DOI:10.1021/jf903303w

JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY
ARTICLE

Production of Fructooligosaccharides by β-Fructofuranosidases from *Aspergillus oryzae* KB

Masahiro Kurakake,* Ryo Masumoto, Kunihiro Maguma, Akiko Kamata, Etsuko Saito, Norihiro Ukita, and Toshiaki Komaki

Faculty of Life Science and Biotechnology, Department of Life and Nutritional Science, Fukuyama University, Sanzou, Gakuenchou 1 banchi, Fukuyama, Hiroshima 729-0292, Japan

Aspergillus oryzae KB produces two types of β-fructofuranosidases: F1 and F2. F1 produces the fructooligosaccharides (FOSs) 1-kestose, nystose, and fructosyl nystose from sucrose through a transfructosylation action, whereas F2 mainly hydrolyzes sucrose to glucose and fructose. F1 and F2 enzymes were more selectively produced from the KB strain in liquid media with a sucrose concentration >2% and <2%, respectively. Immobilization using an anion-exchange resin (WA-30; polystyrene with tertiary amine) and cross-linking with glutaraldehyde depressed the hydrolysis reaction of F2 (high hydrolyzing enzyme) alone and enhanced the thermal stability of F1 (high transferring enzyme). F1 enzyme produced in the high sucrose medium was immobilized, cross-linked, and packed in a tubular reactor for continuous production of FOSs (24.6% 1-kestose, 21.6% nystose, 5.7% and fructosyl nystose). In a long-term operation in which 60% sucrose was inputted at 55 °C, the composition of FOSs produced was 51.9% (transfer ratio: 92%), and production by the immobilized enzyme was maintained for 984 h.

KEYWORDS: β-Fructofuranosidase; fructooligosaccharides; fructosyltransferase; transfructosylation; immobilization; *Aspergillus*

INTRODUCTION

Fructooligosaccharides (FOSs) such as 1-kestose, nystose, and fructosyl-nystose are produced by β-fructofuranosidase. They are used as food ingredients and have beneficial effects on health in humans (*1*) and improve the growth of bifidobacteria in intestinal flora. In general, β-fructofuranosidase hydrolyzes sucrose to glucose and fructose, but some β-fructofuranosidases can transfer the fructosyl residue to the sucrose molecule at a high concentration of sucrose, in which fructosyl residues are transferred to sucrose by β-2,1 glycosidic bonds (*2*−*9*). β-Fructofuranosidase from *Aspergillus niger* is a well-known commercial enzyme for FOSs (*10*, *11*).

β-Fructofuranosidases have differing fructosyl-transferring activities. Hidaka et al. (*11*) investigated the hydrolyzing (U_h) and fructosyl-transferring (U_t) activities of β-fructofuranosidase from *Aspergillus niger*, *Aspergillus oryzae*, *Penicillium nigricans*, and *Saccharomyces cerevisiae*. The ratio of U_t/U_h characterized the transferring reaction by the enzyme of each origin (*11*). Their various characterizations may be attributable to the types of β-fructofuranosidase produced. The two types of β-fructofuranosidase from *Aspergillus niger* or *oryzae* have been separated and their enzymatic properties investigated (*12*−*14*).

The KB strain of *Aspergillus oryzae* produced two types of β-fructofuranosidases: F1 and F2. The former produced 1-kestose, nystose, and fructosyl nystose from sucrose through a transfructosylation action ($U_t/U_h = 22$), whereas F2 hydrolyzed sucrose to mainly glucose and fructose ($U_t/U_h = 0.2$). The coding genes of both enzymes have been clarified (*14*).

The production of F1 and F2 from the KB strain was controlled by the sucrose concentration in a liquid medium. At low concentrations of sucrose (< 2%), the production of F2 (with high hydrolyzing activity) was higher than that of F1 (with high fructosyl-transferring activity); F2 production gradually decreased, whereas F1 increased greatly with increasing sucrose concentration; and F1 production was higher than that of F2 in medium containing > 2% sucrose.

Immobilization of enzymes enables reuse of the enzyme and continuous production of FOSs in a tubular reactor. Some immobilizations were attempted to produce a large amount of FOSs, such as entrapment of strains in calcium alginate or gluten as well as covalent binding of enzymes with silica particles, chitosan particles, and ceramic membranes, and resulted in high production abilities (*15*−*19*).

