Purification and Characterization of an Oat Fructan Exohydrolase That Preferentially Hydrolyzes β-2,6-Fructans

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Oat (Avena sativa cv Fulghum) fructan hydrolase was purified by ammonium sulfate precipitation and anion-exchange, hydrophobic interaction, and size-exclusion chromatography. The enzyme was purified to homogeneity as determined by the presence of a single band (43 kD) on a silver-stained sodium dodecyl sulfate-polyacrylamide gel. A mixture of β-2,6-linked fructan (neokestotraose) isolated from oat was used as the substrate to purify fructan hydrolase. Neokestotriose and small degree of polymerization fructan isomers were used to characterize the substrate specificity of the purified enzyme. The purified fructan hydrolase catalyzed hydrolysis of the terminal β-2,6 linkage of 6G,6-kestotetraose 3.5 times more rapidly than it hydrolyzed the terminal β-2,6 linkage of 6G-kestotriose and approximately 10 times faster than it hydrolyzed the terminal β-2,1 linkage of chicory inulin. Sucrose and 1-kestose were not substrates. The $K_m$ for neokestotriose (β-2,6-linked fructans with a degree of polymerization of 7–14) hydrolysis was 2.8% (w/v), and the $V_{max}$ was 0.041 μmol min⁻¹ mL⁻¹. The $K_m$ for hydrolysis of 6G,6-kestotetraose was 5.6% (w/v), and the $V_{max}$ was 0.138 μmol min⁻¹ mL⁻¹. Catalysis was exolytic and by multiple chain attack. Hydrolysis of neokestotriose was maximal at pH 4.5 to 5.0.

Fructan accumulates in the culms and subterranean tissues of cereals and many forage grasses and is degraded in response to a variety of environmental conditions or management practices (for review, see Suzuki, 1993). Substantial variation in the structures of stored fructan occurs between species. Wheat stores primarily bifurcose-based fructans with extensions at both the β-2,1 and β-2,6 linkages (Bancal et al., 1991). In contrast, oats (Avena sativa) store mainly neokestose-based β-2,6-linked oligomers with a DP of 3 to 5 (Livingston, 1990; Livingston et al., 1993a).

A number of fructan-degrading enzymes have been identified from bacteria, fungi, and higher plants. These are categorized as follows: 2,1-β-D-fructan fructohydrolases (invertase, EC 3.2.1.7), β-fructofuranoside fructohydrolases (invertase, EC 3.2.1.26), 2,6-β-fructan 6-β-D-fructofuranosylfructohydrolases (levanbiohydrolase, EC 3.2.1.64), 2,6-β-fructan fructohydrolases (levanase, EC 3.2.1.65), and β-6-fructan fructohydrolases (FEH, EC 3.2.1.80). The FEHs are the only type that have been convincingly demonstrated in higher plants. Higher plant FEHs that have been characterized exhibit a strong preference for either the β-2,1 or the β-2,6 linkage but were not completely specific for one type of linkage, and many showed some ability to hydrolyze Suc (Yamamoto and Mino, 1985; Henson, 1989; Bonnett and Simpson, 1993). The assignment of one or more of these hydrolytic activities to a particular fructan hydrolase enzyme has not been possible because none of the plant enzymes were purified to homogeneity. The exception is an FEH from chicory roots (Claessens et al., 1990), but the only fructans used to determine substrate specificity were chicory inulin (β-2,1 linked) and Aerobacter levan (highly branched β-2,6 linked). The lack of levan hydrolysis, which Claessens et al. (1990) reported, does not necessarily indicate the absence of β-2,6 hydrolase activity. For example, barley FEH slowly hydrolyzed the β-2,6 linkage present in 6-kestose but did not hydrolyze Aerobacter levan; Henson (1989) and Bancal et al. (1991) hypothesized that the highly branched structure of levan may have resulted in steric hindrance.

Our objective was to purify and characterize a fructan-degrading enzyme from oats with emphasis on its ability to degrade fructans found in situ. Because oats store oligomers that are not commercially available, we purified and identified several oat fructans. These endogenous fructans were then used to characterize an oat FEH.

