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Identification and Characterization of a Novel Arabinoxylanase from Wheat Flour[1]

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An endogenous wheat (*Triticum aestivum*) flour endoxylanase was purified to homogeneity from a crude wheat flour extract by ammonium sulfate precipitation and cation-exchange chromatography. The 30-kD protein had an isoelectric point of 9.3 or higher. A sequence of 19 amino acids at the NH_2 terminus showed 84.2% identity with an internal sequence of the 15-kD grain-softness protein, friabilin. High-performance anion-exchange chromatography and gel-permeation analysis of the hydrolysis products indicated the preferential hydrolysis of highly branched structures by the enzyme; wheat arabinoxylan and rye (*Secale cereale*) arabinoxylan (high arabinose to xylose ratios) were hydrolyzed more efficiently by this enzyme than oat (*Avena sativa*) spelt xylan (low arabinose to xylose ratios). The release of the hydrolysis products as a function of time suggested that the endoxylanolytic activity was associated with the release of arabinose units from the polysaccharides, suggesting that the enzyme action is similar to that by endoxylanases from *Ceratocystis paradoxa*, *Aspergillus niger*, and *Neurospora crassa*. Although the enzyme released arabinose from arabinoxylan, it did not hydrolyze *p*-nitrophenyl-α-L-arabinofuranoside. From the above, it follows that the enzyme, called arabinoxylanase, differs from most microbial endoxylanases and from an endoxylanase purified earlier from wheat flour.

AX, the main NSP in wheat (*Triticum aestivum*) endosperm, consists of a linear backbone of 1,4-linked β-D-xylopyranose units with L-arabinofuranosyl residues attached to the main chain by 1,3- and/or 1,2-α-glycosidic linkages. Its structure was recently intensively studied using ^{1}H-NMR spectroscopy and methylation analysis (Hoffmann et al., 1991, 1992a, 1992b; Gruppen, 1992a, 1992b, 1993; Cleemput et al., 1993, 1995a, 1995c).

NSP-hydrolyzing enzymes are found in wheat grain (Preece and MacDougall, 1958; Kulp, 1968; Lee and Ronalds, 1972; Schmitz et al., 1974; Bremen, 1981; Adlung, 1985; Moore and Hoseney, 1990), in germinated wheat and wheat bran (Beldman et al., 1996), in barley (*Hordeum vulgare*) aleurone layers (Taiz and Honigman, 1976; Dashek and Chrispeels, 1977; Benjavongkulchai and Spencer, 1986), and in germinated barley (Slade et al., 1989). Recently, evidence was presented for the presence of NSP-hydrolyzing enzymes in wheat flour (Cleemput et al., 1995a, 1995b). Such enzymes, upon germination, modify the cell walls surrounding the starch and protein food reserve. At that time, the NSPs, which form the bulk of the endosperm or aleurone wall matrix (Mares and Stone, 1973a, 1973b; Bacic and Stone, 1981), are solubilized and depolymerized to allow access to the cell reserves.

The purification of a β-D-xylosidase and an endoxylanase from wheat flour has been described previously (Cleemput et al., 1995b, 1997). The action pattern of the enzymes on different poly- and oligosaccharides was studied and compared with that of some bacterial and fungal xylanases. Many endoxylanases of bacterial or fungal origin act more efficiently on unsubstituted xylans (Coughlan and Hazlewood, 1993). This was also true for the endoxylanase isolated from wheat flour that preferentially hydrolyzed unsubstituted 1,4-β-xylans. Xylo-oligosaccharides with a degree of polymerization < 6 were not hydrolyzed; therefore, it appeared that this enzyme required at least five consecutive, unsubstituted Xyl units for its action (Cleemput et al., 1997).

In this paper we describe the identification, purification, and characterization of a second endoxylanase enzyme from wheat flour. GPC and HPAEC of the hydrolysis products released by the purified enzyme from AXs with varying degrees of substitution indicated that the enzyme preferentially hydrolyzes highly substituted AXs.

MATERIALS AND METHODS

Substrates and Chemicals for Enzyme Assay and Incubation Experiments

Wheat (*Triticum aestivum*) flour, WAX, RAX, OSX, L-Ara, D-Xyl, xylo-oligosaccharides, and 6-*O*-methyl Gal were as

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Abbreviations: AX, arabinoxylan; GPC, gel-permeation chromatography; HPAEC, high-performance anion-exchange chromatography; NSP, nonstarch polysaccharides; OSX, oat spelt xylan; RAX, rye arabinoxylan; WAX, wheat arabinoxylan.

described by Cleemput et al. (1997). Dinitro(3,5)-salicylic acid and β-mercaptoethanol were from Aldrich.

