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Effects of partial enzymic degradation of sugar beet pectin on oxidative coupling of pectin-linked ferulates *in vitro*

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Abstract

Pectins were extracted from roots and petioles of sugar beet, and treated with α-arabinosidase, 1,4-β-galactanase or polygalacturonase. They were then cross-linked using hydrogen peroxide and peroxidase. The effects on pectin molecular size were monitored by size-exclusion chromatography and viscometry. A decrease in apparent molecular size was observed after α-arabinosidase and polygalacturonase treatment, and all three enzymes caused a decrease in viscosity. The pectins were then cross-linked using hydrogen peroxide and peroxidase, and the effects on dehydrodiferulate formation were monitored by HPLC. Pretreatment with polygalacturonase caused no significant change in subsequent dehydrodiferulate cross-linking, while pretreatment with α-arabinosidase caused a slight change in the ratios of the different dehydrodiferulates formed. Pretreatment with 1,4-β-D-galactanase caused a more significant change in the ratios of the different dehydrodiferulates formed, and also greatly increased the overall recovery of total ferulates (monomers plus dehydrodiferulates), both in root pectin and petiole pectin. The possible effects of polysaccharide microstructure on the dimerisation and further polymerisation of pectin-linked ferulates are discussed.

Keywords: *Beta vulgaris* L.; Chenopodiaceae; Sugar beet; Pectin; Cross-linking; Ferulate; Dehydrodiferulate; α-Arabinosidase; 1,4-β-Galactanase; Polygalacturonase

1. Introduction

Plant primary cell walls contain small but significant amounts of phenolic acids, mainly ferulic and coumaric acids. In the Poaceae, ferulates are mainly linked to arabinose in arabinoxylans. In dicots, ferulates are ester-linked to arabinose and galactose residues in pectin, and they are especially prominent in the Chenopodiaceae. They are linked to the O-6 of galactose in galactan side-chains of rhamnogalacturonan I, and to O-2 and O-5 of arabinan side-chains of rhamnogalacturonan I (Ralet et al., 2005).

Polysaccharide-linked ferulate groups undergo oxidative coupling *in vivo*, to form a range of dehydrodiferulates and -triferulates (Funk et al., 2005; Bunzel et al., 2003; Rouau et al., 2003; Waldron et al., 1997; Ralph et al., 1994), and probably larger coupling products (Fry et al., 2000). This is thought to occur by the action of peroxidase and hydrogen peroxide. As a result, cell-wall polysaccharides become cross-linked (Levigne et al., 2004). These cross-links bring about a substantial increase in molecular weight, which makes it likely that they are inter- rather than intramolecular (Baydoun et al., 2004). Phenolic cross-linking is thought to have a number of important physiological consequences (Waldron et al., 1997). These include decreased cell-wall extensibility, increased intercellular adhesion, and increased protection against digestion by microorgan-

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isms and in the rumen. Dehydrodiferulates and larger coupling products may also act as nucleation sites for lignin biosynthesis (Bunzel et al., 2004).

Cell walls contain at least six different dehydrodiferulate dimers. These occur in different proportions in different plants and in different tissues within the same plant (Wende et al., 2000). Recently, we undertook a study of the conditions required for cross-linking of beet pectins *in vitro*. We reported that the tissue of origin and hydrogen peroxide concentration were the most important factors controlling both the type and the amount of dehydrodiferulates formed (Baydoun et al., 2004). Here, we report the effects of pretreatment of beet pectins with polysaccharidases on subsequent phenolic coupling, and discuss the possible role of polysaccharide microstructure in controlling the coupling reaction.

