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Modification of whey protein concentrate hydrophobicity by high hydrostatic pressure

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Abstract

The objective of this study was to evaluate the influence of high hydrostatic pressure (HHP) treatments on hydrophobicity of whey protein concentrate (WPC). An increase in binding affinity or decrease in apparent dissociation constant indicates increased hydrophobicity, which is positively correlated with functional properties. The effects of HHP treatment (600 MPa, 50 °C, 0 to 30 min) on intrinsic fluorescence of WPC and the binding properties of WPC for aromatic 1-anilino-naphthalene-8-sulfonate (ANS) and aliphatic *cis*-parinaric acid (CPA) probes were studied. HHP treatment of WPC resulted in an increase in intrinsic tryptophan fluorescence intensity and a 4 nm red shift after 30 min of treatment, which indicated changes in the polarity of tryptophan residues microenvironment of whey proteins from a less polar to a more polar environment. There was an increase in the number of binding sites of WPC for ANS from 0.16 to 1.10 per molecule of protein after HHP treatment for 30 min. No significant changes in the apparent dissociation constant of WPC for ANS were observed after HHP treatment, except for an increase from 1.8×10^{-5} M to 3.3×10^{-5} M after 30 min of HHP treatment. There were no significant changes in the number of binding sites of WPC for CPA. However, increased binding affinities of WPC for CPA were observed after the come-up time or 10 min of HHP treatment, with a decrease of apparent dissociation constant from 2.2×10^{-7} M to 1.1×10^{-7} M. The binding sites of WPC may become more accessible to the aliphatic hydrophobic probe CPA after the come-up time or 10 min of HHP treatment. These results indicate that HHP treatment of WPC yields increases in the number of binding sites for an aromatic hydrophobic probe, while aliphatic hydrophobic binding affinity of WPC is enhanced after come-up time or 10 min of HHP treatment.

Keywords: Whey protein concentrate; High hydrostatic pressure; Hydrophobicity; Fluorescence

Industrial Relevance Text: The functionality of protein molecules depends on hydrophobic, electrostatic, and steric parameters of the protein structure. Modifications of proteins that enhance hydrophobicity show promise for improving functional properties of foods. An increase in binding affinity or decrease in apparent dissociation constant indicates increased hydrophobicity. High hydrostatic pressure (HHP) affects the hydrophobicity of beta-lactoglobulin (β-LG), the primary protein in whey, and increases the binding affinity of β-LG for 1-anilino-naphthalene-8-sulfonate and *cis*-parinaric acid. However, little work has been done regarding the effects of HHP on whey protein concentrate (WPC) hydrophobicity and flavor-binding properties, and whether the presence of multiple proteins in WPC has significant influence on the behavior of whey proteins during HHP treatments. WPC is a good candidate for testing the practical utility of the application of HHP to modify the functional properties of a complex protein system since it is in the form that the ingredient is utilized in a number of food applications. The current work describes the effects of HHP on hydrophobicity of WPC and potential applications.

Abbreviations: ANS, 1-anilino-naphthalene-8-sulfonate; BSA, bovine serum albumin; CPA, *cis*-parinaric acid; HHP, high hydrostatic pressure; α-LA, α-lactalbumin; β-LG, β-lactoglobulin; β-ME, β-mercaptoethanol; MG, molten globule; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; WPC, whey protein concentrate.

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1. Introduction

Whey and whey products have been used successfully in the food industry for years. Nutritional value and reasonable cost are key motivators for the food industry to use whey products (Morr & Ha, 1993). Whey protein concentrate (WPC) possesses solubility, creates viscosity, forms gels, emulsifies, facilitates whipping, foaming and aeration, enhances color, flavor and texture, and offers numerous nutritional advantages to formulated products (U.S. Dairy Export Council, 1999).

The impact of hydrophobic interactions of food proteins on protein functional properties has received major attention (Burley & Petsko, 1985; Li-Chan & Nakai, 1989; Semisotnov et al., 1991). The functionality of protein molecules depends on hydrophobic, electrostatic, and steric parameters of the protein structure (Nakai, 1983). Nakai (1983) demonstrated an apparent close relationship between surface hydrophobicity, emulsion capacity and emulsion stability of proteins. Increased fat binding capacity was associated with an increase in hydrophobicity of the protein (Voutsinas & Nakai, 1983). Thus, modifications of proteins that enhance hydrophobicity show promise for improving flavor properties of foods.

Traditional food processing methods rely on high temperatures as a way to ensure prolonged shelf life and food safety. However, the use of high temperatures results in some detrimental changes in nutritional and organoleptic attributes in the processed products (Martin, Barbosa-Cánovas, & Swanson, 2002). High hydrostatic pressure (HHP) presents unique advantages over conventional thermal processing for food product modifications, including application at low temperatures, which permits the retention of food quality attributes (Knorr, 1995). Pressure acts as a physicochemical parameter that alters the balance of intramolecular and solvent–protein interactions (Cheftel, 1992). Low protein concentrations and pressures (up to 200–300 MPa) usually result in reversible pressure-induced denaturation. Higher pressures (above 300 MPa) have irreversible and extensive effects on proteins, including denaturation due to unfolding of monomers, aggregation, and formation of gel structures (Cheftel, 1992).

The major protein components of whey include β-lactoglobulin (β-LG) (50%), α-lactalbumin (α-LA) (20%) and bovine serum albumin (BSA) (5%). Among the three major whey proteins, most research has been focused on β-LG. Funtanberger, Dumay, and Cheftel (1995) reported that HHP (450 MPa, 25 °C, 15 min, pH 7.0) induced partial unfolding and aggregation of β-LG isolate. Electrophoretic patterns also revealed the progressive formation of dimers to hexamers and higher polymers of β-LG, as a function of the type and molarity of buffer and of the pressure level (Funtanberger et al., 1995). HHP (600 MPa, 50 °C, 32 min) induces β-LG into the molten globule (MG) state (Yang, Dunker, Powers, Clark, & Swanson, 2001). Yang, Powers, Clark, Dunker, and Swanson (2003) reported that HHP (600 MPa, 50 °C, 32 min) affected the hydrophobicity of β-LG and observed a significant increase in the binding affinity of β-LG for 1-anilino-naphthalene-8-sulfonate (ANS) and *cis*-parinaric acid (CPA) after HHP treatment.

