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Synthesis of β-D-fructofuranosyl-(2→1)-2-acetamido-2-deoxy-α-D-glucopyranoside (*N*-acetylsucrosamine) using β-fructofuranosidase-containing *Aspergillus oryzae* mycelia as a whole-cell catalyst

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ABSTRACT

Using soft granules consisting of Celite 535 and dried *Aspergillus oryzae* NBRC100959 mycelia containing β-fructofuranosidase as a whole-cell catalyst, *N*-acetylsucrosamine [β-D-fructofuranosyl-(2→1)-2-acetamido-2-deoxy-α-D-glucopyranoside] was produced from sucrose and 2-acetamido-2-deoxy-D-glucose by enzymatic transfructosylation. The isolated yield of *N*-acetylsucrosamine from the reaction mixture was 22.1% (from sucrose). The result of N-terminal amino acid sequence analysis indicated that the enzyme involved in the synthesis of *N*-acetylsucrosamine is a product from gene (NCBI accession number; NW_001884675, locus tag; AOR_1_1114084) encoding putative β-fructofuranosidase on chromosome 6 of strain NBRC100959. The *N*-acetylsucrosamine we produced is highly soluble in water and is more stable in acidic solution than sucrose. The disaccharide was also produced using dried mycelia prepared from another *A. oryzae* strains.

1. Introduction

Various types of mono-and oligosaccharides that exhibit physiologically useful functions have been developed. These carbohydrates are prepared using chemical or enzymatic degradation of biomass polysaccharides or through enzymatic conversion of abundant oligosaccharides. Considerable recent research has focused on 2-acetamido-2-deoxy-D-glucose (GlcNAc), a monosaccharide obtained by the hydrolysis of the biomass polysaccharide chitin, because evidence suggests that intake of this saccharide may prevent skin aging and ameliorate the effects of arthrosis deformans by increasing the production of mucopolysaccharides such as hyaluronic acid and chondroitin.[1–3] Reports also suggest that the oligosaccharides that incorporate GlcNAc may have physiologically beneficial properties. For example, lacto-*N*-biose I [β-D-galactopyranosyl-(1→3)-2-acetamido-2-deoxy-D-glucopyranose], a disaccharide derived from human milk oligosaccharides, is a candidate bifidus factor.[4] Several reports confirmed that various disaccharides that incorporate GlcNAc inhibit the attachment of a range of pathogens to alveolar epithelial cells.[5,6] From these facts, it is expected that various types of oligosaccharides containing GlcNAc will show physiologically useful functions. However, development of such type of functional oligosaccharide has scarcely been performed until now. Then, we planned to develop a new type of GlcNAc-containing functional oligosaccharide, using enzymatic reactions.

Our initial attempts to enzymatically produce oligosaccharide involved attaching fructose to GlcNAc by exploiting the transfructosylation activity of β-fructofuranosidase. Although β-fructofuran osidase is an enzyme that catalyzes sucrose hydrolysis, certain isoforms of the enzyme possess potent transfructosylation activity in solutions containing a high concentration of sucrose. Due to the enzyme's high transfructosylation activity, a number of researchers classified it as a β-fructosyltransferase (EC 2.4.1.99) in order to emphasize the transfructosylation activity. The transfructosylation reaction catalyzed by β-fructofuranosidase can be used to produce several beneficial oligosaccharides. For example, fructooligosaccharides such as 1-kestose [β-D-fructofuranosyl-(2→1)-β-D-fructofuranosyl-(2→1)-α-D-glucopyranoside] and nystose [β-D-fructo furanosyl-(2→1)-β-D-fructofuranosyl-(2→1)-β-D-fructofuranosyl-(2→1)-α-D-glucopyranoside] can be produced from sucrose by enzymes found in several *Aspergillus* strains.[7–12] In addition, the trisaccharide lactosylfructoside [β-D-galactopyranosy-(1→4)-α-D-glucopyranosyl-(1→2)-β-D-fructofuranoside] can be produced from sucrose and lactose by β-fructofuranosidase isolated from *Arthrobacter*.[13–17] These oligosaccharides enhance bowel function by increasing the number of beneficial enterobacteria, such as a bifidobacteia.[18–21]

Here, we report the synthesis of an oligosaccharide consisting of fructose and GlcNAc using dried *A. oryzae* mycelia containing an

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active form of the transfructosylation-catalyzing β-fructofuranosidase as a whole-cell catalyst (Fig. 1). The whole-cell catalysis method reported here has the following advantages: (1) extraction of the enzyme from the culture is unnecessary and (2) the enzyme can be easily removed from the reaction mixture.

