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STUDIES ON FRUCTOSYL TRANSFERASE FROM *AGAVE AMERICANA*

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Key Word Index—Agavaceae; *Agave*; fructosans; fructosyl transferase; biosynthesis.

Abstract—The possible role of fructosyl transferase in the biosynthesis of fructosans in *Agave americana* was investigated. This enzyme was extracted from *A. americana* stem and purified 17.5-fold by salt fractionation and DEAE-cellulose chromatography. The optimum conditions for the enzyme were pH 6.1, temperature 37°, substrate concentration 20% and K_m 3.6×10^{-1} M; Ag^+, Pb^{2+}, Hg^{2+}, Al^{3+}, Sn^{2+}, CN^- acted as inhibitors and Ca^{2+}, Mg^{2+}, Co^{2+} and Li^+ acted as activators. Only sugars of the type F ~ R (R-aldose), e.g. sucrose and raffinose acted as substrates for the enzyme. The donor acceptor specificity of the enzyme was studied extensively. Sugars sucrose. None of the intermediates of fructosan biosynthesis from sucrpse acted as fructose donors. The possible acceptors from sucrose and raffinose. The enzyme was capable of building up oligosaccharides up to $F_{10}G$ from sucrose. None of the intermediates of fructosan biosynthesis from sucrose acted as fructose donors. The possible mechanism of fructosan biosynthesis from sucrose is discussed.

INTRODUCTION

The presence of the enzyme, fructosyl transferase, has been established in plants like *Helianthus tuberosus* [1–5], *Agave vera cruz* [6–8], *Crinum longifolium* [9] and *Cichorium intybus* [10]. The exact role of the enzyme in fructosan biosynthesis is still not established. Bhatia *et al.* [6] reported that the juice of *Agave vera cruz* stem contains an enzyme which could build up short chain glucofructosans, viz. F_2G and F_3G from sucrose. However, they could not demonstrate the biosynthesis of higher fructosans, probably because of the presence of hydrolytic activity in their enzyme preparation. The fructosyl transferases isolated from plants like *H. tuberosus* [4, 11], *C. intybus* [10] and *A. vera cruz* [8] have shown different patterns of donor and acceptor specificity towards different sugars. The present study was therefore undertaken to isolate fructosyl transferase from the stem of *A. americana* and to study its donor acceptor specificity after purification.

RESULTS AND DISCUSSION

The enzyme was purified from the crude extract by ammonium sulphate fractionation and DEAE-cellulose column chromatography. The dialysed protein fractions I and III, obtained by ammonium sulphate fractionation, had very little fructosyl transferase activity whereas dialysed fraction II had the maximum activity.

The dialysed protein fraction II was resolved into two main fractions on a DEAE-cellulose column. The synthetic activity of the enzyme was found to be in the first main fraction which was eluted with NaCl–phosphate buffer of 0.01–0.29 M ionic strength. These eluates were pooled and termed DEAE-cellulose main fraction I or purified enzyme. The fraction obtained with 0.3–0.45 M NaCl–phosphate buffer had weak hydrolytic activity against sucrose. In order to check the purity of dialysed fraction II and DEAE-cellulose fraction I, paper electrophoresis was carried out at 200 V, 8 A for 8 hr in Michaelis Universal buffer (pH 6.99). Dialysed fraction II showed the presence of two bands, one moved 0.9 cm and the other 2.7 cm towards the cathode, whereas DEAE-cellulose fraction I showed only one band at a distance of 0.95 cm towards the cathode. This indicated that the fructosyl transferase activity was confined to the protein fraction obtained by DEAE-cellulose column chromatography. With ammonium sulphate fractionation and DEAE-cellulose column chromatography, 6.6- and

Table 1. Purification of fructosyl transferase from the stem of *Agave americana*

Fraction	Activity glucose formed (mg/ml)	Protein (mg/ml)	Specific activity	Purification (times crude extract)
Crude extract	4.45	7.40	0.60	1
Dialysed 20–50% $(NH_4)_2SO_4$ fraction	13.5	3.41	3.96	6.6
DEAE-cellulose fractionated protein I	23.8	2.25	10.56	17.5

* The work presented here forms part of the thesis submitted by K. S. Nandra to Punjab Agricultural University, Ludhiaha, in partial fulfilment of the requirements for the degree of Doctor of Philosophy (Biochemistry).

