4008 *J. Agric. Food Chem.* **2004,** *52,* 4008–4011

JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Characterization of Soluble Non-covalent Complexes between Bovine Serum Albumin and β-1,2,3,4,6-Penta-*O*-galloyl-D-glucopyranose by MALDI-TOF MS

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β-1,2,3,4,6-Penta-*O*-galloyl-D-glucopyranose (PGG) and soluble complexes of PGG with bovine serum albumin (BSA) were characterized by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS). PGG was also characterized by electrospray ionization mass spectrometry (ESI-MS). Similar fragmentation patterns of PGG were found in ESI-MS and MALDI-TOF MS. The apparent stoichiometries of non-covalent BSA−PGG complexes were determined by MALDI-TOF MS.

KEYWORDS: Hydrolyzable tannin; polyphenolic; protein−tannin interaction; pentagalloyl glucose; MALDI-TOF MS

INTRODUCTION

Polyphenols are found in plants and in plant-derived feeds, foods, and medicines (*1*). A typical Western diet results in the daily ingestion of ∼1 g of polyphenols, >50% of which is polymeric polyphenols (tannins) (*2*). There are three groups of tannins: hydrolyzable tannins, condensed tannins, and phlorotannins (*3*). Hydrolyzable tannins consist of simple phenolic acids such as gallic acid esterified to a core polyol, typically glucose, and condensed tannins are polymers of flavonoid units; both of them are found in terrestrial plants. Phlorotannins are polymeric phloroglucinol derivatives found in marine brown algae.

The defining property of tannins is their ability to form precipitable protein−tannin complexes (*4*), although other activities, such as their potential to serve as biological antioxidants (*3*, *5*) and their potential to chelate metal ions (*6*, *7*), have also received attention. Formation of complexes with protein attenuates the radical scavenging kinetics of the condensed tannin, procyanidin (*8*), indicating that protein−tannin interaction may influence the other activities of tannins. Given that proteins are the main components in most biological matrices, revealing the mechanism of protein−tannin interaction will lead to a better understanding of all the activities of tannins in biological systems.

Characterization of protein−tannin precipitable complexes is the conventional approach for studying protein−tannin interactions (*3*, *9*), but precipitation does not necessarily occur in biological systems when the protein is present in large excess over tannins (*10*). Establishing methods for examining soluble protein−tannin complexes will provide better insights into mechanisms of complex formation and into their biological activities. Equilibrium dialysis has been used to study soluble complexes between proteins and tannins (*11*, *12*), but equilibrium dialysis is slow and thus not suitable for systems sensitive to rapid oxidation. Furthermore, tannins can nonspecifically bind to dialysis membrane, which complicates their quantitative determination. The formation of soluble protein−tannin complexes was demonstrated using size exclusion chromatography (SEC) (*13*), but SEC cannot provide accurate information on the molecular weights and the stoichiometry of the soluble protein−tannin complexes. Nuclear magnetic resonance (NMR) has also been used to study the non-covalent soluble protein−tannin complexes (*14*−*17*), but the size of the protein is a limiting factor for high-resolution NMR analysis; only model peptides are used in those model systems. Recently, microcalorimetry has been used to examine the thermodynamic changes associated with protein−tannin complex formation, but interpretation of the binding isotherms is not straightforward (*18*).

An alternate approach for examining the non-covalent interactions between proteins and ligands is to use methods of mass spectrometry that are dependent on "soft" ionization techniques (*19*−*22*). Recently, electrospray ionization mass spectrometry (ESI-MS) was successfully used to characterize the complexes of polyphenols (M_r 290−940) with peptides (M_r 1060−1189) (*23*, *24*). We have now improved the utility of mass spectrometric approaches by using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) to examine the soluble non-covalent complexes between β-1,2,3,4,6-penta-*O*-galloyl-D-glucopyranose (PGG, M_r 940), a prototypical hydrolyzable tannin, and bovine serum albumin (BSA, M_r 66,000), a globular protein widely used in the study of protein−tannin interactions (*3*, *4*, *8*, *11*).

