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Control of dehydrodiferulate cross-linking in pectins from sugar-beet tissues

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Abstract

Pectins were extracted from roots, petioles and leaves of sugar beet, and cross-linked using hydrogen peroxide and peroxidase. The effects on dehydrodiferulate formation were monitored by HPLC and TLC. Dehydrodimers were formed in different proportions to those found in vivo. There was a net loss of around 50% of the phenolic groups (monomers plus dimers) during dimerisation. Gel filtration showed that root and petiole pectin, but not leaf pectin, increased in molecular weight during cross-linking. The effects of varying the cross-linking conditions were investigated, and it was found that hydrogen peroxide concentration was the most important factor in controlling both the type and amount of dehydrodiferulate formed.

Keywords: *Beta vulgaris* L.; *Chenopodaciaceae*; Sugar beet; Pectin; Cross-linking; Ferulate; Dehydrodiferulate; Hydrogen peroxide; Peroxidase

1. Introduction

A key factor in determining plant cell-wall properties is the manner in which the polymers of the cell walls are cross-linked (Waldron et al., 1997b). Growing cell walls contain phenolic acids, principally ferulic and coumaric acids, ester-linked to pectins in dicots and to arabinoxylans in grasses (Waldron et al., 1997b; Ralph et al., 1994). Some of the ferulic acid moieties are oxidatively coupled to form dehydrodiferulate (DFA) dimers, which cross-link the polysaccharides (Waldron et al., 1996; Ralph et al., 1994). These cross-links are thought to have important roles, such as decreasing cell-wall extensibility during cell maturation (Sanchez et al., 1996) and when dark-grown tissues are exposed to light (Miyamoto et al., 1994). DFA cross-links also promote cell-cell adhesion (Ng et al., 1998) and act as nucleation sites for lignin synthesis (Ralph et al., 1995; Grabber et al., 2002). They may limit cell-wall degradability in forage grasses (Grabber et al., 1998). In foods, they maintain tissue texture during cooking (Waldron et al., 1997a), and (together with other phenolics) they have anti-oxidant properties which are implicated in protecting against major diseases such as cancer, atherosclerosis and cataract (Kroon and Williamson, 1999).

Six different dehydrodiferulate dimers occur in cell walls (Ralph et al., 1994). In most angiosperm tissues, the 8–O–4 and 8–5 benzofuran (8–5B) dehydrodimers are the most abundant, while in the one gymnosperm studied, the 8–8 aryltetralyn (8–8A) dehydrodimer was the most abundant (Sanchez et al., 1996). In beet, the degree of ferulate cross-linking is much greater in roots than in the shoot (Wende et al., 1999). Recently it has been shown that the relative amounts of the dehydrodimers vary in different tissues in the same plant: in leaves, the 8–8A dehydrodimer is the most abundant, while the 8–O–4 predominates in the rest of the plant (Wende et al., 2000). It is not known what causes these variations in the degree of cross-linking and the nature of the cross-links formed. However, both the degree and nature of cross-linking affect the subsequent reaction of these phenolics with monolignols during lignin formation (Grabber et al., 2002).

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Feruloylated pectin can be extracted from the storage roots of sugar beet using hot dilute acid (Thibault and Rombouts, 1986). This pectin increases in viscosity when treated with oxidising agents such as peroxidase/hydrogen peroxide or ammonium peroxysulphate, and forms gels under certain conditions (Thibault et al., 1991). We have used a similar in vitro system to study DFA formation and consequent pectin cross-linking, using pectin extracted from different tissues of beet. The effects of varying the tissue of origin and the cross-linking conditions are reported.

2. Results

2.1. Ferulic acid and coumaric acid content of pectins from different organs of sugar beet

Phenolic acids were hydrolysed from sugar beet pectins using 2 M NaOH, and quantified by HPLC. The amounts of monomeric ferulate and coumarate obtained are shown in Fig. 1 (left-hand data, dark bars). Root pectins contained significant levels of ferulic acid, but relatively little coumaric acid. Petioles contained similar or slightly greater levels of ferulate than roots, and levels of coumarate that were around 20% of ferulate levels, but still an order of magnitude greater than those in roots. In leaves, ferulate levels were significantly lower than in roots or petioles, while coumarate levels were also low, though higher than those in roots. Thus the pectins derived from the major organs in sugar beet had different ferulate:coumarate ratios: 33:1 in root pectin, 5:1 in petiole pectin, 7:1 in leaf pectins.

Ratios of the isomers of ferulic and coumaric acids were similar throughout. The ferulic acid content was made up of 70–80% *trans*-ferulate, with 20–30% *cis*-ferulate. Coumarate was made up of 65–70% *trans*-coumarate and 30–35% *cis*-coumarate.

