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PURIFICATION AND PROPERTIES OF SUCROSE:SUCROSE 1^F-β-D-FRUCTOSYLTRANSFERASE IN ONION SEEDS

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Abstract—A sucrose:sucrose 1^F-β-D-fructosyltransferase (EC 2.4.1.99) has been purified from onion seeds by fractionation with ammonium sulphate and then by chromatography on DEAE-cellulose, CM-cellulose, octyl-Sepharose and Sephadex G-200. The purified enzyme which showed a single protein band on polyacrylamide gel electrophoresis was free from the other fructosyltransferases, catalysed fructosyltransfer from sucrose to another sucrose to form 1-kestose and glucose, and also in some degree transferred a fructosyl residue from sucrose to raffinose and stachyose but did not to 1-kestose and nystose. The enzyme had an M_r of *ca* 68 000, an optimum pH of 5.4, and K_m of 0.083 M, was stable at 20–37° for 10 min, and was inhibited by Hg^{2+}, Ag^+, Mn^{2+} and *p*-chloromercuribenzoate.

INTRODUCTION

In our recent studies on fructosyltransferases involved in the synthesis of fructo-oligosaccharides in liliaceous plants, we have reported the purification and substrate specificities of sucrose:sucrose 1-fructosyltransferase (sucrose:sucrose 1^F-β-D-fructosyltransferase, SST, EC 2.4.1.99) [1], a tentatively termed 6^G-fructosyltransferase [1^F(1-β-D-fructofuranosyl)$_m$ sucrose:1^F(1-β-D-fructofuranosyl)$_n$ sucrose 6^G-fructosyltransferase] [2] and a 1^F-fructosyltransferase [3] from the roots of asparagus (*Asparagus officinalis* L.).

SST that transfers a fructosyl group from sucrose to the 1-hydroxyl of a fructosyl group of another sucrose to produce 1-kestose and glucose is widely distributed in higher plants containing fructose oligomers and polymers. Several workers have studied the SST of onion [4], chicory [5, 6], lettuce [7] and agave plants [8] but did not purify it completely. Recently, Henry *et al.* [9] reported the properties of onion-bulb SST having a small amount of other fructosyltransferase but no detectable hydrolytic activity.

On the other hand, we pointed out [10] that the onion seeds included 1-kestose, nystose, raffinose, stachyose and their related saccharides as well as sucrose, glucose and fructose. Therefore, we attempted to seek an enzymatic system synthesizing the fructosyl oligosaccharides, namely 1-kestose, nystose, raffinose and stachyose. Consequently, we estimated that the SST of onion seeds was concerned in the synthesis of the oligosaccharides. This paper describes purification and properties of SST from onion seeds.

RESULTS

Purification of enzyme

Onion seeds (250 g each; total 2.5 kg) were swollen in 0.05 M phosphate buffer (pH 6.6, 500 ml) for 12 hr at 5° and then homogenized in the same buffer (1.5 litre with cooling with ice by using a polytoron (Kimematica GmbH). The homogenate was filtered through cheese cloth and centrifuged (12 000 × *g*, 10 min). The supernatant was saturated with solid ammonium sulphate and stored overnight in a cold chamber. The resulting precipitate was collected by centrifugation, and a solution in 0.05 M phosphate buffer (pH 6.6) was dialysed for 5 days against the same buffer and then centrifuged to give the 'ammonium sulphate, 0–1 saturation' fraction (1800 ml).

This fraction was concentrated to 500 ml by ultrafiltration (Amicon; Diaflo PM-10 filter), dialysed overnight against 0.01 M phosphate buffer (pH 6.6), and chromatographed on a column (8 × 12 cm) of DEAE-cellulose equilibrated with the same buffer by successive elution with 0.01 M and 0.05 M phosphate buffer, and then 0.15 M phosphate buffer containing 0.25 M sodium chloride. SST was eluted with 0.01 M phosphate buffer, and 0.15 M phosphate buffer containing 0.25 M sodium chloride. The active fractions (10–140), having much more SST activity and almost freed from β-fructofuranosidase activity, were collected and dialysed against 0.005 M phosphate buffer (pH 6.6). The dialysate was the 'DEAE-cellulose' fraction (2320 ml).

This fraction was concentrated to 150 ml, dialysed against 0.01 M acetate buffer (pH 5.0) and was loaded onto a column (3.8 × 24 cm) of CM-cellulose previously equilibrated with the same buffer. Elution was performed successively with 0.01, 0.04 M acetate buffers and 0.15 M acetate buffer containing 0.25 M sodium chloride. The eluate (fractions 160–225) emerging with the final buffer (pH 5.0) was the 'CM-cellulose' fraction (790 ml).

