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Physico-Chemical Characterization of Pectic Polysaccharides from Various Sources Obtained by Steam Assisted Flash Extraction (SAFE)

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Summary: Pectic polysaccharides (PP) have been isolated from a variety of sources and characterized by their yield, anhydrogalacturonic (AGA) content, degree of methyl esterification (DE) and microgel (MG) content. Molar mass and mass distribution (MMD) were analyzed by high performance size-exclusion chromatography (HPSEC) coupled with pressure differential viscosity (PDV) and differential refractive index (DRI) detectors. Results were grouped according to the DE of the PP. Among sources of lower methylated (LM) pectins, apricot pectin had the highest weight average molar mass (M_w) followed by apple pectin. Among high methylated (HM) pectins, pumpkin pectin had the highest value of M_w followed by tangerine and lemon pectins. All pectins studied were found to have bi-modal distribution as indicated by their molar mass calibration curves. Apple pectin was the most polydisperse whereas pumpkin pectin was the least polydisperse as indicated by M_w/M_n, where M_n is the number average molar mass.

Keywords: aggregation; microgels; molar mass; molar mass distribution; pectin

Introduction

Pectin is structurally and functionally the most complex polysaccharide in plant cell walls. Its main chain consists of three types of pectic polymers, which are randomly connected to each other in an undefined manner: homogalacturonan (HG), rhamnogalacturonan I (RG I), rhamnogalacturonan II (RG II) and xylogalacturonan (XGA). HG is a linear polymer consisting of 1, 4-linked α-D-galacturonic acid; RG I consists of a backbone of repeating galacturonic acid and rhamnose disaccharide units with side chains containing various types and amounts of glycans (mainly Arabinan and Galactan) attached to the rhamnose residues; RG II is another branched polymer consisting of a homogalacturonan backbone with attached side chain complexes.[1,2] To complicate matters further, pectin differs from plant to plant and even from the same plant due to a variety of factors. Essentially no two molecules have exactly identical structures and functionalities. Therefore, pectin is often described by the term ''pectic substance''.[3] A high content comprised of uronic acid and its methylester copolymer is the feature, which unifies all pectins.

Pectin as a gelling and stabilizing polymer is used in diverse food products. Moreover it has positive effects on human health and has multiple biomedical applications. To understand better the use of these polysaccharides in food and health systems, their structure–function relationships need to be known in detail.

The technical process of extracting pectic polysaccharides (PPs) from plant materials has a profound effect on their

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molar mass distributions. Here we used high performance size-exclusion chromatography (HPSEC) coupled with a pressure differential viscosity (PDV) and a differential refractive index (DRI) detectors, to reveal and quantify differences in pectin samples from a variety of sources. The degree of esterification and therefore the charge on a pectin molecule is important to the functional properties in the both plant cell wall and commercial products. It would be therefore interesting to see if these differences are also borne out in the hydrodynamic properties of these substances: these are the subject of the current study.

Analysis of Molar Mass and Molar Mass Distributions of Pectin Samples by SEC

Pectin was extracted from apples, apricots, peaches quince pomace, lemon and tangerine pulp, pumpkin fruit, rhubarb plant and sunflower head residue. Pectic substances were isolated from the different sources by the flash extraction method,[4] using hydrochloric acid, purified by the diaultrafiltration method.[5] They were characterized by yield (PP), anhydrogalacturonate (AGA) content,[6] degree of methylesterification (DE)[7] and microgel (MG) content.[8] Molar mass and MMD were analyzed with the aid of a Waters HPSEC (Waters Inc., Milford, MA, USA) delivery system, an inline 2-Channel Vacuum Degasser coupled in series to a ViscoStar model differential pressure viscometer (Wyatt Technology, USA), a Waters 2410 differential refractometer (RI), two PL-Aquagel size exclusion columns (OH-60 and OH-40) and an auto sampler (717 Plus Auto Injector, Waters). Dry samples (2 mg/ml) were dissolved in mobile phase (0.05 M NaNO3), centrifuged at 20,000 g for 30 minutes and filtered through a 0.22 μm Millex HV filter (Millipore Corp., Bedford, MA). The flow rate was 0.8 ml/min and the injection volume was 100 μl. Samples were run in triplicate. Column effluents were detected by ViscoStar, and a RI Detector in series. The electronic outputs from both detectors were connected to separate serial ports in the same personal computer in a manner which permitted data to be collected and processed by ASTRA 5.3.4.13 (Wyatt Technology) and Breez (Waters) software simultaneously. Columns were calibrated using a series of Pullulan standard samples (Showa Denko K.K., Japan) with M_w values of 788KD; 667 KD; 404 KD; 112 KD; 47.3 KDand 22.8 KD respectively. Values of M_w, M_n and M_z for pectin were obtained using universal calibration. The refractive index increment (dn/dc) used for the mobile phase (0.05M $NaNO_3$ [9]) was 0.134 ml/g.

