Effect of homogenization and pasteurization on the structure and stability of whey protein in milk¹

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ABSTRACT

The effect of homogenization alone or in combination with high-temperature, short-time (HTST) pasteurization or UHT processing on the whey fraction of milk was investigated using highly sensitive spectroscopic techniques. In pilot plant trials, 1-L quantities of whole milk were homogenized in a 2-stage homogenizer at 35°C (6.9 MPa/10.3 MPa) and, along with skim milk, were subjected to HTST pasteurization (72°C for 15 s) or UHT processing (135°C for 2 s). Other whole milk samples were processed using homogenization followed by either HTST pasteurization or UHT processing. The processed skim and whole milk samples were centrifuged further to remove fat and then acidified to pH 4.6 to isolate the corresponding whey fractions, and centrifuged again. The whey fractions were then purified using dialysis and investigated using the circular dichroism, Fourier transform infrared, and Trp intrinsic fluorescence spectroscopic techniques. Results demonstrated that homogenization combined with UHT processing of milk caused not only changes in protein composition but also significant secondary structural loss, particularly in the amounts of apparent antiparallel β -sheet and α -helix, as well as diminished tertiary structural contact. In both cases of homogenization alone and followed by HTST treatments, neither caused appreciable chemical changes, nor remarkable secondary structural reduction. But disruption was evident in the tertiary structural environment of the whey proteins due to homogenization of whole milk as shown by both the near-UV circular dichroism and Trp intrinsic fluorescence. In-depth structural stability analyses revealed that even though processing of milk imposed

little impairment on the secondary structural stability, the tertiary structural stability of whey protein was altered significantly. The following order was derived based on these studies: raw whole > HTST, homogenized, homogenized and pasteurized > skimmed and pasteurized, and skimmed UHT > homogenized UHT. The methodology demonstrated in this study can be used to gain insight into the behavior of milk proteins when processed and provides a new empirical and comparative approach for analyzing and assessing the effect of processing schemes on the nutrition and quality of milk and dairy product without the need for extended separation and purification, which can be both time-consuming and disruptive to protein structures.

Key words: whey protein, milk processing, molecular structure, spectroscopy, structural stability

INTRODUCTION

Pasteurization of milk involves of heating to a sufficient temperature and period of time to inactivate and destroy the contaminating pathogenic microorganisms. It is designed to make milk a safe product for human consumption as well as to extend its shelf life. The 2 most commonly used pasteurization processes today are HTST and UHT. In the HTST process, milk is heated to a minimum of 72°C for 15 s, whereas the UHT pasteurization holds the milk at temperatures in the range of 135 to 140°C for a minimum holding period of 2 s. The effect of thermal pasteurization on milk including the quality of protein, fats, minerals, vitamins, appearance, and flavor has been a subject of intense research in the past few decades (Gregory, 1967; Ford et al., 1969; Douglas et al., 1981; Farrell and Douglas, 1983; Burton, 1988; McMahon et al., 1993; Ryley and Kajda, 1994; Li-Chan et al., 1995; Rattray et al., 1997; Carbonaro et al., 1997, 2000; Lacroix et al., 2006; Cattaneo et al., 2008; Al-Attabi et al., 2009).

It is now well established that high temperature processing, especially UHT, causes a series of effects on milk such as loss of available lysine (Mottar and Naudts, 1979; Burton, 1988), and aggregation and de-

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naturation of protein (Cheeseman and Knight, 1974; Douglas et al., 1981; Burton, 1984; Singh and Latham, 1993). As a result, many chemical changes could also occur (Pellegrino et al., 1995; Cosio et al., 2000; Elliott et al., 2005), in addition to modifications on functional properties of milk proteins (Farrell and Douglas, 1983). These changes inevitably affect the renneting, emulsifying, and foaming properties of the dairy products based on the processed milk (Parnell-Clunies et al., 1988; Krasaekoopt et al., 2003; Wang et al., 2007; Borcherdling et al., 2008). It remains equivocal, however, as to whether and how thermal pasteurization treatments would affect the protein digestibility and nutritional values of milk in humans (Burton, 1988; Lacroix et al., 2008; Wada and Lönnerdal, 2014).

Homogenization is a mechanical process that is used to reduce the size of the natural fat globules of milk by pumping milk at high pressure (15–40 MPa) through a small valve. The process breaks fat particles (average diameter approximately 3.5 μm) into much smaller globules (diameter <1 μm ; Sharma and Dalgleish, 1993) and in doing so preventing creaming of the milk. In addition to reduce the size of the fat globules, homogenization also profoundly rearranges casein, serum protein, and milk fat globule membrane (MFGM) protein molecules, alters the structure of MFGM, and changes protein-protein interaction as well as with the MFGM (Sharma and Dalgleish, 1993; Corredig and Dalgleish, 1996; Lee and Sherbon, 2002). Past research has found that homogenization does not significantly affect milk allergy and intolerance in both allergic children (Høst and Samuelsson, 1988) and lactose-intolerant or milk hyper-sensitive adults (Pelto et al., 2000). More recent work seems to suggest that changes in homogenized milk could improve milk digestibility and enhance the bioavailability of MFGM and other health-promoting components (Michalski and Januel, 2006). The effect of homogenization alone or in combination with other treatments such as heating and other treatments on the functional properties of milk and dairy products including cheese making and yogurt making remains to be thoroughly investigated. The objective of this work is to present a spectroscopic approach to analyze and to determine the effect of processing on the apparent molecular structure and thermal stability of milk. In particular, we attempt to provide a semiquantitative understanding of the ramifications of homogenization, pasteurization, or both on whey proteins that were minimally prepared because commonly used chromatographic purification procedure can cause protein to denature, and thereby any subtle structural alterations resulted from processing may be inadvertently lost.

