Effect of Enzymatic and Chemical Oxidation on the Antioxidant Capacity of Catechin Model Systems and Apple Derivatives

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Changes in the chain-breaking activity of catechin model systems and apple derivatives were studied as a consequence of enzymatic and chemical oxidation. Although in different time scales, both enzymatic and chemical oxidations of catechin promoted an initial increase and a following decrease in the chain-breaking activity. The latter was associated with the formation of brown pigments. Similar changes were detected during storage at −18 °C of nonoxidized and enzymatically oxidized apple purees. The initial increase in antioxidant activity upon oxidation was attributed to the formation of procyanidins with larger aromatic structure and to synergy effects among them. The formation of tannins, the reactivity of which is sterically hindered, was proposed as a possible explanation for the decrease in antioxidant capacity in the advanced phases of oxidation.

Keywords: *Chain-breaking activity; polyphenols; polyphenol oxidase; browning*

INTRODUCTION

Fruits and vegetables have recently attracted special attention for their well-documented role in protecting the human body against several degenerative diseases (Potter, 1997; Franceschi et al., 1998; Gardner et al., 1998). An exciting hypothesis is that plant foods may provide an optimal mix of non-nutrient compounds, such as antioxidants and their precursors, which would exhibit synergistic beneficial effects with other bioactive compounds by virtue of their radical-scavenging activity (Ames, 1989; Kinsella et al., 1993; Grisham, 1994).

Due to their wide distribution in many fruits and vegetables, polyphenols represent >75% of the 86 overall natural antioxidants assumed in the diet. Notwithstanding the ability of phenols to scavenge free radicals (e.g., superoxide ions and lipid peroxyl radicals) and to affect a wide range of biological functions, their effective role in the human body remains to be clearly elucidated (Shahidi and Wanasundara, 1992; Block and Langseth, 1994; Gordon, 1996; Aruoma, 1998). This is probably due to the fact that polyphenol antioxidant capacity greatly varies depending on its chemical structure, concentration, and oxidation degree (Gordon, 1990; Nicoli et al., 1999; Lu and Yeap Foo, 2000).

Despite abundant literature data on the relationship between polyphenol chemical structure and antioxidant capacity, very little is known about the effect of oxidative reactions. This aspect is of great importance considering that phenol compounds present in fruits and vegetables can easily undergo enzymatic or chemical oxidation as a consequence of processing and storage. In fact, even if phenol oxidation is generally undesirable due to the unpleasant development of browning and off-flavors, it also represents an important technological step in the production of many foods, such as black tea, alcoholic beverages, and apple juices. Although chemical and enzymatic oxidations have been widely proven to cause a progressive loss in phenol antioxidant capacity, recent experimental data suggest that phenol antioxidant efficiency does not always decrease in oxidative conditions (Ariga and Hamano, 1990; Gordon, 1990; Hagerman et al., 1998; Manzocco et al., 2000). It has been observed that catechin subjected to enzymatic oxidation shows an initial remarkable increase in chain-breaking activity (Cheigh et al., 1995). Accordingly, the antioxidant capacity of some phenol-containing foods, such as tea extracts, orange juices, and alcoholic beverages, has been reported to increase under specific storage conditions (Manzocco et al., 1998a,b; Nicoli et al., 2000).

These contradictory results suggest that a better understanding of the changes in the antioxidant capacity of polyphenols upon oxidation is of great interest in order to minimize losses in the health-protecting value of fruits and vegetables during processing and storage. On the basis of these considerations, the present research was addressed to study the evolution of the antioxidant capacity of catechin and of some apple derivatives as a function of the development of enzymatic and chemical oxidation.

MATERIALS AND METHODS

Catechin Model Systems. An aqueous solution containing 1×10^{-4} M (+)-catechin (C-1251, lot 4100H0586, Sigma, St. Louis, MO) was prepared. The pH of the aqueous mixture was 4.7. No buffer was used to adjust the pH of the solution. The solution was divided into aliquots, which were subjected to enzymatic or chemical oxidation.

