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Studies on the simultaneous determination of acidic and neutral sugars of plant cell wall materials by HPLC of their methyl glycosides after combined methanolysis and enzymic prehydrolysis

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A method which involves enzymic hydrolysis followed by methanolysis and separation of their methyl glycosides by HPLC was applied to complex polysaccharides from three fibre preparations (pea hulls, sugar-beet pulp and wheat bran). The results were compared to those obtained by (1) methanolysis without enzymic prehydrolysis, (2) gas chromatography of the alditol acetates of the neutral sugars released by acid hydrolysis, and (3) colorimetric determination of the uronic acids. Methanolysis alone allows the estimation of non-cellulosic polysaccharides (pectins and hemicelluloses), whereas combined methanolysis and enzyme prehydrolysis also leads to the determination of cellulose, except for wheat bran which is a highly lignified plant cell wall material.

INTRODUCTION

Techniques used to characterize complex polysaccharides such as pectins, hemicelluloses and cellulose in plant cell walls include gas chromatography (Schweizer & Würsch, 1979; Theander & Aman, 1980, 1981; Englyst *et al.*, 1982; Blakeney *et al.*, 1983; Englyst & Cummings, 1984, 1988; Selvendran & Du Pont, 1984; Selvendran *et al.*, 1989) or colorimetry (Englyst & Cummings, 1988; Englyst & Hudson, 1987) for neutral sugars, and colorimetry (Blumenkrantz & Asboe-Hansen, 1973; Ahmed & Labavitch, 1973; Thibault, 1979) for uronic acids. The cell wall polysaccharides are differently resistant to acid hydrolysis and the various stabilities of the liberated monosaccharides require different conditions of hydrolysis (Saeman *et al.*, 1954; Albersheim *et al.*, 1967; Selvendran *et al.*, 1979). Furthermore, the non-specific colorimetric methods give global estimations and cannot account for the individual monosaccharide composition. Uronic acids and neutral sugars are generally separately determined.

In order to simplify the analytical procedures, simultaneous determination of uronic acids and neutral sugars by capillary gas chromatography of the *n*-hexylaldonamide and alditol acetates derivatives has been carried out (Walters & Hedges, 1988) but the procedures were tedious and recoveries of cellulose were only ~20%. Attempts to quantitatively convert uronic acids to the corresponding alditol hexaacetates failed (Blake & Richards, 1968, 1970; Jones & Albersheim, 1972).

Methanolysis presents the advantages of a great stability of released methylglycosides and of a simultaneous analysis of acidic and neutral sugars by capillary gas chromatography (Chambers & Clamp, 1971; Pritchard & Todd, 1977; Ford, 1982; Chaplin, 1982; Preuss & Thier, 1983, Roberts *et al.*, 1987) or high-performance liquid chromatography (Cheetham & Sirimanne, 1981; Hjerpe *et al.*, 1982; Quemener & Thibault, 1990). Recoveries of glucose from cellulose have been increased (Roberts *et al.*, 1987) by using methanolic sulphuric acid instead of methanolic hydrogen chloride; this change was inefficient in the

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case of pectins (Quemener & Thibault, 1990). As we have improved the methanolysis of these acidic constituents by an enzymic prehydrolysis (Quemener & Thibault, 1990), the same approach has been assessed for the estimation of non-starch polysaccharides from some plant preparations.

MATERIALS AND METHODS

Materials

Standard sugars and standard methyl glycosides were from Sigma. Pectins were the samples previously described (Quemener & Thibault, 1990). Pure cellulose CC 31 was from Whatman (UK). Sugar-beet pulp was from the 'Générale Sucrière' (Arthenay, France), wheat bran from Breteau-Aubert (La Varenne, France) and pea hull from Sofalia (Ennezat, France). Proteins and starch were removed by the method of Prosky *et al.* (1984, 1985, 1988). After drying, the samples were ultra-milled with a Spex 6700 freezer mill (Spex Industries Inc., Metuchen, USA) for 2 min and then dried at 40°C over P_2O_5, under vacuum for at least 16 h.

Enzyme preparations

Celluclast (from *Trichoderma reeseii*), Novozym 188 and SP 249 (from *Aspergillus aculeatus*), from Novo Industri (Denmark), were purified by precipitation with $(NH_4)_2SO_4$ (Quemener & Thibault, 1990). The dialysis steps (against water and finally 50 mM acetate buffer, pH 4·5) were performed at 4°C and the dialysis tubing was changed every hour in order to avoid its hydrolysis by cellulases. The resulting solutions were stored at 4°C and diluted in ultrapure water just before use.

