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Neutral sugar side chains of pectins limit interactions with procyanidins

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A B S T R A C T

Interactions between seven hairy regions of pectins, rhamnogalacturonans II and arabinogalactan–proteins and procyanidins with different average degrees of polymerization, low (DP9) and high (DP30), were investigated by isothermal titration calorimetry and absorption analysis to study the impact of neutral sugar side chains of pectins on these associations. Associations between pectic fractions and procyanidins involved hydrophobic interactions and hydrogen bonds. No difference in association constants between various hairy regions and procyanidins DP9 was found. Nevertheless, arabinan chains showed lower association constants, and hairy regions of pectins with only monomeric side chains showed higher association with procyanidin DP30. Only very low affinities were obtained with rhamnogalacturonans II and arabinogalactan–proteins. Aggregation could be observed only with the procyanidins of DP30 and the protein-rich arabinogalactan–protein. Associations were obtained at both degrees of polymerization of the procyanidins, but differed depending on neutral sugar composition and the structure of pectic fractions.

Keywords:

Polyphenol
Condensed tannins
Polysaccharide
Rhamnogalacturonan
Association
ITC

1. Introduction

Pectins are complex polysaccharides of plant cell walls, present mostly in the middle lamella and primary wall. They comprise smooth and hairy regions. The “smooth regions”, also known as homogalacturonans (HGs), consist of a long chain of (1→4) linked α -D-galacturonic acid (GalpA) with various degrees of methylation and/or acetylation. The hairy regions, named rhamnogalacturonans (RGs), are of two types (I and II) (Caffall & Mohnen, 2009; De Vries, Rombouts, Voragen, & Pilnik 1982; Thomas, McNeil, Darvill, & Albersheim, 1987). Rhamnogalacturonans I are formed by the repeating unit $\rightarrow 4$ - α -D-GalpA-(1→2)- α -L-Rhap-(1→), the rhamnose residues of the RG fractions being decorated by long side-chains composed mostly of galactose and arabinose (Ridley, O'Neill, & Mohnen, 2001; Voragen, Coenen, Verhoef, & Schols, 2009). RG I presents this strictly alternating RG backbone (De Vries et al., 1982; Renard, Crépeau, & Thibault, 1995; Schols, Posthumus, & Voragen, 1990), while the misleadingly named RG II possesses a backbone of (1→4) linked α -D-GalpA as

homogalacturonans with side chains containing rhamnose and a variety of rare monosaccharides (Pérez, Rodríguez-Carvajal, & Doco, 2003). Arabinogalactan–proteins (AGPs) are often associated with pectins because both molecules contain a similar structural motif of type II galactans, i.e. (1→3) and (1→6) linked galactans ramified with arabinan oligomers (Voragen et al., 2009). However, in AGPs the arabinogalactan domains are connected via a specific hydroxyproline-rich protein (Clarke, Anderson, & Stone, 1979).

Procyanidins are flavonoids, major polyphenols found in vacuoles of fruits and vegetables. They are a subclass of proanthocyanidins derived from the oligomerization of flavan-3-ols units and form the condensed tannins. They are characterized by their constitutive units, their interflavanic linkages and their degree of polymerization (Hemingway & Karchesy, 1989; Quideau, Deffieux, Douat-Casassus, & Pouységu, 2011). Specifically, apple procyanidins are characterized by the predominance of (–)-epicatechin and of C4–C8 interflavanic linkages (Guyot, Marnet, Laraba, Sanoner, & Drilleau, 1998; Sanoner, Guyot, Marnet, Molle, & Drilleau, 1999; Wojdyło, Oszmianski, & Laskowski, 2008). They can be oligomers or polymers and in cider apples, their number-average degree of polymerization range between DP_n 4.5–50 for varieties Jeanne Renard and Avrolles respectively (Sanoner et al., 1999). They have been reported to exhibit several health effects as antioxidants and anticarcinogens, and to protect against cardiovascular diseases.

Fruit and vegetable processing ruptures cells. This disruption leads to association between constituents of cell walls and vacuoles,

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with a prominent role of pectins and procyanidins (Le Bourvellec, Bouchet, & Renard, 2005). The bioavailability of polyphenols is dependent on their structure and their association with other compounds such as sugars (Parada & Aguilera, 2007). Procyanidins associated with dietary fiber are not bioaccessible in the upper part of the human intestine, and travel to the colon, where they become fermentable substrates for bacterial microflora (Cheynier, Sarni-Manchado, & Quideau, 2012; Palafox-Carlos, Ayala-Zavala, & González-Aguilar, 2011; Saura-Calixto et al., 2010), producing metabolites that are both absorbable and bioactive (Williamson & Clifford, 2010). In vitro, the interaction between procyanidins and dietary fiber reduces the polysaccharide fermentation and increases the production of procyanidin colonic metabolites (Aura et al., 2012; Saura-Calixto et al., 2010). To understand the health effect of polyphenols after binding to polysaccharides such as pectins, earlier work dealt with mechanisms of interactions between these two macromolecules. In our previous work (Watrelet, Le Bourvellec, Imberty, & Renard 2013), interactions between procyanidins and pectins were analyzed, and the impact of the degree of methylation of smooth regions and of the degree of polymerization of procyanidins could be demonstrated. However, the role of the hairy regions of pectins has not been determined. The aim of this study was to quantify the binding of a specific class of proanthocyanidins, the procyanidins, to hairy regions of pectins. Isothermal titration calorimetry and a spectrophotometric method were used to probe thermodynamic parameters and aggregation phenomena in interactions between hairy region fractions and procyanidins with different average degrees of polymerization: low (DP9) and high (DP30).

2. Materials and methods

2.1. Chemicals

Methanol, acetonitrile, and acetone of chromatographic quality were from Biosolve (Distribio, Evry, France). Hexane (Merck, Darmstadt, Germany) was of analytical quality. Ethanol and acetone were from Fisher Scientific (Strasbourg, France).

Silver nitrate was from Merck (Darmstadt, Germany). Sodium borohydride (NaBH_4), *N*-methylimidazole, acetic anhydride, toluene- α -thiol and pectin from apple were from Sigma-Aldrich (Deisenhofen, Germany). Chlorogenic acid, (+)-catechin, and (–)-epicatechin were from Sigma-Aldrich. 4-Coumaric acid was from Extrasynthèse (Lyon, France). Phloridzin and sugar standards were from Fluka (Buchs, Switzerland). Methanol- d_3 was from Acros Organics (Geel, Belgium). Enzymatic cocktail Endozym polifruit liq⁺ was from Spindal groupe AEB (Gretz-Armainvilliers, France). Modified hairy regions (MHR1) from apple were kindly supplied and characterized by Dr. M. C. Ralet (INRA-UR1268 Biopolymères Interactions Assemblages, Nantes, France). Modified hairy regions MHR2, MHR3 and MHR4 from pear, onion and soybean (SSPS) respectively were kindly supplied by Dr. Henk Schols (WU Agrotechnology & Food Sciences, Wageningen, Netherlands). Rhamnogalacturonans of type II monomer and dimer (mRG-II and dRG-II) and arabinogalactan-protein (AGP0 and AGP4) were kindly supplied by Dr. Claire Dufour (INRA-UMR408 Sécurité et Qualité des Produits d'Origine Végétale, Avignon, France).

