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A simple and direct isolation of whey components from raw milk by gel filtration chromatography and structural characterization by Fourier transform Raman spectroscopy

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Abstract

A simple and economical method to isolate whey protein from fresh raw milk is developed by serial defatting, casein eliminating, lactose removing, and separating by gel filtration chromatography. Four major whey components, including immunoglobulin (Ig), bovine serum albumin (BSA), β-lactoglobulin (β-Lg) and α-lactalbumin (α-Lac), and a non-protein of low molecular mass (∼1.7 kDa) but strong absorbance at 280 nm, are detected simultaneously. The small non-protein molecule is rich in aromatic amino acids and thiol groups as supported by the structural characterization with near infrared Fourier transform Raman spectroscopy (FT-Raman). FT-Raman results show that the secondary structure of Ig is dominated by anti-parallel β-pleated sheet; BSA is mainly in α-helix; both β-form and unordered structure are important in β-Lg; while α-Lac is mostly in α-helix coupling with random coil. Differences in the Raman profile for each whey component reflect their intrinsic compositional differences and distinct spatial arrangement. The S–S linkages diverging around 510–540 cm^{-1} indicate that the conformation of disulfide bonds in each whey components is different, which may be responsible for their diversified behaviors in solubility, rheological and functional properties.

Keywords: Whey protein; Raw milk; Gel filtration chromatography; FT-Raman; Secondary structure; Disulfide bonds

1. Introduction

Whey protein represents approximately 20% of the original milk proteins. It exhibits many important functional properties in the manufacture of food. The nutritional values and physiological benefits of whey protein also receive much attention [1–8]. Although whey proteins have versatile functional properties and nutritional values, an estimated 70% of liquid whey is disposed as waste product. Such large volumes of disposed protein will seriously impact environmental pollution due to its high BOD (biological oxygen demand) level. The utilization of whey proteins is confined because there still exists a large variability in the composition and functional properties of commercial whey proteins [9–12]. To date, the factors responsible for the variability remain poorly understood. It is possible that the incongruence of physico-chemical and functional performance of whey protein reported from different groups might result from the compositional variations arising from different whey resources or processing conditions in commercial whey products. To clarify this problem, how to obtain whey protein in its native form, i.e., free from any denaturizing, becomes of utmost importance. Many processes such as ultrafiltration, diafiltration, polyphosphate complex precipitation, heat coagulation, and ion-exchange adsorption technology have been developed for the manufacture of whey protein [13,14]. By these methods, whey protein is prone to the risk of protein denaturation. For protein separation, a number of liquid chromatography methods including reversed-phase [15–18], hydrophobic interaction [19], and ion exchange [20–23] techniques have been employed. For the ion-exchange method, a large amount of mobile phase is usually prepared with various salts at different concentrations or at different pH to wash out the compound of interest, and for reverse-phase HPLC, some organic solvents or an acidic mobile phase have to be applied, all of which potentially denature proteins. In contrast, size exclusion chromatography is relatively amiable to protein molecules and has been frequently used in protein separation [24–27]. Yoshida used a Sephacryl S-200 column to isolated β-Lg and α-La from acid whey protein [24];

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Nakai and Al-Mashikhi isolated whey proteins using Sephacryl S-300 and TSK HW-55 columns [25]. No single method is suitable for all whey proteins and the method of choice is usually based on one or two whey components of interest [17]. Moreover, most previous whey-related studies are sampling from commercial products such as whey concentrates or acid whey proteins, of which properties are closely dependent on the manufacture processes. Little has been reported on fresh raw milk, to the best of our knowledge. Hence, we expect to develop a simple isolation process of whey proteins directly from fresh raw milk.

FT-Raman spectroscopy is a powerful technique for the structural investigation of protein molecules. Compared to other techniques for protein characterization such as differential scanning calorimetry (DSC) and polarizing microscopy, viscosity and turbidity measurements, electron microscopy and chemical modifications, FT-Raman spectroscopy provides direct, non-invasive information to the protein structure in either solid, film or aqueous form. One important factor distinguishing Raman spectroscopy from many other spectroscopic methods is its applicability to in situ systems containing high concentration of proteins, which is critical for the investigation of structural changes during processes such as foaming, emulsifying, and gelling [28]. Bands in the Raman spectrum arising from amide I, amide III, and skeletal stretching modes of peptides and proteins are useful for characterizing backbone conformation [29–32]. Valuable information may also be obtained on SS/SH conversion, CH groups of aliphatic residues, and aromatic rings of amino acid residues [33–36]. Thus, changes in chemical structure and microenvironment of protein side-chains through either intramolecular or intermolecular interactions can be easily probed.

In this work, whey protein was first isolated directly from fresh milk through a simple and economical process including defatting, decaseinating, lactose removing and separating by preparative gel filtration chromatography. It shows that four major components (immunoglobulin, bovine serum albumin, β-lactoglobulin, and α-lactalbumin) and a small non-protein molecule can be easily collected. The molecular structure of these whey components is then characterized by FT-Raman. With its unique spectroscopic advantages, structural information on the backbone conformation of whey components and the microenvironment of important amino acids is elucidated.

