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# Purification and Characterization of a β-D-Xylosidase and an Endo-Xylanase from Wheat Flour[1]

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**A β-D-xylosidase and an endo-xylanase were purified from European wheat (*Triticum aestivum*) flour. The β-D-xylosidase had a molecular weight of approximately 64,000 and an isoelectric point of 5.5. It hydrolyzed *p*-nitrophenyl-β-D-xylopyranoside and xylo-oligosaccharides and released D-xylose units from wheat arabinoxylan and oat spelts xylan. An endo-xylanase with a molecular weight of approximately 55,000 was also obtained and it consisted of a number of isoforms with isoelectric points between 4.0 and 5.0. The action of the isolated endo-xylanase depended on the degree of substitution of the polysaccharide. Unbranched polymers were preferentially hydrolyzed. Since xylo-oligosaccharides were not hydrolyzed, the enzyme appeared to need at least five or more consecutive unsubstituted xylose units. Finally, an α-L-arabinofuranosidase that hydrolyzed *p*-nitrophenyl-α-L-arabinofuranoside was partially purified.**

Hydrolysis of AX, one of the NSPs that makes up plant cell wall material, requires several types of endo- and exo-acting enzymes (Dekker and Richards, 1976). The main chain substituents are liberated by α-L-arabinofuranosidase (EC 3.2.1.55) as α-L-arabinosyl residues. This debranching enzyme assists the depolymerizing 1,4-β-D-xylanhydrolase (EC 3.2.1.8) by creating cleavage sites. The 1,4-β-D-xylanhydrolase, hereafter referred to as endo-xylanase, generates unsubstituted or branched xylooligosaccharides. The mode of action and hydrolysis products of different endo-xylanases vary with the source of the enzymes (Coughlan et al., 1993). Most of the endo-xylanases hydrolyze the main chain linkages at regions where the substrate is not substituted with Ara. β-D-Xylosidase (EC 3.2.1.37) attacks the non-reducing end of xylo-oligosaccharides that are generated by the action of endo-xylanase and liberates Xyl.

NSP-hydrolyzing enzymes of microbial origin have received much attention in recent years, mainly because of their application in the paper pulp (Wong et al., 1988; Nissen et al., 1992), feed (Pettersson et al., 1990; van Paridon et al., 1992), and bread-making industries (McCleary, 1986; Maat et al., 1992; Gruppen et al., 1993; Rouau et al., 1994). The presence of endogenous NSP-hydrolyzing enzymes in wheat (*Triticum aestivum*) have been described in only a limited number of reports (Preece and MacDougall, 1958; Kulp, 1968; Lee and Ronalds, 1972; Schmitz et al., 1974; Bremen, 1981; Adlung, 1985; Moore and Hoseney, 1990; Cleemput et al., 1995b). Recently, Cleemput et al. (1995b) demonstrated the presence of NSP-hydrolyzing enzymes in wheat flour. Low activities of α-L-arabinofuranosidase, β-D-xylosidase, and endo-xylanase were measured in crude extracts of wheat flours, and a progressive depolymerization of WAX, the main NSP in wheat flour, by the flour extracts was observed.

The objective of this work was to study the properties of endogenous wheat flour NSP-hydrolyzing enzymes. To this end, the isolation, purification, and characterization of the wheat enzymes were necessary.

The purification of NSP-hydrolyzing enzymes from wheat has been described in only a few reports. Adlung (1985) isolated and characterized a β-D-xylosidase from whole wheat, and Beldman et al. (1996) recently identified and partially purified some of the arabinoxylanases from wheat bran.

In this study we elaborate on the isolation and purification of an α-L-arabinofuranosidase, a β-D-xylosidase, and an endo-xylanase from the European wheat flour Camp Remy (Cleemput et al., 1995c). Their actions on different substrates were investigated. The hydrolysis products released during incubation were analyzed by GPC and HPAEC. The substrate specificities of the purified wheat flour enzymes were compared with those of similar enzymes of microbial or fungal origin.

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Abbreviations: AX, arabinoxylan; *E*, potential; GPC, gel-permeation chromatography; HPAEC, high-performance anion-exchange chromatography; NSP, nonstarch polysaccharide; OSX, oat spelts xylan; PNP-ara, *p*-nitrophenyl-α-L-arabinofuranoside; PNP-xyl, *p*-nitrophenyl-β-xylopyranoside; RAX, rye arabinoxylan; WAX, wheat arabinoxylan.

## MATERIALS AND METHODS

### Substrates and Chemicals for Enzyme Assays and Incubation Experiments

Substrates for the activity measurements of α-L-arabinofuranosidase and β-D-xylosidase activity were PNP-ara and PNP-xyl (Sigma). Sodium acetate and *p*-nitrophenol were obtained from Merck (Darmstadt, Germany); 3,5-dinitrosalicylic acid was from Aldrich; and Mes (free acid, >99.5%), Trizma base (Tris, reagent grade), and 6-*O*-methyl Gal were from Sigma. Other substrates were WAX (Megazyme, Sydney, Australia), with an Ara/Xyl of 0.55 containing <1.0% Glc, Gal, and Man (measured as described by Cleemput et al., 1993); RAX (Megazyme), with an Ara/Xyl of 0.65 containing 6.5% Man, 2.4% Gal, and 1.5% Glc; and OSX (Sigma) containing 8.4% Ara, 0.9% Gal, and 7.8% Glc. Water-extractable AX with a varying degree of substitution was isolated from wheat (*Triticum aestivum*) flour (Cleemput et al., 1995a). Mono-, di-, and oligosaccharides were obtained from BDH Chemicals (Poole, UK) (L-Ara), Fluka (D-Xyl), and Megazyme (xylobiose, xylotriose, and xylotetraose with purity >95% and xylopentaose with purity >90%).

### Flour

Grain of the wheat cv Camp Remy (1992 harvest) was milled into white flour on a Multomat KOMW0810 mill (Miag, Braunschweig, Germany). The extraction rate was 78% (0.49% flour ash content on a dry matter basis).

### Protein Determination

Protein concentrations were determined according to the Coomassie brilliant blue method of Bradford (1976) with BSA as a standard.

