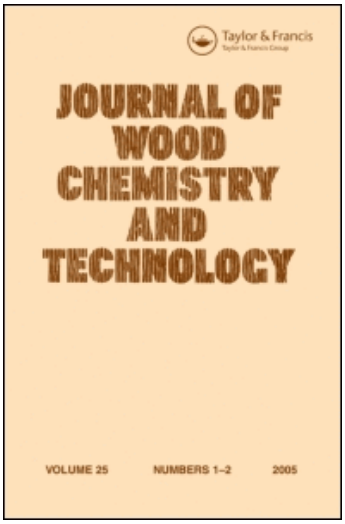

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INTERACTIONS OF STARCH AND OTHER POLYSACCHARIDES WITH CONDENSED TANNINS IN HOT WATER EXTRACTS OF PONDEROSA PINE BARK

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ABSTRACT

Hot water extraction of Ponderosa pine bark yields condensed tannins (polymeric procyanidins, PPC's) and starch. Precipitation of the PPC's as the lead complex coprecipitates the starch, although the latter does not itself form an insoluble lead complex. Ethylene diamine tetraacetic acid (EDTA) treatment of the lead complex removes lead and yields PPC and starch which coprecipitate with ethanol from water as a starch-PPC complex, although the PPC itself is fully ethanol soluble. Chromatography of the starch-PPC complex with water on several polysaccharide media leads to elution of the starch and sorption of the PPC. Similar interactions of PPC and polysaccharides may be responsible for viscosity problems in uses of condensed tannins as tans and as adhesives. Such interactions probably occur in many plant materials.

INTRODUCTION

The extracts of bark, especially with hot water, have many actual and proposed commercial uses. Proposed uses include antioxidants, pigments, clay dispersants, grouting agents, ion-exchange resins and additives for boiler waters and drilling muds. The most promising future uses at present are in wood adhesives[1]

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and the major existing use is as leather tans (tannins)[2] extracted mostly from barks of acacia (wattle), or oak species. The former species yield predominantly condensed tannins and the latter yield gallotannins. It has long been known that condensed tannins can be extracted from softwood barks[3-5] and the chemistry of these tannins is still under active study. Their recent chemistry has been reviewed[6] and they are now known to consist of oligomeric and polymeric proanthocyanidins which are polydisperse, typically with chains of up to twenty flavonoid units. The products from softwood barks are generally polymeric procyanidins (PPC's)[7,8] and the mechanisms of their behavior under acid[9] and alkaline[10] conditions are becoming well understood. Some of the carbohydrate which is frequently associated with the PPC's has recently been shown to be present as ß-glucopyranoside groups, possibly attached to phenolic hydroxyls.[11]

In the use of condensed tannins as adhesives or as tans, excessive viscosity is frequently a problem and may limit their commercial use. This has been a particular problem with tannins from some softwood barks.[12] The viscosity can be reduced by removal of methanol insolubles or by ultrafiltration[12] and it is generally assumed that carbohydrate contamination is detrimental to adhesive and tanning uses of condensed tannins.[13,14] Presumably a major effect of carbohydrate contamination of tannin extracts when the carbohydrate consists of polysaccharides will be to increase viscosity and this effect would almost certainly be amplified by any association (binding) between PPC and polysaccharides. There has been little recent work on polysaccharides of barks, but earlier work revealed that, apart from normal cell wall polysaccharides, pectins and starch are abundant, especially in the phloem[15] and starch metabolism in bark has recently been studied.[16]

The PPC's of course interact (bind) strongly with protein (the basis of the tanning process). However their binding to

polysaccharides, although frequently mentioned, has not been extensively studied in the past. The interactions and pH dependence evidenced by chromatography of various types of tannins on cellulose have been studied[17] and very recently the interactions of PPC's with wood cell walls has been discussed[18] with emphasis on interaction with hemicelluloses. Haslam and coworkers[19] have also recently commenced a study of interactions of gallotannins with proteins and polysaccharides.[20]

EXPERIMENTAL

Bark

A healthy, 12 m, 25 cm base diameter, 49 yr tree (Pinus ponderosa) growing in the Lubrecht Forest of the University of Montana was felled in June and the bark immediately stripped at cambium level from the lower 1·2 m of bole. The bark was returned to the laboratory in sealed black plastic bags and the cream-colored inner bark (ca. 2 mm thick) immediately stripped manually from the outer bark (10-30 mm thick). The separation was 50-70% complete, with the inner bark being completely free from outer bark. Both bark samples were air-dried for 3 days in the dark; yield 249 g inner and 2297 g outer. Both samples were Wiley milled through a 2 mm screen and stored in sealed plastic bags at -20°.

