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Structural features of water-soluble pectins from mung bean hypocotyls

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Physicochemical and enzymic approaches were used to characterize highly methylated pectins isolated from mung bean hypocotyl cell walls. Young cell walls were particularly rich in rhamnogalacturonan-I-like polysaccharides, the galacturonic units of which might be fully methylated. Short, smooth, homogalacturonan blocks separated these hairy regions. The homogalacturonans included acidic and highly methylated blocks, the structures of which have been investigated through computer simulations. Such complex carbohydrates were also detected in the older parts of the hypocotyls but to a lesser extent. Mature cells were characterized by the presence of pure, sparsely methylated homogalacturonan with randomly distributed methyl groups.

INTRODUCTION

Relationships between pectin structures and cell wall mechanical properties have often been suggested since pectin molecules control both the cell wall cation exchange capacity (CEC) and the local pHs within the apoplasm (Sentenac & Grignon, 1981; Demarty *et al.*, 1984). While investigating the growth gradient exhibited by the mung bean hypocotyl, the authors previously observed that with time, highly esterified pectins, soluble in hot water, were gradually replaced by less esterified molecules exhibiting a high affinity for calcium (Goldberg *et al.*, 1986). Significant changes in the structure of the neutral polymers accompanying the pectin molecules, inferred from NMR spectra were also observed (Hervé du Penhoat *et al.*, 1987). These changes in the structure of the polysaccharide network resulted in a significant decrease in the cell wall extensibility (Goldberg *et al.*, 1989). Further investigations were then undertaken in order to fractionate and to characterize the water-soluble pectins extracted from young, plastic cell walls and those isolated from mature, stiff walls. The pectins were fractionated on anion exchange chromatography, the physicochemical behaviour of the recovered fractions and their sensitivity to an endopolygalacturonase were then checked.

MATERIALS AND METHODS

Plant material

Seeds of *Vigna radiata* (L.) Wilczek were soaked in tap water for 2 h, placed on moist vermiculite and covered with a wet cloth. After 3 days at 26°C in the dark, seedlings with hypocotyls 45 m (±5 mm) were selected. Segments were excised from two parts of the hypocotyl. The first ones (Y) corresponded to the young hypocotyl tissues located from the cotyledons to 5 mm below the hook and the second ones (M), 10 mm long, to mature hypocotyl tissues excised 15 mm under the hook.

Preparation of pectins

Cell walls were isolated from young (Y) and mature (M) tissues according to a previously described procedure (Goldberg, 1977). Water-soluble pectins were then

extracted using three successive treatments with boiling water at pH 5.

Ion exchange chromatography

The water-soluble extracts were submitted to ion exchange chromatography on DEAE Sepharose CL 6B (Pharmacia) columns (17 × 1·8 cm) equilibrated by 0·05 M Na-O-Ac buffer, pH 4·7 (Barbier & Thibault, 1982). After sample loading, the columns were washed with 200 ml of the same buffer. Bound polysaccharides were then eluted by successive treatments with 0·05 M Na-O-Ac buffer, pH 4·7, enriched in NaCl (0·2 M, 0·3 M, 0·4 M and 0·5 M NaCl). Fractions (5 ml) were collected and analysed for their galacturonic acid and total sugar contents with corrections for mutual interference. Appropriate fractions were pooled and further analysed.

Analysis

Uronic acids were estimated by the *m*-diphenyl method (Blumenkrantz & Asboe-Hansen, 1973) using polygalacturonic as the standard. Total carbohydrates were detected with anthrone (Dreywood, 1967). In order to analyse the sugar composition of the different pectic fractions recovered after ion exchange chromatography, the samples were methanolysed (24 h at 80°C), methylsililated (4°C, overnight) in 1% trimethylchlorosilane in N,O bis (trimethylsilylfluoroacetamide) and analysed by gas–liquid chromatography on a DB 225 capillary column (J.W. Instrument) as previously described (Morvan *et al.*, 1991).

Potentiometric measurements

After dialysis against distilled water, the pectic fractions were transformed into their H-form by H-exchange chromatography (Amberlite IR 120 H). Potentiometric measurements were then carried out as previously described (Goldberg *et al.*, 1986; Morvan *et al.*, 1989). Parameters such as M_{eq} (the molecular mass of a fictitious repeat unit containing one charge), DE (the degree of esterification) and NS/AS (the ratio of neutral to acidic sugars) were estimated. γCa, the calcium activity coefficient, was measured with a specific electrode (Morvan *et al.*, 1985) and ξ, the linear charge parameter, calculated according to Manning (1978).