In the present study, immobilization by ion-binding and cross-linking was attempted in the production of FOSs by β-fructofuranosidase from *Aspergillus oryzae* KB and the effect of immobilization on the actions of F1 and F2 enzymes investigated. Production of FOSs was evaluated in a tubular reactor using an immobilized enzyme.

MATERIALS AND METHODS

Materials. 1-Kestose, nystose, and fructosyl nystose were purchased from Wako Pure Chemical Industries Limited (Osaka, Japan). All other chemicals were of reagent grade and were available commercially.

*Corresponding author. Tel: 81-084-936-2111. Fax: 81-084-936-2023. E-mail: kurakake@fubac.fukuyama-u.ac.jp.

 Published on Web 12/16/2009

The *Aspergillus oryzae* KB strain was separated from malted rice for the production of the traditional Japanese liquor "sake". Diaion WA-30 of a low basic anion-exchange resin (polystyrene with tertiary amine; particle size, 0.4−0.6 mm) was purchased from Mitsubishikasei Limited (Tokyo, Japan). Lysozyme (origin, egg-white) was purchased from Nagase Chemtex Limited (Osaka, Japan).

Production and Immobilization of β-Fructofuranosidases. The KB strain was cultured in a liquid medium (18 L) containing 2% or 10% sucrose, 2% yeast extract, and 0.2% $Na_2HPO_4 \cdot 12H_2O$ in a 20-L jar fermentor at 30 °C and at 250 rpm for 96 h. Grown mycelial pellets were separated by filtration, washed with distilled water, freeze-dried, and ground to powder by Cyclotec 1093 (Tecator Limited, Hoganas, Sweden). The activities of β-fructofuranosidase prepared from the 2% and 10% sucrose medium were 606 U/g-powder and 234 U/g-powder, respectively. Enzyme activities were determined for the ground-strain suspension in 50 mM acetate buffer (pH 5).

Strain powder (2−4 g) was suspended in 100 mL of 50 mM acetate buffer (pH 5) and 0.2−0.4 g of lysozyme added. The mixture was incubated at 30 °C for 3 h and centrifuged at 1500*g* for 10 min. The clear supernatant contained the β-fructofuranosidase released from the strain. About 100% of the enzyme was recovered. The WA-30 resin was washed several times with distilled water by passing through a 0.22-μm membrane filter. The washed resin (50% water content) of 0.2−1.0 g wet weight was mixed with 5 mL of free enzyme (6−14 U/mL). The mixture was incubated at 125 rpm for 20 h at 4 °C to complete adsorption of the enzyme onto the resin. The immobilized enzyme was washed with distilled water by filtration and stored in the wet state at 4 °C.

For cross-linking with glutaraldehyde, the WA-30-immobilized enzyme (0.25 g wet weight) was mixed with 1−20% glutaraldehyde (0.5 mL) and incubated at 25 °C for 30 min. The cross-linked immobilized enzyme was washed with distilled water by filtration and stored in the wet state at 4 °C.

Determination of Enzymatic Activities. One percent (w/v) sucrose was incubated with the enzymes at pH 5 for 10 min at 40 °C. The reaction (volume, 1 mL) was stopped by the addition of 1 M Na_2CO_3. Glucose produced in the reaction mixture was determined by the glucose oxidase/peroxidase method using a GL-5 kit (Kainos Laboratories Limited, Tokyo, Japan). Enzyme activity was defined as the amount of enzyme that could produce 1 μmol of glucose from sucrose in 1 min.

Production of FOS. The amount of immobilized or free enzyme corresponding to a β-fructofuranosidase activity of 0.3 U or 0.6 U was mixed with 0.5 mL of 50% sucrose dissolved in 50 mM acetate buffer (pH 6). This mixture was incubated at 125 rpm for 24 h at 40 °C. The reaction was stopped by incubating for 10 min in boiling water. The reaction mixture was subjected to high-performance liquid chromatography (HPLC) analysis after dilution and passing through a 0.22-μm membrane filter. The transfer ratio $[(G - F)/G \times 100\ (\%)]$ and the hydrolysis ratio $[F/G \times 100\ (\%)]$ were calculated from the concentration of glucose and fructose.