2.2. Ferulate dehydrodimers present in pectins from different organs of sugar beet

In addition to *trans*- and *cis*-ferulate, small amounts of ferulate dehydrodimers were present in the pectin from all three organs (Fig. 1; right-hand data, dark bars). In roots the dehydrodimers represented 3–8% of the total ferulates (monomers + dimers). In petioles and leaves, the dehydrodimers made up 4–15% and 3–12% of the total ferulates, respectively. In roots and petioles, the 8–O–4 dehydrodimer was the most abundant, and the 8–5B and 8–8A dehydrodimers were the next most abundant. The other three dehydrodimers were present in lower amounts. In leaves, the 8–O–4 dehydrodimer was again the most abundant, but relative amounts of other dehydrodimers could not be assessed due to the low amounts present.

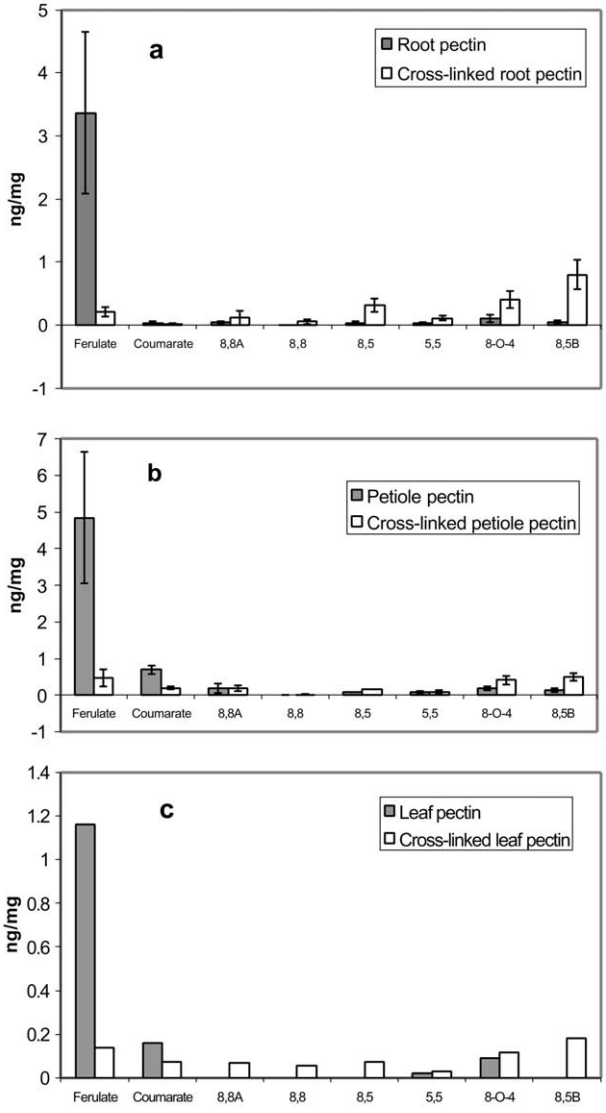

Fig. 1. Ferulate, diferulate and coumarate content of beet tissues before and after cross-linking with hydrogen peroxide and peroxidase. Phenolic compounds were released from pectin by saponification, extracted with ethyl acetate and analysed by HPLC. Figs. 1a, 1b and 1c represent root, petiole and leaf pectin respectively. Dark bars: controls; light bars: cross-linked pectin.

All the pectin preparations contained much lower proportions of dehydrodimers than the intact tissue, which contained 30% and 68% dehydrodimers in shoots and roots respectively (Wende et al., 1999). This is understandable, since dehydrodimers forming intermolecular cross-links would be expected to make pectin relatively insoluble. The acid conditions used to extract the pectins would be expected to break some arabinofuranose bonds. It is likely that pectin would only be extracted when most or all of the arabinose residues participating in intermolecular crosslinks via ferulate dehydrodimers had been released from the pectin molecule by acid hydrolysis.

The relative amounts of the different dehydrodimers were similar in root and petiole pectin to the tissues from which they were extracted. In intact tissues of both root and petiole, the 8–O–4 dehydrodimer was present in greatest amounts, while 8–5B and 8–8A dehydrodimers were the next highest in abundance (Wende et al., 2000). However, in leaf tissue, the 8–8A and 8–8 dehydrodimers were the most abundant (Wende et al., 2000), and thus these dehydrodimers were selectively lost compared to the 8–O–4 dehydrodimer when the pectin was extracted. Presumably the 8–8A and 8–8 dehydrodimers remained in the unextracted tissue.

2.3. Effect of treatment of root pectin with peroxidase and hydrogen peroxide

Treatment of root pectin with peroxidase and hydrogen peroxide caused a decrease of 90–95% in the ferulate monomers (Fig. 1a). Both *trans*- and *cis*-ferulate were affected to the same extent. The decrease in ferulate can also be seen after separation of phenolics by TLC (Fig. 2). In contrast, amounts of dehydrodimers increased following this treatment (Fig. 1a), so that dehydrodimers now formed at least 80% of the recoverable ferulates. Amounts of all the dehydrodimers increased, but by far the largest increase was in the 8–5B dehydrodimer, whose concentration increased by a factor of at least 10. As a result, the 8–5B dehydrodimer became the most abundant one, followed by the 8–O–4 and 8–5 DFAs. Since the 8–O–4 dehydrodimer was the most abundant both in the intact tissue and in the extracted pectin, it is clear that the in vitro cross-linking reactions produced altered relative amounts of the different dehydrodimers, compared to in vivo cross-linking.

