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Quantitative Examination of Oxidized Polyphenol−Protein Complexes

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We quantitatively examined interactions between polyphenols and proteins under oxidizing conditions, using radiolabeled 1,2,3,4,6-penta-*O*-galloyl-D-glucopyranose (PGG) and bovine serum albumin (BSA) as model compounds. We tested $NaIO_4$, 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) radical cation ($ABTS^{\bullet+}$), and 2,2′-azobis(isobutyramidine) dihydrochloride (AAPH) as model oxidants and used sodium dodecyl sulfate to disrupt noncovalent PGG−BSA interactions after the oxidation. We used trichloroacetic acid to isolate the PGG−BSA products after oxidation for radiochemical quantitation. $NaIO_4$ and $ABTS^{\bullet+}$ oxidized PGG−BSA complexes more rapidly than AAPH. Using $NaIO_4$ as the oxidant, we found that soluble oxidized PGG−BSA complexes formed rapidly and were converted to insoluble complexes if PGG was present in excess over BSA.

KEYWORDS: Polyphenol; hydrolyzable tannin; radiolabeled pentagalloyl glucose; polyphenol oxidation; tannin oxidation; protein modification; oxidized tannin−protein interactions

INTRODUCTION

Tannins are polyphenols that are widely found in plant-derived feeds, foods, beverages, and medicines (*1*). For humans consuming a typical western diet, the average dietary consumption of tannins is estimated to be well over 50% of the 1 g of dietary polyphenols consumed daily (*2*). There are three groups of tannins, traditionally classified as the hydrolyzable tannins, the condensed tannins, and the phlorotannins (**Figure 1**) (*1*). Hydrolyzable tannins consist of simple phenolic acids such as gallic acid esterified to a core polyol, typically glucose. Condensed tannins are polymers of flavonoid units. Hydrolyzable tannins and condensed tannins are found in leaves, fruits, flowers, and woody tissues of terrestrial plants. Phlorotannins are phloroglucinol-based polymers found in marine brown algae.

Environmental redox conditions in the surrounding milieu determine the biological activities of tannins (*3*). Under non-oxidizing conditions, tannins form noncovalent complexes with proteins through hydrogen bonding or hydrophobic interactions (*4*). Interactions with tannins may reduce the digestibility of proteins and may contribute to the chemical defenses that reduce the consumption of the plants by herbivores (*5*). Under oxidizing conditions, tannins readily react with toxic reactive oxygen species, such as $OH^{\bullet}$, $O_2^{\bullet-}$, and $ROO^{\bullet}$, leading to the suggestion that tannins may be beneficial antioxidants (*6*, *7*). However, oxidation intermediates (semiquinone radicals) or oxidation products (quinones) of phenols, including tannins, are electrophilic. They are reactive to and can form covalent bonds with nucleophiles, such as amino or thiol groups (*8*). Given that interaction with protein is characteristic of tannins and that proteins have appropriate nucleophilic functionalities, proteins are likely to be the targets of modification by oxidized tannins. In vitro, covalent modifications of proteins were observed in reactions between proteins and oxidized condensed tannins (*9*), oxidized hydrolyzable tannins (*10*), and oxidized phlorotannins (*11*). In vivo, proteins in the diet, in the digestive fluids, or on the surface along the digestive tract are potential targets for modifications by oxidized tannins.

Because tannin is reactive to protein under both nonoxidizing and oxidizing conditions, information on tannin−protein interactions under both conditions is vital to a balanced evaluation of the biological effects of tannin consumption. Tannin−protein interactions under nonoxidizing conditions have been studied extensively, and a two-phase mechanism for complex formation has been developed (*12*): (i) tannin binds to protein forming soluble complexes, and (ii) soluble tannin−protein complexes aggregate forming insoluble complexes. Under nonoxidizing conditions, the molar ratio of tannin/protein determines whether the soluble or insoluble complexes are the predominant species (*13*). Soluble complexes most likely occur under conditions such as those found in the gastrointestinal (GI) tract, where protein is present in large excess over tannin (*13*).

Under oxidizing conditions, tannin reacts with protein to form tannin−protein complexes that are resistant to disruption by protein denaturants (*9*−*11*). However, the interactions between oxidized tannin and protein have not been quantitatively described. Riedl and Hagerman (*9*) and Hagerman et al. (*14*) used radiolabeled protein to trace the protein precipitated by tannin under oxidizing conditions and used protein denaturants to differentiate between noncovalent tannin−protein complexes and oxidized tannin−protein complexes (*9*, *14*). Because their method only allows the examination of insoluble tannin−protein complexes, they promoted precipitation by setting the reaction conditions at high tannin/protein molar ratios. Those reaction