### Enzyme Assay Procedures

#### *Preparation of Enzyme Solutions*

Enzymes were extracted from flour by suspending 1.0 g of flour in 3.0 mL of buffer (50 mM Mes buffer, pH 6.0, for α-L-arabinofuranosidase and β-D-xylosidase activity measurements or 0.1 M sodium acetate buffer, pH 4.8, for endo-xylanase activity measurements). Mixtures were shaken for 15 min at room temperature and centrifuged (3000*g* for 15 min). Supernatants were filtered (0.45 μm) and used for activity measurements. The enzyme solutions of freeze-dried fractions were prepared by dissolving the material in the corresponding buffer.

#### *α-L-Arabinofuranosidase and β-D-Xylosidase Activities*

PNP-xyl or PNP-ara solutions (10 mM) were prepared in 50 mM Mes buffer, pH 6.0. Aliquots of these solutions (100 μL) were incubated with 50 μL of enzyme solution at 40°C. The reaction was stopped after 30 min by adding 1.5 mL of a 1.0% Trizma base solution. In a control assay, Trizma base solution was added before the wheat flour extract. The release of *p*-nitrophenate from the *p*-nitrophenyl-glycoside was determined colorimetrically (410 nm) and the activity was expressed as units of *p*-nitrophenate equivalents released under the experimental conditions. Calibration curves were obtained with *p*-nitrophenol (0–0.05 mM).

#### *Endo-Xylanase Activity*

Endo-xylanase activity was determined by measuring the release of reducing sugars from OSX. The reaction mixture containing 930 μL of the OSX solution (0.5% in 0.1 M sodium acetate buffer, pH 4.8) and 67 μL of the enzyme solution was incubated at 50°C for 60 min with constant stirring. The reaction was stopped by the addition of 1.0 mL of dinitrosalicylic acid solution (1.0 g of 3,5- dinitrosalicylic acid plus 30.0 g of sodium potassium tartrate in 100 mL of 0.4 N NaOH) and boiling for 5 min. After the sample was centrifuged (10,000*g* for 15 min), the colored reaction products were quantified colorimetrically (540 nm). Results are expressed as micromoles of Xyl (Miller, 1959). Calibration curves were determined with Xyl (0.0–0.2 mM). One unit of endo-xylanase activity is defined as the amount of enzyme required to release 1 μmol Xyl $min^{-1}$ under the experimental conditions.

### Enzyme Purification

#### *Step 1. Preparation of a Crude Enzyme Extract from Flour*

Wheat flour (3300 g) was suspended in 9900 mL of 0.1 M sodium phosphate buffer (pH 7.0) and stirred for 30 min. The supernatant, obtained after centrifugation (10,000*g* for 30 min at 4°C) and filtration of the mixture, was dialyzed (3500 $M_r$ cutoff, Spectra/Por 3 membranes, Spectrum, Houston, TX) for 48 h at 4°C against deionized water and lyophilized. The lyophilized material obtained is hereafter referred to as crude enzyme extract.

#### *Step 2. Gradual Ammonium Sulfate Precipitation*

All steps were performed at 4°C. Crude enzyme extract (60.0 g) was dissolved in 1700 mL of 0.1 M sodium phosphate buffer (pH 7.0), and solid ammonium sulfate was slowly added to reach 30% saturation. The solution was stirred for 30 min and left overnight. The precipitate was separated from the above solution by centrifugation (10,000*g* for 30 min), dissolved in deionized water, dialyzed for 48 h (3500 $M_r$ cutoff), and lyophilized. This material is hereafter referred to as AS 0–30. The supernatant fraction was adjusted to 70% saturation in a similar manner, yielding the fraction AS 30–70.

#### *Step 3. Anion-Exchange Chromatography on a Q-Sepharose High-Performance Column*

Fraction AS 30–70 was further fractionated by anion-exchange chromatography on a Q-Sepharose high performance column (HP 35/100, 35 × 100 mm, Pharmacia). A sample (2.0 g) of this fraction was dissolved in 300 mL of 20 mM Tris buffer (pH 8.0, buffer A) and dialyzed (3500 $M_r$ cutoff) overnight against deionized water at 4°C. The volume was adjusted to 400 mL with buffer A, and the mixture

was centrifuged (20,000*g* for 30 min at 4°C). Part of the resulting supernatant (250 mL) was applied to the column (5.0 mL/min flow rate). It was then washed with 5 column volumes of buffer A (5.0 mL/min). Bound proteins were subsequently eluted with a stepwise gradient of buffer B (20 mM Tris-HCl, pH 8.0, plus 1.0 M NaCl) in buffer A as illustrated in Figure 1. Fractions (10.0 mL) were collected and assayed for β-D-xylosidase, α-L-arabinofuranosidase, and endo-xylanase activities.

This experiment was repeated six times and fractions containing glycosidase activity (β-D-xylosidase and α-L-arabinofuranosidase activity) and endo-activity were pooled, dialyzed for 48 h against deionized water at 4°C, and lyophilized, yielding the fractions AEC I (glycosidase activity) and AEC II (endo-xylanase activity) (Fig. 1).

*Step 4. Purification of β-D-Xylosidase and α-L-Arabinofuranosidase Activity by Hydrophobic Interaction and Cation- and Anion-Exchange Chromatography*

Fraction AEC I, which contained both β-D-xylosidase and α-L-arabinofuranosidase activities, was further fractionated by hydrophobic interaction chromatography on a Phenyl Superose column (HR 5/5, 5 × 50 mm, Pharmacia). An aliquot of fraction AEC I (20.0 mg) was dissolved in 5.5 mL of 50 mM sodium phosphate buffer (pH 7.0) containing 1.2 M ammonium sulfate and centrifuged (5000*g* for 15 min). Injection of 5.0 mL of the resulting supernatant was followed by formation of a 60.0-mL linear gradient from 1.2 to 0.0 M ammonium sulfate with a flow of 0.5 mL/min. Fractions (1.0 mL) were collected and assayed for β-D-xylosidase and α-L-arabinofuranosidase activities. Appropriate fractions, resulting from six consecutive separations, were dialyzed (48 h at 4°C) against 50 mM sodium acetate buffer (pH 5.5), yielding fractions HIC Ia, which contained α-L-arabinofuranosidase activity, and HIC Ib, which displayed β-D-xylosidase and α-L-arabinofuranosidase activities.

