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Interactions between globular proteins and procyanidins of different degrees of polymerization

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ABSTRACT

Interactions of proteins with phenolic compounds occur in food products containing vegetable sources, such as cocoa, cereals, or yogurts containing fruit. Such interactions can modify protein digestion and protein industrial properties. Noncovalent interactions between globular proteins (proteins important in industry) and procyanidins (phenolic compounds present in large quantity in fruits) were studied. The affinity constants between procyanidins of various average degrees of polymerization (DP) and lysozyme or α-lactalbumin were measured by isothermal titration calorimetry. The effects of these interactions on protein solubility and foam properties were examined using α-lactalbumin and BSA. Weak interactions were found with epicatechin and procyanidin dimers. Procyanidins of DPn = 5.5 and DPn = 7.4 showed medium (1.5×10^5 M^{-1}) and high (8.69×10^9 M^{-1}) affinities, respectively, for α-lactalbumin at pH 5.5, with n the average number of subunits per oligomer. A positive cooperativity of binding at low procyanidin:protein molar ratios was observed. The affinities of α-lactalbumin and lysozyme for procyanidins increased when the pH was close to the isoelectric pH. Solubility of lysozyme was strongly decreased by procyanidins of DPn = 5.5, whereas α-lactalbumin and BSA were less affected. Protein solubility in the presence of procyanidins was not affected by increased ionic strength but increased slightly with temperature. Procyanidins of DPn = 5.5 and DPn = 7.4 stabilized the average bubble diameter of foam formed with α-lactalbumin but had no effect on foam made from BSA. These results indicate that procyanidins of medium DP can lead to an undesirable decrease of protein solubility, but may play a positive role in foam stability.

Key words: protein, polyphenol, proanthocyanidin, isothermal titration calorimetry

INTRODUCTION

During the past few decades, consumers have shown increasing interest in drinkable milk and fruit juice mixes and, recently, food industries have launched probiotic yogurts with added polyphenols from wine or from green tea. Like traditional hot chocolate in Western countries and masala chai (i.e., spicy tea latte) in India, these products contain dairy proteins and phenolic compounds (**PC**). The effect of PC on some sensory properties of dairy products is known: they induce flavors and off-flavors in milk, cheese, and butter, and induce discoloration in cheeses (O'Connell and Fox, 2001). They can be used as antioxidants in milk (Serafini et al., 2009), as natural pigments for yogurts (Wallace and Giusti, 2008), and as an agent against pathogenic microorganisms (O'Connell and Fox, 2001). An important characteristic of condensed PC is that they are tannins; that is, they are able to interact with and precipitate proteins. Tannins have adverse effects on digestion and reduce the nutritional value of dairy beverages (Boor et al., 1986) and of forage for cattle (Burns, 1978). Protein–tannin interactions are also responsible for the perception of astringency by precipitating proline-rich proteins from saliva (Baxter et al., 1997). Furthermore, these interactions can modify food functional properties: they often result in a decrease of protein solubility (Kumar and Horigome, 1986); increase the heat stability of skimmed milk by addition of tea, cocoa, or coffee extracts; and modify flavor attributes (e.g., decrease flavor of the phenol vanillin in ice cream; O'Connell and Fox, 2001). In contrast to the effects of tannins on astringency and on nutrition, especially on protein digestion, the effect of proanthocyanidins on food functional properties has been less investigated. This investigation requires understanding of the nature

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and the extent of the interactions of proanthocyanidins with the important proteins in food (i.e., globular proteins).

Proanthocyanidins were the PC chosen for this study because they are common in fruits, cocoa, tea, some cereals, some legume seeds, and wine (Santos-Buelga and Scalbert, 2000). In foods, the most common proanthocyanidins are procyanidins, which are composed of units of catechin or epicatechin or both, and prodelphinidins, which are composed of units of gallocatechin or epigallocatechin or both (Santos-Buelga and Scalbert, 2000).

The protein precipitation induced by PC reaches a maximum close to or below the isoelectric point of the proteins (Hagerman and Butler, 1981; Naczk et al., 1996). The precipitation is also influenced by the degree of polymerization (**DP**) of proanthocyanidins; the protein-precipitating effectiveness of mixtures of proanthocyanidins and proanthocyanidin gallates seems to increase with increasing degrees of polymerization until an optimal DP of 12 units of catechin (De Freitas and Mateus, 2001). Above this DP, the precipitating capacity decreases because of steric hindrance (De Freitas and Mateus, 2001).

Proanthocyanidins are believed to bind mainly to proteins via noncovalent interactions. Covalent bonds may also be formed at low pH (Torres and Bobet, 2001) and under oxidation conditions (Pierpoint, 1969). The precise nature of the noncovalent interactions remains unclear. Both hydrophobic interactions and hydrogen bonding have been suggested (Haslam, 1996; Hagerman et al., 1998). Relatively open proteins, such as nonstructured and proline-rich proteins, seem to have a higher affinity for proanthocyanidins than the more closed, globular proteins (Hagerman and Butler, 1981; De Freitas and Mateus, 2001).

