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Characterization of the global structure of low methoxyl pectin in solution☆

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ABSTRACT

Low methoxyl citrus pectin (LMP) and amidated low methoxyl pectin (LMAP) were characterized by high performance size exclusion chromatography (HPSEC) with online light scattering (LS), intrinsic viscosity (η_w), differential refractive index (dRI) and ultra-violet (UV) detection, by amino acid analysis (AAA) and by atomic force microscopy (AFM). HPSEC revealed the following: the weight average molar mass (M_w), ranged 103–288 × 10^3 Da, (η_w), ranged 2.69–4.27 dL/g, radius of gyration (Rg_z), was 28.6–49.5 nm and hydrated radius (Rh_{zv}), was 26.3–41.8 nm. The presence of phenylalanine and tyrosine residues as shown by the UV absorbance at 278 nm in the high molar mass range is indicative of protein or fragments associated with LMP derived from citrus fruit. The ρ value (Rg_z/Rh_{zv}) as a function of molar mass indicated that high molar mass pectin was more compact in shape than intermediate molar mass pectin. AFM images in this study and earlier studies indicate that high methoxyl pectin (HMP) and LMP form network structures in aqueous solution. Unlike HMP networks, LMP networks do not dissociate when dissolved in water at concentrations that are less than 6.6 μg/mL. Furthermore, AFM images, M_w and η_w values for LMP and LMAP indicate that little or no added sugar is bound.

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1. Introduction

Pectin is a complex anionic polysaccharide found in the cell walls of many plants, and is comprised of several different covalently linked polysaccharides (Vincken et al., 2003). It has been found to contain at least 17 different mono-saccharides in various plants. Nonetheless, the major constituent common to all pectin is homogalacturonan (HG), *i.e.* (1–4) linked α-D-galacturonic acid and its methyl ester (Carpita & McCann, 2000). An early study of sodium polygalacturonate in aqueous solution, a fragment of pectin and possibly HG, found that it was highly asymmetric (Fishman, Pepper, & Barford, 1984). The second most abundant constituent in pectin is rhamnogalaturonan I. This constituent contains arabinan, galantan and arabino-galactan side chains. A more detailed description of pectin structure at the mono-saccharide level can be found elsewhere (Kirby, MacDougall, & Morris, 2008).

Electron microscope images obtained by rotary shadowing of peach pectin revealed that pectin isolated from water formed network structures (Fishman, Cooke, Hotchkiss, & Damert, 1993; Fishman et al., 1992). Dissociation of these structures into their component parts in aqueous solution containing 0.05 M NaCl or 50% glycerol revealed that the network subunits were comprised of rods, segmented rods and kinked rods. Electron microscope images confirmed earlier measurements from curve fitting of high performance size exclusion chromatography (HPSEC) chromatograms that pectin structure was comprised of subunits (Fishman, Gillespie, Sondney, & El-Atawy, 1991; Fishman, Gross, Gillespie, & Sondey, 1989). Furthermore, images obtained by Atomic Force Microscopy (AFM) of citrus pectin isolated from water, also revealed that network structures were present at a concentration of 13.1 μg/mL. At a concentration of 6.1 μg/mL the networks were dissociated

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into rods, segmented rods and kinked rods. In addition, dense spherical and "Y" shaped shape structures were observed (Fishman, Cooke, Chau, Coffin, & Hotchkiss, 2007). Similar structures have also been observed in AFM images of tomato (Kirby et al., 2008) and sugar beet pectin (Fishman, Chau, Cooke, & Hotchkiss, 2008).

Commercial pectin, which typically is extracted from apple pomace or citrus peels, has a degree of methyl esterification (DE) of 70–80% (May, 1990; Voragen, Pilnik, Thibault, Axelos, & Renard, 1995) and has been labeled high methoxyl pectin (HMP). HMP has food and pharmaceutical uses some of which include formation of sweet, fruit flavored gels, stabilization of fermented or acidified dairy products, wound bandages and gaskets for receptacles for intestinal fluids from stomatized patients (Rolin, 2002).

Low methoxyl pectin (LMP), DE $\leq$ 50% is obtained by de-esterification of HMP with acid or ammonia in an alcoholic medium (Axelos & Thibault, 1991). Acid deesterification produces LMP with a random distribution of carboxyl groups whereas ammonia produces a blockwise distribution of amide groups and randomly distributed free carboxyl group. Pectin deesterified by salt independent poly methyl esterase from orange produces LMP with a blockwise distribution of carboxyl groups and without significant depolymerization (Hotchkiss et al., 2002; Ström et al., 2007). Both HMP and LMP can undergo gelation but the mechanisms differ. HMP undergoes gelation in the presence of high concentrations of sucrose (e.g. 65%) and low pH (e.g. $\leq$3.5). Gelation has been attributed to a combination hydrogen bond formation and hydrophobic interactions (Li, Al-Assaf, Fang, & Phillips, 2013). In the case of LMP, it has been suggested that the addition of divalent calcium ions holds together two or more polyuronates chains to form side by side aggregates which have been likened to egg boxes (Grant, Morris, Rees, Smith, & Thom, 1973). Presumably, these aggregates are the precursors to gel formation which occurs in the presence of calcium or other divalent cations. It has been found that at low pH (e.g. pH 2.5), that gel formation will occur without the addition of extraneous calcium although the presence of trace amounts of endogenous calcium may play a role (Gilsenan, Richardson, & Morris, 2000).