Cation-exchange chromatography on a Mono-S column (HR 5/5, 5 × 50 mm, Pharmacia) was used subsequently to remove a large part of the contaminating proteins. Aliquots (10.0 mL) of fractions HIC Ia and Ib were applied to the column using 50 mM sodium acetate (pH 5.5) as an eluent (0.5 mL/min flow rate). The pass-through fractions (Mono-S Ia and Ib) containing the activities were pooled and dialyzed against 20 mM Tris-HCl (pH 8.5). After dialysis for 20 h at 4°C, a final purification step was performed with the fraction containing α-L-arabinofuranosidase and the fraction containing both β-D-xylosidase and α-L-arabinofuranosidase activities. An anion-exchange chromatography column (Mono-Q HR 5/5, 5 × 50 mm, Pharmacia) was equilibrated with 20 mM Tris, pH 8.5, and fractions (10.0 mL) were applied to the column (0.5 mL/min flow rate). The adsorbed proteins were eluted from the column in a 14.0-mL linear gradient of 0 to 0.5 M NaCl, followed by a 5.0-mL linear gradient of 0.5 to 1.0 M NaCl. Fractions (Mono-Q Ia and Ib) containing β-D-xylosidase and/or α-L-arabinofuranosidase activities were used for incubation experiments.

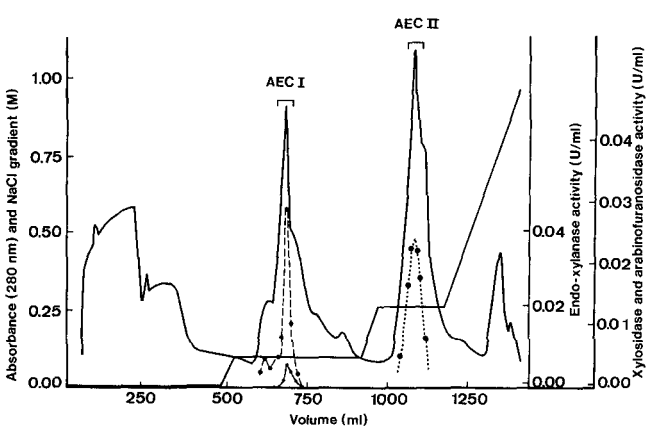


**Figure 1.** Q-Sepharose anion-exchange chromatogram of fraction AS 30–70 eluted at 5.0 mL/min with 20 mM Tris (pH 8.0) and a sodium chloride gradient (——). The eluate was monitored at 280 nm (—) and fractions were assayed for α-L-arabinofuranosidase (——), β-D-xylosidase (— — —), and endo-xylanase activities (· · ·). U, Units.

*Step 5. Purification of Endo-Xylanase Activity by Hydrophobic Interaction and Anion-Exchange Chromatography*

Hydrophobic interaction chromatography was performed with fraction AEC II (containing endo-xylanase activity). This separation step was performed as described for the purification of both the β-D-xylosidase and α-L-arabinofuranosidase activities, except that a 50 mM sodium phosphate buffer (pH 7.0) containing 0.6 M ammonium sulfate was used as the starting buffer. The linear gradient was from 0.6 to 0.24 M in 15.0 mL. Fractions resulting from 10 separations and containing endo-xylanase activity (HIC II) were pooled, dialyzed (48 h at 4°C) against 20 mM bis Tris (pH 6.5), and applied to an anion-exchange column (Mono-Q HR 5/5, 5 × 50 mm, Pharmacia) pre-equilibrated with the buffer (0.5 mL/min flow rate). The column was further eluted with a 12.0-mL linear gradient from 0 to 0.5 mM NaCl and a final step from 0.5 to 1.0 M NaCl in 5.0 mL. Fractions (1.0 mL) were collected and assayed for endo-xylanase activity. The active fractions (Mono-Q II) were used for incubation experiments.

## SDS-PAGE and IEF

The molecular weights of purified enzymes were determined by SDS-PAGE on 12.5 or 20.0% polyacrylamide gels under nonreducing conditions with the PhastSystem unit (Pharmacia), according to the method of Laemmli (1970). The gels were silver-stained according to the instructions of the manufacturer (Biotechnology, Development Technique File no. 210, Pharmacia LKB). Low-molecular-weight range (2,350–46,000) and high-molecular-weight range (14,000–200,000) rainbow-colored protein molecular weight markers (Amersham) were used for calibration.

The pIs of the enzymes were determined with the PhastSystem unit using polyacrylamide gels containing ampholytes (pH 3.0–9.0). The proteins were silver-stained and appropriate standards (Pharmacia calibration kits, pI 3.5–9.3) were used.

### Hydrolysis by the Purified Enzymes

Hydrolysis of the different substrates with the purified β-D-xylosidase and endo-xylanase was examined by incubation of the oligo- and polysaccharides with the obtained enzyme fractions and characterization of the hydrolysis products by GPC and HPAEC. Since the pH and temperature optima of the purified enzymes could not be measured because of a lack of material, a pH of 5.0 and a temperature of 30°C were chosen as incubation conditions for both enzymes. For the three polysaccharides under investigation, controls were performed with a solution of the polysaccharide and buffer instead of the enzyme solution to check the nonenzymatic disintegration of the substrate under the experimental conditions.

#### *Hydrolysis of Xylo-Oligosaccharides, OSX, and WAX by β-D-Xylosidase*

Xylobiose, xylotriose, xylotetraose, and xylopentaose were dissolved in 50 mM sodium acetate buffer (pH 5.0, 0.02% sodium azide, 2.0 mg/mL). An aliquot (1.0 mL) of this solution was incubated at 30°C for 72 h with 100 μL of the active fraction (Mono-Q Ib) obtained after anion-exchange chromatography. Samples (100 μL) were withdrawn after 0, 8, 24, 32, 48, 56, and 72 h and heated at 100°C for 10 min. The resulting samples were subjected to HPAEC after addition of 900 μL of 50 mM sodium acetate buffer (pH 5.0, 0.02% sodium azide) and centrifugation (10,000*g* for 15 min).

