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Populations having different GalA blocks characteristics are present in commercial pectins which are chemically similar but have different functionalities

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Abstract

Two commercially extracted pectins having different physical properties but similar chemical characteristics were fractionated into sub-populations using ion exchange chromatography. Individual sub-populations were characterised using established strategies (galacturonic acid and neutral sugar content, degree of methyl-esterification) including the use of enzymes (*endo*- and *exo*-polygalacturonases) as analytical tool. Some purified populations showed similar degree of methyl-esterification whereas they were eluting at different ionic strength. It was shown that these populations mainly differed in the number of galacturonic acid moieties in '*endo*-polygalacturonase degradable blocks' and in the location of these blocks within the molecule. The size of the blocks present at the non-reducing end of the pectin was also different within the molecules. The separation of pectins on anion exchanger combined with the use of enzymes allowed us to differentiate pectic sub-populations. Commercial pectins appear to be a mixture of several polymers differing in total charge as well as in the distribution of the charges.

Keywords: Pectins; Anion exchange chromatography; Enzymatic degradation; Degree of methyl-esterification; Degree of blockiness; Charge distribution

1. Introduction

Pectins are mainly present in the primary cell wall and in the middle lamella of plants. They constitute around 40% (dry matter basis) of the cell wall of fruits and vegetables (Brett & Waldron, 1996). The nature of pectin depends not only on the origin, the growing and harvesting conditions of the crop and but also on its localisation in the plant tissue and cell wall. Pectins are complex mixtures of polysaccharides composed of a galacturonic acid backbone (homogalacturonan or so-called smooth regions) of which variable proportions can be methyl-esterified. In addition, so-called hairy regions are present, constituted of alternative sequences of rhamnose and galacturonic acid (rhamnogalacturonan I) carrying variously sized neutral side chains (arabinans, arabinogalactans) attached to rhamnose moieties (Pilnik & Voragen, 1991; Voragen, Pilnik, Thibault, Axelos, & Renard, 1995). Pectins are used as food ingredients mainly for their gelling properties while pharmaceutical properties as antidiarrhea, detoxicant, regulation and protection of gastrointestinal tract and anti-tumour activity have also been mentioned (Voragen et al., 1995; Waldron & Selvendran, 1993). Different plant materials are used for the extraction of pectins (e.g. citrus peel, apples pomace and sugar beet pulp) and differences in functional properties are observed according to the process and origin of the raw material. It is known that gelling properties of commercial pectins strongly depend on the degree of methyl-esterification of the galacturonic acid residues (Voragen et al., 1995). Nevertheless, various pectins with similar chemical characteristics (galacturonic acid (GalA) and neutral sugar (NS) content, degree of methyl-esterification (DM) may behave differently in gel formation.

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In addition to the common chemical characterisation of pectins (determination of the GalA content and DM), new parameters to distinguish pectins were introduced (Daas, Alebeek, Voragen, & Schols, 1999; Daas, Arisz, Schols, De Ruiter, & Voragen, 1998; Daas, Meyer-Hansen, Schols, De Ruiter, & Voragen, 1999; Daas, Voragen, & Schols, 2000, 2001; Korner, Limberg, Mikkelsen, & Roepstorff, 1998; Limberg et al., 2000). Daas et al. used enzymatic degradation of the pectins with an *endo*-polygalacturonase of *Kluyveromyces fragilis* and analysed the partially methylated oligogalacturonides released. From these data they defined the degree of blockiness (DB) of pectins represented by the amount of non-methyl-esterified mono-, di- and trigalacturonic acid released by the enzyme relative to the total amount of non-methylesterified galacturonide residues present in the pectin. The higher the DB of pectins having a similar DM, the more blockwise the distribution of the methyl-esters in the pectin. Pectins having similar DM and DB values, may still differ in the size of the blocks. This difference can be characterised by a second parameter: the proportion of mono-, di- and trigalacturonic acid in the *endo*-PG digests. Long degradable blocks will lead to the release of high amounts of di- and trigalacturonic acid compared to monogalacturonic acid upon enzymatic digestion. The third parameter described by Daas et al. is the ratio of the total peak area of oligomers with methyl-esters to the total of peak areas of oligomers without methyl-esters (Me^+/Me^- ratio). This is an indication of the location of the degradable blocks within the backbone: the higher this ratio, the more clustered are the degradable blocks distributed over the pectin molecule (Daas, Alebeek et al., 1999; Daas, Meyer-Hansen et al., 1999; Daas et al., 1998, 2000).