Table 1 is a summary of PP quality characteristics grouped according to DE. For LM pectins, the order of decreasing M_w values was Apricot > Apple > Sunflower > Water Melon. In the case of HM pectins, the order of decreasing M_w values was Pumpkin (1) > Tangerine > Quince > Rhubarb > Lemon > Peach >Pumpkin (2). As expected in each DE grouping, the order of the intrinsic viscosity *[η]* was identical with molar mass only for HM-pectins. Rather surprisingly, the intrinsic viscosity of water melon pectin, 344.4 ml/g, is higher than expected given the values of 105,000 for M_w and 14 nm for Rh_w. Possibly, this is due to a structure, which is less aggregated than the other pectins studied and may be indicated by its low z-average molar mass (Mz). The low M_w value of lemon pectin obtained in this study was due to a longer storage time compared to the other pectins studied.

As indicated by Figure 1, which shows the features of four different PPs by molar mass and MMD, the molar mass against elution volume was non-linear. Non-linearity indicates that the molecular conformation at the high molar end of the distribution is different from that at the low molar end of the distribution.

The relation between the root mean square (RMS) radius and molar mass known as conformation plot is one of basic tools for the characterization of polymers.

Table 1.

Characterization of PP samples from different sources by yield, *AGA*, *DE*, M_w, polydispersity ($\overline{M}_w/\overline{M}_n$), hydrodynamic radius ($R_h$) and exponent a obtained from conformation plot (Log-Log plot of hydrodynamic radius Rh versus molar mass).

Pectins	*PP*, %	*AGA*, %	*DE*, %	*MG*, %	$M_w \times 10^{-3}$	$M_z \times 10^{-3}$	$\overline{M}_w/\overline{M}_n$	[η], *ml/g*	R_h, nm	a
					Low Methoxy (LM) Pectins					
Sunflower	25.1	76.0	46.0	7.4	118.6	1152.0	4.3	61	8	0.44
Water Melon	8.5	46.8	50.0	5.4	105.0	731.0	11.3	344	14	0.30
Apple	20.0	67.2	52.2	15.0	168.0	2820.0	2.5	132	12	0.51
Apricot	7.5	67.2	53.0	19.0	713.0	14790.0	12.1	247	21	0.47
					High (HM) Methoxy Pectins					
Quince	12.4	67.2	58.0	1.7	110.3	607.0	3.2	150	12	0.52
Tangerine	16.4	75.6	71.3	1.6	180.2	840.0	2.3	299	12	0.57
Lemon	20.0	74.0	75.0	15.0	82.0	426.0	9.0		8	
Pumpkin (1)	3.77	75.0	81.7	8.4	769.5	3969.0	3.4	375	33	0.29
Pumpkin (2)				8.4	59.5	72.5	1.2	125	10	0.94
Peach	7.2	66.2	94.8	1.7	66.3	153.0	3.2	142	10	0.60
Rhubarb				19.5	107.4	325.0	2.3	169	13	0.54

All samples were steam assisted flash extracted (SAFE) at120 °C for 5 minutes at pH 2.0 except for lemom which was heated for 7 minutes. Pumpkin (2) is a lower molar mass fraction of Pumpkin (1). The value of molar mass was the average of 3–5 measurements.