Circular dichroism (CD) is a highly sensitive spectroscopic tool for rapid determination of the secondary

structure and folding properties of proteins (Woody, 1995). Briefly, CD is usually defined as the unequal absorption of left-handed and right-handed circularly polarized light. Proteins have CD bands in the far-UV region (190–260 nm) that arise mainly from the amides of the protein backbone and are sensitive to their conformations. In addition, proteins also have CD bands in the near UV (350–260 nm), which arises from aromatic side-chains (Woody and Dunker, 1996).

Fourier transform infrared (FTIR) spectroscopy also provides information about the secondary structure content of proteins (Barth, 2007). It works by shining infrared radiation on a sample and seeing which wavelengths of radiation in the infrared region of the spectrum are absorbed by the sample. Characteristic bands found in the infrared spectra of proteins often fall within the amide I and amide II (1800–1400 cm^{-1}) regions, which arise from the amide bonds that link the amino acids. Because both the C=O and the N–H bonds are involved in the hydrogen bonding that takes place between the different elements of secondary structure, the locations of both the amide I and amide II bands are sensitive to the secondary structure content of a protein.

Fluorescence spectroscopy is one of the most sensitive and versatile techniques for studying protein (Weber, 1960; Edelman and McClure, 1968). Proteins that contain aromatic residues including Trp and Tyr are often fluorescent when excited by UV light. This intrinsic fluorescence is usually strongly dependent on the close environment of the Trp and Tyr side-chains and often serves as a probe for changes in the tertiary structural contact involving these residues.

Although these spectroscopic techniques alone or their combinations have been successfully applied in numerous studies of highly purified proteins, the structural information obtained is far from definitive and quantitative because of the complex nature of protein structure in itself compounded by the challenges involved in the spectral interpretations (Greenfield, 1996; Kelly and Price, 2000; Navea et al., 2006). Therefore, a well-defined and unified procedure for deriving secondary structure of proteins from CD, FTIR, and fluorescence techniques remains yet to be established. Qualitative and comparative studies using these techniques have been attempted for protein–protein interaction (Schirmer and Lindquist, 1997) and protein–ligand interaction (Kim et al., 2004) systems. The applications and analyses of these molecular spectroscopies, empirical or quantitative, on highly mixed and “dirty” systems such as whey proteins have not been published to our best knowledge. Recent attempts have been made, however, to apply these spectroscopic techniques to study qualitatively milk and dairy products with

a particular focus on the detection of heat treatment (Birlouez-Aragon et al., 1998; Chen et al., 2005; Murillo Pulgarín et al., 2005; Schamberger and Labuza, 2006). Highly controlled experiments (e.g., keeping total protein concentration constant) combined with self-consistent and comparative analyses from these complementary techniques could yield more detailed information on the structural changes and thermal stability of whey proteins, assuming complex interactions among various whey protein components are absent. The results may be used as an indicator for the extent of milk processing. In this work, we study the apparent molecular structural changes in soluble whey fractions from various processed milk using highly sensitive spectroscopic techniques including CD, FTIR, and Trp intrinsic fluorescence in combination with an empirical and comparative data analysis routine to shed light on the effect of homogenization, which could have significant implications on the properties of subsequent milk and dairy products.

MATERIALS AND METHODS

Materials

Fresh milk was purchased from a local farm and standardized promptly to prepare whole milk with a 3.25% (wt/wt) fat content. A portion of the fresh milk was also skimmed. The whole milk samples were subjected to the following single or combined processing treatments: standard high-temperature, short-time pasteurization at 72°C for 15 s (HTST) using 1-L quantities of milk in an Armfield Model FT74P/T HTST/UHT (Armfield Inc., Denison, IA) plate and frame continuous pasteurizer as described in Tomasula and Kozempel (2004); homogenization at 35°C using a 2-stage homogenizer (6.9 MPa/10.3 MPa; Universal Pilot Plant, Waukesha Cherry-Burrell, Philadelphia, PA); and UHT sterilization through an indirect plate heat exchanger at 135°C for 2 s. Following pasteurization and sterilization (UHT), the samples were chilled to 4°C. Skim milk was also pasteurized and UHT-treated to obtain **s-HTST** and **s-UHT**, respectively. Some of the homogenized samples were followed by HTST pasteurization (**h-HTST**) or UHT treatment (**h-UHT**). The HTST, homogenization, and UHT treatments were performed in duplicate.

