Identification of histatins as tannin-binding proteins in human saliva

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Tannins have a number of detrimental biological effects and these include interference with normal growth and metabolism if they are present in the feed of various animals. Proline-rich proteins (PRPs) in saliva have been shown to provide protection against tannin, but little is known about the mechanism of protection and interaction of other salivary proteins with tannin. To identify tannin-binding human salivary proteins, parotid and submandibular/sublingual saliva samples were adsorbed with tannin. PRPs, and in particular a group of low- M_r proteins, were readily precipitated by tannin. The low- M_r proteins were purified from parotid saliva and demonstrated to be histatins, a family of well-characterized histidine-rich salivary proteins. The ability of synthetic histatin 5, as well as an acidic PRP (PRP-1) and gelatin to precipitate quebracho condensed tannin and tannic acid was

determined. At pH 7.4 histatin 5 was the most effective precipitant of both condensed tannin and tannic acid and it also precipitated the largest amount of condensed tannin at pH 3.0, but the smallest amount of tannic acid at that pH. In contrast PRP-1 showed a greater ability to precipitate both condensed tannin and tannic acid at pH 3.0 than at pH 7.4. Under most circumstances histatin 5 was therefore more effective in precipitating tannins than proteins with high proline content which generally have been recognized as strong precipitants of tannin. Pre-incubation of tannic acid with α -amylase inhibited the enzyme, but addition of histatin 5 or the acidic PRP PIF-s protected amylase from inhibition by tannin. Similarly salivary proteins may protect other biological activities in the digestive tract from inhibition by dietary tannin.

INTRODUCTION

Tannins are plant-derived polyphenolic compounds which are widely found in foods, particularly fruits and beverages [1,2]. On the basis of their chemical structures, tannins are usually divided into two groups: hydrolysable and condensed tannins [3]. A characteristic property of tannins is their great ability to precipitate protein from aqueous solution [2], and proteins with a high proline content such as gelatin are particularly effective tannin precipitants [4,5]. Studies on animals have demonstrated a number of detrimental biological effects of tannins. These include decrease in both growth rate and body weight gain [6], perturbation of mineral absorption [7,8] and inhibition of digestive enzymes [9–11]. Condensed tannin has also been linked to pulmonary inflammation of cotton-mill and grain-elevator workers [12–14]. Furthermore, systemic toxic effects, including liver necrosis, and fatalities due to tannin absorbed from tannin-damaged mucosa have been reported [7,15,16].

Saliva from many species, including human, monkey, rat, mouse and rabbit, contains a family of so-called proline-rich proteins (PRPs) [6,17–20]. Small amounts of PRPs are also present in human tracheobronchial secretions [21]. They account for approximately 70% of total proteins secreted in human parotid saliva [22], but only small amounts of PRPs are present in the saliva of rats and mice fed a normal laboratory diet [23,24]. Feeding rats and mice high-tannin sorghum leads to an initial weight loss, but within 3 days there is a marked stimulation of PRP synthesis and at the same time weight gain is initiated [2,24–26]. Hamsters also show a weight loss when placed on a diet high in tannin, but in contrast to rats and mice, there is no induction of PRP synthesis, and growth inhibition is observed as long as the animals are kept on this diet [6]. Inclusion of 4% tannin in the diet has no effect on rats and mice, but is fatal to hamsters, with most animals dying within 3 days [2]. The PRPs induced in rats and mice have high affinity for tannin [5,6,27] and the saliva of other animals that naturally consume a diet rich in tannin also contains tannin-binding proteins which are rich in

proline [28–30]. Thus, salivary PRPs have been suggested to play an important role as a first-line defence against dietary tannins [2].

