Fractionation of Whey Protein Isolate with Supercritical Carbon Dioxide To Produce Enriched α-Lactalbumin and β-Lactoglobulin Food Ingredients

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ABSTRACT: An environmentally friendly protein fractionation process using supercritical carbon dioxide (SCO_2) as an acid was developed to produce enriched α-lactalbumin (α-LA) and β-lactoglobulin (β-LG) fractions from whey protein isolate solutions containing from 2 to 10% WPI. This study investigated the effects of pH, temperature, WPI concentration, and residence time on the precipitation kinetics and recovery yields of individual whey proteins and the relative enrichment and composition of both protein fractions. At 5.5−34 MPa and 60−65 °C, solubilized SCO_2 decreased solution pH and induced the formation and precipitation of α-LA aggregates. Gel electrophoresis and HPLC of the enriched fractions demonstrated the production of ≥60% pure α-LA, and ≥70% pure β-LG, under various operating conditions, from WPI containing ∼57% β-LG and 21% α-LA. The enriched fractions are ready-to-use food ingredients with neutral pH, untainted by acids and contaminants.

KEYWORDS: whey proteins, fractionation process, supercritical carbon dioxide, α-lactalbumin, β-lactoglobulin

■ INTRODUCTION

A portion of the whey industrially produced during the cheesemaking process is concentrated through ultrafiltration and ion-exchange chromatography and spray-dried to produce whey protein isolate (WPI). WPI is used as a food ingredient with excellent nutritional and functional properties due to its high protein content (>90 wt %) and low fat, lactose, and ash contents. Commercial WPI consists of a mixture of more than seven different types of proteins, containing more than 50% β-lactoglobulin (β-LG) and 20% α-lactalbumin (α-LA), as well as casein glycomacropeptide (GMP, 15−20%), and bovine serum albumin (BSA), immunoglobulins (Ig), lactoferrin (Lf), and residual caseins (≤10% total), all with different nutritional and functional attributes.[1,2]

The creation of enriched fractions of α-LA and β-LG would emphasize the valuable specific properties of these proteins. For example, β-LG is rich in essential branched-chain amino acids[3,4] and remains soluble at high temperatures under a wide range of acidic pH. β-LG solutions can form gels when their native structure is sufficiently destabilized to allow aggregation,[5] and removal of α-LA from WPI enhances its gelling properties.[6,7] On the other hand, bovine α-LA is prized for its superb nutritional properties[8,9] with potential uses that include specialized foods for infants or the elderly, such as enhanced infant formulas. Human breast milk contains ∼25% human α-LA and no β-LG,[10] and β-LG from added WPI has been deemed a possible cause of infants' allergic reactions.[11−13] Whey proteins enriched with bovine α-LA and depleted in β-LG may bring the amino acid profile of infant formulas closer to that of breast milk.[14]

Various fractionation techniques, such as selective protein aggregation via salting out or heat treatment and pH adjustment, membrane filtration, ion-exchange chromatography, and two-phase partition have been developed to separate α-LA from β-LG in whey protein solutions to take advantage of the specific functional and nutritional properties of the proteins.[2] The separation of α-LA from whey, whey protein concentrate (WPC), or WPI solutions by selective aggregation is based on the loss of solubility of the protein by removal of its stabilizing calcium ions through pH modification and heating.[15] After addition of an organic or mineral acid, α-LA was shown to unfold and aggregate at moderate temperature (50−65 °C) around pH 3.8−4.2, usually accompanied by the precipitation of BSA, Ig, and Lf. β-LG and GMP remained soluble.[16−26] Because subsequent neutralization of the acid introduces salts in the protein fractions and removal of these salts generates extra processing steps and costs, supercritical carbon dioxide (SCO_2) has been investigated as an alternate, clean acid to precipitate α-LA from whey protein solutions.[27−29] When dissolved in aqueous solutions, CO_2 produces carbonic acid that reduces the pH of the solution. The resulting pH depends on the solubility of the gas, which is governed by CO_2 pressure via thermodynamic equilibrium.[30] In its supercritical state, that is, above its critical temperature (31.1 °C) and critical pressure (7.39 MPa), SCO_2 possesses both the properties of a gas and the density of a liquid, which enhances its solubility in water. The main advantage of CO_2 as a protein precipitant is that depressurization of the system releases the dissolved gas and returns the pH of the products to a value close to the initial pH, without the addition of any contaminants.[27,31] In addition, pH overshoots are avoided because the pH is regulated by the thermodynamic equilibrium of the system. For these reasons, CO_2 has successfully been used in the isoelectric precipitation of select soy proteins from aqueous solutions at room temperature and pressures up to 5 MPa (pH as low as

4.5),[32−34] as well as the selective precipitation of casein from milk around 7 MPa (pH 5.4) and 38 °C.[35−39] The properties of SCO_2 as a protein antisolvent were demonstrated with the SCO_2-induced precipitation of various proteins (insulin, trypsin, and others) from organic solvents,[40,41] or with the addition of ethanol or fluorosurfactants to aqueous protein solutions to increase the saturation solubility of CO_2 in the solutions and trigger protein precipitation.[42,43] Besides behaving as an acid, a potential antisolvent, and enabling the production of whey protein fractions clear of additives, another advantage of SCO_2 as a protein fractionaton agent is the possibility for recycling after treatment to design a green process. Treatment of WPI with SCO_2 between 10 and 30 MPa at 40−65 °C can also enhance the gelling properties of the resulting β-LG fraction owing to compositional and slight structural changes.[44]

The purpose of this study was to demonstrate the use of SCO_2 in a pilot-scale process to prepare enriched fractions of α-LA and β-LG from WPI solutions through the acidic precipitation of α-LA and separation of the α-LA-enriched aggregates from the β-LG-enriched solution. The effects of processing parameters on the rates of precipitation of the proteins, the composition and purity of the fractions, and product yields were examined to determine optimal processing conditions and enable the development of a commercial SCO_2 whey protein fractionation process.

■ MATERIALS AND METHODS

Materials. Spray-dried WPI from cheese whey, Provon 190, was purchased from Glanbia Nutritionals Inc. (Richfield, ID, USA) and contained 90.1% (w/w) protein, with 3.6% moisture, 2.9% ash, and 3.4% lactose and fat, total, by difference. Solutions containing 1−28% (w/w) WPI were prepared with distilled water. Liquid carbon dioxide (CO_2) tanks with an eductor tube were purchased from GTS-Welco (Allentown, PA, USA).

