Phytochemistry, 1974, Vol. 13, pp. 1891 to 1896. Pergamon Press. Printed in England.

CARBOHYDRATES OF THE INNER BARK OF *PSEUDOTSUGA MENZIESII*

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(*Received* 10 *December* 1973)

Key Word Index—*Pseudotsuga menziesii*; Pinaceae; carbohydrates; polysaccharides; bark; holocellulose; xylan; galactoglucomannan; glucomannan; starch.

Abstract—A holocellulose fraction was isolated from the inner bark of *Pseudotsuga menziesii* and analyzed. It was composed of acid-insoluble lignin (3·1%), acid-soluble lignin (4·1%), L-arabinose (2·6%), D-xylose (6·3%), D-mannose (9·5%), D-galactose (2·3%), and D-glucose (61·1%). The presence of these sugars and their configurations were positively established by the preparation of crystalline derivatives. The holocellulose was fractionated into its component polysaccharides, a xylan, a galactoglucomannan, a glucomannan, and a glucan-rich residue.

INTRODUCTION

THE CARBOHYDRATES of the bark of Douglas-fir [*Pseudotsuga menziesii* (Mirb.) Franco] have not received a great deal of attention although they merit consideration in regard to utilization of this natural resource.[1,2] The work herein reported includes a systematic isolation and measurement of the various carbohydrate fractions in Douglas-fir inner bark and a report on their nature and properties.

RESULTS AND DISCUSSION

The inner bark was analyzed for the general components shown in Table 1. Immersion of the inner bark in ethanol-water denatured the enzymes and prevented possible alteration of the natural materials. This treatment, known to solubilize monosaccharides,[3] extracted only a small amount of glucose, demonstrating that the sugar residues in the bark existed primarily as part of polysaccharide structures and not as simple sugars.

The hot-water-soluble solids (Table 1) were analyzed to ascertain the carbohydrates which had dissolved. The solids contained nitrogen (3·63%, Kjeldahl) largely due to proteinaceous material, tannins, and starch but not sulfur, phosphorus, or halogen. The polysaccharides contained glucose, arabinose, galactose, xylose, and rhamnose. Separation of the hot-water-soluble solids by cold-water extraction reduced the amounts of protein (nitrogen 0·28%), tannin, and starch in the water-soluble polysaccharide fraction. Further purification with α-amylase enzymes released glucose only, indicating the presence of an

* Taken from the theses submitted by C.-H. Chen and J. V. Zerrudo (1973) in partial fulfillment of the requirements for the Ph.D. degree, and by Y.-C. L. Lai (1972) in partial fulfillment of the requirements for the M.S. degree, Oregon State University, Corvallis, Oregon.

[1] KIEFER, H. J. and KURTH, E. F. (1953) *Tappi* **36,** 14.
[2] HOLMES, G. W. and KURTH, E. F. (1961) *Tappi* **44,** 893.
[3] WOLFROM, M. L., PLUNKETT, R. A., and LAVER, M. L. (1960) *J. Agr. Food Chem.* **8,** 58.

α-D-(1→4)-glucan. An acid hydrolyzate of the purified water-soluble solids contained glucose, galactose, and arabinose in the ratio of 2·9 : 1·0 : 1·3, as well as trace amounts of mannose, xylose, and rhamnose. The glucose was from the residual starch that was known to remain (iodine test). The arabinose and galactose residues undoubtedly were released from an arabino-galactan-type polysaccharide. Water-soluble, highly branched L-arabino-D-galactans have been shown to be present in conifers.[4,5]

TABLE 1. COMPOSITION OF THE INNER BARK OF *Pseudotsuga menziesii*

Component	%oven-dry, unextracted bark
Ethanol–H_2O (4 : 1) soluble	15·4
C_6H_6–ethanol (2 : 1) soluble	4·4
Hot-H_2O soluble	11·1
Ammonium oxalate soluble	2·8
Acidified sodium chlorite soluble (First treatment)	22·0
Holocellulose A residue	44·3
Acidified sodium chlorite soluble (Second treatment)	13·7
Holocellulose B residue	30·6

