Synergistic Effect of BSA on Antioxidant Activities in Model Food Emulsions

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ABSTRACT: Sunflower oil-in-water emulsions containing TBHQ, caffeic acid, epigallocatechin gallate (EGCG), or 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), both with and without BSA, were stored at 50 and 30°C. Oxidation of the oil was monitored by determination of the PV, conjugated diene content, and hexanal formation. Emulsions containing EGCG, caffeic acid, and, to a lesser extent, Trolox were much more stable during storage in the presence of BSA than in its absence even though BSA itself did not provide an antioxidant effect. BSA did not have a synergistic effect on the antioxidant activity of TBHQ. The BSA structure changed, with a considerable loss of fluorescent tryptophan groups during storage of solutions containing BSA and antioxidants, and a BSA–antioxidant adduct with radical-scavenging activity was formed. The highest radical-scavenging activity observed was for the isolated protein from a sample containing EGCG and BSA incubated at 30°C for 10 d. This fraction contained unchanged BSA as well as BSA–antioxidant adduct, but 95.7% of the initial fluorescence had been lost, showing that most of the BSA had been altered. It can be concluded that BSA exerts its synergistic effect with antioxidants because of formation of a protein–antioxidant adduct during storage, which is concentrated at the oil–water interface owing to the surface-active nature of the protein.

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KEY WORDS: Antioxidant activity, bovine serum albumin, emulsions, polyphenols, proteins.

Antioxidants are present in a wide range of plant-derived foods and are also added to foods. Much information exists in the literature about antioxidant mechanisms and structural requirements for activity (1). Although antioxidants have been frequently studied in oils, emulsions, and other foods, there have been few reports of how proteins—which are commonly present in foods—may affect the activity of antioxidants in foods. Most antioxidants of interest for foods have one or more phenolic hydroxyl groups, and several studies have demonstrated that molecules with this structure may bind to proteins (2–5). Polyphenols may associate with proteins through hydrophobic interactions and hydrogen bonding (2), and a range of phenolic antioxidants also has been shown to bind to bovine skin proteins (3). Red wine antioxidants bind to human lipoproteins and protect them from metal ion-dependent and -independent oxidation (4). Plant phenols have been shown to react with whey proteins at pH 9 (5), but such a high pH is not commonly encountered in foods.

This paper describes a study of the effect of BSA on the effectiveness of four antioxidants in model food emulsions. The antioxidants selected for study were TBHQ (**1**), caffeic acid (**2**), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (**3**; Trolox), and epigallocatechin gallate (**4**; EGCG) (Scheme 1). These antioxidants were selected because they are water-soluble and because the molecular structures differ in the number of phenolic hydroxyl groups and their relative locations to each other and to the carboxylic acid groups that are present in two of the structures. This range of structures should allow any possible interactions with the protein to be detected.

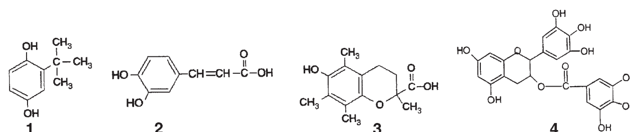
BSA, a minor whey protein with M.W. 66 kDa, was selected for this study because it has been well-characterized (6,7) and is commercially available at high purity. It has surface-active properties and has been used to stabilize model food emulsions (8). Caffeic acid is known to bind to BSA (9), and chlorogenic acid reacts with BSA at alkaline pH to form an adduct (10). However the effect of this protein on the activity of antioxidants in emulsions has not been studied; therefore, this paper describes an investigation of this subject.

MATERIALS AND METHODS

Chemicals. TBHQ, caffeic acid, EGCG, Trolox, BSA, ferric chloride, Tween 20, and 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) were purchased from Sigma-Aldrich Company Ltd. (Gillingham, United Kingdom). Refined sunflower oil of a brand known to lack added antioxidants was purchased from a local retail outlet.

Removal of tocopherols from sunflower oil. Tocopherols were removed from sunflower oil by column chromatography using alumina as described by Yoshida (11).

Preparation of emulsions. Oil-in-water emulsions (20.2 g) were prepared by dissolving Tween 20 (1%) in acetate buffer (0.1 M, pH 5.4) containing antioxidants (5 mmol/kg oil) and



SCHEME 1

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BSA (0.2%). The oil was added dropwise to the aqueous sample cooled in an ice bath while sonicating with a Vibracell High-Intensity Ultrasonic Processor (Sonic & Materials Inc., Danbury, CT). Sonication was for 4 min in total (1 min during oil addition and 3 min more).

