Purification and characterization of a fructosyltransferase from onion bulbs and its key role in the synthesis of fructo-oligosaccharides in vivo

Masaki Fujishima, Hideki Sakai, Keiji Ueno, Natsuko Takahashi, Shuichi Onodera, Noureddine Benkeblia and Norio Shiomi

Department of Food and Nutrition Sciences, Graduate School of Dairy Science Research, Rakuno Gakuen University, 582 Bunkyodai, Midorimachi, Ebetsu, Hokkaido 069-8501, Japan.

Summary

• A fructosyltransferase that transfers the terminal \((2 \rightarrow 1)\)-\(\beta\)-linked \(\alpha\)-fructosyl group of fructo-oligosaccharides \((1^\text{F}(1-\beta-\alpha\text{-fructofuranosyl})_n\text{sucrose}, n \geq 1)\) to HO-6 of the glucosyl residue and HO-1 of the fructosyl residue of similar saccharides \((1^\text{F}(1-\beta-\alpha\text{-fructofuranosyl})_m\text{sucrose}, m \geq 0)\) has been purified from an extract of the bulbs of onion \((\text{Allium cepa})\).
• Successive column chromatography using DEAE-Sepharose CL-6B, Toyopearl HW65, Toyopearl HW55, DEAE-Sepharose CL-6B (2nd time), Sephadex G-100, Concanavalin A Sepharose, and Toyopearl HW-65 (2nd time) were applied for protein purification.
• The general properties of the enzyme, were as follows: molecular masses of 66 kDa (gel filtration chromatography), and of 52 kDa and 25 kDa (SDS-PAGE); optimum pH of c. 5.68, stable at 20–40°C for 15 min; stable in a range of pH 5.30–6.31 at 30°C for 30 min, inhibited by \(\text{Hg}^2\), \(\text{Ag}^+\), \(p\)-chloromercuribenzoic acid \((p\text{-CMB})\) and sodium dodecyl sulfate (SDS), activated by sodium deoxycholate, Triton X-100 and Tween-80. The amino acid sequence of the \(N\)-terminus moiety of the 52-kDa polypeptide was ADNEFPWTNDMLAWQRGCFFRTVRNYMNDSGP-MYYKGYHLYFQYNKDFAYXG and the amino acid sequence from the \(N\)-terminus of the 25-kDa polypeptide was ADVGYXSTSGAATRGTLGPFGLVLANQDLTENTAYFYVSKTDGALTFCQDET.
• The enzyme tentatively classified as fructan: fructan \(6^\text{G}\)-fructosyltransferase \((6^\text{G}\text{-FFT})\). The enzyme is proposed to play an important role in the synthesis of inulin and inulinneo-series fructo-oligosaccharides in onion bulbs.

Key words: fructo-oligosaccharides synthesis, fructosyltransferase, onion \((\text{Allium cepa})\), properties, purification, sequence.


Introduction

Fructo-oligosaccharides (degree of polymerization (DP) 3–c. 10), together with glucose, fructose and sucrose are found in onion, which is one of the most widely distributed liliaceous plants in the world (Bacon, 1957, 1959; Bose & Shirivastava, 1961; Darbyshire & Henry, 1978). The distribution of fructo-oligosaccharides in onion leaf-bases has been studied, and the fructo-oligosaccharide contents were found to be higher in younger (inner) than in older (outer) leaf-bases (Bacon, 1959; Darbyshire & Henry, 1978). The composition of fructo-oligosaccharide isomers in onion plants was also investigated, and the trisaccharide fraction from onion bulbs was shown to comprise 1-kestose (1-kestotriose) and neokestose \((6^\text{G}\text{-kestotriose})\) (Bacon, 1959; Darbyshire & Henry, 1978). The tetra-, penta- and hexa-saccharide fractions from onion...
leaves and bulbs were reported to comprise nystose (1,1-kestotetraose), \( \text{G}^6(1\beta\text{-d-fructofuranosyl})_2\text{sucrose} \) (1,6G-kestotetraose) and \( \text{G}^6(1\text{-d-fructofuranosyl})_3\text{sucrose} \) (1,1,1-kestohexaose) as well as the synthesis of fructo-oligosaccharide isomers from sucrose, 1-kestose or neokestose. The fructosyltransferases (SST, 1-FFT and 6G-FFT) involved in the synthesis of the fructo-oligosaccharides derived from 1-kestose and 6G-kestotetraose could not be enzymologically assigned to a single enzyme because very few fructosyltransferase(s) involved in the synthesis of the fructo-oligosaccharides have been purified from onion bulbs (Shiomi et al., 1997). However recent work of Ritsema et al. (2003) showed that transfer of a fructosyl residue to the terminal glucose C6 and the terminal fructose C1 occurred through the activity of 6G-FFT and that there was no separate 1-FFT activity in onion bulb. Except for the limited investigations of these two activities in onion tissues (Vijn et al., 1997; Ritsema et al., 2003), fructosyltransferase has not been subjected to high purification processes. We describe the purification and properties of the fructosyltransferase possessing activities of 1-FFT and 6G-FFT from onion bulbs.

