Use of Multi-Angle Laser Light Scattering and Size-Exclusion Chromatography to Characterize the Molecular Weight and Types of Aggregates Present in Commercial Whey Protein Products

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ABSTRACT

In this study, a range of commercial whey protein products were characterized by the use of size-exclusion chromatography coupled with a multi-angle laser light scattering (MALLS) detector. The MALLS system detected some very large-sized material that eluted close to void volume in all samples; this material was hardly detected by concentration detector. It was demonstrated by chitosan treatment that this peak was very small lipid globules or phospholipids, which gave the residual “cloudy” appearance in upper layers after ultracentrifugation of whey products. Composition, molecular weight, and the photo diode array (PDA) spectrum (200 to 400 nm) of the major protein peaks, including: β -lactoglobulin (BLG), α -lactalbumin (ALA), bovine serum albumin (BSA), immunoglobulin G (IgG) and some minor components were analyzed. The molecular weight of BLG, ALA, BSA, and IgG peaks in whey protein isolates (WPI) were 2.3 to 3.7×10^4 , 1.4 to 1.6×10^4 , 4.8 to 6.7×10^4 , and 1.2 to 2.5×10^5 Da, respectively. Compared with WPI, WPC has similar major proteins, but more large-sized residual lipid material and different minor constituents such as lactose and nonprotein nitrogen, depending on various commercial samples and protein content. An improved TCA-precipitation method was applied to quantify glycomacropeptide (GMP) in whey proteins, which demonstrated that there was a very low concentration of GMP in WPI manufactured using an ion-exchange process. The molecular weight of GMP was found to be ~ 8600 Da. Size-exclusion chromatography MALLS was demonstrated to be a powerful technique for detailed analysis of the molecular weight of various proteins, aggregates and

minor components, such as GMP, in whey protein products.

(**Key words:** whey protein, size-exclusion chromatography, multi-angle laser light scattering, molar mass)

Abbreviation key: ALA = α -lactalbumin, BLG = β -lactoglobulin, DRI = differential refractive index, GMP = glycomacropeptide, IE = ion exchange, LS = light scattering, MALLS = multi-angle laser light scattering, MF = microfiltration, MW = molecular weight, PDA = photo diode array, SEC = size-exclusion chromatography, UF = ultrafiltration, WPC = whey protein concentrate, WPI = whey protein isolate.

INTRODUCTION

Whey protein products are widely used food ingredients because of their excellent functional (e.g., gelling) and nutritional properties. Whey proteins are valuable byproducts from the cheese-making industry. The manufacture, properties, and uses of whey proteins have been reviewed (Kinsella and Whitehead, 1989; Bottomley et al., 1990; Morr and Ha, 1993; de Wit, 1998). Manufacturing processes and conditions for whey proteins can vary from producer to producer. Nowadays, the manufacturing technology for whey proteins can be classified into two major categories: membrane technology, which includes cross-flow microfiltration (MF) and ultrafiltration (UF), and ion-exchange chromatography (IE). The major proteins present in whey include: β -lactoglobulin (BLG; $\sim 50\%$, wt/wt), α -lactalbumin (ALA; $\sim 20\%$, wt/wt), immunoglobulins (e.g., IgG; $\sim 10\%$ wt/wt), and BSA, ($\sim 6\%$, wt/wt) (Walstra and Jenness, 1984). Two of the most important whey protein products are whey protein concentrates (WPC; 34 to 80%; wt/wt, protein) and whey protein isolates (WPI; $>90\%$; wt/wt, protein). The typical production process for WPC includes UF-diafiltration, (optional step: evaporation) and spray drying. Microfiltration is also used to reduce the fat content for the higher protein WPC products.

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Two different approaches are used to produce WPI, 1) MF, UF-diafiltration, and spray drying and 2) IE coupled with concentration (evaporation or UF) and spray drying. Sweet whey, after separation from cheese curd, is often clarified to remove any remaining fines and pasteurized, and most of the residual fat is removed by centrifugal separation (as whey cream). In the MF/UF process used for WPI production, virtually all the fat remaining in the whey is removed during the MF step because it is effectively retained by this membrane. It should be noted that all the soluble whey proteins are concentrated in the MF/UF process, whereas in the IE process some proteins, e.g., glycomacropeptide (**GMP**), lactoferrin and small peptide fragments are not (usually) recovered (Deshler, 1999). Thus, there are nutritional and possibly functional differences between these two types of WPI.

