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Antioxidant reactivity evaluated by competitive kinetics: Influence of the target molecule concentration

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Abstract

In the present work, a competitive kinetic method using c-phycocyanin (from Arthospira maxima species) as target molecule was employed to estimate the reactivity of several phenols and flavonoids present in the human diet towards peroxyl radicals. The results obtained indicate that the protection afforded by a given compound strongly depends upon the experimental conditions employed and, in particular, on the concentration of the target molecule. This dependence is related to increased role of secondary reactions of the phenol-derived radicals initially formed. Secondary reactions can explain the strong downward curvature observed in R^0/R (where R^0 is the rate of the process in the absence of additive, and R is the rate of the process in the presence of additive) vs. additive concentration plots, for compounds such as kaempferol and protocatechuic acid, particularly at high c-phycocyanin concentration. Also, it can explain the prooxidant role played by phenolic compounds of low reactivity, such as 3-hydroxyflavone at low c-phycocyanin concentrations.

Keywords: Flavonoids; Phenols; c-Phycocyanin; Peroxyl radicals; Antioxidants

1. Introduction

There is consensus that free radical reactions are relevant in many physiological and pathological processes. Reactive oxygen species (ROS) could be important causative agents of a number of human diseases, including cancer and atherosclerosis, as well as the aging process itself (Jurgens, Hoff, Chisolm, & Esterbauer, 1987; Steinberg, 1991).

The antioxidant activity of dietary phytochemicals has been linked to a reduction in human degenerative diseases in populations that consume high amounts of fruits and vegetables. In particular, the ability of plant polyphenolic compounds and/or their metabolites to scavenge oxygen and nitrogen free radicals, has been related to the health benefits of diets rich in fruits and vegetables (Sun, Chu, Wu, & Liu, 2002; Huang, Johanning, & O'Dell, 1986).

Competitive techniques have been widely employed to test the reactivity of antioxidants (XH) and/or free radical scavengers towards free radicals. These methods evaluate how the added substrate protects a reference compound from being degraded by peroxyl radicals (Niki, 1990) using a variety of molecules as reactive targets (phycobiliproteins, crocin, pyrogallol red, etc.) (Bhat & Madyastha, 2000; Chatterjee, Poduval, Tilak, & Devasagayam, 2005; Huang, Ou, & Prior, 2005; Lissi, Pascual, & Del Castillo, 1992; Lissi, Pizarro, Aspée, & Romay, 2000; López-Alarcón & Lissi, 2006; López-Alarcón & Lissi, 2005; Pérez, Leighton, Aspée, Aliaga, & Lissi, 2000; Prior & Cao, 1999; Roginsky & Lissi, 2005; Tubaro, Ghiselli, Rapuzzi, Maiorino, & Ursini, 1998; Tubaro, Rapuzzi, & Ursini, 1999).

In spite of the wide use and advantages of competitive kinetics methods, possible secondary reactions of antioxidant derived radicals with the target molecule can preclude

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a straightforward evolution of the tested compound reactivity. In spite of this, the possibility is generally not considered and only a few works have taken this point into account in the kinetic analysis (Galati, Sabzevari, Wilson, & O'Brien, 2002; Pino & Lissi, 2001). In the present work, we employed a competitive kinetic method using c-phycocyanin (c-Pc) as a target molecule for estimating the reactivity of several natural phenols and flavonoids presents in the human diet towards 2,2′-azo-bis(2-amidinopropane) dihydrochloride (AAPH) derived peroxyl radicals. The results obtained indicate that the relative protection afforded by a given compound strongly depends upon the experimental conditions employed, and emphasize the role of secondary reactions of the phenol-derived radicals initially formed.

