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# Effect of enzymatic deacetylation on gelation of sugar beet pectin in the presence of calcium

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**Abstract**

This study deals with the effect of methyl esters, acetyl groups, and neutral sugar side-chains on the gelation properties of sugar beet pectin with $Ca^{2+}$. Sugar beet pectin was treated in the presence of $Ca^{2+}$ with the enzymes pectin methyl esterase (PE), pectin acetyl esterase (PAE), rhamnogalacturonan acetyl esterase (RGAE), arabinofuranosidase B (AF) and rhamnogalacturonase (RGase) in various combinations.

Addition of RGAE plus PE or PAE plus PE to the pectin–$Ca^{2+}$ mixture significantly increased the release of acetyl groups and methyl esters, in comparison to the addition of only PE or PAE. This indicates that PE activity is hindered by the presence of acetyl groups both in the 'smooth' and in the 'hairy' regions. Also the PAE activity is hindered by the presence of methyl groups in the 'smooth' regions. Treatment with PAE plus PE led to a stiffer gel, as determined by the storage modulus ($G'$), than treatment with PE alone, while RGAE plus PE did not improve the gel forming properties. Addition of only PAE to the pectin–$Ca^{2+}$ mixture did not result in gel formation. A lower stiffness of the gel was found when RGase combined with RGAE and PE were added to the pectin–$Ca^{2+}$ mixture, in comparison to treatment with PE alone. Addition of AF plus PE to the pectin–$Ca^{2+}$ mixture gave similar rheological effects as treatment with only PE.

A fraction representing the 'smooth' homogalacturonan regions, which was obtained after treatment of the beet pectin with RGase and subsequent size-exclusion chromatography, was also able to form a gel with $Ca^{2+}$ and PE. However, the gel formation was much slower, and the stiffness of the gel was lower than when the parental extract was used. Also with the modified pectin the treatment with PAE plus PE gave an increased stiffness of the gel in comparison to PE alone. 

*Keywords*: Sugar beet pulp; Pectins; Pectin methyl esterase; Acetyl esterase; Gelation

## 1. Introduction

Sugar beet pectins are characterized by a high acetyl content, a high arabinose content and by the presence of feruloyl groups attached to the galactose and arabinose side-chains (Colquhoun, Ralet, Thibault, Faulds & Williamson, 1994; Guillon & Thibault, 1988, 1989a,b; Ralet, Thibault, Faulds & Williamson, 1994). The acetyl groups can be located on both the C-2 and C-3 position of the galacturonic acid residues (Keenan, Belton, Matthew & Howson, 1985). It was shown that approximately 75% of the acetyl groups in an acid extracted beet pectin are located in the 'smooth' homogalacturonan regions (Rombouts & Thibault, 1986). The high acetyl content, together with a high arabinose content and a relatively low molecular weight, limit the use of sugar beet pectin in the traditional applications of pectin: gel formation with acid/sugar or $Ca^{2+}$ ions (Pippen, McCready & Owens, 1950).

Several attempts have been made to improve gel formation of beet pectins with calcium by the removal of acetyl groups using acetyl esterases. Pippen et al. (1950) reported that deacetylation of an artificially acetylated citrus pectin with a citrus acetyl esterase was unsuccessful, and suggested that the presence of the methyl groups was the main reason. Another study showed that treatment of sugar beet pectin with a partial purified pectin acetyl esterase (PAE), which also had pectin methyl esterase (PE) activity, led to an improved gel formation with $Ca^{2+}$ (Faulds & Williamson, 1990). Matthew, Howson, Keenan and Bolton (1990) reported an improvement of the gelling properties of sugar beet pectin with $Ca^{2+}$ following treatment with an enzyme preparation derived from *Aspergillus niger*. This treatment resulted in deacetylation, demethylation and in a large reduction of the arabinose content. However, the use of partially purified and poorly defined enzymes or artificially acetylated substrates in these studies make it difficult to draw conclusions.

Recently, two types of acetyl esterases active on beet pectin were purified and characterized in our laboratory. The enzyme PAE specifically removes part of the acetyl

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groups from the 'smooth' homogalacturonan regions of (sugar beet) pectins (Searle-van Leeuwen, Vincken, Schipper, Voragen & Beldman, 1996). The enzyme rhamnogalacturonan acetyl esterase (RGAE) specifically removes part of the acetyl groups present in the 'hairy' regions of pectin (Searle-van Leeuwen, van den Broek, Schols, Beldman & Voragen, 1992). The current study deals with the effect of methyl esters, acetyl groups, and neutral sugar side-chains on the gelation properties of acid extracted sugar beet pectin. For this purpose sugar beet pectin was treated in the presence of $Ca^{2+}$ with the enzymes PE, PAE, RGAE, arabinofuranosidase B (AF) (Rombouts, Voragen, Searle-van Leeuwen, Gereads, Schols & Pilnik, 1988) and rhamnogalacturonase (RGase) (Schols, Gereads, Searle-van Leeuwen, Kormelink & Voragen, 1990) in various combinations.

