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Fructan chemical structure and sensitivity to an exohydrolase

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ABSTRACT

A fructan exohydrolase selective for (2→1)-linked terminal fructosyl linkages, isolated from barley (*Hordeum vulgare* L. cv. Morex) stems and leaf sheaths, was used to elucidate the chemical structures of several oligomeric fructans extracted from liliaceous and graminaceous species. Products released by enzymic and mild acid hydrolysis were separated by reversed-phase high-performance liquid chromatography. Gas-liquid chromatography–mass spectrometry of partially methylated alditol acetates permitted unequivocal deduction of many linkage sequences, first of the hydrolysis products and then of the original oligomers. We found that bifurcose, a tetrasaccharide formed by addition of a fructosyl unit to O-6 of the central fructose residue of 1-kestose, was a central molecule in the generation of the branched, oligomeric fructans of wheat (*Triticum aestivum* L. cv. Fidel). These arise by the extension of both (2→1)- and (2→6)-linked chains from the bifurcose branch-point residue. Some of the (2→6)-linked units that slowly accumulate in oligomers may arise *in vivo* from selective hydrolysis, by fructan exohydrolases, of (2→1)-linked terminal units at branch point residues rather than by the action of (2→6)-specific synthases. Limited hydrolysis by specific exohydrolases *in vitro* coupled with separation of the oligomeric products constitutes an effective approach to the sequence analysis of complex oligosaccharides.

INTRODUCTION

Fructans are important storage polymers in numerous plant species and are involved in both diurnal and seasonal changes in sugar transfer and accumulation[1–3]. The structure of inulins, (2→1)-linked fructans synthesized in storage organs of *Inula*, *Chicorium*, and *Dahlia* is well documented principally because of its simplicity, and separation of the oligomers is readily accomplished by molecular sizing[4]. In many other species, however, fructan and fructan oligomers contain both (2→1)- and (2→6)-linked fructosyl units and even a substantial portion of (2→1,2→6)-linked branch-point

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TABLE I

Oligomeric fructans from asparagus, onion, and wheat

Oligomer	*Hydrolysis products*[a]	*Deduced structure*	*Common name*
3a[b,c]	sucrose	**G ↔ F1←F**	1-kestose
3b[b]	sucrose	**F→6G ↔ F**	neokestose
3c[b,c]	—	**G ↔ F** **6** ↑ F	6-kestose
4a[b,c]	1-kestose, sucrose	**G ↔ F1←F1←F**	nystose
4b[b]	neokestose, sucrose	**F→1F→6G ↔ F**	
4c[b]	neokestose, 1-kestose, sucrose	**F→6G ↔ F1←F**	
4d[c]	1-kestose, 6-kestose, sucrose	**G ↔ F1←F** **6** ↑ F	bifurcose
5a[c]	nystose, 1-kestose, sucrose	**G ↔ F1←F1←F1←F**	inulin pentasaccharide
5b[b]	**4b**, neokestose, sucrose	**F→1F→1F→6G ↔ F**	
5c[b]	neokestose, 1-kestose, sucrose	**F→6G ↔ F1←F1←F**	
5d[b]	neokestose, 1-kestose, sucrose	**F→1F→6G ↔ F1←F**	
5e[c]	bifurcose, 6-kestose, sucrose	**G ↔ F1←F** **6** ↑ F1←F	
5f[c]	bifurcose, 6-kestose, sucrose	**G ↔ F1←F1←F** **6** ↑ F	
5g[c]	bifurcose, **4p**, 6-kestose, sucrose	**G ↔ F1←F** **6** ↑ F 6 ↑ F	
5p[c]	**4p**	**G ↔ F** **6** ↑ F 6 ↑ F 6 ↑ F	phlein pentasaccharide
6e[c]	**5e**, **5f**, bifurcose	**G ↔ F1←F1←F** **6** ↑ F1←F	

6f[c]	**5f**, bifurcose	G ↔ **F**1←F1←F1←F **6** ↑ F	
6g[c]	**5g**, bifurcose	G ↔ **F**1←F **6** ↑ F1←F **6** ↑ F	
6h[c]	**5g**, **5p**, bifurcose	G ↔ **F**1←F **6** ↑ F 6 ↑ F 6 ↑ F	
6p[c]	**5p**	G ↔ **F** **6** ↑ F 6 ↑ F 6 ↑ F 6 ↑ F	phlein hexasaccharide

[a] Products collected from h.p.l.c. were then verified by linkage analysis. [b] Oligomers purified from asparagus or onion. [c] Oligomers purified from wheat leaf blades. We use here a simplified fructan designation of Shiomi[5] that indicates to which position on the sucrose moiety subtending fructosyl units are attached and by which linkage.

residues. Few of these complex oligomeric fructans have been fully characterized.

