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Transglycosylation forms novel glycosides ethyl α-maltoside and ethyl α-isomaltoside in sake during the brewing process by α-glucosidase A of *Aspergillus oryzae*

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Abstract

Sake, the Japanese rice wine, contains a variety of oligosaccharides and glucosides produced by fungal enzymes during the brewing process. This study investigates the effect of knocking out the *Aspergillus oryzae* α-glucosidase (*agdA*) gene on the transglycosylation products in a brewed sake. In addition to α-ethyl glucoside and α-glyceryl glucoside, the amount of two compounds that have molecular masses similar to ethyl maltose decreased by *agdA* gene knockout. Both compounds were synthesized, *in vitro,* from maltose and ethanol with purified *agdA*. Nuclear magnetic resonance analysis identified the two compounds as ethyl α-maltoside and ethyl α-isomaltoside, respectively, which are novel compounds in sake as well as in the natural environment. Quantitative analysis of 111 commercially available types of sake showed that these novel compounds were widely present at concentrations of several hundred mg/L, suggesting that both of them are ones of the common glycosides in sake.

Keywords: α-glucosidase, transglycosylation, glucoside, sake, brewing, *Aspergillus oryzae*

INTRODUCTION

Sake, a Japanese rice wine, is brewed with the "multiple parallel fermentation" process. Enzymes produced by the fungus *Aspergillus oryzae* carry out the saccharification process while the yeast *Saccharomyces cerevisiae* ferments alcohol simultaneously. During the sake-brewing process, fungal hydrolytic enzymes degrade the starch of rice into glucose, which the yeast subsequently uses as the substrate for alcoholic fermentation. Glucose is also an important component of sake that provides sweetness and harmony with other components, which form sake's taste. In general, glucose is the most abundant non-volatile component in sake, ranging in concentration from 1% to 4%, followed by glycerol and isomaltose (6-*O*-α-D-glucopyranosyl-α,β-D-glucopyranose) [1-3]. Yeast produces glycerol, which is also present in wine and beer, as a by-product of alcoholic fermentation [4]. Isomaltose is a product of transglycosylation by fungal enzymes, but yeast does not preferentially assimilate it when glucose is present in the culture medium [5]. Other than isomaltose, kojibiose (2-*O*-α-D-glucopyranosyl-α,β-D-glucopyranose) and sakebiose (3-*O*-α-D-glucopyranosyl-α,β-D-glucopyranose) [5] are generated by transglycosylation by fungal enzymes, suggesting active transglycosylation during the sake-brewing process [5]. Yeast does not use the transglycosylation products in sake during alcoholic fermentation. Besides oligosaccharides, ethyl α-glucoside (α-EG) and glyceryl α-glucoside (α-GG), which transglycosylation generates during the sake-brewing process, are present in sake at concentrations of 0.24%–0.71% and 0.45%, respectively [6, 7]. α-EG adds both sweetness and bitterness to the unique taste of sake [6]. Both α-EG and α-GG have skin moisturizing effects and, thus, are used in the production of cosmetics [8-10]. These glycosides are thought to be synthesized by transglycosylation of a glycosidase only when an acceptor compound (e.g., ethanol or glycerol) and donor maltose are both present, which is a unique situation in the multiple parallel fermentation process [11, 12].

Transglycosylation may be catalyzed by glycosidase derived from *A. oryzae* [11, 13]. However, the glycosidase(s) responsible for generating such transglycosylation products in the sake-brewing process remain unclear. The *A. oryzae* genome codes for several glucosidases, including α-glucosidase A (AgdA), which is encoded by *agdA* [14]. AgdA seems to be a major α-glucosidase because it is highly induced by starch (or maltose), which is also observed in other amylolytic enzymes, such as α-amylase, encoded by *amyA*, *B*, and *C*, and glucoamylase, encoded by *glaA* [15]. Nevertheless, AgdA's involvement in transglycosylation during the sake-brewing process is unknown.

In this study, the *agdA* gene of *A. oryzae* was knocked out to identify which components of sake are produced by AgdA's enzymatic activity. The results identified two novel transglycosylation products (ethyl α-maltoside and isomaltoside) that are generally present in sake.

MATERIALS AND METHODS

Chemicals

All reagents were analytical grade, unless otherwise stated, and purchased from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan).

Construction of an agdA gene disruption (ΔagdA) DNA cassette

A DNA fragment containing the genomic region of the *agdA* open reading frame (ORF) and 500-bp flanking regions were amplified by polymerase chain reaction (PCR) using Platinum™ *Taq* DNA Polymerase (Thermo Fisher Scientific K.K., Tokyo, Japan), and cloned using TOPO TA Cloning® Kits (Thermo Fisher Scientific K.K.). The resulting plasmid, pTAagdA, was linearized by PCR using KOD-Plus-Neo DNA polymerase (Toyobo Co., Ltd., Osaka, Japan). The selective marker gene *sC*, encoding sulfate adenylyltransferase, was amplified from the plasmid pUSC [16] by PCR using KOD-Plus-Neo, and introduced into the linearized plasmid pTAagdA using the InFusion cloning kit (Takara Bio, Inc., Shiga, Japan), generating the plasmid pKSagdA. The DNA cassette for Δ*agdA* was amplified from pKSagdA. Table S1 lists all the primers used for PCR. Plasmid propagation was performed with ECOS™ Competent *E. coli* DH5α (Toyobo Co., Ltd.).

Construction of an AgdA overexpression DNA cassette

A fragment of the *agdA* ORF, amplified by PCR from the genomic DNA of *A. oryzae* strain RIB40, was inserted downstream of the modified enolase A promoter sequence of the PCR-linearized pNENU2512 vector [17] by InFusion cloning. The resulting AgdA overexpression vector pEAGN was used for fungal transformation.

Fungal transformation

In order to generate the Δ*agdA* strain, *A. oryzae* strain NS4Δ*ligD* [18] was transformed with a PCR fragment amplified from the plasmid pKSagdA. Transformation was performed using a standard protoplast method [19]. Czapek–Dox agar (3% glucose, 0.1% K_2HPO_4, 0.05% KCl, 0.05% $MgSO_4 \cdot 7H_2O$, 0.001% $FeSO_4$, and 1.5% agar), supplemented with 0.9% $(NH_4)_2SO_4$ as a nitrogen source, was used as a selective medium. *A. oryzae* strain NS4Δ*ligD* was transformed by PCR-amplification of a DNA fragment of *sC* to generate the control strain NSCS. *A. oryzae* strain *niaD*300 [16] was transformed with the plasmid pEAGN digested with *Bam*HI to generate the AgdA overexpression strain EAGN. Except for addition of 20 U of *Bam*HI into the protoplast and DNA mixture, transformation was performed using a standard protoplast isolation method [19] with Czapek–Dox agar as the selective medium.

