Interaction of Tannin with Human Salivary Histatins

Nicholas Naurato,[†] Peggy Wong,[†] Ying Lu,[‡] Karol Wroblewski,[‡] and Anders Bennick*[,†,‡]

Faculty of Dentistry and Department of Biochemistry, University of Toronto, Toronto M5S 1A8, Canada

The ability of all major human salivary histatins to precipitate condensed tannin was demonstrated, and it was found that histatins 3 and 5 share the same condensed tannin-binding region but less tannin bound to histatin 1. The condensed tannin-binding region of histatin 5 includes both the N- and the C-terminal parts, although more tannin binding occurs in the C-terminal region. Epigallocatechin gallate (EGCG) showed similar binding characteristics as condensed tannin, but much less EGCG was precipitated. Pentagalloyl glucose (PGG) was precipitated equally well by histatins 1, 3, and 5 and bound equally well to the N- and C-terminal regions of histatin 5. In contrast to condensed tannin, cleaving histatin 5 into N- and C-terminal fragments increased their ability to precipitate PGG. Together, these results show a number of differences in the nature of interaction of histatins with condensed tannin, EGCG, and PGG. Most of the condensed tannin−protein complexes remained insoluble under conditions similar to those in the stomach and the small intestine, suggesting that histatins may act as a defense against dietary tannin in humans.

Keywords: *Histatin; proline-rich proteins; condensed tannin; epigallocatechin gallate; pentagalloyl glucose*

INTRODUCTION

Tannins are plant-derived polyphenolic compounds that are widely found in foods, particularly legumes, berries, and grains including sorghum and millet (Kuhnau, 1976). Tannins are usually divided into condensed tannin and tannic acid with the former being prevalent in foods (Freudenberg, 1922). Many studies have demonstrated the harmful effects of tannins on animals such as decreased growth rate and body weight gain (Mehansho et al., 1985), perturbation of mineral absorption (Mitjavila et al., 1977), and inhibition of digestive enzymes (Ahmed et al., 1991). Condensed tannin has also been linked to pulmonary inflammation of cotton mill and grain elevator workers (Laque et al., 1988; Skea et al., 1988; Vuk-Palovic and Rohrbach, 1990), and toxic effects including liver necrosis and fatalities due to absorption from the intestinal mucosa of tannin contained in radiological contrast medium have been reported (Lucke et al., 1963).

Recently, there has been considerable interest in the ability of certain tannins to act as scavengers of free radicals (Yokazawa et al., 1998) and antioxidants (Hagerman et al., 1998), raising the possibility of their medicinal use.

Saliva from many species contains a group of proline-rich proteins (PRPs), which are usually subdivided into acidic, basic, and glycosylated PRPs; in humans they account for 70% of total protein in parotid saliva (Kauffman and Keller, 1979). Several studies have shown that proteins rich in proline, including salivary PRPs, are effective precipitants of tannin, and it has been proposed that salivary PRPs serve as a first line of defense against tannin (Mehansho et al., 1987). In a survey of tannin-binding proteins in human saliva we have shown that histatins, a group of peptides with antibacterial and antifungal activities that are also found in saliva of certain monkeys (Azen et al., 1978), precipitate tannin more effectively than PRPs (Yan and Bennick, 1995). The small size and primary structure of histatins, including the virtual absence of proline and the high content of histidine, are in sharp contrast to the larger size and high proline content of other tannin-binding proteins. Thus, histatins constitute a novel class of tannin-binding proteins. Previously, we have compared tannin binding to histatin 5 (a major component of the histatin family), PRPs, and gelatin, which is generally considered to be a strong tannin binder, and found that on a weight basis histatin 5 bound $1^1/_2$ to 2 times the amount of condensed tannin bound to gelatin (Yan and Bennick, 1995). The major members of the histatin family include histatins 1, 3, and 5. Histatin 3 consists of 32 amino acids from which histatin 5 is derived by removal of the 8 C-terminal amino acids. Other postsynthetic cleavages give rise to several minor histatins (Troxler et al., 1990). In contrast, histatin 1 consisting of 38 amino acids is derived from a separate gene (Sabatini and Azen, 1989), but it shows 68% sequence identity with histatin 3 (Troxler et al., 1990).

The present study was undertaken to compare the tannin-precipitating ability of the major histatins and to locate the tannin-binding region of these proteins involved in tannin precipitation. Moreover, the stability of the condensed tannin−protein complexes under conditions similar to those encountered in the digestive system was evaluated to determine the possibility that such complexes may prevent absorption from the alimentary canal and thus contribute to the defense against tannin.