In fact, the same information about the molecular structure can be obtained from Mark-Houwink (M-H) plot. However, the conformation plot obtained by hydrodynamic or viscometric radius (R_h) is a closer equivalent to the RMS radius conformation plot with the slop approximately equal to that based on the RMS radius. One limitation of the conformation plot based on the RMS radius is the impossibility of characterizing smaller polymers with a majority of molecules with RMS radii of about 10 nm or smaller. The lower limit of size that can be measured depends on the wavelength of light. While, R_h radius can be accurately determined down to about 1 nm, the relation of hydrodynamic radius and molar mass (i.e., R_h conformation plot) may become a suitable alternative.[10] ASTRA software[11] allows one to assess

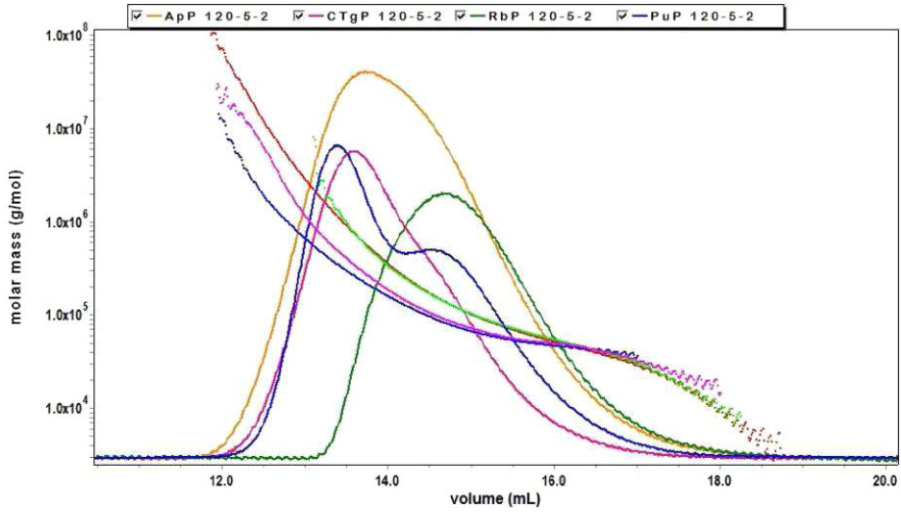

Figure 1.

Molar mass against volume of four pectin samples measured by HPSEC with PDV and DRI detection using universal calibration. Sample calibration curves are superimposed on chromatograms.

the shape of the molecule based on their measured molar mass and hydrodynamic radius obtained from intrinsic viscosity (viscometric radius). The slope of this graph allows one to estimate the shape of a homogeneous polymer. Thus a slope of approximately 0.3 is a sphere. The slope of a random coil is near 0.5–0.6; and that of a rod is consequently span 0.6–1.0. In the case of a heterogeneous polymer such as pectin the relationship between the slope and the molecular shape is somewhat more complicated as indicated below. The conformation plot also may play an important role in the identification of polymer branching, since for example,[10] a slope equal to 0.58 is typical for linear molecules in thermodynamically good solvents, while a slope equal to 0.54 may indicate the presence of a certain amount of branched molecules.

The slope ''a'' of a plot of log R_h against log M_w determined for LM- pectin samples ranged from 0.30 to 0.51 and for HM-pectin from 0.52 to 0.60 excluding Pumpkins (tabl.1). Although such differences in slop may indicates differ in molecular shape of PP, induced mainly by carboxyl group esterification, generic hydrodynamic behaviors of LM and HM-pectins, however this fact involves careful study of individual PP structure in solutions.[8] In similar log RMS–log M_w plots, Malovikova et al [12] have reported a shape factor for pectates between 0.5 and 1, assuming to a rod-like behavior of the molecule.

It should be noted that pectin conformation will depend not only on degree of methyl esterification but also on the distribution of methyl ester groups (i.e. blockwise or random), galacturonan content and on the degree of branching by neutral sugars (e.g. galactose and arabinose). From pectins composition (AGA and DE) and molecular characteristics (Table 1) it was difficult to demonstrate relationship between structure and hydrodynamic properties. Therefore, we may expect to see quite different solution conformations for more heavily branched pectins.

Figure 2 shows the R_h conformation plot for two fractions of pectin from pumpkin fruits. For pumpkin fraction (1) and (2) the slope of 0.29 and 0.94 respectively was observed (Figure 2). This finding indicates the presence of two molecular species of spherical and rod likes polymer conformations in the solution of PPs from Pumpkins.

