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FRACTIONATION OF LIGNIN–CARBOHYDRATE COMPLEXES BY HYDROPHOBIC-INTERACTION CHROMATOGRAPHY

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ABSTRACT

Hydrophobic properties of lignin–carbohydrate complexes (LCC) isolated from *Pinus densiflora* Sieb. et Zucc. have been analysed by hydrophobic-interaction chromatography on Phenyl- and Octyl-Sepharose CL-4B gels. The ability of LCC to be adsorbed by these hydrophobic gels was exclusively dependent on their lignin content. Materials adsorbed on Octyl-Sepharose were desorbed with a lower concentration of 2-ethoxyethanol than those adsorbed on Phenyl-Sepharose. In the adsorption of LCC by Phenyl-Sepharose, π–π interactions between the aromatic ligands and the benzene skeletons of lignin play an important role, whereas hydrophobic interaction is the exclusive driving-force for adsorption in the case of Octyl-Sepharose.

INTRODUCTION

Various studies of the interaction between the hydrophobic parts of biological macromolecules and hydrophobic groups chemically linked to a hydrophilic matrix have been carried out[1–6] and the term "hydrophobic-interaction chromatography" has been introduced[7]. However, for adsorbents containing ionic and hydrophobic groups, electrostatic interaction is superimposed upon the hydrophobic property. In order to minimise the electrostatic interaction, a number of electrically neutral, hydrophobic adsorbents have been synthesised[2,6]. The most widely used adsorbents of this type have been aryl- and alkyl-glycidylagaroses[8].

Previously, we developed a new method for the isolation of lignin–carbohydrate complexes (LCC) from milled wood of *Pinus densiflora* Sieb. et Zucc.[9] and found that LCC have marked affinity for both phenyl- and octyl-glycidylagarose (Phenyl- and Octyl-Sepharose CL-4B) gels. This hydrophobic property of LCC excludes a mere physical mixture of carbohydrate and lignin, and indicates the existence of chemical bonds between lignin and carbohydrate. The hydrophobic property of LCC is considered to be extremely important in elucidating the function of LCC in living plants.

We now report further on the hydrophobicity of LCC and present a new method for the fractionation of LCC from *Pinus densiflora* by hydrophobic-interaction

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chromatography on Phenyl- and Octyl-Sepharose CL-4B gels. The difference in the affinity of LCC for these two adsorbents and the effect of various agents on hydrophobic interaction are also described.

EXPERIMENTAL

General methods. — Unless otherwise specified, materials, methods, and the instrumentation used for g.l.c. were the same as those previously described[9]. The water-soluble, lignin–carbohydrate complex (LCC-W) was isolated from milled wood of *Pinus densiflora*, and W-2 and W-3 fractions having weight-average molecular weights of 5.0×10^5 and 5.0×10^3, respectively, were obtained by gel filtration on Sepharose 4B as described previously[9]. LCC fractions were hydrolysed with 0.5M sulfuric acid at 100° for 6 h and the monosaccharide composition of the hydrolysate was determined by g.l.c. of the derived alditol acetates[10,11] on a column of 3% ECNSS-M at 180°. Configurations of the monosaccharides were determined by g.l.c. on a S.C.O.T. column of SP-1000 at 200° after conversion into acetylated (+)-2-octyl D- and (+)-2-octyl L-glycosides[12]. Lignin content was determined by the acetyl bromide method[13].