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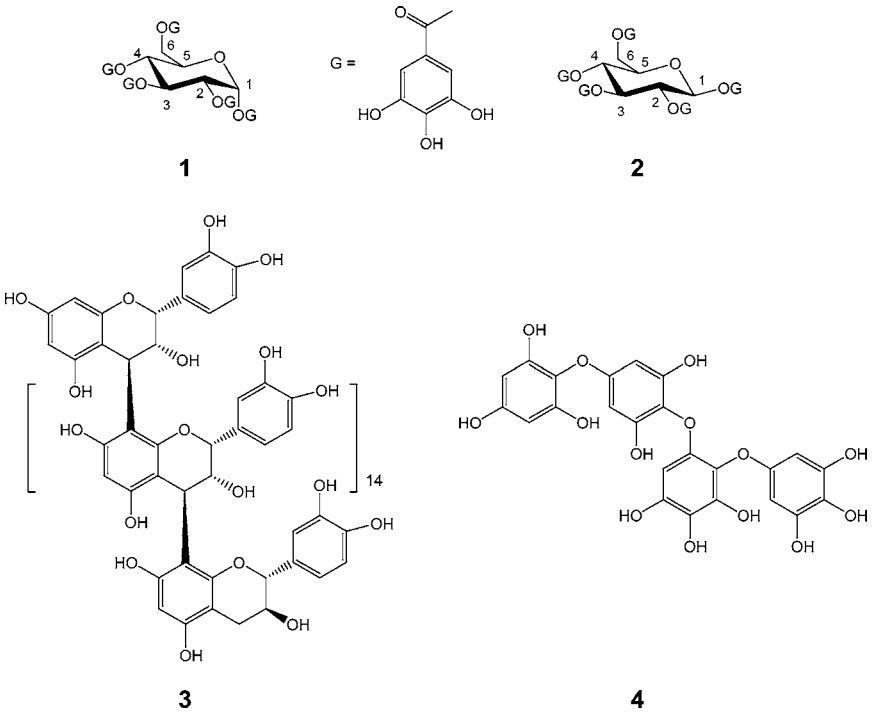

Figure 1. Typical tannins. The two anomers of pentagalloyl glucose (**1**, α; **2**, β) represent hydrolyzable tannins. Procyanidin (**3**) is a typical condensed tannin, while tetrafuhalol A (**4**) is a phlorotannin.

conditions are unlikely to occur in biological systems where protein is in large excess over tannin. To quantitatively examine the interactions between oxidized tannin and protein under a wider range of experimental conditions, new methods are needed.

We hypothesized that by using radiolabeled tannin we would be able to quantitatively examine tannin−protein interactions under oxidizing conditions if appropriate ways to isolate the reaction products could be devised. Our successful synthesis of 1,2,3,4,6-penta-*O*-galloyl[U-^{14}C]-D-glucopyranose ([^{14}C]-PGG) (*15*), a typical hydrolyzable tannin, allowed us to test our hypothesis. We found that we could use a protein denaturant to disrupt noncovalent tannin−protein interactions after oxidation, allowing us to selectively measure insoluble oxidized complexes. Subsequent treatment of the samples with a protein-precipitating agent, such as trichloroacetic acid (TCA), allowed us to differentiate soluble and insoluble oxidized PGG−protein complexes for radiochemical determination.

In our study of oxidized tannin−protein interactions, we used bovine serum albumin (BSA) as a model protein, because it has been widely used in the earlier investigations of noncovalent tannin−protein interactions (*4*, *16*, *17*). We tested three oxidizing systems, namely, $NaIO_4$, 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) radical cation ($ABTS^{\bullet+}$), and 2,2′-azobis-(isobutyramidine) dihydrochloride (AAPH), and selected $NaIO_4$ as the standard oxidizing reagent because its reaction with phenols is well-documented (*18*). Our ultimate goal is to use PGG as a model tannin to trace the biochemical fate of tannins in biological matrices where they work as antioxidants. The pH in the human mouth, stomach, and GI tract is estimated to be 6, 2, and 7.4, respectively (*19*, *20*), so our experiments have been carried out within the pH range of 2−7.4.

MATERIALS AND METHODS

ABTS, BSA (fraction V, fatty acid free), TCA solution (100%, w/v), and electrophoresis grade sodium dodecyl sulfate (SDS) were purchased from Sigma-Aldrich Co. (St. Louis, MO). ScintiVerse BD was purchased from Fisher (Fair Lawn, NJ). AAPH was purchased from Polysciences Inc. (Washington, PA). β-PGG was purified from tannic acid (The Coleman & Bell Co., Norwood, OH) by solvent extraction and was characterized by high-performance liquid chromatography (purity > 98%), negative ion electrospray ionization mass spectrometry (M_r 940) and ^{1}H NMR (*21*). α/β-[^{14}C]PGG was synthesized with a specific radioactivity of 1.19 mCi/mmol^{-1} (*15*). [^{125}I]BSA was prepared by iodination using chloramine T as described previously (*22*). All other chemicals were reagent grade or the best available.

