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Physical Characterisation of the Rhamnogalacturonan and Homogalacturonan Fractions of Sugar Beet (*Beta vulgaris*) Pectin

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28 **Abstract**

29 Acid-extracted sugar beet *(Beta vulgaris)* pectin was subjected to enzymatic hydrolysis using

30 fungal pectin methyl esterase (f-PME) and two endopolygalacturonanases (PGs I and II). From

31 the hydrolysate, the RG-I fraction was separated and purified by chromatographic techniques.

32 This RG-I fraction was shown to be of high weight average molar mass (188000 g/mol), but low

33 intrinsic viscosity (36 ml/g), which is consistent with a random coil conformation (L_p = 1.4 nm).

34

35 The HG fraction was prepared by mild acid hydrolysis of acid extracted pectin. The HG fraction

36 was found to have a relatively low weight average molar mass (20000 g/mol), but a rather high

37 intrinsic viscosity (77 ml/g), which is consistent with the HG fraction being rigid in solution (L_p

38 = 9.8 nm).

39

40 Lower molar mass pectins are richer in HG regions and pectins of higher molar mass are richer

41 in RG-I regions. We conclude that the degradation of the HG region has an important impact on

42 intrinsic viscosity, but less on molar mass and the inverse is true for the degradation of RG-I

43 region. This has important consequences in terms of the functionality of sugar beet pectin

44 molecules.

45

49

1. Introduction

Pectins are a complex family of heteropolysaccharides that constitute a large proportion of the primary cell walls of dicotyledons and play important roles in growth, development and senescence (Ridley, O'Neil, & Mohnen, 2001; Willats, McCartney, Mackie, & Knox, 2001). Pectic polysaccharides are made of several structural elements the important of which are the homogalacturonan (HG) and type I rhamnogalacturonan (RG-I) regions often described in simplified terms as the "smooth" and "hairy" regions respectively. The HG region is composed of (1→4) linked α-D-GalpA residues that can be partially methylated at *C*-6 (Pilnik & Voragen, 1970) and possibly partially acetyl-esterified at *O*-2 and/or *O*-3 (Rombouts, & Thibault, 1986). The degree of methylation (DM) and the degree of acetylation (DAc) are defined as the number of moles of methanol or acetic acid per 100 moles of GalA. The degree of methylation in native pectins is generally in the order of DM ≈ 70-80; whereas degree of acetylation is generally much lower *e.g.* DAc ≈ 35 for sugar beet pectins (Rombouts, & Thibault, 1986). The RG-I region consists of disaccharide repeating unit [→4)-α-D-GalpA-(1→2)-α-L-Rhap-(1→]$_n$ with a variety of side chains consisting of L-arbinosyl and D-galactosyl residues (Voragen, Pilnik, Thibault, Axelos, & Renard, 1995). It has been reported that GalA residues in the RG-I region are partially acetylated (Ishii, 1997; Perrone, Hewage, Thomson, Bailey, Sadler, & Fry, 2002) but not methylated (Komalavilas, & Mort, 1989; Perrone, *et. al.*, 2002). In the case of sugar beet pectin the neutral side chain sugars are substituted with ferulic acid (Fry, 1982; Rombouts, & Thibault, 1986) and there is evidence indicating that pectin chains can be dimerised via diferulic bridges (Levigne, Ralet, Quéméner, & Thibault, 2004a; Levigne, Ralet, Quéméner, Pollet, Lapierre, & Thibault, 2004b; Ralet, Cabrera, Bonnin, Quéméner, Hellin, & Thibault, 2005a). There are a number of different ways in which ferulic acid can dimerise the most common being: 5-5'; 8-O-4'; 8-5' cyclic and 8-5' non-cyclic dimers (Micard, Grabber, Ralph, Renard, & Thibault, 1997).

In order to characterise the physical and chemical properties of the type I rhamnogalacturonan region it is necessary to degrade the homogalacturonan region. This can be achieved in a controlled manner using polygalacturonanses (PGs). PGs catalyse the hydrolytic cleavage of the O-glycosyl bond of α-D-(1→4) polygalacturonate. This degradation proceeds in either a random (*endo*-PG, E.C. 3.2.1.15) or a terminal (*exo*-PG, E.C. 3.2.1.67) fashion. A large number of

81 fungal PGs have been purified and characterised and although they appear to have similar amino

82 acid compositions (Mohamed, Christensen, & Mikkelsen, 2003) they have different chemical

83 and physical properties. *Aspergillus niger* has been shown to posses at least seven *endo*-PGs

84 (Benen, van Alebeek, Voragen, & Visser, 2003). Both methyl and acetyl groups have been

85 shown to affect the mode of action of *endo*-PGs (Renard, & Jarvis, 1999a,b; Mohamed, *et. al.*,

86 2003).

87

88 In the present study, two different *endo*-PGs were used in conjunction with a fungal pectin

89 methyl esterase (f-PME) to generate the RG-I region of sugar beet pectin. The enzyme

90 hydrolysate was fractionated by anion exchange and size exclusion chromatography and the

91 physical and chemical properties of the resultant RG-1 fragment was characterised.

92

93 The homogalacturonan (HG) region was generated by mild acid hydrolysis of sugar beet pectin

94 after saponification with sodium hydroxide at 4 °C in order to prevent β-elimination. The

95 physical characteristics of the HG region were measured without any further purification.

96

97 **2. Experimental**

98 **2.1. Materials**

99 **2.1.1. Enzymes**

100 Recombinant fungal pectin methyl esterase from *A. aculeatus* (f-PME, E.C. 3.1.1.11, UniProt

101 Q12535) and endopolygalacturonase I and II from *A. niger* (PG I and PG II, E.C. 3.2.1.15,

102 UniProt P26213 and P26214) were provided by Novozymes (Bagsvaerd, Denmark). The

103 purification of PGI and PGII are described in detail in Ralet, *et al.* (2008a) and Bonnin, *et al.*

104 (2002a), respectively.

105

106 **2.2. Methods**

107 **2.2.1. Acid extraction of sugar beet pulp (Pectin A)**

108 The alcohol insoluble residue (AIR) (5 g) from sugar beet pulp was obtained as described in

109 Levigne, Ralet, & Thibault (2002) in brief sugar beet roots were immersed in 3 litres of boiling

110 ethanol (96%) and the slurry was filtered through G3 sintered glass and the insoluble material

111 was repeatedly suspended in 70 % ethanol until the filtrate gave a negative reaction to the

112 phenol-sulphuric acid assay (Dubois, Gilles, Hamilton, Rebers & Smith, 1956). The resultant

113 AIR was then dispersed in 150 ml HCl at pH 1.0 and heated for 30 mins at 75 °C with agitation.

114 The residue was separated through G3 sintered glass. The supernatant was adjusted to pH 4.5

115 with 2 M NaOH, concentrated under vacuum at 40 °C, extensively dialysed against distilled

116 water and freeze-dried at condenser temperature of -55 °C and a pressure of 4 millibars (400 Pa)

117 for 48 hours. The resultant pectin (yield 280 mg/g) will be referred to as pectin A. The RG-I

118 and HG fractions of pectin A will prepared by enzymatic and acid hydrolysis, respectively and

119 characterised in terms of molar mass, intrinsic viscosity and confirmation after purification

120 where appropriate (see **Figure 1**).