Protein Determination and Enzyme Assays

Protein was determined according to the method of Bradford (1976) using BSA as the standard. Endoxylanase activity was determined by measuring the release of reducing sugars from WAX or OSX by enzyme solutions prepared as described by Cleemput et al. (1997). The reaction products were quantified colorimetrically (Miller, 1959). Results for determinations with OSX as a substrate were expressed as micromoles of Xyl. Since Xyl and Ara have different molar color yields, calibration curves were made with mixtures of Ara and Xyl (concentration ratio 1:2, range 0.0–2.0 mM) for the determinations with WAX. One unit of endoxylanase activity was defined as the amount of enzyme required to release 1 μmol Ara/Xyl under the experimental conditions.

Enzyme Purification

Step 1. Preparation of a Crude Enzyme Extract from Flour

A crude enzyme extract was prepared from 1500 g of wheat flour suspended in 3000 mL of 0.1 M sodium phosphate buffer, pH 7.0, as described by Cleemput et al. (1997).

Step 2. Fractional Ammonium Sulfate Precipitation

A solution of crude enzyme extract (20.0 g in 550 mL of 0.1 M ammonium sulfate buffer, pH 7.0) was fractionally precipitated with ammonium sulfate by adding solid ammonium sulfate to 70 and 80% saturation successively (Cleemput et al., 1997), yielding fractions AS 70–80 and AS > 80, respectively. A portion of the last fraction (3.0 g) was dissolved in 0.1 M sodium phosphate buffer (pH 7.0, 500 mL) and fractionated by slowly adding ammonium sulfate to 100% saturation. This suspension yielded a precipitate, fraction AS 80–100, and a final supernatant fraction. It was dialyzed and freeze-dried to give fraction AS >100.

Step 3. Gel-Filtration Chromatography

Fraction AS 80–100 was further purified on a Bio-Gel P-10 column (Bio-Rad). The column (39 × 1.0 cm) was equilibrated and eluted (flow rate of 0.25 mL/min) with 0.1 M sodium acetate buffer, pH 4.8, containing 0.02% sodium azide. Fraction AS 80–100 (10 mg/mL) was dissolved in the same buffer. An aliquot (2.0 mL) was separated on the gel-filtration column. Fractions (1.0 mL) were collected, pooled, dialyzed (M_r cutoff 3500; 48 h; 4°C) against deionized water, and freeze-dried.

Step 4. Cation-Exchange Chromatography

Fraction AS > 80 was fractionated on a Mono-S cation-exchange column (HR 5/5, 5 × 50 mm, Pharmacia). An aliquot of this fraction (35.8 mg) was dissolved in 1.5 mL of 25 mM sodium acetate buffer, pH 4.5 (buffer A), and dialyzed against the buffer (M_r cutoff 3500; 4°C; 20 h). The volume obtained after dialysis was 4.5 mL. An aliquot (1.0 mL) was applied to the column (flow rate of 1.0 mL/min). After 5 min of elution with buffer A, a linear gradient of 0 to 500 mM sodium chloride was formed in 20 min, followed by 5 min of elution with 1.0 M sodium chloride. Fractions (1.0 mL) were collected, and this run was repeated several times. Appropriate fractions were pooled, dialyzed (M_r cutoff 3500; 4°C; 45 h) against deionized water, and freeze-dried.

Preparative Enzyme Purification

A large-scale purification was performed by gradual ammonium sulfate precipitation and cation-exchange chromatography on S-Sepharose. A crude enzyme extract was prepared as described in step 1, starting from 3300 g of flour. This material (60.0 g) was subjected to fractional ammonium sulfate precipitation as described in step 2, except that saturation to 70, 80, and 100% ammonium sulfate was carried out to give fractions AS 70–80_b, AS 80–100_b, and AS > 100_b, respectively. Fraction AS 80–100_b was fractionated preparatively on an S-Sepharose cation-exchange column (HP 35/100, 35 × 100 mm, Pharmacia). This fraction (700 mg) was dissolved in 100 mL of 50 mM sodium phosphate buffer, pH 7.0 (buffer A), dialyzed against the buffer (M_r cutoff 3,500; 20 h; 4°C), centrifuged (10,000*g*; 30 min; 4°C), and applied to the column (flow rate 5.0 mL/min). The column was eluted with 300 mL of buffer A, and bound proteins were subsequently eluted from the S-Sepharose column by a 300-mL linear gradient of 0 to 1.0 M sodium chloride. Fractions (10.0 mL) were collected and endoxylanase activity toward WAX was measured in all fractions.

SDS-PAGE and IEF

These techniques were carried out as described by Cleemput et al. (1997).

Hydrolysis of AXs by the Purified Enzyme

The hydrolysis of WAX, RAX, and OSX (10.0 mg/mL in 50 mM sodium acetate buffer, pH 5.0, containing 0.02% sodium azide) by the purified enzyme was studied. The solutions (3.0 mL) were incubated with 350 μL of enzyme solution (7.0 mg of fraction AS 80–100/mL 50 mM sodium phosphate buffer, pH 7.0). Samples were withdrawn after 0, 8, 24, 32, 48, and 56 h, boiled for 10 min, centrifuged (10,000*g* for 15 min), and subjected to GPC. Samples withdrawn after 0, 8, 24, and 32 h were also analyzed by HPAEC. GPC analysis and HPAEC of the enzyme digests was as described by Cleemput et al. (1997).

