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AFM studies of water-soluble wheat arabinoxylans—effects of esterase treatment

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Abstract—The degradation products of water-soluble wheat arabinoxylans treated with *Aspergillus niger* ferulic acid esterase (FAEA-able to cleave 5,5′- and 8-*O*-4′-ferulic acid dimers) have been characterised by atomic force microscopy (AFM) and size exclusion chromatography. The AFM images of arabinoxylans confirmed that a small proportion (~15%) of the population of arabinoxylan molecules contain xylan-based branches attached to the xylan-based backbone. Treatment with FAEA reduced the contour length of the molecules suggesting that certain dimeric ferulic acid linkages may play a previously unconfirmed role in the elongation of arabinoxylans. Overnight treatment with FAEA led to a reduction in the density of branches suggesting that they may also be linked to the backbone through phenolic linkages.

Keywords: Arabinoxylans; Pentosans; Atomic force microscopy; Ferulic acid esterase

1. Introduction

Arabinoxylans are structural components of the cell walls of cereal grains.[1,2] Recent atomic force microscopy (AFM) studies have revealed that a small proportion (~15%) of the population of these supposedly linear polymers are branched.[3,4] As shown in Figure 1 arabinoxylans may contain a range of ferulic acid monomers and/or dimers.[5] Apparent branching of arabinoxylans could result from cross-linking of individual linear arabinoxylan molecules through the formation of ferulic acid dimers. One method of testing whether the apparent branching of the arabinoxylans is due to dimeric ferulic acid cross-links is to treat the sample with specific enzymes that cleave these linkages, and observe whether such treatment eliminates the observed branched structures. This article describes the effect of treating arabinoxylans with a ferulic acid esterase (FAEA) able to cleave 5,5′- and 8-*O*-4′-ferulic acid dimers. The products resulting from incubation with the enzyme have been analysed both by AFM and by size exclusion chromatography (SEC), calibrated against pullulan standards.

2. Results and discussion

The estimated concentrations of ferulic acid (0.89 mg g^{-1}), di-ferulic acid (0.09 mg g^{-1}) and protein (1.8 mg g^{-1}) were found to be consistent with the values reported elsewhere in the literature[6–8] for water-soluble wheat endosperm arabinoxylans. The arabinoxylans were found to contain 5,5′-, 8-*O*-4′- and 8,5′-ferulic acid dimers.

The present studies were undertaken to test whether ferulic acid dimers were responsible for the apparent branching of the xylan backbone of the polymers. HPLC analysis of the ethyl acetate-extracted compounds from FAEA-treated arabinoxylans indicated that, under the conditions of the assay, FAEA released 5,5′-di-ferulic acid and 8-*O*-4′-di-ferulic acid and the amounts released as a function of incubation time are

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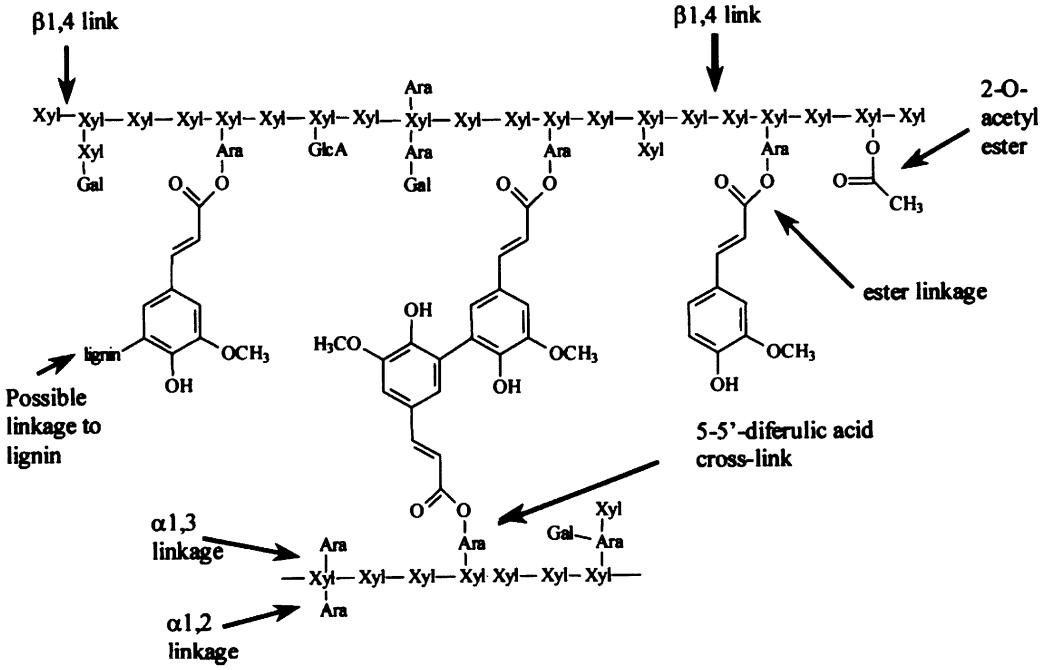