Studies of protein interactions with proanthocyanidins including gallocatechins have been often restricted to turbidimetry, protein precipitation, and ultrafiltration measurements (Oh et al., 1980; Hagerman and Butler, 1981; Asquith and Butler, 1986; Kumar and Horigome, 1986; Artz et al., 1987; Naczk et al., 1996; De Freitas and Mateus, 2001). Using a protein precipitation assay, relatively polar PC, such as procyanidins, have been reported to interact with BSA via hydrogen bonds rather than via hydrophobic interactions (Hagerman et al., 1998). However, because protein-precipitating measurements are related to the solubility of the aggregates and not only to protein–procyanidin affinity, this technique may not be a suitable technique for measuring the real protein–procyanidin affinity. Another technique commonly used is ultrafiltration. According to ultrafiltration measurements, dimers and trimers of catechin were reported to interact mainly with BSA via hydrophobic interactions (Artz et al., 1987). However, because a decrease of solubility could prevent measurement of the true free phenolic concentrations, ultrafiltration might not be a suitable technique for characterizing the affinity and the nature of the main forces driving procyanidin–protein interactions when insolubility occurs.

In this study, isothermal titration calorimetry was used to measure the affinity between procyanidins of various degrees of polymerization and α-lactalbumin and lysozyme at various pH levels. An additional protein, BSA, was used to study the effect of procyanidins on the functional properties of protein. α-Lactalbumin, lysozyme, and BSA were chosen as models for globular protein because α-lactalbumin and lysozyme have highly similar AA sequences but a different isoelectric point (**pI**) and hydrophobicity. On the other hand, α-lactalbumin and BSA have similar pI values but BSA possesses a higher molecular mass than α-lactalbumin. Such comparisons of procyanidin interactions using 3 globular proteins that possess similarities and differences have not been carried out previously, apart from measuring their effects on protein precipitation capacity (Hagerman and Butler, 1981). The present study investigated the effects of these interactions on protein solubility as a function of pH, temperature, and ionic strength, and on protein foam properties.

MATERIALS AND METHODS

Materials

Bovine α-lactalbumin (type I, holo-α-lactalbumin, molecular weight = 14.2 kDa, pI = 4.2–4.5), lysozyme (from chicken egg white, molecular weight = 14.3 kDa, pI = 10.5–11.3), BSA (fractionated by cold alcohol precipitation and essentially fatty acid-free, molecular weight = 66.4 kDa, pI = 4.7–4.9) and (−)-epicatechin were purchased from Sigma Chemical Company (St. Louis, MO) and used without further purification. The BSA consisted of approximately 86% monomers, 12% noncovalent dimers, and 2% higher noncovalent oligomers in 20-m*M* sodium phosphate buffer, pH 7.0, as measured by gel permeation chromatography (Superdex 200, GE Healthcare, Diegem, Belgium).

Purification of Procyanidins

A methanolic extract (320 g) from apples var. 'Jeanne Renard' (using 640 g of fresh parenchyma) and an aqueous acetone extract (8 g) from apples var. 'Marie Ménard' (using 750 g of fresh parenchyma) were ob-

tained by solvent extraction of freeze-dried pulp from cider apples according to Guyot et al. (2001). These fractions were purified using solid-phase extraction. The Jeanne Renard fractions were further purified using normal-phase HPLC. From the eluates, a fraction enriched in dimers and several fractions of medium DP were collected. The dimers-enriched fraction was further purified using reversed-phase HPLC. The Marie Ménard fractions, which were obtained using solid-phase extraction, were further purified on a Toyopearl TSK HW-40 column (Tosohaas, Japan). These fractions were characterized after thiolysis on HPLC using calibration with phenolics standards, as described in Guyot et al. (2001). The freeze-dried fractions were stored in a vacuum dessicator.

Isothermal Titration Calorimetry

The heat effects of successive additions of procyanidins to protein solutions were measured with an MCS isothermal titration calorimeter (MicroCal Inc., Northampton, MA). Solutions of procyanidins and proteins were prepared by weighing both procyanidins and buffer solutions and were degassed during 25 min under vacuum. The reference cell contained degassed water. The sample cell (1.3 mL) contained protein [(0.067–0.27 g/L (i.e., 0.0047 to 0.0187 m*M*)]. A 250-μL syringe was used to perform successive injections of a solution of procyanidins [3.16 m*M* (i.e., 0.927 g/L for epicatechin, 1.820 g/L for $\overline{DP}n = 2$, 4.550 g/L for $\overline{DP}n = 5.5$; and 6.734 g/L for $\overline{DP}n = 7.4$)]. The initial delay was 600 s before the first injection, which consisted of 2 μL of procyanidin solution added to the protein solution for a duration of 5 s. Every 800 s, the syringe injected 5 μL for a duration of 12.6 s for the second and the third injections and 10 μL for a duration of 25.1 s for the fourth until the twenty-sixth injections. To reduce experiment time length, the delay between the injections was reduced to 500 s once at least 26 procyanidin molecules per protein molecule were injected because at these ratios such delay was sufficient to get back to equilibrium. The reference offset was 50%. Samples with only protein or procyanidin were used as blanks, and the data of the sample containing only procyanidins were subtracted from the data of protein with procyanidins. Heat changes were analyzed with the use of Origin software (MicroCal Software, Inc.); the curves were fitted with an iterative modeling (according to the manufacturer protocol) to determine the apparent binding affinities (**K**) and the number of binding sites (**N**). Briefly, the MicroCal software proceeds as follows for the "two sets of interacting sites" model. A broad estimation of the number of K and N are guessed from the curves by the user and entered into the software, and then the following equations are used:

$$F_b = (K_1K_2\ldots K_b[X]^b)/P \text{ and}$$

$$P = 1+K_1[X]+K_1K_2[X]^2+\ldots+K_1K_2\ldots K_b[X]^b.$$

From these equations, [X] (the concentration of free procyanidins) and all F_b (the fraction of total proteins having b-bound procyanidins) are obtained. Then $\Delta Q(i)$ (the heat released from the ith injection) is calculated using the following equation and compared with the measured heat for the corresponding experimental injection:

$Q = M_t\ V_o\ [F_1\ \Delta H_1 + F_2\ (\Delta H_1 + \Delta H_2) + ... + F_b\ (\Delta H_1 + \Delta H_2 + \Delta H_3 + ... + \Delta H_b)]$, where M is the concentration of proteins in V, the working volume; F_b is the fraction of total proteins having b-bound procyanidins; and ΔH is the molar heat of ligand binding. Finally, the initial values of K and N are improved by the Levenberg-Marquardt minimization routine.

