Hydrophobicity of Whey Protein Concentrates Measured by Fluorescence Quenching and Its Relation with Surface Functional Properties

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Surface hydrophobicity of whey protein concentrate (WPC) under heated (85 °C for 5, 10, 20, 30, 40, and 60 min) and unheated conditions was measured using *cis*-parinaric acid (CPA), 1-anilino-8-naphthalenesulfonate (ANS), and a fluorescence quenching method using acrylamide as a quencher. This last method evaluates the degree of exposure of tryptophanyl residues in proteins to the solvent. The initial slope of Stern−Volmer plots, K_{app}, was used as an index of protein hydrophobicity. Surface hydrophobicity of WPC exhibited good relation with surface functional properties such as emulsifying and foaming. Analysis of the data obtained in this work showed that the fluorescence quenching method gave results similar to those obtained using CPA and ANS. Therefore, this simple technique is satisfactory in effectively obtaining information about the hydrophobicity of whey proteins.

Keywords: *Whey protein concentrate; hydrophobicity; fluorescence quenching; heat treatment; surface functional properties*

INTRODUCTION

Because of its high nutritional value and functionality, whey protein concentrate (WPC) obtained from dairy processing has become an important source of functional ingredients used in many formulated foods, including processed meat, bakery, and dairy products (*1*). Functional properties of WPC are primarily dependent on the degree of denaturation of globular whey proteins. Whey proteins have different abilities to denature and aggregate. Ruegg et al. (*2*) used differential scanning calorimetry (DSC) to determine the thermal transition temperature and degree of renaturation after heat treatment of pure *β*-lactoglobulin (*β*-LG), α-lactalbumin (α-LA), and bovine serum albumin (BSA). The transition temperatures near neutral pH were 62 (BSA), 65 (α-LA), and 73 °C (*β*-LG). There was no detectable renaturation of pure *β*-LG and BSA, but ∼80−90% of α-LA renatured. However, denatured molecules of α-LA, BSA, and *β*-LG can interact directly with each other during heating to form aggregates mainly through disulfide binding and hydrophobic interactions (*3*). Hence, the presence of several protein species must be considered when mixed systems containing whey proteins are heated and then cooled to room temperature. These samples are mixtures of native and denatured whey proteins, soluble and insoluble aggregates of denatured *β*-LG cross-linked by disulfide bonds, and complexes of denatured *β*-LG with α-LA and BSA. The composition of the products depends on the ratio of whey protein concentrations, the intensity of heat treatment, and the environmental conditions (*3*, *4*).

Whey protein denaturation can produce a number of undesired effects due to the loss of solubility, but it can also be used to obtain desired effects such as improvements of surface functional properties (foaming and emulsifying) (*5*). One basic requirement in setting operating conditions to produce a desired effect is a knowledge of the denaturation behavior of whey protein in a particular medium. The conformational modifications that lead to denaturation of proteins include the exposure of hydrophobic sites previously buried inside the native structure of protein molecules. Thus, measurements of the surface hydrophobicity may be useful in predicting the extent of whey protein denaturation and the functionality of WPC.

Various techniques have been proposed for evaluation of protein hydrophobicity. Spectroscopic methods are much simpler and quicker than chromatographic and partition in aqueous two-phase system methods, which are generally complex and time-consuming (*6*). Fluorescence spectroscopy is a useful technique to study the structure, dynamics, and binding properties of protein molecules in solution. Fluorescence titration employing hydrophobicity probes such as 1-anilino-8-naphthalenesulfonate (ANS) or *cis*-parinaric acid (CPA) have successfully been used to determine the surface hydrophobicity of whey proteins (*7*−*12*). Hayakawa and Nakai (*8*) classify the surface hydrophobicity of proteins determined by ligand binding into the aliphatic hydrophobicity due to aliphatic amino acid residues and the aromatic hydrophobicity due to aromatic amino acid residues. CPA is composed of an aliphatic hydrocarbon chain, whereas ANS is composed of aromatic rings. Therefore, CPA seems to be useful for determining the aliphatic hydrophobicity and ANS for determining the aromatic hydrophobicity. The protein unfolding increases the exposure of the previously buried inner hydrophobic groups (aliphatic and aromatic amino acid residues). Thus, for the majority of proteins CPA and ANS hydrophobicities increase when the protein molecules unfold due to heat treatment. Both aliphatic and

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aromatic hydrophobicities are important to explain the protein functionalities (*8*).

