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Identification of α-D-Glucosylglycerol in Sake*

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α-D-Glucosylglycerol (GG) was found for the first time in sake (Japanese rice wine) in an amount of about 0.5%. GG was also found in miso and mirin which had been brewed by using koji. GG was hydrolyzed into glucose and glycerol in an equimolar ratio with maltase (EC 3.2.1.20, α-glucosidase from yeast), but not with emulsin (EC 3.2.1.21, β-glucosidase from almond). The retention times and mass spectra of trimethylsilyl derivatives by a GC-MS analysis of GG in sake were comparable to those of various GG samples synthesized by glycol cleavage. It was proven that GG in sake consisted of three components, *viz.*, 2-*O*-α-D-glucosylglycerol (GG-II), (2*R*)-1-*O*-α-D-glucosylglycerol (R-GG-I) and (2*S*)-1-*O*-α-D-glucosylglycerol (S-GG-I). The ratio of the three components in GG was 6:66:28 for sake. It is considered that GG was formed by transglucosylation of the glucosyl groups to glycerol by α-glucosidase from koji in the sake mash.

Key words: α-D-glucosylglycerol; identification; α-glucosidase; transglucosylation; sake

Analytical investigations on the taste of sake by HPLC showed an unknown peak which did not correspond to any component so far reported.[1,2] This unknown peak could not be found in a koji saccharified solution nor in a yeast (*Saccharomyces cerevisiae*) culture with the heat-inactivated koji saccharified solution. However, this unknown peak was found in the yeast culture with the koji saccharified solution which possessed enzymatic activities. Therefore, it was presumed that a yeast product and an enzyme of koji could be concerned with this unknown compound. This same unknown compound was also found in miso and mirin which had been brewed by using koji. The present paper reports the identification of this unknown compound.

Materials and Methods

Reagents. Transglucosidase L-AMANO was provided by Amano Pharmaceutical Co., Ltd. (Nagoya, Japan), and maltase and emulsin were obtained from Oriental Yeast Co., Ltd. (Tokyo, Japan). TMSI-C was from GL Sciences Inc. (Tokyo, Japan), and the other reagents were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

High-performance liquid chromatography. A Shimadzu LC-3A pump and a Showa Denko Shodex RI model SE-11 differential refractive index detector were used. HPLC was conducted in a Shimadzu Shim-pack SCR-101 (N) column (7.9×300 mm, for the ligand-exchange chromatography) placed in a Shimadzu CTO-2A thermostat at 50°C. A short pre-column (4×50 mm) was positioned between a Rheodyne model 7725i sample injector and the separation column. Distilled water was applied as an eluent at a flow rate of 0.6 ml/min with an injection volume of 5 μl to 50 μl.

HPLC with a Merk Hibar Lichrosorb NH_2-5 μm column (4×250 mm, for normal-phase chromatography) was performed at a column temperature of 30°C, using 75%, 80% or 85% acetonitrile as the eluent under the other conditions already described.

Unless otherwise indicated, the ligand-exchange chromatography is simply shown as HPLC in the present paper.

Paper chromatography. Paper chromatography was carried out with Toyo Roshi No. 51A by the descending method at room temperature. *n*-Buthanol–pyridine–distilled water (6:4:3)[3,4] and *iso*-propanol–pyridine–distilled water-acetic acid (8:8:4:1)[5] were used as the developing solvents. After development, each paper was sprayed with an ammoniacal silver nitrate solution or aniline phthalate–n-buthanol solution as the dyeing reagent, and kept at 105°C for 10 min.

Gas chromatography-mass spectrometry. A Hewlett-Packard 5890 series II gas chromatograph was coupled with a Hewlett-Packard 5971 series

* The studies on α-D-glucosylglycerol (Part 1).

