# A Fructooligosaccharide-producing Enzyme from *Aspergillus niger* ATCC 20611

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The fructosyl transfer to sucrose was investigated, and *Aspergillus niger* ATCC 20611 was selected as the most suitable strain for fructooligosaccharide production. This strain showed very high enzyme productivity, and its transfructosylating activity was very strong compared to its hydrolyzing activity. Fructooligosaccharides could be more effectively prepared with a higher concentration of sucrose if use could be made of an enzyme having higher transfructosylating ability. Treatment of 50% (w/v) sucrose with the *A. niger* enzyme afforded a mixture of fructooligosaccharides with inulin-type structures of $1^F(1\text{-}\beta\text{-fructofuranosyl})_n$-sucrose ($n=1$ to 3). The individual saccharides could be separated by the combination of a carbon column chromatography and preparative HPLC.

Various oligosaccharides, *e.g.*, cyclodextrins, maltooligosylsucrose and fructooligosaccharides, prepared through enzymatic transglycosylation, have attracted special attention because of their physiological and physical usefulness.[1] It is known that fructooligosaccharides exist naturally in many kinds of plants.[2] They can also be prepared from sucrose through the transfructosylating action of enzymes,[3] that is, β-fructofuranosidases (β-FFase, EC 3.2.1.26) and β-D-fructosyltransferases (β-FTase, EC 2.4.1.9) from microorganisms and plants, but no method for their industrial production was established until we developed Neosugar (Meioligo®).[4]

Neosugar, a new sweetener, was found to be a nondigestible sugar for humans and to be physiologically useful becouse it improves the intestinal flora.[5] Neosugar is a mixture of fructooligosaccharides, 1-kestose ($GF_2$), nystose ($GF_3$) and $1^F$-β-fructofuranosylnystose ($GF_4$), and it is commercially produced from sucrose using an enzyme obtained from *A. niger* ATCC 20611.

The present paper describes the characteristics and action pattern of the enzymatic activity of the crude enzyme from *A. niger* compared with those of enzymes from other microorganisms. We also present practical methods for the preparation of the oligosaccharides.

## MATERIALS AND METHODS

*Microorganisms and cultivation conditions.* The 11 microorganisms listed in Table I were from the type culture collection of our labolatories. Each strain was subcultured in a medium (80 ml in a 250 ml flask) containing 5.0% sucrose, 0.7% maltoextract, 1.0% polypeptone, 0.5% carboxymethylcellulose (CMC) and 0.3% NaCl, with incubation at 28°C for 28 hr with reciprocal shaking (240 rpm). The subculture was, in turn, inoculated into six flasks (250 ml) containing 80 ml of medium consisting of 5.0% sucrose, 3.5% yeast extract and 0.5% CMC (pH 6.5). Two flasks were incubated at 28°C with reciprocal shaking (240 rpm) for 24, 48 and 72 hr, respectively.

After cultivation, the cultures were centrifuged (5°C), and the supernatant solutions and cells were separated for enzyme assays.

*Enzyme assays.* The reaction mixture for determining the enzyme activities consisted of 25% (w/v) sucrose (1.0 ml) as the substrate, 0.1 M McIlvain buffer (pH 5.0, 1.0 ml) and the enzyme solution (0.5 ml) containing an adequate amount of the cells or the supernatant solution.

The enzyme reaction was carried out at 40°C for 1 hr with moderate shaking in L-type test tubes and terminated by heating in boiling water. Quantitative analysis of trisaccharides, fructose and sucrose in the reaction mixture was performed by high performace liquid chromatography (HPLC). The transfructosylating activity ($U_t$) and the hydrolyzing activity ($U_h$) were determined from the amounts of trisaccharides and fructose, respectively. The β-fructofuranosidase activity ($U_f$) was determined from the amount of sucrose consumed in the reaction. One unit of each of these enzyme activities was defined as the amount of enzyme required to produce (or consume) 1 μmol of the corresponding saccharide per min under the above conditions.

