Tannin interactions with a full-length human salivary proline-rich protein display a stronger affinity than with single proline-rich repeats

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Abstract The protein IB5 has been purified from human parotid saliva. This protein contains several repeats of a short proline-rich sequence. Dissociation constants have been measured at several discrete binding sites using ^{1}H-NMR for the hydrolysable tannins (polyphenols) β-1,3,6-tri-*O*-galloyl-D-glucopyranose, β-1,2,4,6-tetra-*O*-galloyl-D-glucopyranose and β-1,2,3,4,6-penta-*O*-galloyl-D-glucopyranose and the condensed proanthocyanidin (−)-epicatechin. The dissociation constants for trigalloyl glucose and pentagalloyl glucose were 15×10^{-5} and 1.7×10^{-5} M, respectively, which are 115 and 1660 times stronger than those previously measured under the same conditions for a single repeat of a mouse salivary proline-rich protein. The increase in affinity is ascribed to intramolecular secondary interactions, which are strengthened by the rigidity of the interacting molecules.

Key words: ^{1}H-NMR; Tannin; Salivary proline-rich protein; Intramolecular binding; Hydrophobic interaction

1. Introduction

The salivary proline-rich proteins (PRPs) comprise about 70% of the protein content of saliva, and have been divided into glycosylated, acidic and basic classes [1,2]. It has been suggested that the main function of basic PRPs is to bind ingested plant polyphenols (tannins) [3]. Tannins have a range of harmful effects including the inhibition of digestive enzymes [4], and their complexation with salivary PRPs causes them to be precipitated and excreted in a harmless form. In a previous study [5], we have investigated the binding of a tannin to a single PRP repeat sequence and have shown that the binding is mainly a hydrophobic interaction between proline rings and the galloyl rings of the tannin. Here, we have repeated the binding investigation using β-1,3,6-tri-*O*-galloyl-D-glucopyranose (TriGG), β-1,2,4,6-tetra-*O*-galloyl-D-glucopyranose (TetraGG), β-1,2,3,4,6-penta-*O*-galloyl-D-glucopyranose (PentaGG) and (−)-epicatechin complexing with a full-length human salivary PRP, and the results indicate that the association observed is significantly stronger than with the single repeat sequence.

2. Materials and methods

2.1. Protein purification

Parotid saliva (~100 ml) was collected from a male volunteer (A.J.C.) following the procedure described by Steiner and Keller [6]. The subsequent purification was carried out as detailed by Levine and Keller [7] with a few modifications. EDTA was added to the saliva to a final concentration of 5 mM and the protein solution was saturated to 45% with ammonium sulfate. After centrifugation the supernatant was dialysed, lyophilised, dissolved in 0.05 mM Tris buffer at pH 8.5, and applied to a 35×2.5 cm DEAE Sephadex (Whatman) column. Fractions containing protein (assayed from the absorbance at 230 nm, using an extinction coefficient of 75 (mol peptide bond)$^{-1}$ l cm^{-1}), were pooled and lyophilised. The resulting protein was resuspended in 6 ml of 0.05 mM Tris buffer at pH 8.5 and applied to a 35×2.5 cm Sephadex G-150 (Sigma) column. The resulting eluant was pooled into three fractions and lyophilised, and the second fraction was applied to a 35×2.5 cm SP-Sephadex C-25 (Sigma) column equilibrated with 0.14 M sodium acetate/0.05 M NaCl at pH 3.6 and eluted with a gradient (2×400 ml, high salt buffer 0.14 M sodium acetate/0.3 M NaCl). The column was run at a flow rate of 1 ml min^{-1}, and 5 ml fractions were collected. Fractions were pooled and dialysed against deionised water. The fraction containing the desired protein was lyophilised and resuspended in 1 ml 12 mM PBS buffer at pH 7.4. Final purification was performed using a 30×1 cm Sephadex G-50 (Pharmacia) column, equilibrated with PBS at pH 7.4 with a flow rate of 1 ml min^{-1}. Eluant was collected in 2 ml fractions and the fractions containing pure protein were lyophilised and stored. SDS-PAGE experiments (16% acrylamide) were performed following each column chromatography stage to monitor the progress of the purification. The gels were silver stained using a Bio-Rad kit [8].

