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The effect of non-covalent interaction of chlorogenic acid with whey protein and casein on physicochemical and radical-scavenging activity of *in vitro* protein digests

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ABSTRACT

The effects of the interaction of whey protein isolate (WPI) and casein (CS) with chlorogenic acid (CA; 20, 120 and 240 μmol/g protein) on the structural and functional properties of proteins were investigated. CA induced significant structural changes, increased digestibility, and improved functionalities of CS and WPI. Non-covalent association between CA and treated proteins was detected using Fourier transform infrared spectroscopy (FTIR), intrinsic tryptophan fluorescence, and ANS-augmented fluorescence. The CA binding affinity for WPI was superior to that for CS as indicated by higher K_{sv} and lower hydrophobicity. Total sulfhydryl content in CS and WPI decreased, respectively, from 5.4 to 3.2 μmol/g and from 21 to 7.8 μmol/g, and surface hydrophobicity declined by 16.6% and 22.4% with 240 μmol/g CA. Enhanced solubility and foaming capacity of the protein-phenol complex were demonstrated. CA at medium and high concentrations displayed a remarkable synergism of radical scavenging activity with peptides in both protein digests.

1. Introduction

Plant polyphenols have been shown to exert numerous biological functions, for example, antioxidant, anti-cancer, anti-inflammatory, and anti-bacterial activities, and are considered to be possible dietary supplements to treat atherosclerosis, diabetes, and stroke (Ozdal, Capanoglu, & Altay, 2013). In higher plants, polyphenols as secondary metabolites are widely distributed – in the bark, roots, and leaves, as well in fruits and seeds (Guo, Kong, & Meydani, 2009). Plant polyphenols are the main source of antioxidants in human diets (Graf, Milbury, & Blumberg, 2005). As antioxidants, they are often used to improve the quality and shelf-life of food and beverages (Utrera & Estevez, 2013).

In food systems, polyphenols can interact with proteins through covalent and non-covalent associations (Le Bourvellec & Renard, 2012; Maqsood, Benjakul, & Shahidi, 2013). Covalent binding to proteins, an irreversible interaction, normally occurs when a phenolic compound is oxidized to quinone in the presence of polyphenol oxidase or in an alkaline environment (Cao & Xiong, 2015; Wang et al., 2014). Quinone derivatives are highly reactive with nucleophilic groups within a protein to form covalent bonds. Some researchers have suggested that bioactivity and bioavailability of plant polyphenols may be affected by the covalent interaction between polyphenols and proteins (Trombley, Loegel, Danielson, & Hagerman, 2011).

Non-covalent binding of phenolics to proteins refers to interactions that occur through hydrophobic association, hydrogen bonds, electrostatic attraction, and van der Waals forces. Hydrophobic interaction and hydrogen bond are considered as the most important non-covalent driving forces for the phenolic-protein complexation (Cao & Xiong, 2017). The hydroxyl groups of the polyphenols and the carbonyl groups of the peptide chain can form hydrogen bonds. The hydrophobic regions of the protein amino acids (benzene and aliphatic side chains) and the aromatic nuclei of the polyphenols play a key role in the stabilization of the polyphenol-protein complexes (Ozdal et al., 2013).

Studies have found that plant polyphenols can interact with proteins to initiate the inhibition of enzyme activity as well as change the protein solubility, thermal stability, and digestibility (Labuckas, Maestri, Perelló, Martínez, & Lamarque, 2008). It has also been shown that certain structural changes in proteins that are caused by binding with polyphenols can enhance the protein functionality. For example, epigallocatechin gallate (EGCG) improved emulsifying and foaming properties of whey protein isolate (WPI) (Jia et al., 2016), oxidized phenolic compounds increased the gel strength of surimi (Balange & Benjakul, 2009), and EGCG reduced α-lactalbumin denaturation

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temperature and increased its antioxidant capacity (Wang et al., 2014).

The effect of polyphenols on protein digestibility is also of nutritional importance. Some reports suggest that the presence of polyphenols may reduce the digestibility of proteins *in vivo* and *in vitro* because of the formation of insoluble protein aggregates and possible inactivation of digestive enzymes (He, Lv, & Yao, 2007). Arimboor and Arumughan (2011) found that sea buckthorn procyanidins precipitated proteins and inhibited digestive enzymes to reduce protein digestion. However, other researchers have claimed that polyphenols can promote protein digestibility, and this has been demonstrated in a mixed EGCG-WPI system where the presence of EGCG induced structural unfolding, hence, an increased accessibility of the susceptible peptide bonds (Cao & Xiong, 2017). In addition, polyphenols having strong radical scavenging activity are added to dairy products to enhance their antioxidant capacity. Milk proteins, including WPI and casein, are widely used ingredients in protein beverages, such as latte, milk tea, and other milk products flavored with fruit juice. Milk proteins and polyphenols coexisting in protein-based beverages are key compounds to determine the product stability during storage.

The aim of this study was to investigate the effect of chlorogenic acid (CA) addition on the structure and functionality of casein (CS) and WPI, especially the digestibility and antioxidant capacity during digestion. It is hoped that the study will provide a positive basis for the application of plant active ingredients in the development of healthy and nutritious food products.

