Available online at www.sciencedirect.com
ScienceDirect
Food Chemistry 108 (2008) 228–233
Food Chemistry
www.elsevier.com/locate/foodchem

Hydrolysis of epigallocatechin gallate using a tannase from *Paecilomyces variotii*

V. Battestin [a], G.A. Macedo [a,*], V.A.P. De Freitas [b]

[a] *Food Science Department, Faculty of Food Engineering, Campinas State University, P.O. Box 6121, 13083-862 SP, Brazil*
[b] *Center of Investigation in Chemistry, Department of Chemistry, Faculty of Sciences, University of Porto, Porto 4169-007, Portugal*

Received 13 June 2007; received in revised form 17 October 2007; accepted 23 October 2007

Abstract

Epigallocatechin (EGC) and gallic acid (GA) were prepared by the degalloylation of an epigallocatechin gallate (EGCG) extract from green tea. EGCG was entirely hydrolyzed using a tannase (from *Paecilomyces variotii*) at pH 6.0, incubating at 40 °C for 30 min. The antiradical properties and the reducing power of these samples were assessed using the DPPH and FRAP assays, respectively. This work established a relationship between the antioxidant effects of epigallocatechin gallate and the enzymatic reaction products (epigalocatechin and gallic acid). The enzymatic reaction products showed higher scavenging activity and antioxidant capacity when compared to epigallocatechin gallate.

Keywords: Tannase; Antioxidant; *Paecilomyces variotii*; Epigallocatechin gallate; Green tea

1. Introduction

Tea is the second most widely consumed beverage after water worldwide and is rich in polyphenolic compounds known as tea flavonoids. Green tea contains several tea polyphenols, including epigallocatechin gallate (EGCG), epigallocatechin (EGC), epicatechin gallate (ECG), epicatechin (EC) (Suganuma et al., 1999). These flavonoids (also known as catechins) possess strong antioxidant properties (Majchrzak, Mitter, & Elmadfa, 2004). Catechins have been proven to have antioxidant, antimutagenic, and anticarcinogenic properties and can also prevent cardiovascular diseases. The positive health effects of catechins have attracted much attention by chemists and biochemists during recent years (Cao & Ito, 2004).

The considerable antioxidant potential of tea has long been recognized and is dependent on many factors involved in tea preparation. The antioxidant activity of phenolic compounds is mainly due to their redox properties, which allow them to act as reducing agents, singlet oxygen quenchers and metallic ion chelators (Atoui, Mansouri, Boskou, & Kefalas, 2005). Although epigallocatechin (EGC) is an important catechin in tea leaf, it is contained in natural green tea preparations in relatively low amounts compared to its gallate derivative, epigallocatechin gallate (EGCG), which is the most abundant catechin in green tea (Cao & Ito, 2004).

Tannin acylhydrolases, commonly referred to as tannases (E.C. 3.1.1.20), are inducible enzymes produced by fungi, yeast and bacteria. Tannases have mostly been characterized by their activity on complex polyphenolics, and are able to hydrolyze the "ester" bond (galloyl ester of an alcohol moiety) and the "depside" bond (galloyl ester of gallic acid) of substrates such as tannic acid, epicatechin gallate, epigallocatechin gallate, chlorogenic acid, etc (Garcia-Conesa, Ostergaard, Kauppinen, & Williamson, 2001).

In this paper, the activity of tannase on the epigallocatechin gallate extract of green tea was investigated. The aim of this work was to study the potential antioxidant properties of epigallocatechin gallate before and after the enzyme

* Corresponding author. Tel.: +55 193521 2175; fax: +55 193521 1513.
E-mail address: gmacedo@fea.unicamp.br (G.A. Macedo).

0308-8146/$ - see front matter © 2007 Elsevier Ltd. All rights reserved.
doi:10.1016/j.foodchem.2007.10.068

reaction using the tannase produced by *P. variotii* (Battestin, Pastore, & Macedo, 2005). The antiradical properties and the reducing power of these samples were assessed using 2,2-diphenyl-1-picrylhydrazyl (DPPH) and ferric reducing ability power (FRAP) assays, respectively (Bondet, Brand-Williams, & Berset, 1997; Benzie & Strain, 1996).

