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Characterization of flavonoid-protein interactions using fluorescence spectroscopy: Binding of pelargonidin to dairy proteins

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ABSTRACT

In this study, the interaction between the flavonoid pelargonidin and dairy proteins: β-lactoglobulin (β-LG), whey protein (WPI), and caseinate (CAS) was investigated. Fluorescence experiments demonstrated that pelargonidin quenched milk proteins fluorescence strongly. However, the protein secondary structure was not significantly affected by pelargonidin, as judged from far-UV circular dichroism. Analysis of fluorescence data indicated that pelargonidin-induced quenching does not arise from a dynamical mechanism, but instead is due to protein−ligand binding. Therefore, quenching data were analyzed using the model of independent binding sites. Both β-LG and CAS, but not WPI, showed hyperbolic binding isotherms indicating that these proteins firmly bound pelargonidin at both pH 7.0 and 3.0 (binding constants ca. 1.0×10^5 at 25.0 °C). To investigate the underlying thermodynamics, binding constants were determined at 25.0, 35.0, and 45.0 °C. These results pointed to binding processes that depend on the structural conformation of the milk proteins.

1. Introduction

Flavonoids are a class of phenolic compounds that can be categorized into groups such as catechins, anthocyanidins, flavones, or flavonols. Anthocyanins are one of the most important classes of phenolic compounds found in fruits and vegetables because of their ability to generate bright colors (Yang, Koo, Song, & Chun, 2011). However, anthocyanins are also being explored for their utilization as functional food ingredients to prevent chronic diseases, such as cancer, inflammation and diabetes (Sancho & Pastore, 2012). This preventive effect has partly been attributed to the ability of anthocyanins to act as antioxidants and signal inhibitors in biological systems (Chang et al., 2010; Kanakis, Tarantilis, Polissiou, Diamantoglou, & Tajmir-Riahi, 2006; Mandeville, Froehlich, & Tajmir-Riahi, 2009). However, the utilization of anthocyanins as bioactive ingredients in functional food products is currently limited because of their poor chemical stability and low bioavailability. A number of factors are known to contribute to the chemical instability of anthocyanins, such as their chemical structure, polymeric form, concentration, pH, temperature, oxygen levels, light exposure, and the presence of cofactors and/or ascorbic acid (Bridle & Timberlake, 1997). Recent studies suggest these some of these limitations may be overcome by forming flavonoid-protein complexes (Kanakis, Tarantilis, Polissiou, & Tajmir-Riahi, 2013; Neilson & Ferruzzi, 2011). These complexes should be designed to improve the functional performance of the flavonoids, but also to be stable within food matrices and to not negatively impact the physicochemical or sensory properties of food products. It is therefore important to understand the interactions between proteins and flavonoids, and the nature of the complexes formed.

A number of previous studies have characterized the interactions between various types of phenolic compounds and food proteins. For example, the interactions of flavonols and flavonoids with ovoalbumin, lysozyme, phosvitin, gelatin, α-lactalbumin, β-lactoglobulin, and β-casein have previously been studied (Jöbstl, Howse, Fairclough, & Williamson, 2006; Kanakis et al., 2011; Prigent et al., 2009; Yan, Hu, & Yao, 2009; Zorilla, Liang, Remondetto, & Subirade, 2011). These studies have shown that these compounds can interact with proteins and form complexes with different structural and physicochemical properties, such as stoichiometry, size, charge, and solubility.

In the present work, the interaction of an anthocyanin (pelargonidin) with various dairy protein ingredients (β-lactoglobulin, whey protein isolate, and sodium caseinate) was

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studied using fluorescence quenching measurements at different pH and temperature values, and the binding parameters were calculated. Fluorescence quenching is based on the decrease in the quantum yield that occurs from a fluorophore when it interacts with a quencher molecule (van de Weert & Stella, 2011). Fluorescence quenching can be dynamic, owing to the collisions between the fluorophore and quencher, or static, as a result of the formation of a ground state complex between the fluorophore and quencher (Lakowicz & Weber, 1973). Therefore, it is possible to use quenching of the intrinsic tryptophan fluorescence as a tool to study the interactions of flavonoids with dairy proteins. Whey protein isolate (WPI) and caseinate were selected for study because they are widely used functional ingredients in the food industry (Livney, 2010). WPI is isolated from the whey fraction of milk and contains a mixture of globular proteins, such as β-lactoglobulin, α-lactalbumin, and BSA (Morr & Ha, 1993). Caseinate is isolated from the curd fraction of milk and contains a mixture of more disordered flexible proteins (Phadungath, 2005). Previous studies suggest that more disordered proteins have stronger interactions with phenolic compounds than globular proteins, which can be attributed to differences in the exposure of their functional groups (Deaville, Green, Mueller-Harvey, Willoughby, & Frazier, 2007).