Enzymic hydrolysis and methanolysis

Materials used for these assays were as described previously (Quemener & Thibault, 1990). Dried cell wall preparation (~10 mg) was incubated with stirring in the Reacti-Vial at 47°C for 16 h with 0·5 ml of a mixture of purified Celluclast (4 mg of protein/ml, estimated by the method of Lowry *et al.*, 1951), Novozym 188 (2 mg of protein/ml), SP 249 (0·2 mg of protein/ml) and with 0·5 ml of dimethyl L-tartrate at 8 mg/ml as internal standard (Quemener & Thibault, 1990). The hydrolysate was concentrated to dryness with methanol (3 × 500 µl) at 47°C in a stream of air using the Reacti-Vap Evaporator. Methanolysis was carried out with methanolic 2 M HCl (2 ml) at 100°C for 4 h (Preuss & Thier, 1983) with stirring. After neutralization with Ag_2CO_3 (250–300 mg) and centrifugation (5000 *g* for 5 min), the supernatant solution was concentrated to dryness at 40°C, under vacuum. Controls with only the enzymes were carried out using the same conditions.

Analysis of methanolysis products by HPLC

The dry residue was dissolved in water (1 ml), centrifuged and the methyl glycosides were analysed by reverse-phase chromatography. High-performance liquid chromatography was performed on a 590 HPLC (Waters Associates, USA) equipped with Superspher end-capped C_{18} cartridge (4 × 250 mm) and C_{18} guard cartridge (LichroCART^R 4-4) (Merck, Darmstadt, Germany) at 15°C using ultrapure water as eluent at a flowrate of 0·8 ml/min. The eluate was monitored using an Erma ERC-7510 refractive index detector.

Quantification method

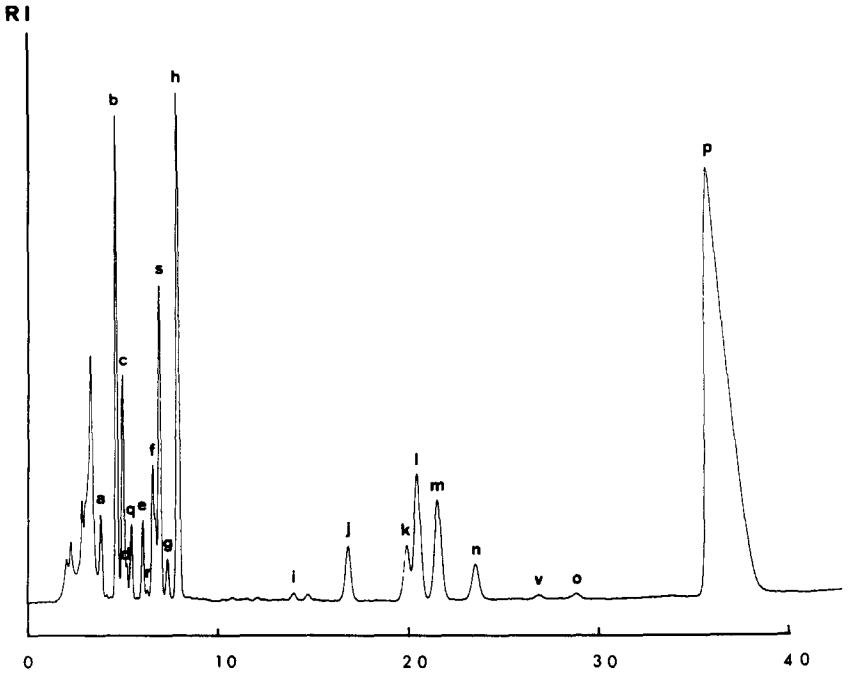
Since methanolysis of each sugar yields a mixture of glycosides, the response factors of standards relative to that of the internal standard (RRF) were calculated from the areas under the main and well-separated peaks as previously described (Quemener & Thibault, 1990), except for arabinose (peak e in place of peak f), for galacturonic acid (k + l + m) and for glucuronic acid (v), (Fig. 1 and Table 1). In the determination of glucose, problems can arise due to the presence of methyl α-D-galactofuranoside and methyl α-D-glucopyranoside in peak b. As the proportions of the areas of the peaks corresponding to the derivatives from the different standard sugars are constant in our conditions, it is possible to determine, from area of the peak a, the area corresponding to methyl α-D-galactofuranoside under peak b and hence to determine the amount of glucose. A similar problem arises for the determination of arabinose from the peak e with the overlapping peak of a derivative from mannose (RRT of 0·166, Table 1). However, these corrections are minor in both cases, as galactose and mannose contents are not very high, and in the second case the respective overlapping peaks correspond to minor isomers (Table 1).

