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Interaction of milk whey protein with common phenolic acids

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HIGHLIGHTS

- Phenolic acids interacted with the structural subunits of whey protein.
- The quenching mechanism was static quenching when phenolics bound to whey protein.
- The conformation of whey protein was altered due to interact with phenolic acids.

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ABSTRACT

Phenolics-rich foods such as fruit juices and coffee are often consumed with milk. In this study, the interactions of α-lactalbumin and β-lactoglobulin with the phenolic acids (chlorogenic acid, caffeic acid, ferulic acid, and coumalic acid) were examined. Fluorescence, CD, and FTIR spectroscopies were used to analyze the binding modes, binding constants, and the effects of complexation on the conformation of whey protein. The results showed that binding constants of each whey protein–phenolic acid interaction ranged from 4×10^5 to 7×10^6 M^{-n} and the number of binding sites n ranged from 1.28 ± 0.13 to 1.54 ± 0.34. Because of these interactions, the conformation of whey protein was altered, with a significant reduction in the amount of α-helix and an increase in the amounts of β-sheet and turn structures.

1. Introduction

Preventing the onset of chronic diseases has become an attractive strategy for improving the cost-effectiveness of public health spending [1]. Increasing intake of bioactive compounds to provide benefits has been proposed as an alternative to classical pharmacology for improving health and lessening the burden of disease [2,3]. Both experimental and epidemiological evidence has demonstrated that an increased intake of phenolic acids is associated with a reduced risk of cardiovascular disease and cancer [4,5]. In human diet, phenolic acids are generally consumed in foods along with other macronutrients. For example, phenolics-rich foods such as fruit juices and coffee are often consumed with milk [6]. The detrimental effects of milk on the antioxidant capacity of tea polyphenols have recently been reported [7]. In our previous study, it was established that milk proteins can affect the absorption of polyphenol [8,9]. Therefore, it shows that the interactions between milk protein and phenolic acids can affect the function of phenolic acids and it is meaningful to study the interactions in vitro.

The abundant proteins in milk whey are α-lactalbumin (α-LA) and β-lactoglobulin (β-LG), which are of major interest in the food industry. α-Lactalbumin is a 14 kDa protein, the native structure of which is divided into two domains: one is largely helical (the α-domain) while the other has a significant content of β-sheets (the β-domain), and these are connected by a calcium binding loop [10]. β-LG exists as a mixture of monomers and dimers, the equilibrium ratio of which depends on the association constant of the dimer and on the protein concentration. Each monomer consists of 162 amino acid residues and has a molecular mass of 18 kDa [7]. A number of investigations have revealed that bovine serum albumin is frequently a "target" of therapeutically active phenolics [11]. Therefore, most researchers focused on the interactions between bovine serum albumin and phenolic acids [12–14]. However, to date, little is known about the interaction modes of phenolic acids

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(chlorogenic acid, caffeic acid, ferulic acid, and coumalic acid) with α-lactalbumin and β-lactoglobulin.

The aim of the present study was to study the interaction of whey protein with phenolic acids. Chlorogenic acid (CHLO), caffeic acid (CAFF), ferulic acid (FERU), and coumalic acid (COUM) are commonly found in human diet. It is of interest to study the interactions of whey protein with these phenolic compounds. The binding constants, binding sites, and main types of binding force were determined by fluorescence experiments. In addition, the conformational changes of the protein were probed based on circular dichroism (CD) and fourier transform infrared spectroscopy (FTIR) data.

2. Materials and methods

2.1. Materials

α-Lactalbumin (purity >85%), β-lactoglobulin (purity >90%), CAFF, CHLO, COUM, and FERU were purchased from Sigma to Aldrich Chemical Co. (St. Louis, MO, USA).

2.2. Preparation of stock solutions

The solution of whey protein was prepared in aqueous solutions containing 10 mM Tris–HCl buffer (pH 7.4), and then measured the concentration by spectrophotometer. The molar concentration was calculated by absorbance divided by the extinction coefficients of protein (optical length is 1 cm^{-1}). The extinction coefficients is 28,840 M^{-1} cm^{-1} at 280 nm for α-LA, and 17,600 M^{-1} cm^{-1} for β-LG [1,10]. Then the solution was diluted to the 0.5 mM with buffer solution. Solutions of phenolic acids (CAFF, CHLO, COUM, and FERU) were prepared in 10 mM Tris–HCl buffer (pH 7.4) and diluted to various concentrations with further buffer (0.5, 0.25, 0.125 mM).