Enzymatic Oxidation. Four hundred units of mushroom tyrosinase (T-7755, lot 48F9610, Sigma) was added to 200 mL of the catechin solution and maintained in a 500-mL capacity screw-capped flask at 25 °C in a water bath with stirring for up to 60 min. The addition of the enzyme did not cause changes in the pH of the solution. To obtain samples with different oxidation levels, at different lengths of time 5 mL of the solution was removed and introduced into plastic tubes. Enzymatic inactivation was achieved by heating the tubes in

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a water bath at 80 °C for 3 min. Samples were then cooled under running water and analyzed. Preliminary experiments indicated that the 3-min heating at 80 °C was sufficient to achieve a complete inactivation of polyphenol oxidase.

Chemical Oxidation. Two hundred milliliter catechin solutions were introduced into a 500-mL capacity screw-capped flask and maintained in a water bath at 25 °C with stirring for up to 20 days in a dark cabinet. At different lengths of time, 5 mL of the solution was removed and immediately analyzed. Similar experiments were carried out excluding the presence of atmospheric oxygen during the reaction. In this case, aliquots of 10 mL of the catechin solution were introduced into 20-mL-capacity vials. Oxygen was removed from the headspace of the vial by flushing nitrogen (5 mL min^{-1}) for 1 min. Samples were then hermetically closed with butyl septa and metallic caps and stirred at 25 °C in a water bath for up to 20 days. At different lengths of time samples were removed and immediately analyzed. To minimize the contact between samples and atmospheric oxygen, the catechin solution was withdrawn from the closed vials using a Hamilton Microliter 700 syringe (Hamilton Bonaduz AG, Bonaduz, Switzerland).

Apple Puree. Apples (Golden Delicious) were purchased from a local market. Apples were peeled, cored, cut into cubes (1 × 1 × 1 cm), and divided in two aliquots, which were subjected to enzymatic or/and chemical oxidation. To favor enzymatic oxidation, apple cubes were homogenized for 2 min (Polytron PT 3000, Kinematica AG, Littau, Switzerland) and maintained in ambient air with stirring for 30 min. The second aliquot of apple cubes was immediately blanched in water at 95 °C for 5 min to prevent enzymatic browning. Blanched apple cubes were then drained, cooled, and homogenized for 2 min (Polytron PT 3000). Volumes of 50 mL of blanched or unblanched apple puree were bottled in plastic tubes and stored at −18 °C for up to 9 months. At different lengths of time samples were thawed, centrifuged (Heraueus, Sepatech Megafuge 1.0, Osterode, Germany) at 6000 rpm for 20 min at 25 °C, filtered through Whatman No. 4 filter paper, and analyzed.

Total Solid Content. Total solid content determinations were carried out by gravimetric method according to AOAC methods (AOAC, 1980).

Chain-Breaking Activity. The chain-breaking activity was measured following the methodology described by Brand-Williams et al. (1995). The bleaching rate of a stable free radical, 2,2-diphenyl-1-picrylhydrazyl (DPPH•) is monitored at a characteristic wavelength in the presence of the sample. In its radical form, DPPH• absorbs at 515 nm, but upon reduction by an antioxidant or a radical species its absorption decreases.

A volume of 1.85 mL of 6.1 × 10^{-5} M DPPH• methanol solution was used. The reaction was started by the addition of 150 μL of sample. In the case of apple derivatives, 150 μL of the filtered supernatant recovered after centrifugation of the apple puree was added. Preliminary trials showed no interactions between DPPH• and the inactivated enzyme. The bleaching of DPPH• was followed at 515 nm (Uvikon 860, Kontron Instruments, Milano, Italy) at 25 °C for at least 60 min. In all cases the DPPH• bleaching rate was proportional to the sample concentration added to the medium. The following equation was chosen to obtain the reaction rate of DPPH• bleaching (Manzocco et al., 1998a):

$$1/A^3 - 1/A_0^3 = 3kt \quad (1)$$

where k is the DPPH• bleaching rate, A_0 is the initial optical density, and A is the optical density at increasing time, t. The chain-breaking activity was expressed as the slope (k) obtained from eq 1 per milligram of dry matter ($-OD^{-3}$ min^{-1} mg of dm^{-1}), assuming that all of the dry matter of the sample possesses antioxidant capacity. The stoichiometric value of catechin was defined as the number of DPPH• moles reduced by 1 mol of catechin. The number of DPPH• moles consumed was calculated from the differences between initial absorbance and the absorbance when the reaction had gone to completion.