The results of this study should provide a better understanding of the interactions between an important flavonoid (pelargonidin) and an important class of food proteins (milk proteins) that are widely used as functional ingredients. This knowledge could facilitate the development of functional food products enriched with flavonoids that have improved stability during storage, and enhanced bioavailability characteristics.

2. Materials and methods

2.1. Materials

Sodium caseinate (CAS) (⩾86%) was obtained from AMCO Proteins (Burlington, NJ). Whey protein isolate (WPI) (⩾87%) and β-lactoglobulin (β-LG) (⩾95%) were kindly donated by Davisco Foods International Inc. (Eden Prairie, MN). Pelargonidin chloride (⩾90%) was purchased from Sigma-Aldrich (St. Louis, MO). All other reagents were purchased from Sigma-Aldrich (St. Louis, MO); they were of analytical grade and used as received.

2.2. Sample preparation

Stock solutions of CAS, β-LG, and WPI (1 mg/mL) were prepared on the day of use by dissolution of the powdered ingredients into the appropriate buffer solution (20 mM sodium phosphate, pH 7.0; 20 mM sodium acetate, pH 3.0). Pelargonidin solutions were made daily by initially preparing a methanolic stock solution (200 μM) at the appropriate pH of study. Protein and pelargonidin stock solutions were filtered before analysis through 0.45 μm filters (Millipore Corporation, Bedford, MA, USA) to remove any large particles that may scatter light strongly. Protein-pelargonidin mixtures with different compositions were prepared by mixing different ratios of stock solutions and buffer solution together. These solutions were subsequently left in the dark at 25, 35 and 45 °C for 1 h prior to measurements.

2.3. Fluorescence spectroscopy

Spectrofluorometric analysis was applied to estimate the interaction of pelargonidin with β-LG, WPI and CAS in aqueous solutions. Samples were made up to a final volume of 3-mL with the appropriate buffer (pH 3.0 and 7.0) solution and placed in 10 × 10 mm cuvettes with controlled temperature (25, 35 and 45 °C) and stirring. The fluorescence emission spectra and fluorescence quenching of the proteins in the presence of different pelargonidin concentrations were then determined. Samples were illuminated using an excitation wavelength of 280 nm and the resulting emission spectra were measured in the wavelength range from 300 to 500 nm. The experiments were made with a K2 Spectrofluorometer (ISS Inc., Champaign, IL). Experimental data were fitted using Origin 7.0 (OriginLab, Northampton, MA, USA). The equation used to determine binding parameters is available in the Supporting Information.

2.4. Circular dichroism measurements

Conformational changes during protein-pelargonidin interaction were monitored by circular dichroism (CD) spectroscopy. CD spectra were registered in a J-715 spectropolarimeter (JASCO, Easton, MD) equipped with a PTC-348WI Peltier-type holder for temperature control and magnetic stirring. The secondary structure of the proteins (β-LG and CAS) was assessed from spectra registered over the 200–250 nm (far-UV) range. Measurements were made in a 1.00-cm cuvette with 10 μg mL^{-1} protein solutions. Recording of spectral data was stopped when the high-tension voltage in the detector reached 750 V. All samples were previously equilibrated against the appropriate buffer (50 mM sodium phosphate, pH 7.0; 50 mM sodium acetate, pH 3.0). Low-temperature spectra were obtained at 25 °C from freshly prepared samples. The results are reported as obtained (raw data).

2.5. Statistical analysis

All measurements were performed on three samples at least three times. Means and standard deviations were calculated from this data using Excel Microsoft (Redmond, WA, USA).

3. Results

The interactions between the anthocyanin (pelargonidin) and different proteins were quantified using fluorescence spectroscopy. Additionally, circular dichroism measurements were carried out to investigate the effect of pelargonidin on the protein secondary structure. Initially, the results for the interactions between pelargonidin and β-lactoglobulin are presented because this is the simplest most well defined system, and is amenable to analysis using theoretical models. The results of the interactions between the pelargonidin and whey protein and caseinate are then discussed because these are more complex multicomponent systems.

3.1. Fluorescence spectroscopy analysis of β-LG–pelargonidin interactions

The dependence of the fluorescence emission spectra for β-LG solutions containing different amounts of pelargonidin (pH 3.0, 45.0 °C) was measured (Fig. 1A). These measurements show that the protein fluorescence intensity, which is dominated by the emission of the two tryptophan residues present in its structure (Lakowicz, 1999), decreases appreciably with increasing pelargonidin concentration whereas there was only a slight wavelength shift (ca. 2 nm) of the spectral maximum. It should be noted that these changes in fluorescence intensity are not accompanied by significant changes in the secondary structure of β-LG (see Fig. S1 in Supplementary Material). To establish which of the two well known quenching mechanisms (dynamic or static) was operative, the intensity data were plotted (Fig. 1B). This data is in agreement with the Stern-Volmer equation for the dynamic quenching of a single emitting chromophore (Lakowicz, 2006):