Differences in chemical composition have been found between batches of whey proteins from the same and different manufacturers (Holt et al., 1999a, 1999b). The source of whey (acid or sweet) and fat content of the original cheese milk can influence composition and functional properties of WPC (Patel et al., 1990; de La Fuente et al., 2002). Because of its high content of disulfide and the free thiol group on BLG, whey proteins can be easily denatured and aggregate or gel upon changing temperature, pH, or ionic strength (Shimada and Cheftel, 1989; de Wit, 1990; Schokker et al., 1999). Some differences have been reported in the size-exclusion chromatography (**SEC**) profiles obtained for different batches of WPC (Morr, 1985; Morr and Foegeding, 1990). Commercial WPC manufactured under similar conditions often have large variations in their physicochemical and functional properties (de Wit et al., 1988; Cayot and Lorient, 1997). Size-exclusion chromatography has been used extensively to study heat-induced aggregation of whey proteins (de Wit, 1990; Parris et al., 1993; Hollar et al., 1995). The functional properties of commercial whey protein products are influenced by processing conditions including heat treatment and mineral content (de Wit, 1990).

Size-exclusion chromatography is widely used to characterize the molecular weight of food materials. The approximate molecular weight of individual peaks can be determined using molecular weight standards. Light scattering (**LS**) has long been used as an absolute method to determine the molecular weight (**MW**), shape, and conformation of polymers (Wyatt, 1993). The use of multi-angle laser light scattering (**MALLS**) detector with a SEC system is a powerful new development in this field. In combination with concentration detectors such as UV and differential refractive index (**DRI**) this type of system can calculate the MW, z-average root mean square radius (*R_g*), and concentra-

tion of material eluting in small, individual slices of the entire SEC chromatogram. Size-exclusion chromatography MALLS has been used to characterize the MW and size distribution of protein particles in heated (pure) BLG solutions (Hoffman et al., 1997; Schokker et al., 2001) and in whey samples during the WPC manufacturing process (de La Fuente et al., 2002).

There do not appear to be any published articles on the weight-average molar mass and size distribution of molecules in whey protein products produced by different processes; this information could be useful in understanding the functional behavior of this important food ingredient. Holt et al. (1999a) used SEC-MALLS on whey products (one 76% protein WPC and two WPI samples produced using IE; however, no MW data were reported for the aggregates, only the composition of some of its major protein species. The aim of this study was to characterize the detailed MW and protein composition of a wide range of commercial whey protein products using SEC coupled with MALLS. With the growing interest in GMP and the differences that should occur in the GMP content in whey products produced by either IE or MF/UF techniques, we also wished to develop a rapid method to quantify GMP and its MW in various whey products.

MATERIALS AND METHODS

Materials

Seven commercial WPI samples and eight WPC samples were obtained from different dairy companies, which originated from United States, New Zealand, and Europe. An agglomerated or instantized WPI was also included in the study. Proximate analyses of these samples were supplied by the manufacturer and well as the principal technologies used in their manufacture (Table 1). IgG, BLG, ALA, BSA, GMP, orotic acid monohydrate, lactose standards, and high MW chitosan (~90% deacetylation) were purchased from Sigma (Sigma Chemical Co., St. Louis, MO). The commercial GMP product (BioPURE-GMP) was obtained from Daisco (Daisco Foods International Inc., Le Sueur, MN). All chemicals used were of analytical grade obtained from Sigma. Filters were obtained from Millipore (Millipore Corp., Bedford, MA).

Sample Preparation

Whey protein solutions (~4.0%, wt/wt, protein) were prepared in 20 mM imidazole, 50 mM NaCl, and 0.1% (wt/wt) NaN₃ at pH 7.0 that was prefiltered through a 0.025- μ m filter. Samples were dissolved for ~4 h at room temperature (~22°C). Whey protein solutions (~12 g) were ultracentrifuged at 100,000 $\times$ g for 90 min at 20°C

Table 1. Proximate chemical composition and processing technology used for the commercial samples of whey protein isolate (WPI) and whey protein concentrate (WPC) (as reported by manufacturers).