Storage and sampling of emulsions. All emulsions were stored in duplicate in 100-mL glass beakers in the dark (inside an oven). Two aliquots of each (0.005–0.1 g, depending on the extent of oxidation) were removed periodically for PV and conjugated diene (CD) analysis, and 1 mL was removed periodically for analysis by GC.

Analytical methods. PV was determined by the ferric thiocyanate method (12) after calibrating the procedure with a series of oxidized oil samples analyzed by AOCS Official Method Cd 8-53 (13). CD content was determined by a method based on AOCS Official Method Ti 1a-64 (13) but using ethanol as a solvent.

Hexanal levels were monitored by headspace solid-phase microextraction (SPME) (14). A manual SPME holder (Sigma-Aldrich Company Ltd.) equipped with a 1-cm-long carboxen-polydimethylsiloxane (75 µm)-coated fiber (Sigma-Aldrich Company Ltd.) was used to adsorb volatiles from the emulsion in a closed vial at 60°C, with a sampling time of 12 min. Volatiles were thermally desorbed (240°C for 12 min) in the injection port of the gas chromatograph. Before use for the first time, the fiber was conditioned by heating at 240°C for more than 2 h in the gas chromatograph injection port.

GC analysis was carried out using an HP 6890 gas chromatograph equipped with an FID. A WCOT capillary column (CPSIL 88, 50 m × 0.25 µm i.d. × 0.25 µm film thickness; Chrompack, London, United Kingdom) was used, and the oven was programmed from 50°C (5 min), increased at 4°C/min to 100°C, and then increased to 150°C (3 min) at 10°C/min. The carrier gas was H_2 (45 mL/min), pressure 37 psi, split ratio 1:50, and detector temperature 260°C. Hexanal was identified by comparison of its retention time with that of an authentic standard (Sigma, Poole, United Kingdom).

Incubation of the antioxidants with BSA (30 and 50°C) and isolation of the protein from the product. To study antioxidant binding by BSA, solutions containing BSA (0.2% w/w) and 10^{-5} mol of antioxidant per 100 mL acetate buffer (pH 5.4) were incubated at 30 and 50°C. Samples were removed periodically during 10 d of storage. Solutions were stored at –20°C.

Incubations were terminated by passing the sample (1 mL) through a micro Bio-Spin P-6 column (Bio-Rad, Richmond, CA) equilibrated with acetate buffer (pH 5.4). The protein, which contained all protein molecules, namely, BSA and BSA–antioxidant adducts, was separated from free antioxidant by elution with buffer for each sample. The protein (2.7 mL) was collected, and the fluorescence attributable to the tryptophan groups was determined by excitation at 280 nm with emission at 331 nm (PerkinElmer LS 3B; Perkin Elmer, Beaconsfield, United Kingdom). Solutions with each antioxidant were stored in triplicate, and duplicate determinations were performed for each sample.

The radical-scavenging activity of the BSA–antioxidant product was also analyzed by the ABTS assay.

Preparation of the $ABTS^{\cdot+}$. ABTS and potassium persulfate (7 mM ABTS and 2.45 mM potassium persulfate, final concentrations) were used to prepare an $ABTS^{\cdot+}$ solution as described by Re *et al.* (15). The $ABTS^{\cdot+}$ solution was diluted with acetate buffer (pH 5.4, 1:100) to an absorbance of 0.7 (±0.02) at 734 nm in a 1-cm cuvette and equilibrated at 30°C.

Acetate buffer solution (pH 5.4) containing BSA (0.07%) or BSA–antioxidant product isolated by gel filtration (56 µL, equivalent to 20 µL of 0.2% BSA protein solution) was added to the $ABTS^{\cdot+}$ solution (2 mL). Buffer solution (pH 5.4) was used as a blank. After mixing, the absorbance at 734 nm was measured immediately and then every minute for 7 min. Duplicate determinations were made for triplicate samples. The percentage inhibition was calculated from the absorbance values at 5 min, and corrected for the solvent and the BSA absorbance.