2. Experimental

Ammonium sulfate, KCl, NaCl, $(NH_4)_2SO_4$, NaN_3, HCl, sodium phosphate, dibasic, 12-hydrate, and sodium sulfate were from Sigma Chem. Co. (St. Louis, MO). Protein standards of α-lactoalbumin (α-Lac), β-lactoglobulin (β-Lg), bovine serum albumin (BSA), immunoglobulin (IgG) were purchased from Bio-Rad Lab. (Richmond, CA). Electrophoresis grade acrylamide, *N*,*N*,*N*,*N*-tetramethyl ethylenediamine (TEMED), Trizma base, *N*,*N*-methylenebisacrylamide, tris-aminomethane, ammonium persulfate, 2-mecaptoethanol, bromophenol blue, coomassie brilliant blue G-250, sodium dodecylsulfate (SDS), and protein markers were purchased from Bio-Rad Lab. (Richmond, CA). All the chemicals for chromatography were analytical grade, and the rest except for electrophoresis were reagent grade. All solutions were prepared with ultra pure water, from an EASYpure RF water system (Barnstead).

2.1. Isolation of whey protein from raw milk

Bovine milk was collected from the Experimental Farm of the National Chiayi University. Raw milk after milking was directly refrigerated (at −18 °C) overnight; the fat floated spontaneously and then froze on that top layer so that it could be easily removed. After removing the frozen fat, the lower portion (mostly lactose and protein compounds) was thawed under cool conditions (4 °C) and then centrifuged (4 °C, 15,000 rpm) for 30 min to isolate protein from other residues. Protein solution was then treated with diluted HCl solution at pH 4.6, the isoelectric point of casein, from which it would be precipitated out. After centrifugation, the pale-greenish transparent solution (whey protein) was subjected to separation by loading on an open column (2.6 cm × 70 cm) filled with Sephadex G-200 (Pharmacia, USA) medium, and the elution was collected by a Gilson FC-205 Fraction Collector (Gilson, France). The flow rate was controlled at 0.8 ml/min, and the volume collected for each tube was 3 ml/tube. The elution buffer was prepared with Tris–HCl at pH 7.2. Each component was collected, dialyzed, and lyophilized for further study.

2.2. Molecular mass determination

The molecular weight of each whey protein component was determined by gel filtration chromatography using a Sephadex G-200 column (2.6 cm × 70 cm) with molecular mass markers (Pharmacia). The molecular mass of each whey component was determined by the simple linear regression, correlating elution volume with molecular weight, on the calibration curve. Both high and low molecular weight calibration curves were quickly constructed by measuring the elution volumes of standard compounds, calculating K_{av} value for each, and plotting K_{av} value versus the logarithm of each standard molecular mass. The standard compounds include ribonuclease A (13.7 kDa), chymotrypsinogen A (25.0 kDa), ovalbumin (43.0 kDa), bovine serum albumin (67.0 kDa), aldolase (158 kDa), catalase (232 kDa), ferritin (440 kDa), thyroglobulin (669 kDa), and blue dextran 2000 (2000 kDa):

$$K_{av} = (V_e - V_0)/(V_t - V_0)$$

where, V_e is the elution volume of protein, V_0 the column void volume, V_t is the total bed volume.

2.3. SDS-PAGE analysis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed in a vertical mini-gel system (Mini-Protean II Dual Slab Cell, Bio-Rad Lab., Richmond, CA) as described by Laemmli [37] with some modifications. polyacrylamide gels (5% stacking and 14% resolving gel) were prepared by co-polymerization of acrylamide and

bis-acrylamide with the aid of initiator TEMED and ammonium persulfate. Buffer solutions for the stacking and resolving gels were prepared from 0.125 M Tris–HCl (pH 6.8) and 0.375 M Tris–HCl (pH 8.9), respectively, incorporating 0.1% SDS. The running buffer (pH 8.6) consisted of 0.1% SDS, 0.1% 2-mercaptoethanol, 0.19 M glycine, 0.025 M Tris–HCl, and 1 mM ethylenediamineteraacetic acid (EDTA). The sample buffer was composed of 0.1% SDS buffer, 10% sucrose, 0.05% bromophenol blue and 20 mM dithiothreitol. The samples (10 μl) were well-mixed with the buffer solution by microcentrifuge at 1200 rpm (Hettich GmbH & Co., Tuttlingen, Germany) and heated at 100 °C in Dry-Bath (Dubuque, IA, USA) for 3 min prior to being loaded to the gels. Electrophoresis was carried out with a fixed voltage of 180 V for 55 min. After electrophoresis, the gel was stained with 0.25% coomassie brilliant blue solution containing 12.5% trichloroacetic acid, 20% methanol, and 7.0% acetic acid for 20 min and destained with a solution of 20% methanol and 7.0% acetic acid overnight. Protein markers for reduced SDS-PAGE were Myosin (200 kDa) phosphorylase b (97.4 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45.0 kDa), carbonic anhydrase (31.0 kDa), trypsin inhibitor (21.5 kDa), and aprotinin (6.4 kDa).

2.4. Identification of whey components by size exclusion chromatography (SEC)

Identification of whey component was carried out by SEC using a TSK gel SW guard column (4 cm × 8 mm) and a TSK G3000 SW (30 cm × 8 mm) (TosoHaas, Japan) loaded on a Hitachi D-7000 HPLC system (Hitachi Ltd., Tokyo, Japan). The system was equipped with a Model L-7100 pump, a Model L-7420 UV–vis detector, and a Rheodyne Model 7725 injector. Peaks were detected at wavelength 280 nm, and acquisition and processing of data were completed by Hitachi B-7000 software with A/D interface. 0.1 M Sodium phosphate buffer solution (pH 6.8) with 0.05% sodium azide driven by the pump system was prepared as mobile phase. Buffer solution was degassed with Branson 2510 ultrasonic system (Branson Ultransonic Corporation, Danbury, CT) right before employing. Each whey component collected from preparative gel filtration chromatography described above was dissolved in the buffer solution (1 mg/ml), filtered using 0.45 μm sterile units (Millipore Co., Bedford, MA), and 10 μl was injected in the chromatographic system. A typical analysis could be completed in 30 min with the flow rate of 0.6 ml/min. Standard proteins including immunoglobulin (IgG), bovine serum albumin (BSA), lactoglobulin (β-Lg), and lactalbumin (α-La) were also run to identify each whey component.

