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Synergistic effect of mixture of two proline-rich-protein salivary families (aPRP and bPRP) on the interaction with wine flavanols

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ABSTRACT

In this study, we have evaluated by HPLC-DAD, DLS and MALDI-TOF a synergic effect of the coexistence of two salivary-PRP fractions (basic-PRPs and acidic PRPs) on the interaction with flavanols. Results obtained showed noticeable enhancement of the interaction between (epi)catechin and PRPs when both types of proteins are blended. Up to 30 soluble aggregates have been tentatively identified with molecular weight from 4680 to 35,851. (epi)Catechins seem to bind preferentially bPRPs than aPRPs, although the medium size aggregates flavanol-bPRPs formed could favour the interaction with aPRPs giving rise to soluble mixed aggregates.

1. Introduction

Astringency has been defined as "the complex of sensations due to shrinking, drawing or puckering of the epithelium as a result of exposure to substances such as alums or tannins" by the American Society for Testing Materials (ASTM, 2004). The precipitation of salivary proteins is one of the most accepted mechanisms to the development of astringency, however not all astringents cause salivary protein precipitation, supporting that there must be other mechanisms implicated (Ferrer-Gallego, Gonçalves, Rivas-Gonzalo, Escribano-Bailon, & de Freitas, 2012). Other authors have proposed different mechanisms such as activation of specific taste receptors (Tachibana, Koga, Fujimura, & Yamada, 2004), or direct interaction between tannins and oral epithelial cells (Payne, Bowyer, Herderich, & Bastian, 2009), or even several of these mechanisms occurring simultaneously (Gibbins & Carpenter, 2013). However, although the bases of the astringency mechanism are not well understood yet, it is broadly assumed the ability of food tannins to interact with some salivary constituents, mainly salivary proteins, resulting in the formation of protein-tannin aggregates (de Freitas & Mateus, 2012; Rinaldi, Gambuti, & Moio, 2012), which, in turn, causes a decrease in salivary lubrication (Canon et al., 2013).

Tannins have been classically divided in two major classes: condensed tannins (proanthocyanidins) and hydrolysable tannins, which include gallotannins and ellagitannins. Proanthocyanidins are polymers composed of four main flavan-3-ol units, that is catechin, epicatechin, gallocatechin and epigallocatechin. These compounds are present in the skins and seeds of grapes (in higher concentrations in the latter (Monagas, Gómez-Cordovés, Bartolomé, Laureano, & Ricardo Da Silva, 2003)) and they are released throughout the maceration process that takes place during red winemaking (Santos-Buelga & De Freitas, 2009).

Salivary proteins (SP) have been classified into six groups attending to their structure and characteristics, namely, basic proline-rich proteins (bPRPs), acidic proline-rich proteins (aPRPs), glycosylated proline-rich proteins (gPRPs), statherin, histatins and cystatins (Castagnola, Cabras, Vitali, Sanna, & Messana, 2011; Soares et al., 2011). Salivary proline-rich proteins (PRPs) are characterized by their high proline, glycine, and glutamine content, in the absence of any hydroxyproline and hydroxylysine. In particular, the content of proline in PRPs ranges between 25 and 40% of all amino acids. They are highly polymorphic and heterogeneous in their primary amino acid sequence, size and post-translational modifications, which could be related with their functional diversity (Castagnola et al., 2003; Oppenheim, Salih, Siqueira, Zhang, & Helmerhorst, 2007). More than 11 human basic-PRPs and five acidic-PRPs isoforms have been identified and the total PRPs represent > 60% by weigh of the total salivary proteome (Inzitari et al., 2005; Messana et al., 2004). The basic PRPs (bPRP) family are expressed by four separate loci including PRB1, PRB2, PRB3, and PRB4. To be precise, PRB1 and PRB2 genes produce the nonglycosylated bPRP whereas PRB3 and PRB4 generate glycosylated bPRP (gPRPs). The main function proposed for the gPRPs is to act as lubricants, whereas bPRPs

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are associated with tannin precipitation (Oppenheim et al., 2007). With regard acidic PRPs (aPRPs), five isoforms have been described, which are encoded by two gene loci named PRH1 and PRH2 Castagnola et al. (2011). PRH1 produces aPRPs Db-s, PIF-s and Pa, and PRH2 encodes for PRP1 and PRP2. In contrast to bPRPs, there are minor structural differences among the aPRPs and their predominant role proposed is related to mineral homeostasis and tooth integrity preservation (Oppenheim et al., 2007).

As aforementioned, the interaction between phenolic compounds and salivary proteins (mainly PRPs) has been described as key process to explain the astringency perception, with or without precipitation of the tannin-protein complexes (Cala et al., 2012; Ferrer-Gallego et al., 2017). There are some works studying protein precipitation in the presence of different tannins in a competitive assay when SP are present simultaneously (Brandão, Soares, Mateus, & De Freitas, 2014; Quijada-Morín, Crespo-Expósito, Rivas-Gonzalo, García-Estévez, & Escribano-Bailón, 2016). Moreover, other authors have studied the interaction between protein fractions (PRPs) separately with selected representative food tannins (Soares et al., 2018). However, to our knowledge, there is no studies focused on the different ability of SP fractions to interact with flavanols when they are isolated or mixed with other SP fractions, which could help for unraveling the astringency process. The aim of this work was to study the effect of the coexistence of basic-PRPs and acidic PRPs salivary fractions on the interaction with flavanols towards the interaction between the same flavanols with these fractions assayed individually, using techniques such as HPLC-DAD, DLS and MALDI-TOF.