Purification of endopolygalacturonase

An endopolygalacturonase was purified from a commercial preparation (a gift from Novo Industries, Denmark) in a three-step chromatographic procedure as previously described (Morvan *et al.*, 1990). The enzymatic solution thus obtained was freed from glucanase, galactanase, pectin methylesterase, pectate lyase and pectin lyase activities. In 0·05 M acetate buffer its pH for optimum activity was close to 5.

Enzymatic degradation of pectic fractions

Enzymatic hydrolysis was carried out under the following standardized conditions: pectin solutions (0·1% in Na-acetate 0·05 M w/v, pH 5) were incubated at 35°C (overnight) with purified polygalacturonase (1 Nkatal ml^{-1}). After neutralization, the amount of reducing sugar equivalents was estimated according to Nelson (1944) using galacturonic acid as the standard.

RESULTS

Composition of the fractions recovered after anion exchange chromatography

Cell walls isolated from young and mature tissues were extracted with boiling water at acidic pH (around pH 5·5). The water-soluble fractions (WSPY and WSPM respectively for pectins extracted from young and mature tissues) were then submitted to anion exchange chromatography. Unbound material was first recovered (fraction a). Bound polysaccharides were eluted using sequential step treatments with increasing NaCl concentrations. Two acidic fractions were separated with 0·2 M NaCl (respectively called b and c), one (d) with 0·3 M NaCl and another one (e) with 0·4 M NaCl. No further pectic material was eluted with 0·5 M and 1 M NaCl solutions. The contents of the recovered fractions are shown in Table 1. As already reported (Goldberg *et al.*, 1986), young cell walls contained more water-soluble uronic acids than mature ones. This difference resulted essentially from the occurrence in young cell walls of large amounts of weakly acid molecules which were eluted from DEAE Sepharose with 0·2 M NaCl or which did not bind to this anion exchanger (fractions b, c and a); these molecules accounted for more than 75% of the uronic acids recovered from the hot-water soluble polymers extracted from young, plastic cell walls but only for 50% of water-soluble pectins extracted from stiff, mature cell walls. In both cases, around 20% of the acids could not be eluted and remained linked to the anion exchanger even after a 1 M NaCl treatment. Neutral sugars were detected in all fractions but the ratio of neutral sugars:acidic sugars decreased noticeably during the elution process.

Physicochemical parameters of the different pectin fractions

Some physicochemical parameters are reported in Table 2. Esterification degrees were inferred from titration plots performed before and after chemical deme-

Table 1. Composition of the fractions recovered after anion exchange chromatography of the polymers extracted with boiling water from young (WSPY) and mature (WSPM) cell walls of mung bean hypocotyls[a]

	Uronic acids		Neutral sugars	
	(1)	(2)	(1)	(2)
WSPY				
a	322	41	631	62
b	157	20	194	19
c	118	15	81	8
d	149	19	91	9
e	39	5	20	2
WSPM				
a	109	24	472	61
b	61	14	126	16
c	62	14	60	8
d	131	29	91	11
e	87	19	31	4

[a]a, unbound fraction; b and c, fractions eluted with 0·2 M NaCl; d, with 0·3 M NaCl and e with 0·4 M NaCl. Uronic acids and neutral sugars expressed as μmoles g^{-1} cell walls (1) and as per cent of recovered acid or neutral sugars (2).

thylation. This parameter was around 65% for all fractions recovered from the water-soluble extracts with the exception of the fraction e recovered from mature hypocotyl tissues. Equivalent mass, representing the mass of one equivalent free charge, decreased significantly from fraction a to fraction e for both pectin samples (from young and mature tissues). As expected from the high value of DE, chemical demethylation induced a large decrease in this parameter. Moreover, it was noticed that M'_{eq}, the mass equivalent of de-esterified pectin fractions decreased markedly from fraction a to fraction e, which indicates that the ratio of neutral to acidic sugars influences significantly the elution process (Fig. 1), a high amount of neutral branches decreasing the strength of the binding of the carboxyl groups on DEAE Sepharose. When the amount of neutral branches was very high, the pectin could not bind to the carboxyl groups of the DEAE (fraction a) as already inferred from the colorimetric estimations reported in Table 1. The values of M'_{eq} obtained for d and e fractions recovered from mature tissues were respectively 200 and 210, indicating that these two fractions mainly consisted of unbranched galacturonans. Besides, it was also observed that the calcium activity coefficients of all pectin fractions except fraction e from mature cell walls, were significantly higher than those obtained with de-esterified pectins (γ'Ca) which indicates that methyl groups play an important role in the pectin selectivity for calcium ions. The lower value exhibited by the e fraction of the mature tissue extract can be related to the low DE of this fraction. After de-esterification, the activity coefficients of calcium ions never reached the theoretical