Solutions of 3.16 m*M* of procyanidins ($\overline{DP}n = 5.5$), α-lactalbumin, and lysozyme were prepared in buffers with an ionic strength (**I**) of 0.023: 26 m*M* of sodium phosphate buffer (pH 3.0), 27 m*M* of sodium acetate buffer (pH 5.5), and 10 m*M* of sodium phosphate buffer (pH 7.5). These protein solutions were diluted to concentrations of 0.0187 m*M* at pH 5.5 and 7.5 and 0.0047 m*M* at pH 3.0 and were then injected into the cell sample. The effect of the procyanidin $\overline{DP}$ on the interactions was studied at pH 5.5 using α-lactalbumin with (−)-epicatechin and procyanidin preparations with $\overline{DP}$ n = 2, 5.5, and 7.4. Experiments were performed at 25°C.

Protein Content

The nitrogen content of the supernatant was measured using the Dumas combustion method (Marcó et al., 2002) on an NA 2100 nitrogen and protein analyzer (Thermo Scientific, Rodano, Italy). After calibration with urea or methionine, the protein concentration was calculated using the nitrogen–protein conversion factors of 6.25 for α-lactalbumin, 5.29 for lysozyme, and 6.02 for BSA.

Protein Solubility

α-Lactalbumin [0.50% (wt/vol)] and $\overline{DP}n = 5.5$ procyanidins [0.29% (wt/vol)] were incubated at a ratio of 5 mol of procyanidins per mol of protein in 26.6 m*M* (I

= 0.023), 50 m*M* (I = 0.043), or 100 m*M* (I = 0.087) of sodium acetate buffer (pH 5.5) at 10°C, room temperature, and 40°C. After 2 h of incubation, samples were homogenized by mixing; aliquots (0.15 mL) were immediately collected and centrifuged for 1 min (12,700 × *g* at 25°C) and the supernatants were analyzed for their protein contents using the Dumas combustion method. In the case of long incubation time (72 h) at room temperature, 1 μL of 10% (wt/vol) of sodium azide was added per mL of solution sodium, and the nitrogen content was accordingly corrected. Blanks consisted of centrifuged and noncentrifuged samples of protein without procyanidins and procyanidins without protein. The protein concentration of the supernatant of the protein blank was set at 100% and defined as 100% solubility. Each ionic strength and temperature was studied in triplicate.

α-Lactalbumin, lysozyme, and BSA [0.5% (wt/vol)], in the absence or presence of procyanidins of DPn = 7.4, were dissolved in 0.01 *M* sodium phosphate buffer (pH 7.5; I = 0.023). The protein solutions were subsequently adjusted to various pH levels (pH 2.0–pH 10.0) with NaOH or HCl (1 *M* or 6 *M*). After 2 h, the samples were centrifuged for 15 min (12,700 × *g* at 25°C). Each pH was studied in triplicate. The protein contents of the samples were measured as described previously and were corrected for the dilution with NaOH or HCl.

Air–Water Interfaces and Foam Properties

The air–water interfacial and foam properties of α-lactalbumin and BSA were studied in the presence of procyanidins of DPn = 2.0, 5.0, and 7.4 at pH 7.0, and with procyanidins of DPn = 5.0 at pH 4.0. The ratios used were 2 and 5 mol of procyanidins per mol of protein. Buffers used were 17 m*M* sodium phosphate buffer (pH 7.0; I = 0.030) and 100 m*M* sodium acetate buffer containing 13 m*M* of sodium chloride (pH 4.0; I = 0.030).

The surface tension of samples was determined using an automatic drop tensiometer (Tracker, I.T. Concept Teclis, Longessaigne, France) according to Benjamins et al. (1996). The shapes of air bubbles in rising configuration in a protein solution [0.01% (wt/vol)] in the absence or presence of procyanidins were analyzed digitally. After 3,600 s, dynamic oscillations of the area of the bubble with a period of 10 s and a relative area deformation of 6.56% were applied. The elastic modulus was calculated from the changes in surface tension and surface area (Benjamins et al., 1996) using the average of 10 oscillations. Each sample was studied in duplicate or triplicate.

Foam was prepared according to the whipping method of Caessens and colleagues (Caessens et al., 1997). A protein solution [1% (wt/vol)] in the absence or presence of procyanidins was whipped for 3 min at 2,500 rpm for samples at pH 4.0 and at 3,500 rpm for samples at pH 7.0. Next, the samples were poured into a cuvette (45 × 57 × 134 mm). The mean bubble diameter (d_{21}) was determined from images of the cuvettes taken in reflection mode via a prism (every 60 s for the duration of 1 h). The scheme of this experiment, set up by TNO Zeist (Bertus Dunnewind, Wim Lichtendonk, and Martin Bos), is described into detail by Wierenga (2005). Each sample was studied in duplicate.

