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# GLUCOFRUCTOSAN METABOLISM IN *CICHORIUM INTYBUS* ROOTS

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**Key Word Index**—*Cichorium*; Compositae; chicory; glucofructosan; fructosyl transferase, fructosan hydrolase; invertase, sucrose metabolism.

**Abstract**—A 10-fold purification of sucrose sucrose fructosyl transferase from *Cichorium intybus* roots was achieved by ammonium sulphate fractionation and DEAE-cellulose column chromatography. The energy of activation for this enzyme was *ca* 48 kJ/mol sucrose. Sucrose sucrose fructosyl transferase and invertase were prominent during early months of growth. Evidence obtained from: (1) the changes in carbohydrate composition at monthly intervals; (2) comparative studies on fructosyl transferase and invertase at different stages of root growth; and (3) incubation studies with [$^{14}$C]glucose, [$^{14}$C]fructose and [$^{14}$C]sucrose revealed that, during the later stages of root growth, fructosan hydrolase is responsible for fructosan hydrolysis. No evidence for the direct transfer of fructose from sucrose to high $M_r$ glucofructosans was obtained.

## INTRODUCTION

Sucrose sucrose fructosyl transferase has been isolated, purified and studied from various plant sources [1–15]. This enzyme forms difructosyl glucose ($F_2G$) and glucose from two molecules of sucrose. It was reported earlier that in *Cichorium intybus* roots during the post flowering stage the amounts of free fructose increases and bound fructose decreases [16]. However, it is still not clear as to whether the hydrolysis of fructosan is due to a specific fructosan hydrolase or invertase. The present study was undertaken primarily to resolve this dilemma.

## RESULTS

### *Purification of sucrose sucrose fructosyl transferase*

Two protein peaks were observed after DEAE-cellulose CC. The second peak, eluted with 0.1 M sodium acetate buffer and 0.5 M sodium chloride, showed fructosyl transferase activity and an 11-fold increase was observed in fraction 28 in the 20–50% ammonium sulphate fraction (Table 1).

### *Effect of temperature on* $V_{max}$ *and* $K_m$

The $V_{max}$ of sucrose sucrose fructosyl transferase increased with increasing of temperature. The $K_m$ varied from 0.15 to 0.25 M and did not show any consistent relationship with temperature. The energy of activation, as calculated from the slope of the data presented in Fig. 1 was *ca* 48 kJ/mol of sucrose.

### *Incubation studies with* [$^{14}$C]*glucose,* [$^{14}$C]*fructose and* [$^{14}$C]*sucrose*

Incubation of crushed roots with [$^{14}$C]glucose, [$^{14}$C]fructose and [$^{14}$C]sucrose in the presence and absence of ATP was carried out in January (early stage of growth) and July (late stage of growth). Autoradiographs showed that [$^{14}$C]glucose and [$^{14}$C]fructose in both the

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Table 1. Purification of sucrose sucrose fructosyl transferase on a DEAE-cellulose column

| Fraction | Total protein fraction | Units per fraction | Sp. act. (per mg protein) | Purification (fold) |
|---|---|---|---|---|
| 20–50% ammonium sulphate fraction | 13.50 mg | 13 23 | 0.98 | 1.0 |
| Fraction 25 | 0.13 mg | 0.30 | 2.30 | 2.3 |
| Fraction 26 | 0.20 mg | 0.54 | 2.70 | 2 7 |
| Fraction 27 | 0.13 mg | 0.92 | 7.07 | 7.2 |
| Fraction 28 | 0.13 mg | 1.39 | 10.70 | 10.9 |

Experiment carried out in March.

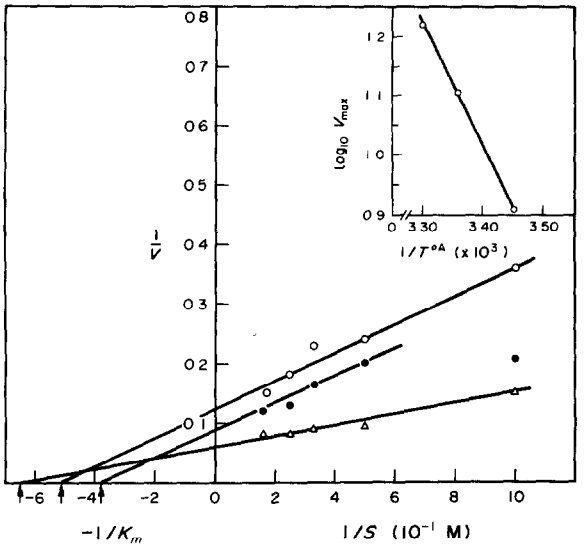


Fig. 1. Lineweaver–Burk plot showing the effect of temperature on $V_{max}$ and $K_m$ (○), 17°; (●) 25° and (▲) 30°. The plot in the top right corner shows the relationship between log $V_{max}$ of sucrose sucrose fructosyl transferase and the reciprocal of the absolute temperature. Velocity was defined as μmol fructose transferred in 100 min by 1 mg protein.