The combined increases in dehydrodimer concentrations were significantly less than the decrease in monomer concentrations. Hence the total amount of ferulates decreased by around 50%. This could be due to the direct conversion of monomers into products other than DFAs, or to further reaction of some of the dehydrodimers, perhaps to form dehydrotrimers or larger complexes. Dehydrotrimers (dehydrotriferulates) have recently been discovered in maize (Bunzel et al., 2003; Rouau et al., 2003).

Increases in dehydrodimer concentrations could also be observed by TLC (Fig. 2). The Rf's of five of the six dehydrodimers were established by eluting spots from a TLC plate, eluting the phenolics, and analysing by HPLC (Wende, Brett and Waldron, unpublished results). This permitted the use of TLC for qualitative assessments of cross-linking. For root pectin, increased amounts of all five dehydrodimers could be detected on TLC after cross-linking. The amount of UV-absorbing material with very low Rf, remaining at the origin, also appeared to increase. This material may correspond to phenolic trimers or larger complexes.

As expected from the work of Thibault et al (1991), cross-linking was accompanied by an increase of viscosity (Table 1). A substantial increase in molecular weight was also observed, with much of the pectin being converted from less than 10 kD to more than 76 kD, as judged by gel filtration on Sepharose CL6B, using dextran standards (Fig. 3a).

Table 1
Viscosity of pectin solutions, measured as a percentage of the flow-time of uncrosslinked pectin from roots

Source of pectin	Before cross-linking	After cross-linking
Root	100	157
Petiole	38	41
Leaf	32	32

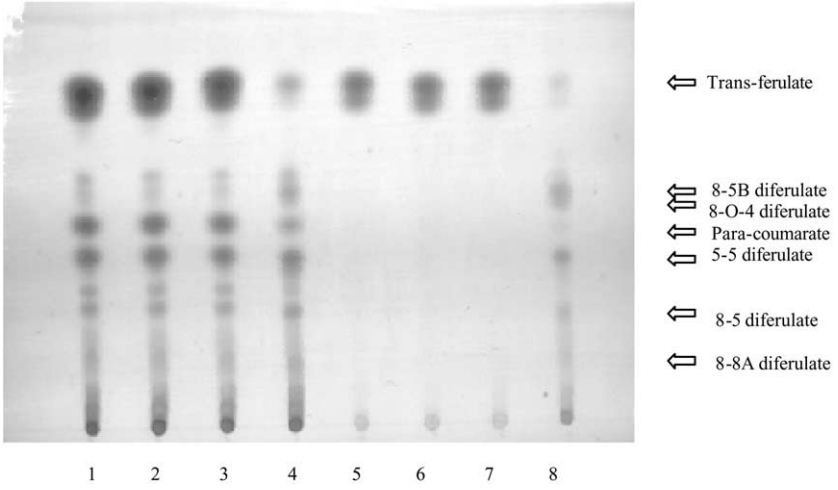

Fig. 2. TLC of phenolics from leaf and root pectin, after treatment with hydrogen peroxide and/or peroxidase. Phenolic compounds were released from pectin by saponification, extracted with ethyl acetate and analysed by TLC. Lanes 1–4: Leaf pectin; lanes 5–8: root pectin. Lanes 1 & 5: no addition; 2 & 6: peroxidase only; 3 & 7: hydrogen peroxide only; 4 & 8: peroxidase and hydrogen peroxide.