2.2. NMR spectroscopy

All experiments were recorded at 276 K using a Bruker AMX-500 spectrometer. Spectra were processed and displayed using the software package FELIX (Biosym Software) running on a Silicon Graphics workstation. One-and two-dimensional spectra were recorded on a 0.5 ml volume of the purified PRP (~0.2 mM) dissolved in H_2O/[$^2H_6$]dimethyl sulfoxide ([$^2H_6$]DMSO) (9:1) or ^2H_2O/[$^2H_6$]DMSO (9:1) in 12 mM PBS buffer at pH 7.4. For the investigation of the PRP/polyphenol interactions, 20 mM solutions of TriGG, TetraGG, PentaGG and (−)-epicatechin were prepared in the same solvent mixture at neutral pH. Since polyphenols do not readily dissolve in aqueous solvent, the solutions were left agitating for at least 24 h to ensure total solubility. The polyphenol solutions were titrated into 0.5 ml volumes of the purified PRP, using at least 5 additions before the onset of precipitation. Changes in chemical shift of the PRP resonances were followed by acquiring one-dimensional spectra after each addition of polyphenol solution. The two-dimensional total correlation spectroscopy (TOCSY) experiment was recorded and processed using similar parameters to those previously described [9]. Referencing of both the one-and two-dimensional spectra was achieved by using the residual dimethyl sulfoxide resonance as a secondary reference at 2.73 ppm from external 3-trimethylsilyl[2,2,3,3-$^2H_4$]propionate (TSP) [9].

2.3. K_d determination

The chemical shift data derived from the PRP/polyphenol titrations were used to determine the dissociation constant (K_d) and the maximum change in chemical shift ($\Delta\delta_{max}$), which is defined as the chemical shift change obtained upon 100% saturation of the binding site, by using a least-squares fitting technique to match the experimental chemical shift data with a theoretical curve generated by the equation:

$$\Delta\delta_i = \frac{\Delta\delta_{max}}{2}\left[\left(1+\frac{K_d}{[P]_i}+\frac{[T]_i}{[P]_i}\right)-\left\{\left(1+\frac{K_d}{[P]_i}+\frac{[T]_i}{[P]_i}\right)^2-4\frac{[T]_i}{[P]_i}\right\}^{1/2}\right] \quad (1)$$

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where $\Delta\delta_i$ is the change in chemical shift, $[P]_i$ is the total concentration of protein, $[T]_i$ is the total concentration of polyphenol, $[P]_o$ is the initial concentration of protein solution, $[T]_a$ is the concentration of polyphenol solution, V_i is the volume of polyphenol solution added, V_o is the initial volume of protein solution, and

$$[P]_i = \frac{[P]_o \cdot V_o}{V_o + V_i}$$

$$[T]_i = \frac{[T]_a \cdot V_i}{V_o + V_i}$$

This expression assumes a 1:1 binding interaction at each fitted proton, which has been justified elsewhere for PRP/polyphenol associations [5]. The curve fittings were performed using EXCEL (Microsoft Corp.).

3. Results

3.1. Characterisation of the isolated PRP

Column chromatography resulted in the isolation of a single basic PRP from parotid saliva. One-dimensional NMR spectra (Fig. 1) showed that a high degree of homogeneity had been attained, because they contain neither the resonances from aromatic and hydrophobic (Leu, Ile, Val) residues found in more typical proteins nor the sharp signals from low molecular weight impurities. In order to characterise the PRP further, a TOCSY experiment (Fig. 2) was acquired, and the crosspeaks were assigned to amino acid residue types using previously tabulated data [9,10]. Only eight amino acid types were identified as being present: proline, glycine, glutamine, asparagine, arginine, lysine, alanine and serine. Sequence-specific assignment was not possible due to very severe spectral overlap. The arginine CδH and lysine CεH diagonal peaks exhibited sufficient resolution for accurate volume measurements to be made. These showed a ratio of 1 arginine residue to 2.3 lysine residues. In addition, peak volumes suggested that the protein contained twice as many proline residues as glutamine residues. The proline residues could be classified into two groups, of roughly equal proportions, on the basis of their CαH chemical shift values. Similarly, the glycine residues were classified into two different groups. Based on previous work [9], these results suggest