Southern blot analysis

Genomic DNA extraction, hybridization, and detection were performed as described previously [20]. Genomic DNA of the candidate Δ*agdA* strain and the host strain were digested with *Sal*I, while that of the EAGN and host strains were digested with *Hind*III. An 877-bp region containing the promoter and ORF of *agdA* and a 2.0-kb region containing the promoter and ORF of pEAGN were used to generate a digoxigenin-labeled probe.

Small-scale sake brewing and evaluation

Koji was prepared in accordance with the method described by Tokuoka et al. [21] using 70% polished ordinary rice and the conidia of strains Δ*agdA* and NSCS. Small-scale brewing was performed with ordinary rice (70% polished), rice-*koji* produced by strains Δ*agdA* and NSCS, and yeast (*S. cerevisiae* K-901) using a three-step mashing processes. As the first step (*Soe*), 24 g of rice and 12 g of rice-*koji* were added to 60 mL of water containing yeast at 10^6 cells/mL. The next day, as the second step (*Naka*), 52 g of rice, 12 g of rice-*koji*, and 92 mL of water were added to the fermentation mash. On day 3, as the third step (*Tome*), 84 g of rice, 16 g of rice-*koji*, and 128 mL of water were added. The mash was fermented at 15°C for 20 days and then centrifuged at 2,000 x g for 10 min to separate the sake cake from the sake.

The sake meter value (a measure of the density of sake relative to water), alcohol content, and total acid content were measured with the use of conventional methods [22]. The amount of fungal cells was estimated based on the amount of *N*-acetyl glucosamine in accordance with the methods described by Reissig et al. [23] and Fujii et al. [24].

Analysis of α-amylase and α-glucosidase

Rice-*koji* (5 g) was soaked in 25 mL of 10 mM acetic acid buffer (pH 5.0) containing 5% NaCl (w/v) for 3 h at room temperature and then filtrated to obtain an enzyme extract solution. The α-amylase activity was measured using a standard method [22], while α-glucosidase activity was measured using a glucoamylase and α-glucosidase assay kit (Kikkoman Biochemifa Co., Ltd., Tokyo, Japan). One unit of α-glucosidase activity was defined as the titer that released 1 μmol of *p*-nitrophenol from *p*-nitrophenyl-α-glucopyranoside in 1 min [25].

Oligosaccharide and glycoside analysis

To detect oligosaccharides and glycosides, high-performance liquid chromatography (HPLC) was performed using a SIL-20A direct injection autosampler, SCL-10Avp controller, and LC-20AD pump (Shimadzu Corporation, Kyoto, Japan), with a Shodex SUGAR SZ5532 column (6.0 × 150 mm) (Showa Denko K.K., Tokyo, Japan). The column temperature was maintained at 80°C using a forced-air circulation-type column oven (CTO-20A; Shimadzu

Corporation). A 5-µL aliquot of the sample was injected into the HPLC system. The flow rate was 0.9 mL/min and the isocratic conditions were 25% Milli-Q water/75% acetonitrile. The system was also equipped with a Shodex RI-501 refractive index detector (Showa Denko K.K.). For detection by mass spectrometry (MS), the ACQUITY UPLC H-Class System (Waters Corporation, Milford, MA, USA) and XevoG2-XS high-performance benchtop quadrupole time-of-flight (QTof™) mass spectrometer (Waters Corporation) were used as described previously [26]. When the Shodex SUGAR SZ5532 column was used, 75% acetonitrile was used as the mobile phase at a flow rate of 0.9 mL/min. Quantification of oligosaccharides and glycosides were performed on HPLC-RI based on the standard curves generated for each compound. For α-EM and α-EiM, compounds purified in this study were used to generate the standard curves.

Protein purification

The EAGN strain was inoculated into 100 mL of YPM medium (yeast extract, peptone, and mannitol at 1%, 1% and 5%) in a 300-mL Erlenmeyer flask, cultured at 30°C for 6 days with shaking at 130 rpm, and then filtered through Miracloth (Merck KGaA, Darmstadt, Germany) to remove the mycelium. The culture was filtered with a 50-kDa cut-off ultrafiltration device (Biomax 50 kDa Ultrafiltration Discs; Merck KGaA) and a 100-kDa cut-off ultrafiltration device (Amicon Ultra–15 mL Centrifugal Filters Ultracel-100K; Merck KGaA). It was then subjected to fast protein liquid chromatography using the ÄKTA start chromatography system (GE Healthcare Japan Co., Tokyo, Japan), equipped with a cation‐exchange column (HitrapTMSP Sepharose Fast Flow; GE Healthcare Japan Co.). Total protein was measured using the method described by Lowry [27]. Proteins in the samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) [28], on 12.5% polyacrylamide gels, and stained with Coomassie brilliant blue R-250. The BlueStar Plus Prestained Protein Marker (MWP03; Nippon Genetics Europe GmbH, Dueren, Germany) was used as a protein standard.

In vitro synthesis and glycoside purification

Purified AgdA (0.18 U/mL) was incubated with 40% (v/v) ethanol and 5% (w/v) maltose in 10 mM acetate buffer (pH 5.0) at 37°C for 48 h. The reaction was stopped by heating at 80°C for 10 min. Residual glucose in the reaction mixture was removed by treating with *S. cerevisiae* K-901 cells. The cells were cultured in a three-fold volume of GYP medium (glucose, yeast extract, and peptone at 2%, 0.5% and 0.5%) of *in vitro* reaction mixture at 30°C and 180 rpm for 24 h, and then centrifuged for 10 min at 900 x g. Afterward, the cells were rinsed with distilled sterile water and then centrifuged twice to remove the medium components. Before adding the yeast, ethanol was removed under reduced pressure and the *in vitro* reaction mixture was diluted with distilled water to adjust the total sugar concentration to 5%. After adding yeast, the solution was incubated at 30°C and 180 rpm for 5 h. Thereafter,

the yeast was removed by centrifugation followed by filtration using a disposable membrane filter unit with a pore size of 0.45 μm (13HP045CN; Toyo Roshi Kaisha, Ltd., Tokyo, Japan). To purify the targeted compounds, yeast-treated reaction solution was fractionated by HPLC with two columns: an Asahipak NH2P-130G 7B (7.5 × 50 mm) (Showa Denko K.K.) and Asahipak NH2P-90 20F (20 × 300 mm) (Showa Denko K.K.). The introduction volume was 100 μL and the flow rate was 5.0 mL/min. The isocratic conditions were 25% Milli-Q water/75% acetonitrile. The collected fractions were concentrated by distillation under reduced pressure and separated by HPLC with the use of 65% acetonitrile as the mobile phase to isolate the target compounds A and B.