2.5. FT-Raman measurement

FT-Raman spectra of each whey component were obtained by using a Bruker RFS-100 FT-spectrophotometer (Bruker Optik GmbH, Lubeck, Germany). Sample was put into the tiny hole of a stainless steel holder for Raman measurement. A continuous wave Nd-YAG laser (Coherent Lubeck GmbH, Lubeck, Germany) with wavelength 1064 nm, pumped by diode laser, was used as the near infrared Raman excitation source. An He–Ne laser beam was overlapped with 1064 nm beam in order to visualize the Raman sampling spot. The laser light with power of 100 mW was introduced and focused on the sample. The scattered radiation was collected at 180° with an ellipsoidal mirror and was filtered, modulated and reflected back into the highly sensitive GaAs detector that was cooled by liquid nitrogen. Raman spectra were produced over the Raman shift 0–3500 cm^{-1}. Typically, 500 interferograms were coadded at 4 cm^{-1} resolution with a sampling period of about 15 min. The intensity ratio of Raman bands 643–621 cm^{-1} ($I_{643/621}$) as well as 855–832 cm^{-1} ($I_{855/832}$) was used to evaluate the microenvironment property of tyrosine [34], and the ratio of 881–758 cm^{-1} ($I_{881/758}$) was used for the analysis of tryptophan, respectively [35]. The spectra in the 1560–1720 cm^{-1} region were subjected to numerical curve fitting (Grams/386; Galactic Ind. Co.). The band shapes were approximated by a Lorentz function. The baseline was approximated by a straight line between two points at 1560 and 1720 cm^{-1}, chosen at both sides of the band envelope. Each numerical calculation of the Raman intensity ratio was based on the average of triplicate measurements atleast. FT-Raman spectra reported in this study were all-original and were not smoothed, normalized, and baseline corrected through data manipulation.

Statistical analysis was conducted with a commercial statistic computing software package (STATISTICA, 1999 ed., StatSoft Inc., Tulsa, OK, USA) in a personal computer. Results were considered statistically significant at $P < 0.05$.

3. Results and discussion

3.1. Isolation and identification of whey components

Although several methods including chromatographic methods, precipitation with chemical control, ultrafiltration with specific membrane material, reverse osmosis, microfiltration, etc. have been developed for isolating of whey protein [13,14], to develop an easy, direct, and economical way to isolate whey protein from raw milk remains full of challenge. As described in Section 2, the method we used is relatively simple, natural, money saving, and free from chemicals abrasive. For instance, organic solvent such as hexane or ethyl acetate is usually used to extract lipid/oil from the system of interest. In contrast, we demonstrate a natural process (without any chemicals usage) to remove lipid/fat from it. Based on the intrinsic physical property of milk, lipid will be frozen and float spontaneously on the top layer when it is put in the freezer. Thus, it is easier to remove it from the remainder. Since all the processes of defatting, delactosing, and casein removing are relatively mild as well as fewer drastic chemicals being used, the risk of protein denaturizing can be minimised. Fig. 1 shows the separation of whey protein by preparative gel filtration chromatography. Five peaks are observed on the chromatogram, which are attributable to Ig, BSA, β-Lg, α-Lac, and a small non-protein molecule. The uncommonly strong absorbance at 280 nm indicates that the small molecule has either high extinction coefficient or many conjugated chromophores. The molecular mass of each

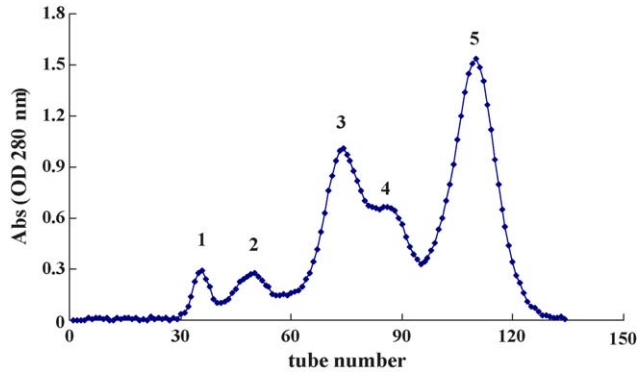

Fig. 1. Gel filtration chromatography pattern of whey protein isolated directly from raw milk after defatting and decaseinating. Peak 1, Ig; peak 2, BSA; peak 3, β-Lg; peak 4, α-Lac; peak 5, small non-protein molecule. Column, Sephadex G-200; flow rate, 0.8 ml/min; absorbance, UV-280 nm; buffer solution, 0.05 M Tris–HCl (pH 7.2).