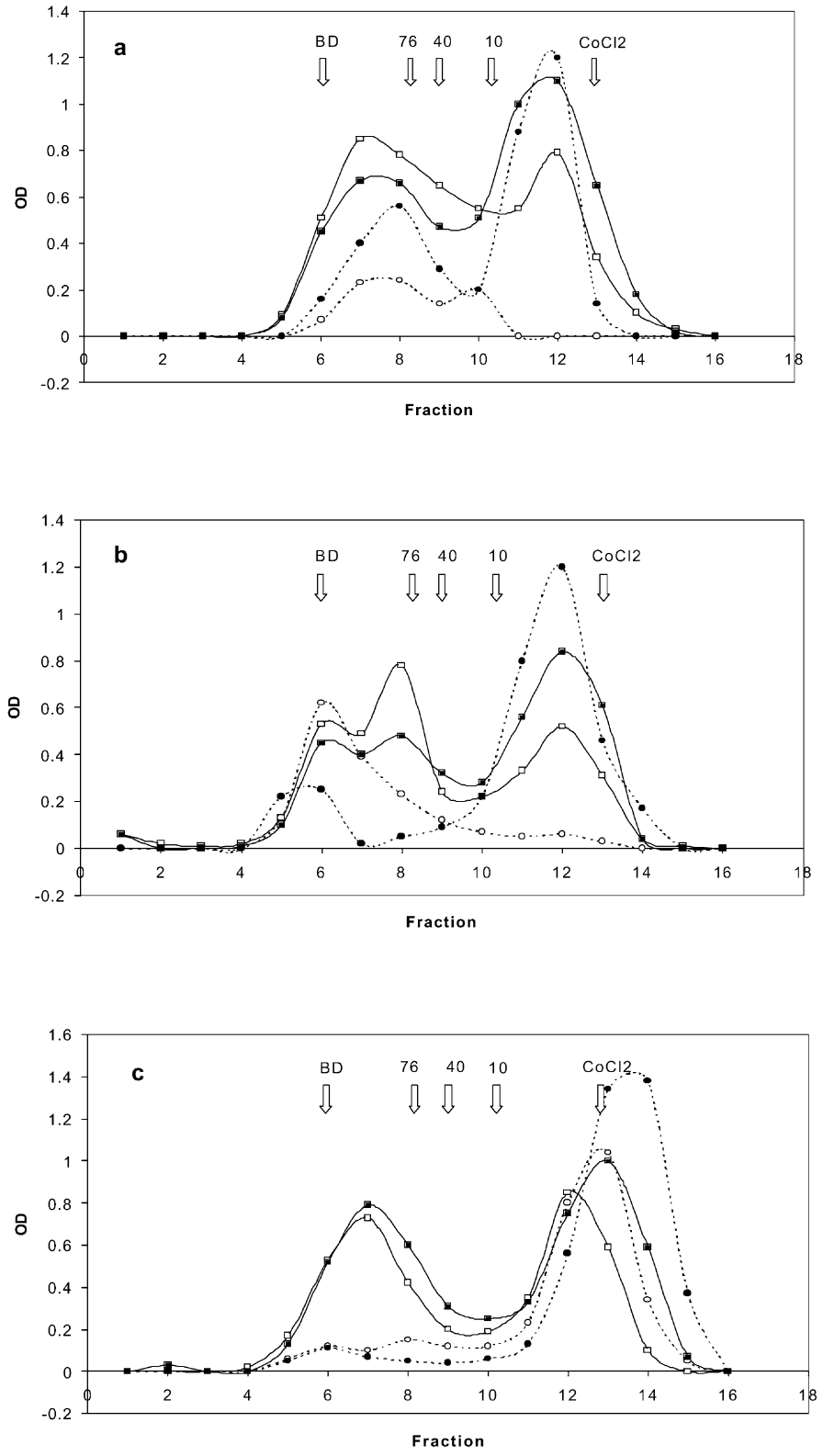

Fig. 3. Gel filtration of pectins on Sepharose CL6B before and after cross-linking with hydrogen peroxide Pectins were passed down a column of sepharose CL6B (40×1.0 cm), and fractions of 3.0 ml were collected. The distribution of phenolics was monitored by measuring the absorbance at 340 nm (circles, dotted lines). Samples of 0.1 ml were then assayed for carbohydrate by the method of Dubois et al (1956), and the absorbance at 490nm recorded (squares, continuous lines). Dark symbols: controls; open symbols: pectin cross-linked with hydrogen peroxide and peroxidase. Figs. 3a, 3b and 3c show pectin from root, petiole and leaf, respectively. Arrows show positions of elution peaks for blue dextran (BD, void volume marker), dextrans of 76, 40 and 10 kD, and cobalt chloride.

2.4. Effect of treatment of petiole pectin with peroxidase and hydrogen peroxide

The effect of hydrogen peroxide and peroxidase on petiole pectins is shown in Figs. 1b and 4. The loss of monomeric ferulates was around 80% (Fig. 1b), and the increase in dehydrodimers was not quite as great as in root pectin, but the changes were qualitatively very similar to those in root pectin. Total ferulates decreased by 50%, and the major dehydrodimer formed was the 8–5B dimer. The loss of monomeric ferulate and coumarate, and increases in some of the dehydrodimers, can be seen by TLC (Fig. 4). It can also be seen that increasing the strength of alkali used to saponify the phenolics had little effect, indicating full extraction of ester-linked phenolics by 2 M NaOH.

In contrast to root pectin, significant amounts of coumarate were present in petiole pectin. Coumarate content also decreased markedly after oxidative treatment, to about 30% of its original level (Fig. 1b).

The viscosity of petiole pectin was considerably less than that of root pectin, and the viscosity increased only slightly after cross-linking (Table 1). However, gel filtration studies indicated a molecular weight increase on cross-linking, to a similar degree to that of root pectin (Fig. 3b).

2.5. Effect of treatment of leaf pectin with peroxidase and hydrogen peroxide

Treatment of leaf pectin with hydrogen peroxide and peroxidase also resulted in the loss of both monomeric ferulate and coumarate, to the same degree as in petiole pectin (Fig. 1c). Dehydrodimer levels also increased. Because of the small amount of dehydrodiferulate initially present, it was hard to compare the relative changes in the different dehydrodimers. The greatest increase again appeared to be in the 8–5B dehydrodimer.