Humans consume a wide range of tannin-containing foods [2,31]. A daily intake of 1.5–2.5 g of dietary tannin in different regions of India has been reported [32]. In the U.S.A., the daily consumption of flavanoids, a broad group of plant-derived natural flavouring components that includes tannin, has been estimated to be 1 g [1]. It seems likely that human salivary PRPs may complex tannins and thereby minimize their detrimental biological effects; however, there have been no studies on the interaction of tannin with human salivary proteins. We have therefore initiated a study in order to characterize the interaction between human salivary proteins and tannin and to evaluate the biological effect of this interaction. In the course of this study, it was found that in addition to PRPs, human saliva contained a group of low- M_r proteins with even greater ability to precipitate tannin than PRPs. These proteins were subsequently shown to be histatins, a group of unique and well-characterized salivary proteins [33]. Since the small size and composition of these proteins were in sharp contrast to proteins that so far have been shown to bind to tannin effectively, they were further characterized and their abilities to precipitate tannin and protect salivary α -amylase from tannin-inhibition were also assessed.

MATERIALS AND METHODS

Materials

Commercial tannic acid and soluble starch were purchased from Fisher Scientific Company, Fair Lawn, NJ, U.S.A. and Sephadex-LH 20, as well as Sephadex-G25, from Pharmacia LKB Biotechnology AB, Uppsala, Sweden. Salivary α -amylase was purchased from Sigma Chemical Company, St. Louis, MO, U.S.A. and gelatin from Anachemia Science, Montreal, Canada. Human acidic PRPs, PRP-1 and PIF-s, were purified as described previously [34]. Standards of various PRPs were purified as

Abbreviation used: PRP, proline-rich protein.

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Figure 1 SDS/15%-PAGE of parotid and submandibular/sublingual proteins precipitated by tannic acid and condensed tannin

Saliva samples (250 μ l) were mixed with 5 μ g (lane 1), 10 μ g (lane 2), 50 μ g (lane 3), 100 μ g (lane 4), 200 μ g (lane 5), 300 μ g (lane 6), 400 μ g (lane 7), and 0 μ g (lane 8) of tannin and incubated at 37 $^{\circ}$ C for 1 h. After centrifugation, the supernatant was removed and the precipitate was dissolved in 100 μ l of SDS sample loading buffer, out of which 5 μ l was used for SDS/PAGE. Lane 0 shows the pattern of parotid salivary proteins (a and b) and submandibular/sublingual salivary proteins (c and d) that had not been exposed to tannin. (a) Parotid proteins precipitated by tannic acid; (b) parotid proteins precipitated by condensed tannin; (c) submandibular/sublingual proteins precipitated by tannic acid; (d) submandibular/sublingual proteins precipitated by condensed tannin. Pa, PRP-2 and PRP-4 are acidic proline-rich proteins; IB-8a and DEAEII-2 are basic proline-rich proteins. 'Low- M_r ' indicates proteins subsequently shown to include histatins. 'X' and 'Y' were not identified. The triangles point to proteins indicated to the right of the illustration.

described [35] and synthetic histatin 4 and 6 were supplied by Dr. J. Pollock, SUNY, Stony Brook, NY, U.S.A. Crude quebracho tannin and standards of various forms of galloyl-glucose were gifts from Dr. A. E. Hagerman, University of Miami, Miami, OH, U.S.A. Parotid and submandibular/sublingual saliva were collected as previously described [17,36]. Small volumes of saliva were collected for individual tannin adsorption experiments from the authors (Q. Y. and A. B.) and, for the purpose of protein purification, 100 ml of parotid saliva was collected at a time from Q. Y. Quebracho condensed tannin and tannic acid were purified on a column of Sephadex LH-20 [37,38]. Purified tannic acid was

analysed by HPLC on a μ Porasil column (Waters Associates, Milford, MA, U.S.A.) [39]. SDS/PAGE on 15% gels was done according to Laemmli [40] and cationic-PAGE was performed at pH 2.75 as described by MacKay et al. [41] and modified by Oppenheim et al. [33].

