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Biosorption Properties of Citrus Peel Derived Oligogalacturonides, Enzyme-modified Pectin and Peel Hydrolysis Residues

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A citrus processing industry priority is obtaining added value from fruit peel. Approximately one-half of each processed fruit is added to the waste stream. Peel residue is composed mainly of water (~80%), the remaining 20% (solid fraction) consists of pectin, soluble sugars, cellulose, proteins, phenolics, etc. Viewing these constituents in light of exploiting potential functionality and creating added value at the same time as diverting material away from the feed mill or land fill, pectin provides enormous opportunity. To create a new technology centered on pectin structure and concomitant functionality, we are investigating methods to precisely engineer pectin structure and correlate it to function. A valuable pectin functionality, resulting from its polyanionic character, is its biosorption capabilities. In the past several years we have developed analytical techniques and biochemical methods to enzymatically modify pectin structure, characterize these structural alterations and determine their effect on rheology and calcium sensitivity. Here we present data on the biosorption properties of modified pectins and pectin fragments using lead as a model cation. The greatest Pb sorption capacity (Mean = 373.3 mg·g^{-1}; S.E. = 1.595; $P > 0.001$) was observed in the Medium DP size-class of galacturonic acid oligomers. A comparison of enzymatically demethylated (blockwise) homogalacturonans indicated that the 60% and 50% DM pectins treated at pH 4.5 had a significantly greater sorption capacity than higher DM or pH 7.5 treated samples.

Polysaccharides, the most abundantly represented class of biomolecules in citrus peel (Grohmann et al., 1995), have well established functionality that is amenable for manipulation and conversion to value-added components. Of the polysaccharides present in peel, pectin offers the greatest potential for enzymatic or chemical conversion to engineer desired properties, i.e. ion exchange capacity, into peel, processing residues or other waste stream components.

Galacturonic acid (GA), the major sugar found in citrus pectin (Cameron et al., 2008; Ridley et al., 2001), is located largely in the linear homogalacturonan region (HG), pectin's dominant structural domain. A variable proportion of these GAs are methyl esterified at the C6 position, effectively masking the negative charge of the carboxyl group. Pectin functionality is associated with the total amount of methylated GAs (degree of methylation; DM) and the distribution of the methylated and unmethylated GAs in the HG regions (Cameron et al., 2008; Luzio and Cameron, 2007, 2008; Willats et al., 2001;). The demethylated GAs may have either an ordered or random distribution. With an ordered distribution the size of a contiguous block of demethylated GAs (demethylated block; DEMB) will vary and its degree of polymerization (DP) can range to greater than 50 GAs in length.

A functional property related to the negative charge imparted by the ionized carboxyl group of GA is its ability to interact with heavy metal cations often associated with various industrial processes that require remediation. Both DM and the DP of a polygalacturonic acid (PGA) oligomer (equivalent to a DEMB in a pectin molecule) have been shown to effect their interaction with cations (Kohn, 1987; Malovikova and Kohn, 1979, 1982). Previous studies testing the effect of pectin DM on metal chelation have used base to saponify the pectin and reduce the DM from its initial higher level (Kohn and Luknar, 1975; Malovikova and Kohn, 1979, 1982). Saponification, or alkaline demethylation, equivalent to a degree of enzyme processivity equal to one (a single GA is demethylated with no contiguous GAs being demethylated), results in a random distribution of methylated GAs and an abundance of small DEMBs (Cameron et al., 2008). Kohn (1987) has demonstrated that oligomer DP affects the binding of cations, the degree of association increasing with DP.

