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Immunological probing of pectins isolated or *in situ*

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SUMMARY

Apple and sugar-beet pectins were extracted with buffers in the absence or presence of EDTA or CDTA as chelators. These pectins were highly methylated and, in the case of sugar-beet, highly acetylated. Neither the extracted pectins nor the alcohol insoluble materials from either species were recognized by a monoclonal antibody directed to calcium-induced pectin dimers ('egg-boxes'), unless chemically de-esterified. It is therefore unlikely that such dimers exist to any large extent *in vivo*.

Key-words: acetylation, apple, egg-box, methylation, pectin, sugar beet.

INTRODUCTION

Pectins are one of the main components of the primary cell walls of dicots. The degrees of methylation (DM) and acetylation (DA), i.e. the proportion (%) of galacturonic acids esterified by methyl groups (on the caboxylic acids) and acetyl groups (on the secondary alcohols) are of paramount importance in explaining their physico-chemical properties, especially their gelling behaviour. The gelation of low-methoxyl pectins (or pectates) with calcium ions has been ascribed to the formation of 'egg-boxes' (Grant *et al.* 1973; Kohn 1975; Powell *et al.* 1982) involving negatively charged blocks on two pectic chains. Nine galacturonic residues has been shown to be the minimum number necessary to obtain stable dimers (Liners *et al.* 1989). A high DA prevents this type of gelation due to steric hindrance (Rombouts & Thibault 1986).

It is commonly assumed that native pectins in the cell walls are cross-linked through calcium ions and some authors have supposed that 'egg-boxes' were present (Jarvis 1984; Selvendran 1985; Fry 1986). As a proof, it is often advocated that calcium-chelating agents have long been known to enhance the extractability of pectins.

Our aim was to study the presence of such 'egg-boxes' in typical cell walls rich in pectins. We have chosen sugar-beet cell walls because they contain pectins with a high DA (Rombouts & Thibault 1986) and apple cell walls which present more conventional pectins. The cell walls and their pectins, extracted with buffer and chelating agents, were chemically and physico-chemically characterized, especially for their calcium-binding properties. Monoclonal antibodies (Liners *et al.* 1989, 1992) against calcium–pectin systems were also used for this purpose.

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This paper is dedicated to Professor Dr M. M. A. Sassen on the occasion of his retirement.

Table 1. Yields of alcohol insoluble solids (AIS; in % of the plant material) and composition (in % dry weight of the AIS) of apple and beet cell walls

	Apple	Beet
Yield in AIS (%)	1·7	3·8
GalA	23·8	19·3
Rha + Fuc	2·9	2·0
Ara	6·7	18·9
Xyl	5·4	1·5
Man	1·4	1·1
Gal	5·9	5·8
Glc	32·2	26·3
Methanol (DM)	3·4 (80)	2·5 (72)
Acetic acid (DA)	2·1 (26)	4·4 (67)
Phenolic acids	0·0	1·5
Proteins	7·1	8·6
Ash	1·0	4·1

DM: degree of methylation; DA: degree of acetylation.

Table 2. Yields (in % of the AIS) and composition (in % dry weight of the extracted pectin) of pectins extracted from apple and beet AIS

	Apple			Beet		
	Buffer	CDTA	EDTA	Buffer	CDTA	EDTA
Yield (% AIS)	7·9	10·6	7·4	5·6	7·1	5·6
GalA	52·7	55·1	60·2	51·3	48·4	49·8
Rha + Fuc	1·5	1·5	1·6	1·3	1·3	1·5
Ara	3·5	3·5	3·6	10·1	8·2	9·6
Xyl	1·3	1·2	1·2	—	—	—
Gal	4·7	4·6	4·6	5·1	4·6	5·2
Glc	2·3	2·1	2·0	—	0·2	—
Methanol (DM)	7·5 (78)	8·1 (80)	8·3 (76)	5·8 (63)	4·6 (52)	5·1 (56)
Acetic acid (DA)	1·0 (6)	1·1 (6)	1·1 (5)	5·6 (32)	4·4 (27)	4·9 (29)

DM: degree of methylation; DA: degree of acetylation.

MATERIALS AND METHODS

The substrates

1. Pectins in apple and beet cell walls. The cell walls isolated as alcohol-insoluble solids (AIS) (Renard & Thibault 1993) were characterized (Table 1). These cell walls were de-esterified with 0·1 M NaOH in an ice-bath for 1 h.