MATERIALS AND METHODS

Plant Growth Conditions

Seeds (Avena sativa cv Fulghum) were grown for 35 d after sowing in a growth chamber with a day/night temperature regime of 15/10°C. Twelve hours of light were provided by incandescent lamps and were supplemented with fluorescent lamps for 11 h beginning 30 min after photoperiod initiation and ending 30 min before the photoperiod ended. Light was 900 μmol m⁻² s⁻¹ at plant height for the 11 h that both light sources were used.

Abbreviations: DP, degree of polymerization; FEH, fructan exohydrolase; HiC, hydrophobic interaction chromatography; neokestotriose; 3a, 1-kestotriose; 3b, 6G-kestotriose; 4a, 6G,6-kestotetraose; 4c, 6G,1-kestotetraose; 4d, 1 & 6G-kestotetraose; 5b, 6G,1 & 6-kestopentaose.

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Purification and Identification of Oat Fructans

Neokestin (DP 7–14) was aqueously extracted from oat stems and leaves (Livingston, 1990) and analyzed by GC-MS of partially methylated alditol acetates (Livingston et al., 1993a). Oat stem bases and adhering leaf sheaths were extracted with 100% ethanol to obtain DP 3 to 5 fructans and then separated according to size on an Aminex 42a column (Bio-Rad). Size classes were separated into individual isomers on an Absorbosphere H5 column (Alttech, Deerfield, IL) (Livingston et al., 1993a). Structural determination of individual isomers was by (a) GC-MS of partially methylated alditol acetates, (b) analysis of products of partial acid hydrolysis, and (c) co-chromatography with isomers whose structures were previously determined (Livingston et al., 1993a).

Enzyme Extraction and Purification

The lower two nodes and connecting internode of main culms and tillers were ground in 50 mM citrate-phosphate buffer (pH 5.0; 3 mL buffer/g tissue) using a VirTis (Gardiner, NY) homogenizer. The homogenate was filtered through two layers of cheesecloth and then centrifuged for 15 min at 12,000g. The supernatant was fractionated using solid (NH₄)₂SO₄. The pellet from the 50 to 75% cut was resuspended in 10 mM citrate-phosphate buffer (pH 7.0) and dialyzed overnight against the same buffer. Tissue grinds, centrifugation, dialysis, and (NH₄)₂SO₄ precipitations were done at 4°C.

The dialyzed (NH₄)₂SO₄ fraction was separated on a Mono-Q anion-exchange column (HR 10/10) using an HPLC system (Shimadzu, Tokyo, Japan) at a flow rate of 4 mL/min. The column was equilibrated with 10 mM citrate-phosphate buffer at pH 7.0. A 20-mL wash was used to elute unbound proteins. Bound proteins were eluted with a 180-mL linear NaCl gradient (0–0.25 M). Fractions containing neokestin hydrolase activity were pooled, brought to 2.4 M (NH₄)₂SO₄, and separated on a 5-mL methyl-1-HIC column (Bio-Rad No. 732-0051) at a flow rate of 1 mL/min. Eluant A was 100 mM sodium phosphate, pH 6.8, containing 2.4 M (NH₄)₂SO₄. Eluant B was 100 mM sodium phosphate, pH 6.8. After the sample was injected the column was washed with 5 mL of eluant A, followed by a linear gradient to 100% B over 30 min and then 10 min at 100% B. Fractions containing β-2,6-fructan hydrolase activity were pooled. Size-exclusion chromatography was on a Bio-Pac PA-1 column (250 × 4 mm, No. 35391; Dionex, Sunnyvale, CA) at a flow rate of 1 mL/min. The elution was from 98% eluant A (100 mM NaOH) and 2% eluant B (100 mM NaOH plus 600 mM sodium acetate) to 33% A:67% B over 20 min, followed by a 10-min wash with 98% A:2% B. These elution conditions allow the separation of linear polymers up to DP 8 (data not shown). Separated carbohydrates were detected with a pulsed electrochemical detector using a gold electrode and identified by co-chromatography with standards. The starting potential, 0.1 V, was applied for 0.5 s and integration was from 0.3 to 0.5 s. The cleaning cycle was from 0.51 to 0.59 s at 0.6 V followed by −0.6 V from 0.6 to 0.65 s.

