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Antioxidant Activity of Protein-Bound Quercetin

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Bovine serum albumin (BSA) was derivatized by covalent attachment of different amounts of quercetin [ratios of BSA to quercetin were 20:1, 10:1, 7:1, 5:1, and 2:1 (w/w)]. The antioxidant activity of the protein−phenol derivatives was investigated using a modified TEAC assay. The results show 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. The derivative with the highest amount of covalently bound quercetin (the 2:1 derivative) showed an antioxidant activity of only 79% compared to an equivalent amount of free quercetin. After the enzymatic proteolysis of the BSA−quercetin derivatives with trypsin, the total antioxidant activity of the degradation products increases in comparison to the respective undigested derivatives but does not reach the activity of an equivalent amount of free quercetin. Even after 240 min of tryptic degradation, there is still a lack in antioxidant activity (for the 7:1 derivative nearly 33%) as compared to free quercetin.

KEYWORDS: Quercetin; bovine serum albumin; protein derivatization; antioxidant activity

INTRODUCTION

In recent years, antioxidants have been the subject of many epidemiological studies that have related consumption of fruits and vegetables with a reduction in the incidence of cardiovascular diseases and several cancers, whereby especially (cereal) fibers and phenolic compounds play a very important role (*1*−*4*). The beneficial effects of the phenolic compounds (contained in high amounts in all kinds of plant foods) are thought to result from their ability to scavenge reactive oxygen and nitrogen species, which are formed in high amounts during intracellular oxidative stress induced by the extraneous attack of prooxidants (*5*, *6*). However, little attention has been directed to interactions of the phenolic compounds with the other components also present in food or in the organism (e.g., proteins), which may be in position to influence the antioxidant acitivity. Different works have discussed a "masking" (diminution) of antioxidant activity of flavonoids that may result from interactions with proteins (*7*−*9*), but no specific characterization of the protein−phenol complexes has been performed. In recent investigations it was shown that proteins and phenolic compounds may interact on the basis of non-covalent interactions such as π-bonding, hydrogen bonding, and hydrophobic or ionic interactions (*10*, *11*). Besides these observations, it has been assumed that covalent bonding is likely to play an important role in protein−phenol interactions (*12*).

In model systems using different food proteins [bovine serum albumin (BSA), myoglobin, lysozyme, whey proteins, soy proteins] and enzymes (α-amylase, bromelain, chymotrypsin, lysozyme, trypsin) in combination with phenolic and related compounds (quinic, ferulic, caffeic, chlorogenic, and gallic acid, resorcinol, pyrocatechol, hydroquinone, *p*-benzoquinone, flavone, apigenin, kaempferol, quercetin, rutin, myricetin, selected natural and synthetic isoflavones) it was proved that the mentioned covalent bondings take place (*13*−*15*). The main reactive residues of proteins are the nucleophilic side chains such as lysine, tryptophan, and cysteine (*13*). The formation of quinoid structures, resulting from an oxidation of the phenolic compounds, is the key step for the subsequent reactions. These highly reactive quinones are capable of undergoing covalent reactions with the nucleophilic side chains (*13*). The effect of a derivatization of food proteins as a result of the covalent attachment of phenolic compounds leads to significant changes in selected physicochemical properties and in the structure of the investigated proteins, the latter in turn influencing the activity of enzymes (*13*).

The possible influence of covalent bonds between proteins and phenolic compounds on the antioxidative properties of the latter was not considered, and this should be of great interest from a physiological point of view. In recent publications we have shown that covalent reactions between BSA and phenolic compounds do occur (*13*, *14*). Therefore, the aim of the present study was to investigate the antioxidant activity of covalent protein-bound quercetin (with BSA as a model protein), including the influence of a varying amount of this most common flavonoid. Different degrees of derivatization were tested with regard to their effect on the antioxidant activity, determined with a modified Trolox equivalent antioxidant capacity (TEAC) assay.