Viscometric Analysis of Enzyme Action

The flow times (30°C) of an AX solution incubated with the purified enzyme were determined with a viscometer (no. III, Ostwald, Vel Leuven, Belgium, 100–150 s). WAX

was dissolved (5.0 mg/mL) in 0.1 M sodium acetate buffer (pH 5.0, 0.02% sodium azide), boiled for 10 min, and centrifuged (10,000*g* for 15 min). An aliquot of the supernatant (2.0 mL) was incubated with 100 μL of enzyme solution (3.0 mg freeze-dried material/mL 0.1 M sodium acetate buffer, pH 5.0, 0.02% sodium azide), mixed for 5 min at 30°C, and introduced into the viscometer. Flow times of the incubation mixture (30°C) were measured as a function of time and the relative visocosity (η) was calculated. Enzyme activity was expressed as the increase in reciprocal relative viscosity ($1/\eta$).

N-Terminal Amino Acid Sequencing of Proteins

The sequences of the N-terminal amino acids of the purified proteins were determined with a pulsed liquid sequencer (model 477A, Applied Biosystems), with online phenylthiohydantion-amino acid identification (model 120A analyzer, Applied Biosystems).

RESULTS

Extraction and Ammonium Sulfate Precipitation

The crude phosphate buffer extract resulted in elimination of contaminating proteins and showed an approximately 4-fold increase in specific activity toward both WAX (Table I) and OSX (flour 5.90 milliunits/mg protein; crude extract 24.5 milliunits/mg protein).

The specific activity toward OSX was highest in fraction AS > 80 (45.7 milliunits/mg protein). This fraction also showed a high hydrolytic activity toward WAX (11.4 milliunits/mg protein, Table I), although very low activities of glycosidase were found (*p*-nitrophenyl-β-D-xylopyranoside, 0.06 milliunit/mg protein; *p*-nitrophenyl-α-L-arabinofuranoside, 0.12 milliunit/mg protein, measured as described by Cleemput et al., 1997). This was surprising, since few endoxylanases of bacterial or fungal origin hydrolyze branched substrates such as AX without the synergistic action of debranching enzymes (Richards and Shambe, 1976; John et al., 1979). These results therefore suggested that fraction AS > 80 contained an endoxylanase able to hydrolyze WAX without the synergistic action of an α-L-arabinofuranosidase able to hydrolyze *p*-nitrophenyl-α-L-arabinofuranoside.

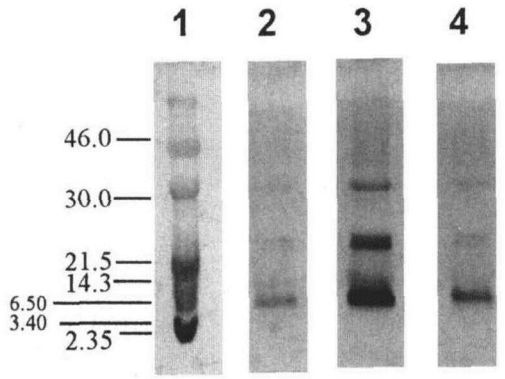

Figure 1. SDS-PAGE (20% polyacrylamide) of the fractions obtained by ammonium sulfate precipitation. Lane 1, Low-M_r marker (2,300–46,000); lane 2, fraction AS > 80; lane 3, fraction AS 80–100; and lane 4, fraction AS > 100.

Electrophoretic analysis of this fraction (AS > 80) showed three major protein bands with M_rs of approximately 30,000, 21,000, and 6,500 (Fig. 1). When fraction AS > 80 was further saturated to 100%, the extra ammonium sulfate precipitation step led to lower specific activity (Table I).

A major band of 6.5 kD was found in fraction AS >100. The latter fraction is further referred to as P-6500. The proteins in fraction AS 80–100 were further fractionated by gel-filtration chromatography.

Gel-Filtration Chromatography of Fraction AS 80–100

Gel-filtration chromatography on Bio-Gel P-10 was not efficient because all proteins eluted in one single peak. Fractions were pooled, dialyzed, and freeze-dried. SDS gel electrophoresis showed (Fig. 2) that a single protein band was present in fractions 15 and 16 with an apparent M_r of 30,000. The same band was present when the SDS gel electrophoresis was carried out under reducing conditions (β-mercaptoethanol). Fraction 17 showed a 6.5-kD band as well as the 30-kD band, whereas fractions 18 to 21 contained 30-, 21.5-, and 6.5-kD proteins. One major protein of M_r 6,500 was present in fractions 22 to 32.