## 2. Experimental

### 2.1. Materials

The acid extracted pectin obtained from sugar beet pulp (ABP) was a gift from Copenhagen Pectin Factory Ltd.

### 2.2. Enzymes

A RGase from *Aspergillus aculeatus* cloned in *Aspergillus oryzae* (Kofod et al., 1994) was used for the large scale treatment of ABP, whereas a RGase purified from a technical preparation of *Aspergillus aculeatus* was used for rheological experiments (Schols et al., 1990). RGAE was purified from *Aspergillus aculeatus* (Searle-van Leeuwen et al., 1992), while PE (Baron, Rombouts, Drilleau & Pilnik, 1980), PAE (Searle-van Leeuwen et al., 1996), and AF (Rombouts et al., 1988) were isolated from *Aspergillus niger*.

### 2.3. Analytical methods

The uronic acid content of the extract was determined by the automated *m*-hydroxy biphenyl assay (Thibault, 1979). The neutral sugar composition was determined after hydrolysis with 2 M trifluoroacetic acid (1 h, 121°C) as described previously (Oosterveld, Beldman, Schols & Voragen, 1996). Feruloyl groups were determined spectrophotometrically at 375 nm in freshly prepared pectin solutions adjusted to pH 10 with a 0.1 M NaOH solution. A molar extinction coefficient of 31,600 was used (Rombouts & Thibault, 1986). Methylation analysis was performed as described previously (Oosterveld et al., 1996), without reduction of galacturonic acid residues to galactose.

### 2.4. Enzyme treatments

ABP was treated with RGase (Schols et al., 1990) in order to degrade the 'hairy' regions. Pectin (0.5%, w/v) was dissolved in 0.04 M sodium acetate buffer pH 5.0. The enzyme was added to obtain a final concentration of 1 μg protein per ml. The sample was incubated for 20 h at 30°C. The reaction was then stopped by heating at 100°C for 5 min. The digest was analyzed by high-performance size-exclusion chromatography (HPSEC).

### 2.5. Gel formation in the presence of $Ca^{2+}$ and enzymes

The acid extracted beet pectin, the pools obtained after treatment with RGase and subsequent separation on Sephacryl S 300, were treated with the following (combinations of) enzymes to investigate their effect on the gel formation with $Ca^{2+}$: PE, PAE, PE plus PAE, PE plus RGAE, PE plus RGase plus RGAE, PE plus AF. The pectins (1%, w/v) were dissolved in 3 ml 0.1 M bis–tris buffer pH 6.0 and 120 μl of 1 M $CaCl_2$ solution in water was added. The enzyme combinations were added to obtain a final concentration of 1 μg of protein per ml and incubated at 25°C for 20 h.

The formation of the gel networks in the preparations was investigated by small amplitude shear strain oscillatory testing. A Bohlin VOR rheometer in oscillatory mode was used to monitor the gel structure development as follows. Immediately after addition of the enzyme(s), the samples were placed in the geometry. A thin layer of mineral oil was added to cover the bob and prevent evaporative losses throughout the measurements. All measurements were carried out at 25°C at a frequency of 1 Hz using a torsion bar of 20 g cm. Ensured was that the strain remained within the linear region of the material at all times.

### 2.6. Concentration of acetyl groups and methyl esters

The enzyme treatments of ABP and the pools obtained after treatment with RGase were repeated as described above. The digests were analyzed for their acetyl and methyl content by HPLC as described (Voragen, Schols & Pilnik, 1986).

