# Purification and Properties of a Fructooligosaccharide-producing β-Fructofuranosidase from *Aspergillus niger* ATCC 20611

Masao HIRAYAMA, Naomi SUMI and Hidemasa HIDAKA

*Bio Science Laboratories, Meiji Seika Kaisha, Ltd.,*
*580, Horikawa-cho, Saiwai-ku, Kawasaki-shi,*
*Kanagawa 210, Japan*

Received September 5, 1988

**A fructooligosaccharide-producing β-fructofuranosidase was purified from cells of *Aspergillus niger* ATCC 20611. The molecular weight was 340,000 by gel filtration. The optimum pH of the enzyme was 5.0～6.0 and the optimum temperature was 50～60°C. The enzyme was rendered inactive by 1 mM $Hg^{2+}$ and the *Km* value for sucrose was 0.29 M. The enzyme catalyzed an almost exclusively fructosyl transfer reaction in a 50% sucrose solution to produce a mixture of fructooligosaccharides and glucose, but both fructosyl transfer and hydrolytic action were observed in a 0.5% solution. The β-fructofuranosidase showed a high regiospecificity to transfer the fructosyl moiety for the 1-OH group of terminal fructofuranosides.**

In a previous paper,[1] we reported fructooligosaccharide production by enzymic fructosyl transfer on sucrose, and *Aspergillus niger* ATCC 20611 was selected as the most suitable strain for the production. This strain showed a very high enzyme productivity and its transfructosylating activity was very strong compared to its hydrolyzing activity. The transfructosylating reaction proceeded smoothly even in a 50% (w/v) solution of sucrose to give a mixture of fructooligosaccharides with an inulin-type structure of $1^F$-(1-β-fructofuranosyl)$_n$sucrose ($n=1\sim3$). This crude enzyme is of particular interest since it appears to be a β-fructofuranosidase which has a high transfructosylating activity and a high regiospecificity for fructosyl transfer to the 1-OH group of terminal fructofuranosides.[1] This paper describes the purification and properties of the fructooligosaccharide-producing enzyme from *A. niger* ATCC 20611.

## Materials and Methods

*Materials.* DEAE-Sephadex A-50, Sepharose 6B and Sephadex G-200 were obtained from Pharmacia. 1-Kestose ($GF_2$), nystose ($GF_3$) and $1^F$-fructofuranosylnystose ($GF_4$) were purified from a mixture of fructooligosaccharides as described in a previous paper.[1] Inulobiose ($F_2$) and inulotriose ($F_3$) were obtained by a modification of the method of Dickerson and Moor.[2] The other saccharides, chemicals and standard proteins were purchased from commercial sources.

*Cultivation conditions. A. niger* ATCC 20611 was cultured at 28°C in a 30-l jar fermentor (Marubishi, Type MSJ-302) under aerated conditions (20 l/min). The cultivation medium (20 l) contained 5.0% sucrose, 0.7% maltoextract, 1.0% polypepton, 0.5% carboxymethyl-cellulose (CMC) and 0.3% NaCl. A subculture (400 ml) grown under similar conditions as described in a previous paper[1] was inoculated. After cultivation for 72 hr, the remaining culture medium was centrifuged at 4°C to obtain the cells (2800 g) and the supernatant solution (20 l). The transfructosylating activities were 1.6 U per mg for the cells and 0.13 U per ml for the solution.

*Cell-free crude enzyme.* The wet cells (880 g, $141\times10^4$ U) were alternately frozen (−20°C) and thawed (20°C) three times, and the resulting material was sonicated (sonifier cell disruptor, model 350) on ice for 5 min in 10 mM McIlvaine buffer (4.0 l), pH 5.0. The sonicated material was centrifuged and the supernatant (4.46 l, $4.84\times10^5$ U) thus obtained was used as a crude extract.

*Analytical methods*

*Enzyme assay.* The enzyme activity was assayed as a *trans*-fructosylating activity by our previous methods.[1] One units was defined as the amount of enzyme required to produce 1 μmol of kestose per min from 10% (w/v)

sucrose at 40°C in 40 mM McIlvaine buffer, pH 5.0.

*Protein estimation.* Protein was measured by the method of Lowry *et al.*,[3] using bovine serum albumin as the standard. Absorbance at 280 nm was used for monitoring protein in column eluates.