121

122 **2.2.2. Mild acid hydrolysis of pectin A**

123 Pectin A (50 mg) was dissolved in distilled water at approximately 10 mg/ml and the pH was

124 increased to pH 12 by the addition of 0.2 M NaOH. The mixture was incubated for 24 hours at 4

125 °C, after which the pH was lowered to pH 1 on addition of 2.5 M HCl. The resultant mixture

126 was heated at 80 °C for 72 hours, during which a precipitate was formed. This precipitate has

127 previously been shown to be the homogalacturonan region (Thibault, Renard, Axelos, Roger, &

128 Crépeau, 1993). The precipitate (AHCl72) was separated by centrifugation and resolubilised by

129 neutralisation with sodium hydroxide (1 M) prior to analysis by HPSEC.

130

131 **2.2.3. Enzymatic hydrolysis of pectin A**

132 Sugar beet pectin A (100 mg) was dissolved in 0.05 M sodium succinate buffer pH 4.5 at a

133 concentration of 7.5 mg/ml overnight at 30 ºC with gentle agitation. The pectin solution was

134 mixed with an enzymatic solution (in 0.05 M succinate buffer pH 4.5) containing f-PME, PG I

135 and PG II. Enzymes were present 25.7, 2.1 and 2.1 nkat/ml respectively in the final reaction

136 mixture. The reaction mixture containing 4.3 mg pectin/ ml was incubated at 30 °C for 24 hours.

137

138 **2.2.4. Analytical**

139 Galacturonic acid and neutral sugar (expressed arbitrarily as arabinose) contents were

140 determined in triplicate by the automated m-hydroxbiphenyl (Thibault, 1979) and orcinol

141 methods (Tollier, & Robin, 1979), respectively, the latter being corrected for interfering

142 galacturonic acid.

143 Individual neutral sugars were obtained by hydrolysis with 2 M trifluoroacetic acid at 121 °C for

144 2 hours and converted to their corresponding alditol acetates (Blakeney, Harris, Henry, & Stone,

145 1983). These alditol acetates were subsequently analysed by gas chromatography using myo-

146 inositol (0.5 mg) as an internal standard on a DB-225 fused-silica capillary (30m x 0.32 mm i.d.)

147 column (J&W Scientific, Courtaboeuf, France) mounted in a DI 200 chromatograph (Delsi

148 Nermag Instruments, Argenteuil, France) with hydrogen as the carrier gas at a constant

149 temperature of 220 ºC.

150

151 Phenolic acids were determined by HPLC after saponification and extraction. Pectin A (8.1 mg)

152 was dissolved in 1 ml of 2 M NaOH and saponified under argon for 30 min at 35 °C in the dark.

153 The internal standard (*o*-coumaric acid) was added, prior to neutralisation with 2 M HCl and the

154 extraction of the phenolic compounds into ether. The ether phase was evaporated and the residue

155 dissolved in 50/50 water/methanol. 20 µl of this material was injected onto a Purospher C18

156 column (Merck, Darmstadt, Germany) and a gradient elution was performed using acetonitrile

157 (A) and pH 4.6 sodium acetate buffer (B) at 60 ml/h and 30 °C: (0 min, A = 15 %; 6 min, A = 15

158 %; 26 mins, A = 35 %; 26.5 min, A = 60 %; 30.5 min, A = 60 %; 31 min, A = 15 %; 35 min, A =

159 15 %). The eluent was detected at 320 nm. Response factors were determined relative to *o*-

160 coumaric acid (ferulic acid = 0.57 and for the diferulic acids 5-5' = 0.50; 8-O-4' = 1.04; 8-5'

161 cyclic dimer = 1.09 and 8-5' non-cyclic dimer = 0.90).

162

163 **2.2.5. Physical**

164 High performance size exclusion chromatography (HPSEC) was performed at room temperature

165 on a system consisting of a Shodex OH SB-G guard column (Showa Denko, Tokyo, Japan)

166 followed by in series (Shodex OH-Pak SB-805 HQ and Shodex OH-Pak SB-804 HQ) eluted with

167 50 mM sodium nitrate buffer containing 0.02 % sodium azide as an antibacterial agent at a flow

168 rate of 42 ml/h. The eluent was detected on-line by:

169

170 i. SpectroMonitor 3000 variable wavelength UV detector at 325 nm (LDC/Milton Roy,

171   Paris, France)

172 ii. MiniDawn light scattering (LS) detector (Wyatt, Santa Barbara, U.S.A.)

173 iii. T-50A differential pressure viscometer (DPV) (Viscotek, Huston, U.S.A.)

174   iv.   ERC 7515A differential refractometer (RI) (Sopares, Gentilly, France)

175

176 Weight average molar masses were calculated using ASTRA (Wyatt, Santa Barbara, U.S.A) and

177 weight intrinsic viscosities using the TRISEC (Viscotek, Huston, U.S.A.). *dn/dc* was taken to be

178 0.146 ml/g (Chapman, Morris, Selvendran, & O'Neill, 1987; Morris, Foster, & Harding, 2000;

179 Levigne, *et. al.*, 2002; Morris, García de la Torre, Ortega, Castille, Smith, & Harding, 2008) for

180 pectin A and its HG and RG-I fractions.

181

182 **2.2.6. Anion Exchange Chromatography (AEC)**

183 Anion exchange chromatography was performed in order to purify the RG-I fraction on a DEAE-

184 Sepharose CL 6B (Pharmacia) column (30 x 2.6 cm) equilibrated with degassed 0.05 M sodium

185 succinate buffer pH 4.5 at a flow rate of 90 ml/h at room temperature. The hydrolysis products

186 of pectin A after 24 hours incubation with polygalacturonase (APG24) (25 ml) were loaded onto

187 the column and the gel was washed with 400 ml of 0.05 M sodium succinate buffer. The bound

188 material was eluted with a linear NaCl gradient 0→0.4 M NaCl in 0.05 M sodium succinate

189 buffer, 1660 ml). Sodium succinate buffer containing 0.4M NaCl (400 ml) was then applied.

190 Fractions (9 ml) were collected and analysed for their galacturonic acid and neutral sugar

191 contents. Fractions (F1 – F10) were combined and concentrated by in vacuum rotary

192 evaporation at 40 °C.

193

194 **2.2.7. Preparative Size Exclusion Chromatography (SEC)**

195 Size exclusion chromatography was performed at room temperature on a Sephacryl S-200

196 column (60 x 1.6 cm) equilibrated with degassed 0.05 M sodium succinate buffer pH 4.5. The

197 anion exchange chromatography fraction containing the RG-I component: APGF10 (1.5 ml) was

198 loaded and eluted at a flow rate of 25 ml/h; 1.5 ml fractions were collected and analysed for their

199 galacturonic acid and neutral sugar contents. Appropriate fractions were combined and

200 concentrated by in vacuum rotary evaporation at 40 °C.

201

202

3. Results and Discussion

3.1. Physico-chemical characterisation of pectin A

Pectin A was first characterised/analysed by HPSEC. The peak at ~19 ml (**Figure 2**) is shown to consist entirely of neutral sugars (99 %) – see section **3.3** (**Table 1**). This "component" is "free" from the main pectic chain and constitutes neutral sugar side chains, which were cleaved from the main pectin chain during acid extraction, but are of sufficiently large molar mass to remain after dialysis. Therefore this component has been ignored when calculating the physical and chemical properties of pectin A.

Pectin A exhibits a typical distribution of sugars (**Table 1**), although the amount of total neutral sugars compared to value calculated by Levigne, *et. al.* (2002) appears to be a lot lower due to the removal of the contribution of the "free" neutral sugar side chains.

