Hemicelluloses

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A convenient biochemical definition of hemicelluloses is given by *Whistler* and *Smart*[1], who state that hemicelluloses are plant cell wall polysaccharides that are insoluble in water and ammonium oxalate solution, but are soluble in dilute alkali. Thus starch and the polysaccharides contained in plant secretions are normally excluded from the hemicellulose group. Whilst this definition is not completely satisfactory from a chemical point of view, it is useful to describe a fraction whose solubility may be related to its biological function.

Chemically, the term hemicellulose covers a larger class of compounds, namely those which contain a chain of D-xylose, D-mannose, or D-galactose units[2], and some of which can be extracted from plant cell walls by water. Other sugars occurring in hemicelluloses are L-arabinose, D-glucuronic acid, 4-*O*-methyl-D-glucuronic acid, L-galactose and L-fucose[2]. Hemicelluloses have low molecular weights with a degree of polymerization between 50 and 300 (*e.g.* the latter in wheat bran[3]). They occur in plant cell walls together with cellulose, pectic substances, lignin, lipids, proteins, pigments and mineral matter. They are physically and possibly chemically bound with cellulose and lignin. Therefore the analysis of hemicelluloses requires their preliminary isolation.

A further complication in the determination of hemicelluloses is the wide variety of chemical structures that exist. The most common hemicelluloses are xylans, which are found in large amounts in woody tissues of plants and particularly in agricultural residues such as cereal stalks, hulls and hay. The majority of xylans consist of a chain of 1,4-linked D-xylose residues, although in the red seaweed, *Rhodymenia palmata*, 1,4- and 1,3-bonds occur in the same molecule[4]. Some hemicelluloses of esparto grass contain a xylan composed solely of xylose residues which may have a single branching chain of one or more xylose units joined to the main chain. Other hemicellulose fractions of esparto grass, the arabino-xylans, consist of about 20% arabinose and 80% xylose[5], while a xylan prepared from wheat flour contained 37% arabinose and 63% xylose[6]. The arabinose residues can be completely removed by mild hydrolysis leaving a true xylan. Similar compounds have been extracted from barley and rye flours[7]. Wheat straw xylan can be divided into fractions in which the arabinose content varies from nothing to almost one arabinose residue per xylose residue[7]. Some hemicelluloses in corn cobs have a main chain of β-1,4-linked xylose residues with side chains of arabinose, D-glucuronic acid and 4-*O*-methyl-D-glucuronic, acid, while certain chains may also be terminated by D-glucuronic acid[7]. Other xylans from beechwood or Norwegian spruce[8] are free from arabinose, but contain 4-*O*-methyl-D-glucuronic acid in the side chains.

Plant polysaccharides from the seeds of the ivory nut palm *Phytelephas macrocarpa* are composed almost entirely of D-mannose residues, while softwoods such as pine and spruce contain glucomannans consisting of 1,4-linked-β-D-mannose residues and glucose units. Similarly, there occur water soluble arabino-galactans consisting of D-galactose and L-arabinose units, whereas the true galactans are free of arabinose[2].

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1) *R. L. Whistler* and *C. L. Smart:* Polysaccharide Chemistry. Academic Press, New York 1953.
2) *G. O. Aspinall*, Adv. Carbohydrate Chem. *14*, 429 [1959].
3) *G. A. Adams*, Canad. J. Chem. *33*, 56 [1955].
4) *S. K. Chanda* and *E. G. V. Percival*, Nature [London] *166*, 787, [1950].
5) *G. O. Aspinall*, *E. L. Hirst*, *R. W. Moody* and *E. G. V. Percival*, J. chem. Soc. [London] 1631 [1953]; *E. L. Hirst*, J. chem. Soc. [London] 2974 [1955].
6) *A. S. Perlin*, Cereal Chem. *28*, 370, 382 [1951].
7) *E. L. Hirst* in *F. Bolam:* Fundamentals of Papermaking Fibres. Technical Section British Paper and Board Manufacturers' Association, Surrey, England 1958.
8) *I. R. C. MacDonald*, J. chem. Soc. [London] 3183 [1952]; *G. O. Aspinall*, *E. L. Hirst* and *R. S. Mahomed*, *ibid.* 1734 [1954]; *G. O. Aspinall* and *M. E. Carter*, *ibid.* 3744 [1956].