Studies have been done to understand the effect of HHP on the functional properties of single whey proteins, such as gel formation (Famelart, Chapron, Piot, Brule, & Durier, 1998), emulsifying capacity (Galazka, Sumner, & Ledward, 1996) and foamability (İbanoglu & Karatas, 2001). However, little work has been done regarding the effects of HHP on certain functional properties of WPC, such as hydrophobicity and flavor-binding properties, and whether the presence of multiple proteins in WPC has significant influence on the behavior of whey proteins during HHP treatments. WPC is a good candidate for testing the practical utility of the application of HHP to modify the functional properties of a complex protein system since it is in the form that the ingredient is utilized in a number of food applications. The current work describes the effects of HHP on hydrophobicity of WPC and potential applications.

2. Materials and methods

2.1. Materials

RT-80 Grade A whey protein concentrate (WPC RT-80) was provided by Main Street Ingredients (La Crosse, WI). WPC RT-80 with the same lot number was used throughout the experiments. The product contained 84.9% protein, 3.9% fat, 3.4% ash, 3.5% lactose, and 3.7% moisture (measured by standard proximate analysis procedures). The pH of a 0.2% solution at 20 °C was 6.4. Standard proteins (β-LG, α-LA, BSA) were obtained from Sigma Chemical Co. (St. Louis, MO). All of the chemicals used were of analytical grade, obtained from Fisher Chemicals (Fairlawn, NJ), unless otherwise specified.

Concentrations of WPC solutions were determined spectrophotometrically by using the molecular absorption coefficients: α-LA: ε_{278}=28 542, β-LG: ε_{278}=17 600, BSA: ε_{278}=44 488. The following molecular absorptions were used to calculate ligand concentration: ANS: ε_{350}=5000, and CPA: ε_{304}=71 400.

2.2. Methods

2.2.1. High pressure treatment

WPC solutions, at concentrations of 0.2% or 1% (w/v) in sodium phosphate buffer (0.01 M, pH 7.0), were treated with HHP of 600 MPa at 50 °C for holding times of 0 (come-up time), 2.5, 5, 7.5, 10, 15, or 30 min in a warm isostatic press with yoke (Engineered Pressure Systems, Inc., Haverhill, MA) with a cylindrical pressure chamber (height=0.25 m, dia=0.10 m). Samples were equilibrated in the chamber for 5 min to reach 50 °C. The come-up

time (4.5 min) is the compression time required to reach a pressure of 600 MPa. Pressure release is within 30 s. Three batches of WPC solutions were used for all HHP treatments. After exposure to high pressure, WPC solutions were studied immediately, or stored at 4 °C for less than 1 month.

2.2.2. Polyacrylamide gel electrophoresis

The WPC samples were analyzed using a Mini-Protean Π Dual Slab Cell (Bio-Rad Laboratories, Hercules, CA). Polyacrylamide gel electrophoresis (4–20%) in the presence of sodium dodecyl sulfate (SDS-PAGE), with or without β-mercaptoethanol (β-ME), was used according to the instruction manual of Ready Gel Precast Gels (catalog number 161-0993, Bio-Rad Laboratories, Hercules, CA).

One milliliter of supernatant, obtained from the untreated, heated or pressurized WPC solutions was diluted with 3 ml of 62.5 mM Tris–HCl (pH 6.8), containing 25% glycerol, 0.01% bromophenol blue, 10% SDS, and/or 5% β-ME. Prior to analysis, solutions were heated for 5 min in a 100 °C water bath, followed by cooling to room temperature with running tap water. Electrophoresis was run at ambient temperature for 35 min at 200 V. The gels were stained with a Coomassie blue solution containing 40% methanol, 10% acetic acid, and 0.1% Coomassie Blue R-250, destained with 40% methanol/10% acetic acids solution, and preserved with a 10% glycerol solution to prevent drying and deterioration. Each sample was analyzed in triplicate. Prestained SDS-PAGE standards (catalog number 1610318, Bio-Rad Laboratories, Hercules, CA) were used to calibrate the gels. The protein standards included myosin (203 kDa), β-galactosidase (120 kDa), bovine serum albumin (90.0 kDa), ovalbumin (51.7 kDa), carbonic anhydrase (34.1 kDa), soybean trypsin inhibitor (28.0 kDa), lysozyme (20.0 kDa), and aprotinin (6.4 kDa).

2.2.3. Intrinsic and extrinsic fluorescence spectra

Conformational changes of WPC solutions were monitored by intrinsic tryptophan and extrinsic fluorescence spectra. Intrinsic fluorescence was assayed using an excitation wavelength of 295 nm and observing an emission wavelength of 350 nm. Extrinsic ANS fluorescence of WPC solutions was assayed using an excitation wavelength of 390 nm and observing emission at a wavelength of 470 nm. Extrinsic CPA fluorescence was assayed using an excitation wavelength of 325 nm and observing emission at a wavelength of 420 nm. For these assays, 36 μl of ANS (5.0 mM in 0.1 M phosphate buffer, pH 7.0) or 20 μl of CPA (2.5 mM in absolute ethanol containing equimolar butylated hydroxytoluene) solution were added to 3 ml of untreated or HHP treated WPC solutions (0.02%). Intrinsic and extrinsic fluorescence were collected with a Fluoro-Max-3 Spectrofluorometer (Jobin Yvon Inc., Edison, New Jersey), and fluorescence intensity was expressed as arbitrary units (a.u.).

