

1 **Calcium binding and calcium-induced gelation of low-methoxyl pectin modified by**  
2 **low molecular-weight polyuronate fraction**

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4 Makoto Nakauma<sup>a,\*</sup>, Takahiro Funami<sup>a</sup>, Yapeng Fang<sup>b,\*\*</sup>, Katsuyoshi Nishinari<sup>b</sup>, Kurt I.  
5 Draget<sup>c</sup>, Glyn O. Phillips<sup>d</sup>

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7 <sup>a</sup> Texture Design Laboratory, San-Ei Gen F.F.I., Inc., 1-1-11, Sanwa-cho, Toyonaka,  
8 Osaka 561-8588, Japan

9 <sup>b</sup> Glyn O. Phillips Hydrocolloid Research Centre at HUT, School of Food and  
10 Pharmaceutical Engineering, Faculty of Light Industry, Hubei University of Technology,  
11 Wuchang, Wuhan 430068, China

12 <sup>c</sup> Norwegian Biopolymer Laboratory, Department of Biotechnology, Norwegian  
13 University of Science and Technology, N-7491 Trondheim, Norway

14 <sup>d</sup> Phillips Hydrocolloids Research Ltd., 45 Old Bond Street, London W1S 4AQ, UK

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16 \* Corresponding author. [m-nakauma@saneigenffi.co.jp](mailto:m-nakauma@saneigenffi.co.jp)

17 \*\* Corresponding author. [fangypphrc@163.com](mailto:fangypphrc@163.com)

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19 **Abstract**

20 The functions of low molecular-weight polyuronate fraction in the calcium binding  
21 and calcium-induced gelation of normal low-methoxyl pectin (LMP) were investigated.  
22 Pectin fractions with different degrees of esterification (DE) and alginate fractions with  
23 different mannuronate/guluronate (M/G) ratios were prepared. Weight average  
24 molecular-weight ( $M_w$ ) of each low molecular-weight polyuronate fraction ranged from  
25 ca. 40,000 to 65,000 g/mol. In the mixtures of LMP and each low molecular-weight  
26 polyuronate fraction, changes in the relative viscosity ( $\eta_r$ ) of dilute solutions and in  
27 rheological properties of gels were examined in the presence of calcium. The addition  
28 of low molecular-weight pectin fraction, regardless of DE, increased  $\eta_r$  of dilute  
29 solutions and increased dynamic storage modulus ( $G'$ ) of gels with greater effects at  
30 lower DE. On the contrary, the addition of low molecular-weight alginate fraction,  
31 regardless of M/G ratio, shifted the critical threshold calcium concentration required to  
32 steepen  $\eta_r$  of dilute solutions higher and decreased  $G'$  of gels with greater effects at  
33 lower M/G ratio (i.e. rich in G). Gelation behavior of the mixture was schematically  
34 presented, and the functions of low molecular-weight polyuronate fraction were  
35 compared on the molecular level between pectin and alginate.

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37 **Keywords:** Calcium binding; Egg-box dimer; Gelation; Low-methoxyl pectin; low  
38 molecular-weight polyuronate

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## 40 **1. Introduction**

41 Calcium-binding behavior of polysaccharides with polyuronate backbone has been  
42 investigated extensively as in the case of pectin and alginate. For pectin, it has been  
43 reported that degree of esterification (DE) and weight average molecular-weight ( $M_w$ )  
44 both influence pectin gelation in terms of gel strength and the kinetics of gel formation,  
45 and the functions relate to affinity and sensitivity to calcium (Hotchkiss et al., 2002;  
46 Luzio & Cameron, 2008; Ralet, Dronnet, Buchholt, & Thibault, 2001; Thibault &  
47 Rinaudo, 1985). Our research team investigated previously (Nakauma et al., 2016) the  
48 calcium binding and calcium-induced gelation of normal sodium alginate modified by  
49 low molecular-weight polyuronate fractions. It was clarified that the addition of the  
50 alginate fraction shifted the critical threshold calcium concentration required to steepen  
51 the relative viscosity ( $\eta_r$ ) of dilute solutions higher and decreases dynamic storage  
52 modulus ( $G'$ ) of gels and that these effects of the alginate fraction depended both on  $M_w$   
53 and mannuronate/guluronate (M/G) ratio. It was also clarified in the same report that  
54 the addition of low molecular-weight G-rich alginate fraction improved the water

