Food Chemistry 136 (2013) 1263–1271

Contents lists available at SciVerse ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Binding affinity between dietary polyphenols and β-lactoglobulin negatively correlates with the protein susceptibility to digestion and total antioxidant activity of complexes formed

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ARTICLE INFO

Article history:
Received 6 July 2012
Received in revised form 5 September 2012
Accepted 11 September 2012
Available online 18 September 2012

Keywords:
Antioxidant
β-Lactoglobulin
Digestion
Interaction
Quenching
Polyphenol

ABSTRACT

Non-covalent interactions between β-lactoglobulin (BLG) and polyphenol extracts of teas, coffee and cocoa were studied by fluorescence and CD spectroscopy at pH values of the gastrointestinal tract (GIT). The biological implications of non-covalent binding of polyphenols to BLG were investigated by *in vitro* pepsin and pancreatin digestibility assay and ABTS radical scavenging activity of complexes formed. The polyphenol–BLG systems were stable at pH values of the GIT. The most profound effect of pH on binding affinity was observed for polyphenol extracts rich in phenolic acids. Stronger non-covalent interactions delayed pepsin and pancreatin digestion of BLG and induced β-sheet to α-helix transition at neutral pH. All polyphenols tested protected protein secondary structure at an extremely acidic pH of 1.2. A positive correlation was found between the strength of protein–polyphenol interactions and (a) half time of protein decay in gastric conditions ($R^2 = 0.85$), (b) masking of total antioxidant capacity of protein–polyphenol complexes ($R^2 = 0.95$).

1. Introduction

Polyphenols, including phenolic acids, flavonoids, proanthocyanidins and resveratrol, are a large and heterogeneous group of phytochemicals abundantly present in foods and beverages such as tea, coffee, wine, cocoa, cereal grains, soy, fruits and berries.

Non-covalent interactions between polyphenols and globular proteins may result in complexation (Chaudhuri, Chakraborty, & Sengupta, 2011; Li et al., 2010), stabilization of protein structure (Kanakis et al., 2011), protein unfolding and precipitation (Ma, Yin, Liu, & Xie, 2011; Papadopoulou & Frazier, 2004; Siebert, Troukhanova, & Lynn, 1996). The strength of interactions depends on the size of polyphenols, polyphenol structure and amino-acid sequence of proteins (Frazier et al., 2010).

Many *in vitro*, *in vivo* and epidemiological studies have suggested that dietary polyphenols induce beneficial effects on human health, treatment and prevention of cardiovascular disease and cancer (Lambert & Elias, 2010; Yang, Wang, Lu, & Picinich, 2009). These effects are mainly due to their antioxidant and anti-inflammatory properties (Tachibana, 2009; Wu, Guo, Ren, Guo, & Meydani, 2009). The inhibition of digestive enzymes involved in carbohydrate, lipid and protein metabolism by dietary polyphenols may be another important mechanism for the health benefits attributed to a diet rich in fruit and vegetables (Hanhineva et al., 2010; McDougall, Kulkarni, & Stewart, 2008).

The structural characterization of the interactions between globular food proteins and polyphenols is a major step in elucidating the effect of polyphenols on globular protein structure. Tryptophan residues are intrinsic fluorophores in proteins and spectroscopic techniques measuring quenching of Trp fluorescence in proteins have been applied to studies of polyphenol binding to various proteins, such as milk caseins and β-lactoglobulin (BLG), lysozyme, hemoglobin, serum albumins and gamma globulins (Chaudhuri et al., 2011; Rahimi Yazdi & Corredig, 2012; Wang et al., 2011; Xiao et al., 2011; Yuksel, Avci, & Erdem, 2010; Zorilla, Liang, Remondetto, & Subirade, 2011). The quantum yield and emission maximum wavelength associated with intrinsic Trp fluorescence are very sensitive to the polarity of the environment and the structural changes in biomacromolecules (Chaudhuri et al., 2011; Ma et al., 2011).

Complexation of polyphenols and proteins can affect the antioxidant activity of polyphenols by affecting their electron donating capacity and reducing the number of hydroxyl groups available

Abbreviations: BLG, beta-lactoglobulin; GIT, gastrointestinal tract; PE, polyphenol extract; BTPE, black tea PE; GTPE, green tea PE; CFPE, coffee PE; CCPE, cocoa PE.

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0308-8146/$ - see front matter © 2012 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.foodchem.2012.09.040

in solution (Arts et al., 2002; Niseteo, Komes, Belščak-Cvitanović, Horžić, & Budeč, 2012). However, due to the prolonged life of polyphenols in complexes, the effect of complexation may be beneficial for the overall antioxidant activity of the polyphenols.

Some recent studies have demonstrated new important biological effects of protein–polyphenol non-covalent interactions. Polyphenols exhibited a potent dose-dependent inhibitory activity on alpha Synuclein aggregation and were capable of disaggregating the pre-formed alpha Synuclein oligomers (Caruana et al., 2011). A positive correlation was also found between the degree of polyphenol oligomerization and inhibition of elastase due to an increased number of protein interacting groups (Brás et al., 2010). In addition, protein–polyphenol complexation may reduce IgE binding of allergens due to irreversible precipitation of allergens (Chung & Champagne, 2009; Chung & Reed, 2012).

Most of these reports focused on the interactions of pure polyphenol compounds and a model protein. In the GIT, dietary protein encounters mixtures of polyphenolic compounds present in various foods. Therefore, our approach was to examine the interactions between a model globular whey protein (β-lactoglobulin) and complex polyphenol mixtures as isolated from various polyphenol rich sources (green and black tea, coffee, cocoa). All the mixtures were analyzed for their exact compositions by high resolution mass spectrometry.