This fraction was concentrated to 50 ml and dialysed against 0.01 M acetate buffer (pH 5.0). The dialysate was added to a column (3.8 × 22 cm) of CM-Sepharose previously equilibrated with the same buffer and eluted successively 0.01, 0.02, 0.06, 0.1 M acetate buffers (pH 5.0), 0.15 M acetate buffer containing 0.25 M sodium chloride and 0.2 M acetate buffer containing 0.5 M sodium chloride. SST was eluted with 0.2 M acetate buffer containing 0.5 M sodium chloride, collected (fractions 225–265) and dialysed against 0.01 M phosphate buffer

(pH 6.6) to give the 'CM-Sepharose' fraction (540 ml).

This fraction was concentrated to 100 ml, was saturated to 30% with solid ammonium sulphate and stored overnight in a cold chamber. The resulting precipitate was removed by centrifugation and the supernatant was applied to a column (2.1 × 27 cm) of octyl-Sepharose (CL-4B) previously equilibrated with 0.01 M phosphate buffer (pH 6.6) saturated to 30% with ammonium sulphate. The column was eluted with 0.01 M phosphate buffer (pH 6.5, 500 ml each) saturated with ammonium sulphate to 30, 25, 20, 15 and 10%, respectively, and then with the buffer alone (1000 ml). As the final eluate showed SST activity, it was dialysed for 2 days against 0.01 M phosphate buffer to give the 'octyl-Sepharose' fraction (1010 ml).

This fraction was concentrated to 5 ml, dialysed overnight against 0.01 M phosphate buffer (pH 6.5) containing 0.25 M sodium chloride, and chromatographed on a column (2.64 × 100 cm) of Sephadex G-200 with the same buffer. SST active fractions (fractions 37–48) were combined to give the 'Sephadex G-200, 1st' fraction (118 ml). This fraction was concentrated to 3 ml and rechromatographed. The eluate (fractions, 37–48) was concentrated and dialysed against 0.005 M phosphate buffer (pH 6.6), to give a highly purified enzyme preparation, the 'Sephadex G-200, 2nd' fraction (20 ml).

The purification is summarized in Table 1. SST was purified about 317-fold with a recovery of 5.4% from the 'ammonium sulphate, 0–1 saturation' fraction. The SST preparation had no detectable activities of β-D-fructofuranosidase, 1^F-fructosyltransferase [3] and 6^G-fructosyltransferase [2], because it could not hydrolyse 1-kestose, nystose and inulin, nor catalyse fructosyltransfer of fructosyl group between 1-kestose or nystose, and could not synthesize $1^F,6^G$-di-β-D-fructofuranosylsucrose from 1-kestose.

Properties of SST

The purified SST preparation was subjected to electrophoresis on polyacrylamide gel, pH 9.4, when the enzyme migrated as a single protein band. The M_r of the enzyme was estimated to be *ca* 68 000 in comparison with those of the reference proteins, by Sephadex G-200 gel filtration. The optimum pH was found to be *ca* 5.4. The enzyme solutions at pH 4.0, 4.6, 5.0, 5.4, 6.0, 6.6 and 7.0 were pre-incubated at 45° for 10 min, cooled to 0°, and adjusted to pH 5.4, and then assayed for the residual enzyme activity. The residual activities were as follows: *ca* 52% at pH's 4.6 and 5.4; *ca* 37 and 35% at pH's 4.0 and 6.0; and *ca* 5% at pH 7.0. The enzyme solutions in McIlvaine buffer (pH 5.4) were heated for 10 min at 20, 30, 37, 40, 45, 50 and 60°, and then the residual enzyme activity was measured. The enzyme was stable at 20–37° but inactivated at 50–60°.

The effect of various reagents on the enzyme activity was examined as follows. A mixture of enzyme (1.0 U, 0.25 ml) and 0.4 M sucrose in McIlvaine buffer (pH 5.4, 0.5 ml) was incubated at 30° for 1 hr in the presence of water of one of the inhibitors (0.25 ml). Residual activity of the enzyme was measured and expressed in terms of relative activity. Mercuric chloride (2.5×10^{-5} M), *p*-chloromercuribenzoate (3×10^{-5} M) and manganese sulphate (2.5×10^{-4} M) and silver nitrate (2.5×10^{-4} M) inhibited the enzyme by *ca* 53.8, 63.5, 45.7 and 32.2% respectively. At 2.5×10^{-4} M, cobalt chloride, calcium chloride and copper sulphate were slightly effective, 12.7, 10.6 and 11.1% respectively and lithium sulphate, zinc sulphate, barium chloride and aluminium chloride were almost ineffective.