Figure 1. Schematic depiction of the main structural features considered present within water-soluble wheat arabinoxylans. The diagram also indicates the phenolic linkages cleaved by a variety of enzymes that are active against arabinoxylans.

Table 1.

Sample	Ferulic acid (FA) (mg g^{-1})	5,5′ Di-FA (mg g^{-1})	8-*O*-4′ Di-FA (mg g^{-1})
Untreated sample	0.00	0.000	0.000
45 min incubation	0.35	0.004	0.001
1 h incubation	0.39	0.004	0.003
2 h incubation	0.49	0.005	0.006
4 h incubation	0.73	0.005	0.007
Overnight	0.64	0.006	0.013
NaOH treatment	0.89	0.012	0.044

given in Table 1. No free 8,5′-benzofuran di-ferulic acid was formed after overnight incubation and the detection limit of the assay was 5 μg g^{-1}. As a result of the FAEA treatment a new low molecular weight peak (Fig. 2, arrowed B) was observed in the SEC chromatogram: based on the use of pullulan standards this new peak would correspond to a molecular mass of 184×10^3. Prolonged incubation with FAEA resulted in an increase in the area under this low molecular weight peak, accompanied by a progressive decrease in the number average contour length of the arabinoxylans, as determined from analysis of AFM images (Table 2). The FAEA used in the present study was a recombinant enzyme, which should possess no xylanase activity. To ensure that the degradation of the arabinoxylan was not due to contamination with a xylanase, during isolation or use, an assay was made for xylanase activity using the DNS method. For overnight incubation at 37 °C no xylanase activity was detected. The detection limit of the DNS method was 2 mU mL^{-1}.

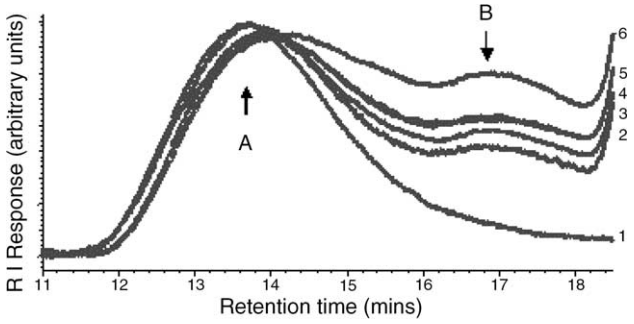

Figure 2. Presentation of SEC data, which illustrates the effect of FAEA treatment on arabinoxylans. The sample used in the present studies corresponds to the fraction 13 isolated using the methods described in the article by Adams et al.[3] Samples are labelled from 1 to 6 corresponding to untreated samples and samples treated for 45 min, 1, 2, 4 h and an overnight incubation (37 °C at pH 6.0), respectively. Continued treatment with the enzyme leads to the progressive loss of high molecular mass material (A) and the appearance of a low molecular mass peak to values corresponding to an estimated pullulan molecular mass of 184×10^3.

The measured ferulic acid content of the arabinoxylans corresponds to about 1 phenolic substitution per 120 arabinose residues. This is equivalent to about 1 ferulic acid substituent per 220 xylose residues in the backbone of the polymers. The measured quantity of 8-*O*-4′ and 5,5′ dimeric linkages corresponds to about 1 linkage per 1400 backbone xylose residues. The measured density of branches detected by AFM can best be expressed as the number of branch points per 1 μm length of the molecule. From the AFM data the calcu-

Table 2. The effect of FAEA treatment on the arabinoxylan extract

Treatment time (min)	'AFM' number average length (nm)	Standard deviation (nm)
0	770	36
45	640	28
60	650	31
120	580	27
180	570	21
Overnight	550	31

The 'AFM' number average length is the number average contour length of the molecules calculated from the AFM images.

