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The Role of Cyclodextrins in ORAC-Fluorescence Assays. Antioxidant Capacity of Tyrosol and Caffeic Acid with Hydroxypropyl-β-Cyclodextrin

Marcos García-Padial, María Cristina Martínez-Ohárriz, Iñigo Navarro-Blasco, and Arantza Zornoza*

Departamento de Química y Edafología, Facultad de Ciencias, University of Navarra, Irunlarrea s/n, Pamplona 31080, Navarra, Spain

ABSTRACT: Tyrosol and caffeic acid are biophenols that contribute to the beneficial properties of virgin olive oil. The influence of hydroxypropyl-β-cyclodextrin (HPβ-CD) on their respective antioxidant capacities was analyzed. The ORAC antioxidant activity of tyrosol (expressed as μM Trolox equivalents/μM Tyrosol) was 0.83 ± 0.03 and it increased up to 1.20 ± 0.11 in the presence of 0.8 mM HPβ-CD. However, the ORAC antioxidant activity of caffeic acid experienced no change. The different effect of HPβ-CD on each compound was discussed. In addition, the effect of increasing concentrations of different cyclodextrins in the development of ORAC-fluorescence (ORAC-FL) assays was studied. The ORAC signal was higher for HPβ-CD, followed by Mβ-CD, β-CD, γ-CD and finally α-CD. These results could be explained by the formation of inclusion complexes with fluorescein.

KEYWORDS: *cyclodextrin, tyrosol, caffeic acid, oxygen radical absorbance capacity*

INTRODUCTION

High levels of free radicals such as reactive oxygen species and reactive nitrogen species play an important role in the oxidative damage of biomolecules [1] such as DNA, proteins and membrane lipids and also in many of the chemical mechanisms associated to the degradation of food and chemical materials.[2] In relation to human health, they are involved in processes related to the pathogenesis of several chronic diseases and aging.[3]

During the past years, evidence of the health benefits associated to the intake of fruits and vegetables has led to investigate a great variety of phenolic compounds from the plant kingdom due to their antioxidant properties, which could reduce the damage caused by free radicals.[4−6] The antioxidant capacity of these compounds is based on their capability as radical scavengers, which can stop radical chain reactions and also inhibit the formation of reactive oxygen species.[7] In addition to the possible health benefits associated to the consumption of phenolics, there is a growing interest in the food industry to substitute synthetic antioxidants by natural alternatives such as phenolic compounds.

The antioxidant activity of phenolic compounds can be modified in the presence of cyclodextrins (CDs). Native cyclodextrins, namely, α-CD, β-CD, and γ-CD, are cyclic oligosaccharides with six, seven, and eight glucopyranose units, respectively. These compounds present a hydrophilic external part and a relatively hydrophobic cavity in which guest molecules can be encapsulated by inclusion complex formation.[8,9] Native cyclodextrins can be chemically modified to improve their solubilities and hydrophilic/hydrophobic properties. Complexation with cyclodextrins can improve properties such as water solubility, bioavailability, stability and the antioxidant activity of the included compound.[10]

Several methods have been described for the assessment of antioxidant capacity for radical scavenging in vitro,[11] although the ORAC (oxygen radical absorbance capacity) method that employs fluorescein as fluorescent probe is one of the most widely used.[12,13] The quantification in ORAC assays, based on AUC (area under the curve) measurements, combines both inhibition time and inhibition percentage of the free radical damage by the antioxidant in a single value.[14] Cyclodextrins are commonly used in ORAC assays to enhance the solubility of hydrophobic compounds, but in recent years a number of ORAC-fluorescein studies have been carried out to study the effect of cyclodextrins on the antioxidant activity of different phenolic compounds.[15−18]

The present study analyzed the influence of hydroxypropyl-β-cyclodextrin (HPβ-CD) on the antioxidant capacity of tyrosol (TY) and caffeic acid (CA), biophenols that contribute to the well-known beneficial properties of virgin olive oil. These compounds have been chosen as model compounds because we are working on different applications of cyclodextrins in the field of olive oil antioxidants[19] and also to compare the effect of cyclodextrins on the antioxidant properties of two compounds with markedly different antioxidant capacity, caffeic acid being more potent than tyrosol.

