Tannin−Protein Complexes as Radical Scavengers and Radical Sinks

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The 2,2′-azinobis(3-ethylbenzothiazoline 6-sulfonic acid) radical cation ($ABTS^{\bullet+}$) decolorization assay has been used to determine the antioxidant activity of the polyphenol epicatechin$_{16}$ (4 → 8) catechin (procyanidin, PC) alone or in complex with the model proteins bovine serum albumin (BSA) or gelatin. PC had a molar antioxidant capacity of approximately 54, 92, or 108 radicals at pH values of 3.0, 4.9, or 7.4, respectively. Radical scavenging occurred via a rapid step followed by a slow step. Interaction with gelatin reduced the rate of rapid scavenging by 50% (PC−BSA mixtures reduced by 15%). Inhibition paralleled formation of precipitable PC−protein complexes over a range of protein/PC ratios. However, inhibition was virtually overcome in 10 min. Reaction with $ABTS^{\bullet+}$ converted the PC−protein complexes from a dissociable form to a form resistant to dissociation by strong denaturants such as SDS. This study demonstrates that PC is a potent $ABTS^{\bullet+}$ scavenger even when bound to protein and that the complexes may act as a radical sink within the gastrointestinal tract.

Keywords: *Procyanidin; condensed tannin; flavonoid; oxidation; recalcitrant; antioxidant capacity; decolorization assay*

INTRODUCTION

Tannins are plant secondary metabolites that were originally recognized because they interact strongly with collagen, converting animal skin to leather. Tannins were subsequently defined as high molecular weight polyphenols that precipitate protein from solution (*1*). Within the broad group of compounds known as tannins or polyphenols, a variety of chemical structures are possible, and the number of plant species containing these compounds is immense (*2*, *3*). Hydrolyzable tannins consist of simple phenolic acids such as gallic acid esterified to polyols, typically glucose, and condensed tannins are polymers of flavonoid units. Procyanidins (PC) are typical condensed tannins composed of catechin and related flavan-3-ols linked by carbon−carbon or ether bonds. Phlorotannins, found only in marine brown algae, contain ether-linked or carbon−carbon linked phloroglucinol groups (*4*). Several common human foods including fruits, beverages, and some grains contain condensed and hydrolyzable tannins, leading to an estimated daily intake for humans of one gram of tannin (*5*).

As evidence has accumulated that reactive oxygen species are contributors to disease and aging (*6*, *7*), searches for natural dietary antioxidants have intensified. The ability of polyphenols to serve as antioxidants (*8*−*11*) has led to the proposal that dietary tannin may be beneficial (*12*). Unlike other antioxidants that are water-soluble (e.g., ascorbic acid) or lipid-soluble (e.g., tocopherols), tannins are renowned for binding proteins in soluble or precipitable complexes (*2*). There is great potential for tannin−protein complexes to form in the gastrointestinal (GI) tract since diets and the GI lining are replete with protein. Furthermore, tannin−protein complexes prepared in vitro are stable at gastrointestinal pH and in the presence of proteases (pepsin, trypsin, chymotrypsin, elastase, carboxypeptidase A, B) and bile acids (*13*, *14*). Increased fecal nitrogen, which is symptomatic of consumption of high tannin diets, is the consequence of formation of stable tannin−protein complexes in the GI tract (*15*). We postulated that protein binding would affect polyphenol reactivity and influence its biological fate just as the localization of other antioxidants is critical to their function (*7*). In previous studies (*8*−*11*), the antioxidant activity of tannin has always been determined in the absence of protein. By examining mixtures of protein with tannin, we hoped to evaluate activity in a context closer to in vivo conditions. In this study, the antioxidant activity of isolated or protein-bound PC (epicatechin$_{16}$ (4 → 8) catechin) was studied using the $ABTS^{\bullet+}$ decolorization assay. PC was chosen as our model polyphenol because it is common in the human diet, it is well characterized, and can be purified to homogeneity. The protein-to-PC ratio and pH were manipulated since these conditions are known to affect PC−protein complexation (*2*).

MATERIALS AND METHODS

Chemicals and Reagents. Procyanidin (epicatechin$_{16}$ (4 → 8) catechin) was purified from *Sorghum bicolor* (Moench) grain (IS8260, provided by Dr. John Axtell, Purdue University) as described by Hagerman and Butler (*16*). Its molecular weight and composition were established by HPLC analysis of the phloroglucinol reaction products (*17*) and confirmed by MALDI-TOF mass spectrometry (personal communication, Anders Bennick). 2,2′-Azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) and fatty acid-free BSA − fraction V, were obtained from Sigma-Aldrich Co. (St.Louis, MO). Calfskin gelatin was obtained from Eastman Kodak (Rochester, NY). Radiolabeled BSA ([^{125}I]BSA) was prepared by iodination using

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chloramine T as described previously (*18*). All solutions were prepared in 5 mM buffers containing 4.25 mM NaCl (citrate/phosphate, pH 3.0; acetate, pH 4.9; phosphate, pH 7.4). All other chemicals used were reagent grade or best grade available.