Adsorption of human saliva samples with tannin

Purified condensed tannin and tannic acid were dissolved in 50% methanol at a concentration of 1 mg/ml. Volumes of tannin solution varying from 5 to 400 μ l were added to microcentrifuge tubes and dried on a rotary evaporator. To each tube was then added 250 μ l of freshly collected saliva and the tubes were incubated at 37 $^{\circ}$ C for 1 h. After centrifugation at 15000 g for 15 min at room temperature, the supernatant was transferred into another tube and the pellet was washed three times with 250 μ l of water and centrifuged at 15000 g for 5 min at room temperature. The pellet was dried on a rotary evaporator and 100 μ l of SDS sample loading buffer was added to dissolve the tannin-protein precipitate, out of which 5 μ l was subjected to SDS/PAGE.

Protein purification

Freeze-dried parotid saliva (100 ml) was dissolved in 10 ml of 0.022 M ammonium bicarbonate, pH 7.8, and applied to a Sephadex G-25 column (425 ml bed volume) which was developed with the same buffer at a flow rate of 0.5 ml/min. The eluate was monitored for A_{220} and 5 ml fractions were collected. Pooled fractions containing the low- M_r proteins were subjected to reversed-phase chromatography on a Pharmacia PeP RPC HR 5/5 C_{18} column (1 ml bed volume) using a Pharmacia fast protein liquid chromatography system. Solvent A was 0.1% trifluoroacetic acid in water and solvent B was 0.1% trifluoroacetic acid in acetonitrile. The sample was dissolved and the column was equilibrated in solvent A. The column was then developed with a gradient of solvent A and B at a flow rate of 0.7 ml/min. The 50 min gradient in respect to buffer B was as follows: 0–5 min, 0%; 5–30 min, 0–40%; 30–35 min, 40–100%; 40–45 min, 100%; 45–50 min, 0%. Three major peaks, named prot #1, #2 and #3 were resolved and collected. Subsequent analyses demonstrated that proteins #1 and #2 were in fact histatin 5 and 3 respectively. Cationic PAGE profile of the proteins showed that each contained a minor component. Prot #3 was not further purified and analysed in the study. Prot #1 and #2 were further purified on the C_{18} column under isocratic conditions of 15% solvent B and subjected to protein analyses.

Protein analyses

Protein samples were hydrolysed under vacuum in 6 M HCl containing 1% phenol at 110 $^{\circ}$ C for 24 h. Amino acid analyses were performed on a Waters PICO-TAG system. For N-terminal analysis, automated Edman degradation of the protein was done on a Porton gas-phase microsequencer using an on-line phenylthiohydantoin (PTH) program. Mass spectroscopic analyses of protein samples were performed on a PE SCIEX API-III mass spectrometer using electrospray ionization.

Peptide synthesis

Histatin 5 (Asp-Ser-His-Ala-Lys-Arg-His-His-Gly-Tyr-Lys-Arg-Lys-Phe-His-Glu-Lys-His-His-Ser-His-Arg-Gly-Tyr) was custom-synthesized using solid-phase strategy on a Novasyn Crystal peptide synthesizer (Nova Biochem Ltd., Nottingham, U.K.) and purified on a Waters μ -Bondapak C_{18} column (Waters Associates, Milford, MA, U.S.A.).

Tannin-binding assay

Purified tannic acid and condensed tannin were dissolved at a concentration of 4 mg/ml in an isotonic barbital buffer, VBS (pH 7.4), or VBS that had been adjusted to pH 3.0. Freshly prepared tannin solutions were used owing to the sensitivity of tannin to oxidation. A volume of 25 μ l of synthetic histatin 5 or gelatin or human salivary acidic PRP PRP-1 (1 mg/ml of water) was added to volumes of tannin solution ranging from 2.5 to 200 μ l. The total volume of each tube was brought up to 300 μ l and the mixture was incubated at 37 °C for 1 h followed by centrifugation at 15000 *g* for 15 min at room temperature. The supernatant was removed and the pellet in each tube was washed twice by adding 300 μ l of VBS and centrifuging at 15000 *g* for 5 min at room temperature. The amount of tannin in the pellet was determined as described [42]. A volume of 600 μ l of 0.1% SDS in 5% (v/v) triethanolamine was added to dissolve the pellet. Subsequently, 100 μ l of the colour reagent (0.1 M FeCl₃ in 0.01 M HCl) was added and the A_{510} value was measured.