17.5-fold purification of the enzyme was achieved (Table 1).

The optimum conditions for fructosyl transferase enzyme were pH 6.1, temperature 37°, sucrose substrate concentration 20% and K_m 3.6×10^{-1} M (calculated from the Lineweaver–Burk plot). Ag^+, Pb^{2+}, Hg^{2+}, Al^{3+}, Sn^{2+} and CN^- inhibited the enzyme activity by 96.6, 84.5, 96.6, 82.3, 95.2 and 100%, respectively, while Ca^{2+}, Mg^{2+}, Co^{2+} and Li^+ increased the enzymic activity by 44, 69.5, 24.2 and 6.6%, respectively, at 0.01 M concentration in the reaction mixture incubated for 24 hr.

Substrate specificity

When sucrose was used as a substrate for the enzyme, 5 oligosaccharides with R_f values less than that of sucrose along with a saccharide with zero R_f value were detected by PC. With the help of different spray reagents, such as naphthoresorcinol–TCA (specific for fructose and fructose-containing sugars), aniline hydrogen phthalate (specific for glucose and glucose-containing sugars) and ammoniacal silver nitrate (specific for reducing sugars), it was concluded that these oligosaccharides were made up of glucose and fructose residues and were non-reducing in nature. The fructose to glucose ratios of the acid hydrolysates of these oligosaccharides showed them to be F_2G, F_3G, F_4G, F_5G, F_6G and $F_{10}G$. All were hydrolysed to glucose and fructose by invertase showing that these were of the inulin type ($\beta 2 \rightarrow 1$). For confirmation of the nature of these oligosaccharides, sucrose-[U-^{14}C] was incubated with the enzyme. The PC autoradiograph of the separated oligosaccharide showed that 6 oligosaccharides were synthesized. The individual oligosaccharides were hydrolysed by acid and the hydrolysate subjected to chromatography and autoradiography. Only labelled glucose and fructose were detected. From the ratio of the radioactivity of fructose and glucose, it has been concluded that these oligosaccharides from 1 to 6 were F_2G, F_3G, F_4G, F_5G, F_6G and $F_{10}G$ (Table 2). All could also be hydrolysed with invertase to labelled glucose and fructose showing therefore that the linkages between the sugars of these oligosaccharides are of the inulin type. This was further confirmed by comparing the R_f values of the oligosaccharides of the reaction mixture with those of the oligosaccharides obtained by the acid hydrolysis of inulin.

Table 2. Structural make-up of isolated oligosaccharides

Oligo-saccharide	Total reducing sugar (mg/ml)	Fructose (mg/ml)	Glucose (total reducing sugar − fructose)	Fructose/glucose ratio
1	7.45	4.96	2.49	1.99:1
2	5.40	4.18	1.22	3.34:1
3	4.96	3.92	1.04	3.78:1
4	4.38	3.60	0.78	4.62:1
5	3.51	3.04	0.47	6.43:1
6*	9.92	9.02	0.90	10.01:1

* Did not move from the origin.

When raffinose (20% concentration) was used as the substrate, the spots corresponding to galactose, sucrose and melibiose, and two spots having R_f values less than that of raffinose were detected on the chromatogram. Fructose-containing sugars, such as F_2G, F_3G, F_4G, melizitose, inulin and sugars, which do not contain fructose, such as maltose, lactose, trehalose, melibiose and cellobiose did not act as substrates for this enzyme.

It is evident that sucrose and raffinose (F ~ R type compounds where R = glucose or melibiose) donate their β-fructofuranose unit linked to the anomeric carbon of the aldose. The replacement of fructosyl group of sucrose with F_n(F_2G, F_3G, F_4G and inulin) rendered them inert as donors. It is also observed that the addition of glucopyranose to the fructosyl group of sucrose at carbon-3 (melizitose) led to the loss of transferase activity.

Donor ability of fructose-containing substrates was further confirmed by using glucose-[U-^{14}C] as an acceptor. When sucrose, raffinose, F_2G, F_3G, melizitose and inulin were incubated with the enzyme in the presence of glucose-[U-^{14}C], the labelled sucrose spot (G*-F) was detected on the PC autoradiograph only in the case of sucrose and raffinose. It is, therefore, apparent that only F ~ R type of sugars act as fructosyl donors. The formation of labelled sucrose has been reported by Allen and Bacon [12], when unlabelled sucrose and glucose-[U-^{14}C] were incubated with sugar beet leaf enzyme.