2.2. Plant material

Apple fruits (*Malus domestica* Borkh.) of the Avrolles and Marie Ménard varieties were harvested at commercial maturity during the 2000 season in the experimental orchard of the Center Technique des Productions Cidricoles (CTPC, Sées, France). Fruits were mechanically peeled and cored as previously described by Guyot,

Doco, Souquet, Moutounet, and Drilleau (1997) and cortex tissues were freeze-dried. Apple pomace was from Val de Vire Bioactives (Condé-sur-Vire, France). Fibrex 615 (Sugar beet pulp) was from Nordic Sugar (Copenhagen, Denmark).

2.3. Procyanidin extraction and purification

Procyanidins were extracted from “Marie Ménard” and “Avrolles” freeze-dried pulps by successive solvent extractions and purified as described by Watrelet et al. (2013). The purified procyanidin fractions were designated DP9 (from “Marie Ménard”) and DP30 (from “Avrolles”). In the two fractions, (–)-epicatechin was the predominant constitutive unit, accounting for more than 95% and 88% in DP30 and DP9 respectively. For details, see Watrelet et al. (2013).

2.4. Rhamnogalacturonan preparation

Rhamnogalacturonans were prepared as described by Renard and Jarvis (1999) using 15 g/L of apple pectins saponified at 4 °C, pH > 12 and left overnight. The solution was then brought to pH 6 and precipitated with three volumes of an ethanol/water/hydrochloric acid mixture (96:3:1, v/v/v) with stirring at 4 °C and left overnight. This suspension was filtered on a GO sintered glass filter. After filtration the pectic acid precipitate was washed thoroughly with an ethanol/water mixture (70:30, v/v) until all remaining chloride ions were eliminated (as verified by silver nitrate precipitation). The resulting powder was dried by solvent exchange (ethanol 96% and acetone), followed by drying at 40 °C in an oven overnight. The pectic acid was dissolved (6 g/L) and hydrolyzed with hydrochloric acid (0.25 N) for 72 h at 80 °C (Thibault, Renard, Axelos, Roger, & Crepeau, 1993). The supernatant was concentrated on a rotary evaporator before dialyzing against 0.1 M NaCl followed by water to eliminate monomers and neutral oligomers (galactose and arabinose). The rhamnogalacturonan oligomers (RG) were finally freeze-dried.

Apple hairy regions were obtained by a modified alkaline peroxide treatment as described by Renard, Lahaye, Mutter, Voragen, & Thibault (1997). Apple pomace was suspended overnight at room temperature with stirring at 50 g/L in water, and pH was stabilized at >13. Polyphenols were then eliminated by adding 200 mL of hydrogen peroxide (30%). The medium was left to react with stirring for 2 h. The pH was adjusted to 5 with acetic acid, and enzymatic cocktail Endozym polifruit liq⁺ (1 mL) was added and left to act overnight with stirring at room temperature. The suspension was filtered on a nylon membrane (250 μm pore size) under vacuum. The filtrate was dialyzed against 0.1 M NaCl, and then water at 4 °C. Finally, the filtrate was freeze-dried to give the apple hairy region fraction (AHR).

Acid-treated hairy regions (HR-H) were obtained from sugar beet pulp as described by Ralet, Tranquet, Poulain, Moise, and Guillon (2010).

Modified hairy regions (MHR1) from apple were prepared by the method of Schols et al. (1990) and were a kind gift from Dr. M.-C. Ralet. Modified hairy regions (MHR2 and MHR3) from pear and onion were prepared by the method of Schols and Voragen (1994). MHR4 from soybean was prepared by Coenen (2007). MHR2, MHR3 and MHR4 were a kind gift from Dr. Henk Schols. All MHRs resulting from drastic enzymic hydrolysis of plant cell walls were isolated as high molar mass polymers with a rhamnogalacturonan backbone. However, the structure of the side chain depends on the botanical origin (Table 1). MHR1 from apple was mostly composed of β - $(1\rightarrow4)$ linked galactans and arabinans (Renard, Voragen, Thibault, & Pilnik, 1991; Schols & Voragen, 1994). They were characterized by a high xylose content with a molar ratio Xyl: GalA of 0.4, which corresponds to xylogalacturonan chains (Schols, Bakx, Schipper, and

Table 1

Composition (mg/g dry matter) and partition coefficient of the different rhamnogalacturonan subfractions.

Samples (mg/g)	K_{av}	Rha	Fuc	Ara	Xyl	Man	Gal	Glc	GalA	Characteristic structure
RG	0.26	49	3	45	15	10	236	128	458	
AHR	0.28	23	2	251	16	–	30	19	167	Arabinan
HR-H	0.13	143	–	25	–	–	199	–	437	RG with monomeric galactans
<i>Pooled SD</i>	–	1.7	0.1	9.6	0.7	0.9	9.5	5.7	4.5	
a MHR1	–0.06	89	9	83	113	–	134	–	385	Arabinan Type I galactan Xylogalacturonan
a MHR2	–0.03	79	–	240	42	–	101	–	209	Arabinan Type II galactan
a MHR3	0.01	132	–	40	17	7	208	–	283	Type I galactan
bMHR4	nd	41	33	188	52	–	351	27	165	Galactan Xylogalacturonan
c AGP0	0.08	11	1	358	2	18	583	16	28	Type II Arabinogalactan
c AGP4	0.02	93	2	250	18	23	354	3	180	

RG, rhamnogalacturonans; AHR, apple hairy regions; HR-H, acid-treated hairy regions; MHR, modified hairy regions (1 from apple, 2 from pear, 3 from onion and 4 from soybean); AGPs, arabinogalactan–proteins; K_{av} , partition coefficient; Rha, rhamnose; Fuc, fucose; Ara, arabinose; Xyl, xylose; Man, mannose; Gal, galactose; Glc, glucose; GalA, galacturonic acid. *Pooled SD*: pooled standard deviation, $n = 11$. a: Schols and Voragen (1994); b: Coenen (2007); c: Pellerin et al. (1995).

Voragen, 1995). MHR2 from pear was rich in arabinose present as (1→5)-linked chains (with proportion of 71%). Its galactans were predominantly highly ramified β-(1→3) and β-(1→6) type II galactan and galactose residues were terminally linked (with proportion of 36%) (Schols & Voragen, 1994). MHR3 from onion was rich in galactose with high proportion of terminal galactose residues (58%) and in β-(1→4) linked type I galactan (19%) (Schols & Voragen, 1994), as was MHR4 from soybean with the additional presence of β-D-Xyl side chains substituted to (1,4)-linked galacturonic acid, i.e. also xylogalacturonans (Coenen, Bakx, Verhoef, Schols, & Voragen, 2007; Nakamura, Furuta, Maeda, Nagamatsu, & Yoshimoto, 2001).

Polysaccharides isolated from red wine were kindly donated by Dr. C. Dufour (INRA-UMR408 Sécurité et Qualité des Produits d'Origine Végétale, Avignon, France). Rhamnogalacturonans, RG-IIs, were purified as described in Pellerin et al. (1996). Fractions mRG-II and dRG-II were predominantly monomeric (90% pure) and dimeric (87% pure). Monomer and dimer (mRG-II and dRG-II) were rich in arabinose and composed of rare neutral sugars. The dimer was distinguished by the presence of boron.

