

# Fructan Precipitation from a Water/Ethanol Extract of Oats and Barley<sup>1</sup>

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## ABSTRACT

Fructan was precipitated from a water and ethanol extract of oat (*Avena sativa* L.) and barley (*Hordeum vulgare* L.). The degree of polymerization and response on a differential refractometer, based on peak area and height, was compared to fructan collected from a lead-based HPLC column and to commercially available inulin. Statistically significant differences are discussed.

Fructan is a fructose polymer of varying size that accumulates in temperate and cool zone grasses during periods when photosynthesis exceeds demand (9). It is reportedly used as short term carbohydrate storage, for osmoregulation of cellular activity, adaptation to low temperature photosynthesis (7), and indirectly for protection from freezing stress (7, 8).

A simple way to identify and quantitate most carbohydrates after separation with HPLC is by refractive index (RI) detection and cochromatography with external standards. The inability to purchase pure fructan isolated from different plants makes quantification (by cochromatography with external standards) of this carbohydrate difficult. Inulin ( $\beta$ -1,2-linked fructan [2] obtained from dahlia (*Dahlia variabilis*), jerusalem artichoke (*Helianthus tuberosus*), and chicory (*Chicorium intybus*) is available commercially, but sufficient quantities of levan ( $\beta$ -2,6-linked fructan [2]) from cereals such as wheat (*Triticum aestivum*), barley (*Hordeum vulgare*), rye (*Secale cereale*), and oats (*Avena sativa*) are not available. Fructan can be collected from an analytical HPLC column, lyophilized, and used as an external standard (4), but this collection method is low yielding, and due to the number of injections necessary, can prematurely degrade analytical columns. Somewhat costly, but extremely durable preparative columns are available packed with the same material used in analytical columns; this allows a more concentrated sample to be injected and gives a higher yield (3).

Phelps (10) reportedly obtained fructan using ethanol and water recrystallization (at low temperatures) but gave no procedural details. Prasník *et al.* (11) obtained analytical-reagent grade inulin from Jerusalem artichoke by storing an extract at 4°C for 1 week. Archibald (1) reviewed methods used in the late 1800s and early 1900s for obtaining large quantities of fructan based on its insolubility in pure ethanol. Procedural

details were not provided, but significant quantities of pure fructan were apparently obtained by precipitation from a solution of fructan dissolved in water (1).

The purpose of this study was to develop a procedure for collecting fructan by precipitation and to compare response (on a differential refractometer using peak area and height) and degree of polymerization (DP) of precipitated fructan to that of fructan (an average DP greater than 6 (DP>6)) collected from a lead-based, analytical column and to commercially available fructan from chicory and dahlia.

## MATERIALS AND METHODS

### Plant Growing Conditions

The barley (*Hordeum vulgare* L.) cultivar 'Dicktoo' and oat (*Avena sativa* L.) cultivar 'Fulgum' were both grown in a seminutritive system previously described (6). Seeds were planted 3 cm deep in plastic tubes (2.5 cm top, i.d. by 11.5 cm deep) filled with a thoroughly mixed medium of one-third (by volume) sand:one-third sphagnum peat moss:one-third perlite. Thirty-six tubes were placed in racks with a spacing of 4.5 cm between centers and suspended in plastic pans. A half-strength Hoagland nutrient solution was added to the pans and covered the bottom one-third of the tubes. Small holes in the bottom of the tubes allowed nutrient solution to enter. The solution was completely renewed every 7 d.

Plants were grown at 13°C day and 10°C night with a 12-h photoperiod in a growth chamber with 300  $\mu\text{mol m}^{-2} \text{s}^{-1}$  light (80% cool fluorescent and 20% incandescent). After 5 weeks, pans containing plants were transferred to a chamber at 2°C with an 18-h photoperiod at 200  $\mu\text{mol m}^{-2} \text{s}^{-1}$  for a 3-week hardening period.

### Carbohydrate Extraction

After hardening, individual plants were removed from tubes, washed in ice water, and trimmed of roots and shoots. Approximately 1 cm of the stem base closest to the roots (crown) was cut with scissors and left in ice water until 10 plants were cut. Forty plants total were used. The remaining stem (approximately 3 cm of the stem just above the crown) was left in ice water until all 40 crowns were ground. Plant crowns were ground (10 at a time) at room temperature in 20 mL of 80% (v/v) EtOH using a stainless steel grinder developed specifically for grinding crown tissue (5). Plant stems were bulked and approximately 30 g fresh weight ground at a time in the same grinder as the crowns. The slurry (containing

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pulp and ethanol) from the stems and crowns were both heated at 70°C for 15 min (to inactivate invertase), the supernate was decanted into a 125 mL Erlenmeyer flask; a fine-grated screen was used to prevent pulp from washing into the flask. The remaining plant pulp was washed two times, each time with 30 mL of deionized water and the washings combined with the original EtOH extract. Approximately 10 mL of Amberlite MB3 resin was added to the flask containing the combined extract (to remove salts and other ionic compounds which precipitate with fructan), and the slurry (extract plus resin) was shaken on a rotary shaker at 250 rpm for 15 min. Samples were transferred to 50 mL centrifuge tubes and centrifuged for 15 min at 900g. The clear supernates were transferred to tared 250 mL evaporator flasks and dried under vacuum on a rotary evaporator at 38°C. The dried extract was weighed and brought to a concentration of 120 mg/mL for one treatment and 200 mg/mL deionized water for another treatment.