On the other hand, intrinsic fluorescence is a useful technique to study the structure and dynamics of protein molecules in solution. The intrinsic fluorescence of tryptophanyl residues is both particularly sensitive to their microenvironments and provides appropriate methods to perform this kind of studies. The degree of exposure of tryptophanyl residues in protein molecules can be evaluated by following the external quenching of the intrinsic protein fluorescence by acrylamide (*13*−*15*). In that sense, the objectives of this study were to establish a simple method for determining the extent of denaturation of whey proteins by measuring the fluorescence quenching of whey proteins after the addition of acrylamide and to relate it with surface functional properties of WPC submitted to different heat treatments.

MATERIALS AND METHODS

Materials. WPCs were supplied by Milkaut S.A., Santa Fe, Argentina. WPC contained 41.8% protein (60% β-LG, 20% α-LA), 3.0% lipids, 1.0% calcium, 1.6% potassium, 0.8% sodium, 1.8% chloride, 2.2% moisture, and 39.1% lactose, on a dry basis. β-LG, α-LA, and BSA were purchased from Sigma-Aldrich (St. Louis, MO). The ammonium salt of ANS was also purchased from Sigma-Aldrich. CPA was purchased from Molecular Probes (Eugene, OR). All other chemicals used in this study were of analytical grade.

Heat Treatment of WPC. WPC samples were dispersed in 10 mM sodium phosphate buffer, pH 7.0, at a protein concentration of 1.2%. A series of test tubes containing 5 mL of the whey protein dispersion was heated in a water bath at 85 °C. After various time periods, a tube was taken and cooled in ice−water.

Measurement of WPC Surface Hydrophobicity Using CPA. We used the method proposed by Kato and Nakai (*7*). Ethanolic solutions of CPA (3.6 mM) were purged with nitrogen, and equimolar butylated hydroxytoluene was added as an antioxidant. Whey protein dispersion (1.2% protein) was serially diluted to provide samples ranging in protein concentration from 0 to 0.06%. CPA in ethanol was then added to give a final concentration of 15 μM. Then, 3 mL of the mixture was excited at 325 nm, and the relative fluorescence intensity was measured at 420 nm in a Jasco FP-770 spectrofluorometer. The relative fluorescence intensity reading was adjusted to 1 when 10 μL of CPA solution was added to 3 mL of 10 mM sodium phosphate buffer, pH 7.0, in the absence of proteins. The slope at the origin of the fluorescence intensity versus protein concentration plot was used as the index of surface hydrophobicity (S_0).

Measurement of WPC Surface Hydrophobicity Using ANS. Three milliliters of whey protein dispersion (0.13 mg/mL in protein) was placed in the cell of the spectrofluorometer, and small aliquots of 5 mM ANS were successively added. Titration with ANS was monitored at $\lambda_{ex} = 390$ nm and $\lambda_{em} = 480$ nm. The relative fluorescence intensity reading was adjusted to 1 when 50 μL of ANS solution was added to 3 mL of 10 mM sodium phosphate buffer, pH 7.0, in the absence of proteins. Analysis of binding data was performed to obtain the maximum fluorescence attainable at saturating ANS concentration (F_{max}). F_{max}, expressed per milligram of sample proteins, is a function of the number of hydrophobic sites accessible to the spectroscopic marker in the system under investigation. The ANS concentration required to obtain half the value of F_{max} is considered to be the apparent dissociation constant of the fluorescent ANS−protein complex (K_d). The ratio F_{max}/K_d represents a cumulative index of surface hydrophobicity of protein, taking into account both the number and affinity of hydrophobic sites. This ratio is defined as the protein surface hydrophobicity index (PSH) (*9*, *10*).