RESULTS AND DISCUSSION

Procyanidin Characterization

From the Jeanne Renard variety, a fraction enriched in dimers and 2 fractions of medium DP were purified. The fraction of purified dimers consisted of 91.5% procyanidin dimers, 6.0% procyanidin oligomers, and 1.8% epicatechin and minor impurities [caffeoylquinic acid (i.e., chlorogenic acid) and phloridzin (i.e., dihydrochalcones)] (Table 1). The 2 fractions of medium DP contained only epicatechins and procyanidins and had DP n = 5.0 and 5.5. The purified Marie Ménard fraction contained only epicatechins and procyanidins and had DPn = 7.4 (Table 1). As expected for extracts of cider apple (Sanoner et al., 1999), the procyanidin fractions mainly consisted of (−)-epicatechin units and the dimeric fraction mainly consisted of procyanidin B2 (epicatechin-(4β→8)-epicatechin).

Effect of Procyanidin Size on Protein–Procyanidin Interactions

Protein–procyanidin interactions were studied using isothermal titration calorimetry by measuring the heat released by procyanidin additions to solutions of α-lactalbumin and lysozyme. The biphasic shape of the curves indicated the existence of at least 2 sets of sites. A "two sets of interacting sites" model could fit the data, and not a "two independent sites" model. Such model takes into account interactivity between binding sites (i.e., it permits cooperative effects and allows protein structural changes). When procyanidins of DPn = 5.5 and 7.4 were injected to a cell containing only buffer, a positive enthalpy change (ΔH_{obs}) was measured, reaching a maximum of about 300 J per mol of procyanidins (data not shown). Because procyanidins are known to be able to aggregate with each other, presum-

Table 1. Weight and composition of the purified phenolic fractions

Item	Jeanne Renard (methanolic extract)			Marie Ménard (acetonic extract)
	Fraction 1	Fraction 2	Fraction 3	Fraction 4
Fraction weight (% of the fresh parenchyma weight)	0.011	0.051	0.035	0.111
DP	2.0	5.0	5.5	7.4
n[1]				
Composition (% of the fraction)				
Epicatechins, catechins, and procyanidins	99.3	100	100	100
Caffeoylquinic acid	0.6	ND[2]	ND	ND
Phloridzin	<0.2	ND	ND	ND

[1]Average degree of polymerization.

[2]ND = not detectable.

ably via hydrophobic interactions (Riou et al., 2002), such a positive (endothermic) enthalpy change upon dilution into the buffer-containing cell may indicate disaggregation of PC, as also proposed for hydrolysable tannins by Frazier et al. (2003). Enthalpy changes caused by protein–procyanidin interactions were corrected for this positive enthalpy.

The titration curves showing the enthalpy changes upon titration of α-lactalbumin with epicatechin, procyanidin dimers, and procyanidins of DPn = 5.5 and 7.4 are presented in Figure 1. Procyanidins of DPn = 5.5 and 7.4 induced enthalpy changes, which occurred in 2 stages (Figure 1). In the first stage (i.e., at ratios lower than about 5 mol of procyanidins/mol of protein), the enthalpy change became more and more negative with each addition of procyanidins, indicating that more and more energy was released (Figure 1). This first stage represents the strongest binding sites. In the second stage of protein–procyanidin interactions, the exothermic effect decreased until reaching a plateau, indicating saturation of the protein (Figure 1).

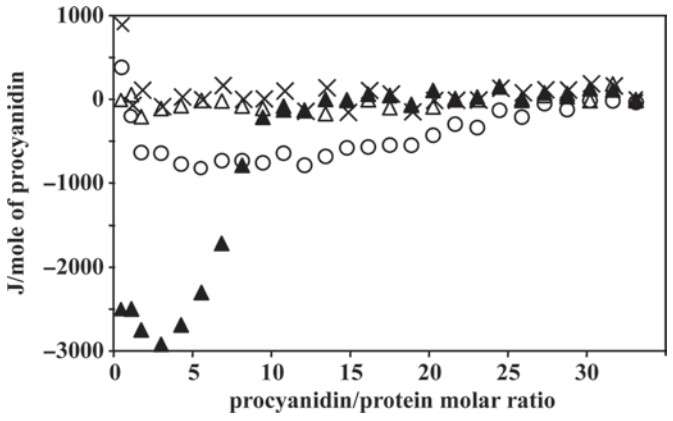

Figure 1. Isothermal titration calorimetry (25°C, pH 5.5) of α-lactalbumin titrated with procyanidins of various average degrees of polymerization (DPn): epicatechin (×); procyanidin dimer (△); procyanidins of DPn = 5.5 (○); procyanidins of DPn = 7.4 (▲).

The K and N values obtained from the titration curves are presented in Table 2. The affinities corresponding to the first stage of interactions (i.e., at low molar ratios) are not presented because too few data points were available to obtain a satisfactory curve fitting; however, the strength and the N values of the second stage reflected the state of the first stage (e.g., a high N in the second stage was correlated with a high N in the first stage). α-Lactalbumin had a medium affinity for procyanidins of DPn = 5.5 (1.10^5 M^{-1} at pH 5.5) and a high affinity for procyanidins of DPn = 7.4 (9.10^9 M^{-1} at pH 5.5). The sigmoidal curve for DPn = 7.4 reveals a specific interaction with α-lactalbumin. In contrast, the affinities for epicatechin and procyanidin dimers were aspecific and were too low to be quantified. To compare these values with other macromolecule–ligand interactions, one can notice that the affinity between α-lactalbumin and procyanidins of DPn = 7.4 is in the same range of magnitude as the one between nicotine and neuronal receptors (2.10^9 M^{-1}; Flammia et al., 1999), whereas α-lactalbumin binds Ca^{2+} with 1 binding site of 3.10^6 M^{-1} and 1 binding site of 3.10^4 M^{-1} (Kronman et al., 1981). The very low affinity of globular proteins for monomers and small procyanidins is in agreement with studies in which BSA aspecifically interacted with epicatechin with an affinity $<10^3$ M^{-1} at pH 5.0, as measured by capillary electrophoresis (Papadopoulou and Frazier, 2004) and an affinity of 3.10^3 M^{-1} for procyanidin dimers at pH 7.0, according to ultrafiltration measurements (Artz et al., 1987). The higher ability of large procyanidins to bind to proteins compared with small procyanidins may be explained by their multidentate character, which allows them to simultaneously bind several protein sites (Haslam, 1996). However, whereas affinity and specificity increase with larger procyanidins, fewer sites are available for these