The relative change in sample absorbance, ΔA_{sample}, was calculated according to the following equation to correct for the solvent and the protein:

$$\Delta A_{\text{sample}} = \frac{A_{t=0(\text{sample})} - A_{t=5(\text{sample})}}{A_{t=0(\text{sample})}} - \frac{A_{t=0(\text{BSA})} - A_{t=5(\text{BSA})}}{A_{t=0(\text{BSA})}} - \frac{A_{t=0(\text{solvent})} - A_{t=5(\text{solvent})}}{A_{t=0(\text{solvent})}} \quad [1]$$

Percent inhibition values were obtained by multiplying ΔA_{sample} values by 100.

Data analysis. Data from the PV measurements were plotted against time. The times to 40 meq/kg (PV) were determined as the induction period for each stored sample. Synergism was calculated by comparing the induction periods, modifying the equations of Satue-Gracia *et al.* (16) and Alaiz *et al.* (17),

$$\%\text{synergism} = 100\frac{[\text{IP}(a+p) - \text{IP}(c)] - [(\text{IP}a - \text{IP}c) + (\text{IP}p - \text{IP}c)]}{[(\text{IP}a - \text{IP}c) + (\text{IP}p - \text{IP}c)]} \quad [2]$$

where a = antioxidant, p = protein, and c = control. Antioxidant capacity by the $ABTS^{\cdot+}$ test, fluorescence values, and PV induction times were analyzed by one-way ANOVA to determine the pooled SD. The mean values within each test were compared with a two-sample t-test by using the pooled SD to determine significant differences ($P < 0.05$).

RESULTS AND DISCUSSION

Sunflower oil-in-water emulsions (10%) containing caffeic acid, EGCG, TBHQ, and Trolox, both with and without BSA (0.5 mM), were stored at 50 and 30°C. Oxidation of the oil was monitored by determination of the PV, CD content, and hexanal formation.

At 50°C in the absence of BSA, the antioxidants showed significant ($P < 0.05$) activity, with the order of activity being Trolox < TBHQ, EGCG, caffeic acid when assessed by the

TABLE 1
Times (d) for Oil-in-Water Emulsions Stored at 50 or 30°C to Reach PV = 40 meq/kg, and Synergism Between BSA and Antioxidants Calculated from These Data[a]

Additive	50°C Without BSA (d)	50°C With BSA (d)	50°C % Synergism	30°C Without BSA (d)	30°C With BSA (d)	30°C % Synergism
Without antioxidant	1.6 ± 0.07[x]	1.8 ± 0.02[x]		6.9 ± 0.01[a]	9.4 ± 0.33[a]	
Caffeic acid	5.1 ± 0.21[y]	9 ± 0.71[z]	100	39.2 ± 7.61[c,d]	70.9 ± 1.68[e]	84
EGCG	5.1 ± 0.21[y]	9.8 ± 0.35[z]	122	34.3 ± 2.23[c,d]	81.2 ± 6.9[e]	148
TBHQ	4.9 ± 0.67[y]	5.3 ± 1.19[y]	<0	29 ± 4.63[c,d]	18 ± 3.0[b,c]	<0
Trolox	2.4 ± 0.42[x]	6.9 ± 0.71[y]	430	19.9 ± 0.2[b,c]	43.4 ± 7.47[d]	135

[a]Range of times for duplicate samples. Values with the same roman superscript letter are not significantly different ($P > 0.05$). EGCG, epigallocatechin gallate; Trolox, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid.

time required to reach PV = 40 meq/kg (Table 1). BSA itself did not have significant antioxidant activity ($P > 0.05$) (Fig. 1). However, in the presence of BSA, the stability of the samples containing caffeic acid, EGCG, and Trolox increased strongly with the time to PV = 40 meq/kg, increasing by more than 75% for these antioxidants (Table 1). For TBHQ, there was no significant increase ($P > 0.05$) in stability in the presence of BSA. In the absence of BSA, caffeic acid was the most effective antioxidant [although it was not significantly different ($P > 0.05$) from EGCG]. In the presence of BSA, the antioxidant activity of EGCG increased more strongly than that of caffeic acid (Table 1), and the order of the mean induction times was reversed; however, there was still no significant difference ($P > 0.05$) in stability between the samples containing these two antioxidants (Fig. 1). The synergistic effect of BSA on the antioxidant activity of Trolox was similar to that with caffeic acid, but the combination of BSA and Trolox was still less effective than the combination of BSA and EGCG. The synergistic effect on the antioxidant activity of BSA combined with EGCG, caffeic acid, or Trolox was confirmed by analysis of CD and the hexanal formed during storage. At 5 d, the relative hexanal peak area for emulsion samples containing no additive, EGCG, caffeic acid, and Trolox was in the ratio 1:0.60:0.65:0.88, whereas for the analogous samples containing BSA, the relative peak area was 1.0:0.25:0.18:0.26.

