Phytochemistry, Vol. 22, No. 7, pp. 1567–1571, 1983.
Printed in Great Britain.

0031–9422/83 $3.00 + 0.00

ENZYMATIC DEGRADATION AND β-ELIMINATION OF THE PECTIC SUBSTANCES IN CHERRY FRUITS

JEAN-FRANÇOIS THIBAULT

Institut National de la Recherche Agronomique, Centre de Recherches Agro-Alimentaires, Laboratoire de Biochimie et de Technologie des Glucides, 44072 Nantes Cedex, France

(*Revised received* 26 *October* 1982)

Key Word Index—*Prunus avium*; Rosaceae; cherry fruit; pectic substances; endopolygalacturonase; β-elimination.

Abstract—Pectic substances from cherry fruits (*Prunus avium*) were studied after extraction and purification. They were subjected to β-elimination by heat treatment and depolymerized by endopolygalacturonase from *Aspergillus niger*. The degraded pectins were fractionated by gel permeation chromatography (Sephadex G-100, Bio-gel P2). The results suggest that the pectic substances largely consist of an α-D-galacturonic backbone interspersed with occasional L-rhamnosyl residues. Neutral sugars as side-chains of varying lengths appear to be concentrated along certain regions of the polymer ('hairy') in contrast to side-chain free ('smooth') parts.

INTRODUCTION

The structure of pectic substances is based on a backbone of 1,4-linked α-D-galacturonic acid (or its methyl ester) interspersed by 2-linked L-rhamnosyl residues [1–3]. Neutral sugars, as side-chains, are covalently linked to the rhamnogalacturonan via the C-3 of galacturonosyl [4–6] and/or the C-4 of the rhamnosyl residues [2, 3, 7, 8]. Side-chain sugars are predominantly galactose and arabinose forming galactan, arabinan and/or arabinogalactan polymers. Covalent bonds between galactose and galacturonic acid and between arabinose and galacturonic acid [9–11] have been found. Xylose [4, 5, 12–14] is also covalently attached to the C-3 of galacturonic acid residue and rare sugars, such as apiose [15, 16], fucose, methyl-fucose or methyl-xylose [10, 16] have been identified in some pectic substances. These structural features have been extensively studied by methylation analysis of aldobiuronic acids obtained after mild acid hydrolysis and of pseudo-aldobiuronic acids obtained from degradation by crude [4, 12] or purified enzymes [5, 7, 8, 16]. In contrast, little attention has been paid to two important structural aspects of pectic substances. It is not known how the methyl esters are distributed along the backbone and the literature is scanty on the number of different side-chains, their length and their distribution along the main chain.

In a preceding paper [17], extraction and characterization of pectic substances from cherry fruits (*Prunus avium*) were described. Four pectic fractions were obtained by extraction successively with water at room temperature, by oxalate at room temperature, by hot (100°) 0.05 M hydrochloric acid and cold (4°) 0.05 M sodium hydroxide from an alcohol-insoluble residue. This paper deals with the enzymatic and alkaline (β-elimination) degradation of these fractions.

RESULTS AND DISCUSSION

Purification

Water-soluble pectins (WSP) and alkali-soluble pectins (OHP) were purified by preparative ion-exchange chromatography on DEAE-Sepharose-CL-6B in order to eliminate the contaminating neutral polysaccharides. The recoveries, expressed in percent of original galacturonides were 90.1 and 55.8 for the WSP and OHP, respectively. Analytical ion-exchange chromatography showed that their content in neutral contaminating polysaccharides is lower then than 4%. Oxalate-soluble pectins (OXP) and acid-soluble pectins (HP) were used as extracted since their content in contaminating polysaccharides is lower than 1% [17].

