The interaction of the pectic polysaccharide Rhamnogalacturonan II with heavy metals and lanthanides in wines and fruit juices

P. Pellerin[1] and M.A. O'Neill[2]

[1] *Unité de Recherches des Polymères et des Techniques Physico-Chimiques, Institut des Produits de la Vigne, INRA, 2 place Viala, 34060 Montpellier Cedex, France*

[2] *Complex Carbohydrate Research Center, University of Georgia, 220 Riverbend Road, Athens, GA 30602-4712, USA*

The complexation of divalent cations with the carboxylic acid groups of uronic acids from pectins is well established. However, one pectic polysaccharide, Rhamnogalacturonan II, exists as a borate ester cross-linked dimer that forms coordination complexes with selected di- (Pb^{2+}, Ba^{2+} and Sr^{2+}) and trivalent (lanthanides) cations. The cation-rhamnogalacturonan II complex accounts for the majority of lead in wines and fruit juices and may also account for some of the heavy metals present in plant cell walls.

The speciation of metals with biological compounds (proteins, peptides, amino acids, etc.) is the subject of increasing interest since the complexation of heavy metals may reduce their toxicity and their bioavalaibility. However, there are a limited number of studies concerning the speciation of metals with plant polysaccharides. This is somewhat surprising since plant cell walls contain polysaccharides (pectins) that contain a high proportion of negatively charged glycosyl-residues. In this article, we discuss the cation-binding characteristics of pectins and in particular describe the selective binding of heavy metals and lanthanides by the structurally complex pectic polysaccharide rhamnogalacturonan II (RG-II).

Article available at http://analusis.edpsciences.org or http://dx.doi.org/10.1051/analusis:199826060032

Pectic polysaccharides and the primary plant cell walls

The cells of growing plant tissue and of parenchyma of fruits and vegetables are surrounded by the primary cell wall. The primary walls of dicots and non-graminaceous monocots are believed to consist of a rigid, rod-like cellulose/xyloglucan load-bearing network that is embedded in and interacts with a compression-resistant, hydrated pectin network [1]. Small quantities of structural glycoproteins, enzymes, and phenolic esters are also intercalated into these networks. The pectin network is cross-linked by divalent cations (mainly Ca^{2+}) and one of its functions is to determine the cation-exchange capacity of the wall.

To date, only three pectic polysaccharides (homogalacturonan, rhamnogalacturonan, and substituted galacturonan) have been isolated from primary cell walls and structurally characterized [2]. Homogalacturonan is a linear chain of 1,4-linked α-D-galactopyranosyluronic acid (Gal*p*A) residues in which some of the carboxyl groups are methyl esterified. HG may, depending on the plant source, be partially *O*-acetylated and contain other, as yet, unidentified esters.

Rhamnogalacturonans I (RG-I) are a group of pectic polysaccharides that contain a backbone of the repeating disaccharide [→2)–α –L–Rha*p*– (1 →4) – Gal*p*A– (1]. The backbone Gal*p*A residues may be *O*-acetylated on C–2 and/or C–3 but there is no evidence that the Gal*p*A residues are methyl esterified. Between 20 and 80% of the rhamnosyl (Rha*p*) are, depending on the plant source and the method of isolation, substituted at C–4 with neutral and acidic oligosaccharide side-chains.

Rhamnogalacturonan II (RG-II) is a substituted galacturonan that is present in the walls of all higher plants predominantly as a dimer that is cross-linked by a borate ester [3]. RG-II contains eleven different glycosyl-residues including the unusual sugars 3–C–carboxy–5–deoxy–L–lyxose (aceric acid), 3–deoxy–2–keto–D-*manno*–octulosonic acid (Kdo), 3–deoxy–2–keto–D–*lyxo*–heptulosaric acid (Dha), apiose, 2–*O*–methyl–xylose, and 2–*O*–methyl–fucose. The RG-II backbone is composed of at least seven 1,4-linked α–D–Gal*p*A residues of which some may be methyl esterified. Two structurally different disaccharides are attached to C–3 of the backbone and two structurally different octasaccharides are attached to C–2 of the backbone (Fig. 1).