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Abbreviations: GG, α-D-glucosylglycerol; GG-II, 2-*O*-α-D-glucosylglycerol; GG-I, 1-*O*-α-D-glucosylglycerol; R-GG-I, (2*R*)-1-*O*-α-D-glucosylglycerol; S-GG-I, (2*S*)-1-*O*-α-D-glucosylglycerol

mass-selective detector for GC-MS. A J&W Scientific DB-225 column (0.25 mm × 30 m, 0.15 μm film) was used. The carrier gas was helium at a flow rate of 0.8 ml/min, and a 0.2-μl to 1-μl aliquot of a sample was injected at a split ratio of 50:1. The oven temperature was programmed from 100°C to 200°C at 5°C/min. The inlet and interface temperatures were set at 240°C and 280°C, respectively. The ionization method was EI (70 eV), and fragment ions were scanned from 70 amu to 650 amu at 1.5 cycles/sec.

Assay of enzyme activity. The activities of α-glucosidase (maltase and transglucosidase L-AMANO) and β-glucosidase (emulsin) were assayed by using *p*-nitrophenyl α-D-glucoside[6] and *p*-nitrophenyl β-D-glucoside, respectively. Each enzyme solution was prepared by dissolution or dilution with a 100 mM acetate buffer (pH 5.0). To 100 μl of 20 mM *p*-nitrophenyl α-D-glucoside or *p*-nitrophenyl β-D-glucoside, 200 μl of a 100 mM acetate buffer (pH 5.0) and 100 μl of the enzyme solution were added. The mixture was incubated at 37°C for 15 min. The reaction was stopped by adding 400 μl of 0.2 M sodium carbonate, and the absorbance at 400 nm was measured. One unit of enzyme activity was defined as the amount of enzyme which liberated 1 μmol of *p*-nitrophenol per 1 min under these conditions.

Hydrolysis with an acid or enzyme. The unknown compound in sake was fractionated by HPLC, and concentrated *in vacuo*. To about 1 mg of the resulting syrup, 100 μl of 2 N sulfuric acid was added. Hydrolysis was performed at 95°C for 6 h. After neutralizing with 100 μl of 2 N sodium hydroxide, the hydrolyzate was analyzed by HPLC.

The activities of maltase and emulsin were adjusted to 0.25 U/ml with a 100 mM acetate buffer (pH 5.0). To about 1 mg of the syrupy sample of the unknown compound, 200 μl of each enzyme solution was added. The mixture was incubated at 40°C for 24 h and then analyzed by HPLC.

Addition of glycerol in both the saccharification of koji and the reaction of maltose with α-glucosidase. To 5 g of koji, 10 ml of 5% glycerol was added. The mixture was saccharified at 50°C for 24 h and then filtered. On the other hand, 100 μl of a 100 mM acetate buffer (pH 5.0) containing 4 U of α-glucosidase (transglucosidase L-AMANO) was added to 2 ml of 20% maltose containing 10% glycerol. The mixture was incubated at 40°C for 24 h and then filtered. Each of these filtrates was analyzed and fractionated by HPLC. Each resulting fraction was concentrated *in vacuo*, trimethylsilylated with TMSI-C, and analyzed by GC-MS.

Synthesis of GG-II. To 1 ml of 4% maltitol, 10 ml of 2% sodium periodate was added. The reaction was performed at room temperature. To 1.1-ml aliquots of the reaction mixture, 0.2 ml of 6% barium chloride was added at 2-min intervals to stop the reaction. The deposit was removed by filtration. The filtrate (1 ml) was applied to an ion-exchange column (Amberlite MB-2, 1 × 20 cm) which was eluted with 100 ml of distilled water. The resulting eluate was concentrated *in vacuo*, and to the residue, 200 μl of 4% sodium borohydride was added. The reduction was performed at 37°C for 4 h. One drop of acetic acid was added to the solution, the generation of gas being confirmed. The solution was concentrated *in vacuo*. To the syrup obtained, 5 ml of methanol was added, and the solution was evaporated *in vacuo*. The excess boric acid was removed as methyl borate by repeating this treatment several times. The residue was dissolved in 0.5 ml of distilled water, and applied to a charcoal column (charcoal:Celite, 1:1; 1 × 15 cm). The column was eluted with 100 ml of distilled water and 150 ml of 2% ethanol in that order. The eluate from 2% ethanol was concentrated *in vacuo* and then purified by HPLC.