component was determined as: α-lactoalbumin (∼14 kDa), β-lactoglobulin (∼18 kDa), bovine serum albumin (∼66 kDa), immunoglobulin (∼150 kDa), and small non-protein molecule (∼1700 Da). The resolution between α-Lac and β-Lg is not good enough; however, it is acceptable to collect each sample at the highest absorption for further usage. Fig. 2 shows the electrophoretic profile of whey components by SDS-PAGE. Six bands (Lane 6) clearly shown on the SDS-PAGE gel indicate the distribution of Ig, BSA, β-Lg, and α-Lac in whey proteins. The molecular mass of non-protein molecule is too small to be detected as evidenced by the absence of any bands (Lane 1) on the SDS-PAGE. Lane 2 and Lane 3 represent the SDS-PAGE of α-Lac and β-Lg, respectively. Since the two components are not well-resolved by preparative gel filtration chromatography and due to the large amount of β-Lg, the band with molecular mass of about 18.5 kDa in Lane 2 is a residue of β-Lg. The molecular mass of α-Lac is further confirmed to be ∼14.0 kDa by SDS-PAGE. Based on SDS-PAGE, the molecular mass of BSA (Lane

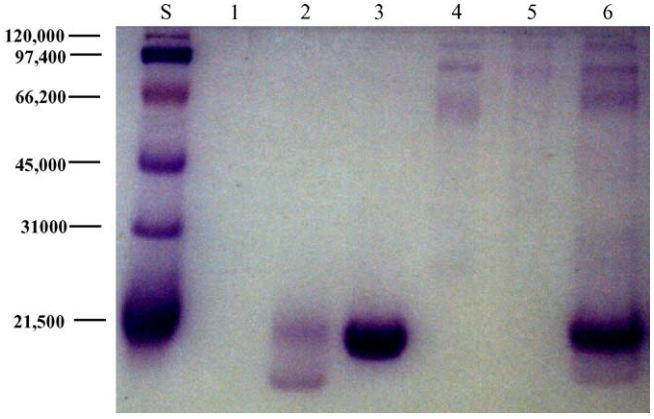

Fig. 2. The SDS-PAGE of whey protein and whey constituents collected from preparative gel chromatography. Lane S: standard protein markers including myosin (200 kDa) phosphorylase b (97.4 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45.0 kDa), carbonic anhydrase (31.0 kDa), trypsin inhibitor (21.5 kDa), and aprotinin (6.4 kDa). Lane 1, non-protein molecule; Lane 2, α-La; Lane 3, β-Lg; Lane 4, BSA; Lane 5, Ig; Lane 6, whey protein. Electrophoresis was carried out at a constant voltage of 180 V for 55 min.

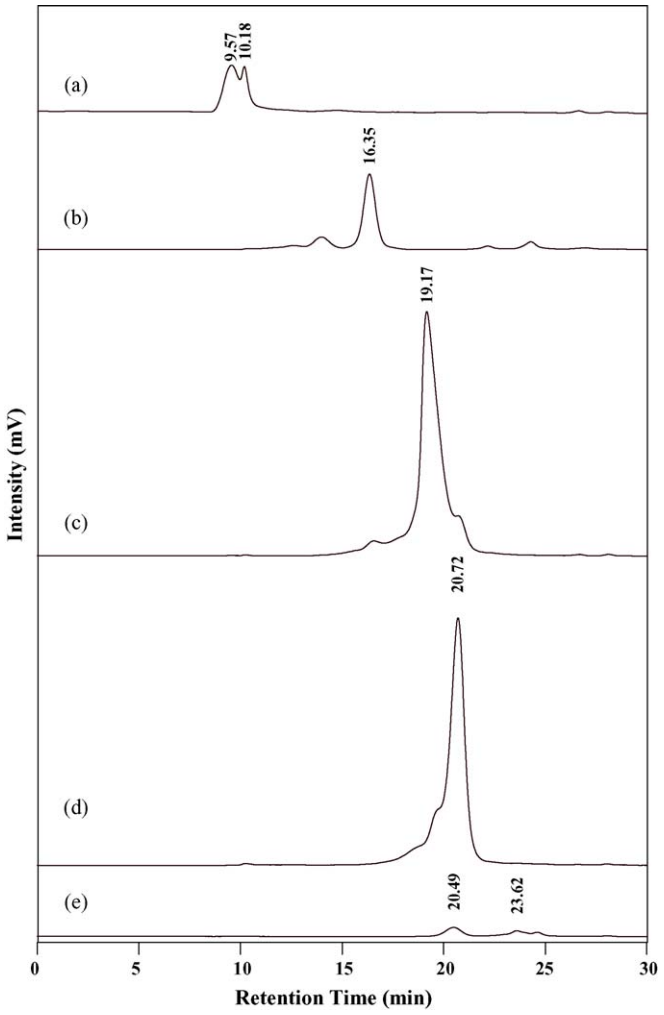

Fig. 3. Size exclusion chromatography patterns of each whey component collected from preparative gel chromatography. (a) Immunoglobulin, (b) bovine serum albumin, (c) β-lactoglobulin, (d) α-lactoalbumin, and (e) small non-protein molecule. Separation was conducted on a Hitachi D-7000 HPLC system. Column, TSK G3000; flow rate, 0.6 ml/min; absorbance, UV-280 nm; buffer solution, 0.1 M sodium phosphate (pH 6.8).

4) is easily obtained as ∼66 kDa. The two light bands of higher molecular mass are attributed to the residue of IgG. Three bands with molecular mass larger than 97 kDa appeared for IgG (Lane 5) on SDS-PAGE indicate three different classes of Ig in the raw milk.