Table 2. Physicochemical parameters of water-soluble pectin fractions solubilized from young (WSPY) and mature (WSPM) cell walls

	M_{eq}[a]	DE[b]	M'_{eq}	AS[c]	γCa[d]	γ'Ca[d]	$(NS/AS)_p$[e]
WSPY							
a	1 695	67	570	30	0·50	0·22	0·2
b	1 310	68	420	42	0·48	0·22	0·2
c	1 025	68	325	55	0·52	0·24	0·3
d	675	56	300	63	0·48	0·24	0·3
e	700	62	270	71	—	—	—
WSPM							
a	1 850	73	500	34	0·50	0·21	0·1
b	1 305	70	385	45	0·50	0·20	0·1
c	850	60	340	53	0·52	0·21	0·2
d	910	78	200	100	0·52	0·21	0·2
e	315	35	210	91	0·35	0·20	0·1

Pectins solubilized with boiling water were submitted to anion exchange chromatography and fractionated from a to e, as described in Table 1.

[a]M_{eq} and M'_{eq} represent the equivalent mass before and after pectin de-esterification, i.e. the molecular mass of a fictitious unit that contains one free charge.

[b]DE is the degree of esterification calculated after titration of native and de-esterified pectins.

[c]AS, estimated from M'_{eq}, is the number of acidic sugars for 100 sugars.

[d]γCa and γ'Ca reppresent the activity coefficient of calcium before and after de-esterification.

[e]$(NS/AS)_p$ the ratio of neutral/acidic sugars in the principal chain corresponds to the ratio $(b - 4{\cdot}35)/4{\cdot}35$, b being the average distance between two charges after de-esterification ($b = 7{\cdot}15/\xi$), ξ being the linear charge density of pectins calculated from the relation $\ln \gamma Ca = -0{\cdot}5 - \ln 2.\xi$ (Goldberg *et al.*, 1986).

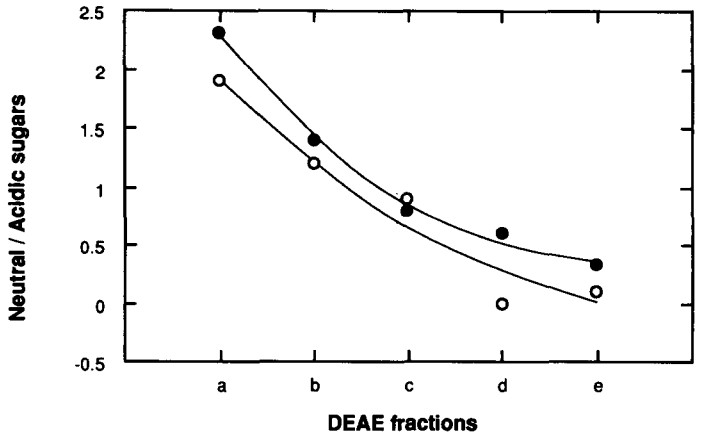

Fig. 1. The ratio of neutral to acidic sugars in successive fractions recovered from water soluble pectins after chromatography on DEAE Sepharose CL 6B. ●, pectins extracted from young cell walls; ○, pectins extracted from mature cell walls.

value (0·18) expected for polygalacturonic acid. These values (γ'Ca) were close to 0·20 which corresponded to an average distance between two free charges (b') equal to 4·7 Å, a value slightly higher than the reference value (4·35 Å) obtained for an homogalacturonan chain. These data showed that some rhamnose units were present in all fractions, randomly distributed inside the galacturonic backbone. The mean distances between two free charges (b'), calculated from γ'Ca, allowed then to estimate the proportion of neutral sugars inside the principal chain, NS/AS, which is equal to (b' − 4·35)/4·35 (Table 2). This proportion varied from 10 to 30%, slightly higher in WSPY (25%) than in WSPM (15%).