Enzymic assays

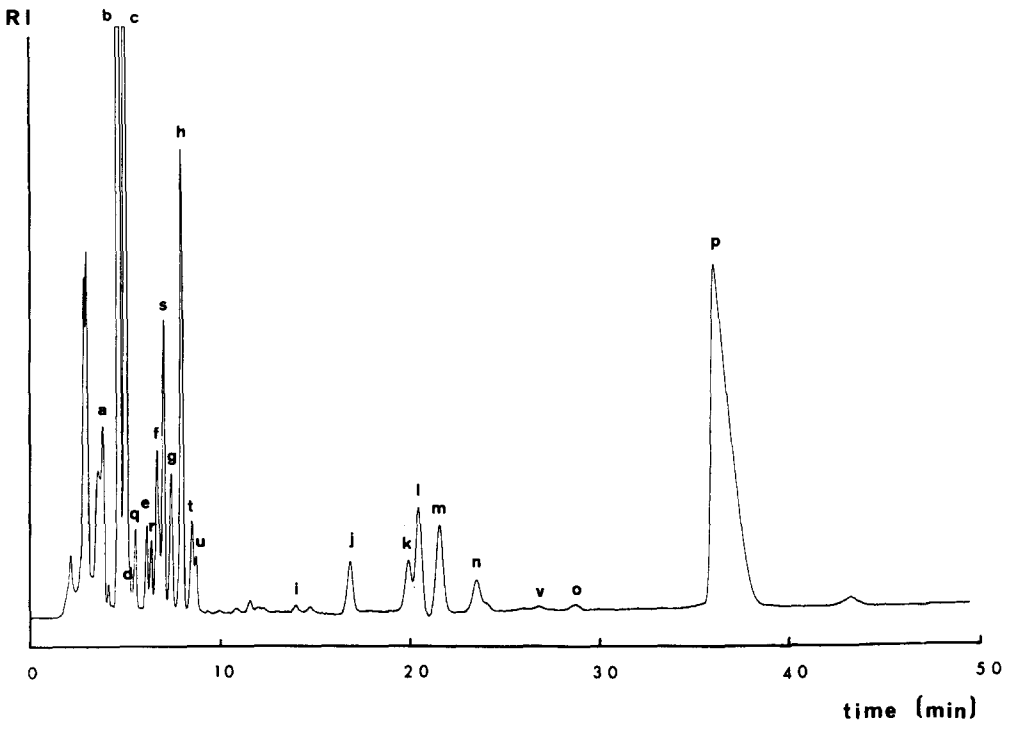
Pea hulls and cellulose CC31 (~10 mg dry wt) were stirred at 47°C ± 2°C up to 24 h in 0·5 ml of enzymic solution (Celluclast alone or in concert with Novozym 188 and/or SP249); the protein/substrate ratios were in the range 10–200% (w/w) for Celluclast, 10–20% for Novozym 188, and 10% for SP 249. Sugars released were analysed on an Aminex HPX 87P column (Biorad Labs, Richmond, California, USA) by elution with ultrapure water (0·6 ml/min, 85°C).

Carbohydrate analysis of preparations

Uronic acids were determined by the *m*-hydroxydiphenyl method (Blumenkrantz & Asboe-Hansen, 1973;



(a)



(b)

Fig. 1. HPLC of methanolysis products from purified pea hull (a) after methanolysis alone and (b) after enzymic prehydrolysis and subsequent methanolysis: a, methyl α- and β-D-galactopyranosides; b, methyl α-D-galactofuranoside and methyl α-D-glucopyranoside; c, methyl β-D-glucopyranoside; d, methyl α-L-arabinofuranoside and methyl α-D-xylofuranoside; q, methyl β-D-galactofuranoside, methyl β-D-xylofuranoside and methyl β-D-glucofuranosidurono-6,3-lactone; e, methyl β-L-arabinofuranoside and (?) methyl β-D-mannoside; r, methyl α-D-glucofuranoside; f, methyl α(?)- and β-L-arabinopyranosides and methyl β-D-glucofuranoside; s, methyl β-D-xylopyranoside; g, methyl α-D-mannopyranoside and (?) methyl α- and β-D-mannofuranosides; h, methyl α-D-xylopyranoside; t,u, methanolysis products of glucose; i,j, methyl L-rhamnosides; k,l, methyl (methyl α- and β-D-galactofuranosid)uronates; m,n, methyl (methyl α- and β-D-galactopyranosid)uronates; v, methyl (methyl α-D-glucopyranosid)uronate; o, unknown; p, dimethyl L-tartrate (internal standard).