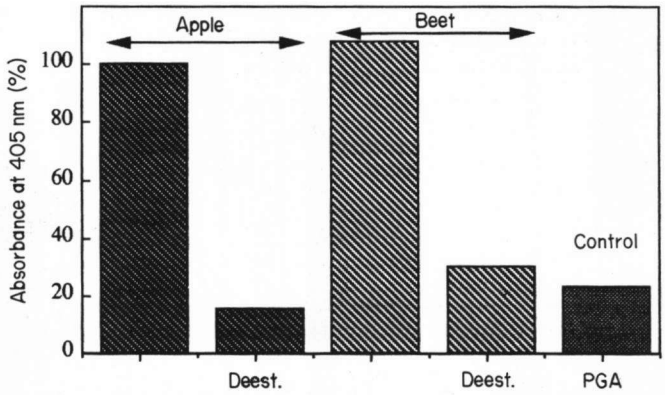

Fig. 1. Alcohol insoluble solids from apples and sugar beets were incubated with the 2F4 monoclonal antibody directed to calcium-associated dimers of pectin ('egg-boxes'). The centrifuged supernatants were then used as primary antibodies in a direct ELISA test performed with pectin-coated microwells. Only de-esterified samples bound the antibody and inhibited the response of the test.

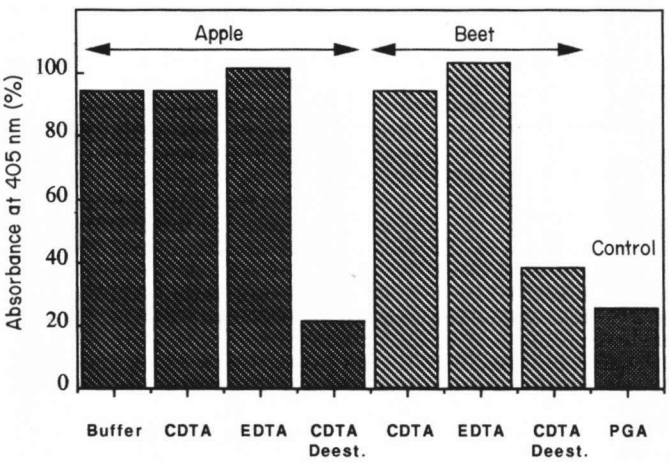

Fig. 2. Apple and sugar beet pectins extracted by different solutions were incubated with the anti- 'egg-box' antibodies directly in pectin-coated microwells. Only the de-esterified extracts were able to inhibit the binding of the antibodies to the microwells.

2. Pectins extracted from the apple and beet cell walls. Apple and beet pectins were extracted at 20–22°C by (1) acetate buffer pH 4·5, (2) the same buffer + 50 mM CDTA, Na_2 or (3) the same buffer + 100 mM EDTA, Na_2. The ionic strength of the extracting solutions were adjusted to 0·2 M (in acetate) and the concentration of CDTA and EDTA was calculated to give the same effective complexing power at pH 4·5. The conditions of pH and temperature were chosen to avoid β-elimination reactions (Renard & Thibault 1993). The characteristics of the extracted pectins are shown in Table 2. The pectins were de-esterified with 0·1 M NaOH in an ice-bath for 1 h.

ELISA tests

The ELISA experiments were performed as previously described (Liners *et al.* 1989, 1992).

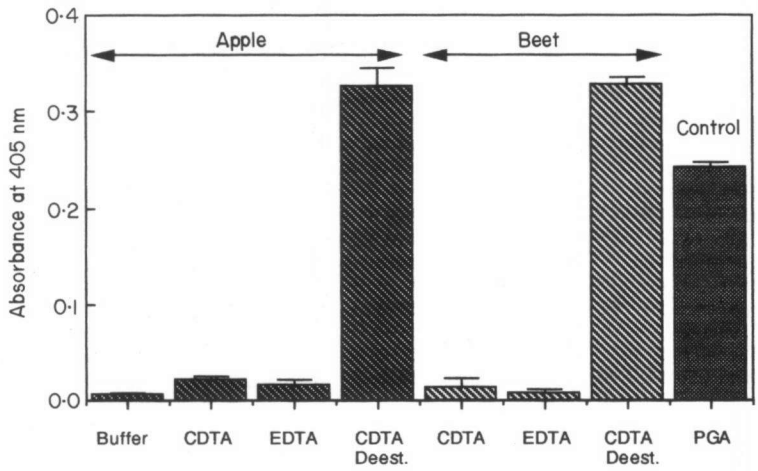

Fig. 3. Microwells of an ELISA titre plate were coated with pectins extracted from apples and sugar beets by different solutions. Only the de-esterified extracts were recognized by the anti-'egg-box' antibodies.

