Studies on Bound *trans*-4-Hydroxy-L-proline in Sandal (*Santalum album* L.)

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1. *trans*-4-Hydroxy-L-proline was found to occur in the bound state in the leaves of sandal (*Santalum album* L.), in which large amounts of free *cis*-4-hydroxy-L-proline are also present. 2. Bound *trans*-4-hydroxy-L-proline was present, associated mainly with a 'wall' fraction and a 'soluble' fraction roughly in equal proportions. 3. Bound proline is present only in small amounts in the 'soluble' fraction but is mostly associated with the 'wall' fraction and the other sedimented fractions. 4. In the free amino acid fraction more than 98% of the hydroxyproline had the *cis*-configuration, whereas in the 'wall' and 'soluble' fractions more than 90% of the bound hydroxyproline was in the *trans*-configuration. 5. Various extraction procedures indicated heterogeneity of the hydroxyproline-containing components. Hot 5% (w/v) trichloroacetic acid extracts about 25% of hydroxyproline and *m*-NaOH extracts an additional 25%. 6. Incorporation of [^{14}C]proline into the bound hydroxyproline was demonstrated. The hydroxyproline component of the 'soluble' fraction does not appear to be the precursor of that of the 'wall' fraction.

The presence of *trans*-4-hydroxy-L-proline as a normal constituent of collagen has been known for a long time. Evidence has accumulated to suggest its presence in the bound state in plants (Steward & Thompson, 1950; Lamport, 1965). Because of a possible role in auxin-induced cell elongation the protein containing hydroxyproline was named 'extensin' (Lamport & Northcote, 1960). Bound hydroxyproline-containing components were also reported to be associated with cytoplasm (Cleland, 1968), nucleus (De & Ghosh, 1967), chloroplasts (Keser, 1955) and membranes (Chrispeels, Doerschug & Sadava, 1969). The available evidence suggests that the biosynthesis of *trans*-4-hydroxyproline in plants takes place by hydroxylation of peptidylproline, a mechanism similar to that in mammalian systems (Steward & Pollard, 1958; Lamport, 1963).

Hydroxyproline very rarely occurs in the free state. However, large amounts of free *cis*-4-hydroxy-L-proline are known to occur in sandal (*Santalum album* L.) (Radhakrishnan & Giri, 1954). *cis*-4-Hydroxy-L-proline is a potent inhibitor of auxin-induced cell elongation (Cleland, 1967) and it has been reported to be directly incorporated into the wall protein (Cleland & Olson, 1968). It was therefore decided to find out whether there was any bound hydroxyproline in sandal. In the present paper, critical evidence is provided for the presence of bound *trans*-4-hydroxy-L-proline in sandal. Also

included are some results on its biosynthesis from proline and on its distribution in various subcellular fractions. A preliminary report of this work has appeared (Kuttan & Radhakrishnan, 1969).

MATERIALS AND METHODS

All chemicals used were of analytical reagent or the best commercially available grade. 4-Dimethylaminobenzaldehyde was purchased from Matheson, Coleman and Bell, Norwood, Ohio, U.S.A., and ninhydrin from Dougherty Chemicals, Richmond Hill, N.Y., U.S.A. [^{14}C]Proline was purchased from The Radiochemical Centre, Amersham, Bucks., U.K.

Radioactivity was measured by using a liquid-scintillation counter (Beckman model LS-100). The scintillation mixture contained 2,5-diphenyloxazole (1g), 1,4-bis-(5-phenyloxazol-2-yl)benzene (50mg) and naphthalene (20g) in 100ml of dioxan (Ramaswamy & Radhakrishnan, 1964).

The following determinations were carried out as described by the authors indicated: hydroxyproline, Neuman & Logan (1950); proline, Schweet (1954); protein, Lowry, Rosebrough, Farr & Randall (1951); total sugars, Dubois, Gilles, Hamilton, Rebers & Smith (1956). Both *trans*- and *cis*-4-hydroxy-L-proline gave identical colour yields in the Neuman-Logan assay (Radhakrishnan, Gopalkrishnan & Giri, 1961).