Synthesis of S-GG-I. To 500 μl of 10% isomaltose, 5 ml of acetic acid and 140 mg of lead tetraacetate (2 mol eq of isomaltose) were added. The reaction was performed at room temperature, until this solution gave a negative result in the starch-iodine test. After the reaction, 1 ml of acetic acid containing 5% oxalic acid was added. The deposit was removed by filtration, and the resulting filtrate was evaporated *in vacuo* to remove the excess acetic acid. The residual syrup was dissolved in 1 ml of distilled water and, like the synthesis of GG-II, deionized, reduced, and purified.

Synthesis of GG-I (1-O-α-D-glucosylglycerol). To 500 μl of 10% trehalulose, 5 ml of 1% sodium borohydride was added. The reduction was performed at 37°C for 4 h. One drop of acetic acid was added to the solution, the generation of gas being confirmed. Like the synthesis of GG-II, the excess boric acid in this solution was removed. The residue obtained was dissolved in 1 ml of distilled water, deionized, concentrated, and dissolved in 500 μl of distilled water. To this solution, 5 ml of acetic acid and 70 mg of lead tetraacetate (1 mol eq of trehalulose) were added. The same procedure as that for the synthesis of S-GG-I was then performed.

Identification of GG by GC-MS. The unknown compound in sake was fractionated by HPLC, and concentrated *in vacuo*. The resulting syrup was kept in a drying desiccator overnight and then trimethylsilylated with TMSI-C. In the same way, various synthesized GG samples were trimethylsilylated. This unknown compound was compared with the various

synthesized GG samples for its retention time and mass spectrum by the GC-MS analysis.

Results

Concentration and occurrence of GG in sake

The HPLC chromatogram of sake is shown in Fig. 1. The unknown compound was eluted between glucose and some disaccharides which seemed to be mainly isomaltose. Although the retention time of each saccharide and alcohol in sake so far reported[1,2] was examined, this unknown compound did not correspond to any reported compound (Table 1).

The unknown compound was hydrolyzed into glucose and glycerol in an equimolar ratio with both acid and maltase, but not with emulsin. Therefore, this unknown compound seemed to be α-D-glucosylglycerol (GG). The concentration of GG was 0.45% in sake (Fig. 1) as calculated from the glucose concentration in the hydrolyzate.

GG was found by an HPLC analysis, when glycerol had been added both in the saccharification of koji and in the reaction of maltose with α-glucosidase (transglucosidase L-AMANO). Therefore, it was considered that GG was formed in the transglucosylation to glycerol of the yeast product by α-glucosidase of *Aspergillus oryzae* in the sake mash.

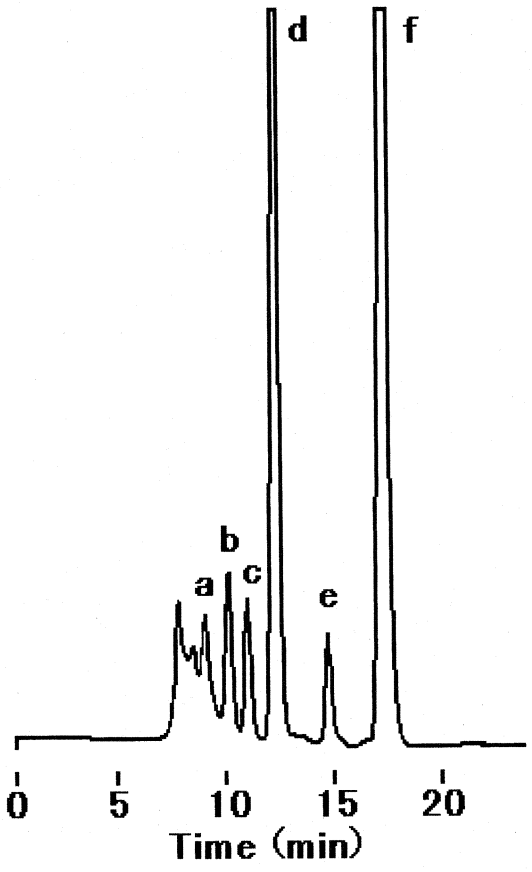


Fig. 1. HPLC Chromatogram of Sake.
HPLC conditions were described in Materials and Methods. a; trisaccharides, b; disaccharides, c; GG, d; glucose, e; glycerol, f; ethanol.

