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TYROSINE RESIDUES ENHANCE CROSS-LINKING OF SYNTHETIC PROTEINS INTO LIGNIN-LIKE DEHYDROGENATION PRODUCTS

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Abstract—Synthetic proteins composed of lysine (polylysine, PL) and a random co-polymer of lysine and tyrosine (polylysine/tyrosine, PLT) were incorporated into lignin-like dehydrogenation polymers (DHPs) formed by the peroxidase-catalysed polymerisation of coniferyl alcohol. The yield of water-insoluble DHPs was greater in the presence of the tyrosine-containing PLT than PL. Indeed, the increase in total yield with PLT was often greater than could be accounted for if all the protein added had become incorporated into the DHPs. A comparison of the Fourier transform infra-red spectra of the DHPs formed in the absence and presence of synthetic proteins provided evidence that the presence of PLT had enhanced the incorporation of coniferyl alcohol, albeit in a less cross-linked form, into DHPs. The insolubilisation of PL and PLT was suggested by the presence of absorption bands in the infra-red spectra of their respective DHPs that are characteristic of protein/amide moieties. The insolubilisation of PLT was confirmed by the release of material from PLT-DHPs by digestion with trypsin. Therefore, this study provides evidence that the presence of tyrosine residues may enhance the cross-linking of proteins into lignin.

INTRODUCTION

Lignin is formed by the polymerisation of free radicals derived from phenylpropanoid cinnamyl alcohols within the cell wall space [1]. During this free radical cascade, linkages are formed with the polymers of the wall. These linkages may be made directly to polysaccharides [2, 3] or via linkages with ferulic acid groups that are ester-linked to the polysaccharides [4–6]. These lignin-polysaccharide cross-links are important in determining the extractibility of the lignin and the utility of the wall as a bioresource for animal feed or industrial use [7, 8]. Previous work has suggested that linkages may also be formed between cell-wall proteins and lignin [9]. Keller *et al.* [10] proposed that linkages involving the phenolic side-chains of tyrosine residues in certain glycine-rich cell wall proteins existed and were involved in directing the deposition of lignin. High tyrosine content is a characteristic of a number of cell wall proteins [11, 12] and recent work by Domingo *et al.* [13] has suggested that a tyrosine-rich cell wall protein, which is structurally different from the tyrosine-rich protein identified by Keller *et al.*, is specifically located in secondary cell walls and may also be involved in directing lignin deposition. Although of considerable interest, evidence that tyrosine residues can participate in lignin-protein linkages has not been sought. To test the hypothesis that tyrosine groups aid the cross-linking of proteins to lignin, we have studied the incorporation of two synthetic proteins, one containing and the other lacking tyrosine, into dehydrogenation polymers (DHPs) formed by the peroxidase-catalysed polymerisation of coniferyl alcohol.

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RESULTS AND DISCUSSION

Formation of DHPs

All of the DHPs obtained by the peroxidase-catalysed polymerisation of coniferyl alcohol were insoluble in water but were completely soluble in dimethyl sulphoxide at 20 mg ml^{-1}. On average, the presence of polylysine/tyrosine (PLT) increased the yield of DHPs to 142%, whereas polylysine (PL) increased the yield of DHPs to 114% (Table 1). The yields differed in different experiments but the increases in yields were in the range 136–157% for PLT and 106–119% for PL. In four out of six experiments, the yield of DHPs in the presence of PLT was greater than the control value plus 10 mg, the weight of the synthetic protein added. This suggests that the increase in yield may, in part, be to increased incorporation of coniferyl alcohol into DHPs.

A small amount of insoluble material (< 1 mg) was formed in the reaction of PLT with peroxidase-H_2O_2, presumably by tyrosine-tyrosine phenolic coupling [14]. However, as this material was also insoluble in dimethyl

Table 1. Yield of DHPs in presence of synthetic proteins

	Yields of DHPs*		
	Control	+ PL	+ PLT
1	39.2	46.2 (117.8)	56.6 (144.4)
2	22.1	24.7 (111.7)	30.9 (139.8)
3	41.2	43.6 (105.8)	57.8 (140.3)
4	35.6	42.3 (118.8)	54.1 (152.0)
5	30.6	N.D.	43.1 (141.2)
6	25.6	N.D.	35.1 (137.1)
Averages	100%	113.5 ± 6.0 (4)†	142.5 ± 3.6 (6)†

* First figure, weight in mg; the figure in parenthesis, percentage yield compared to control reaction. † Average of percentage yield ± standard deviation. N.D., not done.

sulphoxide, it is unlikely to have contributed to the yield in the reaction containing coniferyl alcohol.