Optical Density. Enzymatic and chemical browning was followed by means of measurement of optical density at 390 nm using a Uvikon 860 spectrophotometer. This wavelength was chosen in order to follow the formation of brown catechin polymers.

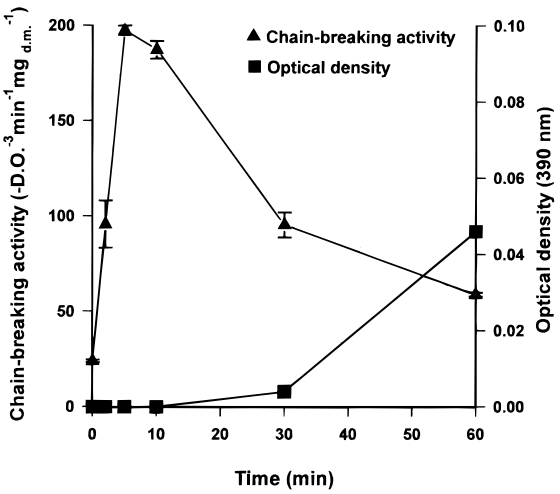

Figure 1. Chain-breaking activity and optical density of catechin undergoing enzymatic oxidation as a function of reaction time.

Statistical Analysis. The results reported in this work are the average of at least three measurements, and the coefficients of variations, expressed as the percentage ratio between standard deviations (SD) and the mean values, were found to be <5 for total solid content, <2 for phenol content, <8 for chain-breaking activity, and <5 for optical density. One-way analysis of variance was carried out, and significance between means was determined using the Tukey test (JMP 3.2.5, SAS Institute Inc., Cary, NC).

RESULTS AND DISCUSSION

The stoichiometric value of catechin, defined as the number of DPPH• moles reduced by 1 mol of catechin, was found to be 2 ± 0.03. According to Brand-Williams et al. (1995), the stoichiometry corresponds approximately to the numbers of hydrogens available for donation on hydroxyl groups. In fact, two DPPH• molecules can be reduced by the hydrogens of the two catechin hydroxyls, leading to the formation of the corresponding *o*-quinones, as occurs during the oxidation of *o*-diphenolic compounds in the presence of polyphenol oxidase (Vamos-Vigyazo, 1981).

Changes in the chain-breaking activity of catechin as affected by enzymatic oxidation are shown in Figure 1. Corresponding values of optical density at 390 nm are also reported. A fast and remarkable increase in the chain-breaking activity during the first 10 min of the reaction was detected. This variation did not correspond to any appreciable change in optical density. When the reaction time was prolonged, the chain-breaking capacity of the solution decreased concomitantly with the increase in optical density. These results are consistent with previous observations by Cheigh et al. (1995). As it is known, catechin oxidation leads to the formation of complex mixtures including colorless intermediate products as well as yellow pigments. The predominant reaction products in the early stages of oxidation are represented by C−C and C−O−C linked dimers (Oszmianski et al., 1996; Guyot et al., 1995, 1996). These procyanidins are expected to be more efficient antioxidants than the original monomers by virtue of the increased capacity of their aromatic structure to support the unpaired electron throughout delocalization around the π electron system. For example, the C−C dimer of

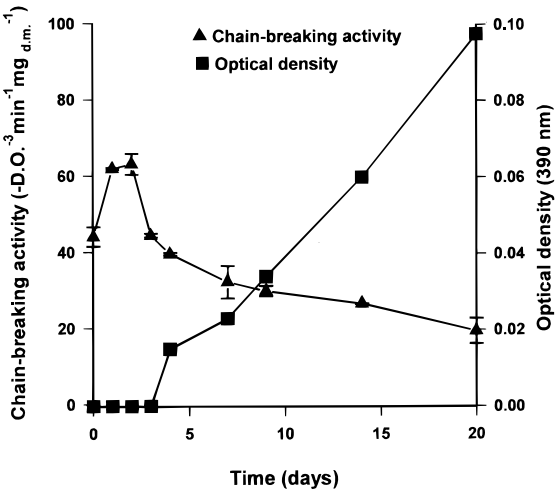

Figure 2. Chain-breaking activity and optical density of catechin undergoing chemical oxidation as a function of reaction time.

catechin has been shown to be ~3 times more active than catechin in scavenging free radicals (Saint-Cricq de Gaulejac et al., 1999a). Moreover, the increased antioxidant activity in the early phases of catechin oxidation may find an additional explanation in the synergy effect between procyanidins. In fact, mixtures of different dimers can exert higher antioxidant capacity than can only one pure procyanidin in the same concentration (Saint-Cricq de Gaulejac et al., 1999a).