Table 1. Retention Times of the Saccharides and Alcohols in Sake

Compound	t_R (min)
Panose, Isomaltotriose	9.0
Nigerose, Kojibiose	10.0
Maltose, Isomaltose	10.3
Unknown (GG)	11.2
Glucose	12.6
α-Ethyl glucoside	12.7
Mannitol	12.6
Arabitol	13.4
Erythritol	13.8
Glycerol	15.2
Ethanol	17.9

HPLC conditions were described in Materials and Methods.

Separation and identification of GG by GC-MS

It was presumed that GG had three kinds of constituent components because α-glucosyl groups were able to combine with any hydroxyl group of *sn*-glycerol.[7] As expected, the trimethylsilyl derivatives of GG in sake were separated into three peaks which were found at 17.5 min, 18.1 min and 18.3 min by the GC-MS analysis (Fig. 2).

It is conjectured that each component of GG behaved differently in the enzyme reaction because the area ratio of the three peaks corresponding to the composition ratio of the three components was uneven. Therefore, in a series of reports, the distinguishing feature of GG-I isomers whose glucosyl group combines with C1-OH or C3-OH of *sn*-glycerol[7] has been shown to have a C2 absolute configuration on the glycerol side in order to compare with other saccharides in the enzyme reaction.

GG-II was synthesized from maltitol by the Malaprade reaction whose time was limited (Scheme 1), *i.e.*, the sorbitol side of maltitol was cleaved more rapidly than the glucosyl side because each adjacent

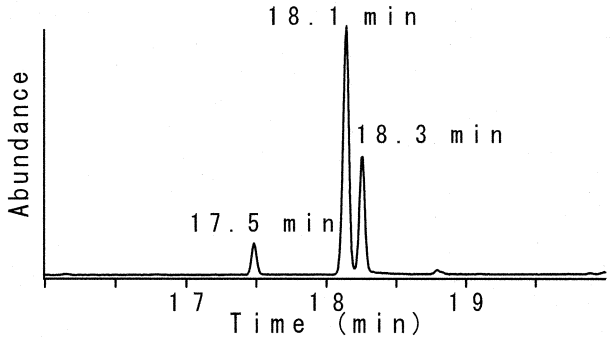
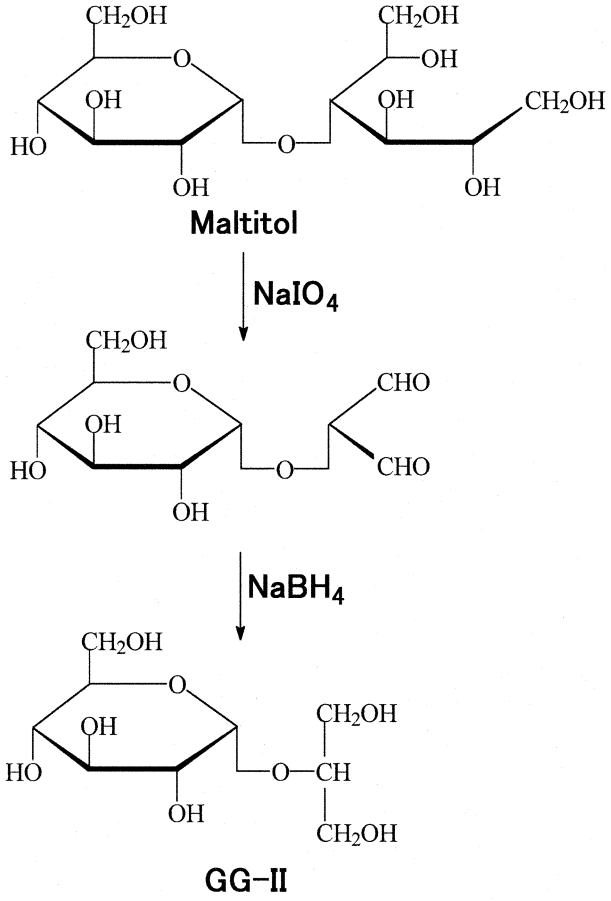


Fig. 2. Total Ion Chromatogram of the Trimethylsilyl Derivatives of GG in Sake.
GC-MS conditions were described in Materials and Methods.