Materials and Methods

Materials

Bulbs of onion (Allium cepa L., cv. Tenshin) were harvested at the experimental farm of Rakuno Gakuen University on 28 July 2002. The dry outer scales were removed and bulbs were stored at \(-40^\circ\text{C}\) until use. All the experiments were performed in triplicate. Standard sugars were prepared as follows. Crystalline 1-kestose (3a: \( \text{F}(1\beta\text{-d-fructofuranosyl})_2\text{sucrose} \), 1-kestotriose) and nystose (4a: \( \text{F}(1\beta\text{-d-fructofuranosyl})_2\text{sucrose} \), 1,1-kestotetraose) were prepared from sucrose using a Scopulariopsis brevicaulis enzyme (Takeda et al., 1994). Neokestose (3b: \( \text{G}^6(1\beta\text{-d-fructofuranosyl})_2\text{sucrose} \), 6G-kestotetraose) and \( \text{F}(1\beta\text{-d-fructofuranosyl})_m\text{G}^6(1\beta\text{-d-fructofuranosyl})_n \) sucrose (4b: \( m = 0, n = 2 \) (1,6G-kestotetraose); 4c: \( m = 1, n = 1 \) (1 and 6G-kestotetraose); 4a: \( m = 1, n = 0 \) (1,1,1-kestotetraose); 4b: \( m = 0, n = 3 \) (1,1,6G-kestotetraose); 4c: \( m = 2, n = 1 \) (1,1 and 6G-kestohexaose); 4d: \( m = 2, n = 2 \) (1 and 1,6G-kestohexaose); 4e: \( m = 3, n = 1 \) (1,1,1,6G-kestohexaose); 4f: \( m = 4, n = 1 \) (1,1,1,1,6G-kestohexaose); 4g: \( m = 5, n = 1 \) (1,1,1,6G-kestohexaose); 4h: \( m = 6, n = 2 \) (1,1,1,1,6G-kestohexaose); 4i: \( m = 7, n = 2 \) (1,1,1,1,1,6G-kestohexaose); 4j: \( m = 8, n = 2 \) (1,1,1,1,1,6G-kestohexaose); 4k: \( m = 9, n = 2 \) (1,1,1,1,1,1,6G-kestohexaose) were obtained from asparagus roots as described in previous papers (Shiomi et al., 1976, 1979; Shiomi, 1981). \( \text{F}(1\beta\text{-d-fructofuranosyl})_m\text{sucrose} \) (6a: \( m = 4 \) (1,1,1,1-kestohexaose) and 7a: \( m = 5 \) (1,1,1,1,1-kestohexaose)) were prepared from Jerusalem artichoke tubers. Fructan nomenclature is not simple since the structures are very complex; the nomenclature for fructo-oligosaccharides proposed by Lewis (1993) and Waterhouse & Chatterton (1993) were also used herein.

Quantitative determination of sugars and proteins

Total sugars were determined by the anthrone method (Morris, 1948) and reducing sugars were measured by the method of Somogyi and Nelson (Nelson, 1944; Somogyi, 1945). Proteins were determined by measuring absorbance at 280 nm with reference to \( E_{1%}^{1\%} = 9.38 \) in aldolase (Babul & Stellwagen, 1969).

Analysis of sugars by high-performance anion-exchange chromatography (HPAEC)

The synthesized or extracted saccharide mixture was separated on a HPAEC carbohydrate column (PA 1: Carbo Pack, Sunnyvale, CA, USA) with a Dionex Bio LC series HPAEC using pulsed amperometric detection (PAD) (Rocklin & Pohl, 1983; Johnson, 1986). The gradient was established by mixing eluent A (150 mm NaOH) with eluent B (500 mm sodium acetate in 150 mm NaOH) in the following two ways,

System I: 0–1 min, 25 mm; 1–2 min, 25–50 mm; 2–20 min, 50–200 mm; 20–22 min, 500 mm; 22–30 min, 25 mm
System II: 0–1 min, 5 mM; 1–2 min, 25–50 mM; 2–14 min, 50–500 mM; 14–22 min, 500 mM; 22–30 min, 25 mM. The flow rate through the column was 1.0 ml min\(^{-1}\). The applied PAD potentials for E1 (500 ms), E2 (100 ms) and E3 (50 ms) were 0.01, 0.60 and −0.60 V, respectively, and the output range was 1 µC.

Purification of the enzyme

All operations throughout the purification were performed at 0–2°C. The enzyme solutions were concentrated with collodion bags.

**Extraction of the enzyme** Onion bulbs (300 g each, total 3 kg), previously washed with water and chopped, were homogenized in 50 mM phosphate buffer (pH 7.0, 600 ml) containing 2 mM 1,4-dithiothreitol (DTT). The homogenate was filtered through cheesecloth and centrifuged (10 000 g, 30 min). The supernatant (6075 ml per 3 kg of onion bulbs) was filtered through cheesecloth and centrifuged (10 000 g) for 30 min, ammonium sulfate was added to bring the supernatant solution to 80% saturation. A solution of the precipitate in 10 mM phosphate buffer (pH 7.0) containing 2 mM DTT was dialysate for 30 h against the same buffer, to give fraction B (\((\text{NH}_4)_2\text{SO}_4, 0.5–0.8\) saturation fraction (205 ml)).