When a tubular reactor was used, cross-linked immobilized enzyme (5.5 g wet weight) was packed into a 10 cm × 1.0 cm i.d. column and 60% sucrose loaded into the column reactor at a flow rate of 0.03−0.34 mL/min. The reactor was placed in a water bath incubator at 55 °C. The bed volume was 7.2 mL. The output from the reactor was sampled and subjected to HPLC analysis.

Separation of β-Fructofuranosidases. F1 and F2 enzymes were separated after chromatography steps at 4 °C. Free enzyme (produced in 2% sucrose medium) was salted-out by saturated 80% ammonium sulfate. The generated precipitate was separated by centrifugation (1500*g*, 10 min) and dissolved in 0.1 M sodium acetate buffer (pH 5). The crude enzyme solution was subjected to gel filtration on a 50 cm × 2.5 cm i.d. Sephadex G-25 column pre-equilibrated with 10 mM sodium acetate buffer (pH 5). Proteins were eluted with the same buffer at a flow rate of 1.3 mL/min. The fractions with β-fructofuranosidase activity were pooled and subjected to anion-exchange chromatography on a 50 cm × 1.0 cm i.d. Super Q Toyopearl column (Toso Limited, Japan) pre-equilibrated with 10 mM sodium acetate buffer (pH 5). Proteins were eluted with a linear gradient of 0−0.4 M NaCl at a flow rate of 0.16 mL/min.

Sodium Dodecyl Sulfate−Polyacrylamide Gel Electrophoresis (SDS−PAGE). SDS−PAGE was performed with an Atto model AE-6400 using 7.5% polyacrylamide gels (pH 8.8). SDS molecular weight markers (Sigma-Aldrich Co., USA) consisting of carbonic anhydrase (29,000), egg albumin (45,000), bovine albumin (66,000), phosphorylase b (97,400) and β-galactosidase (116,000) were used as the standard proteins.

Analysis of Sugars. FOSs were analyzed by HPLC under the following conditions: column, 250 mm × 4.6 mm i.d. NH2P-50 (Asahi Chemical Industry Company, Limited, Kawasaki, Kanagawa, Japan); mobile phase, acetonitrile−water (70:30 v/v); flow rate, 1.0 mL/min; detector, Hitachi model L-3300 differential refractive index monitor (Hitachi Limited, Tokyo, Japan).

Table 1. Sugar Composition (%) of Reaction Mixture by Free and Immobilized Enzyme

enzyme	fructose	glucose	sucrose	1-kestose	nystose	fructosyl nystose	FOSs
free	15.4	40.1	8.0	21.2	15.3	0.0	36.5
immobilized	2.9	24.8	23.4	33.2	14.6	1.1	48.9

RESULTS AND DISCUSSION

Immobilization of Enzyme on WA-30 Resin. Two types of enzyme were prepared in a liquid culture of *Aspergillus oryzae* KB using 2% and 10% sucrose medium. In the 2% sucrose medium, F1 and F2 were produced to a similar extent, but F1 was mainly produced in the 10% sucrose medium (*14*). In the adsorption of KB β-fructofuranosidase on WA-30 resin, the maximal amount of enzyme (300 U) was adsorbed on 1 g of resin, but the activity of the adsorbed enzyme was reduced to about 15% (immobilization efficiency). With an increasing ratio of resin to free enzyme in the adsorption, the adsorbed enzyme activity on the resin was decreased (50−15 U/g-resin), whereas the immobilization efficiency was increased (15−40%). The conditions for adsorption on WA-30 resin were designed to produce an immobilization efficiency of 30−40%.

Immobilization of Enzyme Produced in 2% Sucrose Medium. The enzyme produced from the KB strain in the 2% sucrose medium was immobilized on WA-30 resin, in which the enzymatic activity was 30 U/g-resin (immobilization efficiency: 38%). The production of FOSs was compared with that of the free (native) enzyme in a reaction with 1.2 U/mL enzyme and 50% sucrose for 24 h. The free enzyme enhanced the composition of fructose through a hydrolysis reaction (**Table 1**). In the 2% sucrose medium, the F2 enzyme was also produced with F1 (which has a higher hydrolyzing activity) and hydrolyzed sucrose, and FOSs to glucose and fructose more efficiently. When free enzyme was immobilized on WA-30 resin, the characteristics of the F1 enzyme were more greatly exposed (F1 has higher transferring activity). The composition of FOSs (1-kestose, nystose, and fructosyl nystose) was, therefore, enhanced to 48.9%, in which the transfer ratio $[(G - F)/G \times 100)]$ was 88%. This transfructosylation was relatively high in comparison with that of other high-transferring enzymes (*2*−*4*). We considered that a decrease in fructose composition and hydrolysis reaction by immobilization is attributable to a reduction in the activity of the F2 enzyme.