Sugar composition of the pectin fractions

The different pectin fractions recovered from the water-soluble pectins extracted from young cell walls were methanolysed and their sugar composition estimated from GLC analysis (Table 3). As previously reported (Goldberg *et al.*, 1986), arabinose, galactose and rhamnose were the main neutral monomers. The proportions of galacturonic acids and neutral sugars so obtained were slightly lower than those calculated from M'_{eq} (Table 2), this discrepancy resulting probably from incomplete splitting of the galacturonic backbone during the methanolysis. The ratio of rhamnose (thought to be in a RG I like block) to galacturonic acid is close to the ratio NS/AS calculated from γ'Ca (see above) and increased slightly from fraction a to fraction e. The amount of neutral sugars decreased with the saline concentration used to solubilize the pectins bound to the anion exchanger, which fits in with the decrease of M'_{eq} (Table 2).

Table 3. Sugar composition of pectin fractions recovered after chromatography of water-soluble pectins extracted from young cell walls

	GalU	Rha	Ara	Xyl	Man	Gal	Glu
a	33	3	11	—	1	45	2
b	38	5	10	1	—	46	1
c	55	4	4	—	—	36	1
d	58	4	4	1	—	27	2
e	65	7	7	1	—	19	1

Pectins solubilized with boiling water were fractionated by ion-exchange chromatography, dialysed, methanolysed, sylilated and analysed by GLC, as described in the material and methods section. The values represent molar ratios (%) with respect to total sugars. Ara. arabinose; Gal, galactose; GalU, galacturonic acid; Glu, glucose; Man, mannose; Rha, rhamnose; Xyl, xylose.

Hydrolysis of pectin fractions by endopolygalacturonase (endoPG)

An endoPG was purified from an enzymatic mixture purchased by Novo Industries (Morvan *et al.*, 1990) and checked first on Sigma polygalacturonic acid and citrus pectin (Fig. 2). The time course of the release of reducing sugar equivalents from these two pectic poly-

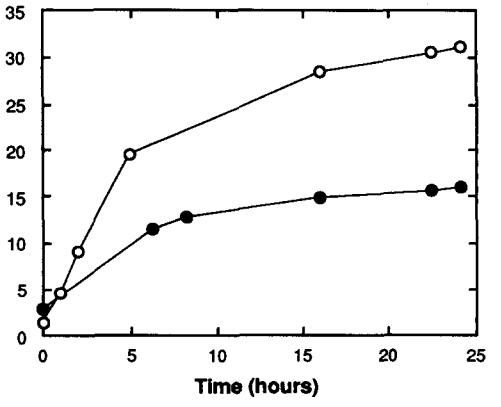

Fig. 2. Time course of hydrolysis of polygalacturonic acid (○) and citrus pectin (●) by a purified endopolygalacturonase. Hydrolysis expressed as the number of reducing sugar equivalents released from a polygalacturonan with DP equal to 100.

mers showed that the enzyme could cleave only one-third of the glycosyl linkages of polygalacturonic acid and around 15% of those present in citrus pectin, i.e. a 50–55% methylated polygalacturonic acid. These data suggest, on the one hand, that the endopolygalacturonase required at least three binding sites and, on the other hand, that it could not act on methylated galacturonic acids. Incubation of endoPG with most of the native pectin fractions induced the release of about seven reducing sugar equivalents from a polysaccharide containing 100 acidic sugar units. One exception was noticed and corresponded to the e fraction recovered from mature tissues. In this case, the percentage of hydrolysis reached 20% with native pectins which confirmed that this fraction, the most strongly bound to the anion exchange gel, contained mainly unbranched, poorly methylated, rhamnogalacturonans. When the endopolygalacturonase was incubated with de-esterified pectin fractions, 30 reducing sugars were released from 100 galacturonic acids whatever the fraction tested. This value, close to the one obtained with commercial polygalacturonic acid, reveals that most of the methyl groups were located on homogalacturonans blocks.