Table 3. Calcium activity coefficients of pectins extracted from apple or beet cell walls

		Calcium activity coefficients		
	Extraction	Experiment	Theory	Published values
Apple	Buffer	0·60	0·70	
	CDTA	0·58	0·72	0·47–0·61
	EDTA	0·52	0·68	
Beet	Buffer	0·38	0·51	
	CDTA	0·54	0·39	0·27–0·35
	EDTA	0·58	0·43	
PGA	—	0·10	0·19	0·09–0·11

The precisions of the measurements are of about 10%.

Direct ELISA. After incubation with poly-L-lysine (0·05 mg ml^{-1} in water), the microwells were coated with PGA or extracted apple and beet pectins at 0·2 mg ml^{-1} in a Ca/Na solution (0·5 mM $CaCl_2$, 150 mM NaCl) for one night at 4°C. Non-specific binding was blocked by incubation with gelatin at 0·2 mg ml^{-1} in the same ionic solution. After removal of the gelatin, 2F4 antibodies (ascite purified on protein G, 10 µg/ml^{-1} in the Ca/Na solution) were added to the wells.

The microplates were then washed with the ionic solution and incubated with peroxidase-labelled sheep anti-mouse Ig. After a second washing cycle, the binding of the antibodies was revealed by a chromogen (ABTS)-substrate (H_2O_2) solution. The absorbance was measured after 15 min at 405 nm.

Competition assays (1) Cell-wall (insoluble) samples. One volume of the 2F4 antibodies at 10 μg ml^{-1} in a Tris/Ca/Na solution (20 mM Tris, 0·5 mM $CaCl_2$, 150 mM NaCl, pH 8·2) was preincubated with the same volume of cell-wall samples (1 mg ml^{-1} in the Tris/Ca/Na solution) and left overnight at 4°C. The cell-wall samples were omitted in the blanks. The mixtures were then centrifuged and the supernatants dispensed into the microwells coated with PGA and blocked as described in the direct procedure.

(2) Soluble pectic samples. Identical volumes of the pectic samples (400 μg ml^{-1} in the Ca/Na solution) and the 2F4 antibodies (10 μg ml^{-1} in the Ca/Na solution) were added directly to the microwells coated with PGA and blocked as previously described. The soluble pectic samples were omitted in the blanks.

In all cases, incubations with secondary antibodies, washings and detection were performed as described above.

RESULTS AND DISCUSSION

Recognition of cell walls by the antibody

Figure 1 shows that the antibody does not recognize in apple or beet cell-walls any pectic sequence able to associate into dimers. After chemical de-esterification, the cell walls bind the antibody to a similar extent.

Recognition of extracted pectins by the antibody

Figures 2 and 3 show that both buffer and chelating agents extract pectins which cannot be recognized by the antibody. After chemical de-esterification, the antibody binds the samples.

Calcium-binding properties of beet and apple pectins

The calcium-binding properties of the extracted pectins were determined in dilute solutions by potentiometry (calcium-selective electrode) and give the calcium activity coefficient. The values were compared with theoretical values for purely electrostatic interactions (Manning 1969) and with previously published (Thibault & Rinando 1985; Racape *et al.* 1989) values for pectins with similar characteristics (Table 3).

The results show that the pectins extracted from beet or apple cell walls with the buffer or with chelating agents are characterized by calcium activity coefficients which are in acceptable agreement with theoretical or previously published values for pectins with similar DMs and random distributions of the free carboxyl groups. The values for PGA are very low (half that expected from the theory) and are explained by the formation of 'egg-boxes'

It can be concluded that the pectins extracted either by a buffer or by a chelating agent have similar and low calcium-binding properties and that they are not able to form 'egg-boxes' in dilute solution.

CONCLUSIONS

Significant amounts of pectins can be obtained from apple or beet cell walls by extraction with buffer either containing or not containing a chelating agent. EDTA extracts the same quantity of pectins as the buffer alone; CDTA increases slightly the quantity of pectins extracted, although EDTA and CDTA were used at the same effective complexation

power. The presence of acetyl groups in beet pectins apparently did not affect their extraction. The chelation of calcium does not appear to be a determining factor.

Whatever their mode of extraction, the pectins have very similar compositions. In particular, all the pectins are highly methylated and the beet pectins have a high degree of acetylation. The calcium-binding properties of these pectins indicated that they have a random distribution of the free carboxyl groups: they are not able to bind strongly to calcium and form 'egg-boxes' in solution.

Accordingly, the monoclonal antibody is not able to recognize pectins either in the cell-walls or after their extraction. It can react with these substrates only if they are de-esterified.

It is therefore highly unlikely that beet and apple pectins can be extensively cross-linked *in situ* by calcium ions in structures similar to the 'egg-box'. Similar conclusions have been drawn from an immunocytochemical study of suspension-cultured carrot cells (Liners & Van Cutsem 1992). However, our results do not rule out the possibility of isolated calcium bridges between pectic chains.

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