Chromatography performed on an anion exchange column (Schols, Reitsma, Voragen, & Pilnik, 1989) or size exclusion column (Kravtchenko, Berth, Voragen, & Pilnik, 1992) showed that commercial pectins were not composed of one single pectic population but they are constituted of various populations with different features. Anger and Dongowski (1984), Kravtchenko, Voragen, and Pilnik (1992) and Schols et al. (1989) suggested that elution on anion exchange column may vary according to the degree of methyl-esterification but as well as to the distribution of the charges. More recently, Ralet and Thibault (2002) studied the effect of charge distribution on the behaviour on an anion exchanger of pectins demethylated by Plant PME or fungus PME using conductometric measurements. In their study, they could not show any influence of the charge distribution on the elution on an anion exchanger.

Until now, the approach of Daas, Alebeek et al. (1999), Daas, Meyer-Hansen et al. (1999), Daas et al. (1998, 2000, 2001) using the DB, mono-, di- and trigalacturonic proportions and the Me^+/Me^- ratio of a pectin preparation 'as is' has not been extended with fractionation of the pectin into sub-populations and characterisation of these sub-populations. This approach would enable us to make an approximation of the intramolecular distribution (distribution of methyl-esters within one pectic molecule) as well as the intermolecular distribution of methyl-esters (distribution of methyl-esters over several pectic molecules). These distributions are expected to be related to the gelling behaviour in the presence of calcium. In this research, two pectins having different calcium reactivity and extracted from the same raw material with similar chemical characteristics are studied using these state-of-the-art approaches and tools.

2. Experimental

2.1. Samples

The samples were kindly provided by Degussa Texturant Systems (Baupte, France). Pectins A and B were selected for having nearly the same degree of methyl-esterification (DM of 74 and 72%, respectively), galacturonic acid content (GalA of 82 and 74%, respectively, in w/w) and intrinsic viscosity, but different calcium sensitivity (Laurent & Boulenguer, 2003). The intrinsic viscosity and the calcium sensitivity have been published already (Laurent & Boulenguer, 2003). Pectin A, also called Calcium sensitive (CS) pectin and pectin B, also called Non-Calcium Sensitive (NCS) have an intrinsic viscosity of 723 and 739 ml/g and a calcium sensitivity of 297 and 39 mPa, respectively (Laurent & Boulenguer, 2003).

2.2. Size exclusion chromatography of pectins

High-performance size exclusion chromatography (HPSEC) was performed with three Tosoh Biosciences TSK gel columns (G 4000, 3000, 2500 PWXL, each 300 × 7.5 mm) in series and in combination with a PWXL guard column (Tosoh Biosciences; 40 × 6 mm). Elution was performed at 30 °C with 0.2 M sodium nitrate at 0.8 ml/min. The eluate was monitored using a Shodex SE-61 refractive index detector. Twenty microliters of pectin (5 mg/ml) were injected.

2.3. Preparative chromatography of commercial pectins

An Akta explorer system was used for separation of pectins on a preparative scale. Pectin (0.5 g) was dissolved in 100 ml of 0.03 M of sodium phosphate buffer. Elution was performed on a Source-Q column (115 × 60 mm; Amersham Biosciences) using 'Millipore' water during four column volumes (CV) followed by a linear gradient in steps: 0–0.12 M of sodium phosphate buffer (pH6) in 13 CV at 60 ml/min; 0.12–0.42 M of sodium phosphate buffer (pH6) in 44 CV; 0.42–0.6 M sodium phosphate (pH6) in 2 CV and finally 8.5 CV of 0.6 M sodium phosphate pH6. The column was washed with 1 M sodium hydroxide for

5 CV. Detection was accomplished with an UV detector set at 215 nm.

The fractions (250 ml) were pooled and ultrafiltrated with a Pellicon 10 kDa membrane (size of 50 cm^2) till a conductivity of <10 μs. After ultrafiltration, the fractions were freeze-dried. Then the different pools were resuspended and dialysed with dialysis tubing (cut of 12–14 kDa for proteins) against 'Millipore water' to remove last traces of salts prior to freeze-drying.