2. Results

2.1. Effects of polysaccharidases on the molecular size of pectins

Beet root pectin was passed through a Biogel P-10 column, and was found to contain both high- and low-molecular weight carbohydrate. However, the ferulate-containing pectin was found to elute principally at the void volume, with an apparent molecular weight of at least 4 kD. This is in agreement with previous analyses, in which the majority of the ferulate-containing pectin was fully- or partially excluded by Sepharose CL6B (Baydoun et al., 2004) or Sephacryl S 500 (Oosterveld et al., 2000), both of which have a higher exclusion limit than Biogel P-10. When the pectin was treated with α-arabinosidase, *endo*-1,4-β-D-galactanase or polygalacturonase prior to passing through Biogel P-10, α-arabinosidase was found to have the greatest effect in decreasing the apparent molecular size, both for total carbohydrate and for ferulate-containing polymers (Fig. 1). A smaller effect was seen after polygalacturonase treatment, and relatively little effect after 1,4-β-D-galactanase treatment. When the effect of enzymic pretreatment on pectin viscosity was investigated, similar results were obtained (Table 1), with alpha-arabinosidase again having the greatest effect, and 1,4-beta-galactanase the least, though in this case a significant effect of galactanase was seen. It was surprising that the two *endo*-polysaccharidases had less effect on molecular size and viscosity than alpha-arabinosidase, an *exo*-polysaccharidase. However, sugar analysis indicated that the pectin contained at least 70% arabinose, and it is possible that this large amount of arabinan side-chains partially hindered access of the other enzymes to the galacturonan and galactan.

2.2. Effect of enzymic pretreatment on changes in molecular size after cross-linking

In order to study whether an increase in molecular weight occurred as result of treatment with peroxidase and hydrogen peroxide, it was necessary to use a gel filtration medium with a larger pore size than Biogel P-10. As reported previously (Baydoun et al., 2004), gel filtration on Sepharose CL-6B shows an increase in the average molecular weight of pectin after treatment with peroxidase and hydrogen peroxide, together with a decrease in the amount of UV-absorbing material recovered (Fig. 2a; see

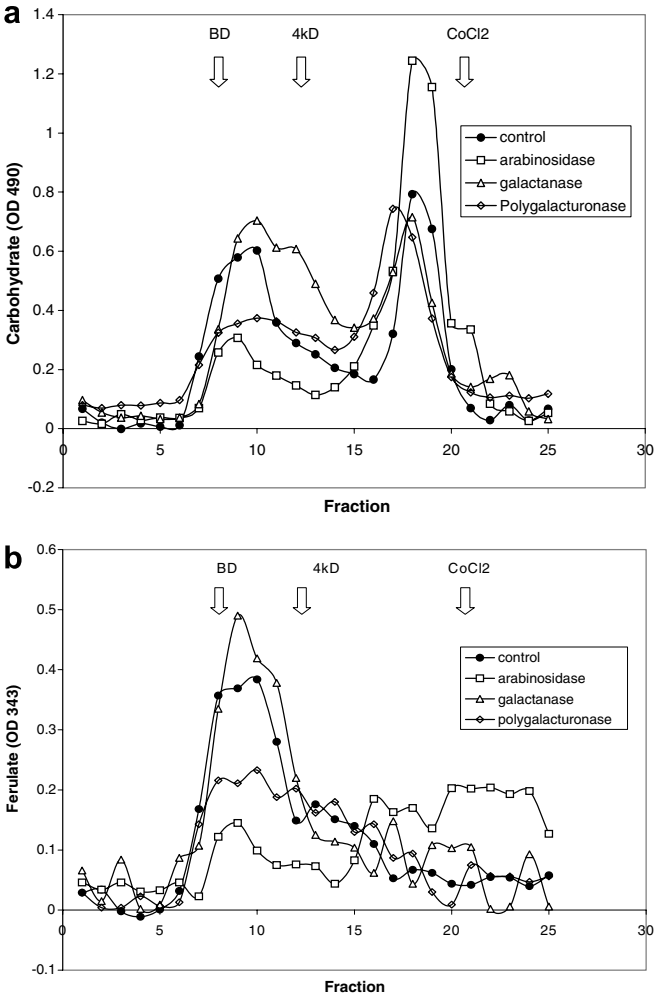

Fig. 1. Gel filtration of root pectins on Biogel P10 before and after treatment with polysaccharidases. Pectins were passed down a column of Biogel P10 (15 cm × 1.0 cm), and fractions of 0.5 ml were collected. (a) Samples of 0.1 ml were assayed for carbohydrate by the method of Dubois et al. (1956), and the absorbance at 490 nm recorded. (b) The distribution of phenolics was monitored by measuring the absorbance at 340 nm. Arrows show positions of elution peaks for blue dextran (BD, void volume marker), 4 kD dextran and cobalt chloride.