The above description gives a brief outline of the structure of hemicelluloses, including some which have been used as substrates for microbial enzymes (see below). The description also illustrates how a relatively simple basic unit, the anhydroxylose chain (to name only one sugar) can give rise to complex mixtures of hemicelluloses. Alternative structures can be based on mannose and galactose with the consequent numerous possibilities for the formation of complex mixtures.

Hemicellulose fractions prepared in the laboratory are complex mixtures of closely related compounds which are not easily separated from one another by existing techniques. These mixtures may already exist as such in plant cell walls. In the isolation of hemicellulose from plant material, it is necessary to remove maximum amounts of non-hemicellulose components with minimum degradation of all the polysaccharides present (celluloses, hemicelluloses, pectins). Otherwise the hemicellulose fraction will be contaminated with its own degradation products and those of other polysaccharides. For example, it has already been mentioned that xylans with an arabinose content of between nothing and a high percentage have been obtained, but it is not certain whether all these compounds occur naturally or whether they represent degradation products from a few parent compounds.

Because of this structural complexity, it is only possible at present to analyse the simplest hemicellulose fractions. In a few cases, simple xylans have been completely hydrolysed by enzymes from micro-organisms, whereas in other instances, oligosaccharides have been obtained indicating incomplete hydrolysis. However, most of the work has been carried out with impure hemicellulose fractions of unknown composition and with crude enzyme preparations. Under such conditions it is not always clear from where the hydrolysis products originate.

Culture filtrates of soil bacteria (*Streptomyces* sp., *Micromonospora* sp., and *Chaetomium globosum*) hydrolyse straw or its derived xylans, to xylose, xylobiose and xylotriose[9]. An arabino-xylan of wheat straw is similarly degraded by culture filtrates of *Myrothecium verrucaria* to a series of oligosaccharides (xylobiose up to heptasaccharide), each of which (except xylobiose) has at least one arabinose unit directly linked to a xylose residue[10]. Extracellular enzymes from *Aspergillus foetidus* hydrolyse a xylan from corn cobs to xylobiose, while the organism itself continues the process to give xylose[11]. Other workers[12] have used micro-organisms from the rumen of the bull to hydrolyse similar xylans to mixtures of xylotetraose, -triose,- biose and xylose. Complete hydrolysis of a water soluble arabinoxylan from wheat flour and a xylan from red seaweed, *Rhodymenia palmata*, is achieved by sheep rumen micro-organisms acting under toluene[13].

It is evident that the difficulties associated with the physico-chemical properties of hemicelluloses have restricted the application of enzymes to the simplest types of these polysaccharides. Therefore no standard methods for the determination of water soluble or insoluble hemicelluloses exist. If suitable enzymes capable of hydrolysing insoluble hemicelluloses to soluble products were available, then an enzymatic assay similar to that described for cellulose (p. 64) would be possible. The following method, based on the work of *Howard*[13], can serve to determine simple, soluble hemicelluloses.

Principle

Certain simple, soluble hemicelluloses of the arabino-xylan or xylan type are completely hydrolysed to their constituent pentoses, *i.e.* to arabinose and xylose, or only xylose, by washed suspensions of toluene-treated bacteria from rumen of sheep.

Soluble pentosan	——→	pentoses
(non-reducing polysaccharide)		(reducing sugars)

9) *H. Sorensen*, Nature [London] *172*, 305 [1953]; *176*, 74 [1955].
10) *C. T. Bishop* and *D. R. Whitaker*, Chem. and Ind. 119 [1955].
11) *R. L. Whistler* and *E. Masak*, J. Amer. chem. Soc. *77*, 1241 [1955].
12) *J. H. Pazur, T. Budovich, E. W. Shuey* and *C. E. Georgi*, Arch. Biochem. Biophysics *70*, 419 [1957].
13) *B. H. Howard*, Biochem. J. *67*, 643 [1957].

After deproteinization of the hydrolysate[14] the reducing value is determined colorimetrically[15] with arsenomolybdate reagent[16].

