Development of a direct ESI-MS method for measuring the tannin precipitation effect of proline rich peptides and *in silico* studies on the proline role in tannin-protein interactions

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Abbreviations: TA, tannic acid; PGG, penta-O-galloyl-β-D-glucose; PRPs, proline rich proteins; BK, bradykinin; BSA, bovine serum albumin.

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Abstract

Tannins are a heterogeneous class of polyphenols that are present in several plants and foods. Their ability to interact and precipitate proline-rich proteins leads to different effects such as astringency or antidiarrheal activity. Thus, evaluation of the tannin content in plant extracts plays a key role in understanding their potential use as pharmaceuticals and nutraceuticals. Several methods have been proposed to study tannin-protein interactions but few of them are focused on quantification. The purpose of the present work is to set up a suitable and time efficient method able to quantify the extent of tannin protein precipitation. Bradykinin, chosen as a model, was incubated with increasing concentrations of 1,2,3,4,6-penta-O-galloyl-β-D-glucose and tannic acid selected as reference of tannic compounds. Bradykinin not precipitated was determined by a mass spectrometer TSQ Quantum Ultra Triple Quadrupole (direct infusion analysis). The results were expressed as PC_{50}, which is the concentration able to precipitate 50% of the protein. The type of tannin-protein interaction was evaluated also after precipitate solubilisation. The involvement of proline residues in tannin-protein interactions was confirmed by repeating the experiment using a synthesized peptide (RR-9) characterized by the same bradykinin sequence, but having proline residues replaced by glycine residues: no interaction occurred between the peptide and the tannins. Moreover, modeling studies on PGG-BK and PGG-RR-9 were performed to deeply investigate the involvement of prolines: a balance of hydrophobic and H-bond contacts stabilizes the PGG-BK cluster and the proline residues exert a crucial role thus allowing the PGG molecules to elicit a sticking effect.

Keywords: tannins, proline rich proteins, mass spectrometry.

1. Introduction

Tannins are a heterogeneous class of polyphenols that are present in several plants and foods, such as fruits, cereals, wine, tea, cocoa and vegetables [1]. According to Bate-Smith and Swain, tannins are water-soluble phenolic compounds with a molecular weight between 500 and 3000 Da, characterized by many hydroxyl groups and capable of forming cross-linkages with proteins. These compounds are classified as hydrolysable and condensed tannins. Hydrolysable tannins are made of a monosaccharide core, such as glucose, partially or completely esterified with an organic acid, such as gallic acid (gallotannins) or ellagic acid (ellagitannins) [2]. Condensed tannins are more complex than hydrolysable tannins: they are polymers of flavan-3-ols linked through acid-labile carbon-carbon bonds [3].

As secondary metabolites of plants, tannins have a role in plant defense, discouraging herbivores from feeding on the plant. These compounds are also used in the production of leather and their levels need to be controlled in wine production to reduce astringency. All these actions are related to the effect of tannins in inducing protein precipitation. In particular, the interaction is known to occur between tannins and proteins rich in proline residues (PRPs) [4]. More recently, the protein precipitation effects induced by some plant components have also been considered as a possible explanation for some bioactive actions. Such effects occur when the precipitating protein (protein target) has a damaging effect or it is contained in infectious organisms. Based on such a mechanism, tannins and plant extracts rich in tannins have been proposed as antidiarrheal [5], antiviral [6,7], antibacterial agents [8–10] and to neutralize the toxic activities of snake venoms [11]. Moreover, a fully detailed study on the molecular interaction of tannins and in particular of penta-O-galloyl-d-glucopyranose with bradykinin has been carried out by NMR [12]. Bradykinin is a proline-rich peptide (Pro residues accounting for 30% of the residues) which acts as an inflammatory mediator and its complexing by tannins can partially explain the well-known anti-inflammatory properties of this class of compounds. More recently, the molecular interaction between tannins and different wheat-derived peptidic fractions, which contain a high content of proline residues and which are responsible for the onset of celiac disease, has been studied [13]. This study indicates that the aggregation between tannins and immunoreactive peptides could represent an important field in the potential protective effect of tannins on the cytotoxicity and/or the immunogenicity of gluten peptides.