* To whom correspondence should be addressed (telephone (416) 978-5400; fax (416) 978 8548; e-mail anders.bennick@utoronto.ca).

[†] Faculty of Dentistry.

[‡] Department of Biochemistry.

10.1021/jf981044i CCC: $18.00
Published on Web 05/04/1999

MATERIALS AND METHODS

Crude quebracho tannin (condensed tannins) was a gift from Dr. A. Hagerman, Miami University, Oxford, OH. The crude tannin was further purified by chromatography on Sephadex LH-20 and ethyl acetate extraction (Asquith and Butler, 1985). Most of the purified condensed tannins had characteristics of high molecular weight procyanidin polymers as shown by HPLC. Pentagalloyl glucose (PGG) was purified from *Rhus typhina* leaves after solvent extraction and methanolysis by the method of Haddock et al. (1982). The purity of the preparation was established by HPLC and NMR. Epigallocatechin gallate (EGCG) was a gift from Dr. M. Harbowy at the Lipton Tea Co. Trypsin, chymotrypsin, elastase, and carboxypeptidase A and B were obtained from Sigma Chemical Company, St. Louis, MO, and glycodeoxycholic acid from Calbiochem, Windsor, ON, Canada. All other chemicals were reagent grade.

Peptide Synthesis. Histatin 1 (DS*HEKRHHGYRRKFHEKHHSHREFPFYGDYGSNYLYDN), M_r = 4929, histatin 3 (DSHAKRHHGYKRKFHEKHHSHRGYRSNYLYDN), M_r = 4063, histatin 5 (DSHAKRHHGYKRKFHEKHHSHRGY), M_r = 3037, a peptide corresponding to residues 1−11 of histatin 5 named Nt-histatin 5 (DSHAKRHHGYK), M_r = 1336, and histatin 7, corresponding to the C-terminal residues 12−24 of histatin 5 (RKFHEKHHSHRGY), M_r = 1719, were synthesized on a Novasyn Crystal automatic peptide synthesizer (Nova Biochem Ltd., Nottingham, U.K.) at the Biotechnology Service Centre, University of Toronto. Phosphoserine is shown as S* and was incorporated in the peptide using fmoc-Ser(po(oblz)-OH)-OH. The identities of the naturally occurring and synthetic histatins were demonstrated by amino acid analysis, partial N-terminal amino acid sequencing, mass spectroscopy, and circular dichroism (Yan and Bennick, 1995). Additionally, preliminary experiments showed identical precipitation of the naturally occurring and synthetic histatins by condensed tannin.

Tannin-Binding Assay. *Precipitation by Condensed Tannins*. Synthetic histatins 1, 3, and 5 as well as Nt-histatin 5 and histatin 7 were assayed for formation of insoluble complexes as described by Yan and Bennick (1995). Purified condensed tannins were dissolved immediately before use in isotonic barbital buffer, pH 7.4 (VBS), in concentrations up to 2.8 μg/μL. To these solutions were added samples of 10 μg of histatin 1, 3, or 5 in a final volume of 120 μL. For comparison of tannin-binding to Nt-histatin 5, histatin 7, and histatin 5, each assay contained 3.15 nmol of peptide. The samples were incubated at 37 °C for 1 h under gentle stirring to minimize oxidation of the tannin. The resulting pellets were redissolved in 0.5% triethanolamine containing 0.1% SDS and assayed for condensed tannin as described by Hagerman and Butler (1978). All assays were done in triplicate and repeated at least once.

Precipitation by Pentagalloyl Glucose (PGG). This assay, including determination of PGG in the tannin−histatin pellets, was done as described for condensed tannin except that the buffer contained 3.5% dimethyl sulfoxide (DMSO) to ensure solubility of PGG and 3.10 nmol of the peptides were used in each assay (15.2 μg of histatin 1, 12.5 μg of histatin 3, and 9.3 μg of histatin 5). The assays were done in triplicate and repeated once. The results were evaluated by Student's *t* test.

Precipitation by Epigallocatechin Gallate (EGCG). The same procedure as described for condensed tannin was used including determination of EGCG in the pellet. Experiments were performed in VBS and in 100 mM oxalate buffer, pH 3.0. The amount of peptide in each assay was 240 nmol, precipitation was only measured in the presence of 550 μg of EGCG due to the large amount of protein required, and the insoluble complexes were sedimented by centrifugation on a Beckman Tl-100 ultracentrifuge at 284 000 g for 30 min. The assays were done in triplicate and repeated once.