2. Experimental

2.1. Materials

2-Acetamido-2-deoxy-D-glucose (GlcNAc) was purchased from TCI Fine Chemicals (Tokyo, Japan). Sucrose, glucose, fructose, 1-kestose, nystose, and diatomaceous earth Celite 535 were obtained from Wako Pure Chemical Industries (Osaka, Japan). The mold cell wall dissolution enzyme Yatalase was purchased from Takara Bio (Shiga, Japan). All *A. oryzae* strains used in this study were obtained from the Biotechnology Section of the National Institute of Technology and Evaluation (Chiba, Japan).

2.2. Preparation of soft granules containing dried *A. oryzae* mycelia

A 500-μL suspension of *A. oryzae* spores was mixed with 100 mL of fresh medium (pH 5.6) containing 2% sucrose, 0.5% peptone, 0.2% $NaNO_3$, 0.1% K_2HPO_4, 0.05% $MgSO_4{\cdot}7H_2O$, 0.05% KCl, 0.001% $FeSO_4{\cdot}7H_2O$, and 4% Celite 535, and mycelia were cultivated by shaking (135 rpm) for 72 h at 28 °C. Wet granules consisting of *A. oryzae* mycelia and Celite 535 were collected by suction filtration using no. 5A filter paper (Kiriyama, Tokyo, Japan), suspended in 50 mL of cold acetone, and then incubated on ice for 30 min. After the granules were collected by suction filtration using no. 5A filter paper, they were re-suspended in 50 mL of cold acetone again. Dehydrated granules were collected by suction filtration using no. 5A filter paper and then dried under decompression at room temperature using an aspirator to produce soft granules consisting of dried *A. oryzae* mycelia and Celite 535 (Fig. 2A). The content of dried mycelia in the granules was about 20% (w/w) with all *A. oryzae* strains used in this study. These values were calculated by subtracting the weight of Celite 535 used from the weight of the granules. Granules were observed under a TM-1000 tabletop electron microscope (Hitachi High-Technologies, Tokyo, Japan).

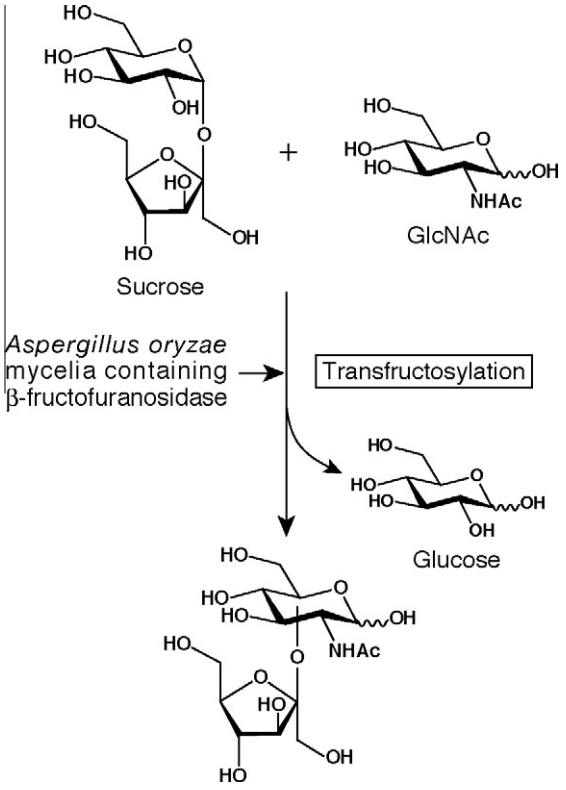

Figure 1. Schematic representation of the transfructosylation of sucrose and GlcNAc.

2.3. Oligosaccharide analysis

Oligosaccharide production was monitored using thin layer chromatography (TLC) and high performance liquid chromatography (HPLC). The TLC plates (Silica Gel 60 N, 230–400 mesh, E. Merck, Darmstadt, Germany) were developed twice using *n*-BuOH/pyridine/H_2O (8:3:1) as the mobile phase. Compounds were visualized by spraying the plates with an aqueous solution containing 2.4% $H_3(PMo_{12}O_{40}){\cdot}nH_2O$, 5% H_2SO_4, and 1.5% H_3PO_4, followed by heating. The HPLC analyses were performed using a LC-10AS pump (Shimadzu, Kyoto, Japan) equipped with a Shodex RI-71 differential refractometer (Showa Denko, Tokyo, Japan) and a COSMOSIL Sugar-D column (size; φ4.6 × 250 mm, Nacalai Tesque, Kyoto, Japan). Oligosaccharides were separated under isocratic conditions using 77% (v/v) CH_3CN in water (flow rate = 0.8 mL min^{-1}) as the mobile phase. The amount of oligosaccharide in the reaction mixture was estimated based on comparison of peak area with a standard curve. The transfructosylation product was characterized using ^1H and ^{13}C NMR spectrometry as well as mass spectrometry (MS). The ^1H and ^{13}C NMR spectra were recorded in D_2O at 25 °C using a VXR-400 spectrometer (Varian, Palo Alto, CA, USA). Chemical shifts were expressed as downfield shifts (ppm) from 3-(trimethylsillyl)propionate-d_4. Mass spectra were recorded using a ZQ4000 LC–MS instrument (Waters, Milford, MA, USA) under positive-ion direct electrospray ionization (ESI) conditions. The optical rotation was measured in H_2O at 20 °C using a P-1030 polarimeter (Jasco, Tokyo, Japan). The stability of the oligosaccharide was measured at several temperatures in the following buffers: 20 mM glycine–HCl (pH 3), 20 mM Na citrate (pH 5), 20 mM Na phosphate (pH 7), and 20 mM glycine–NaOH (pH 9). Buffer solutions containing each oligosaccharide (1%, w/v) were maintained at various temperatures (25, 50, 75, and 100 °C) for 1 h, and then the compound in the solution was subjected to TLC and HPLC analysis.