MATERIALS AND METHODS

Materials. All reagents were of analytical grade or better. Type V fatty acid free BSA was obtained from Sigma-Aldrich (St. Louis, MO)

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10.1021/jf035536t CCC: $27.50 © 2004 American Chemical Society
Published on Web 05/19/2004

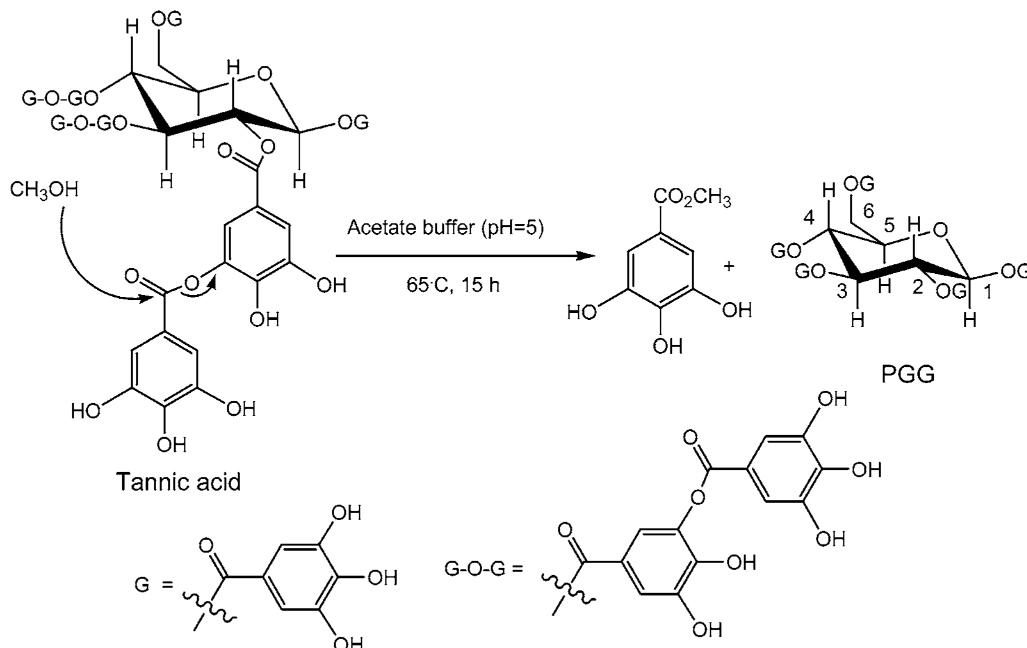

Figure 1. Methanolysis of tannic acid yields pentagalloyl glucose (PGG) and methyl gallate. Tannic acid (0.5 g) was methanolyzed in 10 mL of 70% methanol in 0.1 M acetate buffer (pH 5.0) at 65 °C for 15 h.

and used without further purification. SDS-PAGE with Coomassie brilliant blue staining was used to establish that the sample was at least 99% serum albumin. MALDI-TOF confirmed that there were fewer than six contaminating proteins comprising <1% of the total sample.

Preparation of PGG. A sample of 0.5 g of tannic acid (Coleman & Bell Co., Norwood, OH) was methanolyzed in 10 mL of 70% methanol in acetate buffer (0.1 M, pH 5.0) at 65 °C for 15 h. The pH of the reaction mixture was immediately adjusted to 6.0, and methanol was removed by evaporation under reduced pressure at <30 °C. Water was added to maintain the volume as the methanol was removed. The resulting aqueous solution was extracted with 3 volumes of diethyl ether and 3 volumes of ethyl acetate. The ethyl acetate extracts were combined and evaporated under reduced pressure. Water was added to maintain the volume as the ethyl acetate was removed. The resulting white suspension was centrifuged, and the precipitate was redissolved by gentle heating in 10 mL of a 2% methanol solution. PGG precipitated as the solution was slowly cooled to room temperature and was again collected by centrifugation and washed twice with 10 mL of an ice-cold 2% methanol solution and once with 10 mL of ice-cold double-distilled water. The final material was lyophilized to yield a white powder with an overall mass yield of 34%. This protocol has been scaled up to accommodate larger scale purifications starting with 25 g of tannic acid.