Table 1
Specific viscosity of control and enzyme-treated beet root pectin solutions, before and after cross-linking

Pectin treatment	Before cross-linking	After cross-linking
Control	2.21 ± 0.04	4.19 ± 0.22
1,4-β-D-Galactanase	1.32 ± 0.13	1.98 ± 0.39
Polygalacturonase	1.21 ± 0.05	1.53 ± 0.08
α-Arabinosidase	0.74 ± 0.05	0.79 ± 0.05

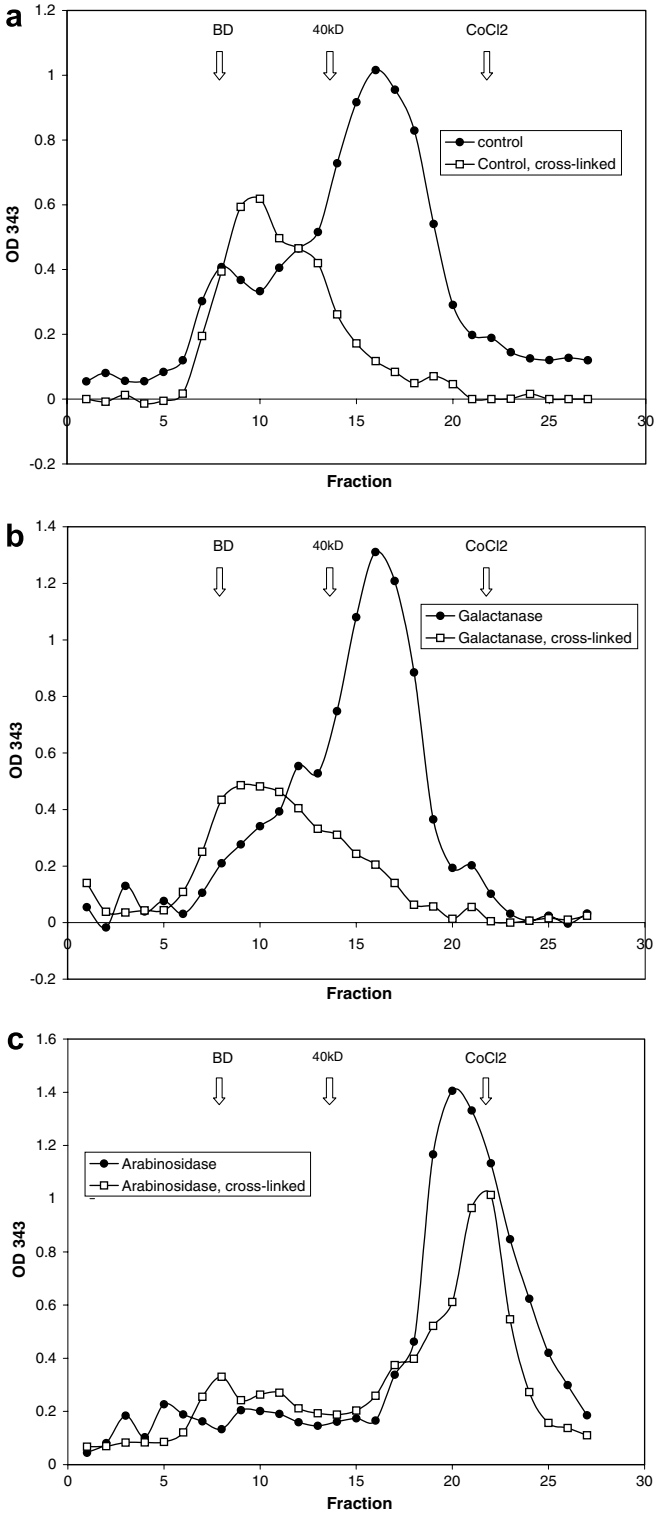

Fig. 2. Gel filtration of polysaccharidase-treated root pectins on Sepharose CL6B before and after cross-linking with hydrogen peroxide and peroxidase. Pectins were treated with buffer only (a), 1,4-β-D-galactanase (b), or α-arabinosidase (c). Part of the sample was then treated with H_2O_2 (0.88 M) and peroxidase (10 μg/ml). Cross-linked and un-cross-linked samples were then passed down a column of Sepharose CL6B (16 cm × 1.0 cm), and fractions of 0.75 ml were collected. The distribution of phenolics was monitored by measuring the absorbance at 340 nm. Arrows show positions of elution peaks for blue dextran (BD, void volume marker), 40 kD dextran, and cobalt chloride.

Fig. 3 of Baydoun et al., 2004). Similar results were seen after 1,4-β-D-galactanase pretreatment (Fig. 2b), and the effects of polygalacturonase pretreatment (not shown) were also similar. Very little increase in apparent molecular weight was seen after α-arabinosidase pretreatment, though there was still a significant decrease in the amount of UV-absorbing material recovered (Fig. 2c). Any increase in molecular size that occurred might have been hard to detect by gel filtration on Sepharose CL-6B, in view of the extremely small size of the products of arabinosidase digestion of the feruloylated carbohydrate (Fig. 1). Enzymic pretreatment also affected the change in viscosity observed on cross-linking (Table 1).