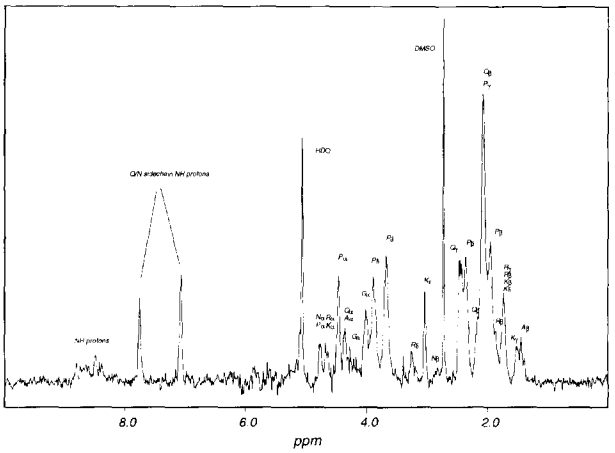

Fig. 1. One-dimensional ^{1}H-NMR spectrum of the purified basic PRP. Proton resonances have been labelled with the one-letter amino acid code and carbon atom position.

roughly equal proportions of Pro-X and Pro-Pro sequences, and the presence of both Gly-X and Gly-Pro sequences. Table 1 presents the chemical shift values for the protons of the amino acid residues identified in the purified PRP.

N-terminal sequence analysis (Applied Biosystems ABI 476A protein sequencer) of the purified PRP resulted in the sequence SPPGKPQGPPQQ..., and electrospray mass spectroscopy (Fisons/VG) gave a molecular weight of 6949.2±16.6 Da. Comparison with the primary structures of previously characterised salivary PRPs identified the purified protein as basic PRP IB5 (P-D) [11,12] (calculated mass 6949.7 Da), whose sequence is shown in Fig. 3. The residue composition and sequence of IB5 is in agreement with the NMR results listed above. The detection of alanine and the absence of aromatic residues in the spectra of the purified PRP also confirmed the identity of the protein.

3.2. PRP/polyphenol binding investigations

On titration of the tannin solutions into the purified PRP solutions, cloudy precipitates were formed during the NMR

Table 1
Proton assignments (ppm) for the amino acid residues present in the purified basic PRP

Amino acid	Proton				
	CαH	CβH	CγH	CδH	CεH
Proline^1	4.77	1.96	2.08	3.67	
		2.38		3.87	
Proline^2	4.46	1.96	2.07	3.67	
		2.35		3.87	
Glycine^1	3.99–4.01				
Glycine^2	4.02				
	4.24				
Glutamine	4.36	2.02	2.47		
		2.17			
Asparagine	4.74	2.76			
		2.86			
Arginine	4.67	1.78	1.75	3.26	
		1.86			
Lysine	4.67	1.73	1.51	1.74	3.04
		1.86			
Alanine	4.32	1.45			
Serine	4.44	3.91			

Chemical shift values were measured on a 0.2 mM sample dissolved in $H_2O/[^2H_6]$DMSO (9:1) at 276 K from external TSP in the same solvent. Superscripts for proline and glycine refer to the categories defined on the basis of their chemical shift values: Proline^1=Pro-Pro sequences, Proline^2=Pro-X sequences, Glycine^1=Gly-X sequences, Glycine^2=Gly-Pro sequences.