Shiomi[5] isolated several tetra- and penta-saccharides from plants of two liliaceous genera, *Asparagus* and *Allium*, by liquid chromatography on carbon–Celite columns. The oligomers were identified by partial acid and enzymic hydrolysis and gas liquid chromatography of the per-*O*-methylated alditols obtained from the hydrolysis products. These sugars were either oligoinulin or neokestose derivatives formed by the addition of (2→1)-linked fructosyl chains to both the glucosyl and fructosyl residues of the initial sucrose starting unit (Table I).

Fructans synthesized by wheat and barley are highly branched molecules containing both (2→1)- and (2→6)-linked fructosyl units[1,2]. Schlubach and colleagues[6–9] found considerable variation in the chemical structures of the fructan oligomers from many cereal grasses. They deduced that different kinds of fructans, namely (2→1)- and (2→6)-linked polymers, were made in different plant organs and at different times

during development. Wheat leaf blades induced to form fructan by excision and incubation under high intensity light formed principally 1-kestose and the tetrasaccharide nystose and its branched isomer bifurcose (Table I)[10]. Penta- and hexa-saccharides were also a mixture of linear and branched structures, but with increasing proportions of (2→6) linkages. Bancal and Gaudillère[11], using reversed-phase, high-performance liquid chromatography, separated several fructan tri-, tetra-, penta-, and hexa-saccharides from growth chamber-grown plants induced to form fructans, or from field-grown plants which had accumulated fructans for weeks.

Although separation of the tri- and tetra-saccharides and analysis by g.l.c.–m.s. of partially methylated alditol acetates derived from their hydrolysis products gave unequivocal identification of their chemical structure, there was ambiguity in the position and length of the branch constituents of the penta-saccharides and higher oligomers. Henson[12] purified a fructan exohydrolase (FEH) from the stems of barley that we thought could be useful in the sequence analysis of oligomeric fructans. The nearly homogeneous enzyme readily hydrolyzed inulin but not phlein or sucrose, suggesting a specificity for (2→1)-linked terminal fructofuranosyl units. By using this enzyme *in vitro* we were able to partially hydrolyze the wheat penta- and hexa-saccharides to smaller oligomers that could be characterized by methylation analysis. This provided unequivocal determinations of the structures of several of the pentamers and hexamers. Our studies now make possible more precise determination of the pathways of synthesis of the highly branched cereal fructans.

MATERIALS AND METHODS

Fructan oligomer samples. — Oligofructan samples purified from asparagus (*Asparagus officinalis* L.) roots and onion (*Allium cepa* L.) bulbs were obtained from Dr. N. Shiomi (Hokkaido University). Fructan oligomers were extracted from wheat (*Triticum aestivum* L. cv. Fidel), either from field-grown plants or induced leaf blades of seedlings excised and incubated under continuous illumination with photosynthetically active light, supplied by a high pressure sodium lamp, at 800 μE $m^{-2}s^{-1}$. Fifty g of blades were cut into smaller pieces and extracted twice with 1 L each of boiling aqueous 40% ethanol. Activated charcoal was added, and the combined extracts were stirred for 1 h. The charcoal was removed by centrifugation followed by ultrafiltration. The extract was then concentrated to 10 mL.

Oligomers were separated according to d.p. by gel-permeation chromatography. One mL of extract was applied to a 1.6-cm × 90-cm column of Bio-Gel P-2 (200–400 mesh; BioRad) equilibrated in water at 40° and eluted with water at 0.5 mL min^{-1}. The oligomeric fractions were collected after detection by refractometry, concentrated under vacuum, and rechromatographed on the same column.

Isomers in the oligomeric fractions were separated and further purified by h.p.l.c. on two 0.4-cm × 25-cm analytical C_{18} columns (RSil C18HL 5 μm; Alltech RSL), connected in tandem and eluted with water at 0.8 mL min^{-1} at ambient temperature. Peaks were detected by refractometry (Erma ERC 7511). Fractions were concentrated

and rechromatographed until single peaks were obtained, then dried *in vacuo* to residues weighing 1.0 to 4.6 mg.

Purification of a barley 1-*fructosyl* $(2\rightarrow1)$-β-D-*fructan exohydrolase (FEH).* — The FEH was extracted from stems and leaf sheaths of barley (*Hordeum vulgare* L. cv. Morex) according to the method of Henson[12]. Briefly, stems were homogenized in ice cold 50mM McIlvaine's buffer[13], pH 5.7 (50mM citric acid adjusted with disodium phosphate), the homogenate was filtered through cheesecloth, and the fraction precipitating between 50 and 75% saturation with ammonium sulfate and containing a major portion of the activity was dialyzed against ice cold 10mM McIlvaine's buffer, pH 7.0. The enzyme was purified further through ion-exchange chromatography on a DEAE-Sephacel column by gradient elution with NaCl, concanavalin A affinity chromatography by gradient elution with D-mannose, and gel-permeation chromatography on a 1.5-cm × 115-cm column of Sephacryl S-200. The enzyme was stored frozen in 50mM McIlvaine's buffer, pH 5.7, containing 0.5M NaCl, mM $CaCl_2$, mM $MnCl_2$, and 1% bovine serum albumin. The enzyme hydrolyzes 5% (w/v) chicory inulin at an initial rate of about 200 nmol fructose/h·100 μL enzyme solution, but exhibits essentially no invertase activity[12].