Characterization of PGG. PGG was characterized by ^{1}H NMR spectroscopy (Bruker 200 MHz Avance spectrometer) in acetone-d_6. ESI-MS of PGG was carried out on a Bruker Esquire 3000 ion-trap mass spectrometer with an electrospray interface (Bruker, Billerica, MA). The ESI-MS was run in negative ion mode under the following conditions: direct infusion of PGG (1.4 mg/mL in 5% acetonitrile) at 400 μL/min, nebulizer at 50 psi, N_2 dry gas at 9 L/min, dry temperature at 365 °C, high voltage (HV) of capillary at 3800 V, HV of end plate offset at −500 V, skimmer 1 at −59.2 V, capillary exit at −86.8 V, and spectral averages at 8. The purity of PGG was determined by high-performance liquid chromatography (HPLC). A Hewlett-Packard 1050 HPLC system with two pumps, an autosampler, and a UV detector was equipped with a C-18 column (Adsorbosphere XL C18 90 Å, 3 μm, 100 × 4.6 mm, Alltech, Deerfield, IL). Mobile phase A was water containing 0.1% acetic acid (v/v), and mobile phase B was acetonitrile containing 0.1% acetic acid (v/v). The flow rate was 1 mL/min. The UV detector was set at 220 nm. PGG (0.045 μg/μL in 5% B, 10 μL) was injected. The gradient started at 5% B, increased to 100% B in 3 min, and then returned to 5% B in 3 min, and the column was re-equilibrated for 7 min.

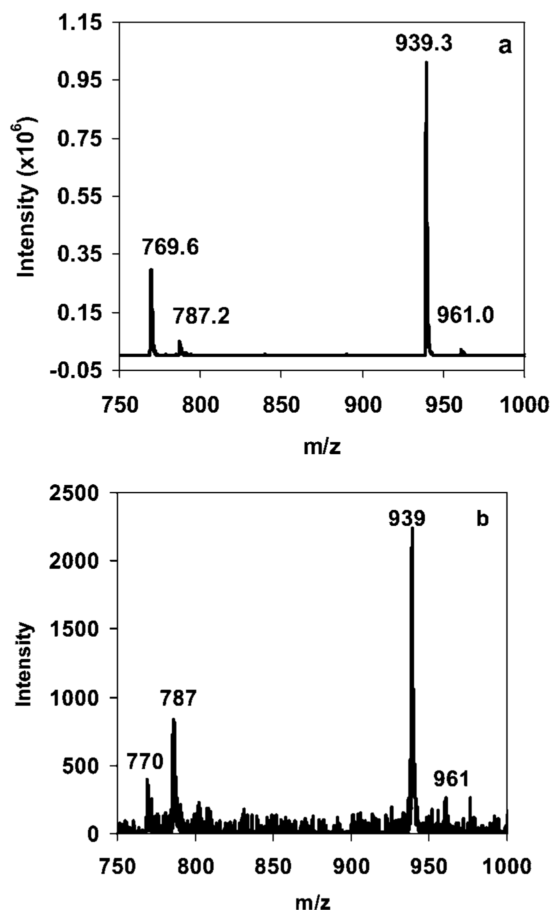

Figure 2. Mass spectra of PGG: (**a**) ESI mass spectrum of PGG taken at negative mode; (**b**) MALDI-TOF mass spectrum of PGG taken at negative mode.

MALDI-TOF MS of PGG and PGG−BSA Soluble Non-covalent Complexes. For studies of PGG, the PGG stock solution was prepared by dissolving 1.5 mg of PGG in 100 μL of acetonitrile. An aliquot (5 μL) of the PGG stock solution was mixed 1:1 with α-cyano-4-hydroxycinnamic acid [10 mg/mL, in 50% (v/v) acetonitrile containing