TLC analysis indicated that a number of unidentified phenolic compounds were present in leaf pectins (Fig. 2). Some decreases in these phenolics were also seen after treatment with peroxidase and hydrogen peroxide. The presence of these phenolics to some extent masks any changes that may be occurring in dehydrodimer concentrations, though an increase in the 8–O–4 dehydrodimer can be seen.

The viscosity of leaf pectin was lower than that of either root or petiole pectin (Table 1). No increase in viscosity was observed after oxidative treatment. Gel filtration on Sepharose CL6B indicated very little change in molecular weight after this treatment (Fig. 3c).

2.6. Effect of pH and temperature on ferulate cross-linking in root pectin

The pH of cross-linking under standard conditions was 4.0. This was due to buffering effect of the pectin itself. The pH was adjusted by addition of HCl or NaOH to give final pH values of 3–9. These changes in pH caused relatively little change in the pattern or extent of cross-linking (results not shown).

In order to test the effect of temperature on cross-linking, the temperature of each of the reactants was adjusted to the temperature to be investigated, and the reactants were then mixed and maintained at the same temperature for 5 min. The results (Fig. 5) showed that there was a slight increase in the amount of the major dehydrodimers formed (8–O–4 and 8–5B) up to 30 °C, after which the amounts formed dropped sharply. At 40 °C, the relative amounts of the dehydrodimers were

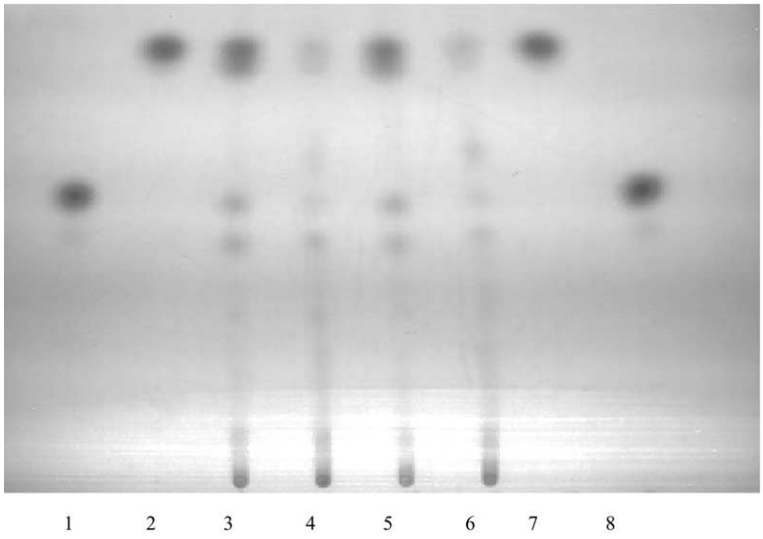

Fig. 4. TLC of phenolics from petiole pectin. Phenolic compounds were released from pectin by saponification, extracted with ethyl acetate and analysed by TLC. Lanes 1 & 8: *p*-coumaric acid standard. Lanes 2 & 7: *trans*-ferulic acid standard. Lanes 3 & 4: pectin extracted with 4 M NaOH. Lanes 5 & 6: pectin extracted with 2 M NaOH. Lanes 3 & 5: controls. Lanes 4 & 6: pectin treated with hydrogen peroxide and peroxidase.

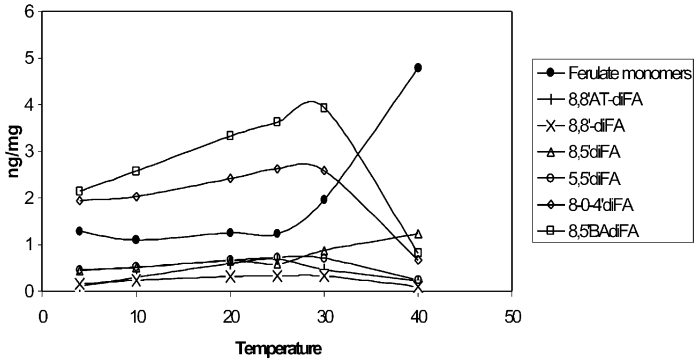

Fig. 5. Effect of temperature on ferulate dimerisation. Root pectin was cross-linked with peroxidase and hydrogen peroxide at the temperature indicated, and the ferulates released by saponification, extracted into ethyl acetate and monitored by HPLC.

different, with the 8–5 dehydrodimer becoming the major DFA formed. At this temperature, the amount of monomeric ferulate remaining was much greater than at lower temperatures, with the percentage dimerisation dropping from 87% at 25 °C to 41% at 40 °C.