Separation of cis- and trans-4-hydroxy-L-proline. For qualitative work the original copper-salt procedure of Friedberg (1954) was used. In other cases the reaction was carried out in aqueous solutions and then the mixture was directly applied on to the paper.

cis- and *trans*-4-Hydroxy-L-proline were separately determined in an automatic amino acid analyser with PA-28 resin (Beckman) under the conditions described by Benson & Patterson (1965) with 1 cm light-path flow cells. *cis*-4-Hydroxy-L-proline emerges about 20 min later than *trans*-4-hydroxy-L-proline, thus effecting a clear separation of the two isomers.

Preparation of cell-wall fraction. The cell-wall fraction was prepared as described by Dougall & Shimbayashi (1960) with slight modifications. Tender leaves (40 g) from the apical regions of sandal shoots were homogenized in water (300 ml) with an Omni-Mixer (Sorvall) and the homogenate was centrifuged at 1000 g for 15 min. The pellet thus obtained was again blended with 100 ml of water by using a mortar and pestle and centrifuged at 1000 g for 15 min; this operation was repeated twice. The 1000 g pellet fraction obtained as described above was further purified by centrifugation in sucrose medium (Zilling & Hölzel, 1958). The concentrations of sucrose used were 2, 1.5, 1 and 0.5 M respectively and centrifugation was at 1000 g. The main cell-wall fraction was sedimented at a sucrose concentration of 2 M and was designated the 'wall' fraction. The sucrose adhering to the pellet was removed by repeated washing with water.

The combined 1000 g supernatant fraction was centrifuged as indicated in Table 1. The 20000 g supernatant fraction was concentrated and dialysed against tap water for 48 h, and then against deionized water for 24 h. It was subsequently found that the bulk of the hydroxyproline contained in this fraction was not sedimented at 100000 g for 30 min and is referred to as the 'soluble' fraction.

All the sedimented fractions were dried in a desiccator *in vacuo* by using P₂O₅ and their weights were recorded.

Hydrolysis. The samples were usually hydrolysed in sealed tubes with 6 M-HCl at 140°C for 3 h unless otherwise stated. The coloured samples were treated with activated charcoal to remove interfering materials before chemical determination. It was found that even with a large excess of charcoal there was no decrease in the hydroxyproline assay.

RESULTS

Presence of bound trans-4-hydroxy-L-proline in sandal leaves. Fresh leaves were dried at 50°C for

24 h and then powdered (100–200 mesh). The leaf powder after repeated extraction with aq. 75% (v/v) ethanol was hydrolysed. Paper chromatography of the copper salts showed the presence of a band in the position of *trans*-4-hydroxy-L-proline.

The identity of the material was further confirmed as follows. The leaf powder was thoroughly washed with aq. 75% (v/v) ethanol until the extract was free of *cis*-4-hydroxy-L-proline and other free amino acids. The residue (10 g) was then hydrolysed with 6 M-hydrochloric acid at 100°C under reflux for 24 h. The hydrolysate was filtered and concentrated to a syrupy liquid. The total bound hydroxyproline as determined by the chemical method was found to be 14.9 mg. The α -amino acids in the hydrolysate were destroyed by nitrous acid (Levin, 1959) and after this treatment there were only very small amounts of amino acids other than proline and hydroxyproline, as checked by paper chromatography. The solution was then desalted on a column (23 cm $\times$ 2.4 cm) of Dowex 50 (X8, H⁺ form), and the imino acids were eluted with M-ammonia and concentrated to a small volume. Proline and hydroxyproline in the eluate were separated on a column of Dowex 50 (X12, H⁺ form) by the method of Manning & Meister (1966). The pooled fractions contained 5.7 mg of hydroxyproline. Paper chromatography of the copper salt of the isolated isomer of hydroxyproline suggested that although it might be either *trans*-4-hydroxy-L-proline or *trans*-4-hydroxy-D-proline (both having the same *R_F* value) it was not *cis*-4-hydroxy-(L or D)-proline. It was also found that the isolated material was not oxidized by a D-amino acid oxidase preparation from sheep kidney under conditions where the D-isomers of hydroxyproline were oxidized (Radhakrishnan & Meister, 1957; Corrigan, Wellner & Meister, 1963), and therefore it was concluded that it was *trans*-4-hydroxy-L-proline.