Pectins demethylated with plant pectin methylesterases, which demethylate processively (contiguous methylated GAs are demethylated until an interference point or intrinsic limit is reached) producing much larger DEMBs, have not been tested for their ability to bind metal ions. In order to evaluate the effect of an ordered distribution of DEMBs, differences in average block size ($\overline{BS}$), and number of DEMBs per molecule of pectin ($\overline{BN}$), treated samples were tested for their effectiveness in Pb chelation. Samples consisted of a series of demethylated pectins from 80% to 50% DM generated at either pH 4.5 or 7.5 (Cameron

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et al., 2008), a series of narrow-range size classes of PGA oligomers produced by limited digestion with endo-polygalacturonase (Cameron et al., 2005) as well as individual, purified oligomers, and peel materials in which the pectin had been released and then demethylated. Additional samples tested were a demethylated, commercially extracted pectin; a non-calcium-sensitive-pectin (NCSP) and a sample of hydrolyzed, fermented citrus peel in which the ethanol had not been stripped.

Materials and Methods

Materials. Unless other wise noted all chemicals were purchased from Sigma-Aldrich (St. Louis, MO) and at least ACS reagent grade.

Biosorbent preparation

Demethylation series. Pectin samples and methods for the pectin demethylation series at pH 4.5 and 7.5 were described previously (Cameron et al., 2008). Briefly, a model pectin with a DM of 94%, an HG consisting of ~97% GA, was demethylated with the citrus salt-independent PME until the desired DM was obtained. Demethylations were run with a radiometer PHM290 pH-Stat controller at 30 °C using 1 M LiOH as the titrant. The reaction was stopped by allowing the demethylated pectin to rapidly flow into a container holding two volumes of ethanol (37 °C). The precipitated pectin was lyophilized. A 0.2% (w/v) solution of the lyophilized pectin was used for a limited endo polygalacturonase (EPG) digest to free DEMBs from the larger molecule. $\overline{BS}$ and $\overline{BN}$ were calculated as previously described (Cameron et al., 2008).

Oligomer narrow-range classes. Narrow-range size-classes of GA oligomers were produced by limited digestion of PGA with EPG followed by differential precipitation and lyophylization (Cameron et al., 2005).

Oligomer purification. Individual oligomers were purified by semi-preparative chromatography of the narrow-range size-classes. A CarboPac PA1 (Dionex Corporation, Sunnyvale, CA) semi-preparative column was loaded with oligomers from either the Low-DP or Medium-DP size class. An elution gradient from 20% to 60% 1 M ammonium formate was used to separate oligomers in the Low-DP size class and from 40% to 80% for the Medium DP size-class. All samples were lyophilized after collection.

Synthesis of block demethylated citrus peel

A mixture was prepared from 1 kg of comminuted, pre-washed orange peel and 3 L of deionized water and the pH adjusted to 1.8 with concentrated nitric acid. This mixture was heated for 3 h at 70 °C to extract the pectin component. The temperature was reduced to 29 °C and 40 g of sodium chloride was added with stirring. The pH was adjusted to 7.0 with dilute aqueous sodium hydroxide and 2 mL of a papaya extract containing PME activity (Collupulin® RTM, Liquid product code 5045 (Gist-Brocades International B.V., Charlotte, NC) was added. The solution was titrated using 1.000 N sodium hydroxide and the pH maintained between 6.8 and 7.2 until the DM of the pectin reached approximately 55% assuming the extracted pectin component had an initial DM was 72%. The pH of the solution was adjusted to pH 3.5 with dilute nitric acid and the solution heated to 85 °C on a stirring hot plate for 2 to 5 min to inactivate the enzyme. The reaction mixture was cooled to room temperature and precipitated with 8 L of 70% (v/v) iPrOH in water . The mixture was collected by filtering through fine nylon mesh cloth and the material pressed to remove excess iPrOH. The peel mixture was pre-dried in a hood for 2–3 h and then dried in a vacuum oven for 16–20 h under vacuum at 50 °C. The dried mixture was milled through a 20-mesh screen on a Wiley Mill Intermediate Model 4276-N.