Samples	Chemical composition % (wt/wt)					Comments
	Protein	Fat	Lactose	Ash	Moisture	
WPI						
A	92.0	1.0	1.0	3.0	4.0	MF/UF
B	90.5	0.4	0.8	3.1	4.4	MF/UF
C	97.4	0.4	0.6	1.6	4.0	IEA/UF
D	97.7	0.3	0.5	2.0	4.9	IEA/UF
E	94.0	1.0	1.0	3.0	5.0	MF/UF
F	92.0	0.4	1.6	2.7	4.5	MF/UF
G	91.0	1.0	1.0	3.0	4.0	MF/UF (instantized)
WPC						
H	73.2	9.5	6.0	4.0	4.0	UF
I	34.0	4.5	48.5	8.0	5.0	UF
J	35.7	3.2	51.1	6.0	4.0	UF
K	36.4	3.4	56.0	1.6	2.6	UF
L	34.5	3.5	52.5	7.0	2.5	UF
M	80.0	7.0	5.5	3.0	4.5	UF
N	33.8	4.0	51.2	7.0	4.0	UF
O	80.5	7.0	4.5	3.0	5.0	UF

in a temperature-controlled centrifuge (L7 ultracentrifuge, Beckman, Fullerton, CA), and about 2 to 3 ml of the supernatant was collected carefully using a 10-ml syringe by puncturing through the wall of centrifuge tube. The supernatant was then filtered through 0.22- μ m filter for WPI or 0.1- μ m filter for WPC samples prior to injection into the SEC columns. Ultracentrifugation could be omitted, but the intensity of light scattering signal from the first peak that eluted close to the void volume required the use of a very dilute solution (i.e., <1%, wt/wt, protein).

Chitosan Clarification

Chitosan was used to remove residual lipids in whey (Hwang and Damodaran, 1995). One percent chitosan solution (in 10%, vol/vol, acetic acid) was added to whey protein solution to a final concentration of 0.01% (vol/vol). The pH of this solution was adjusted to 4.5 by the addition of 1 M HCl. The mixture was incubated for 30 min at room temperature (~22°C) and centrifuged at 1200 $\times$ g for 5 min (CR3i, Jouan Inc., Winchester, VA) at 22°C. The supernatant was collected, and the pH of solution was readjusted to 7.0 by the addition of 1 M NaOH. The whey solution was then ultracentrifuged and collected as described previously.

Size-Exclusion Chromatography with Multi-Angle Laser Light Scattering

Separation of whey protein supernatant solution was carried out by SEC using Superose 6HR 10/30 and Superose 12HR 10/30 columns (Amersham Pharmacia Biotech AB, Uppsala, Sweden) connected in series and

attached to a Waters HPLC system (Waters Corp., Milford, MA) operating at room temperature (~22°C). A solution containing 20 mM imidazole, 50 mM NaCl and 0.1% (wt/wt) NaN₃ at pH 7.0 (filtered through 0.025 μ m) was used as an eluent buffer. All samples were filtered before injection. Sample injection volume was 50 μ l, nominal flow rate was 0.4 ml/min and separation was performed at room temperature (~22°C). Spectrum analysis was conducted by a photo diode array (PDA) detector (Waters Corp., Milford, MA, model 996) operating over the range 200 to 400 nm, with a scanning rate of 1.0/s and a resolution of 1.2 nm. A DAWN-EOS MALLS photometer (Wyatt Technology, Santa Barbara, CA) fitted with a helium-neon laser (λ = 690 nm) and a K5-flow cell, combined with a differential refractive index (DRI) detector (Waters Corp; model 2410) was used for calculation of MW of different whey proteins. Light scattering data from MALLS were processed with ASTRA (version 4.73 04) and HPLC data including RI, UV (280 nm), and PDA spectrum were processed by Waters Millennium version 3.20.

Determination of the Amount of GMP in WPI

Three milliliters of each WPI sample (10%, wt/vol) were prepared in 15-ml centrifuge tubes and vortexed at full speed for 20 s and allowed to stand for 30 min before vortexing again for another 20 s. An equal volume of 14% (wt/wt, 14 g of TCA + 86 g of water) TCA solution was added to the protein solution and the mixture was vortexed for another 10 s at full speed. The TCA-protein mixture was allowed to stand overnight (~14 h) at room temperature (~22°C) before centrifugation at 3000 $\times$ g at 6°C (CR3i, Jouan Inc., Winchester,

VA). The supernatant was collected and filtered through 0.2 μm before injection into the SEC-MALLS system. A phosphate buffer (pH 5.6) was prepared according to Lieske and Konrad (1996), filtered through 0.025 μm and was used as eluent for SEC-MALLS.

A standard curve of GMP was made by injecting a series of GMP (obtained from Sigma Chemical Co.) solutions of known concentrations. The DRI detector was used as concentration detector since the MW of GMP could be calculated simultaneously as this peak eluted from the SEC columns.

