Binding Affinity of Hydrolyzable Tannins to Parotid Saliva and to Proline-Rich Proteins Derived from It

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Proline-rich proteins (PRP) in human parotid saliva have a high affinity for dietary polyphenolic compounds (tannins), forming stable complexes that may modulate the biological and nutritional properties of the tannin. The formation of such complexes may also have an important role in the modulation or promotion of the sensation of oral astringency perceived when tannin-rich foods and beverages are consumed. The major classes of PRP (acidic, basic, and glycosylated) have been isolated from human saliva, and the relative binding affinities of a series of hydrolyzable tannins, which are found in a number of plant-derived foods and beverages, to these PRP classes have been determined using a competition assay. All of the classes of PRP have a high capacity for hydrolyzable tannins. Within the narrow range of binding affinities exhibited, structure/binding relationships with the levels of tannin galloylation, hexahydroxydiphenoyl esterification, and degree of polymerization were identified. No individual class of human salivary PRP appears to have an exclusive affinity for a particular type of hydrolyzable tannin.

Keywords: *Parotid saliva; proline-rich protein; hydrolyzable tannins; polyphenol complexation*

INTRODUCTION

Polyphenolic compounds (tannins) in plant-derived foods and beverages have been shown to have important physiological properties and may be responsible for both beneficial and detrimental effects on human health (Chung et al., 1998). For example, tannins have been shown to have anticarcinogenic and antimutagenic potential, possibly due to their antioxidant activities, and to have antimicrobial properties. Conversely, foods rich in tannins have been reported to be of relatively poor nutritional value and to be linked to incidences of certain cancers. The intake and type of tannin may, therefore, be critical to their overall effect on health. The parotid saliva of herbivorous and omnivorous mammals, including humans, contains proline-rich proteins (PRP) that have been demonstrated to have a high affinity for tannins due to the ability of prolyl residues to provide multiple hydrophobic binding sites and subsequently to hydrogen bond phenolic groups to the tertiary amide carbonyl group, N-terminal to the prolyl residue (Haslam, 1998). It is postulated that the salivary PRP−tannin complexes formed are stable through the digestive tract, hence modulating any physiological effects of the tannin. The consumption of tannin-rich foods and beverages is also associated with the sensation, known as astringency, of dryness and roughness felt in the mouth. The role that PRPs have in inhibiting or promoting the sensation of polyphenol-induced astringency is unclear. It has been postulated that astringency is a result of the loss of lubrication due to PRP−tannin complex formation (Haslam, 1998), but such complex formation may have a protective role against the binding of tannins to mucosal proteins and polysaccharides and palate epithelial cell surface proteins. Human saliva contains proline-rich proteins; these are present as three main classes, acidic, basic, and glycosylated PRPs (Bennick, 1982), which may constitute up to 70% of the total protein. The precise physiological role of each PRP class has as yet not been clearly identified, although functions in maintaining oral homeostasis and bacterial agglutination have been attributed to some PRP classes and all appear to have some level of affinity for dietary tannin (McArthur, 1995).

In a previous paper (Bacon and Rhodes, 1998) we described the development of a competitive binding assay, in which the relative ability of tannins to bind to human parotid salivary proteins could be measured. Preliminary data on the binding of condensed tannin monomers (flavan-3-ols), which are ubiquitous in plant foods, were presented and revealed some structural features important in binding to salivary protein. A major class of dietary polyphenol, the hydrolyzable tannins, that is, complex galloyl and hexahydroxydiphenoyl (HHDP) polyesters of polyols, such as D-glucose, may also accumulate in substantial quantities in some plant tissues. Unlike the condensed tannins, their taxonomic distribution is limited to woody and herbaceous dicotyledons. They have, however, been identified in many edible plants, for example, persimmon, pomegranate, chestnuts, and fruits of the Rosaceae (Haslam, 1998), and may be found in significant levels in some herbal medicines and beverages, such as those derived from eucalyptus, witch hazel, and bramble, raspberry, and wild strawberry leaves (Bisset, 1994), and in wines and spirits aged in oak barrels. The binding affinities of the three classes of PRPs to a range of chemically well-characterized hydrolyzable tannins will be investigated here.

