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First observation of non-covalent complexes for a tannin–protein interaction model investigated by electrospray ionisation mass spectroscopy

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Abstract—Non-covalent complexes for a tannin–protein interaction model have been analyzed by mass spectrometry. The model is the polyphenol penta-*O*-galloyl-D-glucopyranose (PGG), a representative member of the hydrolysable tannin family and the nonapeptide hormone bradykinin (BDK). This is the first observation by electrospray ionisation mass spectrometry of non-covalent complexes for a tannin–protein interaction model. The technique should prove to be a powerful tool of investigation in this field.

Polyphenols, especially tannins, are present in many higher plants and are consumed by humans in food and beverages. Among the properties of tannins, one is particularly important because of its impact on health: the formation of complexes with proteins. These interactions might be important at the biological level,[1] even though at present it is extremely difficult to extrapolate the established in vitro activity of polyphenols to the suspected in vivo activity because of the question of their bioavailability. To study these interactions, model molecules were tested: on the one hand, the linear nonapeptide hormone bradykinin (H-Arg[1]-Pro[2]-Pro[3]-Gly[4]-Phe[5]-Ser[6]-Pro[7]-Phe[8]-Arg[9]-OH) (BDK), which has a wide biological activity[2–5] and on the other, 1,2,3,4,6-penta-*O*-galloyl-β-D-glucopyranose (PGG), a representative polyphenol of the hydrolysable tannin family, which is well known to bind to a variety of substrates.[6–9]

Using NMR, tannin–protein complexes have been observed in solution.[10–12] In the literature, various hypotheses to explain the tannin–protein interaction were proposed: π–π stacking, π–σ attraction, hydrophobic stacking and H bonds.[1,6] Electrospray ionization mass spectrometry (ESI-MS) can be used to observe non-covalent biomolecular complexes.[13] To gain other insights into the nature of the interactions involved in complex formation in the absence of solvent molecules, we attempted to produce such complexes in the gas phase by ESI-MS. To our knowledge, detection of non-covalent tannin–protein complexes has never been performed by mass spectrometry and this method could give additional help for studying such interactions in this important field which has a large impact on human health. However, it will be necessary to show the specificity of the interaction in the gas phase and to be cautious in the structural analogy between gas-phase and solution complexes: this is a general debate in mass spectrometry of non-covalent complexes.[13]

Bradykinin salt and arginine were purchased from Sigma Company and were used without further purification. The synthesis of PGG was done in two steps by galloylation of β-D-glucose in a 83% overall yield[14] and 1 mM water solutions of BDK and PGG were prepared. The 1:1 complex was prepared by mixing an equal volume of each solution. The mixture was then diluted 10 times to obtain a final concentration of 0.05 mM. The same procedure was used for the 2PGG:1BDK complex by mixing 2 volumes of PGG

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with 1 volume of BDK followed by a 10-fold water dilution. For comparison, the same experiments were realized in a 2 mM ammonium formiate buffer (pH 7.0±0.1). Moreover, to investigate the possible occurrence of a simple 'cationisation' reaction in the formation of the complexes, mixtures 1:1:1 and 1:1:20 in BDK, PGG and arginine, respectively were also prepared.

Solutions of the complexes were analysed on a Perkin–Elmer Sciex API 365 (Université Paul Sabatier, Toulouse, France) fitted with an ion spray source working in the positive ion mode. The upper mass range of the quadrupole mass analyser was m/z 3000. Samples were infused into the electrospray interface by a Harvard 11 syringe pump at a flow rate of 5μL/min. The air nebulizer flow and the nitrogen curtain gas were at room temperature. The extraction cone voltage value was set to 3 volts. Scanning was performed from $m/z=$ 400 to 1200 for pure solutions of BDK and PGG and from $m/z=500$ to 3000 for the complex solutions. The data system was operated as a multichannel analyzer and 10 scans were summed to obtain the final spectrum. Resolution was set to 0.5 u over the scanned mass range to separate the isotope peaks for both the single and doubly-charged ions.

