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Study of human salivary proline-rich proteins interaction with food tannins

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ABSTRACT

In this work, saturation transfer difference-NMR, isothermal microcalorimetry and molecular dynamics simulations have been used to study the individual interactions between basic, glycosylated and acidic proline-rich proteins (bPRPS, gPRPs, aPRPs) and P-B peptide with some representative food tannins [procyanidin B2, procyanidin B2 3′-*O*-gallate (B2g) and procyanidin trimer (catechin-4–8-catechin-4–8-catechin)]. Results showed that P-B peptide was in general the salivary protein (SP) with higher affinity whereas aPRPs showed lower affinity to the studied procyanidins. Moreover, B2g was the procyanidin with higher affinity for all SP. Hydrophobic and hydrogen bonds were present in all interactions but the major driving force depended on the procyanidin-SP pair. Furthermore, proline clusters or residues in their vicinity were identified as the probable sites of proteins for interaction with procyanidins. For bPRP and aPRP a significant change to less extended conformations was observed, while P-B peptide did not display any structural rearrangement upon procyanidins binding.

1. Introduction

Condensed tannins are polymers of flavan-3-ol units, namely (epi) catechin or (epi)gallocatechin, which are among the most abundant flavonoids in the human diet (e.g. red grapes, chocolate, red wine). These compounds have received high attention due to their health benefits, e.g. antioxidant and anticancer properties, neurodegenerative and cardiovascular protection (de la Iglesia, Milagro, Campión, Boqué, & Martínez, 2010). Furthermore, these compounds are also related to the sensory properties of vegetable-derived food such as astringency and bitterness (Soares, Brandão, Mateus, & De Freitas, 2015).

Astringency is usually a non-pleasant sensation mainly when perceived with high intensity. However, in some foodstuffs like red wine it is a quality parameter and desired in balanced levels. Astringency has been described as dryness, tightening and puckering sensations perceived in the oral cavity during the ingestion of foodstuffs rich in tannins (ASTM., 1989). Several mechanisms have been proposed for the astringency onset but the most accepted one relies on the interaction/precipitation of salivary proteins (SP), mainly proline-rich proteins (PRPs), by tannins (de Freitas & Mateus, 2012).

SP are usually divided into several major classes including PRPs, statherin (stat), cystatins (cyst), P-B peptide and histatins that account for almost 50% of all SP (Messana, Inzitari, Fanali, Cabras, & Castagnola, 2008). PRPs, as the name suggests, are characterized by a high content in proline residues (25–42%). This family is divided in basic (bPRPs), acidic (aPRPs) and glycosylated (gPRPs) classes (Manconi et al., 2016). The differences between these classes depend on their charge and presence or absence of carbohydrates. aPRPs are characterized by a highly acidic *N*-terminal region, rich in aspartic and glutamic acid residues whilst its *C*-terminal is similar to bPRPs. They also present some phosphate groups throughout their structure (Ser 24, 33 and 38). Regarding gPRPs, they are bPRPs *N*- and *O*-glycosylated that present carbohydrates in 50% of their structure. These carbohydrates are composed of highly fucosylated *N*-linked

Abbreviations: aPRPs, acidic proline-rich proteins; bPRPs, basic proline-rich proteins; B2g, procyanidin B2 3′-*O*-gallate; gPRPs, glycosylated proline-rich proteins; ITC, isothermal microcalorimetry; MD, molecular dynamics; PRPs, proline-rich proteins; STD-NMR, saturation transfer difference-nuclear magnetic resonance; SP, salivary proteins

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saccharides; the major structure is a biantennary asialosaccharide containing 2 fucose residues on one antenna and an unsubstituted terminal lactosamine sequence on the other (Vitorino et al., 2011). Salivary P-B peptide is usually included in the PRPs family due to high content in proline residues (near 50% of its sequence) but it shows higher similarities with salivary statherin (Inzitari et al., 2006). P-B peptide structure is not similar to the PRPs structure as it shows the presence of several hydrophobic amino acid residues (such as Phe, Leu and Ile) and three tyrosine residues. Furthermore, P-B peptide is the product of a specific gene very close to STATH gene and is secreted as a mature protein not a degradation product of larger proteins. Besides this genetic correlation between the P-B and statherin, these two peptides show other highly significant correlations in whole saliva, such as a concentration dependence, as well as both being secreted by the same salivary glands (parotid and submandibular/sublingual glands). On the other hand, none of the two peptides shows a significant correlation with other PRPs. In this work P-B peptide is examined independently of bPRPs.

Most of astringency studies with SP are focused in bPRPs, which has been referred along the years as the one with the highest affinity for food tannins (Lu & Bennick, 1998). In fact, the major biological function attributed to bPRPs is the precipitation of food tannins impeding the subsequent deleterious effects of these compounds in the digestive system, whereas other PRPs have been described to mainly accomplish other functions, such as maintaining oral homeostasis in the case of aPRPs (Shimada, Saitoh, Sasaki, Nishitani, & Osawa, 2006). However, it seems that despite the main function attributed to the different families of the PRPs, most of them are able to precipitate tannins. In fact, some works (*in vitro* and *in vivo*) have shown that other SP different than bPRPs are highly reactive toward polyphenols, namely aPRPs and P-B peptide (Brandão, Soares, Mateus, & de Freitas, 2014; Quijada-Morín, Crespo-Expósito, Rivas-Gonzalo, García-Estévez, & Escribano-Bailón, 2016). Both works studied the protein precipitation in the presence of different tannins in a competitive assay when SP are present simultaneously (saliva). Therefore, the aim of this work was to study the interactions between the fractions of bPRPs, gPRPs, aPRPs and P-B peptide separately with selected representative food tannins [procyanidin B2, procyanidin B2 3′-*O*-gallate (B2g) and a procyanidin trimer (catechin-4-8-catechin-4-8-catechin) (Fig. S1)]. These tannins are present in a wide range of vegetables, fruits and derived products, namely, red wine, tea and beer (Rothwell et al., 2013).