Similar effects occurred for emulsions stored at 30°C (Fig. 2). However, oxidation was much slower than that observed at 50°C. Samples containing the most effective combination, BSA and EGCG, were about eight times more stable at 30°C than at 50°C, with an induction time of 81 d compared to 10 d at the higher temperature. The order of antioxidant activity for combinations of BSA with antioxidants at 30°C was EGCG, caffeic acid > Trolox > TBHQ (Table 1). Again, BSA did not have a synergistic effect on the antioxidant activity of TBHQ, and a prooxidant effect ($P < 0.05$) of the protein was detected at this temperature when the sample was compared with TBHQ alone. BSA acted as a strong synergist to caffeic acid, EGCG, and Trolox for samples stored at 30 and 50°C (Table 1). The synergistic effect of BSA on the antioxidant activity of EGCG, caffeic acid, or Trolox was confirmed by analysis of CD content and the hexanal formed during storage.

To study interactions between the BSA and antioxidants in more detail, solutions containing BSA and antioxidant were stored in a buffer (pH 5.4) at 30 and 50°C. Changes in the BSA structure were monitored by changes in the fluorescence of the tryptophan groups, which are located at positions 134 and 212 on the protein (6).

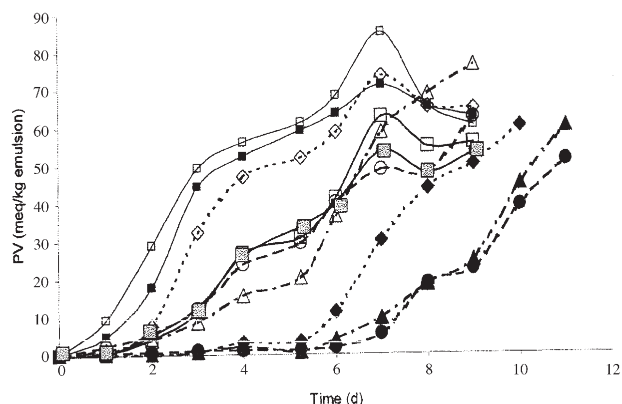

FIG. 1. Changes in PV of emulsions containing antioxidants during storage at 50°C: (–●–) caffeic acid with BSA; (–▲–) epigallocatechin gallate (EGCG) with BSA; (–■–) TBHQ with BSA; (--◆--) 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) with BSA; (–■–) control with BSA; (–○–) caffeic acid; (–△–) EGCG; (–□–) TBHQ; (--◇--) Trolox; (–□–) control.

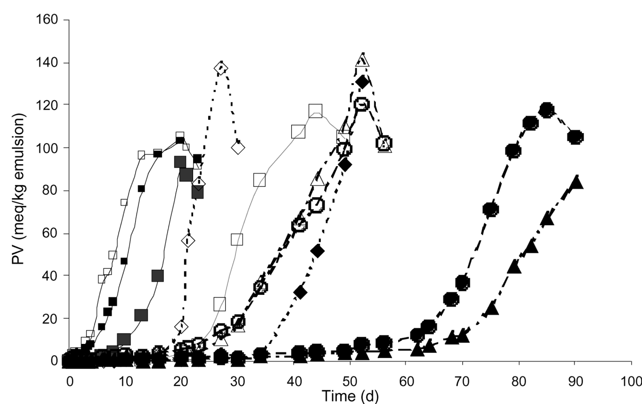

FIG. 2. Changes in PV of emulsions containing antioxidants during storage at 30°C. Sample symbols are as in Figure 1.