Scheme 1. Synthesis of GG-II.

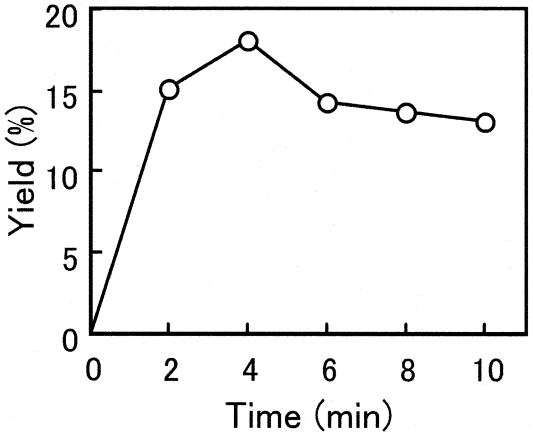
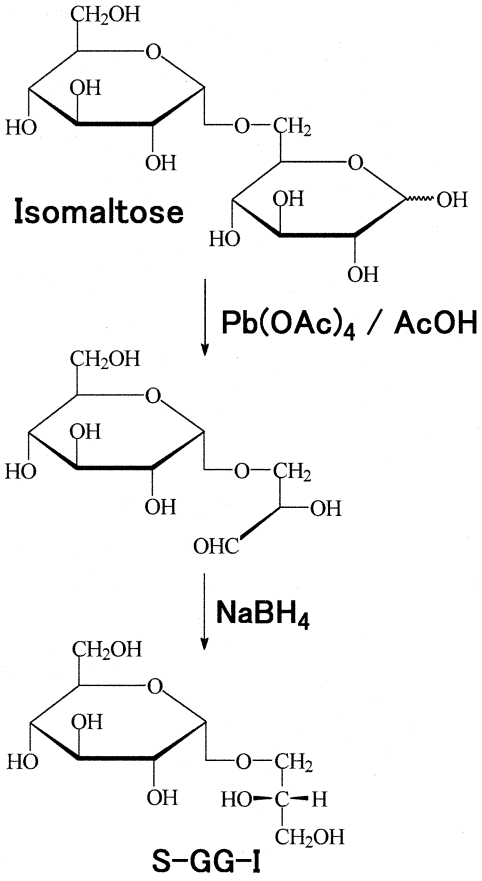


Fig. 3. Relationship between the Yield of GG and the Malaprade Reaction Time for Maltitol.

hydroxyl group of the glucosyl unit had the *trans* configuration.[8] When the reaction time at room temperature was examined by HPLC, the GG peak became a maximum for 4 min (Fig. 3). This yield calculated from the peak area on the HPLC chromatogram was 18%. The GC-MS analysis showed the peak of a trimethylsilyl derivative of GG-II at 17.5 min (Fig. 4a).