2.4. Oligosaccharide production and purification

Soft granules containing 700 mg of dried *A. oryzae* NBRC100959 mycelia were added to a mixture of sucrose (10 g, 29.2 mmol) and GlcNAc (19.4 g, 87.7 mmol) in 100 mL of 20 mM Na citrate buffer (pH 5.5) and the mixture was incubated at 30 °C with gentle shaking. It should be noted that this reaction solution is almost saturated with carbohydrates. At specific intervals during the enzymatic reaction, the progress of oligosaccharide production was monitored using TLC and HPLC. After 8 h of incubation, the granules were removed by filtration using no. 5A filter paper. The resulting supernatant containing the reaction products was loaded onto a charcoal column (particle size: 63–300 mm, Wako Pure Chemical Ind.; column size: ϕ5.7 × 41 cm; solvent: H_2O), and the monosaccharides (GlcNAc, glucose, and fructose) contained in the reaction mixture were eluted with water. The oligosaccharides that adhered to the charcoal were eluted with 5% EtOH in water. Fractions containing the products were collected and concentrated by evaporation, and the target oligosaccharide was further purified by chromatography using an UltraPack NH-40C column [Yamazen, Osaka, Japan; size: ϕ5 × 30 cm; solvent: 80% (v/v) 2-PrOH in water; flow rate: 8 mL min^{-1}]. After the fractions containing the

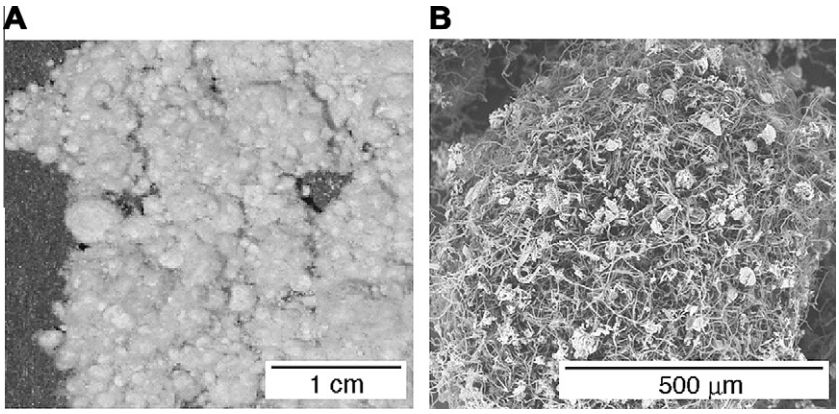

Figure 2. Soft granules composed of dried *A. oryzae* NBRC100959 mycelia and Celite 535. (A) Photograph of soft granules. (B) Electron micrograph of a soft granule.

oligosaccharide were collected and concentrated by evaporation, the sample was lyophilized to produce a white powder as the final product.

2.5. Assay of β-fructofuranosidase hydrolytic activity

The hydrolytic activity of β-fructofuranosidase was assayed using sucrose as a substrate, and was monitored by measuring the amount of reducing sugar released from sucrose. The enzyme solution (50 μL) was added to 50 μL of 0.4% sucrose in 20 mM Na citrate buffer (pH 5.5) and the mixture was incubated at 30 °C. After incubation, the enzymatic reaction was stopped by heating the sample at 95 °C for 5 min. The amount of reducing sugar in the reaction mixture was determined by the Somogyi–Nelson method using glucose as a standard.[22] One unit (U) of enzyme activity was defined as the amount of enzyme required to hydrolyze 1 μmol of sucrose per minute under the assay conditions.