Due to the more significant area of charge delocalization, the inhibition of radicals by phenols tends to increase in the order of progressive polymerization (Ariga and Hamano, 1990; Hagerman et al., 1998). This indication apparently contradicts with the decrease in antioxidant activity observed during the advanced phases of catechin oxidation (Figure 1). However, although it has been observed that the scavenging activity of procyanidin fractions from one monomer to four monomers increases with the degree of polymerization, this trend was found to be reversed for tannins with a polymerization degree superior to four monomers (Saint-Cricq de Gaulejac et al., 1999b; Lu and Yeap Foo, 2000). It must be noted that the scavenging activity of phenols closely depends on both chemical structure and spatial conformation. In fact, with increasing molecular complexity, it is likely that the antioxidant activity decreases as a consequence of the steric hindrance, which reduces the availability of the hydroxyl groups. The lower capacity to donate a hydrogen atom from the hydroxyl group to a free radical could account for the low antioxidant capacity of the catechin-derived pigments (Figure 1). The latter are actually expected to be larger and less polar molecules that retain only to a minimum extent the original monomer structure (Oszmianski and Lee, 1990; Guyot et al., 1995).

Changes in the chain-breaking activity and optical density of catechin model systems undergoing chemical oxidation are plotted in Figure 2. Also in this case, an initial sharp increase in the chain-breaking activity was followed by a further decrease toward values similar to those found for the nonreacted catechin. In addition, as already observed for enzymatic oxidation (Figure 1), a notable decrease in chain-breaking activity was associated with the development of browning. In our experimental conditions, brown pigments were detected after 4 days of storage, as shown by the increase in the absorbance value at 390 nm.

Table 1. Chain-Breaking Activity[a] and Optical Density of Catechin Solution Bottled under Nitrogen Atmosphere as a Function of Storage Time at 25 °C

time (days)	chain-breaking activity ($-OD^{-3}$ min^{-1} mg of dm^{-1})	optical density (390 nm)
0	23.48 ± 0.31	0.000
1	21.81 ± 1.62ab	0.000
3	25.85 ± 0.52a	0.000
7	26.63 ± 0.58bcd	0.002
14	22.55 ± 0.26c	0.018
20	22.95 ± 0.55d	0.020

[a] Chain-breaking data are presented as the mean ± SD ($n = 4$). Means with a common letter differ significantly ($p > 0.05$).

It is evident that, although enzymatic oxidation (Figure 1) proceeds to its latest phases in a shorter time as compared to chemical oxidation (Figure 2), both seem to follow a common evolution leading to similar changes in color and antioxidant activity. A simple explanation can be formulated by considering that the initial oxidation of phenol compounds results in the formation of their quinones (Demmin et al., 1981). Once the quinones are formed, whether enzymatically or chemically, their further oxidation proceeds through the same reaction pathway (Cilliers and Singleton, 1989), reasonably leading to similar antioxidant capacity changes. Similar experiments were carried out but avoiding the presence of oxygen during the reaction. In particular, the catechin solution was bottled under nitrogen atmosphere and subjected to the same storage conditions of samples bottled in air. The effects of such a storage condition on the optical density at 390 nm and on the chain-breaching activity are shown in Table 1. It can be observed that these samples showed only slight changes in the radical scavenging properties and in optical density values, indicating that no significant oxidation occurred. The results acquired clearly demonstrate the critical role of oxygen in promoting changes to the chain-breaking activity. Similar results were reported by Manzocco et al. (1998a), who observed that oxygenation of green tea caused a rapid increase in radical-scavenging activity, whereas no changes were detected in samples stored under a nitrogen atmosphere.