Structural analysis of target compounds by nuclear magnetic resonance spectroscopy

Each saccharide samples was dissolved in 0.5 mL D_2O (99.8 %), and exchangeable protons were substituted with deuteriums by lyophilization. ^{1}H, ^{13}C, and two-dimensional (2D) NMR spectra were recorded using a RESONANCE ECZ 600R nuclear magnetic resonance spectrometer (JEOL Ltd., Tokyo, Japan) at 14.1 T with D_2O (99.8%) as the solvent. To analyze compound A, residual ^{1}H signals in D_2O (known data: 3.34 ppm) [29] or ^{13}C signals (known data: 69.7 ppm) [29] were used as an internal standard. To analyze compound B, acetone (δ_H: 2.225 ppm, δ_C:30.5 ppm) was used as an internal standard. Signal assignments were based on unambiguous chemical shifts and heteronuclear multiple bond coherence (C–H HMBC), heteronuclear single quantum coherence (C–H HSQC), and correlation spectroscopy (H–H COSY).

Content of novel compounds in commercial sake

Following types of 111 commercial sakes were used: *jummai-daiginjo* (8 samples), *jummai-ginjo* (33 samples), *jummai* (58 samples), *daiginjo* (1 sample), *honjozo* (4 samples), regular sake (4 samples), aged sake (1 sample), *kijo-shu* (2 samples). Sake samples were filtered through a disposable membrane filter unit with a pore size of 0.45 μm, and analyzed by HPLC under the same conditions described in the section titled “oligosaccharide and glycoside analysis.” For statistical analysis, student’s *t*-test was performed on Microsoft Excel 2016.

RESULTS AND DISCUSSION

Production of the control and ΔagdA strains

Oligosaccharides and glucosides formed by transglycosylation are present in sake. However, the enzymes responsible for forming these compounds remain unclear. During the sake-brewing process, rice-*koji* made of rice and filamentous fungus *A. oryzae,* provides enzymes in mash (*moromi*). AgdA is the main glucosidase produced by *A. oryzae*; thus, AgdA is most likely involved in transglycosylation during the sake-brewing process. Therefore, the transglycosylation products in sake were identified by comparing the compounds in sake brewed with rice-*koji*

made of *A. oryzae* Δ*agdA* and control strains. A DNA fragment for Δ*agdA* was constructed and transformed into the *A. oryzae* recipient strain NS4Δ*ligD*. Among several candidate transformed strains, one was confirmed by Southern blot and PCR analyses as an Δ*agdA* strain (Figures S1A and S1B, respectively). To eliminate the auxotrophic effect of the Δ*agdA* strain on rice growth, a control strain, named NSCS, was also constructed by transformation of strain NS4Δ*ligD* with the selective marker gene *sC* (Figure S2). Both these strains were used for rice-*koji* production. The hydrolytic enzyme activities and amounts of mycelia in the resulting rice-*koji* were analyzed. While the hydrolytic enzyme activities and amount of mycelia were similar between the Δ*agdA* and NSCS strains, α-glucosidase activity of the Δ*agdA* strain decreased significantly ($p<0.05$) by about 50% of that of the NSCS strain, indicating AgdA is responsible for at least half of the α-glucosidase activity in rice-*koji* (Figure S3). Although AgdA is considered the main glucosidase, this is the first report to demonstrate AgdA's contribution to α-glucosidase activity in the sake-brewing process.

Analysis of oligosaccharides in sake brewed using the ΔagdA strain

To investigate AgdA's role in forming transglycosylation compounds in sake, sake was brewed on a small scale using the rice-*koji* of the Δ*agdA* and NSCS strains. Curves of cumulative weight loss, which reflect the loss of CO_2 gas accompanied with alcoholic fermentation, were similar in both fermentations, but the sake meter value and the alcohol content were slightly higher with the use of the Δ*agdA* strain (Table S2). The increased alcohol level observed in the sample of the Δ*agdA* strain may be due to reduced formation of unfermentable sugars by transglycosylation. However, hydrophilic interaction liquid chromatography/TOF-MS analysis showed increases in the isomaltose and kojibiose levels from 800 mg/L and 270 mg/L to 2,409 mg/L and 692 mg/L, respectively, in the sake brewed with the Δ*agdA* strain (Figure 1A), suggesting that AgdA plays a role in degrading isomaltose and kojibiose during the sake-brewing process. Because AgdA is the main α-glucosidase produced by *A. oryzae*, it was expected to be one of the factor(s) responsible for formation of isomaltose, the most abundant oligosaccharide. However, the sake brewed with rice-*koji* of *A. oryzae* strain Δ*agdA* contained larger amounts of isomaltose indicated that AgdA's contribution for isomaltose formation is not important. Notably, α-EG and α-GG levels in the sake brewed with the Δ*agdA* strain were markedly decreased from 3,180 mg/L and 2954 mg/L to 824 mg/L and 1389 mg/L, respectively, (Figure 1A), which clearly indicates that AgdA is responsible for glycoside formation during the sake-brewing process. This is the first report to identify the enzyme responsible for α-EG and α-GG formation during the sake-brewing process. Other than those, a peak observed at 1.64 min (m/z=213.07) was increased in the sake brewed with the Δ*agdA* strain, which we could not identify so far.

Interestingly, two peaks were observed at 4.4 min and 5.2 min, which we inferred as compounds A and B. However, the amounts of compounds A and B decreased significantly in the sake brewed with the Δ*agdA* strain (Figure 1A). TOF/MS analysis showed the exact masses of lithium adduct ions ($[M+Li]^+$) of both compounds were 377.16 (Figure 1B), suggesting the compounds were structural isomers. The *m/z* value of 377.16 coincides with those of the dehydration and condensation products of maltose and ethanol. This finding is consistent with the observation that the loss of AgdA, which likely catalyzes the dehydration and condensation of maltose and ethanol during the sake-brewing process, resulted in decreased amounts of compounds A and B. Comparisons of MS/MS fragmentation peaks of α-EG with those of compounds A and B showed several overlapping peaks (Figure 1C). The *m/z* values of the fragmented peaks generated from compounds A and B were almost identical, but with different intensities. Reportedly, the MS/MS fragment intensities differ between structural isomeric saccharides, supporting the idea that compounds A and B are structural isomers. Detailed assignment of the fragment of α-EG indicated that overlapped fragments $^{0,3}A_1^+$ or/and $^{1,4}A_1^+$, $^{0,2}A_1^+$, and B_1^+ are derived from the nonreductive ends of glucose residues (Figure 1D). Taken together, these findings strongly suggest that compounds A and B are glycosyl products of α-EG; namely, ethyl α-maltoside. In addition, compounds with the exact masses of the lithium adduct were identical to ethyl maltotorioside and maltotetraoside (*m/z* = 539.22 and 701.27, respectively), suggesting that transglycosylation by AgdA contributes to sake's component complexity.

