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Arabinoxylan fractionation on DEAE-cellulose chromatography influenced by protease pre-treatment

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Abstract

An arabinoxylan mixture was extracted with saturated barium hydroxide from a water unextractable residue of rye bran. The mixture was fractionated on an anion exchange column which was eluted with water, 0.0025 M sodium borate, 0.025 M sodium borate and 0.4 M sodium hydroxide. It was possible to fractionate the arabinoxylan mixture into fractions with different structural features. The fractionation was repeated with prior protease treatment of the arabinoxylan mixture, but most of the arabinoxylan did not bind to the column by any mechanism that the protease treatment affected, As the largest fraction was still eluted with 0.4 M sodium hydroxide. Protease treatment affected the proportion of disubstituted xylose residues (dXyl) in the water, 0.0025 M sodium borate and 0.025 M sodium borate fractions, indicating that protein associated with arabinoxylans with a high dXyl content is more liable to the protease treatment or that protein is mainly associated with these structures. This study gives indications that protein is involved in the separation mechanism of rye arabinoxylan on a DEAE–cellulose column.

Keywords: Rye; Arabinoxylan; Protease; DEAE; ^{1}H NMR

1. Introduction

In rye grain (*Secale cereale* L.) the most abundant dietary fiber polysaccharide is arabinoxylan of which 62–80% is water-unextractable (Pettersson & Åman, 1987; Saini & Henry, 1989; Nilsson et al., 1997). Investigations of water unextractable arabinoxylan have been facilitated by the method presented by Gruppen, Hamer and Voragen (1991), in which saturated barium hydroxide was used as a selective arabinoxylan extractant. Vinkx, Stevens, Gruppen, Grobet and Delcour (1995) divided the water-unextractable rye arabinoxylans into three groups with different extractability, solubility and structural features. One group was extractable with saturated barium hydroxide, or with water after barium hydroxide treatment and had an arabinose to xylose (A/X) ratio of 0.55–0.79. A second group that was extractable by alkali had an A/X ratio of about 1.1 and a third that was precipitated after neutralization of a 1 M potassium hydroxide extract had an A/X ratio of about 0.2. In a previous study we extracted water-unextractable material from rye bran, flour and an intermediate milling fraction with a sequential extraction of saturated barium hydroxide, water, 4 M potassium hydroxide, water and hot 2 M sodium hydroxide (Nilsson, Saulnier, Andersson & Åman, 1996). The barium hydroxide extract contained the major part of arabinoxylan with an A/X ratio of 0.54–0.67. The water extract after the barium hydroxide treatment mainly contained mixed-linked (1 → 3),(1 → 4)-β-D-glucan (β-glucan) but also an arabinoxylan with an A/X ratio of 0.60–0.93. An arabinoxylan with an A/X ratio of about 0.1 was obtained as the precipitate after neutralization of the potassium hydroxide extract while an arabinoxylan with an A/X ratio of 0.27–0.63 remained in solution. After hot alkali extraction a more complex heteroxylan was obtained. Vinkx et al. (1995) made a similar extraction and got a high proportion of a similar heteroxylan in the barium hydroxide extract. One reason for the different behavior of the extractions could be that Vinkx et al. used a protease to aid the extraction of polysaccharides and we therefore decided to investigate how protease treatment could influence the extractability and separability of the water-unextractable polysaccharides. In this study we have separated and characterized an arabinoxylan mixture extracted with saturated barium hydroxide.

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Table 1
Fractionation of barium hydroxide extract and protease treated bariumhydroxide extract on a DEAE-cellulose column in duplicate (I and II). Recovery (%) of arabinose and xylose residues with different eluents

Fraction	Ara	Xyl	mXyl[a]	dXyl[b]	uXyl[c]
Non-protease treated					
I Water	2	3	3	< 1	3
0.0025 M sodium borate	4	5	5	3	4
0.025 M sodium borate	21	17	16	26	15
0.4 M sodium hydroxide	44	47	48	40	49
Total[d]	74	75	77	71	75
II Water	8	11	14	1	12
0.0025 M sodium borate	12	11	11	13	11
0.025 M sodium borate	6	5	4	9	4
0.4 M sodium hydroxide	50	53	43	56	58
Total[d]	78	83	75	81	88
Protease treated					
I Water	6	8	10	3	9
0.0025 M sodium borate	9	9	9	10	8
0.025 M sodium borate	7	5	4	10	4
0.4 M sodium hydroxide	35	40	40	30	43
Total[d]	58	63	63	54	65
II Water	12	15	19	5	16
0.0025 M sodium borate	8	7	6	9	6
0.025 M sodium borate	14	10	8	19	8
0.4 M sodium hydroxide	40	44	38	42	48
Total[d]	73	76	71	76	78