Pectic polysaccharides in the human diet

Pectins are important in the human diet and health since they are a major component of dietary fiber and have been reported to lower serum cholesterol levels, to bind heavy metals, and to have immunostimulating and anti-ulcer activities. Pectins affect the texture and processing characteristics of fruits and vegetables. The ability of pectins to form gels has been exploited by man for many centuries and these

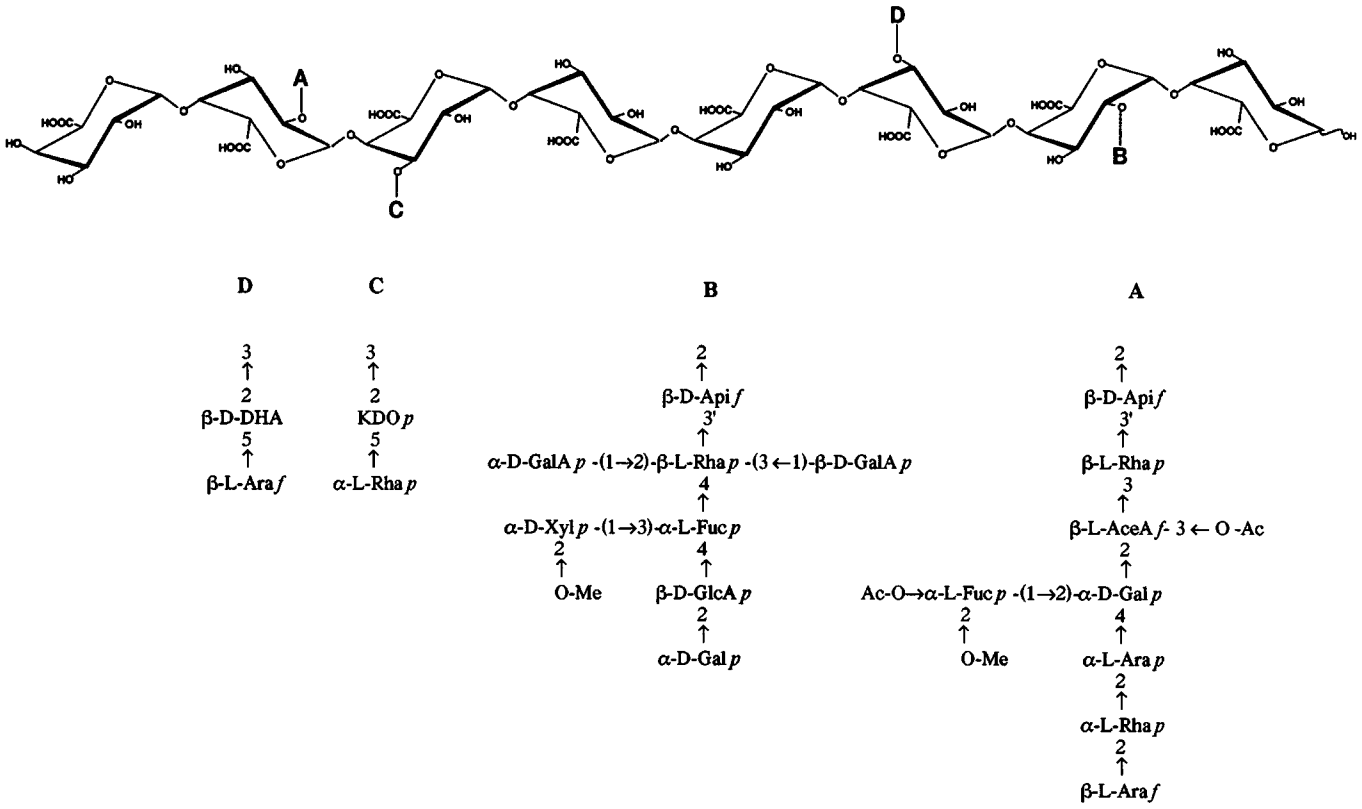

Figure 1. Hypothetical structure of mRG-II. The attachment sites of side chains on the homogalacturonan backbone have not been determined.

polysaccharides have numerous applications; especially in the food industry [4]. Pectins are extracted from plant tissues (e.g. fruits and vegetables) with chelators and acids, by heat treatment, or enzymic (endo-polygalacturonase, pectin-lyase) treatments.