Production of an AgdA by agdA overexpression strain

To identify the structures, compounds A and B were purified from sake to obtain sufficient amounts for NMR analysis. However, due to sake's complex components, it was impossible to purify these compounds directly. Therefore, *in vitro* synthesis by enzymatic reactions was attempted. To prepare AgdA, an AgdA overexpression strain of *A. oryzae* was constructed, where *agdA* was expressed under a strong promoter containing multiple copies of *cis*-elements for amylase induction[17]. *A. oryzae* strain *niaD*300 was transformed with the overexpression vector and a strain harboring two copies of the expression vector, named as EAGN, was selected by Southern blot analysis (Figures S4A and B). EAGN was cultured in YPM liquid medium for 6 days, and α-glucosidase activity of the culture filtrates reached 0.298 U/mL, which was 75-fold greater than the α-glucosidase activity of the wild-type *A. oryzae* strain RIB40 (Table 1). AgdA was prepared by ultrafiltration and fast protein liquid chromatography, which finally yielded a purified AgdA protein (Table 1). SDS-PAGE analysis showed a single band corresponding to the size of AgdA (ca. 109 kDa)[14] in lane B, while several signals were observed in the culture filtrate of EAGN in lane A, suggesting that AgdA was successfully purified (Figure 2).

Enzymatic synthesis and purification of the target compounds

Compounds A and B were synthesized *in vitro,* using ethanol and maltose as substrates, and purified AgdA at concentrations of 20%–60% (v/v), 1%–10% (w/v) and 0.01–0.18 U/mL, respectively, to determine the optimal reaction conditions. Consequently, the optimal substrate and enzyme concentrations for synthesizing the target compounds were 40% ethanol, 5% maltose, and 0.18 U/mL of purified AgdA. HPLC analysis of the products of *in vitro* reaction showed several peaks (Figure 3B). There were obvious peaks of maltose, glucose, and α-EG on a chromatogram. In addition, there were peaks of the *in vitro* reaction solution with elution times corresponding to those of compounds A and B in sake (Figures 3A and B). LC-qTOF/MS confirmed that the *m/z* values ($[M+Li]^+$) of the compounds corresponding to the peaks were 337.16, and MS/MS fragmentation peaks were identical to the fragment peaks of compounds A and B (Figures 4A and B), indicating that the compounds synthesized *in vitro* were identical to compounds A and B. Subsequently, attempts were made to purify compounds A and B; however, there was a large peak corresponding to glucose, as a by-product, which was eluted immediately after compound B, but prevented fractionation of compound B. Because yeast readily consumes glucose in medium, it was added to eliminate glucose from the reaction solution for 5 h at 30°C. As a result, glucose was removed successfully from the solution below the detection limit, and a peak corresponding to compound B was quite distinct (Figure 3C). On the other hand, along with glucose consumption, glycerol was produced in the solution, although the peak was overlapped with that of compound A. Nonetheless, glycerol and compound A were separated with the use of a preparative column (Asahipak NH2P-130G 7B and Asahipak NH2P-90 20F). Finally, compounds A and B were fractionated and lyophilized. Figures 3D and E show chromatograms of the purified fractions of compounds A and B, respectively. The amounts of purified compounds A and B were 10.6 and 128.1 mg, respectively.

Structural determination by NMR

The purified compounds were subjected to determine the structures. Each compound showed two glycosyl residues and one ethoxy segment. ^{1}H and ^{13}C NMR data of compound A (Table S3, Figure S5) were identical to those of the synthetic ethyl α-maltoside (Figure 5A) [29]. While no NMR data associated with compound B was previously reported, further analysis including DQF-COSY, HSQC, HSQC-TOCSY, and HMBC was performed. Analysis was initiated from two anomeric protons at 4.94 and 4.95 ppm (Table S4, Figure S6). After confirmation of C-H correlation using HSQC, each signal was classified into three residues, two saccharide residues, and one ethyl group by HSQC-TOCSY (Figure S7B, C). Spin networks in each residue were confirmed mainly by DQF-COSY (Figure S7A). In both glycosyl residues, proton that showed correlation peak with anomeric proton was assigned as 2-H. Another proton showed correlation with 2-H was 3-H, and the other coupling partner of 3-H was assigned as 4-H. 5-H showed correlation peaks with 4-H and two 6-H. $^{3}J_{H\text{-}H}$ coupling constants between 2-H/3-H, 3-H/4-H, and 4-H/5-H were all in range of 9.0–10.5 Hz. This finding suggested that these protons have axial orientation and the

saccharide residues are glucopyranosyl. Pyranose form was also confirmed by a HMBC cross peak between H-1/C5 (Figure S7D). α-Glycosidic linkages were suggested by gauche (3.9 Hz) coupling constants of anomeric protons. Inter-residual orientation was determined by HMBC correlation peaks between one of the anomeric protons (1-H: 4.94 ppm) and carbon of ethyl group (2"C: 64.4 ppm), and the C6 carbon (C6: 65.8 ppm) and 1-H (1': 4.95 ppm) of the other glucosyl residue (Figure 5C). As a result, the structure of compound B was determined to be ethyl α-isomaltoside (Figure 5B) [30]. This is the first report of these compounds in sake. Organic synthesis of α-EM [29] and enzymatic synthesis of α-EiM [30] were reported previously, but this is the first study to report that both are natural products. In addition, the results of the present study also demonstrated that transglycosylation activity of AgdA synthesizes these glycosides during the sake-brewing process because the amounts of compounds A and B were low in sake brewed with *A. oryzae,* lacking a functional AgdA, while both were synthesized successfully *in vitro* with purified AgdA. It is likely that AgdA transfers the glucose residue at non-reducing end of maltose to 4-OH or 6-OH of the glucose residue in α-EG.

Content of novel compounds in commercial sake

The amounts of these novel compounds in commercial sake products were determined. We determined concentrations of α-EM and α-EiM in 111 sake samples. The amounts of them varies among sakes but they are commonly contained in sake (Figure 6A, B, Table 2), The average concentrations of α-EM and α-EiM were 100 and 498 mg/L, respectively. Comparing the average concentrations of glucose and isomaltose (2.22% and 0.31%), which are appropriate levels as sake [1, 3], the concentration of α-EM and α-EiM were one-tenth to one-hundredth of those sugars. However, it is notable that several sakes contained α-EiM at over 1,000 ppm. Although the sensory properties of both α-EM and α-EiM have not been clarified in this study, these glycosides may affect the taste of sake.

