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GLUCOFRUCTOSAN BIOSYNTHESIS IN *FUSARIUM OXYSPORUM*

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Key Word Index—*Fusarium*; Ascomycetes; fructosans; fructosyl transferase; biosynthesis.

Abstract—Low MW glucofructosans have been detected in the medium of *Fusarium oxysporum*. A 53-fold purification of fructosyl transferase has been achieved by ethanol precipitation, DEAE-cellulose and Sephadex G-100 column chromatography. Maximum fructosyl transferase activity coincided with maximum glucofructosan concentration in the medium. Invertase showed greatest activity in the later stages of growth when glucofructosans were absent. Fructosyl transferase and invertase have been separated by DEAE-cellulose column chromatography. On the basis of kinetic studies and effect of nucleotides on fructosyl transferase in the presence and absence of $MgCl_2$, a two site active centre linked through a nucleotide bridge is proposed. Fructosyl transferase and invertase are highly phosphorylated.

INTRODUCTION

Glucofructosan biosynthesis in plants has been extensively studied [1–12]. A theory of integrated action of certain fructosyl transferring enzymes for fructosan biosynthesis in Jerusalem artichoke had been proposed [13]. In *Agave*, self transfer is reported to be the main pathway for fructosan biosynthesis [14]. Yeast invertase under conditions of high sucrose concentrations could transfer fructose to another sucrose molecule to yield a difructosyl glucose [15–17]. In the recent past, fructose nucleotides have been isolated from various plant sources [18–21] but their physiological role is still not certain. The present study aims at elucidating the mechanism of glucofructosan biosynthesis in *Fusarium oxysporum*.

RESULTS

Detection of glucofructosans in the medium

An examination by partition PC of the growth medium taken on the sixth day after inoculation showed the presence of spots with R_f values less than that of sucrose. Analysis of these spots showed that these correspond to the empirical formulae F_2G, F_3G and F_4G, respectively (Table 1).

Comparative studies on glucofructosans, fructosyl transferase and invertase during various stages of growth

Carbohydrate from the medium on different days was isolated and estimated (Fig. 1). Sucrose concentration showed a progressive decrease from the first day onward. The glucofructosans which initially showed an increase subsequently declined. On the sixth day, when the specific activity of fructosyl transferase was maximum, the concentration of glucofructosans in the medium was also maximum (Fig. 2). After the sixth day, fructose predominated over glucose (Fig. 1). A crude extract of the mycelium had maximum invertase activity on the sixteenth day when glucofructosans were absent (Fig. 2).

Crude extract from 5-day-old mycelium was purified by precipitation with ethanol (200%) followed by

Table 1. Analysis of glucofructosans

Spots	1 Reducing sugar(μg)	2 Glucose (μg)	3 Fructose (μg)	4 Fructose = *RS–glucose (μg)	5 Glucose = RS–fructose	Ratio of fructose/glucose (3/2)	(4/2)	(3/5)	(4/5)	Mean
1(F_2G)	126.5	43.5	88.1	83.0	37.4	2.02	1.91	2.35	2.22	2.12
2(F_3G)	112.9	28.0	83.3	84.9	29.6	2.97	3.03	2.81	2.86	2.92
3(F_4G)	65.5	13.3	52.4	52.2	13.1	3.94	3.93	4.00	3.99	3.94

* RS = reducing sugar.

Glucofructosans F_2G, F_3G and F_4G were isolated from the medium by partition PC, hydrolysed separately with N HCl at 70° for 15 min, neutralized with N sodium carbonate and thereafter, glucose, fructose and RS were estimated.

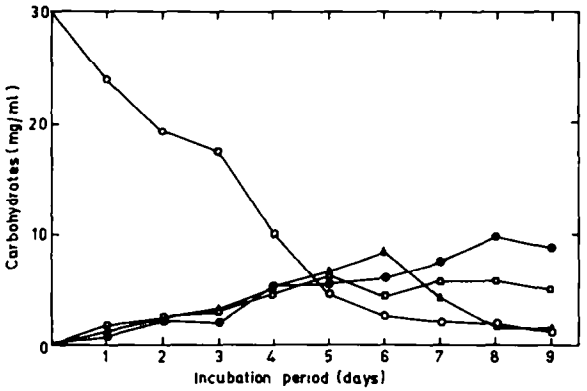

Fig. 1. Changes in the concentration of carbohydrates in the medium of different days of growth. ○—○, sucrose; □—□, glucose; ●—●, fructose; ▲—▲, glucofructosans.