2. Material and methods

2.1. Reagents

All solvents used in this study were of analytical grade and purchased from Sigma Chemicals. Epigallocatechin gallate (EGCG, 95%), epigallocatechin (EGC, 98%) and gallic acid (GC) were purchased from Sigma–Aldrich, Steinheim, Germany.

2.2. Enzyme

The tannase from *P. variotii* was obtained according to a previously published procedure (Battestin & Macedo, 2007). A 250 mL conical flask containing the following constituents: 5 g of wheat bran and 5 g of coffee husk, 10 mL of distilled water and 10% of tannic acid (w/w) (Ajinomoto OmniChem Division,Wetteren, Belgium), was used for the fermentation process. The culture medium (pH 5.7) was sterilized at 120 °C for 20 min. After sterilization, the flasks were inoculated with 2.5 mL (5.0×10^7 spores/mL) of the pre-inoculum suspension and incubated at 30 °C for 120 h. After fermentation, 80 mL of 20 mM acetate buffer, pH 5.0 were added and shaken at 200 rpm for 1 h. The solution was filtered and centrifuged at 9650*g* for 30 min at 4 °C (Beckman J2-21 centrifuge, Beckman–Coulter, Inc. Fullerton, CA, USA). The supernatant was than treated with solid ammonium sulphate (80% saturation) and stood overnight at 4 °C. The precipitate was collected by centrifugation (9650*g* – 30 min), re-suspended in distilled water and dialysed against distilled water. The dialysed preparation was used as crude freeze-dried tannase.

2.3. Preparation of EGCG extracts (substrate)

EGCG was extracted from 1 g of green tea (Té verde-Pompadour tee) with 20 mL of ethanol/water/chloroform (1:1:2, v/v/v) using a blender (Ultra-Turrax) for 5 min according to the procedure described by De Freitas, Carvalho, & Mateus (2003). The 50% ethanolic upper aqueous layer was separated from the chloroform layer containing the chlorophylls, lipids and other undesirable compounds. The ethanol was removed using a rotary evaporator and the resulting aqueous solution containing catechins was dissolved in acetate buffer (pH 6.0, 0.2 M) for the enzymatic hydrolysis. The final concentration of the extract for the enzymatic reaction hydrolysis was 0.3 mg of EGCG/mL of acetate buffer.

2.4. Quantification of EGCG, EGC and gallic acid in the green tea extract

The quantitative determination of individual catechins was performed by HPLC (280 nm) using the equations of the calibration cuves obtained using standards. Standard solutions were prepared by dissolving catechins (EGCG, EGC and GA) in trifluoracetic acid at different concentrations (0.1–1.5 mg). Linearity ($r^2 > 0.98$) was calculated by plotting the peak area of each concentration against the respective catechin concentration.

2.5. Enzymatic hydrolysis

The EGCG obtained from the green tea extract (0.3 mg/mL) was used as the substrate for the enzymatic hydrolysis by tannase, isolated from *P. variotii* (Battestin & Macedo, 2007). The commercial substrate (EGCG Sigma) (1 mg/mL) was also used for an enzymatic reaction. EGCG (0.1 mL obtained from both sources) was prepared in acetate buffer (pH 6.0, 0.2 M) and incubated with 0.01 mL of tannase (1–10 mg/mL) at 40 °C for 30 min. The hydrolysis process was paralyzed by placing in an ice bath for d15 min. The released EGC and gallic acid moieties were identified by analytical HPLC by comparison with authentic standards.

2.6. HPLC conditions

The products formed during the enzymatic reaction were analyzed by HPLC (Elite Lachrom-Merck Hitachi, Darmstadt, Germany) using a 150 × 4.6 mm i.d. reversed-phase C18 column (Merck, Darmstadt, Germany), and detection at 280 nm with a Knauer WellChrom Diode Array Detector K-2800 (Knauer – ASI, Berlin, Germany). The solvents were A: 1% trifluoracetic acid in H_2O, and B: 20% solvent A in 80% acetonitrile. A linear gradient from 7% to 25% B in 40 min was used with a flow rate of 0.5 mL/min.