procyanidins (probably because of steric hindrance; Table 2). This means that, at high procyanidin:protein ratio, procyanidins of medium DP might affect protein solubility as much (or maybe even more) than procyanidins of high DP.

At low molar procyanidin:protein ratios (lower than 5), the changes in enthalpy became more negative with increasing molar procyanidin:protein ratios; that is, once 1 procyanidin molecule is bound to the protein, the affinity of the protein to bind another procyanidin molecule increases. The mechanism, therefore, shows positive cooperativity, as has also been observed for the interactions between gelatin and gallotannins and ellagitannins (Frazier et al., 2003). A cooperativity mechanism may be the result of a change in protein conformation upon ligand binding (Fersht, 1999; e.g., binding of a ligand on one site could unmask a neighboring site). Whether procyanidins affect only conformational freedom (e.g., favoring one conformation) or modify secondary structure of globular proteins remains to be clarified.

Effect of pH on Protein–Procyanidin Interactions

Titration curves showing the enthalpy changes with procyanidins of DPn = 5.5 at pH 3.0, 5.5, and 7.5 are presented in Figure 2a for α-lactalbumin and in Figure 2b for lysozyme. As observed previously, the changes in enthalpy occurred in 2 stages.

When α-lactalbumin was titrated with procyanidins, the exothermic effect, and therefore the affinity, at low ratios increased upon lowering the pH (Figure 2a; Table 2). In contrast, the affinities between lysozyme and procyanidins become stronger at higher pH levels, with a specific binding observed at pH 7.5 (Figure 2b; Table 2). These 2 observations are in agreement with Naczk et al. (1996), who have found that the optimum pH for complex formation generally is 0.3 to 3 units below the pI of the protein. However, the higher affinity at high pH corresponded to fewer binding sites in lysozyme (Figure 2b; Table 2).

To reach saturation of α-lactalbumin by procyanidins, the protein concentration used at pH 3.0 was 4 times lower than at higher pH levels. Because more phenolics are required to precipitate protein in diluted protein solutions than in concentrated protein solutions (Haslam, 1996), the affinity observed at pH 3.0 is likely to be underestimated compared with the affinity measured at higher pH levels.

Effect of Procyanidins as a Function of Protein Type

Protein solubility was studied at a procyanidin:protein molar ratio (i.e., a ratio at which the proteins were not

Table 2. Apparent binding affinity (K) constant and number of binding sites per protein molecule (N) of the second stage of interactions between proteins and procyanidins of various degrees of polymerization (DP; DPn = 2, 5.5, and 7.4) and epicatechin (DPn = 1)[1]

	Lysozyme			α-Lactalbumin					
	pH 3.0	pH 5.5	pH 7.5	pH 3.0	pH 5.5			pH 7.5	
Item	5.5	5.5	5.5	5.5	1	2	5.5	7.4	5.5
K (M^{-1})	$<1 \times 10^3$	$5 \times 10^5 \pm 2 \times 10^5$	$9 \times 10^5 \pm 3 \times 10^5$	ND[2]	ND[3]	ND[3]	$1.5 \times 10^5 \pm 0.5 \times 10^5$	$8.7 \times 10^9 \pm 1 \times 10^2$	ND[3]
N	ND[3]	24.3 ± 0.4	11.6 ± 0.6	ND[3]	ND[3]	ND[3]	19.4 ± 0.6	11 ± 3	ND[3]

[1]The SD correspond to the discrepancy between experimental data points and the theoretical fitting of the curve.
[2]Not determined because of an insufficient number of data points for curve fitting.
[3]Not determined because of a low slope (very low affinity).

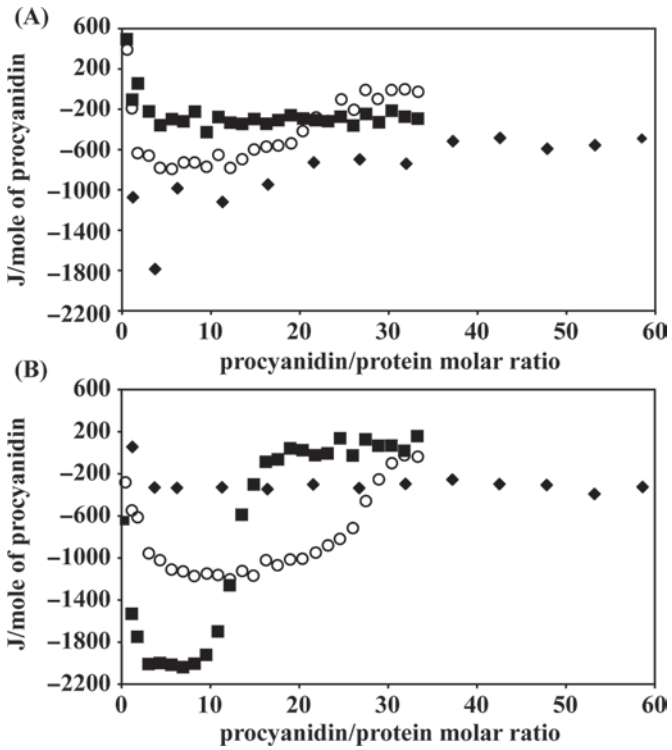

Figure 2. Isothermal titration calorimetry (25°C) of proteins titrated with procyanidins of an average degree of polymerization of 5.5 at different pH values for (a) α-lactalbumin and (b) lysozyme: pH 3.0 (◆); pH 5.5 (○); pH 7.5 (■).