RG-I and RG-II are both solubilized during the processing of fruits and vegetables and are present in fruit-juices and fermented beverages including cider or wine [5]. The concentration of RG-I and RG-II in these products is increased by enzymic maceration and liquefaction of plant material during processing [6]. Such treatments hydrolyze the homogalacturonan portion of pectin in the cell wall and thereby increase the solubility of pectic polysaccharides.

Complexation of metals with pectic polysaccharides

The ability of pectins to bind cations is due to the presence of non-methyl esterified galacturonosyl residues. Demethyl esterified pectins form gels in the presence of calcium due to the formation of ionic cross-links between homogalacturonan chains. The mechanism of gelation is not fully understood, although one model (the "egg-box" model) has received much attention. In this model, calcium cross-linked junction zones are proposed to form between homogalacturonan chains that contain at least six contiguous and non-esterified galacturonosyl residues (Fig. 2). This results in the formation of a polymer network in which water molecules are entrapped. The gelation of pectins and the physical properties of the gel are controlled by the extent of methyl esterification of the galacturonosyl residues and their interaction with divalent cations -(mainly Ca^{2+}).

Due to their anion character, pectins are used as cation-exchangers for the removal of metal cations from aqueous solutions [7]. The divalent cations are exchanged with monovalent counter-ions (Na^+ or K^+). The complexation capacity increases between pH 3 and 7 due to the dissociation of the carboxylic acid groups. Divalent cations can be classified according to the selectivity (affinity, ratio metal/free carboxylic groups) of their complexation [7]:

$$Cu^{2+} \sim Pb^{2+} >> Zn^{2+} > Cd^{2+} \sim Ni^{2+} > Ca^{2+} .$$

Such differences in specificity allow the use of pectins or plant biomass for the removal of heavy metal cations from aqueous solutions. The total capacity of a pectic polysaccharide to complex metal cations is directly related to its degree of methyl-esterification, degree of polymerisation, and its glycosyl-residue composition.

Complexation of specific metal cations with the dimers of rhamnogalacturonan II

Rhamnogalacturonan II is present in the primary walls of all higher plants predominantly as a dimer (dRG-II-B) that is cross-linked by a 1:2 borate-diol ester [3]. The ester is believed to be located between two of the four 3'-linked apiosyl residues present in the dimer. The dimer forms slowly *in vitro* by treating the monomer (mRG-II) at

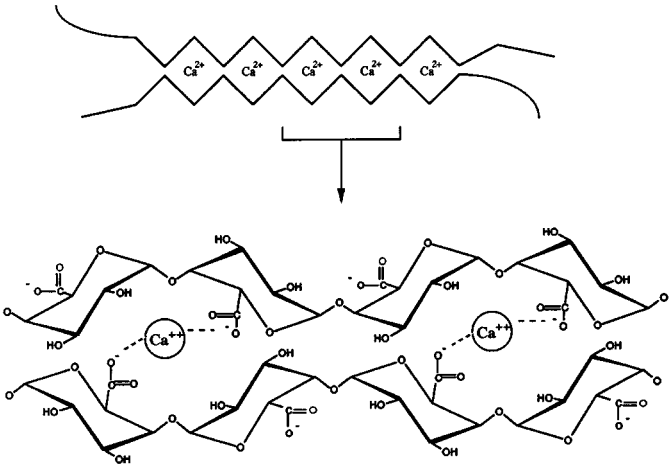

Figure 2.The "egg-box"model for the cross-linkage of two non-esterified galalacturonan chains.