*High performance liquid chromatography (HPLC).* HPLC analysis was carried out with a Shimadzu model LC-5A under the following conditions: column, Shimadzu $PNH_2$ column (4.6 mm × 15 cm); temperature, ambient; mobile phase, acetonitrile–water (75 : 25 v/v); flow rate, 1.0 ml/min; and detector, Shodex RI model SE-31 differential refractive index monitor. The samples were filtered through a membrane filter (pore size, 0.45 μm) before injection.

Preparative HPLC was performed with a Waters model S500A under the following conditions: column, $C_{18}$ 15～20 PrePak Cartridge [(57 mm × 30 cm) × 2]; temperature, ambient; mobile phase, water; flow rate, 100 ml/min; and detector, differential refractive index monitor. The samples (10～20 g as solid weight) in a *ca.* 30% solution were charged after filtration.

*Effect of the sucrose concentration on the formation of fructooligosaccharides and analysis of the products.* All reactions were performed at 40°C with the cells ($6U_h$ of β-FFase per g sucrose) in 0.04 M McIlvain buffer (pH 5.0) containing 0.5, 5.0 and 50% (w/v) sucrose. Samples taken at appropriate times during the reaction were inactivated by boiling and then the saccharide composition was analyzed by HPLC.

When cells of *Saccharomyces cerevisiae* were incubated in 50% sucrose for 8 hr, five saccharides in addition to monosaccharide were detected on HPLC analysis. These saccharides were separated from each other by HPLC, showing retention times of 8.84, 10.67, 12.71, 13.71 and 15.47 min, respectively, and the PMR (400 MHz, $D_2O$) spectral data [$\delta$ ($J$ in Hz) of H-$3^F$ and H-$4^F$] of the purified samples were identical with those of sucrose [4.25 (d, 8.7), 4.09 (t, 8.5)], β-D-fructofuranosyl (2→6)-D-glucose ($F_{2\to6}G$) [4.23 (d, 7.5), 4.18 (t, 7.4)], neokestose [4.26 (d, 8.7) and 4.23 (d, 7.4), 4.18 (t, 7.4) and 4.10 (t, 8.7)], 1-kestose [4.31 (d, 9.0) and 4.23 (d, 8.4), 4.12 (t, 8.2) and 4.08 (t, 9.0)], and 6-kestose [4.24 (d, 8.7) and 4.20 (d, 8.2), 4.15 (t, 8.5) and 4.10 (t, 8.5)], respectively.

*Preparation of fructooligosaccharides.* A mixture containing 50% (w/v) sucrose and cells (2.5 units of $U_t$ per g sucrose) of *A. niger* ATCC 20611 in 0.04 M McIlvain buffer (pH 5.0) was stirred at 40°C for 72 hr and then heated for 15 min in boiling water. HPLC analysis indicated that the carbohydrates in the resulting mixture were glucose (33.4%), fructose (trace), sucrose (9.0%), $GF_2$ (17.3%), $GF_3$ (32.0%) and $GF_4$ (7.2%).

The resulting mixture (20 ml) was applied to an activated charcoal column (5.0 × 30 cm, Takeda Pharmaceutical Co.). After washing with distilled water to remove the monosaccharides and sucrose, the column was successively eluted with 8 l each of 5, 10 and 20% ethanol, and 200 ml fractions were collected at a flow rate of 600 ml/min. The elution of oligosaccharides was monitored, and their homogeneity was examined by thin-layer chromatography (TLC) on silica gel 60 plates (Merck) with development with chloroform–acetic acid–water (6 : 7 : 1)[6] and spraying with diphenylamine-aniline-phosphoric acid reagent.[7] Purified $GF_2$ (200 mg) and $GF_3$ (500 mg) were obtained from the 5% and 10% ethanol eluent fractions, respectively. $GF_4$ (50 mg) was obtained on further purification of the 20% ethanol fractions by preparative HPLC.