The polysaccharide hydrolysis by purified β-D-xylosidase was examined by incubation of 6.0 mL of a solution of OSX (5.0 mg/mL) and 6.0 mL of WAX (5.0 mg/mL) with 600 μL of the enzyme fraction (Mono-Q Ib). Samples were withdrawn as described above and examined by GPC and HPAEC.

#### *Hydrolysis of Xylo-Oligosaccharides, OSX, Wheat, and RAX by Endo-Xylanase*

Incubation of the purified endo-xylanase with xylo-oligosaccharides was performed as described for xylosidase. OSX, WAX, and RAX were used as polysaccharide substrates, and incubation and analysis of the samples were as described for β-D-xylosidase.

### GPC

Changes in the apparent molecular weight distributions of the AX substrates during incubation with the purified enzymes were studied by GPC. Samples of reaction mixtures were separated on a Shodex B-806 column (50 × 0.8 cm, Showa Denko, Tokyo, Japan) that was equipped with a precolumn (5 × 0.6 cm) and eluted with 50 mM sodium acetate, pH 5.0 (1.0 mL/min at 30°C). The eluate was monitored using an ERC-7510 refractive index detector (Erma, Tokyo, Japan). Molecular weight markers were dextran (1.0 mg/mL), with a molecular weight range of 40 × $10^6$ to 5 × $10^6$, and Shodex standard P-82 pullulan (1.0 mg/mL), with molecular weights of 8.53 × $10^5$, 3.80 × $10^5$, 1.86 × $10^5$, 1.0 × $10^5$, 4.80 × $10^4$, 2.37 × $10^4$, 1.22 × $10^4$, and 0.58 × $10^4$.

### HPAEC

The release of Ara, Xyl, and Ara- and Xyl-containing oligosaccharides during incubation of (arabino-) xylan with the purified enzymes was investigated by HPAEC. Samples of the reaction mixtures (30 μL) were injected onto a Dionex CarboPac PA-1 column (4 × 250 mm, Dionex, Sunnyvale, CA) with a CarboPac PA-1 column (4 × 50 mm). Elution was with 14 mM NaOH for 15 min (1 mL/min), followed by a 5-min linear gradient of 14 to 100 mM NaOH and a final gradient of 0 to 350 mM $CH_3COONa$ in 100 mM NaOH (35 min). The eluate was monitored using a pulsed-electrochemical detector (Dionex) in the pulsed-amperiometric detection mode with the following pulse potentials and durations: $E_1$, 0.1 V and 0.5 s; $E_2$,0.6 V and 0.1 s; and $E_3$, −0.6 V and 0.1 s. The internal standard was 6-*O*-methyl-Gal (100 μg/mL sample). A mixture of Ara, Xyl, xylobiose, xylotriose, xylotetraose, and xylopentaose (0.025–0.050 mg/mL) was used as the standard. The concentrations of mono- and oligosaccharides were calculated and expressed in microgram/milliliter of sample, although the impurity of the oligosaccharides results in small errors. The concentrations of the unknown hydrolysis products were estimated by making use of arbitrary response factors. The latter are the response factors of the last identified product eluting before the product under investigation. These concentrations are further referred to as concentrations in arbitrary units.

## RESULTS

### Enzyme Purification

Three endogenous wheat NSP-hydrolyzing enzymes (i.e. an α-L-arabinofuranosidase, a β-D-xylosidase, and an endo-xylanase) were purified from cv Camp Remy wheat flour

**Table I.** *Purification of α-L-arabinofuranosidase from wheat flour*

| Purification Step | Total Protein | Total Activity | Specific Activity | Purification | Recovery |
|---|---|---|---|---|---|
| | *mg* | *units* | *milliunits/mg* | *-fold* | *%* |
| Flour | 269,387 | 20.11 | 0.08 | 1 | 100 |
| Crude extract | 24,660 | 45.37 | 1.84 | 23 | 226 |
| AS 30-70 | 10,247 | 15.37 | 1.50 | 19 | 76 |
| AEC I | 482 | 30.05 | 6.32 | 79 | 15 |
| HIC Ia | 38 | 1.08 | 29.3 | 366 | 5 |
| Mono-S Ia | 4.37 | 0.53 | 123 | 1538 | 3 |
| Mono-Q Ia | 2.47 | 0.44 | 173 | 2163 | 2 |

**Table II.** *Purification of β-D-xylosidase from wheat flour*

| Purification Step | Total Protein | Total Activity | Specific Activity | Purification | Recovery |
|---|---|---|---|---|---|
| | *mg* | *units* | *milliunits/mg* | *-fold* | *%* |
| Flour | 269,387 | 113.88 | 0.42 | 1 | 100 |
| Crude extract | 24,660 | 121.82 | 4.94 | 12 | 107 |
| AS 30-70 | 10,247 | 67.73 | 6.61 | 16 | 60 |
| AEC I | 482 | 20.34 | 42.2 | 101 | 18 |
| HIC Ib | 31 | 13.15 | 431 | 1026 | 12 |
| Mono-S Ib | 14.51 | 6.04 | 416 | 991 | 5 |
| Mono-Q Ib | 2.78 | 5.31 | 1910 | 4548 | 5 |

by a multiple-step purification procedure using extraction of the flour, ammonium sulfate precipitation, anion-exchange, hydrophobic interaction, and cation- and/or anion-exchange chromatography.