All reagents were prepared in metal-free distilled water from a Barnstead Nanopure system (Dubuque, IA). Buffers (10 mM, phosphate, pH 2.1; citrate/phosphate, pH 3.0; acetate/citrate/phosphate, pH 4.0; acetate, pH 4.9; phosphate, pH 6.0 and pH 7.4) containing 8.5 mM NaCl were diluted to a final concentration of 5 mM buffer and 4.25 mM NaCl in the reaction mixtures. PGG was quite water insoluble but could be dissolved in water by incubating the mixture at 100 °C for 1 min. If radiochemical quantitation of PGG was needed, a small amount of [^{14}C]PGG was added to the solution when it was still at elevated temperature. The concentration of PGG stock solution was spectrophotometrically determined ($\epsilon^{280} = 5.4 \times 10^4$ M^{-1} cm^{-1}) vs water. The PGG stock solution was diluted with buffer stocks to yield the desired final PGG and buffer concentration. To prepare BSA solutions, BSA was dissolved in water. If radiochemical quantitation of BSA was needed, [^{125}I]BSA was added to the solution. The concentration of BSA was spectrophotometrically determined ($\epsilon^{280} = 4.37 \times 10^4$ M^{-1} cm^{-1}) vs water. The BSA stock solution was diluted with buffer stocks to yield the desired final BSA and buffer concentration. Reaction mixtures were centrifuged at 11000*g* at 4 °C for 10 min. For radiochemical measurement of PGG, the samples were mixed with scintillation fluid (5 mL) and were counted on a Wallac 1409 liquid scintillation analyzer (Turku, Finland). The oxidants were prepared in appropriate buffers. $ABTS^{\bullet+}$ was prepared by chemical oxidation of ABTS (*23*). For $NaIO_4$ or $ABTS^{\bullet+}$, reactions with PGG and BSA were carried out at room temperature; for AAPH, the reactions were carried out at 37 °C to promote production of the oxidizing species.

The reaction between PGG and BSA under nonoxidizing or oxidizing conditions produced different species of complexes that could be isolated by centrifugation following various treatments with SDS and/or TCA (**Figure 2**).

Quantitation of PGG−BSA Insoluble Complexes Formed without Oxidation. At pH 4.9, BSA or [^{125}I]BSA (0.45 nmol, 300 μL)

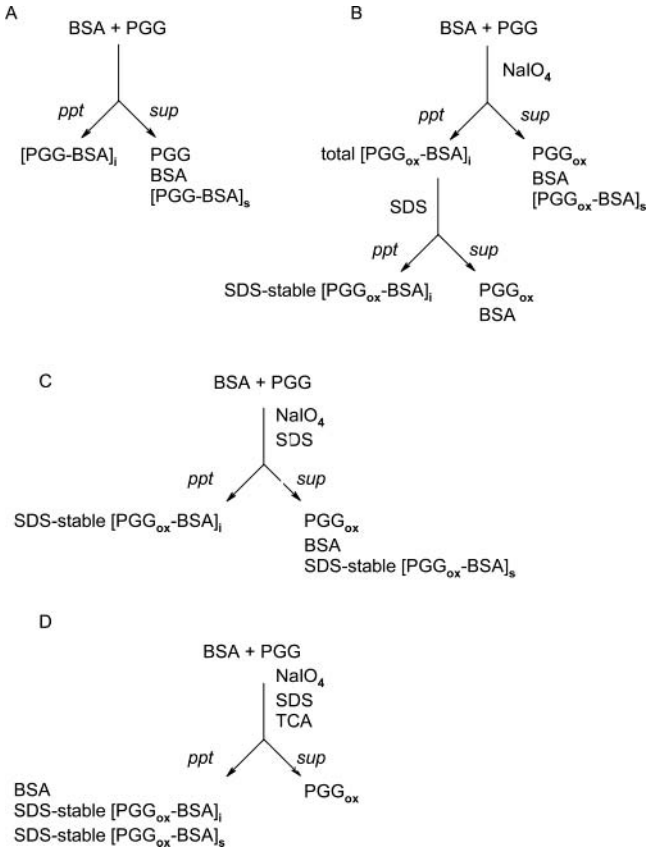

Figure 2. Speciation of PGG–BSA complexes by combining the treatments of centrifugation, SDS, and TCA under various conditions. (**A**) Separating insoluble and soluble PGG–BSA complexes formed in the absence of oxidizing agent. (**B**) Isolating total insoluble PGG–BSA complexes formed in the presence of oxidizing agent and distinguishing between SDS stable and SDS dissociable $[PGG_{ox}–BSA]_i$. (**C**) Isolating SDS stable $[PGG_{ox}–BSA]_i$ without preliminary isolation of total insoluble complexes. (**D**) Isolating SDS stable soluble and insoluble complexes by coprecipitation with BSA using TCA.

was incubated with PGG or [^{14}C]PGG (1.32−10.6 nmol, 100 μL) at room temperature for 30 min, and the mixture was centrifuged to collect insoluble BSA−PGG complexes ($[PGG–BSA]_i$, **Figure 2A**). The supernatant was removed by aspiration. To quantitate BSA, the precipitate was directly counted with a γ-counter (Cobra-Auto-Gamma, Packard Instrument Company, Downers Grove, IL). To quantitate PGG, the precipitate was dissolved by SDS (1%, w/v, 300 uL), mixed with scintillation fluid, and counted.