2.7. Effects of peroxidase and hydrogen peroxide concentrations on cross-linking in root pectin

Varying the peroxidase concentration between 4 and 20 μg/ml had very little effect on the amounts of dehydrodimers formed (Fig. 6). The only significant change was that the amount of monomeric ferulate remaining at the lowest concentration was almost three times that present at the highest concentration.

Variations in hydrogen peroxide concentration had a greater effect, especially at low concentrations (Fig. 7a). The total amounts of dehydrodimers formed rose to a maximum at about 0.3 mM H_2O_2, and then fell at higher concentrations to a level which was around two-thirds of the maximum. Increasing the concentration of H_2O_2 up to 35 mM produced no further significant change in dimerisation (results not shown). Both *trans*-trans and *cis*-ferulic acid decreased in concentration from 0 up to 0.3 mM H_2O_2. However, the decrease in overall ferulate concentration (momomers + dimers) was much less at the lowest H_2O_2 concentrations. This suggests that the decrease in overall ferulate concentration at 0.3 mM and above was due to further reaction of dehydrodimers with excess H_2O_2, producing larger complexes.

When the effects of varying H_2O_2 on the concentrations of individual dehydrodimers was examined, it was found that amounts of five of the six DFAs increased to a maximum at between 0.1 and 0.3 mM, and then decreased (Fig. 7b). However, there were variations in the exact position of the maximum and the shape of the curve, suggesting a different balance between dehydrodimer formation and further reaction of the dehydrodimer in each case. The concentration of the sixth DFA, the 8–8A form, hardly changed at all, suggesting that any dehydrodimers formed underwent further reaction extremely quickly.

2.8. Effect of pectin concentration on cross-linking in root pectin

Decreasing the pectin concentration had little effect on either the absolute or relative amounts of dehydrodimers formed (Fig. 8). Likewise, doubling the pectin concentration had little effect (results not shown). This apparent insensitivity of the reaction to pectin concentration was probably due to the rapidity with which the reaction reached completion. The changes seen by TLC

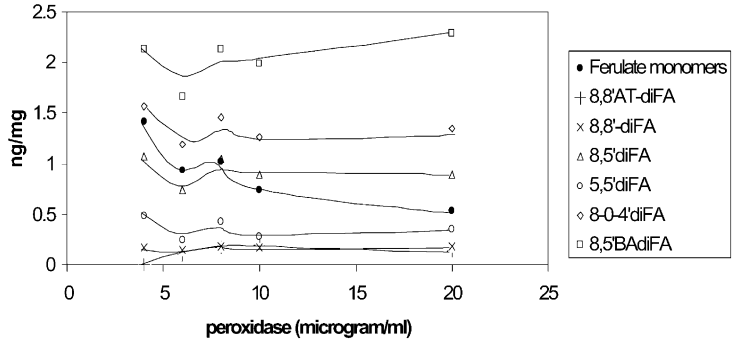

Fig. 6. Effect of varying peroxidase concentration on ferulate dimerisation. Root pectin was cross-linked with hydrogen peroxide and peroxidase at the peroxidase concentration indicated. The ferulates were released by saponification, extracted into ethyl acetate and monitored by HPLC.

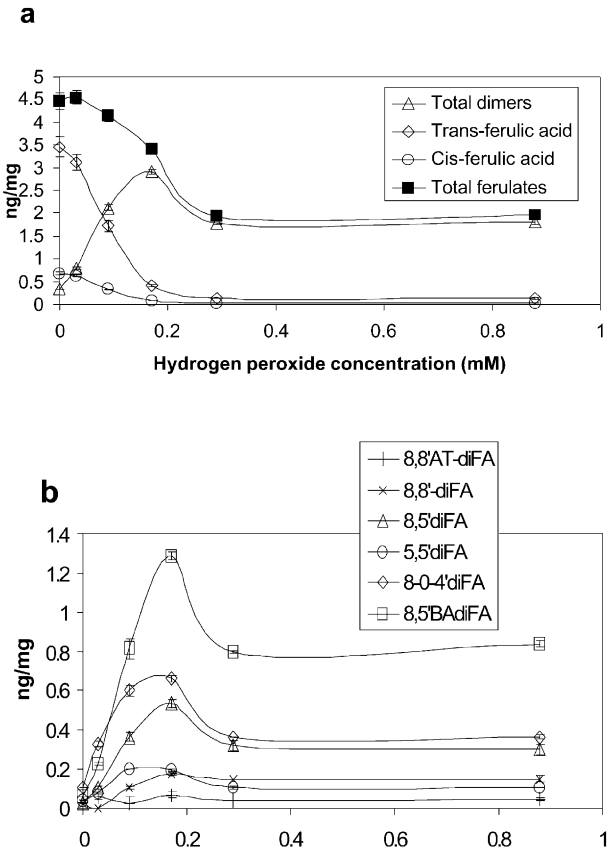

Fig. 7. Effect of varying hydrogen peroxide concentration on ferulate dimerisation. Root pectin was cross-linked with peroxidase and hydrogen peroxide at the hydrogen peroxide concentration indicated. The ferulates were released by saponification, extracted into ethyl acetate and monitored by HPLC.

were compete within 30 sec. Changes in viscosity were complete within one minute, the shortest reaction time that could be studied by viscosity measurements. Since the standard incubation time was 5 min, the results indicate that changes in pectin concentration had no effect on the final ferulate and DFA concentration. Changes in the rate of reaction would be hard to study using our methods.

