Continuous Production of 4^G- β -D-Galactosylsucrose (Lactosucrose) Using Immobilized β -Fructofuranosidase

Katsuhiko Mikuni,* Wang Qiong, Koki Fujita, Kozo Hara,
Shusaku Yoshida¹ and Hitoshi Hashimoto

Bio Research Corporation of Yokohama (13–46, Daikoku-cho, Tsurumi-ku, Yokohama 230–0053, Japan)

¹Central Research Laboratories, Japan Organo Co., Ltd. (1–4–9, Kawagishi, Toda 335–0015, Japan)

β -Fructofuranosidase from *Arthrobacter* sp. K-1 was immobilized onto a carrier resin (FE4611) with glutaraldehyde to effectively produce 4^G- β -D-galactosylsucrose, which increased *Bifidobacterium* in the human intestine. Maximum immobilized enzyme was approximately 30 U/g·wet on carrier resin and its immobilized enzyme yield was 76.6%. Optimal pH broadened and optimum temperature increased in response to immobilization. The pH stable range for immobilized enzyme was wider than that for the native enzyme. Both immobilized and native enzymes were stable at temperatures lower than 45°C, whereas the immobilized enzyme was less stable than the native enzyme at temperatures higher than 65°C. The column reactor was successfully operated for 35 days to produce more than 30% LS using a constant conversion flow system on a pilot plant scale. The present results suggest the possibility of LS production on an industrial scale using a column reactor.

Indigestible oligosaccharides, which increase *Bifidobacterium* in the intestine, have been the subject of recent research interest due to the fact that *Bifidobacterium* play an important role in the prevention of disease and the maintenance of the health of their host.¹⁾ Fructo-oligosaccharides,²⁾ galacto-oligosaccharides,^{3,4)} xylo-oligosaccharides⁵⁾ and raffinose⁶⁾ are known to be effective in causing the proliferation of *Bifidobacterium*. 4^G- β -D-galactosylsucrose (Lactosucrose, LS), one example of an indigestible oligosaccharide, is synthesized by transfructosylation of β -fructofuranosidase from sucrose and lactose.⁷⁾ LS increases the number of *Bifidobacterium*⁸⁾ and improves constipation through improvement of fecal conditions.⁹⁾ LS is very important for human health and is used in food for specified health use.

LS is produced in batch reactions on an industrial scale. The batch reaction requires a large amount of the enzyme, which makes the produc-

tion cost very high. We conducted the immobilization of β -fructofuranosidase to decrease the amount of required enzyme. Few investigations on the immobilization of β -fructofuranosidase have been reported, however, we succeeded in the immobilization of cyclomaltodextrin glucanotransferase using hydrophilic macro porous polymer (FE4611), which has an amine group and can be bounded with enzymes using glutaraldehyde.¹⁰⁾ In the present study, we attempted to construct a bioreactor to effectively produce LS using immobilized β -fructofuranosidase from *Arthrobacter* sp. K-1 on the carrier resin.

MATERIALS AND METHODS

Enzyme. β -Fructofuranosidase¹¹⁾ from *Arthrobacter* sp. K-1 was donated by Ensuiko Sugar Refining Co., Ltd. The enzyme solution used was concentrated from fermentation broth by ultrafiltration.

Substrate. The substrate used for the synthesis

*To whom correspondence should be addressed.

of LS was a mixture of 20% (w/w) sucrose (Ensuiko Sugar Refining Co., Ltd.) and 20% (w/w) lactose (Wako Chemicals) dissolved in 20 mM phosphate buffer (pH 6.5).

Carrier. A previously reported¹⁰⁾ carrier resin (FE4611), produced by Organo Co., Ltd., was used.

Analytical method. Native enzyme activity was assayed as follows. Two-hundred microliters of enzyme solution was added to 200 μ L of substrate solution [20% sucrose and 20% lactose in 20 mM phosphate buffer (pH 6.5)]. The mixture was reacted for 10 min at 40°C. Next, 0.1 mL of reaction mixture was added to 0.9 mL of distilled water in a boiling bath and inactivated for 20 min. After cooling, glucose and fructose in the solution were measured using an F-kit (glucose/fructose; Boehringer Mannheim Yamanouchi, Tokyo).

Immobilized enzyme activity was assayed as follows. Two grams (wet base) of immobilized enzyme and 10 mL of substrate solution [15% sucrose and 15% lactose in 20 mM phosphate buffer (pH 6.5)] were shaken at 100 rpm for 10 min at 40°C. All subsequent procedures were performed in the same manner as described above.