Based on these recent findings it seems that, beside salivary rich peptides such histatins, interaction of tannins with bioactive proline rich peptides deserves some interest; in particular a valuable method able to measure the precipitating effect of tannins towards damaging peptides could be useful in order to identify potential bioactive compounds and/or the plant extracts containing them.

The aim of the present work is 1) to set-up an analytical method able to measure the ability of tannins and/or plant extracts to interact with and precipitate peptides rich in proline and 2) to evaluate the importance of proline in tannin-protein interaction. Bradykinin (BK) was chosen as a target for tannin binding, not only because it is a peptide rich in proline residues, but also due to its involvement in inflammatory disorders such allergies and the common cold, hence representing a potential target peptide of the tannin precipitation effect. Tannic acid (TA) and penta-O-galloyl-β-D-glucose (PGG) were used as reference compounds of tannins.

2. Materials and Methods

2.1. Chemicals and reagents

HPLC-grade water was prepared with a Milli-Q water purification system (Millipore, Milan, Italy). Ammonium acetate was from Riedel-de Haën (Seelze, Germany). Bradykinin acetate, pure tannic acid (TA, CAS number 1401-55-44), 1,2,3,4,6-penta-O-galloyl-β-D-glucose (PGG), formic acid and LC-grade and analytical-grade organic solvents were from Sigma-Aldrich (Milan, Italy). The internal standard peptide LVNEVTEF was custom synthesized by Sigma-Aldrich (Milan, Italy). The peptide RR-9 RGGGFSGFR was synthesized by PRIMM (Milan, Italy).

2.2. Bradykinin-tannin co-precipitation assay

100 µM bradykinin was dissolved in 50 mM acetate buffer pH 7.4 and 25 µL samples were spiked with of PGG or TA at the following concentrations: 10 (only for PGG), 50, 100, 150, 200, 250, 500, 1000 (only for TA) µM performed in 3 replicates. The mixtures were incubated for 10 minutes at 37°C under gentle shaking (1400 rpm on a Thermomixer) and then centrifuged for 10 minutes at 14000 rpm. The supernatant (25 µL) was diluted 1:10 with H_2O/CH_3CN/HCOOH (70/30/0.1, % v/v), spiked with the peptide LVNEVTEF as internal standard (10 µM final

concentration) and analyzed by direct infusion MS as below detailed in order to quantify the amount of bradykinin not precipitated by tannins. The precipitates were then dried, dissolved in $H_2O/CH_3CN/HCOOH$ (70/30/0.1, % v/v) and analyzed by MS in order to verify the presence of bradykinin.

2.3. Supernatant and precipitate analyses by mass spectrometry

30 µL of the supernatant was injected by an automated sample injection into a TSQ Quantum Ultra Triple Quadrupole (Thermo Finnigan, Milan, Italy) equipped with an ESI-source. An HPLC Surveyor MS Pump (Thermo Finnigan, Milan, Italy) pumped the sample at 25 µL/min with an isocratic phase $H_2O/CH_3CN/HCOOH$ (70/30/0.1, % v/v). The analyses were performed in positive ion mode and with the following ion source parameters: capillary temperature 270° C; spray voltage 4.5 kV; capillary voltage 35 V; tube lens voltage 114 V. The flow rate of the nebulizer gas (nitrogen) was 15 a.u. The mass spectrometer operated in full mass scan and the Q3 was used as detector with a scan range 450-1300 *m/z*.

Xcalibur 2.0.7 version was used for the relative quantification of bradykinin. A processing method was set up in order to obtain an automated integration of the areas of the z = 2 (*m/z* 530.9) peak of BK and z = 1 (950.5 *m/z*) peak of the internal standard. The ICIS peak integration parameters set were: smoothing points 7; baseline window 40; area noise factor 5; peak noise factor 10; minimum peak high (S/N) 3. This processing method was applied in the Quan Browser window of Xcalibur for the analysis of all the samples. The results were verified and manually corrected where the integrations were not appropriate.

