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Interactions between Flavonoids and Proteins: Effect on the Total Antioxidant Capacity

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Flavonoids are potent antioxidants. It is also known that flavonoids bind to proteins. The effect of the interaction between tea flavonoids and proteins on the antioxidant capacity was examined. Their separate and combined antioxidant capacities were measured with the Trolox equivalent antioxidant capacity (TEAC) assay. It was observed that the antioxidant capacity of several components of green and black tea with α-, β-, and κ-casein or albumin is not additive; that is, a part of the total antioxidant capacity is masked by the interaction. This masking depends on both the protein and the flavonoid used. Components in green and black tea, which show the highest masking in combination with β-casein, are epigallocatechin gallate and gallic acid. The results demonstrate that the matrix influences the efficacy of an antioxidant.

KEYWORDS: Antioxidant capacity; tea; flavonoids; casein; albumin; catechin

INTRODUCTION

The intake of flavonoids has been associated with lower incidence of various diseases such as cancer, stroke, and cardiovascular diseases (*1*−*3*). The positive health effects of these secondary plant metabolites are probably related to their strong antioxidative properties (*4*, *5*). Tea, the second most commonly consumed beverage in the world after water, is rich in flavonoids (*6*). Tea is produced predominantly in two forms, both originating from *Camellia sinensis*: green tea, which is prepared from the fresh tea leaf and contains mainly catechins, and black tea, which contains mainly thearubigins and theaflavins due to an extra enzymatic oxidation step during manufacturing (*7*).

The way tea is consumed varies among populations. In the United Kingdom, Ireland, and Canada tea is consumed with a substantial amount of milk in it (*8*). Milk contains various proteins, and interactions between polyphenols and proteins have been reported (*9*, *10*). The question arises as to what the effect of such an interaction on the antioxidant capacity is. The aim of the present study is to determine the effect of the interaction between flavonoids and proteins on the total antioxidant capacity [Trolox equivalent antioxidant capacity (TEAC)].

MATERIALS AND METHODS

Chemicals. 2,2′-Azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), bovine serum albumin (BSA fraction V), and α-, β-, and κ-casein (from bovine milk) were obtained from Sigma. The tea and tea fractions were gifts from Lipton (Englewood Cliffs, NJ) except epicatechin, which was from Aldrich (Zwijndrecht, The Netherlands). All other chemicals were of analytical grade purity.

TEAC Assay. To measure the antioxidant capacity, the TEAC assay, described by Re et al. (*11*), has been used with minor modifications. This method is based on the reaction of the blue/green stable ABTS radical ($ABTS^{\bullet}$), which is formed through the reaction between ABTS and $K_2O_8S_2$, with antioxidants. In the reaction with an antioxidant, the blue/green color disappears because the $ABTS^{\bullet}$ reacts with the antioxidant. This decolorization is determined spectrophotometrically at 734 nm after 30 min. The reduction in absorbance is related to that of Trolox, a synthetic, hydrophilic vitamin E analogue, which gives the TEAC value. The TEAC is calculated as moles of Trolox equivalents per gram of solid. The relative contribution of an ingredient to the TEAC of tea is calculated from the TEAC of this ingredient multiplied by its relative content (%w/w) and divided by the TEAC of tea.

Masking of total antioxidant capacity is, as in Arts et al. (*10*), defined as percent difference between the measured increase in antioxidant capacity due to addition of an antioxidant compared to the calculated increase based on an additive effect at $t = 30$ min.

Stock solutions of all tea components and Trolox were prepared in ethanol. BSA and the caseins were dissolved in Milli-Q water. The antioxidant and the protein were added to phosphate-buffered saline [(PBS), a 0.01 M phosphate buffer, pH 7.4, containing 154 mM NaCl] buffer and incubated for 10 min at 37 °C. For the determination of the antioxidant capacity, a concentrated $ABTS^{\bullet}$ solution was added, resulting in an absorbance of the blank between 0.68 and 0.72. Immediately after the addition of the $ABTS^{\bullet}$ solution to the incubation mixture, the absorbance was followed in time. Experiments were performed on a Perkin-Elmer spectrophotometer with cell changer fitted