Process Setup and Experimental Protocol. The pilot-scale process design was adapted from previous works by Tomasula et al.[27,28,31,45] A 1 L high-pressure reactor with bolted closure, floor stand, and MAG 075 MagneDrive stirring assembly and with a maximum working pressure of 38 MPa was purchased from Autoclave Engineers (Erie, PA, USA). Accessories were included or added for control of the temperature and pressure, liquid sampling, and safety of operations.

A 500 mL WPI solution of desired concentration, C, was placed in the reactor base and the reactor was tightly closed. The solution was stirred with a turbine stirrer and then heated through the reactor base using an electric heating mantel (both Autoclave Engineers) powered by a programmable PID temperature controller (Cole-Parmer, Vernon Hills, IL, USA). The temperature, T, was measured with a thermocouple dipped in a well inside the reactor and recorded on a computer with the temperature controller software. The temperature was increased slowly, to prevent overshooting, to the desired temperature, T_R (60−65 °C) and could be quickly reduced as needed using an internal cooling coil connected to the cold-water line. Toward the end of the heating stage, CO_2 was pumped into the reactor from a liquid-CO_2 tank using an air-driven liquid pump (Haskel, Huntington Beach, CA, USA) and dispersed throughout the WPI solution by the turbine stirrer. CO_2 pressure, P, was monitored with a manual gauge and a pressure transducer and recorded on the computer with LabView software (National Instruments Corp., Austin, TX, USA). P was carefully and manually adjusted until both T and P reached the target steady-state values, T_R and P_R, and then the system was kept constant at T_R and P_R for the duration of the experiment (up to 4 h). Variations of ±0.4 °C and ±0.35 MPa were allowed. Infrequent out-of-range variations were corrected quickly by either venting or pumping CO_2 or using the heating or cooling setups. The time, t_0, when T reached $T_R − 1$ °C was considered to be the starting time of the fractionation reaction: $t_0 = 0$.

During an experiment, safety devices included a rupture disk (38 MPa blow-out pressure), heavy rubber curtains, Plexiglas shield, overtemperature alarm, noise-reducing earmuffs, safety goggles, and leather gloves.

At the end of a run, the sample was quenched by removing the heating mantel and lowering T to ~40 °C with the cooling coil. The sample was then extracted through a dip-tube using the pressure inside the reactor as a driving force. During depressurization to atmospheric pressure, dissolved CO_2 evolved from the sample, producing a cooling effect and the formation of a dense, white foam. After collapse of the foam, the sample was collected in centrifuge bottles for post-treatment. The reactor was depressurized, cleaned with hot water, and sterilized with steam, and then the reactor base was disassembled and thoroughly washed. Experiments were performed in triplicate, and the data were analyzed using ANOVA on Microsoft Excel software.

Mixing with the Turbine Stirrer. The turbine stirrer (Autoclave Engineers) was composed of a hollow shaft with an intake hole at its top and a turbine impeller at its bottom. Depending on the rotation rate, the vacuum created between the blades of the turbine forced CO_2 circulation through the hole/shaft/turbine assembly and propelled small CO_2 bubbles to the core of the WPI solution, enabling fast CO_2 dissolution. The effect of the rotation rate on the amount and size of bubbles in 10% WPI solutions was tested systematically with air between 500 and 2000 rotations per minute (rpm). The range of 800−1200 rpm produced an adequate amount of small bubbles without creating a large head of foam, keeping the whey proteins in solution; thus, a constant stirring rate of 1000 rpm was used in all experiments.

pH Measurement. The pH of WPI solutions pressurized with SCO_2 was measured with a high-pressure probe resistant to 13.8 MPa (Innovative Sensors, Inc., Anaheim, CA, USA) hermetically inserted into the lid of the reactor. The pH probe was calibrated before each run at atmospheric pressure and 20 °C using three standard calibration buffer solutions and a pH/Ion Analyzer 350 (Corning, Lowell, MA, USA) with ±0.001 precision. The pH of untreated WPI solutions varied from 6.14 to 6.37 depending on WPI concentration. WPI solutions were treated as described above, and the pH was tracked versus time, T, and P. At the end of a run, most of the CO_2 evolved quickly from the sample and the final pH was ~6.0.

After a standard curve and a model for the calculation of pH as a function of CO_2 pressure and WPI concentration, pH(P,C), had been established, the pH probe was permanently removed so that operating pressures of >13.8 MPa could be routinely used.

Real-Time Kinetic Samples Collection. To quantify the rate of α-LA precipitation as a function of T, P, C, and t, small homogeneous samples were extracted from the reactor during fractionation at predetermined time intervals. During a typical run, samples were extracted at t_i = 10, 20, 30, 60, 90, 120, 150, and 180 min. The procedure for sample extraction was as follows: (1) Six hundred milliliter WPI solutions were used during kinetic experiments, stirred, and brought up to T_R and P_R at $t = 0$, as described above. (2) About 30 s before each time t_i, stirring was stopped to let CO_2 bubbles rise to the surface, and then ~25 g of solution was extracted from the bottom of the reactor through the dip-tube. Samples were cold and foamed due to vaporization of CO_2 during extraction. (3) Stirring was resumed. T dropped by up to 1 °C during extraction and was automatically corrected by the T controller. P dropped by as much as 7 MPa and was adjusted back to P_R by pumping additional CO_2 in the reactor.

The dip-tube was purged before each sample extraction to discard the small volume of material (~2 mL) sitting in the tube between t_i and t_{i+1}. Immediately after foam collapse, each slurry sample was divided between two preweighed 10-mL centrifuge tubes for duplicate analysis, then covered and store in the refrigerator.

CO_2 Solubility. The solubility of CO_2 in WPI solutions as a function of P was measured using the foam-sampling method of Bonnaillie,[46] which was designed to easily and precisely measure the solubility of a gas in a liquid with high foaming properties.

Separation and Quantification of the Protein Fractions. Kinetic samples were centrifuged with a Sorvall EconoSpin centrifuge with bucket holders (Thermo Fisher Scientific Inc., Waltham, MA,

USA) at 2000g (3600 rpm) for 60 min at room temperature. The supernatant enriched with β-LG was collected into glass vials and labeled the β fraction. The aggregate fraction enriched with α-LA was lyophilized, then collected in plastic jars and labeled the α fraction. Large (final) samples were centrifuged in a large-capacity refrigerated benchtop centrifuge with bucket holders (Fisher Scientific, Pittsburgh, PA, USA) at 4000g for 60 min. Both protein fractions were quantified by mass differences before centrifugation, after removal of the supernatant, and after lyophilization.