The amount of α-EiM was five-fold greater than α-EM; there was a similar tendency in the *in vitro* reaction by AgdA (Figure 3B). These results probably reflect transglycosylation's preference for the 6-OH of the glucose rather than the 4-OH of α-EG, and/or the preference of hydroxylation of α-EM over α-EiM. There was weak correlation between the concentrations of α-EM and α-EG (r=0.63), while no correlation was observed between α-EiM and α-EG (r=-0.10), and α-EM and α-EiM (r=0.19), indicating that formation of α-EM and α-EiM did not simply depend on the amounts of the acceptor substrate. Comparison of the average concentrations of the α-EM, α-EiM and α-EG among sake types is shown in Table 2. Statistical analysis showed that the averages concentration of α-EM between *jummai* and *jummai-daiginjo*, are significantly different (p<0.05), but other than that, we could not find any significant differences in the concentration of the glycosides among sake types. Future studies using sake samples

296 with more detailed information on production process are highly desirable to identify the factor(s) involved in
297 forming novel glycosides.

298

299 **Abbreviations Used**

300 HPLC, High-performance liquid chromatography; MS, Mass spectrometry; ORF, Open reading frame; PCR,
301 Polymerase chain reaction; DQF-COSY, double quantum filter-correlation spectroscopy; HSQC, heteronuclear
302 single quantum coherence; HSQC-TOCSY, heteronuclear single quantum coherence-Total correlation
303 spectroscopy; HMBC, heteronuclear multiple bond coherence

304

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308

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311

312 **Supporting Information**

313 Figure S1, Construction of strain *ΔagdA*.; Figure S2, Confirmation of the control strain, NSCS, by PCR analysis.;
314 Figure S 3, Activities of α-amylase and α-glucosidase of the α-*agdA* and control (NSCS) strains.; Figure S4,
315 Confirmation of introduction of the AgdA overexpression cassette.; Figure S5, ^{1}H NMR and ^{13}C NMR spectra of
316 compound A in D_2O.; Figure S6, ^{1}H NMR and ^{13}C NMR spectra of compound B in D_2O.; Figure S7, 2D NMR data of
317 compound B.; Table S1, Primers used for PCR in this study.; Table S2, Analytical values of the sakes produced
318 using the rice-*koji* of *ΔagdA* and NSCS strains.; Table S3, NMR analysis of compound A.; Table S4, NMR analysis
319 of compound B. Table S5, Average concentrations of the α-EM, α-EiM and α-EG among sake types.

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Figure Legend

Figure 1. Analysis of sake, brewed with strain Δ*agdA* by hydrophilic interaction liquid chromatography/TOF-MS. (A) MS chromatogram (TIC) of sake, brewed with the Δ*agdA* and control NSCS strains.1, α-EG; 2, compound A; 3, compound B; 4,α-GG; 5, sakebiose; 6, kojibiose; and 7, isomaltose. The peak intensities of compounds A and B in the sake brewed with strain Δ*agdA* were reduced significantly. (B) MS spectrum of compound A (RT; 4.430 min) and B (RT; 5.172 min). (C) MS/MS fragmentation peaks of compound A, B, and α-EG. (D) Detailed assignment of the MS/MS fragment of α-EG.

Figure 2. SDS-PAGE of culture filtrates of strain EAGN (day 6) (lane a) and purified AgdA (lane b) after ultrafiltration and fast protein liquid chromatography.

Figure 3. Chromatogram of (A) commercial sake, (B) *in vitro* reaction mixture, (C) yeast-treated reaction solution, (D) purified compound A and (E) purified compound B by HPLC-RI. 1, α-EG; 2, compound A; 3, compound B; 4, α-GG; 5, sakebiose; 6, kojibiose; 7, isomaltose; 8, glycerol; 9, glucose; and 10, maltose.

Figure 4. MS/MS analysis of sake components and synthetic products. (A) compound A (RT; 4.430 min), (B) compound B (RT; 5.172 min).

Figure 5. Chemical structure of (A) compound A (ethyl α-maltoside) and (B) compound B (ethyl α-isomaltoside), and (C) DQF-COSY and key HMBC correlations of compound B.

Figure 6. Histogram of the distribution of (A) ethyl α-maltoside and (B) ethyl α-isomaltoside content in 111 commercial sakes.

Tables

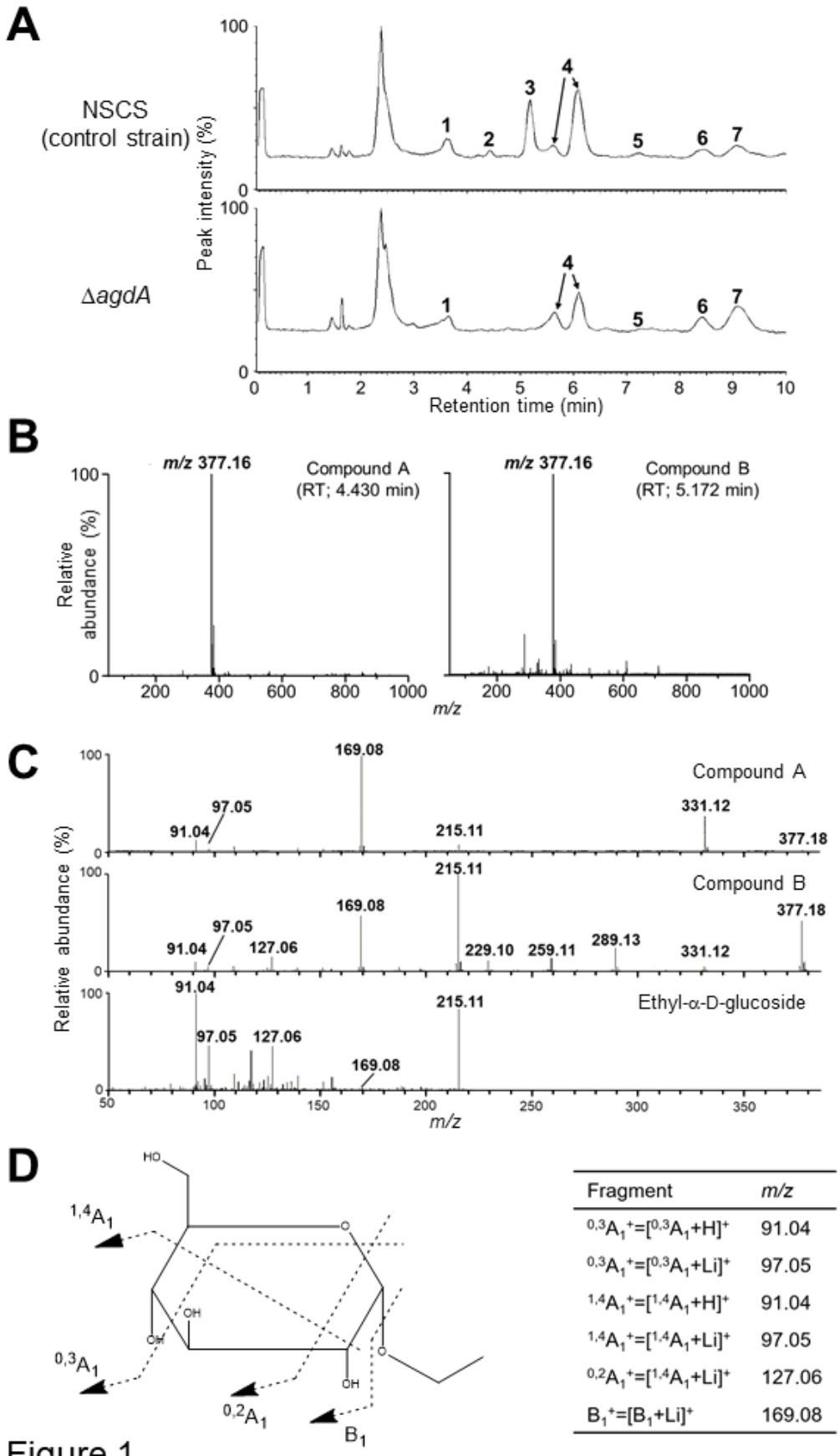
Table 1. α-Glucosidase activities in the culture broths and purified AgdA