2.3. Effect of enzymic pretreatment on the oxidative coupling of pectin-linked ferulate

The ferulates attached to pectin were analysed by saponification, partitioning into ethyl acetate, and separation by HPLC. As previously reported (Baydoun et al., 2004), the ferulates attached to pectin were predominantly in the monomeric form, with only 6% present as dehydrodimers (Fig. 3). Treatment of pectins with hydrogen peroxide and peroxidase *in vitro* led to a substantial increase in the amount of dehydrodiferulate dimers present, and a more than equivalent drop in the amount of ferulate monomers (Fig. 3). Hence the percentage dimerisation greatly increased, while the recovery of ferulates was only around 50%, compared to non-cross-linked controls. In our previous paper (Baydoun et al., 2004), evidence was presented which suggested that this low recovery of ferulates after oxidative coupling was due to the formation of larger coupling products, e.g. trimers, which were not detected by HPLC. The effect of enzymic pretreatment on dimerisation and recovery was now studied. Neither polygalacturonase

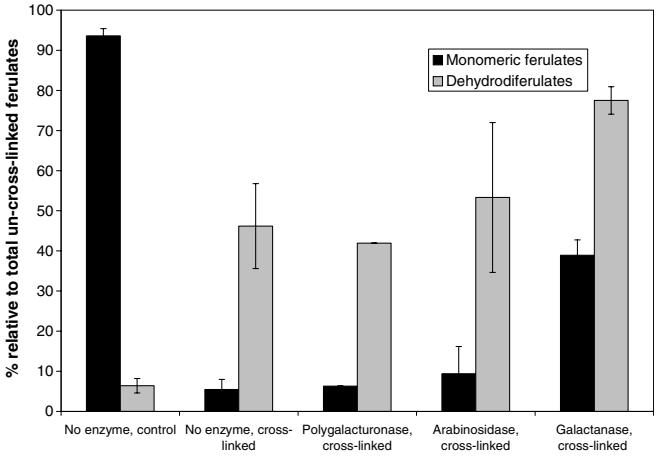

Fig. 3. Effect of cross-linking with hydrogen peroxide and peroxidase on the amount of monomeric ferulates and dehydrodiferulates in root pectins pretreated with polysaccharidases. Phenolic compounds were released from pectin by saponification, extracted with ethyl acetate and analysed by HPLC. Where error bars are shown, two samples were analysed, and results are shown as mean ± the difference between the experimental values and the mean.