Recovery rate of GMP by this TCA procedure was estimated by precipitating relatively pure GMP (BioPURE-GMP) prepared with a similar concentration as the GMP concentration present in WPI samples. The purity of BioPURE-GMP was first estimated by injecting unprecipitated GMP (dissolved in water) into the SEC-MALLS system and the purity of the mass injected was obtained from the standard curve. Then, BioPURE-GMP was precipitated by the same TCA procedure described previously. Finally, the recovery rate of GMP was calculated by determining the concentration of GMP in the TCA-treated sample. A typical specific refractive index increment (dn/dc) value of protein (~ 0.185 ml/g) was used to estimate the MW of GMP.

Data Treatment

The DAWN-EOS MALLS detector simultaneously provides up to 16 LS chromatograms, each at a different scattering angle, for a polymer solute as it emerges from the SEC column (Wyatt, 1993). Additional chromatographs from a concentration detector have to be used for the determination of MW. By dividing the LS signals with the concentration signals obtained from either the DRI or UV detectors, which used a dn/dc value or extinction coefficient (ϵ), respectively, and assuming a second virial coefficient (A_2) of zero (Wen et al., 1996), the MW and R_g of polymer solute eluting in each angle were calculated with a first-order Debye fit (higher order polynomials were also tried but gave a poorer fit to the data). Extrapolating this fitted curve to Y-intercept provides MW and the slope of this curve at low angles gives R_g . With the ASTRA software it was also possible to realign (overlay) the SEC profiles from the different detectors caused by volume delays between units such as the UV and DRI detectors. The coefficient of variation between replicate MW measurements was typically <3%; within a single run the ASTRA software calculated averages and standard deviations of the MW and R_g for each peak.

The calibration of LS detectors was performed by measuring the light scattering intensity of filtered HPLC quality toluene, which has a known Rayleigh

Figure 1. Multi-angle laser light scattering (MALLS) 90° detector responses during size-exclusion chromatography (SEC) of a 4% commercial whey protein isolates sample A (•••) and its supernatant after ultracentrifugation (—). Numbers indicate peaks.

ratio at 90° angle. The responses of LS intensity of the photodiodes arrayed around the scattering cell were normalized to the diode at 90° with an isotropic BSA sample (monomeric BSA with a nominal molecular weight of 66 kDa). The dn/dc value is a constant for whey proteins, which is ~ 0.185 ml/g (Huglin, 1972) and ϵ values are 661, 960, and 2030 cm^2/g for BSA, BLG and ALA, respectively (Pace et al., 1995).

Statistical Analysis

Calculated MW data from SEC-MALLS were analyzed using the general linear models (GLM) of SAS program, and least significant differences ($P < 0.05$) between samples were determined using Scheffe test (SAS, 1999). Each sample was analyzed at least three times.

RESULTS AND DISCUSSION

Typical SEC-MALLS (responses from the 90° LS detector) profiles for a 4% (wt/wt) WPI and WPC solution are shown in Figures 1 and 2, respectively. During SEC the MALLS system clearly detected large aggregated material that eluted close to the void volume (36 to 38 min, peak 1) in both WPI (Figure 1) and WPC (Figure 2) samples. The size of this peak varied with different samples, but it was much larger in WPC samples (probably due to their higher fat content). A similar peak was observed by Lucey et al. (2000) and de La Fuente et al. (2002) in sodium caseinate and whey samples, respectively. In ultracentrifuged samples, the size of peak 1, which is probably due to some residual lipid

Figure 2. Multi-angle laser light scattering (MALLS) 90° detector responses during size-exclusion chromatography (SEC) of a 4% commercial whey protein concentrate (WPC) sample I subnatant after ultracentrifugation (•••) and its chitosan-clarified counterpart (—). WPC samples prior to MALLS-SEC without ultracentrifugation were not tested (due to their high residual lipids content). Numbers indicate peaks.

material, was greatly reduced. All subsequent SEC-MALLS experiments therefore were performed on ultracentrifuged samples. Since residual lipids decrease the foaming properties of whey proteins (Morr and Ha, 1993), methods such as precipitation using chitosan have been developed for the selective removal of these lipids (Hwang and Damodaran, 1995), which we also performed here and the results are shown in Figure 2. Peak 1 nearly disappeared completely after chitosan treatment, which indicates that materials in this peak were residual lipids that were mainly negatively charged, e.g., phospholipids. These lipids formed a complex with chitosan at pH 4.5, where the lipids were negatively charged and chitosan was positively charged.

