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Changes in the antioxidant properties of protein solutions in the presence of epigallocatechin gallate

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Abstract

β-Casein and α-casein showed radical-scavenging activities in aqueous solution, whereas bovine serum albumin (BSA), α-lactalbumin and β-lactoglobulin showed much weaker antioxidant activity, when assessed by the 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) radical-scavenging assay. However, β-casein and α-casein showed reduced antioxidant activity after storage at 30 °C. An increase in radical-scavenging activity and a fall in fluorescence of the protein component were evident after 6 h, when BSA, β-lactoglobulin or casein were mixed with EGCG, and excess EGCG was removed, indicating the formation of a complex with this protein on mixing. Storage of all the proteins with EGCG at 30 °C caused an increase in the antioxidant activity of the isolated protein component after separation from excess EGCG. This showed that EGCG was reacting with the proteins and that the protein-bound catechin had antioxidant properties. The reaction of EGCG with BSA, casein and β-lactoglobulin was confirmed by the loss of fluorescence of the protein on storage, and the increase in UV absorbance between 250 and 400 nm. The increase in antioxidant activity of BSA after storage with EGCG was confirmed by the ferric reducing antioxidant potential (FRAP) and the oxygen radical antioxidant capacity (ORAC) assays.

Keywords: ABTS; Antioxidant; Epigallocatechin gallate; Protein

1. Introduction

Green tea is an important beverage with beneficial health attributes that have been described in many publications (Afaq & Mukhtar, 2002; Ahmad & Mukhtar, 1999; Ahmad & Mukhtar, 2001; Chung, Schwartz, Herzog, & Yang, 2003; Higdon & Frei, 2003). The antioxidant components in green tea include epigallocatechin gallate (EGCG), epicatechin gallate (ECG), epigallocatechin (EGC) and epicatechin (EC). All of these components have antioxidant properties but EGCG has been described as being a more active antioxidant than the other components (Graham, 1992). There is much information in the literature about antioxidant mechanisms and structural requirements for activity (Yanishlieva-Maslarova, 2001).

However, 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 there are several studies demonstrating that molecules with this structure may bind to proteins (Ivanov, Carr, & Frei, 2001; Kroll & Rawel, 2001; Oda, Kinoshita, Nakayama, & Kakehi, 1998; Wang & Goodman, 1999). Polyphenols may associate with proteins through hydrophobic interactions and hydrogen bonding (Oda et al., 1998) and several phenolic antioxidants have also been shown to bind to bovine proteins (Wang & Goodman, 1999). Green tea phenolics may be added to foods containing proteins and have been shown

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to interact with proteins (Arts et al., 2002; Salvi, Carrupt, Tillement, & Testa, 2001) although the data are limited.

Plant phenols have been shown to react with whey proteins at pH 9 (Kroll & Rawel, 2001) but such a high pH is not commonly encountered in foods. It is also known that red wine phenolic antioxidants bind to human lipoproteins in vivo, and protect them from metal-ion dependent and independent oxidation (Ivanov et al., 2001). Bovine serum albumin (BSA) can be derivatized by covalent attachment of quercetin and the antioxidant activity of the protein–phenol derivatives shows that the covalent attachment of quercetin to BSA decreases the total antioxidant activity in comparison to an equivalent amount of free quercetin, depending on the degree of derivatization (Rohn, Rawel, & Kroll, 2004).

Some proteins have been shown to exhibit antioxidant properties (Bourdon & Blache, 2001; Kouoh et al., 1999; Manzanares et al., 2001; Viljanen, Kivikari, & Heinonen, 2004) and EGCG has also been shown to cause a synergistic increase in the oxidative stability of oil-in-water emulsions in the presence of the minor whey protein BSA (Almajano & Gordon, 2004).

This study investigated the effect of EGCG on the antioxidant properties of solutions containing one of five proteins, namely BSA, α-lactalbumin, β-lactoglobulin, α-casein and β-casein.

2. Materials and methods

2.1. Chemicals

EGCG, BSA, α-lactalbumin, β-lactoglobulin, α-casein, β-casein, ferric chloride, potassium persulfate, sodium fluorescein and 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), tripyridyltriazine (TPTZ) were purchased from Sigma–Aldrich Company Ltd. (Gillingham, UK). Casein was isolated from milk in our laboratory.