2.3. Fluorescence spectroscopy

Fluorescence spectra were measured with a spectrofluorophotometer (RF-5301 PC; Shimadzu, Tokyo, Japan). Fluorescence emission spectra were recorded in the range 290–500 nm (excitation wavelength 280 nm) using 3 nm/3 nm slit widths. The interactions between whey protein and phenolic acids were quantitatively analyzed by fluorimetric titrations as follows: 3 mL of a 30 μm protein solution was titrated by successive addition of portions of a phenolic acid solution to reach a final concentration of 90 μM (0,10,20,30,40,50,60,70,80,90 μM). Upon excitation at 280 nm, the maximum emission wavelengths were 324 nm for α-LA and 332 nm for β-LG. The fluorescence intensities at these wavelengths were measured in triplicate. Appropriate blanks corresponding to the buffer were subtracted to correct for background fluorescence. Quenching constants were calculated using the Stern–Volmer equation [15,16].

2.4. Circular dichroism

Circular dichroism measurements were made at 25 °C on a JASCO-810 (JASCO Corporation, Tokyo, Japan) spectrophotometer, using sample solutions with path length 1 mm, over the range 200–250 nm with a scan rate of 100 nm/min and a response time of 4 s. Three scans were accumulated for each spectrum. The protein concentration was kept constant (12.5 μM), whilst varying the concentration of each phenolic acid (12.5,25,50 μM) [7]. The induced ellipticity was taken as the ellipticity of each protein–phenolic acid mixture minus the ellipticity of the phenolic acid alone at the same wavelength, and the results were expressed as molar ellipticity ($[\theta]$) in deg cm^2 $dmol^{-1}$ [17].

2.5. FTIR spectroscopic measurements

Infrared spectra with a resolution of 4 cm^{-1} were recorded on an FTIR spectrophotometer (Perkin–Elmer, UE, Massachusetts, USA) by the attenuated total reflection method, acquiring 60 scans. A phenolic acid solution was added dropwise to the protein solution with constant stirring to ensure the formation of a homogeneous solution and to reach the target phenolic acid concentrations of 125, 250, or 500 μM, with a final protein concentration of 250 μM. Spectra were collected from hydrated films after incubation of the protein/phenolic acid solution for 2 h at room temperature (25 °C) [18]. Spectra of the sample solutions and the buffer solution were collected under the same conditions. The absorbance of the buffer solution was then subtracted from the spectrum of the sample solution to obtain the FTIR spectrum of the protein [19]. Baseline correction was carried out in the range 1600–1700 cm^{-1} to obtain the amide I band. The numbers, positions, and widths of the component bands were estimated by performing a Fourier self-deconvolution and obtaining the second derivative of the protein infrared amide I band. Based on these parameters, a curve-fitting process was carried out by means of Origin 8.0 software (OriginLab, Northampton, UK) to obtain the best Gaussian-shaped curves that fitted the original protein spectra. After identifying individual bands that were diagnostic of certain secondary structures, the percentages of each secondary structure of the protein were calculated by comparing the relative areas of their respective component bands [20,21].

3. Results and discussion

3.1. Fluorescence spectra of whey proteinphenolic acid complexes

Most proteins can emit intrinsic fluorescence after absorbing ultraviolet light owing to the presence of certain residues such as Trp, Tyr, and Phe in their molecular structures, and this is the case for whey protein [22]. Upon excitation at 280 nm, strong emission (λ_{em} = 324 nm for α-LA; λ_{em} = 332 nm for β-LG) from whey protein and no emission from the phenolic acids were recorded in the emission range 290–500 nm. From Fig. 1, it can be seen that the fluorescence intensity of the whey protein diminished as CHLO solution was gradually added. The results of other phenolic acids are shown in supplementary material Fig. S1. The fluorescence intensity of protein can be weakened by a variety of molecule interaction, which is called fluorescence quenching [17]. Fluorescence quenching may result from various processes, such as molecular collision, ground-state complex formation, excited-state reaction, molecular rearrangement, or energy transfer. The mechanisms of these processes are usually classified as either dynamic or static quenching [23]. To elucidate the quenching mechanism induced by phenolic acids, fluorescence quenching data are analyzed with the Stern–Volmer equation [24]:

$$F_0/F = 1 + K_{sv}[Q] = 1 + k_q t_0[Q] \quad (1)$$

where F_0 and F are the fluorescence intensities before and after addition of the quencher, respectively; K_{sv} is the Stern–Volmer dynamic quenching constant, a direct measure of the quenching efficiency; k_q is the quenching rate constant of the biomolecule; t_0 is the average lifetime of the biomolecule; and [Q] is the concentration of quencher. K_{sv} was obtained as the slope of the linear regression, as shown in Table S1 and the insert in Fig. 1, the values were of the order of 10^4 M^{-1} for each phenolic acid. The fluorescence lifetime of the biopolymer was 10^{-8} s so the quenching rate constant k_q