The SEC-MALLS profiles for all the WPI samples are shown in Figure 3. The size of the first peak varied in the WPI samples, presumably reflecting differences in how efficiently residual lipids were removed during commercial processing (e.g., clarification, MF). The SEC-UV-DRI profiles for 4% (wt/wt) WPI solutions are shown in Figure 4. There did not appear to be any very large whey protein aggregates in any of the WPI samples, which suggests that relatively mild processing conditions (e.g., heat treatment) and extra purifications were used in their manufacture (or that any large aggregates were removed by the ultracentrifugation/filtration steps). Immunoglobulin G, BSA, BLG, and ALA were identified using their HPLC standards as peaks 2, 3, 4, and 5, respectively. Because all whey protein

Figure 3. Size-exclusion chromatography, multi-angle laser light scattering (SEC-MALLS) elution profiles for different 4% commercial whey protein isolate samples (A to G) for the LS response at 90°. All samples were ultracentrifuged. Numbers indicate peaks.

solutions were prepared based on same total mass concentration, the height of major protein peaks: BLG (peak 4) or ALA (peak 5) either from UV or DRI, represents sample concentration. The peak heights of BLG in WPI (>90%, wt/wt, protein) and high protein (~80%, wt/wt, protein) WPC were between three to two times as high as those in low protein (~34%, wt/wt, protein) WPC, respectively. These results agree with their corresponding protein content reported by the manufacturers. The peak (from DRI detector) height ratio of BLG to ALA, which indicates the concentration distribution of BLG and ALA, was in the range of 2.21 to 2.97. The average concentration ratio of BLG to ALA was 2.61, which is similar to the ratio of BLG to ALA in milk (Walstra and Jenness, 1984). The BLG and ALA contents of WPI samples ranged from 48 to 73% and 15 to 21% (of total area in DRI chromatographs), respectively. These results are similar to those previously reported by Morr and Foegeding (1990). Higher BLG and ALA contents were found in WPI samples produced using IE due to the very low GMP content.

Because it is possible that there was some adsorption of whey protein by the SEC columns (Holt et al., 1999a; 1999b), IgG, BSA, BLG, and ALA standards were not

Figure 4. Concentration detectors, refractive index and UV responses for the different whey protein isolate (4%, wt/wt) subnatants (samples A to G) after ultracentrifugation. The UV (280 nm) response is the upper curve in each graph. Numbers indicate peaks.

used for quantification of the absolute concentration of these proteins in our samples. Instead, the relative content of the individual whey proteins in the WPI samples was estimated from the area under the SEC-DRI profile (Table 2). Due to the poor separation between IgG, lactoferrin, and BSA, their contents were calculated together.

The SEC-UV-DRI profiles for 4% (wt/wt) WPC solutions are shown in Figure 5. In all WPC samples peak 1 (detected by UV at 280 nm), i.e., mostly residual lipid fraction (e.g., phospholipids), was largely due to the higher fat content in WPC compared to WPI. Besides the major proteins in the whey: IgG (peak 2), BSA (peak 3), BLG (peak 4), and ALA (peak 5), a number of minor components, unknown (peak 6), lactose (peak 7), orotic acid (peak 8), and unknown (peak 9) were present in the low protein content WPC (~34%, wt/wt, protein). Similar results were reported for fresh Cheddar cheese whey (Law et al., 1993). These minor components were either absent or only present at very low concentrations in the high protein content WPC (~80%, wt/wt, protein) and WPI (>90%, wt/wt, protein). Presumably, they were removed due to the extra purification procedures involved in the manufacturing of higher protein content WPC or WPI. The peak (from DRI detector) height ratio of BLG to ALA was in the range of 1.71 to 2.84. The average concentration ratio of BLG to ALA was 2.16. A relatively high ALA content was observed in sample H. No large MW aggregates were found; similar trends were reported for whey samples taken during the WPC manufacturing process (de La Fuente et al., 2002).

The PDA spectrum analysis of ~34% (wt/wt) protein WPC components in peaks 1, 2, 3, 4, 5, 6, 8, and 9 are shown in Figure 6. The spectrum analysis of peak 1 clearly indicates that it had a unique profile compared to the other peaks with one maximum absorbance at 237 nm, which is similar to the smaller maximum peak observed in the usual whey protein spectrum. The typical whey proteins, such as IgG (peak 2), BSA (peak 3), BLG (peak 4), ALA (peak 5), and unknown (peak 6), NPN: orotic acid (peak 8), have two maxima at 234 to 237 nm and 278 to 281 nm. Their overall spectra were also very similar. Unknown peak 9 has MW <174 (larger retention time than orotic acid, MW = 174 Da), one maximum at 291 nm and different spectrum profile from other proteins, which may suggest that it was another NPN. Glycomacropeptide had the same retention time as BLG, but totally different spectrum profile in pH 7.0 buffer (results not shown).