Preparation of block demethylated commercial pectin

Four liters of a 1% pectin solution (70% DM, BB Rapid Set Pectin DK-4623, CP Kelco, Inc., Lille Skensved, Denmark) was prepared by dissolving at 80 °C and then cooling to room temperature. Forty grams of sodium chloride was added, the pH was adjusted to 7.0 with dilute aqueous sodium hydroxide with stirring, and then 2 mL of PME containing papaya extract (Collupulin® RTM, Liquid product code 5045, Gist-Brocades International B.V.) was added. The solution was titrated using 1.000 N sodium hydroxide and the pH maintained between 6.8 and 7.2 until the pectin reached 55% DE as determined by the volume of base added and the initial DM. The pH of the solution was adjusted to 4 with dilute HCl and the solution heated to 85 °C on a stirring hot plate for 2 to 5 min to inactivate the enzyme. The pectin solution was cooled to room temperature and precipitated with 70/30 iPrOH water solution. The pectin was collected by filtering through fine nylon mesh cloth and pressed to remove excess iPrOH/water. The block-deesterified pectin was pre-dried in a hood for 2–3 h and then dried in a vacuum oven for 16–20 h under vacuum at 50 °C.

Extraction of NCSP pectin from citrus peel

NCSP was extracted from pre-washed orange peel according to methods previously described (Joye and Luzio, 2000)

Peel hydrolysis and fermentation

The sample consisted of the residue left after hydrolysis of the complex carbohydrates in citrus processing waste (CPW) and conversion of fermentable sugars to ethanol. Fresh CPW was obtained from a commercial juice processing plant, pretreated by steam explosion to reduce the peel oil concentration to under 0.15 wet weight percent, cooled to 38 °C using vacuum, and hydrolyzed with simultaneous fermentation (SSF) in a 300-L batch using *Saccharomyces cerevisiae* and a hydrolyzing enzyme mixture of Pectinex Ultra SP, Celluclast 1.5 L and Novozyme 188 (Wilkins et al., 2007).

Biosorption studies

The biosorption of Pb^{+2} by different forms of pectin from aqueous solutions was carried out in batch biosorption-equilibrium studies. Desired concentrations of Pb^{+2} solutions were prepared by diluting 1000 ± 2 mg·L^{-1} standard Pb^{+2} stock solution [$Pb(NO^3)^2$, VWR, West Chester, PA]. pH of the solution was adjusted to 4.5, using 0.1 M NaOH. The adsorption capacity of different forms of biosorbent was determined in triplicate (except for individual GA oligomers) by equilibrating 50 mg of biosorbent with 50 mL of metal solution (2.41 meq·L^{-1} or 500 mg·L^{-1}) in 250 mL Erlenmeyer flasks (pre-washed with 1% HNO_3) on an orbital shaker at 120 rpm and 25 ± 2 °C for 6 h. After filtration through Whatman No. 40 filter paper, the Pb^{+2} concentrations in the filtrate was determined by atomic absorption spectrophotometer (AAnalyst 300, Perkin-Elmer, Waltham, MA). The amount of Pb^{+2} adsorbed per unit biosorbent mass (milligrams of metal per gram dry biosorbent) was determined using the following expression:

$$q: \frac{V(C_i - C_{eq})}{M}$$

where q is the metal uptake (mg $Pb^{+2} \cdot g^{-1}$ dry weight of biosorbent), V is the volume of metal solution (mL), C_i is the initial concentration of Pb^{+2} in solution ($mg \cdot L^{-1}$), C_{eq} is the residual concentration of Pb^{+2} in solution at equilibrium, and M is the dry weight of biosorbent.

Statistical analysis

Statistical differences between treatment means was performed using GraphPad Prism version 4.03 for Windows (Graph-Pad Software, San Diego, CA; www.graphpad.com). Tukey's multiple comparison test was used to compare treatment means.

Results and Discussion

Of all the biosorbents tested the greatest amount of Pb^{+2} was chelated by the Medium DP size-class of GA oligomers which was also greater than the other two DP size-classes (Fig. 1; F = 450.9, $P < 0.001$). This greater sorption capacity by these completely demethylated substrates, compared to the partially demethylated substrates discussed later, is in agreement with the report by Kohn (1987) where the author determined that Pb binding was stoichiometric in relation to the amount of demethylated galacturonan present. The small reduction in sorption capacity by the large DP size-class may be due to an increase in cross linking with these larger oligomers as previously observed for very low DM pectins (Kohn and Luknar, 1975).