Quantitation of PGG−BSA Complexes after Oxidation. At pH 4.9, samples containing BSA or [^{125}I]BSA (0.45 nmol, 300 μL) and PGG or [^{14}C]PGG (10.6 nmol, 100 μL) were incubated at room temperature for 30 min before the addition of $NaIO_4$ (0−159 nmol, 75 μL). After the mixtures were incubated for appropriate periods of time, they were centrifuged to collect precipitable PGG−BSA complexes ($[PGG_{ox}–BSA]_i$, **Figure 2B**). The supernatants were removed by aspiration, and SDS (1%, w/v, 100 μL) was added to the precipitates with vortexing to dissolve SDS dissociable BSA−PGG complexes (**Figure 2B**). The samples were centrifuged, and the supernatants were removed by aspiration so that the amount of BSA in the SDS stable precipitates could be determined by γ-counting. For control samples, Nanopure water (100 μL) was added instead of SDS. If isolation of total $[PGG_{ox}–BSA]_i$ was not necessary, SDS was added to the mixture before centrifugation, and only the SDS stable $[PGG_{ox}–BSA]_i$ was determined, either by γ-counting the precipitate or indirectly by scintillation counting 150 μL of the supernatant (**Figure 2C**).

To quantitate both soluble and insoluble oxidized PGG−BSA complexes, TCA (100%, w/v, 100 μL) was added with vortexing after the SDS treatment to precipitate all BSA. SDS stable $[PGG_{ox}–BSA]_i$ and SDS stable $[PGG_{ox}–BSA]_s$ were coprecipitated with the BSA (**Figure 2D**). The mixtures were incubated on ice for 10 min and were centrifuged. The amount of PGG bound to BSA was indirectly determined by counting 150 μL from the supernatants.

Table 1. Oxidation Converts PGG–BSA Complexes to an SDS Stable Form[a]

	precipitated BSA (nmol)	
	without SDS treatment	SDS stable precipitate
no oxidant added	0.42 ± 0.01	0.01 ± 0.01
oxidant added	0.39 ± 0.01	0.09 ± 0.01

[a] [^{125}I]BSA (0.45 nmol, 300 μL) and PGG (10.6 nmol, 100 μL) were incubated at pH 4.9 without or with $NaIO_4$ (53 nmol, 75 μL) (as in **Figure 2A** or **B**, respectively). Samples were centrifuged to isolate $[PGG–BSA]_i$ or $[PGG_{ox}–BSA]_i$. After BSA was determined by γ-counting, the precipitates were treated with 1% SDS (100 μL), recentrifuged, and γ-counted again. Values are the means of three determinations; errors are standard deviations.

RESULTS AND DISCUSSION

Synthetic [^{14}C]PGG as a Suitable Tracer. PGG has a galloyl ester at each of the five hydroxyls on the glucose core with an anomeric carbon at position 1 (**Figure 1**). PGG purified from natural sources comprises only the β-anomer, but synthetic [^{14}C]PGG is a mixture of equal amounts of the α- and β-anomers (*15*). Experiments using equilibrium dialysis have suggested that α- and β-PGG have slightly different relative affinities toward proteins (*17*). Rather than purify the β-anomer of the synthetic radiolabeled compound, we evaluated whether the α/β-[^{14}C]PGG precipitated protein differently from natural β-PGG. Under conditions where optimal precipitation of PGG−BSA complexes was achieved, the same amount of BSA (0.42 ± 0.01 nmol) was precipitated by 10.6 nmol of natural β-PGG or synthetic α/β-[^{14}C]PGG. Similarly, the specific radioactivity of PGG recovered from the PGG−BSA precipitates was the same as the specific radioactivity of PGG used in each reaction. Because α/β-[^{14}C]PGG and the natural β-PGG had the same BSA precipitating activity and were interchangeable in the BSA precipitation reaction, α/β-[^{14}C]PGG is a suitable tracer for following the reaction between PGG and BSA.

Oxidation Changes PGG−BSA Complexes. Under non-oxidizing conditions, noncovalent interactions including hydrogen bonds and hydrophobic forces stabilize tannin−protein complexes (*4*). Spectroscopic evaluation of soluble complexes indicates that the hydroxyl groups of the tannin are hydrogen bond donors, and the carbonyl groups in the protein are hydrogen bond acceptors (*24*). NMR studies suggest hydrophobic interactions involving the hydrophobic faces of aromatic rings of the tannin and the hydrophobic side chains of amino acids in the protein (*12*, *25*). To quantitatively describe noncovalent tannin−protein interactions, conditions are used that promote formation of tannin−protein precipitates, which are easily isolated and analyzed (**Table 1**) (*4*, *26*, *27*). These precipitates are typically completely solubilized by SDS (1%, w/v, final concentration) with release of unmodified tannin and protein (**Table 1**) (*9*, *27*), suggesting that they are extended networks of tannin and protein that are insoluble only because of their size and polarity, not because of any irreversible covalent bond formation.