ABTS$^{\bullet+}$ Preparation. ABTS radical cation (ABTS$^{\bullet+}$) was prepared as described by Re et al. (*19*). Briefly, 15 mL of 7.01 mM ABTS (3.84 mg/mL) prepared in the appropriate buffer was mixed with 1 mL of 39.2 mM $K_2S_2O_8$ (10.6 mg/mL) dissolved in the same buffer. The mixture was incubated at room temperature in a dark bottle overnight and was then diluted with buffer to obtain the desired working solution of radical cation. The λ_{max} for visible wavelengths was 734 nm and at that wavelength a linear absorbance response was obtained through an absorbance of at least 2.0. Assuming complete reaction with a stoichiometry of one mol of potassium persulfate per 2 mol of ABTS, the extinction coefficient for ABTS$^{\bullet+}$ at 734 nm was established to be 12867 M^{-1} cm^{-1}. The extinction coefficient and UV/Vis spectrum of ABTS$^{\bullet+}$ are the same at pH values ranging from 3 to 7.4.

ABTS$^{\bullet+}$ Assay. Protein and PC were mixed in 1.5-mL cuvettes by combining 450 μL of protein solution (0−180 μg/mL) with 450 μL of PC solution (4−8 μg/mL). The mixtures were inverted to mix and incubated for 10 min at room temperature before the mixture was used to zero the spectrophotometer. The decolorization reaction was then initiated by adding 100 μL of 65 μM ABTS$^{\bullet+}$ and immediately mixing by inversion. The cuvette was immediately placed in a thermostated (30 °C) spectrophotometer (CARY 1E), and absorbance readings were collected at 30 s intervals at 734 nm using CARY WinUV software 01.00(6) (Varian, Australia). Using these conditions at the beginning of the reaction the A_{734} was about 0.7.

ABTS$^{\bullet+}$ Decolorization Capacity. To maintain a large excess of radical, we added 163 nmol (150 μL of 1.09 mM ABTS$^{\bullet+}$) to 900 μL of solution containing 0.203 nmol (1 μg) of PC at the desired pH. The starting absorbance was approximately 2.0 at 734 nm. The absorbances of both control and PC-containing samples were monitored during the reaction to account for spontaneous decolorization of ABTS$^{\bullet+}$, which increases as pH is increased. The amount of ABTS$^{\bullet+}$ scavenged at any time corresponds to the difference in absorbance between the control and the PC sample. The difference can be converted to nanomoles of ABTS$^{\bullet+}$ using the extinction coefficient of the radical to determine the number of radicals scavenged per PC molecule.

Precipitation of PC. The amount of PC precipitated by protein was determined by the method of Hagerman and Butler (*20*). PC−protein mixtures (450 μL of solution containing 2−4 μg of PC, 450 μL of solution containing 0−90 μg of protein and 100 μL of buffer) were incubated for 10 min at room temperature and then centrifuged at 21000*g* for 2 min to collect precipitable complexes. The supernatants were removed by aspiration and the pellet was gently washed with 1 mL of 25% MeOH/buffer followed by centrifugation and aspiration as above. The precipitates were dissolved by adding 70 μL of SDS (1% w/v)/triethanolamine (5% v/v) and vortexing vigorously. Color was developed by adding 13 μL of 0.01 M $FeCl_3$ in 0.01 M HCl followed by immediate vortexing. The absorbance at 510 nm was recorded after 15 min. Standards consisting of 10 μL solutions containing appropriate amounts of PC were treated the same way as the washed precipitates.

Precipitation of Radiolabeled BSA. The amount of BSA precipitated was determined with [^{125}I]BSA as described by Hagerman and Butler (*18*). Reactions were performed as described above except [^{125}I]BSA (6.6 mCi/mg) was substituted for the BSA. In some experiments, washed precipitate was vigorously agitated in common protein-resolubilizing agents including 1% SDS (w/v), 0.1 M NaOH, 6 M urea, 6 M guanidine HCl (GuHCl) or dimethylformamide, recentrifuged, and the amounts of redissolved and precipitated protein were individually determined by gamma counting (Cobra-Auto-Gamma, Packard Instrument Company, Downers Grove, IL).