2.6. β-Fructofuranosidase purification and oligosaccharide production

Soft granules containing 5.82 g of dried *A. oryzae* NBRC100959 mycelia were suspended in 600 mL of 10 mM Na phosphate buffer (pH 6.0) containing 0.6 M $(NH_4)_2SO_4$ and 500 mg of Yatalase, and the suspension was incubated with gentle shaking at 30 °C for 3 h, after which insoluble material was removed from the suspension by suction filtration using no. 5A filter paper. Proteins in the resulting supernatant were precipitated by adding $(NH_4)_2SO_4$ (30–90% saturation) and collected by centrifugation (9100×*g*, 4 °C, 15 min). The precipitate containing β-fructofuranosidase was dissolved in 10 mL of 10 mM Na citrate buffer (pH 6.0), dialyzed against the same buffer, and the resulting solution was filtered using a Dismic-25CS syringe filter unit (pore size: 0.2 μm, Advantec MFS, Tokyo, Japan) to obtain crude enzyme solution. The crude enzyme solution was loaded onto a TOYOPEARL SuperQ-650 M ion exchange resin (Tosoh, Tokyo, Japan) column (size: ϕ1.5 × 27 cm) pre-equilibrated with 10 mM Na citrate buffer (pH 6.0), and the enzyme was eluted with a linear gradient of 0–0.25 M NaCl in the same buffer. Two β-fructofuranosidase fractions were collected and concentrated separately using an Amicon Ultra-15 10 K centrifugal filter device (Millipore, Billerica, MA, USA) to obtain a solution of partially purified enzyme. The β-fructofuranosidase in the first fraction was purified by gel filtration column chromatography using a Bio-Gel P-100 fine resin (Bio-Rad, Hercules, CA, USA) [size: ϕ2 × 78 cm; elution: 20 mM Na citrate buffer (pH 5.5)]. The active fractions were collected and the enzyme was again subjected to ion exchange column chromatography using a TOYOPEARL SuperQ-650 M resin [buffer: 10 mM Na citrate (pH 6.0); column size: ϕ1.5 × 20 cm; elution: linear gradient of 0–0.15 M NaCl]. The enzyme was further purified by affinity chromatography using a Con A sepharose 4B (size: ϕ1 × 5.5 cm, GE Healthcare, Buckinghamshire, England) column pre-equilibrated with 50 mM McIlvain buffer (pH 6.5) containing 0.5 M NaCl. The enzyme was eluted from the column with a linear gradient of 0–0.6 M methyl α-D-mannoside in 100 mM McIlvain buffer (pH 6.5) containing 0.5 M NaCl. The homogeneity of the enzyme preparation was confirmed by SDS–PAGE. Proteins in the polyacrylamide gels were stained using Coomassie Brilliant Blue R250 (TCI Fine Chemicals). The N-terminal amino acid sequence of the purified enzyme was determined using a Procise 492 cLC protein sequencer (Life Technologies, Carlsbad, CA, USA).

A 100-μL aliquot of the partially purified enzyme solution was added to a mixture of sucrose (100 mg, 292 μmol) and GlcNAc (194 mg, 877 μmol) in 1 mL of 20 mM Na citrate buffer (pH 5.5), and the mixture was incubated at 30 °C with gentle shaking. At specific intervals during the enzymatic reaction, the progress of oligosaccharide production was monitored using TLC.

2.7. Oligosaccharide production by various strains of *A. oryzae*

Granules containing 90 mg of dried mycelia of each *A. oryzae* strain were added to a mixture of sucrose (1 g, 2.92 mmol) and GlcNAc (1.94 g, 8.77 mmol) in 10 mL of 20 mM Na citrate buffer (pH 5.5). The mixture was incubated at 30 °C with gentle shaking for 8 h, after which the reaction solution was centrifuged (3800×*g*, 4 °C, 5 min) and the transfructosylation product in the resulting supernatant was quantified using HPLC.

3. Results and discussion

3.1. Preparation of soft granules containing dried *A. oryzae* mycelia

β-Fructofuranosidases of several species of *Aspergillus*, including *A. oryzae*, have been extracted from mycelia after cultivation in liquid medium.[12,23–27] In the present study, we confirmed that β-fructofuranosidase is released from *A. oryzae* NBRC100959 mycelia grown in liquid medium containing sucrose by treating mycelia with the mold cell wall dissolution enzyme Yatalase. These results indicate that β-fructofuranosidase is contained within the *A. oryzae* mycelia grown in liquid culture. Thereupon, to synthesize the oligosaccharide through a β-fructofuranosidase-catalyzed transfructosylation reaction, we decided to use *A. oryzae* mycelia that incorporate this enzyme as a whole-cell catalyst.

Through preliminary investigations to determine the state of mycelia that would promote the efficient β-fructofuranosidase reaction, it was found that the soft granules (Fig. 2A) consisting of dried *A. oryzae* NBRC100959 mycelia and Celite 535 show high activity. The soft granules were prepared by performing acetone treatment followed by drying to mycelia grown in liquid medium containing sucrose and Celite. Although strain NBRC100959 died out as a result of the manipulations, the intracellular β-fructofuranosidase remained in an active form. Electron microscopy confirmed that a considerable amount of space exists within the soft granule in which the dried mycelia and the Celite were dispersed (Fig. 2B). We hypothesize that this structure provides for optimal contact between the substrate and the enzyme, which is contained in mycelia, in the reaction solution. Based upon these findings, we decided to use the soft granules for production of the oligosaccharide.