2.2.4. Fluorescent probe binding study

Extrinsic aromatic hydrophobic ANS and aliphatic CPA fluorescence probes are often selected to determine the hydrophobicity of proteins (Nakai, 1983). Due to aromatic structures, ANS probe was used to study aromatic hydrophobic binding. The aliphatic probe CPA was used to study aliphatic hydrophobic binding.

In the binding study, 4 μl of CPA (2.5 mM in absolute ethanol containing 2.5 mM butylated hydroxytoluene) was titrated to the untreated or HHP treated WPC solutions (0.02%) to reach a final concentration at 7 μM for CPA. The hydrophobicity of WPC was assayed as extrinsic CPA fluorescence using an excitation wavelength of 325 nm and observing emission at a wavelength of 420 nm.

CPA binding properties were evaluated with the Cogan method (Cogan, Kopelman, Mokady, & Shinitzky, 1976). The number of accessible binding sites and apparent dissociation constants of CPA with WPC were calculated with the equation $[P_0\alpha=(1/n)(L_0\alpha/(1-\alpha)(K'_d/n)]$, where P_0 is protein concentration, L_0 is a given ligand concentration, n is the number of binding sites per molecule of protein, K'_d is the apparent dissociation constant, and α is the fraction of binding sites remaining free, assuming $\alpha=(F_{max}-F)/F_{max}$. F_{max} is defined as the fluorescence intensity when protein molecules are saturated by the ligand.

Since β-LG exhibits only low affinity for ANS, the binding parameters for ANS can not be calculated from Cogan or Scatchard equations (Laligant, Dumay, Valencia, Cuq, & Cheftel, 1991). As suggested by Laligant et al. (1991), ANS binding parameters were evaluated according to the method of Wang & Edelman (1971). Two experiments were conducted for the ANS binding study (Yang et al., 2003). In experiment one, 4 μl of ANS (5.0 mM in 0.1 phosphate buffer, pH 7.0) were titrated to the untreated or HHP treated WPC solutions (0.02%) to reach a final concentration at 55 μM for ANS. In experiment two, untreated and HHP treated WPC solutions (1%) were diluted with phosphate buffer (0.01 M, pH 7.0) to obtain WPC solutions with concentrations varying from 0.1% to 1%. Twenty microliters of ANS (100 μM in 0.1 phosphate buffer, pH 7.0) were added to 2 ml WPC solutions to obtain ANS concentrations of 1 μM. After mixing, extrinsic ANS fluorescence was determined with the spectrophotofluorometer, using an excitation wavelength of 390 nm and observing emission at a wavelength of 470 nm. The apparent dissociation (K'_d) constants of ANS to WPC solutions were calculated with the equation $1/F=1/F_{max}+[(K'_d/F_{max})(1/L)]$ by varying ANS concentration, where F and F_{max} are the observed and final fluorescence intensities, respectively, and L is the free ligand concentration. In experiment one, $L_0 \gg P_0$ (total concentration of protein) and the total (L_0) and free ligand concentration are rationally equal. Therefore, K'_d can be obtained by plotting $1/F$ vs. $1/L$. The number of binding sites (n) on WPC was

calculated by varying WPC concentration with the equation $L_0/F=(1/\varepsilon)+K'_d/[\varepsilon(nP_0-P_L)]$, where P_L is the concentration of the ligand–protein complex, and ε is a proportionality factor relating F to P_L. In experiment two, since $nP_0 \gg L_0$ and $nP_0 \gg P_L$, the equation becomes $L_0/F=(1/\varepsilon)+(K'_d/\varepsilon nP_0)$. L_0/F was plotted vs. nP_0. nP_0 was calculated using the obtained value of K'_d.

2.2.5. Statistical analysis

All experiments and analyses were done in triplicate (3 independent runs). The analysis of variance test for significant effects of treatments and assay samples were determined using the General Linear Model procedure (PROC GLM) in SAS. Main effect differences were considered significant at the $p<0.05$ level. Mean separations were determined by Fisher's Least Significant Difference (LSD) for multiple comparisons (SAS Institute Inc., 1993).

3. Results and discussion

3.1. Polyacrylamide gel electrophoresis

The SDS-PAGE gels exhibited four major regions for untreated WPC from bottom to top (Fig. 1): monomers of α-LA and β-LG (Region I), dimers of α-LA and β-LG (region II), BSA (Band III), and large aggregates (Region IV, with ~200 kDa molecular weight). The presence of aggregates may have resulted from the ultrafiltration procedure for WPC preparation. After the come-up time (H0) for HHP treatment, dissociation of aggregates and formation of dimers were observed (Fig. 1). Cheftel (1992) reported that pressures greater than 100–200 MPa often result in: (a) dissociation of oligomeric structures into their subunits, (b) partial unfolding and denaturation of monomeric structures, (c) protein aggregation. Region II, containing β-LG and α-LA dimers, was not well separated as treatment time increased. Region IV (Fig. 1) contained proteins with apparent molecular

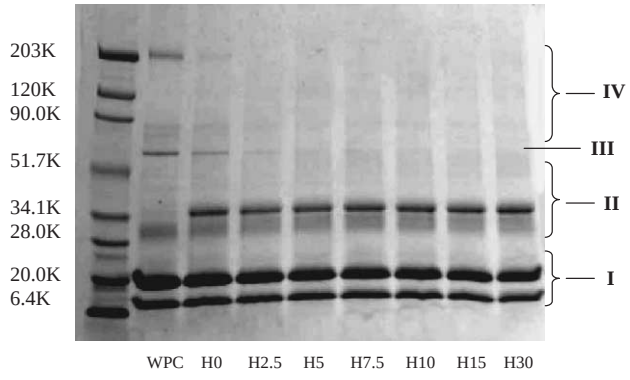

Fig. 1. SDS-PAGE (without β-mercaptoethanol) patterns of untreated and HHP treated (600 MPa, 50 °C, for various holding times: 0, 2.5, 5, 7.5, 10, 15, 30 min; H0–H30) 0.2% WPC solutions. I: SDS-monomeric α-LA and β-LG; II: dimeric and trimeric α-LA and β-LG; III: SDS-monomeric BSA; IV: large aggregates.