Physiological Stability of Insoluble Histatin−Tannin Complexes. This was evaluated as described by Lu and Bennick (1998). Samples of 10 μg of histatin 1, 3, or 5 and 160 μg of condensed tannins were incubated in 120 μL of 50 mM phosphate buffer, pH 7.4, containing 250 mM NaCl for 1 h at 37 °C. The pellet was isolated by centrifugation and the amount of tannin determined.

To approximate physiological conditions in gastric juice, the pellet was suspended in 0.01 M HCl containing 0.09 M NaCl, or in the same solution to which had been added 1 mg/mL pepsin for 210 min, which is twice the estimated half-time of food in the stomach (Fordtran and Locklear, 1966). The amount of tannin remaining in the pellet and supernatant was determined. To simulate the composition of duodenal juice, in other experiments the pellet remaining after incubation at pH 2.0 was reincubated in 29 mM phosphate buffer, pH 7.4, containing 76.5 mM NaCl. To this buffer in another incubation was added 1 mg/mL trypsin, 1 mg/mL chymotrypsin, 0.6 mg/mL elastase, 0.14 mg/mL carboxypeptidase B, and 0.05 mg/mL carboxypeptidase A (Fordtran and Locklear, 1966). Additional incubation was also done in the same enzyme-containing buffer to which had been added 20 mM sodium glycodeoxycholate to evaluate the effect of physiological concentration of bile acids (Rinderknecht et al., 1978; DiMagnone et al., 1972). The samples were incubated for 150 min, which is twice the estimated half-time of food in the small intestine (Hunt and MacDonald, 1954). Following these incubations, the tannin−protein pellet was isolated by centrifugation and assayed for tannin. All assays were done in triplicate. The amount of tannin remaining in the pellet after the various incubations was calculated as a percentage of insoluble tannin present at the beginning of the experiment.

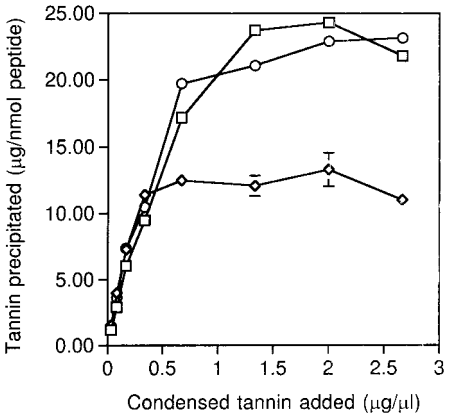

Figure 1. Condensed tannin binding to histatins 1, 3, and 5, where the amount of tannin precipitated per nanomole of peptide in the assay has been plotted as a function of condensed tannin added: (◇) binding to histatin 1; (□) binding to histatin 3; (○) binding to histatin 5. The curves are based on mean values of three assays. Error bars are shown, or they are too small to be seen.

RESULTS

The ability of histatins 1, 3, and 5 to precipitate condensed tannin is illustrated in Figure 1. All three histatins bound condensed tannin. On a molar basis, histatin 1 bound only about half the amount of condensed tannin bound to histatins 3 and 5, but there was no difference between histatins 3 and 5 in their ability to precipitate condensed tannin. Since the only difference between histatins 3 and 5 is the presence of eight additional amino acids at the C-terminal end of histatin 3, it can be concluded that the condensed tannin-binding sites in histatins 3 and 5 are located in the same regions of the proteins.

Figure 2 shows that both the N- and the C-terminal regions of histatin 5 are part of the condensed tannin-binding site. Both Nt-histatin 5 and histatin 7 bound condensed tannin, although more bound to histatin 7. It is also apparent that cleavage of histatin 5 changes

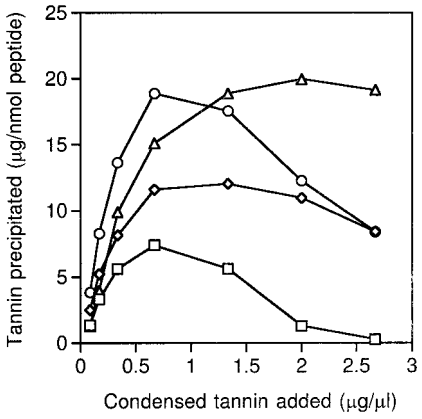

Figure 2. Comparison of condensed tannin binding to peptides constituting the N- and the C-terminal halves of histatin 5 (Nt-histatin 5 and histatin 7, respectively) and histatin 5: (□) binding to Nt-histatin 5; (◇) binding to histatin 7; (○) sum of tannin binding to Nt-histatin 5 and histatin 7; (△) binding to histatin 5. The curves are based on mean values of three assays. Error bars are too small to be seen.