Composition of the Protein Fractions. The protein distributions of the starting WPI and the α and β fractions were determined using sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) on a PhastSystem (Amer Pharmacia Biotech, Piscataway, NJ, USA) with homogeneous gels containing 20% acrylamide and 8 lanes, using the method of Parris et al.[47] A low molecular weight marker (Bio-Rad, Hercules, CA, USA) was added to the first gel lane. Room temperature gel treatment included staining of protein bands with Coomassie brilliant blue dye for 20 min and destaining overnight. The location, size, and intensity of the protein bands were analyzed with a Personal Densitometer SI (Molecular Dynamics, Sunnyvale, CA, USA) and ImageQuant TL 7.0 software (GE Healthcare Bio-Sciences, Piscataway, NJ, USA). Major bands were identified as α-LA, β-LG, residual caseins, Ig, BSA, and Lf. Protein distributions calculated by the software were corrected as needed using an α-LA/β-LG calibration curve.

Because GMP was not visible on SDS-PAGE gels, the starting WPI and all of the liquid (β) fractions were also analyzed with reverse-phase high-pressure liquid chromatography (RP-HPLC) using the method of Bonnaillie et al.,[48] to measure the GMP as well as the β-LG and α-LA contents. RP-HPLC was run on a Varian ProStar 230 HPLC (Varian, Palo Alto, CA, USA) unit equipped with a Varian UV–vis 325 ProStar detector and a polymeric RP column C4 with polystyrene–divinylbenzene copolymer packing (250 mm length, 4.6 mm diameter, particle size = 3.5 μm, pore size = 300 Å). The acetonitrile solvent contained 0.1% trifluoroacetic acid. The flow rate was 0.8 mL/min, and UV absorbance was monitored at 214 nm. Protein solutions were diluted to 1% (w/w) concentration, and then 20 μL β samples were injected in the column following calibration with an α-LA/β-LG standard.

Data analyses were performed in duplicates.

■ RESULTS AND DISCUSSION

CO$_2$ Solubility. After pressurization at constant T, the thermodynamic equilibrium, or saturation of the WPI solution with CO_2 as observed by stabilization of the CO_2 pressure, was reached within 3–7 min (increasing with pressure). The solubility, S, of CO_2 in a 10% WPI solution at thermodynamic equilibrium was linear when measured between 1 and 11 MPa: $S \sim 2.03P$, with P in MPa and S in cm^3 of CO_2 per gram of solution (cm^3/g). S was difficult to measure safely and accurately at higher pressures and, for extrapolation purposes, we considered that S remained proportional to pressure on the entire range of study (0.7–34 MPa). Tomasula et al.[49] showed that the solubility of CO_2 in milk also increased linearly with pressure. At 50 °C and 5.86 MPa, the solubility of CO_2 in cows' milk containing ~3 wt % proteins was ~20 cm^3/g and similar to that in water. In this study, $S \sim 11.9$ cm^3/g at 5.86 MPa and 60 °C in a solution containing 10% WPI, that is, 9% proteins. This significant loss of CO_2 solubility with increased protein concentration may be tied to solvation of the whey proteins by water and the consequently reduced availability of water molecules to solvate CO_2.[49]

pH Calibration of WPI Solutions Saturated with SCO$_2$. pH in the high-pressure reactor was calibrated as a function of CO_2 pressure and WPI concentration to obtain a model for pH(P,C), used for experimental design and optimization of the fractionation process. After the pressure stabilized at a desired

value between 0.28 and 13.8 MPa at 60 °C, the pH of solutions with C = 1–28% WPI was monitored until the pH also stabilized (with ±0.001 precision).

Figure 1 shows the equilibrium pH of solutions containing 1, 2, 5, 10, 15, and 28% WPI and saturated with CO_2, as a
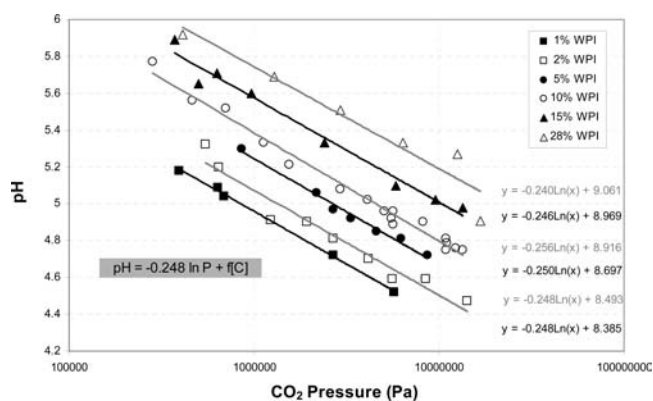

Figure 1. Equilibrium pH of WPI solutions with C = 1, 2, 5, 10, 15, or 28% WPI, T = 60 °C, and CO_2 pressure P = 0.28–13.8 MPa.

function of P. At constant pressure, saturation with CO_2 lowered the pH of the solutions significantly less when the WPI concentration was increased, due to decreased CO_2 solubility and the buffering properties of whey proteins. At constant WPI concentration, the pH decreased logarithmically with increasing CO_2 pressure and was well described by the following equation over the range of concentration studied:

$$\text{pH}(P, C) = -0.248 \ln(P) + f(C) \tag{1}$$

P is reported in Pa, and

$$f(C) = 0.216 \ln(C) + 9.365 \tag{2}$$

with C in g/g, valid for WPI concentrations between 1 and 28 wt %; that is, $0.01 \leq C < 0.28$ g/g.

Between 60 and 65 °C, temperature showed no notable effect on pH.

Prior WPI fractionation studies by Bonnaillie and Tomasula,[16] using hydrochloric acid, showed that α-LA and β-LG optimally separated at T = 60–62 °C and pH 4.0–4.2. The lowest pH value we obtained with a CO_2 pressure of 13.8 MPa was pH 4.47 with C = 0.02 g/g (2% WPI). To maximize the efficiency of the fractionation process (e.g., maximize product throughput while minimizing reactor size and water usage), it is desirable to process more highly concentrated WPI solutions, and therefore pressures >13.8 MPa are needed to lower the pH sufficiently. Due to the proportionality of CO_2 solubility to P and the consistent, logarithmic shape of pH(P) up to 13.8 MPa, we assumed continued validity of eqs 1 and 2 at greater pressures and extrapolated pH(P,C) past the maximum operating pressure of the reactor (38 MPa), as shown in Figure 2.

In theory, pH 4.2 should be obtained for WPI concentrations of ~2 wt % and CO_2 pressures of ~34 MPa or higher. In this study, we examined the kinetics of WPI aggregation under SCO_2 treatment at 60–65 °C, for C = 2–10% WPI and P = 5.5–34 MPa, corresponding to a pH range of 4.2–5.0. In the following discussion, all pH values were calculated from P and C using eqs 1 and 2.