Table 1. Relative retention times and relative response factors of main methyl glycosides to dimethyl-L-tartrate (Internal Standard)

Sugar	Relative retention time	Peak	Relative area to major peak(s)	Relative response factor (mean ± SD)	Anomer
D-Galactose	0·106	a	100·0	0·89 ± 0·03 (a)[c]	α- and β-Pyranoside[a]
	0·132	(b)[b]	9·0		α-Furanoside
	0·151	(q)	25·1		β-Furanoside
D-Glucose	0·128	(b)	100·0	0·64 ± 0·02 (b + c)	α-Pyranoside[a]
	0·137	c			β-Pyranoside[a]
	0·173	r	1·4		α-Furanoside
	0·182	(f)	0·9		β-Furanoside
	0·233	t	0·8		?
	0·238	u	1·0		?
L-Arabinose	0·144	(d)	13·0		α-Furanoside
	0·166	(e)	35·0	2·69 ± 0·03 (e)	β-Furanoside
	0·179	(f)	100·0	0·94 ± 0·02 (f)	β-Pyranoside[a]
D-Mannose	0·166	(e)	9·0		
	0·199	g	100·0	0·68 ± 0·01 (g)	α-Pyranoside[a]
	0·213	g			
D-Xylose	0·144	(d)	5·8		α-Furanoside
	0·155	(q)	5·0		β-Furanoside
	0·195	s	55·0		β-Pyranoside[a]
	0·221	h	100·0	1·22 ± 0·03 (h)	α-Pyranoside[a]
L-Rhamnose	0·289		1·3		
	0·377	i	11·0		
	0·447	j	100·0	0·76 ± 0·01 (j)	α-Pyranoside[a]
	0·643		2·4		
D-Galacturonic acid	0·132		3·4		Methyl ester of galacturonic acid
	0·140		2·9		Methyl ester of galacturonic acid
	0·541	k	100·0	0·83 ± 0·01 (k + l + m)	Methyl (methyl α- or β-D-galactofuranosid) uronates
	0·555	l			Methyl (methyl α- or β-D-galactofuranosid) uronates
	0·584	m		0·70 ± 0·02 (k + l + m + n)	Methyl (methyl α- or β-D-galactopyranosid) uronates
	0·641	n	15·0		Methyl (methyl α- or β-D-galactopyranosid) uronates
	0·786	o	2·1		Unknown
D-Glucuronic acid	0·152	(q)	100·0		Methyl β-D-glucofuranosidurono-6,3-lactone
	0·648		23·5		Methyl (methyl β-D-glucopyranosid) uronate
	0·700		6·5		Methyl (methyl α-D-glucofuranosid) uronate
	0·732	v	67·1	2·26 ± 0·09 (v)	Methyl (methyl α-D-glucopyranosid) uronate
	0·895		6·2		Methyl (methyl β-D-glucofuranosid) uronate

[a]Standards available.
[b]Overlapping peaks are in parentheses.
[c]Peak(s) on which calibration was based.

Thibault, 1979), after dispersion in 72% sulphuric acid at 25°C (Ahmed & Labavitch, 1977) for 1 h and hydrolysis in 2 N sulphuric acid at 100°C for 2 h. Galacturonic acid was used as standard.

Dry preparations were hydrolysed with 2 M trifluoroacetic acid at 120°C (Blake & Richards, 1968) for 3 h (Quemener & Thibault, 1990). Assays were also performed with combined enzymic prehydrolysis (Celluclast and Novozym 188) as described above except Internal Standard (inositol instead of dimethyl L-tartrate). The cellulosic glucose content was determined after presolubilization in 72% sulphuric acid for 2 h 30 at 25°C followed by dilution to 2 M and heating at 100°C for 2 h (Blake & Richards, 1970; Hoebler *et al.*, 1989). Reduction, acetylation of the neutral sugars (Sawardeker *et al.*, 1965) and GC were carried out according to the procedure of Blakeney *et al.* (1983) as modified by Hoebler *et al.* (1989).

Delignification treatment

Wheat bran was delignified by sodium chlorite as previously described (Thibault & Rouau, 1990).

RESULTS AND DISCUSSION

Methanolysis assays

In our previous work (Quemener & Thibault, 1990), methanolysis was performed with methanolic 1 M HCl

for 16–24 h. In order to reduce the length of analysis, we examined methanolic 2 M HCl at 100°C for 4 h (Preuss & Thier, 1983).

The stabilities of standard methyl glycosides and of dimethyl L-tartrate were shown by the constancy of the values of the relative response factors (mean coefficient of variation: 2·3%) obtained after HPLC analysis in triplicate (Table 1). These values fitted well with those previously determined in methanolic 1 M HCl at 85°C for 16 h, except for xylose whose RRF was significantly higher in methanolic 2 M HCl than in 1 M HCl (1·22 ± 0·03 and 1·03 ± 0·03 respectively) indicating that some degradation of this sugar seemed to occur under the latter conditions. When the methyl glycoside standards were not available, the identification of the peaks (Table 1) was based on literature data (Cheetham & Sirimanne, 1981; Quemener & Thibault, 1990) using retention times relative to α-methyl-D-glucopyranoside. For the α- and β-methyl-D-xylopyranosides anomers and the α-methyl-D-mannopyranoside a discrepancy was found between our results and those of Cheetham and Sirimanne (1981): β-methyl-D-xylopyranoside was eluted before the corresponding α anomer and the α-methyl-D-mannopyranoside was eluted at a relative retention time of ~0·199 instead of 0·166 as expected (Cheetham & Sirimanne, 1981).