Quenching of Protein Intrinsic Fluorescence by Acrylamide. Three milliliters of a sample, 0.13 mg/mL in protein, was placed in the cell of a Jasco FP-770 spectrofluorometer, and the fluorescence intensity at 337 nm using excitation at 295 nm was measured (F_0). Aliquots of 5 M acrylamide were mixed into the cell content, and the fluorescence intensity was redetermined (F). Final acrylamide concentrations ranged from 0 to 0.2 M. The F_0/F ratio was plotted versus the quencher concentration (Stern−Volmer plot). At the concentrations used, no corrections for acrylamide absorption were necessary. The Stern−Volmer equation relates the drop in protein fluorescence to the concentration of a collisional quencher, Q. For the quenching of a heterogeneously fluorescent protein, the appropriate form of the Stern−Volmer equation is

$$\frac{F_0}{F} = \left(\sum_{i=1}^{n} \frac{f_i}{(1 + K_i[\mathrm{Q}])\, \mathrm{e}^{V_i[\mathrm{Q}]}} \right)^{-1}$$

where K_i and V_i are the dynamic and static quenching constants for fluorescent component i (each one of tryptophanyl residues present in the protein) and f_i is the fractional contribution of component i to the total fluorescence (*13*). The initial slope of Stern−Volmer plots was defined as K_{app}.

A mixture of pure β-LG, α-LA, and BSA (60:20:20) was prepared in 10 mM phosphate buffer, pH 7.0, at a protein concentration of 0.13 mg/mL in order to simulate a dispersion of commercial WPC (WPCs). Both nontreated WPC and WPCs were also prepared in buffered 8 M urea. All of these samples were titrated with 5 M acrylamide.

Measurement of Emulsifying Properties of WPC. The determination of the emulsion activity was performed according to the method of Pearce and Kinsella (*16*). Two milliliters of WPC dispersion 0.1% in protein was emulsified with 0.5 mL of corn oil. This emulsion was diluted 1/100 with a 0.1% SDS solution, and the absorbance at 500 nm was measured. The emulsifying ability is expressed as the emulsifying activity index (EAI). The EAI was calculated by the formula $EAI = 2T/\phi c$, where T (turbidity) $= 2.3\ A/l$. A was the absorbance at 500 nm, l (light path) $= 10^{-2}$ m, and c was the concentration of protein (10^3 g/m^3), with ϕ (oil phase volume) $= 0.2$. The emulsifying activity was measured just after the formation of the emulsion (EAI_0) and after a 30-min heating at 80 °C following a 24-h storage at room temperature (EAI_{80}). The stability of the emulsions was calculated as follows: $\Delta EAI = 100\ (EAI_0 - EAI_{80})/EAI_0$. ΔEAI is the variation of the EAI relative to the initial EAI (*17*). The higher is ΔEAI, the more unstable is the emulsion formed.

Measurement of Foaming Properties of WPC. Dispersions (20 mL; 1.2% protein) were held at room temperature (22 °C) in a calibrated 100-mL test tube for 15 min. Air was introduced through a glass filter into the dispersion at a constant flow rate, 150 mL/min. After the air was introduced into the protein solution for 15 s, the volume of foam produced was recorded. Foamability was expressed using the the following formula: foam expansion (percentage) $= (V_f/V_s) \times 100$, where V_f = volume of foam after passing air through the solution for 15 s (mL) and V_s = volume of original sample (20 mL). The foam stability was measured as the time taken for half the foam to collapse, $t_{1/2}$.

RESULTS AND DISCUSSION

Figure 1 shows the effect of heating on whey protein hydrophobicity determined fluorometrically using CPA and ANS. The decreasing surface hydrophobicity using CPA as a probe is a consequence of the particular behavior of β-LG, the major whey protein component. β-LG may be classified in the superfamily of the hydrophobic molecule transporters termed *lipocalins*. These small globular proteins share a three-dimensional structural pattern consisting of eight strands of antiparallel β-sheet twisted into a cone-shaped barrel, which