3.2. Oligosaccharide production, identification, and characterization

Oligosaccharide was synthesized by adding soft granules containing 700 mg of dried *A. oryzae* NBRC100959 mycelia to 100 mL of 20 mM Na citrate buffer (pH 5.5) containing a high concentration of substrate carbohydrates. The reason this buffer solution was used is that optimum pH of *A. oryzae* β-fructofuranosidase is around 5.5. Considering low heat stability of this enzyme, oligosaccharide synthesis was performed at a comparatively low temperature (30 °C). A TLC analysis of the reaction mixture in which only sucrose (10%, w/v) was added as a substrate revealed that fructooligosaccharides such as 1-kestose and nystose were generated as the transfructsylation products (data not shown). This result indicates that the β-fructofuranosidase contained in the dried *A. oryzae* mycelia catalyzed transfructosylation reaction. In contrast, no reaction products were detected in the mixture in which only GlcNAc (19.4%, w/v) was added as a substrate (data not shown). On the other hand, in the reaction mixture containing sucrose (10%, w/v) and GlcNAc (19.4%, w/v) (sucrose/GlcNAc molar ratio 1:3), an unknown reaction product was generated in addition to the fructooligosaccharides (Fig. 3). This unknown product (compound **1**, indicated by arrow **1** in Fig. 3) was purified from the reaction mixture, and its structure was identified using MS and NMR spectrometry.

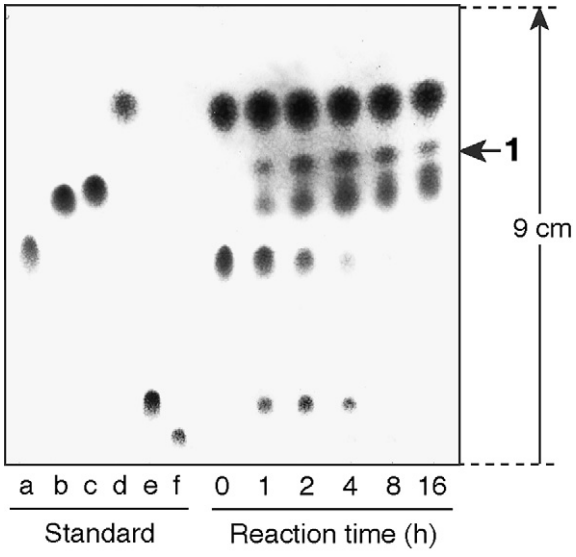

Figure 3. TLC of the compounds formed in the transfructosylation reaction. Carbohydrates in the mixture were identified based on comparison with standards: sucrose (a), glucose (b), fructose (c), GlcNAc (d), 1-kestose (e), and nystose (f). Arrow **1** represents the unknown reaction product (compound **1**).

The ESIMS spectra of compound **1** corresponded to a $[M+H]^+$ species at *m*/*z* 384, suggesting that this compound is a disaccharide consisting of fructose and GlcNAc. The ^1H and ^{13}C NMR assignments of compound **1** are shown in Figure 4A. The position of the glycosidic linkage between fructose and GlcNAc was determined using ^1H-detected multiple bond connectivity (HMBC) NMR experiments. The anomeric hydrogen of GlcNAc (H-1) exhibited cross peaks with C-3 and C-5 of GlcNAc and C-2 of fructose (C-2′) (Fig. 4B). The anomeric hydrogen (H-1) of GlcNAc was found to have a coupling constant ($J_{1,2}$) of 4.0 Hz. These results enabled us to identify compound **1** as a *N*-acetylsucrosamine [β-D-fructofuranosyl-(2→1)-2-acetamido-2-deoxy-α-D-glucopyranoside].