Precipitation of Gelatin. Precipitated gelatin was determined according to Makkar et al. (*21*). PC−gelatin precipitates were prepared and washed as described above. Precipitates were then oven-dried at 80 °C for 1 h before 300 μL of 13.5 M NaOH was added. Samples were incubated in a 120 °C oven for 20 min to hydrolyze the protein, and then the solution was neutralized with 500 μL of glacial acetic acid. After cooling of the sample, 700 μL of ninhydrin reagent was added and samples were placed in a boiling water bath for 20 min before reading the absorbance at 570 nm. The assay was standardized with known amounts of gelatin.

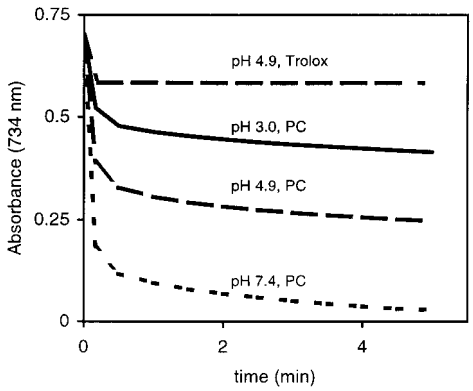

Figure 1. PC scavenges ABTS$^{\bullet+}$ via a fast and slow step. ABTS$^{\bullet+}$ (54.4 nmoles, 100 μL) was added to 900 μL of buffered solution containing either 0.811 nmoles PC (4 μg) or 4.00 nmoles (1 μg) Trolox at $t = 0$ and A_{734} was monitored at 30 °C. Each line represents the average of three determinations.

RESULTS

Antioxidant Activity of PC. Antioxidant activity was determined according to the method of Re et al. (*19*) in which the decolorization of ABTS$^{\bullet+}$ by a test compound is determined after six minutes of reaction. After establishing the linear response range for a given compound, scavenging is expressed as Trolox equivalent antioxidant capacity (TEAC). For example, if a test compound scavenges twice as much radical per mole as Trolox, then its TEAC is 2. This method assumes scavenging by the test antioxidant is complete within six minutes as it is for Trolox (Figure 1).

The original method is valid for many antioxidants, but PC represents a new class of antioxidant that exhibits both fast and slow scavenging. The magnitude of these steps increases with increasing pH (Figure 1). We defined the fast step as the decolorization occurring in the first 10 s, the time it takes to efficiently mix ABTS$^{\bullet+}$ with the sample and obtain the first absorbance reading. Since the slow step continues for much more than 6 min, reporting radical scavenged at 6 min does not account for all of the activity of PC. For example, the TEAC per mole of PC is about 22 based on a 6-min reaction at pH 4.9. When calculated after 1 h of reaction, the Trolox equivalence of PC increased to about 27 and it continued to increase ever more slowly for several hours. The amount of ABTS$^{\bullet+}$ scavenged by Trolox did not change over the same time period.

To accommodate the slow scavenging by PC, we modified the original ABTS$^{\bullet+}$ decolorization method. The scavenging capacity of PC was determined by adding a large excess of radical to solutions of PC at various pH values and spectrophotometrically monitoring loss of the radical cation (Figure 2). Spontaneous decolorization of ABTS$^{\bullet+}$ is accounted for by running parallel control reactions that do not contain the antioxidant. After 4 h at pH 7.4, the reaction of ABTS$^{\bullet+}$ with PC is finished as evidenced by the similar slope of the

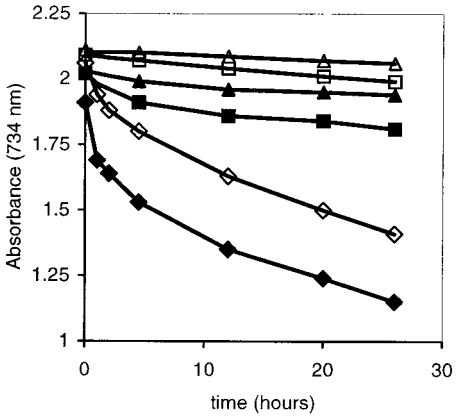

Figure 2. Capacity of PC as an $ABTS^{\bullet+}$ scavenger at pH 3.0, 4.9, and 7.4. $ABTS^{\bullet+}$ (163 nmoles, 150 μL) was added to 900 μL of PC-containing (filled symbols; 0.203 nmoles, 1 μg) or control solution (open symbols) at the desired pH and A_{734} was monitored. The points are the means of three determinations; standard deviations are smaller than the symbols used. △, ▲ pH 3.0; □, ■, pH 4.9; ◇, ◆, pH 7.4.