### Precipitation

Absolute EtOH was added to the solution containing the dissolved extract. In one treatment, the concentration was brought to 900 mL EtOH/L and in another treatment to 950 mL EtOH/L. The flask was swirled once by hand and its contents quickly transferred to 50 mL centrifuge tubes. The solution turned cloudy when EtOH was added, and if it was not transferred quickly to centrifuge tubes, large fragments of white precipitate adhered to the flask. The tubes were covered with parafilm and stored overnight at 2°C to help complete precipitation. The following day the solution was centrifuged at 900g for 15 min. The supernate containing simple sugars and smaller fructan was discarded. The white precipitate was dissolved in water and transferred quantitatively back to the evaporator flask used previously. Reconstituting and precipitating again as above (two precipitations total) gave fructan and 0.5% (percent by weight of total carbohydrate) sucrose but no detectable glucose, fructose, or smaller fructan (DP 3–5). Three total precipitations resulted in fructan and no detectable sucrose, glucose, fructose, or smaller fructan (DP 3–5). The final fructan solution was filtered using a 0.45  $\mu$  filter and freeze dried.

### Fructan Collection from an Analytical Column

A crude extract of barley was concentrated to approximately 25 mg/mL under vacuum at 37°C, and injected into a Bio-Rad<sup>2</sup> (Richmond, CA) Aminex HPX-87P analytical column with an autosampler. The DP>6 fructan fraction was collected directly from the column using a fraction collector. Approximately 10 mg of barley fructan was collected from 27 injections of crude extract.

Inulin from dahlia and chicory was purchased from Sigma Chemical Co. (St. Louis, MO).

<sup>2</sup> Mention of a trademark, vendor, or proprietary product does not constitute a guarantee or warranty of the product by the U.S. Department of Agriculture and does not imply approval to the exclusion of other products that also may be suitable.

### Hydrolysis

Fructan, precipitated and collected from the analytical column, was hydrolyzed with washed Amberlite IR 120H ion exchange resin (strongly acidic, gel type) from Aldrich (Milwaukee, WI). Galactose was not present either before or after hydrolysis, so it was used as an internal standard. Hydrolysis was complete (no detectable fructan was present) after 2 h at 95°C and resulted in peaks which cochromatographed with glucose and fructose. Total area of all unknown peaks after hydrolysis accounted for less than 1% of total peak area except for fructan (inulin) from dahlia and chicory; both had a compound after hydrolysis (accounting for at least as much area as glucose) which eluted at 10.5 min (between sucrose and glucose). Average recovery (calculated as: mg fructose + mg glucose after hydrolysis/mg fructan before hydrolysis + H<sub>2</sub>O incorporated during hydrolysis) after hydrolysis of all samples was 88%, with the highest recovery at 106% and the lowest at 77%. Small samples (1.5 mg/mL) were hydrolyzed because so little DP>6 fructan could be collected from the column. A 0.25 mg original weighing error would therefore account for the variability in recovery.

A randomized complete block design was used to analyze DP and RI response. Each hydrolysis was repeated three times, and eight treatments (Table I) were compared. Assuming a single terminal glucose for each fructan molecule, then after hydrolysis mg fructose + mg glucose/mg glucose represents the average DP of the sample.

### Chromatography

Carbohydrates were separated by HPLC using a Bio-Rad (Richmond, CA) Aminex HPX-87P (lead-based) column (0.78 × 30 cm) at 80°C. A guard column was also used, consisting of two (one cation and one anion) 5 × 30 mm cartridges arranged in series. The mobile phase was degassed HPLC grade water and had a flow rate of 0.6 mL/min. A Waters 410 (Milford, MA) RI detector was used to identify and quantify carbohydrates. RI response was a linear function of the amount of each compound injected. Retention times in minutes were: DP>6 fructan, 7.6; sucrose, 11.0; glucose, 13.2; galactose, 15.1; and fructose, 18.0. For the fructan RI response measurement, two samples (each sample was considered one replication in the analysis of variance) were weighed separately. The average response for each sample (the slope when fructan concentration verses RI response is plotted) was based on five injections of varying amounts. Peak area and height was measured by a Waters (Milford, MA) Baseline 810 chromatography workstation on a microcomputer.