saturated) of 5 (Figure 3). Protein solubility decreased upon the addition of procyanidins (Figure 3). The solubility of BSA decreased by a maximum of 50% and only a narrow pH range (pH 4.0–6.0) was affected (Figure 3). The most drastic effects were obtained for α-lactalbumin and lysozyme: only 0 to 10% of α-lactalbumin remained soluble between pH 3.0 and pH 6.0 and lysozyme became totally insoluble at pH $\leq$ 6.0. Because the solubility of the complexes can differ as a function of various factors (e.g., size of the aggregates, nature of the protein, and type of the interactions), isothermal titration calorimetry was chosen to clarify pH effect on the interactions. This was performed on α-lactalbumin and lysozyme, which possess very similar sequences. At pH 5.5 and pH 7.5, lysozyme had a higher affinity for procyanidins than α-lactalbumin and, at pH 5.5, possessed more binding sites per protein molecule for DPn = 5.5 than α-lactalbumin (24 vs. 19 binding sites; Figure 2; Table 2). At these pH levels, lysozyme (pI = 10.5–11.3) and α-lactalbumin (pI = 4.2–4.5) are positively and negatively charged, respectively, whereas procyanidins are weakly acidic (Vernhet et al., 1996); therefore, electrostatic interactions cannot directly explain the stronger affinity of procyanidins for lysozyme than for α-lactalbumin. Because lysozyme has a much lower hydrophobicity value than α-lactalbumin (Li-Chan, 1990), this contributes to the identification of hydrogen bonds as the main forces driving the interactions between procyanidins and globular proteins rather than hydrophobic interactions. The involvement of hydrogen bonds was proposed for epicatechin–BSA interactions by Frazier et al. (2006). As proposed by Haslam (2006) for interactions of PC with proline-rich proteins, it is possible that, at least when specific binding is observed, initial association is driven by hydrophobic interactions in hydrophobic regions and later enhanced by hydrogen bonds.

Procyanidins decrease the solubility of BSA to a lesser extent than the solubility of α-lactalbumin, which possesses a pI similar to that of BSA (Figure 3). However, BSA has a higher affinity for proanthocyanidins than α-lactalbumin (Hagerman and Butler, 1981). Therefore, the changes in solubility cannot entirely be ascribed to the strength of the interactions between proteins and procyanidins. To reach insolubility, it is likely that many more procyanidin molecules are required to mask hydrophilic binding sites of the high-molecular weight BSA than the low-molecular weight α-lactalbumin. This is in agreement with the fact that BSA can bind up to 178 molecules of tara tannins (i.e., mainly ellagitannins) at pH 5.0 as observed by Frazier et al. (2006), whereas α-lactalbumin was saturated by 19 molecules of procyanidins of $\overline{DP}n = 5.5$, which possess similar molecular weight to tara tannins (pH 5.5; Table 1).

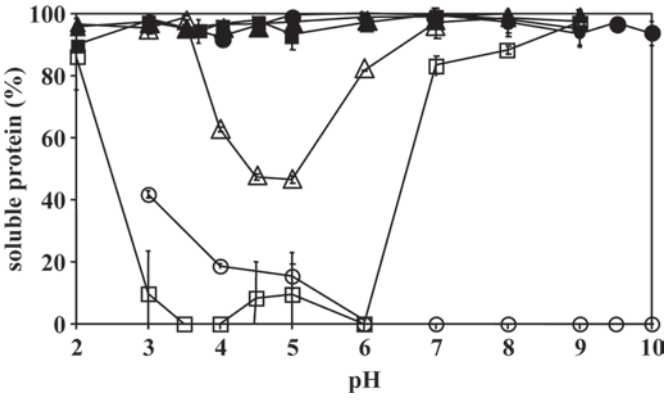

Figure 3. Protein [0.5% (wt/vol)] solubility as a function of pH, in the absence or presence of 5 mol of procyanidins per mol of protein: α-lactalbumin (■), lysozyme (●), and BSA (▲) in the absence of procyanidins; α-lactalbumin (□), lysozyme (○), and BSA (△) in the presence of procyanidins of an average degree of polymerization of 7.4.

Table 3. Protein solubility (%)[1] of α-lactalbumin in the presence of procyanidins of an average degree of polymerization (DP; DPn = 5.5) as a function of temperature and ionic strength

	10°C	25°C		40°C
Buffer molarity	2 h	2 h	72 h	2 h
0.027	16 ± 1	20 ± 2	21 ± 5	27 ± 1
0.050	ND[2]	18 ± 1	18 ± 4	ND
0.100	ND	20 ± 1	18 ± 1	ND

[1]Percentage as a function of protein concentration (defined as 100% in the absence of procyanidins).
[2]ND = not determined.