MATERIALS AND METHODS

Materials. All chemicals were of AnalaR grade and purchased from BDH. Poole, U.K. All column chromatography media, 1,4-butanediol diglycidyl ether, and peroxidase (from

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horseradish) were purchased from Sigma Chemical Co., Poole, U.K. Gallic acid was supplied by Aldrich, Gillingham, U.K. (−)-Epigallocatechin was provided by Unilever Research, Colworth, U.K. 1,2,3,4,6-Penta o-galloyl-D-glucose and vescalagin were supplied by Dr. A. Scalbert, INRA, Avignon, France, and all other hydrolyzable tannins were supplied by Prof. E. Haslam, University of Sheffield, U.K. Water was purified by a Millex Q-plus system (Millipore, Watford, U.K.).

Human Parotid Saliva Collection. Parotid saliva (total of 65 mL) was collected from five healthy, nonsmoking volunteers (two females and three males, ages 25−45 years) using parotid saliva collection cups manufactured in the workshops at IFR to the specifications described by Stephen and Speirs (1976). Saliva flow was induced by applying small quantities of lemon juice onto the volunteers' tongues. After collection, EDTA was added to a final concentration of 5 mM, and after the removal of small samples for SDS−PAGE analysis, the saliva samples from the five individuals were bulked and stored at −20 °C. A sample of the collected saliva was dialyzed against water and lyophilized.

Isolation of Proline-Rich Protein Classes from Saliva. Salivary proteins were fractionated using a modified method based on the work of Levine and Keller (1977) and Kauffman and Keller (1979). All operations were performed at 2 °C. The parotid saliva was thawed and dialyzed overnight against 50 mM KH_2PO_4 buffer, pH 6.8. The dialysate was divided into two batches, equivalent to 30 mL of saliva, and each was processed separately as follows: $(NH_4)_2SO_4$ (biochemical grade) was added slowly with stirring to achieve 45% saturation. The resultant protein suspension was stirred overnight and then centrifuged at 20000*g* for 30 min.

The supernatant was dialyzed against several changes of 50 mM Tris-HCl buffer, pH 8.6, and then applied to a 17 × 1.6 cm column of DEAE-Sephadex A25 equilibrated in the same buffer. The column was eluted with starting buffer at 15 mL/h. Five milliliter fractions were collected, and their absorbance was monitored at 230 nm. The fraction eluted from the column following washing with 125 mL of starting buffer was designated the basic/glycosylated fraction. Fifty millimolar Tris-HCl, 0.5 M NaCl buffer, pH 8.6, was applied to the column to elute the acidic I PRP fraction. The basic/glycosylated fraction was concentrated to 5 mL in a 50 mL Amicon stirred ultrafiltration cell with a 3 kDa cutoff membrane. This concentrate was applied to a 60 × 1.6 cm column of Sephadex G200 equilibrated in 50 mM Tris-HCl buffer, pH 8.6, and eluted at a flow rate of 7.5 mL/h. Fractions (2.5 mL) were collected, and their absorbance was monitored at 230 nm. Elution of the column yielded two peaks, the glycosylated and basic PRP fractions.

The pellet from the ammonium sulfate precipitation described above (containing α-amylase as a major component) was resuspended, dialyzed exhaustively against water, and lyophilized. The residue was dissolved in 50 mM KH_2PO_4 buffer, pH 8.0, and applied to a 17 × 1.6 cm column of DEAE-Sephadex A50 equilibrated in the same buffer. The column was eluted with starting buffer at 15 mL/h, 5 mL fractions were collected, and their absorbance was monitored at 230 nm. This procedure eluted the α-amylase fraction from the column. When all unbound protein had been eluted (100 mL), 50 mM KH_2PO_4, 0.5 M NaCl buffer, pH 8.0, was applied to the column and the eluate collected to yield the acidic II PRP fraction. The four PRP fractions (acidic I, acidic II, basic, and glycosylated) and the α-amylase fraction so obtained were dialyzed exhaustively against water, lyophilized, and stored at −20 °C.

Characterization of Saliva and PRP Fractions. The amino acid composition of each of the PRP fractions was analyzed by Alta Bioscience, University of Birmingham, U.K. Polyacrylamide gel electrophoresis was performed on a Pharmacia PhastSystem on PhastGel homogeneous 20 with SDS under reducing conditions and developed according to the manufacturer's instructions. Molecular masses were estimated using protein markers (Sigmamarkers, low range). Gels were stained with Brilliant Blue R and destained in 10% acetic acid as described by Beeley et al. (1991).