Enzyme activity was expressed as transfructosylation activity, and calculated by subtracting the amount of fructose from that of glucose. One unit of enzyme activity was defined as the amount of enzyme required to transfer fructose at 1 μ mol/min under the above conditions.

Sugar composition in the reaction mixture was determined by high-performance liquid chromatography (HPLC) on a column of TSK-gel Amide-80 (4.6 i.d. $\times$ 250 mm, Tosoh Co., Ltd.) using a solvent of acetonitrile–water (71: 29, v/v), at a flow rate of 1.0 mL/min, with a RI detector.

Preparation of immobilized enzyme. The carrier resin was treated by a 2-fold volume of 5% glutaraldehyde for 60 min at room temperature with slow shaking. After washing with 5 mM phosphate buffer (pH 6.5), the carrier resin was shaken with enzyme solution in a flask at 80 rpm for 2 h at room temperature. Immobilized enzyme was collected and then washed in 5 mM phosphate buffer (pH 6.5) until no protein was detected in the effluent by Lowry's method.¹²⁾

Effects of pH and temperature on immobilized enzyme in a batch reaction. One-hundred microliters of native enzyme solution (3 U) was added to 0.9 mL of 50 mM McIlvaine buffer (pH 3.0–8.0), Tris-HCl buffer (pH 9.0), NaHCO₃/NaOH buffer (pH 10.0–11.0) and Na₂HPO₄/NaOH buffer (pH 11.5–12.0). Enzyme activity was assayed using 0.2 mL of the enzyme solution. Two grams of immobilized enzyme was incubated at 40°C with 10 mL of substrate solution in the same buffer as described above for 10 min.

The optimum temperatures of both native and immobilized enzymes were obtained by assaying activity at 30, 40, 45, 55, 60, 65, 70, 75 and 80°C.

pH Stability and thermostability of immobilized enzyme in a batch reaction. One-hundred microliters of native enzyme solution (3 U) and 0.4 mL of the same buffer as described above were kept for 2 h at 40°C. The mixture was added to 1.0 mL of 100 mM phosphate buffer (pH 6.5) and the residual activity was assayed. Two grams of immobilized enzyme and 10 mL of the same buffer as described above were kept under the same conditions. After washing with 10 mM phosphate buffer (pH 6.5), the residual activity was assayed.

Thermostability was measured as follows. Both native and immobilized enzymes in 50 mM phosphate buffer (pH 6.5) were incubated for 30 min at 30, 40, 45, 55, 60, 65, 70, 75 and 80°C. The residual activities were assayed.

Column reactor system. Laboratory-scale column reactors were prepared by packing immobilized enzyme (12.4 U/g·wet) onto a column (10 mm i.d. $\times$ 300 mm). Substrate solution was allowed to pass through the column reactor at an appropriate flow rate at 60°C. Sugar composition was assayed by HPLC as described above.

A column reactor was prepared by packing immobilized enzyme (12.4 U/g·wet) onto a pilot plant-scale column (46 mm i.d. $\times$ 602 mm). The substrate solution was continuously passed through the column reactor at an appropriate flow rate at 55°C. In other words, the column reactor was operated using a constant conversion method. Sugar composition was assayed by HPLC as described above.

RESULTS AND DISCUSSION

Immobilizing ratio onto the carrier resin.

The amount of enzyme added onto carrier resin (1 g·wet) that was found to be the most effective was 38.1 U. With this amount added, the immobilized enzyme activity and immobilized enzyme yield were 29.2 (U/g·wet) and 76.6%, respectively (as shown in Fig. 1). When 76.3 U/g·wet of enzyme was added, immobilized enzyme yield was 39.3% and so the enzyme was largely lost. When 304.5 U/g·wet of enzyme was added, the immobilized enzyme activity decreased. It is believed that the protein density in the pores was too high when adding 304.5 U/g·wet of enzyme and

Fig. 1. Effect of amount of β -fructofuranosidase (β -FFase) added on immobilized enzyme activity (●) and immobilized enzyme yield (◆).

Immobilized enzyme yield was calculated as follows:
 $\{(\text{immobilized enzyme activity}) / (\text{total added enzyme activity})\} \times 100 (\%)$.

the immobilized enzyme activity decreased because of its steric hindrance.

Effects of pH and temperature on immobilized enzyme in batch reaction.

The optimal pH of the native enzyme was 5.5–7.0, whereas that of the immobilized enzyme was 5.0–9.0, and this range was broadened by immobilization (Fig. 2A).