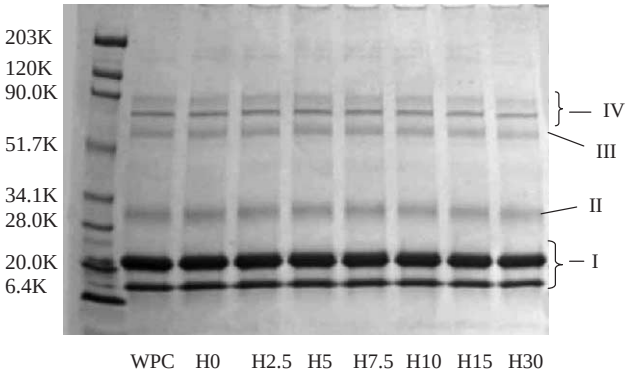

Fig. 2. SDS-PAGE (with β-mercaptoethanol) patterns of untreated and HHP treated (600 MPa, 50 °C, for various holding times: 0, 2.5, 5, 7.5, 10, 15, 30 min; H0–H30) 0.2% WPC solutions. I: SDS-monomeric α-LA and β-LG; II: dimeric α-LA; III: SDS-monomeric BSA; IV: aggregates.

weights larger than 60 kDa, corresponding with trimers and tetramers of whey proteins (Fig. 1). The absence of single bands for dimers and other oligomers in these regions indicates a considerable extent of inter- and intramolecular disulfide bond diversity (Manderson, Hardman, & Creamer, 1999).

Instead of the large aggregates seen in untreated WPC solutions (top of region IV, Fig. 1), intermediate sized aggregates were observed in HHP treated WPC (the whole Region IV, Fig. 1), with molecular weights from approximately 70 to 200 kDa. Due to the presence of free sulfhydryl groups in whey protein aggregates, aggregation is not limited to the formation of linear aggregates; branched aggregates can be formed (Schokker, Singh, Pinder, Norris, & Creamer, 1999), which may explain the presence of intermediate sized aggregates after HHP treatments.

WPC samples were also analyzed in the presence of β-ME, which allowed evaluation of the contribution of disulfide-stabilized aggregates in the soluble protein fraction of both untreated and pressurized WPC solutions (Fig. 2). There were no differences among the samples and most of the aggregates observed in Fig. 1 dissociated into the monomer form of α-LA, β-LG (Region I) and BSA (Region III; Regions III and IV shifted a little) in the presence of β-ME, which indicated that these aggregates were stabilized by disulfide bonds. Dimers of β-LG (Region II, Fig. 2) disappeared in the presence of β-ME, while dimeric α-LA (Region II, Fig. 2) was still observed. β-LG and α-LA were identified based on molecular weight standards. Havea, Singh, and Creamer (2000) also reported the presence of dimeric α-LA after heat treatment in the presence of β-ME, which may indicate the presence of a small quantity of nonreducible dimers of α-LA after both heat and HHP treatments in both the present study and in their study.

3.2. Intrinsic tryptophan fluorescence

At atmospheric pressure (0.1 MPa), β-LG (in 50 mM Tris buffer, pH 7.0) and α-LA (in 10 mM Tris buffer, pH

7.0) display typical fluorescence emission spectra, with a maximum at 332 nm (excitation: 295 nm) (Dufour, Hoa, & Haertlé, 1994; Tanaka, Nakajima, & Kunugi, 1996). The recorded β-LG and α-LA fluorescence emission maximum (332 nm) is characteristic of tryptophan residues in a relatively hydrophobic environment, such as the interior of the globulin (Dufour et al., 1994). The hydrophobic character of the β-LG and α-LA tryptophan neighborhood is additionally suggested by comparison with the fluorescence of free D,L-trptophan in aqueous solution (maximum near 355 nm) (Dufour et al., 1994; Lakowicz, 1999).

The intrinsic tryptophan fluorescence spectra of WPC in the present study were predominated by the spectra of β-LG and α-LA because of their high concentrations (~73% and ~23% of the major whey proteins, respectively) in the WPC (Fig. 3). The fluorescence intensity increased and a red shift was observed after HHP treatment, which indicates changes in the polarity of tryptophan residues microenvironment of whey proteins from a less polar to a more polar environment. HHP treatment for come-up time (H0) resulted in a 2 nm red shift of the maximum emission wavelength from 335 nm to 337 nm (Fig. 3). After HHP treatment of 2.5 min, there was a 1.1-fold increase in fluorescence intensity and the maximum emission wavelength shifted from 337 nm to 338 nm. Further HHP treatment up to 10 min did not cause further changes in the maximum emission wavelength, and the fluorescence intensity showed smaller increases per unit of time, indicating major changes in the tryptophan environment during pressurization occurred within 2.5 min. Thirty minutes of HHP treatment (H30) resulted in a further shift of maximum emission wavelength to 339 nm, and a final 1.2-fold increase in intrinsic fluorescence (Fig. 3).

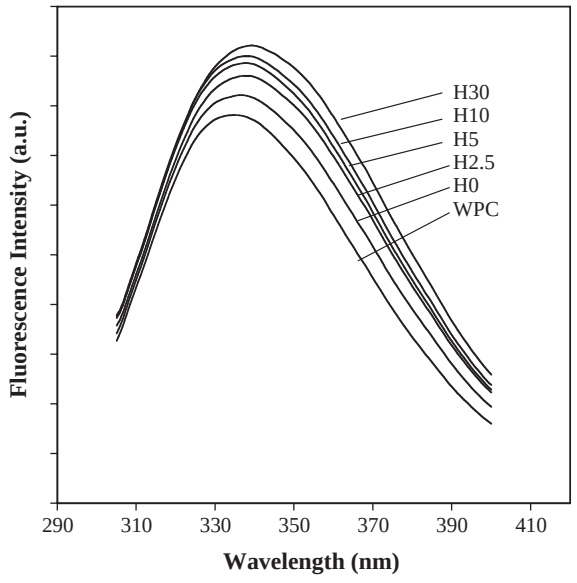

Fig. 3. Mean of three independent intrinsic tryptophan emission spectra of WPC solutions affected by HHP at 600 MPa and 50 °C for various holding times from 0 to 30 min (H0–H30).