Acid extracted pectin is heterogeneous with respect to molar mass, intrinsic viscosity and composition (**Tables 1, 2** and **Figure 2**). The use of UV absorbance (325 nm) allows the visualisation of those populations of pectic molecules (or constituents), which are substituted with ferulic acid *i.e.* the arabinan, galactan or arabinogalactan side chains present on the RG-I region of sugar beet pectin. We can see from the differences between the RI and UV profiles that there are distinct populations of pectic molecules; the components eluting at the lowest elution volumes (12 – 14 ml) appear to be richer in ferulic acid than the species, which elute between 14 – 18 ml and should therefore also be richer in neutral sugars. We propose that this neutral sugar/ ferulic acid rich component is the result of dimerisation of pectins via diferulic bridges. Diferulic acids are known to be present in this pectin sample (11 % of total ferulic acids) and have recently been shown to bridge arabinose residues of Driselase® degraded sugar beet pectin (Levigne, *et. al.*, 2004a) and to have peripheral locations on pectin hairy regions (Levigne, *et. al.*, 2004b).

UV detection also allows a global estimate of the total % of ferulic acid in pectin A, which was found to 0.8 % (**Table 1**), and is in good agreement with the value of 0.7 % found from C18 HPLC after saponification and organic extraction. As ferulic acid only forms ester-linkages with the neutral sugar side chains of the RG-I region, therefore an increase in the % of ferulic acid is

8

234 therefore the result of a loss of galacturonic acid and can be used as an indication of the purity of
235 the RG-I region.
236
237 **3.2. Enzymatic hydrolysis of pectin A**
238 After 24 hours of enzymatic hydrolysis of pectin A by PG and PME, the weight average molar
239 mass and weight average intrinsic viscosity were reduced considerably as can be seen by the loss
240 of higher molecular weight material this is consistent with previous estimates (Oosterveld,
241 Beldman, & Voragen, 2002) and was confirmed after purification (**Table 1**). As can be seen the
242 peak at 19 ml is unaffected by enzymatic hydrolysis by PG and PME this is consistent with this
243 being a fragment of a neutral sugar side chain and being free of GalA (**Figure 3**). Furthermore
244 100 % of the ferulic acid substituted material is conserved in the pectic fragment at 16-17 ml
245 (excluding the peak at 19 ml) and the amount of ferulic acid has increased to 2.4 %. This is
246 consistent with the purification of the RG-I region of sugar beet pectin, as this is the result of the
247 loss of galacturonic acid and therefore concentration of neutral sugars.
248
249 It is however difficult to calculate the molar masses of the RG-I fragment using light scattering
250 due to the presence of galacturonic acid oligomers (Ralet, *et. al*., 2005b) contributing to the light
251 scattering (LS), refractive index (RI) and differential pressure viscometer (DPV) signals (but not
252 the UV signal), therefore a better estimate of the molar mass and intrinsic viscosity can be made
253 after chromatographic separation on a preparative scale. This will also allow chemical analyses.
254
255 **3.3. Anion exchange chromatography (AEC)**
256 After AEC it can be seen (**Figure 4**) that there are 10 components most of which (F2 – F9) are
257 oligogalacturonides of various DPs (1 – 10), DMs and DAcs (see Ralet, *et. al*., 2005a for a full
258 characterisation). The unbound fraction (F1) is the neutral sugar fraction (99 %) described
259 earlier. The fraction, which is retained the longest (F10) is richer in neutral sugars and is
260 presumed to be the RG-I fraction, although it has been shown previously (Ralet, *et. al*., 2005a)
261 that this fraction also contains larger oligogalacturonides.
262
263

3.4. Preparative Size Exclusion Chromatography (SEC)

This fraction (F10) was subjected to SEC on Sephacryl S-200 (**Figure 5**) and two sub-fractions (F10a and F10b) were separated. The higher molecular weight the sub-fraction (F10a) originates from the RG-I region and the lower molecular weight sub-fraction consists of oligogalacturonides (Ralet, *et. al*., 2005a). Fraction F10a which has not previously been examined in detail, was dialysed, freeze-dried and physico-chemical properties characterised.

3.5. Physico-chemical characterisation of RG-I fraction (APG24F10a)

The RG-I region was found to contain 22 mol% GalA; 17 mol% Rha and 62 mol% neutral sugars (GalA: Rha: NS ratio of 1.3: 1.0: 3.6) suggesting that this fraction is indeed the RG-I region and the HG component has been almost entirely degraded, this was also supported by a further increase in the amount of ferulic acid to 2.4 %, which is 3 times that present in pectin A and is slightly larger than the increase in total neutral sugars ~ 2.6 times (**Table 1**). This fraction was found to have a weight average molar mass of 188000 g/mol and the weight average intrinsic viscosity is 36 ml/g (**Table 2**), which is relatively low for a molecule of this molar mass and is reflective of a highly compact or random coil conformation. This value is much higher than previous estimates of 50000 g/mol (Oosterveld, *et. al*., 2002) and 12000 g/mol (Guillon, & Thibault, 1990) calculated from light scattering and viscosity measurements respectively, but it should be noted that in both cases the values are likely to be underestimated due to the presence of contaminants *e.g.* galacturonic acid oligomers, salts and enzymes. In our case the weight average molar mass of RG-I is greatly influenced by presence of "aggregates" appearing as shoulders on the UV chromatogram at 13 – 14 and 14 – 15 ml (**Figure 3**), the exact nature of these aggregates is not clear, but we believe that any diferulic bridges present in the pectin A would also be present in the purified RG-I region as enzymatic hydrolysis and chromatographic separation appear to have no affect on ferulic acid residues. Another factor influencing the molar mass is that at least some of the lower molar mass RG-I molecules may have co-eluted with the larger oligogalacturonides during SEC fractionation.

293 **3.6. Physical characterisation of HG fraction (AHCl72)**

294 During 72 hours acid hydrolysis of pectin A at 80 °C a precipitate is formed (~ 98 % GalA) this

295 is consistent with previous measurements (Thibault, *et. al.*, 1993). After neutralisation both the

296 insoluble and soluble fractions were characterised by HPSEC. The soluble fraction did not

297 consist of any polymeric material; all components eluted near the total volume (results not

298 shown) and probably contained fragments of neutral sugar side chains. The insoluble fraction

299 also contains low molar mass material eluting near the total volume and represent the salts

300 formed after neutralisation. There is however a polymeric component with a peak at an elution

301 volume at ~17 ml (x in **Figure 3**). This component was found to have a weight average molar

302 mass of 20000 g/mol (**Table 2**). This value is good agreement with the values of 17000 – 21000

303 g/mol estimated previously for an acid hydrolysed beet pectin HG region (Thibault, *et. al.*, 1993;

304 Hellin, Ralet, Bonnin, & Thibault, 2005; Yapo, Lerouge, Thibault, & Ralet, 2007; Ralet, *et al.*,

305 2008b) and slightly higher than the estimated molar mass of 16000 g/mol for an enzymatically

306 hydrolysed sugar beet pectin HG region (Bonnin, Dolo, Le Goff, & Thibault, 2002b). The

307 weight average intrinsic viscosity of 77 ml/g (**Table 2**) is again in general agreement with value

308 found previously (75 - 92 ml/g) for the homogalacturonan region of pectins (Thibault, *et. al.*,

309 1993; Yapo, *et. al.* 2007; Ralet, *et al.*, 2008b).