2.4. Uronic acid content

Pectins (60 μg/ml) were boiled (1 h), cooled and then saponified with sodium hydroxide (40 mM). The uronide content was determined by the automated colorimetric *m*-hydroxydiphenyl method (Ahmed & Labavitch, 1977; Blumenkrantz & Asboe-Hansen, 1973; Thibault, 1979). Total neutral sugars were estimated with the automated orcinol method (Tollier & Robin, 1979), using galactose as a standard. Populations A5 and B5 were not soluble so a pre-hydrolysis step with sulfuric acid (72% in w/w) was performed on these samples prior to the colour reaction.

2.5. Neutral sugar content

The neutral sugar composition was determined by gas chromatography according to Englyst and Cummings (1984) using inositol as an internal standard. The samples were treated with 72% (w/w) H_2SO_4 (1 h, 30 °C) followed by hydrolysis with 1 M H_2SO_4 for 3 h at 100 °C and the constituent sugars released were analysed as their alditol acetates.

2.6. Methyl-ester content

The methyl-ester content was determined by GC head-space analysis of the free methanol released after alkaline de-esterification of pectins (Huisman, Oosterveld, & Schols, 2004).

2.7. Degree of blockiness

The degree of blockiness has been determined as described previously (Daas et al., 1999, 2000). Samples (5 mg/ml) were diluted in sodium acetate 50 mM pH5 and incubated with an overdose of *endo*-polygalacturonase of *K. fragilis* (0.04 units /ml) for 24 h. The specific activity of this enzyme for PGA was 128 U/mg. Pectin digests were prepared by incubation of pectic solution with *endo*-polygalacturonase (0.04 units/ml) for 24 h. As a result of the extended *endo*-polygalacturonase incubation employed, end-products were observed as was demonstrated by the use of an excess of enzymes and longer incubation times. EDTA (0.024 mM) was added to solubilise poorly soluble pectins (populations A5 and B5) prior to enzymatic digestion. Oligomers released were analysed by HPAEC (80 μl injection) using a Dionex CarboPac PA1 anion exchange column (250×4 mm) and a CarboPac PA-100 precolumn (50×4 mm). Elution was performed with sodium acetate at pH5 from 0.05 to 0.7 M in 65 min with a flow of 0.5 ml/min. The gradient was hold at 0.7 M sodium acetate for 5 min. The PAD detector (Dionex) was equipped with a gold working electrode and a Ag/AgCl reference electrode. Detection of the oligomers was possible after post column sodium hydroxide (1 M; 0.5 ml/min). The degree of blockiness (DB) is the amount of mono-, di- and trigalacturonic acid released by the *endo*-polygalacturonase related to the amount of free GalA present in the sample (Fig. 1). The absolute degree of blockiness (DB_{abs}) is the amount of mono-, di- and trigalacturonic acid released by the *endo*-polygalacturonase related to the total amount of GalA (free and methyl-esterified GalA) present in the sample (Fig. 1).

2.8. Free GalA blocks at the non-reducing end

Samples (5 mg/ml) were diluted in sodium acetate 50 mM and incubated with *exo*-polygalacturonase of *Aspergillus tubingensis* (Kester, Someren, Muller, & Visser, 1996). The specific activity of this enzyme for PGA was 118 U/mg. Pectin digests were prepared by incubation of pectic solution with *exo*-polygalacturonase (0.04 units/ml) for 24 h. Mono-GalA from the *exo*-polygalacturonase digests samples was analysed on HPAEC at pH12 equipped with a Dionex Carbopac PA1 column (250×4 mm) and a CarboPac PA-100 precolumn (50×4 mm). Sample (50 μl of 5 mg/ml pectin digest) was injected in the column and elution started with a pre-equilibration step of 15 min with 0.1 M NaAcetate in 0.1 M NaOH (1 ml/min) followed by a linear gradient of 1 M NaAcetate in 0.1 M NaOH (0.01–1 M during 60 min) and washing step of 5 min with 1 M NaAcetate in 0.1 M NaOH. Oligomers were detected with a PAD-detector (Dionex) equipped with a gold working electrode and an Ag/AgCl reference electrode. During each series, the PAD response area of a standard amount of monoGalA (0.2 mg/ml) was determined. The amount of free GalA present at the non-reducing end related to the amount of total GalA in the sample is determined and defined as the so-called Block Size at the Non-Reducing end; BS-nr (Fig. 1). The amount of GalA present interior and/or at the reducing end of the sample is determined as well (so-called Block Size Interior and/or at the Reducing end; BS-ir, Fig. 1).