2. Materials and methods

2.1. Materials

Whey protein isolate (WPI, purity > 90%) was purchased from Davisco Foods (Le Sueur, Minn., U.S.A.). Casein (CS, from bovine milk, purity > 90%) was purchased from Sigma-Aldrich (St. Louis, Mo., U.S.A.). Chlorogenic acid (CA, purity > 98%) was obtained from J & K Chemical Technology (Shanghai, China). All chemical reagents were at least of analytical grade and purchased from Sigma-Aldrich (St. Louis, Mo., U.S.A.).

2.2. Incubation of WPI and CS with CA

WPI, CS, and CA stock solutions were freshly prepared in phosphate buffer (100 mM, pH 7.0). WPI and CS solutions (10 mg/mL) were kept at 4 °C overnight to ensure complete hydration. The protein solutions in the pH 7.0 buffer were thoroughly mixed with CA (20, 120, and 240 μmol/g protein) and incubated at room temperature (25 °C) for 2 h to initiate interaction according to Ferraro, Madureira, Sarmento, Gomes, and Pintado (2015). Structural and functional properties of the reacted protein samples were analyzed subsequently. For fluorescence intensity measurement, the protein-CA interaction was carried out in a dilute protein solution, which is described later.

2.3. Solubility

The solubility of CS and WPI before and after the incubation with different concentrations of CA was determined according to Jiang, Chen, and Xiong (2009). The samples were centrifuged at 10,000 × *g* for 20 min. The protein concentration in the supernatant and in the original samples were measured using the Biuret method. Solubility was defined as the percentage of the protein concentration of the supernatant over that of total protein (before centrifugation).

2.4. Fourier-transform infrared spectroscopy (FTIR)

Untreated and CA-treated protein samples were freeze dried. Then 1–2 mg sample and 200 mg KBr were mixed evenly and compressed to prepare transparent film. FTIR spectra were obtained using an infrared spectrophotometer (Nicolet, WI, U.S.A.) from 4000 cm^{-1} to 400 cm^{-1} at a data acquisition rate of 2 cm^{-1} per point. Each sample was scanned 32 times.

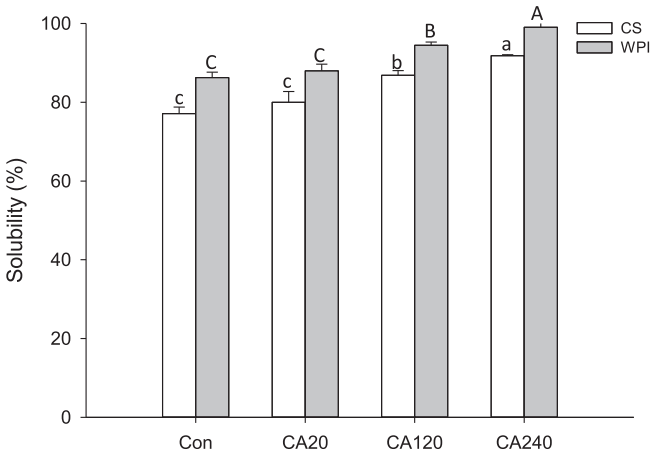

Fig. 1. Solubility of CS and WPI treated with different concentrations of CA (CA20: 20 μmol/g protein, CA120: 120 μmol/g protein, CA240: 240 μmol/g protein) at pH 7.0. Con: WPI or CS without phenolic treatment. Means (n = 3) with different letters for CS (a–c) and WPI (A–C) differ significantly ($P < 0.05$).

2.5. Fluorescence spectroscopy

Different amounts of the CA stock solution were added to 4 mL protein solution (0.5 mg/mL) and mixed thoroughly. The mixtures were incubated in constant temperature water baths at 18 °C, 25 °C or 38 °C for 5 min. The fluorescence spectroscopy of CS and WPI incubated with different levels of CA were recorded using a F7000 spectrofluorometer (Hitachi, Tokyo, Japan). The excitation wavelength was 280 nm and the emission spectra were scanned in the range of 300–500 nm with a scanning speed of 12,000 nm/min.

To further study the interaction mechanism, the fluorescence quenching of CA for CS and WPI was examined. Fluorescence quenching occurs in different modes, including dynamic quenching and static quenching. Stern-Volmer equation was applied to determine the type of quenching (Dobreva et al., 2014): $F_0/F = 1 + K_q \tau_0 [Q] = 1 + K_{sv} [Q]$. Here, F_0 and F are the fluorescence intensities of the protein solution without and with CA, respectively, [Q] is the concentration of CA, K_q is a bimolecular quenching process rate constant, and K_{sv} is the quenching constant. The maximum quenching constant of all kinds of fluorescent quenchers for biological macromolecules is about 2.0×10^{10} L/(mol·s). τ_0 is the lifetime of the phosphor when the quencher is not present. The average life expectancy of the macromolecule is about 10^{-8} s (Wang et al., 2012).

2.6. Sulfhydryl content

Total sulfhydryl content of CA-treated and untreated proteins was analyzed according to Beveridge, Toma, and Nakai (1974). Samples (1 mL) were mixed with 2 mL of 8 M urea and reacted with 0.5 mL of 10 mM 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB). The absorbance (412 nm) of the reacted samples after 30 min were recorded. Samples not reacted with DNTB were used as controls. The sulfhydryl content was calculated using the molar extinction coefficient of 13,600 M^{-1} cm^{-1}.

2.7. Surface hydrophobicity

The variations in surface hydrophobicity of samples were measured using 1-anilinonaphthalene-8-sulfonic acid (ANS) as a fluorescence probe as described by Kato and Nakai (1980). Samples were diluted