310

311 **3.7. Conformational analysis**

312 **3.7.1. Global analysis method (HYDFIT)**

313 The linear flexibility of polymer chains can also be represented quantitatively in terms of the

314 persistence length, L_p of equivalent *worm-like chains* (Kratky, & Porod, 1949) where the

315 persistence length is defined as the average projection length along the initial direction of the

316 polymer chain. In the case of a theoretical perfect random coil $L_p = 0$ and for the equivalent

317 extra-rigid rod (Harding, 1997) $L_p = \infty$, although in practice limits of ~ 1 nm for random coils

318 (*e.g.* pullulan) and 200 nm for a extra-rigid rod (*e.g.* DNA) are more appropriate (Tombs, &

319 Harding, 1998).

320

321 The persistence length, L_p and mass per unit length, M_L can be estimated using Multi-HYDFIT

322 program (Ortega, & García de la Torre, 2007) which considers data sets of intrinsic viscosity and

323 molar mass. It then performs a minimisation procedure (see Ortega, & García de la Torre, 2007)

324 finding the best values of M_L and L_p satisfying the Bushin-Bohdanecky (Bushin, Tsvetkov,

325 Lysenko, & Emel'yanov, 1981; Bohdanecky, 1983) equation (equation 1).

326

327
$$\left(\frac{M_w^2}{[\eta]}\right)^{1/3} = A_0 M_L \Phi^{-1/3} + B_0 \Phi^{-1/3} \left(\frac{2L_p}{M_L}\right)^{-1/2} M_w^{1/2} \qquad (1)$$

328

329 and

330
$$M_L = \frac{m}{l} \qquad (2)$$

331

332 m and l are the average molar mass and length of the average monomeric unit.

333 From **Figures 6A-C** and **Table 2** we can see that the persistence lengths for pectin A and the HG

334 fraction are larger than that of the RG-I fraction. This would suggest a random coil

335 conformation for the RG-I region and a less flexible conformation for both pectin A and HG

336 regions. These results are consistent with previous findings which suggest that sugar beet; apple

337 and flax pectins have flexible structures (Ralet, *et al*., 2008b; Axelos, & Thibault, 1991; Cros, *et*

338 *al*., 1996) and that HG–rich regions (or pectins) are considerably stiffer (Cros, Garnier, Axelos,

339 Imbery, & Perez, 1996; Braccini, Grasso, & Perez, 1999; Noto, Martorana, Bulone, & San

340 Biagio, 200; Morris, *et al*., 2008). The values of the mass per unit lengths (**Figures 6A-C** and

341 **Table 2**) indicate that both sugar-beet pectin A and the RG-I fraction are highly branched (hairy)

342 and the HG region is essentially unbranched (smooth), although we would expect the value for

343 the RG-I region to be higher than that of pectin A. Overall flexibility can be estimated from the

344 ratio of L_p/M_L (nm^{2}mol/ g) which decreases with increasing flexibility (Patel, Morris,

345 Ebringerová, Vodenicarová, Velebny, Ortega, García de la Torre, & Harding, 2008). Therefore

346 we can see that in terms of flexibility RG-I > pectin A > HG $\approx$ citrus pectin (**Table 2**).

347

348 **3.7.2. Conformation zoning (Normalised scaling relations)**

349 Pavlov, *et. al.* (1999) described a new procedure to represent the conformation of polymers in

350 solution based on the relationship between their molar mass, intrinsic viscosity and mass per unit

351 length, M_L. In this case we have taken the mass per unit length calculated previously using the

352 HYDFIT algorithm (Ortega, & García de la Torre, 2007). As we can see from **Figure 7** both

353 pectin A and the HG fraction have conformations which fall in either the rigid rod or semi-
354 flexible coil zones this is in agreement with findings on citrus pectins (Morris, *et. al.*, 2008) (N. B.
355 the high value of M_L for pectin A may lead to an overestimation of the rigidity). Whereas the
356 RG-I region clearly adopts a random coil conformation, which is consistent with a persistence
357 length of 1.4 nm (Tombs, & Harding, 1998). This is again consistent with a more flexible
358 structure for the RG-I fraction.
359

360 **4.Conclusions**

361 Acid extracted sugar beet pectin was shown to be heterogeneous in terms of composition and it is
362 proposed that a high molar mass ferulic acid-rich fraction may be the result of diferulic acid
363 bridging between pectin molecules and more specifically between the RG-I regions of pectin
364 molecules.

365

366 The enzymatic treatment of pectin A followed by AEC and SEC resulted in a RG-I fraction
367 which was now free of the HG component. This RG-I fraction was shown to be of high weight
368 average molar mass (188000 g/mol), but low intrinsic viscosity (36 ml/g), which is consistent
369 with a random coil conformation. This RG-1 fraction has a GalA: Rha ratio of 1.3: 1.0 and is
370 composed of almost 80 % neutral sugars and 2.4 % ferulic acid.

371

372 The HG fraction of acid extracted pectin was also characterised, although without further
373 purification and was found, to have a relatively low weight average molar mass (20000 g/mol),
374 but a rather high intrinsic viscosity (77 ml/g), which is consistent with previous molar mass and
375 intrinsic viscosity estimates (Thibault, *et. al.*, 1993; Bonnin, *et. al.*, 2002b; Ralet, *et al*., 2008b)
376 and with the HG region being rigid in solution.

377

378 In general we can conclude that the degradation of the HG region(s) has an important impact on
379 intrinsic viscosity, but less on molar mass and the inverse is true for the degradation of RG-I
380 region.

381

385

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521

522 **Table 1** Chemical composition of pectin A and its RG-I fraction

Sample	Sugar composition, mol %			Ferulic acid content, %[1]
	Gal A	Rha	Other neutral sugars	
Pectin A[2]	68.6	8.4	22.2	0.8
APG24F1 (Pectin A neutral sugar fraction)	n.d.	n.d.	99	2.0
APG24F10a (RG-I fraction)	21.9	16.7	61.5	2.4

523

524 [1]% FA = 100 % x ($UV_{calculated\ mass}$/ $RI_{calculated\ mass}$)

525 [2]does not include the peak shown to consist almost entirely (99 %) of neutral sugars *i.e.*

526 APG24F1 after anion exchange chromatography

527

528 N.B. For the chemical composition of low molecular weight enzymatic hydrolysis products

529 (APG24F2 - APG24F9) see Ralet, *et. al.* (2005a)

530
531

532   **Table 2** Physical and conformational properties of sugar beet pectin A and its HG and RG-I
533   fractions. Values for a "typical" low methoxyl citrus pectin (P_{19}) are shown for comparison[1].

Sample	M_w, g/ mol	$[\eta]_w$, ml/ g	M_L, g/ mol nm	L_p, nm	L_p/M_L, nm^{2}mol/ g
A[2]	286000	285	620	7.3	0.0118
RG-I	188000	36	555	1.4	0.0025
HG	20000	77	395	9.8	0.0248
P_{19}	165000	395	330	10.0	0.0303

534

535   [1]from Morris, *et al*., 2008

536   [2]does not included the peak shown to consist entirely of neutral sugars *i.e.* APG24F1 after anion