Kinetics of Aggregate Formation. Previous research has shown that, at temperatures $T \geq 60$ °C and acidic pH, α-LA
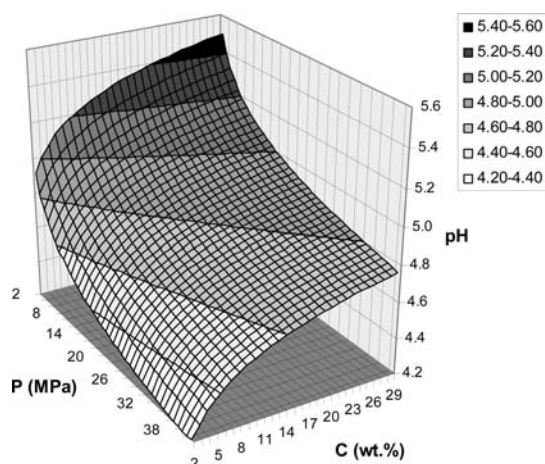
Figure 2. Three-dimensional model for pH(P,C) drawn according to the pH calibration equations (eqs 1 and 2) and extrapolated to 40 MPa.

begins to denature and form aggregate particles[18] and then precipitate from the WPI solution as a slow and kinetically limited phenomenon.[16] A large portion of the minor whey proteins, which are the most sensitive to thermal and pH changes,[16] precipitates quickly, whereas fractions of the soluble β-LG and GMP are entrapped in the aggregate phase due to water holding by the aggregates.[16,50,51] The aggregation reaction being kinetically limited, the yields and rates of precipitation of α-LA, β-LG, and the minor whey proteins with SCO$_2$ are expected to vary greatly as a function of pH, temperature, and solution concentration in this work.[16,52,53] Figure 3 shows that the rate of aggregate formation of a 10% WPI solution between pH 4.9 and 5.0 is slow and rate-limited and depends strongly on temperature.
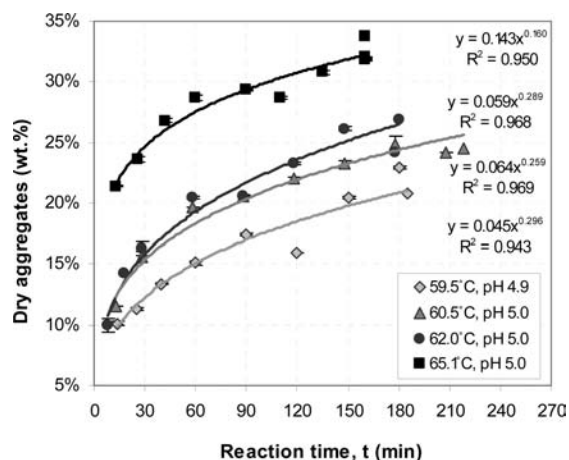

Figure 3. Evolution of the mass fraction of dried aggregates versus initial WPI contents, for 10% WPI solutions treated at pH 4.9–5.0 (P_R = 5.5–9 MPa) and T_R = 59.5, 60.5, 62, 65.1 °C for 160–220 min.

Power-law regressions of the form $Y_{agg} = at^b$ (where Y_{agg} is the aggregate yield, i.e., the mass fraction of dry aggregates after centrifugation and lyophilization relative to the initial mass of WPI in the solution, and t is the reaction time spent at T_R and P_R) were found to fit the experimental data well and were used for data regressions and analyses throughout this work. At constant pH, the rate of total protein aggregation increased significantly with temperature between 59.5 and 65.1 °C

(Figure 3), as well as with decreasing pH and increasing WPI concentration, as will be shown below.

Compositions of the Fractions and Protein Recoveries. The composition of the starting WPI measured with SDS-PAGE (five replicates) was 67.8% β-LG, 25.0% α-LA, and 7.2% minor whey proteins (Ig, BSA, Lf, casein, and casein fragments). After identification and quantification of the GMP with mass spectrometry and RP-HPLC,[48] the composition of the WPI was corrected to 16% GMP, 57% β-LG, 21% α-LA, and 6% minor whey proteins.

The compositions of the aggregate and liquid fractions during the SCO$_2$ fractionation of WPI solutions were tracked as a function of time. For calculation purposes, we considered that most of the lactose and ash, which are highly soluble, remained in the liquid fraction. Figure 4 shows the typical composition of
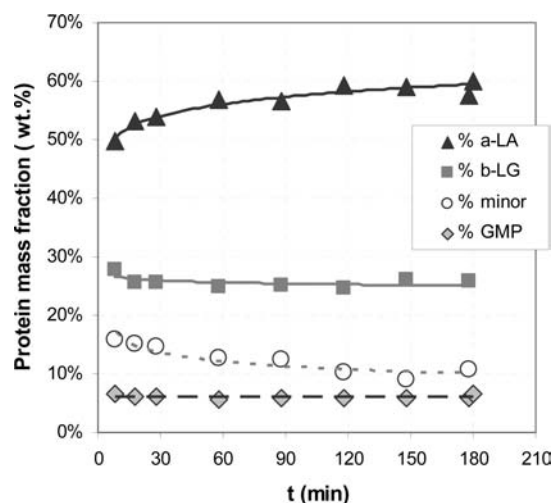

Figure 4. Typical evolution of the composition of the precipitate fraction as a function of reaction time. Operating conditions: 10% WPI solution treated at 62 °C and pH 5.0 (5.5 MPa).

the aggregate fraction and its evolution with time. Depending on pH, α-LA generally precipitated more quickly than the other proteins and the α-LA content of the α fraction increased slowly with time, whereas the β-LG, GMP, and minor proteins contents generally decreased with time. α-LA purities up to 62% (w/w) were obtained in this work, with GMP contents ranging from 2 to 7%, whereas the β-LG and minor protein contents of the α fraction varied greatly with operating conditions.

