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Purification and properties of glucosyltransferase from *Aureobasidium*

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SUMMARY

Purification and properties of glucosyltransferase, which produces panose (Glcα1 → 6Glcα1 → 4Glc) and isomaltose (Glcα1 → 6Glc) from maltose (Glcα1 → 4Glc), are reported. The enzyme, from *Aureobasidium*, was purified to homogeneity by fractionations involving ammonium sulfate and DEAE-Cellulofine, S-Sepharose Fast Flow and Sephadex G-200 chromatography. Molecular mass of the enzyme was estimated to be 395 kDa by gel filtration. The enzyme was identified as a glycoprotein which contains 32% (w/w) carbohydrate. The optimum pH for the enzymatic reaction was 4.5–5.5 and the enzyme was stable over a pH range of 4–6. The optimum reaction temperature for the enzyme was 65 °C and the enzyme retained more than 96% activity at 60 °C after 15 min. The enzyme produced panose from maltose by means of a high efficiency (45.5%) glucosyl-transfer reaction. The enzyme was inhibited by metal ions, such as those of mercury, silver and aluminum, and also by organic inhibitors, especially nitrilotriacetic acid.

INTRODUCTION

Investigations about glucosyltransferase (EC 2.4.1.24: 1,4-α-Glucan 6-α-D-glucosyltransferase) that produce panose and isomaltose from maltose have become important because its products support large numbers of *Bifidobacterium* and so are useful in some health foods [6]. Panose is also a mildly sweet trisaccharide which is suited as an anti-cariogenic sweetening sugar in foods because it inhibits synthesis of water-insoluble glucans by *Streptococcus mutans* and is not fermentable by many oral bacteria [7,10]. Microbial glucosyltransferases from *Aspergillus* spp. [9,11,13,16] have been purified and their enzymatic characteristics have been reported. There is, however, no report on the purification of glucosyltransferase from *Aureobasidium*.

Previously we reported the production of glucosyltransferase, which produces panose from maltose, by *Aureobasidium* [5]. In the present paper, we describe the purification and properties of glucosyltransferase from *Aureobasidium* sp. ATCC 20524.

MATERIALS AND METHODS

Microorganism

Aureobasidium sp. ATCC 20524 which was maintained on agar slants (maltose 1%, yeast extract 0.2%, agar 1.8% and pH 7) was used for this investigation.

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Cultivation and preparation of crude enzyme

Cultivation of the strain for enzyme production was carried out in liquid culture (maltose 2.5%, yeast extract 1.5%, K_2HPO_4 0.75%, $MgSO_4 \cdot 7H_2O$ 0.05%, pH 7) at 30 °C for 2 days with the same conditions described in a previous report [5]. Cells were harvested by centrifugation and lyophilized. The enzyme was solubilized from 5 g of dried cells by 20 mg of Kitalase (2000 U endo-β-1,3-glucanase g^{-1}, Wako, Osaka, Japan) in 75 mM McIlvain buffer (pH 5) at 40 °C for 2.5 h and then centrifuged. The supernatant was used for enzyme purification.

Purification of the enzyme

Ammonium sulfate was added to the crude enzyme solution to 80% saturation and then centrifuged. The supernatant was then dialyzed against 20 mM acetate buffer (pH 4.5). The above purification steps were carried out on ice.

The dialysate was passed through a S-Sepharose Fast Flow column (3.2 × 22 cm) equilibrated with 20 mM acetate buffer (pH 4.5) and then dialyzed against 20 mM phosphate buffer (pH 6.5). The dialyzate was applied on a DEAE-Cellulofine A-800 column (3.2 × 22 cm) equilibrated with 20 mM phosphate buffer (pH 6.5). The column was washed with 20 mM phosphate buffer (pH 6.5) and then eluted with the same buffer containing 0–0.5 M NaCl. The fractions were collected and concentrated with a membrane filter (Amicon UM 10).