### 2.7. Chromatography

High-performance size-exclusion chromatography was performed on three Bio-Gel TSK columns in series (60XL–40XL–30XL) and analyzed with a combined RI detector and viscometer (Viscotek, model 250), a Right Angle Laser Light-Scattering detector (RALLS, Viscotek, LD 600) and a UV detector (Kratos, Spectroflow 773) as described before (Oosterveld, Beldman, Schols & Voragen, 2000). Preparative size-exclusion chromatography of the extract after degradation with RGase was performed on a column (75 × 2.6 cm) of Sephacryl S 300 (Pharmacia) using a Hiload system (Pharmacia). Samples (0.05 g) were eluted with 0.05 M NaOAc buffer pH 5.0 at a flow rate of 2.5 ml/min. The procedure was repeated seven times. Fractions (2.5 ml) were assayed for total neutral sugar (Tollier & Robin, 1979) and uronic acid (Thibault, 1979) content using arabinose and galacturonic acid as standards. The presence of ferulic acid was monitored spectrophotometrically at 335 nm (Ralet et

Table 1
Composition of ABP and fractions thereof obtained by DEAE anion-exchange chromatography, and by size-exclusion chromatography after treatment with the enzyme RGase

| | ABP | DEAE | | SEC (S 300) Rgase | |
|---|---|---|---|---|---|
| | | Pool A | Pool B | Pool I | Pool II |
| Rha[a] | 5.4 | 2.1 | 9.1 | 2.4 | 20.2 |
| Ara | 7.1 | 4 | 11.8 | 5.4 | 15.4 |
| Xyl | 1.2 | 0.5 | 1 | 0.7 | 0.2 |
| Man | 2.0 | 2.8 | 1.9 | 2.6 | 0.9 |
| Gal | 11.2 | 5.5 | 21.4 | 8.5 | 30.1 |
| Glc | 0.4 | 0.9 | 3.6 | 0.5 | 0.6 |
| UA | 72.9 | 84.3 | 51.1 | 79.9 | 32.7 |
| DA[b] | 15.3 | 18.2 | 20.1 | 17.1 | 26.7 |
| DM[c] | 57.7 | 71.4 | 48.8 | 54.3 | 25.8 |
| DF[d] | 1.2 | 0.8 | 1.9 | 1.4 | 2.1 |
| Relative weight[e] | – | 74 | 26 | 76 | 24 |
| Mw[f] | 271 | Nd | Nd | 97 | 14 |
| [n][g] | 4.5 | Nd | Nd | 2.2 | 0.1 |

[a] Sugar composition (mol%).
[b] Degree of acetylation (moles of acetyl groups/100 moles anhydrogalacturonic acid residues).
[c] Degree of methylation (moles of methyl groups/100 moles anhydrogalacturonic acid residues).
[d] Degree of feruloylation (moles of feruloyl groups/100 moles arabinose + galactose residues).
[e] Expressed as weight percentage of the polysaccharides recovered.
[f] Apparent molecular weight (kDa).
[g] Intrinsic viscosity (dl/g).

al., 1994). Corresponding fractions were pooled, dialyzed and freeze-dried.

Preparative anion-exchange chromatography was performed on a column (54 × 2.6 cm) of DEAE Sepharose Fast Flow (Pharmacia) using a Hiload System (Pharmacia) as described previously (Oosterveld et al., 2000). Fractions (20 ml) were assayed for total neutral sugar (Tollier & Robin, 1979) and uronic acid (Thibault, 1979) content. Pooled fractions were dialyzed and freeze-dried.

## 3. Results and discussion

### 3.1. Composition of the acid extracted pectin

The acid extracted sugar beet pectin consisted for 72.9 mol% of galacturonic acid (Table 1). Galactose was the main neutral sugar, besides arabinose and rhamnose. This composition was in agreement with several other acid extracted sugar beet pectins reported in literature (Phatak, Chang & Brown, 1988; Thibault & Rombouts, 1986; Thibault, 1988; Thibault, Renard, Axelos, Roger & Crepeau, 1993). The high galacturonic acid content shows that it predominantly consists of 'smooth' homogalacturonan regions. The degree of methylation was 57.7 and the degree of acetylation was 15.3. These values are slightly lower than those found by Guillon and Thibault for acid extracted sugar beet pectin (Guillon & Thibault, 1990).

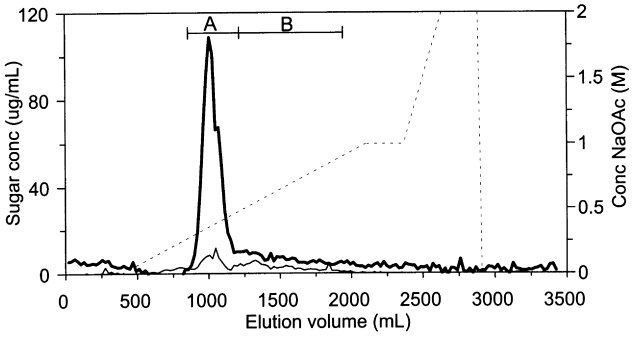


Fig. 1. DEAE anion-exchange chromatography of ABP. Thick line: uronic acid, thin line: neutral sugars, dotted line: NaOAc. A and B: pooled fractions.