TABLE 2
Loss of Fluorescence of the Protein Fraction After Storage of BSA with Antioxidant in Buffer (pH 5.4) at 50 and 30°C[a]

	Time	Loss of fluorescence (%)			
		BSA–caffeic acid	BSA–EGCG	BSA–TBHQ	BSA–Trolox
Incubation at 50°C	3 h	1.2[a] (± 1.5)	33.8[b] (± 20.3)	10.5[a] (± 14.6)	1.0[a] (± 14.6)
	24 h	59.5[a] (± 2.0)	64.2[a] (± 3.1)	91.4[b] (± 0.6)	3.1[c] (± 21.6)
	5 d	86.3[a] (± 5.7)	93.7[a] (± 0.6)	96.5[a] (± 0.6)	47.6[b] (± 10.8)
Incubation at 30°C	24 h	35.6[a] (± 9.5)	52.9[a] (± 11.6)	45.8[a] (± 13.3)	14.5[a] (± 17.1)
	5 d	48.8[a] (± 4.7)	74.7[b] (± 6.5)	89.3[c] (± 2.5)	–5.5[d] (± 8.5)
	10 d	74.5[a] (± 8.7)	95.7[b] (± 6.1)	97.8[b] (± 0.1)	27.1[c] (± 5.5)

[a]Values with the same roman superscript letter in each row are not significantly different ($P > 0.05$). For abbreviations see Table 1.

A notable decrease in fluorescence occurred immediately on mixing antioxidants with BSA. For example, for EGCG a fall from about 400 to 10 was observed. Since this could be due to physical quenching of the fluorescence, the protein component of stored solutions was separated from free antioxidants with a gel-filtration cartridge.

After passing the samples incubated at 30 and 50°C (for various times) through a gel-filtration cartridge, the fluorescence of the protein component was measured again. The data obtained are shown in Table 2. At 50°C, BSA solutions without antioxidants retained their original fluorescence almost completely during 5 d of storage. In contrast, a progressive loss of fluorescence was observed for all the antioxidants incubated with BSA; this effect was most rapid for TBHQ, which exhibited a very low fluorescence at 24 h. The observed order ($P < 0.05$) for fluorescence loss at 24 h was TBHQ > EGCG, caffeic acid > Trolox. At 30°C, loss of fluorescence was slower, and there were no significant differences ($P > 0.05$) in the samples collected before 24 h (30°C). However, the reduction in fluorescence reached 48.8 ± 4.7%, 74.7 ± 6.5%, and 89.3 ± 2.5% after 5 d for BSA stored with caffeic acid, EGCG, and TBHQ, respectively, at this temperature. Following storage at 30°C, loss of fluorescence was almost complete for EGCG and TBHQ stored with BSA for 10 d, but BSA stored with caffeic acid showed a smaller reduction in fluorescence.

Although there was poor reproducibility in the fluorescence measurements for stored Trolox–BSA samples, most of the BSA fluorescence was clearly retained during storage of these samples.

The radical-scavenging activity of the isolated protein of the incubated BSA–antioxidant solutions was also determined using the $ABTS^{\cdot+}$ radical-scavenging assay (Table 3). The antioxidant activity of the BSA–antioxidant product clearly increased progressively for all antioxidants at 50°C, and the radical-scavenging activity of the BSA–antioxidant product was in the order ($P < 0.05$) of EGCG ≈ caffeic acid > TBHQ > Trolox after 5 d of incubation. At 30°C, only the BSA–antioxidant product incubated with EGCG showed a large increase in antioxidant activity with incubation time.

Remarkably, the highest radical-scavenging activity was reached for EGCG–BSA stored at 30°C for 10 d. This activity was equivalent to a concentration of free Trolox of 0.713 mM. The reduction in the $ABTS^{\cdot+}$ was 26% at 5 min for this sample incubated at 30°C, compared with a maximum reduction of 9% for EGCG incubated with BSA at 50°C for 0–5 d.

The presence of BSA caused a marked increase in antioxidant activity for emulsions containing EGCG and caffeic acid. The incubation of BSA with the antioxidants in buffer showed that a considerable loss of tryptophan groups occurred during storage. The antioxidant molecule had clearly bound to the protein, since the separated protein product from stored BSA–antioxidant solutions showed a progressive increase in $ABTS^{\cdot+}$ scavenging with storage time for several days. All the antioxidants used in this study were polar water-soluble compounds,

TABLE 3
ABTS Radical Cation Scavenging Activity of Protein Fraction After Storage of BSA with Various Antioxidants in Buffer (pH 5.4) at 50 and 30°C[a]