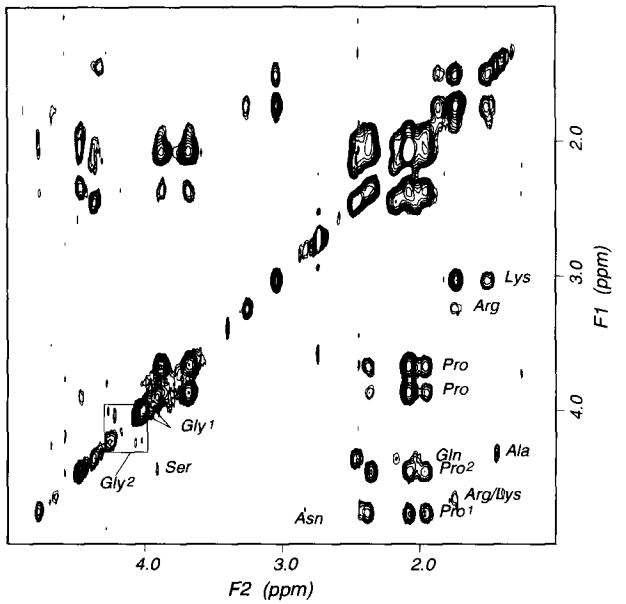

Fig. 2. The aliphatic region of a TOCSY spectrum of the purified PRP. Superscripts for proline and glycine refer to the categories defined on the basis of their CαH chemical shift values (Table 1).

studies which became denser on subsequent additions of the tannin. The precipitation was found to be reversible, the precipitate being solubilised by an increase in temperature. For the series of hydrolysable tannins, PentaGG, TetraGG, TriGG, the precipitate had become significant by 5, 6, and 9 μl of polyphenol solution, respectively, whereas for (−)-epicatechin, a precipitate began to form after 59 μl of tannin solution. During the later stages of the PRP/tannin titrations, broadening of the PRP signals was noted, which is consistent with exchange broadening between free and bound chemical shifts. These results imply strong PRP/tannin binding, with PentaGG being the most effective ligand.

Least-squares fitting of Eq. 1 to the chemical shift changes observed on titration of the protein with the polyphenols gave the results listed in Table 2. The quality of the fitting is illustrated by Fig. 4.

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S P P G K P Q G P P Q Q E G N
      K P Q G P P
  P P G K P Q G P P P P G G N
    P Q Q P Q A P P A
      G K P Q G P P P P
P Q G G R P P R P A Q G Q Q P P Q
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Fig. 3. The amino acid sequence of basic PRP IB5 (P-D) [11,12] aligned to highlight the internal repeat sequences.

4. Discussion

The upfield changes in chemical shift seen on titration of the protein with tannins can be ascribed to ring current shifts, caused largely by face-to-face stacking of the prolyl rings of the protein with the galloyl rings of the tannins [5]. Each proton within the protein experiences different upfield shifts due to local tannin binding, and therefore the curve fitting procedure essentially yields individual microscopic dissociation constants for each site in the protein. As shown in Table 2, for each tannin studied all of the individual dissociation constants were similar suggesting that the binding interactions are of similar strengths. Since almost all of the major chemical shift changes observed involve prolyl protons, the implication is that each proline residue forms an independent and equivalent binding site. Similar conclusions were reached in our earlier investigation [5]. We have therefore averaged the individual dissociation constants to obtain a mean constant for each polyphenol studied.

In our previous study [5] we investigated the binding of PentaGG to a single 22-residue repeat of the tandemly repeated mouse MP5 PRP sequence. This peptide has the sequence GPQQRPPQPGNQQGPPPQGGPQ, which is similar to that of the currently studied IB5, but roughly one third of the length. Since we have shown that the tannin binding sites on both proteins almost entirely consist of independent proline residues, we are confident that the essential elements of binding are common to both proteins. More recently, we have studied the binding of TriGG, (−)-epicatechin and other tannins to the mouse MP5 repeat sequence (Baxter et al., in

Table 2
The dissociation constants (K_d) calculated using Eq. 1 for the interaction of the polyphenols with basic PRP IB5