3. Results and discussion

3.1. Fractionation of commercial pectin preparations in sub-fractions on preparative anion exchange chromatography

Two commercial HM pectins (pectins A and B) originating from the same citrus variety showed totally

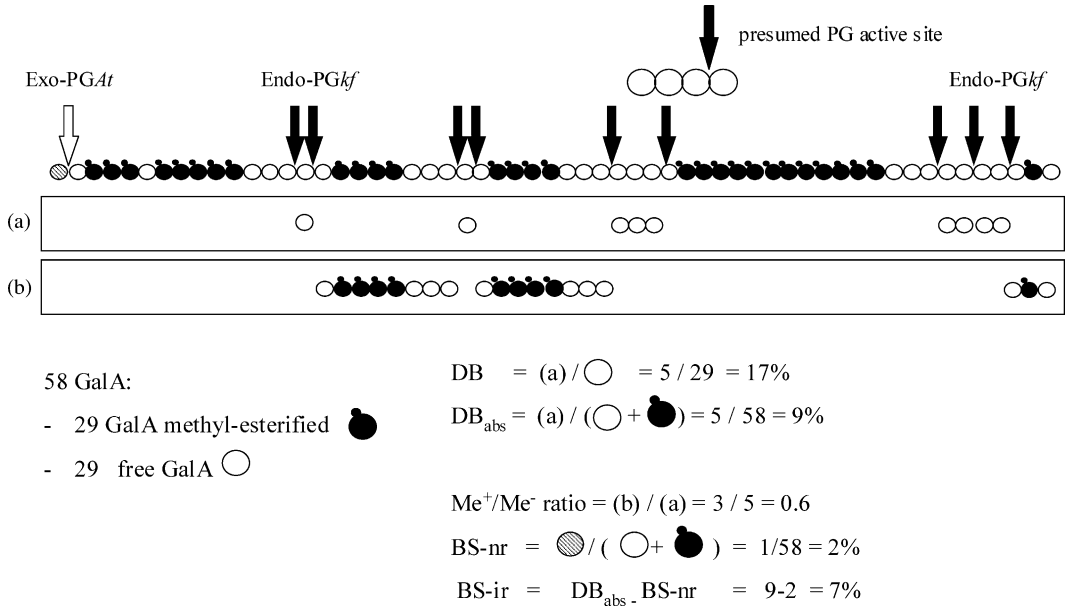

Fig. 1. Schematic representation of enzymatic digestion with *endo*-PG from *Kluyveromyces fragilis* (*endo*-PG*kf*) and *exo*-PG from *Aspergillus tubingensis* (*exo*-PG*At*) on a 50% DM pectin. Description of the parameters: DB, DB_{abs}, Me^{+}/Me^{-} ratio, BS-nr, BS-ir. It is assumed that *endo*-PG needs 4 adjacent non-esterified GalA residues to act (Daas et al., 1999). White and black arrows indicate the action of the *endo*-PG and *exo*-PG, respectively. Oligomers released by *endo*-PG and small enough to be analysed on HPAEC pH5 are indicated as (a) for the non-methylesterified ones and (b) for the methyl-esterified ones. GalA molecules released by *exo*-PG are indicated in ▨.

different gelling properties in presence of calcium. The chemical characteristics (GalA, NS, DM; Table 1) of these pectins did not explain the different gelling behaviour since they were similar. Schols et al. (1989) were able to separate pectic populations present in commercial pectins with different degree of methyl-esterification (DM) according to their charges, using an HPLC system equipped with an anion exchange column (MA7P column). The charge level and charge distribution seemed to have an influence on the elution behaviour of the pectins. Since the column used by Schols et al. was not commercially available, a column giving similar results was used (Propac™ WAX-10 column, Dionex). Pectins A and B showed totally different elution profiles (results not shown). To enable a detailed characterisation of the individual populations and to understand the different physical behaviour, pectins A and B were

Table 1
GalA content, Yield, degree of methyl-esterification (DM) and degree of blockiness (DB) and methyl to non-methyl-esterified peak area ratio of pectins A and B and corresponding fractions obtained by chromatography over Source-Q