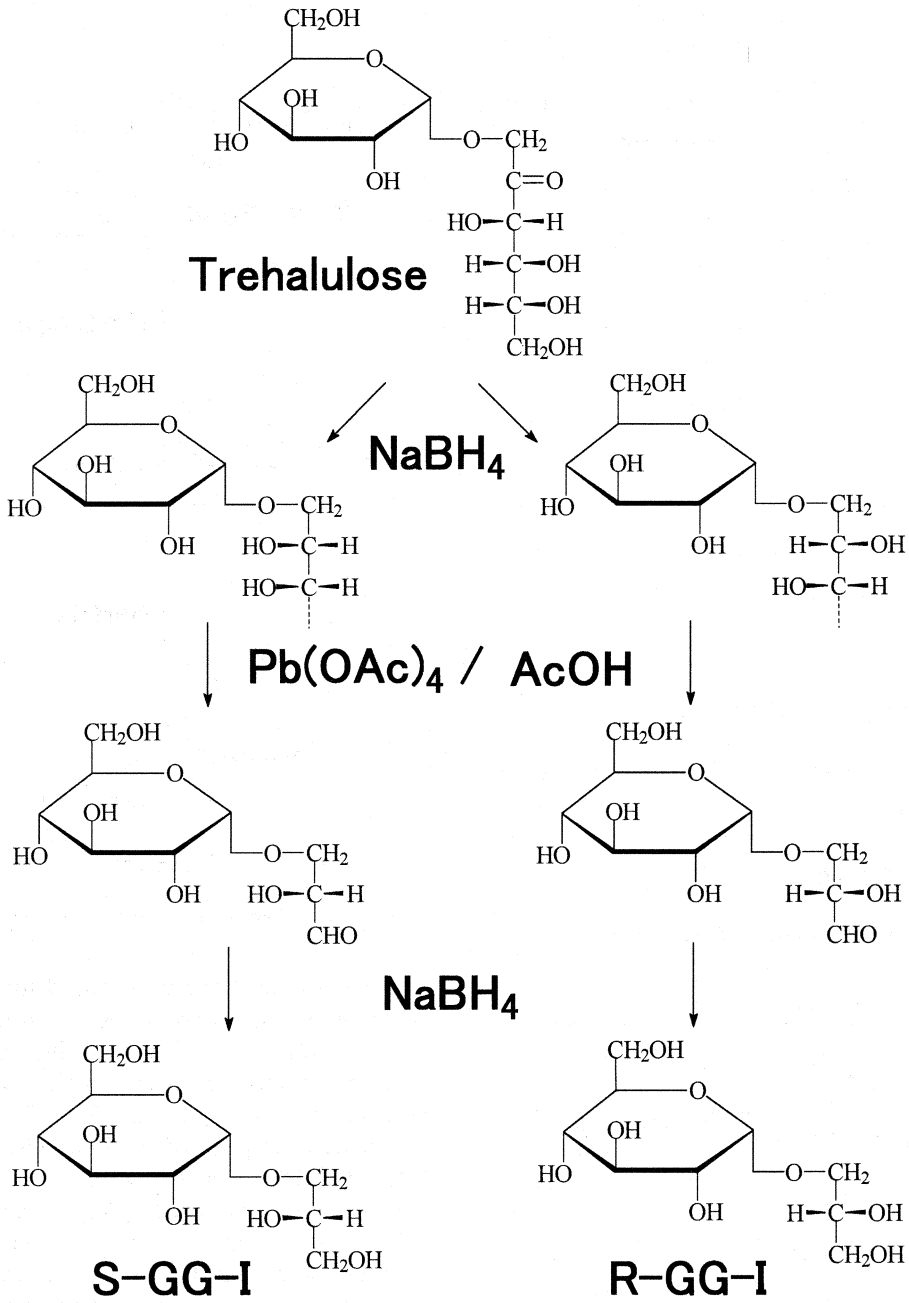
Scheme 2. Synthesis of S-GG-I.

Kaneda *et al.* have synthesized lilioside D (1-*O*-β-D-glucopyranosyl-*sn*-glycerol) from gentiobiose.[9] In the same way, S-GG-I was synthesized from isomaltose by glycol cleavage with lead tetraacetate (Scheme 2). This yield was 12%. The GC-MS analysis showed the peak of a trimethylsilyl derivative of S-GG-I at 18.3 min (Fig. 4b).

GG-I was synthesized by glycol cleavage with lead tetraacetate from trehalulose which had been reduced beforehand in order to make C2 of the aglycone take *R* and *S* configurations (Scheme 3). This yield was 5%. The GC-MS analysis showed two peaks at 18.1 min and 18.3 min (Fig. 4c), the latter peak corresponding to a trimethylsilyl derivative of S-GG-I. The former peak was similar to the latter in the mass spectra (Figs. 5b and 5c). Therefore, the former peak seemed to be a trimethylsilyl derivative of R-GG-I. Moreover, the ratio of S-GG-I:R-GG-I (73:27) in synthesized GG-I may have been the anomeric ratio (α:β) of trehalulose in an aqueous solution.

In addition to the retention times, all the synthesized GG samples were identical with GG from sake in the mass spectra of their trimethylsilyl derivatives (Fig. 5).