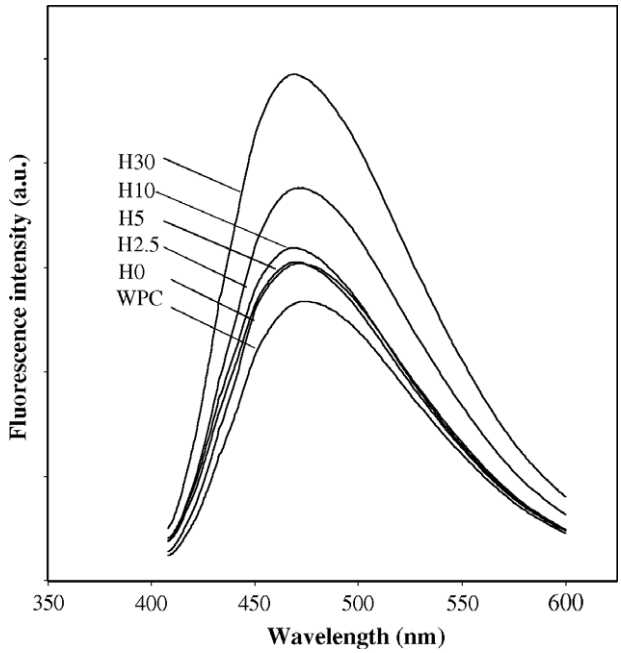

Fig. 4. Mean of three independent extrinsic ANS emission spectra of WPC solutions affected by HHP at 600 MPa and 50 °C for various holding times from 0 to 30 min (H0–H30).

The 4 nm red shift and the increases in the fluorescence intensity of WPC after HHP treatments are consistent with the results of HHP treatment of β-LG (Yang et al., 2001). Both the results from the present study and Yang et al. (2001) report less than the 1.9-fold intensity increase and 13 nm red shift found for β-LG in 8 M urea (Yang et al., 2001). A reasonable explanation, consistent with pressure studies on other proteins (Mohana-Borges, Silva, & de Prat-Gay, 1999), is that unfolding is more or less complete in 8 M urea but that partially folded, perhaps molten globular forms, remain after HHP treatment (Yang et al., 2001).

3.3. Extrinsic ANS and CPA fluorescence spectra

In spite of relatively long-standing knowledge that β-LG tightly interacts in vitro with retinol (Futterman & Heller, 1972), the exact physiological role of β-LG is unknown. However, β-LG can bind fatty acids and triacylglycerols (Brown, 1984), aromatic hydrocarbons (Farrell, Behe, & Enyart, 1987), retinoic acid (Dufour & Haertlé, 1991), CPA (Dufour, Roger, & Haertlé, 1992) and ANS (Hamdan, Curcuruto, Molinari, Zetta, & Ragona, 1996). ANS and CPA fluorescence is very weak in aqueous solutions, but it is greatly enhanced when bound to β-LG (Dufour et al., 1994; Hamdan et al., 1996). Thus, ANS and CPA probes are widely used to assay hydrophobicity of food proteins (Li-Chan & Nakai, 1989; Nakai, 1983; Yang et al., 2001).

The ANS extrinsic fluorescence of HHP treated WPC exhibited three distinct regions (Fig. 4). HHP treatment up to 2.5 min resulted in a 1.4-fold increase in the ANS extrinsic fluorescence intensity. A decrease in the fluorescence intensity to 1.1-fold was observed after further HHP treatment to 5 min. HHP treatment for 30 min resulted in a 2-fold increase in the ANS fluorescence intensity.

ANS fluorescence intensity of β-LG, α-LA, and BSA responded differently to HHP treatments (Galazka et al., 1996; Tanaka et al., 1996; Yang et al., 2001). Increases in the ANS fluorescence intensity were observed for β-LG and α-LA after HHP treatment above 200 MPa (Tanaka et al., 1996; Yang et al., 2001). However, HHP treatment of BSA up to 1000 MPa resulted in gradual decreases in ANS fluorescence intensity (Galazka et al., 1996). Thus, the ANS fluorescence spectrum of HHP treated WPC was observed as the overall changes of individual whey proteins during pressurization. Within 2.5 min of HHP treatment, the increases in ANS fluorescence intensity from β-LG and α-LA overcame the decrease in the intensity of BSA, which resulted in an overall increase in the ANS fluorescence intensity for WPC. However, during further HHP treatments up to 5 min, the WPC ANS fluorescence spectrum was dominated by the characteristic decrease in the ANS fluorescence intensity of BSA. When HHP holding time increased above 10 min, the WPC ANS fluorescence spectra showed gradual increases in fluorescence intensity, which indicated the domination of the spectra by the increases in the ANS fluorescence intensity by β-LG and α-LA over the decrease in the intensity from BSA.