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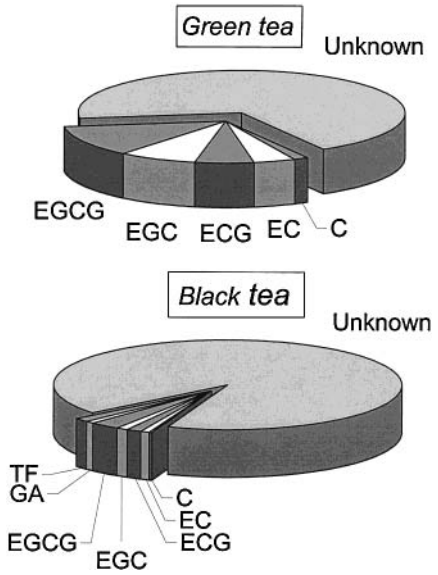

Figure 1. Relative content of several ingredients in green and black tea. The values, expressed as percentage (w/w) in, respectively, green and black tea, are as follows: catechin (C), 1.3 and 0.3%; epicatechin (EC), 4.2 and 0.8%; epicatechin gallate (ECG), 5.8 and 1.4%; epigallocatechin (EGC), 7.9 and 1.2%; epigallocatechin gallate (EGCG), 11.8 and 2.8%; gallic acid (GA), 0.2% (not shown) and 0.8%. The theaflavin (TF) content of black tea is 1.2%, whereas this type of compound is not present in green tea.

with Peltier temperature control. The final concentration of tea and tea components was 1 μg/mL for green tea and the mixed theaflavins and 0.75 μg/mL for black tea. The final concentration of the proteins (α, β, and κ-casein and albumin) was 25 μg/mL. The results are expressed as mean ± SD

Proline Ratio. The amino acid sequences of the different caseins are presented in the Swissprot database [by the Swiss Institute of Bioinformatics (SIB) and the European Bioinformatics Institute (EBI), Switserland]. The proline ratio of a protein was calculated by dividing the total number of proline groups by the total number of amino acids of the protein.

RESULTS

The composition of the freeze-dried green and black tea extracts used in this study is given in **Figure 1**. In the production of black tea, most catechins are oxidized, giving other types of polyphenols such as theaflavins and thearubigins (*7*). Therefore, the catechin content of green tea is higher than that of black tea. The oxidation is probably also the reason for the higher antioxidant capacity (7.3 mmol of TEAC/g of freeze-dried tea) of green tea compared to black tea (5.8 mmol of TEAC/g of freeze-dried tea).

Figure 2 shows the relative contribution of several ingredients to the TEAC values of green and black tea. It can be calculated that the antioxidant capacity of green tea is 93% due to catechins. Other tea components that make up 69% of the total weight hardly contribute to the antioxidant capacity (7%). In black tea, only a small part of the antioxidant capacity is due to catechins (34%). The other tea components in black tea, which make up 91.5% of the total weight, have a much higher contribution to the TEAC value (66%) than they have in green tea. Probably tannins, polymers of oxidized polyphenols (*9*), have a significant contribution to the antioxidant capacity of black tea. These tannins are not present in green tea. The relative contribution of the various components of green tea on the total antioxidant capacity is in accordance with the results previously reported by Rice-Evans (*12*).