[a] (1,3,4)-linked β-D-Xylopyranosyl residue.
[b] (1,2,3,4)-linked β-D-Xylopyranosyl residue.
[c] (1,4)-linked β-D-Xylopyranosyl residue.
[d] Including residues eluted with saturated barium hydroxide.

2. Experimental

2.1. Starting materials

Rye bran (38.4% dietary fibre, 19.8% starch, 14.8% crude protein) was extracted with hot water during enzymatic starch degradation and unextractable material (63.1% dietary fiber, 20.2% crude protein) was isolated by centrifugation (Nilsson et al., 1996). The water-unextractable material was extracted overnight at room temperature with saturated barium hydroxide, neutralized, centrifuged and the supernatant dialyzed and lyophilized.

2.2. Enzyme purification

Protease (main component Subtilisin A) from *Bacillus lichenformis* (Alcalase 2.4 L, Novo Nordisk, Copenhagen, Denmark) (1 ml) was diluted with the same volume of water and precipitated with 1.04 g ammonium sulphate. A pellet was isolated by centrifugation and washed with 2 ml ammonium sulphate (55%). The pellet was then solubilized in water (2 ml) and desalted on a PD-10 column (Pharmacia, Uppsala, Sweden). This enzyme has previously been shown to be devoid of xylanase activity (Faurot, Saulnier, Bérot, Popineau & Thibault, 1995). To check for any arabinofuranosidic activity, 10 μl of purified protease was added to 10 ml wheat arabinoxylan (Megazyme) solution (2 mg/ml) and incubated overnight in 50°C in a shaking water bath. The enzyme was inactivated in a boiling water bath for 30 min. Ethanol was added to give a concentration of 80% and the polysaccharides precipitated in an ice–water bath for 2 h and centrifuged (3000 rpm for 10 min). The supernatant was evaporated in a speed vac concentrator 100H (SAVANT) and analyzed for arabinose. The enzyme was considered devoid of arabinofuranosidic activity as no arabinose was found in the supernatant.

2.3. Enzyme treatment

Purified protease (500 μl) was added to 500 mg barium hydroxide extract dissolved in 240 ml water and the mixture was incubated overnight at 50°C in a shaking water bath. The enzyme was inactivated in a boiling water bath (30 min), after which the mixture was dialyzed against running de-ionized water and concentrated to 225 ml in a rotary evaporator.

2.4. Fractionation

Barium hydroxide extract solution (approximately 100 ml at about 2 mg/ml), with or without protease treatment, was applied to a Whatman DE23 cellulose column (50 × 150 mm) and eluted sequentially with water, 0.0025 M sodium borate, 0.025 M sodium borate, 0.4 M sodium hydroxide and saturated sodium borate (Neukom, Deuel, Heri & Kundig, 1960). Fractions of about 8 ml were collected and analyzed for carbohydrates using phenol–sulfuric acid (Dubois, Gilles, Hamilton, Rebers & Smith, 1956). Fractions containing carbohydrates were pooled, dialyzed against running de-ionized water and lyophilized. All fractionations were run in duplicate.

2.5. Dietary fiber polysaccharide analysis

Dietary fiber polysaccharide residues were analyzed according to Theander, Åman, Westerlund, Andersson and Pettersson (1995) with the following modifications: the starch removal step was omitted and 2–10 mg of fractions were weighed into 15 ml tubes, 500 μl 0.8 M sulphuric acid and 500 μl water containing 0.2 mg myo-inositol as internal standard was added to each tube and thereafter the samples were autoclaved and analyzed in the same manner as described in the method.

