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Interactions of caseins with phenolic acids found in chocolate

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ABSTRACT

To investigate the interactions between caseins and phenolic acids, such as the ones present in chocolate, casein was incubated with protocatechuic acid or *p*-coumaric acid at 55 °C. In addition, casein was isolated from chocolate and the phenolic compounds within these caseins were quantified. Electrophoresis results revealed that casein–phenolic interactions were induced by incubation; minor aggregation of casein subunits was observed after incubation of casein with protocatechuic acid. Minor aggregation of casein isolated from milk chocolate was also observed. *In vitro* hydrolysis of casein control, casein–protocatechuic acid, casein–*p*-coumaric acid, caseins isolated from milk chocolate and white chocolate using trypsin showed degree of hydrolysis of 19.3, 18.6, 17.7, 10.4 and 17.8% respectively. The presence of protocatechuic acid and *p*-coumaric acid in the model system and the presence of phenolic compounds in milk chocolate, in addition to the structural changes occurring during processing, affected the peptide profiles of casein hydrolysates.

1. Introduction

Among minor food components, phenolics or polyphenols have recently received considerable attention because of their functional properties such as antioxidant, antimutagenic, anti-inflammatory, antiatherogenic and antitumour activities (González-Sarrías, Li, & Seeram, 2012; Nahar, Driscoll, Slitt, & Seeram, 2014; Ortega et al., 2008; Tanaka, Kojima, Kawamori, Yoshimi, & Mori, 1993; Živković et al., 2014). Furthermore, it has been demonstrated that many phenolic compounds play important roles in preventing certain human diseases, such as osteoporosis, cancers, and cardiovascular diseases (Kaume, Gbur, DiBrezzo, Howards, & Devareddy, 2014; Morton, Caccetta, Puddey, & Croft, 2000; Oliveras-López, Berná, Jurado-Ruiz, López-García de la Serrana, & Martín, 2014). Black tea, green tea, red wine and cocoa are good sources of phenolics as they are rich in phenolic phytochemicals (Lee, Kim, Lee, & Lee, 2003). Protocatechuic acid is a hydroxybenzoic acid that can be found in many foods such as olives, flaxseed, and wine (Minussi et al., 2003; Van Hoed, 2010). It is also the most important phenolic acid (69.16%) found in cocoa liquor (Ortega et al., 2008). It has been reported to have several physiological functions including antioxidant, antibacterial activity, antimutagenic activity, antitumour activity, and anticancer effects (Yin, Lin, Wu, Tsao, & Hsu, 2009). Coumaric acids, hydroxy derivatives of cinnamic acid, are another important group of phenolic compounds found in cocoa (2.65%) with antioxidant and antigenotoxic properties (Ferguson, Zhu, & Harris, 2005; Kikugawa, Hakamada, Hasunuma, & Kurechi, 1983).

In addition to providing essential nutrients, food proteins are important functional ingredients due to their effect in maintaining the quality and sensory properties of foods (Belitz, Grosch, & Schieberle, 2009). Casein, which accounts for 80% of milk protein, is one of the principal functional food proteins (Fox, 2001; Marchesseau et al., 2002). It is present as large protein complexes incorporating milk salts (Marchesseau et al., 2002). Milk casein is in the form of colloidally dispersed particles (calcium caseinate) which are known as micelles (Marchesseau et al., 2002; McMahon & Brown, 1984). Caseins are heterogeneous proteins whose main types are α_{s1}-casein (38%), α_{s2}-casein (10%), β-casein (36%) and κ-casein (13%) (Fox, 2001; Tuckey, 1963).