## RESULTS AND DISCUSSION

Differences were not statistically significant between treatments for RI response based on peak area (Table I). This is not surprising since the RI for inulin is reportedly identical to that of sucrose (12). It is unlikely that fructan with a DP intermediate to inulin and sucrose would have a different RI.

Significant differences in RI response based on peak height were found between treatments. In addition, response based on peak height was negatively correlated ( $R = -0.875$  signif-

**Table I.** DP and Differential Refractometer Responses for Fructan Obtained from Different Sources and under Different Conditions

Species	Plant Organ	Extract Concn	EtOH Concentration	DP	Differential Refractometer Response	
					Area ( $\times 10^{-4}$ )	Height ( $\times 10^{-4}$ )
		mg/mL <sup>a</sup>	mL/L <sup>b</sup>		$\mu V \cdot s/mg$	$\mu V/mg$
Oat	Crown	120	950	12.3 d <sup>c</sup>	34.0 a	11.9 a <sup>c</sup>
Barley	Crown	120	950	14.1 c	32.0 a	13.0 ab
Barley	Crown	Collected		14.7 c	34.7 a	14.0 bc
Barley	Crown	200	950	16.8 b	33.2 a	15.1 cd
Barley	Stems	200	900	17.6 b	33.8 a	15.4 cd
Barley	Stems	200	950	18.2 b	36.6 a	16.4 de
Dahlia	Tuber	Sigma		27.7 a	32.8 a	18.4 e
Chicory Root		Sigma		28.0 a	33.1 a	18.4 e

<sup>a</sup> mg dry crude extract/mL water to which EtOH was subsequently added to precipitate fructan. <sup>b</sup> mL absolute EtOH/L extract. Fructan precipitated in this mixture immediately upon addition of EtOH. <sup>c</sup> Means within a column followed by the same letter on the line are not significantly different from each other at  $P = 0.05$  according to Tukey's multiple range test.

icant at  $P = 0.001$ ) with DP (when only levan from barley and oats were analyzed, the correlation was  $R = -0.966$  significant at  $P = 0.001$ ). These results indicate that peak spreading increases as the average DP in fructan decreases. This is probably due to the higher intrinsic diffusivity of smaller molecules and the fact that smaller molecules remain in this column longer than larger molecules; this gives smaller molecules more time to diffuse and thus increase peak spreading. Heterogeneous peak fractions with a lower average DP would therefore be expected to spread more than those with a higher average DP. The DP of fractions collected from the beginning of a precipitated oat fructan peak was higher than the DP of fractions collected from the end of the peak, confirming that the fraction was heterogeneous.

Because it is difficult to completely resolve fructan composed of different sized molecules with currently available HPLC columns, using peak height in quantification would probably give incorrect results because more peak spreading will occur in fructan with a smaller average DP. Therefore, the DP of standards should be similar to the DP of fructan in unknowns. However, if peak area can be used accurately in quantification, then any sized fructan could probably be used as an external standard in RI detection.

The average DP of the precipitated oat fructan was significantly lower than any other treatment (Table I). The majority of carbohydrate in winter oat cultivars tested in this laboratory was DP3 to DP5 fructan as opposed to primarily DP>6 fructan in barley (our unpublished data). It is possible that a different mechanism for carbohydrate storage exists in oats which causes smaller sized fructan to predominate. Studies are continuing to investigate this possibility. Oat fructan also had the lowest RI response based on peak height (it was, however, not significantly different than the peak height response for barley crown also at 120 mg extract and precipitated with 95% EtOH). Because a small amount of DP>6 fructan was present in oats, not enough DP>6 fructan could

be collected from the analytical column to be compared with precipitated oat fructan.

No statistically significant difference was found between DP or RI response of barley crown fructan (precipitated from a 120 mg/mL extract) and that collected from the column. Precipitation under these conditions would therefore provide fructan suitable for accurate quantification (using external standards and peak height) of barley DP>6 fructan using a lead based column and RI detection.

Fructan precipitated from the 200 mg/mL extract was significantly different in DP and RI height response from that of oats and barley precipitated from the 120 mg extract. Fructan precipitated from barley crowns and stems were of the same DP and RI height response. This suggests that crowns could be used for analytical measurements of different carbohydrates while stems are used to collect additional fructan with a composition similar to that of crown fructan. It may also be possible to precipitate a range of different sized fructan molecules by starting with different extract concentrations.

While no significant difference was found between the two inulin samples for DP or RI height response, both were considerably different from all other samples for DP and RI height response. This fructan would therefore be unsuitable for quantification of DP>6 fructan found in barley and oats using external standards and RI response based on peak height. However, using peak area, it would make an excellent, readily available standard.

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