More effective separation could be achieved by preparative HPLC. Similar application of the mixture (160 ml) to a carbon column (8.0 × 43 cm) gave rich fractions of $GF_2$ [9.22 g; sucrose (21.8%), $GF_2$ (70.6%) and $GF_3$ (7.6%)], $GF_3$ [16.3 g; $GF_2$ (29.3%), $GF_3$ (68.8%) and $GF_4$ (1.9%) and $GF_4$ [8.29 g; $GF_2$ (2.9%), $GF_3$ (49.1%), $GF_4$ (43.7%) and $GF_5$ (4.3%)]. After concentration, these rich fractions were independently subjected to preparative HPLC to afford purified $GF_2$ (6.3 g, 98% purity), $GF_3$ (10.8 g, 97.3% purity) and $GF_4$ (3.44 g, 99.1% purity). The physical data were as follows: $CF_2$, mp 179～200°C (5% methanol) (lit.[8] 199～200°C), $[\alpha]_D^{20}$ +28.0° (lit.[8] +28.5°); $GF_3$, mp 134°C (5% methanol) (lit.[9] 134°C), $[\alpha]_D^{20}$ +10.0° (lit.[9] +10.1°); $GF_4$, amorphous $[\alpha]_D^{20}$ −1.6°.

Methylation analysis was carried out according to the reported method, which involves complete methylation,[10] hydrolysis[11] and $NaBH_4$ reduction followed by acetylation.[12] All of the samples gave mixtures of alditol acetates derived from the 2,3,4,6-tetramethylglucosyl (**1**), 3,4,6-trimethylfructosyl (**2**) and 1,3,4,6-tetramethylfructosyl (**3**) moieties. Their composition ratios were as follows; $GF_2$, **1** (37%), **2** (31%) and **3** (32%); $GF_3$, **1** (31%), **2** (42%) and **3** (27%); and $GF_4$, **1** (23%), **2** (57%) and **3** (21%). Furthermore, their PMR and CMR data corresponded well with those reported by Kamerling *et al.*[13] and Heyraud *et al.*,[14] respectively.

## RESULTS

### *Distribution of transfructosylating activity ($U_t$) and hydrolyzing activity ($U_h$) in microorganisms*

In order to determine the relationship

TABLE I. DISTRIBUTION OF TRANSFRUCTOSYLATING ($U_t$) AND HYDROLYZING ACTIVITIES ($U_h$) IN CELLS OF VARIOUS MICROORGANISMS[a]