The degrees of methylation of WSP and OHP were not significantly changed after purification. The same sugars were present in the four pectins, arabinose being the most important one, followed by galactose. An unknown sugar was found in the hydrolysates with the same retention time in GC as 2-deoxyglucose [17]. OXP is characterized by a low content in neutral sugars (0.335 mols/mol galacturonic acid residues) in contrast with the WSP (0.858 mols/mol galacturonic acid residues), while HP and OHP contain *ca* 0.47 mols of neutral sugar residues per mol of galacturonic acid residues. The level of rhamnose in OXP and OHP is half that of WSP and HP indicating that OXP and OHP are more typically linear macromolecules since the rhamnose residues kink the polygalacturonic backbone. O'Beirne *et al.* [18] reported also that the EDTA-soluble pectic substances from apple were lower in neutral sugar and particularly in rhamnose than the water-soluble ones. However, pectic substances isolated from cherry are richer in neutral sugars as a whole than apple pectic substances [18, 19].

β-Elimination

WSP, OXP and HP underwent β-elimination through heat treatment [1] at 80° and pH 6.8. The resultant 4,5-unsaturated galacturonic acid residues in the reaction mixture were then estimated by *A* at 235 nm. The *A* increased linearly up to 5–6 hr reaction time and after 9 hr the solutions were cooled to room temperature and the

pH was brought to 4 by 0.1 M hydrochloric acid in order to stop the reaction. Assuming that the molar extinction coefficient for the esterified 4,5-unsaturated galacturonic acid residue is 5500 M^{-1} cm^{-1} [20], it can be calculated that the percentage of α-1,4-linkages split was about 3 for the WSP and about 4 for the OXP and HP. These low values can be explained by the fact that at high temperatures and neutral pH, β-elimination and saponification are competitive reactions. β-Elimination occurs next to an esterified galacturonic acid residue and is, therefore, hindered when methylated carboxylic groups are saponified [21, 22].

The reaction mixtures were fractionated on Sephadex G-100 as shown in Fig. 1. The chromatograms obtained for WSP, OXP and HP show two quite different polysaccharide populations (Table 1). The fraction excluded from the gel is weakly acidic (2.9–11.2% of total galacturonides) and contains mainly neutral sugars (53.1–71.6% of total neutral sugars). The fraction eluted in the fractionation range of the column, is composed mainly of galacturonides (88.8–97.1% of total galacturonides). The former fraction represents, therefore, higher MW molecules with 3.1 (HP) to 11.7 (WSP) mols of neutral sugar residues per mol of galacturonic acid residues and the latter fraction is a degraded galacturonan containing only 0.1 (OXP) to 0.3 (HP) mols of neutral sugar residues per mol of galacturonic acid residues. These results suggest that neutral sugars are not regularly distributed along the pectic backbone, since they are mainly concentrated on less than 11.2% of the galacturonides, forming blocks along the galacturonan backbone. The structure of cherry pectic substances could be, therefore, idealized by the amalgamation of 'hairy' and 'smooth' regions. The fact that the 'hairy' regions are eluted at the void volume of the Sephadex G-100 column suggests the presence of long side-chains. Similar results were found for pectic substances heat-degraded by β-elimination from apple [1] and from soy sauce [5].

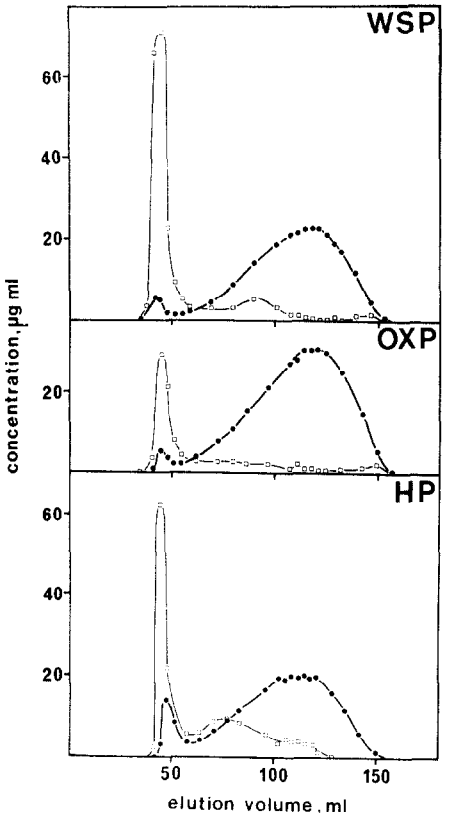

Fig. 1. Gel-permeation chromatography of heat-eliminated pectins on Sephadex G-100. Heat-eliminated pectins were applied to a Sephadex G-100 column (92 × 1.5 cm; V_t = 147 ml; V_0 = 48 ml) equilibrated with 0.1 M acetate buffer pH 4. (●) Galacturonic acids, (□) neutral sugars. WSP, Water-soluble pectins; OXP, oxalate-soluble pectins; HP, acid-soluble pectins.