Enzyme activity toward WAX was measured in the freeze-dried fractions. Fractions 15 and 16 had most of the

Table I. *Purification of arabinoxylanase from wheat flour*

Fraction[a]	Total Protein	Total Activity[b]	Specific Activity[b]	Purification	Recovery
	mg	*units*	*milliunits mg^{-1}*	*-fold*	*%*
Flour	130,650	232.56	1.78	1	100
Crude extract	19,470	32.59	6.81	4	57
AS > 80	560	6.38	11.4	6	2.8
AS 80–100	136	0.64	4.69	3	0.3
15 to 16[c]	17	0.08	4.48	3	0.03
29 to 31[c]	31	0.09	2.74	2	0.04

[a] Abbreviations as in text. [b] Endoxylanase activity toward WAX. [c] Protein concentrations were determined by measuring the A_{280} using an extinction coefficient (1%, 1 cm) of 10.0.

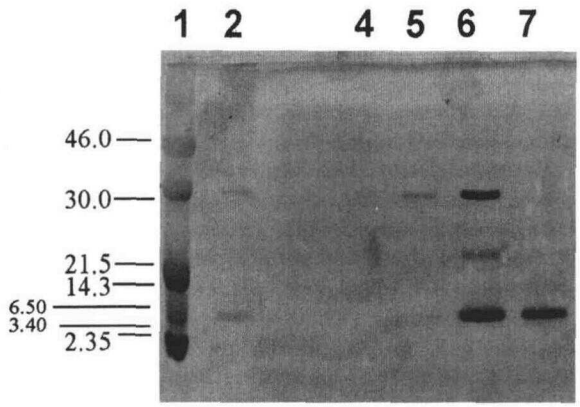

Figure 2. SDS-PAGE (20% polyacrylamide) of the fractions obtained by gel-filtration chromatography of fraction AS 80–100. Lane 1, Low-M_r marker; lane 2, fraction AS 80–100; lane 4, fraction 15 to 16; lane 5, fraction 17; lane 6, fractions 18 to 21; and lane 7, fractions 22 to 32.

activity (Table I). This purification step did not increase the specific activity. In spite of the poor purification and recovery, these results identified the 30-kD protein band as the enzyme able to hydrolyze WAX. The protein is hereafter referred to as "arabinoxylanase." An additional purification method was developed to obtain material for amino acid sequencing more efficiently.

Cation-Exchange Chromatography of Fraction AS > 80

Figure 3A shows the protein profile obtained by Mono-S cation-exchange chromatography of fraction AS > 80 with fractions pooled as indicated. The activity of the fractions was measured after freeze-drying. Fraction 29 to 31 contained hydrolytic activity toward WAX (Table I). Electrophoretic analysis by SDS-PAGE and IEF is shown in Figure 3, B and C, respectively. The lack of protein bands in lanes 4 to 6 and 13 was due to insufficient protein material loaded onto the gel. Fraction 29 to 31 demonstrated a major band with a M_r of 30,000 and a pI of 9.3 or higher. This purification route was therefore successful in obtaining the arabinoxylanase, but, again, low purification and recovery were obtained. The fraction containing the active protein, referred to as P-30,000, was subjected to N-terminal amino acid sequencing and its effect on the viscosity of an AX solution was studied.

Preparative Purification of Arabinoxylanase

The activities, purification factors, and recoveries (for activity toward WAX) of the ammonium sulfate fractions were similar to those listed in Table I (results not shown). Although S-Sepharose efficiently separated AS 80–100_b into two peaks (30-kD protein adsorbed), after 12 h of storage at 4°C all enzyme activity was lost; however, the only differences with the successful Mono-S chromatography were the eluting buffer and the matrix.

Hydrolysis by Arabinoxylanase

Changes in Viscosity by Incubation of WAX with P-30,000

The progressive decrease in viscosity with incubation time is illustrated in Figure 4. This decrease in viscosity further supported our previous findings obtained by the different enzyme activity assays (release of reducing sugars from AX) after the purification steps, which showed that arabinoxylanase is a true endo-acting enzyme.

Changes in M_r Distribution of Different Polysaccharides Incubated with Fraction AS 80–100_b

Because of the lack of sufficient amounts of purified active arabinoxylanase, the incubation was performed with AS 80–100_b, a mixture of three proteins.

The gel-permeation profiles, obtained by incubation of WAX, RAX, and OSX with AS 80–100_b, are shown in Figure 5, a, b, and c, respectively. A clear shift in retention time of the high M_r peak in time was observed for all three polysaccharides. The largest change was found with WAX as a substrate, when a shift in M_r from 100,000 to 12,200 was observed. These decreases in M_r clearly indicate the presence of an endo-acting enzyme in fraction AS 80–100_b. This enzyme preferentially hydrolyzes branched polysaccharides rather than linear structures and is therefore different from the endoxylanase previously isolated from wheat flour (Cleemput et al., 1997). The arabinoxylanase released different mono- and oligosaccharides and higher concentrations of these hydrolysis products from the polysaccharide compared with the endoxylanase characterized previously.