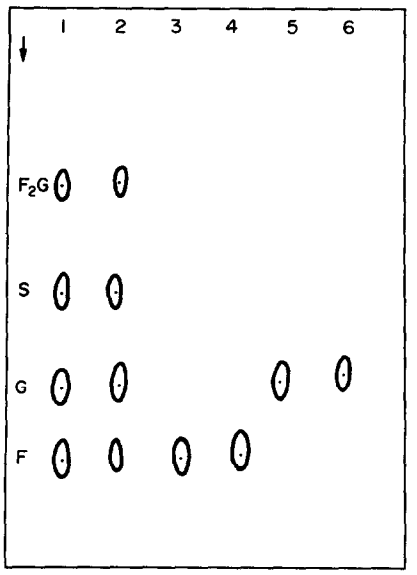


Fig. 2. Diagram of an autoradiograph showing the fate of [$^{14}$C]glucose, [$^{14}$C]sucrose and [$^{14}$C]fructose in the early stages of development (January). In the six experiments (1–6) 1 g crushed roots was incubated with 1 ml 1 μCi carbohydrate solution and 1 ml 0.1 M sodium acetate buffer, pH 5.4, for 24 hr at 30°. Carbohydrates were extracted and separated by PC. X-ray film was kept in contact with the chromatogram for 30 days and was then developed. (1) Incubation with [$^{14}$C]sucrose; (2) with [$^{14}$C]sucrose and 30 mg ATP; (3) with [$^{14}$C]fructose; (4) with [$^{14}$C]fructose and 30 mg ATP; (5) with [$^{14}$C]glucose; and (6) with [$^{14}$C]glucose and 30 mg ATP.

early and late stages of growth remained unchanged (Figs 2 and 3). During the later stages of growth, [$^{14}$C]sucrose was converted to [$^{14}$C]difructosylglucose and spots corresponding to [$^{14}$C]glucose and [$^{14}$C]fructose were not observed whereas, in the early stage of growth, [$^{14}$C]sucrose was converted to [$^{14}$C]difructosylglucose, [$^{14}$C]glucose and [$^{14}$C]fructose (Figs 2 and 3).

*Carbohydrate changes during growth of* Cichorium intybus *roots*

Flowering started in mid-June and carbohydrate composition was determined until mature seed formation, i.e. until September. The bound fructose concentration was maximum 5 months after sowing and, thereafter, bound fructose started to decline, reaching 27% by the end of month 10. Bound glucose varied from 1.7% to 6.9%. Free fructose, which was as high as 25.2% at the end of month 2, was reduced to 3.8% by the end of month 3 and its level again increased during the last 3 months. Free glucose levels remained insignificant. The bound fructose–bound glucose ratio increased to 23 by the end of month 5 and, thereafter, started to decline reaching 6.0 after month 10. Similarly, the ratio of bound fructose–free fructose, after reaching a maximum value of 58 in May, declined to 1.2 by the end of month 10 (Table 2).

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

The ratio of fructosyl transferase–invertase was maximum in April, i.e. 5 months after sowing. In July, neither fructosyl transferase nor invertase activities could be detected (Table 3).

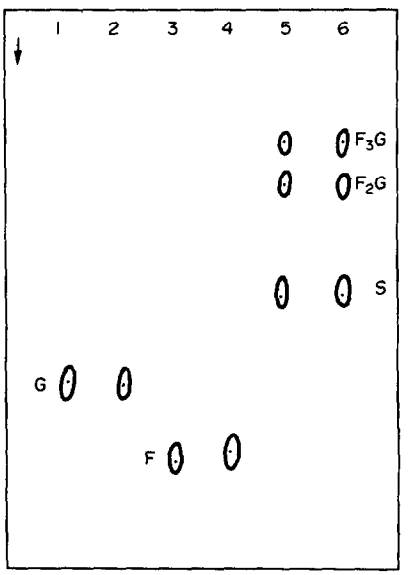


Fig. 3. Experimental conditions were as in Fig. 2, except that this experiment was performed during the late stages of growth (July). (1) Incubation with [$^{14}$C]glucose; (2) with [$^{14}$C]glucose and 30 mg ATP; (3) with [$^{14}$C]fructose; (4) with [$^{14}$C]fructose and 30 mg ATP; (5) with [$^{14}$C]sucrose; and (6) with [$^{14}$C]sucrose and 30 mg ATP.