Methanolic 2 M HCl at 100°C for 4 h released 27–33% more galacturonic acid from apple, citrus or sugar-beet pectins than methanolic 1 M HCl at 85°C for 16 h (Quemener & Thibault, 1990). The corresponding yields were again improved (10–27% according to the sample) by enzymic prehydrolysis with SP 249. The values obtained with this combined method, i.e. 59·7, 75·7 and 76·6% for sugar-beet, apple and citrus pectins respectively, fitted well with those obtained colorimetrically and the amounts previously reported (Quemener & Thibault, 1990). As the yields of neutral sugars (especially rhamnose) by methanolysis alone were also in good agreement with our previous results (Quemener & Thibault, 1990), these conditions may be used instead of those previously used for the determination of sugars in pectins (Quemener & Thibault, 1990) and were therefore examined for the methanolysis of the purified cell wall materials.

The yields of the sugars released from pea hulls, sugar-beet pulp and wheat bran are shown in Table 2. The amounts of the main neutral sugars (except glucose) were close to those determined by GC after 2 M TFA hydrolysis. In contrast, the yields of glucose were very significantly lower than those obtained by GLC after sulphuric acid hydrolysis. The content of galacturonic acid of pea hulls was slightly lower than

Table 2. Yields (% ± SD) of the sugars released from cell wall preparations and from pure cellulose by different methods

Cell wall preparations	Method	Gal A	Glc A	Rha	Ara	Xyl	Man	Gal	Glc
Pea hull	A[a]	12·1 ± 0·2	0·6 ± 0·1	1·8 ± 0·1	3·8 ± 0·3	13·6 ± 1·2	0·6 ± 0·1	1·1 ± 0·1	8·4 ± 0·1
	B[b]	11·6 ± 0·4	0·4 ± 0·1	1·8 ± 0·1	3·7 ± 0·1	13·5 ± 0·5	0·9 ± 0·1	0·8 ± 0·1	52·0 ± 1·2
	C[c]	11·6 ± 0·3	0·4 ± 0·1	1·8 ± 0·1	3·4 ± 0·1	13·0 ± 0·2	0·3 ± 0·1	1·2 ± 0·3	51·0 ± 0·4
	D[d]	13·5 ± 0·6							
	E[e]			1·9 ± 0·3	3·9 ± 0·4	13·4 ± 0·8	0·6 ± 0·1	1·2 ± 0·1	3·9 ± 0·3
	F[f]			2·0 ± 0·0	3·7 ± 0·1	14·4 ± 0·4	0·8 ± 0·2	1·3 ± 0·1	50·7 ± 2·5
	G[g]			0·9 ± 0·1	3·2 ± 0·6	10·2 ± 1·3	0·8 ± 0·1	1·3 ± 0·1	52·1 ± 1·9
Sugar-beet pulp	A	17·8 ± 0·4	0·6 ± 0·0	2·5 ± 0·0	18·7 ± 0·8	1·9 ± 0·2	1·4 ± 0·1	6·1	3·3 ± 0·6
	B	18·1 ± 1·3	0·7 ± 0·1	2·4 ± 0·1	17·7 ± 1·8	1·9 ± 0·2	1·4 ± 0·3	4·5	19·5 ± 0·8
	C	17·1 ± 0·4	0·7 ± 0·1	2·4 ± 0·0	17·7 ± 0·7	1·9 ± 0·1	1·2 ± 0·1	5·7 ± 0·3	19·6 ± 0·2
	D	17·4 ± 0·6							
	E			2·4 ± 0·4	18·5 ± 2·3	1·6 ± 0·1	1·2 ± 0·1	4·8 ± 0·3	2·1 ± 0·2
	F			2·4 ± 0·0	17·8 ± 0·0	1·7 ± 0·0	1·6 ± 0·0	4·9 ± 0·0	18·1 ± 0·4
	G			1·6 ± 0·0	18·7 ± 2·2	1·4 ± 0·3	1·6 ± 0·1	5·0 ± 0·5	20·9 ± 2·9
Wheat bran	A	0·6 ± 0·0	0·8 ± 0·1	0·2 ± 0·1	15·6 ± 0·8	30·4 ± 1·8	0·7 ± 0·1	1·3 ± 0·1	7·4 ± 0·4
	B	0·6 ± 0·1	0·8 ± 0·1	0·1 ± 0·1	14·4 ± 0·4	28·3 ± 1·4	0·5 ± 0·1	1·3 ± 0·1	10·2 ± 0·7
	D			0·1 ± 0·1	15·3 ± 2·5	25·9 ± 1·7	0·6 ± 0·1	1·3 ± 0·1	6·2 ± 0·4
	E			0·2 ± 0·1	13·9 ± 0·0	27·6 ± 0·8	0·5 ± 0·1	1·4 ± 0·1	8·5 ± 0·1
	F			0·1 ± 0·0	13·4 ± 1·1	21·7 ± 1·6	0·7 ± 0·1	1·3 ± 0·1	19·8 ± 1·6
Pure cellulose	A								8·0 ± 0·1
	B								80·1 ± 1·2
	F								95·4 ± 0·5