Distribution of bound trans-4-hydroxy-L-proline

Table 1. *Subcellular distribution of hydroxyproline-containing components in sandal leaves*

The leaf tissue (40 g) was fractionated as described in the Materials and Methods section and the values calculated for 100 g of fresh leaf tissue. Fraction I(b) sedimented at 1000 g in 1.5 M-, 1.0 M- and 0.5 M-sucrose, and fraction II, consisting of material sedimented at 2500, 5000, 10000 and 20000 g, were pooled because of their relatively low concentrations compared with the total hydroxyproline content.

Fraction	Dry wt. (g)	Hydroxyproline		Proline		Hydroxyproline/ proline ratio
		μ mol	% of total	μ mol	% of total	
I. 1000 g pellet						
(a) 'Wall' fraction	10.9	100	49	782	49	0.13
(b) Other 1000 g pellet fraction (sucrose medium)	2.6	22	11	490	31	0.05
II. Pellet fraction (2500–20000 g)	0.9	6	3	205	13	0.03
III. 'Soluble' fraction* (20000 g supernatant)		76	37	90	6	0.84

* The bulk of the hydroxyproline component in this fraction was not sedimented at 100000 g.

in the subcellular fractions. The results on the hydroxyproline and proline contents in different fractions of the sandal leaf homogenate are given in Table 1. Hydroxyproline in the bound state was mainly associated with the 'wall' and 'soluble' fractions, together constituting nearly 86% of the total bound hydroxyproline in the leaves. The rest was distributed in the other fractions, and the values ranged from 0.15 to 3.6% of the total in the fractions. It was also noted that the amount of bound hydroxyproline/g of dried fraction was almost the same in all the pellet fractions.

In contrast, bound proline was mainly concentrated in the 'wall' fraction (49%) and in the other pellet fractions (44%). Very little proline (6%) was associated with the 'soluble' fraction. The hydroxyproline/proline ratio was 0.13 in the 'wall' fraction and 0.84 in the 'soluble' fraction. In the other pellet fractions the value was in the range 0.03–0.05; the decreased ratio may be due to the associated proline-rich proteins.

Distribution of cis- and trans-4-hydroxy-L-proline in the different fractions of the leaves. Since bound *trans*-4-hydroxy-L-proline in the leaves was associated mainly with two fractions, the percentages of each isomer in these fractions as well as in the free amino acid fraction were next determined (Table 2). Preliminary purification of the hydroxyproline from these fractions was effected by chromatography on Whatman no. 3 papers (streaking), with phenol–0.2M-potassium chloride adjusted to pH 1 with hydrochloric acid (50:7, v/v) as solvent. The *cis*- and *trans*-isomers of 4-hydroxy-L-proline in the fractions were determined with an automatic amino acid analyser. More than 98% of the hydroxyproline in the free amino acid fractions was of the *cis*-configuration. In the 'wall' fraction and in the 'soluble' fraction, more than 90% of the total bound hydroxyproline was in the *trans*-configuration. At present we do not know if the traces of *cis*-4-hydroxy-L-proline in the latter fractions are due to a contamination carried over from the very

large pool of *cis*-4-hydroxy-L-proline in the leaves, in spite of exhaustive washing, or whether it is actually present in the bound state.

Extraction of hydroxyproline-containing components from the wall fraction. Various methods were used to solubilize the hydroxyproline-containing components from the 'wall' fraction. The extractions at room temperature were carried out with 200mg of dried 'wall' fraction and 20ml of extractant each time, with a gyratory shaker. The extractions were repeated three times, each lasting 1h. The hot extractions were carried out at 90°C in a water-bath with frequent shaking, for 1h. Samples were autoclaved at 15lb/in² for 15min. Completion of the extraction with each procedure was verified by measuring the bound hydroxyproline after hydrolysis of the last batch of the extract, and extraction was continued if necessary. The extracts were then dialysed against tap water for 24h and then against deionized water for 48h.