Samples	GalA (w/w%)	Yield (%)[a]	NS (w/w%)	DM (%)	DB (%)[b]	DB_{abs} (%)[c]	Methyl- to non-methyl-esterified area ratio	BS-nr (%)[d]	BS-ir (%)[e]
A	82		7	74	16	4.2	0.1	1	3.2
A1	82	39	1	86	3	0.4	1.5	0.1	0.3
A2	59	6	4	85	15	2.2	0.7	0.6	1.7
A3	62	6	5	86	18	2.5	0.4	0.4	2.1
A4	75	44	4	69	15	4.6	0.2	1.2	3.4
A5	57	5	3	44	40	22.4	0.0	1.7	207
B	74		12	72	5	1.4	0.9	0.8	0.6
B1	70	32	2	92	4	0.3	2.8	0.2	0.2
B2	69	26	2	78	6	1.3	1.9	0.4	0.9
B3	75	11	4	59	3	1.2	0.2	0.8	0.41
B4	65	29	5	64	34	12.2	0.1	5	7.2
B5	32	2	4	40	35	21	0.1	nd	Nd

nd, Not determined.
[a] GalA yield is expressed as percentage of all GalA residues recovered.
[b] Amount of mono-, di- and triGalA released by the enzyme related to the amount of free GalA present in the sample.
[c] Amount of mono-, di- and triGalA released by the enzyme related to the total amount of GalA: [(100−DM)×DB/100].
[d] Free GalA present at the non-reducing end also called Block Size at the Non-Reducing end (BS-nr) related to the total amount of GalA in the sample.
[e] Free GalA present interior and/or on the reducing end also called Block Size Interior and/or on the Reducing end (BS-ir) related to the total amount of GalA.

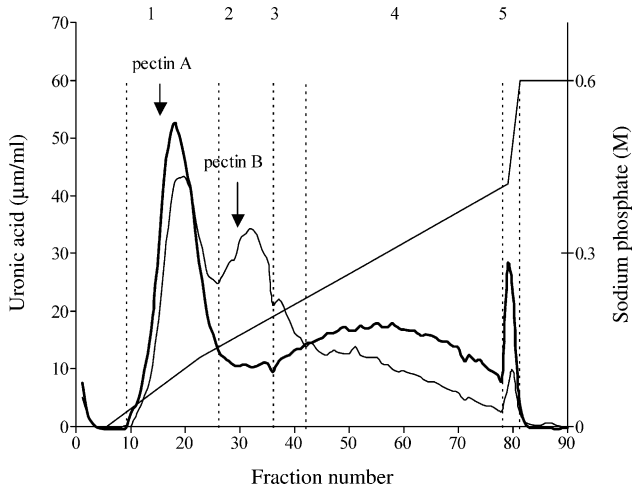

Fig. 2. Preparative anion-exchange chromatography of pectins A and B on Source-Q column. The elution profiles were obtained after determination of the uronic acid content in each fraction. The fractions (250 ml) were pooled as indicated.

fractionated on preparative scale using a Source-Q column (Fig. 2).

Elution profiles obtained with the Source-Q column (Fig. 2) were similar to those obtained with the analytical WAX-10 column: pectins A and B showed several pectic populations in different relative amounts. Next to a detailed characterisation of the individual populations also the mechanism for the different elution behaviour of the various pectic sub-fractions was subject for further studies. So fractions were pooled, ultra-filtrated before freeze-drying and further analysed.

3.2. Chemical characterisation of the different populations obtained after preparative Source-Q chromatography

3.2.1. Galacturonic acid and neutral sugar content in pectic populations

The recovery of pectin was measured by comparison of the GalA content of the injected sample and the GalA content measured in all fractions. Pectins A and B were recovered after chromatography for 76 and 79%, respectively. These values are not uncommon in this scale of chromatography (Kravtchenko et al., 1992). Fractions eluted from the column were analysed for GalA content, DM and DB. The length and distribution of the blocks were also studied (Fig. 1). It is shown in Table 1 that the GalA content was quite high for populations 1–4 from both pectins A and B and lower for the populations eluted with a higher ionic strength (populations A5 and B5). This phenomenon was observed previously by Kravtchenko et al. (1992). The neutral sugar content was low for all the populations (from 1 to 5%, w/w). The non-carbohydrate material may be due to insufficient removal of salts by ultrafiltration.