*Carbohydrate estimation.* The carbohydrate in the enzyme was estimated by the phenol-sulfuric acid method[4] using D-glucose as the standard. High performance liquid chromatography (HPLC) and thin-layer chromatography (TLC) analysis were done as described in a previous paper.[1]

*Polyacrylamide gel electrophoresis.* SDS-Polyacrylamide gel electrophoresis (SDS-PAGE) was done using a Phast system (Pharmacia) with a PhastGel Gradient 8~25 (Pharmacia). Samples containing 1 mM EDTA, 2.5% SDS, 5% 2-mercaptoethanol, 10 mM Tris-HCl buffer, pH 8.0, and 0.1% protein were left for 5 min in boiling water. After separation, proteins were stained with PhastGel Blue R (Coomassie Brilliant Blue).

*Chromatofocusing chromatography.* A column of polybuffer exchanger 94 (Mono P HR5/20, Pharmacia) was equilibrated with 25 mM imidazole buffer, pH 7.4. The purified enzyme (800 $\mu$g of protein) was put on the column and eluted with polybuffer 74 adjusted to pH 3.0 by hydrochloric acid. The eluent was collected in 2-ml fractions at a flow rate of 40 ml per hr.

*Estimation of the molecular weight by gel filtration.* The molecular weight of the purified enzyme was estimated by gel filtration with Sephadex G-200.

*Examination of substrate specificity*

*Self-transfer of fructose moiety, Michaelis constants and molecular activities.* In each case, the level of the enzyme was regulated so that not more than 10% of the substrate was transformed in the course of incubation. The self-transfer reaction was done using an enzyme mixture (1.0 ml) containing 10% of each substrate in a 40 mM McIlvaine buffer, pH 5.0. After incubation for 1 hr at 40°C the transferred products were analyzed by HPLC. Then, similar incubations were done for the substrates which gave transferred products with the exception that eight different substrate concentrations (0.05 ~ 1.5 M) were used. A Lineweaver-Burk plotting of the resulting data obtained by HPLC analysis gave each *Km* value and molecular activity ($V_{max}/e_0$) for the self-transfer reaction.

*Fructosyl transfer from sucrose to several acceptors.* This reaction was done using an enzyme solution (1 U, 1 ml) containing sucrose (10%) and acceptor (10%) in 40 mM McIlvaine buffer, pH 5.0, during 1 hr. The fructosyl-transferred products from sucrose to acceptor were investigated by analyzing the reaction mixture at 20 min intervals by HPLC or TLC and comparing them to their authentic samples.

## Results

### *Purification of the enzyme*

All of the operations for enzyme purification were done at 1 ~ 6°C, and centrifugation was conducted at 6000 × *g* for 15 min. The enzyme solutions were concentrated by a Hollow Fiber System (model DC4 equipped with H1P 10-20: Amicon Corporation).

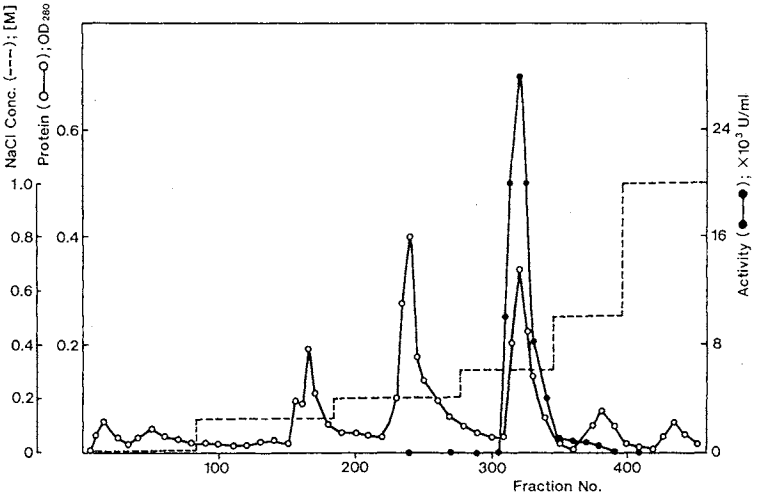


**Fig. 1.** DEAE-Sephadex A-50 Column Chromatograph of the Crude Extract from *A. niger* ATCC 20611.
The crude extract was put onto a DEAE-Sephadex A-50 column (5.0 × 35 cm) equilibrated with 20 mM phosphate buffer, pH 6.0. A step-wise elution was done with 0 M, 0.15 M, 0.2 M, 0.3 M, 0.5 M and 1.0 M NaCl in 20 mM phosphate buffer, as indicated by the dotted line. Ten-ml fractions of eluent were collected at a flow rate of 120 ml per hr. ●—●, transfructosylating activity; O—O, absorbance at 280 nm; ------, NaCl concentration.