Pure neutral water solutions of pentagalloyl-*O*-D-glucose ($C_{41}H_{32}O_{26}$) and peptide bradykinin were examined separately by ESI-MS. Using the experimental conditions described above, PGG gave a relatively weak MH^+ ion at m/z 941.2, an abundant sodium-attached molecule, $(M+Na)^+$ at m/z 963.2 and a weaker potassium-attached molecule $(M+K)^+$ at 979.2. The main fragment ions corresponded to loss of gallic acid (170 u) from MH^+ at m/z 771.0 and from $(M+Na)^+$ at m/z 793.0. The peptide bradykinin produced an abundant doubly protonated molecule $(M+2H)^{2+}$ at m/z 530.9, a weaker singly protonated molecule $(M+H)^+$ at m/z 1060.7 and a very weak triply charged molecule $(M+3H)^{3+}$ at m/z 354.4. This distribution of singly and multiply protonated species can be explained by the presence of three sites of high proton affinity in bradykinin, the two guanidinium groups of Arg1 and Arg9 and the NH_2 terminus. Electrostatic interaction between the two protonated sites of Arg1 destabilizes the triply charged ion, thus explaining its low abundance. At the low cone voltage used (3 volts), only very weak fragment ions emerged from the background, namely Y6, Y7, Y8 and B6.

The electrospray mass spectrum of the 1:1 molar ratio PGG/BDK mixture in water is presented in Fig. 1a and 1b. Mixtures of 2:1 molar ratio produced the same spectrum. Whereas these spectra still exhibited abundant ions due to the protonated peptide alone, protonated PGG-BDK molecular complexes were clearly seen. Thus, the relatively intense peak at m/z 1001.0 was attributed to the 1:1 complex $(BDK+PGG+2H)^{2+}$. The corresponding singly protonated complex gave a smaller peak at m/z 2001.7. Moreover, the doubly charged ion at m/z 1471.7 was attributed to the doubly protonated 1:2 complex $(BDK+2PGG+2H)^{2+}$. The corresponding singly protonated species was also present but at a very low intensity (Fig. 1b). Other low intensity peaks could also be seen: as these peaks could not be interpreted as arising from combinations between both species or their fragments, we believe they came from impurities.

Using an ammonium formate buffer 2 mM, similar results were observed but the complex-derived ions have slightly smaller intensities. In addition, the ammonium-attached PGG molecular ion $(PGG+NH_4)^+$ at m/z 958.4 was formed at a rather low abundance. This is probably due to the large excess of ammonium ions that are able to bind to electron-donating species through H-bonds.

In the gas phase, the external medium (vacuum) is widely considered as being hydrophobic. This reinforces the strength of H bonds comparatively to hydrophobic and van der Waals interactions. To see whether the stability of the gas phase complexes was due only to the solvation of protonated guanidinium parts of the arginine residues of BDK by electron-donating groups of PGG, as in the well known 'cationization' reactions, or whether other groups participate thanks to a steric complementarity, thus creating the specificity of such interactions, competition experiments by adding arginine to the PGG–BDK solutions were done. Using an equimolar solution (1:1:1) of the three species, arginine gave an abundant singly protonated ion $(M+H)^+$ at m/z 174.9. However, although the ions derived from the PGG–BDK complexes were seen, no ion derived from PGG–arginine could be detected (expected at m/z 1115.2). Increasing the amount of arginine up to a 20 fold molar ratio (1:1:20 PGG:BDK:arginine mixture) did not permit to detect the protonated PGG–arginine complex, at an intensity higher than the background (that is about 100 times less than the intensity of the doubly charged PGG–BDK, $2H^+$ ion). So, despite a large excess, protonated arginine was not able to 'cationize' PGG in our electrospray conditions.

Thus the stability of the PGG–BDK complex species cannot be attributed to a simple electrostatic interaction between a protonated arginine residue of BDK and some electron-donating group of PGG, and other groups do participate. These results could be compared to the Penn et al. data about the interactions in gas-phase bradykinin–cyclodextrin complexes.[15] In such complexes, a phenylalanine residue is included into the cyclodextrin cage and linked by hydrophobic effects and a guanidinium group interacts with the cyclodextrin ring via ion-dipole interactions. They conclude that (i) in solution the ion-dipole interaction become weaker while the hydrophobic effect become more important; (ii) it is the contrary in the gas phase, (iii) the forces that keep the complex bound in solution will not necessarily have the same effects in the gas phase.