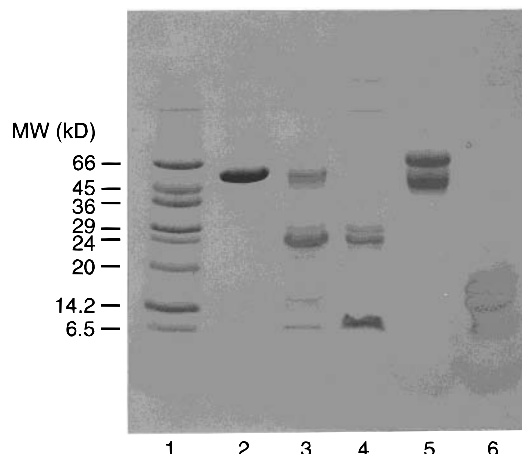

Figure 1. SDS−PAGE of salivary protein fractions: (lane 1) M_r markers [molecular masses (kDa) as marked]; (lane 2) α-amylase fraction; (lane 3) acidic I PRP fraction; (lane 4) acidic II PRP fraction; (lane 5) glycosylated PRP fraction; (lane 6) basic PRP fraction.

Synthesis of Epigallocatechin−Peroxidase Conjugate. Conjugate (HRP−EGC) was synthesized by linking horseradish peroxidase (HRP) via a spacer molecule, 1,4-butanediol diglycidyl ether, to epigallocatechin using the method of Lommen et al. (1995) with the modifications described by Bacon and Rhodes (1998). The HRP−EGC solution (final concentration = 10 mg/mL) was stored at −20 °C until required for use.

Tannin−Protein Binding Competition Assay. Competition assays were performed between HRP−EGC conjugate and hydrolyzable tannins as described previously (Bacon and Rhodes, 1998) on microtiter plates coated either with 0.1 μg/mL solutions of parotid saliva protein or PRP fractions or with coating buffer (0.05 M $NaHCO_3$, pH 9.6) as a blank. The plate development times were varied from 5 to 20 min according to the HRP activity. The HRP activity ($A_{450}\cdot min^{-1}$) values obtained, after the values obtained for the blank plate were subtracted, were plotted against the concentration of test tannin added. From these plots, a displacement constant ($A_{0.5}$) was calculated, equivalent to the molarity (micromolar) of test tannin that was required to displace 50% of the HRP−EGC conjugate from the protein coating on the microtiter plate relative to the control to which no tannin was added. This displacement constant is inversely proportional to the binding affinity of the test tannin to the protein coating.

RESULTS AND DISCUSSION

PRP Fractionation and Analysis. The fractionation procedure described above yielded four PRP fractions and an α-amylase fraction, with a total protein yield of 102 mg, equivalent to 1.7 mg of protein/mL of parotid saliva. This was resolved as follows: acidic I PRP, 22.5%; acidic II PRP, 10.9%; basic PRP, 16.3%; glycosylated PRP, 22.7%; α-amylase fraction, 28.5%. Kauffman and Keller (1979) obtained similar proportions in their study, but they obtained a higher yield of basic PRP (23%) and a lower yield of glycosylated protein (17%). Their saliva samples were, however, obtained from a single subject, and interindividual variation in isoform patterns between and within the different PRP classes is well documented (Beeley et al., 1991).

Figure 1 shows SDS−PAGE of the resolved parotid salivary protein fractions. As a result of their unusual amino acid compositions and their levels of glycosylation, salivary PRPs migrate relatively slowly on SDS−PAGE, and molecular masses calculated using this

