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Cereal Fructosans: Part 2—Characterization and Structure of Wheat Fructosans

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ABSTRACT

Wheat fructosans were prepared from two different flours and separated according to degree of polymerization by gel permeation chromatography.

The different fractions were quantified and characterized with regard to monomer carbohydrate composition. The tri-, tetra- and pentasaccharides made up about 50% of the total fructosan content.

The structures of the trisaccharides were identified by gas chromatography/mass spectrometry. Two fructosan trisaccharides were found, neokestose and 6-kestose, along with raffinose.

INTRODUCTION

Fructosans are oligo- or polysaccharides composed of one glucose unit and a number of fructose units. They are present in all cereals at a concentration of 1–4% of dry matter (MacLeod & Preece, 1954). Several other plants also contain fructosans, such as onions (Darbyshire & Henry, 1978), timothy, rye grass, Jerusalem artichoke, *Dahlia*, etc. (Hirst,

1957). Often, these fructosans are larger than the cereal fructosans. Inulin from *Dahlia* has a degree of polymerization (DP) of about 30 and phlein from timothy about 80 (Suzuki, 1968), whereas cereal fructosans contain from three up to about twenty hexose units (MacLeod & Preece, 1954). The physiological role of fructosans in plants has been suggested to be as a reserve of carbohydrates (Hirst, 1957), but they may also play a role in the resistance of the species to freezing and desiccation (Edelman & Jefford, 1968).

In general, the structures proposed for cereal fructosans contain a glucose unit in the end position of the primary chain and branches at various positions along the chain. The linkages between the fructose units in the primary chain can be of the 'inulin type', $(2\rightarrow 1^1)$-glycosidic linkages, or the 'levan type', $(6\rightarrow 2^1)$-glycosidic linkages. The occurrence of cereal fructosans of each chain type has been reported (Medcalf & Cheung, 1971). Most of the structural identifications have been concerned with the larger fructosans and only a few studies have been performed on the trisaccharide fructosans. The results of these latter studies are neither consistent nor complete regarding the structures (Schlubach, 1965).

In an earlier publication (Dahlqvist & Nilsson, 1984) different extraction procedures for cereal fructosans were compared. The purpose of the present study was to characterize different fractions of wheat flour fructosans and to identify the structures of trisaccharide fructosans. This is of importance as a base for further studies of their hydrolysis during fermentation in bread baking and for investigations of their bioavailability.

MATERIALS AND METHODS

Preparation and purification

Fructosan preparations were made from two different wheat flours. Flour number one was a bakery wheat flour (extraction rate, 80%) obtained directly from a mill (Kungsörnen, Malmö) and flour number two was a wheat flour, from the same manufacturer, intended for home use (extraction rate, 72%). Two hundred grams of wheat flour were refluxed with 650 ml 80% ethanol for 45 min. After centrifugation the supernatant was concentrated by evaporation to a volume of about

100 ml. Proteins in the concentrated supernatant were precipitated when left overnight in a refrigerator and were removed by centrifugation. Lyophilization of the water phase yielded a crude fructosan preparation.

Preparative gel permeation chromatography was performed at room temperature using a Sephadex G-15 (Pharmacia) column (5 × 200 cm; void volume, 1350 ml; flow rate, 45 ml/h). Forty millilitres of a water solution of the crude fructosan, containing up to 2·5 g carbohydrate, were applied at each run. Distilled water was used as eluent and 180 fractions of 15 ml each were collected. Eluted fractions were assayed for total hexoses by the anthrone method (see below). Fractions containing carbohydrates were pooled within the same peak to give seven main fractions: I–V, A and B. Two or three fractions were left out between each peak, I–V, to minimize contamination between peaks. The pooled fractions were then lyophilized.

Analytical gel permeation chromatography was performed at room temperature using a Biogel P2 (Bio-Rad Laboratories) column (1·5 × 90 cm; void volume, 63 ml; flow rate, 6 ml/h). Two millilitres, containing 2 mg or a lyophilized fraction from the preparative gel column, were applied each time and eluted with distilled water. Fractions of each 2 ml were collected and analyzed for total hexoses using the anthrone method (see below).