Release of Mono- and Oligosaccharides by Incubation of Different Polysaccharides with Fraction AS 80–100_b

The levels of mono- and oligosaccharides released during the incubation of WAX, RAX, and OSX by fraction AS 80–100_b are listed in Table II. These data illustrate that, apart from Ara and Xyl, high levels of xylo-oligosaccharides with a degree of polymerization of two to five are formed, along with some unidentified products. The hydrolysis products released from WAX and RAX and their concentrations found were quite similar apart from the unidentified components. More different products were formed during hydrolysis of OSX, but most of them were in lower concentrations than in the enzymatic breakdown of WAX and RAX. This supports our previous observations that this endo-acting enzyme can hydrolyze AX even better than xylan, in direct contrast to the wheat flour endoxylanase described previously (Cleemput et al., 1997) and endoxylanases from germinated barley (Slade et al., 1989), which require substrates with at least three consecutive Xyl units having no Ara substituents in their chain.

The concentration of hydrolysis products released by the arabinoxylanase is much higher than that released by the previously described wheat flour endoxylanase. The arabinoxylanase is able to release Ara during incubation despite the low *p*-nitrophenyl-α-L-arabinofuranoside-hydrolyzing

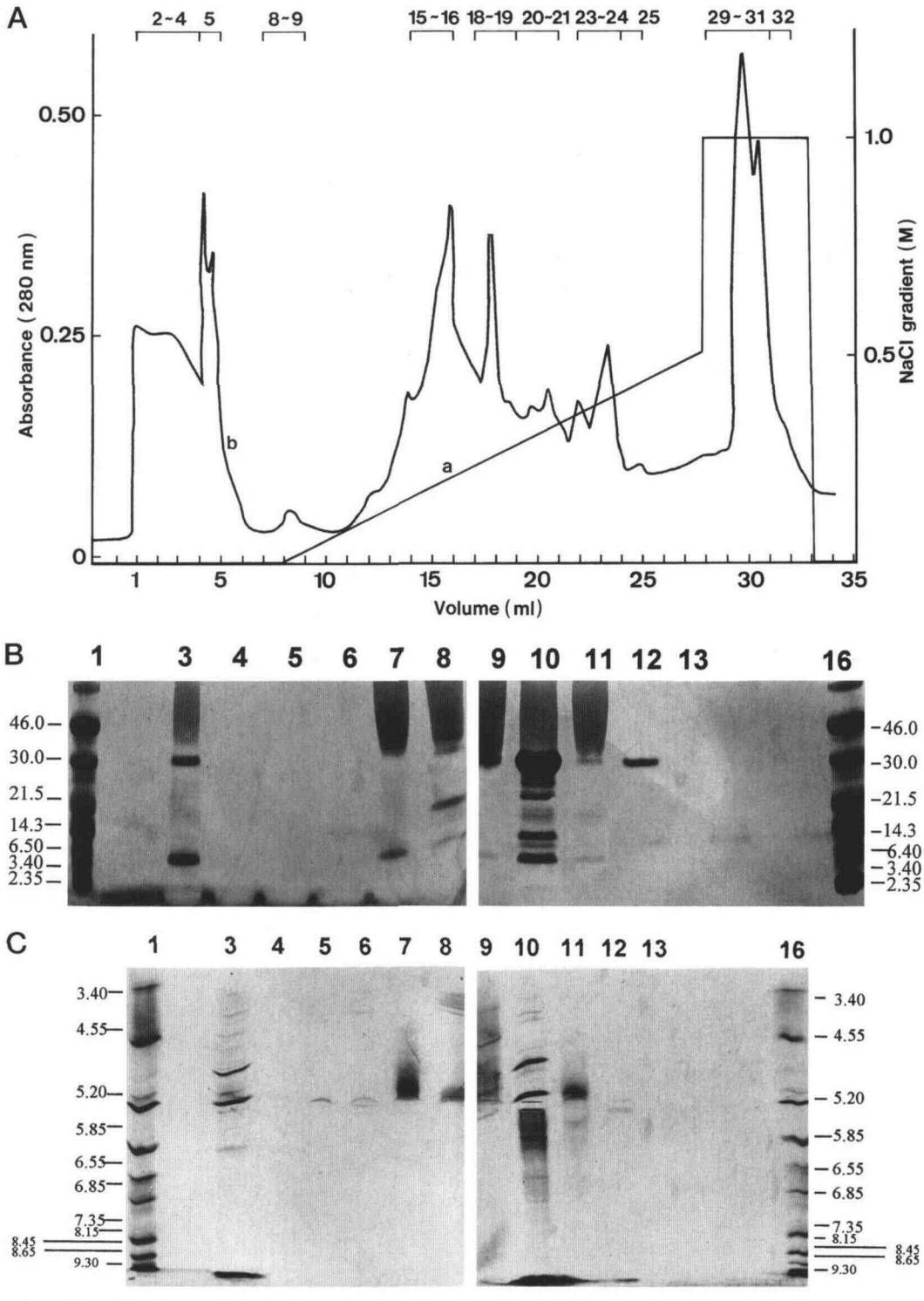

Figure 3. A, Mono-S cation-exchange chromatogram of fraction AS > 80 eluted at 1.0 mL/min with 25 mM sodium acetate buffer, pH 4.5, and sodium chloride gradient (a). The eluate was monitored at 280 nm (b) and fractions were collected as indicated. B, SDS-PAGE (20% polyacrylamide) of the