Oxidation alters the bonding in tannin−protein complexes. For example, free radical oxidation of procyanidin−BSA or epigallocatechin gallate−BSA yields insoluble aggregates that are not solubilized by protein denaturants, including SDS, urea,

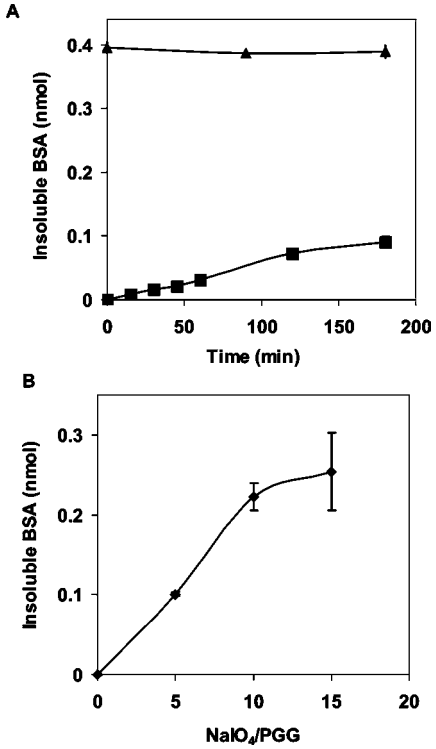

Figure 3. Oxidation changes PGG–BSA complexes. [^{125}I]BSA (0.45 nmol, 300 μL) and PGG (10.6 nmol, 100 μL) were incubated at pH 4.9 without or with $NaIO_4$ (53 nmol, 75 μL) (as in **Figure 2A** or **B**, respectively). The samples were centrifuged to isolate $[PGG–BSA]_i$ or $[PGG_{ox}–BSA]_i$. After BSA was determined by γ-counting, the precipitates were treated with 1% SDS (100 μL), recentrifuged, and γ-counted again. (**A**) The formation of $[PGG_{ox}–BSA]_i$ is independent of time of exposure to oxidant (▲), but conversion of those precipitates to SDS stable $[PGG_{ox}–BSA]_i$ depends on time of exposure to oxidant (■). (**B**) The amount of SDS stable $[PGG_{ox}–BSA]_i$ complex formed is dependent on oxidant concentration up to about 15 mol of periodate per mol of PGG. Values are the mean of three determinations; error bars indicate the standard deviations. If no error bars are shown, the standard deviations are smaller than the markers used in the graphs.

guanidine chloride, or concentrated NaOH, suggesting the formation of stable covalent linkages (*9*, *14*). We found that oxidizing mixtures of PGG and BSA with $NaIO_4$ converted about 25% of the PGG−BSA precipitates to an SDS stable form (**Table 1**). Procyanidin and epigallocatechin gallate precipitate more BSA under oxidizing conditions than in the absence of oxidant (*9*), but PGG precipitated similar amounts of BSA under oxidizing and nonoxidizing conditions (**Table 1**) (*14*).

$NaIO_4$ is a two-electron oxidant. It is a good model oxidant for studying the mechanism of polyphenol oxidation, because it has no absorbance above 260 nm, which allows direct spectrophotometric monitoring of polyphenol oxidation (*28*). Within our experimental pH range (pH 2.1−7.4), the oxidizing species is IO_4^-, which is reduced to IO_3^- by phenols (*18*, *29*). Periodate, not iodate or other more reduced forms of iodine, is the reactive species as demonstrated by the lack of reaction between PGG and IO_3^- under experimental conditions that yielded instant oxidation of PGG by IO_4^- (data not shown). When the $NaIO_4$/PGG molar ratio was set at 5 and the pH was set at 4.9, about 25% of the precipitate $[PGG_{ox}−BSA]_i$ was converted to an SDS stable form by oxidation for 180 min (**Table 1**). Holding the $NaIO_4$/PGG ratio constant, the formation of SDS stable $[PGG_{ox}−BSA]_i$ increased with reaction time (**Figure 3A**). When the reaction time was fixed at 180 min,

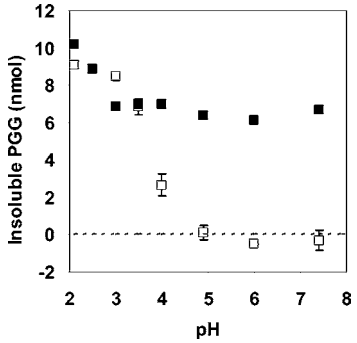

Figure 4. Insoluble oxidation products of PGG (□) interfere with the quantitation of SDS stable $[PGG_{ox}–BSA]$ (■) at pH values below 4.9. [^{14}C]PGG (10.6 nmol, 100 μL) was incubated with appropriate buffer (100 μL) with (■) or without (□) BSA (10.6 nmol) for 30 min before $NaIO_4$ (159 nmol, 75 μL) was added to initiate oxidation. After 2 min, SDS (1%, w/v, 100 μL) and then TCA (100%, w/v, 100 μL) were added with immediate vortexing (**Figure 2D**). After 10 min on ice, the mixtures were centrifuged and precipitated PGG was determined radiochemically. Values are the means of three determinations; error bars indicate the standard deviations. If no error bars are shown, the standard deviations are smaller than the markers used in the graph.

more SDS stable $[PGG_{ox}−BSA]_i$ formed as the ratio of $NaIO_4$/PGG was increased (**Figure 3B**). The amount of precipitate reached a plateau at around $NaIO_4$/PGG = 15 (**Figure 3B**). In the following experiments, the molar ratio of $NaIO_4$/PGG was held at 15. In control experiments, when BSA or PGG was treated individually with $NaIO_4$ at pH 4.9, no precipitate formed.