Digestion of DHPs using trypsin

Digestion with trypsin reduced the amount of insoluble material recovered from 10 mg portions of PLT-DHPs to 8.43 ± 0.15 (3) mg compared with 9.65 ± 0.07 (2) mg recovered from the trypsin-free treatment; these figures are averages ± standard deviation for the number of replicates in parentheses. This represents a loss of 12.64% compared with the trypsin-free control. When 10 mg portions of the control DHPs were subjected to a similar treatment, the difference between the recoveries was negligible, i.e. 9.63 ± 0.11 (3) mg recovered after trypsin digestion and 9.7 ± 0.07 (2) mg recovered from the trypsin-free treatment. This represents a loss of 1.03% compared with the control. These results confirm that proteolytic digestion of the PLT-DHPs removed insoluble material and suggest that protein, presumably PLT, was linked in to the DHPs.

Fourier transformation infra-red spectroscopy (FT-IR)

Control DHPs gave an FT-IR spectrum (Fig. 1a) that is characteristics of DHPs derived from coniferyl alcohol (see [15–19] for background). Each of the major absorption bands denoted on Fig. 1 can be assigned to structural features of guaiacyl-type lignins [18]. The spectrum also shows the relative and intensities expected of DHPs derived from coniferyl alcohol, i.e. 1279 > 1515 > 1230 = 1163 = 1147 = 1037 > 1470 > 1335 > 972 > 1420 > 1609 > 817 > 857 cm^{-1} [18]. In fact, the spectrum has more features in common with the spectrum obtained from DHPs made from coniferyl alcohol *via* the bulk polymerisation or 'Zulauf' method than the endwise polymerisation or 'Zutropf' method [15]. This is to be expected as the method of polymerisation used was similar to the 'Zulauf' method. The higher absorption of the band at 1279 cm^{-1} than that at 1230 cm^{-1} [16] and the peak at 1147 cm^{-1} [18] are diagnostic assignments of guaiacyl-type DHPs derived from coniferyl alcohol.

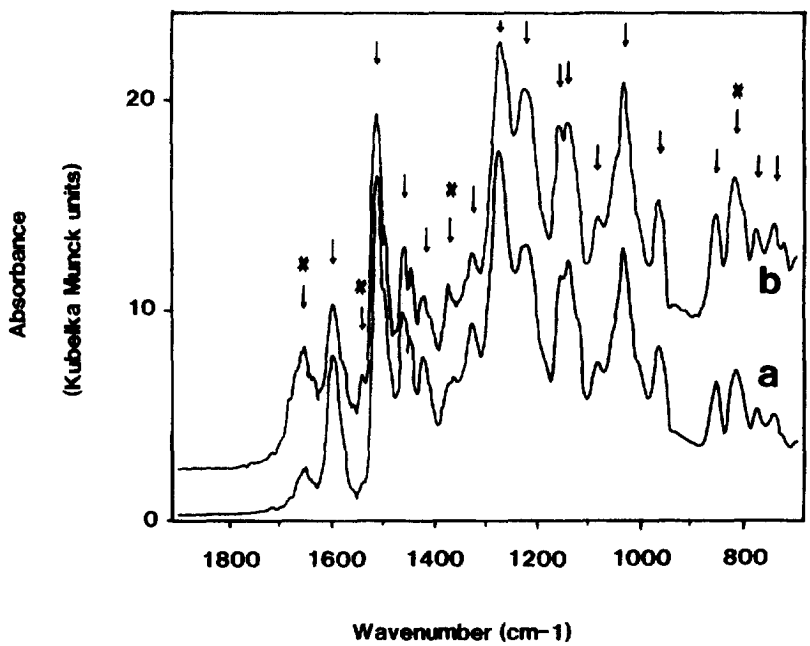

Fig. 1. FT-IR spectra of DHPs. a, Control DHPs derived from coniferyl alcohol; b, DHPs derived from incubation containing PLT. Arrows denote absorption band mentioned in text. These are, from highest to the lowest cm^{-1} value at 1695–1600#, 1609, 1545#, 1515, 1470, 1420, 1378#, 1335, 1279, 1230, 1163, 1147, 1088, 1037, 972, 867, 835#, 775 and 744 cm^{-1}. Absorption bands further highlighted by # are either absent or present at low intensity in control spectra. For clarity, the PLT-DHP spectrum has been raised by 2 absorption units. Peaks at 1515 cm^{-1} had virtually the same absorbance in the original spectra.