Extraction of Bark

A sample of the inner bark (22.90 g) was extracted in a Soxhlet with acetone and dried (18.17 g). The extracted bark was added to water (250 ml), degassed in vacuum and flushed with argon several times and then boiled under reflux for 30 min in the dark. The mixture was cooled, centrifuged (2,000 x g, 20 min) and the pellet futher extracted with water (120 ml, 90 min boiling) as

above, finally centrifuging and washing the pellet with water at room temperature. The first extract (115 ml) was treated with 5% lead acetate solution (100 ml), kept at 2° in the dark for 2 days, the precipitated lead complex isolated by centrifuging, washed with water and finally freeze-dried to a cream-colored powder (1.41 g). The second extract and washings similarly yielded a further 0.74 g corresponding to a total yield of 9.4% of lead complex from the bark.

The same treatment of the outer bark yielded 13% acetone solubles and 6.1% of water solubles precipitated by lead acetate (0.05% Triton X-405 was added to the water to facilitate "wetting" of the bark).

Removal of Lead from Lead Complex

The lead complex from the inner bark (1.00 g) was wetted with ethanol (0.1 ml) and then ultrasonicated with 0.1 M disodium EDTA (50 ml) for 20 min at room temperature before dialyzing against 0.05 M disodium EDTA (2 l, 1 day) followed by water (3 x 4 l, 1 day each). All dialyses were carried out at 2° in the dark under argon in Spectrapor 4 tubing (MWCO 12,000-14,000). The final solution from the dialysis tube was centrifuged from some precipitated starch and freeze-dried to a cream colored sponge (0.23 g) (PPC-starch complex, A).

Attempted Fractional Precipitation of PPC-Starch Complex

The complex (A, 0.25 g) dissolved in water (25 ml) to form an opalescent solution which gave no pellet when centrifuged (2,000 x g, 20 min). The solution cleared when heated in a boiling-water bath for 10 min, but became opaque again on cooling. Ethanol was added with stirring at room temperature until a precipitate formed, which was removed by centrifugation and more ethanol added. Pellets from centrifugation were dissolved in water,

TABLE 1

Fractionation of PPC-Starch Complex by Precipitation with Ethanol from Water.

Fraction	Ethanol Content (v/v, %)	Precipitate (%)	Anhydro-Glucose Content of Precipitate (%)
	0	0	
Ia	28.5	36	76
Ib	37.5	9	79
	44.4	trace	
	62.5	trace	
II	75.0	28	40
	78.0	0	
III	(supernatant evaporated to dryness)	21	7 (+ 1% arabinose)

concentrated to remove ethanol and freeze-dried. Results are shown in Table 1.

Carbohydrate Analyses

Apparent glucose contents were measured by the phenol sulfuric acid method[21] by reference to a glucose standard. When a significant "normal" blank reading was obtained (due to PPC) a phenol-free blank was used. Accurate glycose contents were determined by addition of inositol as internal standard and hydrolysis in 2 M trifluoroacetic acid (120°, 1.5 hr) followed by borohydride reduction, acetylation and gas chromatography,[22] with correction for losses determined with authentic glycose samples.

Gel Column Chromatography

Generally, columns of 25 cm x 2.5 cm diameter were used with water elution at ca. 0.5 ml/min. Blue dextran (Pharmacia) was used to determine V_0 and fractions were assayed by phenol sulfuric acid[21] and by optical density at 280 nm. Results are described in the discussion section.

RESULTS AND DISCUSSION

The hot water extraction of the bark was carried out in the dark and in absence of oxygen to minimize oxidative degradation of flavonoids. Barks, especially the phloem, normally contain significant amounts of starch.[15] It was anticipated that some starch would be extracted by the boiling water and the precipitation of the polyphenol components as lead complexes was intended to separate them from this starch. The inner bark gave a higher yield of lead complex (9.4%) than the outer bark (6.1%) and the former was therefore used in further studies.