The aim of our investigation involved two aspects: first, to determine the antioxidant capacity of tyrosol and caffeic acid in the presence of cyclodextrins and second, to study the influence of different cyclodextrins in the development of ORAC-fluorescence assays, because we consider that there are some points that need to be clarified in relation to both the signal produced by cyclodextrins and the interaction of cyclodextrins with the compounds involved in the ORAC-fluorescence assay.

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MATERIALS AND METHODS

Chemicals. Fluorescein sodium salt and Trolox C [6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid, 97%] were obtained from Sigma-Aldrich (Barcelona, Spain). AAPH [(2,2′-azobis(2-methylpropionamidine)dihydrochloride, 98%] was from Acros (Barcelona, Spain). Tyrosol, 98%, and caffeic acid, 97%, were purchased from Aldrich (Barcelona, Spain). α-CD, β-CD and γ-CD were from Wacker (Burghausen, Germany); methyl β-cyclodextrin (Mβ-CD) and HPβ-CD with average substitution degree of 4 were purchased from Cyclolab (Budapest, Hungary). Potassium dihydrogen phosphate and potassium monohydrogen phosphate of analytical grade were from Panreac (Barcelona, Spain).

ORAC-Fluorescence (ORAC-FL) Assay. The ORAC assays were performed in a Fluostar Omega BMG LABTECH multidetection microplate reader, using 96 well optical Bottom Plates purchased from Thermo Fisher Scientific. Fluorescence was read through the clear bottom, with an excitation wavelength of 485 nm and an emission wavelength of 520 nm. The software used was Multiuser Reader Control and Mars Data Analysis. The oxygen radical absorbance capacity was determined as described by Dávalos et al.[12] with slight modifications.

One of the aims of our study was to evaluate the ORAC-FL signal of different concentrations of α-, β-, γ-, hydroxypropyl-β-, and methyl-β-cyclodextrin ranging from 0.05 to 1 mM. The solubilization of cyclodextrins was carried out by sonication (Ultrasons, Selecta). The experimental procedure was the same as that described below for the antioxidants tyrosol and caffeic acid.

The stock solutions of fluorescein, tyrosol, caffeic acid and HPβ-CD were prepared in 10 mM sodium phosphate buffer (pH 7.4) and were stored at 4 °C for a maximum of 4 weeks. A Trolox stock solution in 10 mM sodium phosphate buffer (pH 7.4) was readied and aliquoted into a small vial for storage at −20 °C until use. All stock solutions were stored in absence of light.

The volume of the final reaction mixture was 200 μL in well. The final concentration of fluorescein in well was 80 nM; those of tyrosol and caffeic acid ranged from 1.5 to 15 and from 0.5 to 5 μM, respectively, in the absence or presence of different concentration of HPβ-CD solutions (0.1, 0.2, 0.4, and 0.8 mM). The AAPH solution was readied in situ in pH 7.4 buffer and quickly added to the mixture (60 mM final concentration in well) using a multichannel pipet and starting with those wells that contained higher antioxidant concentration. The microplate was immediately placed in the reader and the fluorescence recorded every 0.75 min for 160 cycles; therefore, each assay time was 2 h. The microplate was automatically shaken prior to each reading.

Trolox was used as reference standard. It was analyzed in the absence of cyclodextrins to enable the comparison of our results with those reported by other authors. The final concentrations in well ranged from 5 to 20 μM.

Blanks (fluorescein + AAPH) with phosphate buffer were prepared for the assays of antioxidants without cyclodextrins. The blanks corresponding to antioxidant-HPβ-CD mixtures contained also the same concentration of cyclodextrin as the corresponding samples.

All reaction mixtures were prepared in triplicate, and at least three independent assays were performed for each sample. To avoid a temperature effect, only the inner 60 wells were used for experimental purposes, while the outer wells were filled with 200 μL of distilled water.

The results were expressed as relative fluorescence with respect to the initial reading. The area under the fluorescence decay curve (AUC) was calculated by the equation reported by Cao et al.,[20] and data processing was performed using Excel. Final results were expressed in μM of Trolox equivalent/μM of phenolic compound. The relative Trolox equivalent ORAC values were calculated according to the following equation:

$$\mathrm{ORAC}_{\mathrm{value}} = \frac{(\mathrm{AUC}_{\mathrm{sample}} - \mathrm{AUC}_{\mathrm{blank}}) \times [\mathrm{Trolox}]}{(\mathrm{AUC}_{\mathrm{Trolox}} - \mathrm{AUC}_{\mathrm{blank}}) \times [\mathrm{sample}]}$$

where $\mathrm{AUC}_{\mathrm{sample}}$ is the area under curve in the presence of the tested compounds; $\mathrm{AUC}_{\mathrm{blank}}$ is the area under curve of control; $\mathrm{AUC}_{\mathrm{Trolox}}$ is the area under curve in the presence of Trolox, and [Trolox] and [sample] are the molar concentrations of Trolox and tested compounds, respectively.[14]