We had confirmed that *N*-acetylsucrosamine was produced most efficiently in the solution containing 10% (w/v) sucrose and 19.4% (w/v) GlcNAc. Of course, the quantity of production of *N*-acetylsucrosamine may increase if GlcNAc concentration is still higher. However, concentration of GlcNAc cannot be raised any more, because this monosaccharide is mostly saturated in the solution. The production efficiency of *N*-acetylsucrosamine was not influenced so much by the shaking speed of the reaction mixture. *N*-Acetylsucrosamine was generated as a major transfructosylation product through the reaction (Fig. 5), and cumulative dosage of this disaccharide was the highest at the reaction for 8 h. By performing 2 types of column chromatography, 2.47 g (22.1% yield from sucrose) of the disaccharide was isolated from 100 mL of the reaction mixture after 8-h shake. The optical rotation ($[\alpha]_D^{25}$) of *N*-acetylsucrosamine obtained in our experiment were 80.7° (*c* 0.96, H_2O) [lit. $[\alpha]_D^{25}$ 83.6° (*c* 0.5, H_2O)[28]]. More than 8 g of *N*-acetylsucrosamine was dissolved in 2 mL of water to form colorless high-viscosity syrup, indicating that this disaccharide is highly soluble in water. Although neither *N*-acetylsucrosamine nor sucrose decomposed upon incubation at 100 °C for 1 h in buffers of pH 5, 7, or 9 (data not shown), both disaccharides were hydrolyzed to monosaccharides when incubated at 75 °C and 100 °C for 1 h in buffer solution of pH 3. However, the degree of *N*-acetylsucrosamine hydrolysis in buffer of pH3 was lower than that of sucrose, indicating that *N*-acetylsucrosamine is more stable under acidic conditions than sucrose. The data about the stability of this disaccharide are presented in Supplementary Figure S1.

Römer et al. reported the sucrose synthase-mediated synthesis of *N*-acetylsucrosamine from sucrose and uridine diphosphate GlcNAc (UDP-GlcNAc) [overall molar yield: 59% (from UDP-GlcNAc)],[29] while Lichtenthaler et al. synthesized *N*-acetylsucrosamine from sucrose using a seven-step chemical synthesis (overall molar yield: 5.76%).[28] In our method, *N*-acetylsucrosamine was synthesized in a one-step reaction using low-cost carbohydrate substrates and dried *A. oryzae* mycelia, which can be prepared easily, as a catalyst. Our results demonstrate that the method described here is a simpler and lower cost alternative for the production of *N*-acetylsucrosamine.

3.3. Confirmation of *N*-acetylsucrosamine-producing enzyme

We also investigated the enzyme involved in the synthesis of *N*-acetylsucrosamine from sucrose and GlcNAc. The enzyme was extracted from dried *A. oryzae* NBRC100959 mycelia by treating the granules with the mold cell wall dissolution enzyme Yatalase, and was partially purified using anion exchange chromatography on a TOYOPEARL SuperQ-650 M column. The β-fructofuranosidase activity was separated into 2 fractions, Fa and Fb (Fig. 6A). Each partially purified enzyme fraction was added to a solution containing high-concentration sucrose and GlcNAc, and the reaction product was analyzed using TLC. The enzyme contained in the first anion exchange fraction (Fa) synthesized *N*-acetylsucrosamine