1) *Calcium acetate and ammonium sulfate fractionation.* Solid calcium acetate was added to the crude extract (3.4 l) to 6% saturation. After standing overnight, the solution was centrifuged to remove the precipitates and the supernatant (3.4 l) was condensed to about one-fifth by ultrafiltration. Solid ammonium sulfate to 75% saturation was added to the condensate, and the resulting solution was left overnight. The supernatant separated by centrifugation was concentrated again to about one-tenth by ultrafiltration.

2) *DEAE-Sephadex column chromatography.* The protein was eluted from the DEAE-Sephadex column with a stepwise NaCl concentration and transfructosylating activity was detected in 0.5-l fractions (No. 308～358) (Fig. 1). The active fractions were combined and concentrated by ultrafiltration to give a "DEAE-Sephadex conc." fraction.

3) *Sepharose 6B column chromatography.* The "DEAE-Sephadex conc." fraction was further purified by gel filtration on Sepharose 6B column. Rechromatography of the active fractions showed a single symmetrical peak (Fig. 2). The active fractions No. 74～83 were combined, concentrated and dialyzed overnight against a 10 mM phosphate buffer, pH 6.0, to give a "Sepharose 6B (2nd) conc." fraction, the final purified β-fructofuranosidase. The purification process of the enzyme is summarized in Table I. The enzyme was purified approximately 52-fold with a 10% yield recovery from the crude extract. The purified enzyme was homogenous by SDS-PAGE and chromatofocusing (Fig. 3).

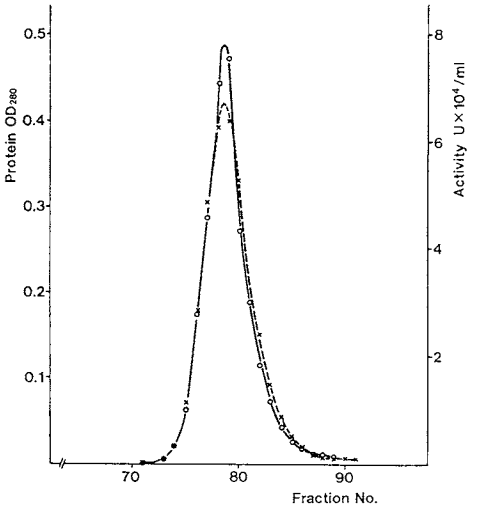


**Fig. 2.** Sepharose 6B Re-chromatography of "Sepharose 6B (1st) Conc." Fraction.

"The 1st conc." fraction was put onto a Sepharose 6B column (5.0 × 62 cm) equilibrated with a 20 mM phosphate buffer (pH 6.0) containing 100 mM NaCl, and eluted with the same buffer. Eluates were collected in 9.8-ml fractions. ○—○, transfructosylating activity; ×---×, absorbance at 280 nm.

*Properties of β-fructofuranosidase*

1) *Molecular weight.* As shown in Fig. 4, the molecular weight of the enzyme was estimated to be 340,000 from gel filtration with Sephadex G-200. In SDS-PAGE analysis the

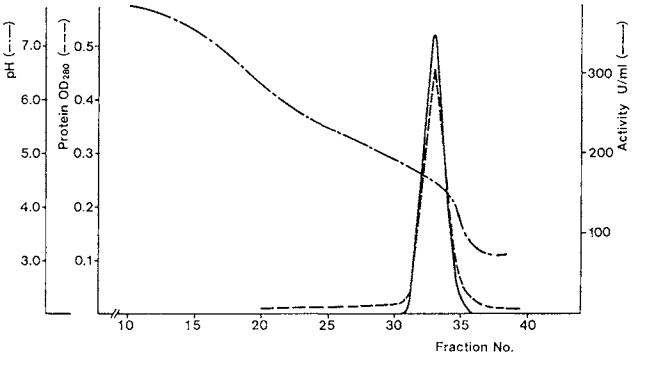


**Fig. 3.** Chromatofocusing of the Purified Enzyme on PBE 94.

——, transfructosylating activity; – – – –, protein at 280 nm; —·—, pH.