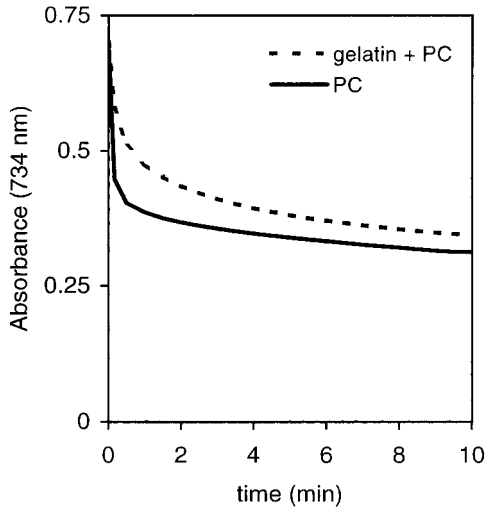

Figure 3. Effect of gelatin on $ABTS^{\bullet+}$ scavenging by PC at pH 4.9. $ABTS^{\bullet+}$ (54.4 nmoles, 100 μL) was added to 900 μL of PC (0.609 nmoles, 3 μg) or a mixture of 3 μg of PC and 0.092 nmoles (6 μg) of gelatin at $t = 0$ and A_{734} was monitored at 30 °C. Each line represents the average of three determinations.

PC and control plots (Figure 2). At pH 3.0 and pH 4.9, the capacity reached a plateau within 24 h after the start of the reaction (Figure 2). At all three pH values, the capacity calculated at 24, 48, and 96 h after the start of the reaction were statistically indistinguishable (t-test, 95% C.L), so capacity was routinely determined after 24 h of reaction. At all three pH values, after the decolorization reaction was complete all of the original $ABTS^{\bullet+}$ could be recovered by adding a stoichiometric amount of oxidizing agent ($K_2S_2O_8$) to the samples, demonstrating that the reaction involved simple radical scavenging and not other pathways of $ABTS^{\bullet+}$ decomposition.

The capacity of PC determined at 24 h after the start of the reaction was 55 ± 5 $ABTS^{\bullet+}$ per PC at pH 3.0, 92 ± 15 $ABTS^{\bullet+}$ per PC at pH 4.9, and 108 ± 5 $ABTS^{\bullet+}$ per PC at pH 7.4 (Figure 2). Trolox capacity is approximately 2 mol of $ABTS^{\bullet+}$ scavenged per mol of Trolox at all three pH values. Therefore, relative to Trolox, PC scavenged 27 times as much radical at pH 3.0, 46 times as much at pH 4.9, and 54 times as much at pH 7.4. Since sorghum PC is a polymer of approximately 17 flavonoid units, its capacity can be expressed as approximately 3 radicals (or electrons) scavenged per monomer at pH 3.0, 5 radicals per monomer at pH 4.9, and 6 radicals per monomer at pH 7.4. Using the same methods, we determined that epicatechin, the major flavonoid monomer that comprises PC, scavenges 7 mol of radicals per mol of epicatechin at pH 3.0, 12 at pH 4.9, and 16 at pH 7.4 (data not shown).

Antioxidant Activity of PC−Protein Complexes. To determine the effect of protein on scavenging by PC, we added $ABTS^{\bullet+}$ to mixtures of PC and protein that had been preincubated for sufficient time to allow complexes to form. The decolorization of $ABTS^{\bullet+}$ was monitored spectrophotometrically at 734 nm. The antioxidant activity of PC in the presence of protein was studied at two pH values so the PC−protein interaction could be manipulated. PC−protein precipitation is maximal near the isoelectric point for many proteins (*20*), so one set of experiments was done at pH 4.9, the pI of BSA and gelatin. Experiments were also conducted at pH 7.4 since at this pH BSA does not precipitate significant amounts of PC (*20*). The molar ratio of protein to PC was also varied to manipulate the precipitability of the PC−protein complexes.

At pH 4.9, both BSA and gelatin inhibited $ABTS^{\bullet+}$ scavenging by PC even though protein itself was a weak antioxidant (TEAC ≤ 0.03 obtained for either BSA or gelatin). When gelatin was mixed with PC at an optimal molar ratio for precipitation, 0.15 mol of gelatin per mole PC, the initial rate of scavenging was reduced by 50% (Figure 3). After 10 min, $ABTS^{\bullet+}$ scavenging was inhibited by 5−10%. However, after hours of reaction with $ABTS^{\bullet+}$, the amount of scavenging by PC and PC−gelatin mixtures differed by less than 5%. A similar result was obtained using the method for capacity determination, indicating only a small irreversible loss of capacity due to gelatin binding (data not shown). In contrast, inhibition by BSA (0.77 mole of BSA per mole of PC) was short-lived, and 2 min after the start of the reaction the rate of scavenging by BSA−PC mixtures was indistinguishable from scavenging by PC alone (data not shown). The degree of inhibition corresponded to the amount of precipitated PC with both measures exhibiting a bell-shaped trend with increasing molar ratio (Figure 4a,b) when the pH equaled the protein pI.