nor α-arabinosidase pretreatment showed any significant effect on the subsequent percentage of dimers or overall recovery after oxidative coupling (Fig. 3). However, 1,4-β-D-galactanase pretreatment had a marked effect. The percentage dimerisation was lower after cross-linking than in untreated controls, but the overall recovery of ferulates was greatly increased, to more than 100%. Hence it seems likely that 1,4-β-D-galactanase pretreatment prevented the formation of larger coupling products. Since the recovery of ferulates was slightly greater than in the controls, it also seems likely that the efficiency of release of ferulates from pectin by alkali was increased by 1,4-β-D-galactanase treatment or cross-linking or the combination of the two. This implies that some ferulates were resistant to saponification in the control, but released more easily after 1,4-β-D-galactanase treatment and cross-linking. Cross-linking by itself is unlikely to increase saponification efficiency, since two ester bonds require to be broken to release a dehydrodimer, while only one must be broken to release the monomer. Hence this effect is more likely to be due to 1,4-β-D-galactanase treatment than to the subsequent cross-linking. This conclusion was confirmed by the observation that overall recovery of ferulates after 1,4-β-D-galactanase treatment alone was also greater than in the controls, and very similar to the recovery after 1,4-β-D-galactanase treatment followed by cross-linking (results not shown). However, the mechanism of this effect of 1,4-β-D-galactanase is unclear. The high recovery of ferulate was not seen if the pretreatment with 1,4-β-D-galactanase was carried out for 0 min rather than 60 min (results not shown). Hence it was due to the action of the enzyme on the pectin rather than to the presence of the enzyme molecule during subsequent cross-linking.

The data were therefore analysed to show how enzyme pretreatment affected the amounts of the individual dimers. Pretreatment with polygalacturonase caused no detectable change in the amounts or proportions of the individual dehydrodimers (data not shown). However, pretreatment with 1,4-β-D-galactanase was found to increase the amounts of all the dehydrodimers, compared to controls in which oxidation was carried out without previous 1,4-β-D-galactanase treatment (Fig. 4). Furthermore, the ratios of the different dimers were altered as a result of 1,4-β-D-galactanase pretreatment, so that the 8-O-4 dehydrodimer was now the major coupling product, rather than the 8,5B dehydrodimer.

Pretreatment with α-arabinosidase also affected the proportions of the different dehydrodimers recovered, though not to the same extent. The amounts of the 8-O-4 and the 8,5B dehydrodimer were approximately equal following α-arabinosidase pretreatment (Fig. 4). However, the total amount of dehydrodimers formed was not significantly increased by α-arabinosidase pretreatment.

Pectin was also extracted from the petioles of sugar beet plants, treated with polysaccharides, cross-linked with hydrogen peroxide and peroxidase, and analysed by HPLC. In this case also, 1,4-β-D-galactanase pretreatment

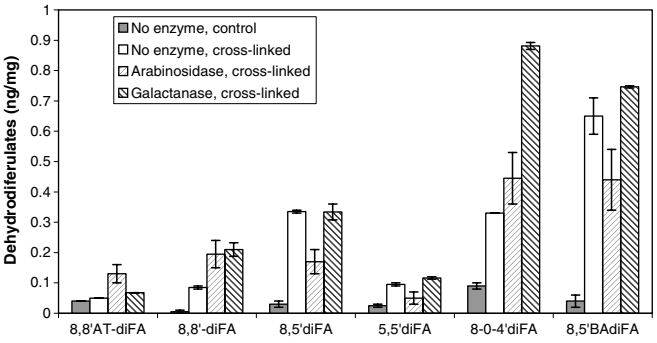

Fig. 4. Effect of cross-linking with hydrogen peroxide and peroxidase on the amount of the different dehydrodiferulates in root pectins pretreated with polysaccharidases. Phenolic compounds were released from pectin by saponification, extracted with ethyl acetate and analysed by HPLC. Two samples were analysed, and results are shown as mean ± the difference between the experimental values and the mean. Data are shown as ng dehydrodiferulate per mg fresh weight of tissue.

greatly increased the recovery of total ferulates after cross-linking (Fig. 5). It also caused a similar shift in the pattern of dehydrodiferulates formed (Fig. 6), though the