Table 3. The effect of FAEA treatment on the branching of the arabinoxylan extract

Treatment time (min)	Branches per μm
0	0.25
45	0.24
60	0.24
120	0.22
240	0.20
Overnight	0.12

lated value is 0.25 branches per 1 μm (Table 3). This is approximately equivalent to 1 branch per 8×10^3 xylose backbone residues, although the observed distribution of branches is heterogeneous. Clearly there are more dimeric phenolic linkages than observed branches. The treatment with the FAEA led to an overall decrease in the contour length of the arabinoxylan molecules (Table 2), observed both by SEC and AFM, but did not appear to release the branched structures (Table 3). In Table 3 the branching is recorded as the number of branches per 1 μm unit of backbone: it is not sufficient to record branching as the number of observed branches per number of chains measured because any de-polymerisation of the chains would then appear to reduce the branching density by generating new low molecular mass chains. The data in Table 3 show clearly that there is no significant decrease in the branching density except after overnight incubation with the enzyme. AFM images (Fig. 3) show branched arabinoxylans are still present in FAEA-treated arabinoxylan samples.

The AFM images obtained in the present study provide further evidence for the existence of branched

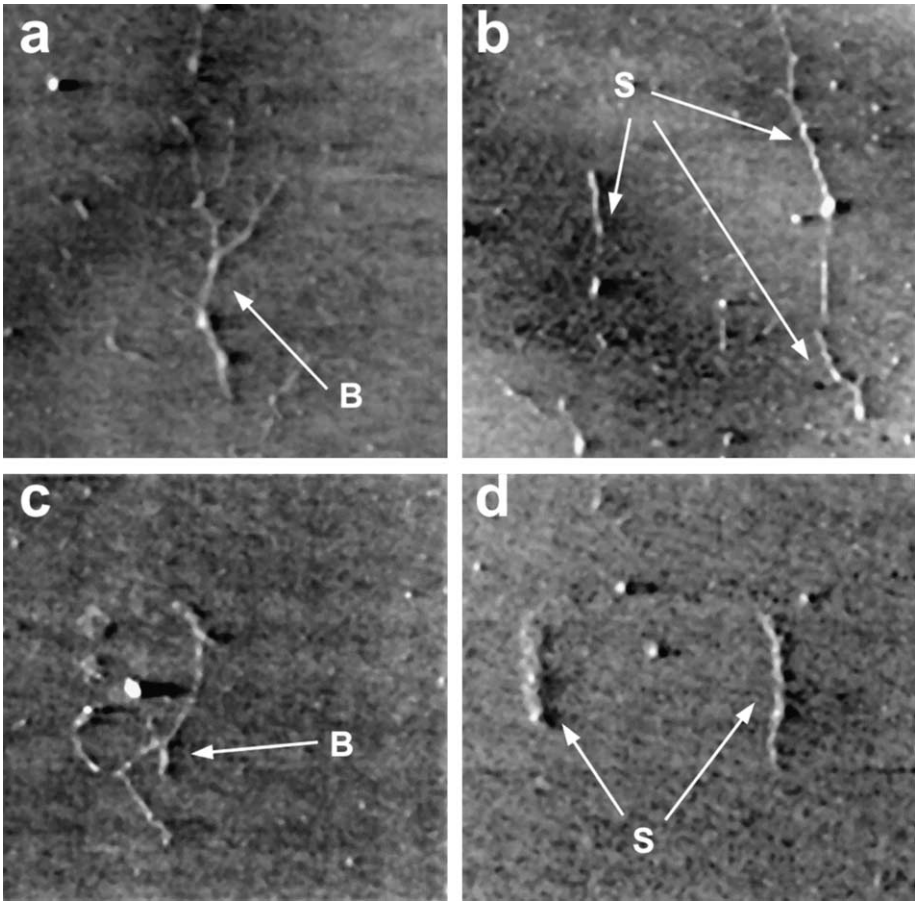

Figure 3. AFM images showing arabinoxylan structures following FAEA treatment for 2 h (a and b) and 4 h (c and d). The data show error signal mode images with an image size of 1 μm × 1 μm. Branched structures (B) are shown in images 'a' and 'c' and linear molecules (S) can be seen in images 'b' and 'd'.