HPLC separation. Dried material (0·5 mg) of each of the fractions III–V, A and B, was dissolved in 50 μl of a water solution containing 1 mg glucose per millilitre as an internal standard and was subjected to HPLC using a Waters instrument (U6K injector, M45 solvent delivery system, RCM 100 compression module). Conditions were as follows: column, Dextropac (Waters); solvent, millipore filtered water; flow rate, 1 ml/min. The elutions were monitored with an interference detector (Optilab 5902).

Characterization of fructosan fractions

Acid hydrolysis was performed on fractions II–V, A and B. Five milligrams were dissolved in 10 ml 1 M HCl. Standard solutions of glucose and fructose were prepared in the same way. The solutions were boiled for 30 min, chilled with water (0°C) and neutralized with 1 M NaOH. Total hexose and fructose contents, reducing power, liberated glucose and, for fraction III also, liberated galactose, were measured after hydrolysis (methods, see below).

γ-Amylase digestion was performed on fractions II–V, A and B to measure the content of malto-oligosaccharides. Tubes containing 0·5 mg of total carbohydrate, 50 μg of amyloglucosidase in a total volume of 1 ml 0·01M Na–acetate buffer, pH 4·75, were incubated for 30 min at 60°C (water-bath). Free glucose was measured with glucose oxidase (see below). The amyloglucosidase (1,4-α-D-glucan glucohydrolase EC 3.2.1.3 from *Aspergillus niger*; crystal suspension, 10 mg/ml) was obtained from Boehringer AG Mannheim GmbH, Germany.

Total hexoses were measured with the anthrone method as described by Scott & Melvin (1953). Total fructose was determined according to Dische & Beurenfreund (1951). Reducing carbohydrates were assayed with the method of Park & Johnson (1949) or with the dinitrosalicylate reagent (Hostettler *et al.*, 1951), depending on the required sensitivity.

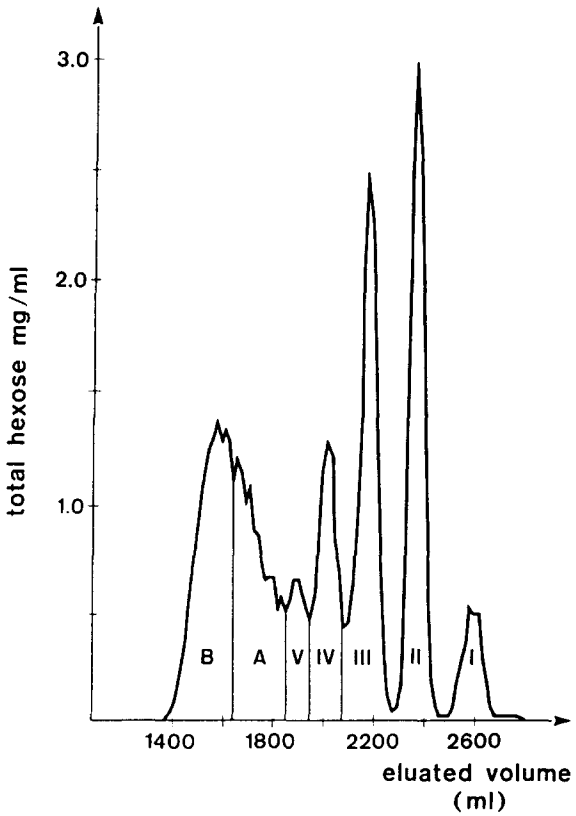


Fig. 1. Sephadex (G-15) gel chromatogram of a wheat-flour fructosan preparation. The Roman numerals indicate the presumed degree of polymerization.

Free galactose was measured enzymatically using galactose dehydrogenase (Dahlqvist, 1984*b*), and free glucose with TGO reagent as described by Dahlqvist (1984*a*). TGO reagent was obtained by dissolving 5·6 g Glox (Kabi Diagnostica AB, Stockholm, Sweden) in 100 ml 0·5M Tris buffer, pH 7·0. Assay of protein was performed by the method of Lowry *et al.* (1951) with the modification reagent B introduced by Eggstein & Kreutz (1955).

Gas chromatography/mass spectrometry

The trisaccharide-containing fraction (III, Fig. 1) was permethylated according to Hakomori (1964). Partially methylated alditolacetates were prepared as previously described (Björndal *et al.*, 1970). Gas chromatography/mass spectrometry (GC/MS) was performed on a VG 12-250 instrument fitted with a SE-30 fused silica capillary column (25 m × 0·2 mm). Separations were carried out at 180°C for partially methylated alditolacetates and at 180–330°C for permethylated oligosaccharides and oligosaccharide alditols.