Table 1. Characterization of the fractions obtained by β-elimination of pectic substances from cherry extracted with water (WSP), oxalate (OXP) and hydrochloric acid (HP)

	WSP	OXP	HP
Fraction excluded from the Sephadex G-100 column ($K_{av} \leq 0.1$)			
% of total AGA	4.5	2.9	11.2
% of total NS	71.6	55.5	53.1
Molar ratio NS–AGA	11.7:1	5.0:1	3.1:1
Included fraction ($K_{av} > 0.1$)			
% of total AGA	95.5	97.1	88.8
% of total NS	28.4	44.5	46.9
Molar ratio NS–AGA	0.2:1	0.1:1	0.3:1

AGA, Anhydrogalacturonic acid.
NS, Neutral sugar (as anhydroglucose).

Enzymic degradation

WSP, OXP, HP and OHP were submitted to the action of an endopolygalacturonase (endoPG) from *Aspergillus niger*. The appearance of reducing groups was quantified and the extent of hydrolysis of α-1,4-linkages was calculated as a function of reaction time (Fig. 2). The course of hydrolysis was fast in the first stages of the reaction (< 1 hr) and became very slow after 10 hr of reaction time. The final percentages of hydrolysis were 26.6, 19.1, 14.2 and 12.2% for the OHP, OXP, WSP and HP, respectively. This order corresponds to increasing degrees of methylation [17] and it is known that endoPG splits α-1,4-linkages between non-esterified galacturonic acid residues [23]. The extent of degradation of HP and WSP is close to that reported for commercial apple pectin with a similar degree of methylation (64.6%) but the final percentage of hydrolysis of OXP is higher than that obtained with a commercial apple pectin of a degree of methylation of 52.4% [24]. The reasons could be either a blockwise distribution of the methoxyl groups for the OXP in comparison with commercial pectin or a different distribution of side-chains along the rhamnogalacturonan. The low degradation of the non-methylated OHP can only be ascribed to the presence of side-chains which apparently hamper the action of the enzyme.

After heat inactivation of the endoPG, the reaction mixtures were dialysed against water. The dialysates were concentrated under vacuum at 35° and chromatographed

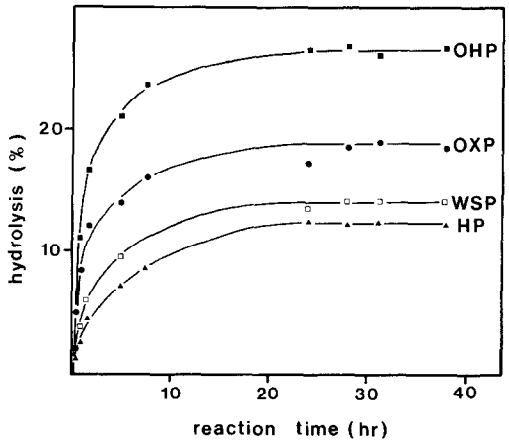

Fig. 2. Hydrolysis of pectins by endopolygalacturonase. Reaction mixtures contain pectic substances (0.2%, w/v), acetate buffer (0.05 M, pH 4.2) and enzyme (2 nkat/ml). Incubation was carrried out at 30°. Percentage of hydrolysis was measured from the increase of reducing groups. WSP, Water-soluble pectins; OXP, oxalate-soluble pectins; HP, acid-soluble pectins; OHP, alkali-soluble pectins.