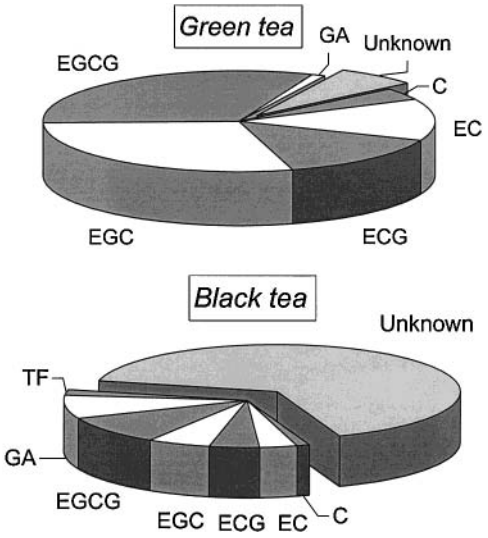

Figure 2. Relative contribution of the polyphenols to the TEAC of green and black tea. The values are as follows: catechin (C), 3.9 and 1.2%; epicatechin (EC), 13.4 and 3.2%; epicatechin gallate (ECG), 14.5 and 4.5%; epigallocatechin (EGC), 28.8 and 5.6%; epigallocatechin gallate (EGCG), 31.6 and 9.6%; gallic acid (GA), 1.2 and 8.0%. Theaflavins (TF) contribute 2.0% to the TEAC black tea. TEAC values of green and black tea are 7.3 and 5.8 mmol of TEAC/g, respectively.

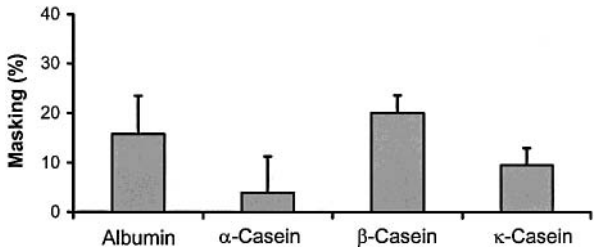

Figure 3. Masking of the antioxidant capacity of catechin with different proteins.

The effect of the interaction between flavonoids and proteins on the total antioxidant capacity was examined. It was observed that addition of catechin to β-casein increases the antioxidant capacity of the β-casein solution, but the increase is smaller than the antioxidant capacity of catechin itself. Twenty percent of the antioxidant capacity was masked (**Figure 3**).

This masking has been determined for four different proteins in combination with catechin (**Figure 3**). The masking of the antioxidant capacity of catechin was most pronounced with albumin (21.1 ± 4.1%) and β-casein (20.0 ± 3.5%), whereas the masking with α- and κ-casein was less. The high masking observed with β-casein compared to α- and κ-casein can be explained by the relatively large amount of proline groups in the protein. Proline groups have a strong affinity for the hydroxyl groups of catechin (*13*). β-Casein, which consists of 224 amino acids of which 35 are prolines, has a proline ratio of 0.156. The proline ratios of α-casein and κ-casein are 0.075 and 0.105, respectively. The rank order of masking observed with these three proteins is identical to the rank order of the proline ratio of the proteins, indicating that the proline groups are indeed involved in the masking.

Masking not only depends on the protein, it also greatly depends on the flavonoid (**Figure 4**). Different tea components display different extents of masking with β-casein. A gallate group coupled to the flavonoid on position 3 increases the extent of masking (epicatechin gallate, 30.0 ± 2.3%); however, a 5′-OH group, converting ring B into a pyragollol group similar to

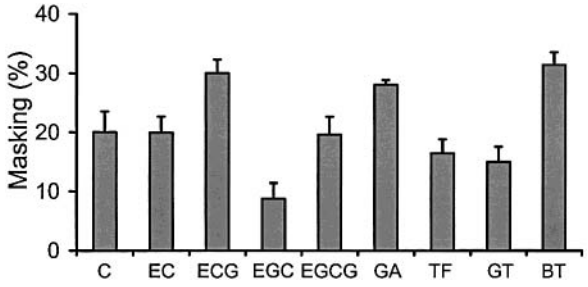

Figure 4. Masking of tea and several tea fractions with β-casein. Tea components examined are catechin (C), epicatechin (EC), epicatechin gallate (ECG), epigallocatechin (EGC), epigallocatechin gallate (EGCG), gallic acid (GA), theaflavonoids (TF), green tea (GT), and black tea (BT).