**DEAE-Sepharose CL-6B chromatography (1st)** Fraction A was added to a column (4 × 40 cm) of DEAE-Sepharose CL-6B equilibrated with 10 mM phosphate buffer (pH 7.0) containing 2 mM DTT. After removing nonadsorbed protein, the elution was achieved with a linear gradient from 0 to 1.0 M sodium chloride in the same buffer. The active fractions (Fr. 48–54, 160 ml) were combined and dialysed against 10 mM phosphate buffer (pH 7.0) containing 2 mM DTT to give a solution as described earlier. The active fractions (Fr. 38–42, 15 ml) were pooled to give fraction E.

**DEAE-Sepharose CL-6B chromatography (2nd)** Fraction E was concentrated to 1 ml and dialysed for 10 h against 10 mM phosphate buffer (pH 7.0) containing 2 mM DTT. The dialysate was loaded onto a column (4 × 40 cm) of DEAE-Sepharose CL6B equilibrated with the same buffer. The elution was accomplished with a linear gradient from 0 to 1 M sodium chloride in the same buffer. The active fractions (Fr. 269–328, 180 ml) were pooled to give fraction F.

**Sephadex G-100 chromatography** Fraction F was concentrated to 1 ml and dialysed for 10 h against 100 mM phosphate buffer (pH 7.0) containing 100 mM sodium chloride and 2 mM DTT. The dialysate was filtered on a column (2.2 × 65 cm) of Sephadex G-100 equilibrated with the same buffer solution as described earlier. The active fractions (Fr. 52–55, 12 ml) were combined to give fraction G.

**Concanavalin A Sepharose chromatography** Fraction G was concentrated to 1 ml and dialysed for 10 h against 10 mM phosphate buffer (pH 7.0) containing 2 mM DTT. The dialysate was loaded on a column (1.2 × 6 cm) of Concanavalin A Sepharose equilibrated with the same buffer. The elution was achieved with the linear gradient from 0 to 0.6 M methyl-α-0-mannopyranoside. The active fractions (Fr. 24–32, 9 ml) were pooled to give fraction H.

**Toyopearl HW65 chromatography (2nd)** Fraction H was concentrated to 1 ml and dialysed for 10 h against 10 mM phosphate buffer (pH 7.0) containing 2 mM DTT and saturated with ammonium sulfate to 45%. The dialysate was applied to a column of Toyopearl HW65. The running conditions were the same as those used for Toyopearl HW65 chromatography (1st). The active fractions (Fr. 48–54, 21 ml) of the Toyopearl chromatogram (2nd) of onion fructosyltransferase were combined and dialysed against 10 mM phosphate buffer containing 2 mM DTT to give a finally purified enzyme preparation, fraction J.

**Toyopearl HW55 chromatography** Fraction D was concentrated to 1 ml and dialysed for 10 h against 100 mM phosphate buffer (pH 7.0) containing 100 mM sodium chloride and 2 mM DTT. The dialysate was filtered on a column (2.2 × 65 cm) of Toyopearl HW55 (superfine) equilibrated with the same buffer solution as described earlier. The active fractions (Fr. 38–42, 15 ml) were pooled to give fraction E.

**Toyopearl HW65 chromatography (1st)** Fraction C was dialysed for 10 h against 10 mM phosphate buffer (pH 7.0) containing 2 mM DTT and saturated with ammonium sulfate to 45%. The dialysate was loaded on to a column (1.9 × 18 cm) of Toyopearl HW65 (fine) equilibrated with the same buffer saturated with ammonium sulfate to 45%. The elution was achieved with a linear gradient from 45% to 0% in the same buffer. The active fractions (Fr. 48–54, 21 ml) were combined to give fraction D.

**Toyopearl HW55 chromatography** Fraction D was concentrated to 1 ml and dialysed for 10 h against 100 mM phosphate buffer (pH 7.0) containing 100 mM sodium chloride and 2 mM DTT. The dialysate was filtered on a column (2.2 × 65 cm) of Toyopearl HW55 (superfine) equilibrated with the same buffer solution as described earlier. The active fractions (Fr. 38–42, 15 ml) were pooled to give fraction E.

**Toyopearl HW65 chromatography (2nd)** Fraction E was concentrated to 1 ml and dialysed for 10 h against 10 mM phosphate buffer (pH 7.0) containing 2 mM DTT. The dialysate was loaded onto a column (4 × 40 cm) of DEAE-Sepharose CL6B equilibrated with the same buffer. The elution was accomplished with a linear gradient from 0 to 1 M sodium chloride in the same buffer. The active fractions (Fr. 269–328, 180 ml) were pooled to give fraction F.