The cell-free aqueous phase obtained after treatment of bacteria from sheep rumen with aqueous butanol and centrifugation, contains an enzyme which hydrolyses soluble carboxymethylcellulose[17] and also acts as hemicellulase.

Reagents

1. Sodium hydrogen carbonate, $NaHCO_3$, A. R.
2. Potassium chloride, A. R.
3. Calcium chloride, $CaCl_2 \cdot 6\ H_2O$, A. R.
4. Potassium dihydrogen phosphate, KH_2PO_4, A. R.
5. Magnesium sulphate, $MgSO_4 \cdot 7\ H_2O$, A. R.
6. Carbon dioxide
7. Zinc sulphate, $ZnSO_4 \cdot 7\ H_2O$, A. R.
8. Barium hydroxide, $Ba(OH)_2 \cdot 8\ H_2O$, A. R.
9. Copper sulphate, $CuSO_4 \cdot 5\ H_2O$, A. R.
10. Sodium carbonate, anhydrous, Na_2CO_3, A. R.
11. Potassium-sodium tartrate, Rochelle salt, A. R.
12. Sodium sulphate, anhydrous, Na_2SO_4, A. R.
13. Ammonium molybdate, $(NH_4)_6Mo_7O_{24} \cdot 4\ H_2O$, A. R.
14. Sulphuric acid, conc., A. R.
15. Disodium hydrogen arsenate, $Na_2HAsO_4 \cdot 7\ H_2O$, A. R.
16. Xylose
17. Sodium sulphide, $Na_2S \cdot 9\ H_2O$
18. Bacterial suspension
    from sheep rumen[13]. Preparation, see Appendix, p. 77.
    or cell-free, aqueous extract
    preparation, see Appendix, p. 78.

Preparation of Solutions (for 200 determinations)

I. Bicarbonate-salts buffer*) (pH 6.8):

Dissolve each of the following in 100 ml. portions of distilled water:

a) 1.68 g. $NaHCO_3$ (0.2 M)

b) 1.15 g. KCl (0.154 M)

c) 2.41 g. $CaCl_2 \cdot 6\ H_2O$ (0.11 M)

d) 2.10 g. KH_2PO_4 (0.154 M)

*) When working with cell-free bacterial extracts use 0.2 M acetate buffer (pH 5.5).

[14] *M. Somogyi*, J. biol. Chemistry *160*, 69 [1945].
[15] *M. Somogyi*, J. biol. Chemistry *195*, 19 [1952].
[16] *N. Nelson*, J. biol. Chemistry *153*, 375 [1944].
[17] *G. Halliwell*, J. gen. Microbiol. *17*, 166 [1957].

e) 3.8 g. $MgSO_4 \cdot 7\ H_2O$ (0.154 M)

Mix 100 ml. solution a), 4 ml. solution b), 3 ml. solution c), 1 ml. solution d) and 1 ml. solution e), and saturate the mixture with CO_2. Check the pH (6.8). Dissolve 0.02 g. $Na_2S \cdot 9\ H_2O$ in the mixture.

II. Deproteinizing agents:

a) Zinc sulphate (5% $ZnSO_4 \cdot 7\ H_2O$ w/v):
Dissolve 20 g. $ZnSO_4 \cdot 7\ H_2O$ in 400 ml. distilled water.

b) Barium hydroxide (*ca.* 0.3 N):
Dissolve 19 g. $Ba(OH)_2 \cdot 8\ H_2O$ in 400 ml. distilled water. Protect the solution from atmospheric CO_2.

Dilute 5 ml. $ZnSO_4$ solution to 20 ml. with distilled water and add a little phenolphthalein solution. Titrate with $Ba(OH)_2$ solution from a 5 ml. burette, shaking continually during the titration, until a definite pink colour is obtained which remains for at least 1 min.; 4.7 to 4.8 ml. $Ba(OH)_2$ solution should be required. Otherwise dilute the stronger solution accordingly.