Proton	K_d (M)			
	PentaGG	TetraGG	TriGG	(−)-Epicatechin
Proline[1] CαH	1.85×10^{-5}	*	1.02×10^{-4}	*
Proline[2] CαH	*	1.56×10^{-4}	*	1.51×10^{-4}
Proline CδH[a]	8.65×10^{-6}	*	1.40×10^{-4}	1.14×10^{-4}
Proline CδH[b]	1.55×10^{-5}	*	*	1.37×10^{-4}
Arginine CδH	4.00×10^{-5}	*	1.89×10^{-4}	1.24×10^{-4}
Lysine CεH	*	6.51×10^{-5}	2.38×10^{-4}	*
Proline CβH[c]	1.01×10^{-5}	1.92×10^{-4}	9.97×10^{-5}	1.16×10^{-4}
Proline CγH	1.11×10^{-5}	3.54×10^{-5}	*	1.23×10^{-4}
Average	1.73×10^{-5}	1.12×10^{-4}	1.54×10^{-4}	1.28×10^{-4}
Standard deviation	1.17×10^{-5}	7.39×10^{-5}	5.94×10^{-5}	1.41×10^{-5}

The initial protein concentration $[P]_o$ was treated as a variable during the curve fitting. Proline[1] and Proline[2] correspond to Pro-Pro and Pro-X sequences, respectively (Table 1) and * denotes either that the chemical shift values did not change for the respective proton resonance or that the data obtained were not suitable for fitting against the theoretical curve.
[a]Downfield Proline CδH.
[b]Upfield Proline CδH.
[c]Downfield Proline CβH.

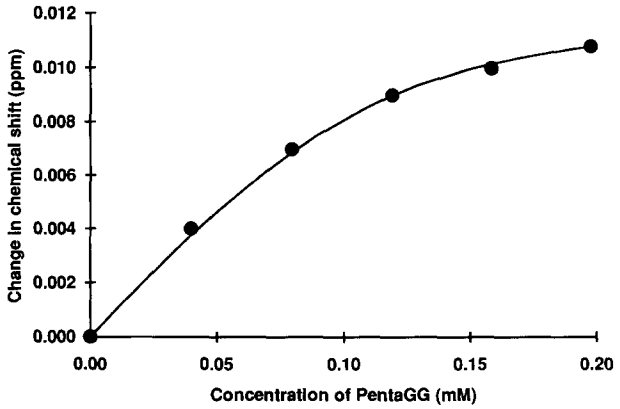

Fig. 4. Observed (●) and fitted (—) chemical shift differences ($\delta_{free}-\delta_{bound}$) of the downfield CβH resonance of proline for the PRP/PentaGG titration experiment with increasing concentration of PentaGG.

preparation). In this work it was shown that under the conditions of the present study the dissociation constants for TriGG and PentaGG with the single repeat were 17.8 and 28.7 mM, respectively. The onset of precipitation was consistent with these figures, in that TriGG resulted in precipitation of the peptide at a lower concentration than when PentaGG was used. However, the dissociation constants calculated in the present work indicate that the interactions are significantly stronger, 115-fold for TriGG and 1660-fold for PentaGG, with IB5/PentaGG precipitating at a lower concentration of tannin than IB5/TriGG.

In understanding these results it is important to note that the dissociation constants are derived from individual proton binding sites. Therefore, the explanation cannot be merely that the longer protein contains more binding sites, since this would produce an identical result for the dissociation constant. The most likely explanation for the much stronger binding observed with the longer protein is that the increased length of the protein allows it to fold and 'wrap around' the tannin, thereby increasing the association by cooperative intramolecular interactions [13]. This observation provides a function for the multiple tandem repeats seen in salivary PRPs.

From a consideration of the energetics of the system, it is clear that the strongest overall interaction will result when the peptide and tannin are relatively rigid, and therefore little internal rotational entropy is lost on forming secondary interactions [14]. Proline-rich proteins are particularly suitable in this respect, as the proline-rich sequences provide rigid regions favorable for effective tannin binding, which are linked by other residues that probably act as flexible hinges. These non-proline residues enable the protein to wrap around and make multiple contacts with bound polyphenol molecules. It is also significant that a much greater increase in affinity was seen for PentaGG than for TriGG, since PentaGG is much more sterically restricted than TriGG, and is therefore better able to form secondary binding interactions.

In conclusion, we have shown that full-length human salivary PRPs precipitate with tannins much more readily than the single shorter repeats studied up until now. This is probably due to favorable intramolecular binding to secondary galloyl binding sites on the tannins. This study emphasises the importance of multiple weak interactions in forming multimolecular complexes.

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