Chromatographic procedures. — Hydrophobic-interaction chromatography was carried out with columns of Phenyl-Sepharose CL-4B (14 × 1.8 cm) and Octyl-Sepharose CL-4B (15 × 1.8 cm) at ambient temperature. The columns were equilibrated with 25mM sodium phosphate buffer (pH 6.8) containing 0.8M ammonium sulfate. The sample (100 mg) was dissolved in 20–30 ml of the equilibrating buffer and applied to the column, which was then thoroughly washed with the same buffer to obtain the unadsorbed LCC fraction (fraction I). In order to characterise the nature of LCC-adsorbent interaction, various agents were tested for their ability to elute the adsorbed LCC. The effect of ionic strength on the affinity of LCC for the hydrophobic gels was analysed by chromatography in the absence of ammonium sulfate. An attempt was made to separate the adsorbed LCC into four fractions (II–V) by sequential elutions with 25mM sodium phosphate buffer containing (*a*) 15% of 2-ethoxyethanol and 0.6M ammonium sulfate, (*b*) 30% of 2-ethoxyethanol and 0.4M ammonium sulfate, (*c*) 45% of 2-ethoxyethanol and 0.2M ammonium sulfate, and (*d*) 50% of 2-ethoxyethanol. Fractions (6 mL) were collected at a flow rate of 40 mL/h and their lignin content was determined by measuring the absorbance at 280 nm. The carbohydrate content in each fraction was determined by the phenol–sulfuric acid method[14] after evaporation of the 2-ethoxyethanol. Fractions I–V were extensively dialysed against distilled water and lyophilised.

RESULTS AND DISCUSSION

When LCC-W was passed through a Phenyl-Sepharose column in the absence of ammonium sulfate, 80.4% of the material was adsorbed. If the eluent contained 0.8M ammonium sulfate, 90.0% of the material was adsorbed. The corresponding figures

TABLE I

DESORPTION OF LCC-W FROM PHENYL- AND OCTYL-SEPHAROSE CL-4B GELS[a]

Phenyl-Sepharose CL-4B

Eluent	*Recovery*[b] *(%)*	*Lignin content*[d] *(%)*	*Neutral sugar*[c] *(%)*				
			L-*Arabinose*	D-*Xylose*	D-*Mannose*	D-*Galactose*	D-*Glucose*
25mM Sodium phosphate buffer	7.1	22.7	30.7	15.6	28.0	15.4	10.3
6M Urea	31.5	42.1	16.8	16.1	34.9	24.9	8.2
6M Guanidine hydrochloride	33.7	42.2	17.9	13.5	35.2	22.3	11.1
80% 1,4-Dioxane	70.8	64.7	15.4	14.7	32.9	24.3	12.7
80% 1,2-Ethanediol	43.8	51.8	13.0	21.1	31.1	21.8	12.9
50% 2-Ethoxyethanol	86.3	45.0	19.0	23.1	28.3	20.4	9.2

Octyl-Sepharose CL-4B

Eluent	*Recovery*[b] *(%)*	*Lignin content*[d] *(%)*	*Neutral sugar*[c] *(%)*				
			L-*Arabinose*	D-*Xylose*	D-*Mannose*	D-*Galactose*	D-*Glucose*
25mM Sodium phosphate buffer	9.5	23.7	38.3	16.7	28.8	11.5	4.7
6M Urea	47.7	38.8	20.2	16.7	36.2	26.9	11.9
6M Guanidine hydrochloride	58.9	39.7	18.2	21.8	28.8	15.3	15.8
80% 1,4-Dioxane	71.0	60.4	16.3	14.8	31.3	25.3	12.2
80% 1,2-Ethanediol	62.3	56.5	19.3	16.7	29.8	25.2	9.0
50% 2-Ethoxyethanol	83.7	46.0	15.2	15.3	31.1	25.0	13.4

[a]Details of the procedure are given in the Experimental section. [b]Percentage of the dry weight of the lignin–carbohydrate complex applied on the column. [c]Percentage of the total neutral sugar. [d]Percentage of the eluted lignin–carbohydrate complex.

for Octyl-Sepharose were 77.6 and 79.9%. The slight increase in adsorption caused by ammonium sulfate may be due to the fact that hydrophobic interactions are promoted by salts[15-19].