The apparent molecular weight of the sample as determined by light-scattering detection (271 kDa) was rather high in comparison to values in the literature as determined by viscometry and universal calibration (46 and 141 kDa, respectively) (Fishman, Gillespie, Sondey & El-Atawy, 1991; Guillon & Thibault, 1990), but was in good agreement with light-scattering results obtained previously for a different acid extracted beet pectin (247 kDa) (Oosterveld, Beldman & Voragen, 2000a). A possible explanation is the fact that light-scattering detection might reveal small amounts of high molecular weight populations (or aggregates), which contribute (much) more than proportional to the average molecular weight and are not easily detected by other methods, as has been shown by Berth et al. for citrus pectin (Berth, Dautzenberg & Rother, 1994). The intrinsic viscosity (4.6 dl/g) was high as compared with literature values (1.1–2.5 dl/g) (Arslan, 1995; Guillon & Thibault, 1990; Fishman et al., 1991).

### 3.2. DEAE chromatography

To determine the homogeneity of the ABP with respect to charge, the sample was separated using DEAE anion-exchange chromatography (Fig. 1). Most of the material eluted in a major peak at 0.35 M NaOAc (pool A), and consisted for 84 mol% of galacturonic acid (Table 1). Its degree of methylation (DM) was 71.4 and the degree of acetylation (DA) was 18.2. The degree of feruloylation (DF) was relatively low (0.8). These findings show that this pool predominantly consists of homogalacturonans. Furthermore, a tailing population (pool B) eluted between 0.45 and 0.85 M NaOAc. This pool contained a relatively high amount of neutral sugars (40.9 mol%), indicating that it contained relatively more 'hairy' regions than pool A. Since pool B eluted later from the anion-exchange column than pool A, it was concluded that it had a higher charge or charge density than the 'smooth' regions.

This was confirmed by the lower DM of this pool. Both pool A and B had a higher DA than the original extract. This is possibly caused by a loss of material with a low ester content on the anion-exchange column, as the total recovery

Table 2
Neutral sugar linkage composition of ABP and ABP pool B (mol%)

| | | ABP | ABP pool B |
|---|---|---|---|
| Rhamnose | T-Rha *p*[a] | 1.2 | 1.2 |
| | 1,2-Rha *p*[a] | 10 | 11 |
| | 1,2,4-Rha *p* | 8 | 10.5 |
| | | 19.2 | 22.7 |
| Arabinose | T-Ara *f* | 18.2 | 13.8 |
| | 1,3-Ara *f* | 1.6 | 0 |
| | 1,5-Ara *f* | 9.6 | 10 |
| | 1,2,5-Ara *f* | 1.7 | 1.9 |
| | 1,3,5-Ara *f* | 4.1 | 4.5 |
| | 1,2,3,5-Ara *f* | 2.7 | 0.4 |
| | | 37.9 | 30.6 |
| Xylose | T-Xyl *p* | 1.2 | 0 |
| | 1,2-Xyl *p* | 0 | 0 |
| | 1,4-Xyl *p* | 0.6 | 1.4 |
| | 1,2,4-Xyl *p* | 0.4 | 0 |
| | 1,3,4-Xyl *p* | 0.3 | 0 |
| | | 2.5 | 1.4 |
| Galactose | T-Gal *p* | 16.1 | 11.1 |
| | 1,4-Gal *p* | 12.9 | 11.9 |
| | 1,6-Gal *p* | 8.4 | 8 |
| | 1,3,6-Gal *p* | 0.2 | 0 |
| | 1,4,6-Gal *p* | 0.1 | 4.6 |
| | | 37.7 | 35.5 |
| Glucose | 1,4-Glc *p* | 0.7 | 3.4 |
| | 1,4,6-Glc *p* | 1.8 | 4.6 |
| | | 2.5 | 8 |
| Mannose | 1,4-Man *p* | 0 | 1.7 |
| | | 0 | 1.7 |

[a] T, terminal; 1,2-linked Rha, etc.

of polysaccharides after anion-exchange chromatography was approximately 85%. The DF in pool B was higher than in pool A.