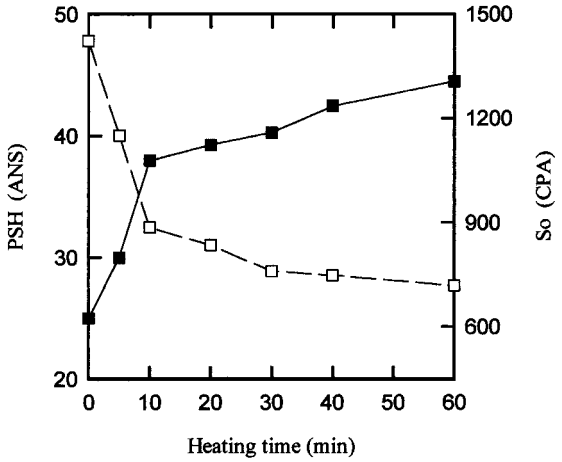

Figure 1. Effect of heating time on whey protein hydrophobicity measured with (■) ANS and (□) CPA probes. Heating temperature was 85 °C. All points were the mean of three measurements.

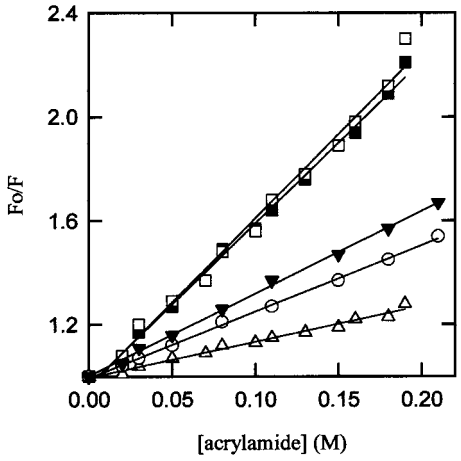

Figure 2. Stern−Volmer plots of whey protein fluorescence quenching by acrylamide: (△) WPCs; (○) nontreated WPC; (▼) WPC heated for 30 min at 85 °C; (■) WPC in 8 M urea; (□) WPCs in 8 M urea. Conditions: temperature, 22 °C; pH 6.8; protein concentration, 0.13 mg/mL. All points were the mean of three measurements.

constitutes a hydrophobic calyx. β-LG is known to bind retinoids, fatty acids, and other compounds with aliphatic hydrocarbon chains (*18*). Although the binding constants have been determined, the region of the protein involved in the interaction remains unclear. β-LG has two potential sites for binding hydrophobic ligands: one in the calyx formed by the β-barrel (*19*) and the other in an external hydrophobic pocket between the α-helix and the β-barrel (*20*). However, most of the experimental evidence points to the calyx as the major binding site for hydrophobic ligands (*18*, *21*−*23*). Wu et al. (*23*) have cocrystallized β-LG with palmitic acid at nearly neutral pH. The carboxyl group of the fatty acid binds to both Lys-60 and Lys-69 at the entrance to the central cavity, whereas the hydrophobic tail stretches in an almost fully extended conformation into the center of the protein. Therefore, it is possible that CPA, an anionic fluorescence probe analogue to fatty acids, principally binds to the main hydrophobic pocket. Laligant et al. (*24*) determined the proportion of residual native β-LG in preheated β-LG solutions (1%) at nearly neutral pH by hydrophobic interaction chromatography. The loss in native β-LG was negligible for solutions heated at 75 °C for 6 min, whereas for solutions heated at 90 °C for 6 min, native β-LG decreased by 70%. The diminution in the presence of native β-LG coincides with the formation of soluble dimers, trimers, and higher oligomers of denatured β-LG. From these various data, it seems that the decrease in the surface hydrophobicity determined by CPA with the intensity of heat treatment (Figure 1) may reflect the loss of CPA binding site as a consequence of irreversible protein unfolding.

The contribution of WPC relatively less abundant proteins (α-LA and BSA) to CPA hydrophobicity could follow opposite patterns, at pH 7.0. Whereas denatured α-LA (complexed or not) presents higher CPA hydrophobicity than the native protein, BSA denaturation leads to the loss of CPA binding sites, similarly to β-LG (*8*, *12*).