Partial hydrolysis of fructan oligomers. — The fructan oligomers (1.0 mg in most cases but, because of scarcity of material, 0.36, 0.18, and 0.14 mg of **5b**, **5c**, and **5d**, respectively) were dissolved in 1 mL of water, and 100 μL of the enzyme solution was added. Mixtures were incubated at 37° for up to 24 h, and 50 μL samples were withdrawn intermittently for determination of fructose released[14] and for h.p.l.c. analysis of oligomer composition. After substantial conversion of the oligomers to smaller molecules, reactions were terminated by freezing until each mixture could be separated by h.p.l.c. To permit more rapid separation a single C_{18} column was used at a constant flow of 1 mL min^{-1}. Minor fractions (yield below 50 μg) were characterized by retention time on the C_{18} column and their fructose:glucose ratio following complete acid hydrolysis.

One hundred μg of **5e** and **5f** were partially acid hydrolyzed in 0.05M HCl at 60° for 5 min, then neutralized by the addition of 0.1M NaOH and analyzed by h.p.l.c. for carbohydrate composition.

Chemical analyses. — For structure determinations, lyophilized materials (50 μg to 3 mg) were dried over P_2O_5 in a vacuum desiccator and *O*-methylated in Me_2SO solution[15] in the presence of Li methylsulfinylmethanide prepared by the addition of *n*-butyllithium, as described by Carpita and Shea[16]. The *O*-methylated fructan oligomers were partitioned into chloroform, and the chloroform was washed extensively with water and evaporated under a stream of N_2. The derivatives were acid hydrolyzed, and the sugars were reduced to alditols with $NaBD_4$ (98%) and acetylated as described in the companion paper[17]. Derivatives were separated in a 0.2-mm × 30-m vitreous silica capillary column coated with SP-2330 (Supelco), temperature-programmed from 160 to 200° at 1.5 deg min^{-1} and then to 240° at 5 deg min^{-1}, with a 6 min hold at the final temperature. Three μL were injected, in the splitless mode, into a helium carrier flow of 1.5 mL min^{-1}. Injection port and transfer line were at 260°. E.i.m.s. was performed with

TABLE II

Linkage analysis of oligomeric fructans from liliaceous and graminaceous species[a]

Oligomer	t-*Fru*	t-*Glc*	*1*-*Fru*	*6*-*Fru*	*6*-*Glc*	*1,6*-*Fru*
3a	1	1	1			
3b	2				1	
3c	1	1		1		
4a	1	1	2			
4b	2		1		1	
4d	2	1				1
4p	1	1		2		
5a	1	1	3			
5c	2		2		1	
5d	2		2		1	
5e	2	1	1			1
5f	2	1	1			1
5g	2	1		1		1
5p	1	1		3		
6b	2	1	1	1		1
6e	2	1	2			1
6f	2	1	2			1
6g	3	1				2
6h	2	1		2		1
6p	1	1		4		

[a] Response factors, relative to terminal glucosyl units, for fructosyl nonreducing terminal units (0.66), internal units (1.07), and branch-point units (1.12) were calculated from total ion-counts of derivatives from 1-kestose, 6-kestose, nystose, and bifurcose standards.

a Hewlett–Packard quadrupole spectrometer Model 21 MSD with an autotuned voltage of 70 eV and a source temperature of 250°. Derivatives were detected and quantified as described in the companion paper[17], and discussed previously[16]. The proportions of (2→1)- and (2→6)-linked fructosyl units were deduced primarily from the ratios of ion counts at *m/z* 161 to *m/z* 162 and *m/z* 190 to *m/z* 189, after correction for borodeuteride purity and ^{13}C natural abundance[16,17]. From integration of total ion counts the molar amount of each fructose derivative, relative to the single derivative of the terminal or 6-linked glucosyl unit, was determined and rounded off to the nearest integer for ease of presentation (Table II). From 1-kestose, 6-kestose, nystose, and bifurcose standards, response factors for derivatives, relative to terminal glucosyl units, were calculated for fructosyl nonreducing terminal units (0.66), internal units (1.07), and branch-point units (1.12).