The enzyme solution from the previous stage was applied on a Sephadex G-200 column (1.5 × 85 cm) equilibrated with 20 mM phosphate buffer (pH 6.5) containing 0.1 M NaCl. After application of the enzyme to the column, it

TABLE 1

Purification of glucosyltransferase from *Aureobasidium*

Step	Activity (nkat)	Protein (mg)	Specific activity (nkat mg protein^{-1})	Yield (%)
Crude extract	115333	1320.5	87	100.0
Ammonium sulfate	101233	202.4	500	87.8
S-Sephadex Fast Flow	81717	89.9	909	70.9
DEAE-Cellulofine A-800	58017	15.7	3695	50.3
Sephadex G-200 (1st)	37333	9.2	4058	32.4
Sephadex G-200 (2nd)	24833	4.9	5068	21.5

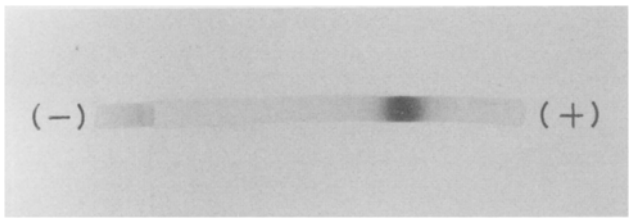

Fig. 1. Disc-PAGE of the purified enzyme from *Aureobasidium*.

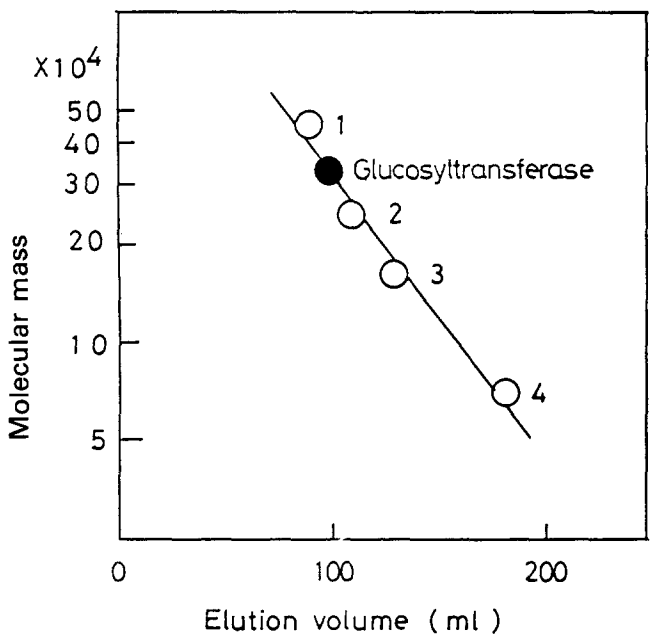

Fig. 2. Plot of logarithm of molecular masses of proteins against elution volumes on Sephadex G-200. Standard proteins: (1) ferritin (450 kDa); (2) catalase (240 kDa); (3) aldolase (158 kDa); (4) albumin (bovine serum; 68 kDa).

was eluted with the same buffer at a flow rate of 10 ml h^{-1}. The above chromatographic steps were carried out at 20 °C.

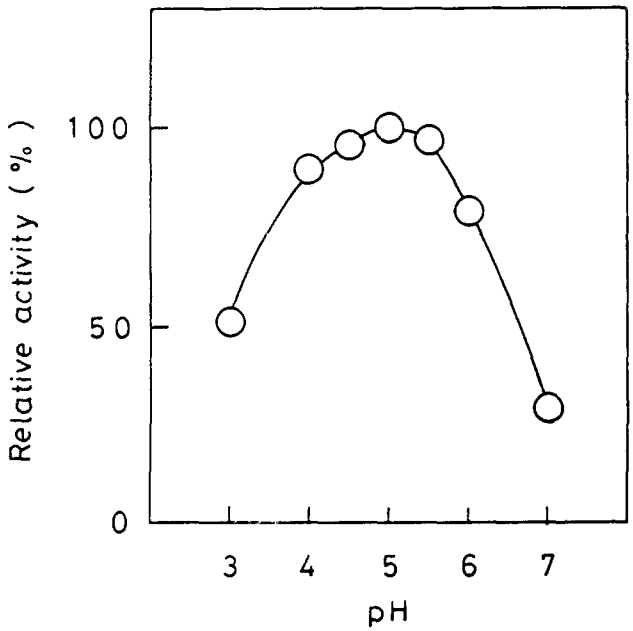

Fig. 3. The effect of pH on glucosyltransferase activity from *Aureobasidium*.

Molecular mass estimation

The molecular mass of the enzyme was estimated by Sephadex G-200 (1.5 × 85 cm) gel filtration according to the method of Andrews [1]. The column was equilibrated with 20 mM phosphate buffer (pH 6.5) containing 0.1 M NaCl. After application of the purified enzyme on the column, it was eluted with the same buffer at a flow rate of 10 ml h^{-1}. The elution of protein standards (ferritin, catalase, aldolase and bovin serum albumin; Boehringer-Mannheim) was carried out in the same manner.