Assay for human salivary α -amylase

A solution of 0.01 μ g/ μ l α -amylase was prepared in 1 mM CaCl₂. To 25 μ l of amylase was added 0–50 μ l of 0.005 μ g/ μ l tannic acid dissolved in 0.02 M sodium phosphate, pH 7.0, containing 0.15 M NaCl (PBS). The total volume was brought up to 100 μ l by adding the same buffer and the mixture was incubated for 15 min at 37 °C. An aliquot of 16 μ l was assayed for α -amylase activity as described [43]. The experiment was repeated by mixing 25 μ l of PIF-s or synthetic histatin 5 (0.01 μ g/ μ l in PBS) with the α -amylase solution before addition of the tannin solution.

RESULTS

HPLC of purified tannic acid showed that it consisted mainly of pentagalloyl glucose, hexagalloyl glucose and heptagalloyl glucose with smaller amounts of tetra- and octa-galloyl glucose present. These components were identified by comparing their elution position with those of standards of galloyl esters. Because

Figure 2 Chromatography of human parotid saliva on a Sephadex G-25 gel filtration column

Dialysed and freeze-dried human parotid saliva (containing about 200 mg of protein) was dissolved in 10 ml of 0.02 M ammonium bicarbonate buffer (pH 8.0) and applied to a Sephadex G-25 column. The column was developed with the same buffer at a flow rate of 0.4 ml/min and 5 ml fractions were collected. (a) Elution profile. (b) SDS/PAGE on a 15% gel of selected fractions from the gel filtration column. Fractions 47 to 72 containing the low- M_r proteins were pooled, freeze-dried and subjected to reversed-phase chromatography.

Figure 3 Fractionation of the low- M_r proteins on a reversed-phase C₁₈ column

Freeze-dried low- M_r proteins (50 μ g) contained in the pooled fractions from the Sephadex G-25 column were dissolved in 100 μ l of water containing 0.1% trifluoroacetic acid (TFA) (pH 2.3) and applied to a C₁₈ column (1 ml bed volume). The column was developed with an acetonitrile (ACN) gradient containing 0.1% TFA at a flow rate of 0.7 ml/min. (a) Elution profile. (b) Cationic PAGE of prot #1 (lane 1), prot #2 (lane 2), and synthetic histatins 4, 5 and 6 from top to bottom (lane 3).

Table 1 Amino acid composition of purified prot #1 and prot #2

Results are from one amino acid analysis.

Amino acid	Residues per 100 residues			
	Prot #1	Histatin 5	Prot #2	Histatin 3
Asp	4.2	4.2†	13.4	12.5†
Glu	4.4	4.4	5.4	3.1
Ser	7.1	7.5	10.1	9.4
Gly	9.3	9.0	9.6	6.3
His	27.2	29.4	20.9	21.9
Arg	13.9	13.0	13.4	12.5
Ala	4.1	4.1	3.5	3.1
Tyr	7.1	7.9	4.3	12.5
Leu	0.0	0.0	3.9	3.1
Phe	4.3	4.1	3.0	3.1
Lys	16.2	16.0	10.1	12.5

† Calculated from the amino acid sequences of histatin 5 and histatin 3 as determined by Oppenheim et al. [33].

of the structural complexity of quebracho condensed tannin, no analysis of the purified material was performed.