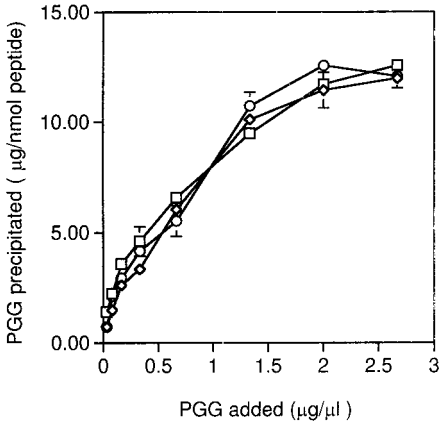

Figure 3. Pentagalloyl glucose (PGG) binding to histatins 1, 3, and 5, where the amount of PGG precipitated per nanomole of protein in the assay has been plotted as a function of PGG added: (◇) binding to histatin 1; (□) binding to histatin 3; (○) binding to histatin 5. The curves are based on mean values of three assays. Error bars are shown, or they are too small to be seen.

the interaction of the protein with tannin, since at higher concentrations of tannin the sum of tannin-binding to 1 mol of Nt-histatin 5 and 1 mol of histatin 7 is lower than that which is bound to 1 mol of histatin 5.

The ability of histatins 1, 3, and 5 to precipitate pentagalloyl glucose (PGG) is illustrated in Figure 3. In contrast to condensed tannin there was no difference in the ability of these molecules to form insoluble complexes with PGG. Moreover, it can be seen from Figure 4 that histatin 7 and Nt-histatin 5 both precipitated approximately 20% less PGG than histatin 5 per mole. When the amount of PGG bound to Nt-histatin 5 and histatin 7 is added, it can be seen that together they bind about 1.5 times the amount bound histatin 5, the molecule from which they are derived.

No precipitation was observed if histatins 3 and 5 were incubated with epigallocatechin gallate (EGCG) at neutral pH. Table 1 illustrates the precipitation observed at pH 3.0. In contrast to condensed tannin, on a molar basis a larger amount of EGCG was precipitated by histatin 3 than 5, indicating that the additional eight amino acids in the C-terminal end of histatin 3 are part

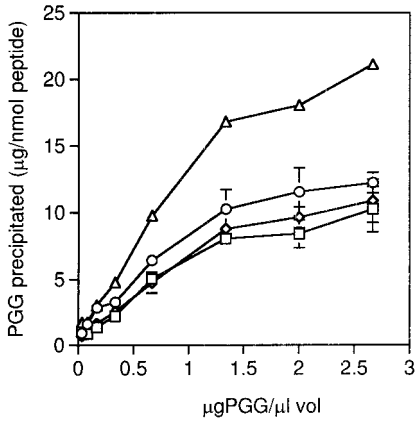

Figure 4. Comparison of pentagalloyl glucose (PGG) binding to peptides constituting the N- and the C-terminal halves of histatin 5 (Nt-histatin 5 and histatin 7, respectively) and histatin 5: (□) binding to Nt-histatin 5; (◇) binding to histatin 7; (△) sum of tannin binding to Nt-histatin 5 and histatin 7; (○) binding to histatin 5. The curves are based on mean values of three assays. Error bars are shown, or they are too small to be seen.

Table 1. Amount of Epigallocatechin Gallate (EGCG) Precipitated by the Peptides Indicated[a]

peptide	μg of EGCG precipitated per nmol of peptide mean ± sd (n = 3)	moles of EGCG precipitated per mole of peptide
histatin 3	1.78 ± 0.07	3.9
histatin 5	1.37 ± 0.04	3.0
Nt-histatin 5	0.07 ± 0.01	0.15
histatin 7	0.47 ± 0.02	1.0

[a] The assay was done in at pH 3.0 in a volume of 120 μL containing 550 μg of EGCG and 240 nmol of peptide.

of the EGCG binding site. EGCG bound to both the Nt-histatin 5 and histatin 7, but as seen with condensed tannin, there was less binding to the Nt-histatin 5 peptide. This experiment was repeated with essentially the same results.

The stability of insoluble histatin−tannin complexes in vitro under conditions similar to those of the intestinal system is shown in Figure 5. At pH 2.0, 93−96% of the insoluble complexes added to the incubation mixture remained insoluble, and if pepsin was present, 97% of condensed tannin could be recovered from the pellet. When the insoluble pellets were subsequently incubated at neutral pH under ionic conditions found in the small intestine, 78−81% of the protein−tannin pellet added to the pH 2.0 incubation mixture remained insoluble, and if proteolytic enzymes were present, 92−96% was recovered in the pellet. Addition of glycodeoxycholic acid resulted in a lowering of condensed tannin that remained insoluble. In the absence of pancreatic enzymes 55−64% of condensed tannin was found in the pellet, and in the presence of enzymes 64−67% could be recovered.