2.7. LC–MS analysis

A Finnigan Surveyor series liquid chromatograph, equipped with a 150 × 4.6 mm i.d., 5 μm s LicroCART® (Merck, Darmstadt, Germany) reversed-phase C18 column maintained at 25 °C by a thermostat, was used. Mass detection was carried out using a Finnigan LCQ DECA XP MAX (Finnigan Corp., San José, CA, USA) mass detector with an API (Atmospheric Pressure Ionization) source of ionization and an ESI (ElectroSpray Ionization) interface. The solvents were A: formic acid in H_2O (1%, v/v), and B: acetonitrile. The capillary voltage was 4 V and the capillary temperature 275 °C. The spectra were recorded in the positive ion mode between 120 and 1500 m/z. The mass spectrometer was programmed to carry out a series of three scans: a full mass, a zoom scan of the most intense ion in the first scan, and a MS–MS of the most intense ion using relative collision energy of 30 and 60.

2.8. Ferric reducing ability power (FRAP)

The FRAP assay developed by Benzie and Strain (1996) was a measure of antioxidant power was performed. In short, the FRAP reagent (10 vol of 300 mM acetate buffer, pH 3.6 + 1 vol of 10 mM TPTZ (2,4,6-tripyridyl-*S*-triazine) in 40 mM HCl + 1 vol of 20 mM $FeCl3 \cdot 6H_2O$) was diluted to one-third its concentration with methanol and pre-warmed at 37 °C. Two hundred and ninety seven microliter of this reagent were mixed with 3 μL of extract (enzyme reaction products). This mixture was shaken and the absorbance read at 593 nm. The test was performed at 37 °C and the 0–4 min reaction time window was used. The results were expressed as Trolox equivalents determined using a calibration curve.

2.9. Radical DPPH scavenging activity

Following the method described by Bondet et al. (1997) with some modifications, the radical activities were determined using DPPH as the free radical. The reaction for scavenging DPPH radicals was performed in polypropylene tubes at room temperature (22–23 °C). In each tube, an aliquot of 297 μL of extract (enzyme reaction products) was added to 3 μL of DPPH solution (60 μM in methanol). The decrease in absorbance was measured at 520 nm at time = 0 and then every 5 min for 20 min. Methanol was used as the blank solution, and DPPH solution with no sample extract served as the control. The anti-radical activity was calculated from the equation determined from the linear regression after plotting known solutions of Trolox