Type II arabinogalactan–protein fractions, AGP0 and AGP4, were isolated by anion exchange chromatography according to Pellerin, Vidal, Williams, and Brillouet (1995). Fractions were similar in terms of neutral sugar composition except for arabinose and galactose with higher content in AGP0 (35.2% and 57.3%, respectively) than AGP4 (27.1% and 38.3%, respectively), while uronic acid content was higher in AGP4 than AGP0. Also, non-reducing arabinose and rhamnose were observed in both AGPs, but rhamnose was 2,4-linked in AGP4. Galactose was 3,6-linked more in AGP4, but 3,4,6-linked more in AGP0 (Pellerin et al., 1995).

2.5. Phase diagram

To identify aggregate formation during pectin–tannin interactions, a spectrophotometric method was used at 25 °C. Systematic variation of concentrations and relative amounts was carried out on a 96-well “microplate”. This was done by varying the concentrations of procyanidins (0, 0.015, 0.03, 0.06, 0.117, 0.23, 0.47, 0.94, 1.875, 3.75, 7.5, 15 mmol/L (–)-epicatechin equivalent) along the rows, and varying the concentrations of hairy regions (0, 0.12, 0.23, 0.47, 0.94, 1.875, 3.75, 7.5 mmol/L galacturonic acid equivalent) along the columns. Solutions were prepared in citrate/phosphate buffer at pH 3.8 and ionic strength 0.1 mol/L. Equal amounts (50 μL) of procyanidin and hairy region fraction solutions were mixed before each spectrum measurement for 20 s. Absorbance spectra (200–650 nm) were recorded, and absorbance at 650 nm was analyzed for each solution using a SAFAS flx-Xenius XM spectrofluorimeter (SAFAS, Monaco). Control spectra were obtained using wells containing hairy regions alone in buffer and procyanidins alone in buffer. After spectra recording, microplates were centrifuged for 10 min at

2100 × g, and supernatants of control wells (hairy regions at 7.5 mmol/L in buffer (S1A) and procyanidins at 15 mmol/L in buffer (S1B)) and supernatants of wells containing procyanidins at a concentration of 15 mmol/L with hairy regions at a concentration of 7.5 mmol/L (S2) were analyzed by high pressure size exclusion chromatography (HPSEC) and high-performance liquid chromatography–diode array detection (HPLC-DAD) to define changes in partition coefficient ($\Delta K_{av} = S2-S1A$) of hairy region fractions and in average degree of polymerization of flavan-3-ols ($\Delta DP_n = S2-S1B$).

2.6. Isothermal titration microcalorimetry

To measure enthalpy changes associated with hairy region–tannin interactions at 25 °C, a VP-ITC microcalorimeter (Microcal®, GE Healthcare, Northampton, MA, USA) was used. To allow comparison between the different pectic substrates, and given the polydispersity in molecular weight for both pectic fractions and procyanidins, all concentrations are expressed relative to the main monomers, i.e. galacturonic acid for pectins and (–)-epicatechin for procyanidins. Procyanidins and hairy region fractions were dissolved in the same citrate/phosphate buffer pH 3.8, ionic strength 0.1 mol/L, and filtered on 0.45 μm membrane. All the solutions were degassed before measurements. The reference cell was filled with water. To obtain a hyperbolic curve as recommended for low affinity systems (Turnbull & Daranas, 2003), different concentrations of compounds were tested. The pectic fraction solution was placed in the 1.448 mL sample cell of the calorimeter, and the procyanidin solution was loaded into the injection syringe and titrated into the sample cell by 30 injections of 10 μL aliquots. The duration of each injection was 20 s, with a separating delay of 5 min. The contents of the sample cell were stirred throughout the experiment at 307 rpm to ensure mixing.

Raw data obtained as a plot of heat flow (microcalories per second) against time (minutes) were then integrated peak-by-peak and normalized to obtain a plot of observed enthalpy change per mole of injectant (ΔH , kJ mol^{–1}) against the molar ratio ((–)-epicatechin/galacturonic acid). Peak integration was performed using Microcal Origin 7.0 (Microcal Software, GE Healthcare). Control experiments included the titration of procyanidin fractions into buffer, and were subtracted from titration experiments. The experimental data were fitted to a theoretical titration curve using Microcal Origin 7.0, with ΔH (enthalpy change), K_a (association constant), and n (number of binding sites per molecule) as adjustable parameters, from the relationship

$$Q_i = \frac{nP_t \Delta H V_0}{2} \left[1 + \frac{A_t}{nP_t} + \frac{1}{nKaP_t} - \sqrt{\left(1 + \frac{A_t}{nP_t} + \frac{1}{nKaP_t} \right)^2 - 4 \frac{A_t}{nP_t}} \right] \quad (1)$$

where P_t is the total galacturonic acid concentration, A_t is the total concentration of the ligand, V_0 is the volume of the cell, and Q_i is the total heat released for injection i .

The other thermodynamic parameters (ΔG and ΔS) were calculated from the van't Hoff equation.

$$\Delta G = -RT \ln K_a = \Delta H - T\Delta S \quad (2)$$

where ΔG is free enthalpy, K_a is the association constant, ΔH is the enthalpy and ΔS is the entropy of interaction.

2.7. Analysis methods

Polyphenols were measured by high-performance liquid chromatography (HPLC)/diode array detection (DAD) after thioacidolysis as described by Guyot, Marnet, and Drilleau (2001) and Le Bourvellec et al. (2011). Analyses were performed using an ultra fast liquid chromatography Shimadzu Prominence system (Kyoto, Japan) comprising two LC-20AD pumps, UFLC Prominence liquid chromatograph, a DGU-20A5 Prominence degasser, a SIL-20AHT Prominence autosampler, a CTO-20AC Prominence column oven, a SPD-M20A Prominence diode array detector, and a CBM-20A Prominence communication bus module, and was controlled by LC Solution software (Shimadzu, Kyoto, Japan). Separations were achieved in the conditions of Le Bourvellec et al. (2011). The average degree of polymerization (DP_n) of procyanidins was measured by calculating the molar ratio of all the flavan-3-ol units (thioether adducts plus terminal units) to (-)-epicatechin and (+)-catechin corresponding to terminal units. Neutral sugars, galacturonic acid and methanol were analyzed as described by Renard and Ginies (2009).

The molecular weight distribution of polysaccharides was determined using a high pressure size exclusion chromatography (HPSEC) system comprising a Jasco LC-NET II/ADC interface, a Jasco PU-2080 plus intelligent HPLC pump, a Jasco RI-2031 plus intelligent RI detector, and a degasser, and was controlled by ChromNav software (Jasco, Tokyo, Japan). Separations were achieved using two columns in series: a 8.0 mm × 300 mm i.d. OH-pack SB-802 HQ column (Showa Denko Europe, Munich, Germany) and a 300 × 7.8 mm i.d. TSK-Gel PWXL column (Tosohaas, Stuttgart, Germany) at 35 °C and a 40 × 6.0 mm i.d. guard column TSK-Gel PWXL (Tosohaas, Stuttgart, Germany). Solutions (20 μL) of the extracts (10 g/L) were injected and eluted with 0.4 mol/L sodium acetate buffer pH 3.5 at 0.8 mL/min. Dextrans T500, T70, T40 and T10 (Pharmacia BioProcess Technology, Uppsala, Sweden) and glucose (Sigma-Aldrich, Deisenhofen, Germany) were used to calibrate the column system.