Oxidation of samples containing PGG and BSA yields mixtures of free oxidized PGG, free BSA, and soluble and insoluble PGG−BSA complexes (**Figure 2B**). We have devised ways to use SDS and TCA to aid in speciation of the major constituents of these mixtures (**Figure 2B−D**).

TCA and SDS for Speciation of Oxidized PGG−BSA Complexes. TCA is a widely used protein-precipitating reagent that we selected for isolation of SDS stable $[PGG_{ox}−BSA]$ complexes. Because no pure SDS stable $[PGG_{ox}−BSA]$ complexes were available, we examined precipitation of BSA by TCA in the presence of SDS and PGG under various conditions. We found that, at pH 7.4, TCA (20%, w/v, final concentration) quantitatively precipitated BSA (0.1−3.16 μg/μL, final concentration; BSA recovery = 100.3 ± 2.6%) in the presence of SDS. BSA was quantitatively precipitated in oxidized mixtures of PGG and BSA at PGG/BSA molar ratios varying from 0.25 to 2 (BSA recovery = 97.1 ± 1.2%). In our experiments, quantitative BSA precipitation was reached where the final concentration of BSA was at least 0.1 μg/μL. For experiments when concentrations of BSA fell below that limit, carrier protein could be added right after the SDS treatment. Quantitative precipitation of BSA by TCA at other pH values was also confirmed (data not shown).

We found a significant side reaction of PGG when the oxidation was carried out at pH values less than 4.9. When PGG was oxidized in the absence of BSA at pH < 4.9, its oxidation products were precipitated by TCA. The formation of TCA precipitable PGG oxidation products was pH-dependent, with more precipitation at lower pH values and no precipitation when the oxidation was carried out at pH values ≥ 4.9 (**Figure 4**). The TCA precipitable oxidation products of PGG could be trapped on 0.45 μm nylon filters (data not shown), suggesting that they were high molecular weight polymers of PGG. The interfering background precipitation of oxidized PGG made it impossible to use our method to quantitate oxidized PGG−BSA

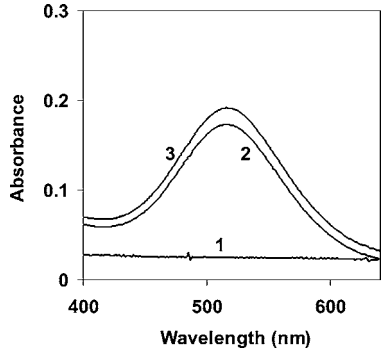

Figure 5. SDS does not inhibit PGG oxidation. At pH 7.4, 38.5 μM PGG was oxidized with a 15 molar excess of $NaIO_4$ in either water (**2**) or 0.33% (w/v) SDS (**3**). The control (**1**) is unoxidized PGG.

complexes from pH 2.1 to pH 4.9 but not from pH 4.9 to pH 7.4 (**Figure 4**). Other tannins may not oxidatively polymerize at low pH, and this method may thus be applicable over a wider range of pH values with some other tannins.

To accurately determine PGG−BSA complexes after oxidation, the bonds in PGG−BSA complexes must be stable in the TCA treatment. To test the stability of the bonds between PGG and BSA, PGG (10.6 nmol, 100 μL) and BSA (10.6 nmol, 100 μL) were mixed and oxidized by $NaIO_4$ (159 nmol, 100 μL) for 15 min. SDS (1%, w/v, 100 μL) was then added to dissolve SDS dissociable complexes. The mixture was suspended in TCA solution (20%, w/v, final concentration) for 4−20 min. There was no change in the amount of PGG (8.67 ± 0.07 nmol) bound to BSA over this period of time, indicating that prolonged exposure to TCA did not change bond formation between PGG and BSA.

It was also important to evaluate the reactivity of oxidized PGG and BSA in SDS solution. SDS did not inhibit the oxidation of PGG by $NaIO_4$ (**Figure 5**). When we added SDS (1%, w/v, 100 μL) to the mixture of PGG (10.6 nmol, 100 μL) and BSA (10.6 nmol, 100 μL) after 15 min of oxidation by $NaIO_4$ (159 nmol, 75 μL), we found that a constant amount of PGG (8.67 ± 0.15 nmol) was bound to BSA with either short (25 s) or long (30 min) exposure of the preoxidized mixture to SDS. When we added SDS to the mixture of PGG and BSA before the addition of $NaIO_4$, we found that PGG reacted with BSA very slowly, with less than 15% of the total PGG bound after 30 min of reaction. These results suggest that SDS can be used to disrupt noncovalently bonded PGG−BSA complexes without altering preexisting oxidized complexes but that SDS effectively inhibits new interactions between oxidized PGG and BSA, just as it inhibits interactions between nonoxidized PGG and BSA (*27*).

We also examined how much SDS was sufficient for complete dissolution of the SDS dissociable PGG−BSA complexes. Noncovalent protein complexes are routinely disrupted using 2% SDS in SDS−PAGE gels, so we tested a range of SDS concentrations from 0.26 to 2.6% in the reaction mixture. We found that a constant amount of PGG (8.82 ± 0.24 nmol) was bound to BSA over the entire range of SDS, indicating that the amount of SDS that we used was sufficient for complete dissociation of the noncovalent BSA−PGG complexes.