The raw whole milk, HTST, homogenized, h-HTST, and h-UHT milk samples (50 mL) were skimmed immediately following the pasteurization treatment by centrifugation at $5,000 \times g$ at 25°C for 30 min. All milk samples, including s-HTST and s-UHT, were then acidified to pH 4.6 with 1 *N* HCl. All acidified samples were cooled to 4°C after the pH (at 4.6) was held at

room temperature (~ 20 – 22°C) for 10 min. The coagulated samples were then centrifuged at $24,000 \times g$ in a Sorvall RC-5B centrifuge (DuPont Co., Wilmington, DE) at 4°C for 40 min. Both the top layer (residual fats) and the precipitate (casein) were discarded, and the centrifugation procedure was repeated one more time to ensure a complete separation of soluble whey, casein, and fats. Only the middle portion of the supernatant was taken and neutralized carefully with 1 and 0.1 *N* NaOH to pH ~ 6.7 . To minimize or eliminate the interference of water-soluble vitamins on the Ellman's assay, CD, FTIR, and fluorescence experiment, the soluble whey was dialyzed [molecular weight cut-off (MWCO) = 5,000 Da, Spectra/Por, Spectrum, Houston, TX] against Milli-Q water (filtered and purified by reverse osmosis using the Millipore water purification system, Bedford, MA) for 36 h at 4°C. All whey protein samples were filtered with a 0.45- μm syringe filter (Millex-HV, PVDF, Millipore Corp., Bedford, MA) and kept frozen (at -20°C) before analysis.

Diluted HCl solutions were prepared from a 12.1 *N* HCl solution (Fisher Scientific, Fairlawn, NJ). All other chemicals including sodium hydroxide (NaOH), sodium phosphate (monobasic and dibasic), and 5,5'-dithio-bis-(2-nitrobenzoic) (**DTNB**, Ellman's reagent) were purchased from Sigma-Aldrich (St. Louis, MO) reagent grade and used directly.

Purified bovine β -LG and α -LA (purity at $\geq 90\%$) were gifts from Harold M. Farrell Jr., of the same laboratory, and used as reference materials in the spectroscopic studies.

Estimation of Protein Concentration in Whey

The protein concentration of each whey solution (before and after dialysis) from the various processed milk samples was estimated by measuring the absorbance at 278 nm (Cary 100 Bio UV-VIS spectrometer, Agilent Technologies, Böblingen, Germany) and corrected for any light scattering at 440 nm when necessary (Leach and Scheraga, 1960; Grimsley and Pace, 2004). An extinction coefficient $\epsilon_{278} = 1.046 \text{ L}\cdot\text{cm}^{-1}\cdot\text{g}^{-1}$ (Mahmoudi et al., 2007) was used in the calculation. Unless otherwise noted, all whey protein samples were kept at a concentration of $0.370 (\pm 0.005) \text{ mg/mL}$ for all spectroscopic experiments using Milli-Q water.

Gel Electrophoresis

Sodium dodecyl sulfate-PAGE of all neutralized and dialyzed whey samples (in water and MWCO = 5,000 Da), pH ~ 6.7 , was carried out using pre-cast mini gels (10 wells) and reagents supplied by Invitrogen Life Tech-

nologies (Carlsbad, CA). The NuPAGE Novex 12% Bis-Tris Gel (NP0341BOX) was used under reducing conditions (50 mM dithiothreitol). All samples were diluted 3× using the sample buffer NuPAGE LDS (4×; NP0007), dithiothreitol stock solution (0.5 M), and water (Milli-Q) before loading onto the gel. Fifteen microliters (~10 µg) of each whey sample (in the sample buffer) were loaded into each well. The running buffer was NuPAGE Tris-Acetate SDS Running Buffer (20×; LA0041) according to the procedures by the manufacturer. Gel was stained with SimplyBlue SafeStain (LC6060) for 3 h followed by destaining in a solution containing 30% methanol and 10% acetic acid to the desired color density level.

Determination of Sulfhydryl and Disulfide Bond Contents

Total free sulfhydryl concentration was determined according to the method by Ellman (1959) in combination with a modified procedure provided by the supplier (Sigma, St. Louis, MO). The reaction buffer contained 1.0 M sodium phosphate and 1.0 mM EDTA (pH 8.0). Two hundred fifty microliters of each dialyzed whey protein sample (1.5–2.70 mg/mL) and 50 µL of 1.0 mM DTNB assay solution were pipetted into 2.5 mL of the reaction buffer. The reaction mixture was incubated in the dark for 20 min at room temperature, and the absorbance was then recorded at 412 nm against a blank of reaction buffer (2.5 mL) containing 50 µL of 1.0 mM DTNB reagent and 250 µL of MilliQ water. A set of 7 solutions containing L-cystine hydrochloride monohydrate (molecular weight = 175.6) at concentrations of 0.0, 0.1, 0.2, 0.3, 0.4, 0.6, and 1.0 mM was used to establish a linear standard curve for the determination of free sulfhydryl content. The final free sulfhydryl concentration (mM) was normalized against the total amount of protein (mg) contained in each sample. All assay experiments were performed in triplicate.

The free sulfhydryl concentration of pure β -LG (gift from H. M. Farrell Jr.) was determined to be 168.5 ± 11.2 µM/mg of β -LG and used as an independent check of the method used in this work.