Adsorption of tannin to human saliva

Upon incubation of tannic acid with parotid saliva, a group of low- M_r proteins running at the dye front in the gel were predominantly precipitated, especially when small amounts of tannin were present (Figure 1a, lanes 1 and 2). When parotid saliva was incubated with larger amounts of tannic acid, acidic PRPs Pa, PRP-2 and basic PRP IB-8a were also precipitated (Figure 1a, lanes 1–7). Incubation of parotid saliva with condensed tannin precipitated predominantly the low- M_r proteins (Figure 1b, lanes 1–7). Small amounts of acidic PRP Pa were precipitated when 50 μ g and 100 μ g of tannic acid were present. Incubation of tannic acid with submandibular/sublingual saliva resulted in precipitation of the low- M_r proteins, acidic PRP PRP-

Figure 4 Tannin-binding assays of histatin 5, PRP-1 and gelatin

Samples (25 μg) of histatin 5, PRP-1 or gelatin were incubated with various amounts of tannin at 37 °C for 1 h. The supernatant was removed from the pellet after centrifugation and the amount of tannin in the pellet was determined by a colorimetric assay. Points shown are values of three individual assays and lines drawn are averages of the three values. (a) Condensed tannin, pH 7.4; (b) condensed tannin, pH 3.0; (c) tannic acid, pH 7.4; (d) tannic acid, pH 3.0.

2, and a protein (labelled 'Y') which was not identified (Figure 1c, lanes 1–7). Incubation of condensed tannin with submandibular/sublingual saliva caused precipitation predominately of the group of low- M_r proteins (Figure 1d, lanes 1–7). Small amounts of acidic PRP PRP-2 and the 'Y' protein were also precipitated. The results shown were obtained with saliva from Q.Y. Identical results were obtained with saliva from A.B., taking into account the differences in the PRP phenotypes of Q.Y. and A.B.

Purification and characterization of the low- M_r proteins

Chromatography of human parotid saliva on a Sephadex G-25 column resulted in one broad peak and the low- M_r proteins eluted in fractions 46 to 72 (Figure 2). Fractionation of pooled fractions containing the low- M_r proteins on a C_{18} column resulted in three major peaks named prot #1, #2 and #3 (Figure 3). Each protein contained a minor component as revealed by cationic

PAGE (result not shown) and prot #1 and prot #2 were further purified on the C_{18} column under isocratic conditions. Prot #3 was not further purified and analysed in the study.

Protein analyses

Amino acid compositions of purified prot #1 and #2 are presented in Table 1. The most prevalent residue in both proteins was histidine, followed by lysine and arginine. These three residues accounted for 57% and 44% of all amino acids of prot #1 and prot #2 respectively. Both proteins lacked proline, threonine, valine, cysteine, methionine and isoleucine and prot #1 also lacked leucine. There was a high degree of similarity between the amino acid compositions of prot #1 and histatin 5, as well as prot #2 and histatin 3. Prot #2 had a much lower level of tyrosine than histatin 3 and this is probably due to the oxidation of tyrosine during hydrolysis. Amino acid sequencing of prot #1 detected only one major amino acid sequence which was identical

to the N-terminal sequence of histatin 5. Mass spectrometry analysis of prot #1 detected one major compound with an M_r of 3035.99, which was within one mass unit of that of histatin 5 (mass 3037). Mass spectrometry of prot #2 also detected one major compound with an M_r of 4062.97, which was within one mass unit of that of histatin 3 (mass 4063). The purified proteins also had the same electrophoretic mobility as histatin 3 and histatin 5 on cationic PAGE (results not shown).

Examination of synthetic histatin 5

The synthetic histatin 5 was identical to native histatin 5 as shown by mass spectrometry and amino acid analysis. CD spectra of native and synthetic histatin 5 were in good agreement and both were characteristic of a random coil structure.