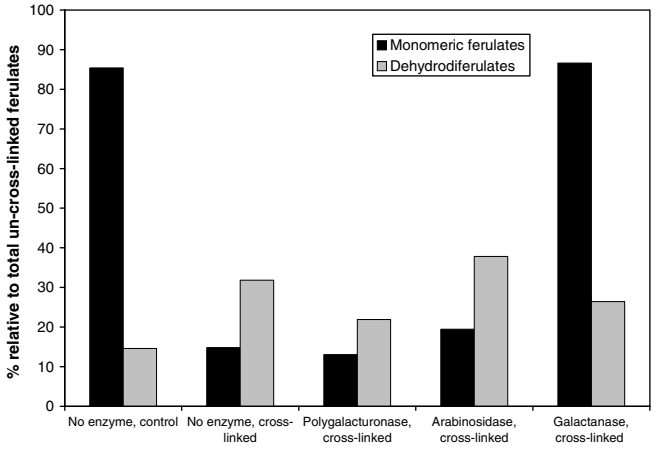

Fig. 5. Effect of cross-linking with hydrogen peroxide and peroxidase on the amount of monomeric ferulates and dehydrodiferulates in petiole pectins pretreated with polysaccharidases. Phenolic compounds were released from pectin by saponification, extracted with ethyl acetate and analysed by HPLC.

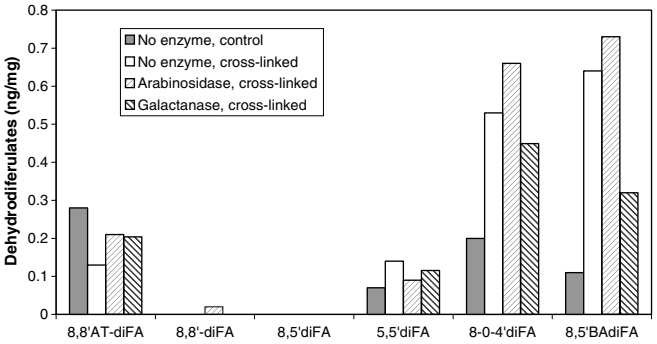

Fig. 6. Effect of cross-linking with hydrogen peroxide and peroxidase on the amount of the different dehydrodiferulates in petiole pectins pretreated with polysaccharidases. Phenolic compounds were released from pectin by saponification, extracted with ethyl acetate and analysed by HPLC. Data are shown as ng dehydrodiferulate per mg fresh weight of tissue.

total amount of dehydrodiferulates did not increase following 1,4-β-D-galactanase pretreatment.

3. Discussion

All three polysaccharidases studied caused either an decrease in apparent molecular weight of beet root pectin, as judged by gel filtration, or a decrease in viscosity, or both. Hence all were able to act enzymically on pectin. The very great decrease in size caused by α-arabinosidase is consistent with the high level of arabinose found in this pectin. This *exo*-polysaccaridase would not be expected to cleave feruloylated arabinose residues from pectin. Examination of the products of α-arabinosidase treatment by paper chromatography in butanol/acetic acid/water (12:3:5) (Wende and Fry, 1997) confirmed that neither ferulate nor feruloyl-arabinose was released from the pectin by the enzyme. However, we cannot rule out the possibility that larger feruloylated oligosaccharides were released. If the decrease in size was solely due to α-arabinosidase activity, then the feruloyl groups must only be present on a minority of the arabinan side-chains, or be located mostly towards the inner ends of these side-chains, or both.

Treatment of the pectin with peroxidase and hydrogen peroxide, after pretreatment with α-arabinosidase, did not cause any detectable increase in apparent molecular weight. Cross-linking still occurs, as shown in Fig. 3, though the proportions of the different dehydrodimers formed changes (Fig. 4). It is possible that the fragments were too small for the increase in size to be seen on Sepharose CL6B. Alternatively, the cross-links formed after α-arabinosidase treatment may be predominantly intramolecular.