RESULTS

Purification of Oat 6-FEH

The reaction products of neokestin hydrolysis by crude enzyme extracts were examined on the Dionex HPLC system. This allowed detection of all endo- and exolytic degradation products of fructan polymers. Fructan polymers that had been decreased by one monomeric unit and Fru were the only products detected; this indicated exolytic rather than endolytic action. Hence, measurement of substrate-dependent Fru production was used as the standard assay for purification of an exolytic neokestin-hydrolyzing enzyme.

During enzyme purification all fractions were assayed for hydrolysis of Suc, chicory inulin, and oat neokestin. The fraction of the extracts precipitating between 50 and 75% (NH₄)₂SO₄ contained approximately 80% of the neokestin-hydrolyzing activity, approximately 70% of the Suc-hydrolyzing activity, and approximately 65% of the inulin-hydrolyzing activity (data not shown). Chromatography on a Mono-Q anion-exchange column resulted in one peak of neokestin-hydrolyzing activity that co-eluted with the only peak of inulin-hydrolyzing activity and with one of the three Suc-hydrolyzing peaks (Fig. 1A). When all neokestin-hydrolyzing fractions from the Mono-Q column were pooled and applied to the HIC column, separation of Suc-hydrolyzing and neokestin-hydrolyzing activity was incomplete. However, when only the tubes containing max-
Oat Fructan Exohydrolase

Figure 1. Elution profiles of oat hydrolytic activities from Mono-Q (HR 10/10) anion-exchange (A) and methyl-HIC (B) columns. Enzyme activities were assayed using neokestin, chicory inulin, and Suc as substrates. Two fractions eluting from the Mono-Q column that contained maximal neokestin-hydrolyzing activity were applied to the HIC column. The inulin-hydrolyzing activity co-eluted with the neokestin-hydrolyzing activity as it did from the Mono-Q column (Fig. 1B). The inulin- and neokestin-hydrolyzing activities were not resolved by chromatography on Con A, on a W-Porex size-exclusion column, or on octyl-, tert-butyl-, and phenyl-HIC (high and low substitution) columns (data not shown). Inulin-hydrolyzing activity was consistently between 10 and 15% of the neokestin-hydrolyzing activity throughout all chromatographic steps. SDS-PAGE analysis of the enzyme preparation after separation by anion-exchange, HIC, and size-exclusion chromatography revealed a single protein of 43 kD (Fig. 2).

Figures not available.

Hydrolysis of neokestin by the purified hydrolase was maximal at pH 4.5 to 5.0 (Fig. 3). When 4a (Fig. 4) was used as the substrate over a pH range of 4.0 to 6.0, activities were again maximal at pH 4.5 to 5.0 (data not shown).

The substrate specificity of the purified hydrolase was first surveyed by determining rates of Fru production from three fructans and Suc. Relative rates of hydrolysis were: 4a, 100%; Cichorium inulin, 11.9%; Aerobacter levan, 8.5%; and Suc, 0%. The substrate specificity of the purified hydrolase was examined in greater detail using the fructans shown in Figure 4; it did not hydrolyze the β-2,1 linkage of 3a but did hydrolyze the β-2,6 linkage of 3b (Table I). The terminal β-2,6 linkage of 4a was rapidly cleaved and released 3b and Fru. Hydrolysis of the terminal β-2,6 linkage of 4a was 3.5 times faster than hydrolysis of the terminal β-2,6 linkage of 3b. Hydrolysis of 5b yielded 4c, 3b, Fru, and a trace of 4a (Table I).

The hydrolysis of neokestin (Fig. 5A) and of 4a (Fig. 6A) followed Michaelis-Menten kinetics. Hydrolysis was apparently saturated at a neokestin concentration of 8%. Be-
cause neokeston is a mixture of chemical species with multiple molecular weights, the calculation of $K_m$ for neokeston in molar units was not done. Saturation with 4a occurred between 6 and 8% (90–120 mM). Rate parameters for hydrolysis of neokeston and of 4a were determined from Lineweaver-Burk plots (Figs. 5B and 6B, respectively). The hydrolase affinity for neokeston was twice that of 4a, but the $V_{max}$ for hydrolysis of neokeston was 30% of the $V_{max}$ for 4a hydrolysis. The specificity constant ($V_{max}/K_m$) for neokeston was 0.015 and for 4a it was 0.024, indicating that 4a may be a slightly better substrate than neokeston.