20 µL of the precipitate dissolved was injected by an automated sample injection into a LTQ - Orbitrap XL mass spectrometer (Thermo Scientific, Milan, Italy) equipped with an ESI-source. An HPLC UltiMate 3000 (Thermo Scientific, Milan, Italy) pumped the sample at 25 µL/min with an isocratic phase $H_2O/CH_3CN/HCOOH$ (70/30/0.1, % v/v). The analyses were performed in positive ion mode and with the following ion source parameters: capillary temperature 270° C; spray voltage 4.5 kV; capillary voltage 35 V; tube lens voltage 114 V. The flow rate of the nebulizer gas (nitrogen) was 15 a.u. After mass spectrometry analysis, the deconvolution was carried out with MagTran 1.02 software.

2.4. Modeling studies

The PGG conformational space was explored by a MonteCarlo procedure which produced 10000 optimized geometries by randomly rotating the peptide backbone torsions as implanted in the VEGA program [14]. The resulting 10000 PGG conformations were clustered according to their structural similarity; in detail, two geometries are considered non-redundant if they differ by more than 60° in at least one rotor. The so derived best five PGG conformations underwent docking simulations based on the recently resolved NMR structure of bradykinin (BK, PDB Id: 6F3V). All 10 frames included in the deposited NMR structure were considered in docking simulations which were performed using PLANTS and considering the entire bradykinin structure [15]. For each BK frame, 10 PGG poses were generated and ranked using the ChemPLP score with the speed equal to 1. The BK frame affording the best docking results (frame #6) was then utilized to generate the RR-9 peptide by manually replacing the proline by glycine residues. The conformational profile of the obtained RR-9 peptide was explored by the MonteCarlo procedure as described above by generating 100000 optimized conformations. The best five obtained RR-9 geometries underwent docking calculations with the PGG ligand as already described for BK. Next, and by applying the same docking procedures in an incremental way, the best performing BK frame (#6) was also utilized to generate two clusters: the first was composed of three BK molecules, while the second cluster comprised three BK molecules in complex with three PGG ligands. The optimized clusters were then neutralized and inserted in a 80 Å side cubic box including about 3750 water molecules. After an initial minimization to optimize the relative position of the solvent molecules, the two systems underwent 50 ns MD simulations using Namd [16] and adopting the same MD characteristics described elsewhere [17]. The produced trajectories were finally wrapped using PBCTools [18] and analyzed by performing MM-GBSA calculations.

3. Results

3.1. Set-up of a ESI-MS method to measure the tannin precipitation effect

Several ESI-MS approaches to obtain a better understanding of the non-covalent interaction between tannins and peptides and which in particular have been found suitable for the study of the (1) molecular interactions, (2) the binding stoichiometry and (3) the evaluation of the complex stability, have been reported.

To our knowledge, no studies based on MS have been reported for the measurement of the precipitating effects of tannins towards proline rich peptides which is the aim of this paper. Bradykinin was selected as target peptide and tannic acid (TA) and penta-O-galloyl-β-D-glucose (PGG) as reference compounds of tannins.

Figure 1 summarizes the assay. After incubating the target peptide with tannin at different final concentrations, the sample is centrifuged and the relative content of the target peptide in the supernatant determined by ESI-MS using LVNEVTEF as internal standard. The precipitating effect is determined by calculating the residual amount of the target peptide in the supernatant in relation to a sample containing the target peptide and prepared in the absence of tannins (100% of the peptide).

Figure 2 shows the ESI-MS spectra of BK spiked with the internal standard. The ions at *m/z* 530.9 and *m/z* 1060.6 refer to the $[M+2H]^{2+}$ and $[M+H]^{+}$ of BK, respectively, while the ion at *m/z* 950.5 is attributed to the $[M+H]^{+}$ of the internal standard.

In preliminary experiments carried out by LC-ESI-MS/UV, neither TA nor PGG up to a concentration of 1 mM significantly precipitated the IS. As shown in ***Figure 3A***, the intensity of BK peaks (but not that of the internal standard) decreases as the concentration of TA increases. ***Figure 3B*** shows the dose-dependent precipitating effect of TA which started at 50 μM (% of residual bradykinin = 87.88±12.52) and reached a plateau at 500 μM (13.00±12.36%). ***Figure 3C*** reports the % of residual BK values for each concentration tested. A similar precipitating effect was observed for PGG (***Figure 4A, B*** and ***C***). The protein precipitation potency for each tannin was then quantified as the concentration able to precipitate the 50% of BK 100μM (PC_{50}). The calculated values are 112.3 μM and 84.6 μM for TA and PGG, respectively.