### 3.3. Methylation analysis

The nature of the glycosidic linkages of the neutral sugar residues in the acid extracted beet pectin and in the DEAE pool B was determined by methylation analysis (Table 2) in order to obtain information about the presence and degree of branching of the side-chains of the 'hairy' regions. Rhamnose was found in these samples in almost equal amounts of (1 → 2)- and (1 → 2,4)-linked residues, as was also observed for sugar beet pectins obtained by autoclaving (Oosterveld et al., 1996, 2000). Terminally linked rhamnose was present in small amounts. Arabinose was mainly terminally and (1 → 5)-linked. (1 → 3,5)- and (1 → 2,3,5)-linked residues were found in relatively small amounts as compared with pectins obtained by autoclaving (Oosterveld et al., 1996, 2000). This suggests that on average the arabinan side branches are relatively short and more linear, which is probably caused by the acid extraction conditions. High amounts of terminally linked galactose were found in both

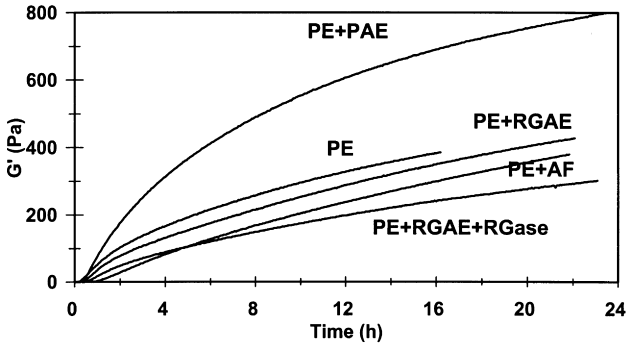


Fig. 2. Development in time of the storage modulus ($G'$) of ABP + $Ca^{2+}$ during treatment with several enzyme combinations.

extracts, as well as (1 → 4)- and (1 → 6)-linked residues. The high amount of terminally linked galactose is in agreement with the presence of rhamnogalacturonans with single galactose residues attached to it, as described by Schols, Voragen and Colquhoun (1994) for apple modified 'hairy' regions (MHR). A difference in the neutral sugar linkage composition between ABP and ABP pool B was the relatively high amount of glucose in the latter, which had mainly (1 → 4)- and (1 → 4,6)-linkages. Probably this glucose originates either from glucans or xyloglucans. The (1 → 4,6)-linked glucose, which is clearly present, can originate either from xyloglucan or from amylopectin in starch. Since starch has not been found in sugar beet pulp we assume that it originates from xyloglucan. However, no (1 → 2)- and terminally linked xylose (typical for xyloglucan) was found in the sugar linkage analysis. This might be due to the fact that their presence is so small that they could not be detected. It can be speculated that the presence of xyloglucans in a population which binds to the DEAE column might indicate that xyloglucans and rhamnogalacturonans are linked together. So, further investigation is needed to confirm this observation. Part of the (1 → 4)-linked glucose probably originates from (1 → 4)-linked glucans, since it is known that in xyloglucans the ratio (1 → 4)-linked glucose: (1 → 4,6)-linked glucose is 1:3 (Oosterveld et al., 2000), whereas we found a much higher ratio (0.74). The (1 → 4)-linked xylose in pool B probably originates from xylans.

### 3.4. Effect of enzymatic modification on gel formation of ABP with $Ca^{2+}$

The acid extracted beet pectin was treated with the following (combinations of) enzymes to investigate their effect on the gel formation with $Ca^{2+}$: PE, PAE, PE + PAE, PE + RGAE, PE + RGase + RGAE, PE + AF. $Ca^{2+}$ was necessary to obtain a gel with pectin upon treatment with PE. Previously it was shown that $Ca^{2+}$ is also necessary for optimal activity and stability of PAE (Searle-van Leeuwen et al., 1996). The formation of the gel networks was investigated by small amplitude shear strain

Table 3
Storage modulus ($G'$), loss modulus ($G''$) and tan $\delta$ of gels formed with ABP or ABP pool I in combination with $Ca^{2+}$ and various enzyme combinations (16 h) (–: no gel formed)

| | | $G'$ (Pa) | $G''$ (Pa) | tan $\delta$ |
|---|---|---|---|---|
| ABP | $Ca^{2+}$ | – | – | – |
| | PE | – | – | – |
| | PAE + $Ca^{2+}$ | – | – | – |
| | PE + $Ca^{2+}$ | 383 | 21.9 | 0.057 |
| | PE + PAE + $Ca^{2+}$ | 690 | 32.6 | 0.047 |
| | PE + RAGE + $Ca^{2+}$ | 350 | 18.8 | 0.054 |
| | PE + RGase + RAGE + $Ca^{2+}$ | 240 | 14.3 | 0.060 |
| | PE + AF + $Ca^{2+}$ | 306 | 16.6 | 0.054 |
| ABP pool I | PE + $Ca^{2+}$ | 131 | 8.1 | 0.062 |
| | PE + PAE + $Ca^{2+}$ | 86 | 4.6 | 0.053 |

oscillatory testing. Fig. 2 shows the development of the storage modulus ($G'$) in time, which is indicative for the amount of elastically effective cross-links formed, as well as for the stiffness of the gel. Table 3 summarizes the rheological parameters of the networks formed after enzyme treatment for 16 h and Table 4 shows the percentage of acetyl groups and methyl esters released after 20 h.