*Extraction, Ammonium Sulfate Precipitation, and Anion-Exchange Chromatography*

Extraction of the flour with the phosphate buffer resulted in a large increase in specific activity for the glycosidases (Tables I and II) and a 3-fold purification for the endo-xylanase (Table III). Fractionation of the crude enzyme extract by precipitation with ammonium sulfate concentrated the activities in the AS 30–70 fraction. Subsequent anion-exchange chromatography resulted in the separation of the glycosidase (fraction AEC I) and endo-xylanase activities (fraction AEC II), as demonstrated in Figure 1. The β-D-xylosidase activity eluted with the α-L-arabinofuranosidase activity at 0.1 M NaCl, and the endo-xylanase activity eluted at 0.25 M NaCl. The other fractions did not show any of the three activities. Arabinofuranosidase and β-D-xylosidase activities were enriched 4- and 6-fold, respectively, by this separation technique. No significant activity enrichment for endo-xylanase was found. The latter can be explained by the separation of the glycosidase activities from the endo-xylanase activity by the anion-exchange chromatography step. The hydrolysis of xylan by the endo-xylanase in fraction AEC II without the cooperative action of the glycosidases, therefore, becomes more difficult.

*Partial Purification of α-L-Arabinofuranosidase and Purification of β-D-Xylosidase*

It was possible to partly separate the α-L-arabinofuranosidase activity from the β-D-xylosidase activity by hydrophobic interaction chromatography of fraction AEC I. The α-L-arabinofuranosidase activity eluted in two peaks, partly before and partly together with the β-D-xylosidase activity. This purification step was very effective in increasing the specific activity of the glycosidases.

Cation-exchange chromatography of fractions HIC Ia and Ib on Mono-S was used to adsorb the contaminating proteins onto the column. The activity in pass-through fractions bound to a Mono-Q column and were eluted in one fraction. The α-L-arabinofuranosidase activity was finally enriched approximately 2000-fold with a yield of about 2%. The fraction containing the β-D-xylosidase activity also showed activity against PNP-ara (specific activity: 107.5 milliunits/mg protein). It seems that this α-L-arabinofuranosidase activity is a side activity of the β-D-xylosidase because it proved to be impossible to separate the two activities by the chromatographic methods that we used. The β-D-xylosidase activity was enriched approximately 4500-fold, with an overall recovery of approximately 5%.

*Purification of Endo-Xylanase*

Hydrophobic interaction chromatography of fraction AEC II contaminated proteins and resulted in a 6-fold increase in specific activity. The active fractions were concentrated using a Mono-Q column and, overall, the endo-xylanase was 15-fold purified, with a yield of about 0.2%.

**Purity, Molecular Mass, and pI**

Purified β-D-xylosidase migrated as a single protein band on SDS-PAGE under nonreducing conditions. It had an apparent $M_r$ of 64,000. Figure 2a shows that it had a high degree of purity. The pI of the enzyme, estimated by IEF, was 5.5 (Fig. 2c).

The homogeneity of the purified endo-xylanase was confirmed by SDS-PAGE (Fig. 2b), with which a single band of

**Table III.** *Purification of endo-xylanase from wheat flour*

| Purification Step | Total Protein | Total Activity | Specific Activity | Purification | Recovery |
|---|---|---|---|---|---|
| | *mg* | *units* | *milliunits/mg* | *-fold* | *%* |
| Flour | 269,387 | 1592.08 | 5.91 | 3 | 100 |
| Crude extract | 24,660 | 453.74 | 18.4 | 3 | 28 |
| AS 30-70 | 10,247 | 166.00 | 16.2 | 3 | 10 |
| AEC II | 1,053 | 12.00 | 11.4 | 2 | 0.8 |
| HIC II | 103 | 6.75 | 65.5 | 11 | 0.4 |
| Mono-Q II | 41 | 3.51 | 85.6 | 15 | 0.2 |

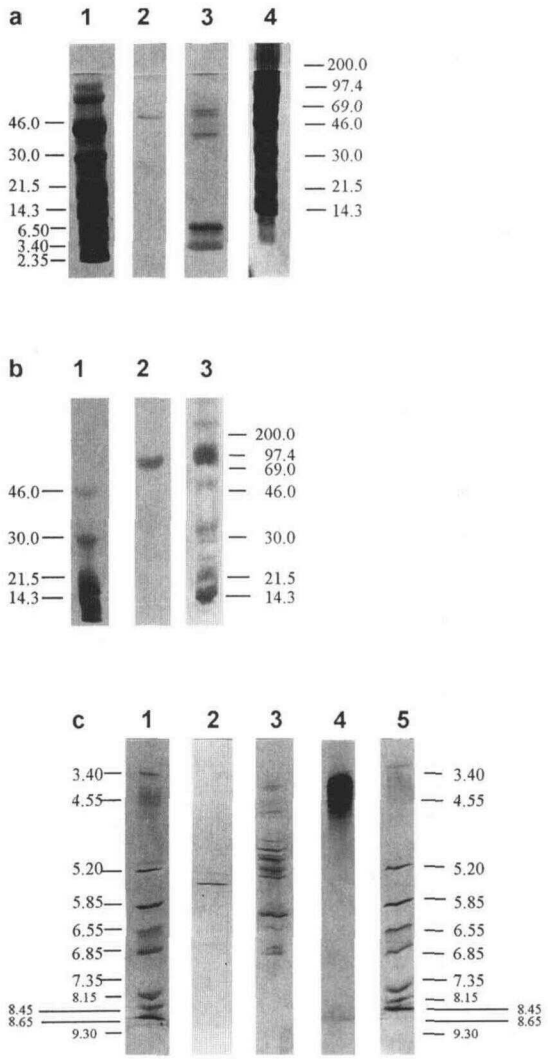


**Figure 2.** a, SDS-PAGE (20% polyacrylamide) of the purified enzymes. Lane 1, Low $M_r$ marker; lane 2, β-D-xylosidase; lane 3, α-L-arabinofuranosidase; and lane 4, high $M_r$ marker. b, SDS-PAGE electrophoresis (12.5% polyacrylamide) of the purified enzymes. Lane 1, Low $M_r$ marker; lane 2, endo-xylanase; and lane 3, high $M_r$ marker. c, IEF of the purified enzymes. Lane 1, Marker; lane 2, β-D-xylosidase; lane 3, α-L-arabinofuranosidase; lane 4, endo-xylanase; and lane 5, marker.

approximately $M_r$ 55,000 was found. IEF showed a number of protein bands with pIs between 4.0 and 5.0 (Fig. 2c).