2.6. ^1H NMR

Dried samples (2–10 mg) were dissolved in D_2O and evaporated to dryness. This procedure was repeated four to five times, and samples were finally dissolved in 1 ml D_2O. Proton nuclear magnetic resonance (^1H NMR) spectra (400 MHz) were recorded at 85°C on a Bruker DRX 400 instrument. Pulse repetition time was 4.2 s and radio

Table 2
Fractionation of barium hydroxide extract and protease treated barium hydroxide extract on a DEAE-cellulose column in duplicate (I and II). Content of arabinose, xylose and total sugar residues (% of dry fraction), ratio of arabinose to xylose residues(A/X) and relative composition of differently linked xylose residues

Fraction	Content			Relative composition			
	Ara	Xyl	Total Sugar	A/X	mXyl[a]	dXyl[b]	uXyl[c]
Barium hydroxide extract	16.0	28.4	47.7	0.56	29	14	57
Fractions eluted							
Non-protease treated							
I Water	14.8	40.8	61.6	0.36	33	2	66
0.0025 M sodium borate	18.3	34.1	54.8	0.54	35	9	56
0.025 M sodium borate	22.5	32.3	57.3	0.70	27	21	52
0.4 M sodium hydroxide	11.7	22.4	39.4	0.52	29	12	59
II Water	18.3	47.2	68.8	0.39	36	2	63
0.0025 M sodium borate	24.0	40.0	66.6	0.60	28	16	56
0.025 M sodium borate	13.7	19.0	38.2	0.72	25	23	51
0.4 M sodium hydroxide	15.5	29.5	51.9	0.53	23	15	62
Protease treated							
I Water	18.0	42.3	62.2	0.43	33	5	62
0.0025 M sodium borate	14.3	24.1	40.5	0.59	28	16	56
0.025 M sodium borate	14.0	17.1	33.4	0.82	25	28	47
0.4 M sodium hydroxide	14.6	29.6	49.6	0.49	28	10	61
II Water	20.3	45.5	68.1	0.44	36	4	60
0.0025 M sodium borate	23.3	34.8	62.6	0.67	27	20	53
0.025 M sodium borate	22.5	28.4	53.6	0.79	24	28	49
0.4 M sodium hydroxide	14.8	29.2	53.6	0.51	25	13	62

[a] (1,3,4)-linked β-D-Xylopyranosyl residue
[b] (1,2,3,4)-linked β-D-Xylopyranosyl residue
[c] (1,4)-linked β-D-Xylopyranosyl residue

frequency pulse angle was 30°. The relative distribution of (1,4)-linked (uXyl), (1,3,4)-linked (mXyl) and (1,2,3,4)-linked (dXyl) xylose residues was calculated from the sugar analysis and integrals of the ^{1}H NMR spectra as described by Westerlund et al. (1990).

3. Results

A fraction rich in arabinoxylan was prepared from rye bran and further fractionated, with or without prior protease treatment, on an Whatman DE23 anion exchange cellulose column. The total recoveries of arabinose and xylose residues varied from 58–83% while recoveries of xylose residues were higher than for arabinose residues in all cases (Table 1). For all analyzed samples the highest recovery was found for the fraction eluted with 0.4 M sodium hydroxide. The total recoveries of m, d and u-Xyl ranged from 54 to 88%. The non-protease treated samples had lower recoveries of dXyl in the water fraction compared to the protease treated samples. Generally the m, d and u-Xyl residues were differently distributed over the fractions eluted. For example, a larger proportion of dXyl was recovered in the 0.025 M sodium borate fraction as compared to mXyl and uXyl.

The barium hydroxide extract had an arabinoxylan content of 44.3%, calculated as the sum of arabinose and xylose residues, with an A/X ratio of 0.56 (Table 2). This arabinoxylan fraction had a relative composition of m, d and u-Xyl of 29, 14 and 57%, respectively. The A/X ratio and the relative composition of m, d and u-Xyl residues revealed differences in arabinoxylan structure between the fractions eluted as well as between protease and non-protease treated samples. The differences were mainly in the relative proportion of dXyl. In the fraction eluted with water, there was a low amount of dXyl. However, the protease treated samples had twice the amount of dXyl compared to the non-protease treated ones. Fractions eluted with 0.0025 M sodium borate had a relative dXyl content of 9–20%, with a tendency of higher proportions in the protease treated samples. All fractions eluted with 0.4 M NaOH were similar to the barium hydroxide extract in terms of the relative composition of m, d and u-Xyl.