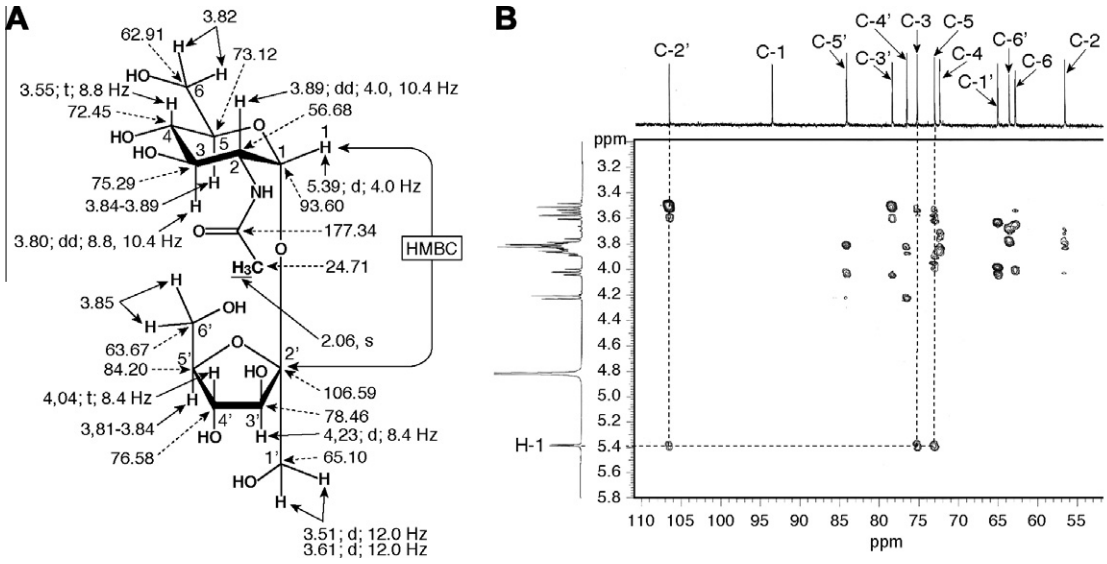

Figure 4. Identification of the transfructosylation product. (A) ^{1}H and ^{13}C NMR shifts and the structure of compound **1**. ^{1}H NMR data (chemical shift in ppm; multiplicity; coupling constant in Hz) and ^{13}C NMR chemical shifts (ppm) are designated with arrows and dashed line arrows, respectively. The bent double-headed arrow indicates the HMBC cross peak. (B) HMBC NMR cross peaks with the GlcNAc anomeric hydrogen of compound **1**. Peak H-1 is the anomeric hydrogen of the GlcNAc residue of compound **1**. Peaks C-1, 2, 3, 4, 5, and 6 are ring carbons of the GlcNAc residue of compound **1**. Peaks C-1′, 2′, 3′, 4′, 5′, and 6′ are ring carbons of the fructose residue of compound **1**. Dashed lines indicate crossing between the peak of H-1 and those of C-3, C-5, and C-2′.

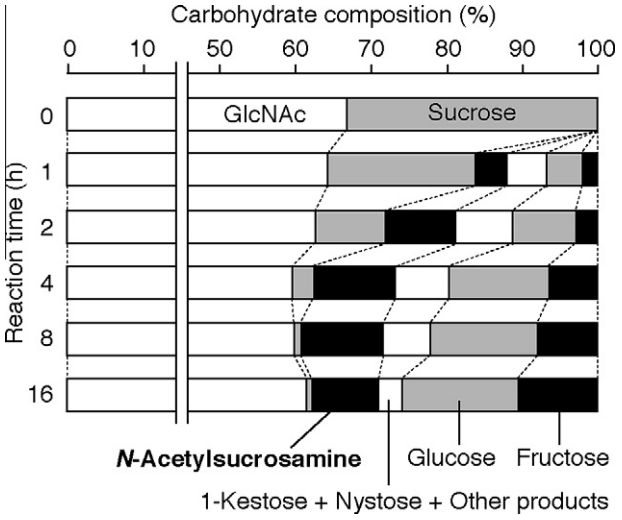

Figure 5. Carbohydrate composition in reaction mixture. The total amount of carbohydrate in the reaction mixture at each time point was taken as 100%.

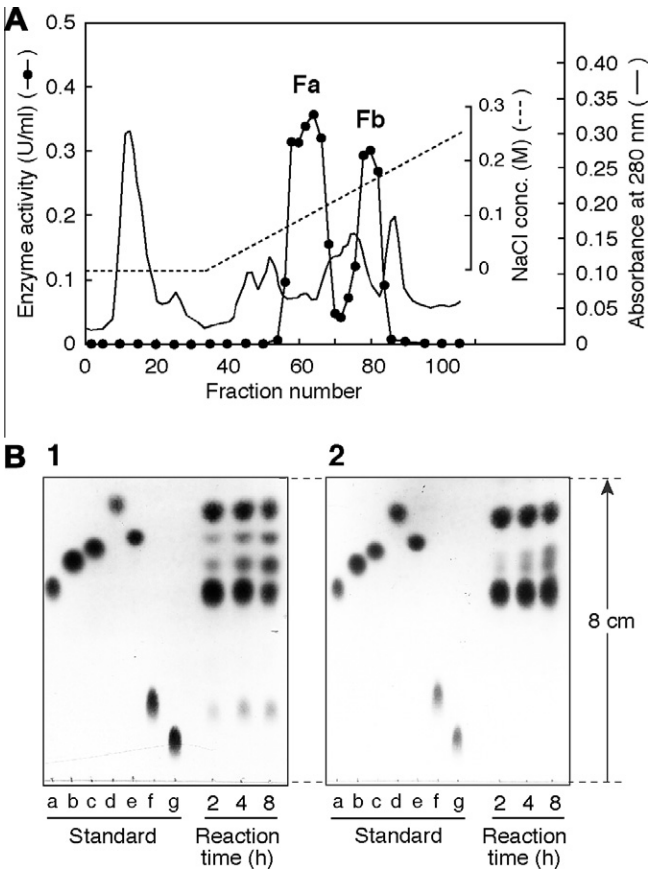

Figure 6. Enzyme purification and *N*-acetylsucrosamine production by partially purified β-fructofuranosidases. (A) Chromatogram showing separation of two kinds of β-fructofuranosidases by ion exchange column chromatography. (B) TLC of the carbohydrates in the reaction mixture. β-Fructofuranosidase (0.65 U) contained in Fa and that (1.55 U) contained in Fb were added separately to a substrate solution. Carbohydrates in the mixture were identified based on comparison with standards: sucrose (a), glucose (b), fructose (c), GlcNAc (d), *N*-acetylsucrosamine (e), 1-kestose (f), and nystose (g).

(main product) and 1-kestose (minor product) (Fig. 6B1), while the enzyme contained in the second anion exchange fraction (Fb) catalyzed only the hydrolysis of sucrose (Fig. 6B2).