Whey proteins in the native state show low surface hydrophobicity values when measured using ANS as a probe. This may be related with the fact that hydrophobic groups are buried inside the native structure of protein molecules. Analysis of ANS−β-LG binding at nearly neutral pH gave a low number of sites ($n = 0.03-0.4$) and a high dissociation constant [$K_d = (2.0-6.5) \times 10^{-5}$ M], suggesting low affinity of β-LG for ANS (*24*). The low affinity of native β-LG for ANS may be due to the planar configuration of the ANS molecule. Such a rigid configuration could hinder the suitable fit of the ligand to the hydrophobic central calyx, in contrast to the behavior of fatty acids described by Wu et al. (*23*). When whey proteins are heated at temperatures >70 °C, protein molecules begin to unfold, causing the surfacing of hydrophobic sites and promoting thus the ANS binding (*25*). In that sense, addition of similar quantities of ANS to heat-treated samples of β-LG resulted in a greater increase in fluorescence intensity. Titration of heat-treated β-LG with ANS gave a curve that approached a maximum in fluorescence intensity at a 1:1 molar ratio of ANS/β-LG (*26*), indicating that a moderately strong binding site had been created by the heat treatment of the protein. Following the β-LG behavior, the surface hydrophobicity index of WPC measured by ANS (PSH) increased as the heat treatment became increasingly severe (Figure 1). Alizadeh-Pasdar and Li-Chan (*12*) have recently reported that when heating BSA at pH 7.0 the surface hydrophobicity measured by ANS decreased, contrary to β-LG behavior. However, this effect was not observed in the ANS hydrophobicity of WPC, suggesting that BSA contribution was negligible, probably because of its low concentration in these products.

If the heat treatment is carried out beyond a given time and temperature regimen (>100 °C), aggregation phenomena and structural collapse occur, resulting in decreased surface hydrophobicity of proteins. In this range, PSH decreases as the severity of treatments increases (results not shown).

Figure 2 shows that for all of the samples assayed, plots of F_0/F appeared approximately linear at acrylamide concentrations up to 0.2 M. In this range, the Stern−Volmer equation can be expressed as

$$\frac{F_0}{F} = 1 + K_{app}[Q]$$

where K_{app} is an apparent constant because of the heterogeneity of protein sample. Moreover, whey proteins possess more than a tryptophanyl residue that can be quenched by acrylamide.

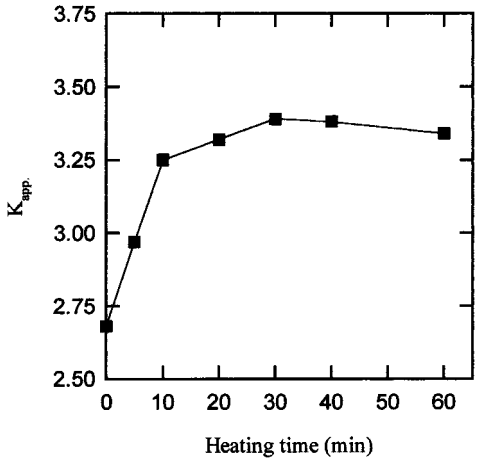

Figure 3. K_{app} calculated from the Stern−Volmer plots of WPC dispersions heated at 85 °C for various periods of time. Conditions: temperature, 22 °C; pH 6.8; protein concentration, 0.13 mg/mL.