	Volume	α-Glucosidase activity	Total activity	Total protein amount	Specific activity	Purification degree
	(μL)	(U/ml)	(U)	(mg)	(U/mg protein)	(fold)
Culture broth (NGAG)	290.0	0.298	86.28	1662.17	0.052	1.0
Purified AgdA	53.4	0.230	12.08	2.15	5.631	108.5
Culture broth (niaD300)	-	0.004	-	-	-	-

Table 2. Average concentrations of the α-EM, α-EiM and α-EG among sake types.

	α-EM	α-EiM	α-EG	Number of samples
		mg/L		
jummai-daiginjo	58	503	3,675	8
jummai-ginjo	102	563	4,954	33
jummai	111	467	5,565	58
daiginjo	31	389	4,366	1
honjozo	84	515	2,784	4
regular sake	60	422	4,010	4
aged sake	34	316	2,568	1
kijo-shu	79	581	3,323	2
Average	100	498	5,012	

Figures



Fragment	*m/z*
$^{0,3}A_1^+=[^{0,3}A_1+H]^+$	91.04
$^{0,3}A_1^+=[^{0,3}A_1+Li]^+$	97.05
$^{1,4}A_1^+=[^{1,4}A_1+H]^+$	91.04
$^{1,4}A_1^+=[^{1,4}A_1+Li]^+$	97.05
$^{0,2}A_1^+=[^{1,4}A_1+Li]^+$	127.06
$B_1^+=[B_1+Li]^+$	169.08