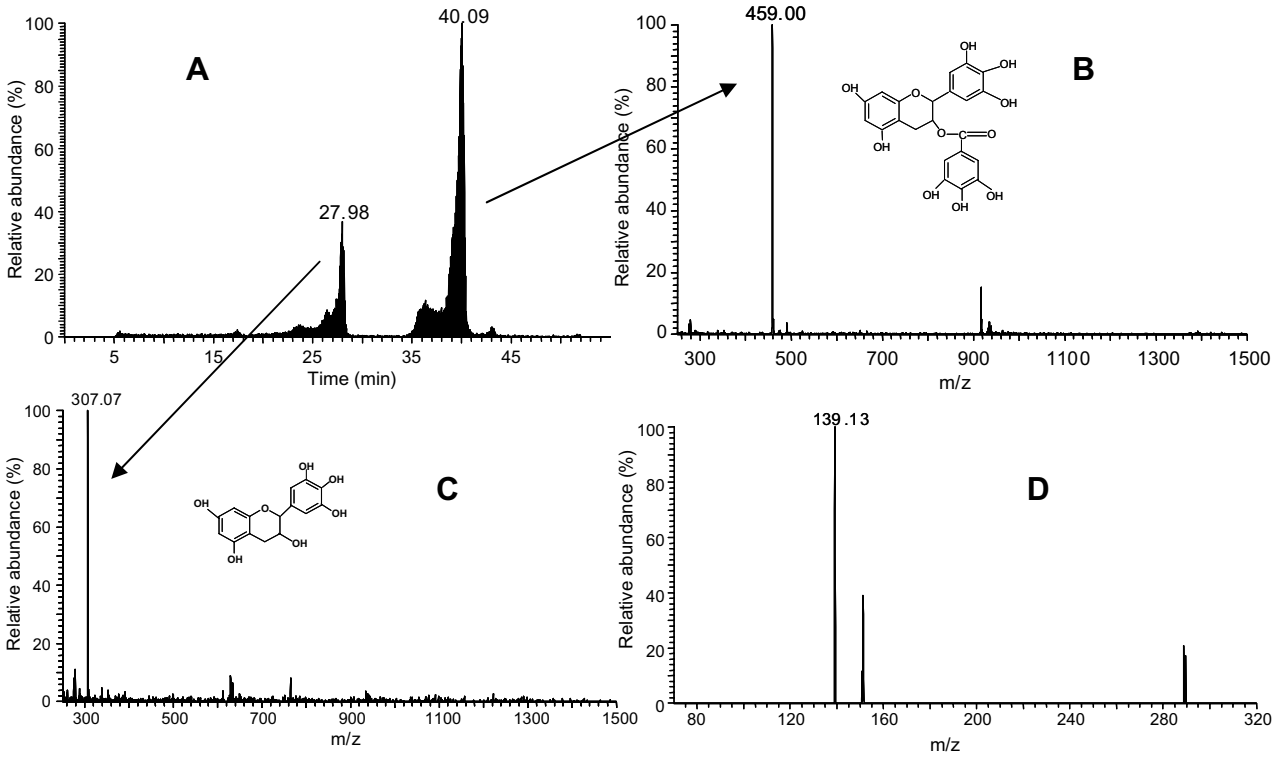

Fig. 1. LC-ESI mass spectrometry analysis of green tea extract (positive-ion mode): (A) total ion chromatogram; (B and C) MS spectrum; (D) MS^2 spectrum of the ion at *m/z* 307.

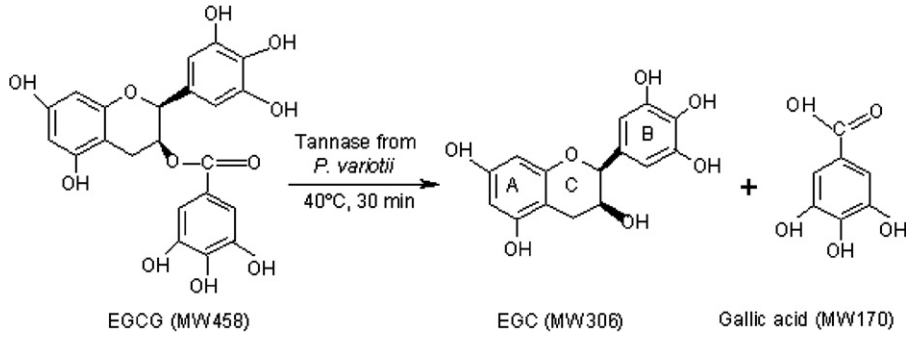

Fig. 2. Degalloylation of EGCG by the tannase from *Paeciloyces variotii*.

with different concentrations. For the final results, the 0–20 min reaction time window was used. Antiradical activity was expressed as micromolar Trolox equivalents (Faria et al., 2005).

2.10. Calculations and statistics

The values are expressed as the arithmetic mean. Statistical significance of the difference between the groups was