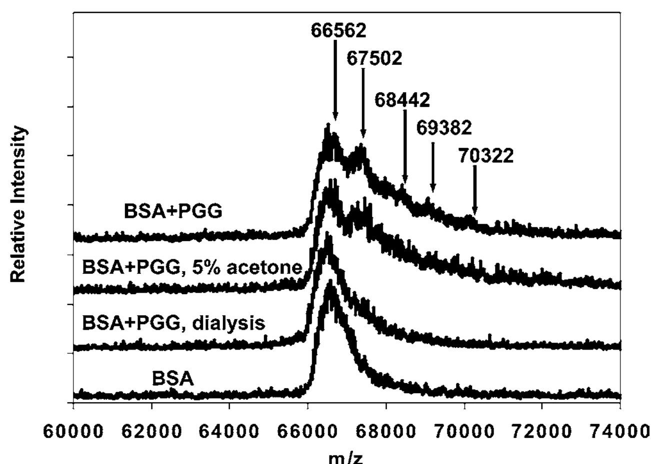

Figure 3. MALDI-TOF mass spectra of BSA–PGG soluble non-covalent complexes. Spectra of positive ions were taken in the linear mode. Peaks at *m/z* 66562, 67502, 68442, 69382, and 70322 were assigned to $[BSA]^+$, $[BSA–PGG]^+$, $[BSA–PGG_2]^+$, $[BSA–PGG_3]^+$, and $[BSA–PGG_4]^+$, respectively.

0.05% (v/v) trifluoroacetic acid in water]. An aliquot (1 μL) of the final mixture was spotted onto a stainless steel target. MALDI-TOF MS was performed on a Bruker Reflex III TOF mass spectrometer (Bruker, Billerica, MA) equipped with a nitrogen laser (λ 337 nm, Laser Science, Franklin, MA) and a reflector. Laser-desorbed negative ions were analyzed after acceleration by 26 kV in the reflector mode.

For studies of BSA−PGG soluble non-covalent complexes, BSA and PGG were prepared in 10 mM acetate buffer, pH 4.9, containing 8.5 mM NaCl. BSA (53 nmol, 500 μL) was incubated with PGG (53 nmol in buffer or in 10% acetone/acetate buffer solution, 500 μL) or the acetate buffer (500 μL) at room temperature for 1 h. The mixtures were centrifuged at 11000*g* for 10 min to remove the trace amount of precipitate that was in the BSA−PGG mixture, but not in the BSA−PGG (5% acetone/buffer solution) or BSA−buffer control. An aliquot (5 μL) of the supernatant of each sample was mixed 1:1 with 3,5-dimethoxy-4-hydroxycinnamic acid [10 mg/mL, in 50% (v/v) acetonitrile in 0.05% (v/v) trifluoroacetic acid]. An aliquot (1 μL) of the final mixture was spotted on the target. A portion (1 mL) of the BSA−PGG mixture was dialyzed against two portions (1 L) of the acetate buffer for 4 h at room temperature and was prepared for MALDI-TOF MS analysis as described above. Laser-desorbed positive ions were analyzed after acceleration by 26 kV in the linear mode.

RESULTS AND DISCUSSION

PGG was purified after methanolysis of tannic acid (**Figure 1**) and was identified by ^{1}H NMR (acetone-d_6): δ 4.4 (dd, 2H, glucose, C_6−H), 4.6 (d, 1H, glucose C_5−H), 5.6 (q, 2H, glucose C_2−H and C_4−H), 6.1 (t, 1H, glucose C_3−H), 6.4 (d, 1H, glucose C_1−H), 7.0−7.38 (5s, 10H, galloyl group). A repurification step was added to the conventional workup (*25*), producing PGG with high purity (>98%, determined by HPLC). This modification also improved the stability of PGG in storage. There was no detectable degradation of PGG during storage at −20 °C for 3 years.

PGG was further analyzed by mass spectrometry. In the negative mode, ESI-MS and MALDI-TOF MS produced similar mass spectra (**Figure 2**). The ion at *m/z* 939 was attributed to the $[M - H]^-$ ion of PGG. It is reasonable to postulate that the other ions represent fragments of the $[M - H]^-$ ion such as tetragalloyl glucose (*m/z* 787) and the corresponding dehydroglucitol (*m/z* 770). The ion at *m/z* 961 was the PGG−Na^+ adduct ion ($[M + Na^+ - 2H]^-$).