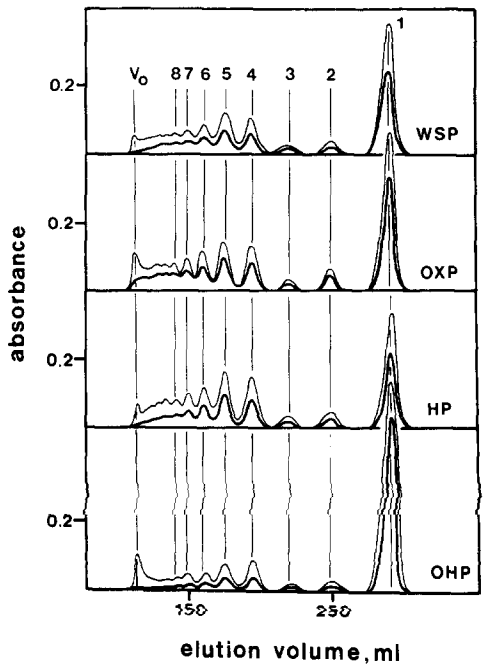

Fig. 3. Gel-permeation chromatography on Bio-gel P2 of low-MW fragments of endopolygalacturonase-degraded pectins. Degraded pectins were dialysed. The dialysates were concentrated and aliquots (2 mg) were injected on the Bio-gel P2 column (203 × 2 cm) equilibrated at 65° with 0.1 M acetate buffer, pH 3.6. The eluate was continuously analysed; (——) *A* at 520 nm (*m*-hydroxydiphenyl method); (——) *A* at 425 nm (orcinol method). V_0, Void volume; *1–8* refers to degrees of polymerization of oligogalacturonides. WSP, Water-soluble pectins; OXP, oxalate-soluble pectins; HP, acid-soluble pectins; OHP, alkali-soluble pectins.

on Bio-gel P2 while the material which cannot dialyse was chromatographed on Sephadex G-100.

Effluents from Bio-gel P2 (Fig. 3) were continuously detected for their neutral sugars and galacturonide contents (see Experimental) without correction for the interference due to acidic sugars during the determination of neutral sugars. The chromatograms show material excluded from the gel and a series of peaks 1–8 with the same elution volumes as oligogalacturonides of degree of polymerization of 1–8. For each peak, the ratio between the area obtained from the galacturonides detection and the area obtained from the orcinol detection is constant and equal to the ratio calculated under the same conditions for a galacturonic acid standard. This shows that these peaks correspond to true oligogalacturonic acids. The content in these oligogalacturonides varies from 29.3% (OXP) to 50.6% (OHP) of the total galacturonides. The materials eluted at the void volume of the column contain anhydrogalacturonic acids (1.5–6.8% of the total galacturonides) with neutral sugars (4.7–14.3%).

Higher MW fragments of endoPG-degraded pectic substances were applied to a Sephadex G-100 column and fractionated into two chemically different populations (Fig. 4). By combination of results from Bio-gel P2 and Sephadex G-100 fractionations the enzyme treated pectic substances can be separated into three classes (Table 2). The first one is an homogalacturonan which arises from 29.3% (OXP) to 50.6% (OHP) of total galacturonides; the second one, eluted at the void volume of Bio-gel P2 or in the fractionation range of the Sephadex G-100 column, contains 0.8 (WSP), 0.3 (OXP and HP) or 1.3–9.1 (OHP) mols of neutral sugar residues per mol of galacturonic acid residues. The third one, excluded from the Sephadex G-100 column, is weakly acidic (2.3–7.5% of total galacturonides) and contains mainly neutral sugars (26.2–50.6% of total neutral sugars), these values leading to the number of mols of neutral sugar residues per mol of galacturonic acid residues vary from 3.1 (OHP) to 14.6 (WSP). These