pH 2.5 – 4.0 with an equimolar amount of boric acid. Typically, the yield of dRG-II-B is < 30% after 24 h, although the rate and extent of dimer formation is increased by using higher concentrations of boric acid. The addition of an equimolar amount of Pb^{2+}, Ba^{2+}, Sr^{2+}, La^{3+}, Eu^{3+} Ce^{3+}, Pr^{3+}, or Nd^{3+} dramatically increases the reaction velocity and complete conversion of the monomer to the dimer occurs in 30 min at pH 3.5 in presence of Pb^{2+} [3]. The role of the cations in dimer formation has not been determined although it appears to be catalytic. The cation most likely forms a 1:1 coordination complex with the dimer and this may involve coordination to the oxygens of glycosyl residues in the proximity of the borate esters or to the oxygens of the borate ester. The cation coordinated to the dimer is not removed by treatment with chelex resins, although it is partially removed at a slow rate by using a large molar excess (~ 100 fold) of CDTA or EDTA.

A limited survey of the cations that promote dimer formation *in vitro* has indicated that they have certain common characteristics including:

- a valence of 2+ or 3+;
- a crystal ionic radius > 0.95 Å;
- an electronic configuration with an incompletely filled sub-shell;
- a low ionization energy;
- an affinity for oxygen-donor ligands.

Physiologically important metal cations (e.g. Cu^{2+}, Fe^{2+}, Mg^{2+} or Zn^{2+}) do not promote dimer formation. Calcium which has an ionic radius of 0.99 Å has a low affinity for dRG-II-B. Mercury which has a crystal ionic radius of 1.10 Å does not promote dimer formation and this may be due to the fact that Hg^{2+} has a low affinity for oxygen-donor ligands. Thus, dRG-II-B can have the potential to be used for the complexation of Pb^{2+}, Ba^{2+}, Sr^{2+}, La^{3+}, Eu^{3+} Ce^{3+}, Pr^{3+}, and Nd^{3+} since these cations readily displace Na^+, K^+ and Ca^{2+} from the dimer.

Speciation of selected metal cations in plant cell walls

The complexation of specific metal cations with the dRG-II-B has been demonstrated *in vitro* by treating mRG-II with boric acid and cations [3]. Such complexes are also likely to be present in the plant cell wall since treating walls with endo-polygalacturonase solubilizes dRG-II-B that contains Sr^{2+} (~0.3 moles/mole dimer), Ba^{2+} (~0.1 moles/mole dimer), and Pb^{2+} (~0.1 moles/mole dimer) [3,8,9]. The mRG-II from the same plants contain little if any of these cations. These results are consistent with previous studies that reported the presence of lead in the insoluble fraction (cell wall) of plants. The physiological significance of the ability of dRG-II-B to bind heavy metals *in muro* is not known, although it may allow plants to grow in the soils that contain elevated amounts of toxic cations. The potential application of these findings for bioremediation of contaminated soils has been proposed [10].

Speciation of selected metal cations in wines and fruit juices

RG-II accounts for less than 5% of the primary cell wall but its resistance to fragmentation by all known pectolytic enzymes is responsible for its unusually high concentration in products obtained by pectolytic enzyme treatment of plant material. Wines and fruit juices may, depending on the source, contain between 50 – 400 mg L^{-1} of dRG-II-B [6,8]. This concentration (5 – 40 µM) is sufficient to complex all of the Pb^{2+}, Ba^{2+}, Sr^{2+}, and lanthanide cations present in most wines and juices. Moreover, boron is also present in relatively high concentration in fruits and vegetables (20 – 50 µg g^{-1} dry wt) and thus is not a limiting factor for the formation of dRG-II-B.