RESULTS AND DISCUSSION

Separation of oligomers by h.p.l.c. — Separation by h.p.l.c. of the size-fractionated oligomers from gel-permeation chromatography provided the material for this study

(Fig. 1). The structures of the major oligomers were deduced as described subsequently, but examination of the distribution of the various oligomers in induced as compared to field-grown plants also revealed some interesting features. Induction of synthesis results in elaboration of the (2→1)-linked inulin oligomers and branched structures starting from bifurcose, a tetrasaccharide formed by the addition of a fructosyl unit to O-6 of the internal fructosyl unit of 1-kestose (Fig. 1). In field-grown plants, the (2→6)-linked oligomers initiate from 6-kestose or bifurcose and accumulate concomitantly with the loss of much of the store of (2→1)-linked inulin oligomers (Bancal and Gaudillère, unpublished data). These data correlate with changes documented in the proportion of several unidentified oligomers from the leaves of induced and field-grown wheat[17]. The major tetramers, pentamers, and hexamers were collected for analysis of chemical structure.

G.l.c.–m.s. analysis of wheat and other fructan oligomers. — Unambiguous identification of the tri- and tetra-saccharides of wheat was achieved by g.l.c–m.s. analysis alone. The partially methylated alditol acetates from terminal, linked, and branch-point units were easily resolved[17]. Neokestose, formed by the liliaceous species, was distinctive in having a 6-substituted glucosyl unit rather than a terminal glucosyl unit. By m.s. of the derivatives from linked residues, the ratios of fragments having *m/z* 161 and *m/z* 190 to *m/z* 162 and *m/z* 189 permitted easy identification of (2→1)- and (2→6)-linked fructosyl units, respectively. As reported in the companion paper[17], fructose methylated at O-1 [from (2→6)-linked units] yields predominantly the mannitol epimer on reduction whereas about equal amounts of mannitol and glucitol derivatives arise from (2→1)-linked fructosyl units[17]. This made deduction of the chemical structures of subsequently studied oligomers possible from gas chromatography alone, although all structures were verified by m.s. Trimers **3a**, **3b** and **3c** were 1-kestose, neokestose, and 6-kestose, respectively (Table II). Tetramer **4a** contained two (2→1)-linked fructosyl units and one terminal fructosyl unit per terminal glucosyl unit, and hence was nystose, whereas **4b** and **4c** were neokestose-containing oligomers having a single (2→1)-linked fructosyl unit added to the glucosyl side and the fructosyl side, respectively (Table II). Oligomer **4d** from wheat leaf blades possessed one branch-point residue and two terminal fructosyl units per terminal glucose, and hence was bifurcose (Table II). Oligomer **4p**, also from wheat, contained two (2→6)-linked fructosyl units and a single terminal fructosyl unit per terminal glucose and was easily recognized as phlein tetrasaccharide. All five tetrasaccharides were easily separated by h.p.l.c. on C_{18} columns eluted with water.

The linkage structures of the various pentamers and hexamers were easily deduced, but there remained ambiguities in sequence that could not be resolved by g.l.c.–m.s. alone. Pentamer **5a** was inulin pentasaccharide, whereas **5b**, **5c**, and **5d** from asparagus and onion were neokestose-containing oligomers having linkages consistent with the structures deduced by Shiomi[5] (Table II). Pentamers **5e** and **5f** from wheat each contained a single branch-point residue and a single (2→1)-linked fructosyl unit per terminal glucosyl unit, whereas **5g** contained a branch-point residue and a single (2→6)-linked fructosyl unit per terminal glucosyl unit (Table II). Pentamer **5p** had only