2.2. Incubation of EGCG with BSA at 30 °C and isolation of protein from the solution

In order to study antioxidant binding by proteins, solutions of each protein (BSA, α-lactalbumin, β-lactoglobulin, α-casein, β-casein) containing protein (0.2% w/w) and antioxidant (0.5 mM) in distilled water, were incubated at 30 °C. Samples were removed at 0 h, 6 h, 1 day, 3 days and 7 days. The samples were stored at −20 °C before investigation.

Before any determination, free EGCG (not bound to the protein) was removed by passing the solution (1 ml) through a Bio-Spin P-6 column (Bio-Rad, Richmond, CA) equilibrated with distilled water. The protein solution, which contained unreacted protein and protein–antioxidant adducts, was separated from EGCG by elution with water. After column calibration, the protein solution (2.7 ml) was collected. Solutions with each protein were stored in triplicate and duplicate determinations were performed for each sample.

For the samples with BSA, casein and β-lactoglobulin, the fluorescence due to the tryptophan groups was determined by excitation at 280 nm with emission at 331 nm (Perkin–Elmer LS 3B, Beaconsfield, UK).

Antioxidant activity was determined by two different methods, namely the ABTS radical-scavenging assay and the oxygen radical absorbance capacity (ORAC) method and expressed as trolox equivalent antioxidant capacity in both cases (TEAC value). The reducing potential of the adduct with BSA was also determined by the ferric reducing antioxidant potential (FRAP) method.

2.3. ABTS radical-scavenging assay

2.3.1. Preparation of the $ABTS^{\bullet+}$

2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) and potassium persulfate (7 mM ABTS and 2.45 mM potassium persulfate final concentration) were used to prepare an $ABTS^{\bullet+}$ solution in phosphate-buffered saline (PBS) (pH 7.4) as described by Re et al. (1999). The $ABTS^{\bullet+}$ was prepared 24 h before use. Just before use, the solution was diluted with PBS (pH 7.4, 1:100) to an absorbance of 0.7 (±0.02) at 734 nm (Perkin–Elmer UV/Vis Lambda Bio 20) in a 1 cm cuvette and equilibrated at 30 °C.

The protein, including protein–EGCG adduct, was separated by gel filtration as a 0.07% solution, and 20 μl of the solution was added to $ABTS^{\bullet+}$ solution (2 ml) (dilution factor 1:100). PBS (pH 7.4) was used as blank. After mixing, the absorbance at 734 nm was measured immediately, and then every minute for 7 min. Duplicate determinations were made for samples, which were stored in triplicate. 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:

$$\Delta A_{(\text{sample})} = \frac{A_{t=0(\text{sample})} - A_{t=5(\text{sample})}}{A_{t=0(\text{sample})}} - \frac{A_{t=0(\text{solvent})} - A_{t=5(\text{solvent})}}{A_{t=0(\text{solvent})}}.$$

Percent inhibition values were obtained by multiplying ΔA_{sample} values by 100. The values were calculated as trolox equivalents (TEAC values).

2.3.2. ORAC

The procedure was based on a previous report of Wang, Cao, and Prior (1995). In the final assay mixture (3.00 ml total volume), sodium fluorescein (7.0×10^{-8} M) was used as the target for free radical damage, AAPH (17 mM) was used as a peroxyl radical generator, and trolox was used as a control antioxidant standard. The test

components were added to the assay mixture at 1:60 dilution. The fluorescence was measured every 5 min at an excitation wavelength of 493 nm and an emission wavelength of 513 nm after the addition of AAPH. The final ORAC values were calculated by using a regression equation relating trolox concentration and the net area under the fluorescein decay curve. The relative ORAC values (trolox equivalents) were calculated as the ORAC value relative to trolox (20 μM)

$$\text{Relative ORAC value} = 20 * K\frac{\text{AUC}_{\text{sample}} - \text{AUC}_{\text{blank}}}{\text{AUC}_{\text{trolox }20} - \text{AUC}_{\text{blank}}},$$

where K is the dilution factor.