Reference substances

The carbohydrates used—glucose, fructose, galactose, sucrose and raffinose—were commercial preparations of analytical grade purity, obtained from Merck.

RESULTS

Isolation and purification

The crude fructosan preparation contained, as well as fructosans, monosaccharides, sucrose, raffinose and a small amount of maltose. By preparative gel filtration the crude preparation was separated into seven main fractions: I–V, A and B (Fig. 1). The Roman numerals indicate the presumed degree of polymerization (DP). The fructosans with a higher DP than five were divided into two main fractions, A and B, of which B contained the largest fructosans.

Chromatograms from several preparations of the two different flours showed that flour number two contained relatively more of the tri- and

TABLE 1
The Relative Amounts of the Main Fractions of Flours Numbers 1 and 2

Fraction	*Per cent of total Flour 1 (n = 3)*		*Per cent of total Flour 2 (n = 5)*	
	Mean	*Range*	*Mean*	*Range*
I	4·5	(3·6–5·1)	4·2	(3·6–4·6)
II	20·7	(20·0–21·7)	18·6	(17·6–19·8)
III	15·6	(14·8–16·2)	21·9	(20·0–24·0)
IV	8·8	(8·4–9·3)	10·0	(9·2–10·6)
V	4·7	(4·6–4·8)	5·0	(4·4–5·7)
A	24·2	(23·0–25·6)	20·9	(18·1–23·0)
B	21·5	(19·7–22·7)	19·3	(18·8–20·0)

tetra saccharides (Table 1). It was therefore used for further separations and characterization of fructosans.

To check for any contamination between the main fractions, a sample of each was rechromatographed on an analytical gel column. Each sample gave rise to one single peak.

Samples of fractions III to B were further separated by HPLC. The column used was able to separate peaks in fraction III and fraction IV (Fig. 2). The larger fructosans resulted in complex chromatograms of poor resolution. The chromatograms from fractions III and IV contained four and six major peaks, respectively. In both chromatograms, one peak was from the internal standard glucose and one was a non-hexose peak eluted before glucose. The remaining peaks, two in the chromatogram of fraction III and four in the chromatogram of fraction IV, all had different retention times. One of the peaks in fraction III had the same retention time as raffinose.

Characterization of the main fraction

The results from the monomer characterization of the seven fractions obtained from gel chromatography are shown in Table 2.

Fraction I, the monosaccharide fraction, consisted of about one-third glucose and two-thirds fructose.

Fraction II, the disaccharide fraction, contained almost equal amounts of glucose and fructose and consisted mainly of sucrose. The presence of some maltose was indicated by the reducing power. Incubation with