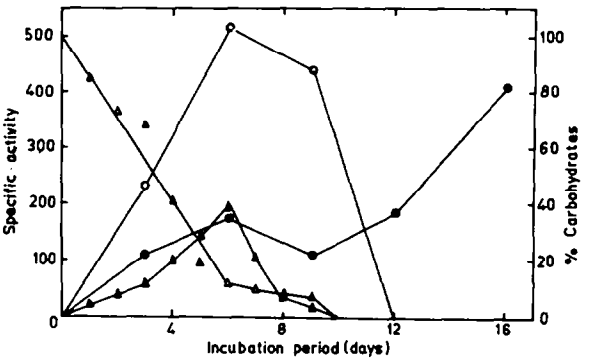

Fig. 2. Relationship between specific activities of fructosyl transferase and invertase and the concentration of glucofructosans and sucrose on different days of growth. ○—○, fructosyl transferase; ●—●, invertase; △—△, sucrose; ▲—▲, glucofructosans.

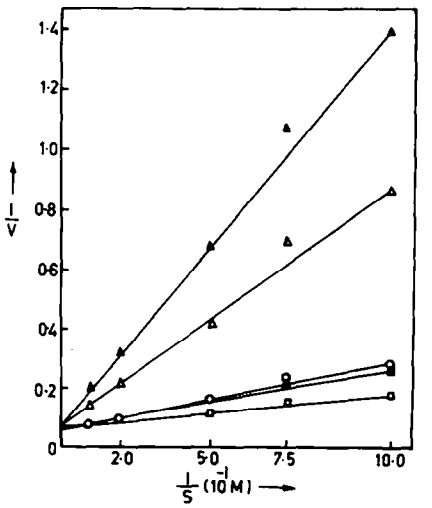

Fig. 3. Lineweaver–Burk plot showing the effect of glucose and fructose on the velocity of fructosyl transferase. ○—○, control; ■—■, 0.05 M fructose; □—□, 0.1 M fructose; △—△, 0.05 M glucose; ▲—▲, 0.1 M glucose. Assay system consisted of 1 ml sucrose, 0.1 ml of [^{14}C]-sucrose (1 μCi), 0.1 ml of enzyme and the required amount of glucose or fructose in 0.1 M sodium acetate buffer pH 5. It was incubated at 25° for 80 min. Two fractions, one of glucofructosans and other of spots with R_fs higher than those of glucofructosans were isolated by partition PC and radiactivity in these two fractions measured in a scintillation counter. Per cent incorporation in glucofructosans was calculated from:

$$\frac{\text{Counts observed in glucofructosan fraction}}{\text{Total counts incorporated in the two fractions}} \times 100.$$

Velocity was calculated as:

$$V = \frac{\%\text{ incorporation in glucofructosans}}{\text{minimum concentration of sucrose used in the assay system}} \times \text{concentration of sucrose used in the test system.}$$

chromatography on DEAE-cellulose and Sephadex G-100 columns. Three peaks were obtained after DEAE-cellulose column chromatography. Peak 1 was due primarily to a fructosyl transferase, although it also showed a slight invertase activity. Peak 2 was due to invertase activity. A 53-fold purification of fructosyl transferase has been achieved using the procedure described in Scheme 1 (Table 2).

MWs of both fructosyl transferase and invertase were found to be 70000 by the method of Whitaker [22] on Sephadex G-100. This may be compared with the MWs of fructosyl transferase from *Lactuca sativa* and *Agave cruz* which have been reported to be 100000 [23] and 62000 [11] respectively.