It was proven that GG in sake consisted of GG-II, R-GG-I and S-GG-I. The ratio of these three com-



Scheme 3. Synthesis of GG-I.

ponents calculated from each peak area in Fig. 2 was 6:66:28, but it varied a little in different samples of sake. When glycerol was added, the GG components ratios in the koji saccharified solution and in the reacted solution of maltose with α-glucosidase were 8:58:34 and 9:52:39, respectively. Accordingly, it is considered that the acceptor specificity of α-glucosidase was mainly related to the ratio of these three components.

Behavior of GG in general saccharide analyses

GG was developed near glucose by paper chromatographies.[3-5] Since GG is a non-reducing sugar, it does not take on color with a certain dyeing reagent (Table 2). GG was separated into two peaks by an HPLC analysis with an amino column (Table 3). Each fraction was concentrated, trimethylsilylated, and analyzed by GC-MS. The result verified that the former and latter peaks corresponded to GG-II and GG-I, respectively.

Discussion

It seems that GG has not previously been reported in sake because it was not separated from the other components in general saccharide analyses by paper

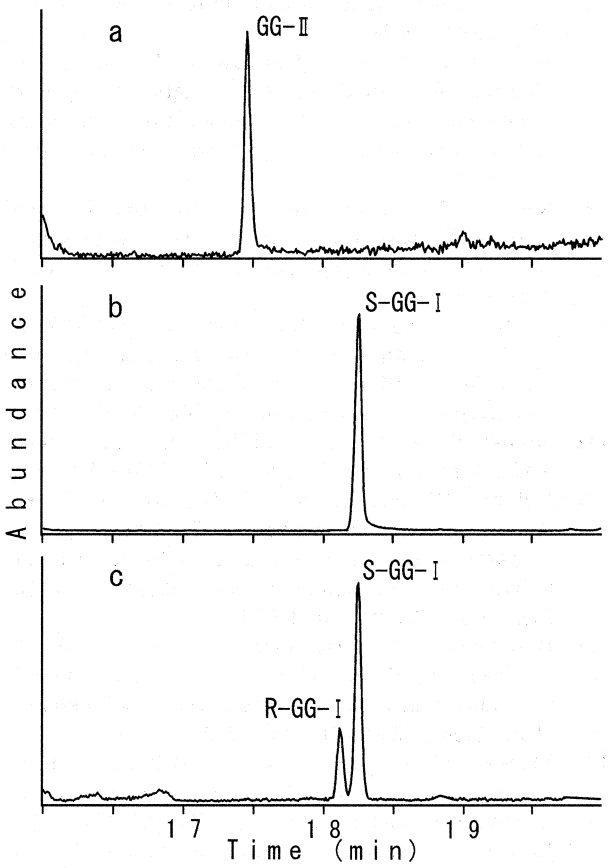


Fig. 4. Total Ion Chromatograms of the Trimethylsilyl Derivatives of Synthesized GG.
GC-MS conditions were described in Materials and Methods.
a; GG-II, b; S-GG-I, c; GG-I.