We concluded that appropriate conditions for using our method to characterize PGG−protein interactions included incubating PGG (10.6 nmol, 100 μL) with a desired amount of BSA (100 μL) and then oxidizing the mixture with an oxidant (75 μL). SDS (1%, w/v, 100 μL) was added to dissolve the SDS dissociable [PGG_{ox}−BSA]. TCA (100%, w/v, 100 μL) was added to quantitatively precipitate all BSA achieving the isolation of SDS stable [PGG_{ox}−BSA]$_i$ and [PGG_{ox}−BSA]$_s$ complexes for radiochemical measurement.

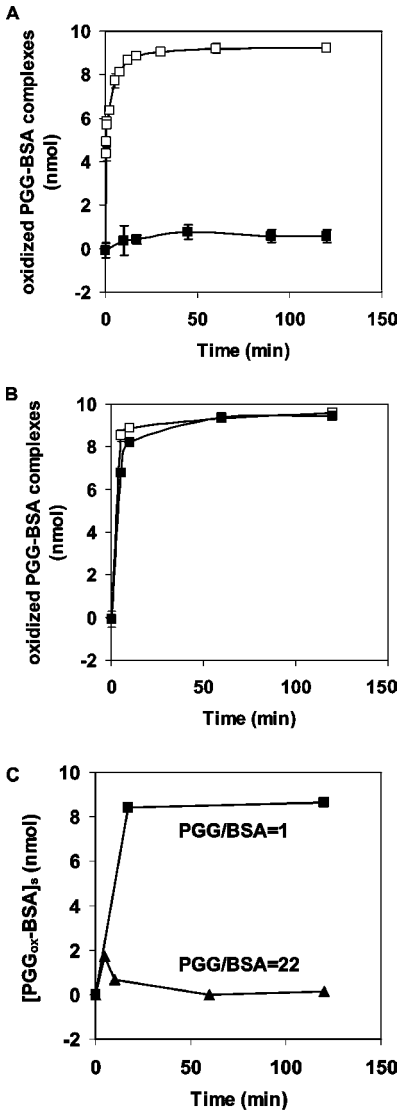

Figure 6. Kinetics of the formation of oxidized PGG–BSA complexes. At pH 4.9, [^{14}C]PGG (10.6 nmol, 100 μL) was incubated with 100 μL of solution containing BSA. After 30 min, $NaIO_4$ (159 nmol, 75 μL) was added to initiate oxidation, and after appropriate periods of time, SDS (1%, w/v, 100 μL) was added with immediate vortexing before radiochemically determining the amount of PGG in SDS stable insoluble complexes ([PGG_{ox}–BSA]$_i$) (■). TCA was used to isolate the soluble and insoluble SDS stable complexes ([PGG_{ox}–BSA]$_i$ and [PGG_{ox}–BSA]$_s$) (□). (**A**) Complexes isolated when BSA = 10.6 nmol. (**B**) Complexes isolated when BSA = 0.48 nmol. (**C**) The amount of PGG in [PGG_{ox}–BSA]$_s$ was calculated by the difference for PGG/BSA = 1 (■) and for PGG/BSA = 22 (▲). Values are the mean of three determinations; error bars indicate the standard deviations. If no error bars are shown, the standard deviations are smaller than the markers used in the graphs.

PGG−BSA Interactions under Oxidizing Conditions. Our new method provided a tool for studying PGG−BSA interactions under oxidizing conditions. In our experiments, the PGG/BSA molar ratio was set at 1 or 22 in order to cover a broad range that could be present in biological systems. At either PGG/BSA ratio, essentially all of the added PGG formed SDS stable [PGG_{ox}−BSA] complexes within 30 min of the addition of $NaIO_4$ (**Figure 6A**,**B**). When PGG was equimolar to BSA,

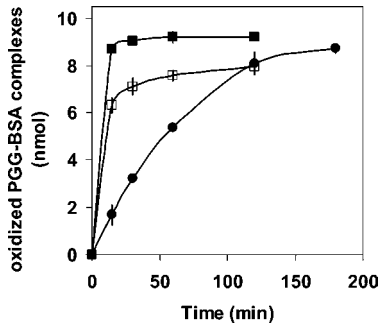

Figure 7. Kinetics of the formation of SDS stable [PGG_{ox}–BSA] with several oxidizing agents. At pH 4.9, [^{14}C]PGG (10.6 nmol, 100 μL) was incubated with BSA (10.6 nmol, 100 μL) for 30 min before adding $NaIO_4$ (■) (159 nmol, 75 μL), $ABTS^{\bullet+}$ (□) (397.5 nmol, 75 μL), or AAPH (●) (10.6 μmol, 75 μL). After appropriate periods of time, SDS (1%, w/v, 100 μL) was added with immediate vortexing to dissociate SDS instable complexes, and TCA (100%, w/v, 100 μL) was added. Values are the mean of three determinations; error bars indicate the standard deviations. If no error bars are shown, the standard deviations are smaller than the markers used in the graph.

virtually all of the SDS stable complexes were soluble (**Figure 6A**). When PGG/BSA = 22, almost all of the SDS stable complexes were insoluble (**Figure 6B**). The kinetics of the reaction when PGG/BSA = 22 suggested that $[PGG_{ox}-BSA]_s$ formed first and were converted into $[PGG_{ox}-BSA]_i$ (**Figure 6C**).