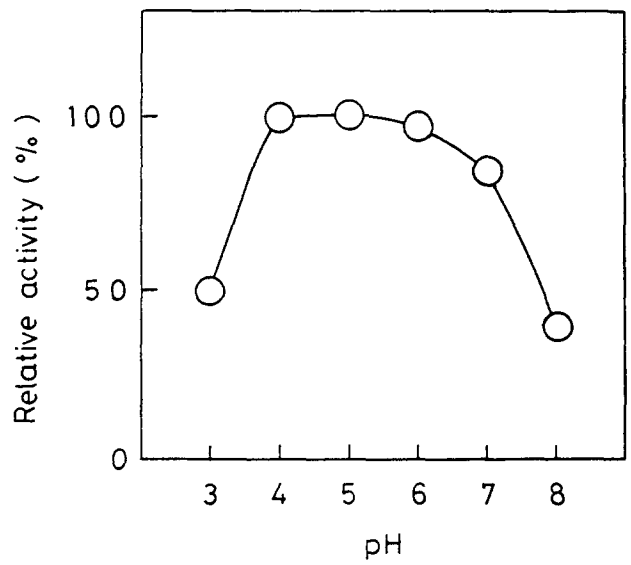

Fig. 4. The effect of pH on the stability of glucosyltransferase from *Aureobasidium*. The activities were measured after 3 h incubation at each pH.

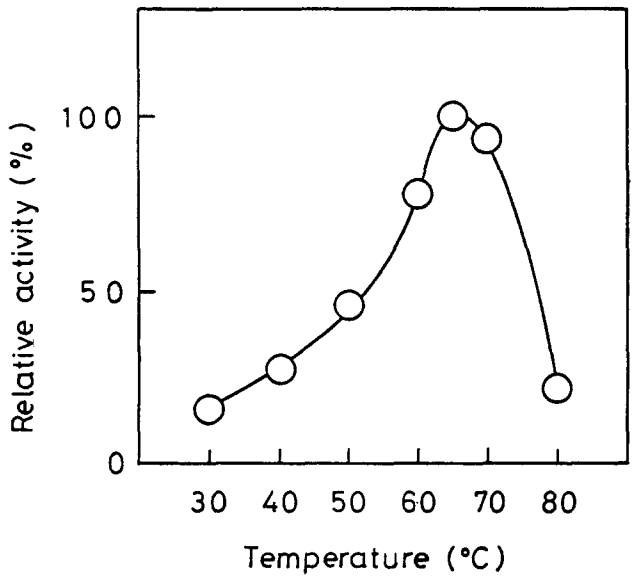

Fig. 5. Effect of temperature on the activity of glucosyltransferase from *Aureobasidium*.

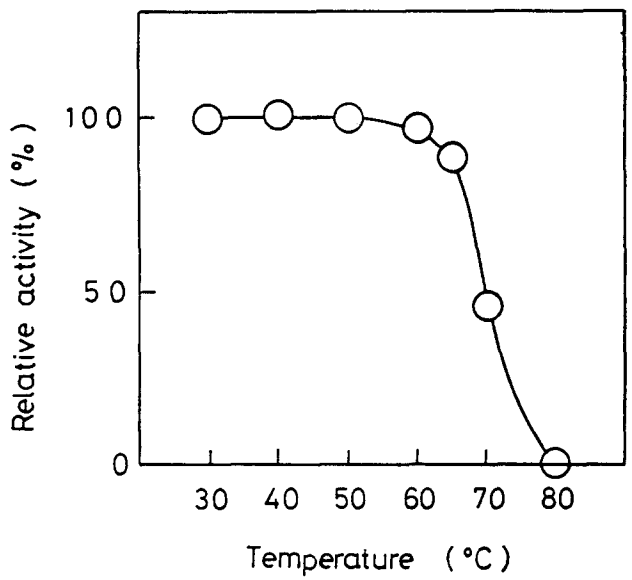

Fig. 6. Effect of temperature on the stability of glucosyltransferase from *Aureobasidium*. The activities were measured after 15 min incubation at each temperature.

Electrophoresis

Disc-electrophoresis of the purified enzyme was performed at a constant current of 3 mA per gel on a 7.5% (w/v) polyacrylamide gel at pH 8.3 according to the method of Davis [2]. After electrophoresis, the gel was stained with coomassie blue.