Trichloroacetic acid (5%, w/v) at 90°C was found to extract 23% of the total bound hydroxyproline (Table 3). Treatment with M-sodium hydroxide extracted about 53% of the total bound hydroxyproline. However, sequential extraction with sodium hydroxide and hot trichloroacetic acid did not yield more than 53%, suggesting that the sodium hydroxide extract included the fraction extractable by hot trichloroacetic acid. Cold trichloroacetic acid, as used by some earlier workers, and the other extraction procedures using cold and hot (90°C) M-acetic acid, autoclaving and 2M-sodium chloride yielded only 5.0–6.3% of the total bound hydroxyproline.

The hydroxyproline/proline ratios in these fractions show that the ratio was higher in the hot trichloroacetic acid extract (0.4) than in the sodium hydroxide extract (0.12) or in the 'wall' fraction (0.13). This probably indicates a selective extraction by hot trichloroacetic acid of one of the hydroxyproline-rich components from the 'wall' fraction.

Table 2. *Distribution of cis-4-hydroxy-L-proline and trans-4-hydroxy-L-proline in various fractions*

The procedure for obtaining 'wall' and 'soluble' fractions is described in the Materials and Methods section. To obtain the free amino acid fraction the leaves were extracted with aq. 75% (v/v) ethanol and the ethanolic extract was shaken with 3 vol. of chloroform and the upper aqueous phase was used for analysis with the automatic amino acid analyser.

Fraction	Hydroxyproline (%)	
	<i>trans</i> -isomer	<i>cis</i> -isomer
Free amino acid fraction (ethanolic extract)	Trace	98.0
Free amino acid fraction after acid hydrolysis	3.7	96.2
'Wall' fraction (1000g sediment in 2M-sucrose)	94.0	6.0
'Soluble' fraction (20000g supernatant)	92.0	8.0

Table 3. Composition of trichloroacetic acid and sodium hydroxide extracts of the 'wall' fraction

The extractions were carried out with 200 mg of dried 'wall' fraction. (The original samples of the 'wall' fraction contained 1.9 μ mol of hydroxyproline and 15.1 μ mol of proline in 200 mg; the hydroxyproline/proline ratio was 0.12.) The components were determined as described in the Materials and Methods section. Values are expressed for 200 mg of 'wall' fraction.

Fraction	Total protein (mg)	Total sugar (mg)	Hydroxyproline (μ mol)	Proline (μ mol)	Hydroxyproline extracted (%)	Hydroxyproline		
						(μ mol/mg of protein)	(μ mol/mg of sugar)	Hydroxyproline/proline ratio
Hot 5% (w/v) trichloroacetic acid extract	2.44	11.9	0.44	1.1	23.4	0.18	0.037	0.4
M-NaOH extract	23.9	13.0	1.0	8.2	53.1	0.041	0.077	0.12

A glycoprotein nature of the hydroxyproline-containing components was suggested by Lampert, (1967), Pusztai & Watt (1969) and Boundy, Wall, Turner, Woychik & Dimler (1967). In the present study it was found that 'wall' extracts (Table 3) were associated with bound sugars. The values of the total sugar extracted in these fractions remained almost the same, whereas the total protein extracted was about ten times more in the sodium hydroxide extract than in the hot trichloroacetic acid extract. The content of hydroxyproline (μ mol/mg of protein) was 0.041 and 0.18 for the sodium hydroxide and hot trichloroacetic acid extracts respectively. Thus the hydroxyproline component appears to be enriched in the trichloroacetic acid extract with respect to protein or proline. The results also suggested that trichloroacetic acid was preferentially extracting a hydroxyproline-containing glycoprotein. The content of hydroxyproline (μ mol/mg of sugar) was 0.037 in the hot trichloroacetic acid extract and 0.077 in the sodium hydroxide extract. The above results thus show heterogeneity of hydroxyproline-containing components in the 'wall' fraction.