DISCUSSION

The data reported above reveal that anion exchange chromatography fractionated the water-soluble pectins as a function of their content of neutral sugars rather than relative to the esterification degree of their uronic acids. Lateral chains of arabinogalactan play then a major role in this fractionation, the number of rhamnose molecules intercalated in the galacturonan chains increasing only slightly from fraction a to fraction d. M_{eq}, the equivalent mass, is then the dominant charge parameter instead of ξ, the charge structural parameter of the principal chain. Although the DE is not the main parameter determining the fractionation on DEAE, its high value in all fractions except fraction e recovered from mature tissues, might be considered as a characteristic of water-soluble pectins from mung bean as already reported (Goldberg *et al.*, 1986). The distribution of the methylated galacturonic acid molecules inside the pectin still raises some questions. According to Komalavilas and Mort (1989), methylesterification is confined to galacturonan regions whereas acetylation occurs in the RG 1 regions. Moreover, according to Jarvis (1984), homogalacturonans are constituted by fully methylated blocks alternating with acidic ones. The experimental data obtained from enzymatic and physicochemical assays can be used for investigating the composition of some pectic fractions. The authors have focussed attention on specific fractions of young and mature cell walls, respectively, fraction a from young tissues and fraction e from mature ones.

Fraction a from young tissues

Fraction a accounts for more than 50% of the water-soluble pectins. GLC analysis, potentiometric measurements and colorimetric estimations showed that the ratio of neutral to acidic sugars was around 30 galacturonic acid molecules for 70 neutral sugars (three rhamnose inside the chain and 67 galactose plus arabinose in lateral branches). The high proportion of neutral sugars reveals the presence in this fraction of a rhamnogalacturonan-1-like region (RG 1, McNeil *et al.*, 1980). According to Lau *et al.* (1985), the backbone of this polysaccharide is composed of approximately equal amounts of linear 2-linked and branched 2,4-linked α-L-rhamnosyl residues, strictly interspaced with 4-linked α-D-galactosyluronic residues. RG 1 occurring in the a fraction is then constituted by repetitive blocks containing three rhamnose/ three galacturonic acids/ 67 arabinose + galactose. The remaining 27 galacturonic acids are then contained in a partially methyl-esterified homogalacturonan chain. EndoPG, shown to solubilize oligouronides with a degree of polymerization equal to 3 from polygalacturonic acid and unable to act on RG 1, released 30 reducing groups from 100 galacturonic acids when incubated with de-esterified fraction a and only six with the native one. The percentage of hydrolysis obtained with native pectins revealed that the homogalacturonan fragment contained blocks of five unesterified galacturonic acids and blocks of 22 more or less methyl-esterified ones. Three regions may then be recognized in the a fraction

(1) a small acidic region constituted by five unesterified galacturonic acids,
(2) a highly methyl-esterified block of 22 galacturonic acids,
(3) a RG 1 fragment containing three rhamnose–galacturonic acid residues, the DE of which is still unknown.

Since the mean DE of fraction a was equal to 67%, it might be deduced that 20 galacturonic acids were esterified among the 30 comprised in fraction a. These 20 molecules can be either in RG 1 or in the highly methylated block. Among the 10 unesterified acid molecules, five are located in block 1 and the others might be in blocks 2 or 3. Information can be inferred from the γCa values which will help to explain the methyl-distribution in blocks 2 and 3. The measured γCa represents an average value depending on the activity coefficients and the DE of each block. If n_1, n_2 and n_3 represent respectively the number of unesterified galacturonic acids in the three blocks and γCa_1, γCa_2, γCa_3 the activity coefficients, it can be assumed that:

$$\gamma\text{Ca measured} = n_1\gamma Ca_1 + n_2\gamma Ca_2 + n_3\gamma Ca_3/n_1 + n_2 + n_3.$$

In block 1, γCa is equal to 0·18 (activity coefficient of polygalacturonic acid), n to 5. In block 2, γCa can be calculated according to Manning (1978) who reported that: Ln γCa = ξ (charge density) = 7·15 Å/b; $b = b'/1 - DE$. In block 3, γCa is equal to 0·3 (the value estimated by Patte-Boucrel (1992) for RG 1 blocks) and $n_3 + n_2$ to 5. In order to determine the value of n_3 compatible with the measured γCa, the authors have calculated the mean γCa corresponding to n_3 equal to 0, 1, 2 and 3. As illustrated in Fig. 3, only $n_3 = 0$ fits with the estimated γCa (0·50). For other n values, the theoretical mean value of the calcium activity coefficient would be less than 0·4. These data imply that RG 1 is quite totally methyl-esterified, contrary to the hypothesis of Komalavilas and Mort (1989). This also suggests that the remaining 17 esterified galacturonic acids are all in block 2 which also contains five unesterified acid molecules (i.e. DE = 70%). Moreover, in this block, the methylesters should be randomly distributed otherwise the unmethylated acids would have been hydrolysed by the endopolygalacturonase. Physicochemical and enzymatic data allow us to suggest that the a fraction isolated from young tissues contained at least three different blocks:

- a RG 1 like polysaccharide (73% of the a fraction) with branched arabino-galactans. The backbone of this hairy region is composed of methyl-esterified galacturonic acids. A similar methylated RG 1 like polymer was described by De Vries *et al.* (1983) for apple pectic substances;
- an unmethylated homogalacturonan (around 5% of the a fraction); and
- a smooth region of highly methylated homogalacturonan (DE equal to 70%) with randomly distributed methylesters, a structure very similar to that reported by De Vries *et al.* (1983) for apple pectic substances.

However, such a scheme agrees only partly with that proposed by Jarvis (1984) who postulated the occurrence of fully methylated blocks. A computer simulation was then undertaken in order to test the different possible sequences along this methylated homogalacturonan. The construction consisted of creating a random chain of five unesterified and 16 esterified galacturonic acids. The program postulated the impossibility of two successive unesterified acid molecules along the chain, which led to 10^5 different sequences. Calculations performed with a large number of sequences (5000 to 20 000) showed that (Fig. 4): (i) no sequence including more than 12 successive methylated acids was possible; (ii) methylated blocks containing more than nine methylated acids seldom occurred (less than 5% of the possible sequences); (iii) most of the sequences (75%) contained a maximum of five to seven successive esterified acids which corresponds to a disperse distribution of the unesterified acid molecules along the chain. Models constructed on the basis of the threefold helix model assumed for both acidic and esterified pectins (Walkingshaw & Arnott, 1981; Cros *et al.*, 1992) are reported in Fig. 5. In most cases (65% of the sequences) at least one unesterified acid was located on each of the three generatrixes whereas the occurrence of all the unesterified acids on the same generatrix was a very low possibility (less than 5%). A completely esterified generatrix was found in 35% of the sequences, giving rise to a lateral asymmetric structure.

Lastly, it may be assumed that the hairy regions were separated by short homogalacturonan blocks since after de-esterification the value of γCa was equal to 0·22, whereas, according to Kohn and Luknar (1977), galacturonan with high DP (higher than 20) would have calcium activity coefficients around 0.

Fraction e from mature tissues

The e fraction was characterized by a very high level of acidic sugars (91%) and a low degree of esterification

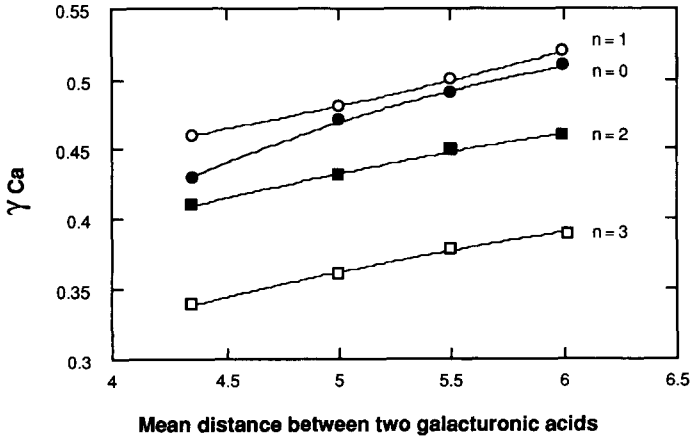

Fig. 3. Theoretical values of calcium activity coefficient (γCa) calculated for different distances between two galacturonic acids when the number of unesterified acids (n) varies from 0 to 3. Distances between two galacturonic acids were chosen close to b' (mean distance after de-esterification of fraction a), the value of which was estimated from γ'Ca, the calcium activity coefficient measured after de-esterification.