Thereafter, F1 and F2 were separated and the effect of immobilization on WA-30 resin on each enzyme investigated. Both enzymes were readily separated by column chromatography. In anion-exchange chromatography of β-fructofuranosidase from the crude enzyme on the Toyopearl Super Q column, β-fructofuranosidase was eluted at about 0.3 M NaCl (**Figure 1A**). The peak fractions (50−56) were used in a reaction with 20% sucrose at pH 5 for 24 h. Two enzymatic fractions (**Figure 1B**) were divided in which the high transfer ratio and high hydrolysis ratio fractions corresponded to F1 and F2, respectively. SDS−PAGE

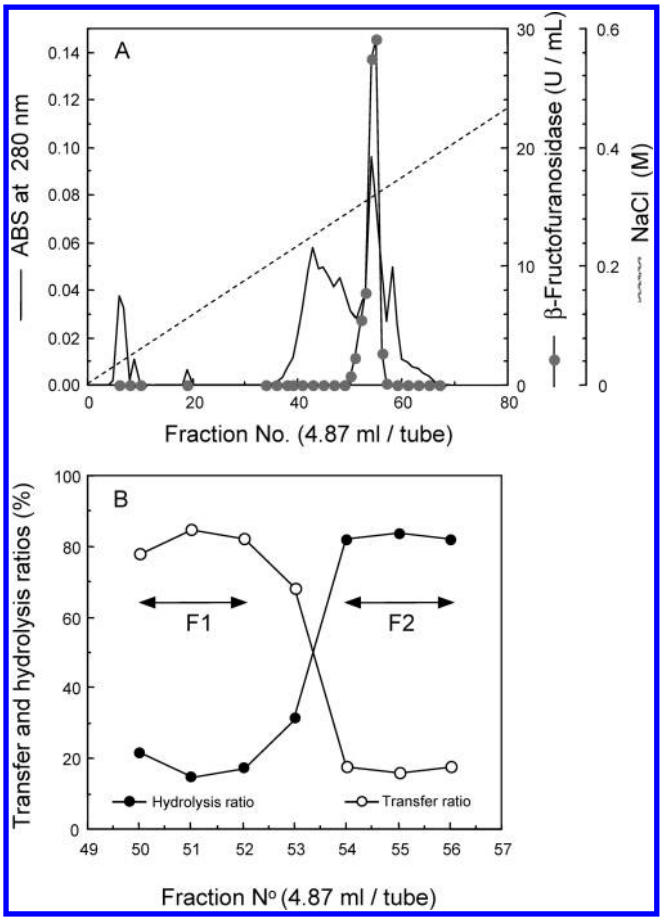

Figure 1. Separation of β-fructofuranosidase on anion-exchange chromatography on a Super Q Toyopearl column.

confirmed that the molecular weights of F1 and F2 were approximately 96 kDa and 79 kDa, respectively (**Figure 2**).

F1 and F2 (adjusted to 0.6 U/mL) were applied to a reaction with 50% sucrose at pH 5 for 24 h and changes in sugar composition analyzed. F1 produced FOSs more efficiently through a high transferring action (**Table 2**). The influence of immobilization on WA-30 resin on the transfructosylation reaction was not observed. The composition of FOSs produced by free and immobilized enzymes was 58.3% and 58.5%, respectively. In the case of the F2 free enzyme, the hydrolysis reaction progressed more efficiently, and glucose and fructose were the main products. The immobilization strongly depressed the hydrolysis reaction of F2, in which 90.1% of sucrose was residual. This suggests that the immobilization of KB β-fructofuranosidase on WA-30 resin is very important for the production of FOSs. For production of the enzyme from the KB strain, even if a small amount of F2 is contaminated with F1, the hydrolysis action is stopped by the immobilization on WA-30 resin, and the high transfer of F1 is maintained.