537   exchange chromatography

538
539

Legends to figures

"Native" Sugar Beet Pectin

HCl

HG fraction

PG / PME

+

etc

AEC / SEC

RG-I fraction
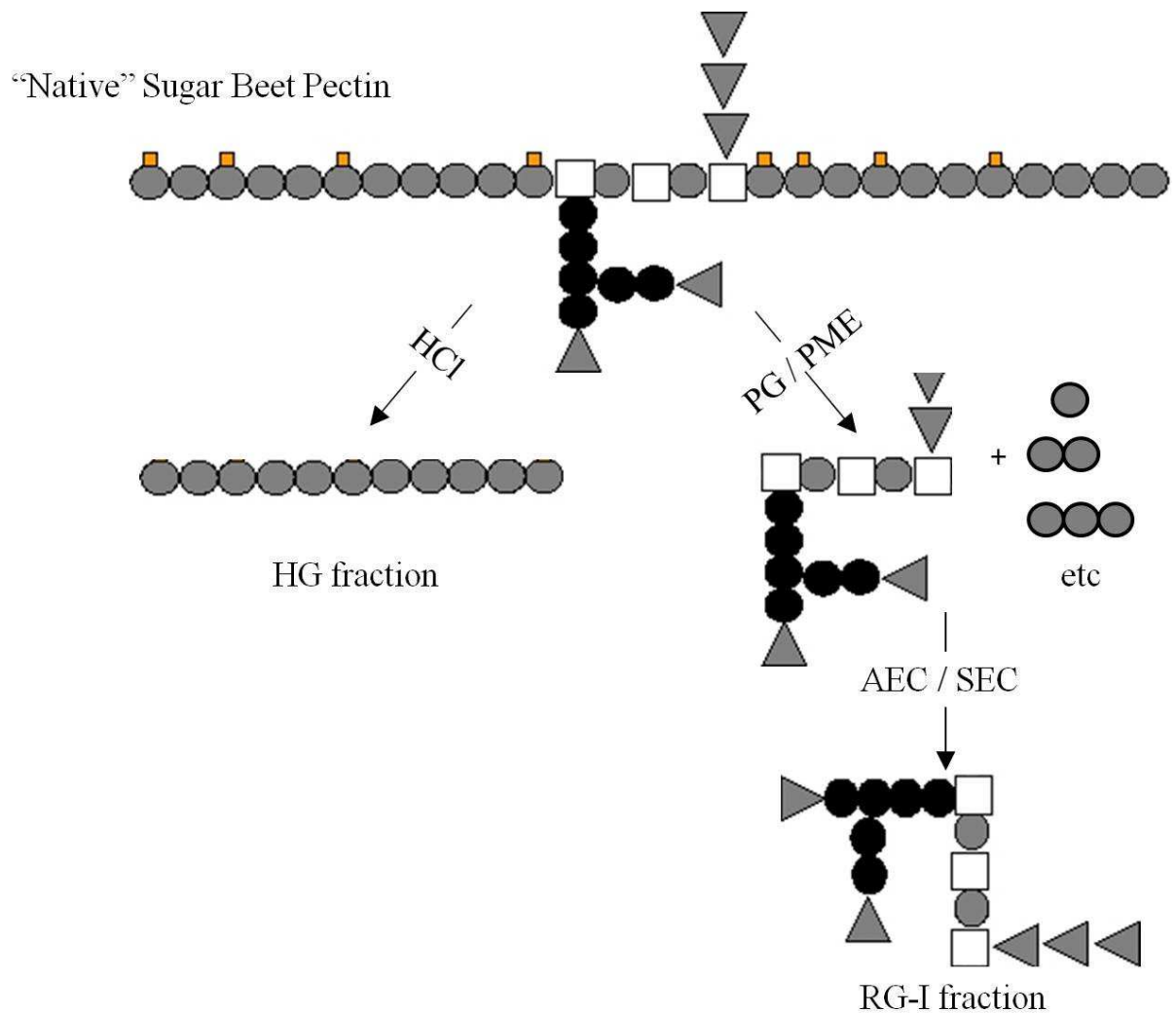
541
542 **Figure 1 -** Schematic structure for generalised sugar beet pectin: galacturonic acid (●); galactose
543 (●); arabinose (▼); rhamnose (□) and methyl groups (■). Adapted from Perez et al., 2003. PG -
544 endopolygalacturonase I and II from *A. niger*; PME - fungal pectin methyl esterase from *A.*
545 *aculeatus*; AEC – anion exchange chromatography and SEC – size exclusion chromatography.
546