At pH 7.4, BSA and gelatin were much less effective inhibitors of $ABTS^{\bullet+}$ scavenging by PC than at pH 4.9. For both proteins, very little PC−protein precipitate formed at pH 7.4 over the entire range of protein:PC molar ratios studied (data not shown). BSA had no effect on PC antioxidant activity at pH 7.4, whereas gelatin inhibited the rapid scavenging step of PC by 20%. However, in contrast to the inhibition observed at pH 4.9, inhibition by gelatin at pH 7.4 lasted only a few minutes after adding $ABTS^{\bullet+}$.

The Effect of Oxidation on PC−Protein Interactions. The same amount of protein was precipitated by PC before or after oxidizing PC−protein complexes with $ABTS^{\bullet+}$ (Table 1). This was true for oxidation of 0.4 nmoles of PC (equivalent to 6.8 nmol of flavonoid monomer) with 8 nmol, 27 nmol, or 54 nmol of $ABTS^{\bullet+}$. Various detergents and denaturing or chaotropic solvents were used in an attempt to disrupt the PC−protein complexes. In the absence of oxidation by $ABTS^{\bullet+}$, SDS, NaOH, urea, or GuHCl solubilized 80−

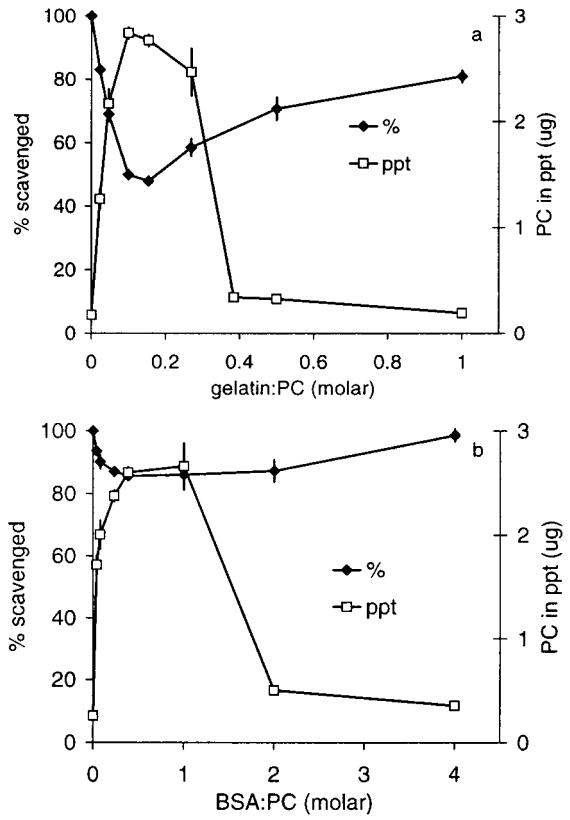

Figure 4. ABTS$^{\bullet+}$ scavenging and formation of precipitable PC−protein complexes at various protein/PC ratios. ABTS$^{\bullet+}$ (54.4 nmoles, 100 μL) was added to 900 μL of protein and PC at pH 4.9 and A_{734} was recorded immediately. That A_{734} was compared to the absorbance of a similar reaction containing only PC to calculate % scavenged. In similar solutions not containing ABTS$^{\bullet+}$, the amount of PC precipitated was determined colorimetrically (*20*). Points represent the means of three determinations, and error bars are the standard deviations. (a) Gelatin (b) BSA.