The optimum temperature of the native enzyme was 55–60°C, whereas that of the immobilized enzyme was 65–70°C. The optimum temperature of the immobilized enzyme had increased when compared to that of the native enzyme (Fig. 2B).

pH Stability and thermostability of immobilized enzyme in batch reaction.

Native enzyme was stable within a pH range of 5.5 to 11.0, whereas the immobilized enzyme was stable between 3.0 and 12.0. Immobilized enzyme

Fig. 2. Effects of pH (A) and temperature (B) on transfructosylation activity of native (■) and immobilized (●) β -fructofuranosidase.

Fig. 3. Effects of pH (A) and temperature (B) on the stability of transfructosylation activity of native (■) and immobilized (●) β -fructofuranosidase.

Fig. 4. Effect of space velocity (SV) on LS production on a laboratory scale.

×, glucose; ●, fructose; ◆, sucrose; ■, lactose; △, LS; □, 1-kestose.

Fig. 5. Continuous production of LS (B) using immobilized β -fructofuranosidase on a pilot plant scale with changing space velocity (A).

was stable within a wider pH range than native enzyme (Fig. 3A).

Both native and immobilized enzymes were stable at temperatures below 45°C and each showed the same level of residual activity at 60°C (Fig. 3B). Furthermore, native and immobilized enzymes had 2 plateaus in the thermostability curve, likely as a result of the crudeness of the enzyme. *Arthrobacter* sp. K-1 really produced 3 kinds of β -fructofuranosidase.¹¹⁾ Native enzyme was more stable than immobilized enzyme at temperatures greater than 65°C. Immobilized enzyme is generally more stable than native enzyme against heat¹³⁻¹⁵⁾ as a result of the superior stability

of the stereo structure. However, the saccharase, a type of β -fructofuranosidase, was unstable against heat as a result of immobilization.¹⁶⁾ β -Fructofuranosidase from *Arthrobacter* sp. K-1 has the same property of the enzyme mentioned above against heat. Hence, the fact that the column reactor was operated at 55°C was likely not problematic.

Effect of space velocity (SV) on LS production.

Optimum SV⁻¹ (h) was 0.6 using 12.4 U/g·wet of immobilized enzyme (Fig. 4). LS was hydrolyzed at a reaction condition of more than 1.0 of SV⁻¹ (h), and 1-kestose, glucose and fructose increased with increasing SV⁻¹. LS hydrolysis was observed in the case of continuous reaction in the column, whereas LS hydrolysis was not observed in the case of batch reactions. This appeared to be the reason why the contact time with enzyme and substrate was too long with increasing SV⁻¹.

Continuous production of LS using a column reactor.

The column reactor was successfully operated for 35 days to produce more than 30% LS, which is the same level as on industrial batch reaction (Fig. 5). No microbial contamination was detected at any point during operation of the column reactor. Space velocity should be controlled definitely to maintain the level at 30% LS.

The present results suggest the possibility of LS production on an industrial scale using a column reactor. We intend to study the combination of immobilized enzyme and yeast,¹⁷⁾ the latter of which digests only glucose because of a deficiency of β -fructofuranosidase and accelerates transfructosylation, lowering the product inhibition to produce LS more effectively.

REFERENCES

- 1) T. Mitsuoka: Bifidobacteria and their role in human health. *J. Ind. Microbiol.*, **6**, 263-267 (1990).
- 2) H. Hidaka, T. Eida, T. Takizawa, T. Tokunaga and Y. Tashiro: Effects of fructooligosaccharides on intestinal flora and human health. *Bifidobact. Microflora*, **5**, 37-50 (1986).
- 3) K. Otsuka, Y. Benno, K. Endo, H. Ueda, O. Ozawa, T. Uchida and T. Mitsuoka: Effects of 4'-galactosyl-