Table 2. Changes in the percentage composition of carbohydrates in *Cichorium intybus* at monthly intervals

| Carbohydrates estimated | Months after sowing | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
| Total fructose | 40.9 | 68.2 | 43.0 | 42.3 | 52.3 | 47.7 | 44.6 | 52.0 | 33.8 | 50.0 |
| Bound fructose | 25.0 | 43.0 | 39.2 | 40.7 | 50.7 | 46.9 | 42.3 | 40.0 | 26.9 | 27 2 |
| Free fructose | 15.9 | 25.2 | 3.8 | 1.6 | 1.6 | 0.8 | 2.3 | 12.0 | 6.9 | 22.2 |
| Total glucose | 7.1 | 7.5 | 3.4 | 2.9 | 2.4 | 2.7 | 3.0 | 1 8 | 2.6 | 5 7 |
| Free glucose | 0.7 | 0.6 | 0.4 | 0.3 | 0.2 | — | — | 0 1 | 0.1 | 1 0 |
| Bound glucose | 6.4 | 6.9 | 3.0 | 2.6 | 2.2 | 2.7 | 3.0 | 3.0 | 2.5 | 4.6 |
| Total carbohydrates | 48.0 | 75.7 | 46.4 | 45.2 | 54.7 | 50.4 | 47 6 | 55.0 | 36 4 | 55.7 |
| Bound fructose/bound glucose | 3.9 | 6.2 | 13.0 | 15.6 | 23.0 | 17.3 | 14.0 | 13 3 | 10.7 | 5 9 |
| Bound fructose/free fructose | 1.5 | 1.7 | 10.3 | 25.4 | 31.6 | 58.6 | 18.4 | 3.3 | 3.9 | 1.2 |

Table 3. Variation in the invertase and fructosyl transferase ratio at different stages of root growth

| Month after sowing (date of sample collected) | $^{14}C$ Incorporation in various carbohydrate fractions | | | | | |
|---|---|---|---|---|---|---|
| | Total incorporation (cpm) | Difructosylglucose + trifructosylglucose | Fructose | Fructosyl transferase activity (%) | Invertase activity (%) | Ratio fructosyl transferase–invertase |
| 1½ (20 December) | 146 000 | 14 500 | 17 700 | 9.91 | 12.1 | 0.82 |
| 2½ (20 January) | 113 000 | 7080 | 23 700 | 6.28 | 21.0 | 0.29 |
| 3½ (20 February) | 111 000 | 2060 | 21 800 | 1.85 | 19 6 | 0.09 |
| 4½ (20 March) | 219 000 | 1030 | 25 700 | 0.47 | 11 7 | 0.04 |
| 5½ (20 April) | 228 000 | 82 710 | 32 000 | 35.80 | 14.0 | 2.55 |
| 6½ (20 May) | 167 000 | 2700 | 9940 | 1.61 | 5.9 | 0.27 |
| 8½ (20 July) | — | — | — | No fructosyl transferase activity detected | No invertase activity detected | |

After developing the chromatogram, strips, 0.5 cm from the base, were cut and radioactivity determined. Total counts were calculated by adding the counts from each strip.

## DISCUSSION

The energy of activation for sucrose sucrose fructosyl transferase was 48 kJ/mol sucrose. Data for the energy of activation for this enzyme from other sources is not available, but invertases from *Cichorium intybus* roots treated with 2,4-dichlorophenoxyacetic acid had energy of activations of 48.1, 46.9 and 21.8 kJ/mol of sucrose [17]. Fructosan hydrolase activity could not be detected during the later stages of growth in the soluble fraction from fresh roots, whereas Flood *et al.*, have shown hydrolase activity to be present in the soluble fraction from chicory roots which had been stored in moist peat at 3° at least 4 weeks [18]. During this storage period, the enzyme activity in the soluble fraction may be due to the loosening of bound enzyme from the cell wall. Invertases are known to be bound to the plant cell wall [19–23]. Sucrose sucrose fructosyl transferase and invertase in *Fusarium oxysporum*, on the other hand, are very easily extractable [9, 10].

