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The interaction of cocoa polyphenols with milk proteins studied by proteomic techniques

Monica Gallo [a], Giovanni Vinci [b], Giulia Graziani [b], Carmela De Simone [b], Pasquale Ferranti [b,c,*]

[a] Department of Molecular Medicine and Medical Biotechnology, University of Naples Federico II, via Pansini 5, 80131 Naples, Italy
[b] Department of Agriculture, University of Naples Federico II, via Università 100, 80055 Portici, Naples, Italy
[c] Institute of Food Science, National Research Council, via Roma 52, 83100 Avellino, Italy

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ABSTRACT

The molecular interaction of cocoa polyphenols with milk proteins were investigated in vitro by combined proteomic and biochemical strategies. Mass spectrometry and antioxidant activity assays allowed monitoring the binding of casein and whey protein fractions to cocoa polyphenols. In particular, the nature of interaction of β-lactoglobulin (β-Lg) with catechin and epicatechin was characterized and the amino acid residue at the binding site was identified. On the other side, antioxidant activity assays also showed a significant effect of the various milk protein fractions in decreasing the in vitro antioxidant activity of polyphenols, suggesting the existence of other types of protein–polyphenol interactions, probably weaker non-covalent bonds. From a nutritional point of view, these data indicate that the β-Lg covalent modification by polyphenol alone do not support the hypothesis of a decrease in the bioavailability of polyphenols themselves (Scalbert & Williamson, 2000). This might also explain the maintenance of the antioxidant properties of cocoa polyphenols in cocoa-based beverages. These results suggest the perspective use of the model system developed to study other complex food matrices.

1. Introduction

Phenolic compounds are one of the most represented groups of substances in the plant kingdom: there are currently more than 8000 known phenolic structures produced by secondary metabolism of plants (Bravo, 1998). Among these compounds, phenolic acids and flavonoids account for 30% and 60% of total polyphenols ingested with diet, respectively (Manach, Scalbert, Morand, Rémésy, & Jiménez, 2004; Ramos, 2007; Xiao et al., 2011). Phenolic compounds are raising great interest in medical and scientific research for their health benefits, which include anti-carcinogenic, anti-atherogenic, anti-inflammatory, anti-microbial, anti-hypertensive activities (Gryglewski, Korbut, Robak, & Swies, 1987; Kaul, Middleton, & Ogra, 1985; Mascolo, Pinto, & Capasso, 1988).

Until now, the polyphenols in green tea, black tea, grape and wine (especially red) have been extensively studied and characterized. Only recently attention has also focused on the characterization of the phenol components of cocoa (Sánchez-Rabaneda et al., 2003; Zumbea, 1998). Cocoa beans are a rich source of polyphenols contributing to about 10% of the dry weight of the whole seed. Cocoa polyphenols have a short half-life in plasma and a rapid excretion rate and, therefore, have a relatively low bioavailability (Manach, Williamson, Morand, Scalbert, & Rémésy, 2005). Among the different classes of flavonoids, procyanidins appear to be from 10 to 100 times less absorbed of their monomeric constituents (Tsang et al., 2005).

If assumed daily in the diet, beneficial effects of phenolic compounds can be cumulative (Cooper, Donovan, Waterhouse, & Williamson, 2008), especially at high doses (Heiss et al., 2007). However, Manach et al. (2005) suggested differences in individual adsorption of polyphenols probably linked to specific polymorphisms. This could explain the high variability in reported flavonoid absorption rates in vivo (Lamuela-Raventós, Romero-Pérez, Andrés-Lacueva, & Tornero, 2005). Absorption and metabolism of polyphenols are determined by their chemical structure, which is correlated with the degree of glycosylation, acylation, polymerization, conjugation with other phenolic compounds, molecular size and solubility (Bravo, 1998).

When ingested, the first stage in the interaction between food polyphenols and proteins occurs in the mouth, where flavanols react first with proline-rich salivary proteins forming insoluble complexes responsible for the perception of astringency and for the characteristic taste of various food products (e.g. fruit, cocoa, coffee, tea, beer and wine) (Baxter, Williamson, Lilley, & Haslam, 1996; Jobstl, O'Connell, Fairclough, Mike, & Williamson, 2004).

