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

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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.

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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 flavonols 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,
& 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, Poliissiou, & 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 (Schoeter, 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-tetramethylchroman-2-carboxylic acid (Trolox), the ammonium salt of 2,2’-azino-bis-(3 ethylbenzothiazoline-6-sulfonic acid) (ABTS), potassium persulfate (K2O8S2) 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-Cyanohydroxy-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 trifuoroacetic 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 supernantant 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 Na2CO3 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 μm. The eluents were: A, water 0.2% formic acid; B, CH3CN/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 (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 H2O 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 H2O/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
concentrations of 5.4 mg/mL, 5.7 mg/mL, and 2.9 mg/mL for caseins, whey proteins and β-Lg, respectively. The cocoa polyphenol extract and individual polyphenols were dissolved in methanol and added at a 10-fold molar excess compared to that of protein. Protein–polyphenol mixtures were incubated at 37 °C and the interaction monitored for 0–48 h by antioxidant activity assays and mass spectrometry analysis.

2.6. Alkylation and tryptic hydrolysis of β-Lg

Alkylation of β-Lg (IAA-β-Lg), β-Lg (3 mg/mL) was dissolved in 5 mM ammonium bicarbonate, pH 8.4, containing iodoacetamide (IAA) in molar ratio 10:1, to obtain alkylation of free cysteine residues. The reaction was carried out for 45 min in the dark. IAA excess was eliminated by dialfiltration with 3 kDa cut-off filters Centricon (Amicon, Italy).

Tryptsin hydrolysis was carried out by adding proteomic-grade trypsin and incubating at 37 °C for 4 h.

2.7. MALDI-TOF-MS analysis

MALDI-TOF-MS experiments were carried out on a Voyager DE-Pro® (Applied Biosystems, Framingham, USA) instrument. Spectra were acquired either in linear or reflector mode using the delayed extraction technology; external calibration was performed with a mixture of peptides standard low molecular masses. Spectra of proteins incubated the polyphenols were acquired by loading 1 μL of the protein sample and using sinapinic acid (10 mg/mL in 50% ACN/TFA 0.1%) as matrix. For peptide analysis, 1 μL of peptide mixture was loaded on the appropriate target steel using α-cyano acid as matrix (10 mg/mL in 50% ACN/TFA 0.1%). MS/MS analysis of selected ions was made with Post-Source Decay (PSD). The identification of the peptides was performed by comparing the measured m/z values with those obtained by simulating the theoretical tryptic digestion of β-Lg using General Protein/Mass Analysis for Windows software (GPMAW).

2.8. ESI–MS/MS analysis

ESI–MS/MS data were obtained using a Q-STAR MS (Applied Biosystems, Foster City, CA) equipped with a nanospray interface (Protana, Odense, Denmark). The dried samples were resuspended in H₂O and TFA 0.1%, were purified by the use of micro ZipTip C18 columns (Millipore, Milan, Italy), and sprayed through gold-coated borosilicate capillaries (Protana). The capillary voltage used was 800 V. The collision energy ranged from 20 to 40 eV, depending on the size of the peptide. The collision induced dissociation spectra were processed using the Analyst 5 software (Applied Biosystems). LC/ESI-MS analysis of β-Lg tryptic peptides was carried out using a C18 reversed-phase column (218TPS2, 5 μm, 250 × 2.1 mm, Vydac, Hesperia, CA, USA) with a flow rate of 0.2 mL/min on an Agilent MSD 1100 system. A flow was 0.03% TFA in water, and solvent B was 0.02% TFA in ACN. Separation was carried out in a linear gradient of 5–70% solvent B for 60 min. MS acquisition was in positive selected ion monitoring mode.

2.9. Antioxidant activity assays

Antioxidant activity was determined on samples prepared as described in paragraph 2.5 using the ABTS method (Pellegrini et al., 2003). In particular, different samples were opportune diluted with methanol, centrifuged for 5 min at 4000 rpm and used for the determination of antioxidant activity. The absorbance at 734 nm of the ABTS discolored solution was recorded on a Spectrophotometer UV/VIS UV2100 (Shimadzu, Japan). The values were expressed in mmol equivalent of Trolox/L.

2.10. Statistical analysis

Data are expressed as mean (SD). Significance of differences was assessed by one way analysis of variance (ANOVA) and (when the F value was significant) by the Tukey–Kramer test for multiple comparisons or by Student’s t test for comparison between two means. Differences were considered to be significantly different if p < 0.05.