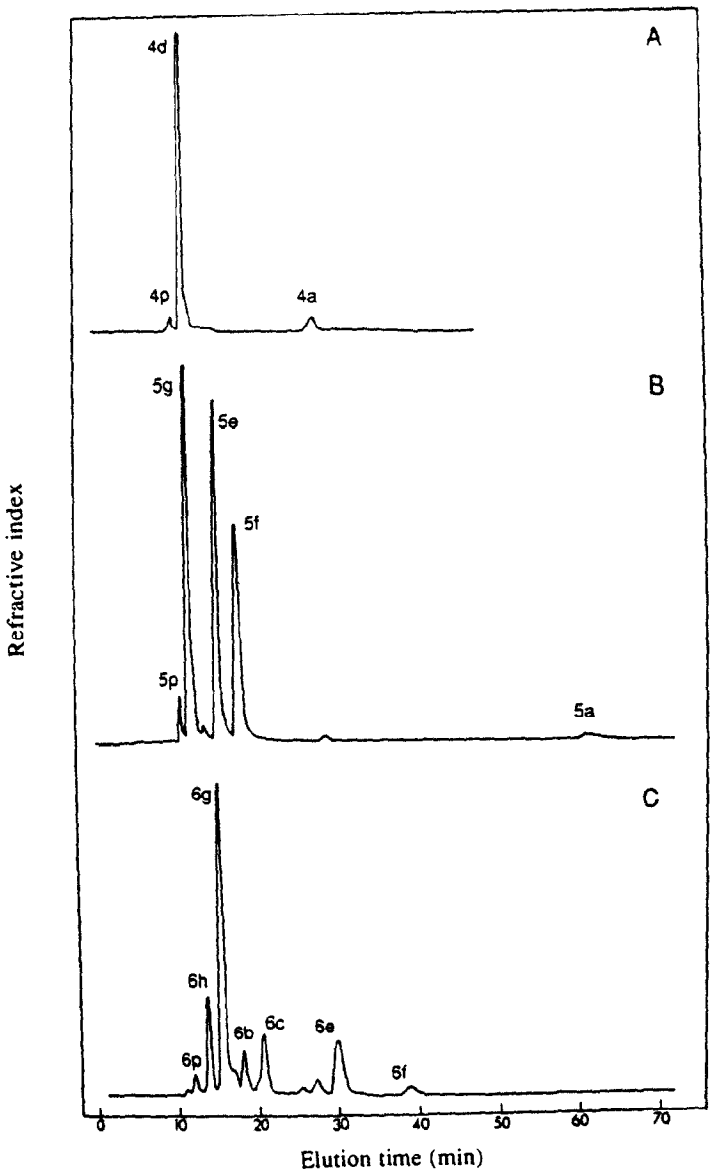

Fig. 1. Separation of wheat-leaf fructan oligomers extracted from induced plants. Oligomers were first size-separated by gel permeation chromatography before fractionation by h.p.l.c. with two 0.4-cm × 25-cm C_{18} columns connected in tandem. A, tetramers; B, pentamers; C, hexamers.

(2→6)-linked units in addition to the appropriate terminal residues, revealing its structure as phlein pentasaccharide (Table II).

Six hexamers were resolved by h.p.l.c. in sufficient purity and amount for investigation of their chemical structures, some of which contained (2→1)-linked fructosyl units and others mostly (2→6)-linked units. Hexamer **6b** was the single oligomer

containing both (2→1)- and (2→6)-linked fructosyl units in addition to a branch-point residue, and hexamer **6g** contained two branch-point residues and three terminal fructosyl units (Table II). Hexamers **6e** and **6f** contained single branch-point residues and two (2→1)-linked fructosyl units per terminal glucosyl unit and were related structurally to pentamers **5e** and **5f**, whereas hexamer **6h** contained a single branch-point residue and two (2→6)-linked fructosyl units per terminal glucosyl unit and was considered related to pentamer **5g** (Table II). Hexamer **6p** contained only (2→6)-linked fructosyl units, consistent with the structure of phlein hexasaccharide. Hexamer **6c** (Fig. 1) turned out to be a mixture of two oligomers that require further purification before their structures can be deduced.

Digestion of the fructan oligomers with (2→1)-fructan exohydrolase. — The rates of hydrolysis of several fructan oligomers varied from 8 to 244 nmol fructose/h·mg (Table III). The reactions were arrested after 8 to 24 h to ensure recovery of sufficient amounts of intermediate hydrolysis products to deduce linkage sequences. Although specific reaction kinetics were not obtained, these preliminary data indicated the number of susceptible terminal fructosyl units per molecule. Subsequent h.p.l.c. analysis permitted detection of the principal intermediate and limit digestion products. The rate of release of fructose from 5 mg mL^{-1} Dahlia inulin was constant for at least 5 d, and h.p.l.c. demonstrated a heterogeneous population of fructan oligomers (not shown). A 5 mg mL^{-1} solution of bifurcose was slowly hydrolyzed by the exohydrolase, but the reaction was far from complete after a 3 day incubation (Table III).

TABLE III

Rates of enzymic cleavage of fructosyl units from liliaceous and graminaceous oligomeric fructans

Oligomer	*Fructose release*[a]	
	(nmol h^{-1} mg^{-1})	*(nmol h^{-1} μmol^{-1})*
Sucrose	15	5
3a (1-kestose)	151	76
3b (neokestose)	12	6
3c (6-kestose)	8	4
4a (nystose)	126	84
4b	121	81
4c	158	105
4d (bifurcose)	13	9
5a (inulin pentasaccharide)	99	82
5b	167	139
5c	203	168
5d	244	202
5e	50	41
5f	51	42

[a] Under the conditions described under *Partial hydrolysis of fructan oligomers.*