The fraction with α-L-arabinofuranosidase activity contained a number of protein bands when analyzed by SDS-PAGE. The approximate $M_r$ values were 6,000, 8,000, 40,000, and 65,000. Unfortunately, it was not possible to identify the α-L-arabinofuranosidase. The IEF gel also showed a range of protein bands between pI 5.0 and 7.0. The α-L-arabinofuranosidase activity was, therefore, not purified to homogeneity.

## Substrate Specificity

The specificities of the purified endogenous wheat NSP-hydrolyzing enzymes toward xylo-oligosaccharides, OSX, RAX, and WAX with varying degrees of substitution were examined.

### *Substrate Specificity of β-D-Xylosidase*

The hydrolysis by the purified endogenous wheat β-D-xylosidase of xylo-oligosaccharides of different degrees of polymerization and polysaccharides of different degrees of substitution was investigated. All xylo-oligosaccharides that were studied were partly hydrolyzed (Table IV), with a consistent release of Xyl and intermediate oligosaccharides. Under the experimental conditions, most of the hydrolysis occurred in the first 8 h of incubation. The enzyme was possibly inactivated or there may have been an inhibition by Xyl (Dekker, 1983; Rodionova et al., 1983; Poutanen and Puls, 1988). The enzymatic hydrolysis of xylotriose resulted in the release of xylobiose and Xyl. From the data it is not clear whether the formed xylobiose was further hydrolyzed. Because after 8 h more Xyl was released than xylobiose, a possible breakdown of xylobiose occurred during the latter period. The concentrations of the hydrolysis products formed did not, however, correspond to the concentrations of xylotriose that were hydrolyzed. The hydrolysis products of xylotetraose were xylotriose, Xyl, and low levels of xylobiose. The concentrations of Xyl that were detected were very low when compared with those of xylotriose that were formed and xylotetraose that were hydrolyzed. This observation is, at present, not understood. The same was noted when xylopentaose was

**Table IV.** *Concentrations (μg/mL) of D-Xyl, xylobiose ($X_2$), xylotriose ($X_3$), xylotetraose ($X_4$), and xylopentaose ($X_5$) released during incubations of xylo-oligosaccharides with β-D-xylosidase*

Experimental conditions are as described in the text.

| Substrate | Time | D-Xyl | $X_2$ | $X_3$ | $X_4$ | $X_5$ |
|---|---|---|---|---|---|---|
| | *h* | | | | | |
| $X_2$ | 0 | 56.5 | 1597.2 | 6.0 | –[a] | – |
| | 8 | 636.7 | 1296.5 | 16.1 | – | – |
| $X_3$ | 0 | 18.8 | 189.6 | 2706.4 | 16.2 | – |
| | 8 | 160.1 | 304.1 | 2109.3 | 18.6 | – |
| $X_4$ | 0 | 15.2 | 81.5 | 43.0 | 1832.3 | 0.6 |
| | 8 | 162.6 | 112.1 | 430.2 | 1334.6 | 7.0 |
| $X_5$ | 0 | 12.3 | 127.8 | 58.0 | 318.9 | 1710.5 |
| | 8 | 115.6 | 99.9 | 109.2 | 577.6 | 1201.6 |

[a] –, Not detected.

**Table V.** *Concentrations (μg/mL) of L-Ara and D-Xyl released by incubation of WAX and OSX with β-D-xylosidase*

Experimental conditions are as described in the text.

| Substrate | Time | L-Ara | D-Xyl |
|---|---|---|---|
| | *h* | | |
| WAX | 0 | 0.57 | 0.48 |
| | 8 | 0.75 | 12.74 |
| OSX | 0 | 0.45 | 0.61 |
| | 8 | 0.48 | 16.10 |

used as a substrate. Here, xylotetraose was the main hydrolysis product and only small concentrations of xylotriose and Xyl were formed. These data show that the purified wheat flour β-D-xylosidase is able to attack xylodimers, trimers, tetramers, and pentamers.

The pure β-D-xylosidase was also incubated with OSX and WAX. Whereas the control samples did not release any low-molecular-weight components, Xyl was released from both substrates by β-D-xylosidase but in higher concentration from OSX than from WAX (Table V). This indicates that the Ara branches in AX hinder the enzyme. Despite the α-L-arabinofuranosidase activity that was found to be associated with this enzyme, only very low levels of Ara were released during incubation. This demonstrates that, in contrast to its activity on PNP-ara, the enzyme can hardly debranch the xylan polymer and does not assist in the hydrolysis of OSX and WAX. In agreement with what was observed when incubating xylo-oligosaccharides (above), the enzymatic action also stopped between 0 and 8 h of incubation.

The gel-permeation profiles of the polysaccharides at different stages of the incubation showed no clear shift in molecular weight (results not shown), indicating that the wheat β-D-xylosidase attacks at either the reducing or nonreducing end of the substrate and, therefore, can be identified as an exo-acting enzyme.

### *Substrate Specificity of Endo-Xylanase*

Wheat flour endo-xylanase, purified as described before, was unable to hydrolyze any of the xylo-oligosaccharides that were used (results not shown). On the other hand, the gel-permeation profiles that were obtained by incubation of OSX, RAX, and WAX with this enzyme (Fig. 3) showed a shift in the molecular weight of the polysaccharide, and OSX was hydrolyzed to the greatest extent, as detected by HPAEC (Tables VI, VII, and VIII). Apart from the low levels of Xyl and Ara, xylo-oligosaccharides (xylotriose, xylotetraose, and xylopentaose) were released in high concentrations, together with other hydrolysis products that could not be identified (blank samples did not show any release of these products). One particular product, eluting between xylotriose and xylotetraose (retention time of 27.5 min), was detected in high concentrations. The hydrolysis of RAX also resulted in the release of Ara, Xyl, xylotriose, and some other oligosaccharides. WAX was a poor substrate for this endo-xylanase, because none of the xylo-oligosaccharides formed from RAX were detected and only low levels of Ara, Xyl, and other products were released.