Donor and acceptor specificity

When the enzyme was incubated with sucrose plus any of the sugars D-mannose, D-xylose, D-ribose and L-arabinose, some additional spots (other than those formed from sucrose alone) having R_f values between sucrose and F_2G, xylose and glucose, ribose and glucose, glucose and sucrose, respectively, were detected by PC. All these new disaccharides were found to contain a fructose moiety and were non-reducing in nature. PC of the acid and invertase hydrolysates of these new disaccharides (other than those formed from sucrose alone) showed the presence of mannose and fructose, xylose and fructose, ribose and fructose, and arabinose and fructose in cases when sucrose was incubated with either mannose, xylose, ribose and arabinose, respectively. The new disaccharides synthesized were therefore mann-fructose, xyl-fructose, rib-fructose and arab-fructose, all having sucrose-type linkages. No such transfer of fructose from sucrose to rhamnose, ribulose, maltose, lactose, trehalose and cellobiose was observed. When the enzyme was incubated with raffinose plus mannose, xylose, ribose or arabinose, new disaccharides (other than those formed from raffinose alone) were detected by PC. These new disaccharides were structurally similar to those formed when sucrose was used as the fructosyl donor. Similarly, no such transfer of fructose from raffinose was observed when incubated with rhamnose, sorbose, maltose, lactose, trehalose and cellobiose. When the enzyme was incubated with unlabelled sucrose plus glucose-[U-^{14}C] or fructose-[U-^{14}C], and unlabelled raffinose plus glucose-[U-^{14}C] or fructose-[U-^{14}C], PC autoradiography showed the presence of labelled sucrose and F_2G only in case of sucrose + glucose-[U-^{14}C] and not in the case of sucrose + fructose-[U-^{14}C], when incubated with the enzyme; the formation

of labelled sucrose was detected on the PC autoradiograph only in case of raffinose + glucose-[U-^{14}C]. This clearly demonstrates that glucose, and not fructose, acts as the acceptor of the fructose molecule from sucrose and raffinose. Our results are in agreement with those of Hestrin *et al.* [13] who have also shown that glucose can act as an acceptor of the fructosyl moiety from sucrose and raffinose in the presence of levan sucrase from *B. levanicum*. Edelman [14] has also reported that, under the influence of mould sucrase, free glucose and glucose combined in the sucrose molecule is exchangeable by means of the transfer of fructose residue from sucrose to free glucose to form reducing compounds, probably a fructosyl glucose disaccharide. On the other hand, he has also shown the formation of difructose with the transfer of fructose to free fructose.

When sucrose-[U-^{14}C] was incubated with the enzyme in the presence of melibiose, melizitose or raffinose and the reaction mixtures were subjected to paper partition chromatography, spots of new saccharides corresponding to raffinose and a spot with R_f value less than that of raffinose were detected in the case of added melibiose and raffinose, respectively. The PC autoradiogram showed the newly synthesized saccharide to be radioactive. The chromatographic analysis of the acid hydrolysates of these synthesized saccharides in case of melibiose and raffinose showed the presence of glucose, galactose and fructose. The radioactivity was only detected in the fructose spot in both cases. This shows that melibiose and raffinose act as acceptors of fructose from labelled sucrose for the synthesis of their higher oligosaccharides.

Mechanism of biosynthesis of oligosaccharides from sucrose

It can be inferred from the above results that only sugars having the F ~ R linkage (R = aldose), e.g. sucrose and raffinose (R = glucose and melibiose, respectively), act as donors of the fructosyl group. These sugars should also possess a free hydroxyl group at C-1 of fructose residue as in the case of sucrose or sucrose-type sugars, i.e. raffinose and a free aldose (glucose, mannose, xylose, ribose, arabinose and melibiose) to act as the acceptors of the fructosyl moiety. Experiments were further designed to elucidate the mechanism of oligosaccharide biosynthesis by fructosyl transferase from sucrose.