Sequencing of the *A. oryzae* NBRC100959 genome (37 Mb) was accomplished in 2005.[30] Then, in order to identify the β-fructofuranosidase involved in the synthesis of *N*-acetylsucrosamine, we purified the enzyme contained in Fa and determined its N-terminal amino acid sequence. Each chromatographic step produced a single peak showing β-fructofuranosidase activity. The purified protein gave a single band on SDS–PAGE analysis (Supplementary Fig. S2). We confirmed that this purified protein catalyzed *N*-acetylsucrosamine production in a reaction mixture containing high-concentration sucrose and GlcNAc (data not shown). The N-terminal amino acid sequence of this protein was determined as IDYNA. This sequence exists in N-terminal region of the predicted protein from gene (NCBI accession number; NW_001884675, locus tag;

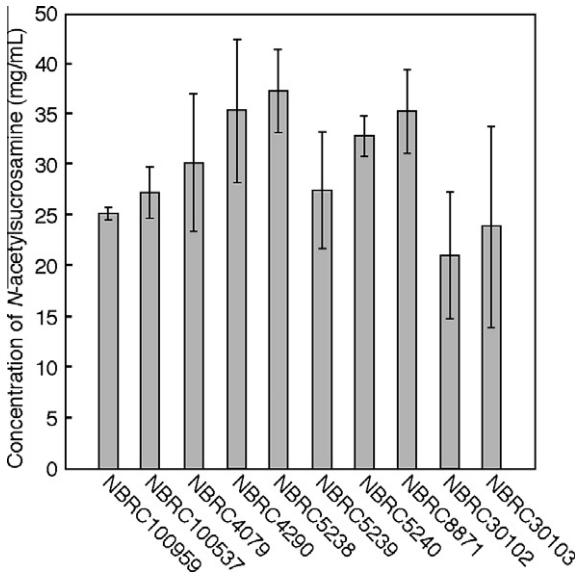

Figure 7. *N*-Acetylsucrosamine production by various strains of *A. oryzae*. This experiment was conducted using three lots of granules prepared separately with each strain. The concentration of *N*-acetylsucrosamine in each reaction mixture was determined. Values are represented as average ± SD of three independent experiments using different granule samples.

AOR_1_1114084) encoding putative β-fructofuranosidase on chromosome 6 of strain NBRC100959. A query of the genome database DOGAN [database of the genomes analyzed at the National Institute of Technology and Evaluation (NITE), http://www.bio.nite.go.jp/dogan/top] indicated that two β-fructofuranosidase genes are located on chromosome 5 of *A. oryzae* NBRC100959. The sequence IDYNA does not exist in the N-terminal regions of the putative proteins from these two genes. Based upon these findings, we concluded that the β-fructofuranosidase involved in the synthesis of *N*-acetylsucrosamine in our experiments was the product from gene AOR_1_1114084 on chromosome 6 of *A. oryzae* NBRC100959.

3.4. *N*-Acetylsucrosamine production by various strains of *A. oryzae*

Soft granules composed of dried mycelia containing β-fructofuranosidase and Celite 535 were prepared using various strains of *A. oryzae* isolated from different sources, and the production of *N*-acetylsucrosamine by each strain was compared. There were no remarkable differences between any of the strains, including strain NBRC100959, with respect to production of *N*-acetylsucrosamine (Fig. 7), indicating that *A. oryzae* strains produce similar quantities of the intracellular β-fructofuranosidase that catalyzes the transfructsylation of sucrose and GlcNAc to produce *N*-acetylsucrosamine.

4. Conclusion

We described the synthesis of *N*-acetylsucrosamine using *A. oryzae* mycelia as a whole-cell catalyst and identified the enzyme involved in the synthesis of this disaccharide. *N*-Acetylsucrosamine was efficiently produced from sucrose and GlcNAc via transfructosylation in the presence of soft granules composed of Celite 535 and dried *A. oryzae* NBRC100959 mycelia containing an active form of β-fructofuranosidase. We believe this methodology represents a low-cost and easy alternative for the production of *N*-acetylsucrosamine. From the result of N-terminal amino acid sequence analysis, it was indicated that the enzyme involved in the synthesis of this disaccharide is a product from gene AOR_1_1114084 encoding putative β-fructofuranosidase on chromosome 6 of strain NBRC100959. We clarified that *N*-acetylsucrosamine is highly water soluble and more stable than sucrose in acidic solution. Our results also indicate that dried mycelia prepared from a variety of *A. oryzae* strains produce about the same quantity of *N*-acetylsucrosamine.

We plan to develop *N*-acetylsucrosamine as a functional oligosaccharide. Because *A. oryzae* is widely used in the Japanese food industry, we believe that from a safety standpoint it would be suitable to use this microbe in the production of *N*-acetylsucrosamine. In the near future we will begin investigating the possible physiological uses of this disaccharide.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.carres.2012.03.037.

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