fractions obtained by cation-exchange chromatography of fraction AS > 80 (fractions numbered as in A). Lane 1, Low-M_r marker; lane 3, fraction AS > 80; lane 4, fraction 2 to 4; lane 5, fraction 5; lane 6, fraction 8 to 9; lane 7, fraction 15 to 16; lane 8, fraction 18 to 19; lane 9, fraction 20 to 21; lane 10, fraction 23 to 24; lane 11, fraction 25; lane 12, fraction 29 to 31; lane 13, fraction 32; and lane 16, low-M_r marker. C, IEF of fractions obtained by cation-exchange chromatography of fraction AS > 80 (fractions numbered as in A). Lane 1, Marker; lane 3, AS > 80; lane 4, fraction 2 to 4; lane 5, fraction 5; lane 6, fraction 8 to 9; lane 7, fraction 15 to 16; lane 8, fraction 18 to 19; lane 9, fraction 20 to 21; lane 10, fraction 23 to 24; lane 11, fraction 25; lane 12, fraction 29 to 31; lane 13, fraction 32; and lane 16, marker.

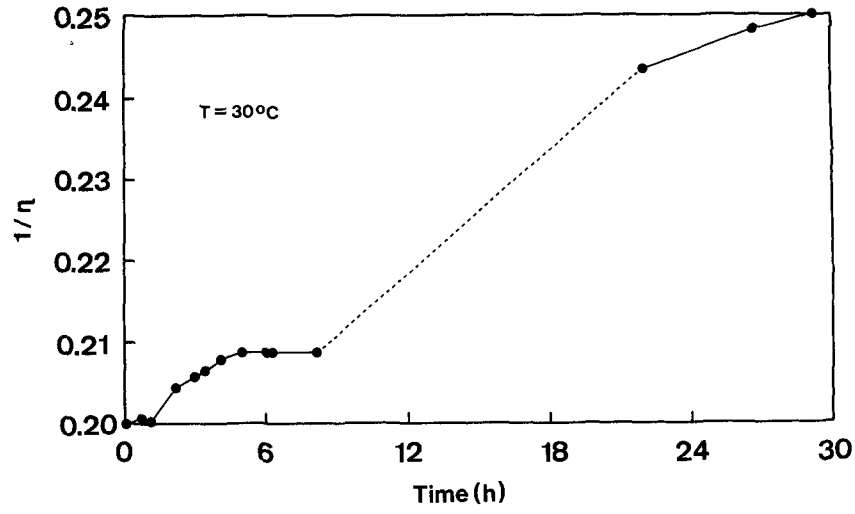

Figure 4. Change of reciprocal of the relative viscosity of a WAX solution incubated with P-30,000 in time.

activity found in this fraction (4.22×10^{-4} units/mg protein). However, this cannot be taken as absolute proof of the presence of arabinofuranosidase activity in fraction AS $80–100_b$, because a wheat bran-associated AX arabinofuranohydrolase of M_r 40,000, which is unable to hydrolyze *p*-nitrophenyl-α-L-arabinofuranoside, was recently described (Beldman et al., 1996).

N-Terminal Amino Acid Sequence

The N-terminal amino acid sequence obtained for P-30,000 is VAIAXSASGFENXEEEQPK. The sequence showed significant homology (84.2% identity in a 19-amino acid overlap) with an internal sequence VAIAPSASGSENCEEEQPK (amino acids 41–59) of grain softness protein (S. Rahman, unpublished data). This 15-kD starch granule

