucuronopyranosyl]-liquiritic acid (**2**), and their derivatives glucosylated at the C-4 position of the terminal glucuronopyranose with additional one (**3** and **4**, respectively) and two (**5** and **6**, respectively) glucose moieties. Compounds **1** and **2** are the major and minor sweet constituents in licorice extract, respectively. Compounds **3**−**6** are new compounds isolated for the first time. Compound **2** was sweeter than compound **1**. Interestingly, compound **3**, which is a monoglucosylated derivative of compound **1**, was sweeter than compound **4**. The sweetness of both compounds was lower than that of the parent compounds, while the lingering sweet aftertaste was markedly improved. Compounds **5** and **6**, which have two additional glucose moieties, showed only slight sweetness.

Keywords: *Glycyrrhiza; food additive; sweetener; enzymatically modified licorice; saponin*

INTRODUCTION

Licorice is the name applied to the roots and stolons of some *Glycyrrhiza* species and has been used as a crude drug worldwide from ancient times (Gibson, 1978). It has also been used as a sweetener and a flavor enhancer for foods in Japan and other countries (for example, it is registered as CFR 184.1408 in the USA). Therefore, its chemical constituents have been extensively investigated (Hiraga and Kajiyama, 1997).

In recent years, the enzymatic transglucosylation reaction has been used to improve the taste or to increase the solubility and stability of natural compounds (Kometani et al., 1996; Suzuki et al., 1991). Enzymatically modified licorice extract (EMLE), which is prepared by the treatment of licorice extract with cyclodextrin glucanotransferase (CGTase) (Kitahata, 1974; Suzuki et al., 1991), is a natural sweetener. It is used in Japan due to its unique properties such as higher solubility and better taste than those of licorice extract itself. However, its constituents have not been fully identified. Since licorice extract is a mixture of many constituents, the enzyme treatment may produce a variety of compounds. In the present paper, six major constituents of EMLE were isolated, and their sweetness was tested to evaluate its quality as a food additive and to get information on the structure−sweetness relationship.

MATERIALS AND METHODS

Instrumentation. The NMR spectra were measured on an Alpha 600 spectrometer (600 MHz for ^{1}H, 150 MHz for ^{13}C; JEOL, Tokyo, Japan) with dimethyl sulfoxide-d_6 as the solvent and tetramethylsilane as the internal standard, and the chemical shifts are given as δ values. Liquid secondary-ion mass spectrometry (LSI-MS) spectra were taken with a ZAB-2SE double-focusing mass spectrometer (VG Analytical, Manchester, U.K.) in the positive mode.

Sample and Reagents. The EMLE product was obtained through the Japan Food Additives Association. For TLC, silica gel F60 254 (Merck, Darmstadt, Germany) was used, and the spots were detected by spraying with 10% H_2SO_4 and heating the plates. All chemicals used were of reagent grade and used without further purification.

Isolation of Constituents. A 1 g sample was dissolved in MeOH−H_2O (1:2) (20 mL). The solution was subjected to preparative HPLC with an ODS column (J'sphere ODS-H80, 20 mm i.d. × 250 mm; YMC, Kyoto, Japan) and eluted with MeOH−2% AcOH (3:2) to yield compounds **1** (78 mg, retention time 28 min), **2** (8 mg, 36 min), **3** (10 mg, 21 min), **4** (4 mg, 25 min), **5** (6 mg, 17 min), and **6** (5 mg, 19 min).