**Table I.** PURIFICATION OF β-FRUCTOFURANOSIDASE FROM *A. niger* ATCC 20611

| Step | | Volume (ml) | Total activity (U) | Total protein (mg) | Specific activity (U/mg protein) | Recovery (%) |
|---|---|---|---|---|---|---|
| Crude extract | | 3400 | $36.9 \times 10^4$ | 6770 | 54.5 | 100 |
| $Ca(OAc)_2$ (6%) | sup. | 3400 | — | — | — | — |
| | conc. | 725 | $19.2 \times 10^4$ | 1430 | 135 | 52 |
| $(NH)_2SO_4$ (75%) | sup. | 880 | $16.6 \times 10^4$ | 739 | 225 | 45 |
| | conc. | 80 | $16.4 \times 10^4$ | 198 | 828 | 44 |
| DEAE-Sephadex conc. | | 8.0 | $9.27 \times 10^4$ | 47.7 | 1940 | 25 |
| Sepharose 6B (1st) conc. | | 11.1 | $4.93 \times 10^4$ | 19.0 | 2600 | 13 |
| Sepharose 6B (2nd) conc. | | 8.6 | $3.85 \times 10^4$ | 13.7 | 2810 | 10 |

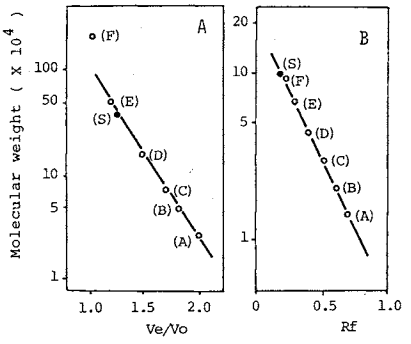


**Fig. 4.** Estimation of Molecular Weight of the Purified Enzyme by Gel Filtration [A] and SDS-PAGE [B].

A: Gel filtration on Sephadex G-200. Column size, 1.6 × 95 cm; eluent, 20 mM phosphate buffer (pH 6.0) + 0.1 M NaCl; flow rate, 4.5 ml/hr; temp., 5°C; protein, 50 ~ 100 μg each; detector, UV (280 nm); fraction, 0.75 ml/tube. Standard proteins: (A), chimotripsinogen (25,000); (B), hen egg albumin (45,000); (C), bovine serum albumin (67,000); (D), aldolase (158,000); (E), ferritin (450,000); (F), blue dextran (2,000,000); (S), purified enzyme.

B: SDS-PAGE. Standard proteins: (A), α-lactalbumin (14,400); (B), tripsin inhibitor (20,100); (C), anhydrase (30,000); (D), ovalbumin (43,000); (E), albumin (67,000); (F), phospholylase (94,000); (S), purified enzyme.

enzyme gave a single protein band, which was estimated to be of molecular weight 100,000.

2) *Carbohydrate content.* The carbohydrate content of the enzyme was found to be 20% (carbohydrate/protein, w/w) by the phenol-sulfuric acid method.

3) *Effects of pH.* The activity of the purified enzyme and cells of *A. niger* were measured at various pHs from 3.0 to 11.0 (Fig. 5A). The optimal pH was pH 5.0 ~ 6.0. The purified enzyme had almost no activity under

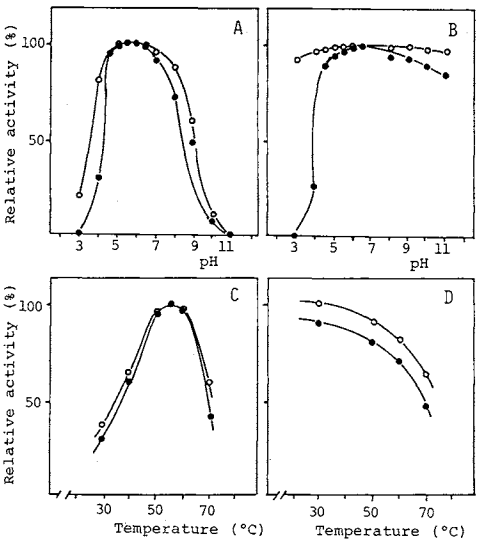


**Fig. 5.** Effects of pH and Temperature on the Transfructosylating Activity and Stability.

A: pH–activity. The reaction mixtures containing 2.5 ml of 10% sucrose solution (0.8 unit) were incubated for 1 hr at 40°C and at various pHs.