In some foods, proteins and polyphenols combine to form soluble complexes, which can reach colloidal size, causing turbidity of beverages and limiting the shelf life of these products. In the beer, wine and juice production, for example, polyphenols may induce formation of protein precipitates with the appearance of negative sensory characteristics for the consumer (Baxter, Lilley, Haslam, & Williamson, 1997; Siebert, 1999; Siebert, Carrasco, & Lynn, 1996a; Siebert, Troukhanova,

* Corresponding author at: Department of Agriculture, University of Naples, Italy. Tel.: +39 081 2539359; fax: +39 081 7762580.
E-mail address: ferranti@unina.it (P. Ferranti).

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& Lynn, 1996b). Polyphenols, in general, interact with globular proteins and can cause structural and conformational changes of the protein. It has been shown that binding affinity depends on the molecular size of the polyphenol molecule: the higher the molecular size of the polyphenol, the greater the tendency to form complexes with proteins (De Freitas & Mateus, 2001).

Structural changes in globular proteins have already been investigated in milk/tea mixtures. Interaction of milk proteins with tea polyphenols induces structural changes in both whey proteins and caseins (Hasni et al., 2011; Hudson, Ecroyd, Dehle, Musgrave, & Carver, 2009; Jobstl, Howse, Fairclough, Mike, & Williamson, 2006; Kanakis, Hasni, Bourassa, Polissiou, & Tajmir-Riahi, 2011). These changes may explain the effect of milk addition on the antioxidant activity of tea as well as other food polyphenols (Stojadinovic et al., 2013). However, despite the numerous studies, the binding mechanism of tea polyphenols with milk proteins has neither been clarified yet, nor is it known whether this interaction is preferential to certain amino acids (Stanner, Hughes, Kelly, & Buttriss, 2004). With regard to the interactions between milk proteins and polyphenols of cocoa and chocolate, it has been shown that, following intake of chocolate, the absorption of epicatechin (one of the major cocoa flavanols) is very low, and is still lower if cocoa is assumed together with milk. This suggested that milk proteins sequester cocoa polyphenols limiting their adsorption in the gastrointestinal tract (Serafini et al., 2003). However, data in the literature are still conflicting, as later studies have found no reduction in the bioavailability of epicatechin when cocoa was consumed with milk (Schroeter, Holt, Orozco, Schmitz, & Keen, 2003). In the light of these contrasting reports on the bioavailability of cocoa polyphenols in the presence of milk, the purpose of this study was to investigate the interaction between polyphenols and proteins in cocoa/milk systems, by means of complementary mass spectrometry techniques. A simplified model system was then developed, in which the polyphenols were incubated with isolated milk protein fractions. The time-dependent protein–polyphenol in vitro interactions were studied by either MALDI-TOF or ESI mass spectrometry, showing a major reactivity of β-lactoglobulin (β-Lg) towards cocoa polyphenols. The data acquired allowed us to characterize the molecular basis of the interaction between the main cocoa flavanols (catechin and epicatechin) with β-Lg by MS/MS structural analysis. The effect of non-enzymatic glycosylation, always occurring in commercial milk–cocoa products due to thermal treatments, on β-Lg interaction with polyphenols was studied. These data were complemented by total antioxidant capacity assays on the protein–polyphenol mixtures in order to correlate polyphenol activity changes to structural interactions with proteins. Finally, the model system in vitro results were verified on a commercial chocolate/milk beverage.

2. Materials and methods

2.1. Reagents and standards

The phenol standards catechin (CAT), epicatechin (EPI), procyanidins, β-Lg, ferulic acid, caffeic acid, coumaric acid, protocatechuic acid, chlorogenic acid, gallic acid as well as trypsin (proteomic grade), 6-hydroxy-2,5,7,8-tetramethylchromano-2-carboxylic acid (Trolox), the ammonium salt of 2,2′-azinobis-(3 ethylbenzothiazoline-6-sulfonic acid) (ABTS), potassium persulfate ($K_2O_8S_2$) and formic acid were supplied from Sigma-Aldrich (Italy). Oligomeric proanthocyanidins (OPCs) were purified as previously reported (Rigaud, Escribano-Bailon, Prieur, Souquet, & Cheynier, 1993). α-Cyano-4-hydroxycinnamic acid (α-cyano), 3.5-dimethoxy-4-hydroxycinnamic acid (sinapinic acid) and 2.3 dihydroxybenzoic acid (DHB) were supplied by Fluka (Italy). Ultrapure water and all solvents of chromatographic purity were purchased from Romil (Italy). Hydrochloric acid and Folin reagent were supplied by Riedel de-Haën (Seelze, Germany), ethanol and trifluoroacetic acid were supplied from Carlo Erba (Italy).