**DISCUSSION**

The purification of oat FEH to homogeneity (as determined by SDS-PAGE) and the use of well-defined, individual fructan oligomers allows unambiguous determination of the types of linkages this enzyme cleaves. The hydrolase described here hydrolyzed both $\beta$-2,6 and $\beta$-2,1 linkages, although $\beta$-2,6 linkages were preferred. Other studies of plant fructan-degrading enzymes have been done with impure enzymes; thus the ability to hydrolyze $\beta$-2,1- and $\beta$-2,6-fructans could not be attributed to a single enzyme. An exception was an inulin-hydrolyzing enzyme isolated from *Cichorium*, which was purified to homogeneity (Claessens et al., 1990). However, a test of this inulin-hydrolyzing enzyme’s ability to hydrolyze *Aerobacter* levan was insufficient to conclude that the enzyme could not hydrolyze $\beta$-2,6 linkages (see the introduction). The oat

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**Table 1. Hydrolysis of neokestose-based fructan oligomers from oats by 6-FEH**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Reaction Rate</th>
<th>Reaction Products</th>
</tr>
</thead>
<tbody>
<tr>
<td>3a</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>3b</td>
<td>12.2</td>
<td>Suc, Fru</td>
</tr>
<tr>
<td>4a</td>
<td>41.5</td>
<td>3b, Fru</td>
</tr>
<tr>
<td>4d</td>
<td>3.5</td>
<td>3a, Fru</td>
</tr>
<tr>
<td>5b</td>
<td>24.7</td>
<td>4a (trace), 4c, 3b, Fru</td>
</tr>
</tbody>
</table>

* Nanograms of fructose produced h$^{-1}$ µL$^{-1}$ oat 6-FEH. Eighteen-microliter assays contained 12 µL of substrate (1 mg/mL) and 6 µL of purified 6-FEH. Reactions were terminated by addition of 200 µL of buffer, pH 8.0. Twenty microliters of each assay were separated via HPLC. Products were detected by pulsed electrochemical detection.
Oat Fructan Exohydrolase

A hydrolyase described here hydrolyzed Aerobacter levan at 8.5% of the rate it hydrolyzed 4a, and such low rates can easily be overlooked. Fructan hydrolases from species that store fructan with only one type of linkage, such as Cichorium intybus, which stores only inulin, may hydrolyze only one type of linkage as reported by Claessens et al. (1990). However, without using purified enzymes and well-defined, appropriate substrates, the catalytic capabilities of these enzymes are not yet clear. The problems associated with characterization of impure plant proteins and the use of nonspecific or nondiagnostic substrates are also evident in the literature concerning the microbial enzymes generally referred to as levanases (Uchiyama, 1993).

Whether plant fructan hydrolases can hydrolyze Suc is not clear, and fructan-hydrolyzing activities have often been classified as $\beta$-fructofuranosidases along with invertases. This confusion also results from the many published characterizations of impure fructan hydrolases and invertases and because most studies of invertases purified from plants did not assess their ability to degrade fructans as is often done for microbial invertases. However, the purified hydrolyase described here and the highly purified hydrolyase from Dactylis glomerata reported by Yamamoto and Mino (1985) hydrolyze both $\beta$-2,6- and $\beta$-2,1-fructans but do not hydrolyze Suc. Simpson and Bonnett (1993) summarized the Suc-hydrolyzing activity of hydrolases from various plant species. There are clear examples of a single enzyme that hydrolyzes Suc, $\beta$-2,1- and $\beta$-2,6-linked fructans in the microbial literature (Xiao et al., 1989; Blatch and Woods, 1993). To our knowledge no enzymes have yet been purified from a higher plant that can hydrolyze all three linkages.