55 holding capacity of calcium-induced alginate gels and made the gels more rheologically  
56 deformable represented by increased yield strain. These results indicated the potential  
57 usage of the G-rich alginate fraction as a novel texture modifier. On the other hand,  
58 effects of low molecular weight low-methoxyl pectin fraction were quite different from  
59 those of the G-rich alginate fraction, and in the mixture of the pectin fraction and  
60 normal sodium alginate, viscosity increase of dilute solutions was detected at a calcium  
61 feed even below the stoichiometry of egg-box dimers. Also, mechanical strength of  
62 calcium-induced sodium alginate gels was increased by the addition of the pectin  
63 fraction as represented by increased  $G'$ . As a series of the study, the functions of low  
64 molecular-weight polyuronate fraction in the calcium binding and calcium-induced  
65 gelation of normal low-methoxyl pectin (LMP with  $M_w$  of ca. 150,000 g/mol) was  
66 investigated in the present study, and the effects of the polyuronate fraction on the  
67 molecular association with LMP were compared between pectin and alginate.

## 68 **2. Materials and methods**

### 69 *2.1. Materials*

70 Pectins from citrus with different DE values (SAN-SUPPORT<sup>®</sup> P-160 for  
71 high-methoxyl pectin and SAN-SUPPORT<sup>®</sup> P-161 for low-methoxyl pectin) and  
72 sodium alginate (SAN-SUPPORT<sup>®</sup> P-80) were provided as commercial products by

73 San-Ei Gen F.F.I., Inc. (Osaka, Japan). Other materials used and the definition of  
74 enzyme unit were the same as previous study (Nakauma et al., 2016). The following  
75 abbreviations were used for convenience throughout this study:

76 LMP low-methoxyl pectin; HMP high-methoxyl pectin; SAL sodium alginate; MAN  
77 polymannuronate; GUL polyguluronate.

## 78 *2.2. Preparation of pectin fractions*

79 Low molecular-weight pectin fractions with different DE values was prepared using  
80 HMP (SAN-SUPPORT<sup>®</sup> P-160) as a starting material and combination of enzymatic  
81 hydrolysis and de-esterification in the same procedure as reported previously (Nakauma  
82 et al., 2016). In brief, HMP with pectinase treatment but without esterase treatment  
83 was identified as  $LM_w$ -HMP, whereas that with both treatments was identified as  
84  $LM_w$ -LMP.

85 Macromolecular characteristics of the pectin fractions and LMP (SAN-SUPPORT<sup>®</sup>  
86 P-161), including  $M_w$ , number average molecular-weight  $M_n$ , radius of gyration  $R_g$ ,  
87 polydispersity index defined by  $M_w/M_n$ , and the Flory exponent  $\nu$ , were determined by  
88 size-exclusion chromatography coupled with a multiangle laser light scattering  
89 photometer (SEC-MALS) as reported previously (Nakauma et al., 2016). As  
90 physicochemical characteristics, constitutional sugars were identified by

91 high-performance anion-exchange chromatography coupled with pulsed amperometric  
92 detection (HPAEC-PAD), whereas DE was determined spectrophotometrically as  
93 reported previously (Nakauma et al., 2016). These characteristics were summarized in  
94 Table 1.

### 95 *2.3. Preparation of alginate fractions*

96 Low molecular-weight alginate fractions with different M/G ratios were prepared  
97 using SAL (SAN-SUPPORT® P-80) as a starting material and combination of acid  
98 hydrolysis and pH-based fractionation in the same procedure as reported previously  
99 (Nakauma et al., 2016). Exceptions from previous report were heating condition for  
100 hydrolysis ; 1 h and pH conditions for recovery of G-rich fraction (identified as  
101  $LM_w$ -GUL);3.8 and for recovery of M-rich fraction (identified as  $LM_w$ -MAN); 2.4.

102 Macromolecular characteristics of the alginate fractions and SAL were determined by  
103 SEC-MALS as reported previously (Nakauma et al., 2016). As physicochemical  
104 characteristics, G content and G-block length (the length of G-block larger than 1) were  
105 determined by a nuclear magnetic resonance NMR spectrometry as reported previously  
106 (Nakauma et al., 2016). These characteristics were summarized in Table 2.