2.8. Statistical analysis

Results are presented as mean values, and the reproducibility of the results is expressed as pooled standard deviation. Pooled standard deviations were calculated for each series of replicates using the sum of individual variances weighted by the sum of individual degrees of freedom (Box, Hunter, & Hunter, 1978).

3. Results

3.1. Composition of the hairy regions

The characterization of hairy regions is shown in Table 1. RG contained a high proportion of galacturonic acid, with a molar ratio GalA:Rha > 7, indicating the predominance of homogalacturonans in the backbone. Its main neutral sugars were galactose and glucose. AHR contained a substantial portion of homogalacturonans (GalA:Rha = 6), but unlike RG, it also contained a high

proportion of arabinan (molar ratio Ara:Rha > 10). HR-H was the simplest rhamnagalacturonan fraction, with the lowest neutral sugar content. The molar ratio GalA:Rha of 2.5 is consistent with the rhamnagalacturonic backbone, while the low (Gal + Ara):Rha ratio (<2) corresponds to very short side chains of arabinogalactan (Voragen et al., 2009). MHR1 and MHR4 contained more homogalacturonic backbone (GalA:Rha = 3.6:3.4, respectively) than MHR2 and MHR3 with a molar ratio GalA:Rha of 2.2 and 1.8, respectively.

The partition coefficients of RG and AHR were identical (K_{av} of 0.26 and 0.28 respectively with retention times of 17.2 min and 17.4 min), corresponding to small polymers obtained after acid hydrolysis from commercial pectins or after enzymatic reaction from apple pomace. The partition coefficient of HR-H fraction was lower (K_{av} of 0.13 with a retention time of 16.3 min), indicating a higher hydrodynamic volume. Partition coefficients were close between RG-IIs (K_{av} of 0.27 for mRG-II and 0.22 for dRG-II) and were in good agreement with calculated molecular weight (Pellerin et al., 1996). Partition coefficients of AGPs were low (K_{av} of 0.02 for AGP4) and similar to commercial apple pectins (Watrelet et al., 2013).

3.2. Interactions with procyanidins of DP_n 9

3.2.1. Hairy regions

Thermograms of titration of hairy region fractions (3.75 mmol/L galacturonic acid equivalent) by procyanidins DP9 (7.5 mmol/L (-)-epicatechin equivalent) are shown in Fig. 1.

The blank experiment (injections of procyanidins DP9 in buffer) yielded small endothermic peaks that were subtracted before integration. The thermogram of titration of RG by procyanidins DP9 (Fig. 1a) showed small exothermic peaks for the first injections, and stronger endothermic peaks after saturation. The thermogram of AHR titration by procyanidins DP9 (Fig. 1b) showed strong exothermic peaks (-8 μJ/s) with clear titration.

Thermodynamic parameters are shown in Table 2. Stoichiometry (defined as ratio of (-)-epicatechin/galacturonic acid) was fixed at 0.01 for RG (1 molecule of (-)-epicatechin binds with 100 molecules of galacturonic acid) and 0.02 for other fractions (1 molecule of (-)-epicatechin binds with 50 molecules of galacturonic acid) using a one-site model. The association constant ($K_a = 2.53 \times 10^3 \text{ M}^{-1}$) observed for RG and DP9 was of the same order of magnitude as that measured for AHR, HR-H, MHR1 and MHR3 (K_a from 1955 M^{-1} to 2225 M^{-1}). The value of K_a was lower for interaction between MHR2 and procyanidins DP9 ($K_a = 872 \text{ M}^{-1}$). Analysis of the thermodynamic contributions indicated an enthalpy contribution ($\Delta H = -18 \text{ kJ mol}^{-1}$, -16 kJ mol^{-1} and -26 kJ mol^{-1} for HR-H, MHR2 and MHR3, respectively) related to the exothermic interaction. The entropy term between HR-H, MHR2 and procyanidins DP9 was almost null ($-T\Delta S = -0.3$ to -0.5 kJ mol^{-1} , respectively), indicating a weak role of hydrophobic interactions. The analysis of the thermodynamic contributions for MHR3 gave a negative entropy term ($-T\Delta S = 7 \text{ kJ mol}^{-1}$) indicating that the interaction was driven by enthalpy, which may result from hydrogen bond association. A favorable entropy contribution was observed for RG, AHR and MHR1 ($-T\Delta S \sim -13 \text{ kJ mol}^{-1}$), indicating that the interaction was driven mostly by entropy, which may result from hydrophobic interactions (Poncet-Legrand, Gautier, Cheyner, & Imbert, 2007; Frazier et al., 2010). The titration of MHR from soybean (MHR4) by procyanidins DP9 (data not shown) displayed strong exothermic peaks, but no thermodynamic parameters could be defined.

The absence of differences in affinity between hairy regions and procyanidins DP9 was confirmed by UV-vis absorbance at 650 nm. Fig. 2a and c shows the variation of absorbance at 650 nm between hairy regions (RG, AHR, HR-H, MHR1, MHR2 and MHR3) and procyanidins DP9 at 15 mmol/L (-)-epicatechin equivalent with the

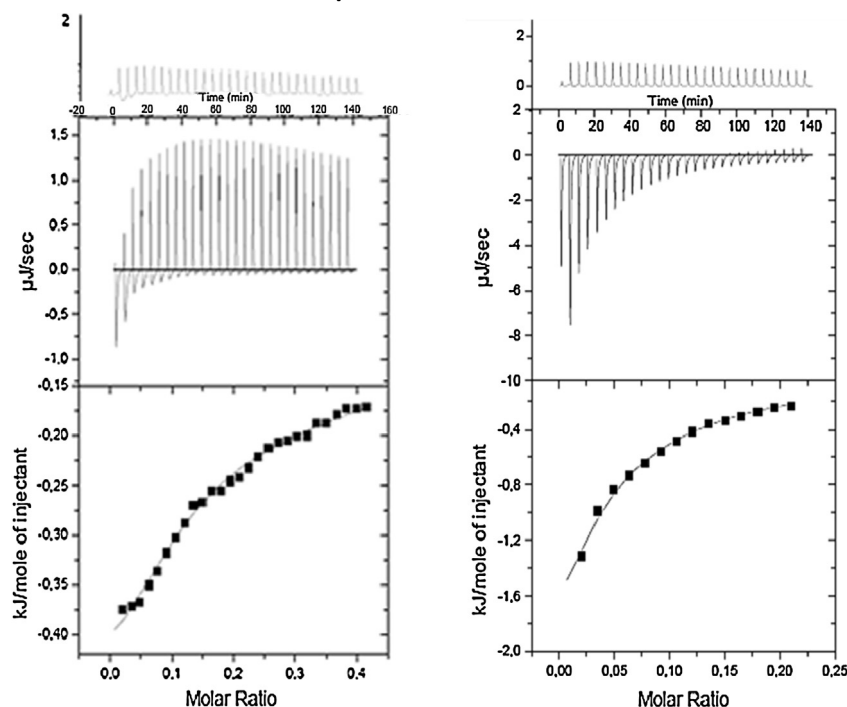


Fig. 1. Thermograms of titration of hairy region fractions (a) RG (b) AHR (3.75 mmol/L galacturonic acid equivalent) by procyanidins DP9 (7.5 mmol/L (-)-epicatechin equivalent in all cases). (Top) Control data obtained with procyanidins in buffer. (Middle) Measurement of heat release during the titration of hairy regions by procyanidins. (Bottom) Molar enthalpy change against a procyanidin/hairy region ratio after peak integration. The one-site fit curve is displayed as a thin line. Experiments were done in duplicate.