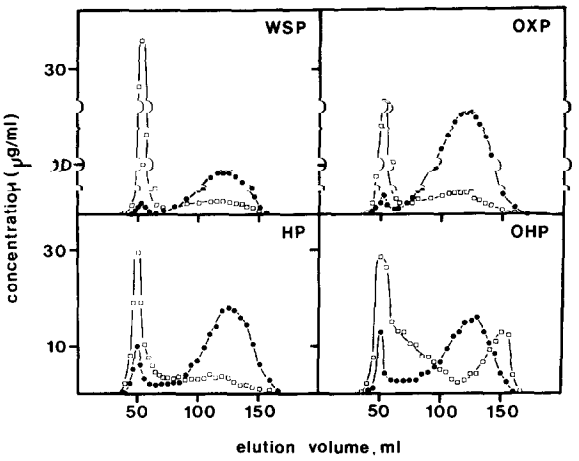

Fig. 4. Gel-permeation chromatography of endopolygalacturonase-degraded pectins after dialysis. Degraded pectins were applied to a Sephadex G-100 column (92 × 1.5 cm; V_t = 147 ml; V_0 = 48 ml) equilibrated with 0.1 M acetate buffer pH 4. (●) Galacturonic acids; (□) neutral sugars. WSP, Water-soluble pectins; OXP, oxalate-soluble pectins; HP, acid-soluble pectins: OHP, alkali-soluble pectins.

Table 2. Characterization of fragments from endopolygalacturonase-treated cherry pectic substances extracted with water (WSP), oxalate (OXP), hydrochloric acid (HP) and sodium hydroxide (OHP)

	WSP	OXP	HP	OHP
Oligogalacturonides* (DP = 1-8)				
% of total AGA	29.8	29.3	42.5	50.6
Low-MW fragments†				
% of total AGA	4.3	5.1	6.8	1.5
% of total NS	4.7	7.4	5.6	14.9
Molar ratio NS–AGA	0.9:1	0.5:1	0.3:1	9.1:1
Intermediate MW Fragments‡				
% of total AGA	63.6	63.0	45.5	40.4
% of total NS	56.1	53.7	43.8	58.9
Molar ratio NS–AGA	0.8:1	0.3:1	0.3:1	1.3:1
Highest-MW fragments§				
% of total AGA	2.3	2.6	5.2	7.5
% of total NS	39.2	38.9	50.6	26.2
Molar ratio NS–AGA	14.6:1	5.7:1	3.5:1	3.1:1

* Obtained from Bio-gel P2 chromatography.

†Obtained from Bio-gel P2 chromatography (fraction excluded from the gel, $K_{av} \leq 0.1$).

‡Obtained from Sephadex G-100 chromatography.

§Obtained from Sephadex G-100 chromatography (fraction excluded from the gel, $K_{av} \leq 0.1$).

AGA, Anhydrogalacturonic acid.

NS, Neutral sugar (as anhydroglucose).

features confirm the results obtained by β-elimination and show that neutral sugars can be found either as long side-chains or as shorter side-chains. EndoPG is not able to hydrolyse trigalacturonic acid [24]. If we assume that there are at least four contiguous galacturonic acid residues between two side-chains, it can be calculated that the long side-chains have an average degree of polymerization of 12–60 and the short side-chains of *ca* 1–4. Studies on pectic substances have not been generally carried out in order to determine whether the neutral sugars are long chains or short side-chains [9]. Nevertheless, the presence of long homo-arabans has been suggested by Talmadge *et al.* [25] in pectic substances from sycamore cell-walls while galactans with degree of polymerization of 8 [13] or 12 [26] to 14 [2] or longer [9] have been obtained. McNeil *et al.* [3] reported that the mean side-chain length of the rhamnogalacturonan I obtained by endoPG treatment of sycamore primary cell-walls was *ca* 6, while Knee *et al.* [27] observed that fragments of endoPG-treated apple cell-walls contained two distinct side-chains: relatively long ones (*ca* 38 residues) composed of arabinose and short ones (less than 6 residues) composed of galactose. Very recently, De Vries *et al.* [28] have degraded purified apple pectic substances by pectin-lyase and pectate-lyase. They show that more than 90% of the galacturonic acid residues are free of neutral side-chains. They conclude that neutral sugars are concentrated in 'hairy' regions, which exist along with 'smooth' (homogalacturonic) regions. Our results are in concordance with this model, but cherry pectins are richer in neutral sugars than apple pectins which have side-chains of a homogeneous average degree of polymerization of *ca* 15. The 'hairy' regions of cherry pectins can be divided in two types distinguishable by the length of the side-chains.

