Glucuronoxylan Xylanohydrolase

A UNIQUE XYLANASE WITH THE REQUIREMENT FOR APPENDANT GLUCURONOSYL UNITS*

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A new category of β-(1→4)-xylan xylanohydrolases that exhibit a specific capacity to hydrolyze glucuronoxylans was characterized using heteroxylans prepared from Vigna (Vigna angularis Ohwi et Ohashi cv. Takara) and maize (Zea mays L.) cell walls together with appropriate derivatives as substrates. Glucuronopyranosyl moieties, as side chains, were prerequisite for enzyme-mediated hydrolysis of the β-(1→4)-xylosyl linkages. The enzyme degraded glucuronoxylans derived from Vigna cell walls to yield a major oligomeric species

Xylβ1→4Xylβ1→4Xylβ1→4Xylβ1→4Xylβ1→4Xylβ1

GlcAcα1

where Xyl represents xylose and GlcA represents glucuronic acid. The enzyme also degraded glucuronoroarabinoxylans derived from maize cell walls to yield a major oligomeric species containing a single glucuronosyl side chain and a single unsubstituted β1→4Xyl pendant terminal. These results indicate that this xylanohydrolase recognizes glucuronosyl moieties inserted as monomeric side chains along the xylan backbone and mediates the hydrolysis of the β-(1→4)-xylosyl linkage of the adjacent unsubstituted xylosyl residue in heteroxylans. This enzyme is the first xylanohydrolase identified that recognizes distinctly different sugars constituting side chains. We propose to designate this new enzyme as a glucuronoxylan xylanohydrolase to be abbreviated as glucuronoxylanase. Use of this unique enzyme demonstrated the presence of repeating units in heteroxylans in cell walls of higher plants.

Glucuronic acid (GlcA),¹ Ara, and feruloyl residues to form complex heteroxylans (2–5). As a component of primary cell walls of plants (6, 7), the polymer is considered to serve an important role in controlling wall functions (7, 8) and is thought to participate in processes responsible for growth and differentiation (7–9).

However, the structure of the polymer has not been resolved, partially because no suitable enzymes have been available to generate appropriate fragments in which structural integrity has been retained. The existence of such enzymes would facilitate the analysis of heteropolymers (2, 11). Two types of xylan-degrading enzymes (i.e., 1,4-β-D-xylan-xylanohydrolase (endo-1,4-β-xylanase) (EC 2.2.1.8) (12–20) and xylan 1,4-β-xylolhydrolase (exo-1,4-β-xylisidase) (EC 3.2.1.37) (12, 14, 18)) have been purified and characterized from various microorganisms. The conventional xylan-degrading enzymes catalyze the hydrolysis of β-(1→4)-xylol linkages in unsubstituted domains along the xylan backbone (2, 11, 21). Although substituents on xylan main chains are generally considered to interfere with the hydrolysis by these xylanases, there is no current evidence to suggest that appendant sugars might be required for activity (11, 17, 21, 22). Consequently, specific enzymic fragmentation of xylans has not been extensively employed for structural studies on heteroxylans.

A Bacillus subtilis enzyme preparation provided a source for the purification of unique xylanohydrolases (23). These enzymes showed the high specificity for degradation of feruloylated glucuronoroarabinoxylans ("feraxans") and were tentatively designated feraxanases (23). Because of the capacity of the enzyme to fragment the xylan, it was employed for structural analysis of feraxan in maize cell walls (4, 7). Although it was suggested that the enzymic action was dependent on certain structural features along the xylan main chain, the nature of the side chain required for recognition and the mode of action of the enzyme was unclear (23). We have now characterized the substrate specificity for this enzyme and identified the recognition site in selected heteroxylans. The enzyme recognizes GlcA side chains along the xylan main chain and mediates the hydrolysis of the β-(1→4)-xylol linkages of the adjacent unsubstituted xylosyl residues.

Xylans are major components of the matrix of most higher plant cell walls and are characterized as β-(1→4)-xylopyranosyl (1). These xylans are often highly substituted with

Xyl, xylose; Ara, arabinose; GC, gas chromatography; GPC, gel permeation chromatography; HPLC, high performance liquid chromatography; RI, refractive index; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; MX, maize xylan; VX, Vigna xylan; MXf and VXf, the major fragments obtained following B. subtilis glucuronoxylan xylanohydrolase digestion of MX and VX, respectively; -CR, poly- or oligosaccharides in which carboxyl groups in uronosyl residues and the hemiacetal at the reducing end is reduced; -A, poly saccharides in which arabinosyl residues are partially hydrolyzed; -OH, poly- or oligosaccharides whose hemiacetals at the reducing ends are reduced.