We have shown, using graphite furnace atomic absorption spectrophotometry (AAS) [11], that the majority of lead in wines is bound with dRG-II-B. Recently, we have extended these studies by using size-exclusion chromatography (SEC) with on-line detection of cations by inductively-coupled-plasma mass spectrometry (ICP-MS) [12] to determine the total, free and bound lead in 20 wine samples (red, white, rosé, and sparkling) from different countries. The co-elution of dRG-II, boron and lead from the SEC column (Superdex 75-HR 10/30) confirmed that lead is indeed complexed with dRG-II-B. Between 45 and 95% of the lead in the wines is bound to dRG-II-B and no free mineral lead is detected. These products provide compelling evidence that much of the lead is bound to dRG-II-B, although it is likely that other lead complexants are also present in some wines. The results of preliminary studies using SEC-ICP-MS indicate that in wines Sr^{2+}, Ba^{2+} and lanthanides are also complexed to dRG-II-B.

One consequence of our results with heavy metals in wines is that the description of wine as a significant source of lead in the human diet needs to be reevaluated. Nutritional studies are required to establish if the complexation of metal cations by dRG-II-B limits their bioavailability and their absorption through the gastro-intestinal tract. The addition of pectic polysaccharides to the human diet has been shown to reduce the uptake of toxic metals and actinides [13]. However, pectins also bind physiologically important cations (Ca^{2+}, Cu^{2+}, Mg^{2+} or Zn^{2+}) and elevated consumption of pectins may result in a decrease in the availability of essential minerals. dRG-II-B has a higher degree of selectivity for cations than does pectin and may have potential applications as a food additive for the removal of toxic cations.

Isolation and characterization of metal-dRG-II-B complexes

Extraction and purification of dRG-II-B

RG-II is solubilized by treating plant cell walls with pectolytic enzymes. In most studies homogeneous purified endo-polygalacturonase are used. This enzyme specifically fragments the homogalacturonan backbone of pectins and releases RG-I, RG-II and low molecular weight oligogalacturonides. These molecules are separated and purified by a combination of successive anion-exchange and size-exclusion chromatographies. Alternatively, commercial pectinases that are used to liquefy plant material can also be used to solubilize RG-II. These enzyme preparations are a mixture of pectinases, cellulases and hemicellulases, but since none of the enzymes are capable of degrading RG-II, they can be used to quantitatively release RG-II from plant material [6]. Fermented beverages including wine and cider can be used as a direct source of RG-II since part of the manufacturing process involves degradation of the fruit cell walls with pectinases produced by the fermentation microflora.

The RG-II is then isolated by ethanol-precipitation or ultrafiltration of the wine or of the fruit juice, and purified by a combination of anion-exchange and size-exclusion chromatographies. These low-resolution chromatographic techniques typically do not resolve dRG-II-B and mRG-II. However, the monomer and the dimer are readily separated using high-resolution SEC columns such as Superdex 75-HR 10/30 (Pharmacia, Sweden) [3,8].

Chemical characterization of RG-II

The glycosyl-residue and glysosyl-linkage compositions of RG-II are determined by GLC analysis. The neutral glycosyl-residue compositions are determined, after acid hydrolysis, by converting the released monosaccharides into their alditol acetates derivatives. The neutral and acidic glycosyl-residue compositions are determined, after solvolysis with methanolic HCl, by converting the methyl glycosides into their corresponding per-*O*-trimethylsilylated derivatives. The identification of some of the monosaccharides present in RG-II (e.g. apiose, 2-0-methyl-xylose, 2-0-methyl-fucose, Kdo, Dha, and aceric acid) requires the use of GLC-MS. However, the simultaneous appearance of these monosaccharides in the same polysaccharide is considered to be diagnostic for the presence of RG-II.

Glycosyl-linkage composition analysis is determined by GLC-MS analysis of the partially methylated alditol acetate derivatives. The procedure involves permethylation of RG-II followed by reduction of the methyl esterified carboxyl

groups, acid hydrolysis, and conversion of the released methylated monosaccharides to their corresponding *O*-methylated alditol acetates derivatives.

Several other analytical techniques including ^{1}H, ^{13}C or ^{11}B NMR spectroscopy, electrospray mass spectrometry, matrix-assisted-laser-desorption time-of-flight mass spectrometry have also been used for the structural characterization of RG-II.