B: pH–stability. The enzyme solutions (12 U, 0.5 ml) were incubated for 30 min at 40°C in an appropriate buffer (0.5 ml), and adjusted to pH 5.0 by adding 0.25 M McIlvaine buffer (5 ml). Then the enzyme activities were assayed by the standard method.

C: Temperature–activity. The reaction mixture containing 2.5 ml of 10% sucrose solution (0.8 unit) were incubated at various temperatures for 1 hr at pH 5.0.

D: Temperature stability. After the enzyme was incubated in a 0.1 M McIlvaine buffer, pH 5.0, at various temperatures for 30 min, the remaining activities were assayed by the standard method. Initial activities before incubation were expressed as 100% (purified enzyme, 0.78 U/ml; cells, 0.81 U/ml).

The buffers used were as follows: pH 3.0 to 8.0, 0.1 M McIlvaine buffer; pH 8.0 to 11.0, Kolthoff buffer. ●—●, purified enzyme; ○—○, cells of *A. niger*.

pH 3.0 and above pH 10.0. The two samples were incubated for an examination of pH stability at various pH values for 30 min at 40°C, and then the remaining activity was assayed. The purified enzyme was stable at pH 4.5 ~ 10.0, but the enzyme in *A. niger* cells was stable from pH 3.0 to 11.0 (Fig. 5B).

4) *Effects of Temperature.* The activity of the purified enzyme was measured at various temperatures from 30 to 70°C. The optimal temperature was 50 ~ 60°C (Fig. 5C). The effects of temperature on the stability of the enzyme and cells of *A. niger* were studied by keeping them at various temperatures for 30 min at pH 5.0, and the remaining activities were then measured. As shown in Fig. 5D, the purified enzyme and the cells both remained above 81% of their initial activities up to 50°C and 60°C, respectively.

**Table II.** Effects of Chemicals on the Transfructosylating Activity

| Compound | Concentration | Relative activity (%) |
|---|---|---|
| $HgCl_2$ | $0.1 \times 10^{-3}$ M | 15 |
| | $0.01 \times 10^{-3}$ M | 75 |
| $AgNO_3$ | $1.0 \times 10^{-3}$ M | 86 |
| $MnSO_4$ | $1.0 \times 10^{-3}$ M | 88 |
| $CuSO_4$ | $1.0 \times 10^{-3}$ M | 93 |
| $CoCl_2$ | $1.0 \times 10^{-3}$ M | 96 |
| $ZnSO_4$ | $1.0 \times 10^{-3}$ M | 96 |
| $MgSO_4$ | $1.0 \times 10^{-3}$ M | 98 |
| Pb $(OAc)_2$ | $1.0 \times 10^{-3}$ M | 100 |
| $AlCl_3$ | $1.0 \times 10^{-3}$ M | 95 |
| $FeCl_3$ | $1.0 \times 10^{-3}$ M | 100 |
| PCMB | $0.1 \times 10^{-3}$ M | 110 |
| EDTA | $1.0 \times 10^{-3}$ M | 97 |
| Aniline | $1.0 \times 10^{-3}$ M | 110 |
| Oligostatin | 0.5 mg/ml | 110 |
| Nojirimycin | 0.5 mg/ml | 100 |
| Control ($H_2O$) | — | 100 |

A mixture of enzyme solution (0.8 U, 0.5 ml), sucrose solution (26% w/v, 1.0 ml) and 0.1 M McIlvaine buffer (pH 5.0) (1.0 ml) was incubated for 1 hr at 40°C in the presence of water or reagent solution (0.1 ml), and the activities were assayed.

5) *Effects of chemicals.* The effects of metal ions and some other organic chemicals on the purified enzyme was investigated and the results are shown in Table II. Only $Hg^{2+}$ inhibited the activity of the enzyme.