The rate of hydrolysis of 6-kestose and neokestose to fructose and sucrose was less than 10% that of 1-kestose, indicating a strong selectivity of the exohydrolase for terminal fructosyl units attached to O-1 of the penultimate unit (Table III). Given the relatively low invertase activity observed, our results substantiate the selectivity of the exohydrolase reported by Henson[12]. Because only terminal fructosyl units are sensitive to the exohydrolase equimolar amounts of all linear oligomers should have similar rates of hydrolysis whereas branched oligomers may have additional susceptible fructosyl units, resulting in increased rates of hydrolysis. This was observed for one of the asparagus fructan oligomers, **5d**, which has two terminal fructosyl units attached to O-1 of fructosyl units (Table I). In general, the longer the oligomer, the faster the rate of hydrolysis (Table III). Slightly different molar amounts of each oligomer were used in our studies, so reaction kinetics for individual substrates remain to be established. Bifurcose and other wheat oligomers (**5e**, **5f**, and **6b**) had reduced rates of hydrolysis. Even though bifurcose has an O-1 linked terminal fructose, the O-6 linked terminal fructosyl unit on the branch-point residue probably exerts steric hindrance to catalysis.

Analysis of the products of partial hydrolysis. — The structures of the asparagus oligofructans of the neokestose series were deduced by Shiomi[5] by methylation analysis and characterization of the products from mild acid hydrolysis. Separation of the products of exohydrolase activity confirmed the putative structures of the asparagus oligofructans and permitted deduction of the structures of the major oligofructans induced in wheat. Inulin pentasaccharide is hydrolyzed progressively to nystose, 1-kestose, and sucrose (Fig. 2A). With the oligomers from the liliaceous species, both **4b** and **4c** yielded primarily neokestose, whereas **5b** yielded **4b**, **5c** yielded **4c**, and **5d** yielded both **4b** and **4c** (Fig. 2B–D). Although bifurcose obtained from wheat was degraded slowly, we found fructose, sucrose, and 6-kestose as hydrolysis products. Cleavage of a terminal fructose from either O-6 or O-1 of the branch-point residue will yield 1-kestose or 6-kestose, respectively, but the 1-kestose will be hydrolyzed further to sucrose and fructose. By comparison of the amounts of 6-kestose and sucrose that had accumulated during hydrolysis, we deduced that cleavage of the terminal fructose linked to the O-1 of the branch-point residue was favored over cleavage of the residue attached to O-6 by a 2:1 ratio.

Wheat oligomers **5e** and **5f** each yielded bifurcose as a hydrolysis-resistant product, indicating that each bore an O-1 linked terminal fructose on one of the two available fructose units attached to the branch-point residue (Fig. 3A and B; Table II). On partial acid hydrolysis, in addition to smaller degradation products, **5f** yielded both bifurcose and nystose, but **5e** gave only bifurcose. Since, in contrast to enzymic hydrolysis, which degrades nystose selectively, mild acid hydrolysis cleaves O-1 and O-6 linked fructosyl units equally, the structures of these two oligomers were established unequivocally. Pentamer **5g** also yielded bifurcose upon hydrolysis (Table I). In addition, small amounts of phlein tetrasaccharide were released, indicating the position of the extra fructosyl unit at O-6 of the fructose linked 2→6 to the branch-point residue of bifurcose.