EXPERIMENTAL

Pectic substances. Four pectic fractions were extracted from cherry fruits as described previously [17] using, successively, H_2O (to give H_2O-soluble pectins, WSP), oxalate (OXP), hot 0.05 M HCl (HP) and cold 0.05 M NaOH (OHP).

Analysis. The anhydrogalacturonic acid and total neutral sugars (expressed as anhydroglucose) were determined by the *m*-hydroxydiphenyl method [29] and the orcinol method [30], respectively. Neutral sugars were corrected for interference from galacturonic acids in the orcinol method. Degrees of methylation were calculated from the OMe content [31] and the anhydrogalacturonic acid content. Individual neutral sugars were determined by GC [32]. Samples were hydrolysed with trifluoroacetic acid (2 M, 120°, 1.5 hr) [33].

Enzymatic hydrolysis. Each reaction mixture contains pectic fraction (0.2%, v/v) NaOAc buffer (0.05 M, pH 4.2) and endopolygalacturanase (endoPG) (2 nkat/ml) from *Aspergillus niger* purified as described elsewhere [24, 34]. 1 nkat produces from polygalacturonic acid 1 nmol of reducing groups (as galacturonic acid) per sec [34]. Incubation was carried out at 30° and periodically, the formation of reducing groups was measured by Nelson's method [35] using galacturonic acid as standard. Percentage of hydrolysis was calculated from the increase of reducing power.

β-Elimination. Pectic fractions (WSP, OXP, HP) (0.5%, w/v) in NaPi buffer (0.1 M, pH 6.8) were treated at 80° [1]. At appropriate intervals, 0.2 ml of the reaction mixture was diluted with 3 ml H_2O and A at 235 nm measured.

Gel chromatography. Ion-exchange chromatography on DEAE-Sepharose CL-6B (Pharmacia) was performed according to ref. [17].

The Sephadex G-100 (Pharmacia) column (92 × 1.5 cm) was eluted in ascending direction with 0.1 M NaOAc buffer pH 4 (ionic strength, 0.1) at 13 ml/hr. The total vol. (V_t = 147 ml) and the void vol. (V_0 = 48 ml) were determined with galacturonic acid and commercial apple pectin (degree of methylation = 72.9%), respectively. The K_{av} was calculated as $(V_e - V_0)/(V_t - V_0)$. Samples of degraded pectic substances in the same buffer (up to 2 mg) were injected and fractions (3.5 ml) were analysed for their galacturonic acid and neutral sugar contents by the *m*-hydroxydiphenyl and orcinol methods, respectively.

Chromatography on Bio-gel P2 (Bio-Rad) was performed according to ref. [36]. Low-MW products (*ca* 2 mg) were injected into the column (203 × 2 cm) and the eluate was analysed continuously by the *m*-hydroxydiphenyl and by the orcinol methods. An aliquot (6 ml/hr) of the effluent was introduced into each analytical line in order to obtain the curves of *A* at 520 nm (*m*-hydroxydiphenyl) and at 425 nm (orcinol) as a function of elution time. No correction was made for the interference of acidic sugars during the orcinol detection.

Acknowledgement—I wish to thank Dr. F. M. Rombouts (*Agricultural University of Wageningen, The Netherlands*) for his help in the preparation of the manuscript.