3. Results

3.1. Analysis of phenolic components in cocoa by LC/ESI–MS/MS

As a preliminary step to study of interaction of polyphenols with milk proteins, the polyphenol composition of the commercial cocoa samples uses in this study was determined by LC–MS/MS (not shown). The TIC chromatogram of cocoa polyphenol extract showed all the expected compounds previously described in cocoa (Cooper et al., 2007; Hammerstone, Lazarus, Mitchell, Rucker, & Schmitz, 1999), including catechin and epicatechin and oligomeric proanthocyanidins. Minor amounts of protocatechuic acid, chlorogenic acid and glucoside esters of apigenin, naringenin, kaempferol, luteolin and quercetin were also detected. Because of the complexity of milk/cocoa mixtures, a simplified model study was preliminary set up mixing single polyphenol compounds (catechin, epicatechin, and oligomeric proanthocyanidins) as well as with those extracted from the cocoa powder with isolated cow milk protein fractions (casein, whey protein and β-Lg). The mixtures were incubated at 37 °C for times ranging from 0 to 24 h and aliquots were taken at time intervals for MS analysis and antioxidant assays.

3.2. Antioxidant activity of polyphenol compounds

To set up the optimal conditions to measure antioxidant activity, ABTS assays (Huang, Ou, & Prior, 2005) were preliminarily carried out testing solutions of pure catechin, epicatechin, OPCs (oligomeric proanthocyanidins) and polyphenol extracts from cocoa.

The ABTS assay results (Fig. 1a) showed that after 4 h incubation at 37 °C, pH 7.4, polyphenol samples showed no significant changes of total antioxidant capacity. This indicated that the incubation conditions did not introduce undesired polyphenol oxidation. OPCs showed a slight but significant (p < 0.05) increase of antioxidant activity, possibly a mere effect of their slow solubilization process, or a consequence of the time/temperature conditions for incubation which induced slow conversion to large-sized polymers with consequent increase of antioxidant capacity. Actually, it has been shown that aged wine, in which the polymerization of polyphenols to tannins increased antioxidant capacity (for a review see Cheynier, 2005). Total polyphenol measurement (Fig. 1b) confirmed the results of ABTS assay. No quantitative variation of total polyphenol content was observed after 24 h. Therefore, based on both ABTS and Folin data, it was possible to conclude that the assay conditions did not affect the polyphenol antioxidant activity.

3.3. Total antioxidant activity of polyphenol/protein mixtures

To measure changes of total antioxidant capacity determined by polyphenol/protein interaction, ABTS assays were performed after incubation of casein, whey proteins or β-Lg with individual polyphenols and total polyphenols extracted from cocoa.

Fig. 2 shows the antioxidant activity by ABTS assay as for polyphenols incubated with the purified milk protein fractions. In the mixture casein/ polyphenol (Fig. 2a), the antioxidant activity decreased significantly (p < 0.05) after 24 h of incubation, thus showing a significant effect of casein on polyphenol activity. This decrease was more pronounced in the case of casein incubated with either catechin or epicatechin.

These results were in agreement with previous studies which showed that incubation of polyphenols affected casein structure, causing a
reduction of α-helix and β-sheet structures and an increase of unfolding (Hasni et al., 2011; Hudson et al., 2009; Jobstl et al., 2006), thus suggesting the existence of interaction with phenols.

Fig. 2b–c shows the results of ABTS assay after incubation of individual phenols with whey proteins and with purified β-Lg, respectively. In both cases, the trend of the antioxidant activity after 24 h of incubation was similar to that observed for caseins, suggesting also in this case that protein/polyphenol interaction reduced antioxidant activity of the polyphenol system by subtracting free polyphenols. Interestingly, this decrease was more pronounced in the case of β-Lg alone. These data were in good agreement with previous reports (Stojadinovic et al., 2013).