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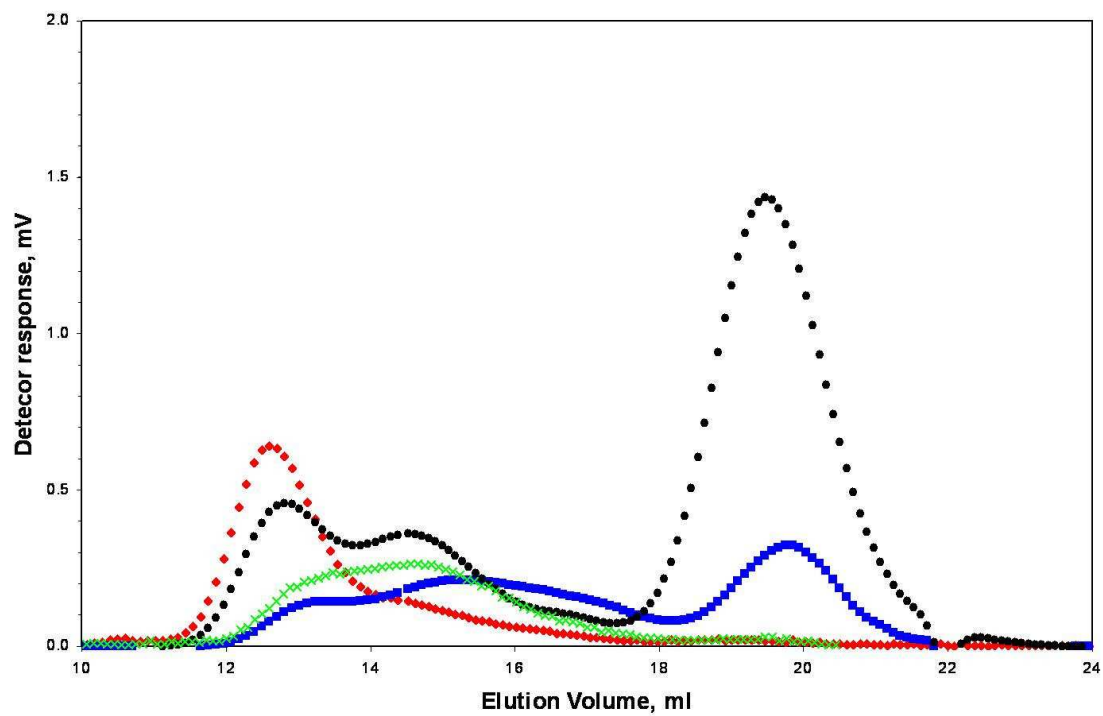
547
548 **Figure 2** - Multi-detector HPSEC chromatogram for pectin A: LS 90° (♦), RI (■), UV 325 nm
549 (•) and DPV (x). N. B. The LS 90 ° and DPV signals have been multiplied by 5 to improve
550 visualisation.
551
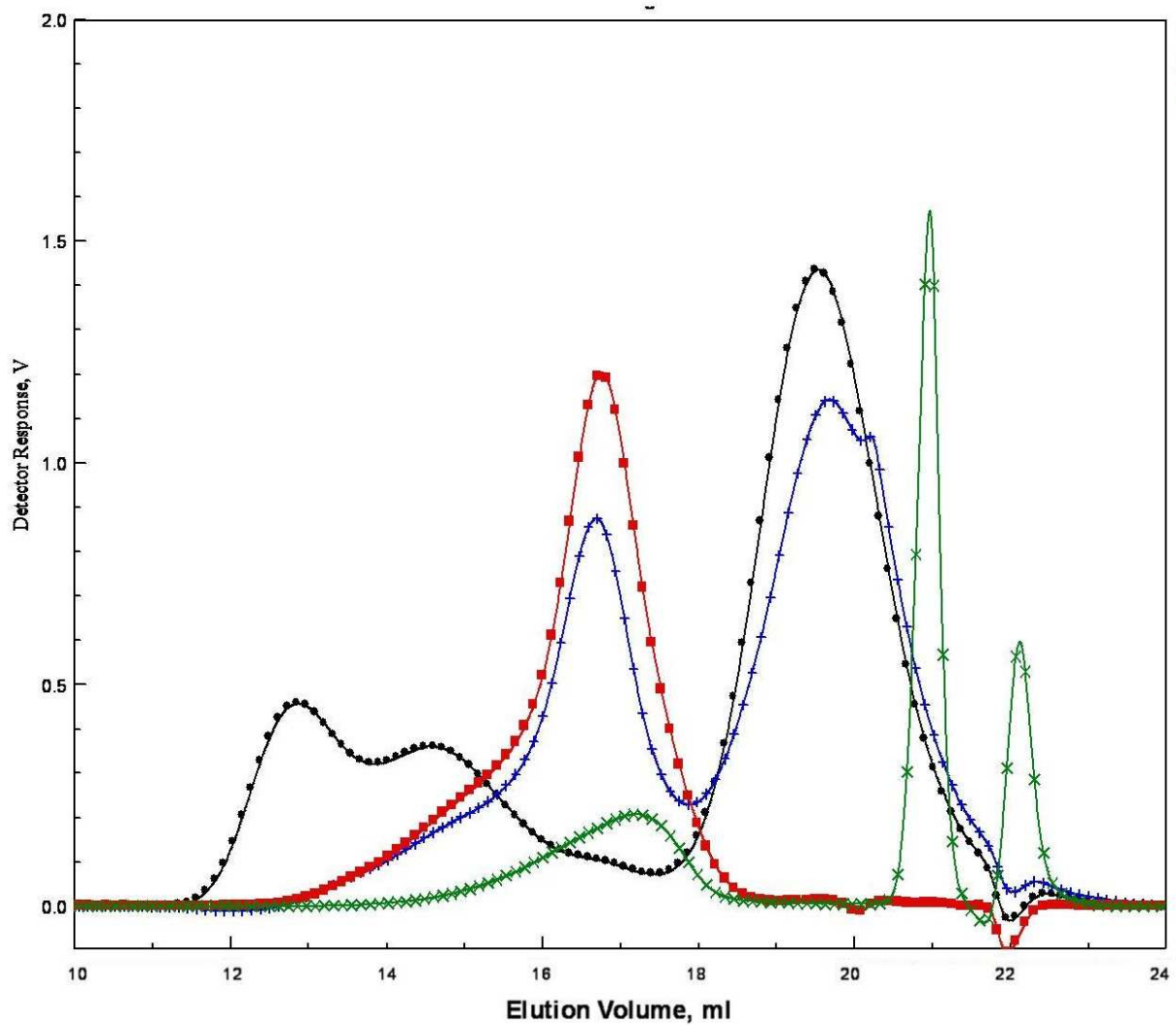
552
Figure 3 - HPSEC chromatogram for Pectin A (●), APG24 (+), APG24F10a (■) and AHCl72
(x). N.B. The chromatogram for AHCl72 is an RI trace; all others are UV absorbance at 325nm.