DISCUSSION

The purpose of the first part of this study was to evaluate the sites on histatins to which various tannins bind and form insoluble complexes. To simulate the interaction of histatin with food tannins, a mixture of condensed tannins purified from quebracho tannin was assayed in isotonic buffer at pH 7.4. While such studies provide biological relevance to protein−tannin interac-

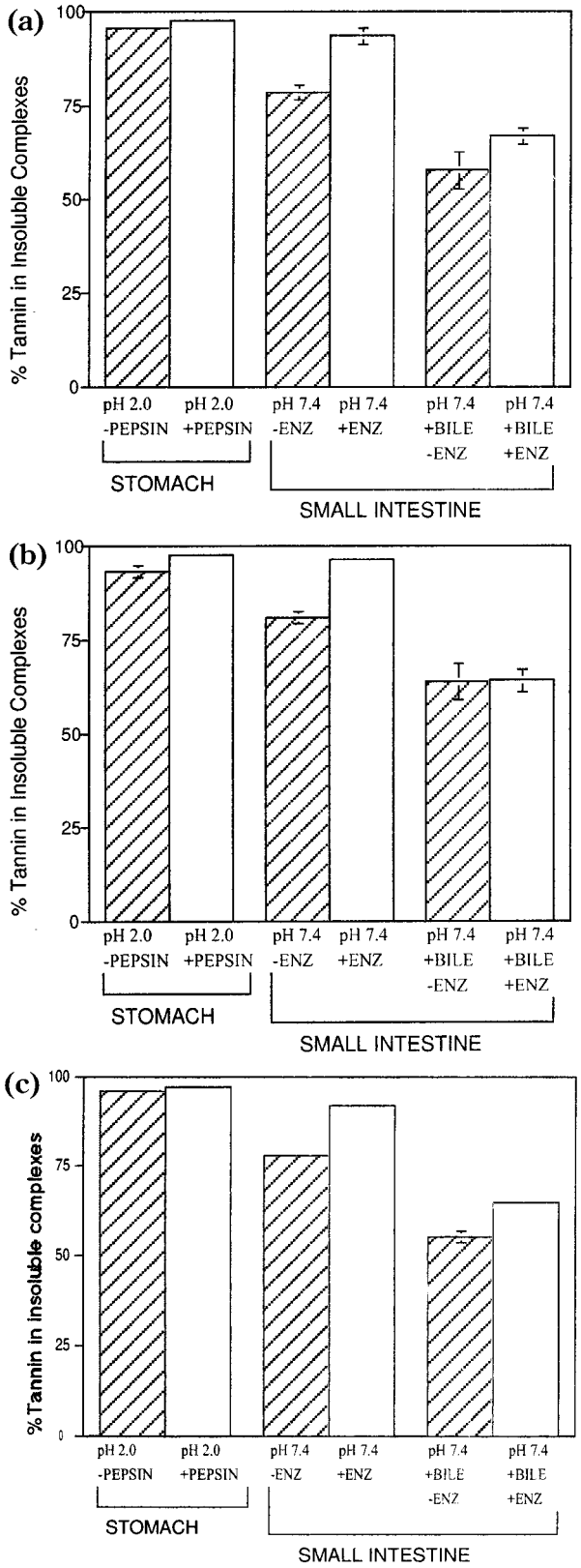

Figure 5. Stability of condensed tannin−histatin complexes under conditions prevailing in the stomach (pH 2.0) in the presence and absence of pepsin and in the small intestine in the absence and presence of proteolytic enzymes and bile (glycodeoxycholic acid): (a) tannin−histatin 1 complexes; (b) tannin−histatin 3 complexes; (c) tannin−histatin 5 complexes. The bar diagram shows the percent of insoluble condensed tannin added to the pH 2.0 incubation mixture that remained insoluble after the last incubation. The bar diagrams are based on three individual assays, and error bars are shown (otherwise they are too small to be seen).

tion, smaller and highly purified tannins are needed to understand the molecular nature of these interactions. PGG is a representative small hydrolyzable tannin that, because of its size, is amenable for use in studies of the detailed molecular interaction with protein. To maintain PGG in solution at sufficient concentration, it was necessary to add 3.5% DMSO to the buffer. Such addition of DMSO has made it possible in other studies to obtain information on the molecular interaction of PRPs and PGG in solution by means of NMR (Baxter et al., 1997), and it is expected that similar studies can be undertaken on histatin−PGG interactions. EGCG is a prominent component of green tea and therefore useful as a model for a small condensed tannin. Experiments at neutral pH failed to produce the precipitate of EGCG with histatin. A pH of 3.0 was therefore chosen. Considering that the pH of the alimentary canal varies from about pH 7.4 to about 1, it is still of physiological relevance to study the interaction at pH 3.0.