arabinoxylans. Previous studies[3,4] have shown that xylanases can almost completely degrade both the linear and branched structures. The isolation of fragments from xylanase-treated arabinoxylans, consisting of two xylose–xylose–arabinose trisaccharides linked through the arabinose residues by a di-ferulic acid cross-link (XXAF–FAXX), led us to believe that di-ferulic acids were cross-linking arabinoxylans and were probably responsible for the observed larger branches. XXAF–FAXX was not degradable by xylanases, suggesting that both the branches and the conventional backbone of the molecules are based on a xylan chain. The fact that we have observed polymerisation of shorter xylan-based chains through di-ferulic acid linkages, that not all the available phenolic linkages have been cleaved even after overnight incubation, and that branching density does start to decrease on prolonged incubation, suggests that phenolic linkages may account for the existence of the branched structures. Phenolic cross-links close to or at the branch points may be difficult to cleave due to steric effects. Although the present enzymatic degradations are inconclusive and did not reveal new information on the nature of the branched structures, the progressive appearance of polymers with an average 'pullulan molecular mass' of 184×10^3 on FAEA treatment was a new and unexpected observation. This would seem to imply that the extracted arabinoxylan molecules contain shorter arabinoxylan subunits linked together by dimeric ferulic acid linkages. Such results would be consistent with the estimated higher number of di-ferulic acid linkages than branch points.

The biosynthesis of the arabinoxylan backbone and the addition of arabinose takes place intracellularly in Golgi vesicles.[9] Cross-linking of arabinoxylans has been implicated[10,11] in the cell wall assembly and the regulation of cell wall expansion and strength. Cell wall polysaccharides and phenolics are both synthesised in the plant cell wall cytoplasm, but the mechanisms of esterification and transportation have not, as yet, been fully elucidated. Both intracellular and extracellular mechanisms have been proposed. Biochemical studies have demonstrated intracellular feruloylation of arabinoxylans.[12] More recent studies[13] suggest that intracellular feruloylation is restricted to the generation of 8,5′-di-ferulic acid linkages, whilst the remaining dimeric linkages are incorporated extracellularly, possibly mediated through the action of peroxidases.

The data presented here suggest that arabinoxylans may be synthesised as shorter oligosaccharides that are later polymerised into longer chains through creation of phenolic cross-links. The linkages cleaved by the FAEA in the present study are of the type that Obel et al.[13] suggest are generated extracellularly, and could be involved in cell wall assembly. Further experiments are needed to establish whether the branches are attached via phenolic linkages and, if so, what types of linkages are involved. This would help establish the time at which such linkages are generated during biosynthesis.

3. Experimental

3.1. Sample preparation and AFM imaging

Water-soluble arabinoxylans were isolated and prepared from wheat flour (variety *Soisson*; Unilever) as previously described.[3,4] Recombinant *Aspergillus niger* esterase A (FAEA) was produced and purified as previously described.[14] SEC studies were made and calibrated as described elsewhere.[3,4] AFM images were obtained using an East Coast Scientific AFM working in the dc contact mode. Both topographic and error signal mode images were recorded. Analysis of contour lengths was made using Image Tool (UTHSCSA, San Antonia, TX, USA) as previously described.[3,4]

3.2. Esterase activity

Recombinant *A. niger* esterase A (FAEA) activity was assayed using methyl ferulate (MFA) as a substrate with the analysis of products by HPLC as previously described.[15] One unit (1 U) of esterase activity was defined as the amount of enzyme releasing 1 μmol of ferulic acid per min at pH 6.0 and 37 °C. The possible contaminating xylanase activity was measured using wheat flour arabinoxylans (medium viscosity arabinoxylan; Megazyme Inc) as the substrate and dinitrosalicylic acid (DNS) as the reagent.[16]

3.3. Enzymatic treatment of arabinoxylan samples

FAEA treatment was performed in the following manner: Arabinoxylan samples (100 μg, prepared in 2 mM ammonium bicarbonate) were incubated with FAEA (37 °C, pH 6.0) in a final volume of 1.2 mL for time periods of 0.75, 1, 2, 4 h and overnight. The samples were divided into two, with half being tested on the SEC column in order to estimate molecular mass and the other half analysed for the presence of ferulic and di-ferulic acids by HPLC.[3,4]