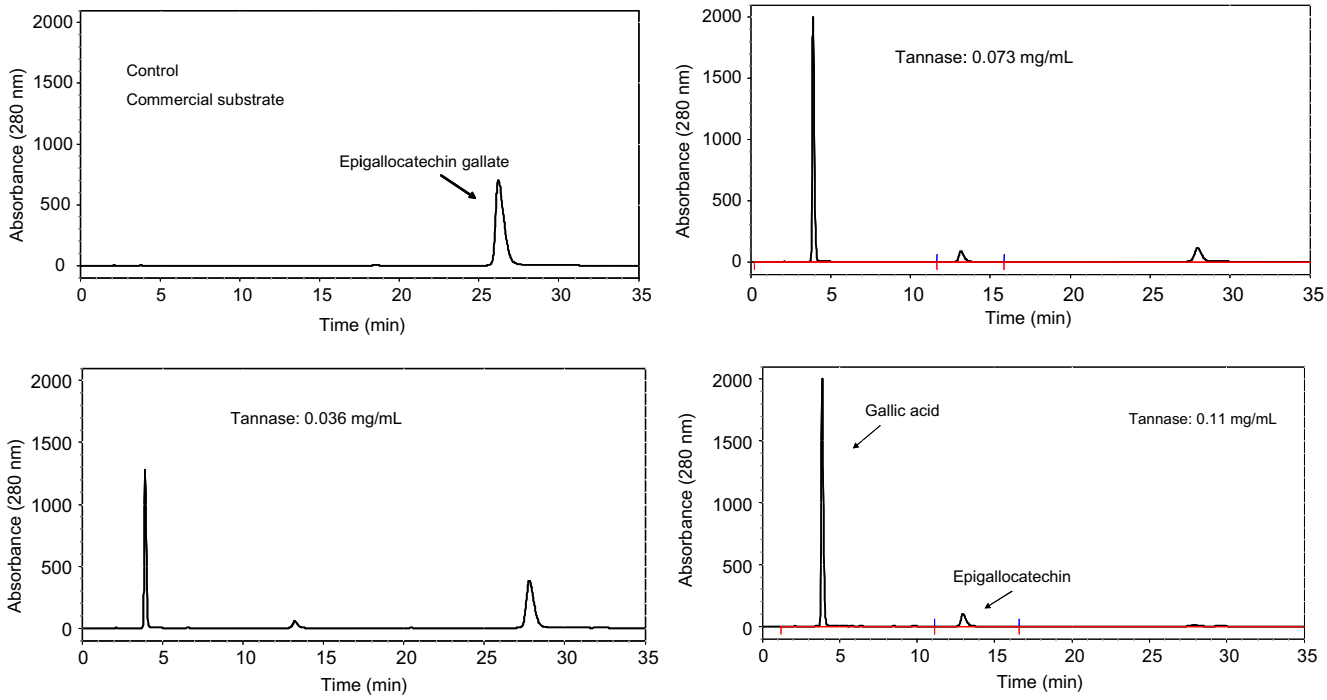

Fig. 3. HPLC analysis of the products formed after the enzymatic reaction using EGCG (commercial substrate) at 3 different concentration of tannase (pH 6.0, 40 °C, 30 min).