Table 2. Purification of fructosyl transferase

Stage of purification	Protein (mg)	Total units of fructosyl transferase	Specific activity of fructosyl transferase (units/mg protein)	Purification	% yield fructosyl transferase
Crude	133	71500	500	1.00	100
Ethanol precipitation	21.5	18000	840	1.6	25
DEAE-cellulose (fraction 6)	3.2	10000	3100	5.9	14
Sephadex G-100	0.278	8000	28700	57.4	11

Table 3. Incorporation of radioactivity in F_2G, using [^{14}C]-glucose, and [^{14}C]-sucrose together with non-radioactive sucrose

Carbohydrates	Sucrose + [^{14}C]-sucrose Counts/100 sec	% incorporation in F_2G	Sucrose + [^{14}C]-glucose Counts/100 sec	% incorporation in F_2G
F_2G	290	2.33	50	0.33
Sucrose	11700		2000	
Glucose	280		12600	
Fructose	200		420	

Two separate experiments were conducted. Assay system consisted of 20 μmol of sucrose, 60 μg of enzyme in 2 ml 0.1 M NaOAc buffer (pH5). In the first case the assay system contained 1 μCi of [^{14}C]-sucrose and in the second 1 μCi of [^{14}C]-glucose. Sugars were isolated by partition PC and radioactivity was measured.

Effect of addition of glucose and fructose on fructosyl transferase activity

At a lower concentration of sucrose (0.1 M) addition of moderate amounts of fructose (0.05 and 0.1 M) led to an increase in the fructosyl transferase activity (Fig. 3). At a higher concentration of sucrose (1 M), however, the added fructose in the above concentration range was without effect on the enzyme activity (Fig. 3). Addition of glucose at 0.05 and 0.1 M to the assay system containing 0.1 M sucrose, caused competitive inhibition of fructosyl transferase (Fig. 3). It was observed by partition PC that addition of either 0.2 M glucose or 1 M fructose to the assay system (0.1 M sucrose) led to complete inhibition of fructosyl transferase. Addition of uniformly labelled [^{14}C]-glucose to the assay system, led to its incorporation into sucrose and F_2G (Table 3).

Table 4. Effect of addition of nucleotides on the percentage of different sugars in the medium

Sugars	Control	ATP	UTP
F_3G	4.3	4.7	6.0
F_2G	11.3	13.0	13.3
Sucrose	27.8	21.4	20.0
Glucose	29.2	25.5	32.2
Fructose	27.4	35.4	29.4

F. oxysporum was grown for 100 hr at 30 ± 2° on a shaker. One set of flasks was kept as control. To the other two sets, 1 mg/ml of ATP in one set and 1 mg/ml of UTP in the other set were added. Flasks were kept for 28 hr. Carbohydrates of the medium were separated by partition PC and estimated.

Effect of added nucleotides on biosynthesis of glucofructosans

Addition of ATP and UTP to the medium at the 100 hr growth stage, enhanced the concentration of glucofructosans in the medium by 13 and 24% respectively as calculated from Table 4.

Effect of magnesium chloride and nucleotides on fructosyl transferase activity

Addition of $MgCl_2$ had very little effect on fructosyl transferase activity. Addition of various nucleotides considerably enhanced the fructosyl transferase activity. When $MgCl_2$ was added together the various nucleotides, in general, a decrease in enzyme activity was observed (Table 5).

Phosphorylated nature of fructosyl transferase and invertase

Purification of fructosyl transferase was accompanied by an increase in the ratio of phosphate: protein (Table 6).

Table 5. Effect of various nucleotides and magnesium ions on the activity of partially purified fructosyl transferase

	μmol of RS*	μmol of glucose	μmol of free fructose	% invertase activity	μmol of fructose transferred	% of fructose transferred
Control	32.1	19.1	13.0	100.0	6.1	100.0
ATP	28.2	18.3	10.0	76.8	8.3	135.7
UTP	29.8	19.6	10.2	78.4	9.4	154.2
$MgCl_2$	31.2	19.0	12.3	94.4	6.7	109.8
ATP + UTP	29.9	19.8	10.1	77.7	9.8	159.9
ATP + $MgCl_2$	32.5	19.1	13.4	103.2	5.7	93.1
UTP + $MgCl_2$	32.3	20.0	12.3	95.1	7.6	124.7
ATP + UTP + $MgCl_2$†	30.6	18.3	12.3	94.8	6.0	98.5
ADP	31.8	20.5	11.3	87.3	9.1	149.8
ADP + $MgCl_2$	32.5	19.7	12.8	98.8	6.8	111.8

Concentration of the components used above in the assay system was 4 mg/ml.