Figure 1

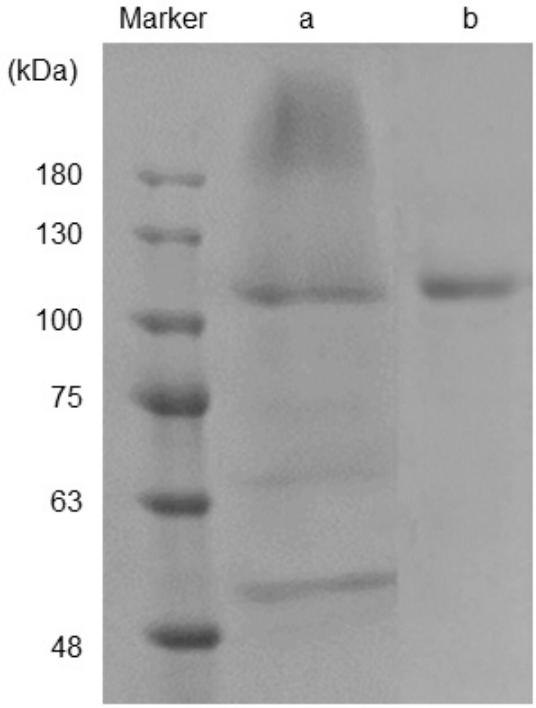


Figure 2

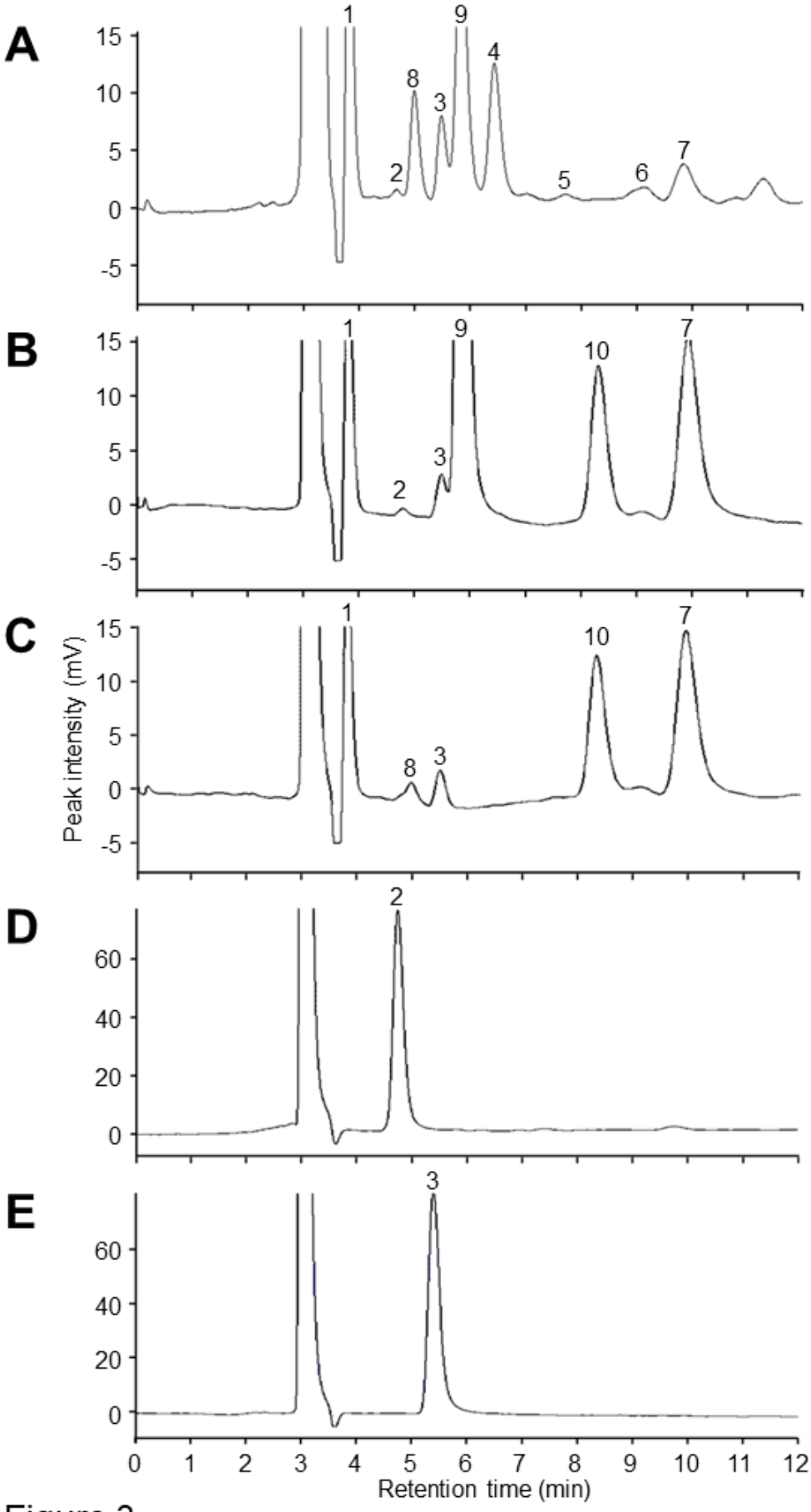


Figure 3

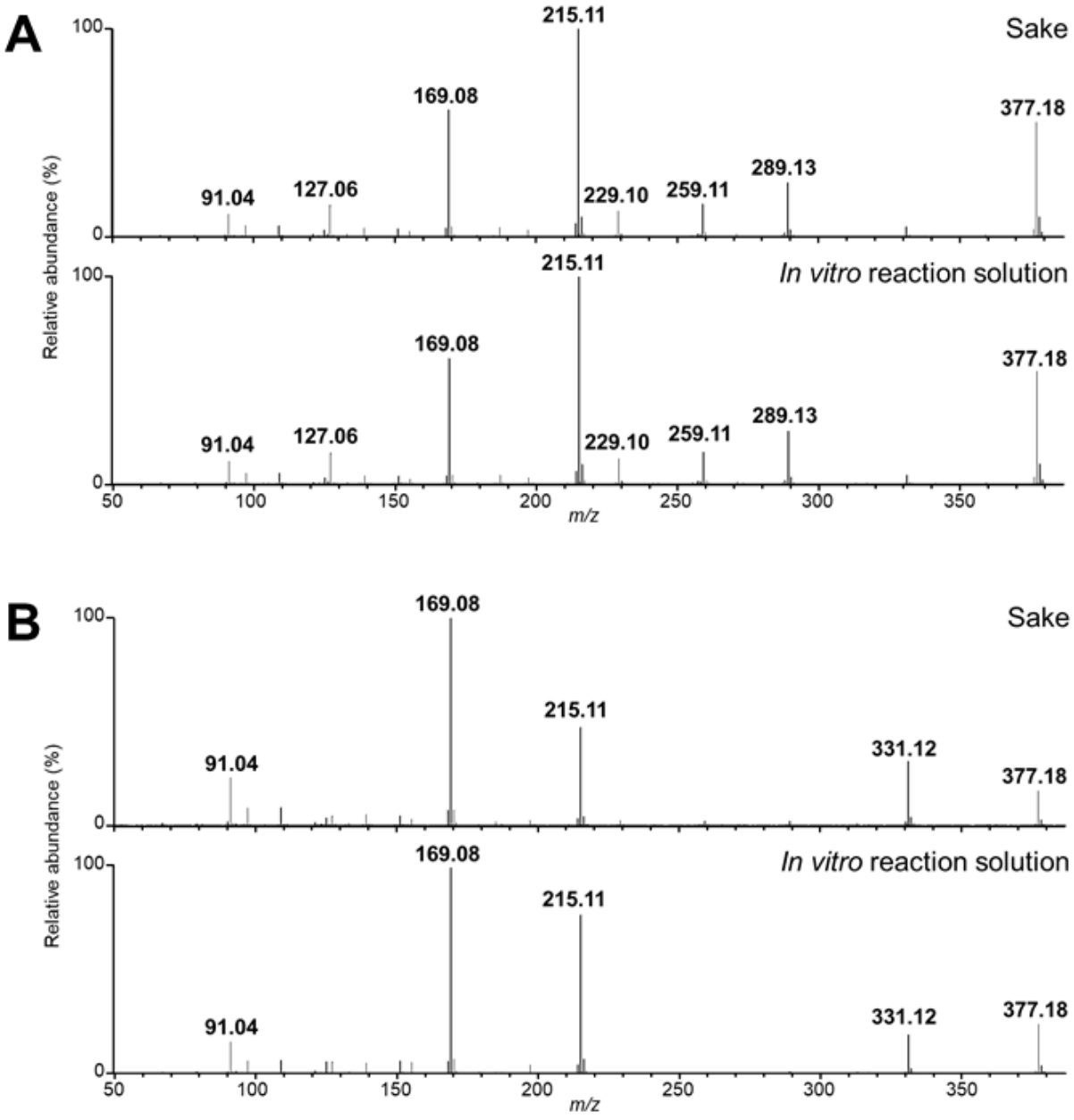
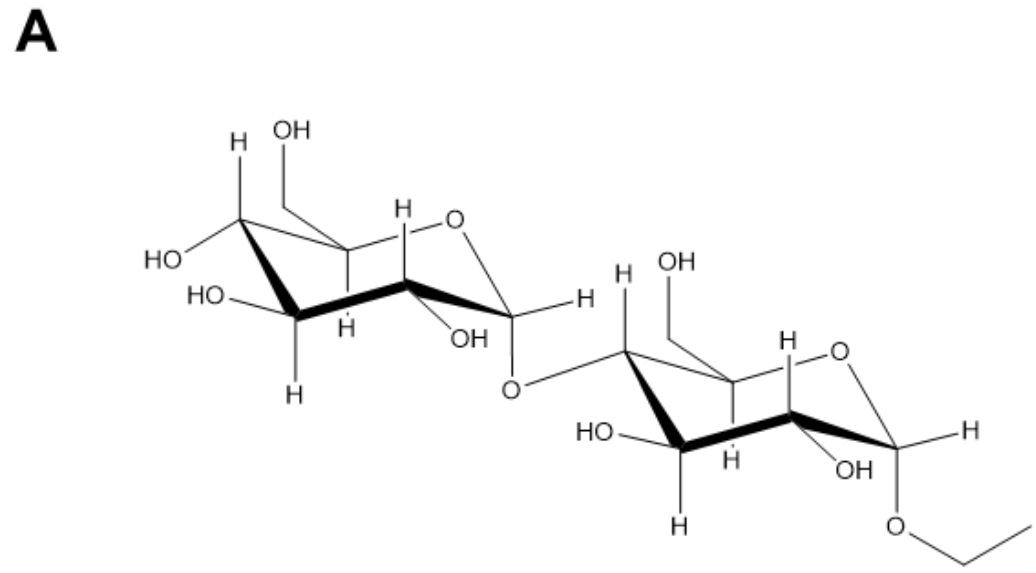