All statistical analyses were run using the program Stata/IC v.12.1 for Windows. Different data groups were compared through nonparametric Kruskal−Wallis and U-Mann−Whitney test for independent or paired groups with statistical significance set at $p < 0.05$.

RESULTS AND DISCUSSION

Effect of Cyclodextrins on the ORAC-FL Assay. One of the objectives of the present paper was to study the influence of cyclodextrins on the ORAC-FL signal. Natural cyclodextrins (α, β and γ-CD) as well as Mβ-CD and HPβ-CD were analyzed by the ORAC-FL method. The former were chosen to analyze the effect of cavity size and the last were selected because they are the most widely applied CD derivatives.

Figure 1 represents a plot of $\mathrm{AUC}_{\mathrm{net}}$ (obtained by subtraction of the blank to each sample) against the concentrations of each

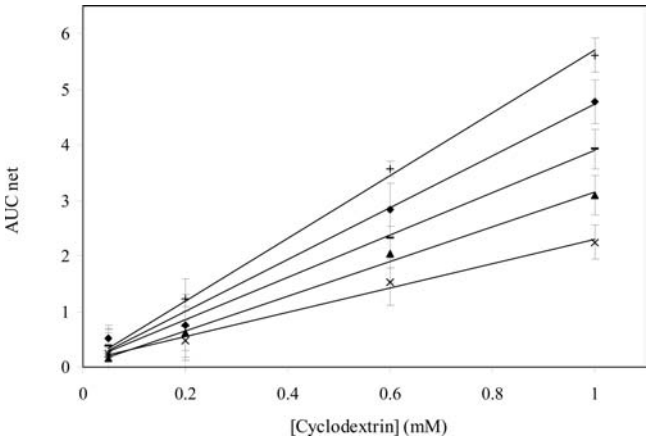

Figure 1. ORAC signal of different cyclodextrins expressed as net area under the curve: HPβ-CD (+), Mβ-CD (♦), β-CD (−), γ-CD (▲), α-CD (×).

cyclodextrin, which ranged from 0.05 to 1 mM. The $\mathrm{AUC}_{\mathrm{net}}$ was higher for HPβ-CD, followed by Mβ-CD, β-CD, γ-CD and finally α-CD. The $\mathrm{AUC}_{\mathrm{net}}$ observed is directly proportional to the concentration of each cyclodextrin, with coefficients of determination (R^2) ranging from 0.996 to 0.998. These results are important for future studies of cyclodextrin complexes with antioxidants in the sense that the effect of cyclodextrins has to be carefully studied under the experimental conditions of the assay before deciding whether to use cyclodextrin blanks in the antioxidant capacity assays of inclusion compounds.

The results obtained can be explained by the formation of inclusion complexes between fluorescein and α, β and γ-CD, since complexation could protect fluorescein from oxidation, hinder its reaction with AAPH and result in an increase of the $\mathrm{AUC}_{\mathrm{net}}$ value. Flamigni[21] determined the stability constants of the complexes of fluorescein with the three natural cyclodextrins; the stability constant of the complex with β-CD was markedly higher (360 M^{-1}) than that obtained for the complexes with α and γ-CD (35 M^{-1}). The values of these constants were associated to a tighter fit of fluorescein in the medium size cavity of β-CD. In relation with the results obtained in the present paper, the higher stability constant

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obtained with β-CD would be in accordance with the higher inhibition of the reaction between fluorescein and AAPH in the presence of β-CDs. The possible complexation of AAPH was discarded because its chemical structure does not seem favorable for inclusion in the CD cavities.

These studies revealed the influence of cyclodextrins in the ORAC-FL assay. Therefore, in subsequent studies, the AUC values corresponding to cyclodextrin were subtracted from each sample in order to obtain the AUC_{net} of the target molecule in the presence of cyclodextrins.

