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ISOLATION AND CHARACTERIZATION OF FRUCTOSYL-TRANSFERASE FROM CHICORY ROOTS*

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Abstract—The occurrence of fructosyltransferase was demonstrated in the roots of chicory. The enzyme was purified 14-fold by ammonium sulphate fractionation and sephadex column chromatography. The optimal conditions for activity were pH 5·6, 37° and substrate (sucrose) concentration of $5{\cdot}84 \times 10^{-1}$ M. The K_m of the enzyme was found to be $2{\cdot}85 \times 10^{-1}$ M. The enzyme was completely inactivated at 62·5°. Neither dialysis nor addition of inorganic phosphate affected its activity. The enzyme was capable of synthesizing glucofructosans from sucrose alone with the simultaneous release of glucose until seven oligosaccharides were synthesized, and with progressive enzyme activity small quantities of fructose were also released. Ag^+ and Hg^{2+} completely inhibited the enzyme action, whereas, Cu^{2+}, Zn^{2+}, Fe^{2+}, Na^+, K^+ and Ca^{2+} had almost no effect. The enzyme activity was significantly increased by Mn^{2+}, Mg^{2+}, Co^{2+} and Ni^{2+}.

INTRODUCTION

THE PRESENCE of fructosyltransferase (sucrose–sucrose 1-fructosyltransferase) was indicated earlier in many plants of the Compositae.[1–7] The nature of the carbohydrates of chicory (*Chicorium intybus* L.) was established to be glucofructosans[8] having glucose and fructose as their structural units and, therefore, the possibility of an enzyme system responsible for their synthesis was sought in the roots of this plant. The presence of the enzyme, fructosyltransferase was confirmed and the present study is mainly concerned with the purification and properties of fructosyltransferase from chicory roots.

RESULTS AND DISCUSSION

Purification

The crude enzyme preparation was first of all subjected to $(NH_4)_2SO_4$ fractionation and the precipitate obtained at 50% concentration was further purified on sephadex. A final purification of 14-fold was obtained as depicted in Table 1.

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[8] RANDHIR SINGH, The role of fructosyltransferase in the biosynthesis of fructosans in the roots of Chicory (*Chicorium intybus* L.), Ph.D.Thesis, Punjab Agricultural University, Ludhiana (1969).

Effect of pH

The optimal pH for enzyme activity was studied by using different buffers and it was found to have a sharp optimum pH at 5·6.

Effect of Temperature

The effect of temperature on the fructosyltransferase activity was studied by incubating the reaction mixture at various temperatures for 24 hr. The maximum activity was observed at 37° and then declined as the temperature was further raised. The low enzyme activity at high temperature appears to be due to its instability as the enzyme was completely inactivated by pre-incubation at 62·5° for about 10 min.

Effect of Substrate

The reaction mixtures consisting of various concentrations of sucrose were incubated at 37° for 24 hr and the glucose released in each case was estimated. At low substrate concentration, the enzyme activity increases with sucrose concentration, reaching a maximum at $5{\cdot}84 \times 10^{-1}$ M sucrose. A further increase in concentration decreases the enzyme activity (Fig. 1). K_m was calculated by plotting the Lineweaver–Burk plot and was found to be $2{\cdot}85 \times 10^{-1}$ M (Fig. 2). The decrease in enzyme activity at high substrate concentration may be attributed to the substrate molecules tending to crowd the enzyme, with the formation of ineffective complexes with two or more substrate molecules attached to the active centre.[9] The shape of the curve in the present studies indicates that inhibition at higher concentration is of a complex type and such inhibition cannot be attributed to any single factor.