The spectrum of the DHPs formed in the presence of PLT (Fig. 1b) shows all of the absorption bands found in the control spectrum. However, the bands at 1663, 1335, 1279, 1230, 1167, 1147, 1037, 972, 857 and 817 cm^{-1} (denoted by arrows in Fig. 1b) have all been enhanced. The enhanced signals at 1279, 1230, 1147, 1037, 857 and 817 cm^{-1} are characteristic of guaiacyl groups [18] and suggest a greater incorporation of these groups in the DHPs, i.e. the enhanced polymerisation of coniferyl alcohol. The enhanced absorption bands at 1279, 1230 and 1147 cm^{-1} can be assigned to the increased abundance of condensed guaiacyl ring structures [18], i.e. a guaiacyl ring structure linked *via* a carbon bond. This is interesting in that the small but significant increase in absorbance at 1335 cm^{-1} can be assigned to the presence of guaiacyl ring structures substituted at position 5. The enhanced absorption at 972 cm^{-1} can be assigned to the presence of carbon-carbon double bonds (–C=C–) and suggests that the presence of PLT increases the prevalence of –C=C–groups in the DHPs [18]. This, in turn, indicates that the PLT-DHPs may be less cross-linked through its propenoic side-chains than the control DHPs.

Therefore, the presence of PLT seems to have enhanced the overall deposition of coniferyl alcohol into DHPs, increased the proportion of 5-linked condensed guaiacyl groups and the relative abundance of –C=C– bonds. It may be that the presence of PLT has encouraged the formation of relatively simple linkages between tyrosine groups and coniferyl alcohol that are not extensively cross-linked to the developing DHP structure. This would explain the increase in the proportion of free –C=C– bonds in the DHPs. A comparison of the molecular mass profiles of the control and PLT-DHPs may confirm this suggestion.

In addition to the absorption bands that are enhanced in the PLT spectra, there are also four absorption bands that are enhanced from negligible levels in the control spectrum to reasonable levels in the PLT spectrum. These are at 1660–1695, 1545, 1378 and 835 cm^{-1}; highlighted in Fig. 1b by asterisks. The enhanced absorption around 1650–1680 cm^{-1} can be assigned to carbonyl (C=O) stretching (band I) and the signals at 1545 cm^{-1} can be assigned to carbonyl (band II) of amide groups [20, 21]. In the spectra of proteins, the absorption of the first band is usually higher than the second band and this is also the case in the PLT-DHPs. Seen together in this ratio, these absorption bands provide evidence for the presence of amide (or protein) structures in the PLT-DHPs and strongly suggest that PLT has been incorporated into the DHPs. The increased absorption around 1375 cm^{-1} can be assigned to the presence of phenolic hydroxyl groups [16] which could arise through the increased incorporation of coniferyl alcohol *or* tyrosine into the DHPs. The enhanced signal at 835 cm^{-1} can be assigned to the presence of 1,4-substituted benzene ring structures [21], such as those found in tyrosine. This suggests that tyrosine and therefore PLT has been insolubilised in these DHPs.

In comparison, the spectrum obtained for the DHPs made in the presence of PL was very similar to that the control spectrum and for this reason, and the sake of clarity, it is not included on Fig. 1. A difference spectrum was obtained by subtracting the control spectrum from the PL spectrum and this highlighted the small differences (Fig. 2). The main differences are absorption bands at 1650 cm^{-1} and 1545 cm^{-1}. These can be assigned to the amide I and II bands discussed above. These absorbances reflect the incorporation of PL into insoluble DHPs.

