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Utilisation of model pectins reveals the effect of demethylated block size frequency on calcium gel formation

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ABSTRACT

Calcium-mediated gelation of LMP is thought to arise from formation of a dense network of Ca^{2+}-cross-linked DMB meeting a required minimum average length along pectin chains. The use of MP containing specific average DMB size ($\overline{BS}$) types, in the range of 3–100 and in varying proportion (0–100%), has afforded further insights into the gelling behaviour of pectins with a certain DM in the presence of Ca^{2+} ions. It clearly appeared that a required minimum $\overline{BS}$ and a required minimum average frequency ($\overline{BSF}$) of the required minimum $\overline{BS}$ are conditions that must be satisfied by a pectin for formation of a highly dense Ca^{2+}-cross-linked DMB network equaling an elastically stable, strong, and cohesive gel. Furthermore, there is a clear contribution of the pectin branched domains to gelation and formation of a firmer and more cohesive gel. The results suggest that this pectin portion may function, not only as a "maintainer" of the pectin molecular weight to a sufficiently high level which fosters good gelation regarding the gelling rate and the strength and nature of the gel formed, but also as junction-zone-terminating structural elements that limit the appearance of undesirable phenomena, notably turbidity, syneresis, and precipitation.

1. Introduction

Pectic substances are a large family of at least eight blocks co-biopolysaccharides from plant origin, of which unbranched HG and neutral sugar-branched RG-I are the most frequently reported (Yapo, 2011). The well-known functional property of pectins, since their first discovery and crude characterisation in 1790–1825, is gel-formation under specified conditions. This functional property is (almost exclusively) conferred by the pectin HG regions, which are made of (1,4)-linked α-GalA*p* residues, partially methylesterified at *C*-6 position and sometimes partly acetyl-esterified at O-2/O-3 positions. The amount and $\overline{DP}$ of the pectin HG domains influence its gelling characteristics and the strength of the gel formed (Yapo, 2009). It is widely believed that the DM of pectin governs its mechanism of gelation. However, it should be kept in mind that extracted (crude) pectins are typically heterogeneous, with respect to individual polymer chains size, charge distribution, and charge density, so that the overall DM is always a mean value.

Depending on DM, pectins are distinguished as low methoxy pectins (LMP; DM < 50) and high methoxy pectins (HMP; DM ≥ 50). In general, LMP are produced from HMP using four kinds of deesterification, viz. by alkali (e.g., NaOH) treatment at cold temperature (LMP with random distribution of deesterified GalA units), by ammonia treatment (amidated LMP with random distribution of deesterified GalA units and amide groups partially substituted for methoxy groups), by fungal PME treatment (LMP with non-blockwise distribution of deesterified GalA residues), and by plant PME (LMP with blockwise distribution of deesterified GalA residues). HMP are believed to form gels at high sugar

Abbreviations: ADLP, alkali-deesterified lemon pectin; BME, beta-mercaptoethanol; BS, demethylated galacturonic acid block size; BSF, demethylated galacturonic acid block size frequency; CCHMP, commercial citrus highly-methylesterified pectin; DB, degree of blockiness; DB_{abs}, absolute degree of blockiness; DE, degree of esterification; DM, degree of methylesterification; DMB, demethylated galacturonic acid block; DP, degree of polymerisation; EndoPG, endopolygalacturonases; GalA, galacturonic acid; GFC, gel filtration chromatography; HG, homogalacturonans; HGA, homogalacturonic acids; HMP, high methoxy pectins; HPAEC-PAD, high performance anion exchange chromatography-pulsed amperometric detection; IEC, ion exchange chromatography; LMP, low methoxy pectins; LP, lemon pectin; MP, model pectins; MRGI, modified type one rhamnogalacturonans; Mw, molecular weight; MWCO, nominal molecular weight cut-off; MWD, molecular weight distribution; OGA, oligogalacturonides; PAE, pectin acetylesterases; PGA, polygalacturonic acids; PME, pectin methylesterases; PME-PDP, partially deesterified pectin with pectin methylesterase; RGI, type one rhamnogalacturonans; SAOS, small amplitude oscillatory shear; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; UMPS, ultra-methylated pectin strands sample.