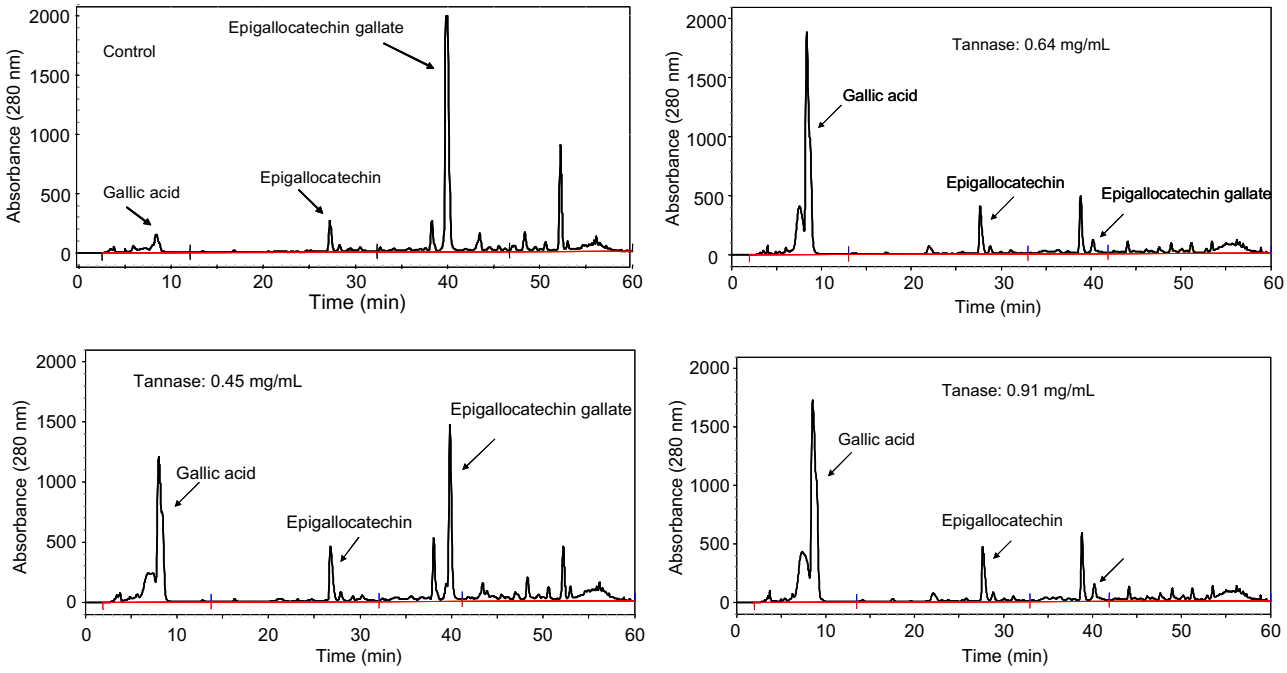

Fig. 4. HPLC analysis of the products formed after the enzymatic reaction using the green tea extract (pH 6.0, 40 °C, 30 min).

analyzed by the Tukey test. Differences were considered significant when $P < 0.05$.

3. Results and discussion

3.1. Detection and identification of the compounds extracted from green tea

The hydroalcoholic extract of green tea containing polyphenolic compounds was thoroughly analyzed by HPLC/DAD-MS. The use of mass spectrometry, coupled to high performance liquid chromatography, allowed the identification of EGCG and EGC (Fig. 1). The mass spectrum showed an ion mass consistent with the structure of epigallocatechin (MW: 306 g/mol). The mass spectrum of this compound revealed a $[M^+]$ molecular ion at *m/z* 307 and a major fragment ion $[M\text{-}168]^+$ at *m/z* 139 which correspond to a retro-Diels–Alder of the catechin moiety (Freitas, Souza, Silva, Santos-Buelga, & Mateus, 2004). The HPLC/DAD-MS analysis also showed the presence of a very significant peak with the same retention time as the EGCG (retention time of 40 min) in the UV–Vis spectrum. The mass spectrum showed an ion mass $[M^+]$ at *m/z* 459 consistent with the structure of EGCG (Fig. 1).

3.2. Quantification of EGCG, EGC and gallic acid

The amounts of EGCG, EGC and GA in the green tea extract were determined by HPLC using the calibration curves of the authentic standards. The catechin concentration, expressed in mg/g of dry weight of the green tea extract, was: EGCG (3.8 mg/g), EGC (0.44 mg/g) and gallic acid (0.35 mg/g).

Leaf composition depends on a variety of factors, including climate, season, horticultural practices, and the type and age of the plant. Chiu and Lin (2005) have studied the concentration of catechins in young green tea leaf from different regions, and the levels of EGCG and EGC obtained were 4.5 and 2.8 mg/g of tea leaf, respectively.

3.3. Enzymatic reaction with commercial EGCG and green tea substrate

The hydrolysis process was carried out using EGCG as the substrate, which was hydrolyzed by the enzyme tannase to form gallic acid and EGC (Fig. 2).