Effect of Procyanidins on Solubility as a Function of Ionic Strength and Temperature

No differences were observed after incubation of α-lactalbumin with procyanidins of DPn = 5.5 mixtures at 3 ionic strengths (Table 3). After 2 h, the solubility of α-lactalbumin was decreased to 18 to 21% by the presence of procyanidins (from 95–100% in the absence of procyanidins). Even after 3 d of incubation, increasing the ionic strength had no effect on the solubility of α-lactalbumin in the presence of procyanidins. Such a long incubation time was tested because it has been observed in apple juice that a higher ionic strength decreases protein solubility after several days of incubation (Tajchakavit et al., 2001). A first step of polymerization or activation (i.e., oxidation) of polyphenols, before reaction with nitrogenous material, has been proposed to explain this lag phase in the development of haze in beverages (Siebert, 2006). No lag for polyphenol–protein interactions is observed in our model,

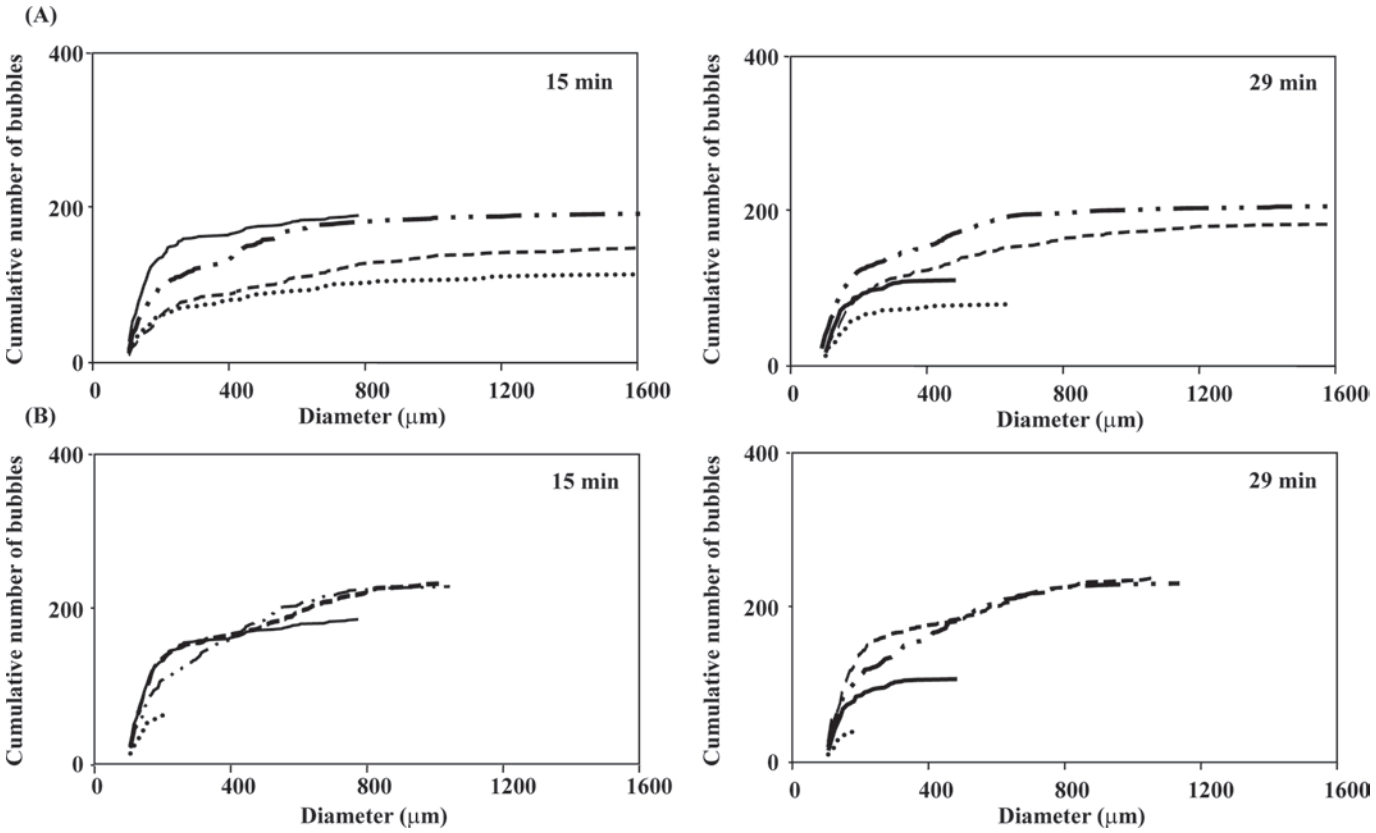

Figure 4. Cumulative number of bubbles as a function of bubble diameter of foam from α-lactalbumin at pH 7.0, after 15 and 49 min of foam formation, in the absence (—) or in the presence of procyanidins of various degrees of polymerization (DP): DPn = 2.0 (. . .); DPn = 5.0 (— . . —); and DPn = 7.4 (– –). (a) Two moles of procyanidin/protein; (b) five moles of procyanidin/protein.