| No. | Organism[b] | Incubation time (days) | Cell growth (mg/ml) | $U_t$ | | $U_h$ | | $U_t/U_h$ |
|---|---|---|---|---|---|---|---|---|
| | | | | Specific (units/mg) | Enzyme (units/ml) | Specific (units/mg) | Enzyme (units/ml) | |
| 1 | *A. niger* ATCC 20611 | 1 | 143 | 0.271 | 38.8 | 0.019 | 2.8 | 14.2 |
| | | 3 | 349 | 0.266 | 92.8 | 0.022 | 7.7 | 12.2 |
| 2 | *A. niger* NRRL 4337 | 1 | 139 | 0.040 | 5.7 | 0.020 | 2.8 | 2.1 |
| | | 3 | 275 | 0.023 | 6.3 | 0.013 | 3.6 | 1.8 |
| 3 | *A. niger* ATCC 9612 | 1 | 114 | 0.124 | 14.2 | 0.016 | 1.7 | 8.0 |
| | | 3 | 264 | 0.016 | 4.2 | 0.005 | 1.3 | 3.1 |
| 4 | *A*. pullulans* ATCC 20612 | 1 | 35 | 0.028 | 1.0 | 0.004 | 0.1 | 7.8 |
| | | 3 | 126 | 0.008 | 1.0 | 0.002 | 1.5 | 0.7 |
| 5 | *A. oryzae* IAM-2609 pp13 | 1 | 73 | 0.038 | 2.8 | 0.011 | 0.7 | 3.5 |
| | | 3 | 268 | 0.013 | 3.5 | 0.003 | 0.5 | 5.0 |
| 6 | *A. oryzae* RIB-143 | 1 | 158 | 0.003 | 0.5 | 0.003 | 0.5 | 0.8 |
| | | 3 | 419 | 0.004 | 2.0 | 0.002 | 0.8 | 2.6 |
| 7 | *A. awamori* K 6611 | 1 | 116 | 0.044 | 5.1 | 0.017 | 1.9 | 2.6 |
| | | 3 | 380 | 0.011 | 4.2 | 0.033 | 12.5 | 0.3 |
| 8 | *C. paradoxa* FERM P6868 | 1 | 85 | 0.007 | 0.7 | 0.021 | 1.8 | 0.4 |
| | | 3 | 129 | 0.010 | 1.3 | 0.022 | 2.8 | 0.4 |
| 9 | *F. lini* IAM-5008 | 1 | 216 | 0.001 | 0.2 | 0.003 | 0.6 | 0.2 |
| | | 3 | 274 | 0 | 0 | 0.003 | 0.8 | 0 |
| 10 | *P. nigricans* IAM-7218 | 1 | 143 | 0.010 | 1.4 | 0.003 | 0.4 | 3.1 |
| | | 3 | 294 | 0 | 0 | 0 | 0 | 0 |
| 11 | *S. cerevisiae* MIS 2-6 9021 | 1 | 45 | 0.007 | 0.4 | 0.166 | 7.5 | 0.04 |
| | | 3 | 78 | 0.005 | 0.4 | 0.130 | 9.6 | 0.04 |
| 12 | *A*. pullulans*[c] ATCC 20612 | 3 | 80 | 0.133 | 10.6 | 0.014 | 1.1 | 9.8 |

[a] Cell growth was expressed as mg of cells per ml of cultured broth. Specific and enzyme activity were defined as units per mg of cells and units per ml of cultured broth, respectively. The enzyme activity indicates the quantitative enzyme productivity of each organism. The $U_t/U_h$ ratio shows the relative strength of the transfructosylating activity.

[b] *A*, *Aspergillus*; *A**, *Aureobasidium*; *C*, *Chalara*; *F*, *Fusarium*; *P*, *Penicillium*; *S*, *Saccharomyces*.

[c] Optimum culture conditions for producing $U_t$ of this strain.

between enzyme activity and transformation of sucrose into fructooligosaccharides, the transfructosylating and hydrolyzing activities in the cells and culture broth of microorganisms, including *A. niger* ATCC 20611, were assayed. The culture conditions used were those determined for the formation of high transfructosylating activity in our previous work.[15] Since the majority of both activities existed in the cells, only the intracellular activities are described. The changes in the two activities, cell growth and pH were followed during the 3 day cultivations, as shown in Fig. 1, which presents two typical examples. The distributions of the two activities in the 11 microorganisms examined after 1 and 3 days incubation are summarized in Table I.

The data shown in Table I indicate that both the transfructosylating and hydrolyzing activities are widely distributed in various strains of microorganisms, but there were remarkable differences among them. High enzyme pro-