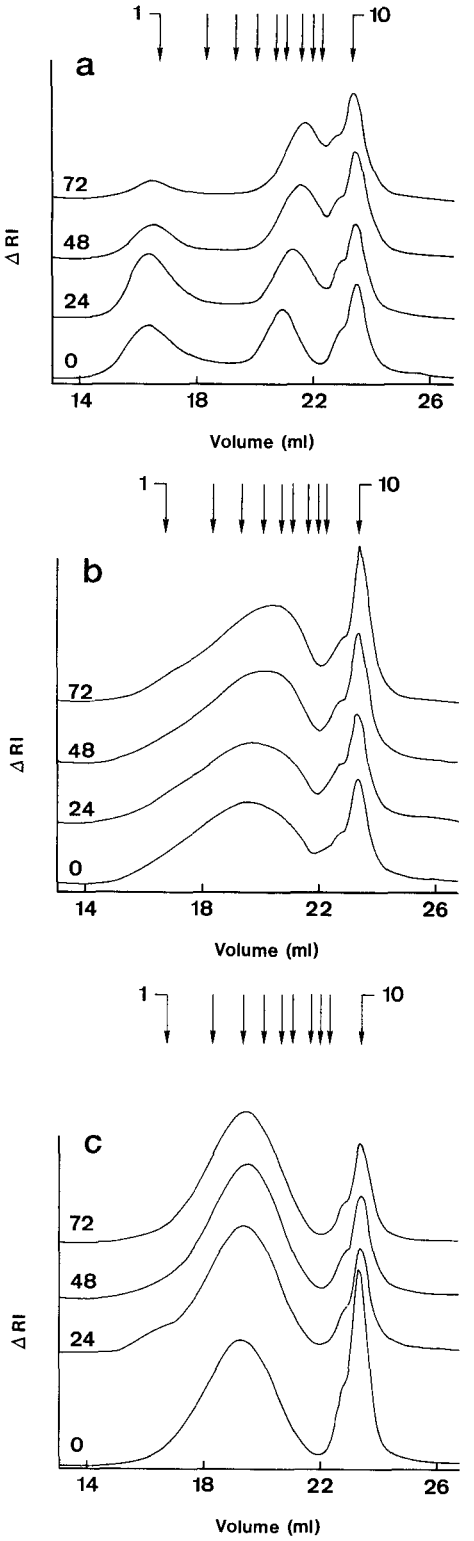


**Figure 3.** Gel-permeation profiles on a Shodex column of an incubation mixture of OSX (a), RAX (b), and WAX (c) with endo-xylanase at 0, 24, 48, and 72 h. The eluate was monitored by refractive index (RI) detection. Elution volumes of dextran ($40 \times 10^6$ to $5 \times 10^6$), pullulan standards of molecular weight $8.53 \times 10^5$, $3.80 \times 10^5$, $1.86 \times 10^5$, $1.0 \times 10^5$, $4.80 \times 10^4$, $2.37 \times 10^4$, $1.22 \times 10^4$, $0.55 \times 10^4$, and Xyl are indicated by numbers (1 through 10, respectively).

**Table VI.** *Concentrations (μg/mL) of L-Ara, D-Xyl, or arbritary concentrations[a] (μg/mL) of other hydrolysis products (indicated by retention time; min) released during the incubation of WAX with endo-xylanase*

Experimental conditions are as described in the text.

| Substrate | Time | L-Ara | 10.9[a] | 13.6[a] | D-Xyl | 25.5[a] |
|---|---|---|---|---|---|---|
| | *h* | | | | | |
| WAX | 0 | 0.78 | –[b] | – | – | – |
| | 8 | 0.78 | – | 0.41 | – | – |
| | 24 | 0.83 | – | 0.56 | 0.09 | – |
| | 32 | 0.97 | 0.07 | 0.72 | 0.15 | – |
| | 48 | 0.94 | 0.08 | 0.81 | 0.16 | 0.15 |
| | 56 | 0.97 | 0.12 | 0.86 | 0.19 | 0.16 |
| | 72 | 1.04 | 0.13 | 0.91 | 0.14 | 0.20 |

[a] Arbitrary concentrations were calculated with the response factor of the preceding identified product. [b] –, Not detected.

The difference in hydrolysis between WAX and RAX was quite surprising because the latter polysaccharide contains more branched zones. The RAX possibly has more Ara units linked 1,3-α and 1,2-α to the same Xyl unit but at the same time has more open segments of unsubstituted Xyl units. To our knowledge, however, the presence of relatively open segments of unsubstituted Xyl units in RAX has not been documented. The above results were also suggested by the fact that the release of the hydrolysis products from WAX were only found after several hours of incubation and, therefore, also after some release of Ara, whereas the hydrolysis of RAX and OSX seems to occur from the beginning of incubation.

We conclude that this purified endo-xylanase has no affinity toward oligosaccharides but clearly shows endo-acting activity toward OSX and RAX and to a lesser degree toward WAX.

## DISCUSSION

A β-D-xylosidase with an $M_r$ of approximately 64,000 and a pI of 5.5 was isolated from flour and purified more than 4000-fold by ammonium sulfate precipitation, anion-exchange, hydrophobic interaction, and cation- and anion-exchange chromatography. This enzyme hydrolyzed the synthetic substrate PNP-xyl and xylo-oligosaccharides (from xylobiose to xylopentaose) and released Xyl units from WAX and OSX. Some previously described microbial β-D-xylosidases have transferase activity (Deleyn et al., 1982; Rodionova et al., 1983; Matsuo and Yasui, 1984; Desphande et al., 1986). Only very low concentrations of xylo-oligosaccharides with a higher degree of polymerization than the original substrate were found during incubation of β-D-xylosidase with xylo-oligosaccharides. After purification the enzyme still displayed activity toward PNP-ara, in agreement with what has been reported for β-D-xylosidases purified from *Penicillium wortmani* (Deleyn et al., 1982), *Aspergillus niger* (Rodionova et al., 1983), and *Trichoderma reesei* (Poutanen and Puls, 1988).

The β-D-xylosidase purified from wheat flour differs in $M_r$ and pI from the β-D-xylosidase purified from whole wheat (Adlung, 1985). That 56.2-kD enzyme with a pI of 6.38 released D-Xyl from water-extractable pentosans, OSX, xylobiose, and xylotriose. A β-D-xylosidase purified from wheat bran (Beldman et al., 1996) was only active toward xylo-oligosaccharides.