Assays

Enzyme activity was determined by a reaction mixture which consisted of 0.2 ml of enzyme solution and 0.8 ml of 37.5% (w/v) maltose in 75 mM McIlvain buffer (pH 5.0). The reaction mixture was incubated at 65 °C for 10 min as previously described [5].

Products in the reaction mixture were measured by high performance liquid chromatography (HPLC; Nippon Bunko, Tokyo, Japan) using a YMC-Pack Polyamine-II column (4.6 × 250 mm, YMC Co. Ltd, Kyoto, Japan) and RI detector under the conditions as described previously [5]. Glucose released in the reaction mixture was assayed by the glucose oxidase method (Glucose test B; Wako, Osaka, Japan). One katal of enzyme activity is defined as the quantity of enzyme responsible for the transfer of 1 mol of glucose per second.

Protein was assayed by the method of Lowry et al. [8]

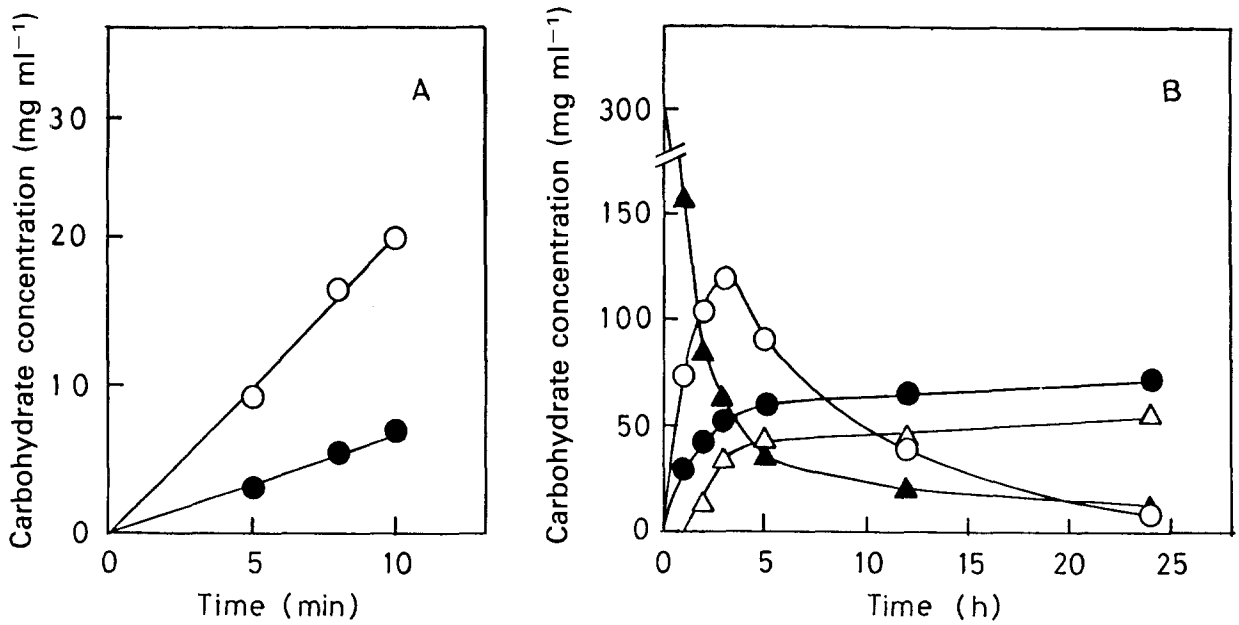

Fig. 7. Time courses of product formation by glucosyltransferase from *Aureobasidium* at 65 °C (A) and 55 °C (B). Figure symbols: panose (○), isomaltose (△), glucose (●) and maltose (▲).

TABLE 2

Effect of various metal ions and organic inhibitors on glucosyltransferase activity from *Aureobasidium*

Compound (1 mM)	Relative activity (%)
$HgCl_2$	0
$AgNO_3$	1
$AlCl_3$	1
$NiSO_4$	20
$CuSO_4$	23
$BaCl_2$	65
$CoCl_2$	72
$ZnSO_4$	78
$MgSO_4$	95
$CaCl_2$	97
$FeSO_4$	115
$Pb(OAc)_2$	124
$MnSO_4$	128
Nitrilotriacetic acid	17
Monoiodoacetic acid	44
Sodium arsenate	44
Sodium fluoride	52
Sodium citrate	89
PCMB	90
EDTA	97
Sodium azide	98
Sodium oxalate	98
Hydroxylamine	98
2NA	107
Control	100

PCMB: *p*-chloromercuribenzoic acid.
EDTA: ethylenediaminetetraacetic acid.
2NA: Ethylenediaminetetraacetic acid disodium salt.
Effect of metal ions on enzymatic activity was tested in PIPES (piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)/NaOH) buffer (40 mM, pH 6.1).

with bovine serum albumin (Nakalai, Kyoto, Japan) as standard.