Circular Dichroism Spectroscopy

Circular dichroism spectroscopy was performed with an Aviv Model 420 CD spectrometer (Aviv Biomedical Inc., Lakewood, NJ). Spectra in the far-UV region (190–260 nm) were measured at the concentration of 0.37 to 0.38 mg/mL for all whey samples in a 0.05-cm path length quartz cell. The CD spectra in the near-UV region (250–320 nm) were acquired using whey

concentration at ~1.0 mg/mL in a path length of 1-cm rectangular cuvette. All spectra were recorded with 0.5-nm step and 5 s average time, and corrected for solvent contribution (MilliQ water).

The analysis of the secondary structural elements of the whey protein from the far-UV CD spectra was carried out using the software CDNN (v.2.1, Applied Photophysics Ltd., Leatherhead, UK) developed by Gerald Böhm (Böhm and Jaenicke, 1992). A database consisting of 33 reference proteins was used in the deconvolution analysis. The standard deviation was $\pm 0.4\%$ based on a set of triplicate CD experiments, and followed by the CDNN analysis.

Temperature dependence of far-UV CD (260–190 nm) spectra was also carried out in a 0.05-cm path length quartz cell. Temperature was adjusted using a solid-state peltier element, and a minimum of 30 min equilibration time was used between each temperature point. Each spectrum at each temperature was corrected for solvent (Milli-Q water) contribution before being subjected to deconvolution using the CDNN program.

FTIR Spectroscopic Measurements and Analysis

Infrared spectra were collected with a FTIR spectrometer (Nexus 670, Thermo Electron, Madison, WI) equipped with a DTGS KBr detector and a KBr beam splitter. All whey protein samples were used without dilution. Two hundred microliters of the solutions investigated in this work were placed on a CaF₂ plate, allowed to air dry overnight, and then vacuum dried for 30 min before measurement. The thin film was scanned from 4,000 to 1,000 cm⁻¹ at 20°C with a nominal resolution of 2 cm⁻¹ and 128 accumulative scans.

The analysis of the secondary structure of whey protein from the FTIR spectra was carried out as previously reported (Byler and Susi, 1986). The baselines were corrected linearly using the built-in software of the spectrophotometer (Omnic v.7.3). By means of the second derivative in the amide I region, 1,700 to 1,600 cm⁻¹, 8 major peaks were resolved respectively and fitted using the Peak Resolve (Omnic v.7.3). A Gaussian function was used during the fitting routine for all peaks corresponding to α -helix (~1,660 cm⁻¹), unordered (~1,550 cm⁻¹), β -sheet (1,620–1,610 cm⁻¹), β -turn (1,690–1,670 cm⁻¹), and β -antiparallel sheet (~1,690 and 1,640–1,630 cm⁻¹) according to the assignments by Barth (2007). A separate Fourier self-deconvolution procedure (Omnic v.7.3; Kauppinen et al., 1981) was also used to independently confirm the positions of the overlapping peaks (± 2 cm⁻¹). The areas of all the component bands assigned to a given secondary structural element were then summed and divided by the total

integrated area to yield the percentage of the element. The standard deviation of this analysis procedure was $\pm 2\%$.

Fluorescence Spectroscopy

Fluorometric experiments were carried out on a FluoroLog-3 spectrometer (Horiba Scientific, Edison, NJ). The intrinsic tryptophan fluorescence spectra of the whey proteins were recorded from 305 to 430 nm at 20°C using excitation wavelength $\lambda_{\text{ex}} = 295$ nm. The excitation spectra were also recorded from 230 to 320 nm when the emission wavelength (λ_{em}) was set at 340 nm. The bandwidths used were 5 nm for both excitation and the emission spectra. The path length of the quartz fluorescence cuvettes used was 1.0 cm in all fluorescence experiments.

Temperature dependence of intrinsic Trp fluorescence was carried out by recording the emission from 305 to 430 nm using $\lambda_{\text{ex}} = 295$ nm, and the excitation from 230 to 320 nm using $\lambda_{\text{em}} = 340$ nm for each whey sample at each temperature. Temperature was adjusted using a solid-state Pelletier element, and a minimum of 20 min equilibration time was used between each temperature point.

RESULTS AND DISCUSSION

Change in Soluble Whey Protein Concentration Caused by Processing of Milk

Whey protein solution samples were dialyzed (MWCO = 5,000 Da) to remove lactose and water-soluble vitamins, which could interfere with the Ellman's assay, CD, FTIR, and fluorescence experiments carried out in this work. The protein concentration was estimated spectroscopically before and post the dialysis. The difference in whey protein concentration is negligible among all the whey solutions before dialysis, which fell roughly within the range of 3.5 to 3.8 mg/mL. This is in close agreement with what was reported in the literature and determined by the Kjeldahl method (Karman and van Boekel, 1986; DePeters and Ferguson, 1992), from 4 to 7 mg/mL, depending on breeds, diet, and season. It should be noted that the soluble whey proteins likely suffered a loss as a result of acidification of milk and the subsequent separation of soluble whey from casein and lipids because of interactions between whey and casein (Haque et al., 1987; Corredig and Dalgleish, 1999), and between whey and milk fat globules (Sharma and Dalgleish, 1993; Corredig and Dalgleish, 1996). In addition, it is possible that the water-soluble vitamins, mainly thiamine (vitamin

B₁), riboflavin (B₂), and niacin (B₃; López-de-Alba et al., 2006) could contribute to the UV-Vis absorption of whey, which would introduce errors in the estimation of protein concentration using the absorption value at 278 nm. However, it is unlikely that the concentrations of these vitamins in milk (100–200 µg/L; Koop et al., 2014) are sufficiently high to cause significant alteration in the estimation of protein concentration.