REFERENCES

1. Barrett, A. J. and Northcote, D. H. (1965) *Biochem. J.* **94**, 617.
2. Eda, S. and Kato, K. (1980) *Agric. Biol. Chem.* **44**, 2307.
3. McNeil, M., Darvill, A. G. and Albersheim, P. (1980) *Plant Physiol.* **66**, 1128.
4. Aspinall, G. O., Craig, J. W. T. and Whyte, J. L. (1968) *Carbohydr. Res.* **7**, 442.
5. Kikuchi, T. and Sugimoto, H. (1976) *Agric. Biol. Chem.* **40**, 87.
6. Ishii, S. (1981) *Phytochemistry* **20**, 2329.
7. Talmadge, K. W., Keegstra, K., Bauer, W. D. and Albersheim, P. (1973) *Plant Physiol.* **51**, 158.
8. Aspinall, G. O., Begbie, R., Hamilton, A. and Whyte, J. N. C. (1967) *J. Chem. Soc. C*, 1065.
9. Darvill, A., McNeil, M., Albersheim, P. and Delmer D. P. (1980) in *The Biochemistry of Plants* (Colbert, N. E., ed.) Vol. 1, pp. 101–116. Academic Press, New York.
10. Foglietti, M. J. and Percheron, F. (1968) *Carbohydr. Res.* **7**, 146.
11. Aspinall, G. O., Gesteiner, B., Molloy, J. A. and Uddin M. (1968) *J. Chem. Soc. C*, 2554.
12. Aspinall, G. O., Cottrell, I. W., Egan, S. V., Morrison, I. M. and Whyte, J. N. C. (1967) *J. Chem. Soc. C*, 1071.
13. Toman, R., Karacsonyi, S. and Kubackova, M. (1975) *Carbohydr. Res.* **43**, 111.
14. Bouveng, H. O. (1965) *Acta Chem. Scand.* **19**, 953.
15. Ovodov, Y. S., Ovodova, R. G., Bondarenko, O. D. and Krasikova, I. N. (1971) *Carbohydr. Res.* **18**, 311.
16. Darvill, A. G., McNeil, M. and Albersheim, P. (1978) *Plant Physiol.* **62**, 418.
17. Barbier, M. and Thibault, J. F. (1982) *Phytochemistry* **21**, 111.
18. O'Beirne, D., Van Buren, J. P. and Mattick, L. R. (1981) *J. Food Sci.* **47**, 173.
19. De Vries, J. A., Voragen, A. G. J., Rombouts, F. M. and Pilnik, W. (1981) *Carbohydr. Polymers* **1**, 117.
20. Edstroem, R. D. and Phaff, H. J. (1964) *J. Biol. Chem.* **239**, 2489.
21. Albersheim, P., Neukom, H. and Deuel, H. (1960) *Arch. Biochem. Biophys.* **90**, 46.
22. Bemiller, J. N. and Kumari, G. V. (1972) *Carbohydr. Res.* **25**, 419.
23. Rexova-Benkova, L. and Markovic, O. (1976) in *Advances in Carbohydrate Chemistry and Biochemistry* (Tipson, R. S. and Horton, D., eds.) Vol. 33, pp. 323–385. Academic Press, New York.
24. Thibault, J. F. and Mercier, C. (1978) *J. Food Biochem.* **2**, 379.
25. Talmadge, K. W., Keegstra, K., Bauer, W. D. and Albersheim, P. (1973) *Plant Physiol.* **51**, 158.
26. Aspinall, G. O. and Jiang, K. (1974) *Carbohydr. Res.* **38**, 247.
27. Knee, M., Fielding, A. M., Archer, S. A. and Laborda, F. (1975) *Phytochemistry* **14**, 2217.
28. De Vries, J. A., Rombouts, F. M., Voragen, A. G. J. and Pilnik, W. (1982) *Carbohydr. Polymers* **2**, 25.
29. Thibault, J. F. (1979) *Lebensm.-Wiss. Technol.* **12**, 247.
30. Tollier, M. T. and Robin, J. P. (1979) *Ann. Technol. Agric.* **28**, 1.
31. Wood, P. J. and Siddiqui, I. R. (1971) *Analyt. Biochem.* **39**, 418.
32. Sawardeker, J. S., Sloneker, J. H. and Jeanes, A. (1965) *Analyt. Chem.* **37**, 1602.
33. Albersheim, P., Nevins, D. J., English, P. D. and Karr, A. (1967) *Carbohydr. Res.* **5**, 340.
34. Thibault, J. F. and Mercier, C. (1977) *J. Solid-Phase Biochem.* **2**, 295.
35. Nelson, N. (1944) *J. Biol. Chem.* **153**, 375.
36. Thibault, J. F. (1980) *J. Chromatogr.* **194**, 315.