Protein unfolding causes a red shift on the fluorescence emission spectrum due to the major exposition of the tryptophanyl residues to the aqueous solvent. This major exposition of tryptophanyl residues to the aqueous solvent promotes an increase in the fluorescence quenching of denatured proteins by acrylamide. For example, bovine β-LG contains two Trp residues, Trp-19 and Trp-61, which are in quite different environments. Trp-19 is in an apolar environment within the cavity of β-LG, whereas Trp-61 protrudes beyond the surface of the molecule. Heat treatment of β-LG results in a number of irreversible, linked, spectrally detectable changes to the structure of the protein. After β-LG has been treated, the wavelength of the maximum of the intrinsic fluorescent emission is shifted from 332 nm to longer wavelengths. This red shift indicates that the major fluorophore (Trp-19) has moved from an apolar environment to a more polar region (*27*). Consequently, in the unfolded state, the solvent exposure of both Trp-19 and Trp-61 could be similarly quenched by acrylamide with a higher efficiency than in the native state of the protein. In conclusion, the more intense the heat treatment, the higher is the degree of denaturation and the more pronounced is the Stern−Volmer plot (*28*). This trend was observed in the quenching of whey protein fluorescence in Figure 2. It can be seen from Figure 2 that the minor quenching efficiency was obtained with WPCs, because the protein molecules are predominantly in the native state. Heat treatment is a necessary step in processing WPC from cheese whey. Thus, the heat lability of the major whey proteins often results in their partial denaturation during processing. For this reason, protein fluorescence quenching by acrylamide increased when commercial WPC dispersions were tested. Moreover, the heat treatment of commercial WPC dispersions resulted in more pronounced Stern−Volmer plots than for nontreated WPC dispersions. The effect of acrylamide on the fluorescence of unfolded species by heat treatment was substantially less than for whey proteins denatured by urea (Figure 2). These results point to the presence in the denatured cross-linked species of a residual structure that hinders ful tryptophan accessibility to the acrylamide. The presence of 8 M urea produced a similar effect on the denaturation of WPC and WPCs, suggesting that both samples possessed a similar degree of protein unfolding.

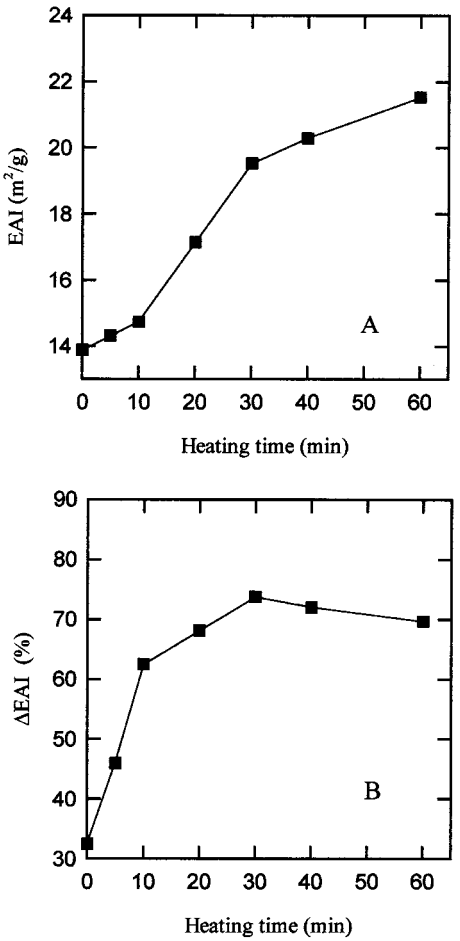

Figure 4. Changes in the emulsifying activity (A) and emulsion stability (B) of WPC dispersions heated at 85 °C for various periods of time. Conditions: temperature, 22 °C; pH 6.8; p rotein concentration, 0.1%.

In conclusion, the slope of Stern−Volmer plots for heated samples depended on the temperature and time of heating process, parameters that defined the extent of protein unfolding. Figure 3 shows the values of K_{app} calculated from the slope of Stern−Volmer plots of WPC dispersions heated at 85 °C for various periods of time. We propose that these K_{app} values can be used as an index of surface hydrophobicity.

On the other hand, a good correlation is obtained between hydrophobicity and surface functional properties of proteins (*7*). The ability of proteins to unfold the tertiary structures is normally considered a positive attribute for an emulsifier. It is therefore not surprising that heat denaturation often improves emulsifier performance, as long as solubility is not lost (*29*). Following this, Figure 4A shows the increase in EAI for WPC dispersions in which heating time at 85 °C was gradually increased.

High foamability has been correlated with a high rate of increase of surface pressure π. π is equal to $\gamma_0 - \gamma$, where γ_0 is the surface tension of the solvent and γ is the observed surface tension (*30*). Proteins with high surface hydrophobicity will increase π rapidly, due to its affinity for the air−water interface, and have a low rate of desorption. Hence, foaming power has been linked with surface hydrophobicity. Therefore, surface hydrophobicity increased by heat denaturation enhances foamability of WPC dispersions (foam expansion), as Figure 5A shows.