It has been demonstrated that different phenolic compounds bind to a variety of proteins, especially to proteins with a high content of proline, such as β-casein with 15.6 mole percentage of proline (Baxter, Lilley, Haslam, & Williamson, 1997; Charlton et al., 2002; Siebert, Troukhanova, & Lynn, 1996). The binding of phenolic compounds to proteins has been suggested to reduce their antioxidative potential due to the reduction in their accessibility (Kilmartin & Hsu, 2003; O'Connell & Fox, 2001; Serafini, Ghiselli, & Ferro-Luzzi, 1996). On the other hand, these interactions between phenolic compounds with caseins have been shown to increase the stability to heat denaturation (O'Connell & Fox, 2001; O'Connell, Fox, Tan-Kinitia, & Fox, 1998), to oxidative degradation (O'Connell & Fox, 1999), and the foaming ability of their micelles (Rosenthal, Bernstein, & Nakimbugwe, 1999; Sausse, Aguie-Beghin, & Douillard, 2003). Although the interaction between

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caseins and larger phenolic compounds have been investigated (O'Connell & Fox, 1999, 2001; O'Connell et al., 1998; Sausse et al., 2003), the interactions between caseins and smaller phenolic acids, such as protocatechuic acid and *p*-coumaric acid that are also present in food matrices such as chocolate, are unknown. The results obtained from such investigation will be important for the understanding of the behavior and characteristics of casein within a food matrix and its effect on the bioavailability of phenolic acids.

Given this, the objective of this research was to study the casein–phenolic acid interactions in a model system. In addition, caseins from milk and white chocolate were isolated and compared with the caseins in the model system. The casein–phenolic interactions were induced by heat incubation and casein was extracted from both milk chocolate and white chocolate. Characteristics of the casein–phenolic complexes were investigated by using a combination of electrophoresis, reversed-phase high performance liquid chromatography (RP-HPLC) and tryptic hydrolysis.

2. Materials and methods

2.1. Materials

Commercial whole casein from bovine milk (Technical Grade), α-casein, β-casein, κ-casein, protocatechuic acid (3,4-dihydroxybenzoic acid), *p*-coumaric acid and trypsin type IX-S from porcine pancreas (EC 3.4.21.4, 13,700 U/mg, 14,800 U/mg protein) were purchased from Sigma-Aldrich, Co. (St. Louis, MO, USA). Samples of milk chocolate and white chocolate were obtained as gifts from Barry-Callebaut (Montreal, Qc, Canada).

2.2. Induction of casein–phenolic interactions

Casein–phenolic interactions were induced using the procedure reported by Ali (2002) with modifications. Casein–phenolic solution mixtures were prepared with 10 mM phosphate buffer (pH 7) and by mixing 1 ml casein solution (5 mg/ml) with 1 ml protocatechuic acid solution (1 mg/ml) or 1 ml *p*-coumaric acid solution (1 mg/ml); the ratio of casein to phenolic acid was 5:1 in order to resemble the natural concentration of these compounds observed in chocolate formulations. Casein control solution (2.5 mg/ml casein) was prepared by mixing 1 ml casein solution with 1 ml phosphate buffer (10 mM; pH 7). The above solutions were heated in a water bath at 55 °C for 2 h then cooled to room temperature.

2.3. Casein extraction from chocolate

Milk chocolate and white chocolate were defatted using the Soxhlet extraction method following the procedure described by the Association of Official Analytical Chemists (1990) with modifications. The fat from 10 g of chocolate sample melted using a water bath at 50 °C was extracted with 100 ml of petroleum ether for 8 h. The defatted chocolate samples were air dried in a fume hood. Casein was isolated from milk chocolate and white chocolate using the procedure described by Veloso, Terxeira, and Ferreira (2002) and Molina (2006) with modifications. Defatted chocolate powder (10 g) was ground using a mortar and pestle and was reconstituted with 100 ml of distilled water and adjusted to pH 4.6 (1 M HCl) to precipitate the casein. The mixture was allowed to stand for 1 h with continuous stirring then centrifuged (8000 ×*g*, 25 min), then the supernatant was discarded. The residue was washed with acetone followed by centrifugation (8000 ×*g*, 25 min). The casein was air dried and stored at 4 °C.