### 107 *2.4. Relative viscosity measurement of dilute solutions*

108 For the mixture of LMP and each low molecular-weight polyuronate fraction,

109 changes in  $\eta_r$  by calcium addition were measured at 25 °C using an Ubbelohde type  
110 capillary viscometer as reported previously (Nakauma et al., 2016). Concentration of  
111 LMP in the mixture was fixed at 0.05%, whereas those of each low molecular-weight  
112 polyuronate fraction were 0.01%, 0.02%, and 0.05%.  $\eta_r$  of dilute solutions was  
113 determined as  $t_s/t_0$ , where  $t_s$  is the flow time for test solutions (either the mixture or  
114 LMP alone) titrated by 7.5 mM CaCl<sub>2</sub> solution, and  $t_0$  is the flow time for the solvent;  
115 20 mM acetate buffer (pH 5.0). To eliminate the dilution effect by the addition of  
116 CaCl<sub>2</sub> solutions during titration,  $\eta_r$  was normalized:

$$117 \quad \eta_r^N = \eta_r^{Ca} / \eta_r^C$$

118 Here  $\eta_r^{Ca}$  is the relative viscosity in calcium titration, and  $\eta_r^C$  is the relative viscosity  
119 in buffer titration (Fang et al., 2008). Data were presented as means  $\pm$  SD of triplicate.

### 120 *2.5. Rheological measurements of gels*

121 For the mixture of LMP and each low molecular-weight polyuronate fraction,  
122 rheological properties of gels were measured at 25 °C using a strain-controlled  
123 rheometer in an oscillation shear mode as reported previously (Nakauma et al., 2016).  
124 Concentration of LMP in the mixture was fixed at 0.8%, where as those of each low  
125 molecular-weight polyuronate fraction were 0.2%, 0.4%, and 0.8%. Dynamic  
126 viscoelasticity measurements, including frequency sweep and strain sweep tests, were

127 applied to gels formed by curing of the mixture at 25 °C for at least 20 min to reach to  
128 pseudosaturation. From the frequency sweep test, some rheological parameters were  
129 determined, including constant  $K_f$  and exponent  $n_f$ , based on the power-law relationship  
130 between frequency  $\omega$  and complex viscosity  $\eta^*$  (Keogh & O' Kennedy, 1998):

$$131 \quad \eta^*(\omega) = K_f \omega^{n_f} \quad (0 < n_f < 1)$$

132 From the strain sweep test, some rheological parameters were determined, including  
133 constant for the higher modulus component  $K_{s1}$ , constant for the lower modulus  
134 component  $K_{s2}$ , exponent for the higher modulus component  $n_{s1}$ , and exponent for the  
135 lower modulus component  $n_{s2}$  in the following dual exponential equation:

$$136 \quad G'(\gamma) = K_{s1} \exp(-n_{s1} \times \gamma) + K_{s2} \exp(-n_{s2} \times \gamma)$$

137 In addition, the yield strain was identified as a peak in the plot of the elastic stress ( $G'$   
138 multiplied by strain) as a function of strain (Walls, Caines, Sanchez, & Khan, 2003).

139 Data were presented as means  $\pm$  SD of triplicate for each rheological parameter.

#### 140 *2.6. Statistics*

141 Data were analyzed by t-test to know the statistical difference from the control with a  
142 significance defined at  $p < 0.05$  or  $0.01$  at both sides using Microsoft Excel 2013  
143 (Redmond, WA).