Immobilization of the Enzyme Produced in 10% Sucrose Medium. Application of F1 enzyme (high transferring activity) for the production of FOSs was investigated. F1 was selectively produced in a liquid culture of the KB strain with the 10% sucrose medium (in which about 90% of the F1 and F2 mixture produced is F1) (*14*). F1 produced FOSs from sucrose more efficiently, but its thermal stability was not high (<40 °C) (*14*). In general, stability at >55 °C is necessary for the application of sugar-related enzymes to prevent contamination by microbes. Improvement of the stability of F1 enzyme was examined by cross-linking with glutaraldehyde. Cross-linking strengthens the binding

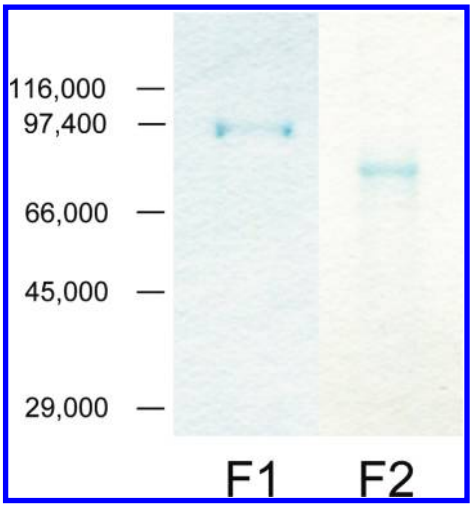

Figure 2. SDS−PAGE of F1 and F2 β-fructofuranosidases.

Table 2. Sugar Composition (%) of Reaction Mixture by F1 and F2 Enzymes

enzyme		fructose	glucose	sucrose	1-kestose	nystose	fructosyl nystose	FOSs
F1	free	1.3	32.0	8.4	22.3	31.3	4.7	58.3
	immobilized	1.0	30.5	10.0	27.0	26.8	4.7	58.5
F2	free	12.6	19.7	64.1	3.6	0.0	0.0	3.6
	immobilized	3.4	5.2	90.1	1.3	0.0	0.0	1.3

between enzyme and WA-30 resin through covalent binding. The free enzyme was immobilized on WA-30 resin and treated with given concentrations of glutaraldehyde. **Figure 3** shows the relationship between the immobilized enzyme activity and the concentration of glutaraldehyde in the cross-linking reaction. Enzyme activity was decreased with increasing concentration of the cross-linking reagent because of the reduction of structural flexibility. During incubation in water at 60 °C for 10 min, the activity of the immobilized enzyme (F1) was decreased to 6.8 U/g-resin from 40 U/g-resin without cross-linking. Enzyme activity was reduced by cross-linking, and the activity of about 20 U/g-resin was maintained by treatments using >4% glutaraldehyde. It is considered that the cross-linking area on the surface of WA-30 resin is local and is dependent on the distribution of the enzyme on the surface. In this experiment, the condition of 12% glutaraldehyde was selected, which reduced the deactivation at 60 °C.

The cross-linked immobilized enzyme (25 U/g-resin) was packed into a tubular reactor and continuous production of FOSs at 55 °C evaluated. Sucrose (60%) was placed into the reactor at a flow rate of 0.04−0.34 mL/min. **Figure 4** shows the change in sugar composition as a function of residence time in the reactor. At the residence time of 21 min, the composition of 1-kestose was maximal and decreased as those of nystose and fructosyl nystose increased. 1-Kestose was used as an acceptor in the following transferring reaction by F1. The production of FOSs over 35 min was almost constant. The immobilized enzyme demonstrated high production of FOSs in the tubular reactor.