3.2.2. Degree of methyl-esterification in the various pectic populations

As charge and charge density are the most important parameters that influence the elution of pectic polysaccharides from an anion exchange column (Schols et al., 1989), the DM of all the pectin pools was determined. In general, pectic molecules with a lower DM were bound more strongly to the column and needed thus higher salt concentrations to be eluted (Table 1). This is in agreement with the findings of Kravtchenko et al. (1992) for lemon pectins eluted from a DEAE-Sepharose column. However, some populations with similar DM were found to elute at different buffer concentrations: sub-population A1, A2 and A3 (all DM 86) eluted at 0.1, 0.17 and 0.21 M buffer, respectively. On the other hand, populations A3 and B3 present different DM values whereas they eluted at the same ionic strength. These observations may be explained by a different distribution of the methyl-esters over the pectin backbone. To check this hypothesis the degree of blockiness, reflecting the distribution of methyl-esters over the pectic backbone, was determined. Some results are in contrast with Kravtchenko et al. (1992) since the latest pectins eluted (A5 and B5) presented a low DM (44 and 40% DM, respectively).

3.2.3. Degree of blockiness of the pectic populations

All pectic populations were digested with polygalacturonase of *K. fragilis* (PG*kf*) and degradation products were analysed and quantified using HPAEC pH5. All oligomers observed in the elution pattern have been previously identified using Maldi-TOF MS (Daas et al., 1998). From these results, two different parameters were determined: the DB and the Me^{+}/Me^{-} ratio (Daas et al., 1999, 2000) (Table 1). A high DB value is indicative for a blockwise distribution of non-esterified galacturonic acid residues in a pectin. The Me^{+}/Me^{-} ratio is indicative for the distribution of the non-esterified GalA 'blocks' over the pectin backbone (Daas et al., 2000). The higher this ratio, the closer the non-esterified GalA 'blocks' are. Parental pectin B presented a more random distribution of the methyl-esters than parental pectin A since the DB is 5% for pectin B and 16% for pectin A (Table 1). These values fit in the range mentioned by Daas et al. (1999): DB of 1% for a random DM70 pectin (R70) and DB of 11% for a blockwise DM70 (B71).

To check whether the populations of pectins A and B had different charge distributions, the DB of each sub-fraction was analysed. For similar DM pectins (populations A1–A3), the DB value is increasing for populations eluting at higher ionic strength (DB of 3% for pectin A1, 15% for pectin A2 and 18% pectin A3). As expected on forehand, the more blockwise the distribution of free GalA residues within the pectin (higher DB), the later the pectin eluted. The DB gives information about the presence of blocks (3–18% of all *non-esterified* GalA residues are grouped for pectin A1–A3). It is obvious that the DB is not enough to explain the elution behaviour of the sub-populations on the anion exchanger.

For example, populations A3 and A4 are both blockwise pectins but the DM is different so the proportion of blocks is different. To take this into account, we introduced the DB_{abs} (Fig. 1). This parameter gives information about the absolute number of blocks in the pectin samples without correction of the DM (Fig. 1). It is clear that blocks of free GalA were influencing the elution behaviour of the pectins: the more blocks of non-methyl-esterified GalA in the pectic sample, the later the elution is (Table 1). The DB_{abs} was increasing from 0.4% for A1 to 22% for A5 and from 0.3% for B1 to 21% for B5. Only fraction B2 was slightly deviating from this rule. This fraction B2 contained a few more blocks or larger ones than pectin B3, while these blocks were closer to each other compared to pectin B3 (Me^+/Me^- ratio is, respectively, 1.9 compared and 0.2). Anger and Dongowski (1984), Kravchentko et al. (1992) and Schols et al. (1989) explained the elution behaviour by the charge distribution. Our results confirm this hypothesis although our findings differ from the results published recently by Ralet and Thibault (2002) using a DEAE-Sepharose CL-6B column for chromatography of pectins.

It has also been noticed above, that pectins A3 and B3 eluted at the same ionic strength whereas their DM were different (86 and 59%, respectively). The DB_{abs} of population A3 (2.5%; Table 1) was higher compared to DB_{abs} of population B3 (1.2%). Also the Me^+/Me^- ratio of fraction A3 was twice as high than that for fraction B3 (0.4 and 0.2%, respectively). So less degradable blocks were present in pectin B3 but more distant from each other compared to the pectin A3. This may explain their similar binding on the anion exchanger. Obviously, the co-elution of a 86% DM pectin having some blocks of GalA residues with a random 59% DM pectin complicated the interpretation of anion exchange patterns but the enzymatic degradation of these populations showed us that these pectins were different concerning the amount and distribution of free GalA blocks. Another surprising finding was the co-elution of populations A2 and B2 with similar DM but different DB_{abs} (2.2 and 1.3%, respectively) and Me^+/Me^- ratio (0.7 and 1.9, respectively). Population B2 contained less '*endo*-PG degradable' blocks more clustered compared to population A2. These data revealed that the anion exchange column does not make any distinction between the 'random' pectin B2 with some clustered but rather short GalA blocks and the blockwise pectin A2 with only few, more distant blocks. Our findings clearly show that the column is not able to distinguish between all different pectin populations present but the enzymatic degradation of the pectins showed that this populations presented different *endo*-PG degradable blocks.