In a previous publication we demonstrated that histatin 5 was an effective precipitator of condensed tannin, showing greater ability to precipitate tannin at neutral pH than gelatin and salivary proline-rich proteins, both considered to be strong tannin binders (Yan and Bennick, 1995). We have now extended this observation and shown that all the major histatins, histatins 1, 3, and 5, are effective precipitators of condensed tannin and PGG. Studies of the interaction of proline-rich proteins and various tannins have shown that it is in particular the basic residues and proline that are responsible for interaction of the protein with tannin (Baxter et al. 1997). Thus, it is not surprising that there is little difference in the interaction of histatins 3 and 5 with condensed tannin, since of the eight additional C-terminal residues found in histatin 3 only one is a basic amino acid, and there is no proline present. The reason for the lower binding of condensed tannin to histatin 1 is not clear at present. In view of the role of basic residues in the interaction of PRPs and tannin, it is interesting to note that in histatin 1, basic amino acids including histidine account for 37% of the residues whereas they constitute 58% of residues in histatin 5. It is unlikely that the presence of phosphoserine in histatin 1 is responsible for the lower tannin binding, since studies on PRPs showed that dephosphorylation had no effect on tannin binding (Lu and Bennick, 1998).

Beside the three major histatins, saliva also contains a number of minor histatins (Troxler et al., 1990), and at least one of these, histatin 7, can also bind tannin as shown in this study. In view of the great similarity of the amino acid sequence of all the minor histatins, it is likely that they all have the ability to bind tannin, although it may be limited by the size of the peptides.

Both Nt-histatin 5 and histatin 7 contain basic residues, and this may in part explain why both the N- and the C-terminal parts contribute to tannin binding to histatin 5. In PRP−tannic acid interaction, there is evidence that part of the interaction occurs by hydrophobic stacking of the pyrrolidine rings in proline and the polyphenol rings of tannins (Baxter et al., 1997). It is possible that histidines in histatin play a similar role whereby the imidazole ring interacts with aromatic components of condensed tannin. While both the C- and the N-terminal regions of histatin 5 take part in tannin binding, variation in their interaction is illustrated by analysis of the protein−tannin precipitates. The C-

terminal region of histatin 5 (histatin 7) is a more efficient precipitant of condensed tannin than the N-terminal part (Nt-histatin 5) (Figure 2), and cleavage of histatin 5 makes it less efficient, since the sum of condensed tannin binding to Nt-histatin 5 and histatin 7 is less than that to histatin 5 at higher condensed tannin concentrations.

While only 3.15 nmol of histatins was necessary for assaying precipitation by condensed tannin and PGG, 240 nmol was needed to determine binding to EGCG. Consequently, these assays were only done at a EGCG/histatin ratio of 5:1 (Table 1). The inability of histatins to precipitate EGCG at neutral pH is in agreement with the weak interaction of epicatechin with PRP (Baxter et al., 1997). Additionally, we have by means of NMR in unpublished experiments found a similar weak interaction in solution at pH 3.0 between histatin 5 and EGCG. The higher gravitational force needed to precipitate histatin 5-EGCG complexes than histatin-condensed tannin complexes suggests that the former complexes are present as smaller aggregates.

In general it has been observed that tannin−protein precipitates are more readily formed when a protein is close to its isoelectric point (Hagerman and Butler, 1978). Histatins 3 and 5 have isoelectric points higher than 9.5 (Peters and Azen, 1977), but since more EGCG was precipitated at pH 3.0 than pH 7.4, factors other than a net charge of zero must be important. In agreement with the results obtained with condensed tannin, less EGCG was bound to Nt-histatin 5 than to histatin 7 (significant at the 1% level) (Table 1). However, differences must exist in the interaction of these tannins with histatins, as illustrated by the larger amount of EGCG bound to histatin 3 than to histatin 5 (significant at the 2% level), in contrast to condensed tannin where the same amount was bound to these two peptides.

Since there was no difference in PGG binding to histatins 3 and 5 (Figure 5), the results suggest that the PGG binding site in histatin 3 is located in the 24 N-terminal residues of histatin 3. In contrast to condensed tannin, histatin 1 bound as much PGG per mole as did histatins 3 and 5, indicating different modes of interaction of condensed tannin and PGG with histatins. That this is likely the case is also shown by the observation that whereas less condensed tannin was bound to Nt-histatin 5 than to histatin 7, these peptides bound equal amounts of PGG. Moreover, cleavage of histatin 5 into Nt-histatin 5 and histatin 7 decreased the amount of condensed tannin but increased binding of PGG (Figures 2 and 5).