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(e.g., sucrose) concentration (>55 wt%) and acid (low pH 2.2–2.8) conditions, thereby yielding the so-called "high methoxy pectin-sugar-acid-gel (HMP-SAG)". The mechanism of gelation of HMP is usually explained by formation of acid-and-sugar-promoted junction zones, between the pectin methylesterified HG domains, which are then stabilised by intermolecular hydrophobic interactions between methylester groups and by intermolecular hydrogen bonds between carboxyl groups of unesterified GalA residues and secondary alcohols (Oakenfull & Scott, 1984). This pectin functionality is currently exploited in the food industry for manufacturing jams, jellies, and marmalades.

Gelation of LMP, in contrast, does not require sugar and acid (low pH) conditions, but does need multivalent cations (especially Ca^{2+}), thereby yielding the so-called "calcium gel". Domains of utilisation of calcium gel are, for instance, formulation of low-calorie jellies and stabilisation of acidic yoghourts. A widely accepted mechanism of gelation for LMP is based on the so-called "egg-box junction zone model", in which deprotonated carboxyl groups of DMB of the pectin HG domains are believed to be cross-linked by Ca^{2+} ion bridges, thus forming intermolecular junction zones, which are then stabilised by van der Waals interactions, hydrogen bonds, and electrostatic interactions (Grant, Morris, Rees, Smith, & Thom, 1973; Kohn, 1975, 1987; Morris, Powell, Gidley, & Rees, 1982). However, the ability of LMP to form egg-box-type junction zones does not only depend on its degree of de-methylation (100-DM), but also on inter- and intra-molecular distribution patterns of unesterified GalA residues of the individual polyelectrolyte chains (Fraeye, Duvetter, Doungla, van Loey, & Hendrickx, 2010; Kohn & Luknar, 1977; Voragen, Pilnik, Thibault, Axelos, & Renard, 1995).

Introduction of two "blocky" parameters, namely DB which represents the ratio of the amount of unesterified (mono-, di- and tri-) GalA residues, released by EndoPG, to the total amount of unesterified GalA residues in pectin, and DB_{abs} which accounts for the ratio of the amount of unesterified (mono, di-, and tri-) GalA residues, liberated by EndoPG, to the total amount of (esterified and unesterified) GalA residues within pectin (Guillotin et al., 2005), have allowed to posit that the more blockwise the distribution of unesterified GalA residues on pectin chains is, the higher the probability of these pectin strands to be cross-linked by Ca^{2+} ions and to form stable egg-box junction zones, which results in stable, strong, and cohesive calcium gels. Thus, strong correlation between the DB_{abs} of pectin and gel strength (or stiffness) has recently been reported by alkali- or plant PME-demethylation of partially methylesterified pectins (e.g., 79% methylesterified commercial apple pectin), thereby confirming the importance of the (de-)methylesterification pattern for gel forming abilities (Fraeye et al., 2010). Nevertheless, acid-extracted (commercial citrus and apple) pectins are a mixture of non-homogenous pectin strands with varying size, DM, DB, and DB_{abs}, and therefore it would not be one study too many to use an ultra (chemically) methylated, size-homogenous, pectin HG as the starting sample to unequivocally substantiate existence of such a strong correlation. This original idea, which has been advanced for the very first time a year earlier (B.M. Yapo, unpublished), is being investigated with amply interesting results.

Furthermore, the minimum DMB required for formation of stable egg-box junction structures has variably been reported to be 6–13, 7, 9, 12, 14, and 15–20 GalA residues as succinctly summarised elsewhere (Fraeye et al., 2010; Taylor, 1982; Vincent & Williams, 2009). The present study aims at reporting on the gelling behaviour of partially demethylated MP, with different specific $\overline{BS}$ types, in the presence of Ca^{2+} ions. By mixing, in the presence of Ca^{2+} ions, a fixed amount of CCHMP (95% DM), with varying amounts of commercial triGalA or laboratory-produced HGA, having a narrow MWD, we have surprisingly observed that good gelation occurred with the HGA/CCHMP mixture under certain conditions, whereas no gelation occurred with the triGalA/CCHMP mixture over the whole range (from 0 to 100% of triGalA) or with CCHMP alone (100% CCHMP). This revealed that the required minimum $\overline{BS}$ and $\overline{BSF}$ in pectins are the key determinants for calcium-promoted gelation of partially demethylated pectins.