2.9. Effect of ammonium peroxysulphate on root pectin

Thibault and Rombouts (1986) examined the effects of several oxidising agents on the viscosity and gelling behaviour of beet root pectins. They found that treatment either with hydrogen peroxide and peroxidase or with ammonium peroxysulphate could increase viscosity. Effects of ammonium peroxysulphate on our root pectin were therefore compared with effects of peroxide/peroxidase. The results showed that ammonium peroxysulphate caused decreases in monomeric ferulates similar to those caused by peroxide/peroxidase (Fig. 9). However, dehydrodimer concentrations generally also decreased, becoming undetectable in most cases. Hence the increases in viscosity and gelling observed with ammonium peroxysulphate were not due to the formation of the same dehydrodimers which are formed with peroxide/peroxidase. This is in accordance with the different mechanisms proposed for the two oxidising agents by Thibault et al. (1991) and Ralph et al. (1994) respectively. Only one significant saponifiable, UV-absorbing compound was formed with ammonium peroxysulphate, with an Rf of approximately 0.4 (Fig. 9). This compound was absent in unreacted pectin and after peroxide/peroxidase treatment. Fig. 9 also shows that the observed effects of both oxidising agents were complete within 5min, with incubation for up to 12 hours producing no further change. This would be expected for peroxide/peroxidase, since no further changes in viscosity were seen after 5 min. However, it is more surprising for ammonium peroxysulphate, since this agent is reported to have effects on viscosity for several hours (Thibault and Rombouts, 1986).

3. Discussion

The results show that the extent and nature of ferulate cross-linking were different in vivo and in vitro. This may have been due to the spatial constraints imposed on the pectin in vivo, compared to the relative flexibility which the molecules are likely to have experienced in

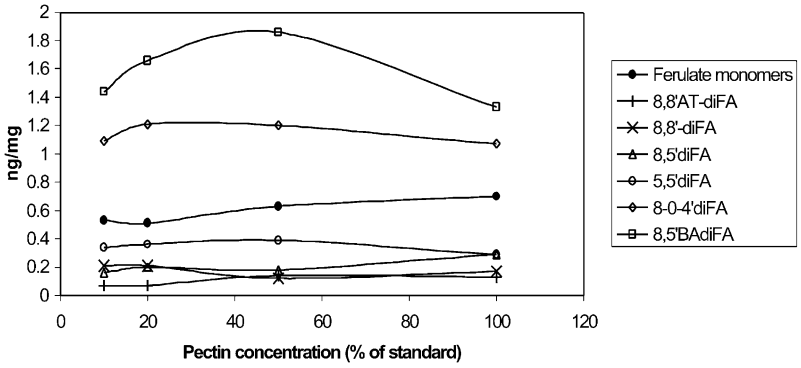

Fig. 8. Effect of varying pectin concentration on ferulate dimerisation. Root pectin at the concentration indicated was cross-linked with peroxidase and hydrogen peroxide. The ferulates were released by saponification, extracted into ethyl acetate and monitored by HPLC.

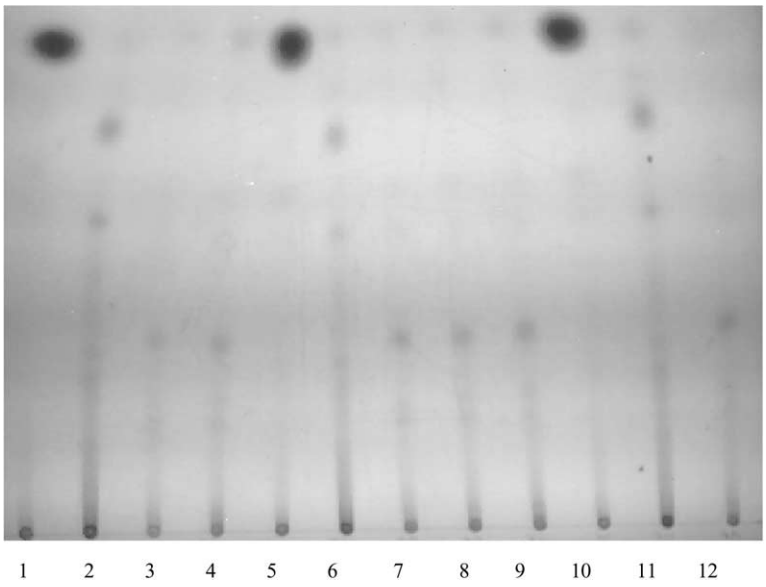

Fig. 9. Effects of ammonium peroxysulphate and peroxidase/hydrogen peroxide on ferulate content. Root pectin was cross-linked with ammonium peroxysulphate or with peroxidase and hydrogen peroxide for various times. The ferulates were released by saponification, extracted into ethyl acetate and monitored by HPLC. Incubation times: lanes 1–3: 12 h; lane 4: 9 h; lanes 5–7: 6 h; lane 8: 3 h; lane 9:1 h; lanes 10–12: 5 min. Lanes 1, 5, 10: controls; lanes 2, 6, 11: peroxidase and hydrogen peroxide; lanes 3, 4, 7, 8, 9, 12: ammonium peroxysulphate.