In light of these findings and considering that phenol compounds present in fruits and vegetables can easily undergo enzymatic or chemical oxidation, a phenol-containing food was chosen to evaluate the role of oxidation on the changes in antioxidant capacity during processing and storage. In particular, an apple puree was chosen because the antioxidant activity of apple derivatives can be essentially accounted for by phenol constituents, whereas other antioxidant species contribute to only a minimum extent (Miller at al., 1995). Actually, the contribution of vitamin C with the total antioxidant activity was calculated to be <15% in raw apple and only 5% in apple juice (Wang et al., 1996; Miller and Rice-Evans, 1997).

To study the effect of enzymatic browning on the antioxidant capacity of apple derivatives, apple puree was stirred in air to favor polyphenol oxidase activity. A blanched apple puree was taken as nonoxidized control. Table 2 shows the optical density and the chain-breaking activity of apple purees produced with minimal enzymatic oxidation (blanched) or from well-oxidized pulps (browned). It is noteworthy that the darkening of the puree was associated with a significant decrease

Table 2. Chain-Breaking Activity[a] and Optical Density of Blanched and Browned Apple Puree

apple puree	chain-breaking activity ($-OD^{-3}$ min^{-1} mg of dm^{-1})	optical density (390 nm)
blanched	0.021 ± 0.003	0.130
browned	0.004 ± 0.001	4.121

[a] Chain-breaking data are presented as the mean ± SD ($n = 4$).

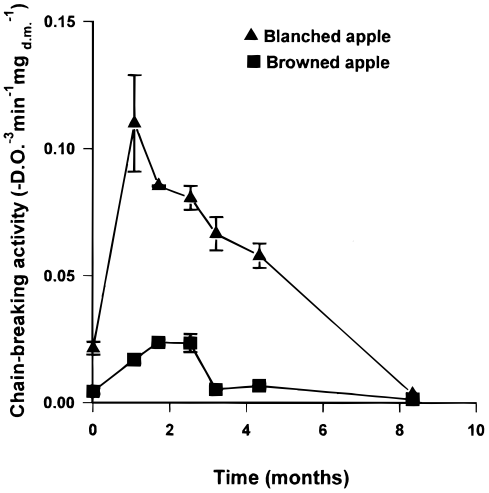

Figure 3. Chain-breaking activity of blanched and browned apple purees as a function of storage time at −18 °C.

in its antioxidant capacity, confirming the lower radical scavenging properties of strongly oxidized phenols.

Figure 3 shows the changes in antioxidant capacity of the nonoxidized and enzymatically oxidized apple purees during storage at −18 °C. Data clearly show that the chain-breaking activity of the apple purees mimicked the evolution already observed for the catechin model system, even if over longer times (Figures 1 and 2). In the case of the blanched puree, in which enzymatic activity was prevented, the initial increase and the following decrease in the radical scavenging properties can be attributed to the chemical oxidation of phenols. The maximum antioxidant activity identifiable at 2 months of storage of the browned puree can be the result of different effects. As shown in Table 2 the oxidation products of apple phenols retain antioxidant activity and hence can be further oxidized both chemically and enzymatically. In fact, despite the development of chemical oxidation, which is evident at low temperatures (see blanched puree), the occurrence of enzymatic oxidation cannot be underestimated because it is well-known to easily proceed even at freezing temperatures (Fennema, 1975; Manzocco et al., 1999).

CONCLUSIONS

Results obtained in this study clearly showed that the antioxidant capacity of catechin is strongly affected by oxidative reactions. Although in different time scales, both enzymatic and chemical oxidations of catechin were found to promote an initial increase in the chain-breaking activity. The subsequent formation of brown polymers in the reacting solution was associated with a decrease in antioxidant capacity. Similar changes in the antioxidant capacity were detected during storage at −18 °C of nonoxidized and enzymatically oxidized apple purees. However, in foods with complex composition instead of model systems, the oxidation of phenols is likely to occur concomitantly with other events, which could further contribute to the evolution of the antioxidant capacity during processing and storage.

It can be concluded that the antioxidant properties of phenol-containing foods are expected to greatly change depending on the oxidative degree of the phenol compounds. The marked fluctuation in their antioxidant properties upon processing indicates that further research is needed to determine those technological conditions able to preserve or even promote a gain in the antioxidant capacity of phenol-containing foods. This, in turn, raises the question of whether a clear correlation between phenol content in foods and their health-protecting capability in vivo could find a possible explanation, suggesting that caution should be used in interpreting epidemiological data relevant to processed fruits and vegetables.

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