Tannin-binding assay

From Figure 4 it is apparent that addition of increasing amounts of tannin to the proteins caused increased precipitation of tannin-protein complexes. However, no maximum plateau of tannin precipitation was achieved by any of the proteins examined under the assay conditions. At pH 7.4 using condensed tannin (Figure 4a), histatin 5 exhibited the highest capacity among the three proteins to bind condensed tannin, while PRP-1 bound only a little condensed tannin over the range of tannin used. At a condensed tannin concentration of $2.7 \mu\text{g}/\mu\text{l}$, $1 \mu\text{g}$ of histatin 5 precipitated approximately $9.2 \mu\text{g}$ of condensed tannin which is about $3.2 \mu\text{g}$ higher than that precipitated by $1 \mu\text{g}$ of gelatin. Under the same conditions, however, $1 \mu\text{g}$ of PRP-1 precipitated only $1 \mu\text{g}$ of condensed tannin. No condensed tannin-precipitation by PRP-1 was seen in solutions containing less than $0.67 \mu\text{g}$ of condensed tannin/ μl . The condensed tannin-precipitation patterns for gelatin and histatin 5 obtained at pH 3.0 (Figure 4b) are similar to those obtained at pH 7.4 (Figure 4a). At pH 3.0, the amount of condensed tannin precipitated by PRP-1 increases and is comparable with that precipitated by gelatin. Both PRP-1 and gelatin precipitated noticeably lower amounts of condensed tannin than histatin 5 when the concentration of condensed tannin was higher than $0.67 \mu\text{g}/\mu\text{l}$. However, when the concentration of condensed tannin was lower than $0.67 \mu\text{g}/\mu\text{l}$, histatin 5 precipitated slightly smaller amounts of condensed tannin than PRP-1 and gelatin.

The binding assay was repeated with tannic acid and slightly different tannin-binding curves were obtained for each protein. At pH 7.4, histatin 5 had the highest capacity to precipitate tannic acid over the range of tannic acid used in the assay (Figure 4c). At a tannic acid concentration of $2.6 \mu\text{g}/\mu\text{l}$, $1 \mu\text{g}$ of histatin 5 precipitated about $5.6 \mu\text{g}$ of tannic acid, but $1 \mu\text{g}$ of gelatin only precipitated about $2.4 \mu\text{g}$ of tannic acid. Both histatin 5 and gelatin precipitated smaller amounts of tannic acid than condensed tannin under the same conditions (Figures 4a–4d). Unlike the result with condensed tannin (Figure 4a), PRP-1 precipitated only slightly smaller amounts of tannic acid than gelatin when the concentration of tannic acid varied from $1.3 \mu\text{g}/\mu\text{l}$ to $2.7 \mu\text{g}/\mu\text{l}$. However, no apparent tannic acid precipitation by PRP-1 was seen when the concentration of tannic acid was lower than $0.67 \mu\text{g}/\mu\text{l}$. At pH 3.0 histatin 5 precipitated much less tannic acid than gelatin and PRP-1 (Figure 4d). Gelatin precipitated the largest amount of tannic acid and PRP-1 only slightly less. There was no precipitation of tannic acid by histatin 5 when the concentration of tannic acid was lower than $1.3 \mu\text{g}/\mu\text{l}$. This is in striking contrast to the extensive precipitation of condensed tannin by histatin 5 at pH 3.0 (Figures 4b and 4d).

Figure 5 Inhibition of amylase by tannic acid and the effect of other salivary proteins

Salivary α -amylase ($0.25 \mu\text{g}$) was preincubated with various amounts of tannic acid in the presence or absence of histatin 5 or PIF-s ($0.25 \mu\text{g}$) at 37°C for 15 min. The incubation mixture was assayed for amylase activity as described in the text. $\square$, Salivary protein absent; $\blacklozenge$, histatin 5 present; $\circ$, PIF-s present. Points shown are averages of three individual assays.