The trends observed were also evident from ^{1}H NMR spectra (Figs. 1 and 2). The signal around 5.4 ppm corresponds to the anomeric proton of the terminal α-L-arabinofuranosyl residue linked to the O-3 position of

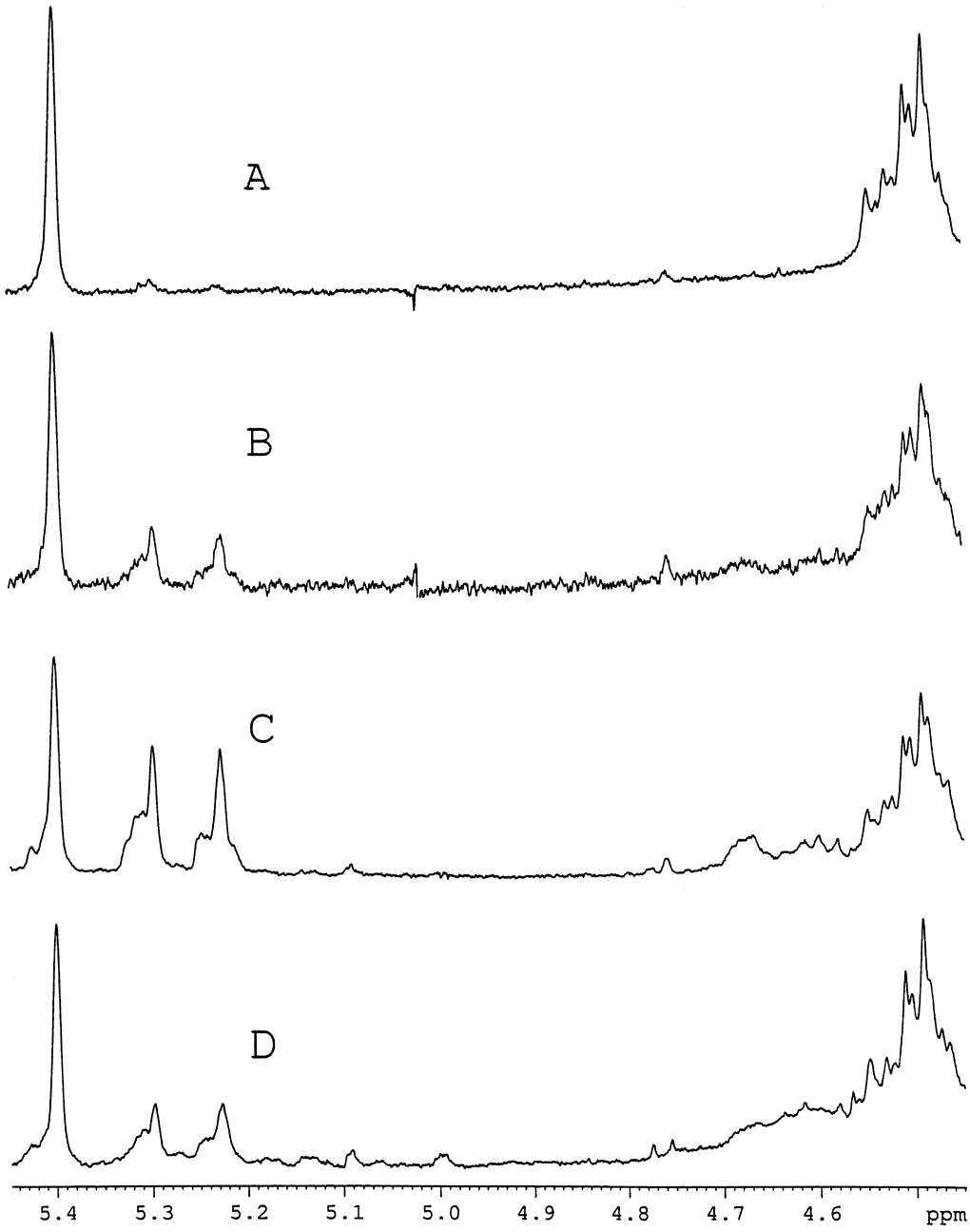

Fig. 1. ^{1}H NMR spectra of eluted fractions from the non-protease treated sample: (A) Water; (B) 0.0025 M sodium borate; (C) 0.025 M sodium borate; (D) 0.4 M sodium hydroxide.

a (1,3,4)-linked β-D-xylopyranosyl residue, the signal around 5.3 ppm corresponds to the anomeric proton of the terminal α-L-arabinofuranosyl residue linked to the O-3 position of a (1,2,3,4)-linked β-D-xylopyranosyl residue and the signal around 5.2 ppm corresponds to the anomeric proton of terminal α-L-arabinofuranosyl residue linked to the O-2 position of (1,2,3,4)-linked β-D-xylopyranosyl residues (Bengtsson & Åman, 1990; Hoffmann, Geijtenbeek, Kamerling & Vliegenhart, 1992). The signals representing dXyl were small in the fractions eluted with water and much larger in the fractions eluted with 0.025 M sodium borate. Differences between protease and non-protease treated samples were also reflected by the ^{1}H NMR spectra.