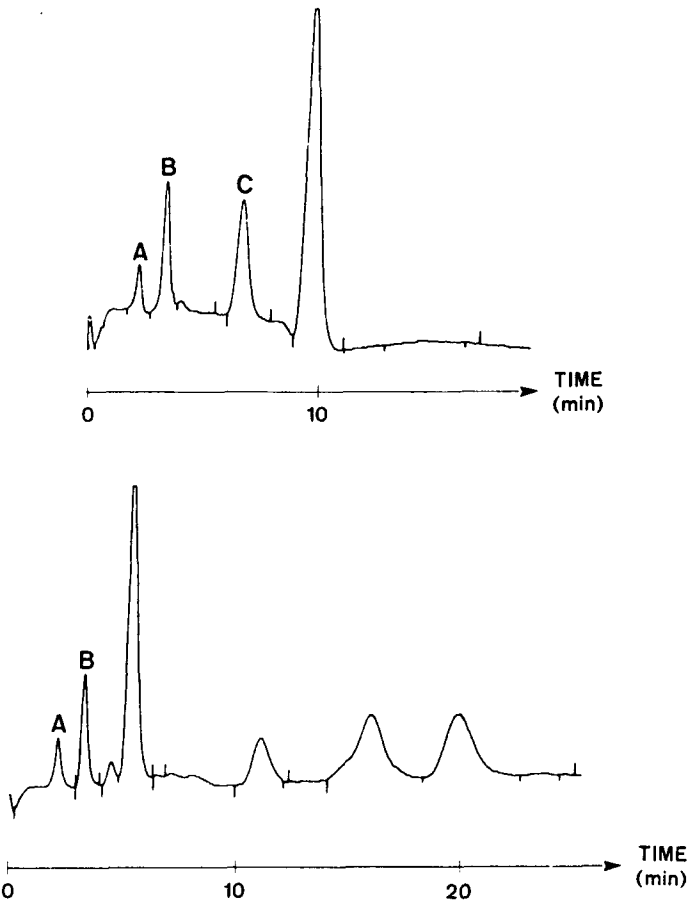


Fig. 2. HPLC chromatograms of fructosan fractions. For chromatographic conditions, see text. Above: fraction III. Below: fraction IV. A is a non-hexose peak. B is the internal standard glucose. C has the same retention time as raffinose.

γ-amylase, which hydrolyzed malto-oligosaccharides as well as starch, liberated about 9% glucose. Also the other fractions contained small amounts of reducing carbohydrates, but the content of malto-oligosaccharides was less than 1% in all of them since practically no glucose was liberated upon incubation with γ-amylase.

Fraction III contained about one-third glucose and two-thirds fructose. Furthermore, it contained 3·4% galactose, indicating that slightly more than 10% of the trisaccharides were raffinose.

The total glucose content of fraction IV (23%) and fraction V (21%)

TABLE 2
Chemical Characterization of Fractions from Flour Number 2

	(*Per cent by weight of total hexose*)						
	I	*II*	*III*	*IV*	*V*	*A*	*B*
Total fructose[a]	61	48	63	70	67	87	91
Total glucose[b]	31	50	32	23	21	11	6
Total galactose[c]	—	—	3·4	—	—	—	—
Free glucose[d]	32	0	—	—	—	—	—
Reducing power[e]	102	7·3	0·9	0·6	1·1	1·1	2·9
Malto-oligosacch.[f]	—	9·2	<1	<1	<1	<1	<1

[a] Detected as total fructose after acid hydrolysis.
[b] Detected as free glucose after acid hydrolysis.
[c] Detected as free galactose after acid hydrolysis.
[d] Detected as free glucose before acid hydrolysis.
[e] Glucose standard.
[f] Detected as free glucose after incubation with γ-amylase.

was consistent with the predicted degree of polymerization, based on the assumption of one glucose unit per molecule. Based on the same assumption, the average degree of polymerization appears to be about 9 for fraction A and about 16 for fraction B. The relative amounts of the different fractions are shown in Table 1.

In both types of flour the higher fructosans, having more than five hexose units, constituted 50–60% of the total fructosans (III–V, A and B). The rest were lower fructosans ($n \leq 5$) where tri- and tetrasaccharides dominated, amounting to 20–25% and 10–15%, respectively.

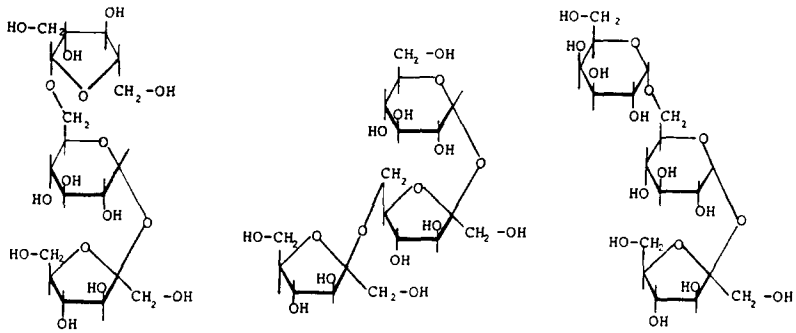
Identification of the trisaccharides

Fraction III was reduced with $NaBD_4$, permethylated and analysed by GC/MS. Three components were observed with retention times of 21·05, 21·35 and 21·50 min. The mass spectra of these components showed no incorporation of deuterium, indicating non-reducing oligosaccharides. One of the components (retention time, 21·50 min) gave a mass spectrum and a retention time identical to raffinose. The mass spectrum showed *inter alia* the following fragments *m/z* 45, 71, 75, 88, 101, 111, 127, 145, 155, 187, 219, 263, 423 and 483. Mild acid hydrolysis (0·1M HCl 80°C, 30 min) to hydrolyze the fructose residue yielded a disaccharide which,

after reduction ($NaBD_4$) and permethylation, could be identified by mass spectrometry as Gal 1–6 Glc. The 1–6 linkage was determined by the fragments *m/z* 134, 178, 293 and 337 formed by cleavage within the alditol.