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MATERIALS AND METHODS

Materials. Bovine serum albumin (1 g, Fluka Chemie AG, Buchs, Switzerland) was dissolved in distilled water, and the pH value of the protein solution was adjusted to 9 using 0.5 M NaOH (final volume = 95 mL). Five BSA−quercetin derivatives were prepared with ratios (w/w) of 20:1, 10:1, 7:1, 5:1, and 2:1 (equivalent to molar ratios of 1:10, 1:20, 1:28, 1:40, and 1:99). In each case after the addition of quercetin solution (Fluka Chemie AG; each in 5 mL of ethanol), the pH was adjusted once more to 9. After 24 h of reaction time under continuous stirring at room temperature with free exposure to air, the samples were dialyzed for 18−20 h against water and finally lyophilized. A nonderivatized control was prepared under the same conditions but without addition of phenolic compound.

Trypsin from porcine pancreas (EC 3.4.21.4, protein content = 98%) was obtained from Sigma Chemical Co., St. Louis, MO (14900 units/mg of solid, one BAEE unit = ΔA_{253} of 0.001/min with BAEE as substrate at pH 7.6 at 25 °C).

For determination of the degree of derivatization and in order to calculate the amount attached covalently to BSA, quercetin was used to calibrate the regression curve by using its absorption at 325 nm ($Y = 6E-07x$; $R^2 = 0.9597$). This analysis was performed by applying RP-HPLC as described in detail by Rawel et al. (*14*).

Proteolytic Digestion of the BSA Quercetin Derivatives. The samples were dissolved in 0.02 M $CaCl_2$ (final concentration = 36 mg of derivative/9 mL). Tryptic hydrolysis (450 μL of enzyme, 1 mg/mL) of BSA, as well as of its derivatized products, was investigated by incubation at 35 °C (enzyme/substrate ratio, E:S, = 1:80). After different durations of hydrolysis (0, 5, 15, 30, 45, 60, 120, and 240 min), 700 μL was removed from the incubation mixture and heated to 100 °C for 10 min. After cooling, 500 μL of trifluoroacetic acid (0.5%) was added to 500 μL of the mixture, allowed to stand for 10 min, and then centrifuged at 9088*g* for 10 min (Megafuge 2.0R, Heraeus, Hanau, Germany). The remaining trichloroacetic acid (TCA) soluble peptides in the supernatant were analyzed by RP-HPLC, which was performed on a Micra-NPS-C18 column (33 × 4.6 mm, 1.5 μm, flow rate = 0.5 mL/min, UV detection at 220 nm) with a column temperature of 25 °C using a Jasco chromatographic system (Gross-Umstadt, Germany). A distilled water/acetonitrile (water acidified with 0.1% trifluoroacetic acid; v/v) gradient was applied under the following conditions: 100% water, 2 min; 0−70% acetonitrile, 10 min; 70% acetonitrile, 4 min; 70% acetonitrile, 100% water, 4 min; 100% water, 10 min (regeneration/equilibration). The injection volume of the samples was 20 μL. The total peak area of the TCA-soluble peptides was used to quantify the extent of enzymatic digestion. Another 100 μL of the removed and heated incubation mixture was, after cooling, directly used for the TEAC assay.

Determination of the Total Antioxidant Activity. The antioxidant activity of the BSA−quercetin derivatives and the degradation products of the proteolytic digestion were determined in vitro with a modified TEAC assay. The TEAC assay, originally described by Miller et al. (*16*), is based on scavenging of long-lived radical anions ($ABTS^{\bullet}$). In this assay the radicals are generated by potassium persulfate and can easily be detected spectrophotometrically at 734 nm (*17*). Trolox (a water-soluble vitamin E analogue) is used as an antioxidant standard. The modification used here was to measure absorbance exactly 6 min after the reaction was started (addition of persulfate to a mixture of ABTS and sample). The exact time period between starting the reaction and measuring the absorbance is very important because of the stoichiometric reaction of ABTS and persulfate, which leads to an incomplete, time-dependent formation of the ABTS radical.