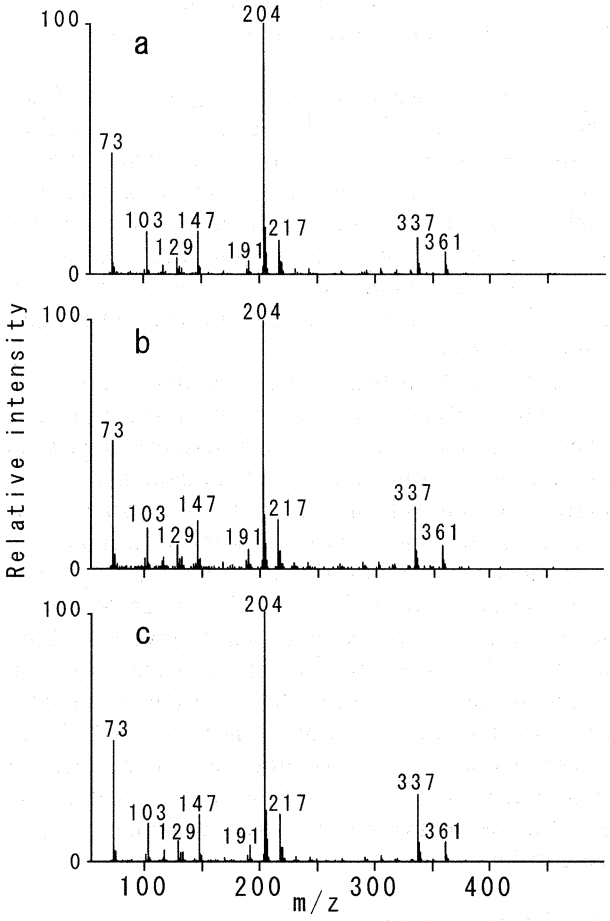


Fig. 5. Mass Spectra of the Trimethylsilyl Derivatives of GG.
GC-MS conditions were described in Materials and Methods.
a; GG-II, b; R-GG-I, c; S-GG-I.

Table 2. R_f Values for GG and Other Compounds

Compound	Developing solvent A		Developing solvent B	
	Dyeing reagent a	Dyeing reagent b	Dyeing reagent a	Dyeing reagent b
Isomaltose	0.22	0.22	0.42	0.42
Maltose	0.27	0.27	0.49	0.49
Glucose	0.38	0.38	0.56	0.56
GG	0.38	N.D.*	0.56	N.D.*
Glycerol	0.57	N.D.*	0.67	N.D.*

(N.D.*: Non-detected)
Paper chromatography conditions were described in Materials and Methods.
Developing solvent: A) *n*-BuOH:pyridine:H_2O (6:4:3)
B) *iso*-PrOH:pyridine:H_2O:AcOH (8:8:4:1)
Dyeing reagent: a) Ammoniacal silver nitrate solution
b) Aniline phthalate-*n*-BuOH solution

Table 3. Retention Times (min) of the Saccharides and Alcohols in Sake by HPLC with an Amino Column

Compound	Concentration of CH_3CN as the eluent		
	75%	80%	85%
Glycerol	6.5	7.1	7.8
α-Ethyl glucoside	6.6	7.4	8.8
GG-II	9.4	12.8	18.6
Glucose	10.4	13.3	18.9
GG-I	10.4	14.0	21.0
Maltose	15.7	23.9	N.D.*
Isomaltose	17.7	28.5	N.D.*

(N.D.*: Non-detected)
The conditions for HPLC with an amino column were described in Materials and Methods.

chromatography and HPLC with an amino column. When HPLC with an amino column was carried out, using 75% acetonitrile as the eluent, GG-II was separated from the other compounds, but glucose interfered with GG-I. This separation of GG-II and glucose was similar to the results reported by Schoor *et al.*[10]

The saccharides and sugar alcohols present in sake are mainly glucose, glycerol, isomaltose, isomaltotriose, panose and α-ethyl glucoside.[1,2] Together with these components, it is anticipated that GG, as much as about 0.5%, is also deeply involved in the taste of sake. Miso and mirin which are traditional Japanese foods made from koji contain about 0.5% and 0.1% of GG, respectively. Other alcoholic beverages such as beer and wine have not contained

GG by an HPLC analysis. Therefore, it is presumed that koji plays an important role in the production of GG.

In case of transglucosylation to glucose as an acceptor by α-glucosidase, the glucosyl unit could be transfered to any hydroxyl group of glucose. Nevertheless, the major product is isomaltose. Accordingly, α-glucosidase seems to recognize the binding site of glucose. Similarly, α-glucosidase seems to recognize the binding site of glycerol in the formation of GG, so that the ratio of these three components is uneven.

GG-II has been found in an alkaline hydrolyzate of teichoic acids of the cell wall of gram-positive bacteria.[11] GG-II also exists in blue-green algae such as marine cyanobacteria which inhabit a high osmotic environment, and it has been reported to be biosynthesized and to be related to intracellular osmoregulation.[12-28] On the contrary, GG of sake seems to be produced *in vitro*. The concentration in sake of GG is almost equal to that of glycerol (about 0.5%), while the amounts of transglucosylation products such as α-ethyl glucoside and isomaltose are less than the acceptors. Therefore, GG is interesting not only for its influence on the taste of sake, but also for the variation of its concentration and for the behavior of its three components in the sake mash.

Acknowledgments

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