2.3.3. FRAP assay

The FRAP assay was performed as previously described (Benzie & Strain, 1996). The FRAP reagent was freshly prepared each day by mixing together 10 mM TPTZ and 20 mM iron(III) chloride in 0.25 M acetate buffer, pH 3.6. The test solution (100 μl) was added to 3 ml of FRAP reagent, and the absorbance was read at 593 nm (Perkin–Elmer UV/Vis Lambda Bio 20), after incubation at room temperature for 15 min, using the FRAP reagent with distilled water as a blank. Data were expressed relative to values obtained for trolox and expressed as trolox equivalents.

2.4. Statistical analysis

Antioxidant capacity by the ABTS assay, ORAC value and FRAP value and the fluorescence determinations were analyzed by one-way analysis of variance (ANOVA) to determine the pooled standard deviation. The mean values within each test were compared by a two-sample t-test by using the pooled standard deviation to determine significant differences ($p < 0.05$).

3. Results and discussion

The antioxidant activities of five proteins, namely BSA, β-lactoglobulin, α-lactalbumin, β-casein and α-casein, were determined by the ABTS radical scavenging assay. β-Casein and α-casein showed strong radical-scavenging activities in aqueous solutions, whereas BSA, β-lactoglobulin and α-lactalbumin showed much weaker antioxidant activities (Table 1). The antioxidant activity of casein has been previously reported (Hu, McClements, & Decker, 2003). However, the antioxidant activities of β-casein and α-casein were markedly reduced after storage in water at 30 °C. The antioxidant activity of α-casein fell rapidly from 18% ABTS inhibition to <4% inhibition within 24 h, whereas the activity of β-casein fell more slowly but inhibition was <4% within 7 days. The reason for the change in antioxidant activity of casein on storage is not clear. The caseins contain a highly solvated, flexible charged domain and a hydrophobic globular domain. The flexible characteristics of the charged domain cause the dimensions of the molecule to be sensitive to ionic strength and pH. Casein micelles are composed of sub-micelles, which tend to dissociate when the environmental conditions change. Changes in the polar region of the casein molecule, as a consequence of change of pH, would have a big effect in the ABTS assay, since cations would strongly repel the positively charged ABTS radical, and steric hindrance toward approach of the radical to a reactive site on the protein may also change. Storage of the proteins with EGCG at 30 °C caused an increase in the antioxidant activity of the isolated protein fraction after separation from free antioxidant for all proteins studied (Fig. 1). Arts et al. (2002) reported that EGCG, mixed with several proteins, caused a reduction of antioxidant activity from the sum of the values determined separately for EGCG and the protein. In our study, the separation of the free EGCG from the protein revealed that the EGCG had bound to the protein. The protein-bound catechin was contributing to the antioxidant properties, but this is consistent with the finding of Arts et al. (2002) because the adduct had less antioxidant activity than the free EGCG. The antioxidant activity of the protein component isolated from mixtures of protein with EGCG was in the order: α-casein^{a} > β-casein^{b} > BSA^{c} > β-lactoglobulin^{c,d} > α-lactalbumin^{d} before storage but the antioxidant activity of all five proteins increased strongly during storage (Fig. 1). This indicated that the reaction of EGCG with proteins progressed during storage.

Some proteins fluoresce due to the presence of tryptophan, and the fluorescences of BSA, casein and β-lactoglobulin were studied during storage with EGCG to gain information about structural changes in the region of the tryptophan group. The proteins retained their fluorescence when stored in the absence of EGCG, but protein isolated from mixtures with EGCG showed falls in fluorescence before storage that increased during storage for each of the three proteins (Table 2). The fall in fluorescence for mixtures of EGCG stored with protein for 24 h was 90% for casein, 56% for BSA and 29% for β-lactoglobulin. In

Table 1
Antioxidant activity assessed by % inhibition of the ABTS radical by protein solutions during storage at 30 °C

	0	6 h	1 day	3 day	7 day
BSA	4.7^{a} (±0.7)	4.2^{a} (±1.0)	5.2^{a} (±1.1)	5.1^{a} (±1.5)	4.9^{a} (±1.2)
β-Lactoglobulin	3.2^{ab} (±0.7)	3.6^{ab} (±0.9)	3.7^{ab} (±1.0)	4.3^{a} (±1.2)	5.0^{a} (±1.1)
α-Casein	17.0^{c} (±1.7)	17.5^{c} (±1.3)	3.0^{b} (±0.7)	2.4^{b} (±0.9)	1.8^{b} (±1.0)
β-Casein	14.3^{d} (±2.8)	12.0^{d} (±2.0)	6.0^{a} (±1.5)	4.2^{a} (±0.8)	2.4^{b} (±0.9)
α-Lactalbumin	2.5^{b} (±0.7)	2.0^{b} (±0.9)	1.8^{b} (±0.6)	1.6^{b} (±0.9)	1.4^{b} (±0.5)

Samples with different superscripts were significantly different ($P < 0.05$).