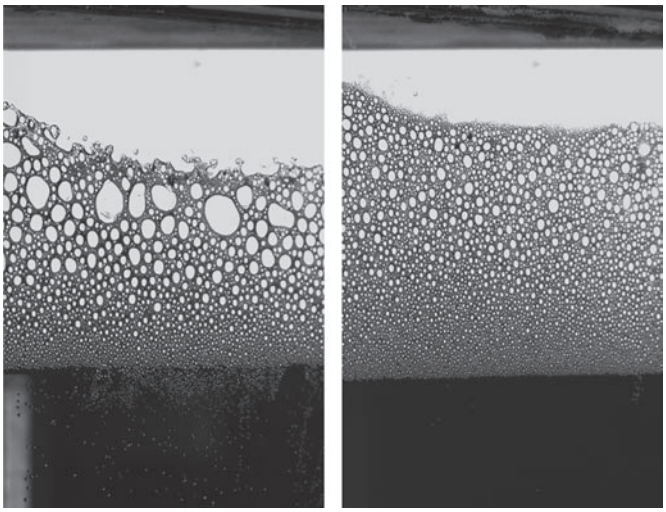

Figure 5. Images of foam from α-lactalbumin in the absence (left) and in the presence (right) of 2 mol of procyanidins (with an average degree of polymerization of 5.0) per mol of α-lactalbumin at pH 4.0 (after 49 min of foam formation).

probably because this model does not contain any molecules (e.g., carbohydrates) that slow down protein–polyphenol interactions (Mateus et al., 2004).

Increasing the temperature to 40°C had no effect on α-lactalbumin solubility in the absence of procyanidins (as expected at this protein concentration). When α-lactalbumin was incubated with procyanidins of $\overline{DP}n = 5.5$ for 2 h at 10, 25, or 40°C, its solubility increased with temperature from 16% at 10°C to 27% at 40°C (Table 3). This difference could indicate that α-lactalbumin interacts less strongly with procyanidins at higher temperatures. Because hydrophobic interactions increase with higher temperature, whereas hydrophilic interactions decrease, this result would indicate that hydrophilic interactions are the main driving force in α-lactalbumin–procyanidin interactions. However, because only solubility was measured and not direct interactions, another possible explanation may be that the solubility of the aggregates increases with temperature.

Effect of Procyanidins on Air–Water Interface and Foam

The low solubility of lysozyme at pH 7.0 in the presence of procyanidins prevented foam properties from being studied with lysozyme. The effects of procyanidins on air–water interface properties and foam properties of α-lactalbumin and BSA were studied at pH 4.0 and 7.0, with 2 or 5 mol of procyanidins per mol of protein. Proteins were whipped at lower speed at pH 4.0 than at pH 7.0 because of their higher foamability at the lower pH. At both pH levels, the presence of procyanidins did not modify the initial foams; the average bubble diameter of foam was not affected (data not shown). Procyanidins also had no effect on surface tension and elasticity at air–water interfaces (data not shown). However, the kinetics of foam disappearance were modified for α-lactalbumin. In the absence of procyanidins or in the presence of low-$\overline{DP}$ procyanidins ($\overline{DP}n = 2$), the total number of bubbles and the average bubble diameter decreased over time (Figure 4). This indicates an overall coarsening of the foam as a result of coalescence of larger bubbles. On the contrary, procyanidins of $\overline{DP}n = 5.0$ and 7.4 were able to provide a rather constant average bubble diameter over time, especially at the high procyanidin:protein molar ratio (5 mol of procyanidin:protein; Figure 4b). This extra-stabilization of foam is even clearer at pH 4.0, as presented in Figures 5 and 6. The effect of medium-$\overline{DP}$ procyanidins on foam stability presumably is an effect against Ostwald ripening. In our system, low-$\overline{DP}$ procyanidins did not seem to possess this property. However, catechin improves foamability and volume stability of protein foam, possibly because of the cross-linking of protein molecules by catechin (pH 7.0; Sarker et al., 1995). Such a mechanism was optimal at a molar catechin:protein ratio of 0.1. The fact that catechin improved foam properties (Sarker et al., 1995), whereas procyanidin dimers did not, can be ascribed to the molar ratio that was used in the former study. But it can also be ascribed to the nature of the protein–procyani-

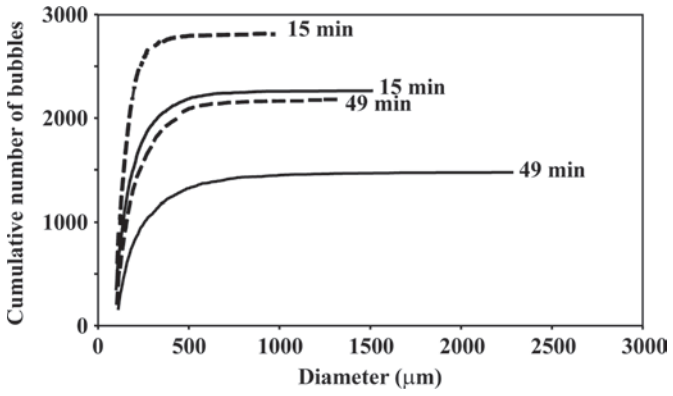

Figure 6. Cumulative number of bubbles as a function of bubble diameter, in the absence (—) or in the presence (– –) of 2 mol of procyanidins (with an average degree of polymerization of 5.0) per mol of α-lactalbumin at pH 4.0 (after 15 and 49 min of foam formation).

din couple studied or to the presence of Tween 20 in the study of Sarker et al. (1995).

CONCLUSIONS

Procyanidins of medium DP were able to stabilize protein foam, whereas those of smaller DP had no effect on protein foam properties. This can be related to the higher affinity of protein for procyanidins of medium DP than for smaller DP. Procyanidins of medium DP can decrease the solubility of proteins but may have a positive role in foam stability. In contrast to noncovalent interactions with hydroxycinnamic acids (i.e., PC that are present in large quantities in food and that interact poorly with globular proteins; Prigent et al., 2003), noncovalent interactions with oligomeric procyanidins affect protein functional properties. By modulating these interactions, food products may be created or improved, especially in the dairy industry.

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