To confirm the presence of BK in the precipitates, the pellet obtained by precipitation was dissolved in H_2O/CH_3CN/HCOOH (70/30/0.1, % v/v) and analyzed by direct infusion in a LTQ-Orbitrap XL mass spectrometer. ***Figures 5*** and ***6*** show the presence of $[M+3H]^{3+}$ and $[M+2H]^{2+}$ ions of BK without any covalent adduct with TA or PGG, as can be appreciated by the deconvoluted spectra.

3.2. Study of the involvement of proline residues in bradykinin precipitation

In order to understand the involvement of Pro residues in BK precipitation an analogue peptide (RR-9), where the Pro residues are replaced by Gly, was tested. ***Figure 7*** shows the MS spectrum of RR-9 characterized by the ions at *m/z* 470.7 and 940.2 which represent the $[M+H]^{+}$

and $[M+2H]^{2+}$ ions, respectively. PGG was then selected as precipitating tannin. As shown in ***Figure 8***, PGG did not induce any significant precipitating effect up to 250 µM while only a weak effect was observed at 500 µM.

3.3. Modelling studies

Figure 9 reports (on the left) the best putative complex between BK and PGG as computed by initial docking calculations and reveals that it is stabilized by a fine balance of both hydrophobic and polar contacts. The former involve two galloyl moieties plus the central glucose carbon skeleton which approach Pro2 and Pro3 and are reinforced by π-π stacking with Phe8. Polar interactions involve almost all galloyl groups which stabilize a rich network of reinforced H-bonds with the charged termini plus Arg1 and Arg9. The reported complex suggests that the proline residues are both involved in direct apolar contacts and, due to their rigidifying effect, are responsible for retaining the charged residues exposed and far enough to optimize their H-bonds with PGG. This supposition finds encouraging confirmation when analyzing the computed complexes between PGG and RR-9 (complexes not shown). Here, the replacement of prolines with glycines markedly increases the peptide flexibility and thus the charged groups are able to stabilize intramolecular salt bridges which shield and render them unavailable for contacting PGG. Indeed, the best obtained PGG-RR-9 complex appears to be almost exclusively stabilized by π-π stacking which involves nearly all galloyl rings with Phe5 and Phe8, reinforced by weak H-bonds between the PGG ester functions and the BK backbone atoms. These clear differences are reflected in the primary ChemPLP scores as seen in the best (-100.31 kcal/mol vs. -90.59 kcal/mol. for BK and RR-9, respectively) and average (-92.35 ± 4.21 kcal/mol vs -82.12 ± 7.01 kcal/mol. for BK and RR-9, respectively) score values. Remarkably, 35 out of the 50 best PGG-BK complexes show scores better than the global minimum as computed by PGG-RR-9 simulations. These notable score differences suggest that even simple docking simulations might be successful in predicting the precipitation effect of tannins.

With a view to gaining deeper insights into the molecular mechanisms by which PGG exerts its precipitation effect, the dynamic behaviours of the two clusters composed of a BK trimer with and without PGG molecules were compared by MD simulations. ***Figure 9*** shows the profile of the interaction energies experienced by the BK peptide in the two MD runs as computed by the

MM-GBSA approach. These energies can be seen as a measure of the complex stability and reveal that the inclusion of the PGG molecules markedly increases such a stability (the energy averages are equal to -69.05 ± 7.60 kcal/mol and -38.67 ± 8.44 kcal/mol with and without PGG, respectively). In detail, the stability increase is almost completely ascribable to a strengthening of the van der Waals interactions (the van der Waals energy averages are equal to -66.01 ± 8.11 kcal/mol and -36.72 ± 7.12 kcal/mol with and without PGG, respectively) while the electrostatic terms are similarly marginal in both simulations (the electrostatic energy averages are equal to -3.04 ± 5.31 kcal/mol and -1.95 ± 4.70 kcal/mol with and without PGG, respectively). The key role of hydrophobic contacts is confirmed by the right complex in ***Figure 9*** which shows the BK-PGG cluster as obtained at the end of the MD run. One may observe that PGG remains precisely inserted between two bradykinin peptides and even at the end of the simulation the contacts between PGG and the proline residues maintain a pivotal role thus emphasizing the relevance of the hydrophobic interactions in determining the PGG precipitation effects.