Both the presence of $Ca^{2+}$ and PE proved to be necessary to obtain a gel from the acid extracted sugar beet pectin. The storage modulus of the gel thus formed leveled of within 16 h. The value of $G'$ at this point was 383 Pa. Approximately 15% of the methyl groups were released in this period. This combination was used as a reference sample for the other combinations.

Treatment of the pectin–$Ca^{2+}$ mixture with PAE alone did not lead to gel formation. Approximately 8.5% of the acetyl groups initially present in ABP was released by this treatment. This acetyl release was much lower than found by Searle-van Leeuwen et al. (1996), who measured an acetyl release of 30% with PAE. The lower acetyl release in the current study was probably due to the use of another buffer system.

The addition of PE + RGAE to the pectin–$Ca^{2+}$ mixture resulted in an increased hydrolysis of acetyl groups as well as methyl esters by approximately 14% in comparison with PE alone. This indicates that the acetyl groups hindered the action of PE, as was already suggested by Pippen et al. (1950). Since RGAE is only active on the 'hairy' regions and the activity of PE increased when RGAE was added, it can be concluded that PE is able to remove methyl groups from these 'hairy' regions. The gel formation curve, however, was similar to the curve without RGAE. Another conclusion from this experiment is that the addition of RGAE did not result in a higher amount of $Ca^{2+}$ cross-links between pectin molecules and that the acetyl groups in the 'hairy' regions do not interfere with the gel formation with $Ca^{2+}$.

Table 4
Percentage of acetyl groups and methyl esters released after 20 h by PE, PAE, and RGAE, alone and in combination with each other and RGase and AF, during treatment of ABP or ABP pool I in combination with $Ca^{2+}$

| | | 20 h | |
|---|---|---|---|
| | | % Ac | % Me |
| ABP | PE | 0 | 10.7 |
| | PAE + $Ca^{2+}$ | 8.5 | 0 |
| | PE + $Ca^{2+}$ | 0 | 14.8 |
| | PE + PAE + $Ca^{2+}$ | 13.8 | 27.2 |
| | PE + RGAE + $Ca^{2+}$ | 13.5 | 28.9 |
| | PE + RGAE + RGase + $Ca^{2+}$ | 20.4 | 24.8 |
| | PE + AF + $Ca^{2+}$ | 5.5 | 14.2 |
| ABP pool I | PE + $Ca^{2+}$ | 0 | 11 |
| | PE + PAE + $Ca^{2+}$ | 15 | 27.9 |

The addition of PE + RGase + RGAE to the pectin–$Ca^{2+}$ mixture led to lower values for $G'$, which was probably caused by partial degradation of the rhamnogalacturonan backbone of the 'hairy' regions by RGase, resulting in a lower apparent molecular weight. The total release of acetyl groups was higher than in the absence of RGase.

The combination of PE + PAE released more acetic acid and methanol from the pectin–$Ca^{2+}$ mixture, originating from the 'smooth' homogalacturonan regions, than PE or PAE alone. The values for $G'$ were twice as high as with PE alone. This can be explained by the fact that the formation of $Ca^{2+}$ cross-links increased with an increasing amount of free carboxyl groups, due to the removal of the methyl groups. Here it is shown that the PE activity increased when it acts together with PAE and that the PAE activity increased when it is combined with PE. This observation suggests that the acetyl groups interfere with the action of PE and that methyl groups interfere with the action of PAE. Searle-van Leeuwen et al. (1996) showed that demethylation of an acid extracted beet pectin with PE also increased the activity of PAE. In literature, it is shown (Pippen et al., 1950) that the acetyl groups interfere with the "egg-box" formation with $Ca^{2+}$, however, this could not be concluded directly from our experiments.

Treatment of the pectin–$Ca^{2+}$ mixture with a combination of PE + AF resulted in similar values for the storage modulus as when AF was omitted. This is in agreement with the assumption made by Hwang et al. that side branches of pectin have a relatively small influence on gel formation with $Ca^{2+}$ (Hwang, Pyun & Kokini, 1993). Furthermore, the arabinose content of pectins obtained by acid extraction is relatively low and will have relatively little influence on the physico-chemical properties (Oosterveld, Beldman & Voragen, 2000b).