Different Oxidants Have Different Efficiencies for Forming [PGG_{ox}−BSA]. Reactive oxygen species can be produced in the digestive tract by oxidation of dietary lipids (*30*), by transition metal-catalyzed Fenton reactions (*31*), or by immune inflammatory metabolism (*32*). Different oxidants may have different influences on tannin−protein interactions. We tested this hypothesis by using $ABTS^{\bullet+}$ and AAPH as alternatives to $NaIO_4$. $ABTS^{\bullet+}$ is a nitrogen-centered radical that has been used to oxidize procyanidin in the presence of BSA (*9*). AAPH is a source for carbon-centered alkyl radicals or oxygen-centered alkyperoxyl radicals (*33*). Upon thermal cleavage, one AAPH molecule generates two alkyl radicals. The resulting alkyl radicals may react with the dissolved oxygen in the solvent and form alkylperoxyl radicals. The oxidizing species from AAPH are generated in situ, and their formation is the rate-limiting step for the subsequent oxidative reactions. To obtain observable results in a reasonable period of time, AAPH was used in a 1000-fold molar excess over PGG. $NaIO_4$ and $ABTS^{\bullet+}$ are "preformed" oxidants, so only a 15-fold molar excess of $NaIO_4$ or a 30-fold molar excess of $ABTS^{\bullet+}$ was used.

More PGG was bound to BSA during oxidation with $NaIO_4$ than with $ABTS^{\bullet+}$ (**Figure 7**). AAPH induced $[PGG_{ox}-BSA]$ complexes more slowly than $NaIO_4$ or $ABTS^{\bullet+}$ despite the higher reaction temperature and large excess of AAPH (**Figure 7**). Our results demonstrated that different oxidants had different efficiencies for inducing oxidized tannin−protein complexes.

Covalent bonds may be involved in the formation of SDS stable $[PGG_{ox}-BSA]$. The formation of covalent bonds likely involves either direct attack by PGG semiquinone radical on susceptible amino acid residues (*34*) or attack of PGG quinone on nucleophilic residues such as lysine, cysteine, methionine, and tryptophan (*8*, *35*). We are continuing to characterize the nature of the linkage in model systems of tannin and protein under oxidizing conditions.

We have described a radiochemical method to quantitate oxidized PGG−BSA complexes. Applying it to examine PGG−BSA interactions under oxidizing conditions, we found that different oxidants had different efficiencies in inducing oxidized tannin−protein complexes. When $NaIO_4$ was used as a model oxidant, the formation of complexes occurred via fast kinetics at both high and low PGG/BSA molar ratios, and PGG/BSA molar ratios determined the solubilities of the products. At low PGG/BSA, oxidation produced SDS stable $[PGG_{ox}-BSA]$ complexes that were soluble; while at high PGG/BSA, oxidation produced SDS stable $[PGG_{ox}-BSA]$ complexes that were insoluble. Given that PGG shares structural features with other hydrolyzable tannins (*1*), the mechanistic information obtained from the PGG−BSA model system is likely to be generally true for other hydrolyzable tannin−protein systems.

The radioactive tracer provides invaluable specificity and sensitivity for our method but limits its application to those polyphenols that can be conveniently labeled. However, the general methods for manipulating the tannin−protein complexes could be used even with unlabeled tannins if other methods for determining protein-bound tannin could be developed. Paz et al. (*36*) have previously used nitroblue tetrazolium with glycine to stain quinoproteins (*36*). Our preliminary experiments revealed that this method could be developed into a quantitative method for analyzing oxidized tannin−protein complexes.

Our results also provided insight into the likely fate of dietary polyphenols in the digestive tract where they might work as antioxidants (*9*). We demonstrated that tannin−protein complexes formed under oxidizing conditions are less easily disrupted than those formed in the absence of oxidants, providing a probable mechanism behind the limited digestibility and uptake of polyphenols from the diet (*2*, *37*). Moreover, we demonstrated that the potential benefits of radical scavenging by dietary polyphenol (*6*, *7*) might be offset by their potential to alter some proteins in oxidizing environments.

ABBREVIATIONS USED

AAPH, 2,2′-azobis(isobutyramidine) dihydrochloride; $ABTS^{\bullet+}$, 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) radical cation; BSA, bovine serum albumin; GI, gastrointestinal; PGG, 1,2,3,4,6-penta-*O*-galloyl-D-glucopyranose; SDS, sodium dodecyl sulfate; TCA, trichloroacetic acid; $[PGG-BSA]_i$ and $[PGG-BSA]_s$, insoluble and soluble complexes of PGG with BSA; $[PGG_{ox}-BSA]_i$ and $[PGG_{ox}-BSA]_s$, insoluble and soluble oxidized PGG−BSA complexes.

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