AHCl72 has contains no ferulic acid and therefore shows no UV signal at 325nm.
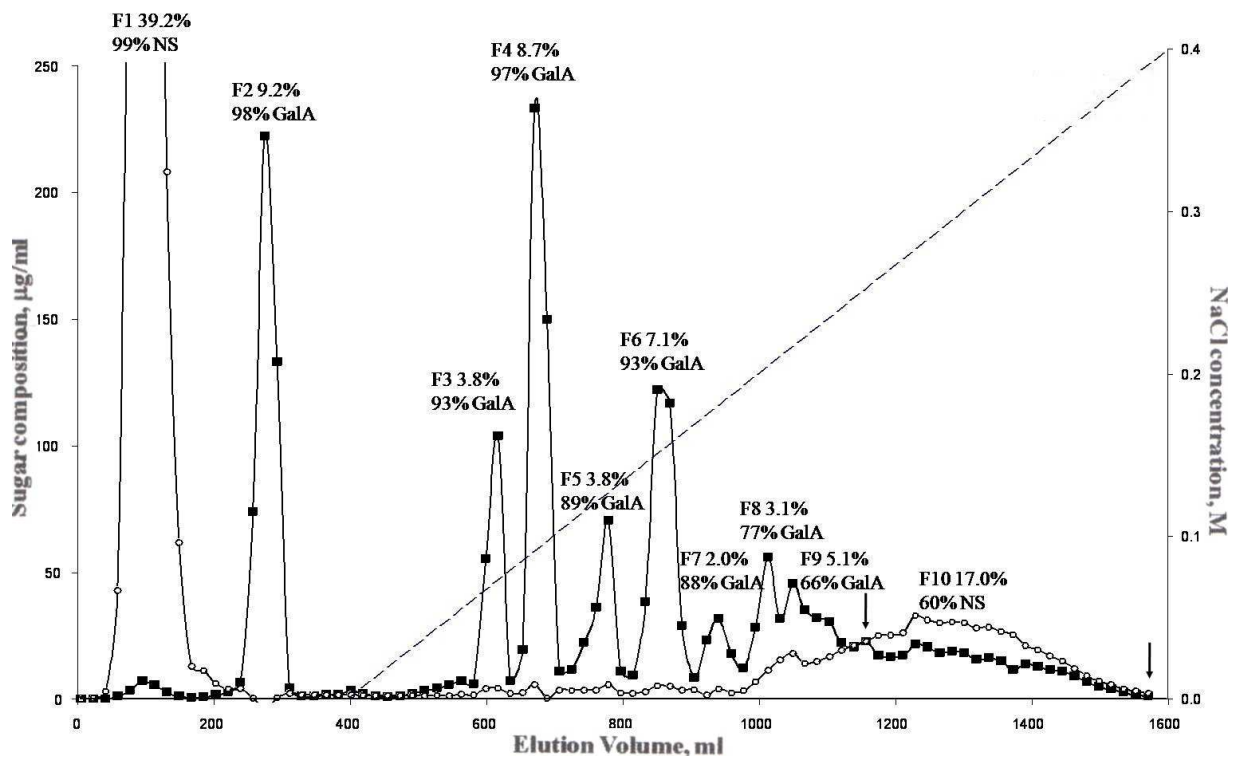
557
558 **Figure 4 -** Anion exchange chromatogram (DEAE-Sepharose CL 6B) for pectin A after
559 enzymatic hydrolysis for 24 hours at 30 °C with f-PME, PGs I and II (-■- Gal A and -○- neutral
560 sugars). The proportion of each fraction present is indicated, together with the major constituent:
561 galacturonic acid (GalA) or total neutral sugars (NS). Fraction APGF10 which will be further
562 purified by SEC is indicated by the arrows.
563
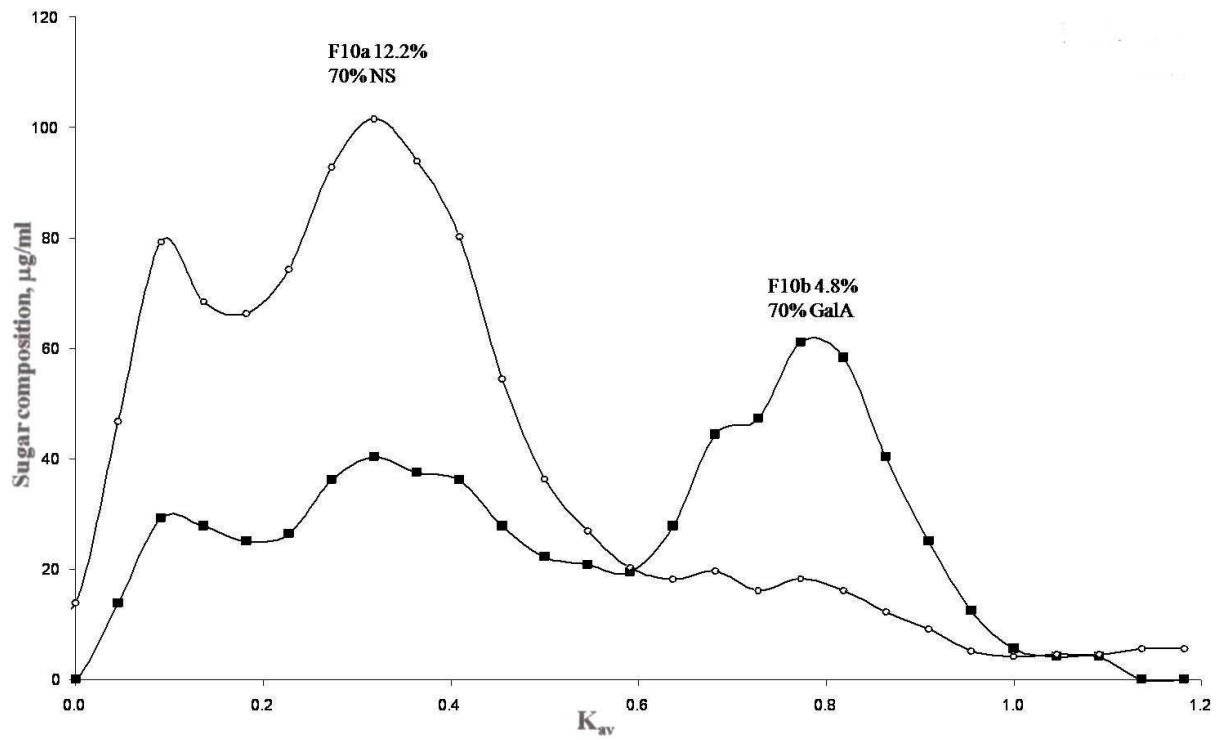
564