4. Discussion

Several methods have been set-up to measure the protein precipitation effect of tannins and the most popular are those based on BSA as protein target and colorimetric assays to measure the unbound protein fraction in the supernatant or tannins in the precipitated protein-tannin fraction. Makkar H.P. [19] determined the precipitated BSA by ninhydrin assay and Haruo Kawamoto [20] quantified the precipitated BSA by HPLC-UV. Two methods were based on the use of filter disk and membrane after tannin fractions or plant extract immobilization on these kinds of surface: the extent of the interaction was determined as reduction of the BSA diffusion [21] or by gamma counting of BSA ^{125}I-labelled adsorbed [22]. Scott H. reported a miniaturized method [23] which aimed to reduce the amount of volume in the assay by using a microplate to measure the unprecipited BSA by the Bradford assay. On the other hand, some methods were based on the analysis of a tannin co-precipitated fraction after solubilisation of the precipitate by reaction with ferric chloride followed by quantification of the total absorbance (510 nm) [24,25].

However, such methods are not suitable for the measurement of the precipitation of target peptides when used at micromolar level and contained in small sample volumes (not higher than one hundred microliters). Such conditions are required when using bioactive peptides, which are expensive or difficult to synthesize and/or isolate. The method here reported fulfils these requirements since the volume of the samples is in the order of hundreds of microliters and the concentration of the target peptides is in a micromolar range, depending on the MS analyzer used. In the present work, by using triple quadrupole, which is quite a common instrument, the peptide was used at a final concentration of 100 micromolar but the concentration could be further reduced when more sensitive analyzers, such as qTOF or orbitraps, are available. Moreover, the sample volumes can be further reduced if micro-wells are used and, in this case, a high throughput method can also be adapted. Despite the fact that the triple quadrupole mass spectrometer is not the best instrument in terms of sensitivity when used in full MS acquisition mode, good reliability and precision can be reached and better results can be obtained if compared to colorimetric assays.

The second part of the paper was aimed at better characterizing the involvement of Pro residues in the tannin precipitation effects of BK. By using the developed method we found that the presence of Pro residues is required for tannins to induce BK precipitation, since the peptide analogue, with Pro residues substituted with Gly (RR-9) was only slightly precipitated at the highest concentration (500 μM), while no effect was observed at 84.6 μM, which is the concentration required to PGG for precipitating BK.

The involvement of Pro was then further investigated by molecular modelling studies. The obtained results confirm that the stabilizing interactions in the BK-PGG cluster comprise a precise balance of hydrophobic and H-bond contacts in which the proline residues exert a crucial role thus allowing the PGG molecules to elicit a sticking effect by shielding the polar groups of the peptide. The analysis of the PGG effects on the bradykinin folding reveals that on average the peptide assumes more extended conformations when interacting with PGG as described by the end-to-end distance averages (here the distance between the charged termini) which are equal to 15.33 ± 0.66 Å and 12.32 ± 0.89 Å for the simulations with and without PGG, respectively. This effect is clearly understandable by considering that the bradykinin peptides tend to assume the extended conformations which maximize their interactions with PGG. As discussed above, the capacity to retain extended conformations is primarily due to the rigidifying effect of the proline residues and is further increased by the PGG itself, which

however does not induce dramatic conformational changes of the peptide as assessed by the rmsd analysis of the superimposed peptide conformations [26]. The so computed rmsd values show indeed almost identical resulting averages (5.21 ± 1.76 Å and 5.13 ± 2.36 Å for the simulations with and without PGG, respectively) thus suggesting that the structural differences observed with PGG are ascribable to fine conformational shifts by which BK optimizes its interactions with PGG without exerting unfolding effects.

