Effects of Acidic and Alkaline Treatments on Tannic Acid and Its Binding Property to Protein

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Effects of acidic and alkaline treatments on tannic acid (TA), one of the hydrolyzable tannins, and its binding property to protein [bovine serum albumin (BSA)] were investigated. Dissociation of TA-BSA complexes was apparent in both highly acidic (pH <3) and alkaline (pH >7) treatments. TA dissociated from the complexes and pure TA was not significantly hydrolyzed in highly acidic (pH 1.0-3.0) media and retained its protein-binding property. TA did not seem to be hydrolyzed even with a more acidic treatment (in 1 M HCl, pH <0.5), although its protein-binding property was much reduced. The higher pH treatment (pH >6.5) faciliated the hydrolysis of TA, as judged by the release of its constituent gallic acid.

INTRODUCTION

Tannins are phenolic compounds that occur widely in the plant kingdom (Bate-Smith and Lerner, 1954; White, 1957). They are broadly classified into two groups based on chemical structure: hydrolyzable tannins (HT) and condensed tannins (CT) (Freudenberg, 1920; Zucker, 1983; Salunkhe et al., 1989). Both groups form insoluble complexes with proteins [i.e., bovine serum albumin (BSA), gelatin] in vitro (Hagerman and Butler, 1981; McManus et al., 1983). This process is suggested to reduce the digestibility of protein in vivo (McLeod, 1974; Feeny, 1976; Rhoades and Cates, 1976).

HT contain a central core of carbohydrates (i.e., glucose and polyhydric alcohol) which are esterified by phenolics (i.e., gallic acid, ellagic acid) and, unlike CT, are readily hydrolyzed by acids, bases, or certain enzymes (Salunkhe et al., 1989). On this basis, HT is expected to be subject to degradation by the extremes of pH which may occur within the gut of mammals.

In their physiological and metabolic study on digestion of tannin-rich foliage of *Eucalyptus* spp. by the koala, *Phascolarctus cinereus*, Cork et al. (1983) speculated that HT contained in the foliage were hydrolyzed in the acid stomach to their constituent phenols and sugars. If this is the case, one could expect that the animal has little need to develop any further mechanisms to overcome the protein-binding problem of dietary HT.

However, a microbiological study recently conducted on intestinal microflora of the koala (Osawa and Walsh, 1983) has provided evidence suggestive of an alternative view: there are unique bacteria which frequently occur in the hindgut and which are capable of degrading tannic acid, one of the HT. The possibility should be considered that dietary HT might not be hydrolyzed under acidic conditions of the stomach but degraded by these bacteria in the hindgut.

In this study, we investigated the in vitro effects of various pH conditions on tannic acid and its binding property to BSA to examine directly whether HT may survive unaltered in conditions resembling those of the stomach. Tannic acid consists mainly of gallotannin, which has a core of glucose esterified to an average of 9-10 gallic acid residues per glucose (White, 1957; Salunkhe et al., 1989). It was expected that, like other hydrolyzable tannins, tannic acid would release those gallic acid residues in free form upon hydrolysis. On this basis, possible hydrolysis of tannic acid, resulting in the release of gallic acid, during the treatments described in our experiments was determined by measuring the amount of gallic acid released.

MATERIALS AND METHODS

Preparation of Tannic Acid-Bovine Serum Albumin Complex. Bovine serum albumin solution, containing 10% BSA (Sigma Chemical Co., St. Louis, MO), and tannic acid solution (TA), containing 4% commercial tannic acid (Kanto Chemical Co., Tokyo, Japan), all with a final pH of 5.5, were prepared.

Equal volumes of TA and BSA were mixed, by vortexing, and allowed to stand at ambient temperature (23 °C) for 15 min. TA-BSA complex was separated by centrifugation (10^4g, 20 min, 4 °C). The supernatant was replaced with diluent and mixed thoroughly, and complexes were reprecipitated by centrifugation (10^4g, 20 min, 4 °C). The "rinsing" procedure was repeated three times to remove any unbound tannic acid or bovine serum albumin from the precipitate. Finally, TA-BSA complex was dispersed in diluent to obtain a solution (TA-BSA) containing the complex (dry weight) at a final concentration of 2.0% (20 mg/1 mL).

It should be noted that, unless otherwise stated, sterile phosphate buffer solution (10 mM Na_2HPO_4; pH 5.5) was used as a diluent and special care was taken throughout the study not to contaminate solutions of the complexes with any bacteria which might degrade the complexes.