We demonstrated that changes in pH that occur in the human GIT (1.2-2.5 in the stomach and 7.2 in the intestine and saliva) affect the binding affinity of polyphenolic compounds to the protein. The most profound effect of pH on the binding affinity was observed for polyphenol extracts composed mainly of phenolic acids. The binding constants obtained were analyzed relative to the known biological effects of polyphenols – i.e., protein digestion inhibition and masking of the antioxidative power of complexes formed. All polyphenol extracts (PE) tested, regardless of chemical composition, protected protein secondary structure at an extremely acidic pH of 1.2. A positive correlation was observed between the binding strength of protein–polyphenol interactions, half time of protein decay during gastric digestion and masking of the total antioxidant capacity of protein–polyphenol complexes.

2. Materials and methods

The details of the experiments are given in the Supplementary material available online (Supplementary material: Materials and methods).

2.1. Isolation of phenolic extracts

Dietary polyphenols were isolated by methanol extraction and quantified using Folin-Ciocalteu reagent. Molar concentration was calculated using averaged molar mass (AMM) of polyphenols (BTPE – 420, GTPE – 459.7, CFPE – 387, CCPE – 487.63 g/mol) calculated from the data obtained from LC–MS analysis of the obtained PE (Supplementary material: Tables S1–S4).

2.2. LC–MS analysis of phenolic extracts

Isolated PEs were characterized by LTQ Orbitrap XL hybrid FTMS (Thermo Fisher Scientific, USA). Compounds were identified by combining exact masses, molecular formulae derived from Mass Frontier Spectral Interpretation Software (Thermo Fisher Scientific, USA) and spectra from LTQ Orbitrap XL hybrid FTMS with findings from the literature and internal database of spectra of standard polyphenol compounds.

2.3. Fluorescence spectroscopy

Fluorescent spectra were recorded on a FluoroMax-4 spectrofluorimeter (Horiba Scientific, Kyoto, Japan). The following buffers were used: 0.1 M HCl with 2 g/L NaCl pH 1.2, 0.1 M glycine-HCl pH 2.5 and phosphate buffered saline (PBS) pH 7.2. To 2.5 mL of 2.5 μg/mL (0.14 μM) protein solution (prepared in a suitable buffer immediately before the experiment) 10 aliquots of PEs (1 mg/mL) were added. Protein solution was titrated with 10×2 μL of PEs at pH 1.2, 2.5 and 10×1 μL of PEs at pH 7.2. After the addition of each aliquot fluorescent spectrum was immediately recorded under the conditions: λ excitation 280 nm, λ emission 290–410 nm. Because some polyphenols possess intrinsic fluorescence, a blank was made for each polyphenol concentration, in which protein solution was replaced with appropriate solvent, as described previously (Soares, Mateus, & de Freitas, 2007). The blank spectrum was automatically subtracted from the emission spectrum of the corresponding solution. Spectra were further analyzed using an OriginPro 8 software package (Northampton, MA, USA).

2.4. Fluorescence quenching analysis

Fluorescent quenching is described by the Stern-Volmer equation:

$$F_0/F = 1 + K_{SV} \times [Q] \quad (1)$$

(Liang, Tajmir-Riahi, & Subirade, 2007) where F_o and F are the fluorescence intensities before and after addition of a quencher; K_{sv} is the Stern-Volmer quenching constant and [Q] is the concentration of the quencher. Polyphenols are able to absorb energy both at the proteins' excitation and to a lower extent emission wavelength. In order to overcome the inner filtering effect, the measured emission fluorescence intensity at 340 nm was corrected according to Eq. (2):

$$F = \mathrm{Fu} \times 10^{QL \times (\varepsilon_{\lambda ex} + \varepsilon_{\lambda em})} \quad (2)$$

(Shpigelman, Israeli, & Livney, 2010)where Fu is the measured, uncorrected, emission intensity, $\varepsilon_{\lambda ex}$ and $\varepsilon_{\lambda em}$ are the molar extinction coefficients of polyphenols at the excitation and emission wavelengths, [Q] is the quencher concentration and L is the path length of the cell.

A linear Stern-Volmer plot indicates that one mechanism of quenching occurs – static (complex formation) or dynamic. The Stern-Volmer plots obtained were linear at all the applied conditions (Fig. 1). Thus, the fluorescence quenching rate constant (k_q) can be calculated according to Eq. (3) if τ_0 (fluorescence lifetime of fluorophore without a quencher) is known.

$$K_{SV} = k_q \times \tau_0 \quad (3)$$

(Lakowicz, 1999; Soares et al., 2007)

The maximum value possible for diffusion-limited quenching in water is $\sim 10^{10}\ M^{-1}\ s^{-1}$.

Trp fluorescence lifetime depends on pH and buffer composition. Trp τ_0 in phosphate buffer pH 7.0 is 3.15 ns. At the acidic pH of 1.2 in hydrochloric acid Trp τ_0 is 0.75 ns and at pH 2.5 Trp τ_0 is 2.33 ns (citric acid-phosphate buffer) (Gudgin, Lopez-Delgado, & Ware, 1981). According to previous studies (Soares et al., 2007), τ_0 of the Trp residues of BLG at neutral pH is 1.28 ns at λ_{ex} 280 nm. The lowest K_{sv} values determined in our study were in the range of $1 \times 10^4\ M^{-1}$ yielding k_q above $10^{12}\ M^{-1}\ s^{-1}$, two orders of magnitude higher than the diffusion-limited quenching.

When the value of the bimolecular quenching rate constant is higher than diffusion-limited quenching, it could mean that there is a complex formation between a protein and a quencher, corresponding to a static mechanism of the fluorophore quenching.