Characteristics of Isolated Compounds. Compound **3**: amorphous powder; LSI-MS (positive mode) *m*/*z* 985.2 $[M + H]^+$, 822.9 $[M + H - Glc]^+$, 646.1 $[M + H - Glc - GlcA]^+$, 451.0 $[M + H - Glc - (2 \times GlcA) - H_2O - H_2]^+$; high-resolution LSI-MS found 985.4648, calcd for $C_{48}H_{73}O_{21}$ $[M + H]^+$ 985.4644; ^{1}H NMR 5.0 (d, J = 3.0 Hz, H-1‴), 4.53 (d, J = 7.5 Hz, H-1″), 4.37 (d, J = 7.5 Hz, H-1′), 3.70 (d, J = 9.0 Hz, H-5″), 3.60 (d, J = 9.0 Hz, H-5′), 3.51 (m, H-6‴), 3.48 (t, J = 9.0 Hz, H-4″), 3.47 (t, J = 9.0 Hz, H-3″), 3.40 (t, J = 9.0 Hz, H-3′), 3.32−3.38 (m, H-4′,3‴,5‴), 3.29 (m, H-2′), 3.16−3.20 (m, H-2‴,4‴), 3.10 (m, H-2″); ^{13}C NMR given in Table 1.

Compound **4**: amorphous powder; LSI-MS (positive mode) *m*/*z* 985.2 $[M + H]^+$, 823.1 $[M + H - Glc]^+$, 646.0 $[M + H - Glc - GlcA]^+$, 450.9 $[M + H - Glc - (2 \times GlcA) - H_2O - H_2]^+$; high-resolution LSI-MS found 985.4663, calcd for $C_{48}H_{73}O_{21}$ $[M + H]^+$ 985.4644; ^{1}H NMR 5.01 (br s, H-1‴), 4.52 (d, J = 7.5 Hz, H-1″), 4.34 (d, J = 7.5 Hz, H-1′), 3.68 (d, J = 9.0 Hz, H-5″), 3.55 (br s, H-5′), 3.42−3.52 (m, H-3″,4″,6‴), 3.23−3.40 (m, H-2′,3′,4′,3‴,5‴), 3.14−3.20 (m, H-2‴,4‴), 3.09 (t, J = 9.0 Hz, H-2″); ^{13}C NMR given in Table 1.

Compound **5**: amorphous powder; LSI-MS (positive mode) *m*/*z* 1147.1 $[M + H]^+$, 985.0 $[M + H - Glc]^+$, 822.5 $[M + H -$

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Table 1. ^{13}C NMR Data for Compounds 1−6[a]

	1	2	3	4	5	6
C-3	88.3	88.1	88.3	88.3	88.3	88.3
C-4	39.1	39.0	39.1	39.1	39.1	39.1
C-5	54.4	54.3	54.5	54.4	54.5	54.4
C-11	199.1	199.5	199.1	199.2	199.1	199.2
C-12	127.4	127.6	127.4	127.7	127.4	127.7
C-13	169.8	169.3	169.8	169.4	169.8	170
C-29	28.5	179.3	28.5	179.3	28.5	179.4
C-30	177.8	19.2	177.7	19.3	177.8	19.3
C-1′	103.5	103.5	103.5	103.5	103.5	103.5
C-2′	82.7	82.4	82.7	82.8	82.9	82.9
C-3′	75.3	74.9	75.9	76.1	76.1	76.1
C-4′	71.3	73.2	73.2	73.3	73.5	73.6
C-5′	76.0	75.9	75.1	75.0	75.0	75.0
C-6′	170.1	171.2	170.6	169.7	169.8	169.4
C-1″	104.8	104.5	104.5	104.5	104.5	104.6
C-2″	75.0	74.5	74.4	74.5	74.5	74.5
C-3″	76.3	76.1	75.5	75.6	75.6	75.6
C-4″	71.6	71.7	80.1	80.1	80.1	80.1
C-5″	75.7	75.7	74.9	75.0	74.9	75.0
C-6″	170.3	172.1	169.6	169.7	169.8	169.8
C-1‴			100.6	100.6	100.9	100.9
C-2‴			72.3	72.4	72.0	72.0
C-3‴			73.2	73.3	73.0	73.0
C-4‴			69.1	69.2	79.0	78.9
C-5‴			71.3	71.5	71.2	71.2
C-6‴			60.0	60.0	59.6	59.5
C-1⁗					100.3	100.3
C-2⁗					72.6	72.7
C-3⁗					73.4	73.4
C-4⁗					70.1	70.1
C-5⁗					71.5	71.5
C-6⁗					61.0	61.0

[a] In DMSO-d_6.