III. Copper reagent:

a) Dissolve 12 g. potassium-sodium tartrate, 24 g. Na_2CO_3, 16 g. $NaHCO_3$ and 144 g Na_2SO_4 in distilled water and make up to 800 ml.

b) Dissolve 4 g. $CuSO_4 \cdot 5\ H_2O$ and 36 g. Na_2SO_4 in distilled water and make up to 200 ml.

Just before use mix 4 parts solution a) with 1 part solution b).

IV. Arsenomolybdate reagent:

Dissolve 25 g. $(NH_4)_6Mo_7O_{24} \cdot 4\ H_2O$ in 450 ml. distilled water, carefully mix in 21 ml. conc. H_2SO_4 and finally add 3 g. $Na_2HAsO_4 \cdot 7\ H_2O$ in 25 ml. distilled water, incubate for 24 to 48 hours at 37°C. Store the solution in brown bottles with glass stoppers.

V. Xylose standard solution (0.03% w/v):

Dissolve 30 mg. xylose in distilled water and make up to 100 ml.

VI. Enzyme preparation:

Use the bacterial suspension*) obtained according to p. 77 or the cell-free extract prepared according to p. 78.

Procedure

Experimental material

Hemicelluloses occur in nature as mixtures and are also closely bound up with other polysaccharides. They must be freed from the latter before the enzymatic hydrolysis. Treat plant material as described in the chapter "Cellulose" (p. 67). Extract the holocellulose with increasing concentrations of KOH or NaOH (0.5 to 24%)[18,18a]. Fractionate the extracts

*) The washed bacteria are suspended in bicarbonate-salts buffer (solution I), because this medium is similar to the natural environment of the bacteria. Another, simpler buffer probably would be satisfactory.

18) *M. H. O'Dwyer*, Biochem. J. *20*, 656 [1926]; *R. L. Whistler*, *J. Bachrach* and *D. R. Bowman*, Arch. Biochem. Biophysics *19*, 25 [1948].

18a) *L. E. Wise*, *M. Murphy* and *A. A. Addieco*, Paper Trade J. *122*, 35 [1946].

(= hemicellulose fractions) further with acetic acid at pH 5 and then with 95% ethanol[18], with ammonium sulphate[19] or with *Fehling*'s solution[20] or by electrophoresis[21]. Identify each fraction by the constituent sugars formed on acid hydrolysis. The simple pentosan, an arabino-xylan, which was analysed by the method described here, was extracted from flour with water and freed from hexosan by treatment with saliva[13]. This pentosan consists of a main chain of xylose residues with single arabinose units attached along it[6].

Enzymatic hydrolysis

Prepare 10 ml. conical centrifuge tubes as follows:

	Experimental tube	*Control tubes*	
		without enzyme	*without substrate*
pentosan	0.03 to 1.5 mg.	0.03 to 1.5 mg.	—
bacterial suspension *) (VI)	0.15 ml.	—	0.15 ml.
buffer *) (soln. I)	to 0.3 ml.	to 0.3 ml.	to 0.3 ml.

Add two drops of toluene to each tube, pass CO_2 through to remove the air and stopper the tubes. Incubate at 38°C, with occasional shaking, until a maximum and constant reducing value is reached (up to 18 hours). If the hydrolysis is complete, the only products detected by paper chromatography are arabinose and xylose.

Colour reaction

To each tube add

1.7 ml. distilled water.

Deproteinize with

2.0 ml. $Ba(OH)_2$ solution (IIb)

and then add

2.0 ml. $ZnSO_4$ solution (IIa).

Shake vigorously and centrifuge or filter. Mix in test tubes (1.6 cm. diameter, with a mark at 25 ml.):

2.0 ml. filtrate

2.0 ml. copper reagent (solution III).

Stopper the tubes with glass marbles and heat for 10 min. in a boiling water bath. Cool and add to each tube

2.0 ml. arsenomolybdate reagent (solution IV).

Shake gently until all the cuprous oxide has dissolved, dilute with

distilled water to 25 ml.

and mix thoroughly (evolution of CO_2). The colour develops quickly and can be measured as soon as the CO_2 evolution is complete. It is stable for several hours.

*) When working with cell-free extracts use 0.15 ml. of the extract and 0.15 ml. 0.2 M acetate buffer (pH 5.5). Anaerobic conditions are then unnecessary.