The chromatographic profiles from the DRI detector overlaid with the calculated MW of WPI and WPC samples are shown in Figure 7. The peaks were identified according to elution time of their corresponding standards. The summary of calculated MW from different

Table 2. Relative major protein concentrations and molecular weights (MW) of commercial whey protein isolate (WPI) samples (using DRI).¹

Samples	Protein component of WPI									
	Immunoglobulin G (IgG) + lactoferrin + bovine serum albumin (BSA)			β -Lactoglobulin (BLG)		α -Lactalbumin (ALA)		Glycomacropeptide (GMP)		
	Rel.% ² (wt/wt)	IgG (MW $\times 10^5$ Da)	BSA (MW $\times 10^4$ Da)	Rel.% ^{2,3} (wt/wt)	MW $\times 10^4$ Da	Rel.% ² (wt/wt)	MW $\times 10^4$ Da	Rel.% ² (wt/wt)	MW Da	
A	4.96 $\pm$ 0.42 ^a	1.42 $\pm$ 0.15 ^a	5.53 $\pm$ 0.24 ^a	55.94 $\pm$ 0.32 ^a	2.33 $\pm$ 0.03 ^a	16.71 $\pm$ 0.35 ^a	1.41 $\pm$ 0.0 ^{ab}	22.37 $\pm$ 1.07 ^a	8796 $\pm$ 245 ^a	
B	6.32 $\pm$ 0.15 ^b	1.27 $\pm$ 0.01 ^a	5.58 $\pm$ 0.44 ^{ab}	49.37 $\pm$ 0.52 ^b	2.27 $\pm$ 0.03 ^a	17.92 $\pm$ 0.42 ^{bf}	1.52 $\pm$ 0.05 ^{ab}	26.38 $\pm$ 0.4 ^b	8667 $\pm$ 300 ^a	
C	14.89 $\pm$ 0.11 ^c	1.33 $\pm$ 0.03 ^a	6.53 $\pm$ 0.13 ^b	68.25 $\pm$ 0.02 ^c	2.99 $\pm$ 0.14 ^b	15.58 $\pm$ 0.12 ^c	1.57 $\pm$ 0.04 ^b	1.36 $\pm$ 0.014 ^c	—	
D	6.17 $\pm$ 0.09 ^b	1.26 $\pm$ 0.10 ^a	6.67 $\pm$ 0.10 ^b	73.4 $\pm$ 0.23 ^d	3.69 $\pm$ 0.44 ^c	20.06 $\pm$ 0.05 ^{de}	1.50 $\pm$ 0.01 ^{ab}	0.25 $\pm$ 0.0 ^d	—	
E	4.46 $\pm$ 0.29 ^{ab}	1.26 $\pm$ 0.04 ^a	4.83 $\pm$ 0.10 ^a	51.46 $\pm$ 0.18 ^b	2.27 $\pm$ 0.01 ^a	19.73 $\pm$ 0.12 ^{def}	1.46 $\pm$ 0.01 ^{ab}	24.33 $\pm$ 0.88 ^e	8529 $\pm$ 119 ^a	
F	4.28 $\pm$ 0.49 ^b	1.70 $\pm$ 0.05 ^b	5.52 $\pm$ 0.22 ^a	48.83 $\pm$ 0.89 ^b	2.27 $\pm$ 0.05 ^a	20.78 $\pm$ 0.54 ^{de}	1.60 $\pm$ 0.07 ^b	24.45 $\pm$ 0.44 ^e	8351 $\pm$ 130 ^a	
G	5.79 $\pm$ 0.03 ^b	2.55 $\pm$ 0.06 ^c	6.67 $\pm$ 0.14 ^b	54.09 $\pm$ 0.02 ^a	2.30 $\pm$ 0.01 ^a	18.97 $\pm$ 0.30 ^{bef}	1.63 $\pm$ 0.02 ^b	20.99 $\pm$ 0.17 ^a	8614 $\pm$ 229 ^a	

¹Values are means $\pm$ standard deviations for n = 3.

²Relative percentage of total area (total protein) under DRI chromatograph.

^{a,b,c,d,e,f} Values in a column with different superscripts are significantly different ($P < 0.05$).

Figure 5. Concentration detectors, refractive index, and UV responses for the different whey protein concentrate (4% wt/wt) subnatants (samples H to O) after ultracentrifugation. The UV (280 nm) response is the upper curve in each graph. Numbers indicate peaks.