$(2 \times \text{Glc})]^+$, 645.4 $[M + H - (2 \times \text{Glc}) - \text{GlcA}]^+$, 451.0 $[M + H - (2 \times \text{Glc}) - (2 \times \text{GlcA}) - H_2O - H_2]^+$; high-resolution LSI-MS found 1147.5148, calcd for $C_{54}H_{83}O_{26}$ $[M + H]^+$ 1147.5173; ^{1}H NMR 5.06 (d, $J = 3.0$ Hz, H-1⁗), 4.98 (d, $J = 3.0$ Hz, H-1‴), 4.54 (d, $J = 7.5$ Hz, H-1″), 4.39 (d, $J = 7.5$ Hz, H-1′), 3.71 (d, $J = 9.0$ Hz, H-5″), 3.27−3.70 (m, 16 H, other sugar moiety protons), 3.20−3.27 (m, H-2‴,2⁗), 3.05 (t, $J = 9.0$ Hz, H-2″); ^{13}C NMR given in Table 1.

Compound **6**: amorphous powder; LSI-MS (positive mode) *m/z* 1147.1 $[M + H]^+$, 985.1 $[M + H - \text{Glc}]^+$, 822.7 $[M + H - (2 \times \text{Glc})]^+$, 645.4 $[M + H - (2 \times \text{Glc}) - \text{GlcA}]^+$, 451.0 $[M + H - (2 \times \text{Glc}) - (2 \times \text{GlcA}) - H_2O - H_2]^+$; high-resolution LSI-MS found 1147.5179, calcd for $C_{54}H_{83}O_{26}$ $[M + H]^+$ 1147.5173; ^{1}H NMR 5.06 (d, $J = 3.0$ Hz, H-1⁗), 4.98 (d, $J = 3.0$ Hz, H-1‴), 4.54 (d, $J = 7.5$ Hz, H-1″), 4.39 (d, $J = 7.5$ Hz, H-1′), 3.71 (d, $J = 9.0$ Hz, H-5″), 3.27−3.70 (m, 16 H, other sugar moiety protons), 3.20−3.27 (m, H-2‴,2⁗), 3.05 (t, $J = 9.0$ Hz, H-2″); ^{13}C NMR given in Table 1.

Sensory Test for Sweetness. The sweetness of the isolated compounds was tested as an aqueous solution at a concentration of 0.5% (w/v). Four persons (two males and two females) ranked the compounds in order of sweetness. Aspartame, acesulfame K, and sucrose were also tested for comparison.

RESULTS AND DISCUSSION

The EMLE sample dissolved in MeOH−H_2O was directly subjected to preparative HPLC. Figure 1 shows the HPLC chromatogram. The six numbered peaks were fractionated. Compound **1** (retention time 28 min) and compound **2** (36 min) were identified as glycyrrihizin and 3-*O*-[*β*-D-glucuronopyranosyl-(1→2)-*β*-D-glucuronopyranosyl]liquiritic acid (Khalilov et al., 1989; Shibata, 1994; Kitagawa et al., 1993), respectively.

Compound **3** (21 min) was obtained as an amorphous powder. High-resolution LSI-MS of compound **3** revealed the molecular formula $C_{48}H_{72}O_{21}$. The LSI-MS