Enrichment of the two protein fractions, the main target of this study, was evaluated by calculating the mass ratio of α-LA to β-LG in each fraction, "ratio α/β". Ratio α/β of the starting WPI was 0.37. Enrichment of the β (liquid) fraction was obtained when its ratio α/β became <0.37, whereas enrichment of the α (aggregate) fraction corresponded to a high value of ratio α/β. Figure 5 demonstrates the depletion of α-LA from the β fraction as it precipitates, and the resulting enrichment of the β fraction with β-LG as its ratio α/β decreased with time. The positive effects of both temperature increase and pH reduction on the rate of α-LA precipitation are also clearly visible in Figure 5. For example, the treatment of 10% WPI solutions with SCO$_2$ at pH 4.6 and ~65 °C resulted in a ratio of α/β = 0.1 after approximately 60 min.
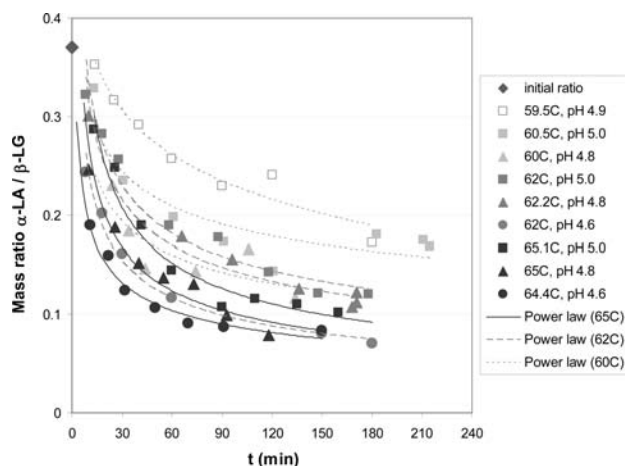
Figure 5. Enrichment of the β fraction as a function of time, temperature, and pH for 10% WPI solutions, as represented by the mass ratio of α-LA to β-LG in the liquid.

The precipitation rates of individual proteins were studied by calculating their recovery yields in the aggregate fraction using the equation

$$\mathrm{Rec}_k = \text{amount of protein } k$$
$$\text{in aggregate/initial amount in}$$
$$\text{solution}$$
$$= x_k \times Y_{\mathrm{agg}}/x_{k,0} \qquad (3)$$

where x_k is the mass fraction of protein k in the aggregate fraction and $x_{k,0}$ is the mass fraction of protein k in the starting WPI. For α-LA, $k = A$, and $k = B$ for β-LG.

The recovery rate of β-LG in the aggregate fraction, Rec_B, was separated between two distinct phenomena: (1) entrapment of β-LG within the aggregates due to water holding, which was proportional to the total volume of the aggregates after centrifugation and before drying, [16] and (2) actual acid-induced or thermally induced denaturation and aggregation of β-LG, causing partial precipitation. The recovery of β-LG in the aggregate due to the aggregation reaction only was calculated as $\mathrm{Rec}_{B/reaction} = \mathrm{Rec}_B - \mathrm{Rec}_{B/water}$, where $\mathrm{Rec}_{B/water}$ was considered to be equal to the fraction of wet aggregates, $Y_{\mathrm{agg}/wet}$. Rec_{GMP}

was considered to proceed from water holding only; thus, $\mathrm{Rec}_{GMP} \sim Y_{\mathrm{agg}/wet}$.

Effects of Temperature. Figure 6 shows the effects of time and temperature on the recovery rates of α-LA and β-LG by aggregation only in 10% WPI solutions treated with SCO_2 at pH 4.9–5.0 ($P_R = 5.5$–9 MPa). Similarly to the aggregation of WPI with HCl,[16] the rate of α-LA aggregation at constant pH was extremely sensitive to temperature in the range of 60–65 °C, whereas the aggregation of β-LG showed little to no effect of temperature below 62 °C and a marked acceleration at 65 °C. The recovery rate of α-LA in the aggregate fraction was much greater than that of β-LG at all temperatures and increased progressively and significantly with T_R, from $\mathrm{Rec}_A \sim$ 50% after 120 min at 59.5 °C to $\mathrm{Rec}_A \sim$ 90% after 120 min at 65.1 °C (Figure 6A). Over the same temperature range at pH ~ 5, the recovery of β-LG via aggregation was <8% after 120 min, enabling the production of α-LA-rich aggregates. However, the purity of the α fraction depended on temperature, because the recovery of β-LG after 120 min doubled, or even tripled, from $\mathrm{Rec}_{B/reaction} \sim 2$–3% between 59.5 and 62 °C to $\mathrm{Rec}_{B/reaction} \sim 6.5\%$ at 65.1 °C (Figure 6B). Thus, increasing temperature accelerated the precipitation of α-LA, its recovery yield, and the rate of production of the desired aggregate fraction, but its purity degraded above 62 °C due to acceleration of the precipitation of β-LG.

Effects of pH. At constant temperature, reducing the pH of the WPI solution by increasing the SCO_2 pressure generally helped destabilize both the α-LA and β-LG proteins and increased their rates of precipitation. Figures 7 and 8 present the effects of pH reduction (pH 5.0, 4.8, and 4.6) on the precipitation of α-LA and β-LG from 10% WPI solutions at $T_R = 60$ °C (Figure 7) and 65 °C (Figure 8).

The acceleration of α-LA precipitation with reduced pH (Figures 7A and 8A) is useful to increase the production yield of the α fraction at shorter residence times or to obtain greater α-LA recovery yields at constant times. The adverse effect is that at both 60 and 65 °C, the rate of β-LG precipitation accelerated more rapidly with reduced pH than that of α-LA (Figures 7B and 8B), thereby decreasing the purity and ratio α/β of the resulting α fractions.

Studies of the acid fractionation of WPI with HCl showed that both the optimal aggregate yield and optimal aggregate composition, in the range of 60–65 °C, were obtained between
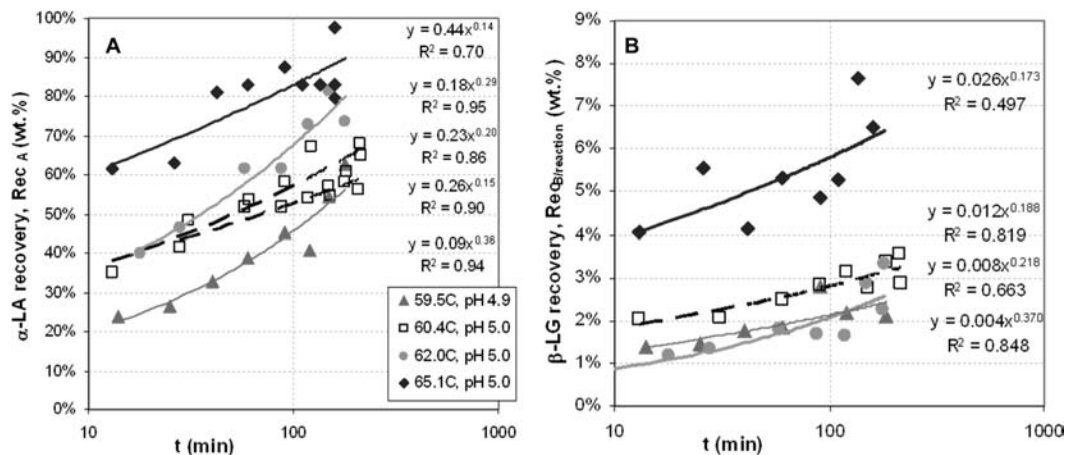