Dialysis caused significant concentration loss, by more than one-third, for all whey solutions due to osmotic pressure except for those from s-UHT and h-UHT milk samples, which decreased even more. In the case of h-UHT whey, the concentration was reduced by almost 60% compared with that of predialysis. The small amount of precipitate formed (in UHT whey) during dialysis was evidently due to denaturation and aggregation of serum proteins. This further showed that UHT treatment significantly changed the chemical and physical properties of the fat globule membranes and serum proteins, and thus their interaction. Homogenization that preceded UHT promoted these interactions, leading to lowered amount of whey proteins in the soluble fraction, as in the case of h-UHT whey. One possible explanation is that homogenization disrupts the fat droplets and causes the globule membrane to rearrange, resulting in an increased level of interactions between whey and casein, and whey and fat globules (Elfagm and Wheelock, 1978; Sharma and Dalgleish, 1994; Oldfield et al., 1998; Corredig and Dalgleish, 1999; Anema, 2008).

Chemical Changes in Whey Caused by Various Processing Treatments of Milk

Chemical changes in the whey from the various milk samples (postdialysis) were assessed by SDS-PAGE and free sulfhydryl analysis by the Ellman's assay method. Results are shown in Figures 1 and 2, respectively. The overall electrophoretic profiles of the whey solutions look quite similar to one another except for the h-UHT and s-UHT whey samples (Figure 1). Densitometry analysis revealed that the relative amount of α -LA remains virtually unchanged across all whey solutions, approximately 22 to 25%, whereas the level of β -LG decreased from approximately 50% in raw and HTST whey to 35 and 25% for the s-UHT and h-UHT whey samples, respectively. Homogenization, HTST pasteurization, and the combination of both treatments did not significantly alter the relative composition of whey.

Results from the Ellman's assay method (Figure 2) demonstrated reduced levels of free sulfhydryl (–SH) in both h-UHT and s-UHT whey samples within ex-

Figure 1. Sodium dodecyl sulfate-PAGE profiles of dialyzed whey (in water, molecular weight cut-off = 5,000 Da, and pH ~6.7) from milks that were treated as follows: (1) raw; (2) high-temperature, short-time pasteurized (HTST); (3) homogenized (H); (4) homogenized and pasteurized (h-HTST); (5) skimmed and pasteurized (s-HTST); (6) homogenized UHT (h-UHT), and (7) skimmed UHT (s-UHT). β-LG = purified bovine β-LG; MW = molecular weight; Lf = lactoferrin; Cas = caseins.

Figure 2. Free sulfhydryl (SH) content (μM) of whey protein determined by the 5,5'-dithio-bis-(2-nitrobenzoic) (DTNB) method. All data were collected in triplicate with standard deviations plotted. HTST = high-temperature, short-time pasteurized; H = homogenized; h-HTST = homogenized and pasteurized; s-HTST = skimmed and pasteurized; h-UHT = homogenized UHT, and s-UHT = skimmed UHT.

perimental error as represented by standard deviation compared with the raw whole and the other processed whey samples, as normalized based on estimated protein concentration. This is consistent with previously published work on the denaturation effect of indirect UHT processing on whey by HPLC analysis (Morales et al., 2000; Elliott et al., 2005). The studies by Carbonaro et al. (1997) on disulfide reactivity (also by Ellman's assay in the presence of dithioerythritol) of whey proteins also arrived at similar conclusion on the inverse relationship between disulfide reactivity and the intensity of thermal treatment of different thermal-treated milk. Homogenization treatment does not appear to affect the level of free sulfhydryl of the soluble whey proteins in milk when compared with their skimmed counterparts (Figure 2). Taken together with the SDS-PAGE results (Figure 1), the loss of β-LG in both h-UHT and s-UHT whey samples is attributable to the reduced level of free sulfhydryl due to the formation of intermolecular disulfide bonds, aggregation of β-LG, or both.

Figure 3. Far-UV circular dichroism spectra of various whey protein samples at room temperature (20°C) using a 0.05-cm path length cell. All protein concentration was adjusted to 0.37 mg/mL by measuring the UV absorption at 278 nm. HTST = high-temperature, short-time pasteurized; H = homogenized; h-HTST = homogenized and pasteurized; s-HTST = skimmed and pasteurized; h-UHT = homogenized UHT, and s-UHT = skimmed UHT.