[a]Methanolysis for 4 h at 100°C in methanolic 2 M HCl according to Preuss and Thier (1983).
[b]Enzymic prehydrolysis with purified Celluclast (20% (w/w)) and Novozym 188 (10%) and subsequent methanolysis in the same conditions.
[c]Enzymic prehydrolysis with Celluclast (20%), Novozym 188 and SP 249 (10% of each) and subsequent methanolysis.
[d]Colorimetric method (Blumenkrantz & Asboe-Hansen, 1973; Thibault, 1979).
[e]Acid hydrolysis with TFA.
[f]Combination of TFA and enzymic prehydrolysis.
[g]Modified Saeman procedure (see text).

that estimated colorimetrically (which corresponds to the total uronic acid content, i.e. galacturonic, glucuronic and 4-*O*-methyl glucuronic acids). All these results confirm that methanolysis with 1 M or 2 M HCl quantitatively releases the neutral sugars from acidic polysaccharides such as pectic substances (Quemener & Thibault, 1990) or from reserve neutral polysaccharides such as galactomannans (Preuss & Thier, 1983) but not from structural polysaccharides such as cellulose (Roberts *et al.*, 1987). Enzymic hydrolysis was therefore explored as a preliminary step.

Enzymic depolymerization

Celluclast preparation contains CMCase, FPase and 1,3-1,4-β-D-glucan 4-glucanohydrolase activities (Massiot *et al.*, 1989; Chanzy & Henrissat, 1983). SP 249 preparation, which is rich in polygalacturonase activity (Massiot *et al.*, 1989), had a synergistic effect with Celluclast. As the combination of a cellobiohydrolase (EC 3.2.1.2.1.) such as Novozym 188 and Celluclast allows an optimum release of glucose, we have examined the degradation of cell wall material with a combination of these three enzymes. Pea hulls, which contained the highest amount of cellulose and a significant level of pectins, were used as model substrate.

These enzymes contain associated galactose (4·2 mg/ml), glucose (1·7 mg/ml) and mannose (0·2 mg/ml) as shown by the chromatography on Aminex HPX 87P of Celluclast preparation. After purification, the level of these associated sugars was less than 0·01 mg/ml. However, methanolysis of purified Celluclast and Novozym 188 revealed a contaminant (cf. peak g in Fig. 1(b)) that may be mannose derived from the enzyme (glycoprotein).

As the optimum temperature, measured by the quantification of the glucose released for 24 h from pea hulls with a Celluclast/substrate ratio of 1% (w/w), was 45–50°C, a temperature of 47°C ± 2°C was chosen for all the assays. The yields of glucose and of cellobiose released by Celluclast alone and combined with Novozym 188 or/and SP 249 are shown in Table 3. Comparison of these results with those determined by GC after sulphuric treatment (Table 2) demonstrate that Celluclast alone can quantitatively depolymerize the cellulose (in ~ 16 h but with a protein/pea hull ratio of 100% (w/w)). The time-course of hydrolysis with a combination of Novozym 188 and Celluclast shows that this maximum value was reached after ~6 h with protein/pea hull ratios of 10% and 20% for these two enzymes respectively. Similar results were obtained on Whatman cellulose: 60·9% and 72·8% of glucose was released after 16 h of incubation with Celluclast alone (protein/cellulose ratio of 200%) and Celluclast (20%) combined to Novozym 188 (10%), respectively.

The synergistic effect of SP 249 on cellulose degradation by Celluclast was low. This poor synergism may be explained by the method of purification of the cell wall materials, which probably dissociated a large part of the pectins from the cellulose, making the latter more accessible to cellulases.

Enzymic prehydrolysis and methanolysis

The yields of sugars released from the three fibre preparations by Celluclast (20%) combined to Novozym 188 (10%) for 16 h and subsequent methanolysis (methanolic 2 M HCl, 4 h, 100°C) are presented in Table 2. This combination significantly increased all the yields of glucose compared with methanolysis alone. For pea hull and sugar-beet pulp, the values obtained were very close to those determined by GC. The amount of glucose released from Whatman cellulose by this enzyme combination was tenfold higher than with methanolysis alone but was lower than that obtained by the Saeman procedure (Table 2). For wheat bran, it was only about half of that determined by GC. It was significantly improved by the delignification treatment. Taking into account the yield of material (~72%), the amounts of glucose released from delignified wheat bran by the proposed method and by

Table 3. Yields (% of the cell wall material) of glucose and of cellobiose released from pea hull by enzymic hydrolysis with different combinations of Celluclast, Novozym 188 and SP 249 at different times of reaction (see text)