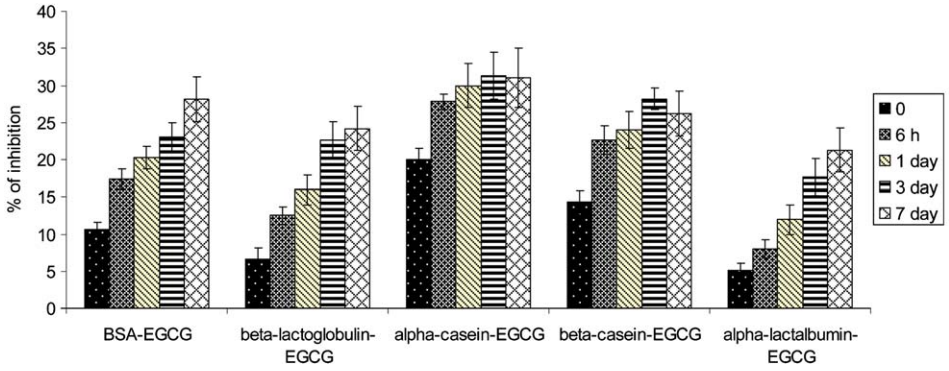

Fig. 1. Antioxidant activity assessed by % inhibition of the ABTS radical by protein–antioxidant complexes separated from free EGCG following storage of protein with EGCG at 30 °C.

Table 2
Fluorescence of protein solutions stored with and without protein at 30 °C

Time of storage (incubation)	0 h	6 h	24 h	3 day	7 day
β-Lactoglobulin	230[a] (±20)	222[a] (±20)	232[a] (±22)	230[a] (±18)	226[a] (±20)
β-Lactoglobulin plus EGCG	220[a] (±12)	168[b] (±9)	119[c] (±5)	51[d] (±5)	15[e] (±3)
BSA	285[f] (±17)	283[f] (±25)	283[f] (±30)	287[f] (±28)	285[f] (±38)
BSA plus EGCG	270[f] (±15)	170[g] (±12)	74[h] (±9)	28[i] (±4)	11[j] (±2)
Casein (total)	430[k] (±29)	427[k] (±45)	400[k] (±40)	443[k] (±50)	379[k] (±40)
Casein (total) plus EGCG	380[k] (±18)	210[l] (±12)	22[m] (±5)	12[m] (±3)	0[n]

Samples with different superscripts were significantly different ($P < 0.05$).

each case, the free antioxidant was separated from the protein before the determination of fluorescence, so it is clear that the antioxidant was chemically bound to the antioxidant. Fluorescence continued to decrease during the 7 day storage period. An increase in UV absorbance between 250 and 400 nm for the protein component after storage with EGCG was demonstrated using BSA (Fig. 2) and this provided further evidence for the binding of the antioxidant to the proteins, since EGCG absorbs at 280 nm. For all proteins, it was clearly shown that the binding of EGCG during storage caused a progressive increase in the antioxidant activity of the protein, which was confirmed by the FRAP and the ORAC assays for BSA–EGCG mixtures. The FRAP value of the BSA–EGCG adduct increased from 100 to 350 μM trolox equivalents during storage (Fig. 3), and the ORAC values increased from 2.6 to 5.1 mM trolox equivalents (Fig. 4). The FRAP assay is measuring the ferric reducing potential, and the ORAC assay is measuring the scavenging of azoperoxy radicals, so it is not surprising that the measured activity differs for the two methods. However, both methods confirm the binding of EGCG to protein. The product formed immediately upon mixing is likely to be due to non-covalent bonding in the complex, but the progressive increase on storage indicates, either that complex formation increases progressively or, more likely, that covalent bonding occurs during storage. The nature of the complex or adduct formed between proteins and antioxidants on storage has not been identified in this paper, but it has been shown that the fluorescence due to tryptophan decreases when proteins are