144 **3. Results and discussion**

145 *3.1. Relative viscosity measurement of dilute solutions*

146 *3.1.1. Mixture of LMP and low molecular-weight pectin fraction*

147  $\eta_r^N$  of LMP alone (i.e. control) increased monotonously with increased concentration  
148 of calcium (in mM), and this was also the case for the mixture with either  $LM_w$ -HMP or  
149  $LM_w$ -LMP (Fig. 1a & b). For the mixture, increasing degree of  $\eta_r^N$  was larger with  
150 increased addition level of low molecular-weight pectin fraction in general, and the  
151 deviation from the control was enlarged with increased calcium feed.  $LM_w$ -LMP was  
152 more effective than  $LM_w$ -HMP in these regards.  $\eta_r^N$  was replotted as a function of  
153  $R_{\text{total fGal}}$ ; the molar ratio of fed calcium to free galactose residues from both LMP and  
154 the pectin fraction (Fig. 1c & d). In this plot,  $\eta_r^N$  reached peaked or saturated at  $R_{\text{total}}$   
155  $f_{\text{Gal}}$  of 2.14 for the control, whereas  $\eta_r^N$  did so at 1.41 for the mixture with 0.05%  
156  $LM_w$ -HMP and at 0.89 for the mixture with 0.05%  $LM_w$ -LMP. It is likely for each low  
157 molecular-weight pectin fraction, particularly  $LM_w$ -LMP, to associate with LMP due to  
158 molecular similarity from thermodynamic point of view. The addition of the pectin  
159 fraction, particularly  $LM_w$ -LMP, can act as a low molecular weight cross-linker to  
160 increase the hydrodynamic size of LMP. This contributes to increased exclusion  
161 volume and thus increased  $\eta_r^N$ . Differed from previous study using SAL (Nakauma et

162 al., 2016), decrease in  $\eta_r^N$  at low calcium feed (i.e.  $R_{\text{total fGal}} < 0.25$ ) was not detected in  
163 the LMP control or the mixture with low molecular-weight pectin fraction. This  
164 indicates that monocomplexation should hardly occur for LMP at low calcium feed, and  
165 thus the pectin fraction has no impact on that molecular event. Calcium-binding  
166 behavior of LMP is less critical than that of SAL due to sequential irregularity of  
167 calcium binding site (Winning, Viereck, Norgaard, Larsen, & Engelsen, 2007), and  
168 egg-box dimer formation can start even when theoretical calcium/galacturonate  
169 stoichiometry (i.e. 0.25; 1 mol calcium/4 mol galacturonate) is not achieved (Fang et al.,  
170 2008). This can be a cause for absence of the initial critical threshold concentration of  
171 calcium in the LMP control or the mixture with low molecular-weight pectin fraction.  
172 The second critical threshold concentration of calcium, which indicates the initiation of  
173 lateral associations of egg-box dimer starting theoretically at the calcium/galacturonate  
174 stoichiometry of 0.55 (Fang et al., 2007), was obscure in the LMP control or the mixture  
175 with low molecular-weight pectin fraction, which is another difference from previous  
176 study using SAL. If the peak in  $\eta_r^N$  corresponds to the second critical threshold  
177 concentration, it is anticipated that low molecular-weight pectin fraction, particularly  
178  $LM_w$ -LMP, can promote the associations of LMP in some way.

179 3.1.2. Mixture of LMP and low molecular-weight alginate fraction

180  $\eta_r^N$  increased monotonously with increased concentration of calcium (in mM)  
181 followed by a peak in some cases for the mixture with either  $LM_w$ -MAN or  $LM_w$ -GUL  
182 at each addition level (Fig. 2a & b). In contrast to low molecular-weight pectin  
183 fraction, increasing degree of  $\eta_r^N$  was smaller with increased addition level of low  
184 molecular-weight alginate fraction, and the deviation from the control was enlarged  
185 with increased calcium feed.  $LM_w$ -GUL was more effective than  $LM_w$ -MAN in these  
186 regards.  $\eta_r^N$  was replotted as a function of  $R_{fGal+Gul}$ ; the molar ratio of fed calcium to  
187 free galacturonate residues from LMP and free guluronate residues from alginate  
188 fraction (Fig. 2c & d). No substantial difference was observed between plots for the  
189 mixture with  $LM_w$ -MAN. On the other hand,  $\eta_r^N$  for the mixture with  $LM_w$ -GUL was  
190 lower than that for the control when the stoichiometry was lower than 0.25 and almost  
191 overlapped with the control within the stoichiometry range from 0.5 to 1.0 at each  
192 addition level of  $LM_w$ -GUL.  $\eta_r^N$  for the mixture with  $LM_w$ -GUL was again lower than  
193 that for the control when the stoichiometry was higher than 1.0, and this effect was  
194 enhanced with increased addition level of  $LM_w$ -GUL. It is unlikely for low  
195 molecular-weight alginate fraction, particularly  $LM_w$ -MAN, to associate with LMP from  
196 thermodynamic point of view, and molecular associations can occur separately and