Identification of metal cations complexed to dRG-II-B

The cations bound to isolated dRG-II-B preparations have been identified and quantified by inductively-coupled-plasma-atomic-emission spectroscopy (ICP-AES) [8] or graphite furnace atomic absorption spectrophotometry (AAS) [11]. SEC-ICP-MS is also a powerful technique for determining the cation contents of dRG-II-B [9,12]. In principle, ^{207}Pb NMR spectroscopy could be used to structurally characterize the dRG-II-B/Pb complex. However, lead has a relatively insensitive nucleus (comparable to ^{13}C) and the chemical shifts of lead are concentration-dependent which is likely to complicate the interpretation of the NMR spectra. Lanthanides are used as chemical shift reagents in NMR spectroscopy and although dRG-II-B is known to bind lanthanides, the use of these interactions for NMR spectroscopy analysis has not been investigated.

Conclusion

The selective complexation of Pb^{2+}, Ba^{2+}, Sr^{2+}, La^{3+}, Eu^{3+} Ce^{3+}, Pr^{3+}, and Nd^{3+} by dimers of RG-II has been documented, although the locations of the glysosyl residues that participate in the formation of the coordination complex have not been identified. The specificity of the complexation for toxic metals and the occurrence of relatively high amounts of dRG-II-B in wines and fruit juices may have implications in human nutrition. The actual bio-availability and toxicity of bound Pb^{2+} in wine and cider need to be determined. The potential use of RG-II as a food additive in contaminated area is now being investigated. The role of dRG-II-B and metals in the normal growth and development of plants is also being studied.

Acknowledgement

Research at the CCRC was supported in part by US Department of Energy Grants DE-FG02-96ER20220 and DE-FG05-93ER20097.

References

1. Carpita, N. C.; Gibeaut, D. M. *Plant. J*. **1993**, *3*, 1-30.
2. Albersheim, P.; Darvill, A. G.; O'Neill, M. A. Schols H. A. Voragen in: *Progress in Biotechnol. 14. Pectins and Pectinases*, Visser, J.; Voragen, A. G. J. Eds., 1996; pp 47-55.
3. O'Neill, M. A.; Warrenfeltz, D.; Kates, K.; Pellerin, P.; Doco, T.; Darvill, A. G.; Albersheim, P. *J. Biol. Chem.* **1996**, *271*, 22923-22930.
4. Visser, J.; Voragen, A. G. J. in: *Prog. Biotechnol.* 14, 1996.
5. Doco, T.; Brillouet, J. M. *Carbohydr. Res.* **1993**, *243*, 333-343.
6. Doco, T.; Williams, P.; Vidal, S.; Pellerin, P. *Carbohydr. Res.* **1997**, *297*, 181-186.
7. Dronnet, V. M.; Renard, C. M. G. C.; Axelos, M. A. V.; Thibault, J. F. *Carbohydr. Polym.* **1996**, *30*; 253-263.
8. Pellerin, P.; Doco, T.; Williams, P.; Vidal, S.; Williams, P.; Brillouet, J. M.; O'Neill, M. A. *Carbohydr. Res.* **1996**, *290*, 183-197.
9. Matsunaga, T.; Ishii, T.; Watanabe-Oda, H. in: *Plant nutrition-for sustainable food production and environment*, Ando, T. Ed., 1997; pp 81-82.
10. O'Neill, M. A.; Pellerin, P.; Warrenfeltz, D.; Vidal, S.; Darvill, A.; Albersheim, P. 1996, US Patent n° 08/755,058.
11. Pellerin, P.; O'Neill, M. A.; Peirre, C.; Cabanis, M. T.; Darvill, A. G.; Albersheim, P.; Moutounet, M. *J. Int. Sci. Vigne Vin* **1997**, *31*, 33-41.
12. Szpunar, J.; Pellerin; Makarov, A.; Doco, T.; Williams, P.; Medina, B.; Lobinski, R. **1998**, *13*, in press.
13. Schlemmer, U. *Z. Lebensm. Unters Forsch* **1986**, *183*, 339-343.