**Sephadex G-100 chromatography** Fraction F was concentrated to 1 ml and dialysed for 10 h against 100 mM phosphate buffer (pH 7.0) containing 100 mM sodium chloride and 2 mM DTT. The dialysate was filtered on a column (2.2 × 65 cm) of Sephadex G-100 equilibrated with the same buffer solution as described earlier. The active fractions (Fr. 52–55, 12 ml) were combined to give fraction G.

**Concanavalin A Sepharose chromatography** Fraction G was concentrated to 1 ml and dialysed for 10 h against 10 mM phosphate buffer (pH 7.0) containing 2 mM DTT. The dialysate was loaded on a column (1.2 × 6 cm) of Concanavalin A Sepharose equilibrated with the same buffer. The elution was achieved with the linear gradient from 0 to 0.6 M methyl-α-0-mannopyranoside. The active fractions (Fr. 24–32, 9 ml) were pooled to give fraction H.

**Toyopearl HW65 chromatography (2nd)** Fraction H was concentrated to 1 ml and dialysed for 10 h against 10 mM phosphate buffer (pH 7.0) containing 2 mM DTT and saturated with ammonium sulfate to 45%. The dialysate was applied to a column of Toyopearl HW65. The running conditions were the same as those used for Toyopearl HW65 chromatography (1st). The active fractions (Fr. 48–54, 21 ml) of the Toyopearl chromatogram (2nd) of onion fructosyltransferase were combined and dialysed against 10 mM phosphate buffer containing 2 mM DTT to give a finally purified enzyme preparation, fraction J.

**Measurement of enzyme activity**

Reaction mixtures were composed of enzyme in 10 mM phosphate buffer (pH 7.0) containing 2 mM (±)dithiothreitol 25 µl, substrate in distilled water 50 µl and disodium hydrogen phosphate–citric acid buffer (McIlvaine buffer, pH 5.5) 25 µl and the reaction was carried out at pH 5.68.

**SST activity**

One unit of SST activity was defined as the amount of enzyme which catalysed fructosyl transfer from sucrose to sucrose.
producing 1 μmol of 1-kestose (isokestose, 3a) in 1 min under the conditions described as follows: A mixture (I) of enzyme (25 μl), 200 mM sucrose (50 μl), McIlvaine buffer (pH 5.5, 25 μl) and toluene (a trace amount) was incubated at 30°C. The reaction was stopped by heating in a boiling water bath for 3.5 min, and the reaction mixture diluted 10–100 times with distilled water, filtered and an aliquot (25 μl) was applied to HPAEC. The SST activity was calculated from the amount of 1-kestose present.

1-FFT activity

One unit of 1-FFT activity was defined as the amount of enzyme which catalysed the fructosyl transfer from 1-kestose to another 1-kestose to synthesize 1 μmol of nystose (4a) in 1 min. A mixture (II) of enzyme (25 μl), 200 mM 1-kestose (50 μl) and McIlvaine buffer (pH 5.5, 25 μl) was used. All enzyme reactions were carried out at 30°C. The reaction was terminated by heating in a boiling water bath for 3.5 min, and the reaction mixture diluted 100–300 times with distilled water, filtered and an aliquot (25 μl) was applied to HPAEC. The 1-FFT activity was calculated from the amount of nystose (4a) detected.

6G-FFT activity

One unit of 6G-FFT activity was defined as the amount of enzyme which catalysed the fructosyl transfer from 1-kestose to another 1-kestose to yield 1 μmol of 1F,6G-di-β-D-fructofuranosylsucrose (4c) in 1 min. The activities of 6G-FFT were tested using mixture (II). All experiments were conducted as done for the 1-FFT activity, except the 6G-FFT activity was calculated from the amount of 1F,6G-di-β-D-fructofuranosylsucrose (4c) detected.

Disc electrophoresis and analysis of N-terminal amino acid sequence

A 7.5% polyacrylamide gel (pH 8.0, Davis, 1964; Orstein, 1964) was used for 2 h at room temperature at 2 mA per tube. α-Lactalbumin (14 kDa), trypsin inhibitor (20 kDa), carbonic anhydrase (30 kDa), ovalbumin (43 kDa), bovine serum albumin (BSA, 67 kDa) and phosphorylase (94 kDa), were purchased from Amersham Pharmacia Biotech (Buckinghamshire, UK) and used as reference proteins. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) was conducted according to the method of Laemmli (1970). Protein bands in the gel were stained with Coomassie Brilliant Blue R-250. After SDS-PAGE of the purified enzyme, the protein band was blotted onto a polyvinylidene difluoride (PVDF) membrane (Bio-Rad Laboratories, Hercules, CA, USA). The N-terminal amino acid sequences were analysed using a HP G1005A protein sequencing system (Hewlett-Packard, Palo Alto, CA, USA) (unknown amino acids are indicated by X).

Determination of molecular mass by gel filtration

The descending method was used with a column (1.8 × 67 cm) of Sephadex G-100 pre-equilibrated with 10 mM phosphate buffer containing 100 mM sodium chloride (pH 6.5) and 2 mM DTT and elution was accomplished with the same buffer at a flow rate of 6 ml h−1. Ribonuclease (13.7 kDa), chymotrypsinogen A (25 kDa), ovalbumin (43 kDa) and bovine serum albumin (BSA, 67 kDa), were purchased from Amersham Pharmacia Biotech.