Influence of HP-β-Cyclodextrin on the Antioxidant Capacity of Tyrosol and Caffeic Acid. The cyclodextrin selected for this study was HPβ-CD because it has a high solubility in aqueous medium and low toxicity, properties well suited for future applications. The AUC_{net} of each tyrosol-cyclodextrin sample has been determined by subtracting a blank which contained the corresponding cyclodextrin concentration, as described in the previous section.

Figure 2 shows the antioxidant activity of different concentrations of tyrosol alone and in the presence of

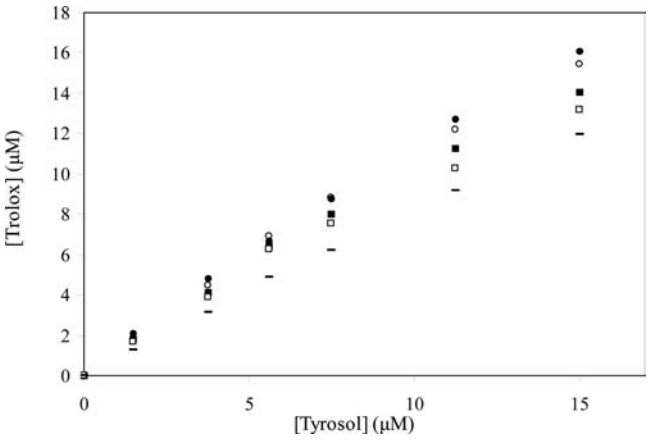

Figure 2. Antioxidant activity of different concentrations of tyrosol in the presence of increasing HP-β-CD concentrations: 0 mM (−), 0.1 mM (□), 0.2 mM (■), 0.4 mM (χ), and 0.8 mM (●). The relative standard deviation (%RSD) of the experimental data ranged from 2% to 11%.

increasing concentrations of HPβ-CD (0.1, 0.2, 0.4, and 0.8 mM). There is a linear relationship between tyrosol concentration and antioxidant activity at each cyclodextrin concentration with R^2 values ranging from 0.989 to 0.999. The addition of increasing concentrations of cyclodextrins resulted in a clear enhancement in the antioxidant activity of tyrosol.

The antioxidant activity of a fixed concentration of tyrosol was plotted against the concentration of HPβ-CD as shown in Figure 3. It can be observed that at high concentrations of HPβ-CD, the slope reaches a plateau because the sites of inclusion between tyrosol and HPβ-CD are being saturated. At the highest HPβ-CD concentration employed, the antioxidant capacity of tyrosol was 1.5 times higher (50% increase) than in the absence of cyclodextrin. This can be related with the formation of an inclusion complex between tyrosol and HPβ-CD, recently reported by our group.[19] A dynamic equilibrium is associated to the formation of inclusion complexes so the antioxidant activity rises as the concentration of complexed tyrosol reaches a maximum. Statistically significant differences (Kruskal–Wallis test, $p < 0.001$; U-Mann–Whitney test, $p <$

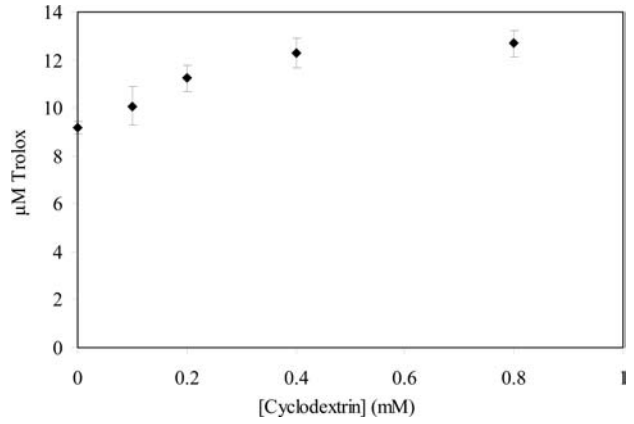

Figure 3. Influence of HP-β-CD in the ORAC antioxidant activity of 11.25 μM tyrosol.

0.05) were obtained for the analytical data up to the beginning of the plateau (0.4 mM HPβ-CD)

The antioxidant activity of pure tyrosol was determined to be 0.83 ± 0.03 μM Trolox equivalents/μM Tyrosol. Our result was in accordance with the value of 0.78 μM Trolox equivalents/μM Tyrosol reported by Madrona et al.[22] The antioxidant capacity of tyrosol in the presence of 0.8 mM HPβ-CD increased markedly up to 1.20 ± 0.11 μM Trolox equivalents/μM Tyrosol. The present study evidence the ability of HPβ-CD to increase the antioxidant activity of Tyrosol, an interesting fact from the point of view of possible applications in the field of antioxidants.