2.2. Phenol extraction, quantitation and characterization by LC/MS/MS

A commercial sample (1 g) of defatted cocoa powder (Perugina, Italy) was extracted with 10 mL of methanol/water (70/30 v/v) in ultrasonic bath for 1 min. The sample was centrifuged at 4000 rpm for 10 min and the supernatant defatted with 10 mL hexane. The polyphenol extract was centrifuged at 13,000 rpm for 3 min and the supernatant filtered with Phenex filters RC 0.22 μm.

Total phenol concentration in the different extracts were determined spectrophotometrically by the Folin–Ciocalteu assay (Box, 1983) using gallic acid as a standard. An aliquot of 125 μL of each extract was mixed with 125 μL of Folin–Ciocalteu phenol reagent and reaction carried out for 6 min. Then, 1.25 mL of saturated Na_2CO_3 solution (7.5%) was added and allowed to stand for 90 min before the absorbance of the reaction mixture was measured in triplicate at 760 nm. The total phenol content was expressed as mg gallic acid equivalents/mL of sample.

The identification of polyphenol compounds was performed by liquid chromatography coupled with tandem mass spectrometry (LC/MS/MS). Chromatographic separations were obtained using a HPLC equipped with two micropumps series 200 (Perkin Elmer, Canada) and a Prodigy ODS3 100 Å column (250 × 4.6 mm, particle size 5 μM) (Phenomenex, CA, USA). The eluents were: A, water 0.2% formic acid; B, CH_3CN/MeOH (60:40 v/v). The gradient used was as follows: 20–30% B (6 min), 30–40% B (10 min), 40–50% B (8 min), 50–90% B (8 min), 90–90% B (3 min), 90–20% B (3 min) at a constant flow of 0.8 mL/min. A flow of 0.2 mL/min was sent into the mass spectrometer. The injection volume was 20 μL. MS and MS/MS analyses of cocoa extracts were performed with an API 3000 triple quadrupole (Applied Biosystems, Canada) instrument equipped with a Turbolonspray source. Acquisition was performed in negative ion mode by multiple reaction monitoring (MRM).

2.3. Extraction of milk proteins

Raw cow milk was defatted by centrifugation at 5000 rpm for 30 min at 4 °C. Separation of the casein fractions was carried out by isoelectric precipitation according to Aschaffenburg & Drewry (1957). Casein was lyophilized and stored for subsequent experiments. Whey proteins (WP) were precipitated from the milk whey with 12% (v/v) trichloroacetic acid (TCA). The pellet was recovered after centrifugation at 4500 rpm for 30 min and washed three times with acetone at −20 °C for 30 min, and finally centrifuging at 4500 rpm for 30 min. The dry residue was used for analysis.

2.4. RP-HPLC analysis of whey proteins

HPLC analysis of WP was performed using a modular HP 1100 (Agilent) instrument equipped with a C4 Reverse Phase 5 μm; 250 mm × 2.1 mm (Vydac, 218TP54) column. Solvent A was 0.1% trifluoroacetic acid (TFA) in H_2O and solvent B 0.1% TFA in acetonitrile (ACN). The chromatographic separation was carried out with a B gradient from 35 to 55% in 65 min, at the constant flow of 1 mL/min. The eluate from the column was monitored 220 and 280 nm. For analysis of WP, 150 μL sample dissolved at a concentration of 2 mg/mL in H_2O/TFA 0.1% were injected. The chromatographic peaks containing α-lactalbumin (α-La) and β-Lg were dried, freeze-dried and finally stored in a freezer at −20 °C.

Lactosylated β-Lg (Lac-β-Lg) was isolated by RP-HPLC separation in the same conditions as a commercial sample of pasteurized whole milk.

2.5. Study of in vitro interaction of polyphenols with caseins and WP

Interactions between polyphenol components of cocoa and milk proteins were studied using a simplified model obtained by incubating milk individual caseins and WP with cocoa polyphenols extracts and with polyphenol standards. Proteins were solubilized in 5 mM ammonium bicarbonate (AMBIC) at pH 6.8 (close to milk pH of the milk), at