2.4. Polyacrylamide gel electrophoresis (PAGE)

Native-PAGE was carried out according to the method reported by Ornstein (1964) and Davis (1964) with some modifications. Acrylamide staking gel (4%) and resolving gel (8%) were selected to perform with a Mini-PROTEAN 3 Cell unit (Bio-Rad, Hercules, CA, USA). Sample solutions were prepared by dissolving 600 μl casein–phenolic mixtures or 15–20 mg casein isolated from chocolate in 300 μl sample buffer (distilled water, 0.5 M Tris–HCl pH 6.8, glycerol and 0.5% bromophenol blue) separately. Sample solutions (10–15 μl) were loaded into each individual sample well. The commercially available high molecular weight calibration kit (Amersham Bioscience, Piscataway, NJ, USA) was used as standard protein markers. The marker proteins were thyroglobulin (669 kDa), ferritin (440 kDa), catalase (232 kDa), lactate dehydroxygenase (140 kDa) and albumin (67 kDa). Gels were run at constant current (7.5 mA/gel) for approximately 1.5 h. After electrophoresis, gels were fixed for about 10 min in fixing solution (distilled water: methanol: acetic acid/7:2:1) followed by staining for 1 h with Coomassie Brilliant Blue R-350. Destaining of gels was done by frequently replacing the fixing solution until the protein bands were clearly visible.

SDS-PAGE was performed following the method reported by Laemmli (1970) with some modifications. A Mini-PROTEAN 3 Cell unit (Bio-Rad) was used with a stacking gel of 4% as well as a resolving gel of 12.5%. Sample solutions were prepared by dissolving 250 μl casein–phenolic mixtures or 15–20 mg casein isolated from chocolate in 250 μl sample buffer (distilled water, 0.5 M Tris–HCl pH 6.8, glycerol, 10% SDS, 0.5% bromophenol blue and β–mercaptoethanol), respectively, followed by heat treatment at 95 °C for 5 min. Sample solutions (10–20 μl) were loaded into each individual sample well. Broad molecular weight standard kit (Bio-Rad) was loaded into the first sample well. The standard protein markers were myosin (200 kDa), β-galactosidase (116.25 kDa), phosphorylase b (97.4 kDa), serum albumin (66.2 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), trypsin inhibitor (21.5 kDa), lysozyme (14.4 kDa) and aprotinin (6.5 kDa). The molecular weight of the standard proteins and their migration distance were used to make a standard curve to estimate the molecular weight of the protein samples. The electrophoresis of gels was carried out at a constant voltage (120 V) for approximately 1.5 h. A mixture of methanol (20% v/v) and acetic acid (10% v/v) was used to fix gels for 10 min. The gels were stained by Coomassie Brilliant Blue R-350 followed by destaining with the fixing solution.

2.5. RP-HPLC

RP-HPLC was performed by following the protocol reported by Alli, Gibbs, Okoniewska, Konishi, and Dumas (1993) with a few modifications to monitor the profiles of casein–phenolic complexes. The system used was equipped with a programmable solvent module (Model 126, Beckman Coulter, Indianapolis, IN, USA) for completing the delivery of solvents and a programmable detector module (Model 166, Beckman Coulter) for detecting the absorbance of the eluted fractions. Samples were prepared by mixing 200 μl casein standard, casein control, phenolic controls, casein–phenolic mixtures with 800 μl sample buffer (0.1% trifluoroacetic acid (TFA) in 10% acetonitrile solution) and then filtered through an acetate membrane filter (0.45 μm, GE, Piscataway, NJ, USA). Solvent A and B were used to generate a gradient elution system with solvent A composed of 0.1% trifluoroacetic acid (TFA) in deionized water and solvent B composed of 0.1% trifluoroacetic acid (TFA) in acetonitrile/deionized water (7:3). Samples (100 μl) were injected into the system by a manual injector through a 100 μl loop. Separation of fractions was done through an Eclipse XDB C18 reversed phase column (5 μm, 4.6 × 250 mm; Agilent, Santa Clara, CA, USA). The fractions of samples were eluted at a constant flow rate of 0.5 ml/min with the following gradient: starting at 10% solvent B and increasing to 70% in 30 min, followed by holding at 70% solvent B for 20 min, and returning to 10% solvent B in 10 min. Chromatographic data were analyzed by the Gold System (Beckman Coulter).