In the case of caffeic acid, no change was observed in the ORAC-FL signal of this compound in the presence of HPβ-CD, although in some of the assays a very slight decrease of antioxidant activity was detected (data not shown). These results did not provide consistent evidence of any effect of HPβ-CD on the antioxidant capacity of caffeic acid, although the formation of the complex is reported in the literature.[23] The antioxidant activity determined was 3.00 ± 0.30 μM Trolox equivalents/μM caffeic acid. This value is within the range of results obtained in previous studies.[24−27]

There are several ORAC studies in the literature about complexes of antioxidants with CDs; some of them observed an enhancement of the antioxidant activity but others reported no effect. For example, the complexation of resveratrol in HPβ-CD showed almost double the antioxidant activity it showed in the absence of CDs.[15] The antioxidant activity of flavonols, kaempferol, quercetin and myricetin increased upon complexation with HPβ-CD.[17] Inclusion complexes of native and two β-cyclodextrin derivatives with some tea polyphenols such as epigallocatechin gallate and gallocatechin gallate increased the antioxidant activity; nevertheless, with catechin the antioxidant activity decreased.[16]

The interaction of tyrosol and caffeic acid with HPβ-CD gives rise to complexes with relatively similar stability constants[19,23] (265 and 279 M^{-1}, respectively). However, our investigation evidenced that the role of inclusion complexation in the antioxidant activity of tyrosol and caffeic acid is markedly different. This fact can be explained from different points of view based on the general mechanisms involved in the oxidation reactions between oxygen and nitrogen-centered radicals and phenolic compounds. Hydrogen-atom transfer (HAT) and electron transfer (ET) from the phenolic moiety to the radical are the most important oxidation pathways for the

antioxidant action of phenolic compounds. These mechanisms are mainly related to the O−H bond dissociation energy (BDE) and the ionization potential (IP) of the particular antioxidant. The predominant mechanisms in ORAC assays are those involving hydrogen atom transfer reactions.[7]

A first approach to explain the results obtained is based on a study of Leopoldini et al.[28] which reported that the values of BDE and IP of tyrosol were lower in a nonpolar medium than in water, whereas in the case of caffeic acid, the opposite tendency was observed. These facts would be in accordance with the increase in the antioxidant activity of tyrosol upon inclusion in the CD cavity, which is less polar than water, and also with the very slight decrease detected in the case of caffeic acid.

In addition, the different effect of cyclodextrins on the antioxidant activity of the compounds studied could be related to the hydrogen-bond pattern of the phenolic antioxidants in water in comparison with that in the inclusion complex. It is well-known that intermolecularly hydrogen bonded phenols cannot react with radicals and only non-hydrogen-bonded phenols are reactive.[29] Caffeic acid presents a catechol structure in which ortho OH groups are able to form an intramolecular hydrogen-bond, which leaves a free hydroxyl group with a decreased BDE.[30] The inclusion of caffeic acid in the cavity of β-CD does not result in the formation of H-bonds with the cyclodextrin rims,[31] so the intramolecular H-bond does not seem to change upon complexation.

On the other hand, the para-substitution of tyrosol may well give rise to an inclusion complex in which the H-atom transfer ability of tyrosol is improved if the hydroxyl group is properly positioned in the cyclodextrin rim, where the formation of H-bonds would be less favored than in bulk water.

AUTHOR INFORMATION

Corresponding Author

*Tel.: +34 948425600 (6378). Fax: +34 948425649. E-mail: azornoza@unav.es.

Notes

The authors declare no competing financial interest.

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ABBREVIATIONS USED

AAPH, [(2,2′-azobis(2-methylpropionamidine) dihydrochloride; ORAC, oxygen radical absorbance capacity; ORAC-FL, oxygen radical absorbance capacity fluorescence; CD, cyclodextrin; Mβ-CD, methyl-beta-cyclodextrin; HPβ-CD, hydroxypropyl-beta-cyclodextrin; AUC, area under curve; AUC_{net}, net area under curve; HAT, hydrogen-atom transfer; ET, electron transfer; BDE, bond dissociation energy; IP, ionization potential

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