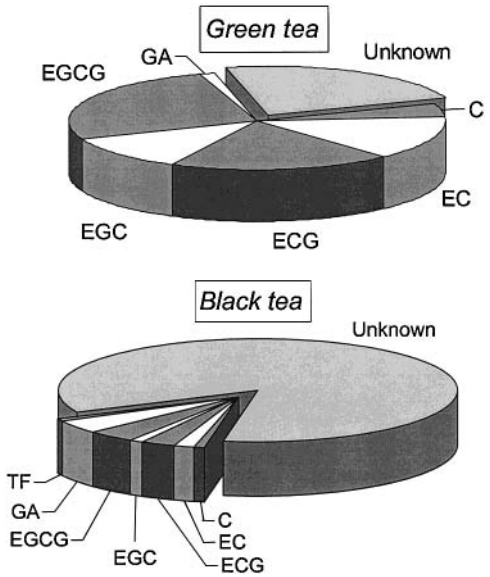

Figure 5. Relative contribution of the polyphenols to the masking of green and black tea with β-casein. The values are as follows: catechin (C), 4.5 and 0.7%; epicatechin (EC), 14.5 and 1.7%; epicatechin gallate (ECG), 19.1 and 2.9%; epigallocatechin (EGC), 11.4 and 1.1%; epigallocatechin gallate (EGCG), 26.2 and 4.0%; gallic acid (GA), 1.2 and 4.2%. The contribution of theaflavins (TF) to the masking of black tea is 0.6%.

that in gallate, decreases the extent of masking (epigallocatechin, 8.8 $\pm$ 2.6%). In epigallocatechin gallate, containing both a gallate group at position 3 and a pyragollol group in ring B, the opposite effect of both structural modifications appears to level out. The masking of the antioxidant capacity of green and black tea by β-casein is, respectively, 14.1 $\pm$ 2.6 and 31.4 $\pm$ 2.1%.

The relative contributions to the masking of green and black tea by the interaction of different tea components with β-casein can be calculated on the basis of the relative abundance of the components and their masking (**Figure 5**). Possible synergistic or antagonistic interactions between the components that may occur are not incorporated in this estimation. The masking of green tea can for almost 80% be explained by the masking of catechins in the tea. In black tea these catechins play a much smaller role (16%). In green tea almost half of the masking is due to epigallocatechin gallate and epicatechin gallate. In black tea gallic acid has the highest contribution of the compounds that were examined.

DISCUSSION

The aim of the present study is to determine the effect of the interaction between flavonoids and proteins on the total antioxidant capacity. It was found that the antioxidant capacity of a mixture of flavonoids and proteins is less than the sum of the antioxidant capacity of flavonoid and protein separately. The degree of this masking depends on both the type of polyphenol and the type of protein.

The result of this masking is in the first place that the antioxidants do not reach their optimum scavenging capacity in the matrix. For example, addition of milk to tea is expected to reduce the antioxidant capacity of the antioxidants present in tea. This might also occur in other products containing both polyphenols and proteins. The decreased antioxidant capacity is expected to result in a faster oxidation of the product.

Another consequence of the interaction with proteins might be a decreased bioavailability of the antioxidants. The interaction, resulting in protein−polyphenol complexes, can be both reversible and irreversible depending on pH, temperature, and protein and flavonoid concentrations (*13*). The fate of these complexes in the gastrointestinal tract is not known. Serafini et al. found that addition of milk to black tea abolishes the increase of the antioxidant potential observed when tea is consumed without milk (*14*). However, other studies show no or no relevant effect of proteins on the bioavailability or on the antioxidant capacity of tea polyphenols (*4*, *15*).

After absorption in the gastrointestinal tract, the antioxidants enter the bloodstream. Here the matrix also affects the antioxidant capacity. Binding to albumin results in a substantial masking of the antioxidant capacity of flavonoids (*10*). Recently, van de Berg et al. reported that the antioxidant capacity correlates well with physiological processes such as protection against lipid peroxidation (*16*). Binding also decreases the free concentration of the antioxidant. This means that in addition to the intrinsic activity of a compound, bioavailability and metabolism, the binding to proteins also has to be considered for the efficacy of a flavonoid in vivo.

In conclusion, the interaction of flavonoids with proteins affects the antioxidant efficacy of the flavonoids. Although the effect on the bioavailability is not equivocal, the interaction will reduce the antioxidant capacity of the flavonoids both in products and in vivo. Apparently, the matrix influences the efficacy of antioxidants.

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