Carbohydrates were assayed by the method of Dubois et al. [3] with glucose (Wako, Osaka, Japan) as standard.

Paper chromatography

Sugars in the reaction mixture were separated on Toyo no. 50 filter paper using a solvent system of *n*-butanol/pyridine/water (6:4:3, v/v) using three ascents and were detected with silver nitrate reagent (2.5 ml of saturated silver nitrate aqueous solution and 500 ml of acetone were mixed, and the resultant precipitate was dissolved by the addition of 15 ml water).

^{13}C-Nuclear magnetic resonance (NMR) analysis

^{13}C-NMR spectra of samples were obtained in solution in D_2O at 250 MHz with a Bruker A-250 spectrometer operated in the Fourier-transform mode, with complete proton decoupling. Chemical shifts were expressed in ppm from the signal of tetramethylsilane.

RESULTS AND DISCUSSION

Purification of the enzyme

The results of purification of glucosyltransferase from *Aureobasidium* are shown in Table 1. The specific activity of the final purified material was 5068 nkat mg protein^{-1} representing a purification factor of 58. The enzyme showed a broad single band by Disc-PAGE (Fig. 1) similar to the glycoprotein invertase [4].

The molecular mass of the enzyme was determined by gel filtration. As shown in Fig. 2, the molecular mass was estimated to be 395 kDa. This value is higher than that reported for the glucosyltransferase from *Aspergillus niger* (116 kDa) [9]. The carbohydrate content of the enzyme was 32% (w/w) and was similar to that reported for the glucosyltransferase from *A. niger* (27.6%) [9].

Effects of pH and temperature on the enzyme

The effect of pH on the enzyme reaction is shown in Fig. 3. Optimum reaction pH of the enzyme was 4.5–5.5 and was slightly higher than that reported for the *Aspergillus* enzymes [17]. The enzyme was stable within the pH range of 4–6 (Fig.4), retaining more than 96% of its original activity. The pH stability data were again similar to the *A. niger* system [9].

The effect of temperature on the reaction of the enzyme is shown in Fig. 5. Optimum reaction temperature for the enzyme was 65 °C and is higher than that reported for enzymes from *Aspergillus* [17] and *Penicillium* [15]. The enzyme retained more than 96% activity at 60 °C, 89% at 65 °C, but was completely inactivated at 80 °C for 15 min (Fig. 6). The thermal stability of the enzyme was similar to a glucosyltransferase from *A. niger* [16].

Time course of the enzymatic reaction

In Fig. 7(A), the time course of enzymatic reaction at 65 °C is shown. The enzyme produced panose from maltose and released glucose into the reaction mixture. The enzyme exhibited no maltose-hydrolyzing activity under these reaction conditions. The time course of enzymatic reaction at 55 °C is shown in Fig. 7(B). The efficiency of panose production to initial maltose concentration reached 45.5% after 3 h. The molecular ratios of panose vs glucose in the reaction mixture after 10 min at 65 °C and 3 h at 55 °C were 1.04 and 0.93, respectively.

An oligosaccharide in addition to panose was produced in the reaction mixture during prolonged incubation. The HPLC retention time of the product (30.8 min) and its paper chromatography R_F value (0.42) were identical to those of isomaltose. The chemical shifts (ppm) for ^{13}C-NMR analysis of the product were also identical to those of authentic isomaltose: 61.1, 66.3, 70.1, 70.2, 70.7, 72.2, 72.5, 73.8, 74.8, 75.0, 76.7, 92.9, 96.6 and 98.6. Although similar enzymes from *A. niger* [14,16] and *A. oryzae* [12] produced isomaltose as the major product, the enzyme from *Aureobasidium* produced more panose than isomaltose.