Table 1. Amino Acid Analysis of PRP Fractions from Human Parotid Saliva

amino acid	acidic I, mol/100 mol	acidic II, mol/100 mol	glycosylated, mol/100 mol	basic, mol/100 mol
Asp/Asn	8.5 *(8–10)*[a]	6.5 *(8–10)*	5.1 *(5)*	8.6 *(5)*
Thr	0.4 *(0)*	1.7 *(0)*	0.5 *(0)*	0.0 *(0)*
Ser	4.3 *(4)*	4.9 *(4)*	6.4 *(4)*	6.5 *(4)*
Glu/Gln	24.0 *(27)*	25.9 *(27)*	18.8 *(19)*	19.2 *(16)*
Pro	25.9 *(23–27)*	22.1 *(23–27)*	33.3 *(37)*	32.4 *(41)*
Gly	18.5 *(19–21)*	16.3 *(19–21)*	19.8 *(23)*	18.0 *(22)*
Ala	2.0 *(1)*	1.4 *(1)*	1.0 *(1)*	2.5 *(0)*
Cys	0.0 *(0)*	0.0 *(0)*	0.0 *(0)*	0.0 *(0)*
Val	1.8 *(2)*	2.9 *(2)*	0.7 *(1)*	1.1 *(0)*
Met	0.0 *(0)*	0.0 *(0)*	0.0 *(0)*	0.0 *(0)*
Ile	1.2 *(1–1.5)*	1.7 *(1–1.5)*	0.5 *(0)*	1.1 *(0)*
Leu	2.8 *(2–3)*	4.1 *(2–3)*	1.0 *(0)*	2.2 *(0)*
Tyr[b]	0.0 *(0)*	0.0 *(0)*	0.0 *(0)*	0.0 *(0)*
Phe	0.9 *(1)*	3.2 *(1)*	0.5 *(0)*	0.0 *(0)*
His[c]	3.3 *(2)*	1.9 *(2)*	2.9 *(1)*	0.0 *(0)*
Lys	2.8 *(1–1.5)*	2.3 *(1–1.5)*	4.8 *(5)*	6.2 *(7)*
Arg	3.7 *(4)*	4.9 *(4)*	4.7 *(5)*	2.3 *(5)*

[a] Literature values for class (Bennick, 1982) are given in parentheses. [b] Tyr is recorded as zero for all fractions. The small amounts detected are likely to be glucosamine. [c] His may be overestimated due to other material coeluting.

technique are likely to be overestimates (Creighton, 1984). PRPs stain poorly with conventional staining procedures, but under the aqueous destaining conditions used, proteins rich in proline may be stained pink or purple (Beeley et al., 1991). The acidic I and acidic II PRP fractions both show protein bands apparently in common, most of which stain purple with Brilliant Blue R. The acidic I PRP fraction has an additional higher molecular mass band (stained purple) and the acidic II PRP fraction a low molecular mass band (stained blue). Thus, acidic II PRP fraction is likely to be contaminated with non-PRP components. The glycosylated PRP fraction shows two major, but slightly diffuse, purple bands of relatively high apparent molecular mass (45–60 kDa) and the basic PRP fraction, a series of lower molecular mass bands (10–20 kDa) which stain poorly but bright pink. The α-amylase fraction showed only one major band (stained dark blue) with an apparent molecular mass of 55 kDa and a similar relative migration to a commercial sample of human salivary α-amylase (not shown). Salivary α-amylase is a glycoprotein that may comprise several isoforms with molecular masses of 60–70 kDa.

Amino acid analysis of the PRP fractions is shown in Table 1. The acidic I and II PRP fractions have similar compositions, and the basic and glycosylated PRP fractions have substantially higher proline contents. The composition of the isolated fractions is similar to that of the examples published by Bennick (1982) with all fractions rich in proline, glutamic acid (glutamine), and glycine (total = 64–72 mol/100 mol). Aspartic acid (asparagine), serine, arginine, and lysine are present in all fractions with other amino acids being either absent or present only at relatively low levels, as is typical for salivary PRPs.

Relative Affinity of Hydrolyzable Tannins to Parotid Salivary Protein. The displacement constants ($A_{0.5}$ values) of a series of well-characterized hydrolyzable tannins to whole parotid saliva are shown in Table 2. These $A_{0.5}$ values, determined for each compound, are inversely related to their ability to displace HRP–EGC binding and hence their affinity for parotid salivary protein. Structures of the compounds tested are shown in Figures 2. All compounds tested,

Table 2. Displacement Constants ($A_{0.5}$) of Hydrolyzable Tannins to Human Parotid Saliva

	mo mass	$A_{0.5}$ (μM)
gallotannins		
Chinese tannin (hepta-, octagalloylglucose)	1321	0.43
tannic acid (mono- to octagalloylglucoses)	940.7	0.12
β-1,2,3,4,6-penta-*O*-galloyl-D-glucose	940.7	0.59
β-1,2,3,6-tetra-*O*-galloyl-D-glucose	788.6	0.33
β-1,3,6-tri-*O*-galloyl-D-glucose	636.5	1.22
aceritannin (β-2,6-di-*O*-galloyl-1,5-anhydro-D-glucitol)	468.4	≫20
taratannin (pentagalloyl quinate)	952.7	0.23
ellagitannins (HHDP esters)		
eugeniin (β-1,2,3-tri-*O*-galloyl-4,6-hexahydroxydiphenoyl-D-glucose)	938.7	0.67
casuarictin (β-1-*O*-galloyl-2,3:4,6-bishexahydroxydiphenoyl-D-glucose)	936.6	0.95
rugosin D (eugeniin dimer)	1875	0.77
sanguin H6 (casuarictin dimer)	1871	0.11
vescalagin	934.6	0.77
neochebulinic acid	956.7	1.08
gallic acid	170.1	23.1