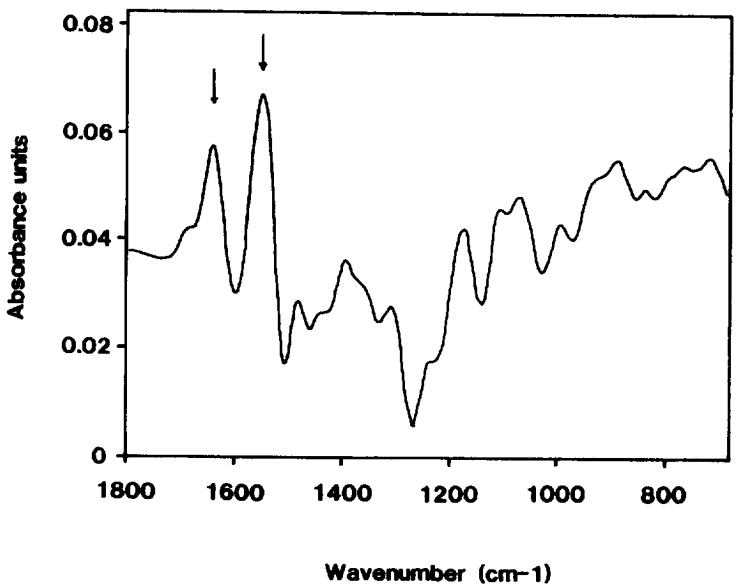

Fig. 2. Difference FT-IR spectrum obtained by subtracting PL-DHP spectrum from control DHP spectrum. The absorption bands denoted by arrows are at 1545 cm^{-1} and 1650 cm^{-1}, respectively.

Our findings strongly suggest that PLT and PL are incorporated into the DHPs. Although the incorporation of the synthetic protein, polyhydroxyproline, into DHPs formed by the action of peroxidase-H_2O_2 on coniferyl alcohol has been noted before [22], the author did not compare the incorporation of this synthetic polypeptide to that of other polypeptides or to a suitable control. The increased yields of DHPs formed in the presence of the synthetic proteins in this study may be the result of these polymers acting as matrices or surfaces upon which the free radical polymerisation of coniferyl alcohol may occur more quickly. This hypothesis was originally presented for the peroxidase-catalysed polymerisation of eugenol by Siegel [23] in 1957. However, the augmentation of polymerisation noted by Siegel was relatively unspecific in that many materials, soluble or insoluble, organic or inorganic, had positive effects. Since the only difference between PLT and PL is the presence of tyrosine residues, the enhanced incorporation of coniferyl alcohol into DHPs formed in the presence of PLT, but not PL, suggests that these tyrosine groups in PLT play an important role in directing the polymerisation of coniferyl alcohol. Previously, tyrosine residues have been implicated in cross-links formed between peroxidase and synthetic lignin DHPs [24]. However, the mechanism by which the phenolic tyrosine residues enhance polymerisation is not yet known but they may act as templates or nucleation sites for the initiation of free radical reactions.

EXPERIMENTAL

Formation of dehydrogenation products. The synthetic proteins used were polylysine (PL) and a random copolymer of lysine and tyrosine (PLT) in the ratio 4:1. Unless otherwise specified, all reagents were obtained from Sigma. DHPs were formed from 50 mg coniferyl alcohol and 300 units horseradish peroxidase type II dissolved in 50 mM phosphate buffer pH 6.7. Reaction conditions were chosen to maximise the yield of DHPs. Dehydrogenation reactions were carried out at pH 5.5, which is probably closer to the physiological pH of the cell wall, yielded less DHPs. This effect has been noted previously [25]. Synthetic proteins (10 mg) were added and the reaction started by the addition of the first of ten aliquots of 15 μmol H_2O_2 over 4 days. Reactions were stirred at 100 rpm. Control reactions lacking coniferyl alcohol were also carried out. After 4 days, insol. DHPs were collected by centrifugation (3500 *g*, 10 min), washed repeatedly with water and freeze-dried.

Digestion of DHPs samples using trypsin. Duplicate 10 mg portions of freeze-dried control DHPs and triplicate 10 mg portions of PLT-DHPs (10 mg) were resuspended in 800 μl of 100 mM Tris HCl pH 8.8 and sonicated for 5 min. Then, 200 μl of 5 mg ml^{-1} trypsin in the same buffer (or 200 μl of buffer for the trypsin-free treatment) was added, mixed well then incubated with end-over-end mixing (60 rpm) for 1 hr at 37°. The samples were then centrifuged (15 800 *g*, 10 min) in a microfuge and the supernatant removed. The pellet was then resuspended in 1 ml of dist. H_2O and washed by centrifugation $\times 4$. Samples were resuspended in 250 μl of H_2O then frozen and freeze-dried.

FT-IR. DHPs were analysed by solid state FT-IR using the diffuse reflectance Fourier transform IR (DRIFT) method. Samples ($\sim$10 mg) were placed in DRIFT cells and the unsmoothed spectra shown were acquired from 3000 scans. The background was obtained using finely ground KBr. Difference spectra were calculated using spectrometer software.

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