In addition to electrophoretic analysis of whey proteins, each whey component was further analysed by gel exclusive chromatography using a TSK G-3000 SW column and identified by running protein standards. As shown in Fig. 3, each whey component (peaks 1–5) has a different elution time. The peak intensity of each component reflects the relative amount present in whey protein with β-Lg and α-La being present in the largest quantities. Ig shown single peak in preparative gel filtration (Fig. 1) can be further separated into three peaks (Figs. 2 and 3) by SDS-PAGE and SEC. This indicates that Ig consists of different classes of immunoglobulin such as IgG, IgA, and IgM. The shoulders shown on the profiles of β-Lg and α-La clearly explain their incompletely resolved by the preparative gel filtration with

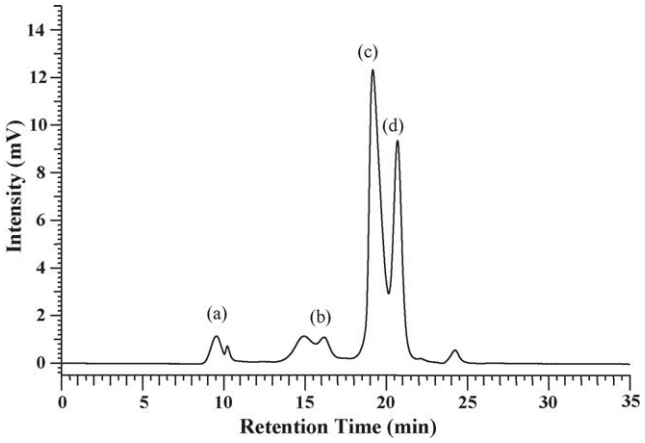

Fig. 4. Size exclusion chromatography pattern of whey protein isolated directly from raw milk after defatting and decaseinating. (a) Immunoglobulin, (b) bovine serum albumin, (c) β-lactoglobulin, and (d) α-lactoalbumin. Separation was conducted on a Hitachi D-7000 HPLC system. Column, TSK G3000; flow rate, 0.6 ml/min; absorbance, UV-280 nm; buffer solution, 0.1 M sodium phosphate (pH 6.8).

Sephadex G-200. This is also seen in the electrophoretic results by SDS-PAGE. The lighter bands shown on the SDS-PAGE gel for β-Lg and α-La are due to the trace residues for each other collected from the incompletely resolved peak. The separation with gel filtration depends on the molecular mass of interest. The molecular mass of β-Lg and α-La are too close to be completely resolved. If necessary, further purification of β-Lg and α-La can be achieved by ion-exchange chromatography as demonstrated by Yoshida [24]. Interestingly, the strong absorption compound, assigned as small non-protein molecule in preparative gel filtration chromatography, only shows little response (intensity even less than α-La) in the SEC profile. This is reasonable for the small molecules may leak out through the dialysis membrane that has molecular mass cut-off at 3000 Da. According to a previous study by Xu et al. [38], the small molecule may be attributed to glycomacropeptide (GMP). To consolidate the above result, an isolated whey protein (1 mg/ml) after dialysis and lyphilization was directly analyzed by SEC, which was demonstrated in Fig. 4. Four major constituents of whey protein, Ig, BSA, β-Lg, and α-La are separated simultaneously. As expected, separation is much better than the one by preparative gel filtration chromatography. The identification of these whey components is accomplished by spiking protein standard in the analysis. Peaks 1–3 are attributed to Ig; peak 4 belongs to BSA; peak 5 is for β-Lg, and peak 6 is assigned to α-La. The retention time increases in order for each whey component being IgG, BSA, β-Lg, and α-La, which is in response to the decrease of molecular mass of these whey constituents. The higher is its molecular mass, the faster a solute will be eluted. Several chromatographic methods have been developed for the analysis of whey protein; however, no single method reported to date is appropriated for simultaneously separating all whey constituents. It has been reported that proteins are readily undergoing structural changes during chromatographic separations [39–43], which may occur in all types of chromatographic columns. As demonstrated above, there is no significant difference in the chromatographic profiles between Figs. 3 and 4. This indicates that there is no protein interaction during the isolation process. Hence, the method we present here is not only a simple and economical way to isolate whey protein directly from fresh raw milk; the whey protein obtained by this method may potentially be used as a cheaper and convenient marker for the molecular mass determination in protein research.

3.2. Structure characterization of whey components by FT-Raman

FT-Raman spectroscopy has been developed to be a simple, direct, and non-invasive tool for exploring molecular structure. It provides a novel method to determine the secondary structure of protein and disclose the local information of protein side groups such as tryptophan, tyrosine, phenylalanine, and sulfhydryl compounds [29–36]. Reviewing previous Raman studies on whey-related protein, few systematic studies on the structure of whey protein had been reported, particularly from the whey components obtained directly from raw milk. Most previous works have been sampling from certain commercial whey products. It is known that there exists a large variability in the composition and functional properties of commercial whey proteins [9–12]. This may result in structural variability. To understand the eccentric molecular structure of whey components, it is necessary to reserve whey protein in native form during the process of isolation. As described above, we use a more gentle process to isolate whey protein directly from raw milk, which will reserve protein mostly in its native form. Figs. 5 and 6 demonstrate the FT-Raman spectra of each whey component in the range of 350–1800 cm^{-1} and curve fitting for the amide I profile in the region of 1560–1720 cm^{-1}, respectively.