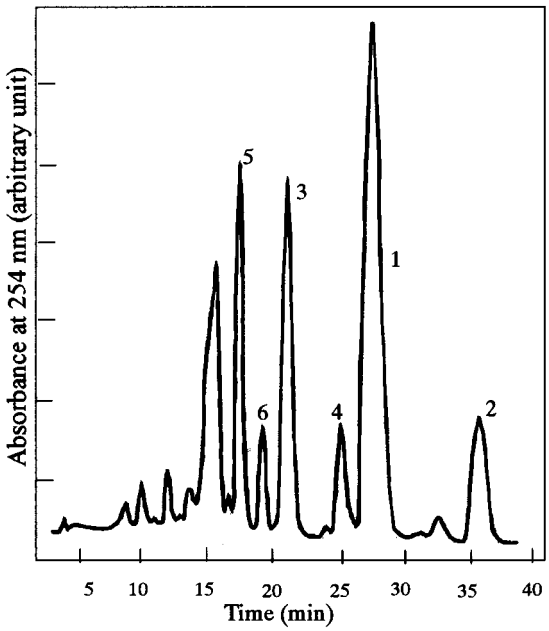

Figure 1. HPLC chromatogram of an EMLE product. HPLC conditions: column, J'sphere ODS-H80 (20 mm i.d. × 250 mm); flow rate, 5 mL/min; eluent, MeOH/2% AcOH (3:2).

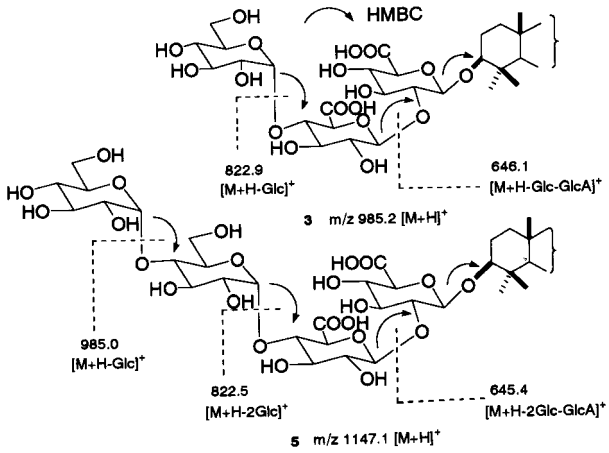

Figure 2. LSI-MS fragments and partial HMBC of compounds **3** and **5**.

spectrum gave fragment ion peaks at *m/z* 822.9 $[M + H - \text{Glc}]^+$ and 646.1 $[M + H - \text{Glc} - \text{GlcA}]^+$, suggesting that the 3-*O*-glucosyl chain contained a glucosyl and two glucuronosyl moieties (Figure 2). The ^{13}C NMR spectrum (Table 1) showed three anomeric carbon signals (δ 103.5, 104.5, and 100.6) together with two signals for carboxyl groups of glucuronosyl moieties (δ 170.6 and 169.6). A comparison of the ^{13}C NMR spectrum with that of glycyrrhizin revealed that compound **3** was a monoglucoside of glycyrrhizin. The assignments of the proton and carbon signals from the sugar moiety of compound **3** were confirmed on the basis of ^{1}H−^{1}H correlation spectroscopy (COSY), heteronuclear multiple quantum coherence (HMQC), and heteronuclear multiple bond connectivity (HMBC) data. By comparison of the ^{1}H and ^{13}C NMR data for compounds **1** and **3** (Table 1), the H-1‴ (δ 5.0, d, $J = 3$ Hz) and C-1‴ (δ 100.6) signals of the terminal glucose moiety in compound **3** were assigned. The C-4″ (δ 80.1) in compound **3** was lower-field-shifted as a glucosylation shift by 8.5 ppm than the corresponding signal (δ 71.6) in compound **1**. This result, with the HMBC correlation observed between H-1‴ and C-4″ in compound **3**, suggested that a