Results

The enzyme was purified from onion extract by successive chromatography using DEAE-Sepharose CL-6B, Toyopearl HW65, Toyopearl HW55, DEAE-Sepharose CL-6B (2nd), Sephadex G-100, Concanavalin A Sepharose and Toyopearl HW65 (2nd). The Toyopearl chromatogram (2nd) of onion fructosyltransferase is shown in Fig. 1. Thus, as shown in Table 1, the enzyme as 6G-FFT could be purified 1327-fold from fraction A and its specific activity was 26.54 U mg−1 protein. The enzyme was accompanied with 1-FFT activity. The relative activity of 6G-FFT compared with 1-FFT in purified enzyme was 2.3. The enzyme preparation was almost free from 1-kestose hydrolysing activity (1/200 of activities of 6G-FFT + 1-FFT; 0.1 m 1-kestose (50 μl), 0.006 U enzyme (25 μl), McIlvaine buffer (pH 5.5, 25 μl), 30°C, 30 min of reaction) and did not show SST and invertase activities (0.1 m sucrose (50 μl), 0.005 U enzyme (25 μl), McIlvaine buffer (pH 5.5, 25 μl), 30°C, 24 h of reaction).

General properties of the enzyme

Disc electrophoresis The purified enzyme (Fraction J) was subjected to electrophoresis on a polyacrylamide gel (Fig. 1). One gel was stained using Coomassie Brilliant Blue, and a second gel was cut into discs (2 mm wide), each of which was homogenized with McIlvaine buffer (pH 5.5, 0.2 ml). A quantity of 0.2 ml of 200 mM 1-kestose was added and each mixture was incubated at 30°C for 12 h. The 6G-FFT and 1-FFT activities were assayed by HPAEC of the reaction products. The 6G-FFT and 1-FFT activities resided in a single disc, which showed a protein band.

Molecular mass estimation Molecular mass was estimated by gel filtration chromatography and by SDS-PAGE. As shown in Fig. 2a, the molecular mass was estimated to be 66 kDa by Sephadex G-100 column chromatography. In SDS-PAGE analysis, two polypeptide bands were detected, and the molecular masses were estimated to be 52 kDa and 25 kDa, respectively (Fig. 2b). The results obtained by SDS-PAGE differed from that obtained by gel filtration chromatography. The reason for the existence of two bands in SDS-PAGE was discussed in the following text.
Optimum pH A pH-dependence curve of the reaction between the enzyme and 1-kestose is given in Fig. 3a. The optimum pH for 6G-FFT or 1-FFT was 5.68. The relative activity curve of 6G-FFT was parallel to that of 1-FFT.

pH-dependence of stability A number of enzyme solutions at pH 5.0, 5.16, 5.28, 5.38, 5.52, 5.68, 6.18, 6.31 and 6.70 were preincubated at 30°C for 30 min, cooled to 0°C, adjusted to pH 5.5 and the residual enzyme activities were measured (Fig. 3b). At pH 5.38–6.31, 80% or more of the initial activities of 6G-FFT and 1-FFT were detected whereas at pH 5.0 and 5.16, 70% or more of the activities were lost. Both of the stability curves showed similar pattern at the range of pH 5.0–6.31.

Thermal stability Enzyme solutions were heated for 15 min at 30, 40, 50 or 60°C (20°C was used as the control), and the residual activity was measured (Fig. 4); the enzyme was stable at 20–40°C, but inactivated at 50°C and 60°C.

Effects of inhibitors A mixture of enzyme (1.29 U ml⁻¹, 25 µl), 400 mM 1-kestose 25 µl and McIlvaine buffer (pH 5.5, 25 µl) was incubated at 30°C for 30 min in the presence of water or one of the inhibitors (4 mM; 0.4 mM for pCMB, 25 µl). After the reaction was stopped by heating at 100°C for 4 min, the mixture was subjected to HPAEC. Residual relative enzyme activity is shown in Table 2. Mercuric chloride, silver nitrate, pCMB, SDS inhibited the enzyme (6G-FFT) by 100, 100, 97.5 and 52.4%, respectively. Calcium chloride, magnesium chloride, manganese chloride, ferric chloride, cobalt chloride, zinc chloride, cupric sulfate and ethylenediaminetetraacetic acid (EDTA) affected 6G-FFT by 17.6–35%. Sodium deoxycholate, Triton X-100 and Tween-80 activated the enzyme by 209.7, 160.7 and 171.3%, respectively. All inhibitors and activators also affected 1-FFT and 6G-FFT equally.

N-terminal amino acid sequences Amino acid sequences of the N-terminus of each of the two polypeptides obtained
by SDS-PAGE derived from the purified enzyme from onion bulbs were determined. Figure 5 aligns these sequences with those of carrot invertase and sucrose:fructan 6-fructosyltransferase (6-SFT) from barley.

Substrate specificity of the enzyme

Self-transfer of fructosyl residue between two identical fructooligosaccharides; 1-kestose, nystose or fructosylnystose.