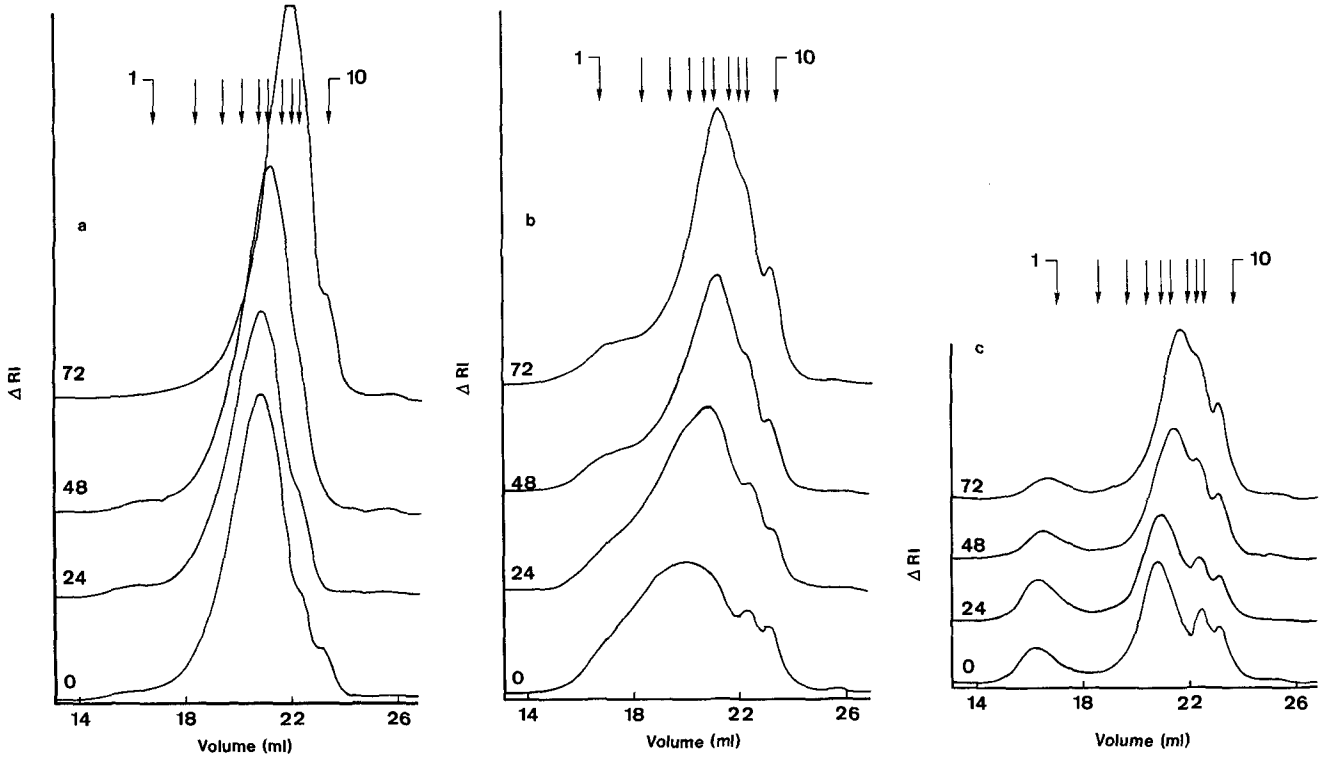

Figure 5. Gel-permeation profiles on a Shodex column of incubation mixture of WAX (a), RAX (b), and OSX (c) with arabinoxylanase at 0, 8, 24, and 32 h. The eluate was monitored with refractive index (RI) detection. Elution volumes of dextran (40×10^6 to 5×10^6), pullulan standards of M_r 8.53×10^5, 3.80×10^5, 1.86×10^5, 1.0×10^5, 4.80×10^4, 2.37×10^4, 1.22×10^4, 0.55×10^4, and Xyl are indicated by numbers (1–10, respectively).

Table II. *Concentrations of L-arabinose (ara), D-xylose (xyl), xylobiose (X_2), xylotriose (X_3), xylotetraose (X_4), and xylopentaose (X_5) or arbitrary concentrations of other hydrolysis products (indicated by retention time; min) released during incubations of WAX, RAX, and OSX with arabinoxylanase*

Experimental conditions were as described in the text.

Substrate	Time	ara	13.3[a]	xyl	X_2	X_3	27.7[a]	X_4	X_5	32.23[a]	29.1[a]	29.7[a]
	h						$\mu g\ mL^{-1}$					
	0	6.89	0.29	2.64	2.27	6.27	0.37	4.44	4.03	1.08		
	8	9.03	4.69	3.75	3.52	7.82	1.29	5.21	5.55	1.98		
WAX	24	11.49	8.28	4.49	4.01	10.11	1.87	6.57	6.72	3.13		
	32	19.33	10.07	8.04	5.34	16.40	2.68	14.34	12.07	6.15		
	0	4.67	5.51	2.11	1.86	3.79	4.12	3.63	1.67	0.28		
	8	10.30	10.22	4.37	2.95	6.49	7.40	3.56	5.04	1.47		
RAX	24	15.43	19.24	6.25	4.01	11.27	8.69	14.24	10.72	4.20		
	32	18.00	22.62	7.32	4.57	14.26	8.26	14.31	28.21	5.27		
	0	3.97	0.20	2.27	2.13	4.91	0.93	2.41	1.81	0.60	2.25	
	8	6.11	5.00	3.14	2.94	6.05	18.85	3.13	2.62	1.05	2.42	
OSX	24	8.71	15.18	4.63	3.21	9.28	43.28	7.99	5.86	3.44	3.56	0.87
	32	10.32	21.77	5.72	3.66	12.08	50.66	10.74	8.86	5.05	3.49	1.42