When MALDI-TOF MS is used to characterize tannins, K^+, Na^+, or Cs^+ is usually added to the matrix to form tannin−metal adducts, and the resulting adducts are detected in positive mode ($[M + \text{metal ion}]^+$) (*26*−*29*). Specific tannins are identified by comparing the measured *m/z* values for the tannin−metal ion adducts to the calculated *m/z* values. This approach provides a powerful tool for identifying tannins in mixtures such as plant extracts, but does not provide fragmentation information because the presence of metal ion adds ambiguity to structural assignment to the fragment ions of tannins. Our method provided direct information about the molecular ions and the fragment ions that could provide structural information of the parent molecules.

We also used MALDI-TOF MS to characterize soluble non-covalent BSA−PGG complexes (**Figure 3**). When BSA was incubated with PGG (molar ratio = 1), BSA−PGG was seen as a distinct shoulder on the main BSA peak. A series of shoulders were assigned to BSA−PGG_2 up to BSA−PGG_4. Dialysis of the BSA−PGG complexes removed PGG from BSA, establishing the non-covalent nature of the association between BSA and PGG.

A two-stage mechanism for precipitating protein by PGG has been proposed (*17*): (1) PGG binds to BSA, forming soluble non-covalent complexes; (2) soluble non-covalent BSA−PGG complexes aggregate, forming precipitates (**Figure 4**). Our results support this mechanism by providing direct evidence for the formation of soluble non-covalent BSA−PGG complexes in aqueous solution. We found that 5% acetone did not prevent formation of soluble BSA−PGG complexes, but did prevent formation of precipitable BSA−PGG complexes. It has previously been noted that low levels of acetone inhibit formation of precipitable non-covalent BSA−PGG complexes (*10*), leading us to suggest that acetone prevents the aggregation step that leads to insoluble precipitates rather than the fundamental binding of PGG to BSA.

We have successfully used MALDI-TOF MS to characterize the soluble non-covalent complexes between a well-defined polymeric polyphenol (PGG) and a typical globular protein (BSA). Our method might be adapted to systems containing other polyphenols and other proteins. Riedl and Hagerman have

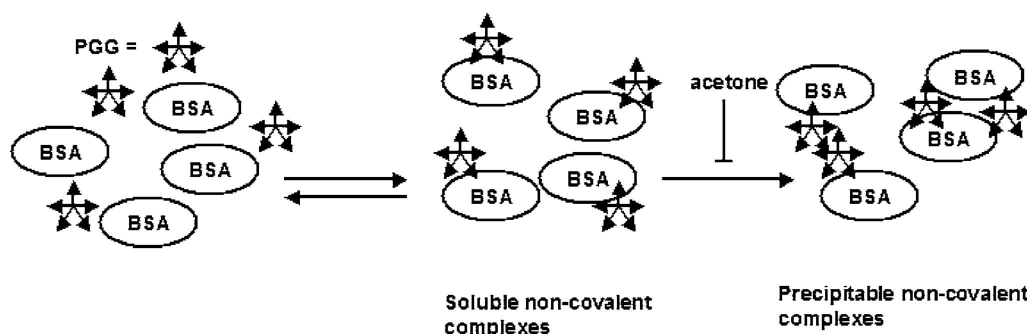

Figure 4. Two-stage mechanism for protein precipitation by PGG.

demonstrated that covalent linkages form when BSA−procyanidin complexes are exposed to oxidants (*8*). We are now using MALDI-TOF MS to characterize the oxidation products of protein−polyphenol complexes, hoping to shed light on the fate of protein−polyphenol complexes that have served as biological antioxidants.

ABBREVIATIONS USED

PGG, β-1,2,3,4,6-penta-*O*-galloyl-D-glucopyranose; BSA, bovine serum albumin; MALDI-TOF MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; SEC, size exclusion chromatography; NMR, nuclear magnetic resonance; ESI-MS, electrospray ionization mass spectrometry.

ACKNOWLEDGMENT

Dr. John Hawes provided invaluable assistance with the operation of and sample preparation for the MALDI-TOF mass spectrometer.