Tannase exhibited high activity on the commercial substrate (EGCG-Sigma) and on the substrate extracted from green tea (Figs. 3 and 4). The ability of tannase to hydrolyze the commercial substrate was compared with its ability to hydrolyze that obtained from green tea extract. The results showed that the tannase from *P. variotii* was able to hydrolyze the ester bonds from natural substrates. EGC and GA can be formed by the degalloylation of this gallate (EGCG) present in the tea extract. The HPLC analysis of the reaction products (Figs. 3 and 4) indicated that commercial EGCG (1.0 mg/mL) could be completely converted to EGC and gallic acid using tannase at the final concentration of 0.11 mg/mL at pH 6.0, 40° and 30 min. For the green tea extract, the EGCG (0.3 mg/mL) could be hydrolyzed using tannase at 0.64 mg/mL. Tannases show high specificity for the phenolic site of the substrate (Garcia-Conesa et al., 2001). These results are in agreement with those of Cao and Ito (2004), who used tannase for the preparation and purification of EGC. There is little data about the enzymatic hydrolysis of green tea with the tannase from *P. variotii*. All the enzymatic reactions were monitored using denatured enzyme as a control.

EGC is present in relatively low amounts in natural green tea preparations as compared to its gallate derivative (EGCG) (Cao & Ito, 2004). The EGC concentration of the green tea doubled after treatment with tannase (Fig. 5).

Increases in the enzyme concentration (0.91 mg/mL) reduced the EGCG concentration in the green tea extract from 0.31 mg/mL to 0.0084 mg/mL. In compensation, the gallic acid and EGC contents increased 16 and 2 times, respectively.

3.4. DPPH and FRAP

The free radical scavenging activity of the extracts was tested using the DPPH method. The substances tested reacted with the DPPH, which is a stable free radical, and induced a decrease in the absorbance measured at 515 nm, which indicates the scavenging potential of the

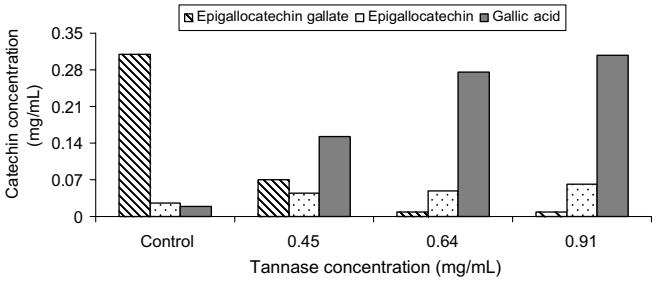

Fig. 5. Quantitative determination of epigallocatechin gallate, epigallocatechin and gallic acid obtained using different tannase concentrations.

Table 1
Antiradical activity and reducing power before and after enzymatic hydrolysis as assessed by the DPPH and FRAP method

Phenolic compounds	Commercial substrate (EGCG)	Green tea extract
Antiradical activity		
Control (without tannase)	25.29 ± 0.56^a	24.09 ± 0.05^a
Enzymatic reaction products (in presence of tannase)	24.60 ± 0.12^a	22.78 ± 0.35^b
Reducing power		
Control (without tannase)	23.95 ± 0.93^a	73.4 ± 1.19^a
Enzymatic reaction products (in presence of tannase)	39.09 ± 1.33^b	85.0 ± 0.59^b

[a,b]The values are means of duplicates and those with different letters are significantly different at $p < 0.05$.

extracts. It can be seen from the results in Table 1 that there is a trend for increasing radical scavenging capacity with the increase in the EGC and gallic acid contents of the green tea extracts. The reducing power of the extracts was subsequently assessed using the FRAP method (Table 1). The products formed after enzyme reaction showed higher scavenging activity and antioxidant capacity.

Catechins (including epicatechins) with three hydroxyl groups in the B ring are the gallocatechins, and those esterified to gallic acid at the 3-OH group in the C ring are the catechin gallates. The antioxidant activity responds broadly to the tenet that the structures with the most hydroxyl groups exert the greatest antioxidant activity, the catechin–gallate esters reflecting the contribution from gallic acid (Rice-Evans, Miller, & Paganga, 1996). Concerning any structure-activity relationships, the *o*-dihydroxy groups in the B-ring and the hydroxyl group in the C-ring are usually related to the antioxidant properties of the flavonoids (Faria et al., 2005).