The formation of tetrasaccharides and higher saccharides was determined by HPAEC when 1-kestose was incubated with the enzyme. As shown in Fig. 6, 1\(\beta\)-fructofuranosylsucrose (4c, 1.239 mg (1.86 µmol) ml\(^{-1}\) of reaction mixture), nystose (4a, 0.530 mg (0.796 µmol) ml\(^{-1}\) of reaction mixture) and a small amount of neokestose were produced with liberation of sucrose at initial reaction time, 0.25 h and then the saccharides 5c and 4b were formed within 1 h. After 24 h of reaction, fructo-oligosaccharides including neokestose, nystose, 4c, 4b, 5a, 5b, 5c, 5d, 6a, 6b, a mixture of 6c, 6d\(_1\), 6d\(_2\) and higher oligosaccharides were detected on chromatograms of HPAEC (Fig. 6a, 100 times dilution). This fructo-oligosaccharide composition was very similar to that of the saccharides of onion bulbs or the saccharides synthesized from 1-kestose by the crude onion enzyme previously reported (Shiomi et al., 1997). An experiment similar to that described above was performed using nystose as a substrate. As shown in Fig. 7, 1\(\beta\)-fructofuranosylsucrose (5c, 1.231 mg (1.488 µmol) ml\(^{-1}\) reaction mixture) and 1\(\beta\)-fructofuranosylsucrose (5a, 0.08 mg (0.097 µmol) ml\(^{-1}\) reaction mixture) were produced at 0.25 h and the fructo-oligosaccharides similar to those of the reaction mixture using 1-kestose as substrate were formed within 24 h (Fig. 7a, 300 times dilution). An experiment similar to that described was also performed using fructofuranosylnystose (1\(\beta\)-fructofuranosylsucrose) as a substrate. 1\(\beta\)-fructofuranosylsucrose (6c, 1.061 mg (1.072 µmol) ml\(^{-1}\)) and a small amount of 1\(\beta\)-fructofuranosylsucrose (6a) with liberation of nystose were produced at 0.25 h and higher oligosaccharides, hexa-decasaccharides were also formed just within 24 h. Thus, the enzyme catalysed 'self-transfer'
between two similar saccharides of the 1-kestose series (1F(1-β-d-fructofuranosyl)ₘ sucrose) to form the oligosaccharide having a DP higher by one α-fructose residue in the initial reaction. Furthermore, the α-fructosyltransfer involved HO-6 of a terminal α-glucosyl residue and also HO-1 of a terminal α-fructosyl residue.

Fructosyltransfer reactions from 1F(1-β-d-fructofuranosyl)ₘ sucrose (ₘ = 1, 2 or 3) or neokestose to sucrose.

Fructosyltransfer from 1F(1-β-d-fructofuranosyl)ₘ sucrose (ₘ = 1, 2 or 3) or neokestose to sucrose was determined from the amount of neokestose or 1-kestose produced with the enzyme by HPAEC. As shown in Table 3, the enzyme catalysed fructosyltransfer most rapidly from 1-kestose and quite rapidly from nystose or 1F-fructofuranosylnystose to sucrose to form neokestose. The enzyme also catalysed the transfer of a single, terminal, α-fructosyl residue linked to α-glucosyl residue of neokestose to HO-1 of the fructosyl residue of sucrose, forming 1-kestose. Furthermore as stated above, no SST activity of the purified enzyme was detected.

**Effect of substrate concentration** The effects of the 1-kestose or nystose concentration on fructosyltransfer were investigated. As shown in Table 4, the data obtained were plotted according to the Lineweaver–Burk model from which Kₘ and Vₘₐₓ values were obtained for 1-kestose and nystose.
Comparison of amino acid sequences

of N-terminus of 52 kDa and 25 kDa polypeptides derived from the purified onion enzyme with deduced amino acid sequences of fructan:fructan 6G-fructosyltransferase (6G-FFT) from onion (Allium cepa), invertase from carrot (Daucus carota), and sucrose:fructan 6-fructosyltransferase (6-SFT) from barley (Hordeum vulgare). 52 kDa and 25 kDa peptides, purified onion enzyme; Ac6G-FFT, onion 6G-FFT (Vijn et al., 1997); DcINV, carrot vacuolar invertase (Unger et al., 1994); Hv6-SFT, barley 6-SFT (Sprenger et al., 1995). The amino acids with asterisks are N-terminals of 43 kDa and 25 kDa polypeptides from carrot invertase. The dotted amino acids are N-terminals of 49 kDa and 23 kDa polypeptides from barley 6-SFT. The identical amino acids are shaded.