6) *Effects of sucrose concentration on the products.* The effects of sucrose concentrations on the reaction products were successively studied by monitorring the changes of the carbohydrate composition in three different sucrose concentrations over 80 hr. Three units of each enzyme per gram sucrose were used in both reactions of 5.0% and 50% of sucrose, but an excess 500 U per gram sucrose was added to 0.5% of sucrose to investigate the final products of the enzyme reaction. As shown in Fig. 6, the total amounts fructooligosaccharides increased to 50% and 60% of the carbohydrate composition after 24 hr of incubation of 5.0% and 50% sucrose solutions, respectively, and then those decreased with an increase of fructose. Treatment of excess enzyme with 0.5% of sucrose solution gave both 50% of glucose and fructose as the final products. At the early stage (2 hr) some fructooligosaccharides (*ca.* 25%) were formed, but they were hydrolyzed over 10 hr into glucose

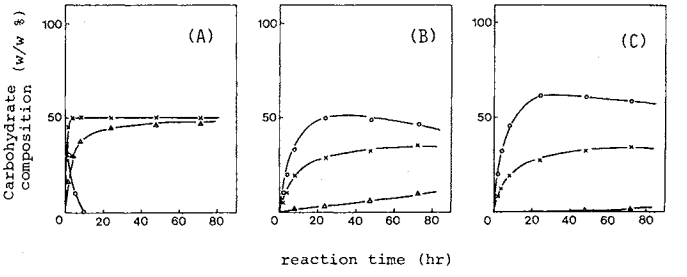


**Fig. 6.** Effects of Sucrose Concentrations on the Reaction Products.

Enzyme reactions were done at 40°C in McIlvaine buffer, pH 5.0, under the following conditions. Sucrose concentration and the enzyme used per g sucrose: (A), 0.5% and 500 U; (B), 5.0% and 3 U; (C), 50% and 3 U. ○—○, total fructooligosaccharides; △—△, fructose; ×—×, glucose.

and fructose.

*Substrate specificity*

1) *Self-transfer of fructoses moiety, Michaelis constants (Km) and molecular activities ($V_{max}/e_0$) for several substrates.* The term "self-transfer of fructose moiety" is used when a substrate acts as both fructosyl donor and acceptor. The transfer was investigated by incubating each substrate shown in Table III with the purified enzyme followed by a HPLC analysis of the product. The enzyme catalyzed fructosyl transfer to produce the transferred products from the substrates having terminal 2-β-D-fructofuranose moiety such as sucrose, 1-kestose, nystose, $1^F$-fructofuranosylnylstose and inulobiose, but failed to cleave turanose, isomalturose and melezitose. Furthermore, the effect of substrate concentration on the self-transfer reaction was studied. The enzyme reaction of each substrate done in eight different concentrations and *Km* and relative $V_{max}/e_0$ were calculated from the results. As shown in Table III, sucrose had the largest $V_{max}/e_0$ of the five substrates studied.

**Table III.** SELF-TRANSFER OF FRUCTOSE MOIETY, MICHAELIS CONSTANTS AND MOLECULAR ACTIVITIES

| Substrate | Product | *Km* (M) | Relative $V_{max}/e_0$ |
|---|---|---|---|
| Sucrose (GF) | $GF_2$ | 0.29 | 100 |
| 1-Kestose ($GF_2$) | $GF_3$ | 0.80 | 52 |
| Nystose ($GF_3$) | $GF_4$ | 0.14 | 0.01 |
| $1^F$-Fructosyl-$GF_3$($GF_4$) | $GF_5$ | 0.37 | 0.01 |
| Raffinose | Raf-F | 0.20 | 0.02 |
| Inulobiose ($F_2$) | $F_3$ | * | * |
| Turanose | None | — | — |
| Isomalturose | None | — | — |
| Melezitose | None | — | — |

* Not tested.

**Table IV.** FRUCTOSYL TRANSFER FROM SUCROSE TO SEVERAL ACCEPTORS

| Acceptor | | Fructose transferred[a] |
|---|---|---|
| Type | Substrate | |
| G1-(2F1)$_n$-2F | None | (+)[b] |
| | 1-Kestose | + |
| | Nystose | + |
| | $1^F$-F-Nystose | + |
| F1-(2F1)$_n$-2F | Inulobiose | + |
| | Inulotriose | + |
| Galn-G1-2F | Raffinose | + |
| | Stachyose | + |
| R-OH | Fructose (F) | + |
| | MeOH, EtOH | + |
| Others | Glucose (G) | − |
| | Galactose (Gal) | − |
| | Turanose (G1-3F) | − |
| | Malturose (G1-4F) | − |
| | Isomalturose (G1-6F) | − |
| | Maltose (G1-4G) | − |
| | Melezitose (G-F3-1G) | − |

[a] The symbols + indicate the presence of the transferred products.

[b] Self-transferred product (1-kestose) of sucrose.