Table 1. Change in Solubility of Protein Precipitated by PC before and after ABTS$^{\bullet+}$ Oxidation[a]

		% of precipitate redissolved			
condition	protein pptd (μg)	1% SDS	0.01 M NaOH	6 M urea	6 M GuHCl
BSA+PC	12.9 ± 0.1	93.6 ± 0.5	80.8 ± 9.9	77.1 ± 6.3	79.4 ± 9.1
BSA+PC+ ABTS$^{\bullet+}$	14.2 ± 0.5	8.3 ± 1.4	9.7 ± 7.6	8.5 ± 1.6	3.9 ± 0.5
Gel+PC	3.9 ± 0.3	19.7 ± 6.6	80.4 ± 6.7	ND	ND
Gel+PC +ABTS$^{\bullet+}$	3.8 ± 0.5	ND	14.1 ± 8.8	ND	ND

[a] Precipitated protein was determined by radioactive counting (BSA) or ninhydrin (gelatin) after incubating PC and protein in the presence or absence of ABTS$^{\bullet+}$ for 20 min at room temperature and pH 4.9. Precipitates were collected and treated with protein resolubilizing agents (SDS, NaOH, urea, GuHCl). Samples were again centrifuged before the amount of protein released from the precipitate was determined. Some resolubilizing agents were incompatible with ninhydrin and therefore were not used (ND). Each value reported is the mean of four replicates and errors are standard deviations.

95% of the BSA found in PC−BSA precipitates (Table 1). In contrast, none of these agents solubilized more than 10% of BSA from PC−BSA complexes which had reacted with ABTS$^{\bullet+}$. For PC−gelatin complexes before oxidation, 0.01 M NaOH was the only agent found to be both compatible with the ninhydrin assay and effective at disrupting (>90%) the complexes. Like PC−BSA, gelatin bound to PC became highly recalcitrant (only ~14% redissolved) after reaction with ABTS$^{\bullet+}$ (Table 1). The monomeric constituents of PC, epicatechin, and catechin did not precipitate BSA before or after reaction with ABTS$^{\bullet+}$ at protein/phenolic ratios similar to those used in the PC−BSA experiments.

DISCUSSION

Because of its apparent low bioavailability (*22*), PC biological activity is believed to be restricted to the gut lumen. It has been suggested that antioxidants not absorbed from the GI tract may serve a beneficial role (*23*) since reactive oxygen species can be produced in the digesta by oxidation of dietary lipids (*24*, *25*) or by transition metal-catalyzed Fenton reactions (*26*). Furthermore, mitochondrial electron transport (*27*) and the immune system in disease states (*28*, *29*) produce reactive oxygen species that may be detrimental to contents or lining of the gut lumen. Papas suggests that the poor bioavailability of γ-tocopherol as compared to α-tocopherol may predispose γ-tocopherol to act as a gut lumen-specific antioxidant since it has equivalent antioxidant activity to the α form (*30*). Similarly, polyphenols may protect the GI lining and contents from oxidative damage or they may spare low molecular weight, bioavailable antioxidants (*12*). The tendency of polyphenols to interact with dietary or endogenous proteins in the GI tract (*13*−*15*) provides a compelling rationale for examining the antioxidant activity of PC in complex with protein. Such a model approach is not only more rigorous than simpler antioxidant screenings containing PC in isolation but can also help predict whether in vivo studies are warranted and may lead to useful biomarkers of activity.

Antioxidant Activity of PC. Finding an unambiguous way to measure the antioxidant activity of tannins and of tannin−protein complexes has been challenging. Tannin, like other phenolics, interferes with metal binding and reduces iron in hydroxyl radical-generating Fenton systems (*11*, *31*) and quenches phycoerythrin fluorescence (unpublished results) in the ORAC assay (*32*). The metmyoglobin assay (*33*) provided useful preliminary data on the antioxidant activity of several polyphenols (*11*), but we have subsequently identified complications with this method. During the metmyoglobin assay, metmyoglobin generates ABTS$^{\bullet+}$ at a constant rate. In the presence of an antioxidant, there is a lag period before ABTS$^{\bullet+}$ accumulates because the antioxidant quenches the radical as fast as it is produced. For a fast-scavenging antioxidant, after the antioxidant capacity is exhausted ABTS$^{\bullet+}$ accumulates at the same rate observed in the absence of antioxidant. The length of the lag period is thus proportional to the antioxidant concentration and the fast-scavenging activity. However, we found that when PC was used as the antioxidant in the metmyoglobin assay, the rate of ABTS$^{\bullet+}$ accumulation after the lag period was inversely proportional to PC concentration, suggesting that either PC interfered with ABTS$^{\bullet+}$ generation or that the initial products of PC oxidation continue to slowly scavenge ABTS$^{\bullet+}$ after the lag period. As described below, results obtained with the ABTS$^{\bullet+}$ decolorization method confirmed the latter hypothesis. Similar problems with the metmyoglobin assay have been described elsewhere (*34*).