3.4. Characterization of protein/polyphenols interactions by MALDI-TOF-MS

The mixtures previously analyzed for antioxidant activity were characterized by mass spectrometry. The time-dependent formation of covalent protein–polyphenol complexes was first investigated by MALDI-TOF-MS on the basis of the protein mass increase. MALDI-TOF mass spectra of incubated caseins did show any stable ad- duct between the individual caseins neither with catechin/epicatechin nor with extracted cocoa polyphenols. In the system whey protein/polyphenol, mass spectra showed no reactivity of α-La with extracts of cocoa polyphenols, catechin or epicatechin over 24–48 h incubation time (Fig. 3). The same spectra, instead, showed a significant reactivity for β-Lg (both variants A and B, molecular mass 18,367 Da and 18,281 Da respectively) towards catechin or epicatechin, either pure or present in the cocoa extract. In fact, after incubation with the cocoa polyphenols (Fig. 4), a 288 Da mass increase (18,655 Da for variant A and 18,569 Da for variant B) was observed in the protein molecular mass, corresponding to addition of a single epicatechin or catechin molecule. In addition, the spectra performed at various incubation times showed that the interaction between β-Lg and polyphenol was detectable starting from 4 h of incubation. At that time, the relative abundance of the β-Lg/polyphenol adducts was still much lower than that of the native proteins. The abundance of the adducts increased with the incubation time. These findings also excluded that the ions observed were due to cluster artifacts of MALDI analysis.

The stable nature of the β-Lg/polyphenol adducts was supported by their persistence in MALDI TOF MS following denaturing RP-HPLC separation.

3.5. In vitro reactivity of catechin and epicatechin with β-Lg

Once proved that β-Lg was the only major milk protein to stably bind cocoa polyphenols, we investigated the nature of the protein binding site.

a) Native β-Lg (constituted by a mixture of the two genetic variants A and B).

b) β-Lg alkylated to block the single free cysteine at position 121, in order to study its possible involvement in polyphenol binding.

c) Lactosylated β-Lg obtained by RP-HPLC purification from total WP extracted from a sample of heat-treated milk. In this latter case, we aimed to define whether heat treatment employed to prepare commercial milk/chocolate beverages might change β-Lg binding to polyphenols. In β-Lg, it is known that the preferential site of lactosylation is located at lysine 100 (Fogliano et al., 1998).

Fig. 5 shows the MALDI-TOF-MS analysis of native β-Lg (control in Fig. 5a) incubated with catechin (Fig. 5b) and an epicatechin (Fig. 5c) for 24 h: both A and B variants added a single polyphenol molecule. On the contrary, β-Lg treated with IAA, in which the free cysteine was blocked, showed a molecular mass of 18,423 and 18,339 Da for variants A and B, respectively, corresponding to the mass increase of 57 mass units for alkylation of the single free cysteine of the protein (Fig. 5d–f). However, upon incubation with polyphenols, no mass increase was
observed, indicating that the polyphenol binds specifically to the free cysteine residue.

In order to confirm this hypothesis, and to assign the reactive site of β-Lg with polyphenols, tryptic hydrolysis was performed on the samples after a diafiltration step to remove polyphenol excess from the reaction mixture. Fig. 6b shows the partial MALDI spectrum of the tryptic digest of native β-Lg before and after incubation with catechin (control in Fig. 6a). The identification of peptides was performed on the basis of the measured peptide masses compared with those expected on the basis of trypsin specificity (Table 1) using the data processing software GPMAW (Applied Biosystems).

The mass spectra obtained after incubation of epicatechin were very similar to that obtained for catechin incubation, demonstrating the same extent of reactivity of the two phenols with β-Lg. These data are in agreement with literature data that demonstrate two flavanols exhibit the same behavior in terms of reactivity, chemical properties, etc. (Mendoza-Wilson & Glossman-Mitnik, 2006).

Peptide mass mapping allowed to verify the protein sequence completely. In particular, signals at m/z 2647.09 and 2675.14 corresponded to peptides 102–124 of β-Lg variants B and A respectively. These signals, in the β-Lg sample incubated with catechin (Fig. 6b), were accompanied by signals at m/z 2935.83 and 2963.89 where the mass of the two peptides was increased by 288 units corresponding to addition of a single catechin monomer. A similar result was obtained for epicatechin. In the case of IAA-β-Lg (Fig. 6c), peptide 102–124 was present only in the alkylated form but no evidence of catechin binding was found. In fact, peptide 102–124 contains three cysteine residues, two of which involved in a disulfide bridge and the third one free. This result supported the hypothesis that the polyphenol could have reacted with the free cysteine residue on the peptide, and alkylation of this residue prevented interaction either flavanols. For unambiguous structural characterization, peptides at m/z 2961.40 and m/z 2933.36 were analyzed by ESI-Q-TOF MS/MS (not shown) confirming binding of polyphenols to cysteine 121. Moreover, analysis showed the absence of these peptides in the case of alkylated β-Lg, confirming, also in this case, the data provided by MALDI-TOF-MS.