Acidic and Alkaline Treatments on TA-BSA. Equal volumes (2.5 mL) of TA-BSA and serial log dilutions of either 2 M HCl or 2 M NaOH (2.0×10^{-4} to 2 M) were mixed; thus, each contained 50 mg of TA-BSA complex. A mixture of TA-BSA (2.5 mL) and diluent (2.5 mL) was also prepared to serve as a control. The mixtures were incubated at 37 °C for 3 days under anaerobic condition using a Bio-bag (Becton and Dickinson Pty. Ltd., Cockeysville, MD).

After incubation, the mixtures were centrifuged (10^4g, 20 min, 4 °C) to separate TA-BSA (TA-BSA/1) from the supernatant. The TA-BSA/1 was vacuum-dried and weighed. The pH of supernatant (S/TA-BSA/1) was measured and then adjusted to 5.5 by adding the appropriate amount of HCl or NaOH solution. After this pH adjustment, re-formation of TA-BSA was noted

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Table I. Amount of TA–BSA (Milligrams in Dry Weight; Mean ± SE) Precipitated in Different Concentrations of HCl or NaOH, Amount of TA–BSA Reprecipitated from Supernatant after pH Adjustment to pH 5.5, and Gallic Acid (GA) Levels (Millimoles per Liter; Mean ± SE) Released as a Result of TA Hydrolysis (for Details, See Text)

assay	sample assayed	concn of HCl added						concn of NaOH added					
		1.0 M	10^{-1} M	10^{-2} M	10^{-3} M	10^{-4} M	control	10^{-4} M	10^{-3} M	10^{-2} M	10^{-1} M	1.0 M	
pH before adjustment	S/TA–BSA/1	0.4	1.2	3.0	4.6	5.0	5.5	5.6	5.8	7.1	9.7	12.5	
TA–BSA precipitated before adjustment	TA–BSA/1 mean	31.2 ± 5.6	0.2 ± 0.1	4.2 ± 2.1	36.2 ± 7.2	38.0 ± 4.6	44.3 ± 3.2	43.8 ± 5.2	42.9 ± 8.9	4.5 ± 1.3	3.1 ± 1.1	0.1 ± 0.1	
TA–BSA reprecipitated after adjustment	TA–BSA/2 mean	0.8 ± 0.7	32.2 ± 4.6	23.9 ± 7.1	0.0	0.0	0.0	0.0	0.0	13.5 ± 5.1	0.0	0.0	
concn of GA released	S/TA–BSA/2 mean	0.1 ± 0.1	0.1 ± 0.1	0.2 ± 0.1	0.5 ± 0.1	0.6 ± 0.1	0.4 ± 0.2	0.4 ± 0.3	0.6 ± 0.1	2.0 ± 0.2	0.2 ± 0.1	0.0	

in many mixtures. This complex (TA–BSA/2) was collected by centrifugation (10^4g, 20 min, 4 °C), vacuum-dried, and weighed while the supernatant (S/TA–BSA/2) was used for gallic acid determination as described below.

Acidic and Alkaline Treatment on TA and Gallic Acid. Equal volumes (2.5 mL) of serial log dilutions of either 2 M HCl or 2 M NaOH and TA (2%) were mixed and incubated anaerobically at 37 °C for 3 days. TA (2%) mixed with an equal volume of diluent served as a control.

After incubation, the pH of each TA solution (5 mL) was measured and then adjusted to 5.5 by adding appropriate concentrations of HCl or NaOH to obtain the secondary TA solution (final volume of 10 mL). The TA solution thus prepared was mixed with 10 mL of 20% BSA solution (pH 5.5). The mixture (total of 20 mL) were vortexed and allowed to stand at 4 °C overnight. Precipitated TA–BSA (TA–BSA/3), if any, was collected by centrifugation (10^4g, 20 min, 4 °C), vacuum-dried, and weighed, while supernatants (S/TA–BSA/3) were used for gallic acid (GA) determination as described below. The test was done in triplicate.

GA solution (Sigma; at a final concentration of 10 mM) was also incubated anaerobically under different acidic and alkaline conditions to determine its possible degradation by the treatments.

Determination of Tannic Acid Hydrolysis. Reversed-phase high-performance liquid chromatography (HPLC) as described by Hoefler and Coggon (1976) was used to quantify GA contained in S/TA–BSA/2, S/TA–BSA/3, and GA solution after the treatments. A Model 2152 HPLC system (LKB Produkter AB, Bromma, Sweden) equipped with a solvent conditioner was used at ambient temperature (23 °C) with flow rate of 1 mL/min. Solvent for the column, 20 mM citrate–phosphate buffer, pH 4.5, and samples were filtered through 0.45-μm Millipore filters prior to use. Samples were injected onto a Phenomenex μBondpak C_{18} (10 μm) column (300 × 3.9 mm) with a 30 × 3.9 mm guard column, through an injector (Model 7125; Rheodyne Inc., Cotati, CA) fitted with a 200-μL loop. Eluent was monitored using a variable-wavelength unit (Model 2151, LKB) set at 254 nm and connected to an integrator (C-R6A Chromatopac; Shimadzu Co., Kyoto, Japan). It should be noted that the TA (1%) solution used in the present study already contained 5.5 mM free gallic acid before the incubation.