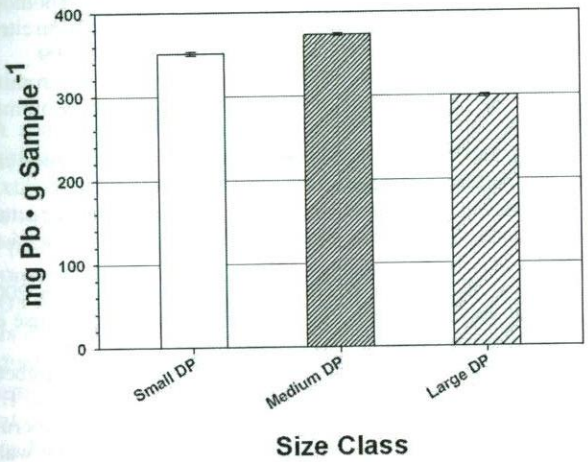

Fig. 1. Biosorption capacities of narrow-range-size classes of GA oligomers (Mean ± SE).

The greatest sorption capacity for the citrus salt-independent PME demethylated pectins was found in the 60% DM sample demethylated at pH 4.5 (Fig. 2). ANOVA indicated the means were significantly different (F = 730.4, $P < 0.0001$) and Tukey's multiple comparison test indicated all means were significantly different from each other ($P < 0.001$ except the 60% DM – pH 4.5 vs. 50% DM – pH 4.5 samples were $P < 0.05$). The 50% DM – pH 4.5 had the next highest sorption value but was still significantly lower than the 60% DM sample ($P < 0.001$). As discussed in Cameron et. al. (2008) demethylation of these model pectins at pH 4.5 resulted in a smaller $\overline{BS}$ compared to the pH 7.5 demethylation series, and also had a larger $\overline{BN}$ per pectin molecule. This suggests that the lower sorption values observed for 80% – pH 7.5 and 70% – pH 7.5 compared to their pH 4.5 counterparts may be related to both $\overline{BS}$ and $\overline{BN}$. Demethylated blocks below a minimal size threshold may not be sufficient for optimal sorption. Once an optimal size is reached a greater number of these blocks present in the pH 4.5 demethylated samples might allow for a greater sorption capacity. This hypothesis could be tested by comparing sorption capacities of purified oligomers equivalent to the $\overline{BS}$ estimated for the different demethylated model HGs.

Of the remaining samples tested the commercial pectin that had been demethylated with a commercial papaya extract containing PME activity demonstrated the best sorption capacity (Fig. 3; ANOVA F = 2100, $P < 0.0001$; Tukey's multiple comparison test $P < 0.001$). The hydrolyzed peel sample was not included in this statistical comparison. The relatively large sorption capacity of the commercial pectin was unexpected. The final DM of the commercial pectin was 55%, a DM value mid way between the 60% and 50% DM – pH 4.5 samples but the sorption capacity was significantly greater than either of those two pectins (Figs. 2 and 3; ANOVA F = 708.2, $P < 0.0001$; Tukey's multiple comparison test $P < 0.001$). The nano-structure of this demethylated, commercial pectin has not been investigated so no conclusions can be drawn regarding average demethylated block sizes and number. The NCSP pectin is also an enigmatic, by definition this pectin should have no large demethylated blocks (Joye and Luzio,