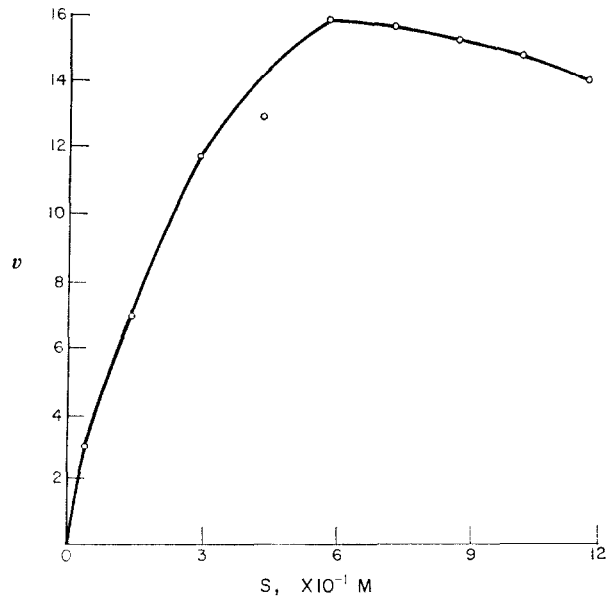

FIG. 1. THE EFFECT OF SUCROSE CONCENTRATION ON FRUCTOSYLTRANSFERASE ACTIVITY.
Velocity (v) is expressed in terms of mg of glucose released per ml of reaction mixture during a 24 hr incubation period.

[9] M. DIXON and E. C. WEBB, in *Enzymes* (2nd Ed.), p 75, Longmans, London (1964).

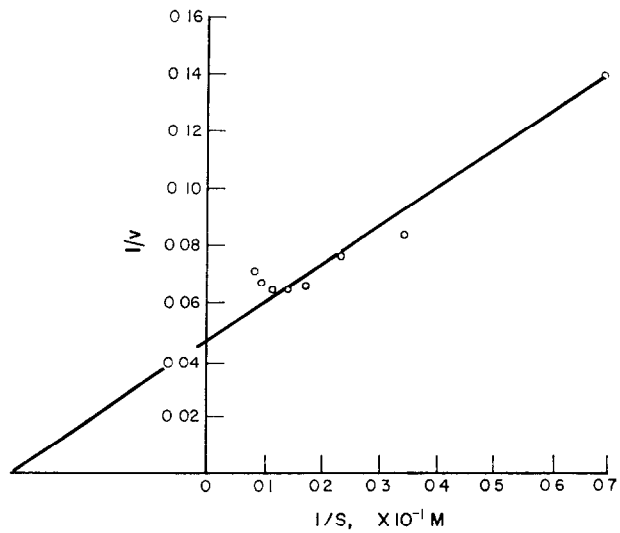

FIG. 2. LINEWEAVER–BURK PLOT FOR DETERMINING THE K_m OF THE FRUCTOSYLTRANSFERASE.

Effect of Dialysis and Inorganic Phosphate

The enzyme preparation did not contain any chromatographically detectable carbohydrate. Addition of boiled, undialysed enzyme did not increase the activity of the dialysed enzyme. Thus, dialysis appears to have no adverse effect on the activity of fructosyltransferase. The dialysed enzyme was free from phosphate when tested with the Fiske and Subbarow[10] reagent. Addition of phosphate did not enhance the transfructosylation activity.

Dedonder[3] implicated an essential role for inorganic phosphate in the synthesis of glucofructosans. On the other hand Bhatia *et al.*[6] observed that inorganic phosphate has no effect on the synthesis of oligosaccharides (glucofructosans) in *Agave vera cruz*. The present study also indicates the non-involvement of inorganic phosphate in the synthesis of glucofructosans.

TABLE 1. PURIFICATION OF FRUCTOSYLTRANSFERASE FROM THE ROOTS OF CHICORY

Fraction	Volume (ml)	Total protein	Total activity (mg glucose liberated at 37° in 24 hr)	Specific activity (mg glucose/ protein)	Purification (times crude enzyme)	Recovery (%)
Crude extract	500	2718·75	2780	1·02	—	100
Ammonium sulphate fraction (20–50%)	140	148·05	1386	9·36	9·17	49·85
Sephadex fraction	125	80·00	1155	14·44	14·15	41·54

[10] G. H. FISKE and Y. SUBBA ROW, *J. Biol. Chem.* **66**, 375 (1925).

TABLE 2. EFFECT OF METAL IONS ON FRUCTOSYLTRANSFERASE ACTIVITY

Metal	Concentration of the ion in reaction mixture (M)	Fructosyltransferase activity (mg glucose liberated at 37° in 24 hr)	Enzyme activity as % of the control
Control	—	9·45	100
Cu^{2+}	0·01	9·24	97·7
Zn^{2+}	0·01	8·40	88·8
Fe^{2+}	0·01	8·10	85·7
Na^{+}	0·01	8·10	85·7
K^{+}	0·01	9·30	98·4
Ca^{2+}	0·01	8·55	90 4
Mn^{2+}	0·01	20·00*	211·6
Co^{2+}	0·01	18·90*	200·0
Ni^{2+}	0.01	12 87*	136·2
Mg^{2+}	0·01	19 35*	204 7
Ag^{+}	0 01	nil	0
Hg^{2+}	0·01	nil	0

* One more oligosaccharide spot with R_f less than that of F_2G was observed presumably F_3G.