The components with retention times 21·05 and 21·35 gave mass spectra containing sequence ions of *m/z* 219, 423 and *m/z* 613; the latter ion was formed from the molecular ions by elimination of 45 ($\cdot CH_2OMe$).

The permethylated trisaccharide mixture was hydrolyzed, reduced and analyzed by GC/MS as partially methylated alditolacetates. Derivatives



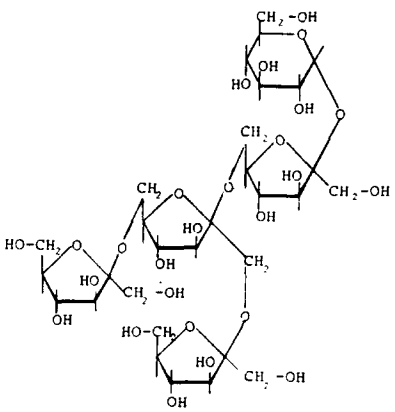


Fig. 3. Above: structures of wheat-flour trisaccharides found in the present study. Below: The main fraction fructosan in Durum wheat flour according to Medcalf & Cheung (1971).

representing 2-*O*-substituted and 2,6-di-*O*-substituted fructose (analyzed as mannitol and glucitol derivatives) were present, as well as non-reducing terminal glucose, galactose and 6-*O*-substituted glucose. The terminal galactose and part of the 6-*O*-substituted glucose are formed from the raffinose.

The fructose and the rest of the 6-*O*-substituted glucose can be combined into the following structures assuming that all structures have a common sucrose structure: 6-kestose, neokestose (Fig. 3).

DISCUSSION

As has been pointed out by several authors (White & Secor, 1953; Medcalf & Cheung, 1971), wheat fructosans occur in homologous series from trisaccharides up to polysaccharides. In our study we have used gel chromatography to quantify fractions of wheat fructosans of different degrees of polymerization (DP). These have shown that fructosans of low DP (<6) make up a substantial part, about 50%, of the fructosans in wheat.

Most of the structural identifications performed in the past have been concerned with larger fructosans (Schlubach & Müller, 1952; Montgomery & Smith, 1957). In our study we have analyzed the structures of the trisaccharides. Two fructosan trisaccharides were found, neokestose and 6-kestose (Fig. 3). The additional finding of a small amount of raffinose confirms earlier reports by, among others, MacArthur & D'Appolonia (1979).

The presence of neokestose and 6-kestose and the absence of 1-kestose (1-fructosylsucrose, the trisaccharide components of 'neosugar') give indications of possible structures of the larger wheat fructosans. There are two structurally different homologous series of fructosans in wheat (White & Secor, 1953; Medcalf & Cheung, 1971). One has primarily (2→1)-glycosidic linkages and one has primarily (6→2)-glycosidic linkages. This was suggested by Medcalf and Cheung to be the reason behind the contradictory reports about wheat fructosan structures. Schlubach & Müller (1952) extracted, from wheat kernels, an oligosaccharide they called 'sitosin'. The results of methylation and hydrolysis indicated (2→1)-glycosidic linkages between the fructose units, a structure commonly referred to as 'insulin type'. From neokestose, which we found in our study, a fructosan polysaccharide with 'inulin-type' structure can

be built. It will obviously not have the glucopyranosyl unit in the end position as in inulin, but it will still be non-reducing and contain only (2→1)-glucosidic linkages between the fructose units. Similar fructosans with non-terminal non-reducing D-glucopyranoside residues, probably formed from neokestose, have been found in *Polygonatum odoratum* var. *japonicum* rhizomes (Tomada *et al.*, 1973). Structures of fructosans representing the other homologous series, containing primarily (2→6)-glucosidic linkages, have been identified by Montgomery & Smith (1957) and (from durum wheat) by Medcalf & Cheung (1971) (Fig. 3). The trisaccharide 6-kestose, which we found, may be an intermediate to any of these structures.

It is possible that the properties, like fermentability and bioavailability, will differ between fructosans of different structure and DP. The fractions characterized here will be subject to further studies with respect to these properties.

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