vitro. Also, the extracted pectin is likely to have been altered structurally by bond breakage during extraction. Another possibility is that neighbouring molecules may have influenced the course of the reaction in vivo. Guillon and Thibault (1987 and 1990) showed that gelation of beet pectin was affected by the structure of the polysaccharide side-chains. Our observations support the idea that the structure of the polysaccharide in the vicinity of ferulic acid affects its cross-linking under oxidative conditions.

The nature of the tissue of origin affected the cross-linking properties of the extracted pectin. Root pectin contained two populations of differing size, <10 kD and >76 kD respectively. Both contained ferulate, and oxidative treatment caused a shift from the smaller sized population to the larger one. Petiole pectin was very similar, in size and ferulate content, and a similar increase in size was observed, but both the increase in viscosity and the extent of dimerisation were lower than for root pectin. Total pectin from leaf tissue had relatively low molecular weight and low ferulate content. However, the feruloylated pectin was similar in size to pectin from root and petiole. It showed little change in molecular weight after oxidative treatment, perhaps because the feruloylated pectin was such a low proportion of the total pectin that it was unable to interact with other feruloylated pectins.

In pectin from all three tissues studied, the dehydrodimer showing the greatest increase after cross-linking was the 8–5B DFA. This contrasted with the situation in vivo, where the predominant dehydrodimer was the 8–O–4 or 8–8A DFA. Hence the constraints imposed on the pectins in muro greatly affected the nature of the dehydrodimers formed during cross-linking.

When other factors which might influence cross-linking in vitro were studied, the concentration of hydrogen peroxide was found to have the greatest effect. This may relate to important controls on cross-linking in vivo, since significant variations in hydrogen peroxide are known to occur in the cell wall under different physiological and developmental conditions (Bestwick et al., 1999). Since dimerisation of ferulates may be a starting point for further polymerisation of phenolics to form lignin (Grabber et al., 2002), the extent and nature of lignification may be greatly influenced by the hydrogen peroxide concentration which induces the initial dimerisation.

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, petioles and storage root were collected, and the root cut into 5 mm slices. Tissues were immersed in ethanol, and then extracted three times with the minimum quantity of 80% EtOH at 80 °C for 10 min. The residue was extracted with the minimum quantity of 0.1 M HCl at 80 °C for 20 min. This extraction method has been shown to extract carbohydrates with a sugar composition characteristic of pectins (Thibault and Rombouts, 1986). The extract was dialysed against three changes of water and concentrated by rotary evaporation at 45–55 °C. The extracts used for cross-linking experiments contained approximately 5 mg/ml carbohydrate, as judged by the phenol/sulphuric

acid method (Dubois et al., 1956) using a glucose standard. They related to the starting tissue wet weights as follows: Roots: 1 ml was derived from 1 g fresh weight; Leaves: 1 ml from 8 g fresh weight; Petioles: 1 ml derived from 3.5 g fresh weight.

4.2. Oxidative treatment of pectin

Oxidation with peroxidase and hydrogen peroxide was carried out for 5 min at 20 °C, using 0.5 ml pectin solution, 5 μl peroxidase (horseradish peroxidase, Sigma Cat. No P-8125, 1 mg/ml in 0.1 M NaH_2PO_4, pH 6), and 5 μl H_2O_2 (3%; final concentration 0.88 mM). Oxidation with ammonium peroxysulphate was carried out in the same way, using 0.5 ml pectin solution and 20 μl ammonium peroxysulphate (0.25 M). For phenolic analysis, the reaction was terminated by addition of an equal volume of 4 M NaOH. Saponification was carried out at room temperature for 18 h. The samples were neutralised with HCl, and the alkali-labile phenols were extracted with ethyl acetate and analysed by reversed-phase HPLC as described (Wende et al, 1999; Waldron et al, 1996). HPLC results are given as ng ferulate per mg fresh weight of tissue. Error bars show the mean of two samples, + the difference between the values and the mean. TLC was carried out on 0.2 mm silica-gel plates (20×20 cm), impregnated with a fluorecent indicator UV_{254} (Camlab), developed with chloroform–acetic acid (9:1). The TLC plates were photographed on a UV-transilluminator. Relative viscosities (for unsaponified pectin) were estimated as the relative flow times through a constricted pasteur pipette at 20 °C, using pectin solutions adjusted to 6 mg carbohydrate. ml^{-1}.

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