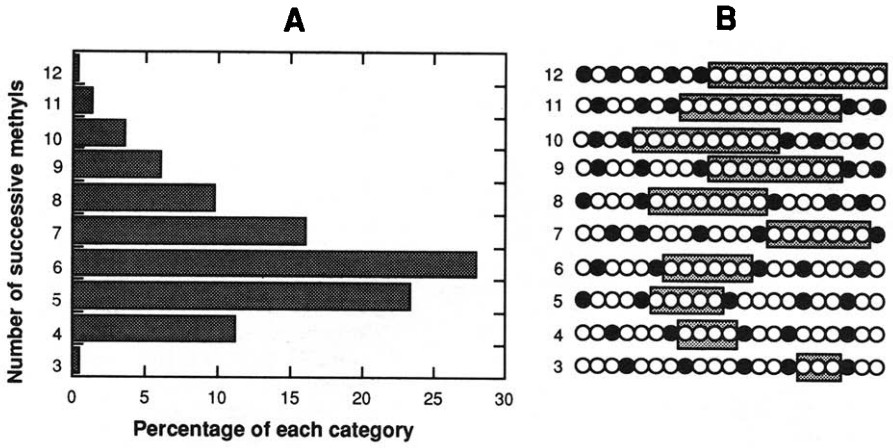

Fig. 4. Maximum possible numbers (categories) of adjacent unesterified galacturonic acids in an homogalacturonan chain constituted by 16 methylated and five unmethylated galacturonic acids. (a) Frequency histogram. (b) Schematic representation of a possible sequence for each category: methylated (○) and unmethylated (●) galacturonic acid. In each sequence, the frame indicates the longest methylated block.

(35%). In its native state it showed the highest affinity for calcium (γCa around 0·3). Using similar calculations as for the a fraction, it is concluded that fully or even partially methylated galacturonan blocks do not occur since the activity coefficient should have been close to 0·2. Moreover, the experimental value of γ'Ca fits in with this scheme. The e fraction should then be considered as a single, partially methylated block, with methylesters randomly distributed along the chain. Data obtained from enzymatic hydrolyses are compatible with two different structures: on the one hand, repetitive sequences of two methylated and six unmethylated acids; on the other one, sequences with one methylated and three unmethylated acids. The pectin-methylesterase (PME) activity present in mung bean hypocotyl cell walls (Goldberg *et al.*, 1992) might be involved in the in-muro formation of such acidic homogalacturonan. The highly methylated homo-

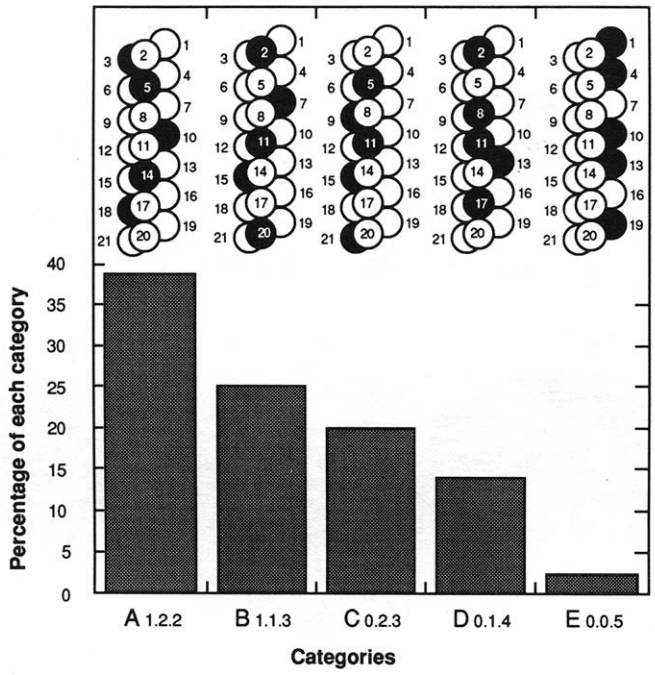

Fig. 5. Distribution of the five possible configurations of the methylated homogalacturonan according to the threefold helix model. $A_{1.2.2}$, $B_{1.1.3}$, $C_{0.2.3}$, $D_{0.1.4}$ and $E_{0.0.5}$ represent the five configurations, the three subscripts indicating the number of unmethylated acids along each of the three generatrixes. Schematic diagrams of the corresponding threefold helixes are represented in the upper part of the figure; the numbers (from 1 to 21) indicate the successive galacturonic acids (○, methylated; ●, unmethylated) along the helix.

galacturonans (DE = 70%), which represented 20% of the a fraction in young cell walls, might have been progressively de-esterified during the cell development, giving rise to the low methylated galacturonans (e) present in mature walls.

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