2. Materials and methods

2.1. Reagents

All reagents used were of analytical grade and all solvents were of HPLC grade. (+)-Catechin (CAT) and (−)-epicatechin (EC) were purchased from Sigma-Aldrich (Saint Louis, MO, USA). Trifluoroacetic acid (TFA) was purchased from Riedel-de Haën (Hanover, Germany). Ultrapure water was obtained from a Milli-Q Gradient water purification system (Millipore, Billerica, MA, USA).

2.2. Saliva collection and treatment

Whole human saliva was collected according to Quijada-Morín et al. (2016) from six healthy, nonsmoker volunteers (three men and three women aged between 24 and 50 years old). They were previously instructed to avoid foods and beverages for at least 1 h before collection. Collection time was set at 10:30 a.m. to reduce variability due to circadian rhythms of saliva secretion (Dawes, 1972). The saliva pool was immediately treated with 10% TFA (final concentration of 0.1%) to inhibit protease activity and to precipitate high molecular weight proteins such as mucins (Messana et al., 2004; Soares et al., 2011). After that, the sample was centrifuged for 10 min at 12,000*g* and the supernatant was dialyzed using a Spectra/Por 3 cellulose membrane (SpectrumLabs, Rancho Dominguez, CA, USA) with an exclusion size of 3.5 kDa. The dialysis process was carried out using ultrapure water at 10 °C for 48 h, renewing the water every 8 h. Salivary proteins (SP) fractions were purified from treated saliva by means of preparative HPLC-DAD. Preparative separation was performed in an Agilent 1260 Infinity series Preparative LC. (Agilent Technologies) consisting of a thermostated autosampler, 2 coupled preparative pumps which formed a binary system, a diode array detector and a thermostated sampler collector, controlled by OpenLab CDS Chemstation Workstation software (version C.01.04; Agilent Technologies). Detection was carried out at 214 nm as preferred wavelength. A Zorbax 300SB-C8 (9.4 mm × 250 mm × 5 μm) column was used. The solvents used were (A) aqueous TFA 0.1% and (B) TFA 0.1% in acetonitrile. The elution profile was as follows: from 8% to 12% B for 7 min, from 12% to 18% B for 18 min, from 18% to 32% B for 35 min, from 32% to 90% B for 4 min, hold and isocratic flow at 90% B for 6 min, from 90% to 8% B for 5 min and hold and isocratic flow at 8% B for 15 min. The flow-rate was 3 mL min^{-1} and the injection volume was 1 mL.

Table 1
Percentage of decrease (−) or increase (+) of the area of the chromatographic peaks assigned to each protein fraction after reaction with flavanols (CAT or EC).

Fraction	CAT	EC
bPRPs	+5.93*	+5.12*
bPRPs + aPRPs†	+5.88	+13.8*
aPRPs	−5.77	+7.10
bPRPs + aPRPs‡	−8.82	+7.46

* Statistical differences ($P < 0.05$) compared to the control.
† Chromatographic area corresponding to bPRPs fraction.
‡ Chromatographic area corresponding to aPRPs fraction.

Basic (bPRPs) and acidic (aPRPs) Proline-Rich-Proteins fractions were collected at the DAD chromatographic-detector outlet. Identification was carried after tryptic digestion using a nano-UPLC system (nanoAcquity, Waters Corp., Milford/MA,USA) coupled to a LTQ-Orbitrap Velos mass spectrometer (ThermoFisher Scientific, San Jose/CA, USA) via a nano-electrospray ion source (NanoSpray flex, Proxeon, Thermo) (nanoAcquity, Waters Corp., Milford/MA,USA) (Quijada-Morín et al., 2016). The obtained fractions were freeze-dried and frozen prior to its use in the interaction assays.

2.3. Interaction assays by HPLC-DAD

bPRPs and aPRPs were dissolved in Milli-Q water and analyzed both isolated (aPRRs or bPRPs) and blended (aPRPs + bPRPs) before (control) and after (samples) the interaction with catechin or epicatechin in a ratio protein:tannin 1:3 (*w/w*). In each case, the SP were blended with water (control) or with the tannin solution (samples), vortexed, incubated for 25 min at room temperature and centrifuged for 5 min at 12,000*g*. The supernatant was injected into the HPLC-DAD (Agilent 1200 Series), using a method previously optimized in our laboratory (Quijada-Morín et al., 2016). All experiments were performed in triplicate and were compared towards the control without flavanol.

2.4. Dynamic light scattering

The size of the complexes formed between purified bPRPs and aPRPs and the flavanol (catechin or epicatechin) was determined by dynamic light scattering (DLS, Zetasizer Nano, Malvern, UK) at a wavelength of 633 nm. The intensity fluctuations of the particles were measured before (control) and after (samples) the interaction with the flavanols. The diffusion coefficients of particles were calculated and then converted into a size distribution. Sample solutions with PRPs and flavanol were prepared in a final volume of 20 μL (1:3 protein:tannin *w/w*). The intensity of the scattered light was detected at an angle of 173^0 (standard measurement) at 25 °C. All the samples were prepared in 0.1 M acetate buffer, pH 5.0, with the ionic strength adjusted with 100 mM sodium chloride.

2.5. MALDI-TOF analysis

As explained with the previous techniques, purified bPRPs and aPRPs were dissolved in Milli-Q water and analyzed both isolated (aPRRs or bPRPs) and blended (aPRPs + bPRPs) before (control) and after (samples) the interaction with catechin or epicatechin in a ratio protein:tannin 1:3 (*w/w*). In each case, the SP were blended with water (control) or with the tannin solution (samples), vortexed and incubated for 20 min at room temperature. After that, 2 μL of sample was overlaid with 2 μL of matrix solution (3 mg/mL of super-DHB matrix (9:1