3.2.1. Immunoglobulin

Although immunoglobulin only represents a small proportion in cow's milk, it plays an important role in immunological functions, which is strongly determined by its molecular structure. X-ray crystallography is the gold standard method to explore protein structure; however, it requires the molecules to form well-ordered crystals. This may weaken its application in exploring the macromolecule of Ig with molecular mass over 160 kDa. Silverton et al. successfully demonstrate the three-dimensional structure of an intact human immunoglobulin as a Y-shape with two antigen binding sites [44]. However, the structure of Ig isolated directly from raw milk is not well documented. Moreover, the secondary structure of Ig and the molecular interaction of its amino acid side groups are still not addressed. Recently, Wasacz et al. [45] used FTIR spectrometer to investigate the structural change of IgG in non-aqueous solvents and show that the secondary structure of IgG is sensitive to the solvent effect. However, the secondary structure of Ig in aqueous solution is unavailable due to the strong infrared absorption band (1650 cm^{-1}) in water that causes detrimental interference in the amide I region. Surprisingly, there remains no FT-Raman spectroscopic study to date on the structure of Ig. We present here the structural investigation of Ig by FT-Raman spectroscopy for the first time. Fig. 5a shows the FT-Raman spectrum of Ig. As

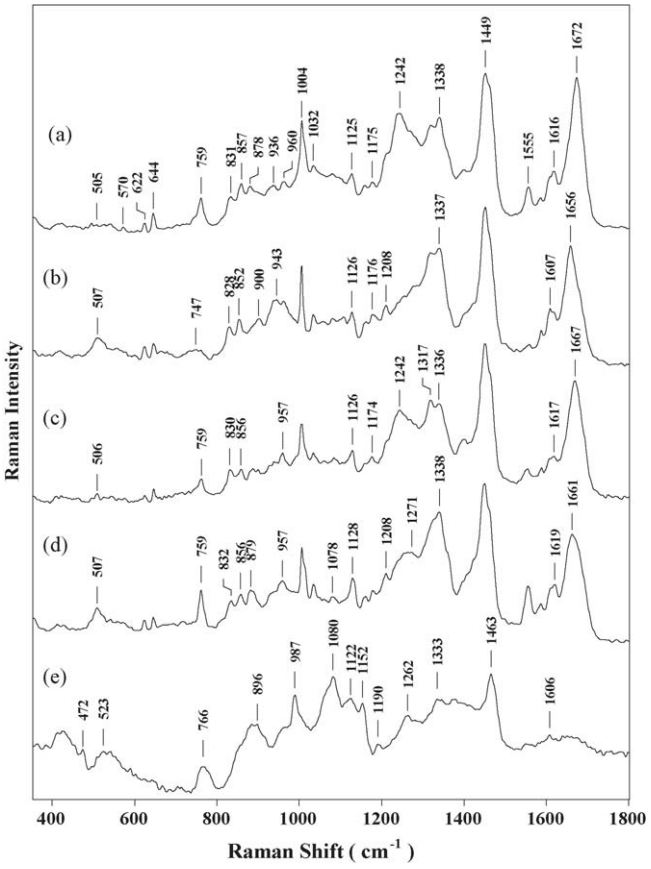

Fig. 5. FT-Raman spectra of whey protein in the range of 350–1800 cm^{-1}. (a) Immunoglobulin, (b) bovine serum albumin, (c) β-lactoglobulin, (d) α-lactoalbumin, and (e) small non-protein molecule. Data acquisition conditions: excitation wavelength, 1064 nm; laser power, 100 mW; spectral resolution, 4.0 cm^{-1}; coadded scan, 500 (~15 min).

illustrated by the amide I at 1672 cm^{-1} coupling with amide III at 1242 cm^{-1}, the secondary structure of Ig is dominated by anti-parallel β-pleated sheet. As shown in Fig. 6a, the curve fitting FT-Raman spectrum of Ig in the range from 1580 to 1720 cm^{-1}, the protein backbone of Ig is mostly in anti-parallel β-pleated sheet. This is consistent with previous studies [44,45] that γ-globulin has largely a β-sheet secondary structure with smaller amounts of turns and disordered or random secondary structure.

The microenvironment of aromatic amino acid and sulfhydryl groups can be easily understood from the specific vibrational modes of Raman spectra in the low-frequency range. Yu et al. [33] report that the intensity change of 644 cm^{-1} is a reflection of tyrosine side-chain conformational changes. The large intensity ratio of $I_{643/621}$ means the higher susceptibility to external stresses. Accordingly, Ig that has the relatively higher Raman intensity ratio of $I_{644/622}$ compared to other components is more susceptible to environmental changes. Siamwiza et al. [35] reported that the tyrosine doublet at around 855 and 830 cm^{-1} was sensitive to the nature of hydrogen bond of the phenol hydroxyl group. If a tyrosine residue is on the surface of a protein in aqueous solution, the phenolic OH will be both an acceptor and a donor of moderate to weak H-bond and the doublet intensity ratio ($I_{857/831}$) will be about 1:0.8 ($I = 1.25$). If the phenolic oxygen is the acceptor atom in a strong H-bond, the intensity ratio will be about 1:0.4 ($I = 2.5$). If the phenolic hydroxyl is the proton donor in a strong H-bond, the intensity ratio will be 1:2 ($I = 0.5$). Accordingly, the phenolic OH in Ig will be both an acceptor and a donor of moderate to weak H-bond because the intensity ratio of $I_{857/831}$ is about 1:0.72 ($I = 1.38$) for Ig. The Raman peak at 760 cm^{-1} is due to the vibrational stretching of tryptophan indole ring. Thus, Ig has a relatively high content of tryptophan. Miura et al. [34] report that the Raman bands around 880 and 1360 cm^{-1} of tryptophan can be used to monitor the strength of H-bonding and the hydrophobicity of the environment of the indole ring. When a tryptophan residue is buried, the relative intensity of the band at 880 cm^{-1} is strong whereas an exposed tryptophan gives a weak feature at 880 cm^{-1}. Hydrophobic interactions between an indole ring of tryptophan residue and the surrounding aliphatic groups cause the 1360 cm^{-1} peak to increase and the 1340 cm^{-1} one to decrease. Based on this description, tryptophan is in a moderately exposure in Ig. Disulfide bonds are very important in supporting the Y-shape of Ig and in connecting the two heavy and light chains. Raman bands at about 510, 525 and 540 cm^{-1} due to the S–S stretching mode can be ascribed to *gauche–gauche–gauche* (ggg), *gauche–gauche–trans* (ggt) and *trans–gauche–trans* (tgt) conformation of disulfide bonds [29]. Accordingly, all the three conformations of S–S stretching are important in Ig.