Statistical Analysis. The determination of the total antioxidant activity was repeated five times, and standard deviation was calculated. A maximum of ±5% standard deviation from the averaged values was generally obtained. The averaged values with respective standard deviation are documented in the respective figures. Student's *t* test was applied to evaluate the significance of the results.

RESULTS AND DISCUSSION

Influence of BSA Derivatization on Total Antioxidant Activity. Different concentrations of quercetin were applied to produce various degrees of BSA derivatization [ratios (w/w) of BSA to quercetin were 20:1, 10:1, 7:1, 5:1, and 2:1, equivalent to molar ratios of 1:10, 1:20, 1:28, 1:40, and 1:99]. With a higher concentration of the quercetin present in the reaction mixture, the formation of the reactive quinones is elevated. **Table 1** illustrates the amounts of quercetin bound covalently to BSA.

Table 1. Amount of Covalently Bound Quercetin in the BSA–Quercetin Derivatives

derivative	quercetin covalently bound [μg of quercetin/mg of protein]
control	
BSA–quercetin 20:1	7.9
BSA–quercetin 10:1	11.2
BSA–quercetin 7:1	12.2
BSA–quercetin 5:1	15.0
BSA–quercetin 2:1	17.5

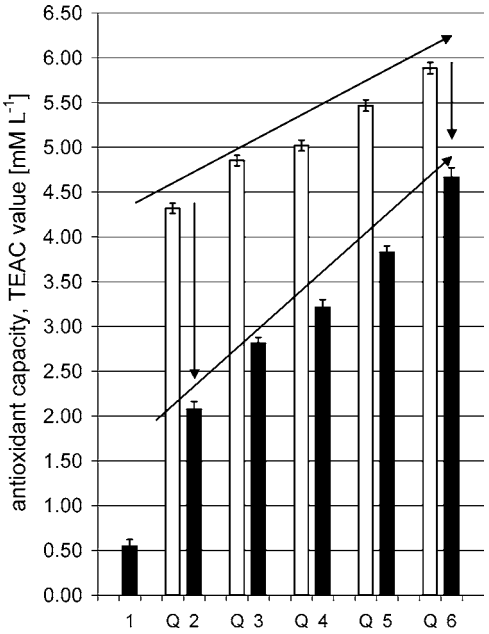

Figure 1. Total antioxidant acitivity of free quercetin and the BSA–quercetin derivatives (TEAC value in mM L^{-1}): 1 = underivatized BSA (control); 2, BSA–quercetin derivative 20:1; 3, BSA–quercetin derivative 10:1; 4, BSA–quercetin derivative 7:1; 5, BSA–quercetin derivative 5:1; 6, BSA–quercetin derivative 2:1; Q, equivalent amount(s) of free quercetin; arrows illustrate the trend in antioxidant activity with increasing degree of derivatization.

As a consequence of the derivatization (covalent binding of quercetin to BSA) the antioxidant activity of quercetin is decreased significantly (**Figure 1**). Nevertheless, the BSA−quercetin derivatives retain, depending on the degree of derivatization, an antioxidant activity. The derivative with the highest amount of covalently bound quercetin (2:1 derivative) has an antioxidant activity of 79% compared to an equivalent amount of free quercetin (**Figure 1**, column 6). It should be noted that also the underivatized BSA has a weak antioxidant activity (0.6 mM L^{-1}, **Figure 1**, column 1). Under consideration of this value the (net) antioxidant activity of quercetin decreases even further (70%). The antioxidant activity of the BSA−quercetin derivatives is reduced with a parallel diminution of the amount of covalently bound quercetin. Consequently, the 20:1 derivative shows a TEAC value of 2.1 mM L^{-1}. This means a decrease in antioxidant activity to an extent of 47% compared to an equivalent amount of free quercetin, under the consideration of