increase of concentrations of hairy regions, standardized here in galacturonic acid equivalent. Absorbance at 650 nm was chosen as an indicator for the formation of cloud and precipitate, as neither pectins nor procyanidins absorb at this wavelength. Absorbance of all hairy regions with procyanidins DP9 was low (0.04), quite stable and similar between hairy regions. Only the MHR3 fraction showed an increase in absorption up to 0.08 from 1.875 mmol/L galacturonic acid equivalent. Fig. 2b and d shows the variation in absorbance between hairy regions (RG, AHR, HR-H, MHR1, MHR2 and MHR3) at 7.5 mmol/L galacturonic acid equivalent and procyanidins DP9 with the increase in concentrations of procyanidins DP9, given in (-)-epicatechin equivalent. Initially, all hairy regions displayed a slight turbidity, which disappeared with addition of a low concentration of procyanidins. With the increase in procyanidin concentrations, absorbance of hairy regions RG, AHR and HR-H

increased slightly (up to 0.04) and in a similar fashion. Among MHRs fractions (Fig. 2d), MHR3 showed an increase in absorption up to 0.08 from 0.94 mmol/L (-)-epicatechin equivalent, while MHR1 and 2 were stable around 0.01.

The ΔK_{av} correspond to the difference in partition coefficient in HPSEC between the pectic hairy regions (at 7.5 mmol/L in galacturonic acid equivalent) that had not formed aggregates with procyanidins (at 15 mmol/L (-)-epicatechin equivalent) (S2) and the initial pectic compounds in buffer (S1A). After centrifugation of hairy regions incubated with procyanidins DP9, values of ΔK_{av} obtained were close to nil irrespective of the pectic fractions (Table 2). There was no difference in hydrodynamic volume for hairy regions before and after association with procyanidins DP9. The ΔDPn corresponds to the difference in degree of polymerization between procyanidins that had not formed aggregates with

Table 2

Thermodynamic binding parameters of interaction between pectic compounds and procyanidin fractions DP9 and DP30 (30 mmol/L in (-)-epicatechin equivalent).

DP 9	RG	AHR	HR-H	MHR1	MHR2	MHR3	MHR4	mRG-II	dRG-II	AGP0	AGP4
<i>n</i>	0.01	0.02	0.02	0.02	0.02	0.02	-	-	-	0.02	0.02
K_a (M ⁻¹)	2530	1955	1850	2105	872	2225	-	-	-	44	57
ΔH (kJ.mol ⁻¹)	-6	-3	-18	-5	-16	-26	-	-	-	-67	-38
ΔG (kJ.mol ⁻¹)	-19	-19	-19	-19	-17	-19	-	-	-	-9	-10
ΔS (J.mol ⁻¹ .K ⁻¹)	45	51	1	47	2	-25	-	-	-	-193	-95
ΔK_{av}	-0.001	0.000	0.006	0.001	-0.002	-0.001	nd	0.002	-0.001	0.001	0.005
ΔDPn	-1.61	-1.06	-1.07	0.17	0.00	-0.55	nd	0.15	-0.16	-0.22	0.15
DP 30	RG	AHR	HR-H	MHR1	MHR2	MHR3	MHR4	RGII2	RGII3	AGP0	AGP4
<i>n</i>	0.02	0.02	0.02	0.02	0.02	0.02	-	0.02	0.02	0.02	0.02
K_a (M ⁻¹)	2620	574	5390	1835	875	1170	-	72	62	112	165
ΔH (kJ mol ⁻¹)	-3	-17	-4	-7	-24	-41	-	-16	-14	-54	-31
ΔG (kJ mol ⁻¹)	-19	-16	-21	-19	-17	-17	-	-11	-10	-12	-13
ΔS (J mol ⁻¹ .K ⁻¹)	56	-4	59	38	-23	-78	-	-18	-12	-142	-61
ΔK_{av}	0.004	0.004	0.015	0.011	0.004	-0.006	nd	0.000	-0.004	0.009	0.011
ΔDPn	-11.82	-10.17	-9.49	-4.89	-5.01	-8.44	nd	0.28	0.27	-3.62	0.53

Average of duplicates for each measurement. Stoichiometry corresponds to the molar ratio value of (-)-epicatechin/galacturonic acid. nd: not defined.