Effects of metal ions and inhibitors on enzymatic activity

Effects of metal ions and other known enzyme inhibitors on the activity of glucosyltransferase from *Aureobasidium* are shown in Table 2. The activity of the enzyme was strongly inhibited by Hg^{2+}, Ag^{+}, Al^{3+}, Ni^{2+} and Cu^{2+}, each at a concentration of 1 mM. The enzyme was also inhibited by nitrilotriacetic acid, monoiodoacetic acid and sodium arsenate.

In spite of the excellent qualities of panose described in the Introduction, little work on an efficient process for its mass production has been reported. While the production of panose from pullulan using the hydrolyzing reaction of neopullulanase from *Bacillus* has been reported in a previous paper [7], the panose concentration detected in the reaction mixture was low because of the low substrate concentration (5%). Results from the above experiments suggest the usefulness of glucosyltransferase from *Aureobasidium* for the production of high concentrations of panose and isomaltose from maltose by the glucosyl-transferring reaction. The above desirable glucosyltransferase may also be useful for the synthesis of favorable oligosaccharides and/or glucosides by glucose transfer to C_6 hydroxyl group.

REFERENCES

1 Andrews, P. 1965. The gel-filtration behavior of proteins related to their molecular weights over a wide range. Biochem. J. 96: 595–606.

2 Davis, B.J. 1964. Disk electrophoresis. II. Methods and application to human serum proteins. Ann. N.Y. Acad. Sci. 121: 404–427.

3 Dubois, M., K.A. Gilles, J.K. Hamilton, P.A. Rebers and F. Smith. 1956. Colorimetric methods for determination of sugar and related substances. Anal. Chem. 28: 350–356.

4 Fujita, K., K. Hara, H. Hashimoto and S. Kitahata. 1990. Purification and some properties of β-fructofuranosidase I from *Arthrobacter* sp. K-1. Agric. Biol. Chem. 54: 913–919.

5 Hayashi, S., T. Hinotani, S. Hayashi, Y. Takasaki and K. Imada. 1993. Development of medium components for the production of glucosyl-transferring enzyme by *Aureobasidium*. World J. Microbiol. Biotechnol. 9: 248–250.

6 Kitahata, S. 1989. Synthesis of oligosaccharides by microbial enzymes. Kagaku to Kogyo 63: 161–169.

7 Kuriki, T., M. Tsuda and T. Imanaka. 1992. Continuous production of panose by immobilized neopullulanase. J. Ferment. Bioeng. 73: 198–202.

8 Lowry, O.H., N.J. Rosebrough, A.L. Farr and R.J. Randall. 1951. Protein measurement with the folin phenol reagent. J. Biol. Chem. 193: 265–275.

9 McCleary, R.V. and T.S. Gibbon. 1989. Purification, properties, and industrial significance of transglucosidase from *Aspergillus niger*. Car. Res. 185: 147–162.

19 Ooshima, T., T. Fujiwara, T. Takei, A. Izumitani, S. Sobue and S. Hamada. 1988. The caries inhibitory effects of GOS-sugar *in vitro* and in rat experiments. Microbiol. Immunol. 32: 1093–1105.

11 Pazur, J.H. and T. Ando. 1961. The isolation and the mode of action of a fungal transglucosidase. Arch. Biochem. Biophys. 93: 43–49.

12 Pazur, J.H. and D. French. 1952. The action of transglucosidase of *Aspergillus oryzae* on maltose. J. Biol. Chem. 196: 265–272.

13 Pazur, J.H., A. Cepure, S. Okada and L.S. Foresberg. 1977. Comparison of the action of glucoamylase and glucosyltransferase on D-glucose, maltose, and malto-oligosaccharides. Car. Res. 58: 193–202.

14 Pazur, J.H., Y. Tominaga, C.W. DeBrosse and L.M. Jackman. 1978. The synthesis of 1,6-anhydro-β-D-glucopyranose and D-glucosyl oligosaccharides from maltose by a fungal glucosyltransferase. Car. Res. 61: 279–290.

15 Saroja, K., R. Venkataraman and K.V. Giri. 1955. Transglucosidation in *Penicillium chrysogenum* Q-176. Biochem. J. 60: 399–403.

16 Tsujisaka, Y. and J. Fukumoto. 1963. Studies on maltose splitting enzymes of mould. Part I. Crystallization of transglucosidase of *Aspergillus niger*. Nippon Nogeikagaku Kaishi 37: 673–676.

17 Vámos, L. and P. Rose. 1973. Investigations into and an enzymic assay method of transglucosidase activity of commercial glucoamylase preparation. Stärke 25: 195–202.