Secondary Structural Changes in Whey from Processed Milk

The apparent secondary structure of whey protein at room temperature (20°C) from the processed milk samples was studied by both far-UV CD and FTIR spectroscopic methods. Figure 3 gives results from the far-UV CD studies. Except for s-UHT and h-UHT whey, the far-UV CD spectra for all whey samples possess similar spectral shape with a broad negative CD band and a maximum spanning from 210 to 218 nm, typical for mixed α -helical (double dip at 208 and 222 nm) and β -sheet structures (single dip at 215 nm). The

CD spectrum of h-UHT shows a gross loss in both apparent α -helix and β -sheet contents, and an increase in random coil structure as the maximum peak shifted to 205 nm. In addition, the intensity of the CD band varies among different whey samples for the same concentration, suggesting secondary structural shift may have occurred.

Quantitative analysis of the individual secondary structural element for purified proteins, β -LG, and α -LA was accomplished by recording their far-UV CD spectra and then followed by the deconvolution procedure using the CDNN program and a database consisting of 33 reference proteins (Böhm and Jaenicke, 1992), and results are presented in Table 1. For β -LG, the secondary structural content analyzed in our recent publication (Qi et al., 2014) is consistent with the analyses in the literature (Dong et al., 1996). Native β -LG contains predominately, over 50% β -sheet with the majority in antiparallel form (~45%). The relative amounts of other structural elements including α -helix, parallel β -sheet, turn, and unstructured are also in close agreement with the analyses by x-ray crystallography (Brownlow et al., 1997). The secondary structural content for α -LA was also analyzed from its far-UV CD spectrum (data not shown for brevity). Table 1 gives the amount of each secondary structural elements of α -LA determined in this work, which is also in close agreement with the published x-ray crystal structure (Chrysina et al., 2000).

Because whey is composed of more than 50% β -LG and about 25% α -LA, its overall CD spectrum should reflect roughly a close composite of these 2 major proteins assuming significant and complex intermolecular interactions between β -LG and α -LA and other whey components that may cause considerable structural

Table 1. Secondary structural analysis (%)¹ from far-UV circle dichroism spectra of whey protein from homogenized, pasteurized, and UHT milk (pH ~6.7)

Sample ²	Antiparallel β -sheet	Turn	α -Helix	Unordered	β -Sheet
Raw whole	34.7	18.0	14.4	27.7	5.2
Pasteurized (HTST)	34.3	18.2	14.5	27.9	5.1
Homogenized	37.8	17.5	13.1	26.7	4.9
h-HTST	33.2	18.3	15.0	28.3	5.2
s-HTST	31.8	18.5	11.2	32.9	5.6
h-UHT	26.5	21.5	11.7	35.3	5.0
s-UHT	29.0	19.3	12.5	33.6	5.6
β -LG ³	43.3	16.1	10.5	25.6	4.5
α -LA ⁴	10.6	21.6	35.0	29.6	3.2

¹Apparent values as analyzed by deconvolution using CDNN program and a database consisting of 33 reference proteins (Böhm and Jaenicke, 1992).

²h-HTST = homogenized and pasteurized; s-HTST = skimmed and pasteurized; h-UHT = homogenized UHT, and s-UHT = skimmed UHT.

³From our published data (Qi et al., 2014). β -LG = purified bovine β -LG.

⁴Studied and analyzed in the same fashion as other whey samples and used as a reference.

changes to whey proteins are absent. We further assume that the composition of all the whey samples studied in this work remained the same except for s-UHT and h-UHT whey. To carry out a self-consistent and semiquantitative analysis to compare various whey samples, it is reasonable to approximate whey as an enclosed and unified protein system with the exception of h-UHT and s-UHT due to their noted loss in β -LG. It should be pointed out that the results obtained for whey proteins in Table 1 are strictly comparative and by no means an accurate measurement of secondary structural content. Nevertheless, we believe the relative composition of each structural element was reasonably estimated.

Table 1 demonstrates that HTST, homogenization, and h-HTST essentially imposed no change on the apparent secondary structural content of whey compared with raw whey. However, whey from s-HTST milk lost over 6% of total secondary structure for both antiparallel β -sheet and α -helix combined compared with raw whole whey given these whey samples have similar protein compositions (Figure 1). As a result, an equal amount of increase in apparent random coil structure was observed.

The UHT treatment, as expected, clearly caused significant apparent secondary structural reductions for both h-UHT and s-UHT whey samples. This denaturation effect was especially severe for the h-UHT whey, which contained $\sim 8\%$ less apparent antiparallel β -sheet and $\sim 2\%$ lowered α -helical content compared with raw whey. This loss is transformed into an approximately 7% increase in random structure and 3% increase in turn structure, and an irregular structural element. Skimmed UHT whey underwent a total loss of about 7% in apparent antiparallel β -sheet and α -helix combined, converting into random structure by almost the same amount. The relative content of α -LA is much

higher in s-UHT and h-UHT whey, as indicated in Figure 1. Therefore, the amount of apparent α -helical content would have been much higher for s-UHT and h-UHT whey samples if α -LA were to remain its native structure. This showed that UHT processing caused more severe damage on both antiparallel β -sheet (predominately in native β -LG) and α -helix (mostly present in native α -LA). Homogenization followed by UHT further aggravated this denaturation effect, as in the case of h-UHT.