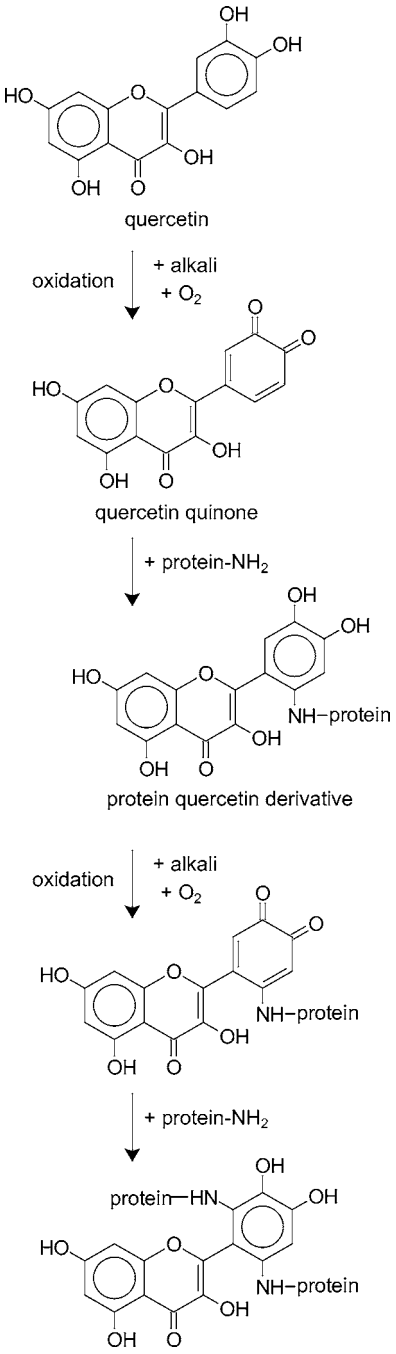

Figure 2. Reaction of quercetin with free amino group(s) of protein(s) after quinone formation.

the antioxidant activity of underivatized BSA, a (net) decrease to an extent of 35% (**Figure 1**, column 2). A good correlation was obtained between the amount of bound quercetin (**Table 1**) and the corresponding increase in total antioxidant activity of the derivatives ($R^2 = 0.994$).

An explanation of the antioxidative ability of the BSA−quercetin derivatives can be derived from the reaction mechanism in **Figure 2**, where the initial step is the oxidation of the quercetin to its respective quinone. The reaction of this quinone with the nucleophilic side chains of proteins such as lysine, cysteine, and tryptophan leads to a protein−phenol derivative. This phenol−protein derivative is still a potential antioxidant because of its capability to become a free radical, which can then scavenge ABTS radicals. This is obviously the mechanism for the antioxidant activity of the BSA−quercetin derivatives.

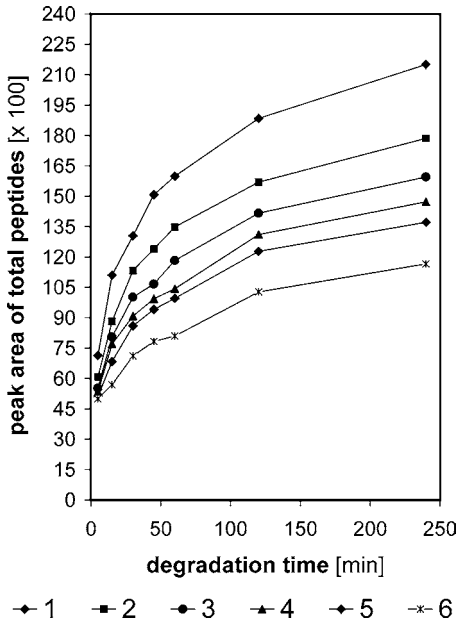

Figure 3. In vitro proteolytic degradation of the BSA–quercetin derivatives with trypsin (peak area of total peptides): see caption of **Figure 1**.