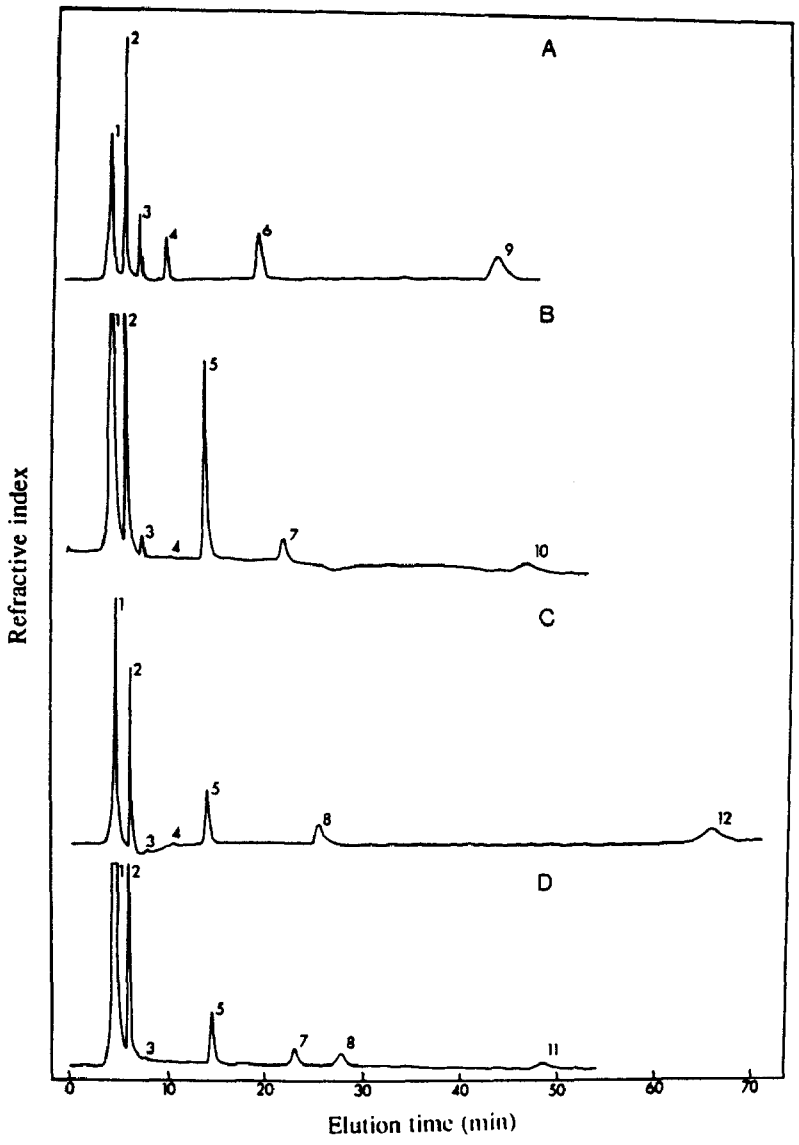

Fig. 2. Separation of hydrolysis products of neokestose-containing pentasaccharides from asparagus. A, inulin pentasaccharide after 24 h hydrolysis; B, pentamer **5b** after 24 h hydrolysis; C, pentamer **5c** after 24 h hydrolysis; D, pentamer **5d** after 8 h hydrolysis. Peaks identified: 1, buffer salts; 2, monosaccharide (fructose); 3, sucrose; 4, 1-kestose; 5, neokestose; 6, nystose; 7, **4b**; 8, **4c**; 9, inulin pentasaccharide; 10, **5b**; 11, **5d**; 12, **5c**.

Once the sequences of **5e** and **5f** had been established, **6e** and **6f** were easily deduced. The exohydrolase yielded both **5e** and **5f** from hexamer **6e**, whereas only **5f** was released by hydrolysis of **6f** (Fig. 3C and D; Table I). There were only two positions where the addition of the O-1 linked fructosyl unit could have occurred, and the hydrolysis products permitted discrimination of the two oligomers. Hexamer **6g** was the

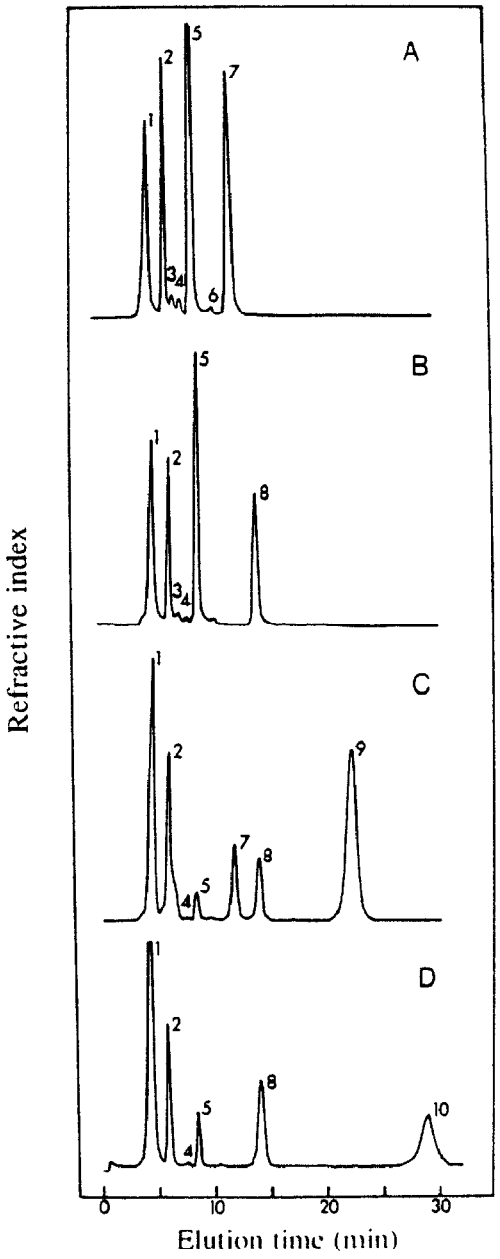

Fig. 3. Separation of hydrolysis products of wheat oligomeric fructans. A, pentamer **5e** after 24 h hydrolysis; B, pentamer **5f** after 24 h hydrolysis; C, hexamer **6e** after 7.2 h hydrolysis; D, hexamer **6f** after 13 h hydrolysis. Peaks identified: 1, buffer salts; 2, monosaccharide (fructose); 3, 6-kestose; 4, sucrose; 5, bifurcose; 6, 1-kestose; 7, **5e**; 8, **5f**; 9, **6e**; 10, **6f**.

only oligomer found containing two branch point units (Table II). Partial enzymic hydrolysis to **5g** indicated that the additional branched structure was attached to O-6 of the bifurcose branch-point residue rather than to O-1. Hexamer **6h** also yielded **5g** upon

hydrolysis, but formation of **5p** as well permitted straightforward identification of sequence (Table I).