Additionally, tan $\delta$ ($= G''/G'$) is a good parameter to describe the nature of a gel. $G''$ is indicative for relaxation of cross-links. A low value for tan $\delta$ ($<0.1$) shows the presence of an elastic system, whereas high values ($>1$) imply a more liquid-like character of the network (Ross-Murphy, 1984). All the values found for tan $\delta$ were lower than 0.060, showing that for all combinations in which gel formation was observed an elastic system was formed.

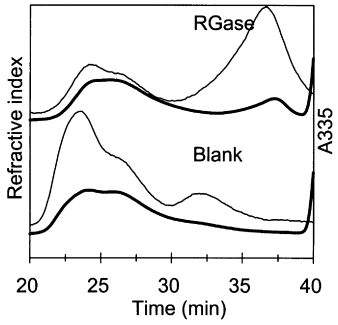


Fig. 3. HPSEC elution pattern of ABP before and after treatment with rhamnogalacturonase (RGase). Thick line: RI, thin line: $A_{335}$.

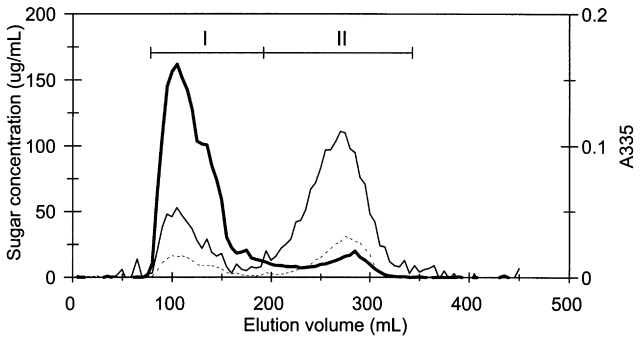


Fig. 4. S 300 SEC elution pattern of ABP after treatment with rhamnogalacturonase (RGase). Thick line: uronic acid; dashed line: neutral sugars; thin line: $A_{335}$. I and II: pooled fractions.

While relatively large differences were found for $G'$, the differences for tan $\delta$ were much smaller. The lowest value for tan $\delta$ was found for PAE + PE, which shows that the gel formed had a somewhat more elastic nature in comparison to the other combinations. It can be concluded that the enzyme treatments did not change the elastic nature of the gels as much as they changed the stiffness of the gels.

### 3.5. Treatment with RGase

To investigate the role of the 'smooth' regions in gel formation with $Ca^{2+}$, the original beet pectin was treated with RGase in order to degrade the 'hairy' regions. Although it is known that the removal of acetyl groups by RGAE is necessary for RGase to work optimally on modified 'hairy' regions from apple (Searle-van Leeuwen et al., 1996), we found that ABP was degraded to the same extent without the use of RGAE based on the HPSEC results (data not shown). This indicates that sufficient acetyl free regions must have been present in beet pectin for RGase to act on. Based on their molecular size, two populations were present after the enzyme treatment, as shown by HPSEC (Fig. 3). A population, containing most of the material as indicated by refractive index readings, eluted at 26 min after the RGase treatment. This population had a low $A_{335}$ absorption (indicating a low ferulic acid content). Since it is known that ferulic acid is only present in the 'hairy' regions (Colquhoun et al., 1994; Guillon & Thibault, 1988, 1989a,b; Ralet et al., 1994), it was concluded that this population contained a low amount of 'hairy' regions and a high amount of 'smooth' regions. Upon RGase treatment a small population was formed, which eluted at 38 min. Based on the high $A_{335}$ absorption, it was concluded that this population contained a relatively high proportion of 'hairy' regions.