The data in Table I show that the desorbing ability (based on the total weight of desorbed material) increases in the following order: urea < guanidine hydrochloride < 1,2-ethanediol < 1,4-dioxane < 2-ethoxyethanol. When based on the weight of lignin, the order is urea < guanidine hydrochloride < 1,2-ethanediol < 2-ethoxyethanol < 1,4-dioxane. Urea and guanidine hydrochloride, which are known to be good splitting agents for hydrogen bonds[20-23], do not desorb the LCC material to the same extent as 1,4-dioxane and 2-ethoxyethanol. These findings suggest that the LCC material is linked mainly by hydrophobic interaction of the lignin part and the gel.

Thus, it was possible to fractionate LCC on the basis of differences in lignin contents. Stepwise elution of the adsorbed materials with increasing concentrations of 2-ethoxyethanol and decreasing concentrations of ammonium sulfate gave five fractions (I–V) (Figs. 1–3). Among the three fractions of LCC, *i.e.*, LCC-W, W-2, and W-3, the adsorbed parts of W-3 were eluted from both columns with the lowest concentration of 2-ethoxyethanol, and the major fractions were P-II, O-II, and O-III.

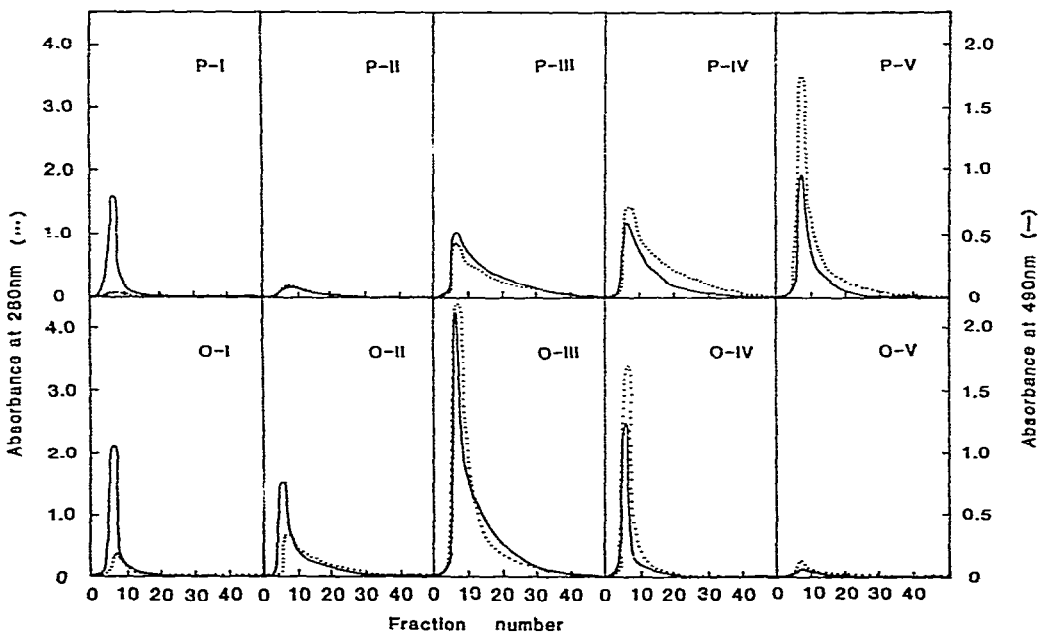

Fig. 1. Hydrophobic-interaction chromatography of the LCC-W fraction on Phenyl-Sepharose (P) and Octyl-Sepharose (O); I–V represent the elution profiles using 25mM sodium phosphate buffers (pH 6.8) containing (I) 0.8M ammonium sulfate, (II) 15% of 2-ethoxyethanol and 0.6M ammonium sulfate, (III) 30% of 2-ethoxyethanol and 0.4M ammonium sulfate, (IV) 45% of 2-ethoxyethanol and 0.2M ammonium sulfate, and (V) 50% of 2-ethoxyethanol as eluents (See Experimental). Each fraction was analysed for carbohydrate (——) and lignin (....).