197 independently between LMP and the alginate fraction. It is thus anticipated that the  
198 decrease in  $\eta_r^N$  by the addition of  $LM_w$ -MAN should be mainly due to its chelating  
199 effect. In the mixture with  $LM_w$ -GUL, monocomplexation and subsequent egg-box  
200 dimer formation of  $LM_w$ -GUL can occur prior to molecular associations of LMP. This  
201 may be reasonable when differences in the chain length, sequential regularity of calcium  
202 binding site, and molecular conformation between LMP and  $LM_w$ -GUL are considered.  
203 Contribution of  $LM_w$ -GUL (even after self-associations) to  $\eta_r^N$  should be lower than that  
204 of LMP, and this can explain the  $\eta_r^N$  behavior at low calcium feed. On the other hand,  
205 macroscopic phase separation between LMP and  $LM_w$ -GUL can explain the  $\eta_r^N$   
206 behavior at high calcium feed.

### 207 *3.2. Rheological measurements of gels*

#### 208 *3.2.1. Mixture of LMP and low molecular-weight pectin fraction*

209 Concentration of calcium fed to the system was 20 mM in theory, corresponding to  
210  $R_{\text{total fGal}} = 1.06$  for 0.8% LMP alone (i.e. control), and the addition of low  
211 molecular-weight pectin fraction increased the content of free galacturonate residues in  
212 the system and thus decreased  $R_{\text{total fGal}}$  (Table 3). From the stoichiometry point of view,  
213 calcium feed should be sufficient for LMP to form egg-box dimers and multimers  
214 except for the mixture with 0.08%  $LM_w$ -LMP, in which  $R_{\text{fGal+Gul}}$  was smaller than 0.55,

215 theoretical calcium/galacturonate stoichiometry for starting lateral associations of  
216 egg-box dimer (Fang et al., 2007). From the strain sweep test, no difference was found  
217 in the yield strain between the LMP control and the mixture with  $LM_w$ -HMP at each  
218 addition level, whereas the sum of  $K_{s1}$  and  $K_{s2}$  (i.e. equilibrium  $G'$  in the linear  
219 viscoelastic regime) for the mixture increased with increased addition level of  
220  $LM_w$ -HMP (Table 3). From the frequency sweep test, no difference was found in the  
221 power-law exponent  $n_f$  between the LMP control and the mixture with  $LM_w$ -HMP at  
222 each addition level (Table 3). Also,  $G'$  for the mixture with  $LM_w$ -HMP was almost  
223 independent of frequency from 0.1 to 100 rad/s and increased with increased addition  
224 level of  $LM_w$ -HMP in the whole frequency range tested (Fig. 3a). These results  
225 indicate that the addition of  $LM_w$ -HMP should not alter the nature of inter-molecular  
226 associations of LMP and strengthen the super-molecular structure of LMP. This  
227 accords qualitatively with the  $\eta_t^N$  profile in dilute solutions. For the mixture with  
228  $LM_w$ -LMP, the yield strain decreased with increased addition level of  $LM_w$ -LMP in  
229 general, whereas the sum of  $K_{s1}$  and  $K_{s2}$  increased with increased addition level of  
230  $LM_w$ -LMP.  $LM_w$ -LMP showed a greater effect in increasing the equilibrium  $G'$  than  
231  $LM_w$ -HMP even though the calcium feed per binding site (represented by  $R_{total} f_{Gal}$ ) was  
232 lower when compared at the same addition level (Table 3). No difference was found in

233  $n_f$  between the LMP control and the mixture with  $LM_w$ -LMP at each addition level  
234 (Table 3). Similar to the case of  $LM_w$ -HMP,  $G'$  for the mixture with  $LM_w$ -LMP was  
235 almost independent of frequency from 0.1 to 100 rad/s and increased with increased  
236 addition level of  $LM_w$ -LMP in the whole frequency range tested (Fig. 3b).  $LM_w$ -LMP  
237 showed a greater effect in increasing  $G'$  than that  $LM_w$ -HMP when compared at the  
238 same addition level. These results indicate that the addition of  $LM_w$ -LMP should not  
239 alter the nature of inter-molecular associations of LMP and strengthen the  
240 super-molecular structure of LMP, similar to  $LM_w$ -HMP. One marked difference is the  
241 structural brittleness provided by the addition of  $LM_w$ -LMP with the LMP system as  
242 presented by decreased yield strain. This also accords qualitatively with the  $\eta_f^N$  profile  
243 in dilute solutions, and decreased yield strain of gels may correspond to the peak shift to  
244 lower calcium concentration (Fig. 1d).