The same analysis was also carried out on the lactosylated form of β-Lg. The tryptic map of Lac-β-Lg presented the same peptides 102–124 with addition of catechin (signals m/z 2933.83 and 2961.89) (Fig. 6d). This demonstrated that lactosylation did not prevent polyphenol binding, and that lactosylated β-Lg covalently bound polyphenols at the same cysteine 121 residue.
3.6. LC ESI MS analysis of polyphenol/β-Lg adducts in commercial products

LC–ESI-MS analysis of the intact whey protein fraction isolated from a commercial pasteurized chocolate/milk did not evidence any adduct possibly because of the low extent of modification (not shown). However, the same analysis carried out on the tryptic digest of the same fraction monitoring the triply charged ions of reacted peptides (Fig. 7d) showed the presence of the same peptides 102–124 of β-Lg with addition of catechin/epicatechin which had been observed in the samples incubated in vitro (Fig. 7a–c) although to a very low extent. These findings proved that covalent interactions β-Lg/polyphenol occur also in real milk and milk/chocolate beverages and adduct formation can be monitored using the proposed peptidomic approach, once a robust quantification method has been developed.

4. Discussion

Several studies have highlighted the nutritional implications of interactions of polyphenols with proteins in food, which may determine a decreased bioavailability of polyphenols. The loss of food nutritional quality has been ascribed to modification of essential amino acid residues and to reduction of protein digestibility due to inhibition of proteolytic and glycolytic enzymes. According to some studies, polyphenols may interact irreversibly with dietary proteins and digestive enzymes in the gut and be transported in vivo bound to plasma proteins (Brunet, Blade, Salvado, & Arola, 2002). This protein binding can affect the physiological effects of polyphenols, depending on their intake and structure. Indeed, binding to food proteins may have implications in terms of bioavailability (Serafini, Ghiselli, & Ferro-Luzzi, 1996; Wollgast & Anklam, 2000), as the antioxidant capacity of polyphenols can be modified by the presence of proteins (Arts, Haenen, Voss, & Bast, 2001; Arts et al., 2002; Riedl & Hagerman, 2001). Therefore, the proteins present in the food matrix, the digestive environment and the blood have the potential to significantly affect the biological activity of polyphenols.

Despite this, the structural definition of the nature of these protein–polyphenol complexes has never been carried out. Several kinds of interactions have been hypothesized: strong (covalent, ionic) or weak (hydrogen bridges, π bonds, hydrophobic) bonds. With regard to the covalent adducts, there are few data in the literature to support
experimentally their formation and to identify binding sites of proteins. This study demonstrates for the first time the formation of the complex protein–polyphenol involving a milk protein, β-Lg and cocoa polyphenols, through covalent binding of free-SH group of the free cysteine residue of the protein.

To this aim, a strategy combining proteomic with biochemical approaches has been applied to the characterization of a simplified model system and then of a commercial product, a chocolate–milk drink. The polyphenol extract of commercial cocoa was characterized by LC–ESI–MS/MS and incubated with milk proteins. The interaction and complex formation at several incubation times was studied by mass spectrometry and antioxidant activity assays. Antioxidant activity of polyphenols was measured by ABTS in a model assay system, which was shown to preserve antioxidant activity of polyphenols under the conditions of time/temperature/medium used for the interaction study.

The measurement of antioxidant activity of casein/polyphenol mixtures revealed a time-dependent decrease of activity. It has been reported that the interaction with polyphenols modifies casein conformation resulting in a reduction of α-helices and β-sheets (Hasni et al., 2011). This decrease of antioxidant activity was observed also for polyphenol/ WP and polyphenol/β-Lg systems. Considering that polyphenols incubated in the absence of proteins did not change significantly their antioxidant activity it was possible to hypothesize that the reduced antioxidant activity of polyphenol–protein system is due to a stable protein–polyphenol interaction, resulting in a decrease of free polyphenol, with a decrease of the antioxidant activity of the system.