4. Conclusions

This study demonstrated that EGC and gallic acid can be successfully formed from the degalloylation of EGCG by tannase in green tea extracts. This work established a relationship between the antioxidant effects of EGCG and those obtained after the enzymatic reaction (EGC and gallic acid). The products of the enzyme reaction showed higher scavenging activity and antioxidant capacity than the EGCG. Further studies are needed to explore the mechanism and the potential of this enzyme for future applications in green tea. These results suggest that tannases are able to improve the antioxidant activity of green tea.

Acknowledgments

The authors acknowledge the financial support from CAPES (*Coordenação de Aperfeiçoamento de Pessoal de Nível Superior-Brasil*) and are grateful to the Department of Chemistry, Faculty of Sciences, University of Porto, Portugal and to Professor Victor A.P. de Freitas for his contribution to this work.

References

Atoui, A. K., Mansouri, A., Boskou, G., & Kefalas, P. (2005). Tea and herbal infusions: Their antioxidant activity and phenolic profile. *Food Chemistry, 89*, 27–36.

Battestin, V., & Macedo, G. A. (2007). Tannase production by *Paecilomyces variotii*. *Bioresource Technology, 98*, 1832–1837.

Battestin, V., Pastore, G., & Macedo, G. (2005). Optimizing the fermentation broth for tannase production by a new isolated strain *Paecilomyces variotii*. In Abstracts of the 12th European Congress in Biotechnology . Denmark, Copenhagen. *Journal of Biotechnology*, 118, S49.

Benzie, I., & Strain, J. (1996). The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": the FRAP assay. *Analytical Biochemistry, 239*, 70–76.

Bondet, V., Brand-Williams, W., & Berset, C. (1997). Kinetics and mechanisms of antioxidant activity using the DPPH free radical method. *LWT-Food Science and Technology, 30*, 609–615.

Cao, X., & Ito, Y. (2004). Preparation and purification of epigallocatechin by high-speed countercurrent chromatography (HSCCC). *Journal of Liquid Chromatography & Related Technologies, 27*, 145–152.

Chiu, F.-L., & Lin, J.-K. (2005). HPLC Analysis of naturally occurring methylated catechins, 3″- and 4″-methyl-epigallocatechin gallate, in various fresh tea leaves and commercial teas and their potent inhibitory effects on inducible nitric oxide synthase in macrophages. *Journal of Agricultural and Food Chemistry, 53*, 7035–7042.

De Freitas, V. A. P., Carvalho, E., & Mateus, N. (2003). Study of carbohydrate influence on protein–tannin aggregation by nephelometry. *Food Chemistry, 81*, 503–509.

Faria, A., Oliveira, J., Neves, P., Gameiro, P., Santos-Buelga, C., De Freitas, V., et al. (2005). Antioxidant properties of prepared blueberry (*Vaccinium myrtillus*) extracts. *Journal of Agricultural and Food Chemistry, 53*, 6896–6902.

Freitas, V., Souza, C., Silva, A. M. S., Santos-Buelga, C., & Mateus, N. (2004). Synthesis of a new catechin–pyrylium derived pigment. *Tetrahedron Letters, 45*, 9343–9352.

Garcia-Conesa, M. T., Ostergaard, P., Kauppinen, S., & Williamson, G. (2001). Hydrolysis of diethyl diferulates by tannase from *Aspergillus oryzae*. *Carbohydrate Polymers, 44*, 319–324.

Majchrzak, D., Mitter, S., & Elmadfa, I. (2004). The effect of ascorbic acid on total antioxidant activity of black and green teas. *Food Chemistry, 88*, 447–451.

Rice-Evans, C. A., Miller, N. J., & Paganga, G. (1996). Structure-antioxidant activity relationships of flavonoids and phenolic acids. *Free Radical Biology and Medicine, 20*, 933–956.

Suganuma, M., Okabe, S., Kai, Y., Sueoka, N., Sueoka, E., & Fujiki, H. (1999). Synergistic effects of (−)-epigallocatechin gallate with (−)-epicatechin, sulindac, or tamoxifen on câncer-preventive activity in the lung câncer cell line PC-9. *Cancer Research, 59*, 44–47.