Figure 6. Aggregation of α-LA and β-LG as a function of time and temperature in 10% WPI solutions at pH 4.9–5.0 ($P_R = 5.5$–9 MPa) and $T_R = 59.5, 60.4, 62,$ and 65.1 °C.
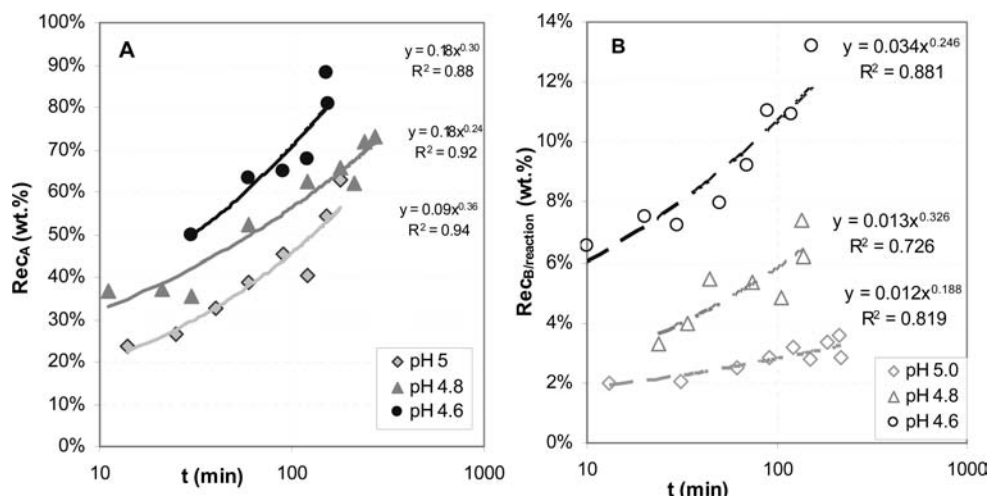
Figure 7. Effect of pH on the recovery yields of α-LA and β-LG in the aggregate fraction for 10% WPI solutions treated with SCO_2 at 60 °C.
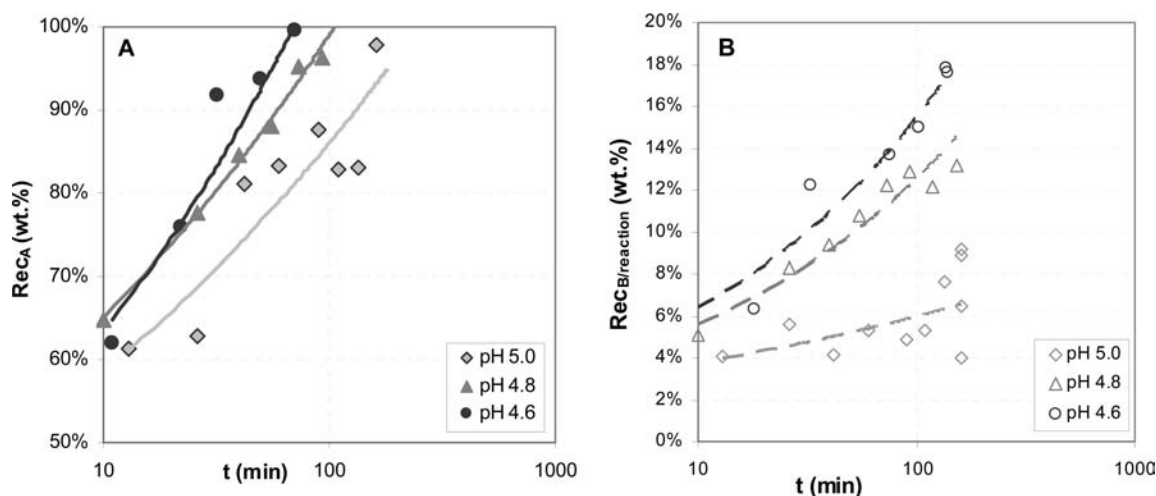

Figure 8. Effect of pH on the recovery yield of α-LA and β-LG in the aggregate fraction for 10% WPI solutions treated with SCO_2 at 65 °C.

pH 4.0 and 4.2 after 60 min of reaction, when the aggregation rate of α-LA was maximized without significantly accelerating β-LG precipitation,[16] thereby increasing the ratio α/β of the α fraction.

Effects of WPI Concentration. According to eqs 1 and 2, pH 4.56 was the lowest value reached with 10% WPI solutions and a maximum operating pressure of 34 MPa. Lower pH values of ~4.4 and ~4.2 were reached by using SCO_2 pressures of 31−34 MPa and reducing the concentration of the WPI solutions to C = 5 and 2% WPI, respectively. Further concentration reductions to obtain pH values around 4.1−4.0 were considered to be impractical because the very small mass of precipitate collected in the samples would lead to large experimental errors during mass difference measurements. Data scattering with C = 2% WPI being already consequent, lower values of C were not employed in this work. Figure 9 shows the evolution of the precipitation rates of α-LA and β-LG as a function of time and temperature at pH 4.6, 4.4, and 4.2 using solutions containing C = 10, 5, and 2% WPI, respectively.

At all values of pH, the rates of aggregation of both α-LA and β-LG increased significantly with temperature, and the recovery of β-LG in the aggregate fraction accelerated more quickly than that of α-LA, resulting in a noticeable decrease of purity of the α fraction from 60 to 65 °C.

At constant C, prior HCl fractionation studies[16] have shown that the aggregation rate of α-LA accelerates with reduced pH between pH 4.6 and 4.0, whereas that of β-LG did not vary much. Under constant SCO_2 pressure (~32 MPa), the rates of aggregation of both α-LA and β-LG decreased significantly with reduced pH at all temperatures, demonstrating a concentration dependence that is strong enough to reverse the expected trend. This effect indicates that the order of the SCO_2-driven aggregation reaction must be >1 for both proteins. Hinrichs et al.[54,55] found that the thermal- and pressure-induced denaturations of α-LA and β-LG at 60−65 °C have reaction orders of 2.5 and 2.0, respectively, in milk[54] and orders of 2 and 3 in WPI solutions;[55] however, they employed hydrostatic pressures about 20−40 times greater (P = 200−800 MPa, no CO_2) than those used in this study (5.5−34 MPa CO_2). Tomasula and Yee[28] utilized pressurized nitrogen to show that, up to 21 MPa, pressure alone did not trigger any whey protein precipitation; however, antisolvent effects of SCO_2 are possible and will be examined in future work.