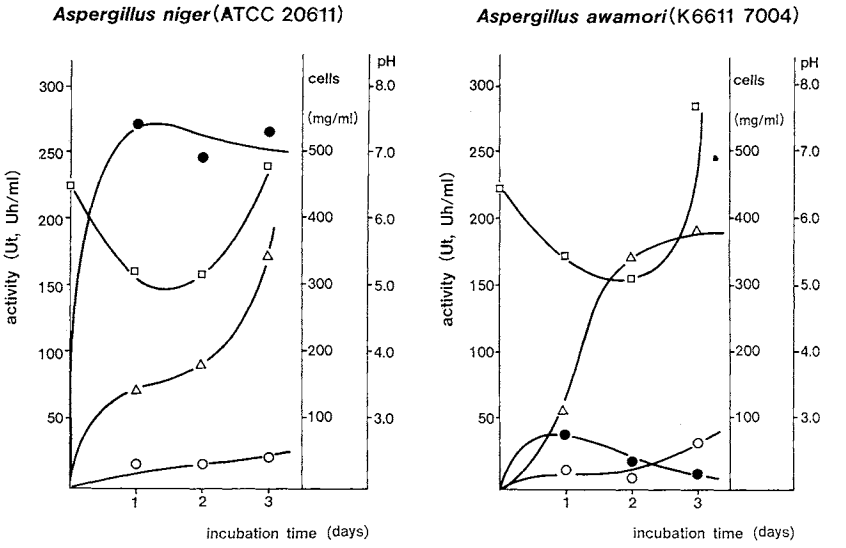


FIG. 1. Time Courses of the Production of Enzymes by Two Typical Organisms.
Transfructosylating activity, $U_t$ (●—●); hydrolyzing activity, $U_h$ (○—○); amount of cells, (△—△); pH of the medium, (□—□).

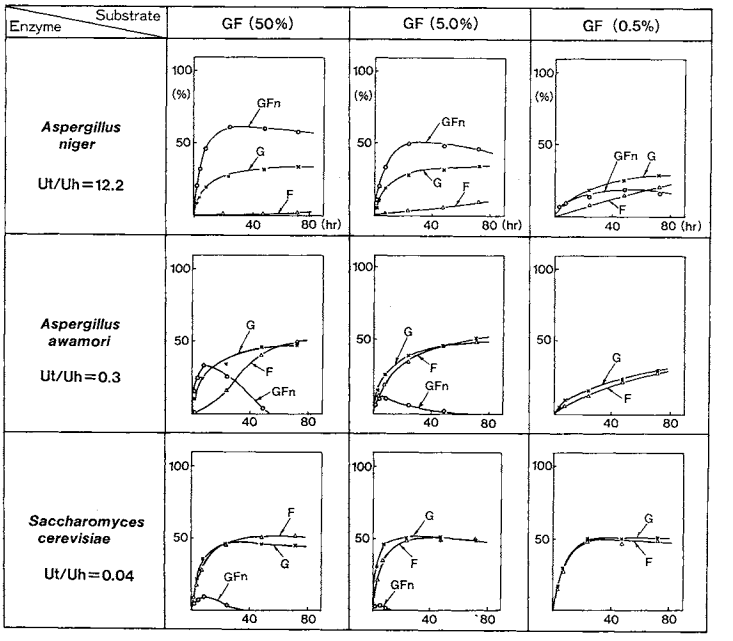


FIG. 2. Effect of the Sucrose Concentration on the Formation of Transfructosylated and Hydrolyzed Products [$GF_n$ and fructose (F)] by three typical enzymes. Six units ($U_t$) of each enzyme was used per gram sucrose.

ductivity as to the transfructosylating activity was detected in strains of *A. niger* (Nos. 1 and 3), *Aspergillus awamori* and *Aureobasidium pullulans* (No. 12), and especially in a strain of *A. niger* ATCC 20611 (No. 1). On the other hand, high productivity as to the hydrolyzing activity was observed in *A. awamori* (No. 7) and *S. cerevisiae* (No. 11). Concerning the

relative strength of the transfructosylating activity to that of the hydrolyzing activity, high $U_t/U_h$ ratios were observed for strains of *A. nigher* (Nos. 1 and 3) and *A. pullulans* (No. 12). In most of the examined strains, this ratio tended to be at maximum at an early stage (1 day incubation) and to decrease at a later stage (3 days incuation). However, *A. niger* ATCC 20611 showed almost the same ratio throughout the incubation.