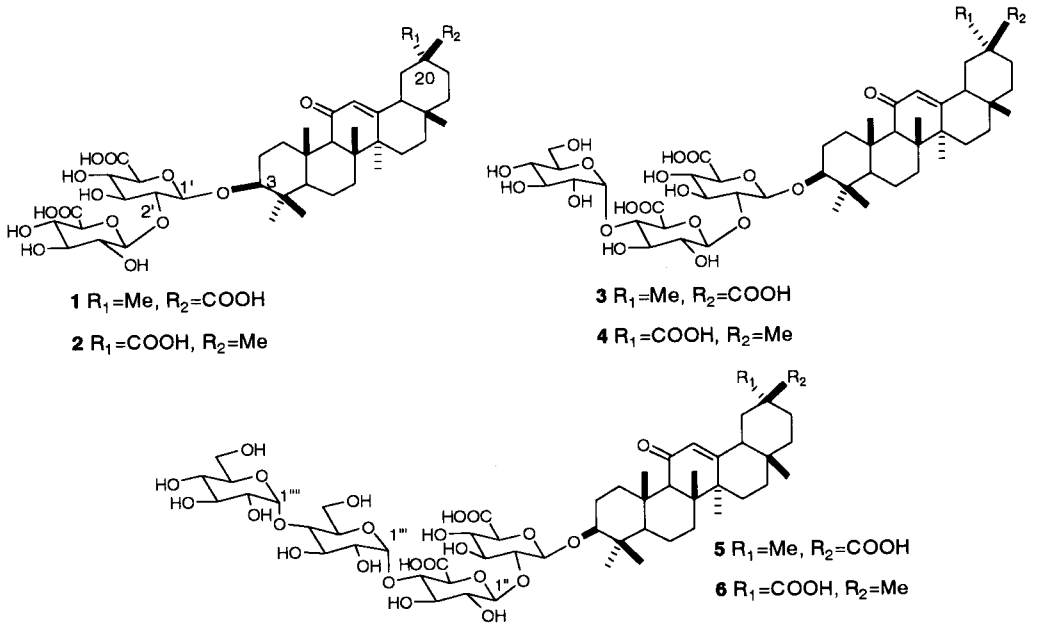

Figure 3. Structures of compounds **1**−**6**.

Table 2. Relative Sweetness of Compounds 1−6

	rel sweetness[a]	concn tested (%)		rel sweetness[a]	concn tested (%)
aspartame	+++++	0.5	compd **3**	+++	0.5
sucrose	+	1.0	compd **4**	++	0.5
acesulfame-K	+++++	0.5	compd **5**	+	0.5
compd **1**	+++	0.5	compd **6**	+	0.5
compd **2**	++++	0.5			

[a] +, slightly sweet; ++ to +++++, sweet (the number of plus signs indicates the order of sweetness).

glucopyranosyl group was connected to the C-4″ position of the terminal glucuronic acid moiety of compound **1**. Consequently, compound **3** was 3-*O*-[α-D-glucopyranosyl-(1→4)-*β*-D-glucuronopyranosyl-(1→2)-*β*-D-glucuronopyranosyl]glycyrrhetinic acid.

The molecular formula of compound **5** (17 min) was $C_{54}H_{82}O_{26}$ on the basis of the high-resolution LSI-MS data. The LSI-MS data (Figure 2) of compound **5** gave fragment ion peaks at *m*/*z* 985.0 $[M + H - Glc]^+$, 822.5 $[M + H - (2 \times Glc)]^+$, and 645.4 $[M + H - (2 \times Glc) - GlcA]^+$, suggesting that the 3-*O*-glucosyl chain contains two glucuronosyl and two glucosyl moieties. Its ^{13}C NMR spectrum showed four anomeric carbon signals (*δ* 103.5, 104.5, 100.9, and 100.3) together with two signals for carboxyl groups of glucuronosyl moieties (*δ* 169.8 and 169.8). A comparison of the ^{13}C NMR spectra of compounds **5** and **3** revealed that compound **5** was a monoglucoside of compound **3**. The assignments of the proton and carbon signals from the sugar moiety of compound **5** were also performed on the basis of the $^1H-^1H$ COSY, HMQC, and HMBC spectral data. By comparison of the ^1H and ^{13}C NMR data for compounds **5** and **3**, the H-1‴″ (*δ* 5.06, d, *J* = 3 Hz) and C-1‴″ (*δ* 100.3) signals of the terminal glucose moiety in compound **5** were assigned. The C-4‴ (*δ* 79.0) in compound **5** was shifted to lower field by 9.9 ppm relative to the corresponding signal (*δ* 69.1) in compound **3**. In addition, an HMBC correlation was observed between H-1‴″ and C-4‴ in compound **5**. These results suggested that the glucopyranosyl group in compound **5** was connected to the C-4‴ position of the terminal glucose moiety of compound **3**. Compound **5** was therefore 3-*O*-[α-D-glucopyranosyl-(1→4)-α-D-glucopyranosyl-(1→4)-*β*-D-glucuronopyranosyl-(1→2)-*β*-D-glucuronopyranosyl]glycyrrhetinic acid.