A feature of fluorescence studies is that conformational changes within a protein can affect the emission wavelength (λ_{max}) and the emission intensity at λ_{max} (I_{Trp}) differently (Stapelfeldt, Petersen, Kristiansen, Qvist, & Skibsted, 1996). For example, Stapelfeldt et al. (1996) examined β-LG and reported that λ_{max} increased at pressures up to 300 MPa, whereas I_{Trp} attained a maximum value at a lower pressure of 200 MPa. The present study also showed that HHP treatment affected the ANS λ_{max} and I_{Trp} differently. In addition to the fluorescence intensity changes, a 6 nm blue shift was observed for the ANS maximum emission wavelength following 10 min of HHP treatment, and a 2 nm red shift appeared after further HHP treatment to 30 min. The blue shift during 10 min of HHP treatment indicates that HHP treated whey proteins bind ANS in a less polar environment compared to native whey proteins, which was also observed for β-LG after HHP treatment by Yang et al. (2001), although to a greater extent. Given that the ligand-free pocket of native β-LG is filled with water (Sawyer, Brownlow, Polikarpov, & Wu, 1998), such a polarity decrease is reasonable and consistent with pressure-induced MG formation. Further HHP treatment to 30 min resulted in a 2 nm red shift, indicating structural change with further pressure treatment. These results indicate that the presence of α-LA and BSA affected the binding of ANS to WPC, probably through the formation of aggregates, as observed in the gel electrophoresis (Fig. 1).

HHP treatment of WPC solutions resulted in decreases in fluorescence intensity of CPA and broadened peaks in CPA fluorescence spectra (Fig. 5). After HHP treatment for 10 min, a 64% decrease in CPA fluorescence intensity was observed and a second peak appeared. The large decrease of CPA fluorescence intensity and the appearance of a second emission peak suggest important conformational changes in the CPA binding environment. Similar results were observed by Dufour et al. (1994) for β-LG retinol fluorescence during HHP treatment.

3.4. Hydrophobic probe binding study

Hydrophobic probe binding study is different from intrinsic and extrinsic fluorescence spectra study. Titration curves are measured here, and apparent dissociation constant and the number of the ligand binding sites are calculated as stated in the methods. Table 1 shows the results of binding study. Titrations of WPC with ANS and CPA were plotted according to Cogan et al. (1976) and Wang and Edelman methods (1971). WPC exhibited 0.16 binding sites per molecule of protein for ANS, indicating one molecule of WPC bound with 0.16 molecules of ANS, with an apparent dissociation constant of 1.8×10^{-5} M (Table 1). Yang et al. (2003) reported that native β-LG exhibited 0.41 binding sites for ANS, with a higher dissociation constant than the present study (4.5×10^{-5} M). The lower value of WPC may indicate that the presence of aggregates in WPC decreased the accessibility of the binding sites for ANS.

WPC exhibited 1.9 binding sites for CPA, with an apparent dissociation constant of 2.2×10^{-7} M (Table 1). Yang et al. (2003) reported similar results for native β-LG (0.95 binding sites for CPA, with a dissociation constant of 2.1×10^{-7} M). The higher number of binding sites of WPC for CPA may indicate the generation of new binding sites by the aggregates present in WPC.

Come-up time of HHP treatment resulted in an increase in the number of binding sites from 0.16 to 0.33, which

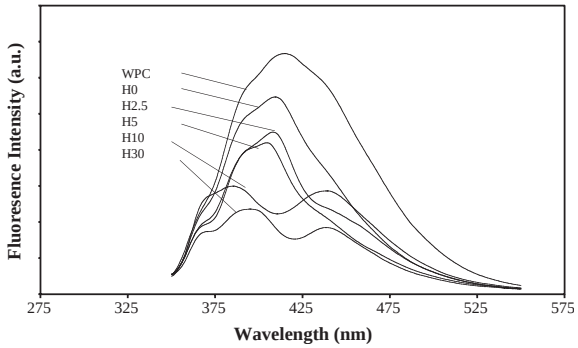

Fig. 5. Mean of three independent extrinsic CPA emission spectra of WPC solutions affected by HHP at 600 MPa and 50 °C for various holding times from 0 to 30 min (H0–H30).

Table 1
Apparent dissociation constants (K'_d) and the number of ligand binding sites (n) of WPC after HHP treatment (600 MPa and 50 °C) for holding time of 0 to 30 min (H0–H30) for 1-anilino-naphthalene-8-sulfonate (ANS), calculated using the method by Wang & Edelman (1971)* and *cis*-parinaric acid (CPA), calculated using the method by Cogan et al. (1976)**

WPC sample	ANS		CPA	
	n	K'_d (M)	n	K'_d (M)
Untreated	0.16^{a}	1.8×10^{-5} ^{a}	1.9^{a}	2.2×10^{-7} ^{a}
H0	0.33^{b}	1.7×10^{-5} ^{a}	1.8^{a}	1.1×10^{-7} ^{b}
H2.5	0.80^{c}	2.3×10^{-5} ^{a}	2.0^{a}	1.9×10^{-7} ^{a}
H5	0.72^{c}	2.1×10^{-5} ^{a}	2.1^{a}	2.0×10^{-7} ^{a}
H7.5	0.85^{c}	2.5×10^{-5} ^{a}	1.9^{a}	1.5×10^{-7} ^{b}
H10	0.97^{d}	2.5×10^{-5} ^{a}	1.8^{a}	1.1×10^{-7} ^{b}
H15	1.0^{d}	2.3×10^{-5} ^{a}	1.6^{a}	2.5×10^{-7} ^{a}
H30	1.1^{d}	3.6×10^{-5} ^{b}	1.7^{a}	4.1×10^{-7} ^{c}

^{a,b}: means with different letters in the column are significantly different ($p<0.05$).
* Data are means of three independent analyses.
** Data are means of three independent analyses.

may relate to the dissociation of the large aggregates present in untreated WPC and exposure of more binding sites for ANS (Table 1). HHP treatments of WPC up to 30 min resulted in even higher number of binding sites for ANS, indicating that structure modifications of WPC and formation of aggregates during HHP treatments generated new binding sites for ANS. Yang et al. (2003) reported that the number of binding sites for ANS interaction with β-LG did not change significantly during HHP treatment at 600 MPa and 50 °C for 30 min. So the presence of α-LA and BSA in WPC may be responsible for the increases in the number of binding sites for ANS through the formation of aggregates during HHP treatments. HHP treatments up to 15 min did not cause significant changes in the dissociation constants of WPC for ANS, but a 1.8-fold increase in the dissociation constant was observed after 30 min of HHP treatment (Table 1).