The influence of sucrose concentration on the velocity was studied. The Michaelis constant was determined by means of the Lineweaver–Burk plots. The K_m value for the enzyme was calculated to be 0.083 M.

In studying substrate specificities, the transfer of a D-fructosyl residue between two identical saccharide molecules was investigated. A mixture of enzyme (5.0 U/ml, 20 μl) and one of the [U-^{14}C]-substrates [0.2 M (sucrose, 0.070 μCi; 1-kestose, 0.064 μCi; nystose 0.050 μCi) in McIlvaine buffer pH 5.4, 20 μl] was incubated in the presence of toluene at 30° for 5 or 10 hr. The reaction was stopped by the addition of 0.1 M mercuric chloride (10 μl) and then the mixture, together with carrier sugars [glucose, fructose, 1-kestose, nystose, 1^F(1-β-D-fructofuranosyl)$_3$sucrose, 100 μg of each] was subjected to paper chromatography. Each fraction thus obtained was concentrated *in vacuo* to dryness. Each residue was dissolved in water (2 ml) and assayed for radioactivity. As shown in Table 2, the enzyme catalysed fructosyltransfer between two similar saccharides of sucrose to form 1-kestose but did not act on nystose or 1^F(1-β-D-fructofuranosyl)$_3$sucrose. Also, fructosyltransfer between two similar saccharide molecules of sucrose, 1-kestose, neokestose, raffinose or nystose was tested by TLC.

A mixture of enzyme (5.0 U/ml, 20 μl) and substrate in

Table 1. Purification of onion SST

Step	Total activity (U)	Total protein (mg)	Volume (ml)	Specific activity (U/mg protein)
$(NH_4)_2SO_4$, 0–1 satn.	5616	15336	1800	0.37
DEAE-cellulose	3184	7649	2320	0.42
CM-cellulose	2379	2234	790	1.06
CM-Sepharose	1805	968	540	1.86
Octyl-Sepharose	621	20.8	1010	29.9
Sephadex G-200, 1st.	392	5.8	118	67.6
Sephadex G-200, 2nd.	305	2.6	20	117.3

See text for details.

Table 2. Transfer of a D-fructosyl residue between two identical saccharide molecules by onion SST

	Fructose transferred (μmoles/ml reaction mixture)	
	Incubation time (hr)	
[U-^{14}C]-Substrate	5	10
Sucrose	3.70	7.10
1-Kestose	Nil	Nil
Nystose	Nil	Nil

See text for details.

McIlvaine buffer (pH 5.4, 0.1 M) was incubated for 10 hr at 30° in the presence of toluene, and then treated with 0.1 M mercuric chloride (10 μl) and subjected to TLC. Only 1-kestose and glucose were produced from sucrose. Traces of the saccharides corresponding to disaccharide, and tetrasaccharide having one additional fructose were formed from neokestose or raffinose but there was no transferred product from 1-kestose or nystose.

Next, fructosyltransfer from sucrose to several oligosaccharides was examined. A mixture of 0.1 M [U-^{14}C] sucrose (0.1 μCi, 10 μl) in McIlvaine buffer (pH 5.4), one of acceptors (0.1 M raffinose, stachyose 1-kestose and nystose, 10 μl) in the same buffer, and enzyme (20 μl) was incubated in the presence of toluene at 30° for 10 hr. 0.1 M mercuric chloride (10 μl) was added to terminate the reaction, and the mixture was subjected to PC (3 developments) together with carrier sugars. Zones corresponding to mono- to penta-saccharide were extracted with water, and the extracts were concentrated *in vacuo* to dryness. Each residue was dissolved in water (2 ml) and assayed for radioactivity. The fructose transferred was calculated from the radioactivity of the synthesized saccharide having a degree of polymerization higher by one fructose unit than that of the acceptor. Table 3 shows that the enzyme catalyses the fructosyltransfer from sucrose to raffinose and stachyose although they were not so preferable acceptor as sucrose.

Table 3. Fructosyltransfer from sucrose to several oligosaccharides by onion SST

		Fructose transferred (μmoles/ml reaction mixture)	
		Incubation time (hr)	
Donor	Acceptor (Unlabeled)	5	10
[U-^{14}C]Sucrose	None	2.38	4.60
[U-^{14}C]Sucrose	Raffinose	0.75	1.44
[U-^{14}C]Sucrose	Stachyose	0.13	0.27
[U-^{14}C]Sucrose	1-Kestose	Nil	Nil
[U^{14}-C]Sucrose	Nystose	Nil	Nil

See text for details.