Delignification with acidified sodium chlorite reagent is known to dissolve polysaccharides[6] and so the solubilized solids (Table 1; first treatment) were analyzed to determine the amount and nature of these dissolved polymers. The solids contained 13·4% ash as sulfate,[7] which was not lowered by dialysis and reprecipitation. The presence of uronic acids in the nondialyzable material was shown by a positive carbazole-sulfuric acid test.[8] IR spectroscopy showed a strong absorption at 1590 cm^{-1} which was attributed to carboxylate ions.[9] The presence of carboxylic acid moieties explained the high ash content and the difficulty in removing the ash. The inorganic materials existed as cations to the carboxylate anions. The acidified-sodium chlorite-soluble materials contained essentially no nitrogen (0·84%, Kjeldahl), and no phosphorus, sulfur, or halogen. An acid hydrolyzate of the nondialyzable solids contained glucose, galactose, mannose, arabinose, xylose, and rhamnose in the ratio of 59·1 : 3·9 : 3·7 : 11·9 : 1·0 : 1·0). These carbohydrates amounted to 56·5% of the total dissolved solids, or 12·4% of the original sample of inner bark. These results are in agreement with Timell's[6] comments that the acidified sodium chlorite method of delignification does not quantitatively separate lignin and plant carbohydrates. Timell[6] also commented that holocellulose isolation by any means can result in alterations of polysaccharide structures and that possible changes from the structures in the native state should be considered when investigating plant polymers.

[4] TIMELL, T. E. (1965) *Advances in Carbohydrate Chemistry* (WOLFROM, M. L., ed.), Vol. 20, pp. 409–482. Academic Press, New York.
[5] WHISTLER, R. L. and RICHARDS, E. L. (1970) *The Carbohydrates Chemistry and Biochemistry* (PIGMAN, W. and HORTON, D., eds.) Vol. IIA, pp. 462–464, Academic Press, New York.
[6] TIMELL, T. E. (1964) *Advances in Carbohydrate Chemistry* (WOLFROM, M. L., ed.), Vol. 19, pp. 247–302. Academic Press, New York.
[7] PAECK, K. and TRACY, M. V. (1956) *Mod. Methoden Pflanzenanal. (Mod. Methods Plant Anal.)* **1**, 468.
[8] DISCHE, S. (1962) *Methods in Carbohydrate Chemistry* (WHISTLER, R. L. and WOLFROM, M. L., eds.), Vol. 1, p. 497, Academic Press, New York.
[9] NAKANISHI, K. (1962) *Infrared Absorption Spectroscopy. Practical* p. 44, Holden-Day, San Francisco.

Holocellulose A (Table 1) isolated by the first treatment with acidified sodium chlorite reagent was slightly yellow in color indicating incomplete delignification. A similar problem of delignifying barks was reported by Timell.[10] A second treatment with acidified sodium chlorite reagent yielded a white holocellulose termed Holocellulose B (Table 1).

TABLE 2. COMPOSITIONAL ANALYSIS OF HOLOCELLULOSE B ISOLATED FROM DOUGLAS-FIR INNER BARK

Component	Holocellulose B (%)
Acid-insoluble lignin	3·1
Acid-soluble lignin	4·1
L-arabinose	2·6
D-xylose	6·3
D-mannose	9·5
D-galactose	2·3
D-glucose	61·1
Total	89

Table 2 shows a thorough compositional analysis of Holocellulose B as demonstrated by a total recovery of 89%. The loss of 11% was undoubtedly caused by acid-degradation during lignin and carbohydrate analyses. The presence of each monosaccharide and its correct configuration was definitely established by the preparation of crystalline derivatives. Sirups of each of the constituent monosaccharides were isolated by preparative paper chromatography. The following crystalline derivatives were prepared from these sirups: penta-*O*-acetyl-D-galactose diethyl dithioacetal[11], m.p. 76·5–77·5°, $[\alpha]_D^{23\cdot5}$ + 10·0° (*c* 4·0, chloroform); tetra-*O*-acetyl-L-arabinose diethyl dithioacetal[11], m.p. 77·0–78·5°, $[\alpha]_D^{23\cdot5}$ − 26·4° (*c* 0·54, chloroform); penta-*O*-acetyl-β-D-glucopyranose,[12] m.p. 133·0–134·0°, $[\alpha]_D^{23\cdot5}$ + 3·9° (*c* 3·4, chloroform); di-*O*-benzylidene-D-xylose dimethyl acetal,[13] m.p. 211·0–212·5°, $[\alpha]_D^{23\cdot5}$ − 8·0° (*c* 1·0, chloroform); D-mannose phenylhydrazone,[14] m.p. 193·5–194·5°, $[\alpha]_D^{23\cdot5}$ + 25° (*c* 0·1, pyridine). The m.p.'s were unchanged on admixture with authentic materials.