Apart from the β-D-xylosidase, an α-L-arabinofuranosidase capable of hydrolyzing PNP-ara was partially purified by following the same purification scheme. This enzyme differs from the AX arabinofuranohydrolase purified from *Aspergillus awamori* by Kormelink et al. (1991). The latter enzyme released terminal, nonreducing α-L-arabinofurano-side residues from AX but showed no activity toward PNP-ara. In the classification of Kaji (1984), the α-L-arabinofuranosidase partially purified from wheat flour as described above may fit in the group of the *A. niger* type of α-L-arabinofuranosidases. However, further purification and more details regarding the mode of ac-

**Table VII.** *Concentrations (μg/mL) of L-Ara, D-Xyl, and xylotroise, ($X_3$), or arbritary concentrations[a] (μg/mL) of other hydrolysis products (indicated by retention time; min) released during the incubation of RAX with endo-xylanase*

Experimental conditions are as described in the text.

| Substrate | Time | L-Ara | 13.6[a] | D-Xyl | 15.8[a] | $X_3$ | 27.7[a] |
|---|---|---|---|---|---|---|---|
| | *h* | | | | | | |
| RAX | 0 | 0.85 | 0.45 | 0.12 | 0.22 | –[b] | 2.42 |
| | 8 | 0.86 | 1.63 | 0.48 | 0.38 | – | 3.43 |
| | 24 | 0.86 | 2.17 | 0.49 | 0.30 | 0.40 | 3.66 |
| | 32 | 0.96 | 2.58 | 0.62 | 0.47 | 0.44 | 3.69 |
| | 48 | 1.04 | 2.94 | 0.63 | 0.49 | 0.46 | 3.73 |
| | 56 | 1.13 | 3.08 | 0.65 | 0.51 | 0.50 | 3.76 |
| | 72 | 1.27 | 3.51 | 0.72 | 0.55 | 0.59 | 4.16 |

[a] Arbitrary concentrations were calculated with the response factor of the preceding identified product. [b] –, Not detected.

**Table VIII.** *Concentrations (μg/mL) of L-Ara, D-Xyl, xylotriose ($X_3$), xylotetraose ($X_4$), and xylopentaose ($X_5$) or arbritary concentrations[a] (μg/mL) of other hydrolysis products (indicated by retention time; min) released during incubation of OSX with endo-xylanase*

Experimental conditions are as described in the text.

| Substrate | Time | L-Ara | 13.4[a] | D-Xyl | $X_3$ | 27.5[a] | $X_4$ | 29.8[a] | $X_5$ | 32.2[a] |
|---|---|---|---|---|---|---|---|---|---|---|
| | *h* | | | | | | | | | |
| OSX | 0 | 0.50 | –[b] | – | – | 58.24 | – | – | – | – |
| | 8 | 0.56 | 0.24 | 0.06 | 0.36 | 132.42 | – | – | – | – |
| | 24 | 0.80 | 0.79 | 0.16 | 0.89 | 129.91 | 0.79 | – | 0.61 | 0.30 |
| | 32 | 0.94 | 1.09 | 0.24 | 1.54 | 140.76 | 1.13 | – | 1.08 | 0.60 |
| | 48 | 1.14 | 1.56 | 0.36 | 2.05 | 132.25 | 2.15 | – | 1.66 | 0.97 |
| | 56 | 1.28 | 1.82 | 0.45 | 2.57 | 134.56 | 3.13 | 0.40 | 2.16 | 1.18 |
| | 72 | 1.57 | 2.39 | 0.63 | 3.86 | 138.84 | 3.65 | 0.80 | 3.94 | 2.02 |

[a] Arbitrary concentrations were calculated with the response factor of the preceding identified product. [b] –, Not detected.

tion of wheat flour α-L-arabinofuranosidase on AX, arabinan, arabinogalactan, and arabino-oligosaccharides would be required to reach a final classification.

Recently, Beldman et al. (1996) characterized a number of α-L-arabinofuranosidases from wheat bran and concluded that these enzymes were active only toward oligosaccharides. An enzyme that is able to release only Ara from polymeric AX has been partially purified by Beldman et al. (1996). The authors suggested a similarity in the substrate specificity of this enzyme with the AX arabinofuranohydrolase purified from *A. awamori* by Kormelink et al. (1991). It therefore seems that none of these α-L-arabinofuranosidases acts similarly to the α-L-arabinofuranosidase described here.

An enzyme with clear endo-activity was purified 15-fold from wheat flour by a four-step fractionation procedure. The wheat flour endo-xylanase, obtained by ammonium sulfate precipitation, anion-exchange chromatography, hydrophobic interaction, and anion-exchange chromatography had an $M_r$ of approximately 55,000. Since multiple protein bands having pIs between 4.0 and 5.0 were found by IEF, this enzyme preparation appeared to be composed of a number of isoforms.

Dekker and Richards (1976) divided the endo-xylanases into those that release Ara from AX and arabinoglucuronoxylan and those that do not release Ara units. An alternative classification by Reilly (1987) characterized the enzymes as either releasing Xyl and xylobiose or releasing larger oligosaccharides. Because the endo-xylanase that we studied here released only very small levels of Ara and Xyl, did not yield any xylobiose, but did release substantial levels of xylo-oligosaccharides with higher degrees of polymerization, it could be classified in the second group of endo-xylanases according to Reilly (1987).

The action of the endo-xylanase isolated from wheat flour depended on the degree of substitution of the polysaccharide. Unbranched polysaccharides were hydrolyzed preferentially. This phenomenon might be explained by the presence of a large substrate-binding site on the enzyme. Since xylo-oligosaccharides such as xylopentaose were not hydrolyzed, the enzyme appeared to require at least five or more unsubstituted Xyl units for hydrolytic action. It is clear that substituted AX needed to be debranched prior to hydrolysis of the backbone. Therefore, the α-L-arabinofuranosidase would likely act synergetically with endo-xylanase.

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