with the exception of taratannin, are related biogenetically to the most widely distributed hydrolyzable tannin, β-1,2,3,4,6-penta-*O*-galloyl-D-glucose. Most of the compounds had high affinities for parotid salivary protein and gave $A_{0.5}$ values of at least the same order or lower than the most strongly binding galloylated condensed tannin monomers assayed by Bacon and Rhodes (1998). The displacement constants ($A_{0.5}$) obtained here for hydrolyzable tannins were, with one exception, in the range 0.11–1.22 μM. This contrasts with the relatively broad range of values obtained previously for condensed tannin monomers, for which the lowest value of $A_{0.5}$ obtained for parotid salivary protein was 0.54 μM with (−)-epigallocatechin gallate, and the highest values obtained were with nongalloylated compounds such as (+)-catechin, in which displacement, in the competition assay, of HRP–EGC was not sufficient even with the highest tannin concentration tested (350 μM) to measure a displacement constant. As almost all of the hydrolyzable tannins tested have low $A_{0.5}$ values for parotid salivary protein, differences between the different compounds are relatively small, although some trends in structure/binding affinity relationships within the data can be elicited as follows:

Galloylation. The more highly substituted galloyl esters of glucose tested (four gallate ester groups and greater) all had low displacement constants ($A_{0.5}$) for parotid salivary protein, but a correlation with the level of galloyl substitution was not observed. A commercial tannic acid, reported by the supplier to be essentially a mixture of mono- to octagalloylglucose, gave a lower $A_{0.5}$ value, indicating a higher affinity of binding to parotid salivary protein, but Chinese tannin (a mixture of hepta- and octagalloylglucose) gave an $A_{0.5}$ value indicative of an affinity of the same order as the tetra- and pentagalloyl derivatives. The galloylglucose with the lowest substitution, β-1,3,6-tri-*O*-galloyl-D-glucose, however, had a higher $A_{0.5}$ value, and aceritannin, with an anhydroglucitol core and only two galloyl groups, had the highest $A_{0.5}$ value, of the compounds tested, for parotid salivary protein, indicating that hydrolyzable tannins with a low degree of galloyl substitution tend to bind less well to parotid salivary protein. The displacement constant for gallic acid to parotid salivary protein was also relatively high, demonstrating that the affinity of all the gallate esters tested could not merely