Sucrose sucrose fructosyl transferase performs the following reaction:

$$\text{sucrose} + \text{sucrose} \rightarrow \text{difructosylglucose} + \text{glucose}.$$

This reaction is believed to occur in the forward direction during fructosan synthesis. [$^{14}$C]Glucose and [$^{14}$C]fructose, in both the presence and absence of ATP, are neither interconvertible nor converted to sucrose under the experimental conditions used (Figs 2 and 3). The primary function of glucose might be to meet the energy needs of the root cells. The autoradiograph showed that the back reaction of sucrose sucrose fructosyl transferase, or the fructosylation of glucose, as mentioned below in the form of equations, was not taking place:

$$\text{difructosylglucose} + [^{14}\text{C}]\text{glucose} \rightarrow [^{14}\text{C}]\text{sucrose} + \text{sucrose}$$

$$[^{14}\text{C}]\text{glucose} + \text{sucrose} \rightarrow [^{14}\text{C}]\text{sucrose} + \text{glucose}$$

$$[^{14}\text{C}]\text{glucose} + \text{glucose–(fructose)}_{n+1} \rightarrow [^{14}\text{C}]\text{sucrose} + \text{glucose–(fructose)}_{n}.$$

Incubation of [$^{14}$C]sucrose with crushed roots during the early stages of growth resulted in its transformation to [$^{14}$C]difructosylglucose, [$^{14}$C]glucose and [$^{14}$C]fructose indicating that, at this stage, both fructosyl transferase and invertase are present in the roots. The presence of these two enzymes should lead to a higher level of glucose

than fructose but, on the contrary, during the first 2 months the level of free fructose was much higher than free glucose (Table 2) indicating that glucose is preferentially utilized over fructose for meeting the energy needs of the organism. However, [$^{14}$C]sucrose during the later stages of growth is only converted to difructosylglucose whereas [$^{14}$C]glucose and fructose could not be detected (Fig. 3) indicating that, at this stage, invertase and sucrose sucrose fructosyl transferase are present in negligible amounts and [$^{14}$C]difructosylglucose is formed from the transfer of fructose from glucofructosan to sucrose by the action of fructosan hydrolase. This reaction can be represented as:

glucose–(fructose)$_{n+1}$ + [$^{14}$C]sucrose → [$^{14}$C]difructosylglucose + glucose–(fructose)$_n$

glucose–(fructose)$_{n+1}$ → glucose–(fructose)$_n$ + fructose.

The possibility that glucose, formed by the action of sucrose sucrose fructosyl transferase during the later stages of growth, might have been utilized is ruled out under the experimental conditions mentioned in Figs 2 and 3; the exogenous glucose is not consumed. Hence, the only possible explanation of the formation of difructosylglucose during later stages of growth is by transfer of fructose from glucofructosans to sucrose by the action of fructosan hydrolase.

The isolation of fructose containing nucleotide from *Dahlia* tubers [24] led Pontis and Fischer [25] to suggest that fructose-2-phosphate may be the intermediate for the synthesis of fructose nucleotide. Uniformally labelled [$^{14}$C]fructose was not incorporated into difructosylglucose or higher fructosans even when ATP was provided for the phosphorylation of fructose (Figs 2 and 3). Therefore, involvement of fructosyl nucleotides is ruled out if it is assumed that fructosan-2-phosphate is the necessary component for the biosynthesis of nucleotide diphosphate fructose.

Incubation of roots with [$^{14}$C]sucrose did not incorporate radioactivity into high $M_r$ fructosans, as no radioactivity was observed at the base of the paper chromatogram (Figs 2 and 3). This shows that fructose transfer to high $M_r$ fructosans from sucrose is not taking place.

The post-flowering stage is characterized by an increase in the free fructose content. Bound fructose, after reaching a peak at the end of month 5, started declining and the ratio bound fructose–bound glucose declined from 23 to 6 at the end of the study (Table 2). A similar trend has been reported previously [16] but this study was confined to a period of 4 months from April to May. Fructosans are hydrolysed by fructosan hydrolase during the post-flowering stage because the contribution of invertase at this stage is negligible. The flowering stage is characterized by the translocation of sugars, obtained by the hydrolysis of fructosans, from the roots to flowers and seeds where they are utilized [16]. A higher level of fructose during the early and late stages of root growth appeared to be due to the presence of invertase and fructosan hydrolase, respectively, at these stages. The ratio fructosyl transferase–invertase was maximum in April, the same time as the level of bound fructose was maximal. (Tables 2 and 3). At this stage it appears that all of the sucrose is channelled towards fructosan synthesis and, finally, in July neither fructosyl transferase nor invertase activity could be detected, again indicating that fructosans were being hydrolysed by fructosan hydrolase.