2. Experimental

2.1. Chemicals

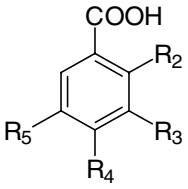
AAPH (2,2′-azo-bis(2-amidinopropane) dihydrochloride) thermolysis in air saturated solutions was used as peroxyl radical source (Niki, 1990). Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), flavonoids, phenolic compounds (Fig. 1) and AAPH were purchased from Sigma–Aldrich (St. Louis, MO, USA) and employed as received. Phycocyanin (c-Pc) was obtained from Arthospira maxima species and purified by the method of Neufeld and Riggs (Neufeld & Riggs, 1969). The c-Pc concentration was estimated by considering a mw of ca. 13,000 per each bilin group.

2.2. Solutions

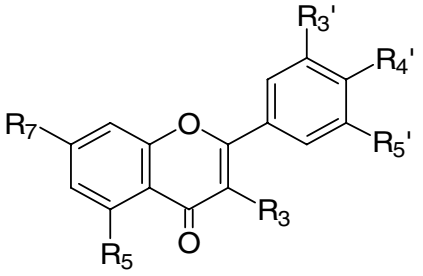
A mixture containing c-Pc (0.8 or 23 μM) with or without the tested compounds (1–50 μM) in phosphate buffer (10 mM) at pH 7.0, was incubated at 37 °C in the thermostated cuvette of either a Perkin Elmer LS-50 (Norwalk, CT, USA) spectrofluorimeter (c-Pc = 0.8 μM) or a Hewlett Packard 8453 (Palo Alto, CA, USA) UV-visible spectrophotometer (c-Pc = 23 μM). The reaction was initiated by adding an aliquot of AAPH (10 mM final concentration). The consumption of the target molecule (c-Pc) was evaluated from the decrease in its fluorescence intensity (excitation: 620 nm; emission: 640 nm) or from the progressive absorbance decrease, measured at 620 nm. Stock solutions of c-Pc (77–230 μM) were prepared daily in phosphate buffer 10 mM, pH 7.0. Stock solutions of the tested phenolic compounds were prepared in ethanol immediately before their use. The final ethanol concentration in the cuvette was below 5%.

2.3. Kinetic analysis

A simple kinetic model for the competitive oxidation of a target molecule (c-Pc) and a given antioxidant (XH) by a radical ($ROO^{\cdot}$) must consider reactions (1)–(4)



Compounds	R_2	R_3	R_4	R_5
Salicylic acid	-OH	-H	-H	-H
Gallic acid	-H	-OH	-OH	-OH
Protocatechuic acid	-H	-OH	-OH	-H



Compounds	R_3	R_5	R_7	R_3'	R_4'	R_5'
Flavone	-H	-H	-H	-H	-H	-H
3-Hydroxy-flavone	-OH	-H	-H	-H	-H	-H
Galangin	-OH	-OH	-OH	-H	-H	-H
Kaempferol	-OH	-OH	-OH	-H	-OH	-H
Quercetin	-OH	-OH	-OH	-OH	-OH	-H
Apigenin	-H	-OH	-OH	-H	-OH	-H
Luteolin	-H	-OH	-OH	-OH	-OH	-H

Fig. 1. Structures of tested polyphenols.

$$AAPH \xrightarrow{O_2} 2ROO^{\cdot} + N_2 \quad (1)$$

$$ROO^{\cdot} + c - Pc \rightarrow \text{bleaching} \quad (2)$$

$$ROO^{\cdot} + XH \rightarrow X^{\cdot} + ROOH \quad (3)$$

$$2ROO^{\cdot} \rightarrow \text{non radicals products} \quad (4)$$

and all the self-reactions and cross-reactions of the radicals produced in steps (2) and (3). In this scheme, and for simplicity, we are not considering the formation of alkoxyl radicals in reaction (4). This oversimplified scheme predicts a monotonous increase in the (R^0/R) values (where R^0 is the initial rate of consumption of the target molecule in absence of antioxidant, and R is the initial rate of consumption of c-Pc in presence of antioxidant) with XH concentration, depending upon the relative rates of processes (2)–(4). If $R_4 \gg R_2$ (where R_4 and R_2 are the rates of reactions (4) and (2), respectively), as expected at low c-Pc concentrations, the consumption of c-Pc in absence of XH follows a first-order kinetics (Pino & Lissi, 2001) and