* RS = reducing sugar.

† In the case of ATP + UTP + $MgCl_2$, 4 mg each of ATP, UTP and $MgCl_2$ were added.

Table 6. Data on phosphorylated nature of fructosyl transferase

Fraction tested	Purification (-fold)	Mol organic phosphate/ mol protein
After passing on DEAE-cellulose column	5.9	80.3
Fraction 13 from Sephadex G-100 column*	54.8	155

* Fractions of 5 ml each was collected.

Table 7. Fructosyl transferase purification by Scheme 2

Stage of purification	Protein (mg)	Total units	Specific activity (units/mg protein)	Purification
Crude	180.0	1980	11	
Supernatant after 100% ammonium sulphate saturation	11.0	1780	162	14.7
Sephadex G-200	2.0	975	477	43.3
DEAE-cellulose	1.5	425	283	25.7

The required quantity of enzyme was incubated with sucrose in 0.1 M sodium acetate buffer pH 5 so that total concentration of sucrose was 0.1 M in a total volume of 1 ml. It was incubated for 1 hr at 25° and reaction was stopped by heating the assay mixture to 100° for 10 min. One unit of fructosyl transferase is defined as the quantity of enzyme responsible for the formation of 1 μmol of glucose at pH5 at 25° in 1 hr.

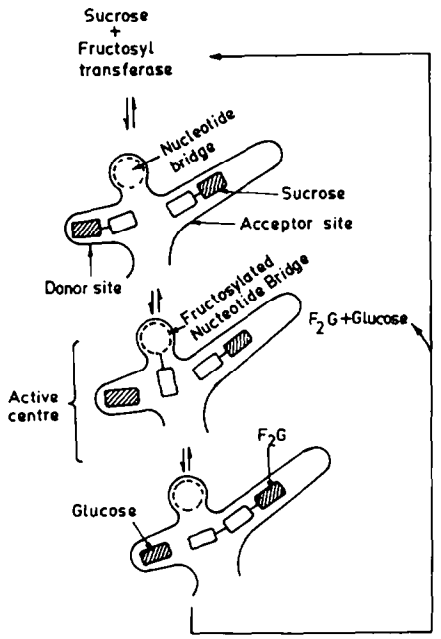

Fig. 4. A proposed model for fructosyl transferase action. ▨—□—, sucrose; ▨—, glucose, □·—, fructose.

In fructosyl transferase purified by Scheme 2 (Table 7), the ratio of organic phosphorus: protein on a molar basis was 73.03. Inorganic phosphate was absent in all the purified fractions. In the final step of purification of [^{32}P]-fructosyl transferase and invertase, two ^{32}P peaks overlapped the two protein peaks (Fig. 5). The phosphate: protein ratio in fructosyl transferase in fractions 6, 7 and 8 from DEAE-cellulose column was 71.5, 60.4 and 48.3 respectively. Fractions 21 and 22 of invertase were highly phosphorylated (Fig. 5).

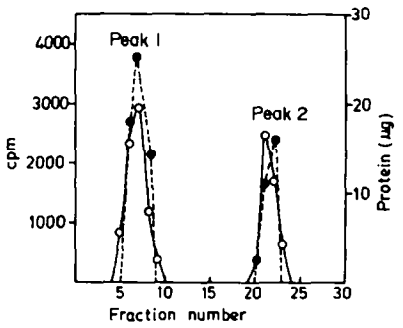

Fig. 5. Incorporation of ^{32}P into various fractions of fructosyl transferase (peak 1) and invertase (peak 2) when purified by DEAE-cellulose column chromatography. ●—·—●, protein; ○- - -○, radioactivity of ^{32}P.