HHP treatments of WPC did not result in significant changes in the number of binding sites for CPA (Table 1), indicating formation of aggregates of WPC during HHP treatments did not significantly alter the number of the binding sites of WPC for CPA. The apparent dissociation constant for CPA with WPC decreased to 1.1×10^{-7} M after HHP treatment for come-up time, indicating an increase in the binding affinity of WPC for CPA. This improvement may result from the dissociation of aggregates (Creamer, 1995) present in untreated WPC. Further HHP treatment up to 5 min resulted in decreases in the binding affinity, indicating some conformational changes around the binding sites during HHP treatment. Changes in secondary structure (around the loosely structured surface loops) may occur during HHP treatment (Creamer, 1995), which could decrease the accessibility to the binding sites for CPA. HHP treatments of 7.5 or 10 min decreased the apparent dissociation constant to 1.5×10^{-7} M and 1.1×10^{-7} M, respectively. Further HHP treatment to 30 min resulted in a significant decrease in the binding affinity, with the apparent dissociation constant increasing to 4.1×10^{-7} M, which may be related to the formation of aggregates.

Yang et al. (2003) reported that the surface hydrophobic site of HHP-induced β-LG dimers were surrounded by hydrophobic amino acid residues, which resulted in an increase of hydrophobic affinity of β-LG for CPA at the surface hydrophobic site (Yang et al., 2003). This is consistent with the current finding, where increase in the binding affinity of WPC for CPA was observed after 10 min of HHP treatment. However, the formation of β-LG dimers (Yang et al., 2003) and aggregates from β-LG, α-LA and BSA monomers by disulfide bonds may decrease accessibility of CPA to the surface hydrophobic binding site. Therefore, it follows that the binding affinity of CPA to WPC decreased after further HHP treatment over 10 min, to 30 min. Overall, the binding of ANS and CPA to WPC is affected by HHP treatments. HHP treatment of WPC resulted in increases in the number of binding sites for aromatic hydrophobic probe, while aliphatic hydrophobic binding affinity of WPC is enhanced after come-up time or 10 min of HHP treatment. Since there is a close relationship between hydrophobicity and other protein functional properties, such as emulsion capacity (Nakai, 1983) and fat binding capacity (Voutsinas & Nakai, 1983), HHP treatment shows potential for improving functionality of WPC and may provide opportunities for improvement of flavor in reduced fat products.

4. Conclusions

The hydrophobic probe binding behavior of WPC is affected by the holding time of pressurization. HHP resulted in an increase in intrinsic tryptophan fluorescence intensity and a 4 nm red shift after 30 min of treatment, which indicate changes in the polarity of tryptophan residues microenvironment of whey proteins from a less polar to a more polar environment. HHP treatment for 30 min resulted in an increase in the number of binding sites for ANS from 0.16 to 1.10 per molecule of protein. No significant changes in the apparent dissociation constant of WPC for ANS were observed after HHP treatment, except for an increase from 1.8×10^{-5} M to 3.3×10^{-5} M after 30 min of HHP treatment. There were no significant changes in the number of binding sites of WPC for CPA. However, increased binding affinities of WPC for CPA were observed after the come-up time or 10 min HHP treatment, with a decrease of apparent dissociation constant from 2.2×10^{-7} M to 1.1×10^{-7} M.

These results indicate that during HHP treatments, conformational changes of whey proteins and aggregation affect the hydrophobicity and binding properties of WPC. HHP treated WPC may display improved functionality and provide opportunities for increasing utilization of WPC in the food industry, which warrants research attention.

References

Brown, E. D. (1984). Interactions of β-lactoglobulin and α-lactalbumin with lipids: A review. *Journal of Dairy Science*, *67*, 713–722.

Burley, S. K., & Petsko, G. A. (1985). Aromatic–aromatic interaction: A mechanism of protein structure stabilization. *Science*, *229*, 23–28.

Cheftel, J. C. (1992). Effects of high hydrostatic pressure on food constituents: An overview. In C. Balny, R. Hayashi, K. Heremans, & P. Masson (Eds.), *High pressure and biotechnology* (pp. 195–209). London: John Libby & Co, Ltd.

Cogan, U., Kopelman, M., Mokady, S., & Shinitzky, M. (1976). Binding affinities of retinol and related compounds to retinol binding proteins. *European Journal of Biochemistry*, *65*, 71–78.

Creamer, L. K. (1995). Effect of sodium dodecyl sulfate and palmitic acid on the equilibrium unfolding of bovine β-lactoglobulin. *Biochemistry*, *34*, 7170–7176.

Dufour, E., & Haertlé, T. (1991). Binding of retinoids and β-carotene to β-lactoglobulin. Influence of protein modifications. *Biochimica et Biophysica Acta*, *1079*, 316–320.

Dufour, E., Hoa, G. H., & Haertlé, T. (1994). High-pressure effects of β-lactoglobulin interactions with ligands studied by fluorescence. *Biochimica et Biophysica Acta*, *1206*, 166–172.

Dufour, E., Roger, P., & Haertlé, T. (1992). Binding of benzopyrene, ellipticine, and *cis*-parinaric acid to β-lactoglobulin: Influence of protein modifications. *Journal of Protein Chemistry*, *11*, 645–652.

Famelart, M. H., Chapron, L., Piot, M., Brulé, G., & Durier, C. (1998). High pressure-induced gel formation of milk and whey concentrates. *Journal of Food Engineering*, *36*, 149–164.

Farrell, H. M., Behe, M. J., & Enyart, J. A. (1987). Binding of *p*-nitrophenyl phosphate and other aromatic compounds by β-lactoglobulin. *Journal of Dairy Science*, *70*, 252–258.