19) *I. A. Preece* and *K. G. Mackenzie*, J. Inst. Brewing *58*, 353 [1952]; *I. A. Preece* and *R. Hobkirk*, ibid. *59*, 385 [1953].

20) *S. K. Chanda*, *E. L. Hirst*, *J. K. N. Jones* and *E. G. V. Percival*, J. chem. Soc. [London] 1289 [1950].

21) *B. J. Hocevar* and *D. H. Northcote*, Nature [London] *179*, 488 [1957].

Read the optical densities at 500 to 520 mμ. The sensitivity of the measurements is increased four-fold by reading at 660 mμ, but at this wavelength errors due to the reagent blank and re-oxidation of the cuprous oxide are greater. A high concentration of sodium sulphate is used here to prevent re-oxidation. It is therefore unnecessary to use test tubes with constrictions (*e.g. Folin Wu* blood sugar tubes) unless high accuracy is required, for example, with amounts of sugar below 5 μg.[16]. It is possible to use 1 to 5 ml. of the deproteinized filtrate with equal volumes of copper reagent, but the arsenomolybdate solution need not exceed 2 ml. The coloured solution can be diluted to a suitable volume before reading the optical density. For example, with amounts of sugar up to 10 μg., the final volume should be 6 ml. (2 ml. filtrate, copper reagent and arsenomolybdate reagent)[15].

Standard curve

Pipette into test tubes (1.6 cm. diameter, with a mark at 25 ml.):

0.03 to 2.0 ml. xylose standard solution (V) (corresponding to 0.01 to 0.6 mg. xylose)
distilled water to 2.0 ml.
2.0 ml. copper reagent (solution III).

Stopper the tubes with glass marbles, heat for 10 min. in a boiling water bath and cool. Add

2.0 ml. arsenomolybdate solution (IV),

shake gently and dilute with

distilled water to 25 ml.

Mix and read the optical densities. Plot the optical densities (ordinate) against mg. xylose/tube (abscissa) (standard curve).

Calculations

Obtain the pentose content of the experimental tube (P_E) and the two control tubes (P_{C1}, P_{C2}) from the standard curve.

It follows that:

$3 \times [P_E - (P_{C1} + P_{C2})]$ = mg. pentosan (calculated as xylose) in the enzymatic hydrolysis mixture

Sources of Error

Reducing compounds, which are not sugars, and proteins interfere with the colour reaction. However, they are removed in the deproteinization with $ZnSO_4$-$Ba(OH)_2$.

Appendix

Preparation of the bacterial suspension[13]

Add 0.02 g. $Na_2S \cdot 9\,H_2O$/100 ml. rumen fluid to maintain anaerobic conditions, filter through six layers of muslin and centrifuge for 1 min. at 300 g. Centrifuge the supernatant for 30 min. at 2°C (5000 to 20000 g). Discard the supernatant and suspend the sediment in buffer (solution I) (volume equal to that of the original rumen fluid).

The bacterial suspension obtained in this way contains in 0.15 ml. the bacteria from 0.15 ml. rumen fluid. This amount of bacteria is sufficient to completely hydrolyse the arabino-xylan mentioned under "Experimental material" in 18 hours at 38°C.

Preparation of cell-free extracts[17,22]

Suspend the bacterial sediment obtained as described above in distilled water instead of buffer (volume equal to half that of the original rumen fluid). To the suspension in an Erlenmeyer flask at 2° C, slowly add 0.4 volumes n-butanol (A. R.) over 5 min., with stirring, and continue to stir for a further 15 min. The rate of stirring should not exceed that required to ensure a homogeneous mixture, so as to avoid excessive aeration of the solution. Centrifuge for 20 min. at 2° C and 20000 g. Suck off the orange-coloured, butanol-containing aqueous layer with a pipette and centrifuge again for 20 min. at 2° C and 20000 g. The aqueous phase contains the hemicellulase. A slight turbidity disappears on the dropwise addition of distilled water (mix thoroughly after each drop). The solution can be dialysed overnight at 1° C against distilled water to remove butanol.

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22) *G. Halliwell*, unpublished.