The pools were separated using a S 300 preparative size-exclusion column (Fig. 4). Only approximately 65% of the polysaccharides originally present in the extract were recovered. 15% of the material was lost during dialysis after the enzyme treatment, which was caused by removal of the rhamnogalacturonan oligomers and by some other oligosaccharides already present in the original pectin. Furthermore, some material was lost during the purification. The high galacturonic acid content of pool I (79.9 mol%) shows that it predominantly consists of homogalacturonans (Table 1). The amount of neutral sugars had decreased from 27.1 to 20.1 mol% as compared with the untreated beet pectin. Pool I had a lower intrinsic viscosity than the original material, which is in contradiction with a previous study (Oosterveld et al., 2000b), in which it was shown that treatment of ABP with a RGase + RGAE decreases the molecular weight but not the intrinsic viscosity. Possibly the solubility of highly viscous pectins is decreased after degradation of the rhamnogalacturonans with RGase + RGAE and subsequent purification and freeze-drying. The relatively high molecular weight of pool I suggested that the original ABP contained on average a low number of 'hairy' regions per molecule. Pool II contained a high proportion of neutral sugars, confirming that this pool mainly consists of 'hairy' regions. Rhamnose was present in a high proportion as compared with the neutral sugar composition of the original material. The DM of pool I was much higher than that of pool II, confirming that the 'smooth' regions have a lower charge or charge density than the 'hairy' regions. The DA in the 'hairy' regions was higher than in the 'smooth' regions. This can be partly explained by the observation that the RGase activity is higher on pectin regions without acetyl groups (Colquhoun, de Ruijter, Schols & Voragen, 1990; Schols et al., 1990). These acetyl free regions are then degraded to oligomers, which are (partially) lost during dialysis. Consequently, the remaining polymeric material will have a higher acetyl content. On the other hand, DEAE anion-exchange chromatography as well as experiments with an autoclave extract from sugar beet pulp (Oosterveld et al., 2000) also showed that the 'smooth' regions had a higher DM and a lower DA than the 'hairy' regions. As was already seen in the HPSEC analysis, most of the ferulic acid was located in pool II.

### 3.6. Effect of enzymatic modification on gel formation of ABP pool I with $Ca^{2+}$

To determine the effect of the 'hairy' regions on gel formation with $Ca^{2+}$ and PE, a mixture of $Ca^{2+}$ and pool

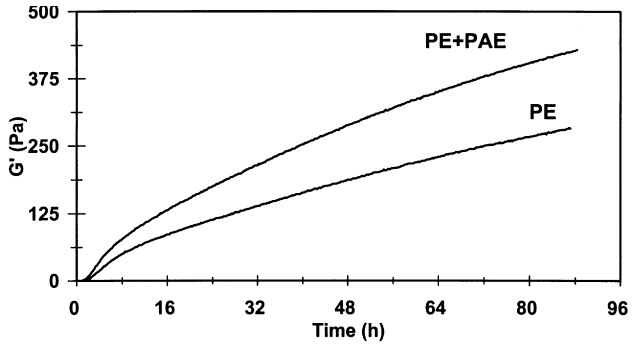


Fig. 5. Development in time of the storage modulus ($G'$) of ABP pool I + $Ca^{2+}$ during treatment with PE or PE + PAE.

I, representing the 'smooth' homogalacturonan regions of ABP was treated with PE or with PE + PAE. Fig. 5 shows the development of the storage modulus ($G'$) in time. The values of $G'$, $G''$ and tan $\delta$ after 16 h are shown in Table 3. A gel was formed after addition of PE to the mixture of pool I and $Ca^{2+}$. The maximum value for $G'$, indicative for the stiffness of the gel, was lower than that of the original pectin, which was probably caused by the lower apparent molecular weight of ABP pool I (Table 1). Furthermore, the gel was formed much slower. The amount of acetyl groups and methyl esters released was of the same level as that released from ABP.

Again, a higher maximum value for $G'$ was found after addition of PE + PAE to the mixture in comparison with PE alone. tan $\delta$ was somewhat lower for the mixture of pool I plus $Ca^{2+}$ with PE + PAE than with PE alone, again showing that the addition of these enzymes gives a more elastic gel.

Also for pool I addition of PAE led to a higher release of both acetyl groups and methyl esters during this experiment, confirming that the acetyl groups interfere with the action of PE (Table 4).

## 4. Concluding remarks

It was shown that removal of acetyl groups from the 'smooth' regions of sugar beet pectin by the enzyme PAE increases the action of PE and therefore improves the gelling capacity of this type of pectin with $Ca^{2+}$. From these results it is concluded that PAE is the most promising enzyme for the enzymatical modification of beet pectins on industrial scale, besides PEs, which can be used to alter the calcium sensitivity of pectins in general. The significant improvement of the gel forming properties of beet pectin after treatment with PAE may lead to a more general use of beet pectins in the pectin industry. In our experiments enzymatic modification with glycanases never led to significantly improved physico-chemical properties of the pectins used. Therefore, glycanases are assumed only to be used for specific purposes such as the production of debranched arabinans (Cooper, McCleary, Morris, Richardson, Marrs & Hart, 1992) or the improvement of the oxidative cross-linking reaction of beet pectins by removal of the arabinan side-chains (Guillon & Thibault, 1990).

An important prerequisite for introduction of enzymatic modification of beet pectins with PAE on industrial scale, is the availibility of sufficient amounts of enzymes at reasonable prices. The use of cloned polysaccharides degrading enzymes as described by Kofod et al. (1994) is assumed to be an important step towards the introduction of these enzymes on industrial scale.

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