4. Discussion

An arabinoxylan and protein-rich fraction was extracted from water unextractable material from rye bran with saturated barium hydroxide. In order to evaluate if protease pre-treatment would influence the fractionation of arabinoxylans, both untreated and protease treated materials

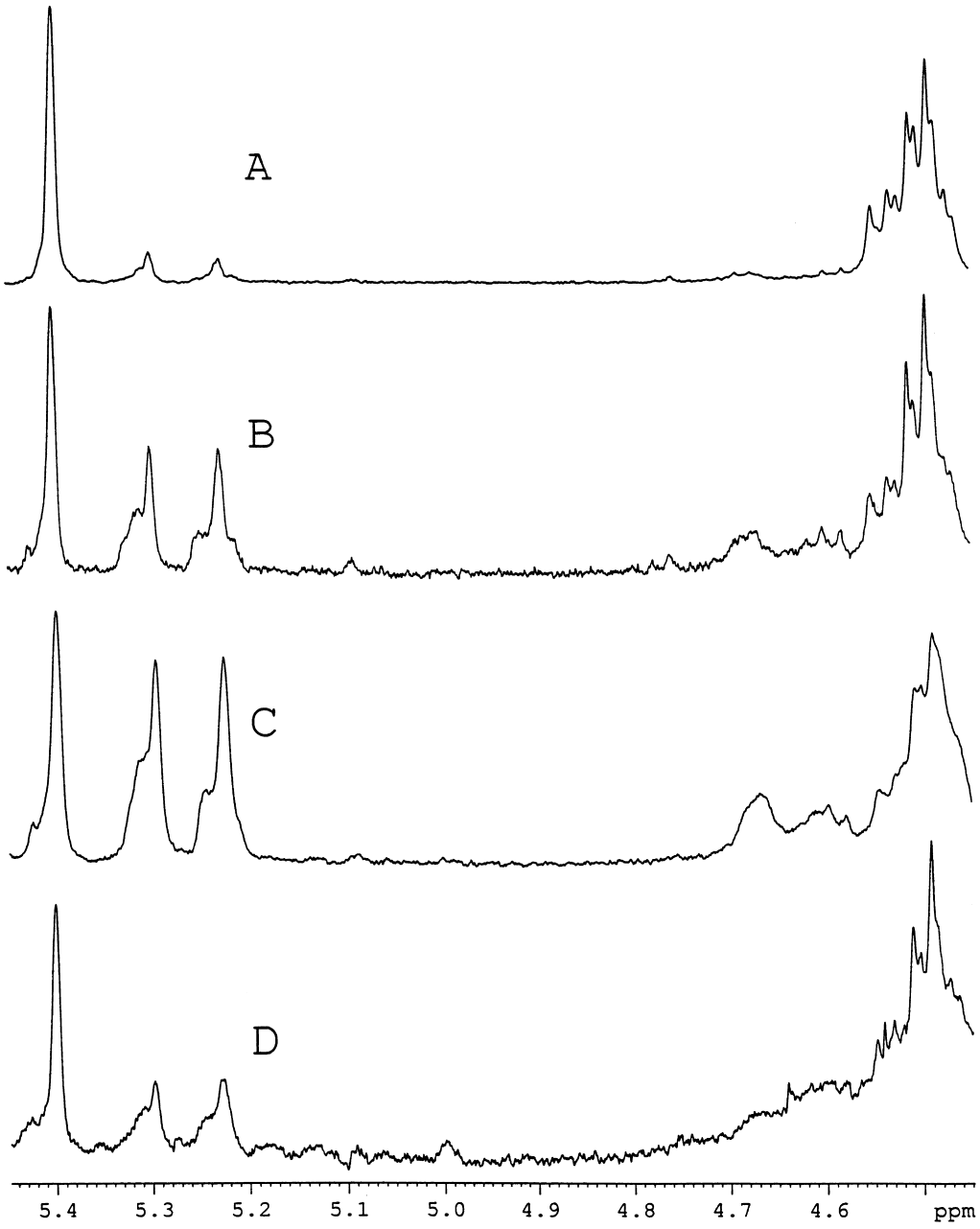

Fig. 2. ^{1}H NMR spectra of eluted fractions from the protease treated sample: (A) Water; (B) 0.0025 M sodium borate; (C) 0.025 M sodium borate; (D) 0.4 M sodium hydroxide.

were fractionated on a DEAE–cellulose column. From the results it was evident that the arabinoxylan in this extract was not homogenous and that it was possible to separate it into fractions with different structural features. The structural differences in the collected fractions were mainly reflected in the proportion of dXyl. The fractions eluted with water with a very low proportion of dXyl, had structural features similar to the most abundant water-extractable polysaccharide denoted Arabinoxylan I (Bengtsson & Åman, 1990). For this fraction the ^{1}H NMR spectra revealed more disubstituted xylose residues in the protease treated sample (Figs. 1 and 2). This was also reflected in the proportion of dXyl while no noticeable difference in the proportion of uXyl was found. As this fraction was eluted with water it must be essentially devoid of anionic compounds. Consequently the protease treatment released a neutral polysaccharide fraction with a higher content of dXyl compared to what was present in the non-protease treated samples.

The fraction eluted with 0.0025 M borate showed spectra with a high ratio of dXyl to mXyl in the protease treated sample (Fig. 1). The fraction eluted with 0.025 M borate also showed spectra with a higher degree of disubstitution compared to the protease treated sample. This could also be

seen in the relative composition of m, d an u-Xyl. In the case of the borate fractions the protease seemed to have partially degraded protein associated with the polysaccharides, but some anionic residues remained as they still bound to the column. These results indicated that the protease treatment mainly affected the fractionation mechanism of a rather small proportion of arabinoxylan that was rich in dXyl.