Biosynthesis of trans-4-hydroxy-L-proline in sandal. In our earlier studies [14 C]proline was found to be a precursor of *cis*-4-hydroxy-L-proline, which occurs free in sandal (Kuttan & Radhakrishnan, 1970). The same techniques were employed to study the incorporation of [14 C]proline into bound *trans*-4-hydroxy-L-proline in intact leaves. After the incubation period, the leaves were homogenized with water. The supernatant fraction obtained after separation from the pellet fraction by centrifugation at 20000g was further fractionated by gel filtration on a column (1.1 cm $\times$ 64 cm) of Sephadex G-25 (bead form). The elution was carried out with 0.01M-sodium chloride. This procedure effectively separated the free amino acid fraction from the 'soluble' fraction containing bound hydroxyproline (Fig. 1), which was eluted in the void volume.

The pellet fraction obtained as described above was thoroughly washed with aq. 75% (v/v) ethanol to remove the adhering free amino acids. The washed pellet fraction and the soluble fraction from Sephadex G-25 chromatography were hydrolysed. Hydroxyproline was isolated after addition of known amounts of unlabelled *trans*-4-hydroxy-L-proline, by paper chromatography followed by chromatography on Dowex 50 (X12, H⁺ form) (Kuttan & Radhakrishnan, 1970) and the specific radioactivity was determined. Approx. 0.37% of the initial proline radioactivity was found to have been incorporated into the hydroxyproline of the 'wall' fraction and 0.18% into that of the 'soluble' fraction, giving a total incorporation of about 0.6%, which is comparable with the results of Cleland & Olson (1968).

Fig. 1. Sephadex G-25 chromatography of the 'soluble' fraction. The details of operation of the column are given in the text. The total radioactivity in the [^{14}C]proline used for the incorporation studies was 800000 c.p.m. The fresh wt. of the leaves was 1.2g. ●, Radioactivity; ○, hydroxyproline.

Fig. 2. Optical configuration of isolated hydroxy[^{14}C]proline by paper chromatography of the copper salt. The isolated hydroxy[^{14}C]proline (10 μmol; 3600 c.p.m.) from the 'wall' fraction obtained after [^{14}C]proline incorporation was mixed with a known amount of unlabelled *cis*-4-hydroxy-L-proline and the copper salts were prepared in an aqueous medium. The mixture was streaked on Whatman no. 3 paper and the chromatogram was developed. Strips (2.5 cm) were then cut out, eluted with water and the radioactivity and hydroxyproline determined in the eluates. ●, Radioactivity; ○, hydroxyproline.

The optical configuration of the hydroxy[^{14}C]proline isolated in the above two fractions was checked by paper chromatography of the copper salts (Fig. 2). The bulk of the radioactivity was found in the region of the *trans*-isomer, thus establishing that the hydroxy[^{14}C]proline formed was *trans*-4-hydroxy-L-proline.

An attempt was then made to study the role of the 'soluble' fraction in the biosynthesis of the hydroxyproline-containing component in the 'wall' fraction. Advantage was taken of the low proline content of the 'soluble' fraction compared with that of the 'wall' fraction. If hydroxyproline is synthesized in the 'soluble' fraction and later incorporated into the 'wall' fraction, as was suggested by Lampport (1965) and Olson (1964), a high labelling of the [^{14}C]proline should occur in the 'soluble' fraction before the 'wall' fraction is labelled. [^{14}C]Proline incorporation with intact leaves was studied at different time-intervals by the infiltration procedure described by Kuttan & Radhakrishnan (1970). After incubation, the leaves were homogenized with water and the homogenate was centrifuged at 20000g for 15 min to obtain the pellet and supernatant fractions. The pellet fraction was washed with aq. 75% (v/v) ethanol to remove the adhering free amino acids. The supernatant fraction was dialysed against distilled water and the diffusate was concentrated *in vacuo*. The total radioactivities in the hydrolysates of the washed pellet fraction, in the dialysed supernatant fraction

Fig. 3. Time-course of incorporation of [^{14}C]proline radioactivity into the leaf fractions. Details of the fractionation method are given in the text. Total radioactivity used for each time-interval was 200000 c.p.m. The weight of leaf tissue varied from 0.13 to 0.18g in the different experiments. The total radioactivities in the respective fractions calculated for 100mg of fresh leaf tissue are shown. ■, Free amino acid fraction; ●, soluble fraction; ○, pellet fraction.