Although it is less sensitive and accurate (standard deviation = $\pm 2\%$) than the far-UV CD spectroscopic method, FTIR analysis can still serve as an independent and confirmative tool to study the secondary structural effect in whey protein caused by milk processing. The FTIR spectra in the amide I region ($1700\text{--}1600\text{ cm}^{-1}$), known for its sensitivity to protein secondary conformational change, were deconvoluted and analyzed following the band assignments by Barth (2007). Figure 4 compares the amide I region of the deconvoluted FTIR spectrum of raw whey with that of h-UHT whey. Table 2 shows the assignments of the deconvoluted IR absorption bands and their corresponding secondary structural content. It is clear that the results are consistent with the analyses by far-UV CD spectroscopy as discussed above. Although differences do exist in the exact structural contents analyzed by the 2 techniques, particularly pertaining to the amounts of apparent antiparallel β -sheet and unordered structures, results from the FTIR analyses also lead to the same conclusion as that from the far-UV CD spectra. The discrepancy is likely because they are derived from 2 distinctively different analysis procedures including sample preparation and spectral deconvolution. Both techniques demonstrated the denaturation effect of homogenization, especially when followed by UHT treatment, on both the apparent antiparallel β -sheet and α -helical structures of whey

Table 2. Secondary structural analysis (%)¹ from the amide I region (cm^{-1}) of the Fourier transform infrared spectra of whey protein (in hydrated films) from processed milk samples

Sample ²	1,690 and 1,640–1,630 Antiparallel β -sheet	1,690–1,670 Turn	$\sim 1,660$ α -Helix	$\sim 1,650$ Unordered	1,620–1,610 β -Sheet
Raw whole	43	20	15	15	8
Pasteurized (HTST)	42	23	13	13	9
Homogenized	41	23	14	16	6
h-HTST	33	22	14	23	8
s-HTST	38	24	10	25	3
h-UHT	40	21	10	22	7
s-UHT	43	22	12	15	8
β -LG ³	45	22	12	13	8
α -LA ⁴	12	20	36	26	6

¹Apparent values and the assignments were made based on the publications by Barth (2007).

²h-HTST = homogenized and pasteurized; s-HTST = skimmed and pasteurized; h-UHT = homogenized UHT, and s-UHT = skimmed UHT.

³From our published data (Qi et al., 2014). β -LG = purified bovine β -LG.

⁴Studied and analyzed in the same fashion as other whey samples and used as a reference.

Figure 4. Fourier transform infrared spectra of whey protein from raw and homogenized UHT (h-UHT) milks in hydrated films. Each spectrum was deconvoluted using a Gaussian function and varying peak width.

protein, and as a result, increased the level of apparent unstructured elements, turn, and random coil. This damaging effect of UHT is somewhat lessened in the s-UHT whey.

Tertiary Structural Disruption in Whey from Processed Milk

The near-UV spectrum of a protein can be used to monitor the rigidity of the aromatic amino acids, Phe, Tyr, and Trp, and thus represents the tertiary structure of the protein (Kelly and Price, 2000). The CD spectra of the whey protein from variously processed milk in the near-UV region are shown in Figure 5. For native β -LG, it has been established (Townend et al., 1967) that the 293 nm peak belongs to Trp residues, the 285 nm to Tyr plus a small contribution by Trp, and the peaks below 270 nm to Phe. Because of the mixed nature of the whey protein, its near-UV CD spectrum reflected approximately a composite form of tertiary structural characteristics of β -LG and α -LA.

Figure 5. Near-UV circular dichroism spectra of various whey protein samples at room temperature (20°C) using a 1-cm path length cell. All protein concentration was adjusted to ~1.0 mg/mL by measuring the UV absorption at 278 nm. HTST = high-temperature, short-time pasteurized; H = homogenized; h-HTST = homogenized and pasteurized; s-HTST = skimmed and pasteurized; h-UHT = homogenized UHT, and s-UHT = skimmed UHT.

Two Trp, 4 Tyr, and 4 Phe are present in β -LG; and 4 Trp, 4 Tyr, and 4 Phe are present in α -LA (Swaigood, 1982), and their distinctive CD bands are as labeled in Figure 5. To examine at least qualitatively the effect of processing, especially homogenization, on the tertiary structural contact in whey, the near-UV CD spectra of all whey samples were compared based on the peak assignments for β -LG and α -LA. Homogenization alone or in combination of HTST does not appear to cause a significant effect on the soluble whey fraction. The

Figure 6. Intrinsic Trp fluorescence excitation and emission spectra of whey proteins in water at 20°C. All protein concentrations were kept at 0.37 mg/mL. HTST = high-temperature, short-time pasteurized; H = homogenized; h-HTST = homogenized and pasteurized; s-HTST = skimmed and pasteurized; h-UHT = homogenized UHT, and s-UHT = skimmed UHT.

UHT treatment, however, disrupted almost all tertiary structural features as their near-UV CD spectra almost lost all distinctive bands with s-UHT being the most severe case.