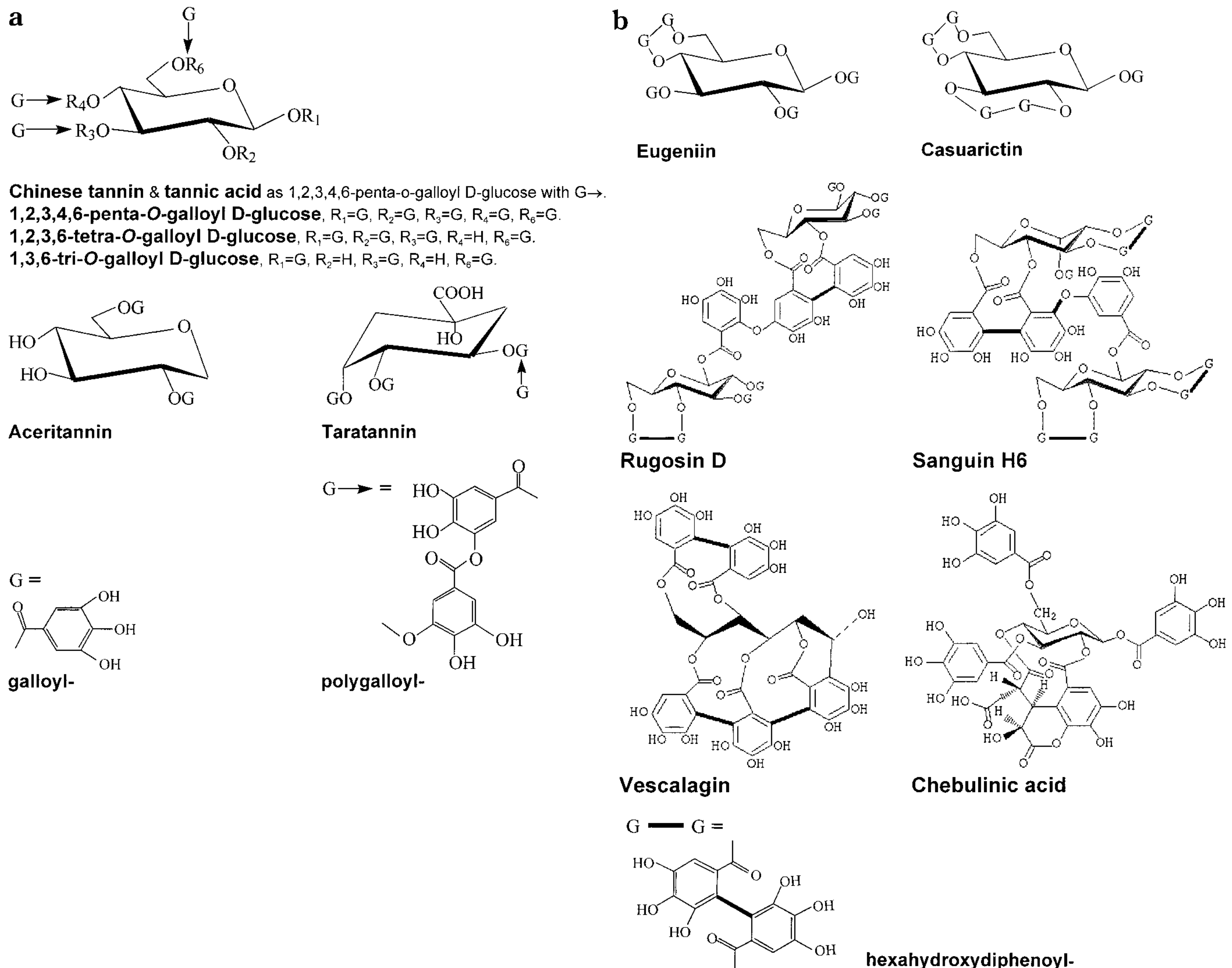

Figure 2. Structures of hydrolyzable tannins: (a) gallotannins; (b) ellagitannins (HHDP esters).

be attributed to gallic acid per se and was as a consequence of esterification in more complex molecules.

The protein binding relationships reported here are in general in agreement with those presented by other workers. Baxter et al. (1997), from dissociation constants determined from NMR measurements, concluded that larger and more complex polyphenols interacted more strongly with a PRP fragment and reported that pentagalloylglucose had a higher binding affinity than trigalloylglucose. It has been postulated (Haslam, 1998) that larger tannins have the ability to form multiple bonds with adjacent proline residues and also to associate and stack with other tannin molecules after binding to the protein, whereas simpler phenols may only have the opportunity to bind to a single proline residue. In addition, the conformations of the larger galloylated glucose molecules can be extremely flexible and poorly solvated in water, which encourages hydrophobic interactions. This property appears to reach a maximum, in terms of affinity to salivary protein, in relation to molecular size and level of galloylation with tetra- and pentagalloyl-substituted glucoses, and further polygalloylation has no additional effect. Although there are few studies of the binding of tannins to PRP, there are some studies relating to binding to other proteins. In studies of the inhibition of β-glucosidase by hydrolyzable tannins, Ozawa et al. (1987) demonstrated that the ranking of the relative binding to the enzyme was penta- > tetra- > trigalloylglucose. McManus et al. (1985) showed that level of galloylation and molecular size were important but that binding capacity with bovine serum albumin (BSA) was at an optimum with the pentagalloyl derivative and did not increase further with larger polygalloyl-substituted glucoses, such as in Chinese tannin. Kawamoto et al. (1995) determined, from the analysis of galloylglucose/BSA coprecipitates, that there was a positive relationship between degree of galloylation and protein affinity and that the level of affinity for esters with fewer than three galloyl groups was relatively low.