Previously, we found that when chromatograms of orange pectin were integrated by parts, M-H plots of the parts indicated that macromolecules at the high molecular mass end of the distribution were spherical whereas macromolecules at the low molecular mass end of the distribution were rods.[4] A follow up study which employed atomic force microscopy

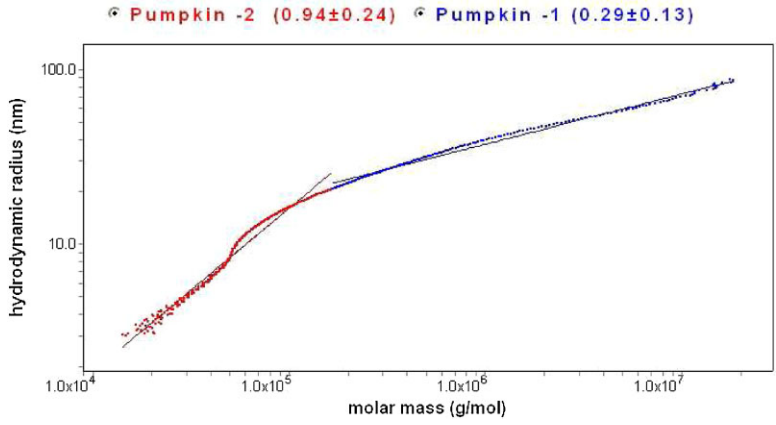

Figure 2.
Hydrodynamic radius (R_h *conformation plot*) versus molar mass of Pumpkin (1) and Pumpkin (2) pectins.

revealed that orange pectin is a mixture of spherical and linear molecules in the form of rods, kinked rods, segmented rods and branched rods.[13] Furthermore, these linear molecules can aggregate into a network which tends to approach the compactness of a sphere. Thus in a general sense, the log M_w against log R_h slope is really a measure of compactness. Moreover M-H exponents over an entire non-linear M-H plot are average values of all the shapes present and do not necessarily measure the molecular conformations of individual molecules present. In this study, the lower fraction of Pumpkin, Pumpkin (2), has rod like shape based on an ''a'' value of 0.94, which is different than the value of 0.29 (spherical shape), the ''a'' value of the high molecular fraction distribution. This indicates that the molecules in that fraction are extended, which agrees with that found for orange pectin.[4] Furthermore, as in the case of the M-H plot, the overall ''a'' value from a distribution, which has a non linear molar mass against elution volume may not necessarily measure the molecular species of individual molecules present.

The order of decreasing polydispersity for the pectins in Figure 1 is Apricot > Lemon > Pumpkin > Rhubarb. Thus all pectins in Figure 1 are bimodal in conformation as indicated by their non-linear calibration curves. Pumpkin has the biggest disparity in size of the two conformations. Consequently, the macromolecular species are partially resolved in the chromatogram into two visible distributions.

As indicated above, the tendency to aggregate is another source of pectin complexity. Degree of aggregation depends on pectin source, storage time, hydrolysis mode and solution temperature.[14]

Other factors which affect the state of aggregation of pectin are its concentration and ionic strength in solution.[13] To determine the M_w of pectin one should separate aggregated species (MG) from the solution by centrifugation.[8] We illustrate this procedural point in Figure 3. This figure shows differences in M_w and MMD of pectin samples extracted by sodium hexamethaphospate (SHMP) followed by centrifugation or without centrifugation. These