Significance of the sequence structure of the wheat oligomeric fructans. — The chemical structures of the highly branched oligomeric fructans of certain grasses have long been a source of confusion. Schlubach and colleagues[6–9] have published several reports of (2→1)-linked and (2→6)-linked fructans in cereal species, first regarded as species-specific, then tissue-specific components, and then as different oligomers within the same tissues. This has led to speculation that different synthases are responsible for the generation of the two distinctive polymers and are under developmental control. The capacity for branching in these graminaceous fructans may at first appear to result from the random attachment of fructosyl units to O-1 or O-6 of the penultimate fructose residue. However, our data here indicate that the synthesis is not so random. There are 12 possible pentamers and well over 50 hexamers derivable solely by chain extension from the sucrose fructosyl moiety using only (2→1) and (2→6) linkages. We found that during both short-term induction and long-term accumulation, 7 pentamers and 8 hexamers accumulated in measurable quantities (Fig. 1). Five pentamers and 5 hexamers were produced in amounts large enough to permit structural determination. The accumulation of 1-kestose was marked just after induction of fructan synthesis in wheat[18], and nystose and bifurcose were produced in nearly equal amounts[10,18]. Bancal and Gaudillère[18] noted that 6-kestose, although always in small amounts, was present from the onset of induction. They suggested that 6-kestose, accompanying huge increases in sucrose, may be formed inadvertently by invertase in a transfructosylation reaction, demonstrated *in vitro* with extracts from barley leaves[19]. As an alternative, nascent exohydrolases could cleave the terminal fructosyl unit attached to the O-1 of the bifurcose branch-point residue to yield 6-kestose, resistant to further hydrolysis, much as we have shown *in vitro* (Table I). Oligomers that accumulate on initial induction of fructan synthesis are predominantly (2→1)-linked structures that, beginning with bifurcose, become branched at specific sites. Nystose, bifurcose, and many oligomers accumulate quickly after the induction of synthesis (Fig. 1)[10,11].

In field-grown plants, the distribution of oligomers changes markedly in the period following initial induction (Fig. 1), perhaps as a result of several diurnal cycles of alternate synthesis and degradation that contribute sugar to sucrose for subsequent transport to growing regions of the plant[20]. The oligomers that accumulate slowly are enriched in (2→6)-linkages relative to (2→1)-linkages (Fig. 1; Table II). Several of the pentamers and hexamers contain (2→6) linkages that emanate from bifurcose rather than 6-kestose (Table I). Just as 6-kestose can be derived from bifurcose, the (2→6)-linked phlein tetra-, penta-, and hexa-saccharides may also result from alternate synthesis and degradation of the branched oligomer rather than from a separate synthase. The (2→1)-specific fructan exohydrolase, in fact, was extracted from barley stems and leaf sheaths that accumulate similar kinds of oligomeric fructans. All structures may begin with bifurcose, formed by a (2→1)-linkage synthase and an O-6 branching enzyme. Contiguous branches formed (*e.g.*, hexamer **6g**) could be trimmed by the exohydrolase to yield phlein tetrasaccharide, just as we have done *in vitro*.

Iteration of the contiguous branching and subsequent trimming could ultimately yield longer (2→6)-linked oligomers without any requirement for enzymes other than a (2→1)-linkage synthase, an O-6 branching enzyme, and a hydrolase. The distribution of oligomers would then depend greatly on sucrose supply. Inhibition of fructan exohydrolase by sucrose, as suggested by Edelman and Jefford[4], would result in accumulation of the (2→1)-linked, highly branched structures, whereas diminution of sucrose and subsequent activation of the exohydrolase would yield the distribution of oligomers found in field-grown plants (Fig. 1). In support of this idea, excised leaves and stems of field-grown plants supplied with sucrose quickly accumulate the same oligomers found in induced leaves (Bancal *et al.*, unpublished results). Other grasses, however, make much longer (2→6)-linked fructan polymers[2,3]. Although (2→1)-linkages and branch point residues are also found in these polymer mixtures[10], specific (2→6)-fructan synthases are probably mandated in these cases. Our ability to purify specific pentamers and hexamers will permit us to test some of these hypotheses *in vivo* and *in vitro*.

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