At the residence time of 105 min, the stability of the immobilized enzyme was investigated in a long-term operation. The transfer ratio and production of FOSs were constantly maintained for 984 h (**Figure 5**). This stability is sufficient for the industrial production of FOSs. The high stability is attributable to the cross-linking with glutaraldehyde and the low water activity in 60% sucrose. **Table 3** shows the sugar composition at 984 h, in which the composition of FOSs (mainly 1-kestose and nystose) was 51.9%, and the transfer ratio was 92%. The productivity was 163 g/h per liter of the bed volume of the

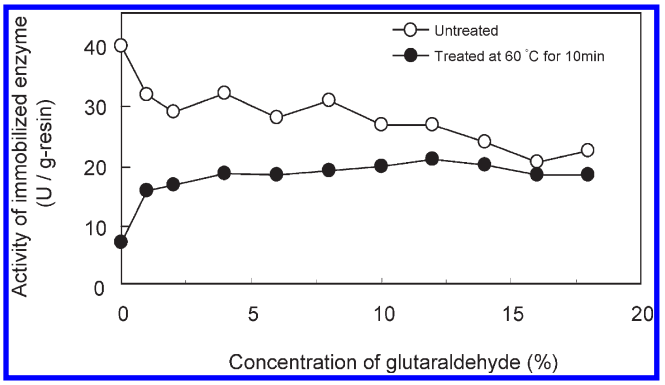

Figure 3. Change in activity and thermal stability of immobilized enzyme as a function of the concentration of glutaraldehyde in a cross-linking reaction.

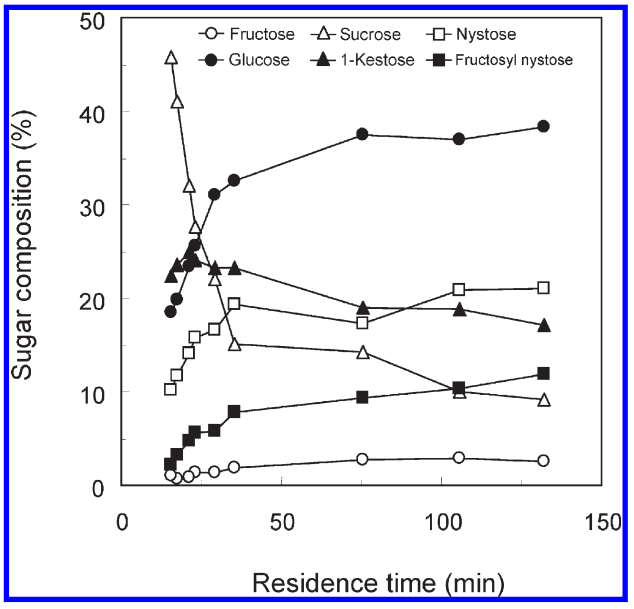

Figure 4. Change in sugar composition as a function of residence time in a tubular reactor packed by the immobilized enzyme.

reactor. Improvement in productivity would be expected by enhancing the immobilization efficiency in immobilization and cross-linking. The chemical and physical properties of the resin (particle size, porosity, and functional group for binding) must be discussed.

Although the immobilized enzyme is readily prepared using an ion-exchange resin through ion binding, the weak binding power causes elimination of the enzyme from the resin in a long-term operation. Cross-linking was, therefore, used to support immobilization in addition to the improvement of the thermal stability of the enzyme. Cross-linking with glutaraldehyde was available to the KB enzyme immobilized on WA-30 anion-exchange resin. Though a decrease in the enzymatic activity by cross-linking was < 50% (**Figure 3**), high production of FOSs by the enzyme-packed reactor was maintained in a long-term operation. As in other methods of immobilization of enzymes from fungi, mycelia can be immobilized without releasing enzymes (*15*, *16*). For example, entrapment of mycelia in calcium alginate is a popular method of immobilization but often causes higher pressure and heterogeneous flow in the packed tubular reactor because of the soft large particles prepared by entrapment. In the enzyme immobilized on WA-30 resin, the small hard particles enable the smooth operation of the reactor.

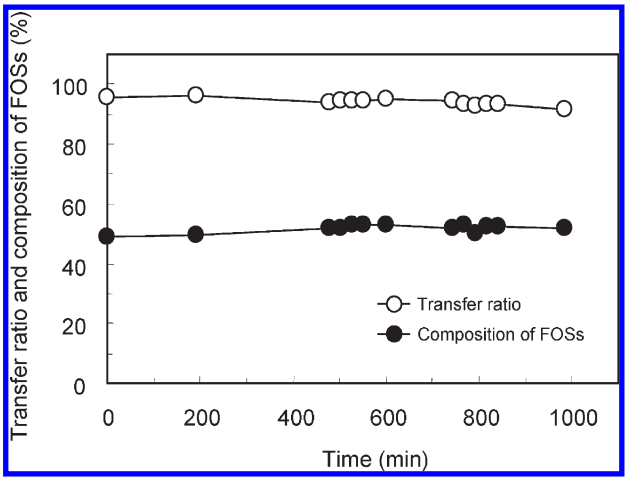

Figure 5. Stability of immobilized enzyme in a tubular reactor in long-term operation.