Low methoxyl and amidated pectin are incorporated in low sugar products, reduced sugar preserves, fruit preparations for yogurts, desert gels and toppings, and savory applications (e.g. sauces and marinades). Furthermore, they are used in low acid, high sugar preserves containing figs, bananas and other confectionary products (Anonymous, 2009).

The objective of this study is to determine the effect of added sucrose and to some extent endogenous proteins on the structure of LMP by employing HPSEC with multi online detectors and by atomic force microscopy (AFM). Furthermore, measurement of molar mass, viscosity, radii and AFM images of LMP in water will provide additional structural information on the tendency of pectin to aggregate in the presence and absence of sugar, and without added calcium or other divalent cations. The ability of pectin to aggregate is vital in the ability of LMP to form gels. Therefore, the knowledge gained in this study should be useful in the formulation of LMP gels.

2. Materials and methods

2.1. Materials

Six low methoxyl pectin samples were gifts from CP Kelco, a Huber Company (www.cpkelco.com). They were labeled LMP 36.1, LMP 36.8, LMP 50.2, LMAP 1, LMAP 2 and LMAP 3. Two low methoxyl pectin samples were gifts from DuPont/Danisco (www.danisco.com), and were labeled LMP 32 and 48. All samples were standardized with sugar by the supplier. Therefore, LMP 36.8 was also characterized with sugar removed by both methanol and isopropanol. Samples labeled as LMP samples were not amidated whereas LMAP samples were amidated. The number following LMP samples is the degree of methyl esterification (DE) whereas in the case of LMAP samples, the end number merely indicates different samples. LMP 36.8 was labeled LMP IPA ppt 36.8 or LMP MeOH ppt 36.8 depending on whether sugar was removed from pectin by precipitating it with isopropyl alcohol (IPA) or methanol (MeOH). Either 2.5 g of pectin was added to a 100 mL of 70/30 MeOH/H_2O solution or 5.5 g of pectin was added to 100 g 70/30 IPA solution, stirred overnight and passed through a Whatman paper filter (Grade 541, Sigma–Aldrich, St. Louis, MO). The precipitated pectin was removed from the filter and dried overnight at room temperature in a vacuum oven.

2.2. High performance size exclusion chromatography

Dried samples of low methoxy pectin (2 mg/mL) were dissolved in mobile phase (0.05 M $NaNO_3$ and 0.01% NaN_3) and filtered through a 0.45 μm Millex HV filter (Merck Ltd, Tullagreen, Ireland). The solvent delivery system consisted of a model 1200 series degasser, auto sampler and a pump (Agilent Technologies, Germany). The flow rate was 0.7 mL/min. The injection volume was 200 μL. Samples were run in triplicate. The column set consisted of three model TSK $GMPW_{xl}$, 7.8 × 300 mm, 13 μm particle size exclusion columns (Tosoh Bioscience, Tokyo, Japan) in series. Placed directly before and after the column set were TSK_{gel} PW_{xL} guard columns (Tosoh Bioscience, Tokyo, Japan), dimensions 6.0 mm I.D. × 4.0 cm, pore size 12 μm. The columns were in a heated water bath set at 35 °C. The column set was connected online and in series to a UV-1260 Infinity spectrophotometer (Agilent Technologies, Germany), HELEOS II multi-angle laser light scattering photometer (MALLS) (Wyatt Technology, Santa Barbara, CA), model 255-V2 differential pressure viscometer (DP) and a differential refractive index detector (RI) (Wyatt Technology, Santa Barbara, CA). The UV/VIS, MALLS, DP and dRI detectors were aligned with Bovine Serum Albumin (BSA) and normalized with Pullulan 50. The extinction coefficient for the UV wavelength at 278 nm was determined from the RI concentration of each individual sample. The d*n*/d*c* value used was 0.132. Electronic outputs from all the scattering angles measured by the UV, MALLS, DP and dRI were sent to a desktop computer directory for processing with ASTRA (Ver. 6.1.1.17) software (Wyatt Technology, Santa Barbara, CA).

2.3. Amino acid analysis

Dehydrated pectin samples were hydrolyzed in 6 N HCl containing a small amount of phenol. The hydrolysis flasks were extensively purged of oxygen using a PicoTag workstation (Waters Corp., Milford, MA), and then incubated at 110 °C for 20 h. Hydrolyzed samples were filtered, dried under vacuum, and derivatized with AccQFluor reagent (Waters) following the manufacturer's directions. Chromatography was performed using procedures described as 'system 1' in Cohen and De Antonis (Cohen & De Antonis, 1994), with α-aminobutyric acid as an internal standard. Separation was achieved using an AccQTag C18 reverse phase column (Waters); detection by fluorescence used 250 nm as the excitation and 395 nm as the emission wavelength. Hydrolysis, derivativzation and analysis of each sample were performed in triplicate. The total percentage of nitrogen and crude protein present in low methoxyl pectin as determined by Kjeldahl analysis has been described previously (Fishman, Chau, Cooke, Yadav, & Hotchkiss, 2009).