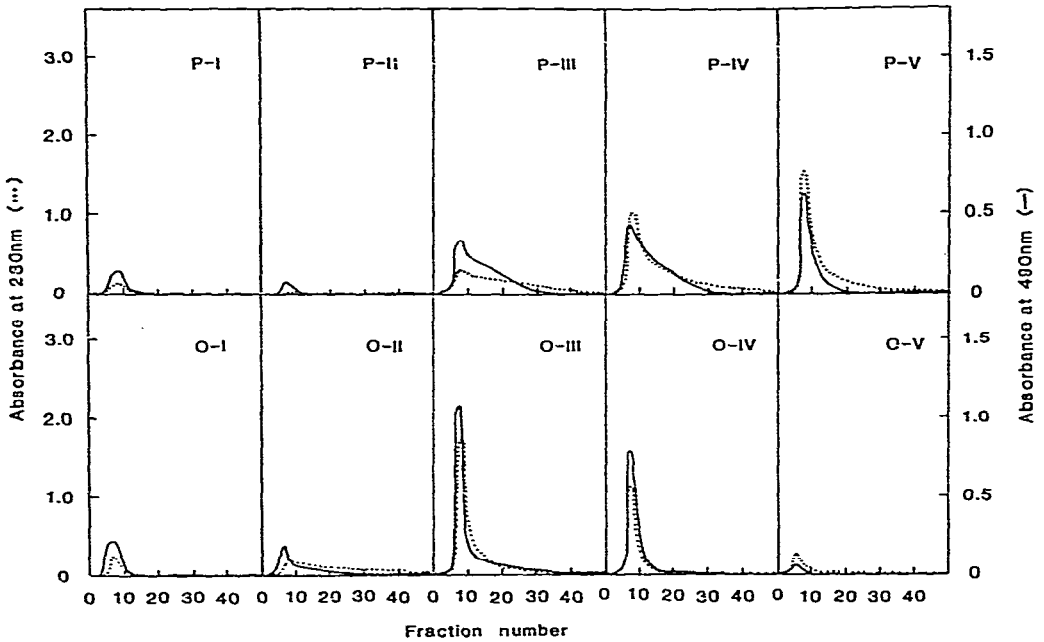

Fig. 2. Hydrophobic-interaction chromatography of the W-2 fraction on Phenyl-Sepharose (P) and Octyl-Sepharose (O); key: as specified for Fig. 1. Each fraction was analysed for carbohydrate (——) and lignin (...).