A mixture of 1.5 ml of the purified enzyme, 0.5 ml of sucrose-[U-^{14}C] (10 μCi) and 0.5 g sucrose was incubated at 37° and 0.1 ml samples were taken at different time intervals. A sample (10 μl) of each reaction mixture was subjected to PC for 48 hr and spots were detected with benzidine–TCA reagent and radioactivity of individual spots measured by autoradiography (Fig. 1).

The concentration of glucose F_2G, F_3G and F_nG increased with the time up to 120 hr and then became constant (Fig. 1). It was thought that after 120 hr of incubation either the sucrose concentration had decreased below the minimum level required for the discharge of donor function, or that the enzyme had been inactivated. Sucrose (25 mg) was again added after 140 hr to the rest of the reaction mixture which was subjected to radioassay as indicated above. Addition of sucrose led to an increase of radioactivity in glucose, F_2G, F_3G and F_nG, indicating that the enzyme was still active and that the inhibition of oligosaccharide synthesis was caused by the exhaustion of the substrate, sucrose. The constancy of the concentration of the oligosaccharides, viz. F_2G, F_3G etc., after 120 hr indicated that either these oligosaccharides cannot act as donors of the fructosyl moiety or that their concentrations were not high enough for the action of fructosyl transferase. Unlabelled F_2G, F_3G and F_4G oligosaccharides isolated from the reaction mixture by preparative-TLC

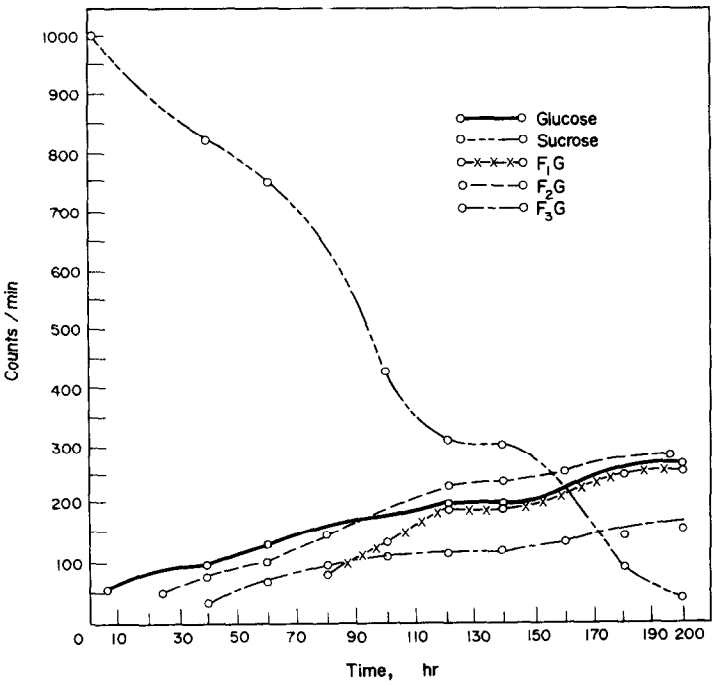

Fig. 1. Time progress curves of transfructosidase activity.

were incubated individually (20% concentration) with purified enzyme for 72 hr and the reaction mixture was subjected to PC for 60 hr. The results showed that fructosyl transferase had neither hydrolytic nor synthetic action on substrates like F_2G, F_3G and F_4G. It was concluded that these intermediates cannot act as donors of the fructosyl moiety for the synthesis of higher oligosaccharides, and might act as acceptors of fructose from sucrose only for the synthesis of higher oligosaccharides.

The ability of F_2G to act as acceptor of the fructosyl moiety from sucrose was further confirmed by incubating (i) sucrose + sucrose-[U-^{14}C] and (ii) sucrose + F_2G + sucrose-[U-^{14}C] with purified enzyme for 18 hr in capillary tubes. Spots of glucose, sucrose and F_2G in the case of reaction mixture (i) and spots of glucose, sucrose, F_2G and F_3G in reaction mixture (ii) were detected by autoradiography.