On the other hand, as also shown in **Figure 2**, a second oxidation leads again to a formation of a BSA−quercetin quinone, which is able to react with further free nucleophilic protein side chains, so it comes to cross-links of protein molecules, resulting in polymerization. In the context of reactions between different proteins (myoglobin, lysozyme, BSA, whey proteins, soy proteins, chymotrypsin) and plant phenolic compounds, we have proved the occurrence of such polymers by SDS-PAGE (*13*). The described polymerization is partly responsible for the loss of the antioxidative ability of covalently bound quercetin. With an increasing amount of proteins present during the derivatization, the reactive quercetin sites are more and more involved in protein−quercetin−protein reactions. Otherwise, steric hindrance could also be responsible for the decreased reactivity against the ABTS radicals.

Another interesting observation is made when we consider the comparison of antioxidant activity depending on the degree of derivatization between the derivatives and the equivalent amounts of free quercetin. With higher degree of derivatization the difference between the TEAC values of the protein-bound quercetin and the equivalent amount of free quercetin decreases significantly (**Figure 1**, arrows). An explanation for this behavior could be a better accessibility for the ABTS radical due to the derivatization and corresponding denaturation.

Proteolytic Digestion of the Derivatives Influencing Antioxidant Activity. In agreement with our former results (*13*) the in vitro tryptic degradation of the BSA−quercetin derivatives is diminished depending on the degree of derivatization (**Figure 3**). Trypsin splits preferentially those peptide linkages, which contain either lysine or arginine (*18*). The phenolic substances react very well with the nucleophilic ϵ-amino groups of lysine (*13*), a consequence of which is that the attack of trypsin against a lysine-involved peptide bond is prevented or at least makes its tryptic degradation difficult. The above-mentioned protein polymerization could be a further reason for the decrease of the tryptic degradation of the derivatives with increasing amounts of quercetin involved.

The formation of protein−phenol derivatives cannot be excluded during harvesting, storage, and processing of plant foods that contain both proteins and phenolic compounds.

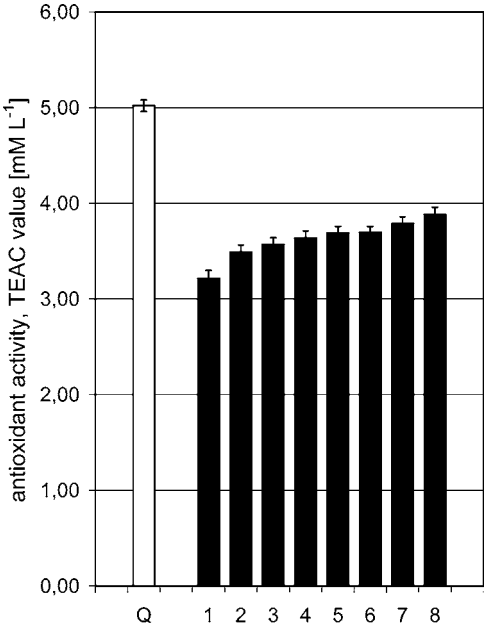

Figure 4. Total antioxidant acitivity of the peptides released from in vitro tryptic degradation of a BSA quercetin derivative (7:1) (TEAC value in mM L^{-1}); 1, without degradation; 2, 5 min of degradation; 3, 15 min of degradation; 4, 30 min of degradation; 5, 45 min of degradation; 6, 60 min of degradation; 7, 120 min of degradation; 8, 240 min of degradation; Q, equivalent amount of free quercetin.

Therefore, such reaction products are consumed. In this connection the gastrointestinal degradation of these derivatives is of great interest from a physiological point of view. In vitro experiments showed that there are relationships between the degree of derivatization, protein structure, and, consequently, the degradation behavior of such protein−phenol derivatives. In most cases the in vitro enzymatic splitting of such products (e.g., myoglobin−phenol derivatives) is diminished (*13*), whereas the derivatization (e.g. of lysozyme chlorogenic acid derivatives) leads to an increasing of the enzymatic degradation (*13*), the latter being due to protein unfolding and, consequently, a better enzymatic attack. In vivo experiments with rats confirm these results (unpublished results).