A
Sake
215.11
169.08
377.18
289.13
91.04
127.06
229.10
259.11
In vitro reaction solution
Relative abundance (%)
m/z
B
Sake
169.08
215.11
331.12
377.18
91.04
In vitro reaction solution

Figure 4



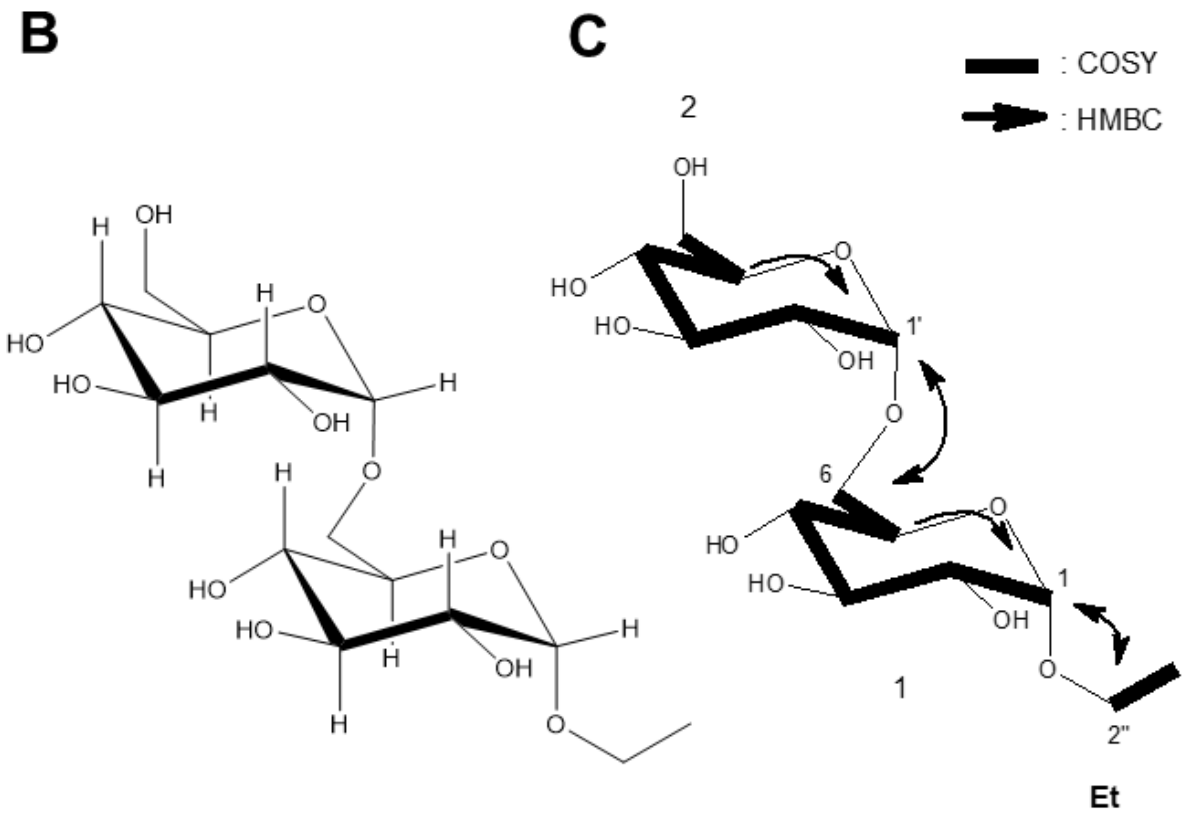


Figure 5

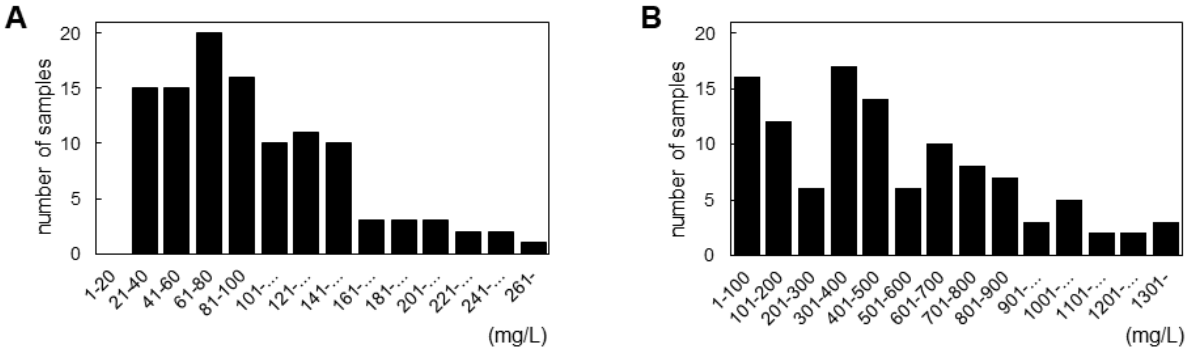
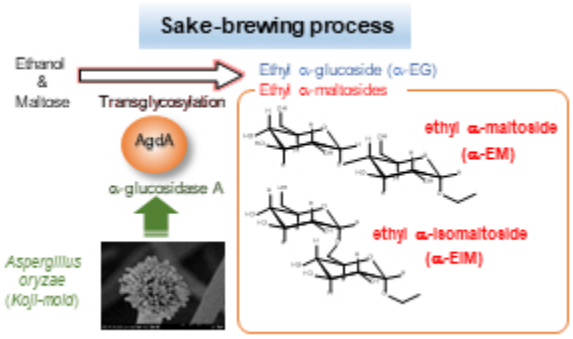

A
number of samples
20
15
10
5
0
1-20
21-40
41-60
61-80
81-100
101-...
121-...
141-...
161-...
181-...
201-...
221-...
241-...
261-
(mg/L)
B
number of samples
20
15
10
5
0
1-100
101-200
201-300
301-400
401-500
501-600
601-700
701-800
801-900
901-...
1001-...
1101-...
1201-...
1301-
(mg/L)

Figure 6



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