Many studies were performed regarding the thermal denaturation kinetics of α-LA and β-LG from milk, whey, or WPC at neutral pH, which mostly agree and attribute reaction orders of 1.5 for β-LG and 1.0 for α-LA between 70 and 150 °C.[52,53,56,57] Another study at pH 5.2 and 80 °C found similar
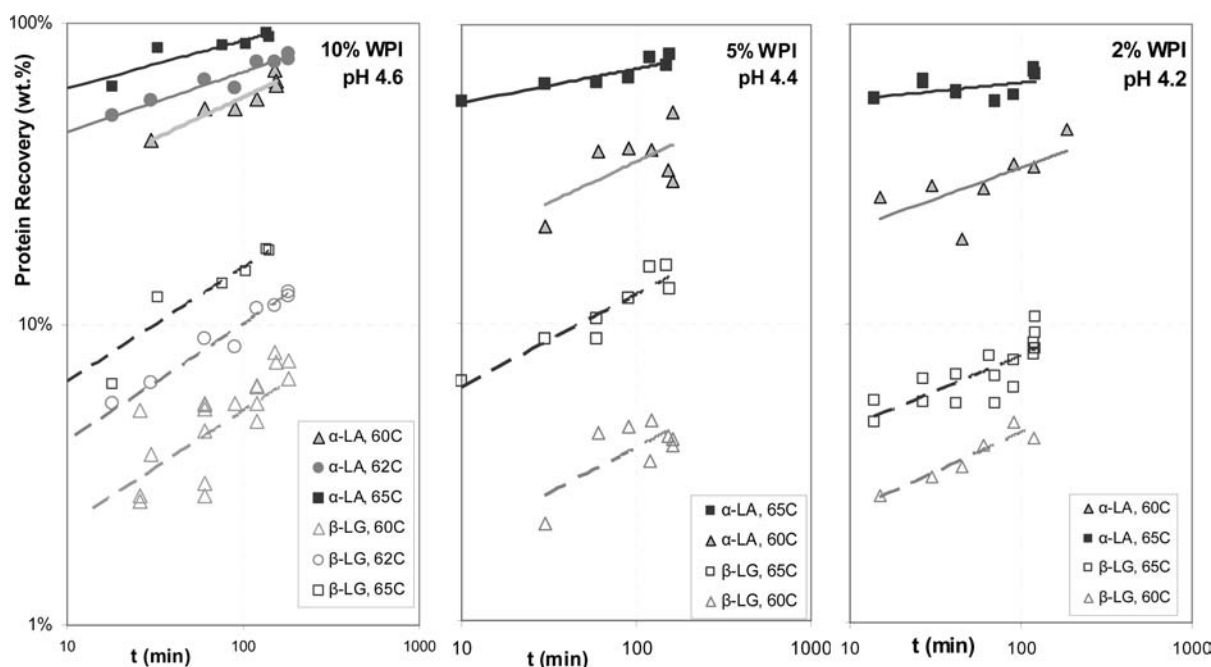
Figure 9. Kinetics of α-LA and β-LG aggregation at 60, 62, and 65 °C and pH 4.6, 4.4, and 4.2 during SCO_2 treatment of WPI solutions with concentrations C = 10, 5, or 2 wt % WPI.

results.[58] However, values for the orders of the aggregation reactions of α-LA and β-LG at 60−65 °C as a function of acidic pH (between pH 4 and 5) were not found in the literature, and therefore a necessary model is being designed in our laboratory to answer this question and will be presented in future work.

Optimal Results and Technical Challenges. The goal of this study is the parametric optimization of the pilot-scale SCO_2 fractionation process to design a large-scale, industrial whey protein fractionation process that is at the same time efficient and economical. Process efficiency is measured in terms of the purity of the two fractions produced or with their ratio α/β (low value for the β fraction, high for the α fraction), and the total aggregate yield, Y_{agg}, which is a function of the individual protein recovery rates, Rec_A and Rec_B. The economy of the scaled-up process will be a complex function of all the processing parameters and more. For example, water usage will depend mostly on the dilution of the starting WPI (spray-dried or liquid; liquid WPI can contain up to 30 wt % solids after ultrafiltration); CO_2 usage will depend on the desired pH, a function of P_R and C, and may be costly unless it is recycled;[59] utility usage to produce heat, to pump and compress CO_2, and to centrifuge and dry the products will depend highly on C, T_R, P_R and the residence time, t_R; the total production rates (e.g., in kg/h) will depend mostly on t_R and C; and the capital costs will be greatly altered by P_R and t_R, both of which affect the design and dimensions of the entire process and equipment, particularly the high-pressure reactor.[29]

Table 1 presents the results of triplicate experimental runs that produced α and β fractions with the highest purities. Table 1 illustrates some of the conflicting effects of the main processing parameters, C, T_R, P_R, and t_R, on the efficiency and economy of the SCO_2 fractionation process and some of the technical challenges faced in optimizing the process. As a whole, β fractions contained as little as 5% (w/w) α-LA and as much as 74% β-LG, with 18−32% GMP, whereas α fractions contained as little as ~2% GMP and 21% β-LG and as much as 62% α-LA.

The highest β fraction enrichment (rows 4 and 7), with ratio α/β = 0.067−0.102, up to 6 times lower than the starting WPI (0.37), was obtained at high WPI concentration (C=10%), high pH (4.8−5), high temperature (65 °C), and long residence time to maximize the recovery of α-LA in the aggregate fraction and, therefore, remove most of it from the β fraction, while moderating the aggregation of β-LG. Even though a fair amount of β-LG coprecipitated with α-LA ($Rec_{B/reaction}$ = 7−12%) or was entrapped in the precipitate via water holding (~15%), >70% of the β-LG was recovered in the β fraction, whereas the α fraction possessed both a high yield (32−37%) and a good purity (ratio α/β ~ 1.44).