The polysaccharide composition of Holocellulose B is given in Table 3. The methods of fractionation and the nomenclature are those of Beélik, *et al.*[15] Hemicellulose A represents the polysaccharide fraction extracted from the holocellulose with 10% NaOH after the holocellulose had been impregnated with $Ba(OH)_2$ to selectively block the dissolution of mannose-containing polysaccharides. Hemicellulose A is customarily rich in anhydroxylose units. Hemicellulose B represents the polysaccharide fraction extracted from the holocellulose with dilute alkali (1% sodium hydroxide) after the $Ba(OH)_2$ had been washed out. The fraction is termed galactoglucomannan[16] and is readily soluble in water. Hemicellulose C represents the polysaccharide fraction extracted from the holocellulose

[10] TIMELL, T. E. (1961) *Svensk Papperstidn.* **64,** 651.

[11] WOLFROM, M. L. and KARABINOSE, J. V. (1945) *J. Am. Chem. Soc.* **67,** 500.

[12] BATES, F. J. and ASSOCIATES (1942) *Polarimetry, Saccharimetry and the Sugars*, p. 448. U.S. National Bureau of Standards, Circular C440, United States Government Printing Office, Superintendent of Documents, Washington, D.C.

[13] WHISTLER, R. L. and BEMILLER, J. N. (1962) *Methods in Carbohydrate Chemistry* (WHISTLER, R. L. and WOLFROM, M. L., eds.), Vol. 1, p. 88, Academic Press, New York.

[14] ISBELL, H. S. and FRUSH, H. L. (1962) *Methods in Carbohydrate Chemistry* (WHISTLER, R. L. and WOLFROM, M. L., eds.) Vol. 1, p. 147, Academic Press, New York.

[15] BEÉLIK, A., CONCA, R. J., HAMILTON, J. K., and PARTLOW, E. V. (1967) *Tappi* **50,** 78.

[16] CASEBIER, R. L. and HAMILTON, J. K. (1965) *Tappi* **48,** 664.

residue with strong alkali (15% sodium hydroxide) after removal of Hemicelluloses A and B. It is water insoluble. The residue after the removal of Hemicelluloses A, B, and C is termed residue C and is generally rich in anhydroglucose units.

TABLE 3. POLYSACCHARIDE COMPOSITION OF HOLOCELLULOSE B

Polysaccharide	Holocellulose (%)*
Hemicellulose A; a xylan	7·0
Hemicellulose B; a galactoglucomannan	1·8
Hemicellulose C; a glucomannan	2·9
Residue C; a glucan-rich fraction	62·6
Total	74·3†

* By isolation.
† The remaining material was lost through dialysis and purification.

The presence of xylose and arabinose in Hemicellulose A was demonstrated by PC and GLC. The presence of a uronic acid was suggested by a positive carbazole-sulfuric acid test.[8] The presence of a uronic acid also was suggested by ash (4·4%), and acidity analyses (2 mol of sodium hydroxide per mol of xylan), and by viscosity measurements in water and aqueous NaCl. In both of these media the reduced viscosity showed a marked increase with dilution below 0·3 g/dl. This behavior is typical of a polyelectrolyte.[17] The uronic acid is thought to be a glucuronic acid moiety because of the numerous xylans isolated from wood and bark which contain this sugar or its derivatives.[4,6,18] However, no evidence of glucuronic acid or 4-*O*-methyl-D-glucuronic acid has been found by PC.[19] The uronic acid moieties may have been lost during hydrolysis and resulting sample preparation, as has been mentioned by others.[20] GLC analysis of the xylan hydrolyzate showed a ratio of xylitol pentaacetate-arabinitol pentaacetate-glucitol hexaacetate of 4·5 : 1·0 : 1·2. The glucitol hexaacetate is considered to be due to incomplete separation of the xylan from glucan polymers (Table 3). A molecular weight of $1·8 \times 10^4$ and a degree of polymerization of 90 were shown by end-group analysis and viscosity measurements.

These data in conjunction with periodate oxidation results (133·5 moles of periodate per mole of xylan) and formic acid formation (22·5 moles per mole of xylan) are consistent with a gross structure for Hemicellulose A consisting of a backbone of 90 xylose units attached by β-D (1→4) glycosidic linkages (negative optical rotation).[21] The arabinose moieties are considered to be branches.