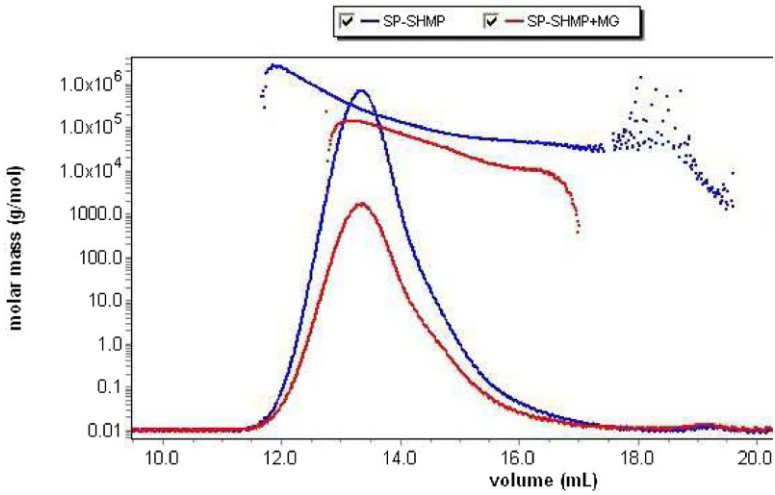

Figure 3.
Molar mass against volume for two sunflower pectin's samples extracted from sunflower head residue by sodium hexamethaphospate (SHMP), measured by HPSEC with differential viscometric detection and universal calibration. Sample calibration curves are superimposed on chromatograms. The sample represented by upper calibration curve and chromatogram has been centrifuged whereas the sample represented by the lower calibration curve and chromatogram have not been centrifuged.

microstructures are constituted by subunits associated with non-covalent forces. Pectin units in fact have been shown to aggregate in the shape of segmented rods and kinked structures of 2–4 nm width. These complex structures, including a combination of helices and overlapping chains have an average length ranging from 20 to 300 nm.[15]

In Figure 4 are plots of cumulative number fraction as a function of molar mass. These plots show, all other things being equal, that molar mass distributions differ greatly with the plant source from which they are extracted.

Conclusion

The physical-chemical characteristics of pectin from different sources have been determined by HPSEC with an online differential refractometer in series with a pressure differential viscometer. Compositional parameters (AGA, DE) in addition to physical parameters (viscosity, weight-, number- and z- average molar mass, MMD, and hydrodynamic radius) were determined. With this methodology, we have shown the vast diversity of pectin macromolecules from various plant sources. MMD plots showed that pectins were a bimolar mixture of at least two kinds of conformations. Since most pectin regardless of biological origin is a mixture of extended or linear molecules and compact or spherical molecules, the relationship between chemical structure and solution properties depends on the molar ratio of these two kinds of moieties. In the cases of orange,[13] sugar beet[16] and peach[15] pectin it has been shown that pectin under appropriate conditions forms networks that can be dissociated into a mixture of rods, segmented rods, kinked rods and spherical or compact molecules. Extended or linear pectin molecules will have larger values of viscosity and hydrodynamic radius or RMS radius than compact or spherical molecules of the same molecular weight. Also molar mass, charged monosaccharide components, solution pH and ionic strength also will affect the solution properties of pectin.

Also, the macromolecules at the lower end of the pumpkin pectin molar mass distribution were rod like, less compact than those at the higher end molar mass were spherical form. Furthermore, it has been shown that HPSEC coupled with PDV and DRI detectors in series gave data with high signal to noise, and produced accurate, rapid analysis of pectic polysac-

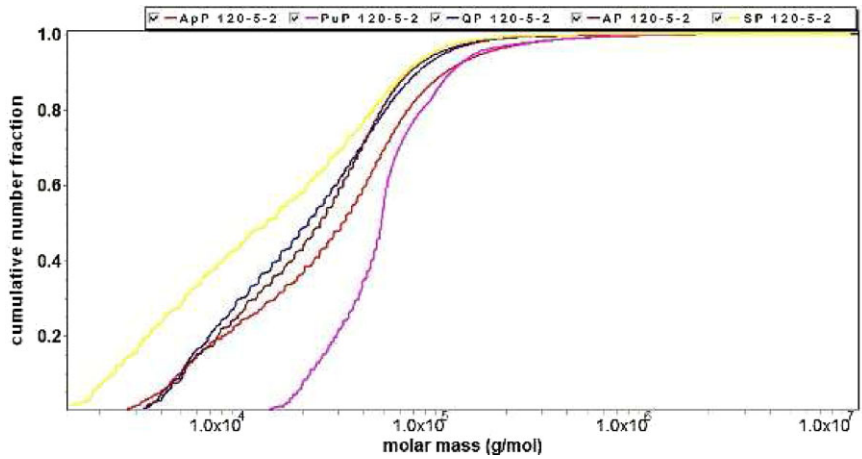

Figure 4.
Cumulative number fraction against molar mass for selected pectin samples (Lines from top to bottom belong to PPs from Sunflower, Quince, Apple, Apricot and Pumpkin).

charides. Moreover, it has been shown that the system herein employed is an important tool in optimizing processes responsible for pectin production.

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