Funtanberger, S., Dumay, E., & Cheftel, J. C. (1995). Pressure-induced aggregation of β-lactoglobulin in pH 7.0 buffers. *Lebensmittel-Wissenschaft und-Technologie*, *28*, 410–418.

Futterman, S., & Heller, J. (1972). The enhancement of fluorescence and the decreased susceptibility to enzymatic oxidation of retinol complexed with bovine serum albumin, β-lactoglobulin, and the retinol-binding protein of human plasma. *Journal of Biological Chemistry*, *247*, 5168–5172.

Galazka, V. B., Sumnerb, I. G., & Ledward, D. A. (1996). Changes in protein–protein and protein–polysaccharide interactions induced by high pressure. *Food Chemistry*, *57*, 393–398.

Hamdan, M., Curcuruto, O., Molinari, H., Zetta, L., & Ragona, L. (1996). Electrospray mass spectrometry: Complexation between 1-anilino-naphthalene-8-sulfonate and proteins. *Journal of Mass Spectrometry*, *31*, 1261–1264.

Havea, P., Singh, H., & Creamer, L. K. (2000). Formation of new protein structures in heated mixtures of BSA and α-lactalbumin. *Journal of Agricultural and Food Chemistry*, *48*, 1548–1556.

İbanoglu, E., & Karatas, S. (2001). High pressure effect on foaming behavior of whey protein isolate. *Journal of Food Engineering*, *47*, 31–36.

Knorr, D. (1995). Hydrostatic pressure treatment of food: Microbiology. In G. W. Gould (Ed.), *New methods of food preservation* (pp. 159–172). New York: Kluwer Academic Publisher.

Lakowicz, J. R. (1999). Protein fluorescence. In J. R. Lakowicz (Ed.), *Principles of fluorescence spectroscopy* (pp. 466–485). New York: Kluwer Acedemic/Plenum Publishers.

Laligant, A., Dumay, E., Valencia, C. C., Cuq, J. L., & Cheftel, J. C. (1991). Surface hydrophobicity and aggregation of β-lactoglobulin heated near neutral pH. *Journal of Agricultural and Food Chemistry*, *39*, 2147–2155.

Li-Chan, E., & Nakai, S. (1989). Effect of molecular changes (SH groups and hydrophobicity) of food proteins on their functionality. In J. E. Kineslla, & W. G. Soucie (Eds.), *Food proteins* (pp. 232–241). Champaign: American Oil Chemists' Society.

Manderson, G. A., Hardman, M. J., & Creamer, L. K. (1999). Effect of heat treatment on bovine β-lactoglobulin A, B, and C explored using thiol availability and fluorescence. *Journal of Agricultural and Food Chemistry*, *47*, 3617–3627.

Martin, M. F. S., Barbosa-Cánovas, G. V., & Swanson, B. G. (2002). Food processing by high hydrostatic pressure. *CRC Critical Reviews in Food Science and Nutrition*, *42*, 627–645.

Mohana-Borges, R., Silva, J. L., & de Prat-Gay, G. (1999). Protein folding in the absence of chemical denaturant. *Journal of Biological Chemistry*, *274*, 7732–7740.

Morr, C. V., & Ha, E. Y. W. (1993). Whey protein concentrates and isolates: Processing and functional properties. *CRC Critical Reviews in Food Science and Nutrition*, *33*, 431–476.

Nakai, S. (1983). Structure–function relationships of food proteins with an emphasis on the importance of protein hydrophobicity. *Journal of Agricultural and Food Chemistry*, *31*, 676–683.

SAS Institute, Inc. (1993). *SAS User's Guide Statistics*. Cary, NC: SAS Institute, Inc.

Sawyer, L., Brownlow, S., Polikarpov, I., & Wu, S. Y. (1998). β-lactoglobulin: Structure studies, biological clues. *International Dairy Journal*, *8*, 65–72.

Schokker, E. P., Singh, H., Pinder, D. N., Norris, G. E., & Creamer, L. K. (1999). Characterization of intermediates formed during heat-induced aggregation of β-lactoglobulin AB at neutral pH. *International Dairy Journal*, *9*, 791–800.

Semisotnov, G. V., Rodionaca, N. A., Razgulyzev, O. I., Uversky, V. N., Gripas, A. F., & Gilmanshin, R. I. (1991). Study of the "molten globule" intermediate state in protein folding by a hydrophobic fluorescent probe. *Biopolymers*, *31*, 119–128.

Stapelfeldt, H., Petersen, P. H., Kristiansen, K. R., Qvist, K. B., & Skibsted, L. H. (1996). Effect of high hydrostatic pressure on the enzymic hydrolysis of β-lactoglobulin B by trypsin, thermolysin and pepsin. *Journal of Dairy Research*, *63*, 111–118.

Tanaka, N., Nakajima, K., & Kunugi, S. (1996). The pressure-induced structural change of bovine α-lactalbumin as studied by a fluorescence hydrophobic probe. *International Journal of Peptide and Protein Research*, *48*, 259–264.

U.S. Dairy Export Council. (1999). *Product specifications*. Arlington, VA, USA: U.S. Dairy Export Council.

Voutsinas, L. P., & Nakai, S. (1983). A simple turbidimetric method for determining the fat binding capacity of proteins. *Journal of Agricultural and Food Chemistry*, *31*, 58–63.

Wang, J. L., & Edelman, G. M. (1971). Fluorescent probes for conformational states of proteins. *Journal of Biological Chemistry*, *246*, 1185–1191.

Yang, J., Dunker, A. K., Powers, J. R., Clark, S., & Swanson, B. G. (2001). β-Lactoglobulin molten globule induced by high pressure. *Journal of Agricultural and Food Chemistry*, *49*, 3236–3243.

Yang, J., Powers, J. R., Clark, S., Dunker, A. K., & Swanson, B. G. (2003). Ligand and flavor binding functional properties of β-lactoglobulin in the molten globule state induced by high pressure. *Journal of Food Science*, *68*, 444–452.