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**EXPERIMENTAL PROCEDURES**

RESULTS AND DISCUSSION

**Substrate Specificity**—Feraxanase degraded MX and VX to yield products resolved by Superose 12 (system A) (Fig. 6). An enzymic degradation pattern as a function of time reveals the progression of fragment release (Fig. 7). The appearance of intermediate sized fragments at an early stage of the reaction is consistent with an endo type action pattern. An in situ structural modification of selected xylans altered their susceptibility to feraxanase. Feraxanase degraded xylans known to possess GlcA side chains (MX, MX-A, VX) (Fig. 6). However, xylan derivatives whose GlcA residues had been converted to Glc residues were no longer substrates for the enzyme (MX-CR, MX-A-CR, and VX-CR) (Fig. 6). Modification of other side chain constituents in xylans, i.e. the deletion of most (83%) of the Ara side chain or reduction of the hemiacetal at the reducing end residue, did not influence the enzyme’s ability to act on substrate derivatives. The results demonstrate that GlcA side chains are essential for feraxanase-mediated hydrolysis.

**Recognition Site**—Exhaustive digestion of VX (30 mg) with feraxanase yielded a major oligomer fraction (14.2 mg) designated as VXf fraction (Fig. 2). The GlcA residue and hemiacetal of the reducing end of VXf was reduced to obtain VXf-CR (Table II, Fig. 3). Hydrolysis of VXf-CR with an endo-β-(1→4)-xylanase gave two fragments, VXf-CR-x1 and -x2. These fragments were identified as Xylβ1→4(Glc1α2)xylitol and Xylβ1→4Xylβ1→4Xyl, respectively, from interpretation of fragments prepared after treatment with purified β-xyllosidase (Fig. 4), methylation analyses (Table II), and 13C NMR spectroscopy. Based on these results, VXf was identified as Xylβ1→4Xylβ1→4Xylβ1→4(Glcα1→2)Xylβ1→4Xyl. On the other hand, digestion of MX with feraxanase gave a major oligosaccharide fraction designated as MXf (1.6 ml) was similar to that for MX-OH (6.9 mg/ml). In MX-OH, Xyl residues located between site B and site C were highly substituted with Ara side chains, whereas the Xyl residue between sites A and C was not substituted. On the other hand, VX-OH was not substituted with Ara. These data indicate that the Ara side chains located between sites B and C do not affect the affinity of the enzyme to the polysaccharide. Based on these results, we conclude that the enzyme recognizes GlcA residue (site C) and splits the β-(1→4)-Xyl linkage (site A) of the adjacent unsubstituted Xyl residue located at the reducing end side of the xylan.

**NEW CATEGORY OF XYLANOXYLANS**—Many 1,4-β-d-xylo-β-d-xylanohydrolases (endo-1,4-β-xylanohydrolases, EC 3.2.1.8) have

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*Footnote:* Portions of this paper (including part of “Experimental Procedures,” Figs. 1–5, and Tables I–III) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are included in the microfilm edition of the Journal that is available from Waverly Press.
been isolated and purified from various organisms and classified into one group of the Enzyme Code (12, 13, 15-20). These endo-β-(1→4)-xylanases degrade β-(1→4)-xylosyl linkages comprised of unsubstituted domains of heteroxylans and liberate xylobiose, xylotriose, and higher oligomers (11, 21, 22, 34). Substituents on xylan main chains hinder enzymatic cleavage (10, 17, 21, 34). Thus, highly substituted native heteroxylans, such as glucuronoxylans derived from maize cell walls, were not effectively degraded by endo-β-(1→4)-xylanase (2, 4). No specific cleavage of heteroxylans by a unique endo-β-(1→4)-xylanase has been confirmed.

Frederick et al. (35) purified and characterized two endoxylanases from *Aspergillus niger* and suggested that these xylanases preferentially attack xylan backbones near branch points. The presence of preferential cleavage sites on heteroxylans is reported for other xylanases (11, 22). However, these xylanases degrade β-(1→4)-xylooligomers (degree of polymerization, 7–9) (11, 35) or unsubstituted domains of heteroxylans (22). In addition, major oligomers liberated by action of these xylanases from heteroxylans are xylohomooligomers such as Xylβ1→4Xyl, Xylβ1→4Xylβ1→4Xyl, and Xylβ1→4Xylβ1→4Xylβ1→4XylXyl. Thus, appendages to the basic structure are not a prerequisite for catalysis by these xylanases.

On the contrary, feraxanase does not act on unsubstituted β-(1→4)-xylan (23) but degrades heteroxylans with GlcA side chains. We detect no xylohomooligomers liberated from xylans during feraxanase-mediated hydrolysis (4, 23) (Fig. 2). Therefore, the action of feraxanase is clearly distinguishable from the conventional xylanases classified as 1,4-P-D-xylanohydrolase (EC 3.2.1.8). For a class of glycanases that have an unprecedented specificity for GlcA appendage-dependent glycanase. Since feraxanase has an unprecedent specificity for GlcA1-2(β(1→4)-xylan), it seems appropriate that this novel enzyme be designated glucuronoxylan xylanohydrolase (EC 3.2.1.15).