Acid hydrolysates of F_2G from both the reaction mixtures were subjected to PC and individual components radioassayed. In the case of F_2G both glucose and fructose were labelled in both cases, whereas in the case of F_3G radioactivity was detected only in fructose. This indicated that F_3G formed in the case of reaction mixture (ii) had label as F*-F-F-G and not as F-F-F*-G* showing that sucrose donated its labelled fructose molecule to unlabelled F_2G for the formation of labelled F_3G (F*-F-F-G). Possibly, the following reactions took place:

$$G^* - F^* + G^* - F^* \xrightarrow[\text{transferase}]{\text{Fructosyl}} G^* - F^* - F^* + G^*$$

$$G^* - F^* + G - F - F \xrightarrow[\text{transferase}]{\text{Fructosyl}} G - F - F - F^* + G^*.$$

It is therefore inferred that synthesis of oligosaccharides from sucrose takes place as follows:

$$F - G + \text{Fructosyl transferase} \rightarrow F - \text{fructosyl transferase} + G$$

$$F - \text{fructosyl transferase} + F - G \rightarrow F - F - G + \text{Fructosyl transferase}.$$

This process is continued by stepwise transfructosylation reaction to give rise to higher glucofructosans.

EXPERIMENTAL

Preparation of crude extract and $(NH_4)_2SO_4$ *fractionation.* Fresh plants were taken from the forest area of Phillaur (Punjab). The crude extract and the dialysed fractions 0–20, 20–50 and 50–70% $(NH_4)_2SO_4$ were prepared by the method of ref. [6] and designated as dialysed protein fractions I, II and III, respectively.

DEAE-cellulose column chromatography. The dialysed 20–50% $(NH_4)_2SO_4$ fraction (protein fraction II) was purified by DEAE-cellulose column chromatography as described in ref. [4] with some modifications. The DEAE-cellulose column with an effective length of 24 cm was equilibrated with 0.5 mM Pi buffer (pH 6.1). The flow rate was adjusted to *ca* 1 ml/min. Dialysed $(NH_4)_2SO_4$ fraction was applied on the column and eluted with NaCl–Pi buffer (pH 6.1), with a stepwise concn gradient of increasing ionic strength (0.01–0.60 M). Fractions (60 × 10 ml) were collected in ice-cooled tubes and analysed for protein content and enzymatic activity. The protein was determined by the method of ref. [15] using BSA as standard.

Enzymic activity was measured in terms of glucose liberation by incubating 20% sucrose with enzyme preparation for specified periods at 37°.

Micro-incubation technique. A known amount of substrate soln was dried *in vacuo* and a measured amount of enzyme was added to make the substrate concns 20% for the donor and 10% for the acceptor sugars. The contents were thoroughly mixed and sucked into capillary tubes. A drop of toluene was introduced as a preservative and the tubes were sealed from the opposite end. The capillary tubes were incubated at 37° for specific periods. After incubation the enzyme was inactivated at 100° for 5 min.

Hydrolysis of oligosaccharides. The oligosaccharides were either hydrolysed with 0.1 N HCl in capillary tubes at 69° for 15 min or by incubation with 50% diluted invertase (B.D.H.) in capillary tubes at 29° for 24 hr.

Paper partition chromatography. Qualitative. Reaction products of fructosyl transferase and their acid and invertase hydrolysate were separated by descending PC [16] using *n*-BuOH–HOAc–H_2O(BAW) (4:1:5, upper) and identified with benzidine–trichloroacetic acid (TCA) [17, 18], naphthoresorcinol–TCA [19], aniline hydrogen phthalate [19] and ammoniacal silver nitrate [20] spray reagents. *Preparative.* The reaction mixture of fructosyl transferase was fractionated by descending PC for 60 hr on 3 MM paper using BAW. After detecting the separated oligosaccharides on the guide strip with benzidine–TCA spray reagent, the corresponding areas of unsprayed chromatogram were marked and cut. The oligosaccharides from these were eluted by micro-elution technique [21].

Preparative-TLC. Various oligosaccharides present in the reaction mixture were isolated by preparative-TLC as described in ref. [22].

Measurement of radioactivity. (i) After autoradiography the corresponding spots on the PC were eluted into the planchets by the technique used in ref. [21] and the radioactivity was measured after drying. (ii) The PC containing the radioactive spots was sprayed with benzidine–TCA reagent. Each spot was cut and its radioactivity measured from both the surfaces using end window Geiger Muller Counter (Nuclear Chicago). The average of two surfaces has been represented as the radioactivity of that spot.

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