Effect of Metal Ions

The effect of 12 metal ions at 0·01 M concentration was investigated on the enzyme activity (Table 2). As is apparent from the table, Ag^{+} and Hg^{2+} completely inhibited the enzyme. Mn^{2+}, Co^{2+}, Ni^{2+} and Mg^{2+} greatly enhanced the activity of the enzyme as one extra oligosaccharide was synthesized, at the same time, the amount of glucose released was almost double when compared to the control. The rest of the metal ions tested had no significant effect on the enzyme activity.

Effect of Time of Incubation

The time course of the enzyme reaction was studied by incubating the enzyme with substrate for different periods. As is evident from Fig. 3, in the very early stages there is very little production of glucose but from 7 hr onwards there is a constant increase in glucose till 120 hr when the reaction reaches a steady state.

Progress of the Reaction

Figure 1 represents the carbohydrate content of the reaction at different time intervals up to 140 hr. In 45 min of incubation, no sugar appeared on chromatogram except sucrose, but after 2 hr, two sugars appeared in the reaction mixture, viz. one with R_f lower than that of sucrose (oligosaccharide, F_2G) and the other with R_f of glucose. Both these spots progressively increased up to 22 hr. At 31 hr another spot with R_f lower than that of the earlier spot appeared and continued to increase in intensity till 54 hr. At 75 hr two more spots appeared making in all 4 spots with R_f lower than that of sucrose. At 96 hr one more spot appeared and two other spots were visible at 120 and 140 hr. Thus a total of seven oligosaccharides were synthesized by the enzyme till the steady state was reached. In analogy with the pattern of oligosaccharides synthesized *in vivo*,[8] these spots were designated as F_2G, F_3G, F_4G, F_5G, F_6G, F_7G and F_8G respectively, starting from the spot just above

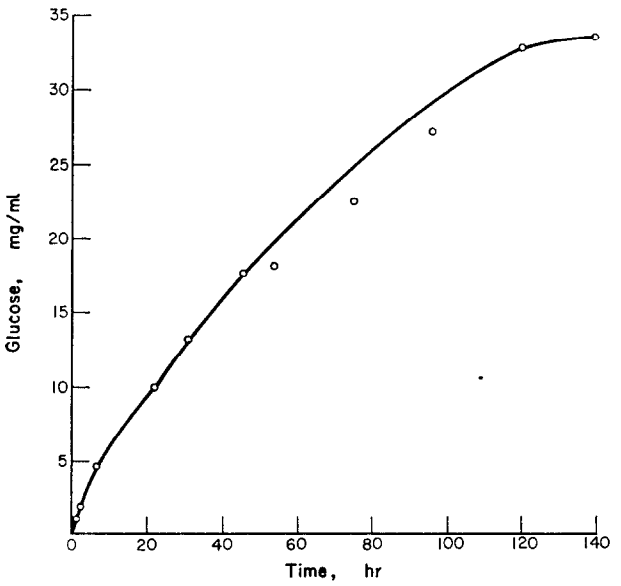

FIG. 3. TIME COURSE STUDY OF THE FRUCTOSYLTRANSFERASE ACTIVITY.
Activity is expressed in terms of mg of glucose released per ml of reaction mixture during different time intervals.

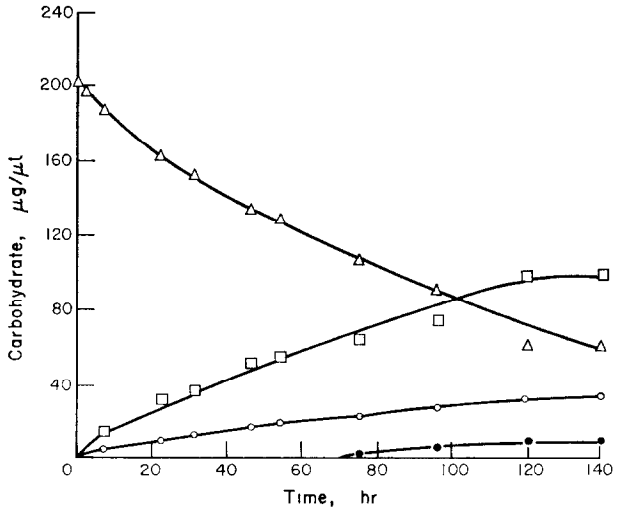

FIG. 4. PROGRESS CURVES OF THE FRUCTOSYLTRANSFERASE ACTIVITY.
Activity is expressed in terms of μg of respective carbohydrate per μl of reaction mixture at different time intervals.
Fructose ●——●; Glucose, ○——○;
Oligosaccharides, □——□; Sucrose, △——△.

sucrose (the R_L* values of the two series were compared and found to be the same). A spot with R_f of fructose was noticeable at 75 hr and persisted till the end.