The antioxidant activity of enzymatically degraded splitting products of protein−phenol derivatives has not been investigated yet and is a subject of interest from the physiological point of view. The splitting products resulting from the above-mentioned in vitro tryptic digestion were investigated with regard to changes occurring in their antioxidant activity. **Figure 4** illustrates exemplarily the antioxidant activity of the time-dependent degradation of the 7:1 derivative. All of the resulting peptides possess antioxidant activity, whereby the antioxidant power of the equivalent amount of free quercetin is not reached. With increasing amount of splitting products, the TEAC values rise significantly (**Figure 4**). The highest antioxidative effect is possessed by the reaction products resulting from the degradation after 240 min (**Figure 4**, column 8). An explanation of this phenomenon can be the elimination of the structural hindrance to the ABTS radical as compared to the undigested derivative, whereby due to proteolysis "buried" quercetin residues are freed and can react with the ABTS radical. A splitting of the protein−quercetin−protein bonds could also account for this phenomenon.

Conclusions. Plant phenolic compounds react with proteins and enzymes under formation of covalent bonds (*13*−*15*). These reactions diminish the antioxidant acitivity of phenolic compounds, as shown in the present study for quercetin. Decisive for the antioxidativity of the BSA−quercetin derivatives is the amount of bound quercetin—in other words, the degree of derivatization. Nevertheless, a covalent attachment to proteins means generally a loss of antioxidant activity of quercetin. These preliminary results are based on TEAC assay. Further studies are planned with other radicals such as DPPH, which may be suitable for protein−polyphenol interactions and their antioxidant activities, to confirm our results.

In this connection the findings of Serafini et al. (*7*) should be taken into consideration; they showed in an intervention study that the plasma antioxidant activity [ferric reducing antioxidant potential assay (FRAP)] increased significantly after the consumption of dark chocolate. In contrast, there was no significant change in plasma FRAP values after the ingestion of milk chocolate alone or dark chocolate consumed with milk. Serafini et al. postulated that "This inhibition could be due to the formation of secondary bonds between chocolate flavonoids and milk proteins which would reduce the biological accessibility of the flavonoids and therefore the chocolate's potential antioxidant properties in vivo." Our results confirm these interpretations, wherby we suggest that the "secondary bonds" could also be covalent bonds between chocolate (milk) proteins and the cacao flavonoids. Also, Arts et al. (*8*) have found that the antioxidative activity (TEAC assay) of green and black tea phenols is diminished in the presence of different proteins (α-, β-, κ-casein, and BSA) due to interactions, which have not been characterized any further. They conclude that the "masking" effect of the antioxidant activity depends on both the protein and the flavonoid used.

Such interactions between proteins and phenols can also take place in vivo. In this respect Arts et al. (*9*) have shown that the plasma proteins can mask the antioxidant activity (TEAC assay) of phenolic compounds (quercetin, rutin, catechin, 7-monohydroxyethylrutoside). The kinds of interactions responsible for this observation were not discussed.

The interpretation of these results from the literature leads us to the conclusion that there are two distinct types of interactions between phenolic compounds and proteins responsible for diminishing the antioxidant activity. It has been clearly shown that covalent protein-bound phenolic compounds contribute to this decrease. The question of the role of the noncovalent bound phenolic compounds is still not clear and requires further study.

With regard to food supplementary products available on the market, such reactions could also be of interest, especially where these products have the intention of combining the positive effects of phenolic compounds (first of all, the antioxidative properties) with the parallel intention of improving the digestion (e.g., by the addition of proteolytic plant enzymes). Here a reaction between both components could lead to a decrease in antioxidant activity. It was already shown that in commercially available preparations, containing bromelain and quercetin, the enzyme activity was inhibited due to the reactions of the bromelain with the quercetin (*19*).

These results are significant with regard to further experiments planned, involving physiological and toxicological effects of proteins and enzymes derivatized with phenolic compounds.

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