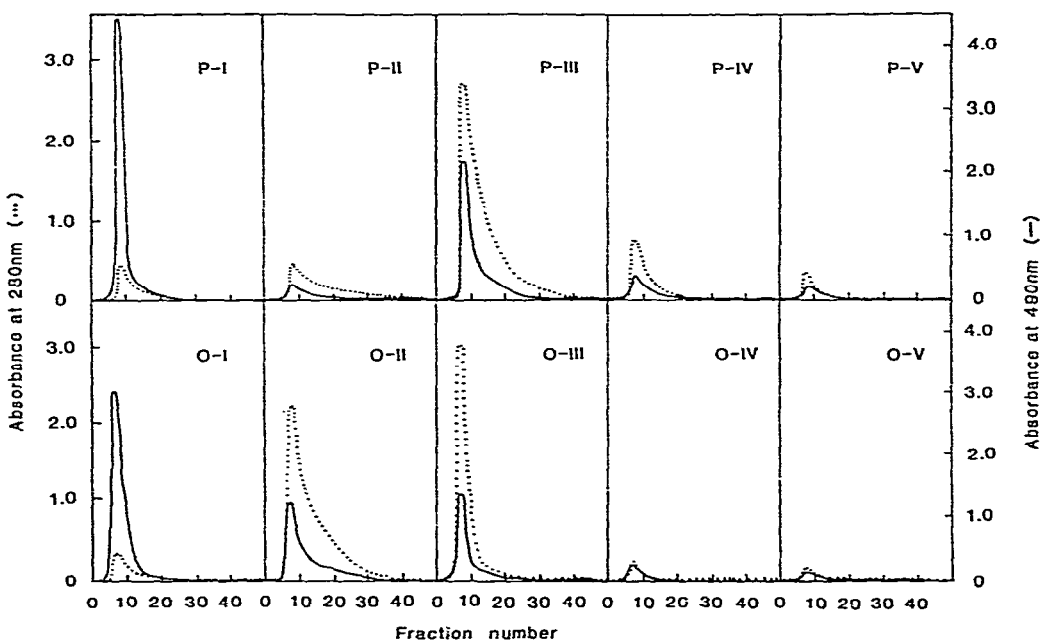

Fig. 3. Hydrophobic–interaction chromatography of the W-3 fraction on Phenyl-Sepharose (P) and Octyl-Sepharose (O); key: as specified for Fig. 1. Each fraction was analysed for carbohydrate (——) and lignin (...).

TABLE II

PROPERTIES OF THE FRACTIONS FROM HYDROPHOBIC-INTERACTION CHROMATOGRAPHY OF THE LCC-W FRACTION[a]

Component	*Original*	*Phenyl-Sepharose*					*Octyl-Sepharose*				
	LCC-W	*P-I*	*P-II*	*P-III*	*P-IV*	*P-V*	*O-I*	*O-II*	*O-III*	*O-IV*	*O-V*
Lignin content[b] (%)	40.5	8.0	22.6	34.0	43.5	50.5	8.8	28.4	38.8	44.9	60.5
Neutral sugar[c] (%)											
L-Arabinose	22.5	23.4	19.0	13.9	17.0	13.4	30.6	14.7	15.0	15.4	24.0
D-Xylose	18.2	24.5	22.6	37.4	22.3	13.7	17.8	16.0	12.4	22.7	15.6
D-Mannose	29.2	25.8	28.8	13.9	28.6	32.5	18.5	31.5	27.7	18.0	32.2
D-Galactose	19.1	15.0	19.3	20.5	23.0	18.0	10.2	25.3	32.1	23.1	21.3
D-Glucose	11.0	11.3	10.4	14.3	9.2	22.4	23.4	12.5	12.6	20.7	7.9
Recovery[d] (%)		17.5	7.8	12.7	29.7	32.3	12.7	25.3	36.5	14.6	10.9

[a]Details of the procedure are given in the Experimental section. [b]Percentage of the dry weight of each lignin–carbohydrate complex. [c]Percentage of the total neutral sugar. [d]Percentage of the eluted lignin–carbohydrate complex.

TABLE III

PROPERTIES OF THE FRACTIONS FROM HYDROPHOBIC-INTERACTION CHROMATOGRAPHY OF THE W-2 FRACTION[a]

Component	*Original*	*Phenyl-Sepharose*					*Octyl-Sepharose*				
	W-2	*P-I*	*P-II*	*P-III*	*P-IV*	*P-V*	*O-I*	*O-II*	*O-III*	*O-IV*	*O-V*
Lignin content[b] (%)	46.6	10.1	n.d.[e]	31.4	63.7	59.4	14.2	28.3	40.6	56.8	n.d.[e]
Neutral sugar[c] (%)											
L-Arabinose	15.8	16.0	12.1	7.0	7.8	8.9	17.9	10.7	7.7	6.8	7.7
D-Xylose	16.2	23.8	25.7	25.6	19.4	31.5	24.4	18.6	25.8	19.0	30.1
D-Mannose	37.3	21.0	13.7	29.4	24.0	11.3	22.3	29.9	30.7	17.7	13.6
D-Galactose	16.7	20.1	13.2	8.3	16.6	11.1	19.1	23.4	11.4	28.7	11.8
D-Glucose	14.0	19.1	35.4	29.7	32.1	37.1	16.3	17.4	24.4	27.8	36.6
Recovery[d] (%)		15.2	3.1	20.8	27.7	32.2	15.6	7.8	33.6	35.0	8.0

[a]Details of the procedure are given in the Experimental section. [b]Percentage of the dry weight of each lignin–carbohydrate complex. [c]Percentage of the total neutral sugar. [d]Percentage of the eluted lignin–carbohydrate complex. [e]Not determined.