DISCUSSION

Sucrose: sucrose 1F-β-D-fructosyltransferase (SST), present in onion seeds, was free from other fructosyltransferases and purified 317-fold by seven steps to give a single protein. The general properties of onion SST (M_r *ca* 68 000; optimum pH 5.4; stable at 20–37°; inhibited by Hg^{2+}, Mn^{2+}, Ag^+, *p*CMB) resembled those of asparagus SST [1] (M_r *ca* 65 000; optimum pH 5.0; stable at 20–37°; inhibited by Hg^{2+}, Mn^{2+}, Ag^+, *p*CMB) except that K_m of onion enzyme (0.083 M) was smaller than that of the asparagus enzyme (0.11 M).

Onion SST catalysed the fructosyltransfer from sucrose to sucrose and specifically producing 1-kestose and glucose. Neokestose and raffinose served as substrates but 1-kestose and nystose did not. Also the enzyme transferred some fructosyl residues from sucrose to raffinose and stachyose but not to 1-kestose and nystose. Thus the fructosyltransfer seemed to occur at the 1-hydroxyl of the fructosyl residue of the sucrose moiety in raffinose and stachyose but not to do so at the corresponding position of the terminal fructosyl residue in 1-kestose and nystose.

We have also found that onion seeds contain fructosyl raffinose and stachyose as well as 1-kestose, nystose, raffinose and stachyose [10]. It is interesting that the synthesis of these fructosyl oligosaccharides occur in onion seeds, although these fructosyl oligosaccharides comprise a small portion of total sugar.

EXPERIMENTAL

Material. Onion seeds (*Allium cepa* L. var. Early yellow globe) were purchased from commercial sources (Sapporo Konoen Ltd.). [U-^{14}C]Sucrose was obtained from The Radiochemical Centre (Amersham, Bucks., U.K.). [U-^{14}C]1-Kestose [*O*-β-D-Fruf-(2 → 1)-*O*-β-D-Fruf-(2 ↔ 1)-α-D-Glcp], and [U-^{14}C]nystose [*O*-β-D-Fruf-(2 → 1)-*O*-β-D-Fruf-(2 → 1)-*O*-β-D-Fruf-(2 ↔ 1)-α-D-Glcp] were prepared from [U-^{14}C] sucrose using a preparation of asparagus enzyme [11], and 1-kestose, neokestose and nystose were isolated from asparagus roots [12] as previously described.

Determination of sugars and proteins. Reducing sugars were determined by the method of Somogyi–Nelson [13–15] and glucose was measured with a commercial Glucostat reagent. Proteins were determined by the method of Lowry *et al.* [16], being calibrated for bovine serum albumin and by measuring absorbance at 280 nm.

PC and TLC. PC: Solvent I, PrOH–EtOAc–H_2O (7:1:2). TLC (silica gel):, Solvent II, *n*-BuOH–*iso*PrOH–H_2O (10:5:4). Sugars were detected by anisidine phosphate [17] and alkaline silver nitrate reagents [18].

Measurement of enzyme activity. Sucrose: sucrose 1F-β-D-fructosyltransferase (SST) was measured in an incubation mixture (1 ml) of enzyme (0.5 ml) and sucrose in McIlvaine buffer (0.8 M, pH 5.4, 0.5 ml). After the incubation at 30° for 1 hr, the reaction was terminated by addition of the Somogyi–Nelson reagent (1 ml) and reducing power was measured. When no invertase activity was detected in the enzyme soln, the amount of fructose transferred was directly obtained from the measured value of reducing power. However, when the invertase activity was detected, glucose was also determined by the Glucostat method to calculate the amount of fructose transferred by the formula 2 × glucose minus reducing sugars. One unit of the enzyme activity was defined as the amount of enzyme transferring a D-fructosyl group from sucrose to sucrose that produces 1 μmole of 1-kestose in 1 hr under the conditions described above.

Disc electrophoresis. Enzyme protein was run on 7.5% polyacrylamide gel for 2.5 hr at pH 9.4 at room temperature and 2 mA/tube according to the method of Ornstein–Davis [19, 20]. The protein band was stained with Amido Black 10B.

Determination of M_r by gel filtration. The ascending method was used with a column (2.64 × 100 cm) of Sephadex G-200 pre-equilibrated with 0.01 M phosphate buffer containing 0.25 M sodium chloride (pH 6.5). Elution was effected with the same buffer at 8 ml/hr. Cytochrome c (M_r, 12400), chymotrypsinogen A (25000), hen-egg albumin (45000), bovine serum albumin (67000) and aldolase (147000) were used as marker proteins.

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