In contrast, Guillon and Thibault (1990) found that when persulphate was used for the oxidative cross-linking of sugar beet pectins, treatment with arabinofuranosidase brought about a significant increase in gelling capacity, and therefore, presumably, of pectin cross-linking and molecular weight. There are two differences between the work of Guillon and Thibault (1990) and our work which might bring about this difference. First, the method used to prepare the pectin was different. Guillon and Thibault (1990) obtained pectin from oxalate-extracted sugar beet pulp, using 0.05 M HCl for 30 min at 85 °C. In the current work, pectin was obtained directly from ethanol-extracted beetroot slices, using 0.1 M HCl for 20 min at 85 °C. The fact that pre-extraction with oxalate had been carried out in the former work means that the detailed structures of the two pectins are likely to have been different. For instance, the pectin used in the current work appears to be of rather lower molecular weight and to have a higher content of arabinose. Secondly, different cross-linking conditions were used. Guillon and Thibault (1990) used 0.04 M ammonium persulphate at 37 °C for 48 h to obtain gellation, while in the current work dimerisation was obtained with 0.88 mM hydrogen peroxide and horseradish peroxidase for 5 min. In our previous work, we found that treatment of our pectin preparation with ammonium persulphate for up to 12 h, followed by saponification and analysis of the ferulates released, did not give rise to any dehydrodiferulates corresponding to those formed using peroxidase and hydrogen peroxide (Baydoun et al., 2004). Hence it is likely that the mechanism of cross-linking is different, which may explain why persulphate brought about gelation after arabinosidase treatment, whereas hydrogen peroxide and peroxidase brought about no apparent increase in molecular weight.

Treatment of the pectin with 1,4-β-D-galactanase produced relatively little effect on the molecular weight of pectin, consistent with the relatively small amount of galactose present in the pectin. However, it did cause a significant change in the relative and absolute amounts of monomeric ferulates and dehydrodiferulates recovered after cross-linking. Total monomers and dehydrodimers both increased, and the relative proportions of the dehydrodimers altered. The results were somewhat similar to those found when the concentration of hydrogen peroxide was decreased below 0.1 M (Baydoun et al., 2004), though the amounts of dehydrodimers formed after 1,4-β-D-galactanase pretreatment were much higher than at such low hydrogen peroxide concentrations. This suggests that a major effect of 1,4-β-D-galactanase treatment was to inhibit the formation of ferulate trimers and larger aggregates during subsequent cross-linking. Hence relatively minor changes to the structure of the pectin caused a major change in the cross-linking patterns seen with peroxide and hydrogen peroxide.

These results suggest that the polysaccharide microstructure is an important factor in controlling the oxidative cross-linking of pectin-linked ferulate. Since ferulate trimers and larger aggregates are thought to act as nucleation points for the initiation of lignin biosynthesis, such fine structural details within pectin molecules may exert a major influence on the lignification process. It will be of considerable interest to determine whether the same effects are observed with ferulated arabinoxylans in cereals.

4. Experimental

4.1. Extraction of pectins from beet

Growth of sugar beet (*Beta vulgaris* L., var. Saxon) plants (for 8–10 weeks) was as described (Wende et al., 2000). Leaves and petioles were collected. Tissues were then extracted with ethanol followed by 0.1 M HCl at 80 °C for 20 min. The HCl extract was dialysed and then concentrated by rotary evaporation. See Baydoun et al. (2004) for details.

4.2. Polysaccharidase treatment of pectin

α-Arabinosidase, *endo*-1,4-β-D-galactanase and polygalacturonase were obtained from Megazyme, Ireland. Incu-

bations were carried out with 0.5 ml pectin (approximately 5 mg/ml carbohydrate) and 10 units enzyme at 40 °C for 1 h. No additional buffer was used due to the high buffering capacity of the pectin (pH 4, close to the pH optima of the enzymes used).

4.3. Oxidative treatment of pectin

Oxidation with peroxidase and hydrogen peroxide, saponification and extraction of phenolics, and HPLC were carried out as described (Baydoun et al., 2004). Relative viscosities were estimated as the relative flow times through a 5 ml syringe at 20°.

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