RESULTS

Effects of Acidic and Alkaline Treatments on TA–BSA. After the incubation, pH values of the primary mixtures of TA–BSA at increasing concentrations of HCl and NaOH were in the range 0.4–12.5. Recoveries of TA–BSA [TA–BSA/1 against TA–BSA (50 mg) initially contained] fell markedly (less than 20%) in most of the mixtures which had been under highly acidic (pH <3 for TA–BSA) of slightly alkaline (pH >7 for TA–BSA) conditions (Table I). This indicates that substantial amounts of the complexes dissociated under these conditions.

In contrast, mixtures under less extreme conditions (pH 5.5–5.8) showed much higher recovery (approximately 80%). The complexes which had been treated with 1.0 M HCl (pH <0.5) had a brownish resin-like appearance, different from their original appearance (gray/white powder-like). It is likely that protein fractions contained in the mixtures were "coagulated" under such extremely acidic condition or that oxidation of some phenolics or aromatic amino acids took place.

When the pH value of supernatants of TA–BSA/1 (S/TA–BSA/1) was adjusted to 5.5, "reprecipitation" of TA–BSA (TA–BSA/2) occurred in most instances. However, this did not occur in TA–BSAs with 10^{-1} M (pH 9.7) and 1.0 M NaOH (pH 12.5) (Table I).

GA was detected in all S/TA–BSA/2 except those from the mixtures which had been treated with 1.0 M NaOH. The concentrations of GA detected in S/TA–BSA/2 ranged

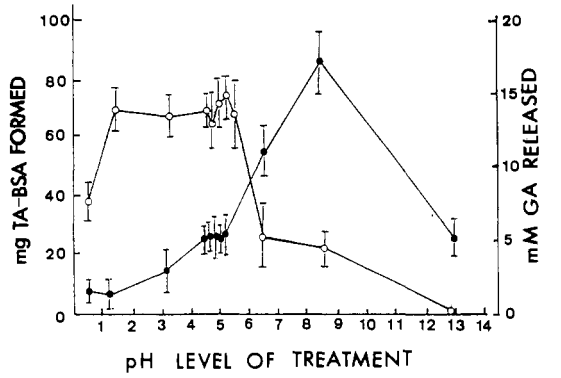

Figure 1. Extent of TA hydrolysis at different pH levels of treatments. Expressed as amount (milligrams) of TA–BSA (TA–BSA/3) formed after addition of 10 mL of 20% BSA solution (pH 5.5) to 10 mL of 0.5% TA (adjusted to pH 5.5) which had been treated at different pH levels, and GA (millimoles per liter) released in supernatant (S/TA–BSA/3) as a result of TA hydrolysis. Bar, standard error; O, TA–BSA precipitated; ●, GA released in supernatants.

from 0.1 to 2.0 mM. There was a trend toward higher levels in supernatants derived from mixtures of higher alkalinity, with the exception that the yield of GA abruptly dropped at 10^{-1} and 1.0 M NaOH for S/TA–BSA/2 (Table I).

Effects of Acidic and Alkaline Treatments on TA and GA. After incubation, pH values of 1% TA at different concentrations of HCl and NaOH ranged from 0.5 to 12.9 (Figure 1). When the solutions were adjusted to pH values of 5.5 and mixed with BSA, formation of TA–BSA (TA–BSA/3) was observed in all mixtures except those containing TA which had been treated with 1.0 M NaOH (pH 12.9). The yields of TA–BSA/3 from the mixtures which had been treated with 10^{-4}–10^{-1} M HCl (final pH 1.4–5.0) and 10^{-4}–10^{-3} M NaOH (final pH 5.1–5.2) were comparable, while the yields were markedly reduced by about 30–60% in the mixtures which had been incubated with 1.0 M HCl (pH 0.5) and 10^{-2}–10^{-1} M NaOH (pH 6.5–8.6); no protein-binding property was observed in TA treated with 1.0 M NaOH (pH 12.9) (Figure 1).

GA was detected in all supernatants of TA–BSA/3 (S/TA–BSA/3). Since 1% TA solution had already contained a fraction of GA (5.5 mM) before the incubation, the true value of GA yield as a result from TA hydrolysis during incubation was obtained by subtracting this concentration from that determined by HPLC. The true yield ranged from 0.2 to 17.2 mM. There was an appreciable linear correlation between the yield of GA and pH up to pH 8.6 (10^{-1} M NaOH), but the yield fell off sharply at 1.0 NaOH (pH 12.9) (Figure 1).