These data indicate that the chemical composition of Douglas-fir bark is quite different from Douglas-fir wood. The extractive content is more than three times greater in the bark than in the wood and the holocellulose content is somewhat less (69% as much as in wood).[22] The polysaccharides that comprise the holocellulose appear to be different from those in the wood of gymnosperms.[4,5] For example, the xylan fraction possesses more arabinose residues than most wood xylans.[4]

[17] GLAUDEMANS, C. P. J. and TIMELL, T. E. (1958) *Svensk Papperstidn.* **61**, 1.
[18] WHISTLER, R. L. and RICHARDS, E. L. (1970) *The Carbohydrates Chemistry and Biochemistry* (PIGMAN, W. and HORTON, D., eds.), Vol. IIA, pp. 452–458.
[19] HAMILTON, J. K. and THOMPSON, N. S. (1957) *J. Am. Chem. Soc.* **79**, 6464.
[20] SHAFIZADEH, F. and MCGINNIS, G. D. (1971) *Carbohyd. Res.* **16**, 273.
[21] WOLFROM, M. L., LAVER, M. L., and PATIN, D. L. (1961) *J. Org. Chem.* **26**, 4533.
[22] GRAHAM, H. M. and KURTH, E. F. (1949) *Ind. Eng. Chem.* **41**, 409.

The data also indicate that the chemical composition of Douglas-fir bark is different from the chemical composition of the barks of other gymnosperms. Timell[10] found the holocellulose content of *Abies amabilis* (Dougl.) Forb to be 57·9%, *Picea engelmanni* Parry to be 58·2%, *Pinus contorta* Dougl. to be 63·8%, and *Ginkgo biloba* to be 56·5% on an extractive-free basis. In contrast, Holocellulose B (Table 1) isolated in the same way from Douglas-fir inner bark amounted to 38·2% of the inner bark on an extractive-free basis. The polysaccharides that comprise Holocellulose B (Table 3) also appear to be different from those in the above-mentioned gymnosperms. For example, the xylan fraction from Douglas-fir inner bark contains more than twice as much arabinose as a xylan isolated from *Abies amabilis* (Dougl.) Forb.[10] The conclusion is that the inner bark of Douglas-fir contains polysaccharides that are quite different from the polysaccharides in the wood and bark of other gymnosperms.

EXPERIMENTAL

Hydrolyses and chromatography. Unless otherwise stated, hydrolyses of the polysaccharides were accomplished with 3% H_2SO_4 under reflux for 5 hr. The solutions were neutralized to pH 5 with aq $Ba(OH)_2$ and the neutral hydrolyzates recovered by centrifuge. PC of the resulting simple sugars was accomplished by the descending method (repeated three or four times for improved resolution) using EtOAc–pyridine–H_2O (8:2:1) and *o*-aminodiphenyl as spray reagent.[23] The sugars were analyzed quantitatively by GLC as their alditol acetates according to Borchard and Piper.[24] Authentic crystalline alditol acetates for use as standards were synthesized.[25,26]

Analyses. Samples were analyzed for ash,[7] N, S, P, and halogens by the usual methods, for tannins by the method outlined by Browning,[27] for starch by iodine solution, and for amino acids according to Clark.[28]

Isolation of carbohydrate fractions. The inner bark was taken 1·5 m height from a standing Douglas fir tree* (later shown to be 130-yr-old). The inner bark (4832 g, moisture content 44·9%) from which the cambium had been removed, was immersed in 95% EtOH (18 l.) H_2O (1207 ml) was added to give a 4:1 EtOH–H_2O soln. After standing for 3 days, the solids were recovered by filtration and the filtrate paper chromatographed. The residue was ground in a Wiley Mill and a part (1500 g dry wt; passed a 35-mesh screen) was extracted successively with C_6H_6–EtOH (2:1), hot water (55 ± 3°), and 0·5% aq. ammonium oxalate soln (75 ± 3°) (Table 1). The extracted bark meal was delignified with acidified sodium chlorite reagent[29] (two treatments) (Table 1).

Hot-H_2O-soluble carbohydrates. The hot-H_2O-soluble solids (Table 1) (74·9 g, 1% slurry) were stirred in H_2O for 2 hr at room temp. and centrifuged (repeated three times). The aq. centrifugate was conc. and the solids were ppt. by the addition of EtOH to 70%. The flocculent precipitate was recovered by centrifugation, washed with 70% EtOH and lyophilized; yield 19·4 g. The cold-H_2O-soluble solids were treated with α-amylase enzymes, dialyzed, and the dialyzate was analyzed for monosaccharides. The non-dialyzables were treated with the proteolytic enzymes chymotrypsin and trypsin, dialyzed, and the non-dialyzables recovered.

Acidified-sodium chlorite-soluble polysaccharides. The acidified-sodium chlorite-soluble solids were recovered by dialysis and precipitation from 70% EtOH (repeated once). IR spectra were obtained in KBr pellets and Nujol mulls.