**Presence of Repeating Structural Units in Heteroxylans**—This study demonstrates for the first time the presence of repeating structural units as major components in xylans derived both from maize and Vigna cell walls; GlcA side chains regularly distributed along β-(1→4)-xylans serve as markers. This finding evokes a consideration of the presence of a regulatory mechanism for processing heteroxylan during synthesis and degradation in plant tissues. The evidence clearly vindicates the usefulness of this enzymic approach as a powerful new tool for specific fragmentation of heteroxylans. The enzymic fragmentation of cell wall macromolecules offers the opportunity for a unique approach for exploring the structure of a major component of plant cell walls.

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**Experimental Procedures**

The Bacillus subtilis enzyme preparation (Novo L-120) was a gift from Novo Industri A/S (Nordborg, Denmark). The enzyme was purified from the Bacillus subtilis enzyme preparation according to a procedure described previously (23). The enzyme was eluted at a flow rate of 1 ml/min and yield was monitored by OD 

**Chemical Modification of Polysaccharides**

Partial hydrolysis of the side chains of the xylose fraction was performed by treatment with 2 M sodium acetate buffer (pH 4.2) at 40°C for 20 h. The hydrolysate was then dialyzed against water and lyophilized. The reduced xylose residues were then converted to the corresponding 2-thiouronium salts for GC analysis. A solution of xylose was prepared and analyzed by GC for comparison. The 2-thiouronium salts were then treated with oxalic acid solution at 60°C for 1 h. After treatment, the resulting solutions were subjected to GC analysis.

**Neutral Sugar Composition**

Neutral sugar compositions of the xylose fraction were determined by GC analysis. The samples were hydrolyzed with 2 M sodium acetate buffer (pH 4.2) at 40°C for 20 h. The hydrolysates were then treated with oxalic acid solution at 60°C for 1 h. After treatment, the resulting solutions were subjected to GC analysis.

**Structural Analysis**

The structural analysis was performed by fragment analysis. The samples were hydrolyzed with 2 M sodium acetate buffer (pH 4.2) at 40°C for 20 h. The hydrolysates were then treated with oxalic acid solution at 60°C for 1 h. After treatment, the resulting solutions were subjected to GC analysis.

**Carbohydrate Analysis**

The carbohydrate content was determined by the phenol-sulfuric acid method (24). The carbohydrate content was measured by the method of Dubois et al. (25). The results are expressed as reducing sugar equivalents.

**Spectral Analysis of Xylose**

Xylose was subjected to fragment analysis. The samples were hydrolyzed with 2 M sodium acetate buffer (pH 4.2) at 40°C for 20 h. The hydrolysates were then treated with oxalic acid solution at 60°C for 1 h. After treatment, the resulting solutions were subjected to GC analysis.
Table III

Neutral sugar compositions and glycosidic linkage compositions of
xylan fragments

| Fragment** | MKK | MkK-C | MkK-CB |
|------------|-----|-------|--------|
| Neutral sugar compositions | mole % | 39.1 | 36.7 | 35.8 |
| Xyl-xylitol | 60.9 | 61.3 | 59.6 |
| Glic | 0 | 0 | 4.3 |
| Assigned glycosidic linkages | mole % | 0 | 17.9 | 8.5 |
| Axy | 36.5 | 34.8 | 35.4 |
| 2(xyl)- | 34.2 | 31.0 | 8.2 |
| 4(xyl)- | 9.4 | 5.4 | 5.1 |
| 2(xyl)- or 3,4xyl- | 35.9 | 36.4 | 17.4 |

** MKK was prepared by digestion of NS with feruloylase, MKK was prepared by reduction of reducing-end of NS, MKK-C was prepared by reduction of both carboxylic groups in such residues and benzoate of the reducing end.

Fig. 1.
Elution profile of the endo-β-(1→4)-xylanase digestion product of VXX-CR when subjected to RPC on Bio Gel P-4 (system B). Carbohydrate was detected by RI. Peak a was identified by xylanase. Peak b was designated VXX-CR-x1-a. Peak c was identified as undigested VXX-CR-x1-a.

Fig. 2.
Elution profile of feruloylase digestion products of NS. NS were treated with feruloylase and then subjected to GPC on Bio Gel P-4 (system B). Fractions were collected and assayed for total sugar content. Axy (open triangles) indicate elution volumes at which Dextran T-500 (Vo) or 1,4-xyloligosaccharides (degree of polymerization is 2 to 81 and glucose appear.

Fig. 3.
Elution profile of MKK and MKK-CB derived from chromatography on a column of Bio Gel P-4. Feruloylase digestion products derived from MK were fractionated by Sepharose CL-2B (system 1). The major fraction was designated MKK-CB. Fractions were collected and assayed for total sugar content. Axy (open triangles) indicate elution volumes for Dextran T-500 (Vo), 1,4-xyl-oligosaccharides of DP 2 to 12 and glucose.