('soluble' fraction) and in the free amino acid fraction (diffusate) were determined. The time-courses of incorporation of [^{14}C]proline into these fractions are given in Fig. 3, which shows that the

maximum incorporation of [^{14}C]proline into the 'soluble' and 'wall' fractions occurred at the same time, at about 8 h. Also, the incorporation of radioactivity into the 'wall' fraction was at least 5 times greater than that in the 'soluble' fraction. The disappearance of radioactivity from the free amino acid fraction ran closely parallel to its appearance in the bound-hydroxyproline-containing fractions. These results indicate that the 'wall' and 'soluble' fractions are probably synthesized separately.

DISCUSSION

The results given in the present paper show that *trans*-4-hydroxy-L-proline is present in the bound state in sandal leaves, in which the occurrence of free *cis*-4-hydroxy-L-proline is also known (Radhakrishnan & Giri, 1954). Sandal is, therefore, a unique system where two diastereoisomers of a rarely occurring amino acid are present. Cleland (1967) reported that *cis*-4-hydroxy-L-proline was a potent inhibitor of cell elongation, and both the *cis*- and *trans*-isomers were directly incorporated into the cell-wall protein (Cleland, 1968). However, it is noteworthy that in sandal only *trans*-4-hydroxy-L-proline is found in the bound state, even in the presence of a large pool of free *cis*-4-hydroxy-L-proline.

Hydroxyproline-containing components associated with the cell-wall are becoming increasingly important in view of their possible role in cell elongation. Lampport (1965) considered that hydroxyproline-containing components were mainly associated with the cell-wall and that they were insoluble in conventional aqueous media. A cytoplasmic hydroxyproline-containing component has been reported in *Avena* coleoptiles (Cleland, 1967) and in algae (Gotelli & Cleland, 1968). Our studies also indicate two major hydroxyproline-containing components, one soluble in water and the other insoluble, and differing in their contents of hydroxyproline and proline.

Differences in the extractability and electrophoretic behaviour of the hydroxyproline-containing components in the cell-wall has been noted (Lyndon & Steward, 1963; Steward & Chang, 1963; Pusztai & Watt, 1969). A fraction insoluble in M-sodium hydroxide has been reported in tobacco suspension cultures by Olson (1964) and a similar trypsin-resistant fraction in sycamore cell-wall preparations (Lampport, 1965). Our studies also established the heterogeneity of hydroxyproline-containing components in the 'wall' fraction on the basis of their extractability. The hydroxyproline components in the 'wall' fraction can be roughly divided into (a) a fraction soluble in hot 5% (w/v) trichloroacetic acid, (b) a fraction soluble in M-sodium hydroxide but insoluble in hot trichloroacetic acid and (c) a

fraction insoluble in M-sodium hydroxide. The nature of the protein components of these fractions needs to be established. Preliminary studies show that they are different in their contents of hydroxyproline, proline, protein and sugars.

It has been reported that [^{14}C]proline is a precursor of free *cis*-4-hydroxy-L-proline in sandal (Kuttan & Radhakrishnan, 1970). [^{14}C]Proline is also a precursor of bound *trans*-4-hydroxy-L-proline, as reported here and elsewhere (Lampport, 1963; Chrispeels *et al.* 1969). These studies have shown that the formation of bound *trans*-4-hydroxy-L-proline from proline in plants is essentially similar to peptidylproline hydroxylation in mammalian systems. Our studies therefore indicate the presence in sandal of two stereochemically distinct hydroxylation systems, giving either free *cis*-4-hydroxy-L-proline or bound *trans*-4-hydroxy-L-proline.

The marked difference in the contents of proline and hydroxyproline between the 'wall' fraction and the 'soluble' fraction and the studies on the time-course of incorporation of [^{14}C]proline indicate that the two fractions are probably separately synthesized. It has been suggested (Lampport, 1965; Olson, 1964) that the hydroxyproline component of the soluble fraction is later incorporated into the cell-wall, although Cleland (1968) obtained only limited transfer of cytoplasmic hydroxyproline-containing protein into wall protein.

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