The changes in the substrate and the products of the reaction with time are shown in Fig. 4. There was an increase in concentrations of glucose and oligosaccharides with time until 120 hr after which the reaction rate was almost constant. Sucrose concentration decreased and that of fructose, which appeared only at 75 hr increased till the end of the incubation period. The decrease in concentration of sucrose is linked to the synthesis of oligosaccharides with the concomitant release of glucose.

From 75 hr onward, fructose started appearing (Fig. 1) indicating that whole of the fructose was not being transferred to sucrose for the synthesis of oligosaccharides. Thus for this period the mechanism of reaction is represented as :

$$\underset{\text{Sucrose}}{\text{F-G}} + \underset{\text{Sucrose}}{\text{F-G}} \longrightarrow \underset{\text{Oligosaccharide}}{\text{F}_2\text{G}} + \underset{\text{Glucose}}{\text{G}}$$

$$\underset{\text{Oligosaccharide}}{\text{F}_2\text{G}} + \underset{\text{Sucrose}}{\text{F-G}} \longrightarrow \underset{\text{Oligosaccharide}}{\text{F}_3\text{G}} + \underset{\text{Glucose}}{\text{G etc.}}$$

From 75 hr onwards, the reaction is of a complex type, and the changes in sucrose concentration hereafter, cannot be reasonably correlated with any single factor, though formation of glucofructosans is still the dominant feature.

Edelman and Jefford[11] have put forward a theory of the integrated action of certain enzymes isolated from Jerusalem artichoke. The synthesis of glucofructosans in this plant has been attributed to the combined action of two enzymes: sucrose–sucrose 1-fructosyltransferase[4] (SST) which synthesizes a trisaccharide (1^F-fructosylsucrose) from sucrose according to the equation: $\text{G-F} + \text{G} \sim \text{F} \rightarrow \text{G} \sim \text{F} - \text{F} + \text{G}$ and β (2→1′) fructan–β(2→1′) fructan 1-fructosyltransferase[12] which is highly specific for the transfer of a single terminal $\beta(2 \rightarrow 1')$ linked fructofuranosyl residue to the same position in another molecule according to the equation: $\text{G} \sim \text{F} - \text{F}_n + \text{G} \sim \text{F} - \text{F}_m \rightleftharpoons \text{G} \sim \text{F} - \text{F}_{n-1} + \text{G} \sim \text{F} - \text{F}_{m+1}$. The number ($n$) of extrasucrosyl residues of the donor molecule can be any number from one (trisaccharide) to about thirty five (inulin) and the number (m) for the acceptor molecule can be any number from zero (sucrose) to about thirty five.

Though the above given mechanism relates only to the artichoke, similar enzymes have been found in other plants, most particularly the onion.[13] As the chicory is a plant closely related to artichoke, there is every possibility that the present enzyme preparation may be a mixture of the known enzymes, one synthesizing the trisaccharide from sucrose and the other transferring the terminal fructosyl group of the trisaccharide to the sucrose molecule and then further transfer of terminal fructosyl groups from trisaccharide molecules to the growing chain.

EXPERIMENTAL

Plant Material

The crop was raised in the fields of department of Botany and Plant Pathology, Punjab Agricultural University, Ludhiana and the samples were taken as and when required.

* $R_L = \dfrac{\text{Distance travelled by the substance}}{\text{Distance travelled by levulose (fructose)}}$.

[11] J. EDELMAN and T. G. JEFFORD, *New Phytol.* **67**, 517 (1968).
[12] J. EDELMAN and A. G. DICKERSON, *Biochem. J.* **98**, 787 (1966).
[13] R. W. SCOTT, Transfructosylation in higher plants containing fructose polymers, Ph.D. Thesis, University of London (1968).