3.2.4. Does the molecular size distribution influence the behaviour of the pectic populations in anion exchange chromatography?

To establish whether the molecular size of the various populations could explain their behaviour on the anion exchange column, each pectic population was analysed by high performance size exclusion chromatography (HPSEC). It can be seen that the molecular size was slightly higher for pectins eluting at high ionic strength (Fig. 3) except for population A5. Since HPSEC elution profiles from most of the pectic populations showed rather similar molecular distribution in the range of 100–43 kDa (18–24 min; Fig. 3), it can be concluded that the size of the pectic polymers from the populations does not explain the different elution behaviour of the sub-fractions on the Source-Q column.

All pectin PG*kf* digests were analysed by HPSEC as well. Next to the amount of mono-, di- and trigalacturonic acid released by the *endo*-PG and taken into account in the DB parameter, larger fragments are released which also give information on the distribution of methyl-esterified carboxyl groups over the galacturonan backbone. In general, the shift in Mw after PG digestion was more

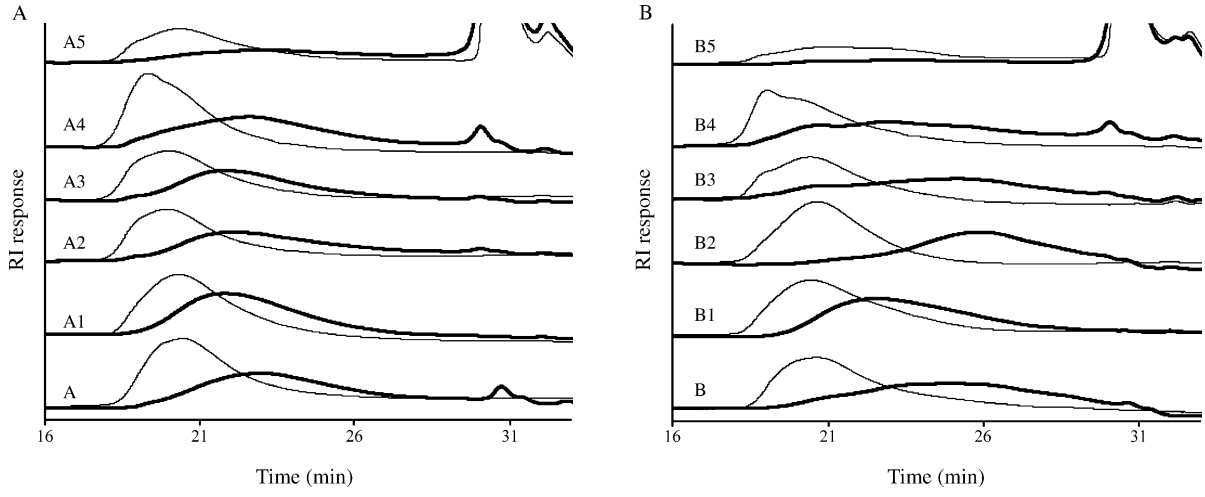

Fig. 3. HPSEC elution profiles of the different populations obtained after preparative anion-exchange chromatography of pectins A (A) and B (B) on Source-Q column before (thin lines) and after degradation of pectins with *endo*-polygalacturonase from *K. fragilis* degradation (thick lines).

pronounced for pectin B populations than for pectin A populations (Fig. 3). This Mw shift seems to be independent from the amount of small oligomers released (29–32 min) and these fragments (64–2 kDa) are not included in the calculation of any parameters described so far. Combining the results obtained from the DB calculation described above for pectins A2 and B2, and the HPSEC PG*kf* digests profiles, it was concluded that these pectins were indeed totally different. Only a part of the pectin A2 molecule was well degradable by the *endo*-PG*kf* and this part was constituted of free GalA clusters. The other part of the pectin A2 was hardly degraded by the PG*kf*, which is subject for further research. For population B2, it was concluded that compared to population A2, smaller amounts of *endo*-PG degradable blocks were present, and that these blocks were more randomly distributed over the pectic backbone. This explains the large decrease in Mw of pectin B2 compared to pectin A2. The same phenomenon is observed for populations A3 and B3.