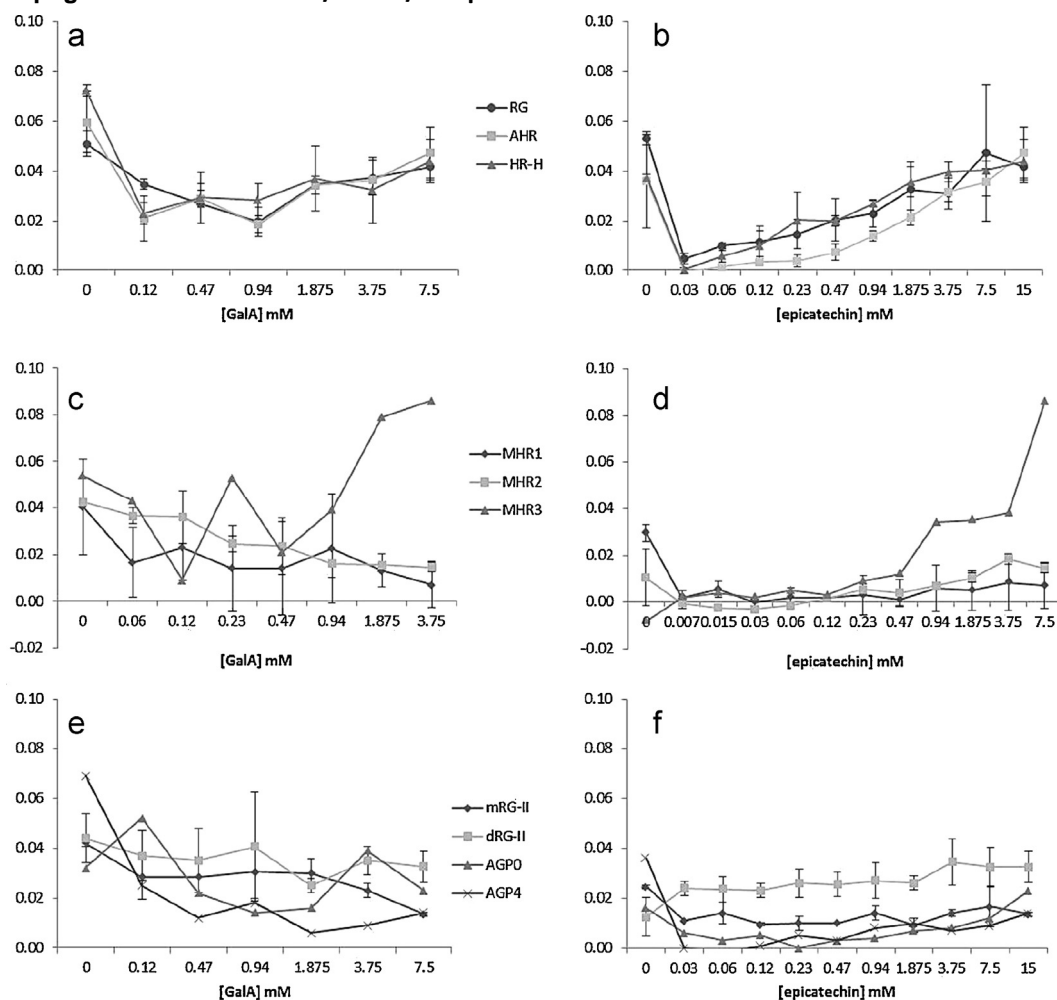


Fig. 2. Absorbance at 650 nm obtained in the phase diagram between pectic hairy regions and procyanidins DP9. Variation of absorbance of RG, AHR and HR-H (a); MHR1, MHR2 and MHR3 (c); mRG-II, dRG-II, AGP0 and AGP4 (e) at different concentrations (galacturonic acid equivalent) with procyanidins DP9 (15 mmol/L or 7.5 mmol/L (–)-epicatechin equivalent). Variation of absorbance of RG, AHR and HR-H (b); MHR1, MHR2 and MHR3 (d); mRG-II, dRG-II, AGP0 and AGP4 (f) (7.5 mmol/L or 3.75 mmol/L galacturonic acid equivalent) at different concentration of procyanidins DP9 ((–)-epicatechin equivalent).

pectins (S2) and the initial degree of polymerization of procyanidins in buffer (S1B). The ΔDP_n was -1.61 for RG and -1.06 for AHR and HR-H. The degree of polymerization of free procyanidins was slightly lower than the initial degree of polymerization of procyanidins, i.e. hairy regions RG, AHR and HR-H precipitated preferentially with highly polymerized procyanidins. The value of ΔDP_n of MHR3 was slightly negative, but there was no difference for MHR2 and MHR1. This means that MHR3, MHR1 and MHR2 were not selective with procyanidins DP9.

3.2.2. Rhamnogalacturonans II and arabinogalactan–proteins

During injection of procyanidins in rhamnogalacturonans type II (mRG-II and dRG-II), peaks were endothermic and heat was absorbed. No titration could be observed. Hence no interaction could be detected by ITC between RGII and procyanidins DP9.

A thermogram of titration of arabinogalactan–protein by procyanidins DP9 displayed only exothermic peaks (data not shown). Thermodynamic parameters were calculated from raw data after subtraction of blank experiments (procyanidins DP9 in buffer) (Table 2). Stoichiometry was fixed at 0.02 using a one-site model. Association constants obtained for AGP0 and AGP4 were very weak ($K_a = 44 \text{ M}^{-1}$ and 57 M^{-1} , respectively) and correspond to the limit of detection by isothermal titration calorimetry.

The variation of absorbance at 650 nm between rhamnogalacturonans II and arabinogalactan–proteins and procyanidins DP9 at

15 mmol/L (–)-epicatechin equivalent with the increase in concentrations of rhamnogalacturonans in galacturonic acid equivalent is shown in Fig. 2e. Whatever the pectic fraction, the absorption was stable at around 0.04. The variation of absorbance between RG-IIs and AGPs at 7.5 mmol/L galacturonic acid equivalent and procyanidins DP9 with the increase in concentrations of procyanidins DP9 in (–)-epicatechin equivalent (Fig. 2f) was low (0.01) and stable between fractions, but the dimer of RGII (dRG-II) showed absorption at 0.03.

The ΔK_{av} obtained was close to nil for all RGII and AGPs. With RGII and AGPs, the ΔK_{av} and ΔDP_n were very low or nil: there was no selective precipitation of either the procyanidins or the polysaccharides.

3.3. Interaction with procyanidins of DPn 30

3.3.1. Hairy regions

ITC measurements were made with 3.75 mmol/L of pectic compound (galacturonic acid equivalent) and 7.5 mmol/L ((–)-epicatechin equivalent) of procyanidins DP30.

Titration of RG, AHR, HR-H and MHR1, MHR2 and MHR3 by procyanidins DP30 showed complex curves characterized by strong exothermic peaks (Fig. 3a and b); to avoid over-fitting, the stoichiometry was fixed at 0.02 for all fractions to allow fit and determination of association parameters.