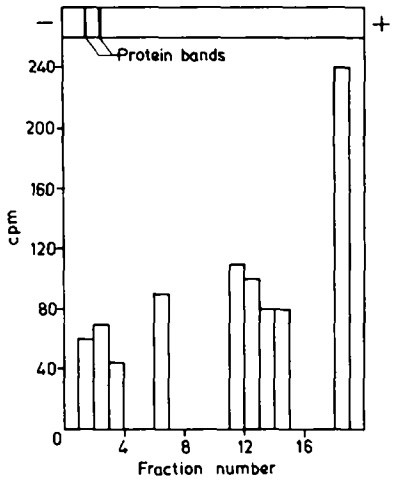

Fig. 6. Incorporation of ^{32}P in protein bands of fructosyl transferase separated by acrylamide gel electrophoresis.

Both enzyme preparations gave two bands by polyacrylamide gel electrophoresis (PAGE) (Figs. 6 and 7). When fructosyl transferase and invertase were mixed and co-PAGE carried out, only two protein bands were observed. Fructosyl transferase and invertase contained, respectively, 11.14 and 24.24% of the total radioactivity applied. The ratio of organic phosphate:fructosyl transferase ranged between 48 and 72. It was calculated that a minimum of 5–8 phosphate molecules were bound to fructosyl transferase.

DISCUSSION

The presence of low MW glucofructosans, i.e. F_2G, F_3G and F_4G similar to those detected in the medium of *F. oxysporum* (Table 1) have already been reported in other fungi [24–29]. It is postulated that the action of invertase and fructosyl transferase on sucrose may be primarily to supply glucose needed for the growth of *F. oxysporum* and that synthesis of glucofructosans may be only a side reaction. Only when sucrose supply in the medium was inadequate were glucofructosans utilized as an energy source. For example, when the sucrose concentration, which was 30 mg/ml at time of inoculation, fell to 2.7 mg/ml on the sixth day (Fig. 1), glucofructosans which at this stage were the major carbohydrate constituents in the medium (Fig. 1), became the principal source of glucose supply. Of the sugars formed on the hydrolysis of glucofructosans, glucose was preferentially utilized by the fungus, leading to an accumulation of fructose (Fig. 1). A similar accumulation of free fructose due to hydrolysis of glucofructosans during later stages of growth has also been observed both in *Claviceps purpurea* [30] and the higher plants, *Agave vera cruz* [31] and *Cichorium intybus* [32].

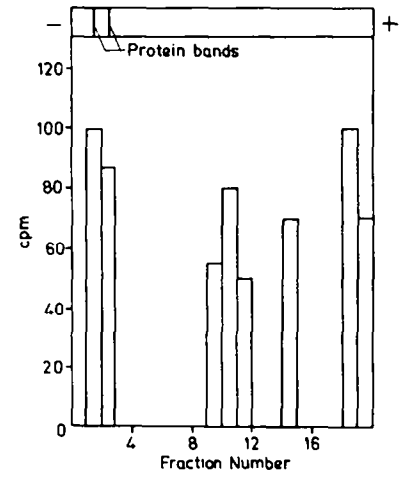

Fig. 7. Incorporation of ^{32}P in protein bands of invertase separated by acrylamide gel electrophoresis.

With yeast invertase a limited synthesis of glucofructosans has been reported only at high sucrose concentration [27]. In the present study, however, with only 3% initial sucrose concentration a very high concentration of glucofructosans accounting for as much as 39% of the total carbohydrates of the medium was observed on the sixth day (Fig. 2). Such a large accumulation of glucofructosans cannot, evidently, be explained on basis of action of invertase on sucrose as suggested earlier [26]. Data presented in Fig. 2 conclusively showed that fructosyl transferase and invertase are different enzymes and that glucofructosans are synthesized mainly by the action of fructosyl transferase. In general, fructosan-storing plants have both the fructosyl transferase and hydrolytic activities [11, 33].

The high degree of competitive inhibition by glucose (Fig. 3) indicates that, although sucrose is the true substrate for the enzyme, the latter may bind glucose more effectively than it does sucrose. It is not unreasonable to assume that the enzyme utilizes the glucose part of the sucrose molecule primarily for binding. With the glucose firmly bound at the active centre, transfer of fructose may become more facile.

Inulin did not act as the acceptor of the fructosyl residues. Presumably because of its much larger size it could not fit into the acceptor site meant for the much smaller sucrose molecule. With an increase in sucrose concentration up to 20% in the medium and *in vitro* too, no higher homologue of glucofructosans than F_4G was formed, suggesting that the acceptor site is perhaps just big enough to accommodate glucofructosans up to F_4G only.