- lactose intake on human fecal microflora. *Bifidus*, **2**, 143-149 (1989).
- 4) M. Ito, Y. Deguchi, A. Miyamori, K. Matsumoto, H. Kikuchi, T. Kobayashi, T. Yajima and T. Kan: Effect of administration of galactooligosaccharides on the human faecal microflora, stool weight and abdominal sensation. *Microb. Ecol. Health Dis.*, **3**, 285-292 (1989).
 - 5) M. Okazaki, S. Fujikawa and N. Matsumoto: Effect of xylooligosaccharide on growth of bifidobacteria. *J. Jpn. Nutr. Food Sci.*, **43**, 395-401 (1990).
 - 6) Y. Benno, K. Endo, N. Shiragami, K. Sayama and T. Mitsuoka: Effects of raffinose intake on human fecal microflora. *Bifidobact. Microflora*, **6**, 59-63 (1987).
 - 7) K. Fujita, T. Osawa, K. Mikuni, K. Hara, H. Hashimoto and S. Kitahata: Production of Lactosucrose by β -fructofuranosidase and some of its physical properties. *Denpun Kagaku*, **38**, 1-7 (1991).
 - 8) Y. Ogata, K. Fujita, H. Ishigami, K. Hara, A. Terada, H. Hara, I. Fujimori and T. Mitsuoka: Effect of a small amount of 4^G- β -D-galactosylsucrose (Lactosucrose) on fecal flora and fecal properties. *J. Jpn. Nutr. Food Sci.*, **46**, 317-323 (1993).
 - 9) M. Kumemura, F. Hashimoto, C. Fujii, K. Matsuo, H. Kimura, R. Miyazoe, H. Okamatsu, T. Inokuchi, H. Ito, K. Oizumi and T. Oku: Effects of administration of 4^G- β -D-galactosylsucrose on fecal microflora, putrefactive products, short-chain fatty acids, weight, moisture and pH, and subjective sensation of defecation in elderly with constipation. *J. Clin. Biochem. Nutr.*, **13**, 199-210 (1992).
 - 10) S. Sakai, N. Yamamoto, S. Yoshida, K. Mikuni, H. Ishigami and K. Hara: Continuous production of glucosyl-cyclodextrins using immobilized cyclomaltodextrin glucanotransferase. *Agric. Biol. Chem.*, **55**, 45-51 (1991).
 - 11) K. Fujita, K. Hara, H. Hashimoto and S. Kitahata: Purification and some properties of β -fructofuranosidase I from *Arthrobacter* sp. K-1. *Agric. Biol. Chem.*, **54**, 913-919 (1990).
 - 12) O.H. Lowry, N.J. Rosebrough, A.I. Farr and R.J. Randall: Protein measurement with the folin phenol reagent. *J. Biol. Chem.*, **193**, 265 (1951).
 - 13) T. Nakakuki, T. Hayashi, M. Monma, K. Kawashima and K. Kainuma: Immobilization of the exo-maltohexaohydrolase by irradiation method. *Biotechnol. Bioeng.*, **25**, 1095-1107 (1983).
 - 14) J.P. Lenders, P. Germain and R.R. Crichton: Immobilization of a soluble chemically thermostabilized enzyme. *Biotechnol. Bioeng.*, **27**, 572-578 (1985).
 - 15) K. Mikuni, K. Iwasaki, K. Hisada, N. Koga and N. Kuwahara: Thermostability of immobilized pullulanase on a ceramic carrier made from sepiolite. *J. Appl. Glycosci.*, **43**, 291-295 (1996).
 - 16) S. Usami, J. Noda and K. Kunihiya: Preparation and properties of water-insoluble saccharase. *J. Ferment. Technol.*, **49**, 598-603 (1971).
 - 17) K. Mikuni, K. Arakawa, H. Hashimoto, K. Hara, M. Ando, S. Shimada and Y. Nakatomi: Manufacture of fructo-oligosaccharides of fructosides with fructosyltransferase and yeast. *Japanese Patent Kokai Tokukaihei*, 4-293494, 1992-10-19 (in Japanese).

(Received December 14, 1999; Accepted April 24, 2000)

固定化 β -fructofuranosidase を用いた 4^G- β -D-galactosylsucrose (ラクトスクロース) の連続生産

三國克彦, 汪 瓊, 藤田孝輝
原 耕三, 吉田収作¹, 橋本 仁

株式会社横浜国際バイオ研究所
(230-0053 横浜市鶴見区大黒町 13-46)

¹ オルガノ株式会社総合研究所
(335-0015 戸田市川岸 1-4-9)

ヒト腸内の *Bifidobacterium* を増加させる 4^G- β -D-galactosylsucrose (ラクトスクロース) を効率的に生産するために *Arthrobacter* sp. K-1 から産生される β -fructofuranosidase をグルタルアルデヒドを用いて担体 (FE 4611) に固定化した。酵素の最大固定化量は約 30 U/g wet 担体であり、その回収率は 76.6% であった。固定化することによって至適 pH は広がり、至適温度は高くなった。固定化酵素の pH 安定領域は Native 酵素より広がった。固定化酵素および Native 酵素とも 45°C まで安定であったが、65°C 以上では固定化酵素より Native 酵素の方が安定であった。パイロットプラントスケールでカラムリアクターをコンスタントコンバージョンで運転したところ、ラクトスクロースの生成率 30% 以上で 35 日間生産可能であった。この結果より、固定化酵素を用いて工業的にラクトスクロースが生産可能であることが示唆された。