	Time	Inhibition (%)			
		BSA–caffeic acid	BSA–EGCG	BSA–TBHQ	BSA–Trolox
Incubation at 50°C	3 h	1.10[a] (± 0.23)	2.85[b] (± 0.27)	1.54[a] (± 0.22)	0.42[c] (± 0.06)
	24 h	2.64[a] (± 0.18)	6.69[b] (± 0.54)	1.73[c] (± 0.25)	1.29[c] (± 0.54)
	5 d	9.19[a] (± 0.71)	9.28[a] (± 0.60)	5.99[b] (± 0.31)	2.68[c] (± 0.90)
Incubation at 30°C	24 h	1.02[a] (± 0.21)	4.25[b] (± 0.16)	1.22[a] (± 0.14)	0.69[a] (± 0.01)
	5 d	1.21[a] (± 0.11)	9.57[b] (±1.79)	3.91[c] (± 0.48)	1.41[a] (± 0.22)
	10 d	4.04[a] (± 0.13)	26.37[b] (± 4.36)	4.02[a] (± 0.51)	2.65[c] (± 0.24)

[a]Values with the same superscript letter in each row are not significantly different ($P > 0.05$). ABTS, 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt; for other abbreviations see Table 1.

and oil-in-water emulsions are known to be stabilized poorly by polar water-soluble antioxidants compared with less polar antioxidants such as tocopherols, which are present at the oil–water interface, where they are more effective (18). This is known as the polar paradox. Since BSA is known to be surface active, as shown by its ability to stabilize emulsions (8), the probable mechanism leading to the increase in antioxidant activity of emulsions containing BSA and antioxidant is that BSA binds the antioxidant and transports it to the oil–water interface, where it is highly effective at reducing the rate of oxidation. Since the protein fraction incorporating bound EGCG has radical-scavenging activity, it is clear that the binding of this compound still leaves free phenolic hydroxyl groups, which have antioxidant activity. EGCG has three neighboring hydroxyl groups on two aromatic rings, and it is likely that even if the hydroxyl groups on one of these rings are involved in binding to BSA, the hydroxyl groups on the second ring are still free to allow the molecule to act as an antioxidant. The lower radical-scavenging activity that the adduct reached following storage of BSA and EGCG at 50°C compared with 30°C shows that the protein–antioxidant adduct may lose scavenging ability, presumably by further loss of phenolic hydroxyl groups by oxidation or binding to a second amino acid side chain at the higher temperature. An additional effect of BSA may be to change the size of the oil droplets in the emulsion, but this was not determined. Any change in interfacial area would have an effect on the rate of oxidation and the interaction between water-soluble antioxidants and lipids, although the effect is expected to be small.

The nature of the binding of caffeic acid and Trolox molecules to BSA is not clear. The reduction of protein fluorescence indicates that binding of antioxidants to BSA occurs for both these antioxidants. Tyrosine-411 has been identified as the site for reversible binding of small, aromatic carboxylic acids to human serum albumin, which is >73% homologous with BSA (7). However, the BSA–antioxidant adduct formed in stored emulsions probably contains a covalent attachment of the antioxidant, since the radical-scavenging activity of stored BSA–antioxidant solutions increased progressively over several days. Involvement of the carboxylic acid group in binding to BSA would leave the *o*-diphenol moiety free to act as an antioxidant as long as the hydroxyl groups had not been oxidized. Similar binding of the carboxylic acid group of Trolox would leave the hindered phenolic hydroxyl group free to confer antioxidant properties on the BSA–Trolox adduct.

TBHQ was different from the other antioxidants. The combination of BSA with TBHQ at 50°C produced a BSA–antioxidant product that had some radical-scavenging activity according to the $ABTS^{\cdot+}$ test, but the combination did not provide increased stability of the emulsions. This highlights a limitation of the ABTS test. The ABTS test is a measure of radical scavenging of the $ABTS^{\cdot+}$, but it does not provide information about the propagation of oxidation in an emulsion by the radical formed from the antioxidant. Hence, unhindered phenols may be very effective in the ABTS test without being effective as antioxidants in retarding the oxidation of edible oils. Thus, the unhindered phenol, tyrosol, was similar to α-tocopherol in its ability to scavenge the $ABTS^{\cdot+}$ (19); however, tyrosol is not effective in stabilizing oils (20). Thus, the lack of antioxidant activity of TBHQ in emulsions containing BSA is due to the inability of the BSA–antioxidant adduct to retard the propagation of lipid oxidation.

Although degradation of BSA was monitored by the loss of tryptophan fluorescence, the amino acid(s) involved in binding the antioxidant to the protein are not known. Changes in conformation following binding to other amino acids may also reduce the fluorescence. Whether it is the antioxidant itself or an oxidation product such as a quinone that binds to the protein is also unclear.

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