Time (h)	Celluclast alone				Celluclast + SP 249 (10%)[a]		Celluclast + Novozym 188 (10%)[a]		Celluclast + Novozym 188 (20%)		Celluclast + Novozym 188 (20%) + SP 249 (10%)	
Celluclast/ Pea hull ratios (% (w/w))	20	50	100	200	20	50	20	50	20	50	10	20
3	18·3 (10·5)	28·5 (8·7)	37·3 (6·0)	35·5 (3·5)[b]			45·0	45·2	43·2	49·1	47·4	47·7
6					32·7	42·6	50·3	49·6			52·3	50·6
16	36·0 (7·7)	44·7 (2·0)	51·8 (0·5)	50·7 (0·7)	42·7 (6·1)	48·0	51·8	49·4			51·2	
24	39·3 (5·3)	47·9 (1·5)	51·0 (0·5)	52·0 (0·5)	43·3 (8·4)	47·8 (1·3)	49·9	49·7				

[a]Protein/pea hull ratios (w/w).
[b]Yields of cellobiose in parentheses.

the Saeman procedure were 13·4 and 15·5%, respectively. These results are in agreement with the well-known fact that lignin hinders the accessibility of cellulose to the enzymes. Arabinoxylans were also partially removed by the delignification treatment (~25–30%) whereas the uronic acid content was only slightly modified. Assays performed on pea hulls and sugar-beet pulp with addition of SP 249 (1%) to Celluclast (20%) and Novozym 188 (10%), gave similar values of glucose and galacturonic acid. The apparent slight discrepancy between the value of galacturonic acid in the case of pea hull and the global estimation obtained by colorimetry may be due to the fact that this method accounts for all the uronic acid and that 4-*O*-methyl glucuronic acid was impossible to quantify by the chromatographic method. However, the quantification of glucuronic acid was possible because thermostatting the column to 15°C led to the resolution of the peak (v) corresponding to that attributed to galacturonic acid (peak o, Fig. 1). The contents of the other sugars, obtained after enzymic prehydrolysis, were slightly lower than after methanolysis alone or TFA determinations. The large variations observed with galactose were due to a contaminant (Fig. 1(b)) making an accurate integration difficult. In the case of mannose, the variations were ascribed to the low amount in the pea hull sample (0·6%) and to the significant proportion of mannose coming from the enzymes used (about 4/1). The highest accuracy was generally obtained for rhamnose determination in sugar-beet pulp and in pea hull. It is interesting to note that the Saeman procedure did not release this sugar as efficiently as the proposed method (Table 2). This result is in good agreement with those previously reported (Quemener & Thibault, 1990). As the methanolysis conditions were standardized and corresponded to an equilibrium for the different isomers, it was generally possible to use isolated peaks for calibration by linear regression. However, after enzymic pretreatment, it was necessary, for an appropriate quantification, to take into account some minor overlapping peaks (Fig. 1(b) Table 1). The relative areas of isomers to major and well-separated peaks (Table 1) were very useful for these minor corrections. The determination of the neutral sugars was therefore easier and more accurate from chromatograms obtained after methanolysis alone (except for glucose).

Enzymic prehydrolysis and TFA

The yields of sugars released from the three cell wall samples by Celluclast (20%) combined with Novozym 188 (10%) and subsequent hydrolysis with TFA are presented in Table 2. As expected, the yields of glucose were improved by enzymic treatment and under these conditions (except for wheat bran) were close to those obtained by combined enzymic prehydrolysis and methanolysis or by the Saeman procedure.

CONCLUSIONS

In conclusion, evidence was presented that methanolysis with methanolic 2 M HCl at 100°C for 4 h results in efficient neutral sugar release from isolated pectins. However, for complete release of galacturonic acid an enzymic prehydrolysis with pectolytic enzymes (SP 249) is necessary. In the same way, a high degree of depolymerization of cellulose from non- or low lignified plant tissues such as pea hulls or sugar-beet pulp can only be achieved by including cellulolytic enzyme treatment (Celluclast in combination with Novozym 188). In the case of wheat bran, which is a highly lignified plant cell wall, this enzymic step was insufficient. The proposed method, which is efficient and convenient for the determination of the monosaccharide composition of pea hull and sugar-beet pulp, overcame the shortcomings of hydrolysis by sulphuric acid. Moreover, even if identification and quantification is a bit more challenging after methanolysis, the mixture of methylglycosides released leads to a more specific detection.