Inhibition of α -amylase by tannic acid and protection from inhibition by histatin 5 or PIF-s

α -Amylase activity was considered to be 100% in the absence of tannin in the preincubation mixture. Preincubation of $0.25 \mu\text{g}$ of α -amylase with various amounts of tannic acid inhibited the enzyme activity in a dose-dependent manner (Figure 5). The presence of $0.5 \mu\text{g}$ of tannic acid caused 75% inhibition of α -amylase and the presence of $1 \mu\text{g}$ of tannic acid completely inactivated the enzyme. Preincubation of $0.25 \mu\text{g}$ of amylase and $0.25 \mu\text{g}$ of histatin 5 or PIF-s with $0.5 \mu\text{g}$ of tannic acid resulted in only 20% inhibition of α -amylase activity; preincubation of $1 \mu\text{g}$ of tannic acid with the same amounts of amylase and histatin 5 or PIF-s caused only 85% inhibition of α -amylase. The presence of histatin 5 or PIF-s therefore prevented inactivation of α -amylase by tannic acid.

DISCUSSION

Gel electrophoresis provides a simple means for surveying tannin-binding proteins in human saliva, because the proteins dissociate from the insoluble tannin-protein complexes in the presence of SDS and can be visualized and identified in the gel. Saliva samples were used without purification or concentration in this study so as to investigate tannin-binding to the mixture of salivary proteins as they are present in saliva. Because of the limited solubility of tannin it was not possible to assay tannin binding to protein at tannin concentrations higher than $2.7 \mu\text{g}/\mu\text{l}$. Within the range of the assay, no maximum plateau of tannin precipitation by any proteins examined in this study was obtained. This indicates that either the concentration of tannin was not high enough to saturate all binding sites on the proteins, or that binding of additional tannin molecules to the tannin in the tannin-protein complexes occurred as observed in previous studies [44,45]. While the mechanism of interaction of histatins with tannin is not clear, it is apparent that histatins are a novel

class of tannin-binding proteins with an avidity for tannin which surpasses that of proteins with high proline content, generally considered to be strong precipitants of tannin.

The results from gel electrophoresis showed that various PRPs were precipitated by tannin and this is consistent with previous studies on animal saliva [24,28,29]. It is interesting to note that Hagerman and Butler [5] demonstrated that a rat salivary PRP and gelatin had similar abilities to precipitate condensed tannin at pH 7.4 and pH 4.9. In contrast we found that one of the human acidic PRPs (PRP-1) precipitated much smaller amounts of condensed tannin than gelatin at neutral pH, but both proteins precipitated comparable amounts of condensed tannin at pH 3.0. A similar pattern was seen when tannic acid was used instead of condensed tannin. Hydrogen bonding between the carbonyl groups of the protein peptide bonds and phenolic hydroxyl groups of tannin as well as hydrophobic interactions are considered the major mechanisms in tannin-protein interaction [4,5,45,46]. In general PRPs have open extended structures in which the peptide carbonyl groups are exposed to hydrogen bonding [5]. Proline residues in a proline-rich peptide also act as binding sites for tannin by hydrophobic interaction of the aromatic portion of tannin with the pyrrolidine structure of proline [45]. The relative inability of PRP-1 to precipitate condensed tannin at neutral pH may be due to the highly asymmetrical charge distribution on human salivary acidic PRPs. The N-terminal segment contains almost all of the negatively charged amino acids in the protein, but the C-terminal segment contains several basic amino acid residues [47]. Studies on calcium binding to human acidic PRPs [48] and on inhibition of hydroxyapatite formation by these proteins [49] suggest that there is an interaction of the N- and C-terminal regions of the proteins, possibly by electrostatic forces. As a consequence, the protein molecule may be less extended at neutral pH and the accessibility of proline residues would be lowered. At pH 3.0, however, most acidic amino acids in the N-terminal part would not be charged and there may be no interaction between the N- and C-terminal regions of PRP-1. Consequently, the proline residues would be more accessible and higher precipitation could be expected.