Taken together, the results show that while all the tannins studied bind to histatins, the nature of their interactions with the peptides varies. The molecular basis for these differences are currently under investigation.

If histatins have a protective role in the alimentary canal, it is important first to evaluate the stability of tannin−histatin complexes under conditions similar to those in the digestive tract. The incubation conditions chosen for these experiments were the same as those in a previous study on the stability of PRP-condensed tannin complexes (Lu and Bennick, 1998), allowing comparison of the two studies. The in vitro experiments demonstrated that the complexes were very stable under conditions similar to those prevailing in the stomach but less stable under conditions approximating those existing in the small intestine, particularly in the presence of glycodeoxycholic acid. Nevertheless, under all conditions most of the complexes remained insoluble. Even if condensed tannin is resolubilized in the intestine, it may subsequently form soluble complexes with bile salts and thereby prevent absorption of tannin from the intestine. It is also possible that the enzymes added to the assays with time were inactivated and contributed to precipitation of tannin. Because of the complex dynamic conditions in the digestive tract, these studies cannot duplicate in vivo conditions, but they show the potential importance of histatin−tannin complexes in intestinal physiology and provides justification for further evaluation of their role under in vivo conditions. By comparison of these results with those previously obtained with PRP, it is apparent that insoluble PRP−tannin and histatin−tannin complexes have very similar stability under the experimental conditions.

Histatins are present in both submandibular/sublingual and parotid saliva (Troxler et al., 1990). The former is predominant under resting conditions, but during chewing, when the secretion of saliva is stimulated, secretion from the parotid gland dominates (Sas and Dawes, 1997), and this is the source of basic PRPs, the other family of tannin-binding proteins (Lu and Bennick, 1998). Histatins may therefore ensure that tannin-binding proteins always are present in saliva, whereas PRPs provide additional tannin-binding protein during eating.

Azen et al. (1978) have demonstrated that there is a correlation between the presence of histatins in monkeys and their diet. Thus, insect-eating monkeys such as the tamarin and squirrel monkey lack histatins, but the histatins are present in the saliva of fruit and plant-eating monkeys such as cercophitecoids. Humans is the only other species in which histatins have been found, and present evidence indicates that the hominid ancestors of humans were predominantly fruit eaters (Andrews and Martin, 1991). Thus, it appears that there is a correlation between the presence of histatin and the diet of primates, supporting a tannin protective role of these proteins.

ABBREVIATIONS USED

PRP, proline-rich protein; PGG, pentagalloyl glucose; EGCG, epigallocatechin gallate; VBS, isotonic barbital buffer, pH 7.4; M_r, molecular weight; SDS, sodium dodecyl sulfate; DMSO, dimethyl sulfoxide.

LITERATURE CITED

Ahmed, A. E.; Smithard, R.; Ellis, M. Activities of the enzymes of the pancreas, and the lumen and mucosa of the small intestine in growing broiler cockerels fed on tannin-containing diet. *Brit. J. Nutr.* **1991**, *65*, 189−197.

Andrews, P.; Martin, L. Hominid dietary evolution. *Philos. Trans. R. Soc. London, Ser. B* **1991**, *334*, 199−209.

Asquith, T. N.; Butler, L. G. Use of dye-labeled protein as spectrophotometric assay for protein precipitants such as tannin. *J. Chem. Ecol.* **1985,** *11*, 1535−1544.

Azen, E. A.; Leutenegger, W.; Peters, E. H. Evolutionary and dietary aspects of salivary basic (Pb) and post Pb (PPb) proteins in anthropoid primates. *Nature* **1978**, *273*, 775−778.

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.

DiMagnone, E. P.; Go, V. L. W.; Summerskill, W. H. J. Impaired cholecystekinin-pancreozymin secretion, intraluminal dilution, and maldigestion of fat in sprue. *Gastroenterology* **1972**, *63*, 25−32.

Fordtran, J.; Locklear, T. W. Ionic constituents and osmolality of gastric and small-intestinal fluids after eating. *Am. J. Dig. Dis.* **1966**, *11*, 503−521.

Freudenberg, H. *Die chemie der natürlichen gerbstoffe (The chemistry of natural tannins*); Springer-Verlag: Berlin, 1920.