HHDP Esters. In ellagitannins, galloyl groups of galloyl esters of glucose are linked by C−C bonds following oxidative coupling to give HHDP esters. In a series of analogues, casuarictin (a bis-HHDP ester) has a higher displacement constant ($A_{0.5}$ value) for parotid salivary protein than eugeniin (a mono-HHDP ester), which has a higher $A_{0.5}$ value than their galloylated analogue, β-1,2,3,4,6-penta-*O*-galloyl-D-glucose, suggesting that HHDP esters bind less well to salivary proteins than galloyl esters. The dimeric forms of these HHDP esters, sanguin H6 and rugosin D, respectively, both bind strongly to parotid salivary proteins. Sanguin H6 has the lowest $A_{0.5}$ value for parotid salivary protein among the compounds tested, much lower than its

Table 3. Displacement Constants ($A_{0.5}$) of Hydrolyzable Tannins to PRP Fractions from Human Parotid Saliva

	$A_{0.5}$ (μM)			
	acidic I	acidic II	glycosylated	basic
sanguin H6 (casuarictin dimer)	0.18	0.13	0.11	0.36
tannic acid (mono- to octagalloylglucoses)	0.14	0.13	0.15	0.27
taratannin (pentagalloyl quinate)	0.26	0.18	0.20	1.12
β-1,2,3,6-tetra-*O*-galloyl-D-glucose	0.34	0.23	0.32	0.82
Chinese tannin (hepta-, octagalloylglucose)	0.54	0.27	0.66	0.91
β-1,2,3,4,6-penta-*O*-galloyl-D-glucose	0.31	0.40	0.56	0.98
eugeniin (β-1,2,3-tri-*O*-galloyl-4,6-hexahydroxydiphenoyl-D-glucose)	0.76	0.30	0.64	1.79
rugosin D (eugeniin dimer)	0.88	0.56	1.28	1.93
vescalagin	1.20	0.70	0.86	4.25
casuarictin (β-1-*O*-galloyl-2,3:4,6-bishexahydroxydiphenoyl-D-glucose)	1.00	0.51	0.81	2.30
neochebulinic acid	>10	3.66	1.00	>10
β-1,3,6-tri-*O*-galloyl-D-glucose	>15	8.01	1.15	>15
gallic acid	27.5	17.9	17.2	>60
aceritannin (β-2,6-di-*O*-galloyl-1,5-anhydro-D-glucitol)	>20	>20	8.2	>20

monomer, casuarictin, and also lower than all of the condensed tannin monomers tested previously (Bacon and Rhodes, 1998). Rugosin D, however, displayed a similar level of binding to its monomer, eugeniin. Other workers have demonstrated that dimerization of HHDP esters may affect their affinities with proteins. In equilibrium dialysis studies with BSA (McManus et al., 1985), rugosin D displayed the highest binding affinity of the compounds tested, higher than its monomer, eugeniin. Ozawa et al. (1987) in studies of the inhibition of β-glucosidase by hydrolyzable tannins, including a series of galloylglucoses as discussed above, showed that the HHDP dimers, rugosin D and sanguin H6, were the most inhibitory compounds, that is, had the highest binding affinity to β-glucosidase. The HHDP monomer, casuarictin, also had a lower binding affinity than its dimer and lower than β-1,2,3,4,6-penta-*O*-galloyl-D-glucose, presumably as a result of some loss of conformational flexibility.

Related Metabolites. Vescalagin is derived from a bis-HHDP glucose ester that has undergone pyranose ring opening and has an additional C−C bond to the galloyl group at C5 to give the most condensed and relatively inflexible structure of the hydrolyzable tannins. This compound has a displacement constant to parotid salivary protein only slightly greater than that of β-1,2,3,4,6-penta-*O*-galloyl-D-glucose (0.77 versus 0.59 μM). Vescalagin has been reported to have only relatively weak affinities for other proteins. For example, McManus et al. (1985), in equilibrium dialysis studies, demonstrated that vescalagin had an order of affinity to BSA similar to that of β-1,3,6-trigalloylglucose, and both bound weakly relative to β-1,2,3,4,6-penta-*O*-galloyl-D-glucose. In this study, however, vescalagin readily displaces HRP−EGC binding in the competition assay and indicates a relatively strong affinity to parotid salivary protein.

Neochebulinic acid, which has probably been formed via ring opening and subsequent aryl ring fission of a HHDP glucose ester, has a higher displacement constant to parotid salivary proteins than the HHDP esters from which it is derived. There are few published data on the binding of chebulinic acid to proteins with which to make any comparisons with these data. However, a relative astringency (RA) value for chebulinic acid, which has been correlated to its binding to hemoglobin, was quoted by Okuda et al. (1985) of the same order as those of vescalagin and 1,2,3,6-tetra-*O*-galloyl-D-glucose.