Preparation of Crude Extract

The roots were washed with distilled water and then blotted dry with filter paper. The outer skin was scrapped and the bottom and top ends were discarded. The remaining portion was then sliced into small pieces, treated with chloroform–water, grated and pressed through muslin cloth. The pressed juice was centrifuged at 5000 rev/min for 10 min. The supernatant, hereafter referred to as the crude extract, was stored under chloroform at 4°.

Enzyme Enrichment

Precipitates obtained from the crude extract with ethanol (20, 50 and 70%, by vol.) did not show any significant enzyme activity. On the other hand, enriched preparations of enzyme could be obtained by the use of $(NH_4)_2SO_4$. To the crude extract of enzyme was added solid $(NH_4)_2SO_4$ (20 g/100 ml), dissolved and kept at 4° for 6 hr. The precipitate formed had little enzyme activity and was removed by centrifuging at 5000 rev/min for 20 min. A further quantity of $(NH_4)_2SO_4$ (30 g/100 ml, original volume) was dissolved in the clear supernantant and left overnight at 4° for complete precipitation. The precipitate so obtained was washed with 50% (w/v) $(NH_4)_2SO_4$ till the washings gave a negative Seliwanoff's test. The washed precipitate was dissolved in ice cold phosphate buffer (pH 5·6) and filtered. This filtrate was referred to as stock enzyme enriched solution.

Dialysis

Dialysis of the stock enzyme solution was carried out in the presence of chloroform in a cellophane bag, against repeated changes of cold diluted phosphate buffer. The process was continued till the liquid in touch with cellophane bag gave a negative test for sulphate ions. The dialyses was accompanied by a sufficient increase in volume in the cellophane bag. A small precipitate appearing during dialysis was filtered off. Its activity in comparison with the crude extract was determined.

Sephadex Column Chromatography

The clear supernatant obtained in the above procedure was further analysed on a sephadex (G-200) column (54 × 3 cm) equilibrated with 0·002 M phosphate buffer (pH 5·6). The flow rate of the column was maintained at 10 ml/15 min. The clear aliquot of the enzyme solution was placed on the top of the column and was eluted with a mixture of phosphate buffer (0·05 M, pH 5·6), and NaCl (0·05 M) in equal volume. 3-ml fractions were collected using the same buffer as eluting solvent and the fractions were further analysed for the protein content and enzyme activity. The purification in comparison to crude extract was then determined.

Assay of the Enzyme Activities

The enzyme was assayed by incubating with sucrose (Analar grade) at 37° in the presence of chloroform and under a layer of toluene. The reaction mixture contained $5{\cdot}84 \times 10^{-1}$M sucrose in a final vol. of 5 ml of buffered enzyme. A boiled control was also incubated everytime under the same conditions. Samples drawn at known intervals were heated in a boiling water bath for 10 min to inactivate the enzyme. The inactivated samples were cooled and filtered. The filtrates were further analysed for released glucose by the method of Klein and Acree.[14]

Protein Estimation

The protein was estimated by the procedure of Lowry *et al.*[15] using ovalbumin as the standard protein.

Chromatography of Carbohydrates

(a) *Qualitative.* Reaction mixtures at known time interval were spotted on a filter paper sheet (Whatman No. 1), the starting line having been previously streaked with 0·01 M $HgCl_2$ solution and dried.[16] A mixture of fructose, glucose and sucrose as reference and the control (sucrose and boiled enzyme) were also spotted. The paper was irrigated with *n*-BuOH–HOAc–H_2O (4:1:5, by vol) (BAW)[17] by descent for 48 hr. Finally, the dried paper was sprayed with benzidine–trichloracetic acid.[18] The constituents on the chromatogram were then identified by comparison with standards.

(b) *Quantative.* A known vol. of the reaction mixtures of different time intervals was micropipetted onto Whatman No. 1 filter paper and the paper was then developed by descent in BAW, triple run, 24 hr for each run. After locating the separated carbohydrates on a guide strip, the corresponding areas of the unsprayed chromatograms were marked and cut.

[14] G. M. Klein and S. F. Acree, *Bur. Std. J. Res. Wash.* **5**, 1063 (1930).
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[18] J. S. D. Bacon and J. Edelman, *Biochem. J.* **48**, 114 (1951).

Estimation of Carbohydrates from Paper

The carbohydrates were extracted from the cut portions of the chromatograms, by the method of Srinivasan and Bhatia.[19] The extracted carbohydrates were further estimated individually according to the method of Dubois *et al.*[20]

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