Uniformly labelled [^{14}C]-fructose was not incorporated into F_2G when added in Czapek's medium. This observation rules out the involvement of fructosyl nucleotide in its biosynthesis if it is assumed that fructose-2-phosphate is the necessary component for the biosynthesis of nucleoside diphosphate fructose. While there is no direct evidence for the involvement of nucleoside diphosphate fructose, added nucleotides in the medium did affect the rate of glucofructosan biosynthesis (Table 4). $MgCl_2$ by itself had very little effect on fructosyl transferase activity (Table 5). However, when added together with nucleotides, it led to a lowering of the activity as compared to cases when only nucleotides were added. Mg^{2+} ions may form a complex with nucleotides and the Mg nucleotide complex may be less effective when compared with the nucleotide alone in increasing fructosyl transferase activity. The observed increase in fructosyl transferase activity was accompanied by a concomitant decrease in invertase activity (Table 5).

A tentative model for fructose transfer is proposed which assumes the presence of two sites on the fructosyl

transferase for sucrose, one acting as the donor and the other as the acceptor site, with a nucleotide acting as a bridge between the two sites (Fig. 4). It is proposed that fructose is transferred from the donor site to the bridge and this fructosylated nucleotide, in turn, transfers the fructose moiety to the sucrose at the acceptor site to form F_2G. In its presence, the fructose residue from a donor sucrose is transferred only to an acceptor molecule sucrose or a glucofructosan and not to water. The nucleotide bridge may thus be a regulatory mechanism for activating either enzyme activity, depending on which carbohydrates are available in the medium for the supply of energy to the organism. On the basis of such a model one can explain the transfer of fructose from the acceptor site to glucose on the donor site via the nucleotide bridge to form sucrose. This model also explains the enhanced activity of fructosyl transferase on addition of fructose into the assay system (Fig. 3). Fructose probably occupies the vacant positions on the acceptor site (it is assumed that the acceptor site is large enough to accommodate up to F_4G) and gives suitable conformation to sucrose at the acceptor site leading to enhanced glucofructosan biosynthesis. Complete inhibition of fructosyl transferase at higher concentrations of fructose is explained by assuming the crowding out of sucrose from the active site.

Data presented in Figs. 5, 6 and 7 show that fructosyl transferase and invertase are highly phosphorylated. The anodic end of the gels contained higher amounts of radioactivity (Figs. 6 and 7). Presumably, phosphate is bound to the enzyme protein in more than one way and the more labile phosphate might be cleaved under alkaline conditions during electrophoresis.

While the present data are insufficient to warrant such a conclusion, the possibility that invertase and fructosyl transferase are related proteins which might differ principally in their phosphate content cannot be ruled out. In fact, in an analogy with the nature of phosphorylase a and b one would be tempted to suspect that this suggestion may be more than a mere speculation.

EXPERIMENTAL

Materials. Peroxidase and ATP were purchased from V. P. Chest Institute, New Delhi. Glucose oxidase was obtained from BDH. UTP was the kind gift of Boehringer, Mannheim. ADP and Sephadex were procured from Sigma. Uniformly labelled [^{14}C]-glucose, -fructose, -sucrose and NaH_2 [^{32}P]-O_4 were obtained from BARC, Trombay. DEAE-cellulose was purchased from Pharmacia.

Preparation of culture. The culture of *F. oxysporum* NCIM 1072 was obtained from the National Chemical Laboratory, Poona, India and was maintained by transfer at 14 day intervals on agar slants in tubes using modified Czapek's medium at an initial pH of 5.5. Medium contained 2 g of $NaNO_3$, 1 g of K_2HPO_4, 0.5 g of KCl, 0.5 g of $MgSO_4$, 0.01 g of $FeSO_4$ and 30 g of sucrose/l. Agar–Agar (20 g/l.) was added to solidify the medium. Liquid cultures of *F. oxysporum* were kept in 1 l. conical flasks containing 250 ml of the medium and pieces of broken glass. The medium was sterilized by autoclaving at 1 bar for 20 min and inoculated from the culture grown on solidified agar slants. The flasks were kept on a shaker at 25° for 4 days, and thereafter kept at 5°. Liquid cultures were used to inoculate the sterilized medium.