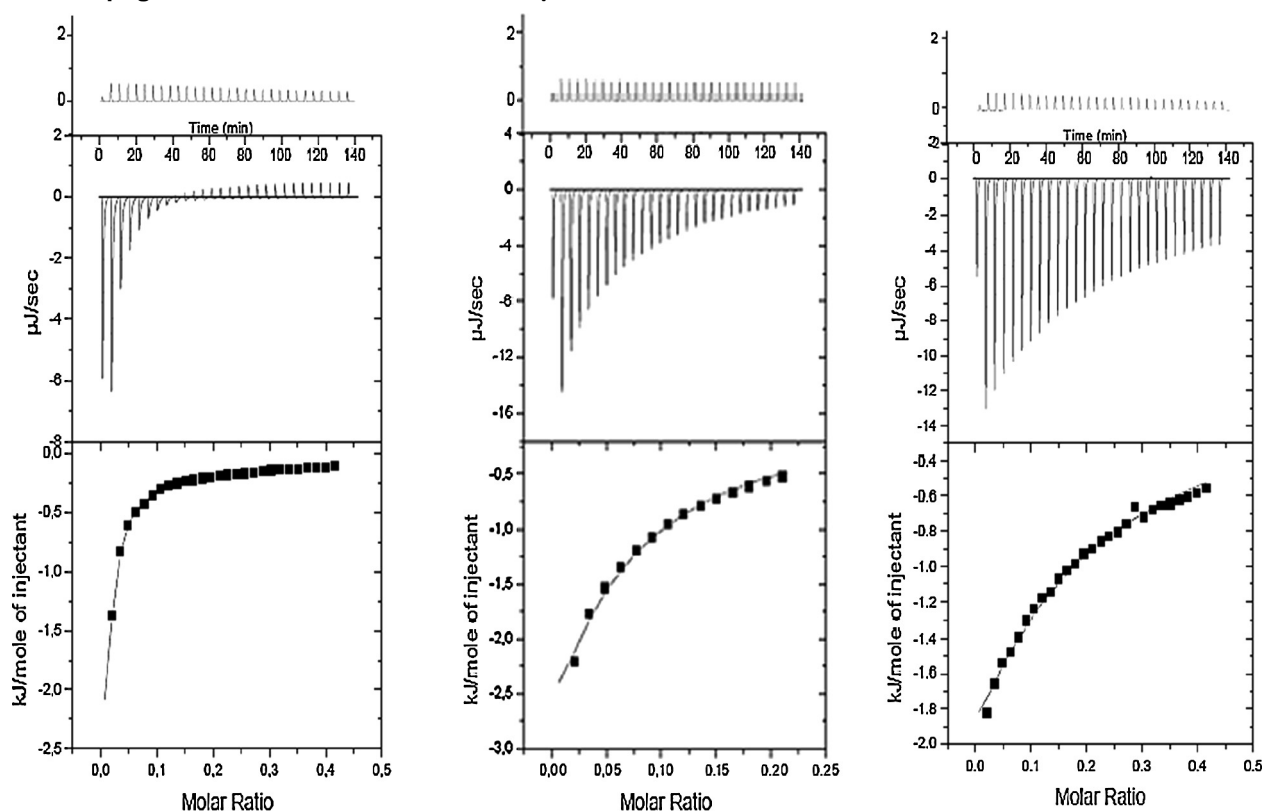


Fig. 3. Thermograms of titration of hairy regions fractions (a) RG (b) AHR (c) AGP0 (3.75 mmol/L galacturonic acid equivalent) by procyanidins DP30 (7.5 mmol/L (–)-epicatechin equivalent in all cases). (Top) Control data obtained with procyanidins in buffer. (Middle) Measurement of heat release during the titration of hairy regions by procyanidins. (Bottom) Molar enthalpy change against a procyanidin/hairy region ratio after peak integration. The one-site fit curve is displayed as a thin line. Experiments were done in duplicate.

Thermodynamic parameters are presented in Table 2. The association constant K_a ranged from 574 M^{-1} to 5390 M^{-1} in the order $\text{AHR} < \text{MHR2} < \text{MHR3} < \text{MHR1} < \text{RG} < \text{HR-H}$. Concerning the interaction between HR-H, RG and MHR1 and procyanidins DP30, thermodynamic contributions ($\Delta H = -4$; -3 and -7 kJ mol^{-1} and $-T\Delta S = -16.1$; -15.3 and $-10.4 \text{ kJ mol}^{-1}$, respectively) indicated that the interaction was driven mostly by entropy, also indicating the role of hydrophobic interactions (Frazier et al., 2010; Poncet-Legrand et al., 2007). However, thermodynamic contributions for the interaction between AHR, MHR2 and MHR3 with procyanidins DP30 showed a very weak entropy ($-T\Delta S = 1$; 6 and 21 kJ mol^{-1} , respectively), but a high enthalpy contribution ($\Delta H = -17$; -24 and -41 kJ mol^{-1} , respectively), which indicates that the association was mostly driven by enthalpy and also involves hydrogen bonds. The titration of MHR from soybean (MHR4) by procyanidins DP30 displayed very strong exothermic peaks, but no thermodynamic parameters can be defined, because it seemed that binding sites could not be saturated in these conditions.

Absorbance at 650 nm obtained between hairy regions and procyanidins DP30 at 15 mmol/L (–)-epicatechin equivalent with the variation of concentrations of hairy regions in galacturonic acid equivalent (Fig. 4a) showed an increase in absorbance from 0.12 mM in galacturonic acid equivalent and from 0.04 to 0.14 absorbance 650 nm for RG and AHR with a very high standard deviation due to the formation of aggregates. The same increase with a high standard deviation was obtained for absorbance at 650 nm with variation of concentrations of procyanidins DP30 and hairy regions at 7.5 mmol/L of galacturonic acid (Fig. 4b). For MHRs fractions (Fig. 4c and d), the absorbance was quite stable as a function of galacturonic acid concentrations, but MHR3 showed a slight increase from 0.94 mmol/L (from 0.04 absorbance) up to 0.09. It was

similar with the increase in (–)-epicatechin concentrations from 0.94 mmol/L (0.02).

The ΔK_{av} corresponds to the difference in partition coefficient in HPSEC between the hairy regions (at 7.5 mmol/L in galacturonic acid equivalent) that had not formed aggregates with procyanidins (at 15 mmol/L (–)-epicatechin equivalent) (S2) and the initial pectic compounds in buffer (S1A). The ΔK_{av} was close to null for all hairy regions. This means there was no selectivity of procyanidins DP30 for hairy regions. The ΔDP_n corresponds to the difference in degree of polymerization between procyanidins that had not formed aggregates with pectins (S2) and the initial degree of polymerization of procyanidins in buffer (S1B). The value of ΔDP_n was negative for every hairy region from -4.89 (MHR1) to -11.82 (RG). This means that hairy regions interacted preferentially with highly polymerized procyanidins.

3.3.2. Rhamnogalacturonans II and arabinogalactan–proteins

Titration of monomer and dimer of rhamnogalacturonans type II (mRG-II and dRG-II) by procyanidins DP30 displayed weak exothermic peaks (data not shown) resulting in weak association constants ($K_a = 72\text{--}62 \text{ M}^{-1}$) (Table 2). The isothermal titration calorimetry method may not be the most appropriate one for such low affinity interactions.

During titration of arabinogalactan–proteins AGP0 and AGP4 by procyanidins DP30, strong exothermic peaks were obtained (Fig. 3c), but the association constants were of the same order of magnitude as for rhamnogalacturonans II. Hence only weak associations could be detected by ITC for rhamnogalacturonans II and arabinogalactan–proteins.

The variation of absorbance at 650 nm of RG-II and AGPs with procyanidins DP30 at 15 mmol/L (–)-epicatechin equivalent