2) *Fructosyl transfer from sucrose to several acceptors.* An enzyme reaction of each 10% (w/v) solution of sucrose and acceptor elucidated the properties of the structures as the fructosyl acceptor. The results showed that the purified enzyme catalyzed the fructosyl transfer from sucrose to terminal 2-β-fructofuranosides, fructose and lower primary alcohols such as methanol and ethanol (Table IV).

## Discussion

A fructooligosaccharide-producing enzyme purified from cells of *A. niger* was a glycoprotein having 20% (w/w) carbohydrates, and its molecular weight was estimated by gel filtration to be 340,000. On the other hand, the value obtained by SDS-PAGE was about one-third of the above value, 100,000. This differnce suggested the presence of subunit structures in the enzyme, like those reported for *Neurospora* and *Fusarium* invertase.[5,6] As no inhibition of the purified enzyme was observed with EDTA, it seems that metals are not essential for the transfer action of the enzyme. An interesting property was found in the effects of sucrose concentration on the products. As shown in Fig. 6, the enzyme reaction in 50% sucrose solution yielded the transferred products almost exclusively accompanied by glucose as the by-product, as if the enzyme was

β-fructosyltransferase, though a small amount of fructose was produced by a prolonged reaction. On the other hand, the reaction with dilute substrate afforded fructose in addition to the transferred products only at an early stage, and finally gave a mixture of completely hydrolyzed products, glucose and fructose. It is well known that some β-fructofranosidases catalyze fructosyl transfer to produce oligosaccharides and their amounts increase with an increase in the substrate concentration.[7,8] These results support the hyothesis that the *A. niger* enzyme is a β-fructofranosidase having a strong fructosyl transfer activity, like *Claviceps* enzyme[9] which produced neokestose from sucrose as the main products. Another property of the enzyme was observed in the substrate specificity including regiospecificity in the transfer site of fructosyl moiety. As shown in Tables III and IV, the purified enzyme transferred fructosyl moiety to terminal 2-β-fructofuranosides with the exception of fructose and lower primary alcohols. Furthermore, the moiety was solely transferred to the 1-OH group of the terminal fructofuranosides. It was reported that β-fructofranosidase from yeast[10] and *Claviceps purpurea*[9] had three acceptor groups (6-OH of glucoside, and 1-OH and 6-OH of furanoside) and two (6-OH of glucoside and 1-OH of furanoside) on sucrose, respectively. In comparison with these data, the *A. niger* enzyme has a higher regiospecificity in the fructosyl transfer reaction. The value of apparent *Km* of the purified enzyme for sucrose was 0.29 M, which seemed to be larger than those of hydrolytic β-fructofranosidase, for example, 0.0061 M of *N. crassa*,[5] 0.0044 M of *F. oxysporium*[6] and 0.025 M of *Saccharomyces cerevisiae*.[11] However, compared with the values for enzymes having fructosyl transfer activity, the *Km* value of *A. niger* enzyme was not significantly different from those of *C. purpurea* enzyme (0.148 M) and SST (0.11 M) of asparagus.[12]

## References


1) H. Hidaka, M. Hirayama and N. Sumi, *Agric. Biol. Chem.*, **52**, 1181 (1988).
2) A. G. Dickerson and J. Moor, *Carbohydr. Res.*, **39**, 162 (1972).
3) O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).
4) M. Dubois, K. A. Gilles, J. K. Hamilton, P. A. Rebers and F. Smith, *Anal. Chem.*, **28**, 350 (1956).
5) R. L. Metzenberg, *Biochim. Biophys. Acta*, **89**, 291 (1964).
6) M. Nishizawa, Y. Maruyama, and M. Nakamura, *Agric. Biol. Chem.*, **44**, 489 (1980).
7) J. Edelman, in "Advances in Enzymology," Vol. 17, ed. by F. F. Nord, Interscience Publishers, Inc., New York, 1956, pp. 189~232.
8) H. Hidaka and M. Hirayama, *Kagaku to Seibutsu*, **23**, 600 (1985).
9) A. G. Dickerson, *Biochem. J.*, **129**, 263 (1972).
10) E. H. Fischer, L. Kothes and J. Felling, *Helv. Chim. Acta*, **34**, 1134 (1951).
11) G. Gascon, N. P. Neumann and J. O. Lampen, *J. Biol. Chem.*, **243**, 1578 (1968).
12) N. Shiomi and M. Izawa, *Agric. Biol. Chem.*, **44**, 603 (1980).