Proteomic analysis, however, confirmed the formation of covalent complexes only in the case of WP, not for caseins. More precisely, while α-La presents no reactivity with polyphenol extracts of cocoa as well as with phenol standards, β-Lg showed a significant reactivity to catechin and epicatechin experimentally measurable starting from 4 h of incubation and remained stable up to 48 h. Taken together, these data suggest that covalent binding occurs with β-Lg, while other weaker, likely non-covalent interactions take place in the case of casein with cocoa polyphenols.

MALDI TOF MS and ESI-Q-TOF MS/MS analyses of tryptic peptides of β-Lg have allowed the identification of the binding site as the free thiol group of cysteine 121. In support of our findings, reactivity of grape flavan-3-ols, including catechins, with cysteine and cysteine derivatives has already been described (Tanaka, Kusano, & Kouno, 1998; Torres & Bobet, 2001; Torres, Lozano, & Maher, 2005; Torres et al., 2002). In any case, this is the first structural report of a food polyphenol covalent adducts with milk proteins. Interestingly, grape polyphenol adducts with cysteine thiol groups showed a higher antiradical capacity than their underivatized counterparts (Torres et al., 2002), and this suggests that functional properties of β-Lg/polyphenol adducts deserve further detailed investigation, possibly on pure compounds obtained by chemical synthesis.

At the same time, our data showed also that only a small part of the protein interacts with the polyphenol; therefore, it can be concluded that the implications of nutritional point of view are not such as to support the hypothesis of a quantitative decrease in the
Fig. 6. Partial MALDI-TOF-MS analysis of the tryptic digest of β-Lg incubated for 4 h with polyphenols: (a) control, and (b) native β-Lg, (c) alkylated β-Lg, (d) lactosylated β-Lg incubated with catechin (CAT).

Table 1

Identification of tryptic peptides of native β-Lg, Lac-β-Lg and IAA-β-Lg after 24 h of incubation with catechin (CAT) or epicatechin (EPI). CM: peptide carboxymethylated. x: peptide detected.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Measured MH⁺</th>
<th>Theoretical MH⁺</th>
<th>Peptide sequence</th>
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<td>x</td>
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<tr>
<td>102–124 A</td>
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<td>2675.20</td>
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<td>x</td>
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<tr>
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<td>2721.111</td>
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<td>2848.30</td>
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<tr>
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<tr>
<td>102–124 B + CAT</td>
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<td>2935.83</td>
<td>YLLFCMENSAPQSLAQCQLVR + CAT</td>
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<tr>
<td>102–124 A + CAT</td>
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<td>2963.44</td>
<td>YLLFCMENSAPQSLVQCLVR + CAT</td>
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<tr>
<td>102–124 B + EPI</td>
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<td>YLLFCMENSAPQSLAQCQLVR + EPI</td>
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<td>2963.40</td>
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<tr>
<td>61–70 A + 149–162 + lactose</td>
<td>3173.29</td>
<td>3173.39</td>
<td>WENGECAKQ + LSNPRTQLEEQCHI + lactose</td>
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<tr>
<td>(102–124B) CM</td>
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<td>2704.33</td>
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<td>2704.37</td>
<td>2704.33</td>
<td>YLLFCMENSAPQSLVQCLVR</td>
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bioavailability of polyphenols themselves. Furthermore, LC ESI MS analysis of a commercial chocolate/milk drink demonstrated the formation of the same covalent complex also in this product.

5. Conclusions

A proteomic approach has been developed to monitor the formation of stable polyphenol/protein complex in either laboratory or commercial milk products. The results obtained allow us to hypothesize the use of this methodology for similar products like chocolate milk drinks, chocolate milk, and formulated protein containing cocoa. The extension of this method to the quantitative level could be useful to understand the type of polyphenol–protein interaction in these products taking into account the characteristics of ingredients and the technological parameters of the production process. At the same time, the observed protein effects on the antioxidant activity decrease measured in vitro have allowed us to assume the presence of other types of weak protein–polyphenol interactions that reduce this activity. Currently, little is known about polyphenol absorption, bioavailability, biodistribution, and metabolism, although there is probably a common path. Consequently, further studies are needed to identify the nature and strength of these interactions, in order to assess the possible structural changes upon food ingestion and digestion. As a matter of fact, the consequences of the interactions in nutritional terms are not so easily measurable and generalizable as it should be taken into consideration a number of other factors, not least the type of polyphenol and the nature of the protein matrix.

References