Haddock, E. A.; Gupta, R. K.; Al-Shafi, S. M. K.; Haslam, E. A. The metabolism of gallic acid and hexahydroxydiphenic acid in plants. Part 1. Introduction. Naturally occurring galloyl esters. *J. Chem. Soc., Perkin Trans. 1* **1982**, 2515−2524.

Hagerman, A. E.; Butler, L. G. Protein precipitation method for quantitative determination of tannins. *J. Agric. Food Chem.* **1978**, *26*, 809−812

Hagerman, A. E.; Riedl, K. M.; Jones, A.; Sovik, A. 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

Hunt, J. N.; MacDonald, I. The influence of volume on gastric emptying. *J. Physiol.* **1954**, *126*, 459−474.

Kauffman, D. L.; Keller, P. J. The basic proline-rich proteins in human parotid saliva from a single subject. *Arch. Oral Biol.* **1979**, *24*, 249−256.

Kuhnau, J. The flavonoids: A class of semi-essential food components: Their role in human nutrition. *Wld. Rev. Nutr. Diet* **1976**, *80*, 117−121.

Lauque, D. E.; Hempel, S. L.; Schroeder, M. A.; Hyatt, R. E.; Rohrbach, M. S. Evaluation of the contribution of tannin to the acute pulmonary inflammatory response against inhaled cotton mill dust. *Am. J. Pathol.* **1988**, *133*, 163−172.

Lu, Y.; Bennick, A. Interaction of tannin with human salivary proline-rich proteins. *Arch. Oral Biol.* **1998**, *43*, 717−728.

Lucke, H. H.; Hodge, K. E.; Patt, N. L. Fatal liver damage after barium enemas containing tannic acid. *Can. Med. Assoc. J.* **1963**, *89*, 1111−1114.

Mehansho, H.; Butler, L. G.; Carlson, D. M. Dietary tannins and salivary proline-rich proteins. Interactions, induction and defense mechanisms. *Annu. Rev. Nutr.* **1987**, *7*, 423−440.

Mehansho, H.; Clements, S.; Sheares, B. T.; Smith, S.; Carlson, D. M. Induction of proline-rich glycoprotein synthesis in mouse salivary glands by isoproterenol and by tannins. *J. Biol. Chem.* **1985**, *260*, 4418−4423.

Mitjavila, S.; Lacombe, C.; Carrera, G.; Derache, R. Tannic acid and oxidized tannic acid on the functional state of rat intestinal epithelium. *J. Nutr.* **1977**, *107*, 2113-2121.

Peters, E. H.; Azen, E. A. Isolation and partial characterization of human parotid basic proteins. *Biochem. Genet.* **1977**, *15*, 925−946.

Rinderknecht, H.; Nagaraja, M. R.; Adham, N. F. Effect of bile acids and pH on the release of enteropeptidase in man. *Dig. Dis.* **1978**, *23*, 332−336.

Sabatini, L. M.; Azen, E. A. Histatins, a family of salivary histidine-rich proteins, are encoded by at least two loci (HIS1 and HIS2). *Biochem. Biophys. Res. Commun.* **1989**, *160*, 495−502.

Sas, R.; Dawes, C. The intra-oral distribution of unstimulated and chewing-gum-stimulated parotid saliva. *Archs Oral Biol.* **1997**, *42*, 469−474.

Skea, K. L.; McAvoy, D.; Broder, I. Grain dust contains a tannin-like material which fixes complement. *Environ. Res.* **1988**, *46*, 181−189.

Troxler, R. F.; Offner, G. D.; Xu, T.; Vanderspek, J. C.; Oppenheim, F. G. Structural relationship between human salivary histatins *J. Dent. Res.* **1990**, *69*, 2−6.

Vuk-Pavlovic, A.; Rohrbach, M. S. Modulation of inflammatory cell function by cotton bract tannin: Changes in the capacity of alveolar macrophages and neutrophils to produce hydrogen peroxide. *Am. J. Respir. Cell Mol. Biol.* **1990**, *3*, 235−243.

Yan, Q.; Bennick, A. Identification of histatins as tannin-binding proteins in human saliva. *Biochem. J.* **1995**, *311*, 341−347.

Yokozawa, T.; Dong, E.; Nakagawa, T.; Kashiwagi, H.; Nakagawa, H.; Takeuchi, S.; Chung, H. Y. In vitro and in vivo studies on the radical-scavenging activity of tea. *J. Agric. Food Chem.* **1998,** *46*, 2143−2150.

Received for review September 16, 1998. Revised manuscript received March 2, 1999. Accepted March 9, 1999. The studies reported in this paper were partly supported by a grant from the Canadian Medical Research Council.

JF981044I