Figure 6. Photo diode array spectrum (200–400 nm) profiles of the main peaks obtained for a typical whey protein concentrate sample (34% protein, sample I) subnanant after ultracentrifugation and separated by size-exclusion chromatography (SEC). The number (e.g., 237 nm) for each peak represents the wavelength for maximum absorbance detected for an individual peak after SEC.

WPI samples is listed in Table 2. Statistical analysis of the relative peak area and MW of proteins indicate some significant differences among these WPI samples ($P < 0.05$). There were some problems in the estimation of the MW of IgG, which had the closest elution time to peak 1. The higher the concentration of lipid residual, the higher the LS signal peak carryover effect was, which resulted in a larger estimated MW for IgG. The MW of IgG was 126 to 171 kDa, and it varied compared to the commercial SEC standard IgG of 158 kDa (Law et al., 1993). One apparently large MW of IgG (255 kDa) from sample G (instantized WPI) may have been caused by the very large carryover from additional lipid or lecithin of the LS signal from peak 1.

No obvious very high molecular protein aggregates were observed, which is similar to results reported previously (Holt et al., 1999a, 1999b). The calculated MW of BSA (48 to 66 kDa) was slightly smaller than the standard monomer BSA (66 kDa). The MW of BLG in different WPI samples ranged from 22 to 37 kDa, which was between the MW of its monomer (18 kDa) and dimer (36 kDa). This may suggest that there is an equilibrium between BLG dimer and its monomer at pH 7 (Schokker et al., 1999). The calculated MW of ALA ranged from 14 to 16 kDa, which was slightly higher than its standard (14 kDa).

A comparison of MW of major proteins from WPI samples calculated using DRI or UV is given in Table 3. Because of the higher sensitivity of UV detector (~100 times higher) than DRI, the MW calculated by UV tended to be more consistent and accurate. The overall MW of BLG and ALA calculated from WPC samples was 28 to 37 kDa and 25 to 29 kDa, respectively, which are not listed here because the relative large carry over of LS signal from lipid residuals (peak 1), which significantly affected the estimation of their MW.

Standard curve of GMP shows a good linear relationship between the peak area and the amount of GMP injected. The result is also reflected by the following equation:

$$DRI(area) = 1.622 \times 10^7 x(mg) \quad r^2 = 0.9999$$

where, *DRI (area)* is the DRI detector response and *x* is the mass of GMP injected with the maximum load of 1.4 mg.

The purity of BioPURE-GMP estimated using this standard curve was ~91%, which was slightly greater than the minimal 90% purity reported by the manufacturer. The recovery rate of GMP investigated using our TCA precipitation method shows a consistent rate of $44 \pm 3\%$ based on an initial GMP concentration of ~13

Figure 7. Size-exclusion chromatography-refractive index (SEC-RI) elution profile of whey protein isolate (a) and SEC-RI-UV (280 nm) whey protein concentrate (b) obtained for a 4% solution that had been ultracentrifuged. The elution profile is overlaid with the calculated molar mass (■) and multi-angle laser light scattering (MALLS) at 90° angle (●●●). The peaks 2, 3, 4, 5, 6, 7, 8, and 9 are IgG, BSA, β -lactoglobulin, α -lactalbumin, unknown, lactose, orotic acid, and unknown NPN, respectively.

mg/ml. It is also found in our experiments that GMP recovery rate varies according to the initial GMP concentration in the solution, e.g., when the GMP concentration was ~2.2 mg/ml, the recovery rate was ~75%.

The recovery rate of GMP was lower compared to the method proposed by Lieske and Konard (1996). This may have been caused by differences in the precipitation step as they analyzed immediately after their sample was mixed and filtered. In our experiment, the TCA-GMP mixture was allowed to stand overnight before centrifugation and filtration, which allowed enough time to avoid the occurrence of any additional precipitate after filtration.

The MW of GMP was also investigated. All WPI samples as well as the BioPURE-GMP had a similar MW of ~8600, which is close to the theoretical MW of 7000 to 8000 Da (Abd El-Salam et al., 1996), although the GMP has an unusual short retention time (close to BLG). This may be due to a large hydrodynamic volume arising from the hydrophilic groups, such as sialic acid, on GMP (Minkiewicz et al., 1996). The determination of MW from our SEC-MALLS technique is not dependent on retention time or hydrodynamic volume but rather the fundamental LS properties of the polymer. The calculated GMP concentrations as well as the corresponding MW are also listed in Table 2. The SEC-MALLS profiles of TCA treated WPI samples are shown in Figure 8, and the absence of any contamination by BLG in our GMP peak was confirmed from the absence of any absorbance peak at 280 nm. Samples C and D show very low GMP concentration and differ from others, which agree with its manufacturing condition, i.e., IE process. In WPI samples manufactured using membrane processing, all proteins including GMP were present.