With LCC-W and W-2, the major fractions eluted from the Octyl-Sepharose gel were O-III and O-IV, and P-V was the major fraction eluted from Phenyl-Sepharose. The chemical properties of the separated fractions are summarised in Tables II–IV. No important differences were observed in the neutral sugar compositions, except that the arabinose contents were particularly high in the unadsorbed fraction. However, there were substantial differences in lignin contents, which increased as the concentration of 2-ethoxyethanol in the eluents was increased. These results indicate

TABLE IV

PROPERTIES OF THE FRACTIONS FROM HYDROPHOBIC-INTERACTION CHROMATOGRAPHY OF W-3 FRACTION[a]

Component	*Original W-3*	*Phenyl-Sepharose*					*Octyl-Sepharose*				
		P-I	*P-II*	*P-III*	*P-IV*	*P-V*	*O-I*	*O-II*	*O-III*	*O-IV*	*O-V*
Lignin content[b] (%)	22.5	9.4	26.1	35.6	41.1	58.8	11.2	28.5	35.9	55.3	61.4
Neutral sugar[c] (%)											
L-Arabinose	27.6	36.3	36.2	23.8	16.1	9.9	35.5	25.6	21.2	9.1	11.1
D-Xylose	16.5	18.3	20.9	22.2	23.4	33.1	18.7	19.5	22.7	28.5	46.1
D-Mannose	26.1	4.0	18.3	27.9	19.8	17.6	22.6	27.4	27.2	20.0	13.2
D-Galactose	19.3	9.5	14.8	26.1	28.5	21.9	14.5	17.8	20.8	20.0	13.2
D-Glucose	10.5	31.8	9.8	12.2	12.2	17.5	8.7	9.7	8.0	16.4	14.9
Recovery[d] (%)		26.9	17.3	34.5	14.5	6.8	34.3	39.0	13.6	6.4	6.7

[a]Details of the procedure are given in the Experimental section. [b]Percentage of the dry weight of each lignin–carbohydrate complex. [c]Percentage of the total neutral sugar. [d]Percentage of the eluted lignin–carbohydrate complex.

that the lignin moiety strongly influences the hydrophobic properties of LCC. Although 2-ethoxyethanol was selected as an eluent because of its high desorbing ability, 1,4-dioxane may give similar results. The stronger adsorption by Phenyl-Sepharose suggests that π–π interactions participate when this gel is used, since the phenyl group is far less hydrophobic than the octyl group and is equivalent to the hydrophobicity of a C_{2-3} unbranched hydrocarbon[17,24,25]. The importance of π–π interactions in hydrophobic-interaction chromatography has been emphasised by Jankowski *et al.*[26] and Homcy *et al.*[27]. For Octyl-Sepharose, however, the hydrophobic interaction is the exclusive driving-force for the binding of LCC. Since the condition of this hydrophobic interaction is extreme, binding with the octyl group might not be significantly affected by the salt concentration.

The importance of the hydrogen bonds between lignin and carbohydrate in plant tissues has been reported[28], and Pew[29] and Goring[30,31] emphasised the immobilisation of the carbohydrate chains by the three-dimensional network structure of lignin in a "snake cage" manner. Our results indicate the importance of hydrophobic and π–π interactions in the structure and function of LCC, and suggest that the benzene skeletons of lignin moieties in LCC are strongly bound to each other in the plant cell-wall, resulting in the immobilisation of the hemicellulose. Besides demonstrating the interactions of LCC with hydrophobic groups, it is clear that hydrophobic-interaction chromatography is a powerful technique for purifying LCC from polysaccharides not linked to lignin, and for the fractionation of LCC variants that have differences in lignin contents.

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