### 245 *3.2.2. Mixture of LMP and low molecular-weight alginate fraction*

246 From the stoichiometry point of view, calcium feed should be sufficient for LMP to  
247 form egg-box dimers and multimers except for the mixture with 0.08%  $LM_w$ -GUL, in  
248 which  $R_{fGal+Gul}$  was smaller than 0.55 (Table 4). From the strain sweep test, the yield  
249 strain for the mixture with  $LM_w$ -MAN increased with increased addition level of  
250  $LM_w$ -MAN in general, whereas the sum of  $K_{s1}$  and  $K_{s2}$  decreased with increased

251 addition level of  $LM_w$ -MAN (Table 4). From the frequency sweep test,  $n_f$  decreased  
252 with increased addition level of  $LM_w$ -MAN (Table 4). Also,  $G'$  for the mixture was  
253 more frequency dependent, particularly within the frequency range from 0.1 to 1.0 rad/s,  
254 with increased addition level of  $LM_w$ -MAN (Fig. 4a). These results indicate that the  
255 addition of  $LM_w$ -MAN should prevent inter-molecular associations of LMP and weaken  
256 the super-molecular structures. This accords qualitatively with the  $\eta_r^N$  profile in dilute  
257 solutions. Similar results were obtained for the mixture with  $LM_w$ -GUL, but these  
258 effects of  $LM_w$ -GUL were much larger than those of  $LM_w$ -MAN when compared at the  
259 same addition level. It is anticipated that  $LM_w$ -GUL should bind with calcium prior to  
260 LMP and should form microgels or clusters which can prevent the inter-molecular  
261 associations of LMP. Rheological data were obtained at a fixed calcium dose not at a  
262 fixed  $R_{\text{total fGal}}$  or  $R_{\text{fGal+Gul}}$  in the present study. It should be noted that the functions of  
263 low molecular-weight polyuronate fraction in calcium-induced gelation of LMP may be  
264 different at lower  $R$  values than theoretical stoichiometry of forming egg box dimer.

### 265 *3.3. Molecular association mechanism between LMP and low molecular-weight pectin* 266 *fraction in comparison with low molecular-weight alginate fraction*

267 Molecular association during calcium-induced gelation of LMP alone was presented  
268 schematically in comparison with that of SAL alone (Fig. 5). For LMP,

269 intra-molecular and inter-molecular associations occur coincidentally upon calcium  
270 addition, which is quite different from multiple steps and critical behaviors of SAL.  
271 Intra-molecular association leads to the reduction of molecular size and volume, while  
272 inter-molecular association leads to the expansion. It may depend on  $M_w$  of LMP and  
273 also the gelation step which association is dominant, but in the case of LMP used in the  
274 present study, inter-molecular association can be dominant over intra-molecular  
275 association even at low calcium feed, causing the increase in  $\eta_r$  (Ralet, Dronnet,  
276 Buchholt, & Thibault, 2001; Fang et al., 2007). This is quite different from SAL, in  
277 which intra-molecular association occurs dominantly at below the stoichiometry  $R =$   
278 0.25. Absence of the second critical threshold concentration for LMP, which is  
279 detected at  $R = 0.55$  in the case of SAL, indicates that lateral association of egg-box  
280 dimers is more difficult to form than in SAL. This difference between LMP and SAL  
281 can be attributed to the degree of molecular homogeneity in terms of monomer  
282 composition and conformation. For SAL, very trace amount of monomers exists in the  
283 molecules other than guluronate and mannuronate, and the sequence of these monomers  
284 is regular with linear molecular conformation. Thus, calcium binding behavior of SAL  
285 is critical through a series of molecular event, including intra-molecular association (i.e.  
286 monocomplexation) and egg-box dimer formation, followed by lateral inter-molecular

287 association of the dimers. In contrast, LMP is characterized by a variety of monomers  
288 and existence of the hairy region, making molecular associations more random and  
289 super-molecular structures less regular than in SAL, thus preventing the lateral  
290 associations (Fang et al, 2008). As a contribution to elasticity enhancement, it is  
291 anticipated that energetic factor due to the strength of crosslinks should be dominant for  
292 SAL, particularly G-rich one (Funami et al, 2009), whereas entropic factor due to the  
293 number of crosslinks plays an additional role for LMP.