The data from low-resolution and high-resolution LSI-MS showed that the molecular formula of compound **4** was the same as that of compound **3**. The ^1H and ^{13}C NMR data of compound **4** were essentially the same as those of compound **3** except for the chemical shifts of C-29 and C-30, which were almost the same as those of compound **2**. These data demonstrated that compound **4** was a monoglucoside of compound **2**, in which C-4″ was glucosylated. Thus, compound **4** was 3-*O*-[α-D-glucopyranosyl-(1→4)-*β*-D-glucuronopyranosyl-(1→2)-*β*-D-glucuronopyranosyl]liquiritic acid.

The structure of compound **6** was elucidated in the same way. Mass spectral data showed that this compound has the same molecular formula as that of compound **5**. Comparison of NMR data of compound **6** with those of compounds **5** and **4** indicated that compound **6** was a monoglucoside of compound **4**. Consequently, compound **6** was 3-*O*-[α-D-glucopyranosyl-(1→4)-α-D-glucopyranosyl-(1→4)-*β*-D-glucuronopyranosyl-(1→2)-*β*-D-glucuronopyranosyl]liquiritic acid.

The identified structures of compounds **1**−**6** are represented in Figure 3. Compounds **1**, **3**, and **5**, which have a common aglycon, glycyrrhetinic acid, are the major constituents of EMLE; Compounds **2**, **4**, and **6**, which have a common aglycon, liquiritic acid, are the minor constituents of this food additive. The approximate ratio of the former to the latter is 5:1. Licorice extract contains **1** and **2** as the major and minor constituents, respectively. Therefore, it is considered that both compounds **1** and **2** were glucosylated to make compounds **3** and **5** and compounds **4** and **6**, respec-

tively. Compounds **3**−**6** are new compounds isolated for the first time.

A broad, large peak that eluted earlier than peak 5 (Figure 1) was studied by LC/MS and was suspected to be a mixture of derivatives of compounds **1** and **2** having triglucosyl groups. Although other small peaks in Figure 1 have not been studied further, these may be derivatives of compounds **1** and **2** having longer sugar chains.

The structure−sweetness relationship in glycyrrhizin derivatives was studied previously (Tanaka, 1997). We investigated the sweetness of the isolated compounds. As shown in Table 2, the sweetness depended on the aglycon and the length of the 3-*O*-glucosyl chain. Compounds **1** and **2** are the major and minor sweet compounds in licorice extract, respectively. Interestingly, compound **2** was sweeter than compound **1**.

When the two compounds were monoglucosylated, however, the order of sweetness was reversed; namely, compound **3**, which is the derivative of compound **1**, was sweeter than compound **4**. Although the sweetness of both compounds was lower than that of the parent compounds, the lingering sweet aftertaste characteristic of licorice extract was markedly improved. Compounds **5** and **6**, which have two additional glucose moieties, showed only slight sweetness, which was comparable to that of sucrose.

With CGTase, natural products with long sugar chains can be produced. For example, we detected rutin derivatives with 32 additional glucose moieties (Akiyama et al., 2000). Several small peaks other than the peaks for **1**−**6** were detected in Figure 1. These seemed to be derivatives with longer sugar chains, and so, they did not seem to be sweet on the basis of the results of the sensory test for compounds **1**−**6**. To produce an EMLE product with a high quality, therefore, the derivatives with longer sugar chains should be removed in the purification process or the glucosylation reaction should be controlled not to produce the derivatives with longer sugar chains.

ABBREVIATIONS USED

EMLE, enzymatically modified licorice extract; CGTase, cyclodextrin glucanotransferase; LSI-MS, liquid secondary-ion mass spectrometry; COSY, correlation spectroscopy; HMQC, heteronuclear multiple quantum coherence; HMBC, heteronuclear multiple bond connectivity.

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