The lead complex was dissolved in and dialyzed against EDTA followed by water. This process removed lead and any low molecular weight material. The resultant polymeric product gave strong responses to both butanol-hydrochloric acid[20] for PPC and phenol-sulfuric acid[21] for carbohydrate. Since the PPC's are soluble in ethanol as well as water, whereas polysaccharides are insoluble in ethanol, an ethanol-water fractional precipitation was attempted. Table 1 shows that the product separated clearly into three fractions; (I) at 28.5-37.5% ethanol, (II) at 75% ethanol and (III) not precipitated up to 78% ethanol. Fraction (I) yielded 79% glucose on acid hydrolysis, a solution gave a strong blue coloration with iodine, but no significant color with butanol/hydrochloric acid and it was assumed to be starch. It had a significant ash content (8.0%), which was apparently due to residual lead salts and accounted for the low glucose content.

Fraction (II) contained 40% glucose after acid hydrolysis and gave a strong butanol/hydrochloric acid reaction due to formation of cyanidin (cellulose TLC identification[23]). This fraction evidently contained both polymeric procyanidin (PPC) and glucan and was further investigated. Fraction (III) contained 7% glucose and 1% arabinose after acid hydrolysis, yielded cyanidin with butanol/hydrochloric acid and is assumed to consist of polymeric PC glycosides.[11]

Gel Chromatogramph of Fraction II

Chromatography on cellulose was carried out with Whatman CF11 fibrous cellulose. A pure carbohydrate peak (strong phenol-sulfuric acid response, no UV or butanol/hydrochloric acid reaction) eluted at V_o (120 ml). After elution of the carbohydrate peak was complete the eluent retained a small but significant UV absorbance beyond V_e 400 ml. Combined phenol-sulfuric acid analyses of the water-eluted peak indicated presence of 69% of the material added to the column, calculated as glucose, and the solution gave a strong blue color with iodine. It was concluded that fraction II contained 69% starch which completely eluted through the cellulose column while the other component of II (PPC) was sorbed by the cellulose. Chromatography on Pharmacia Sepharose CL-2B gave similar results, with a purely carbohydrate peak eluted at about V_o and the UV absorbing material sorbed to the column.

When fraction II (102 mg) was added to a column of Sephadex G100 the starch eluted at about V_o (45 ml, 55 mg) and a small UV absorbing peak (λ_{max} 270 nm) followed at 180 ml. The latter absorbance corresponded to the equivalent of 5 mg catechin and gave a positive phenol-sulfuric acid test for carbohydrate. Further elution with 1 M urea solution (200 ml) yielded a broad "plateau" showing low level UV absorbance corresponding to the equivalent of 6 mg catechin equivalent, with a lower level of

carbohydrate. Subsequent elution with 8 M urea solution (200 ml) yielded more UV absorbing material corresponding to the equivalent of 5 mg catechin with a lower level of carbohydrate. It was concluded that the PPC was only very slowly desorbed from the cross-linked dextran by concentrated urea solution.

Chromatography of Fraction II on Pharmacia Sephadex LH60 gave complete recovery of starch by elution with water and the sorbed PPC was then eluted with ethanol. The PPC's purified in this way will be the subject of further investigations.

It is notable that in earlier work on PPC's from Loblolly pine bark,[24] a water-soluble PPC (tannin B) was purified by elution with water from a cellulose column, i.e. was not strongly sorbed on the cellulose, while a high molecular weight analogue (tannin C) sorbed strongly to the cellulose and required elution with acetone-water. In the same study a lower molecular weight ethyl acetate soluble PPC (tannin A) was resistant to elution with water from Sephadex LH60. It is possible that the strength of binding of the PPC's to polysaccharides is dependent on several factors, such as molecular weight, stereochemistry, extent of their substitution with glucoside groups. In our work the PPC contained 8% carbohydrate and strongly sorbed to all polysaccharides investigated, while tannin B contained 2.4% "reducing sugars (glucose)" and did not sorb strongly to cellulose. These effects will be further investigated.

CONCLUSIONS

Both starches and PPC's are extracted from Ponderosa pine bark with hot water. Some of the starch coprecipitates with the lead complex of PPC although starch itself will not precipitate with lead acetate. The starch-PPC complex precipitates with ethanol from water although the PPC's themselves do not precipitate. When the starch-PPC complex is chromatographed on cellulose, Sepharose or Sephadex the starch is completely eluted

by water, but the PPC is strongly sorbed to the polysaccharide gel and only partly and slowly removed by elution with concentrated urea solutions. It is very probable that strong complexes of PPC's form with polysaccharides such as starch, cellulose and hemicelluloses in the plant.

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