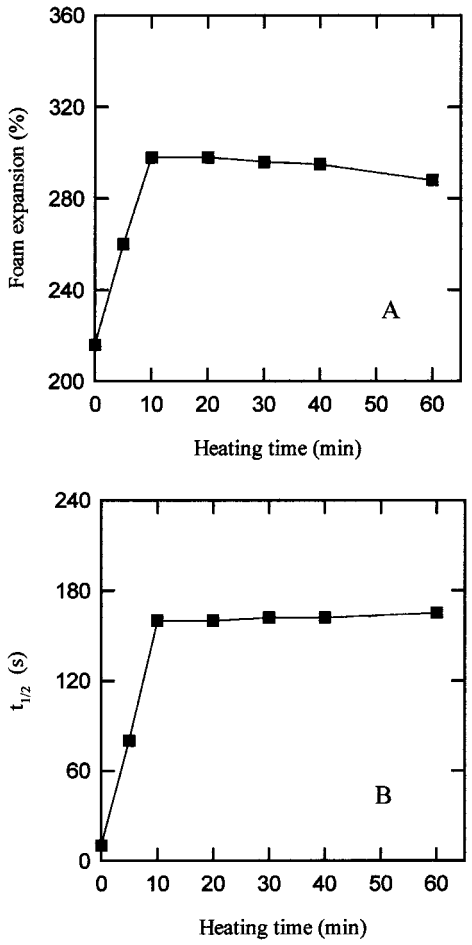

Figure 5. Changes in the foaming power (A) and foam stability (B) of WPC dispersions heated at 85 °C for various periods of time. Conditions: temperature, 22 °C; pH 6.8; protein concentration, 1.2%.

Table 1. Linear Correlation Analysis[a]

	surface hydrophobicity		
	S_0	PSH	K_{app}
EAI	−0.822*	0.847*	0.772*
ΔEAI	−0.990***	0.982***	0.996***
foam expansion	−0.945**	0.926**	0.968***
$t_{1/2}$	−0.981***	0.969***	0.987***

[a] *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

The positive correlation obtained between surface hydrophobicity and emulsion instability (Figure 4B) follows the trend observed for dry-heated β-LG (*5*). Moreover, the emulsifying activity of acylated β-LG is much higher than that of native β-LG, but with reduced emulsion stability at nearly neutral pH (*17*). In acylated β-LG, hydrophobic interactions are strengthened by the introduction of important hydrophobic moieties on lysyl side chains.

The stability of foams formed from WPC dispersions was increased with the increase of heating time at 85 °C, as shown in Figure 5B. The denaturation of whey proteins caused by heating process enhances protein−protein interactions, forming a strengthened viscoelastic interfacial layer of protein having an increased resistance to collapse (*30*).

Table 1 shows the linear correlation coefficients obtained for the relationships between hydrophobicity measured by the three methods assayed in this work and surface functional properties of WPC dispersions. In all the cases, there is a significant linear correlation between each one of the variable pairs assayed. Therefore, the fluorescence quenching method proposed in this work can be satisfactorily used to give information about the hydrophobicity of whey proteins.

Limitations in using anionic probes such as CPA and ANS to determine protein hydrophobicity include the possibility that electrostatic as well as hydrophobic interactions may contribute to the interaction with probes, thus overestimating the hydrophobicity. The use of neutral or uncharged probes such as diphenylhexatriene (DPH) (*6*) or 6-propionyl-2-(dimethylamino)naphthalene (PRODAN) (*12*) may circumvent this problem. In that sense, one of the most attractive features of acrylamide as a neutral quencher has been that it does not interact significantly with proteins (*13*). Another advantage of this simple method is that protein concentration has not been accurately known. However, for a general applicability of this method to other protein systems, more extensive studies should be required.

ABBREVIATIONS USED

α-LA, α-lactalbumin; ANS, 1-anilino-8-naphthalenesulfonate; β-LG, β-lactoglobulin; BSA, bovine serum albumin; CPA, *cis*-parinaric acid; Trp, tryptophan; WPC, whey protein concentrate.

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