3.4. Extraction of ferulic acids and di-ferulic acids

Arabinoxylan samples (0.2 g) were saponified by overnight incubation with sodium hydroxide (1 M NaOH in a final volume of 5 mL). The mixtures were agitated at room temperature in the absence of light. The reaction was terminated by the addition of acetic acid and adjusted to pH = 4.0. *trans*-Cinnamic acid was added as an internal standard (100 μL of 100 nmol mL^{-1} stock solution in MeOH). Phenolic compounds were extracted

with equal volumes of ethyl acetate (three times). The combined extracts were dried (rotary evaporator), re-dissolved in 5:50 (v/v) MeOH–water and filtered (0.2 μm).

3.5. Analysis of ferulic and di-ferulic acids

Samples (0.1 mL) were analysed by HPLC. The phenolics were separated and quantified by reverse phase HPLC using a Prodigy 5 ODS-3 column (phenomenex, 5 μm, 250 × 4.60 mm) with 10% acetonitrile in 1 mM trifluoroacetic acid (solvent A) and a gradient employing increasing MeOH–acetonitrile (1:1 (v/v)) (solvent B) in 1 mM trifluoroacetic acid. The following gradient elution conditions were used: = 30 min, 38% B; time = 41 min, 100% B; time = 51 min, 10% B; flow rate = 1 mL min^{-1}; injection loop 100 μL. A dual wavelength detector was used for monitoring the phenolic profiles at 325 and 280 nm and the peak areas were quantified at 325 nm. Unless otherwise stated all solvents were of HPLC grade. Absorbance spectra were performed with a diode array detector by scanning from 230 to 360 nm.

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References

1. Dervilly-Pinel, G.; Rimsten, L.; Saulnier, L.; Andersson, R.; Aman, P. *J. Cereal Sci.* **2001**, *34*, 207–214.
2. Saulnier, L.; Crepeau, M. J.; Lahaye, M.; Thibault, J.-F.; Garcia-Conesa, M. T.; Kroon, P. A.; Williamson, G. *Carbohydr. Res.* **1999**, *320*, 82–92.
3. Adams, E. L.; Kroon, P. A.; Williamson, G.; Morris, V. J. *Carbohydr. Res.* **2002**, *338*, 771–780.
4. Adams, E. L.; Kroon, P. A.; Williamson, G.; Gilbert, H. J.; Morris, V. J. *Carbohydr. Res.* **2004**, *339*, 579–590.
5. Waldron, K. W.; Parr, A. J.; Ng, A.; Ralph, J. *Phytochem. Anal.* **1996**, *7*, 305–312.
6. Hoffmann, R. A.; Roza, M.; Maat, J.; Kamerling, J. P.; Vliegenthart, J. F. G. *Carbohydr. Polym.* **1991**, *15*, 415–430.
7. Izydorczyk, M. S.; Biliaderis, C. G. *Carbohydr. Polym.* **1995**, *28*, 33–48.
8. Izydorczyk, M. S.; Biliaderis, C. G. *Carbohydr. Polym.* **1992**, *17*, 237–247.
9. Gibeaut, D. M.; Carpita, N. C. *FASEB J.* **1996**, *8*, 904–913.
10. Gibeaut, D. M.; Carpita, N. C. *Plant Physiol.* **1991**, *97*, 551–561.
11. Carpita, N. C. *Annu. Rev. Plant Physiol. Mol. Biol.* **1996**, *47*, 445–476.
12. Fry, S. C.; Willis, S. C.; Paterson, A. E. J. *Planta* **2000**, *211*, 679–692.
13. Obel, N.; Porchia, A. C.; Scheller, H. V. *Planta* **2003**, *216*, 620–629.
14. Juge, N.; Williamson, G.; Puigserver, A.; Cummings, N. J.; Connerton, I.; Faulds, C. B. *FEMS Yeast Res.* **2001**, *1*, 127–132.
15. Kroon, P. A.; Garcia-Conesa, M. T.; Fillingham, I. T.; Hazlewood, G. P.; Williamson, G. *J. Sci. Food Agric.* **1999**, *79*, 428–434.
16. Bailey, J. *J. Biotechnol.* **1992**, *23*, 257–270.