3.2.5. GalA blocks present at the non-reducing end, interior and/or at the reducing end

The presence of *endo*-PG degradable blocks at the extremities of the molecule may also lead to a less pronounced decrease in Mw for pectin A polymers compared to those of pectin B. To obtain more information about the localisation of free GalA blocks in the galacturonan backbone, an *exo*-polygalacturonase (*exo*-PG) was used to degrade the pectins. Since this enzyme is known to release only non-methyl-esterified GalA from the non-reducing end of pectins (Benen, Vincken, & Alebeek, 2002) and needs two non-methyl-esterified GalA to act (only one free GalA at the non-reducing end is not a substrate for the *exo*-PG) (Korner, Limberg, Christensen, Mikkelsen, & Roepstorff 1999; Limberg et al., 2000), it is possible to determine whether pectins contain different block sizes of un-esterified GalA at the non-reducing end. *Exo*-PG digests were analysed with HPAEC at pH12. Only monogalacturonic acid is released by the enzyme during pectin digestion. The amount of GalA released by the *exo*-PG, related to the total amount of GalA present on the pectin (Block Sequence on the Non-Reducing end: BS-nr), was 1% for pectin A and 0.8% for pectin B. So pectin A was slightly more degraded with *exo*-PG (Table 1). This indicates the presence of larger blocks of non-methyl-esterified GalA on the non-reducing end for pectin A. These results can be compared with previous results of Limberg et al. (2000) where the authors also analysed the *exo*-PG digests of a pectin with similar chemical characteristics as pectin A (blockwise pectin with a DM of 76, reference P76 in the publication). Based on their published values, we calculated that they found a BS-nr of 1.4% which is in the same range of the BS-nr of 1% found in our study for pectin A. The BS-nr increased for populations eluting at increasing ionic strength except for populations A3 (BS-nr of 0.4% compared to 0.6% for population A2). In general, we can assume that the longer the block of free GalA on the non-reducing end of pectins, the later is the elution. The DB_{abs} is giving information on the blocks recurrence over the galacturonan backbone. The *exo*-PG is giving information about the GalA blocks present at the non-reducing end. Therefore it is possible to determine the amount of blocks in the interior and/or in the reducing end of the galacturonan backbone by determining the BS-ir (Block Sequence Inside and on the Reducing end) parameter. This parameter was calculated by substracting the DB_{abs} with the BS-nr (Block Sequence on the Non-Reducing end). From the data presented in Table 1, we can deduce that the higher the amount of GalA in block sequences located in the interior and/or on the reducing end, the stronger is the binding of the pectin on the anion exchanger. The BS-ir is 0.3% for population A1 and increased up to 20.7% for pectin A5. Population B3 is deviating from this rule. These results may explain the differences in elution behaviour of the pectic populations. Pectins are shown to be different from each other by different localisation of the free GalA blocks: some pectins presented more blocks on the non-reducing end, others on the reducing end and/or inside the pectic backbone.

4. Conclusions

Commercial pectins showed to be a mixture of different populations which can be separated on a preparative Source-Q column. This leads to differentiations between pectins with similar chemical features but different gelling behaviour. Separation of the pectins was dependant on the DM as observed previously (Kravtchenko et al., 1992; Schols et al., 1989) but the degree of blockiness (DB_{abs}) influenced the elution behaviour as well which is in agreement with previous suggestions (Schols et al., 1989). The position of the non-methyl-esterified GalA blocks is also varying in the populations purified from the parental commercial pectin. Nevertheless, it is important to notice that some populations with similar DM and different distribution of the methyl-esters eluted at the same ionic strength, which makes it difficult to interpret anion exchange elution patterns. The parameters described by Daas et al. (1999, 2000) to characterise pectins in term of size and type of distribution of free GalA blocks over the galacturonan backbone are not fully adequate. The parameters described in this study (DB_{abs}, BS-nr and BS-ir) and elaborated on the method of Daas et al. (1999, 2000), and Limberg et al. (2000), provide further valuable information on the fine structure of more homogeneous pectic populations and on the behaviour of these pectins on an anion exchanger.

The combination of HPAEC and enzymatic digestion allow us to visualise and characterise the different pectic polymers present in commercial pectins.

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