2. Materials and methods

2.1. Materials

CCHMP (95% DM, 85% GalA), citrus PGA (95% purity, $\overline{M_w}$ 25–50 kDa, lot 81325), triGalA, diGalA, and orange peel PME (P5400, lyophilised powder, ≥150 U/mg protein; 25–50% protein) were purchased from Sigma–Aldrich Co. (St. Louis, MO). MonoGalA was bought from Fluka (Buchs, Switzerland). Commercial polygalacturonase preparation (EPG-M2), produced by *Aspergillus aculeatus*, was purchased from Megazyme International Ireland Ldt. (Bray, Co., Wicklow, Ireland). Protein molecular weight makers in the range of 6–200 kDa, viz. aprotinin (6.5 kDa), α-lactalbumin (14.4 kDa), soybean trypsin inhibitor (21.5 kDa), carbonic anhydrase (31 kDa), ovalbumin (45 kDa), bovin serum albumin (66.2 kDa), phosphorylase b (97.4 kDa), β-galactosidase (116.3 kDa), and myosin (200 kDa) were from Biorad Laboratories, Inc. (Hercules, CA). A pullulan kit with a narrow MWD ($\overline{M_w}$~6.0, 10.0, 21.7, 48.8, 113.0, 210.0, 393.0, and 805.0 kDa) was from American Polymer Standards Corp. (Mentor, OH).

LP64 (64% DM, 85% GalA) was produced by dilute citric acid treatment of lemon peel, followed by dialysis and alcohol-precipitation (Yapo, 2009). Two homogenous HGA samples, referred to as HGA60 ($\overline{DP}$ 60) and HGA100 ($\overline{DP}$ 100), were purified from citric-acid-extracted alkali-deesterified pineapple flesh and lemon peel (ADLP) pectins, respectively (Yapo, 2009). The acid-generated RGI oligomers product (7.0%, w/w), obtained concomitantly with HGA100 (90.0%, w/w) from ADLP, is referred to as modified RGI (MRGI). UMPS (98% DM, 82% GalA) was produced from CCHMP as a soluble fraction in cupric sulfate solution containing excess of Cu^{2+} ions as follows. Solution (1%) of CCHMP (95% DM) was gradually added to 7% $CuSO_4 \cdot 5H_2O$ (Yapo, 2010). The mixture was stored at 4 °C to allow complete formation of insoluble Cu-pectinate complexes and was centrifuged to separate the precipitate formed from the supernatant. The precipitate was then purified to give a final pectinate product accounting for ~28% (w/w) and having an average DM of ~90%. This fraction was not further examined in this study. The pectin in supernatant was purified by precipitation-and-washing with ethanol, thereby yielding ~70% (w/w) final pectin product with a slightly increased DM (~98%) compared to the "parent" pectin. This product is referred to as an ultra-methylated pectin strands sample (UMPS). A partially de-methylesterified pectin (to a desired DM) from UMPS by the PME and then purified as Al-pectinate precipitate is referred to as PME-PDP. Table 1 shows some chemical and macromolecular characteristics of the initial pectic samples used.

2.2. Analysis of homogeneity and action patterns of enzyme preparations

2.2.1. Homogeneity

The two commercial enzyme preparations, viz. *A. aculeatus* EPG-M2 and orange peel P5400 were analysed for homogeneity in "native state" by GFC and in denatured state by SDS-PAGE. GFC was performed with a high resolution Superdex-200 HR 10/30 column (Amersham Biosciences Corp., NJ, USA). Elution of proteins was monitored spectrophotometrically at a scanning UV wavelength range of 215–280 nm. MWD of protein eluates was monitored with BioRad protein molecular weight standards as specified above and apparent $\overline{M_w}$ was estimated as described elsewhere (Andrews,