Fig. 5

Fig. 6 (a) High-performance anion-exchange (HPAE) chromatogram and (b) time-course of production of tetrasaccharides and other oligosaccharides from 1-kestose by onion (Allium cepa) fructosyltransferase: closed squares, sucrose; closed, upward pointing triangles, 1-kestose (3a); closed, downward pointing triangles, neokestose (3b); closed diamonds, nystose (4a); filled circles, 4b (6G(1-β-D-fructofuranosyl)2 sucrose); open squares, 4c (1F,6G-di-β-D-fructofuranosyl sucrose); open, upward pointing triangles, 5a (1F(1-β-D-fructofuranosyl)3 sucrose); open, downward pointing triangles, 5b (6G(1-β-D-fructofuranosyl)3 sucrose); open diamonds, 5c (1F(1-β-D-fructofuranosyl)2-6G-β-D-fructofuranosyl sucrose); open circles, 5d (1F-β-D-fructofuranosyl-6G(1-β-D-fructofuranosyl)2 sucrose); multiplication signs, 6(6G(1-β-D-fructofuranosyl)m sucrose); plus signs, 7(1F(1-β-D-fructofuranosyl)n-6G-(1-β-D-fructofuranosyl)sucrose (m + n = 5)). Reaction mixture consisted of enzyme (25 µl), 200 mM 1-kestose (50 µl, 100 mM final concentration), McIlvaine buffer (pH 5.5, 25 µl) and toluene (a trace amount), and was incubated at 30°C. The reaction was stopped by heating in a boiling water bath for 3.5 min, the reaction mixture was diluted 100 times with distilled water, filtered and an aliquot (25 µl) was applied to HPAE chromatography.
Up to now, with the exception of an enzyme from asparagus roots (Shiomi, 1981), no enzyme catalysing fructosyltransfer from a (2 → 1)-β-linked fructo-oligosaccharide to HO-6 of the glucosyl residue of another saccharide molecule has been purified and characterized. An onion enzyme fraction containing activities of SST and 1-FFT was assumed to catalyse fructosyltransfer from 1-kestose to the glucosyl (HO-6) residue of sucrose, producing neokestose (Scott et al., 1966; Edelman & Jefford, 1968). A ‘sucrose:sucrose fructosyltransferase 2.4.1.99.’ and a ‘fructan:fructan fructosyltransferase 2.4.1.100.’ have been separated from the inner leaf bases of onion plants: the latter preparation also produced neokestose from 1-kestose (Henry & Darbyshire, 1980). We describe here for the first time purification and the general properties of fructosyltransferase from onion bulbs.

The onion enzyme was free from SST activity and was purified 1327-fold (6G-FFT) and 1292 (1-FFT)-fold by eight successive purification steps to give a single protein band on native PAGE. Although the molecular mass of the enzyme was estimated to be 66 kDa by gel filtration chromatography, two polypeptide bands, at 52 kDa and 25 kDa, were detected by SDS-PAGE. The amino acid sequence of the N-terminus of each peptide showed very close identities (96% and 90%

### Table 3

<table>
<thead>
<tr>
<th>Donor</th>
<th>Acceptor</th>
<th>Fructose transferred (µmol ml⁻¹ of reaction mixture)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-Kestose (n = 1)</td>
<td>Sucrose</td>
<td>1.48</td>
</tr>
<tr>
<td>Nystose (n = 2)</td>
<td>Sucrose</td>
<td>1.04</td>
</tr>
<tr>
<td>Fructosyl nystose (n = 3)</td>
<td>Sucrose</td>
<td>0.872</td>
</tr>
<tr>
<td>Neokestose</td>
<td>Sucrose</td>
<td>1.14</td>
</tr>
<tr>
<td>Sucrose</td>
<td>Sucrose</td>
<td>nd</td>
</tr>
</tbody>
</table>

nd, Not detected.

### Table 4

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Enzyme activity</th>
<th>$K_m$ (mmol)</th>
<th>$V_{max}$ (mmol min⁻¹ mg⁻¹ protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-Kestose (3a)</td>
<td>6G-FFT</td>
<td>88</td>
<td>10.5</td>
</tr>
<tr>
<td></td>
<td>1-FFT</td>
<td>18</td>
<td>7.51</td>
</tr>
<tr>
<td>Nystose (4a)</td>
<td>6G-FFT</td>
<td>310</td>
<td>34.9</td>
</tr>
<tr>
<td></td>
<td>1-FFT</td>
<td>440</td>
<td>3.66</td>
</tr>
</tbody>
</table>


### Discussion

Up to now, with the exception of an enzyme from asparagus roots (Shiomi, 1981), no enzyme catalysing fructosyltransfer from a (2 → 1)-β-linked fructo-oligosaccharide to HO-6 of the glucosyl residue of another saccharide molecule has been purified and characterized. An onion enzyme fraction containing activities of SST and 1-FFT was assumed to catalyse fructosyltransfer from 1-kestose to the glucosyl (HO-6) residue of sucrose, producing neokestose (Scott et al., 1966; Edelman & Jefford, 1968). A 'sucrose:sucrose fructosyltransferase 2.4.1.99.' and a 'fructan:fructan fructosyltransferase 2.4.1.100.' have been separated from the inner leaf bases of onion plants: the latter preparation also produced neokestose from 1-kestose (Henry & Darbyshire, 1980). We describe here for the first time purification and the general properties of fructosyltransferase from onion bulbs.