Enzyme isolation. Mycelium collected after the required time was washed with chilled H_2O and then crushed with chilled acid-washed sea sand. The crushed mycelium was extracted with 0.1 M NaOAc buffer (pH 5) at 4° for 2 hr and then centrifuged at 2° for 15 min at 10000 *g*.

The supernatant was taken as the crude enzyme extract.

Assay system for invertase and fructosyl transferase consisted of 15 ml 0.1 M sucrose in 0.1 M NaOAc buffer pH 5 and 1 ml of enzyme. It was incubated at 25° for 24 hr and the reaction stopped by heating the assay mixture at 100° for 10 min. Total reducing sugars and free glucose were estimated and free fructose determined from their difference. The value of fructose obtained was ascribed to invertase activity. The excess of free glucose over free fructose in the assay system measures the amount of fructose which has been enzymatically transferred to form F_2G from sucrose. One unit of fructosyl transferase is defined as the quantity of enzyme responsible for the transfer of 1 μmol of fructose at pH 5 at 25° in 24 hr. Similarly, one unit of invertase is defined as the quantity of enzyme responsible for the release of 1 μmol of fructose from sucrose at pH 5 at 25° in 24 hr.

Enzyme purification (*Scheme* 1). The crude enzyme was pptd by adding 2 vol. EtOH. The clear supernatant was washed off and the residue centrifuged at −4° at 15000 *g* for 30 min. The ppt., dissolved in 0.1 M NaOAc buffer (pH 5), was again centrifuged at 15000 *g* for 10 min to obtain the clear supernatant which was loaded on a DEAE-cellulose column. Elution was effected with an increasing gradient of NaCl in 0.1 M NaOAc buffer, pH 5. Peak 1 of fructosyl transferase was eluted with 0.1 M NaOAc buffer pH 5 and peak 2 of invertase was eluted with 0.1 M NaOAc buffer with 0.5 M NaCl at pH 5. The effective length of the column was between 30 and 35 cm. Fractions (10 ml) were collected and relative protein contents were determined by measuring the A 280 nm. The fraction with the highest specific fructosyl transferase activity was further resolved using a Sephadex G-100 column and 0.1 M NaOAc buffer pH 5 as the eluant.

Enzyme purification (*Scheme* 2). Crude enzyme was pptd with 0.1% protamine sulphate for 15 min and the supernatant obtained after centrifugation at 10000 *g* for 10 min was saturated to 100% with $(NH_4)_2SO_4$. On centrifugation at 10000 *g* for 20 min, it was observed that most of the enzyme activity was present in the supernatant. Further purification was achieved by Sephadex G200 and DEAE-cellulose column chromatography.

Preparation of [^{32}P]*-fructosyl transferase and invertase. F. oxysporum* was grown on modified Czapek's medium containing 510 mg of KH_2PO_4, 5.0 mCi (1 mm) of NaH_2PO_4, 1 g of KCl, 1 g of $MgSO_4$ and 70 g of sucrose/2 l. Mycelium was collected on the 6th day. Fructosyl transferase and invertase were purified by Scheme 2.

Acrylamide gel electrophoresis. The method of ref. [34] with the modifications of ref. [35] was used. Coomassie blue was used for developing protein bands. The radioactive gels were sliced, digested with H_2O_2 [36] and radioactivity measured in a scintillation counter.

n-BuOH–HOAc–H_2O (4:1:5) [37] was used for PC. Benzidine CCl_3CO_2H [38] and urea H_3PO_4 spray reagents [39] were used for detection of sugars. The carbohydrates were extracted from the cut portions of the chromatograms by the method of ref. [40] and estimations made by the PhOH–H_2SO_4 method [41]. Glucose oxidase was used to estimate glucose [42]. Fructose was estimated by alcoholic resorcinol and 80% HCl [43]. Reducing sugars were estimated by the method of ref. [44]. Protein was estimated by the procedure of ref. [45]. Bray's reagent [46] was used for measuring radioactivity. Phosphate was estimated by the method of ref. [47].

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