294 Molecular association during calcium-induced gelation of the mixture of LMP and  
295 each low molecular-weight polyuronate fraction was also presented schematically (Fig.  
296 6 for the mixture with  $LM_w$ -LMP and Fig. 7 for the mixture with  $LM_w$ -GUL). As  
297 mentioned, for LMP used in the present study, inter-molecular association can be  
298 dominant over intra-molecular association even at low calcium feed, and  $\eta_t$  increases  
299 gradually without showing critical concentration boundary of calcium, both of which  
300 are different from the behavior of SAL. Moreover, the pectin fraction added can act as  
301 a low molecular weight cross-linker to increase the hydrodynamic size of LMP and can  
302 promote the association of the long chain normal pectin since thermodynamic  
303 incompatibility between LMP and the pectin fraction should not be high considering the  
304 similarity of monomer composition and conformation. Low molecular-weight pectin

305 fraction, particularly  $LM_w$ -LMP, associates with free galacturonate in the LMP  
306 molecules via calcium, and as a result, LMP has longer chain and larger number of  
307 galacturonate site than the original LMP. It is anticipated that these changes should  
308 increase the opportunity for molecular associations but decrease the structural  
309 homogeneity of super-molecular structure at the same time, which may result in the  
310 network formation of various pore sizes (Fig. 6). These may explain larger  $G'$  and  
311 smaller yield strain for the mixture in a concentrated system. Effects of the pectin  
312 fraction relate to the sequentiality of calcium binding site, and a certain length is  
313 necessary for binding, for example consecutive 14-20 M free galacturonate (Rees, 1982;  
314 Axelos, & Thibault, 1991). This is why  $LM_w$ -LMP is more effective than  $LM_w$ -HMP  
315 in modifying the gelation behavior of LMP.

316 Thermodynamic incompatibility between LMP and low molecular-weight alginate  
317 fraction can be higher than in the pectin fraction, and thus the alginate fraction prefers to  
318 bind together rather than binding to LMP. Differed from the pectin fraction, dimers  
319 and multimers of the alginate fraction cannot associate easily with LMP molecules (Fig.  
320 7). It is clear from viscometry in dilute solutions that the addition of low  
321 molecular-weight alginate fraction, particularly  $LM_w$ -GUL, decreases  $\eta_r$  of LMP at  
322 below the stoichiometry  $R = 0.25$ . This may indicate the absence of inter-molecular

323 association between LMP and low molecular-weight alginate fraction, and the alginate  
324 fraction can compete with LMP for calcium at low calcium feed. Decrease in  $\eta_r$  for  
325 the mixture with  $LM_w$ -GUL at above the stoichiometry  $R = 0.55$  can be attributed to  
326 macroscopic phase separation based on the incompatibility between LMP and  
327  $LM_w$ -GUL. Super-molecules from  $LM_w$ -GUL are dispersed within the LMP system  
328 and can prevent molecular associations of LMP, making the pore size of the gel network  
329 larger and rheological nature of the system more flexible and plastic. Effects of low  
330 molecular-weight alginate fraction relate to the sequentiality of calcium binding site,  
331 and a certain length is necessary for binding, for example consecutive 20 M free  
332 guluronate (Kohn, 1975; Kohn & Larsen, 1972). This is why  $LM_w$ -GUL is more  
333 effective than  $LM_w$ -MAN in modifying the gelation behavior of LMP.

#### 334 **4. Conclusions**

335 Low molecular-weight polyuronate fraction modifies the calcium binding and  
336 consequent molecular association behaviors of long chain normal low-methoxyl pectin  
337 and thus rheological properties of the gels. These effects of the polyuronate fraction  
338 are enhanced when the degree of methyl-esterification is low for pectin and the  
339 guluronate content or guluronate-block length is high for alginate, indicating a key role  
340 of calcium binding site. Low molecular-weight pectin fraction, particularly

341 low-methoxyl one, strengthens the gel structure of normal low-methoxyl pectin,  
342 whereas low molecular-weight alginate fraction, particularly guluronate-rich one,  
343 weakens the gel structure of normal low-methoxyl pectin. Different functions between  
344 low molecular-weight pectin and alginate fractions can be attributed to the molecular  
345 compatibility with normal low-methoxyl pectin.