LITERATURE CITED

(1) Haslam, E.; Lilley, T. H.; Cai, Y.; Martin, R.; Magnolato, D. Traditional herbal medicines−the role of polyphenols. *Planta Med.* **1989**, *55*, 1−8.
(2) Gonthier, M. P.; Donovan, J. L.; Texier, O.; Felgines, C.; Remesy, C.; Scalbert, A. Metabolism of dietary procyanidins in rats. *Free Radical Biol. Med.* **2003**, *35*, 837−844.
(3) Hagerman, A. E.; Riedl, K. M.; Jones, G. A.; Sovik, K. N.; Ritchard, N. T.; Hartzfeld, P. W.; Riechel, T. L. High molecular weight plant polyphenolics (tannins) as biological antioxidants. *J. Agric. Food Chem.* **1998**, *46*, 1887−1892.
(4) Hagerman, A. E.; Rice, M. E.; Ritchard, N. T. Mechanisms of protein precipitation for two tannins, pentagalloyl glucose and epicatechin$_{16}$ (4 → 8) catechin (procyanidin). *J. Agric. Food Chem.* **1998**, *46*, 2590−2595.
(5) Bors, W.; Foo, L. Y.; Hertkorn, N.; Michel, C.; Stettmaier, K. Chemical studies of proanthocyanidins and hydrolyzable tannins. *Antioxid. Redox Signaling* **2001**, *3*, 995−1008.
(6) Seibels, B.; Lamberski, N.; Gregory, C. R.; Slifka, K.; Hagerman, A. E. Effective use of tea to limit dietary iron available to starlings (*Sturnus vulgaris*). *J. Zoo Wildl. Med.* **2003**, *34*, 314−316.
(7) Inoue, M. B.; Inoue, M.; Fernando, Q.; Valcic, S.; Timmermann, B. N. Potentiometric and ^{1}H NMR studies of complexation of Al^{3+} with (−)-epigallocatechin gallate, a major active constituent of green tea. *J. Inorg. Biochem.* **2002**, *88*, 7−13.
(8) Riedl, K. M.; Hagerman, A. E. Tannin−protein complexes as radical scavengers and radical sinks. *J. Agric. Food Chem.* **2001**, *49*, 4917−4923.
(9) Kawamoto, H.; Nakatsubo, F.; Murakami, K. Stoichiometric studies of tannin−protein co-precipitation. *Phytochemistry* **1996**, *41*, 1427−1431.
(10) Hagerman, A. E.; Robbins, C. T. Implications of soluble tannin−protein complexes for tannin analysis and plant defense-mechanisms. *J. Chem. Ecol.* **1987**, *13*, 1243−1259.
(11) Feldman, K. S.; Sambandam, A.; Lemon, S. T.; Nicewonger, R. B.; Long, G. S.; Battaglia, D. F.; Ensel, S. M.; Laci, M. A. Binding affinities of gallotannin analogs with bovine serum albumin: ramifications for polyphenol−protein molecular recognition. *Phytochemistry* **1999**, *51*, 867−872.
(12) Mcmanus, J. P.; Davis, K. G.; Beart, J. E.; Gaffney, S. H.; Lilley, T. H.; Haslam, E. Polyphenol interactions. 1. Introduction−some observations on the reversible complexation of polyphenols with proteins and polysaccharides. *J. Chem. Soc., Perkin Trans. 2* **1985**, 1429−1438.
(13) Hatano, T.; Hori, M.; Hemingway, R. W.; Yoshida, T. Size exclusion chromatographic analysis of polyphenol−serum albumin complexes. *Phytochemistry* **2003**, *63*, 817−823.
(14) Simon, C.; Barathieu, K.; Laguerre, M.; Schmitter, J. M.; Fouquet, E.; Pianet, I.; Dufourc, E. J. Three-dimensional structure and dynamics of wine tannin−saliva protein complexes. A multitechnique approach. *Biochemistry* **2003**, *42*, 10385−10395.
(15) Baxter, N. J.; Lilley, T. H.; Haslam, E.; Williamson, M. P. Multiple interactions between polyphenols and a salivary proline-rich protein repeat result in complexation and precipitation. *Biochemistry* **1997**, *36*, 5566−5577.