The onion enzyme was free from SST activity and was purified 1327-fold (6G-FFT) and 1292 (1-FFT)-fold by eight successive purification steps to give a single protein band on native PAGE. Although the molecular mass of the enzyme was estimated to be 66 kDa by gel filtration chromatography, two polypeptide bands, at 52 kDa and 25 kDa, were detected by SDS-PAGE. The amino acid sequence of the N-terminus of each peptide showed very close identities (96% and 90%

respectively) with a part of deduced onion 6G-FFT (Vijn et al., 1997) (Fig. 5). This slight difference of the amino acid sequence observed between our results and those reported by Vijn et al. (1997) could possibly result from the use of different tissues in the two, which were sampled from different growing stages in both investigations.

The sequences of 52 kDa and 25 kDa peptides have also shown high identities with the 43 kDa and 25 kDa polypeptides of soluble invertase of carrot (Daucus carota), respectively (Unger et al., 1992). These authors reported the separation of carrot invertase into three peptides with molecular masses of 68 kDa, 43 kDa and 25 kDa on SDS-PAGE, and the 43 kDa and 25 kDa polypeptides were N-terminal and C-terminal proteolytic fragments of the 68 kDa polypeptide which was eluted as a monomeric protein from a gel filtration column. These results were supported by analysis of the cDNA encoding carrot invertase (Unger et al., 1994). Similar results were described for 6-SFT from barley (Sprenger et al., 1995). These results strongly suggest that the 6G-FFT purified from onion bulbs is also hydrolysed to form 52 kDa and 25 kDa polypeptides. However, for reasons that remain unclear, the sum of the molecular masses of two peptides obtained by

![Fig. 8 Pathway of enzymatic synthesis of fructo-oligosaccharides in onion (Allium cepa) bulbs.](image-url)
SDS-PAGE in onion enzyme is larger than the molecular mass estimated by gel filtration. Additional molecular mass estimation techniques should be considered, such as two-dimensional electrophoresis, to confirm that the eluted protein is a single molecular species.

The optimum pH for 6G-FFT activity was similar to that of 1-FFT activity. The relative activity curves of 6G-FFT and 1-FFT were parallel. Both of the stability curves of 6G-FFT and 1-FFT showed similar pattern at the range of pH 5.0–6.3. The thermal stabilities for the enzyme were the same for the activities of 6G-FFT and 1-FFT. In addition, the tendency of inhibition or activation for both activities of the enzyme showed a similar profile. Sodium deoxycholate, Triton X-100 and Tween-80 activated onion 6G-FFT but did not affect asparagus leaf 6G-FFT (unpublished). Studies on the activation mechanism by the detergents are now in progress. It is well known that ion metals activate various enzymes and heavy-metal ions inhibit most enzymes. Conversely, bile salts, particularly deoxycholate, and nonanionic detergents such as Tweens and certain of the Triton products did not affect asparagus leaf 6G-FFT (unpublished). Studies on the activation mechanism by the detergents are now in progress.

The Michaelis constant ($K_m$) value of 6G-FFT activity for 1-kestose was 88 mM, which was similar to that of onion seed SST for sucrose (83 mM) (Shiomi et al., 1985); or asparagus SST for sucrose (110 mM) (Shiomi & Izawa, 1980). The general properties of the enzyme resemble those of 6G-FFT from asparagus root, but its substrate specificity differs. The enzyme catalysed a ‘self-transfer’ between identical oligosaccharides of the 1-kestose series ($1^F(1\-\beta-d-fructofuranosyl)_m$ sucrose). The terminal d-fructosyl residue is transferred to HO-6 of the glucosyl residue preferentially and HO-1 of the fructosyl residue of another saccharide molecule (Tables 1 and Figs 3, 6 and 7). Sucrose does not serve as a substrate for ‘self-transfer’, but the enzyme acts on sucrose as a fructosyl acceptor from 1-kestose and its derivatives or neokestose to produce neokestose or 1-kestose. Studies on general properties and substrate specificities revealed that the onion fructosyltransferase could be classified as fructan:fructan 6G-fructosyltransferase (6G-FFT), because the onion enzyme showed a higher activity of 6G-FFT than of 1-FFT using $1^F(1\-\beta-d-fructofuranosyl)_m$ sucrose ($m = 1$, 2 or 3) as a substrate. A biosynthetic mechanism of the fructo-oligosaccharides in onion bulb was confirmed, as shown in Fig. 8, but the fructo-oligosaccharides were found to be synthesized by a two-enzyme system, SST and 6G-FFT together with 1-FFT activity, which is different from the three-enzyme system – SST, 1-FFT and 6G-FFT – in asparagus roots. The higher DP of fructo-saccharides in onion bulbs was estimated to be c. 10 (Shiomi et al., 1997) or more (Ernst et al., 1998), whereas that of the saccharides in asparagus roots was c. 22 (Shiomi, 1993) from the HPAEC chromatograms. These observations are in agreement with different specific enzyme systems leading to the relative activities of 1-FFT to 6G-FFT in onion and asparagus extracts which averaged 0.45 (Table 1) and 15 (Shiomi, 1992), respectively.

References


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