The pH values of GA solution at different concentrations of HCl and NaOH ranged from 0.4 to 12.7 after incubation. Unlike TA, GA did not precipitate BSA under any pH conditions. The concentrations of GA after the incubation remained comparable to its original concentration (10 mM) in GA solutions (pH 0.4–8.6). However, the GA which had been incubated at 10^{-1} (pH 9.7) and 1.0 M NaOH (pH 12.7) appeared to have been modified substantially, since very little was detectable by HPLC. This result is interpreted to mean that any decrease in the yield of complexes or increase of GA in the TA-containing media (except those at 10^{-1}–1.0 M NaOH) was the result of TA hydrolysis.

DISCUSSION

It has been demonstrated by several workers (Van Buren and Robinson, 1969; Brenbaum, 1980; Martin et al., 1985) that pH is one of the most important environmental factors affecting the protein-binding property of TA. These workers indicated that there is an optimum pH (pH 4–6) for this property, acidic and alkaline shifts away from the optimum pH caused dissociation of the bond between TA and proteins, which resulted in less yield of insoluble TA–protein complexes. The results of our study were consistent with these findings.

In the present study, dissociation of the binding was observed for TA–BSA in highly acidic media (final pH <3) and in highly alkaline media (final pH >7.0). In many mammals, pH in the stomach is highly acidic (pH 1–3) and pH in the small intestine slightly alkaline (pH 7–8) (Swenson, 1970). Thus, it can be postulated that, even if large amounts of TA were consumed, little digestive difficulty would be experienced since the protein fraction would be readily liberated from the complexes in the acidic stomach or the alkaline small intestine and thus could be available as sources of nutrients.

However, our results suggest that the situation may be more complex and that the nutritional problem still exists, since TA released from the complexes could rebind with dietary and/or endogenous proteins (i.e., mucus, digestive enzymes, etc.) in the lower part of the alimentary tract, where the pH lies in the range 3–7 for many mammals (Swenson, 1970). These complexes would then appear as proteinous nitrogen in the feces. Arguing against this possibility, some workers (Booth et al., 1959; Cork et al., 1983) speculated that tannins based on gallic and ellagic acids would be expected to undergo hydrolysis, in the acidic environment of the stomach, to their constituent phenols and sugars, thereby reducing their protein-binding properties.

While our results showing a lack of binding of protein to free GA are consistent with part of this suggestion, our other results seem to contradict the above speculation: TA placed in the acidic environment of pH between 1.1 and 1.3 for 3 days was not hydrolyzed to release any substantial amounts of GA and sustained its protein-binding property. TA did not seem to be hydrolyzed in even more acidic environment (in 1 M HCl, pH <0.5) although its protein-binding property was much reduced. Such an acidic environment is, however, highly unlikely in the alimentary tract of mammals.

In contrast, alkaline treatment facilitated dissociation of TA–protein complexes, hydrolysis of TA, and apparent alteration of GA. Increases in GA levels detected in TA solutions incubated with increasing pH levels up to 8.6 were indicative of TA hydrolysis, although TA was not entirely hydrolyzed within such pH range and retained an appreciable protein-binding property. The sudden decrease in GA at higher pHs can be explained by the apparent instability of GA itself at these pHs.

The evidence presented here suggests that the protein-binding property of TA is largely unaltered within a pH range of 1.0–6.0. If this situation pertains in vivo, a degree of TA hydrolysis may occur in the slightly alkaline environment of the small intestine, while any TA that was not hydrolyzed may rebind with proteins in the lower part of the intestine, remain undigested, and finally be excreted in feces. This view is compatible with the results of many nutritional studies on chicks (Vohra et al., 1966; Potter and Fuller, 1968) and laboratory rats (Joslyn and Glick, 1969; Glick and Joslyn, 1970), in which the use of dietary TA resulted in reduced nitrogen retention or increased endogenous loss in feces.

Recently, a series of works (Osawa, 1990, 1991, 1992; Osawa and Mitsuoka, 1990; Osawa and Sly, 1992) revealed

that some bacteria (i.e., *Streptococcus bovis* biotype I and facultatively anaerobic Gram-negative rod-shaped bacteria) capable of degrading the TA–protein complex were prevalent in the gut microflora of the koala, which feeds almost exclusively on tannin-rich eucalyptus leaves (Hills, 1966; Macauley and Fox, 1980; Cork and Pahl, 1984). More recently, Osawa and Walsh (1993) reported that these bacteria degraded TA itself, thereby dissociating the TA–protein complex. The prevalence of such bacteria in the koala's alimentary tract may be interpreted as an adaptational digestive strategy employed by the animal against dietary HT.

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