[a] Arbitrary concentrations calculated with the response factor of the preceding identified product.

protein with a pI between 7.5 and 8.0, which is extracted by SDS solutions and is prominent in soft wheats (Greenwell and Schofield, 1986; Morrison et al., 1992; Oda et al., 1992), has been proposed as a marker for grain softness and is referred to as "friabilin" (Greenwell and Schofield, 1989). The present protein is not a dimer of friabilin because its electrophoretic mobility did not change after reduction.

The N-terminal amino acid sequence of P-6500 is IDXGHVDSLVRPXLSYVQGG. Assuming that X stands for C, this N-terminal sequence is identical to that of nonspecific lipid-transfer protein of wheat (Désormeaux et al., 1992). This protein has a M_r of approximately 9000.

In the Edman sequencing determination of both proteins single amino acid derivatives were released at each cycle, indicating purity of the proteins.

DISCUSSION

Ammonium sulfate precipitation of a crude wheat extract resulted in two fractions displaying enzyme activity toward OSX and WAX. Apart from the endoxylanase present in fraction AS 30–70 (Cleemput et al., 1997), a second endo-acting NSP-hydrolyzing enzyme was present in the fraction not precipitating at 80% saturation. The action was different from both that of the wheat flour endoxylanase and that of many purified microbial endoxylanases. The latter enzymes preferentially hydrolyze the xylan backbone at unsubstituted domains and are hindered by Ara substituents.

Following incubation of WAX, RAX, and OSX with fraction AS 80–100_b, the preferential attack by this enzyme on more branched structures was shown by GPC. Analysis of the hydrolysis products of the wheat flour arabinoxylanase acting on AX was performed with HPAEC. The release of Ara, Xyl, xylo-oligosaccharides ranging from xylobiose to xylopentaose, and other unidentified products (presumably Ara-substituted xylo-oligosaccharides) was demonstrated. It remains unclear whether Ara is released directly from the AX or from Ara-Xyl mixed oligosaccharides formed during hydrolysis. However, because the release of Ara is associated with the release of xylo-oligosaccharides, a preliminary debranching of the main chain did not appear to be required for an efficient hydrolysis of the polysaccharide.

Highly purified endoxylanases from *Ceratocystis paradoxa*, *Aspergillus niger*, and *Neurospora crassa* have also been shown to hydrolyze the 1,3-α-L-arabinofuranosyl branch points of AX (Dekker and Richards, 1975a; Takenishi and Tsujisaka, 1975; John et al., 1979; Mishra et al., 1984). It is not known whether the two types of linkages are hydrolyzed at the same active site, but it seems likely that both types of hydrolysis are catalyzed by the same enzyme. Molecular biological studies will be required to obtain firmer evidence for the existence of bifunctional xylanolytic enzymes (Biely, 1993). Exhibition of α-L-arabinofuranosidase activity by a carefully purified product of a cloned endoxylanase gene in an α-L-arabinofuranosidase negative host should be convincing evidence for the existence of Ara-liberating endoxylanases (Biely, 1993).

Only a few endoxylanases with activity toward xylan backbones near branch points have been described, including an extracellular one from *C. paradoxa* (Dekker and Richards, 1975b), two from *A. niger* (Frederick et al., 1985), and a unique xylanase that requires glucuronosyl substituents for its activity (Nishitani and Nevins, 1991).

Purification of the endoenzyme showed that the wheat arabinoxylanase had an apparent M_r of 30,000 and a pI ≥ 9.3. The sequence of the first 19 amino acids of this protein were homologous with those of a 15-kD grain-softness protein, showing that this enzyme is a wheat protein and not a contaminant of the flour.

The major N-terminal sequence and sequences of peptides derived from protease digests of grain softness protein were reported by Jolly et al. (1993). No similarity was found between these sequences and the N-terminal sequence found for P-30,000. Because of the differences in M_r, pI, N-terminal amino acid sequence, and extractability of

P-30,000 and the grain-softness protein, we can conclude that they are different.

Apart from the purification of arabinoxylanase, a nonspecific lipid-transfer protein of wheat was purified. The presence of a nonspecific lipid-transfer protein in fraction AS > 100 was surprising, since Désormeaux et al. (1992) purified this protein from a 40 to 80% ammonium sulfate precipitate of wheat seeds or flour.

We can conclude that arabinoxylanase is a novel plant enzyme with a very specific action pattern. More work is needed to better understand the role of arabinofuranosyl substituents for the mechanism.

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