5. Conclusions

In conclusion, an *in vitro* method was set-up for the quantitative determination of the tannin precipitation effect of proteins and proline rich peptides. The method could be useful to test the ability of tannins to precipitate and hence inactivate damaging peptides and also to evaluate the selectivity of the precipitation effect. Modelling studies confirmed the crucial role of Pro residues for the interaction between tannins and the peptide. Some proline rich peptides can be considered as potential targets of tannins such as bradykinin, an inflammatory mediator which also exerts its pro-inflammatory activity in the g.i. tract, and wheat-derived peptidic fractions which contain a high content of proline residues and which are responsible for the onset of celiac disease. The method could be applied to isolated compounds as well as to complex matrices such as plant derivatives and fractions. The precision of the method was found satisfactory since the CV% was always lower than 20% for all the concentrations tested. Finally, the method is time efficient thus permitting the rapid screening of several plant extracts and compounds able to complex and precipitate damaging target peptides.

Figure legends

Figure 1 – Graphic representation of the bradykinin precipitation assay.

Figure 2 – ESI-MS spectrum of a solution of BK (10 µM) spiked with the IS. Ions at 530.9 *m/z* and 1060.6 *m/z* are the $[M+2H]^{2+}$ and $[M+H]^{+}$ of BK, respectively. The ion at 950.5 *m/z* refers to the $[M+H]^{+}$ of the internal standard.

Figure 3 - Dose-dependent precipitating effect of BK induced by TA. A) Tannic Acid dose-dependently reduces the relative abundance of the ion at *m/z* 530.9 refers to BK $[M+2H]^{2+}$ in

respect to the abundance of the ion at *m/z* 950.5 attributed to the internal standard (LVNEVTEF). TA concentrations: 0 μM (a), 50 μM (b), 100 μM (c), 150 μM (d), 200 μM (e), 250 μM (f), 500 μM (g), 1000 μM (h). B) Plot showing the residual BK in respect to TA concentration. Values are mean ± SD of three replicates. C) Mean % of residual BK for each concentration tested.

Figure 4 - Dose-dependent precipitating effect of BK induced by PGG. A) PGG dose-dependently reduces the relative abundance of the ion at *m/z* 530.9 refers to BK $[M+2H]^{2+}$ in respect to the abundance of the ion at *m/z* 950.5 attributed to the internal standard (LVNEVTEF). TA concentrations: 0 μM (a), 10 μM (b), 50 μM (c), 100 μM (d), 150 μM (e), 200 μM (f), 250 μM (g), 500 μM (h). B) Plot showing the residual BK in respect to TA concentration. Values are mean ± SD of three replicates. C) Mean % of residual BK for each concentration tested.

Figure 5 – Analysis of the precipitate occurred between TA and BK: in the upper panel the spectrum of $[M+3H]^{3+}$ and $[M+2H]^{2+}$ BK ions; in the lower panel the deconvoluted spectrum obtained with MagTran confirms the identity of BK.

Figure 6 - Analysis of the precipitate occurred between PGG and BK: in the upper panel the spectrum of $[M+3H]^{3+}$ and $[M+2H]^{2+}$ BK; in the lower panel the deconvoluted spectrum obtained with MagTran confirms the identity of BK.

Figure 7 - ESI-MS spectrum of a solution of RR-9 (10 μM) spiked with the IS. Ions at 470.7 *m/z* and 940.2 *m/z* are the $[M+2H]^{2+}$ and $[M+H]^{+}$ of RR-9, respectively. The ion at 950.5 *m/z* refers to the $[M+H]^{+}$ of the internal standard.

Figure 8 - Profile of RR-9 reduction in the supernatant by PGG increasing concentration.

Figure 9 - Dynamic profile of the complex stability with (grey line) and without (dashed grey line) PGG molecules as assessed by MM-GBSA calculations based on the two performed MD runs. At the bottom, two representative BK-PGG complexes are shown. On the left the best BK-PGG complex as obtained by docking simulations. On the right, a portion of the last frame of the MD simulations with PGG (in both complexes prolines are coloured in orange for easy identification).

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