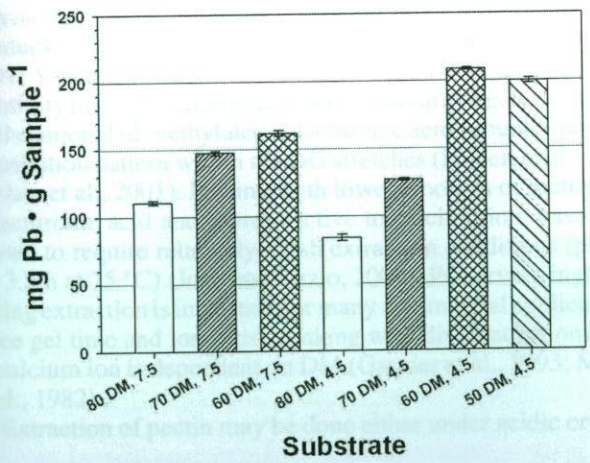

Fig. 2. Biosorption capacities (Mean ± SE) of blockwise demethylated model homogalacturonans demethylated to predetermined DM values at either pH 7.5 or pH 4.5.

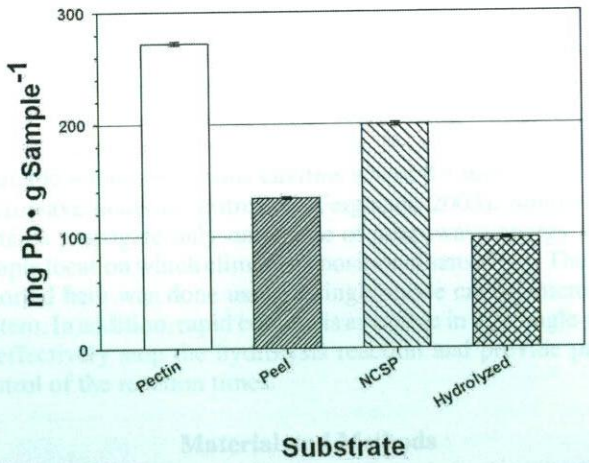

Fig. 3. Biosorption capacities (Mean ± SE) of a commercial pectin demethylated with a papaya extract containing PME activity (Pectin), pectin released from peel residue (Peel), an NCSP and lyophilized material from a single state fermentation of citrus peel (Hydrolyzed).

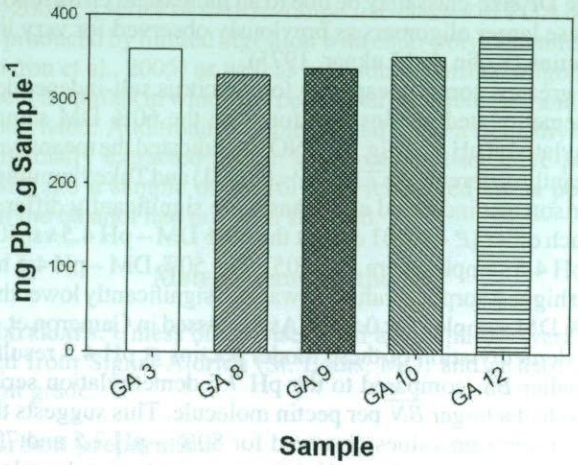

Fig. 4. Biosorption capacities of purified GA oligomers.

2000) but it was equivalent in sorption capacity to the 60% and 50% DM – pH 4.5 samples (Figs. 2 and 3) and not significantly different from the 50% DM – pH 4.5 sample. The demethylated peel samples were close to the values for the pH 7.5 demethylation series and the 70% DM – pH 4.5 HG. The sample of hydrolyzed and fermented peel had the lowest sorption value (Fig. 3).

The sorption capacity of individual oligomers was similar to the narrow-range size-classes of GA oligomers (Fig. 4). Unfortunately the lack of sufficient samples prevented the replication of this analysis and statistical comparison to other samples. The data does suggest that a GA trimer is sufficient to complex with Pb^{2+} but it has been shown (Kohn, 1987) that the DP of an oligomer has a major impact on the solubility of the oligomer-metal ion complex, dependent on the metal species being complexed.

Conclusion

The data presented demonstrate the need for continued studies on the utilization of citrus peel and peel derived pectin for the removal of heavy metal ions from other waste streams requiring remediation. To maximize the sorption capacity of pectic materials it may be necessary to fragment larger molecules and demethylate them either chemically or enzymatically.

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