*Effect of the sucrose concentration on the formation of fructooligosaccharides*

The effect of the sucrose concentration on the formation of fructooligosaccharides, on the incubation of three typical microorganisms showing different $U_t/U_h$ ratios, is shown in Fig. 2. Both an increase in the sucrose concentration and one in the $U_t/U_h$ ratio caused an increase in the formation of fructooligosaccharides and, on contrary, the hydrolyzed products increased with a decrease in the sucrose concentration and one in the $U_t/U_h$ ratio. That is, the largest amount of fructooligosaccharides was produced with 50% sucrose by a strain of *A. niger* and much fructose was liberated with a lower sucrose concentration by *S. cerevisiae*.

The difference in the bond-formation in the products was investigated by quantitative HPLC analysis of the trisaccharides obtained. Table II shows that 8 hr incubation of the *A. niger* cells gave only 1-kestose in a good yield, but a similar reaction with *S. cerevisiae* afforded three kinds of trisaccharide, *i.e.*, neokestose, 1-kestose and 6-kestose.

TABLE II. EFFECTS OF THE ENZYMES OF VARIOUS ORGANISMS ON THE TRISACCHARIDE FORMATION THROUGH THEIR TRANSFRUCTOSYLATION

| Condition[a] | Enzyme | *A. niger* | | *A. awamori* | | *S. cerevisiae* | |
|---|---|---|---|---|---|---|---|
| | time (hr) | 8 hr | 72 hr | 8 hr | 72 hr | 8 hr | 72 hr |
| Product[b] | $F_{2\to6}G$ | 0 | 0 | 0 | 0 | 2 | 0.5 |
| | Neokestose | 0 | 0 | 0 | 0 | 0.7 | 0 |
| | 1-Kestose | 40 | 19 | 32 | 0 | 0.5 | 0 |
| | 6-Kestose | 0 | 0 | 1 | 0 | 4 | 0 |
| | Nystose | 7 | 31 | 4 | 0 | 0 | 0 |

[a] All reactions were carried out in a 50% (w/v) sucrose solution containing 6 units ($U_t$) of enzyme per gram sucrose.

[b] Only oligosaccharides are presented as weight percentages of total sugar, as determined by HPLC calculation.

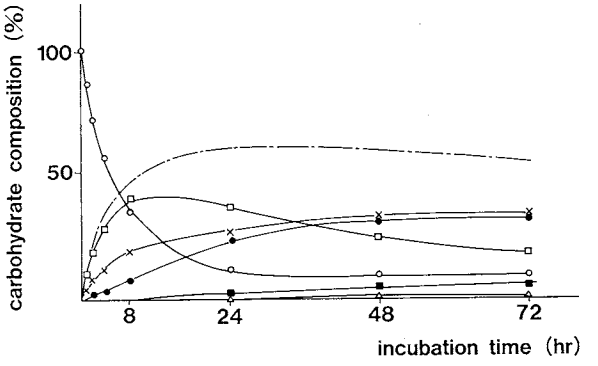


FIG. 3. Time Course of the Formation of Fructooligosaccharides with Cells of *A. niger* ATCC 20611.
Conditions: enzyme activity, $6U_t$/g sucrose; initial sucrose concentration, 50% (w/v). Symbols: ○—○, sucrose; △—△, fructose; ×—×, glucose; □—□, $GF_2$; ●—●, $GF_3$; ■—■, $GF_4$; —·—, $GF_n$.

*Preparation of fructooligosaccharides*

During the transfructosylating reaction on sucrose with cells of *A. niger* ATCC 20611, the changes in the carbohydrate composition were followed (Fig. 3). As the reaction progressed, sucrose was rapidly converted into fructooligosaccharides ($GF_2$ and $GF_3$), and the content of these saccharides reached *ca.* 60% of the total carbohydrates within 24 hr. After 24 hr incubation, the consumption of sucrose seemed to stop at a *ca.* 10% content, and $GF_2$ decreased gradually as $GF_3$ and $GF_4$ increased.