Characterization of holocellulose B. A portion (0·4566 g) of holocellulose B (Table 1) was analyzed for acid-insoluble lignin;[30] yield 3·1%. The acid-soluble lignin was determined in the filtrate by UV absorption [31,32] at 280 nm (absorbance 2·62) and 210 nm (absorbance 3·77); yield 4·1%. A sample of Holocellulose B (27·9 mg) was

* The tree was in the George T. Gerlinger State Experimental Forest, located near Falls City, Oregon, U.S.A., and operated by the School of Forestry, Oregon State University, in cooperation with the State Forestry Department of Oregon.

[23] TIMELL, T. E., GLAUDEMANS, C. P. and CURRIE, A. C. (1956) *Anal. Chem.* **28,** 1916.
[24] BORCHARDT, L. G. and PIPER, C. V. (1970) *Tappi* **53,** 257.
[25] ABDEL-AHKER, M., HAMILTON, J. K. and SMITH, F. (1951) *J. Am. Chem. Soc.* **73,** 4691.
[26] SAWARDEKER, J. S., SLONEKER, H. and JEANES, A. (1965) *Anal. Chem.* **37,** 1602.
[27] BROWNING, R. L. (1967) *Methods of Wood Chemistry*, Vol. 1, p. 227, Interscience, New York, NY.
[28] CLARK, J. M. JR. (1964) *Experimental Biochemistry*, p. 93, W. H. Freeman, San Francisco.
[29] WHISTLER, R. L., BACHRACH, J. and BAUMAN, D. R. (1948) *Arch. Biochem.* **19,** 25.
[30] Tappi Standard T 222 m-54 (1954) *Acid Insoluble Lignin in Wood Pulp*, Technical Association of the Pulp and Paper Industry, New York.
[31] BROWNING, B. L. and BUBLITZ, L. O. (1953) *Tappi* **36,** 452.
[32] GOLDSCHMID, O. (1971) *Lignins, Occurrence, Formation, Structure, and Reactions* (SARKANEN, K. V. and LUDWIG, C. H. eds.), p. 241, Wiley-Interscience, New York.

acid hydrolyzed according to the procedure of Laver *et al.*[33] The reducing power [34] of the hydrolysis soln ceased to increase after 12 hr reflux, indicating that hydrolysis was complete at that time. The hydrolyzate was subjected to PC and GLC (Table 2).

Polysaccharide fractions of holocellulose B. The polysaccharide fractions of Holocellulose B were separated by impregnation with $Ba(OH)_2$ followed by graded alkali extractions[15] (Table 3). The fractions were hydrolyzed and the hydrolyzates subjected to PC.

Characterization of hemicellulose A; a xylan. Hemicellulose A was purified by dissolution in aq. alkali and reprecipitation (repeated three times). The ash content[35] was lowered from 27·9% to a constant 4·4% by careful titration with 2 M H_2SO_4 and dialysis. The purified material was H_2O soluble $[\alpha]_D^{25}$ −30·5° (*c* 1·74, H_2O). It was tested for uronic acids by a carbazole–H_2SO_4 color test.[8] The free acidity was determined by titration with NaOH (0·05 M). Viscosity change with concentration was determined H_2O and aq. NaCl (1·0 M) at 25·0° using a Cannon–Ubbelhode viscometer. Reducing end-group analysis was performed by the Somogyi method[34] and the intrinsic viscosity $[\eta]$ (0·42 dl/g) was determined in diethylenediamine copper II reagent.[36] The number average degree of polymerization (DP) was calculated from the relationship $DP = K[\eta]$ where K = 212.[17] A portion (0·2695 g) of the xylan was oxidized with sodium metaperiodate.[37]

Acknowledgements—We wish to acknowledge financial support from the Environmental Protection Agency, Bureau of Solid Waste Management, Grant Number EP-00276-04.

[33] LAVER, M. L., ROOT, D. F., SHAFIZADEH, F., and LOWE, J. C. (1967) *Tappi* **50,** 618.
[34] HODGE, J. E. and HOFREITER, B. T. (1962) *Methods in Carbohydrate Chemistry* (WHISTLER, R. L. and WOLFROM, M. L., eds.), Vol. 1, p. 380. Academic Press, New York.
[35] Tappi Standard T 211 m-28. (1958) *Ash in Pulp.* Technical Association of the Pulp and Paper Industry, New York.
[36] BROWNING, B. L. (1967) *Methods of Wood Chemistry*, Vol. II, p. 537, Interscience, New York.
[37] SEARS, K. F., BEELIK, A., CASEBIER, R. L., ENGEN, R. J., HAMILTON, J. K. and HERGERT, H. L. (1971) *J. Polymer Sci.*, Part C., p. 425.