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REFERENCES

Ahmed, A.E.R. & Labavitch, J.M. (1977). *J. Food Biochem.*, **1**, 361.
Albersheim, P., Nevins, D.J., English, P.D. & Karr, A. (1967). *Carbohydr. Res.*, **5**, 340.
Blake, J.D. & Richards, G.N. (1968). *Carbohydr. Res.*, **8**, 275.
Blake, J.D. & Richards, G.N. (1970). *Carbohydr. Res.*, **14**, 375.
Blakeney, A.B., Harris, P.J., Henry, R.J. & Stone, B.A. (1983). *Carbohydr. Res.*, **113**, 291.
Blumenkrantz, N. & Asboe-Hansen, G. (1973). *Anal. Biochem.*, **54**, 484.
Chambers, R.E. & Clamp, J.R. (1971). *Biochem. J.*, **125**, 1009.
Chanzy, H. & Henrissat, B. (1983). *Carbohydr. Polym.*, **3**, 161.
Chaplin, M.F. (1982). *Anal. Biochem.*, **123**, 336.
Cheetham, N.W.H. & Sirimanne, P. (1981). *J. Chromatogr.*, **208**, 100.
Cheetham, N.W.H. & Sirimanne, P. (1983). *Carbohydr. Res.*, **112**, 1.
Englyst, H.N. & Cummings, J.H. (1984). *Analyst*, **109**, 937.
Englyst, H.N. & Cummings, J.H. (1988). *J. Assoc. Off. Anal. Chem.*, **71**, 808.
Englyst, H.N. & Hudson, G.J. (1987). *Food Chem.*, **24**, 63.
Englyst, H.N., Wiggins, H.S. & Cummings, J.H. (1982). *Analyst*, **107**, 307.
Ford, C.W. (1982). *J. Sci. Food Agric.*, **33**, 318.
Hjerpe, A., Antonopoulos, C.A., Classon, B., Engfeldt, B. & Nurminen, M. (1982). *J. Chromatogr.*, **235**, 221.
Hoebler, C., Barry, J.L., David, A. & Delort-Laval, J. (1989). *J. Agric. Food Chem.*, **37**, 360.

Jones, T.M. & Albersheim, P. (1972). *Plant Physiol.*, **49,** 926.
Lowry, O.H., Rosebrough, N.J., Farr, A.L. & Randall, R.J. (1951). *J. Biol. Chem.*, **193,** 265.
Massiot, P., Thibault, J.F. & Rouau, X. (1989). *J. Sci. Food Agric.*, **49,** 45.
Preuss, A. & Thier, H.P. (1983). *Z. Lebensm. Unters. Forsch.*, **176,** 5.
Pritchard, D.G. & Todd, C.W. (1977). *J. Chromatogr.*, **133,** 133.
Prosky, L., Asp, N.G., Furda, I., De Vries, J.W., Schweizer, T.F. & Harland, B.F. (1984). *J. Assoc. Off. Anal. Chem.*, **67,** 1044.
Prosky, L., Asp, N.G., Furda, I., De Vries, J.W., Schweizer, T.F. & Harland, B.F. (1985). *J. Assoc. Off. Anal. Chem.*, **68,** 677.
Prosky, L., Asp, N.G., Schweizer, T.F., De Vries, J.W. & Furda, I. (1988). *J. Assoc. Off. Anal. Chem.*, **71,** 1017.
Quemener, B. & Thibault, J.F. (1990). *Carbohydr. Res.*, **206,** 277.
Roberts, E.J., Godshall, M.A., Clarke, M.A., Tsang, W.S.C. & Parrish, F.W. (1987). *Carbohydr. Res.*, **168,** 103.
Saeman, J.F., Moore, W.E., Mitchell, R.L. & Millet, M.A. (1954). *Tappi,* **37,** 336.
Sawardeker, J.S., Sloneker, J.H. & Jeanes, A. (1965). *Anal. Chem.*, **37,** 1602.
Schweizer, T.F. & Würsch, P. (1979). *J. Sci. Food Agric.*, **30,** 613.
Selvendran, R.R. & Du Pont, M.S. (1984). In *Developments in Food Analysis Techniques,* ed. R.D. King. Elsevier Applied Science Publishers, London & New York, pp. 1–68.
Selvendran, R.R., March, J.F. & Ring, S.G. (1979). *Anal. Biochem.*, **96,** 282.
Selvendran, R.R., Verne, A.V.F.V. & Faulks, R.M. (1989). In *Modern Methods of Plant Analysis,* eds H.F. LinsKens & J.F. Jackson. Springer-Verlag, Berlin, pp. 234–59.
Theander, O. & Aman, P. (1980). *J. Sci. Food Agric.*, **31,** 31.
Theander, O. & Aman, P. (1981). In *The Analysis of Dietary Fiber in Food,* eds W.P.T. James & O. Theander. Marcel Dekker, New York, pp. 51–70.
Thibault, J.F. (1979). *Lebensm.-Wiss. U.-Technol.*, **12,** 247.
Thibault, J.F. & Rouau, X. (1990). *Carbohydr. Polym.*, **13,** 1.
Walters, J.S. & Hedges, J.I. (1988). *Anal. Chem.*, **60,** 988.