Table 3. Sugar Composition (%) of Reaction Mixture in Run at 984 h

fructose	glucose	sucrose	1-kestose	nystose	fructosyl nystose	FOSs
2.9	34.9	10.3	24.6	21.6	5.7	51.9

The production of FOSs (51.9%) by the immobilized KB enzyme was less than the 60% produced by β-fructofuranosidase from *Aspergillus niger* (*11*). This may be explained by the composition of 2.9% fructose (**Table 3**). If this amount is transferred to sucrose, production of about 60% is estimated. This property is essentially attributable to the free (native) F1 enzyme; therefore, conditions such as substrate concentration and pH must be studied for reduction of the slight hydrolysis reaction. Removal of glucose from the reaction mixture by oxidase enhances the production of FOSs because glucose is an inhibitor of β-fructofuranosidase (*15*, *20*).

Immobilization on WA-30 resin depressed the hydrolysis reaction of F2 (has high hydrolyzing activity) more efficiently. If the production of F2 from the KB strain was regulated, the enzyme produced in a low sucrose medium (< 2%) would have the same high production of FOSs as that by F1 alone. Other *Aspergillus oryzae* produced β-fructofuranosidase with a high transfer activity in a low sucrose medium (1%) (*21*, *22*). Sequencing of the *Aspergillus oryzae* genome (37 Mb) was accomplished in 2005 (*23*). Two genes encoding the F1 enzyme (amino acid sequence, 593; molecular weight, 64,140; accession number, BR000414) (*14*) and F2 enzyme (amino acid sequence, 524: molecular weight 57,459; accession number AP007164-1378) were identified. Production of high transferring β-fructofuranosidase would be attributable to the destruction of the F2 coding gene. In the future, regulation of F2 production from *Aspergillus oryzae* should be studied by gene manipulation.

In conclusion, β-fructofuranosidase from *Aspergillus oryzae* KB had a high production of FOSs in the tubular reactor using the immobilized enzyme. The immobilization with an anion-exchange resin (WA-30) and cross-linking with glutaraldehyde reduced the activity of F2 (high hydrolyzing enzyme) and enhanced the thermal stability of F1 (high transferring enzyme), and the packed reactor maintained high productivity for about 1000 h.

LITERATURE CITED

(1) Hidaka, H.; Hirayama, M.; Tokunaga, T.; Eida, T. The effects of undigestible fructooligosaccharides on intestinal microflora and