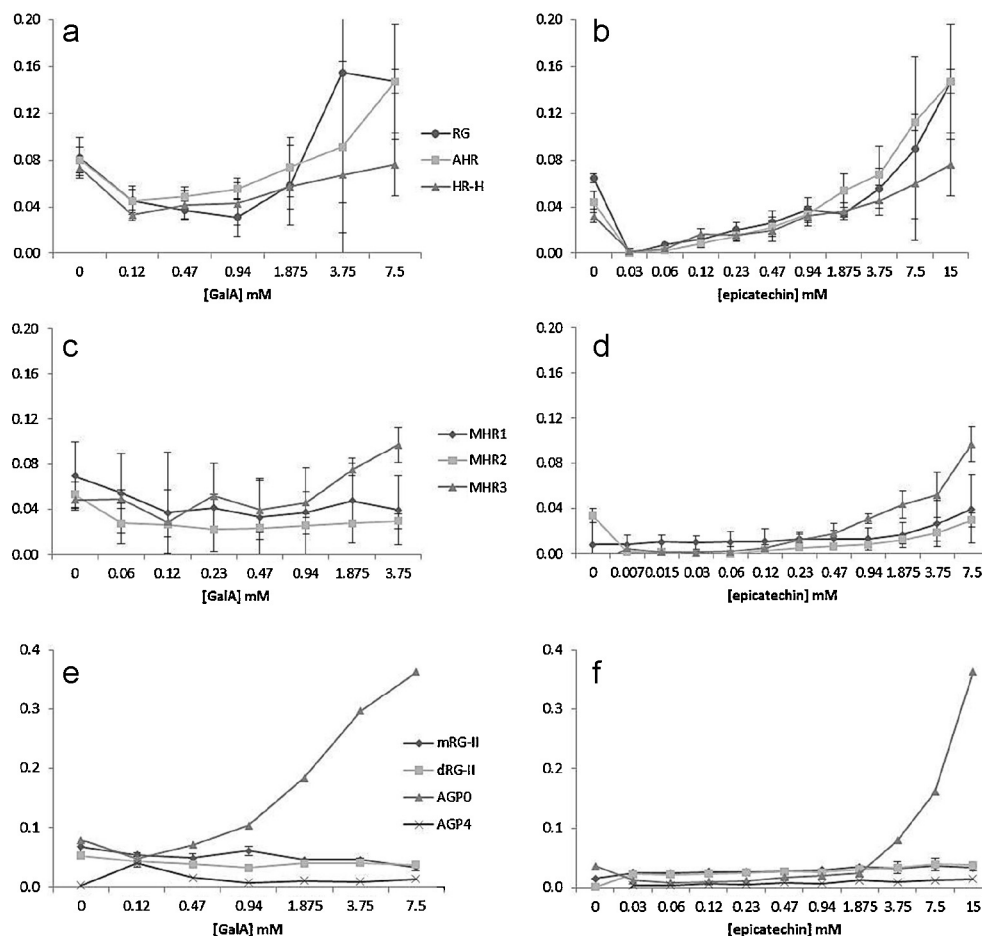


Fig. 4. Absorbance at 650 nm obtained in the phase diagram between pectic hairy regions and procyanidins DP30. Variation of absorbance of RG, AHR and HR-H (a); MHR1, MHR2 and MHR3 (c); mRG-II, dRG-II, AGP0 and AGP4 (e) at different concentrations (galacturonic acid equivalent) with procyanidins DP30 (15 mmol/L or 7.5 mmol/L (–)–epicatechin equivalent). Variation of absorbance of RG, AHR and HR-H (b); MHR1, MHR2 and MHR3 (d); mRG-II, dRG-II, AGP0 and AGP4 (f) (7.5 mmol/L or 3.75 mmol/L galacturonic acid equivalent) at different concentrations of procyanidins DP30 (–)–epicatechin equivalent).

(Fig. 4e) showed no variation with the increase in galacturonic acid concentrations for RG-II and AGP4, but for AGP0 the absorbance at 650 nm increased markedly from 0.12 mmol/L galacturonic acid equivalent, and at a constant 7.5 mmol/L galacturonic acid it also increased markedly from 1.875 mmol/L (–)–epicatechin equivalent (Fig. 4f). For RG-II and AGP4 the absorption was stable.

The ΔK_{av} values were very low to nil, even for AGP0, indicating that there was no selective precipitation of larger or smaller polysaccharides. The ΔDP_n was only markedly negative for AGP0, indicating that the more highly polymerized procyanidins were selectively precipitated by AGP0.

4. Discussion

Using isothermal titration calorimetry and absorbance at 650 nm, rhamnogalacturonans and hairy regions were shown to interact with procyanidins whatever their degree of polymerization. Thermodynamic data measured by isothermal titration calorimetry enable to compare affinity between pectic fractions and procyanidins and to demonstrate that interactions could be driven by either enthalpy or entropy or both, indicating that procyanidins and pectic fractions can interact by different mechanisms, depending on the pectin side chains.

Interactions between hairy region fractions and procyanidins DP9 showed association constants of the order of 10^3 M^{-1} , of the same order as with commercial pectins from apple (Watrelet et al., 2013), whereas interactions with procyanidins DP30 presented

association constants ranging between 10^2 M^{-1} and $5 \times 10^3 \text{ M}^{-1}$. Most of the associations between hairy regions and procyanidins DP9 were driven by entropy, which could indicate a contribution of hydrophobic interactions, while associations with procyanidins DP30 were driven by entropy (with RG, HR-H and MHR1) or by enthalpy for AHR, MHR2 and MHR3, i.e. those fractions might bind with procyanidins mainly through hydrogen bonds (Frazier et al., 2010; Poncet-Legrand et al., 2007). However, the pectic fractions conformation and structure could induce difference of binding systems through aggregation phenomenon for example. The differences in thermodynamic parameters observed between procyanidins were related to differences obtained by analysis of absorbance at 650 nm and analysis of supernatants after associations. With procyanidins DP30, aggregates appeared for some hairy region fractions, while with procyanidins DP9 formation of cloud or aggregate was very limited. The determination of ΔK_{av} in this study showed that procyanidins, whatever their degree of polymerization, bind without selectivity with respect to size of hairy regions. Watrelet et al. (2013) showed that procyanidins DP30 interacted preferentially with smaller molecules of commercial apple pectins. The determination of ΔDP_n showed differences in hairy regions for binding to highly polymerized procyanidins DP30, unlike procyanidins DP9. The length of procyanidins DP30 facilitated aggregates formation (Carn et al., 2012). Procyanidins DP30 are a polymer more non-uniform than procyanidins DP9 (Le Bourvellec, Picot, & Renard, 2006). This facilitated detection of the selectivity of hairy regions of pectins.

HR-H was the simplest structure with rhamnogalacturonan and mono and dimeric side chains. Its association constant with procyanidin DP30 was high and the formation of precipitate showed a marked selectivity for highly polymerized procyanidins. The presence of few neutral sugar side chains on the rhamnogalacturonan backbone could allow a linear structure probably able to "stack" with procyanidins. Low association constants were observed for procyanidin DP30 interacting with arabinan-rich pectic fractions (AHR and MHR2). AHR was mainly composed of arabinan chains that have been suggested to adopt a 2-fold helical conformation (Janaswamy & Chandrasekaran, 2005). Ha, Viëtor, Jardine, Apperley, and Jarvis (2005) demonstrated that arabinan chains were generally more mobile than galactan chains. The high mobility and the conformation of those chains could limit the association with procyanidins. For MHR2, it is concluded that the presence of galactan type II (1,3- and 1,6-linked galactans) with arabinans led to no modification of association with procyanidins (Goubet et al., 1995). Type II galactans possess lateral 1,3-linked β -D-galactan chains which adopt open helical structure and 1,6-linked β -D-galactan side chains with a non-helical linear structure (Pérez, Mazeau, & Hervé du Penhoat, 2000). This combination results in highly branched structures, which limits the association with procyanidins. The same phenomenon may occur with AGPs. MHR1, MHR3 and MHR4 fractions composed of type I galactan chains with 1,4 linkages, associated with procyanidins as efficiently as arabinan chains. However, due to the presence of xylogalacturonans, association constants cannot be defined. It can be suggested that xylogalacturonans are flexible enough to associate with procyanidins.

RGIs do not bind procyanidins efficiently. The present results are not in agreement with aggregation phenomena observed by Riou, Vernhet, Doco, and Moutounet (2002) between dimeric RG-II and tannins. Similarly, conflicting results were obtained for interaction between AGP0 and procyanidins DP30: we observed clear aggregation by absorption analysis, whereas this was not observed by previous dynamic light scattering investigation. It could be explained by the medium used by Riou, such as ionic strength, which induces a high self-aggregation of procyanidins and limits aggregation with AGP0 (Poncet-Légrand, Cartalade, Putaux, Cheynier, & Vernhet, 2003).

5. Conclusion

Interactions between hairy regions of pectins and procyanidins were demonstrated and depend on composition and conformation of neutral sugar side chains of pectins and procyanidins. Capacity of association between procyanidins DP30 and hairy fractions of pectins could be ranked: arabinans < arabinans + galactans II < galactan I < arabinans + galactan I + xylogalacturonans < rhamnogalacturonan. Further investigations are still in progress with the same compounds by the dynamic light scattering (DLS) to measure the aggregates formation.

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