CONCLUSIONS

SEC combined with PDA, MALLS, and DRI detectors is a powerful tool for studying the state of aggregation of commercial whey protein products. It fractionates the protein mixture according to its molecular size and

Table 3. Molecular weight (MW) comparison of major proteins in commercial whey protein isolate samples calculated from differential refractive index (DRI) and UV detectors.¹

Samples	MW from DRI ($\times 10^4$ Da)			MW from UV ($\times 10^4$ Da)		
	BSA	BLG ²	ALA ³	BSA	BLG	ALA
A	5.53 $\pm$ 0.24 ^a	2.33 $\pm$ 0.03 ^a	1.41 $\pm$ 0.0 ^{ab}	5.38 $\pm$ 0.07 ^{ab}	3.18 $\pm$ 0.06 ^a	1.50 $\pm$ 0.07 ^a
B	5.58 $\pm$ 0.44 ^{ab}	2.27 $\pm$ 0.03 ^a	1.52 $\pm$ 0.05 ^{ab}	4.73 $\pm$ 0.05 ^{ab}	3.22 $\pm$ 0.07 ^a	1.56 $\pm$ 0.05 ^a
C	6.53 $\pm$ 0.13 ^b	2.99 $\pm$ 0.14 ^b	1.57 $\pm$ 0.04 ^b	4.57 $\pm$ 0.20 ^{ab}	3.12 $\pm$ 0.09 ^a	1.55 $\pm$ 0.04 ^a
D	6.67 $\pm$ 0.10 ^b	3.69 $\pm$ 0.44 ^c	1.50 $\pm$ 0.01 ^{ab}	5.27 $\pm$ 0.22 ^{ab}	3.23 $\pm$ 0.08 ^a	1.49 $\pm$ 0.05 ^a
E	4.83 $\pm$ 0.10 ^a	2.27 $\pm$ 0.01 ^a	1.46 $\pm$ 0.01 ^{ab}	4.31 $\pm$ 0.11 ^b	3.17 $\pm$ 0.05 ^a	1.50 $\pm$ 0.02 ^a
F	5.52 $\pm$ 0.22 ^a	2.27 $\pm$ 0.05 ^a	1.60 $\pm$ 0.07 ^b	4.52 $\pm$ 0.64 ^{ab}	3.36 $\pm$ 0.06 ^a	1.60 $\pm$ 0.06 ^a
G	6.67 $\pm$ 0.14 ^b	2.30 $\pm$ 0.01 ^a	1.63 $\pm$ 0.02 ^b	6.26 $\pm$ 0.13 ^a	3.28 $\pm$ 0.03 ^a	1.67 $\pm$ 0.06 ^a

^{a,b,c,d,e,f} Values in a column with different superscripts are significantly different ($P < 0.05$).

¹Values are means $\pm$ standard deviations for $n = 3$.

²BLG = β -lactoglobulin.

³ALA = α -lactalbumin.

Figure 8. Size exclusion chromatography, multi-angle laser light scattering (SEC-MALLS) elution profiles for TCA-treated whey protein isolate samples (A to G) for the least squares response at 90°. Peaks indicate glycomacropeptide.

determines the MW and performs a spectrum analysis of the material eluting in small, individual slices of the SEC chromatogram. Our results indicated that there was always some residual lipid material present in commercial whey proteins samples. The concentration of this residual material was greatly reduced by ultracentrifugation and eliminated by using chitosan to form lipid-chitosan complexes. For different WPI samples, there were some significant differences between samples for the MW of BSA, BLG and ALA, depending on the concentration detector used. Glycomacropeptide was present at a very low concentration of WPI obtained by IE and a method to quantify GMP in whey protein products using SEC-MALLS was developed. For WPC samples, there were differences in the concentration of residual lipids (presumably reflecting differences in the efficiency of fat removal during processing). A greater concentration of minor components were found in the

low protein content WPC. In general, higher concentrations of residual lipids in WPC samples made it more difficult to estimate MW of some of the individual whey proteins. The MW calculated by the UV concentration detector tended to be more consistent than DRI. Differences in functionality of commercial WPI and WPC samples could be due to the changes in protein compositions, the concentration of residual lipids and possibly mineral contents.

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