565 **Figure 5 -** Size exclusion chromatogram (Sephacryl S-200) for APGF10 (-■- Gal A and -○-

566 neutral sugars). The proportion of each fraction present is indicated, together with the major

567 constituent: galacturonic acid (GalA) or total neutral sugars (NS).

568

569

570   **Figure 6** - Solutions to the Bushin-Bohdanecky using the HYDFIT algorithm (Ortega and García

571   de la Torre, 2007). The x-axis and y-axis represent L_p (nm) and M_L (g/ mol nm) respectively.

572   The target function, Δ is calculated over a range of values for M_L and L_p. In these

573   representations, the values of Δ function are represented by the full colour spectrum, from the

574   minimum in the target function in blue ($\Delta = 0.5$) to red ($\Delta \geq 1.5$). The calculated minima are

575   indicated ($\odot$).

576
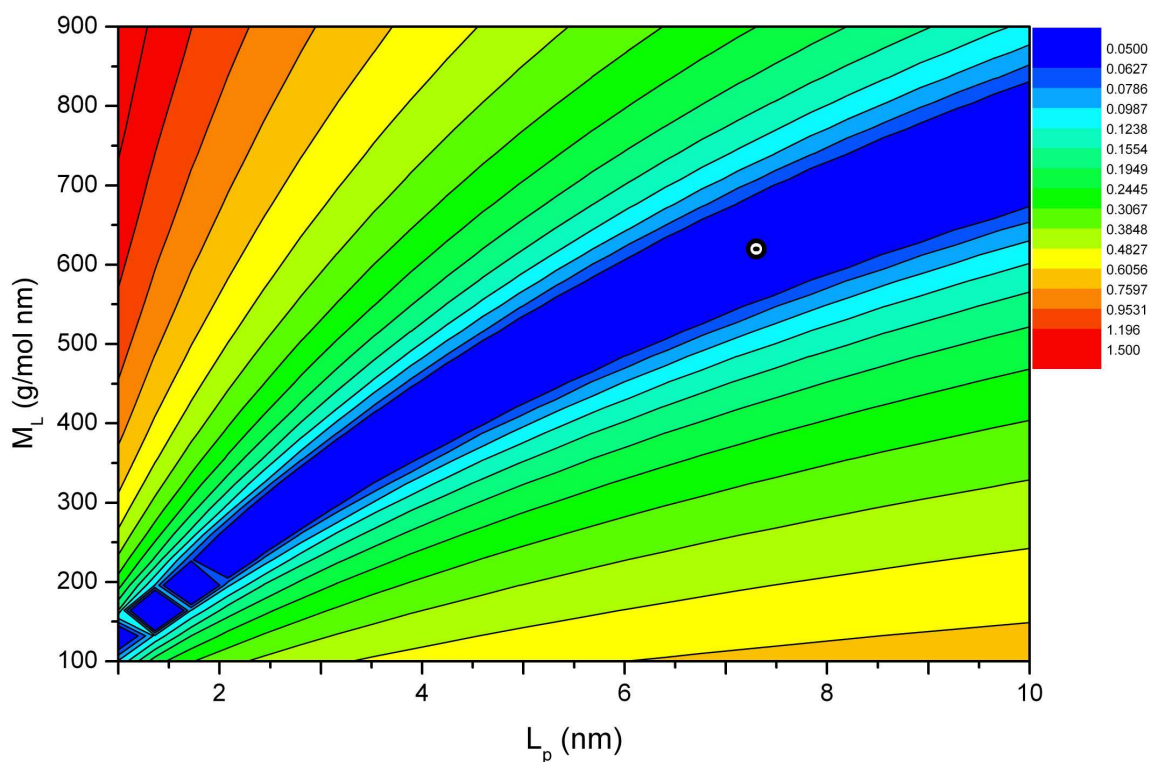
577
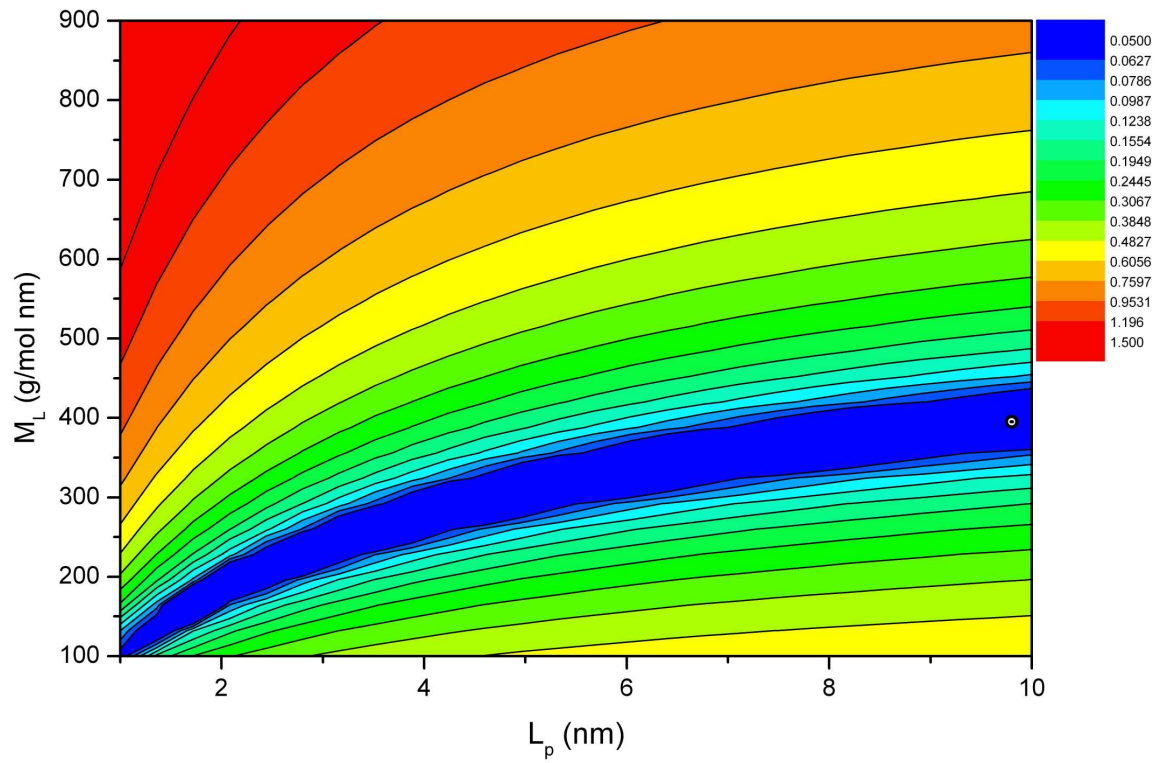578   **A** - Pectin A ($L_p = 7.3$ nm and $M_L = 620$ g/ mol nm)
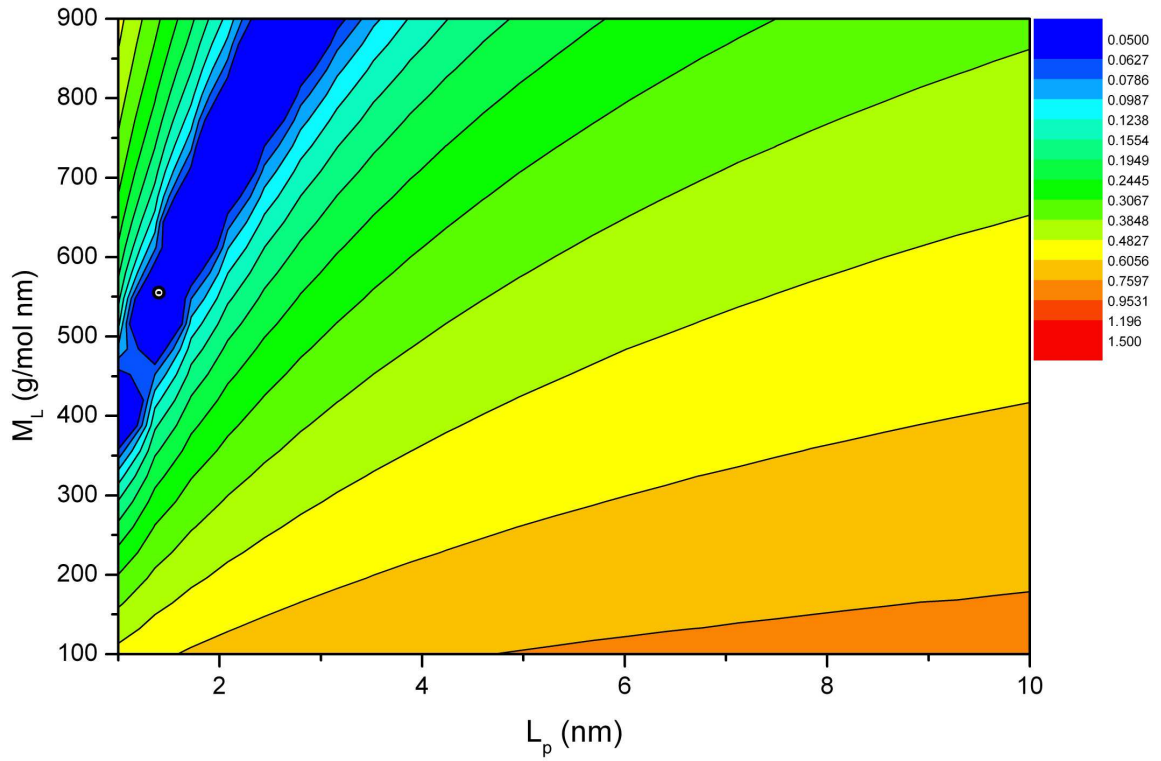
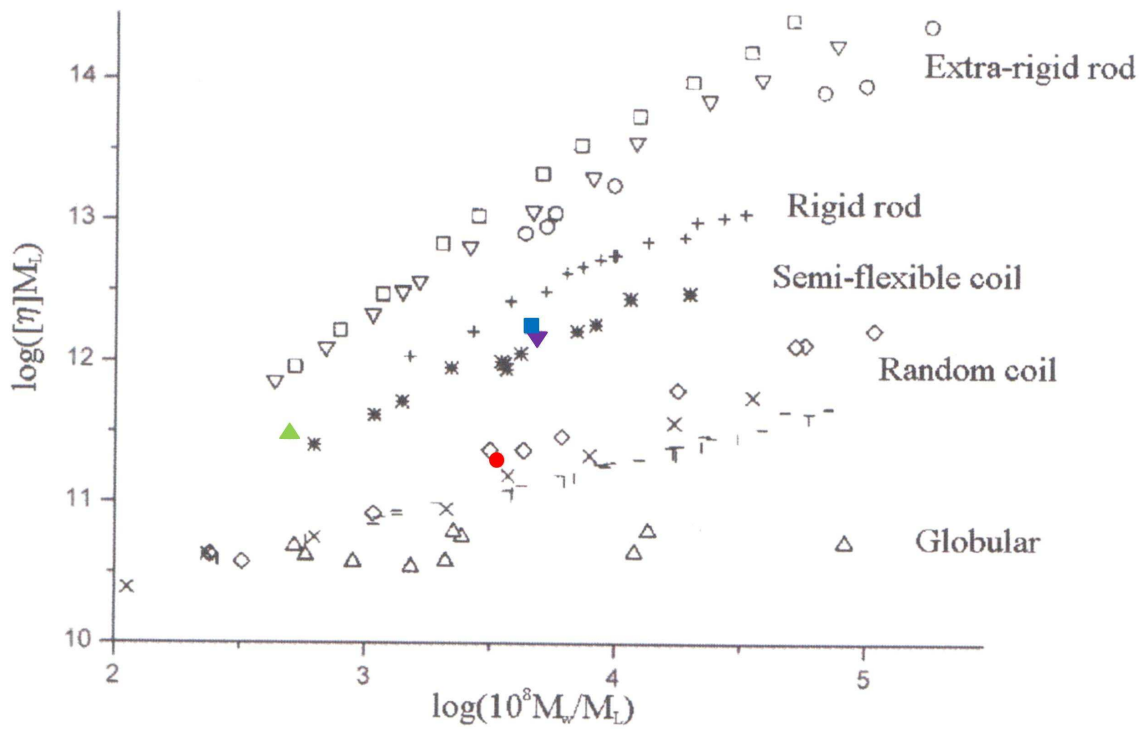581   **B** - RG-I fraction (L_p = 1.4 nm and M_L = 555 g/ mol nm)

584   **C** - HG fraction (L_p = 9.8 nm and M_L = 395 g/ mol nm)

585

Figure 7 - Normalised scaling plot of $[\eta]M_L$ versus M_w/M_L (adapted from Pavlov, *et. al.*, 1999) where the solution conformations for Pectin A (■) and its RG-I (●) and HG (▲) fractions are indicated a typical citrus pectin is shown for comparison (▼). All other symbols are as defined previously (Pavlov, *et. al.*, 1999) in brief: schizophyllan (□), DNA (○), globular proteins (Δ), xanthan (∇), poly(1-vinyl-2-pyrrolidone) (◊), cellulose nitrate (+), pullulan (x), methyl cellulose (*), poly-α-methylstyrene (-) and polystyrene (│).