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349

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419

#### 420 **Figure captions**

421 **Fig. 1.** Changes in normalized relative viscosity  $\eta_r^N$  ( $\eta_r^{\text{Ca}}/\eta_r^{\text{C}}$ ) during titration of 7.5 mM  $\text{CaCl}_2$  for  
422 the mixture of 0.05% normal low-methoxyl pectin (LMP) and low molecular-weight pectin fraction  
423 at 0% (closed circle), 0.01% (open triangle), 0.02% (closed square), and 0.05% (open circle) for  
424 high-methoxyl pectin fraction ( $LM_w\text{-HMP}$ ) (a & c) and low-methoxyl pectin fraction ( $LM_w\text{-LMP}$ ) (b  
425 & d). Data are plotted as a function of calcium concentration (a & b) and the molar ratio  $R_{\text{total fGal}}$   
426 (calcium/total free galacturonate from LMP and pectin fraction) (c & d). See the text for  
427 experimental detail. Data are presented as means  $\pm$  SD of triplicate.

428 **Fig. 2.** Changes in normalized relative viscosity  $\eta_r^N$  ( $\eta_r^{\text{Ca}}/\eta_r^{\text{C}}$ ) during titration of 7.5 mM  $\text{CaCl}_2$  for  
429 the mixture of 0.05% normal low-methoxyl pectin (LMP) and low molecular-weight alginate  
430 fraction at 0% (closed circle), 0.01% (open triangle), 0.02% (closed square), and 0.05% (open circle)  
431 for mannuronate-rich alginate fraction ( $LM_w\text{-MAN}$ ) (a & c) and guluronate-rich alginate fraction

432 ( $LM_w$ -GUL) (b & d). Data are plotted as a function of calcium concentration (a & b), and the molar  
433 ratio  $R_{fGal+Gul}$  (calcium/the sum of free galacturonate from LMP and free guluronate from alginate  
434 fraction) (c & d). See the text for experimental detail. Data are presented as means  $\pm$  SD of  
435 triplicate.

436 **Fig. 3.** Frequency-dependence of dynamic storage modulus  $G'$  for the mixture of 0.8% normal  
437 low-methoxyl pectin (LMP) and low molecular-weight pectin fraction at 0% (closed circle), 0.2%  
438 (open triangle), 0.4% (closed square), and 0.8% (open circle) for high-methoxyl pectin fraction  
439 ( $LM_w$ -HMP) (a) and low-methoxyl pectin fraction ( $LM_w$ -LMP) (b). Concentrations of both  $CaCO_3$   
440 and glucono- $\delta$ -lactone were fixed at 20 mM. See the text for experimental detail. Measurements  
441 were carried out in triplicate, and one representative datum is shown.

442 **Fig. 4.** Frequency-dependence of dynamic storage modulus  $G'$  for the mixture of 0.8% normal  
443 low-methoxyl pectin (LMP) and low molecular-weight alginate fraction at 0% (closed circle), 0.2%  
444 (open triangle), 0.4% (closed square), and 0.8% (open circle) for mannuronate-rich alginate fraction  
445 ( $LM_w$ -MAN) (a) and guluronate-rich alginate fraction ( $LM_w$ -GUL) (b). Concentrations of both  
446  $CaCO_3$  and glucono- $\delta$ -lactone were fixed at 20 mM. See the text for experimental detail.  
447 Measurements were carried out in triplicate, and one representative datum is shown.

448 **Fig. 5.** Schematic presentation of calcium-induced gelation for normal low-methoxyl pectin (LMP)  
449 (a) in comparison with normal sodium alginate (SAL) (b).

450 **Fig. 6.** Schematic presentation of calcium-induced gelation for the mixture of normal low-methoxyl  
451 pectin (LMP) and low molecular-weight low-methoxyl pectin fraction ( $LM_w$ -LMP).

452 **Fig. 7.** Schematic presentation of calcium-induced gelation for the mixture of normal low-methoxyl  
453 pectin (LMP) and low molecular-weight guluronate-rich alginate fraction ( $LM_w$ -GUL).