Separation of the series of purified fructooligosaccharides ($GF_2 \sim GF_4$) from the reaction mixture was attempted by two methods: carbon column chromatography and preparative HPLC. Although simple application of the former gave only low yields of the pure saccharides, the combination of the two methods successfully afforded the series of desired saccharides in good yields.

Methylation, PMR and CMR analyses showed that $GF_2$, $GF_3$ and $GF_4$ were 1-kestose, nystose and $1^F$-fructofuranosylnystose, respectively.

## DISCUSSION

The data presented in this paper show that transfructosylating activity, which is responsible for the production of fructooligosaccharides from sucrose, exists in various microorganisms. However, there were quantitative differences among the latter, the enzyme of *A. niger* ATCC 20611 being the most efficient for the preparation of fructooligosaccharides.

It is known that β-FFases commonly possess both transfructosylating ($U_t$) and hydrolyzing activity ($U_h$).[3] In order to estimate the transfructosylating ability of cultured cells, the changes in cell growth and specific activity of 11 microorganisms were examined during cultivation. $U_t$ and $U_h$ were assayed by HPLC as the amounts of trisaccharides and fructose formed from sucrose. Therefore, the $U_t/U_h$ ratio indicated the relative strength of the transfructosylating activity of each strain, and the enzyme productivity was defined as the cell growth multiplied by the corresponding specific activity. For the efficient production of fructooligosaccharides, it is preferable to have both a high $U_t/U_h$ ratio and high enzyme productivity. As judged from the data in Table I, the cells of *A. niger* ATCC 20611 showed the highest values for both parameters. These data thus indicate that this strain is the most suitable for the preparation of fructooligosaccharides.

With regard to the sucrose concentration, it was confirmed that the transfer reaction proceeded smoothly even in a 50% solution, and that the transferred products increased as the sucrose concentration increased. It is known that transfer to a substrate competes with transfer to water in the reaction catalyzed by β-FFases,[3] and the present results suggested that these enzymes would be β-FFases and that fructooligosaccharides could be effectively prepared with a high sucrose concentration by using enzymes showing a high $U_t/U_h$ ratio. Furthermore, a significant difference was observed in the bond-formation of the trisaccharides. As shown in Table II, the *A. niger* enzyme produced only one trisaccharide, but the *A. awamori* and *S. cerevisiae* enzymes afforded two and three kinds of trisaccharide, respectively. These three trisaccharides were identified as neokestose, 1-kestose and 6-kestose, which revealed that different enzymes have different relative specificities for the three receptor groups (OH-$1^F$, OH-$6^F$ and OH-$6^G$) of sucrose, as shown in Fig. 4. That is, the *S. cerevisiae* enzyme, which showed low regiospecificity for the transfer of fructosyl residues to all three primary alcohol groups of sucrose, most favored the OH-$6^G$ position,

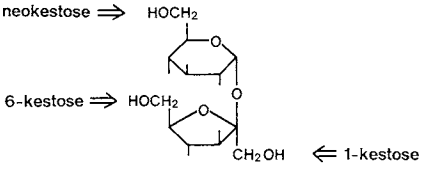


FIG. 4. Sites of Bond Formation in Sucrose for Three Kinds of Trisaccharides.

but the *A. niger* enzyme showed greater specificity and transferred fructose virtually solely to the OH-$1^F$ position.

Through the strong transfructosylating action of the enzyme of *A. niger* ATCC 20611, sucrose was practically converted into a mixture of fructooligosaccharides with inulin-type structure of $1^F(1\text{-}\beta\text{-fructofuranosyl})_n$-sucrose ($n=1$ to 3). The time course of the reaction (Fig. 3) indicated that saccharides showing a higher degree to polymerization increased with the progress of the reaction, although the total amount of oligosaccharides did not change much after reaching *ca.* 69% of the total sugar. Therefore, a mixture with a desired composition can be prepared by controlling the reaction conditions.

The method for the purification and the properties of the enzyme from *A. niger* ATCC 20611 will be reported in a subsequent paper.

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