(16) Verge, S.; Richard, T.; Moreau, S.; Nurich, A.; Merillon, J. M.; Vercauteren, J.; Monti, J. P. First observation of solution structures of bradykinin-penta-*O*-galloyl-D-glucopyranose complexes as determined by NMR and simulated annealing. *Biochim. Biophys. Acta (G)* **2002**, *1571*, 89−101.
(17) Charlton, A. J.; Baxter, N. J.; Khan, M. L.; Moir, A. J. G.; Haslam, E.; Davies, A. P.; Williamson, M. P. Polyphenol/peptide binding and precipitation. *J. Agric. Food Chem.* **2002**, *50*, 1593−1601.
(18) Frazier, R. A.; Papadopoulou, A.; Mueller-Harvey, I.; Kissoon, D.; Green, R. J. Probing potein−tannin interactions by isothermal titration microcalorimetry. *J. Agric. Food Chem.* **2003**, *51*, 5189−5195.
(19) Lin, S. H.; Cotter, R. J.; Woods, A. S. Detection of non-covalent interaction of single and double stranded DNA with peptides by MALDI-TOF. *Proteins* **1998**, *Suppl. 2*, 12−21.
(20) Moniatte, M.; Lesieur, C.; Vecsey-Semjen, B.; Buckley, J. T.; Pattus, F.; Van Der Goot, F. G.; Van Dorsselaer, A. Matrix-assisted laser desorption−ionization time-of-flight mass spectrometry in the subunit stoichiometry study of high-mass non-covalent complexes. *Int. J. Mass Spectrom.* **1997**, *169*, 179−199.
(21) Glocker, M. O.; Bauer, S. H. J.; Kast, J.; Volz, J.; Przybylski, M. Characterization of specific noncovalent protein complexes by UV matrix-assisted laser desorption ionization mass spectrometry. *J. Mass Spectrom.* **1996**, *31*, 1221−1227.
(22) Loo, J. A. Studying noncovalent protein complexes by electrospray ionization mass spectrometry. *Mass Spectrom. Rev.* **1997**, *16*, 1−23.
(23) Verge, S.; Richard, T.; Moreau, S.; Richelme-David, S.; Vercauteren, J.; Prome, J. C.; Monti, J. P. First observation of non-covalent complexes for a tannin-protein interaction model investigated by electrospray ionisation mass spectroscopy. *Tetrahedron Lett.* **2002**, *43*, 2363−2366.
(24) Sarni-Manchado, P.; Cheynier, V. Study of non-covalent complexation between catechin derivatives and peptides by electrospray ionization mass spectrometry. *J. Mass Spectrom.* **2002**, *37*, 609−616.
(25) Hagerman, A. E.; Zhao, Y.; Johnson, S. Methods for determination of condensed and hydrolyzable tannins. In *Antinutrients and Phytochemicals in Foods*; Shahadi, F., Ed.; American Chemical Society: Washington, DC, 1997; pp 209−222.
(26) Pasch, H.; Pizzi, A.; Rode, K. MALDI-TOF mass spectrometry of polyflavonoid tannins. *Polymer* **2001**, *42*, 7531−7539.
(27) Pasch, H.; Pizzi, A. Considerations on the macromolecular structure of chestnut ellagitannins by matrix-assisted laser desorption/ionization-time-of-flight mass spectrometry. *J. Appl. Polym. Sci.* **2002**, *85*, 429−437.
(28) Perret, C.; Pezet, R.; Tabacchi, R. Fractionation of grape tannins and analysis by matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry. *Phytochem. Anal.* **2003**, *14*, 202−208.
(29) Krueger, C. G.; Vestling, M. M.; Reed, J. D. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry of heteropolyflavan-3-ols and glucosylated heteropolyflavans in sorghum [*Sorghum bicolor* (L.) Moench]. *J. Agric. Food Chem.* **2003**, *51*, 538−543.

Received for review December 31, 2003. Revised manuscript received April 7, 2004. Accepted April 14, 2004.

JF035536T