The fraction eluted with 0.4 M NaOH was, by far, the largest fraction and can probably be further fractionated with other methods. The spectra of these fractions were quite similar in all the samples and also similar to the arabinoxylan in the original barium hydroxide extract. As these fractions were strongly bound to the column material it is reasonable to assume that it must be some ionic compounds, probably protein, associated with the polysaccharides in the fractions. The protein fraction in this study was evidently not degraded by the protease treatment in such a way to influence the fractionation. Another explanation could be that the polysaccharides were bound to the column material by other mechanisms or components not involving protein and therefore not affected by the protease treatment.

Other components that could be responsible for the separation on an anionic column are uronic acid residues that have been shown to exist on arabinoxylans, in fact about 1% of the polysaccharide fraction of the barium hydroxide extract was analyzed as uronic acid residues by a colorimetric method. Protease pre-treatment was shown to have an effect on the separation of arabinoxylan on the DEAE-cellulose column and was evidently one of the criteria for fractionation on the column. Any possible effects of the low amounts of uronic acid residues and other ionic compounds remains to be studied but would not influence our conclusion.

The fractionation on the anion column seems to be structure specific, either because the polysaccharide itself interacts differently with the column depending on structural differences or that some other component associated with the polysaccharide interacts with the column material. With the increasing pH in the eluents it is also possible that protein–protein linkages are broken and that part of the protein, formerly associated with the polysaccharide, is still bound to the column when the polysaccharide elutes. Protease treatment may have degraded the polysaccharide associated protein in such a way that it may be more liable to degradation in alkali. Protein may be associated with polysaccharides with several linkages such as hydrophobic, covalent and ionic linkages. A few recent investigations have studied possible covalent linkages between arabinoxylan and protein in cereals. A ‘galactoarabinoxylan–peptide’ with suggested covalent linkage between the peptide and saccharide have been isolated from wheat (Chen, Shelton, D’Appolonia, & Khan, 1992). In a study on wheat gluten, which represents 80% of the protein in wheat, no major arabinoxylan was found to be covalently linked to protein (Saulnier et. al., 1997). Interaction between protein and arabinoxylan is a relatively unknown subject, but the results of this study indicate that protein contributes to the separation of arabinoxylan on an anionic cellulose column.

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