In the ABTS$^{\bullet+}$ decolorization assay, the radical cation is generated before the antioxidant is added so that scavenging can be measured directly instead of via the lag time. If the antioxidant is a fast-acting scavenger,

the capacity can be determined after 6 min of reaction, as recommended by Re et al. (*19*). For antioxidants with slow scavenging kinetics, the entire reaction can be monitored to establish total antioxidant capacity, and for compounds such as PC this can take hours for each reaction. We have monitored PC scavenging over long periods of time to determine accurate capacity values.

Antioxidant Activity of PC − Kinetics and Capacity. PC is unlike any antioxidant studied thus far in terms of its mixed scavenging kinetics and high capacity. Prior to this study, antioxidants were generally classified as fast (e.g., vitamin C, vitamin E) or slow radical scavengers (e.g., protein, glutathione). Although Re et al. recently reported that flavonoid monomers are primarily fast scavengers of $ABTS^{\bullet+}$ with negligible slow scavenging activity (*19*), we found that the monomeric flavonoids catechin and epicatechin display significant slow scavenging activity in addition to their fast activity (unpublished data). Likewise, PC, a flavonoid polymer, displays a fast (complete within 10 s after adding $ABTS^{\bullet+}$) and a significant slow scavenging step (Figure 1). The decolorization data does not fit a first- or second-order function supporting the contention that there are two scavenging phases. The biphasic nature of the scavenging is not a consequence of restricted access to functional groups in the PC polymer since monomeric flavonoids and intermediate-sized polymers (6 units) exhibit similar scavenging kinetics (*35*). We speculate that the slow phase represents scavenging by polyphenol oxidation products, possibly copolymers, which were generated early in the decolorization reaction. Further work is needed to characterize the intermediates and their significance. Residence time for food in the GI tract is about 24 h, so the slow scavenging step by antioxidants commonly found in the diet could have importance during a substantial period of the digestive process.

The complex polyphenolic PC is a more potent antioxidant than simple phenolic compounds such as Trolox. Considering only the fast scavenging step, PC is 9 to 24 times more active than Trolox for pH 3−7.4, corresponding to 1 to 3 electrons donated per flavonoid monomer. Chemically, these initial products are likely semiquinones or quinones. However, we cannot compare the true initial rates of the decolorization by Trolox and PC because we do not have the capability to monitor the reaction at such short time intervals. Nevertheless, the amount of radical decolorized after the fast step is clearly an underestimate of PC capacity since PC exhibits a significant slow step in its scavenging activity. We drove the slow step to completion by adding a large excess of $ABTS^{\bullet+}$ and monitoring the scavenging reaction to completion. The estimated total capacity of PC is 27 to 54 times that of Trolox for pH 3−7.4. The impressive molar antioxidant capacity of PC—approximately 108 radicals per PC molecule at pH 7.4—is more than an order of magnitude higher than that reported for any antioxidant we are aware of and can be rationalized based on the polymeric nature of PC and the activity of its flavonoid subunits. Bors and Michel (*36*) cite the multiple electron-donating sites on flavonoid polymers as the reason for scavenging rates apparently in excess of diffusion-controlled limits and for their superior molar capacities as compared to monomeric flavonoids (*11*). The high activity of monomeric and polymeric flavonoids as radical scavengers could be due to copolymerizing reactions between oxidized flavonoids as witnessed during auto-oxidation of phenolics at high pH or when catalyzed by polyphenol oxidase in the presence of molecular oxygen (*37*).

Since PC is a polymer of 15−17 epicatechin units with a terminal catechin (*17*), one might expect PC to have an activity 15−17 times that of epicatechin, i.e., approximately 120−270 $ABTS^{\bullet+}$ scavenged per molecule of PC at pH 3.0−7.4. The capacity reported here for PC is approximately 40−45% of the predicted values. Therefore, it is likely that some restriction exists in the pathway of PC oxidation that is not present for the monomers. However, the potential for PC−protein complexes to act as radical sinks (discussed below) give PC unique features as an antioxidant that are not possible with the monomer.

The capacity of PC and the rate of scavenging increase with pH. This pH-dependence is not due to decreased stability of the ABTS radical with increasing pH since the simple antioxidant Trolox shows no difference in activity over the same pH range. Therefore, it seems that the degree to which the PC phenolic groups are ionized determines the magnitude of the fast scavenging step. The dependence of scavenging rate by PC on pH could have important biological implications since the pH within various portions of the GI tract ranges from about pH 1 to pH 8. Therefore, the efficacy of PC as an antioxidant may vary depending on the region of the GI tract in which PC resides.

Antioxidant Activity of PC−Protein Complexes. When characterizing an antioxidant, the kinetics and capacity of the free compound are not the only significant measures of bioactivity (*38*). For any presumed dietary antioxidant, scavenging by the isolated compound is not as biologically relevant as scavenging in the presence of other biomolecules that may inhibit or potentiate activity. The tendency of tannins to interact with protein in the GI tract (*13*−*15*) suggests that protein binding may be especially relevant for the bioactivity of PC.