The intrinsic fluorescence of Trp residue primarily reflects its environment and can be used to further probe tertiary structural interruption in whey caused

by various milk processing treatments (Figure 6). It is known (Halder et al., 2012) that the major fluorescence contribution of β -LG comes from Trp19, buried within the hydrophobic β -barrel, whereas Trp61 is located on the surface of the protein, which does not contribute to the overall fluorescence of β -LG. For α -LA, on the other hand, the intrinsic fluorescence arises from all

Figure 7. Temperature dependence of far-UV circular dichroism spectra and the apparent secondary structural elements of whey proteins obtained by spectral deconvolution using the CDNN program. A) Whey from raw milk, B) α -helix, C) antiparallel β -sheet, D) parallel β -sheet, E) β -turn, and F) random coil. All spectra were corrected against solvent (water). Various lines were used to make it easier to visualize the data points. HTST = high-temperature, short-time pasteurized; H = homogenized; h-HTST = homogenized and pasteurized; s-HTST = skimmed and pasteurized; h-UHT = homogenized UHT, and s-UHT = skimmed UHT.

Table 3. Mid-point transition temperature ($^{\circ}\text{C}$) for whey from various processed milk as analyzed from Figure 8 using the 2-state denaturation model

Sample ¹	Maximum fluorescence intensity (Figure 8A)	Maximum fluorescence wavelength (Figure 8B)
Raw whole	81.9 ± 4.6	81.2 ± 5.0
Pasteurized (HTST)	69.7 ± 3.5	66.5 ± 5.6
Homogenized	68.3 ± 2.8	70.4 ± 3.6
h-HTST	69.1 ± 2.6	66.1 ± 2.3
s-HTST	65.2 ± 2.2	66.2 ± 2.2
h-UHT	60.7 ± 1.8	58.1 ± 2.8
s-UHT	63.1 ± 1.2	62.1 ± 1.8

¹h-HTST = homogenized and pasteurized; s-HTST = skimmed and pasteurized; h-UHT = homogenized UHT, and s-UHT = skimmed UHT.

4 Trp residues (numbers 26, 60, 104, and 118) in its native state (Vanhooren et al., 2006). Figure 6 shows that when protein concentration was kept the same (estimated spectroscopically), all processing treatments including homogenization followed by HTST and UHT had a diminishing effect on the intensity of Trp fluorescence relative to raw whey. Skimming before HTST and UHT appeared to alleviate the denaturation of soluble whey in terms of their tertiary structural content, as in the case of s-HTST and s-UHT. Keeping in mind the fact that the relative level of α -LA is higher in the h-UHT and s-UHT whey than in other samples, the fluorescence intensity (both dotted lines in Figure 6) appears to be higher than it should be.

Thermal Stability of the Secondary Structures of Whey from Processed Milk

To investigate the secondary structural stability of whey from the processed milk, we conducted far-UV CD experiments as a function of temperature. A deconvolution procedure was performed on each spectrum at each temperature using the software CDNN (Böhm and Jaenicke, 1992), and a database consisting of 33 reference proteins was used. Figure 7A shows the CD spectra of raw whey (as an example) at different temperatures. Figures 7B to F illustrate changes in the individual secondary structural element of whey as a function of temperature. When the temperature denaturation behavior of the whey is assumed to follow a 2-state model (Zwanzig, 1997), that is, only the native and denatured states are involved in the denaturation process (no chemical reactions): folded $\leftrightarrow$ unfolded, and the analyses yielded useful information on the structural stability in the form of mid-point transition (T_m ; Pace, 1990) for each secondary structural element. The resulting T_m values based on Figures 7B–F fell

Figure 8. Temperature dependence of intrinsic Trp fluorescence intensity of the whey protein samples. A) Normalized maximum emission intensity ($\lambda_{\text{ex}} = 295 \text{ nm}$) against the excitation at $\lambda_{\text{em}} = 340 \text{ nm}$, and B) maximum wavelength (nm). The insert in (A) shows changes in the Trp fluorescence emission spectra of raw whey as a function of temperature. HTST = high-temperature, short-time pasteurized; H = homogenized; h-HTST = homogenized and pasteurized; s-HTST = skimmed and pasteurized; h-UHT = homogenized UHT, and s-UHT = skimmed UHT.

in the range of 74 to 77°C and with standard deviations varying from 2 to 12°C , which would suggest any differences in T_m among all the whey samples cannot be accurately determined. Nevertheless, we believe this analysis procedure yielded similar transition temperature as previously determined by differential scanning calorimetry (Bernal and Jelen, 1985) for WPC at pH 6.5.

Thermal Stability of the Tertiary Structures of Whey from Processed Milk

To further understand the effect of milk processing on the tertiary contact and stability of whey, experiments were carried out on the Trp intrinsic fluorescence using temperature (10 – 90°C) as an environmental perturbation. The results are shown in Figures 8A and B. When a protein containing fluorescent Trp residues is denatured under environmental perturbation such

as temperature, the intensity of the Trp fluorescence decreases and the maximum of the peak undergoes red shift (as shown in the insert of Figure 8A). When the 2-state model was used to analyze the Trp intrinsic fluorescence intensity and maximum peak position (Figure 8) as a function of temperature, as discussed above for the analysis on secondary structural elements. The T_m for each whey sample of processed milk can be derived reasonably accurately. Table 3 gives the T_m values analyzed from Figure 8 using the 2-state model (Pace, 1990). The structural stability of various whey samples was thus expanded as the following: raw whole > HTST, homogenized, and h-HTST > s-HTST and s-UHT > h-UHT.

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