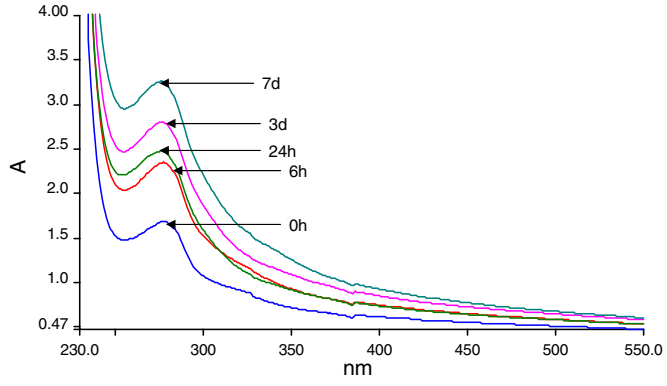

Fig. 2. Change in the absorbance at 280 nm for the protein isolated from mixtures of BSA with EGCG during storage at 30 °C.

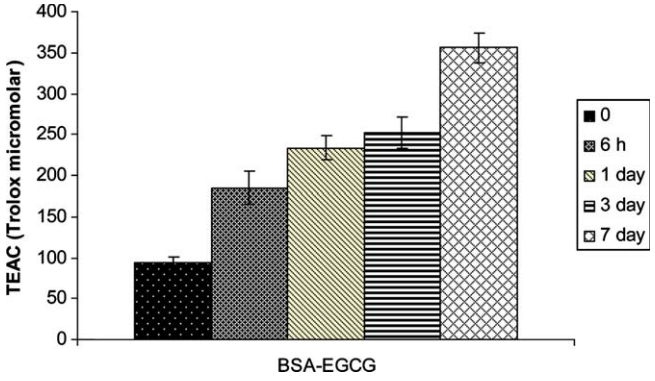

Fig. 3. Change in antioxidant activity in trolox equivalents assessed by the FRAP assay for the protein isolated from mixtures of BSA with EGCG during storage at 30 °C.

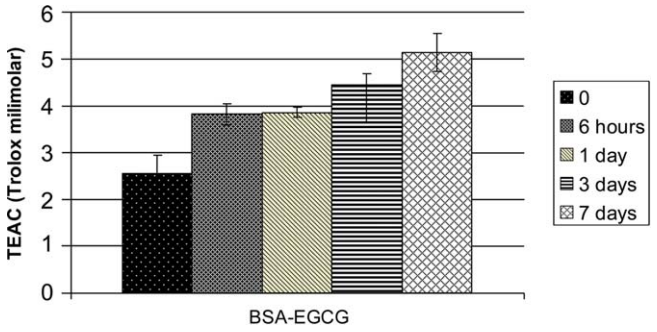

Fig. 4. Change in antioxidant activity in trolox equivalents assessed by the ORAC assay for the protein isolated from mixtures of BSA with EGCG during storage at 30 °C.

stored with EGCG. The occurrence of an oxidation reaction was confirmed by storing antioxidant with BSA under nitrogen, when the decrease in fluorescence was strongly reduced (data not shown). It is known that *o*- or *p*-diphenols are oxidised to quinones, which react with lysine and tryptophan side chains in proteins (Rawel, Kroll, & Hohl, 2001). Since EGCG contains phenolic hydroxyl groups in three rings, loss of aromaticity in one of the rings on oxidation to a quinone and binding to protein would still leave phenolic hydroxyl groups available with antioxidant properties in the protein–EGCG adduct.

4. Conclusions

Mixing of BSA, β-lactoglobulin, α-lactalbumin, β-casein and α-casein with EGCG at 30 °C caused the formation of an adduct with antioxidant activity. The antioxidant activity of the protein component was in the order: α-casein^{a} > β-casein^{b} > BSA^{c} > β-lactoglobulin^{c,d} > α-lactalbumin^{d} before storage. The antioxidant activity of the protein component, which included both unmodified protein and the protein–EGCG adduct, increased with storage time at 30 °C.

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