The rate of $ABTS^{\bullet+}$ scavenging by PC was decreased in the presence of protein under conditions (pH, protein/PC ratio) where PC and protein form substantial amounts of precipitable complexes. However, some inhibition was noted even when tannin was present in a soluble tannin−gelatin complex (high protein/tannin), indicating that soluble complexation can affect scavenging. Therefore, although precipitability is a good predictor of inhibition, formation of soluble complexes could also be biologically relevant (*39*). Factors that affect formation and nature of PC−protein complexes—pH, protein type and stoichiometry, surfactants, ionic strength—could be important determinants of the antioxidant activity of PC in vivo.

Interaction with protein initially decreases the ability of PC to quench $ABTS^{\bullet+}$. The inhibition by PC−protein interaction is transient although the exact mechanism of recovery is not known. Oxidatively induced release of PC from the complex is unlikely since our data show that protein becomes more, not less, resistant to dissolution after oxidation and the characteristic dark brown color of oxidized PC is coincident with the protein precipitate. It seems likely that protein bound to PC partially obstructs collisions between PC and $ABTS^{\bullet+}$ but does not prevent them. Thus, $ABTS^{\bullet+}$ can eventually exploit these restricted collision paths and $ABTS^{\bullet+}$ is decolorized by PC−protein to a similar degree as by free PC. In addition, PC buried in an aggregate may scavenge radicals if oxidative damage to one PC site can be

transferred along the molecule to PC sites that are not directly accessible to $ABTS^{\bullet+}$. Analogous relay behavior has been demonstrated during oxidation of proteins containing multiple redox-active sites (*40*).

Possible Biological Consequences. Enzyme- and nonenzyme-based free radical sinks including superoxide dismutase/catalase, peroxidase/ascorbate, lignin, melanin, and sclerotization have been described in a number of biological systems (*40*−*44*). These sinks provide an irreversible means for organisms to detoxify and dispose of reactive oxygen species that could have detrimental consequences if left unattended. Although the potent radical scavenging activity of PC and other polyphenolics in simple chemical systems might suggest that they behave as radical sinks, the products of phenolic oxidation include the reactive species known as quinones (*45*). Quinones can also be formed by autoxidation of polyphenolics in basic solutions or via enzyme-mediated reactions (polyphenol oxidase) and once formed are prone to nucleophilic attack (*45*, *46*). In this study, $ABTS^{\bullet+}$ may have oxidized PC to a quinone species that reacted with the protein to form the observed recalcitrant complexes (Table 1). The oxidized PC−protein complex is resistant to several strong protein-resolubilizing agents and would likely be unreactive toward other biological processes. We therefore propose that PC−protein complexes may act as radical sinks in the GI tract due to the potent radical scavenging activity of PC combined with the ability of the bound protein to act as a sacrificial target for PC-derived quinone species. Formation of covalent adducts between oxidized tannin and protein (*47*) has been demonstrated for tannins from marine algae (*4*), but the current study is the first to provide evidence for these reactions for a tannin found in human foods. Furthermore, this is the first report of such a process stemming from radical-mediated oxidation of tannin.

As compared to other antioxidants, PC is unique by virtue of its GI tract-specific localization, scavenging kinetics, high capacity, and ability to form complexes with protein. Unlike the intracellular environment where antioxidant radicals can be recycled in a controlled manner, the gut lumen is an extracellular environment where oxidized compounds can persist until evacuation and can damage dietary components or gut lining in the interim. The ability of PC within PC−protein complexes to retain its high antioxidant capacity and the tendency of PC−protein complexes to be converted to recalcitrant complexes upon oxidation may allow PC to effectively reduce oxidative stress in the GI tract without releasing damaging quinones in the process. Furthermore, animals that consume PC need to contribute little to assemble the sink since PC does not need to be absorbed and the protein involved may itself be part of the diet or chemical defense system such as salivary proline-rich proteins (*15*). Moreover, disposal of the sink described here takes advantage of the normal evacuating function of the GI tract.

ABBREVIATIONS USED

ABTS, 2,2′-azinobis(3-ethylbenzothiazoline 6-sulfonic acid); BSA, bovine serum albumin; GI, gastrointestinal; GuHCl, guanidine hydrochloride; ORAC, oxygen radical absorbance capacity; PC, procyanidin; SDS, sodium dodecyl sulfate; TEAC, Trolox equivalent antioxidant capacity.

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