2. Experimental section

2.1. Isolation and identification of salivary proteins

Large volumes of unstimulated saliva were isolated from eighteen healthy volunteers and treated as reported previously (Soares, Sousa, Mateus, & De Freitas, 2011). After this treatment, saliva was dialyzed for 24 h (cellulose membrane, MWCT 3.5 kDa) against water at 4 °C with stirring. Water was replaced several times. Then, saliva was centrifuged and the supernatant was freeze-dried. The resulting powdered saliva was solubilized in the minimal possible volume of water and filtered. The resulting solution was used to isolate the different families of PRPs and P-B peptide by semi-preparative HPLC: HPLC equipped with reversed-phase C8 column (150 × 2.1 mm, 5 μm), solvents were 0.2% aqueous TFA (A) and 0.2% TFA in ACN/water 80/20 (v/v) (B) and gradient applied was 10 to 45% of B in 40 min, at a flow rate of 0.60 mL/min and detection at 214 nm.

The several fractions of PRPs and P-B peptide were freeze-dried and the major proteins present in each fraction were identified by ESI-MS by flow injection analysis in an LTQ Orbitrap XL mass spectrometer (Thermo Fischer Scientific, Bremen, Germany) controlled by LTQ Tune Plus 2.5.5 and Xcalibur 2.1.0 (detailed experimental conditions in Supporting Information). gPRPs molecular weight determination was performed by static light scattering (SLS) measurement with modifications (Puskás, Szemjonov, Fenyvesi, Malanga, & Szente, 2013). Different concentrations of gPRPs were prepared in miliQ water and were measured at 25 °C. The average molecular weight was found to be about 16 kDa.

The study was conducted according to the Declaration of Helsinki and was submitted to Ethics Committee.

2.2. Isolation of procyanidin dimer B2, procyanidin B2-3′-O-gallate and procyanidin trimer

Procyanidin dimer B2 and procyanidin B2 3′-*O*-gallate (B2g) were isolated by preparative HPLC from a procyanidin fraction extracted from grape seeds (*Vitis vinifera*). Grape seeds were extracted as described previously yielding four fractions. Procyanidin B2g was isolated from fraction I and isolated according to the procedure described elsewhere (Teixeira et al., 2013).

Procyanidin trimer (catechin-(4-8)-catechin-(4-8)-catechin) was obtained by chemical synthesis between taxifolin and (+)-catechin as described in the literature (Gonçalves, Mateus, Pianet, Laguerre, & de Freitas, 2011). After the reaction, the mixture was fractionated through a TSK Toyopearl HW-40(s) gel column (300 mm × 10 mm i.d., 0.8 mL/min, methanol as eluent) and the fraction corresponding to the trimer fraction was recovered and analyzed by ESI-MS (Finnigan DECA XP PLUS). Spectroscopical data were in accordance with literature.

2.3. Saturation transfer difference (STD)-NMR

Protein samples were prepared in D_2O containing 3 μM of each protein and the procyanidins were progressively added to the protein samples in the 0.1–3.5 mM ranges. To maintain protein concentration constant throughout the experiments, the procyanidins were lyophilized and added as a powder.

STD-NMR experiments were recorded on a Bruker Avance III 600 HD spectrometer, operating at 600.13 MHz, equipped with a 5 mm PATXI 1H/D-13C/15N (detailed experimental conditions in Supporting Information). To process all the spectra, to baseline and phase corrections and to integrate the areas, TopSpin 2.1 software from Bruker was used. The binding constants (K_A) were determined using the following equations (Viegas, Manso, Nobrega, & Cabrita, 2011):

$$A_{STD} = \frac{I_0 - I_{Sat}}{I_0} \times L/Pmolar\ ratio = \frac{I_{STD}}{I_0} \times L/Pmolar\ ratio = \frac{\propto_{STD} \times [L]}{K_D + [L]} \quad (1)$$

$$K_A = \frac{1}{K_D} \quad (2)$$

where I_0 and I_{Sat} are the signal intensities off-resonance and on-resonance, respectively; and $\propto_{STD}$ is the maximum amplification.

2.4. Isothermal titration microcalorimetry

ITC experiments were conducted at 298 K using a V-P MicroCalorimeter controlled by Origin VPViewer software. Aqueous solutions of each protein (between 20 and 30 μM) and of procyanidins (titrant, between 1 and 10 mM) were prepared and degassed before titration. The sample cell was loaded with 1.4 mL of protein solution and titrant was loaded into the injection syringe. After baseline stability was achieved, procyanidin solution was injected (4–12 μL/injection) into the sample cell 15–35 times until reaching stabilization. Spacing between injections was equal or higher than 350 s. Samples were stirred constantly at 307 rpm to ensure thorough mixing. Raw data obtained from a plot of heat flow *vs.* injection number were transformed using the AFFINIMETER software to construct a plot of enthalpy change *vs.* molar ratio. The resulting data were fitted in order to obtain the binding constant (K_A), the binding sites (n) and the thermodynamic parameters change for all the studied SP-procyanidins interaction.