3.2.2. Bovine serum albumin

Similar to Ig, BSA represents a small proportion in raw milk as proved by the chromatographic results in Figs. 1, 3 and 4. Although BSA is readily crystallized, the crystals have not been amenable to X-ray crystallography and therefore the tertiary structure can only be surmised [46]. It is estimated that ~55% and 16% of the residues of bovine BSA occur in α-helical and β-sheet structures, respectively [46]. Whereas, earlier Raman work by Lord and Chen [47] indicated that the polypeptide backbone was predominant in α-helical form with the remainder of random coil and no evidence for β-pleated-sheet conformation could be detected. In addition, there are also some disagreements in the conformation of disulfide bridges in bovine BSA. BSA is unique with its numerous disulfide bonds (17 S–S), which play an important role in stabilizing its protein structure. Several research groups report that the 17 disulfide bridge conformation of BSA can only stay in ggg form [47,48]. However, Nakamura et al. [49] suggest that the 17 disulfide bridges in the N-form of BSA solution take both the ggg and ggt conformations. The incongruent results from different research groups may come from sample variability, as milk protein is more or less species-specific. Therefore, it warrants further investigation of the structure of BSA, which isolates directly from fresh raw milk under mild conditions that will encounter less protein denaturing. FT-Raman spectrum shown in Fig. 5b clearly reveals that the secondary structure of BSA is largely in α-helix form as supported by the amide I at 1656 cm^{-1} coupling with amide III around 1260–1280 cm^{-1} [29–32]. The strong intensity of 943 cm^{-1} also provides an indicative of high α-helix content. The disulfide bonds in BSA are predominant in ggg

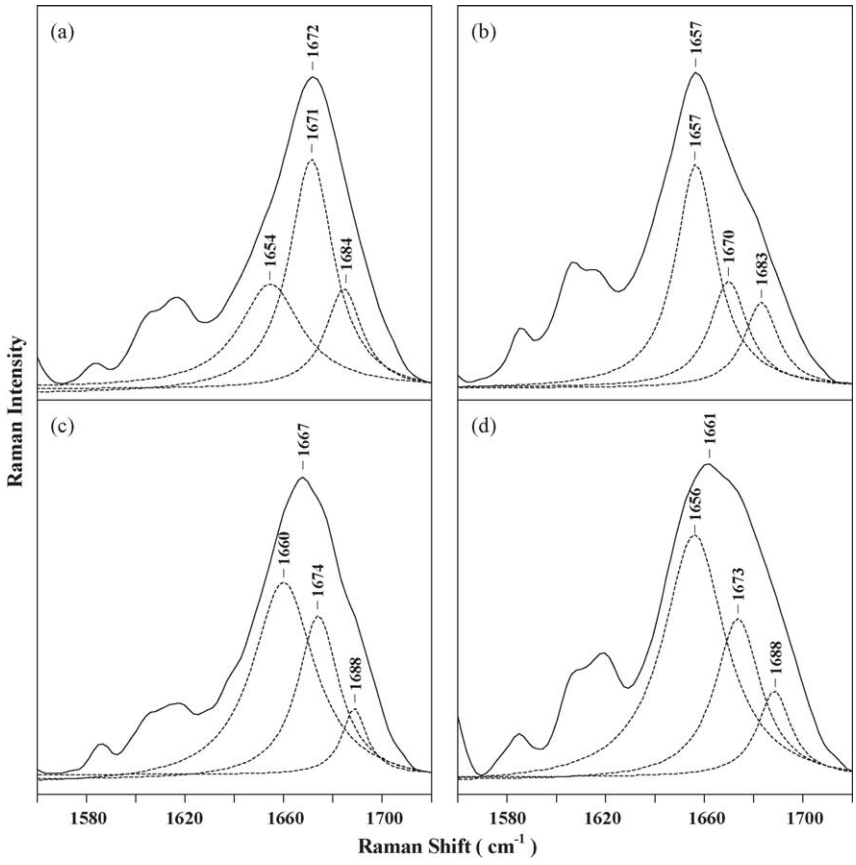

Fig. 6. Curve fitting for the amide I profile in the region of 1560–1720 cm^{-1} of FT-Raman spectrum of whey proteins. (a) Immunoglobulin, (b) bovine serum albumin, (c) β-lactoglobulin, and (d) α-lactoalbumin.

conformation with small amount in ggt form and even less content in tgt. The S–S in bovine BSA is quantitatively estimated: ∼10 in ggg, ∼6 in ggt conformation and no more than one S–S in tgt conformation based on curve fitting manipulation for FT-Raman spectrum in the region of S–S bonds. FT-Raman spectrum also shows bovine BSA has relatively lower content of tryptophan residue compared to other whey components as indicated by the weak Raman response.