Fructan hydrolases have been localized exclusively in vacuoles of barley and Jerusalem artichoke (Wagner et al., 1983; Frehner et al., 1984; Wagner and Wiemken, 1986), the pH of which is approximately 5.0 to 6.0 (Planz and Heber, 1986; Kurkdjian and Guern, 1989). The pH activity optima range from 4.5 to 5.5 for fructan hydrolases isolated from several grass species (Yamamoto and Mino, 1985; Wagner and Wiemken, 1986; Henson, 1989; Jeong, 1991), which is considered evidence of their vacuolar location. The pH activity optimum of oat fructan hydrolase (pH 4.5–5.0) is appropriate for a vacuolar enzyme.

Other physiologically relevant characteristics of the oat hydrolase include its substrate specificity and the kinetic rate parameters for hydrolysis of endogenous substrates. Based on the rates of hydrolysis in Table I and on the $K_m$ and $V_{max}$ of 4a was the preferred substrate among those tested. The $K_m$ of oat fructan hydrolase for hydrolysis of 4a suggests that a fairly high in vivo concentration of this substrate is needed if the enzyme is to operate under conditions that are not substrate limiting. The concentration range of 4a in the crowns and lower stem bases of 8-week-old oat plants of 49 genotypes was 0.95 to 7.8 mg/g fresh weight and the average was 4.8

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**Figure 5.** Kinetics of neokestin hydrolysis by oat fructan hydrolase. A, Plot of reaction velocities ($\mu$mol Fru produced min$^{-1}$ mL$^{-1}$) versus neokestin concentrations ranging from 1 to 10% (w/v). B, Lineweaver-Burk plot of fructan hydrolase reaction velocities at neokestin concentrations used in A.

**Figure 6.** Kinetics of 4a hydrolysis by oat fructan hydrolase. A, Plot of reaction velocities ($\mu$mol Fru produced min$^{-1}$ mL$^{-1}$) versus substrate concentrations ranging from 0.25 to 10% (w/v). B, Lineweaver-Burk of fructan hydrolase reaction velocities at substrate concentrations used in A.
mg/g fresh weight (Livingston et al., 1993a). Because the $K_m$ for 4a was 5.6% (w/v) and the reported average concentration of 4a in extracts of whole oat crowns and stems was 0.48%, we conclude that oat fructan hydrolase probably functions under substrate-limiting conditions. However, the concentration of 4a inside the vacuole where fructans and fructan hydrolases are sequestered would be higher than those concentrations determined by extraction of whole plant tissues.

The observation that 3b is released as a product of 4a hydrolysis indicates that the oat hydrolase functions with a multiple chain attack mechanism rather than catalyzing multiple hydrolytic events on a single fructan chain during one enzyme-substrate encounter. The latter type of attack would release only Fru and SuC. No hydrolysis of the internal β-2,6 bond of 4a was detected, confirming that this enzyme is exolytic.

Hydrolysis of the terminal β-2,6 linkage of 5b resulted in the production of 4c and Fru, and, based on hydrolysis rates of 4a and 3b, this should be a relatively rapid catalytic step. 4c is rapidly released during hydrolysis of 5b. And, since 4c breakdown is slow because of the presence of the terminal β-2,1 linkage, it accumulates during hydrolysis of 5b. Conversely, 4a appears as a minor product of 5b hydrolysis because cleavage of the terminal β-2,1 linkage of 5b is slow. Yet, once 4a is synthesized, it is rapidly broken down to 3b and Fru. Thus, 4a does not accumulate during hydrolysis of 5b. The results of 5b hydrolysis are consistent with the observed rapid hydrolysis of the terminal β-2,6 linkages of 4a and 3b and are consistent with the very slow or zero hydrolysis of the terminal β-2,1 linkages of 4d and 3a.

Neokestin concentrations of oat crowns and lower stem bases range from 35 to 68 mg/g fresh weight (Livingston et al., 1993b), which is as much as 2.5 times greater than the $K_m$ of this oat hydrolase for neokestin (2.8%, w/v). The population of fructan polymers that constitute neokestin likely reach saturating levels at some stages of development or under some environmental conditions. Neokestin concentrations in crowns of oat cv Fulghum increase 10-fold during cold hardening (Livingston et al., 1994).

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LITERATURE CITED