As shown in the tannin adsorption experiments, salivary histatins were even more readily precipitated by tannin than PRPs. When small amounts of tannin were present, histatins were the predominant proteins precipitated by tannin and under most of the assay conditions, histatin 5 had the highest capacity to precipitate tannin. Although tannin-binding to proteins with high proline content is well known, the binding of tannin to proteins with high histidine content has not been reported. The finding that histatins bind tannin efficiently is of interest not only because of their high histidine content but also because of their small size and lack of proline. These characteristics are in sharp contrast to the generally accepted concept that proteins with high affinity for tannin are large, and have an open, loose structure and a high proline content [5].

One of the most striking features of histatins is their high level of basic amino acids, especially histidine. In histatin 5, histidine, arginine and lysine together account for about 60% of total amino acids. Arginine and histidine will precipitate tannins [50], and polylysine shows an affinity for condensed tannins similar to that of polyproline. In addition, the result of a recent NMR study [45] provides further evidence that arginine takes part in tannin-protein interaction. Several factors could contribute to the interaction of tannin with arginine and lysine, including hydrophobic interactions of the side chains of arginine and lysine with the aromatic part of tannin. Moreover amino-aromatic interactions between the side-chain amino groups of arginine, lysine and histidine and the side-chains of aromatic amino acids

have been described [51]. It remains to be demonstrated if similar interactions occur between basic amino acids in histatins and the aromatic rings in tannins. Just as important as the unique amino acid composition of histatin 5, the lack of secondary structure in this protein may make it more accessible to tannin binding. The basis for the difference in precipitation of condensed tannin and tannic acid by histatin 5 at pH 3.0 is not clear at present. To elucidate the mechanisms whereby histatin 5 interacts with tannin, NMR studies have been initiated.

The efficiency of histatin 5 in precipitating tannin shows that a high proline content and large molecular size are not essential for a protein to interact effectively with tannin. The difference in pH dependence of tannin binding to histatin 5 and PRP-1 would ensure tannin binding to salivary proteins over a broad pH range and may also explain the necessity of the presence of both families of proteins.

The inhibition of salivary α -amylase by tannic acid is in agreement with previous studies on pancreatic amylase [9–11]. It is of interest that a mass of histatin 5 or PIF-s equal to that of α -amylase protected the enzyme from inhibition by tannin. This suggests that tannin interacts preferentially with histatin 5 or PIF-s. Considering that the total concentration of PRPs and histatins (1.9 mg/ml) is nearly three times that of α -amylase (0.66 mg/ml) in human parotid saliva, it is reasonable to predict that PRPs and histatins are capable of protecting this enzyme from tannin inhibition. In order to evaluate the extent to which dietary tannin would be complexed by salivary proteins *in vivo*, further characterization of tannin binding to the various members of the histatin and PRP families is necessary. The finding that PIF-s was as efficient as histatin 5 in protecting amylase from inhibition by tannic acid was not expected since PIF-s like PRP-1 precipitated little tannic acid at neutral pH (results not shown). The ability of PIF-s to protect α -amylase from tannic acid inhibition may be attributed to the formation of soluble tannic acid-PIF-s complexes which could not be detected by the assay used in this study. The formation of soluble complexes of tannin with other proteins has been observed [52,53]. Precipitation of tannin by salivary protein may therefore not be necessary for the protein to inactivate tannin. Salivary amylase can assist not only in digestion of food starch, but may also play a role in removal of starch adhering to oral surfaces and it is also known to bind to micro-organisms and may thereby affect the oral microbial ecology [54]. We have chosen amylase as an example of a biological activity that may be affected by tannin and other proteins in saliva. In view of the effect of tannin and salivary PRPs on the growth of rats [24], it will be of interest to evaluate to what extent human salivary proteins can protect other biological activities in the gastrointestinal tract from inhibition by tannin.

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