Evidence obtained from the changes in carbohydrate composition at various stages, comparative studies on fructosyl transferase and invertase at different stages of growth, and incubation studies with [$^{14}$C]sucrose, [$^{14}$C]glucose and [$^{14}$C]fructose clearly establish that fructosans are hydrolysed during the post-flowering stage by a specific fructosan hydrolase and not by invertase.

## EXPERIMENTAL

Materials uniformally labelled [$^{14}$C]glucose, [$^{14}$C]fructose and [$^{14}$C]sucrose were obtained from BARC Trombay. ATP was obtained from Cal. Biochem. DEAE-cellulose and inulin were purchased from Pharmacia and BDH, respectively. The *Cichorium intybus* (chicory variety Kalpa No. 1) was sown in the first week of November in the fields of Department of Biochemistry, Punjab Agricultural University, Ludhiana and samples were taken as and when required.

*Preparation of crude extract.* Isolation was done at 0–4°. The roots, after washing with $H_2O$ were crushed in a pestle and mortar with chilled acid-washed sea sand and extracted with 0.1 M NaPi buffer, pH 7.5 and centrifuged at 10 000 g for 10 min. The first ppt obtained on addition of 20% $(NH_4)_2SO_4$ was discarded after centrifugation at 10 000 *g* for 15 min. To the supernatant, 30% $(NH_4)_2SO_4$ was added. The ppts obtained after centrifugation at 15 000 *g* for 20 min were dissolved in 0.1 M NaOAc buffer, pH 5.4 and dialysed for 60 hr against the same buffer. The dialysed extract, free from sulphate ions and reducing sugars, was again centrifuged at 10 000 *g* for 10 min to remove the white ppts which appeared during dialysis to obtain the crude extract.

*Purification of sucrose sucrose fructosyl transferase.* The crude extract so obtained was loaded onto a DEAE-cellulose column. Elution was effected with a step-wise increasing gradient of NaCl in 0.1 M NaOAc buffer, pH 5.4.

*Assay system for sucrose sucrose fructosyl transferase.* This consisted of 200 μmol sucrose, 50 μmol NaOAc buffer, pH 5.4, and 500 μl enzyme in a total vol. of 1 ml. After incubation at 30°, reducing sugar was estimated as described in ref. [26]. The products were separated by PC in *n*-BuOH–HOAc–$H_2O$ (4:1:5) [27]. Sugars were visualized by the use of suitable spray reagents [28, 29]. One unit of enzyme was defined as the quantity of enzyme responsible for the transfer of 1 μmol fructose at pH 5.4 at 30° in 100 min. Protein was estimated by the method of ref. [30].

*Assay for fructosyl transferase and invertase using* [$^{14}$C]*sucrose.* The assay system consisted of 0.1 ml 20–50% dialysed $(NH_4)_2SO_4$ enzyme extract and 0.1 ml [$^{14}$C]sucrose in NaOAc buffer, pH 5.4. The sucrose concn in the assay system was 0.2 M. The reaction, after 24 hr incubation at 30°, was stopped by heating to 100° for 5 min. Thereafter, the samples in duplicate were applied to Whatman No 3 MM chromatography paper. Strips, 0.5 cm from the base, were cut after developing the chromatogram and the radioactivity determined in scintillation fluid consisting of 4 g PPO and 200 mg POPOP in 1 l. toluene. Radioactivity in fructose was due to invertase and radioactivity in fructosan was due to fructosyl transferase.

*Extraction and estimation of carbohydrates.* Carbohydrates were extracted repeatedly with boiling $H_2O$ as described in ref. [31]. Total fructose was estimated with resorcinol-HCl [32].

Bound fructose was measured after destroying free fructose with 30% KOH. The difference between these two gave the amount of free fructose. Free glucose was measured using glucose oxidase [33]. The carbohydrates in soln after hydrolysis with HCl (overall concn 0.1 M) at 100° for 20 min and neutralization with alkali were taken as an estimate for total glucose. The difference in total glucose and free glucose gave the bound glucose.

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