In solution, the 1:1 and 2:1 complexes between PGG and BDK do exist as demonstrated by NMR,[10–12] in the literature, various hypotheses to explain the tannin–

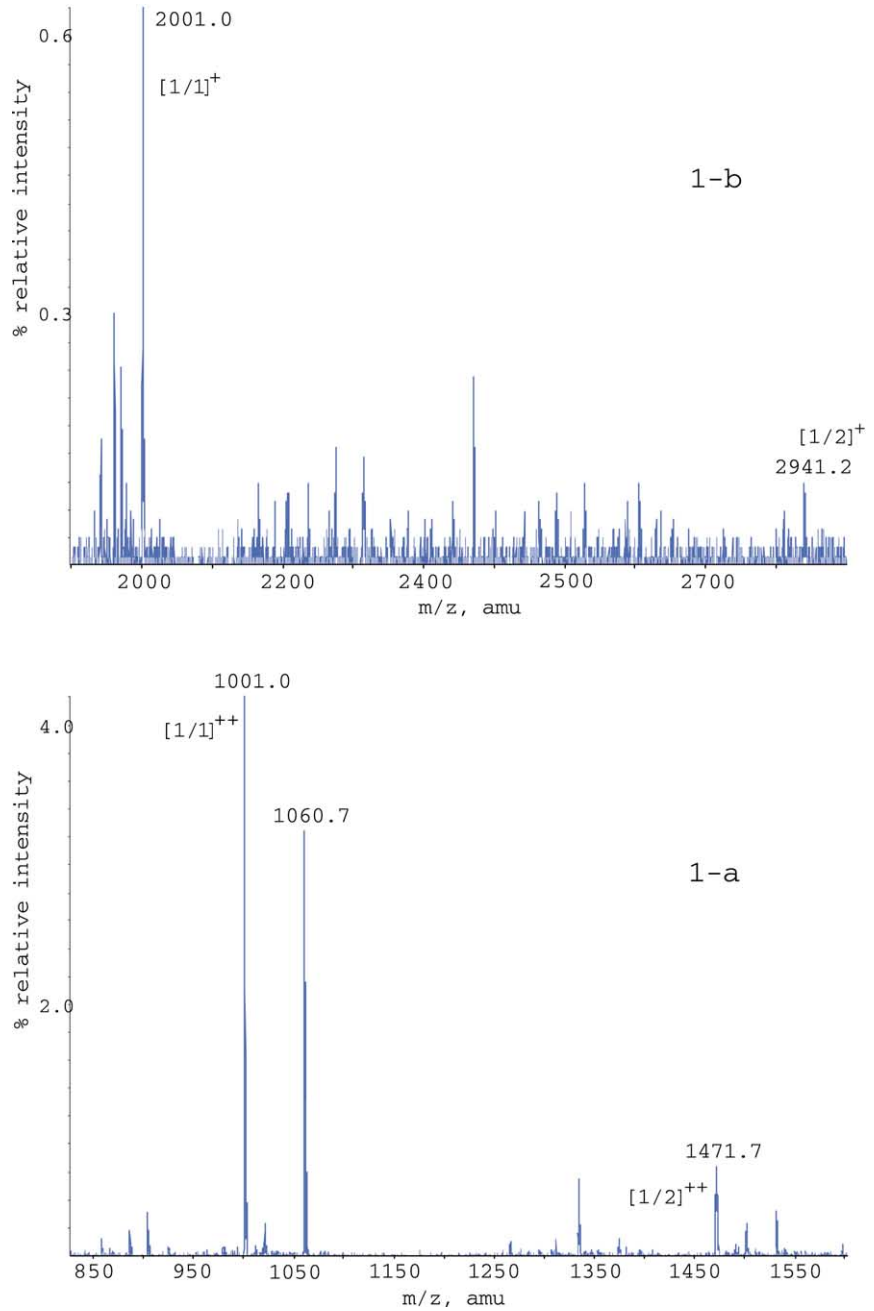

Figure 1. Electrospray mass spectrum of the equimolar mixture of PGG/BDK complex in water. The doubly protonated bradykinin $(M+H)^+$ is the 100% relative intensity.

protein interaction were made: π–π stacking or π–σ attraction for phenylalanine residues, hydrophobic stacking for the proline rings reinforced by hydrogen bonding, and/or hydrogen bonding for arginine guanidinium.[1,6] Moreover, owing to the high flexibility of PGG and BDK molecules, conformational mutual adaptation may allow them to adopt a steric complementarity, and creation of additional van der Waals bonds may be suggested. However, in an aqueous medium and with the fast equilibrium between free and bound molecules,[12] any evaluation of van der Waals bonds is particularly tricky.

In the gas-phase PGG–BDK complexes, the relative strength of the different bonding modes is probably different than in solution, lowering hydrophobic interactions while increasing the relative strength of H-bonds, but, clearly, other interactions that those between the guanidinium part of an arginine residue and PGG should be involved to explain the survival of these complexes in the gas phase.

In conclusion, ESI-MS might provide good information about the tannin–protein interactions. Moreover, the rapidity of the data acquisition and the relative simplic-

ity of interpretation make ESI-MS a powerful tool for screening numerous tannin–protein complexes.

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