various physiological functions on human health. *Adv. Exp. Med. Biol.* **1990**, *270*, 105–117.
(2) Hirayama, M.; Sumi, N.; Hidaka, H. Purification and properties of fructooligosaccharide- producing beta-fructofuranosidase from *Aspergillus niger* ATCC 20611. *Agric. Biol. Chem.* **1989**, *53*, 667–673.
(3) Duan, K. J.; Sheu, D. C.; Chen, J. S. Purification and characterization of β-fructofuranosidase from *Aspergillus japonicus* TIT-KJ1. *Biosci. Biotechnol. Biochem.* **1993**, *57*, 1811–1815.
(4) Chang, C. T.; Lin, Y.; Tang, M. S.; Lin, C. F. Purification and properties of beta-fructofuranosidase from *Aspergillus oryzae* ATCC 76080. *Biochem. Mol. Biol. Int.* **1994**, *32*, 269–277.
(5) Ptel, V.; Saunders, G.; Bucke, C. Production of fructooligosaccharides by *Fusarium oxysporum*. *Biotechnol. Lett.* **1994**, *16*, 1139–1144.
(6) Hang, Y. D.; Woodams, E. E.; Jang, K. Y. Enzymatic conversion of sucrose to kestose by fungal extracellular fructosyltransferase. *Biotechnol. Lett.* **1995**, *17*, 295–298.
(7) Muramatsu, M.; Nakakuki, T. Enzymatic synthesis of novel fructosyl and oligofructosyl trehaloses by *Aspergillus sydowi* beta-fructofuranosidase. *Biosci. Biotechnol. Biochem.* **1995**, *59*, 208–212.
(8) Oliveira, I. M. D. A.; Park, Y. K. Screening of beta-fructofuranosidase producing microorganisms for production of fructooligosaccharides and studies of some enzyme properties. *Rev. Microbiol.* **1995**, *26*, 125–129.
(9) Kurakake, M.; Onoue, T.; Komaki, T. Effect of pH on transfructosylation and hydrolysis by β-fructofuranosidase from *Aspergillus oryzae*. *Appl. Microbiol. Biotechnol.* **1996**, *45*, 236–239.
(10) Hidaka, H.; Hirayama, M. Method for Production of Sugar. Japanese Patent 61-268190, 1988.
(11) Hidaka, H.; Hirayama, M; Sumi, N. A fructooligosaccharide-producing enzyme from *Aspergillus niger* ATCC 20611. *Agric. Biol. Chem.* **1988**, *52*, 1181–1187.
(12) L'Hocine, L.; Wang, Z.; Jiang, B.; Xu, S. Purification and partial characterization of fructosyltransferase and invertase from *Aspergillus niger*. *J. Bacteriol.* **2001**, *81*, 73–84.
(13) Wallis, G. L. F.; Hemming, F. W.; Peberdy, J. F. Secretion of two β-fructofuranosidases by *Aspergillus niger* growing in sucrose. *Arch. Biochem. Biophys.* **1997**, *345*, 214–222.
(14) Kurakake, M.; Ogawa, K.; Sugie, M.; Takemura, A.; Sugiura, K.; Komaki, T. Two types of β-fructofuranosidases from *Aspergillus oryzae* KB. *J. Agric. Food Chem.* **2008**, *56*, 591–596.
(15) Lin, T. J.; Lee, Y. C. High-content fructooligosaccharides production using two immobilized microorganisms in an internal-loop airlift bioreactor. *J. Chin. Inst. Chem. Eng.* **2008**, *39* (3), 211–217.
(16) Chien, C. S.; Lee, W. C.; Lin, T. J. Immobilization of *Aspergillus japonicus* by entrapping cells in gluten for production of fructo-oligosaccharides. *Enzyme Microb. Technol.* **2001**, *29* (4−5), 252–257.
(17) Cheng, T. C.; Duan, K. J.; Sheu, D. C. Immobilization of β-fructofuranosidase from *Aspergillus japonicus* on chitosan using tris(hydroxymethyl)phosphine or glutaraldehyde as a coupling agent. *Biotechnol. Lett.* **2005**, *27* (5), 335–338.
(18) Nishizawa, K.; Nakajima, M.; Nabetani, H. A forced-flow membrane reactor for transfructosylation using ceramic membrane. *Biotechnol. Bioeng.* **2000**, *68* (1), 92–97.
(19) Hayashi, S.; Matsuzaki, K.; Inomata, Y.; Takasaki, Y.; Imada, K. Properties of *Aspergillus japonicus* β- fructofuranosidase immobilized on porous silica. *World J. Microbiol. Biotechnol.* **1993**, *9* (2), 216–220.
(20) Chen, W.; Liu, C. Production of β-fructofuranosidase by *Aspergillus japonicus*. *Enzyme Microb. Technol.* **1996**, *18*, 153–160.
(21) Sangeetha, P. T.; Ramesh, M. N.; Prapulla, S. G. Production of fructosyl transferase by *Aspergillus oryzae* CFR 202 in solid-state fermentation using agricultural by-products. *Appl. Microbiol. Biotechnol.* **2004**, *65* (5), 530–537.
(22) Sangeetha, P. T.; Ramesh, M. N.; Prapulla, S. G. Production of fructo-oligosaccharides by fructosyl transferase from *Aspergillus oryzae* CFR 202 and *Aureobasidium pullulans* CFR 77. *Process Biochem.* (*Amsterdam, Neth.*) **2004**, *39* (6), 753–758.
(23) Machida, M.; et al. Genome sequencing and analysis of *Aspergillus oryzae*. *Nature* **2005**, *438*, 1157–1161.

Received for review May 24, 2009. Revised manuscript received December 3, 2009. Accepted December 04, 2009.