3.3. β-Lactoglobulin and α-lactoalbumin

β-Lg and α-La are the two major constituents of whey protein as the represent with content more than 75% in whey. Although both have been widely studied, there remain some disagreements in their structure by a variety of techniques. Thus, we will briefly re-examine their structure by FT-Raman spectroscopy. β-Lg represents about 50% of the whey protein; it is the one most extensively studied in protein research. Since the structure of β-Lg is well characterized, which contains about 45% β-sheet, 10% α-helical and 45% unordered structures, it can serve a good reference to examine the precision of FT-Raman in evaluating the secondary structure of whey protein. As shown in Figs. 5c and 6c, Raman spectrum of amide I at 1667 cm^{-1} and amide III at 1242 cm^{-1} indicate that both β-sheet and unordered structure are most predominant in the secondary structure of β-Lg, which is consistent with a previous study by Howell and coworkers [32]. Compared to other whey components, the Raman intensity ratio of the doublet tyrosine at 856 and 830 cm^{-1} is relatively small ($I_{856/830} = 0.78$), which implies that tyrosine residues in this protein are in a buried environment or act as proton donors. The two disulfide bridges of β-Lg is mostly in ggg form as revealed by the small Raman intensity at 506 cm^{-1}. This is completely consistent with previous study by Byler et al. that both disulphides adopt a ggg conformation.

For α-La, similar to BSA, α-helix and unordered structure are the main secondary structures as evidenced by the amide I at 1661 cm^{-1} and amide III at 1271 cm^{-1} shown in Figs. 5d and 6d. Contrary to other whey component, it shows that tryptophan in α-La prefers a buried state and predominantly in a hydrophobic interaction due to the explicit Raman response at 879 and 1360 cm^{-1}. The enhanced S–S stretching at 507 cm^{-1} implies that the four intramolecular disulfide bonds are major in ggg form. The homogeneous disulfide conformation and relative hydrophobicity of aromatic amino acids may explain the thermal stability of α-La.

3.3.1. Small non-protein molecules

Fig. 5e shows the FT-Raman spectrum of the small molecule collected from the one shown as peak 5 in Fig. 1. As described above, the molecular mass of this molecule is too small to be collected after dialysis because it will leak out through the dialysis membrane with molecular mass cut-off at 3000 Da.

In order to collect this sample, we replaced phosphate buffer with ammonium bicarbonate buffer and lyophilized it right after fractionation. Its molecular mass is determined to be 1700 Da. Raman profile displayed in Fig. 5e sheds some light on the structure of this small molecule although more detailed work may be needed to resolve its exact structure. Raman peaks at 766, 896, 987, 1080, 1122, 1152, 1262, 1333, and 1463 cm^{-1} are typical patterns of saccharides [50]. According to prior study by Xu et al. [38] plus current Raman result, the small molecule is suggested to be a kind of glycomacropeptide (GMP) of which molecular mass is below 7 kDa. Compared to the Raman profile of pure protein and carbohydrate, the broad Raman bands in Fig. 5e may arise from the fusion of active modes in these two biomolecules. For instance, Raman peak of 766 cm^{-1} may be due to the response of tryptophan and glucose/galactose, and the shoulder band from 830 to 910 cm^{-1} may be from the combination of tyrosine and glucose/galactose. Raman fingerprints in the range from 400 to 1000 cm^{-1} reveals that the glycomacropeptide is rich in disulfide linkage and aromatic amino acids. The abundance of aromatic amino acids in this small molecule further provides a solid evidence for the strong absorptive response as shown in the chromatogram of Fig. 1.

According to current Raman results, it seems that globular proteins exhibit predominately β-structure, while, α-helical form takes large part in the secondary structure of albumins. Although both Ig and β-Lg are commonly high in β-structure, they still exhibit significant difference in the secondary structure, which can be traced by carefully examining the vibrational mode of amide III and the stretching of amide IV (930–980 cm^{-1}). The sharpening of amide III band in β-Lg may indicate the uniformity of H-bond, whereas, the flattening of amide III band in Ig implies the complexity of H-bonds in this macromolecule [32], of which molecular mass is about 160 kDa. Similarly, BSA and α-La also express differently in the secondary structure although most of their structure is α-helix. Differences in the secondary structure for each whey component reflect their intrinsic compositional differences and distinct spatial arrangement.

In summary, we present a simple and economical process for isolating whey protein directly from raw milk. Whey protein isolated by this mild method will have least risk of denaturation. A small molecule of non-protein with molecular mass about 1.7 kDa was separated and identified to be a glycomacropeptide. Raman results clearly indicate that the molecular structure of each whey component is different. The secondary structure of Ig is mainly in anti-parallel β-pleated sheet. For BSA, the secondary structure of protein is mostly in α-helix; for β-Lg, however, both β-structure and unordered structure are important, and α-La is a α-helix with some parts random coil conformation. Variation in the Raman intensity of 621, 643, 760, 829, 855, and 1338 cm^{-1} clearly indicates the different behavior of major amino acids in each whey component. It is apparent that differences in the Raman profile for each whey component reflect their intrinsic compositional differences and distinct spatial arrangement. Protein conformation is greatly influenced by SH/S–S conversion because of intramolecular or intermolecular interaction. The splitting and formation of disulfide bonds has great influence in the function of whey protein. The S–S linking diverging around 510–540 cm^{-1} indicates that the conformation of disulfide bonds in each whey components is different, which is responsible for their distinguished behavior in solubility, rheological and functional properties.

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