Polyol Core. The aliphatic polyol core of hydrolyzable tannins is usually D-glucose, although a small number of others (e.g., quinate, shikimate) have been identified. Taratannin is a complex pentagalloyl ester of quinate and is not homologous to the other tannins tested here in that it does not have a central glucose core. This compound had a very low displacement constant ($A_{0.5}$) for parotid salivary protein, lower than the galloylglucose esters tested, but it is difficult to draw any conclusions as to the absolute significance of the type of polyol core in relation to salivary protein binding as the taratannin galloyl ester substitution pattern is not directly comparable. It is clear, however, that galloylated derivatives of both types of core tested here have a high affinity to salivary proteins and that observed differences are more attributable to the nature of the attached ester ligands.

Relative Binding of Hydrolyzable Tannins to PRP Classes. The HRP−EGC conjugate used as a standard in the competitive tannin binding assay gave a high level of binding to all of the PRP fractions when coated on microtiter plates. In a previous paper (Bacon and Rhodes, 1998), we showed that, under similar conditions, HRP−EGC had a high affinity for parotid salivary protein and, to a lesser extent, to another PRP, gelatin, but had relatively little or no apparent affinity for a number of other proteins tested, including human salivary α-amylase. It is not possible to accurately quantify, in absolute terms, the binding to different proteins as the quantity of protein coated to microtiter plates in each case is not known, and even though the coating conditions for each PRP fraction were identical, it is possible that different PRP fractions may bind to microtiter plates to different extents. It is clear, however, that HRP−EGC binding to all of the PRP fractions is much higher than to most other proteins, indicating that they all have a high affinity to dietary tannin. The displacement constants ($A_{0.5}$ values) of the hydrolyzable tannins to PRP fractions are shown in Table 3. Compounds are ranked in order of their $A_{0.5}$ values to whole parotid salivary protein (as derived from the data in Table 2). All of the PRP fractions show an affinity for hydrolyzable tannins. For any given tannin, the $A_{0.5}$ value for the basic PRP fraction is higher than those for the other three PRP fractions. All of the other PRP fractions had similar $A_{0.5}$ values for a given tannin, with the acidic II fraction tending to have a lower $A_{0.5}$ value in the majority of cases. In general terms, the ranking of the displacement constants of the tannins to each of the PRP fractions is similar (Tables 2 and 3), with sanguin H6 and the commercial sample of tannic acid giving the lowest values with all of the PRP fractions and β-1,3,6-tri-*O*-galloyl-D-glucose, gallic acid, and aceritannin having the highest values. Although there are

some small differences in ranking of the displacement constants to the different PRP fractions to hydrolyzable tannins, these are generally not important, and none of the protein fractions appear to demonstrate a specificity for any particular tannin. The only observed anomaly was that the glycosylated PRP fraction had a relatively low $A_{0.5}$ value for neochebulinic acid and for β-1,3,6-tri-*O*-galloyl-D-glucose, although this does not have a significant effect on the overall ranking of the displacement constants of the compounds tested. There is some evidence that salivary PRP from different herbivorous and omnivorous mammals may have differing tannin binding specificities with PRP binding specifically to different types of tannin (Hagerman and Robbins, 1993). However, no such specificity of binding could be identified within the human parotid PRP classes for the hydrolyzable tannins in the present study, and the different human PRP classes cannot be assigned roles in binding specific tannins, but it is clear that all have a significant affinity for tannins. The classes of PRP in human saliva may have individual roles in terms of some of their other properties such as maintaining oral homeostasis (acidic PRP), lubrication, and bacterial attachment (glycosylated PRP) (McArthur et al., 1995). No specific role has yet been identified for the basic PRP, but they have been shown to be very effective in forming insoluble complexes with both condensed tannins and tannic acid relative to acidic and glycosylated forms (Lu and Bennick, 1998). Yan and Bennick (1995), however, demonstrated that the presence of a human salivary acidic PRP in an incubation mixture of tannic acid and salivary α-amylase was very effective in preventing the inhibition of α-amylase activity, suggesting that, even though the PRP is not precipitated, it may still have a relatively high affinity for tannins. Tannin−protein coprecipitation is a secondary step in a process following an initial complexation stage. Kawamoto and Nakatsubo (1997) reported that environment (pH, temperature, ionic strength) has major effects on the precipitation step but less influence on the initial complexation, and lack of precipitation may not be indicative of a lack of binding of a protein for tannins. Different protein−tannin complexes will also clearly have differing solubility characteristics. The competitive tannin binding assay used here is based on the comparison of tannin/PRP interaction per se and is not reliant on the measurement of secondary precipitation events; hence, it allows the direct comparison of tannin binding to different types of protein.

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