On the other hand, the highest α fraction purity, with ratio α/β = 2.84, was obtained at low WPI concentration (C = 2%), low temperature (60 °C), low pH (4.2), and a long residence time of 120 min (row 1). High α fraction purities (ratio α/β = 2.48 and 2.37) were also obtained at C = 2% and T_R = 65 °C and C = 5% and T_R = 60 °C (rows 2 and 3). The main common factor was that low concentrations produced smaller volumes of wet aggregates compared to C = 10% ($Y_{agg/wet}$~2% when C = 2 and ~6% when C = 5%), which significantly reduced the amount of β-LG entrapped in the α fraction via waterholding. However, processing dilute WPI solutions implies using larger quantities of water, as well as longer residence times due to slower kinetics; therefore, a much larger reactor and other equipments will be needed, and considerably more energy will be used to both heat the solution and dry the β fraction at constant production rates.[29]

Using 10% WPI solutions, ratios of α/β of the α fraction were as high as 1.92 at pH 5 and low to medium T_R (60−62 °C) (rows 5 and 6). Increasing T_R to 65 °C (row 7) accelerated β-LG aggregation more than α-LA while also considerably increasing the volume of wet aggregates and the amount of β-LG entrapped by water holding, thereby raising the total β-LG recovery yield and reducing the purity of the α fraction (ratio α/β = 1.44). The same trend was observed at pH 4.6, where raising T_R from 60 to 62 °C and then 65 °C consistently

Table 1. Selected Results of the Fractionation of WPI with SCO$_2$ as a Function of C, T_R, pH(C,P_R), and t_R: Aggregate Yield, Protein Recoveries, and Compositions and Enrichment Ratios of the α (Solid) and β (Liquid) Fractions[a]

	C (wt %)	T_R (°C)	P_R (MPa)	t_R (min)	calcd pH	$Y_{agg/wet}$ (wt %)	Y_{agg} (wt %)	composition of β fraction				recoveries of α fraction			composition of α fraction		
								α-LA (wt %)	β-LG (wt %)	1-Rec$_B$ (wt %)	ratio α/β	Rec$_A$ (wt %)	Rec$_B$ (wt %)	Rec$_{B/reaction}$ (wt %)	α-LA (wt %)	β-LG (wt %)	ratio α/β
0					~ 6.3			21	57	100	0.37	0	0	0	21	57	0.37
1	2	59.9	31.0	120	4.24	2.1	16.2 ± 1.2	11.6	63.2	93.5	0.156 ± 0.017	50.5	6.5	4.4	59.0	20.8	2.84 ± 0.01
2	2	64.8	31.1	120	4.24	2.0	19.9 ± 1.9	11.0	70.1	90.5	0.158 ± 0.038	65.1	9.5	7.4	62.5	25.2	2.48 ± 0.14
3	5	60.4	5.7	270	4.86	6.2	22.3 ± 0.9	8.4	67.2	88.6	0.126 ± 0.020	73.5	11.4	4.8	60.8	25.7	2.37 ± 0.13
4	10	65.0	11.7	150	4.83	15.2	37.0 ± 3.2	5.0	73.7	73.2	0.067 ± 0.004	96.5	26.8	11.6	53.1	37.1	1.43 ± 0.07
5	10	60.4	5.7	215	5.01	10.5	23.0 ± 1.1	11.2	65.1	87.3	0.172 ± 0.005	66.3	12.7	2.0	55.2	28.8	1.92 ± 0.03
6	10	62.0	5.9	180	5.00	10.9	25.7 ± 1.4	8.0	70.2	84.3	0.114 ± 0.011	77.4	15.7	4.5	56.3	30.7	1.92 ± 0.51
7	10	65.1	5.6	160	5.01	15.2	32.6 ± 1.1	6.9	67.3	77.1	0.102 ± 0.027	88.9	22.9	7.1	51.1	35.7	1.44 ± 0.19
8	10	60.0	32.1	26	4.58	9.3	18.0 ± 3.5	17.3	61.8	87.4	0.280 ± 0.001	41.2	12.6	3.5	45.6	37.6	1.21 ± 0.06
9	10	60.0	31.0	60	4.59	10.7	24.6 ± 0.6	16.5	60.5	84.5	0.273 ± 0.012	70.2	15.5	4.8	53.9	32.4	1.67 ± 0.15
10	10	60.0	29.8	120	4.60	11.2	26.9 ± 3.1	12.3	65.6	83.3	0.188 ± 0.021	74.6	16.7	5.5	53.9	32.9	1.65 ± 0.16
11	10	60.0	30.8	180	4.59	12.7	30.5 ± 0.9	9.2	67.7	80.3	0.142 ± 0.001	88.4	19.7	7.1	54.9	33.3	1.65 ± 0.02
12	10	62.0	32.3	120	4.58	13.3	31.7 ± 2.7	11.3	64.6	76.1	0.176 ± 0.006	80.4	23.9	10.6	49.1	39.3	1.25 ± 0.15
13	10	62.0	33.6	60	4.57	11.4	26.1 ± 2.7	12.2	63.4	81.7	0.193 ± 0.015	73.2	18.3	6.9	52.6	36.3	1.49 ± 0.48
14	10	65.0	32.4	120	4.58	17.1	43.4 ± 1.6	6.7	65.1	58.2	0.102 ± 0.008	82.7	41.8	24.8	36.1	49.5	0.73 ± 0.02
15	7	64.4	17.9	155	4.65	9.5	31.0 ± 1.9	8.4	68.1	79.9	0.125 ± 0.058	89.3	20.1	10.7	54.7	33.2	1.72 ± 0.48
16	10	64.8	29.6	140	4.60	15.9	39.6 ± 2.7	5.1	61.8	65.8	0.083 ± 0.008	94.5	34.2	18.9	46.0	45.0	1.02 ± 0.06
17	7	60.0	18.6	156	4.64	10.5	25.5 ± 3.6	10.5	67.2	82.4	0.157 ± 0.039	71.3	17.6	10.6	52.7	35.5	1.49 ± 0.08

[a]Row 0 corresponds to the starting WPI; C = concentration of the WPI solution; T_R, P_R and t_R = temperature, CO$_2$ pressure, and residence time in the reactor, respectively; $Y_{agg/wet}$ = wet aggregate yield; Y_{agg} = dried aggregate yield; Rec$_A$ and Rec$_B$ = recovery yields of α-LA and β-LG in the α fraction, respectively; Rec$_{B/reaction}$ = recovery of β-LG in the precipitate due to aggregation only; 1-Rec$_B$ = recovery of β-LG in the β fraction; ratio α/β = mass ratio of α-LA to β-LG in the fraction.

improved the aggregation rate of α-LA (rows 10, 12, and 14) and the total aggregate yield, but accelerated the precipitation and recovery of β-LG even more dramatically. This caused a sharp loss of purity in the α fraction with temperature: ratio α/β = 1.65, 1.25, or 0.73 at 60, 62, or 65 °C, respectively. However, higher operating temperatures have practical processing interests because the production rate of the protein fractions is improved and residence times are shortened owing to faster α-LA aggregation kinetics, leading to a higher productivity and lower equipment costs.

Shorter residence times will bring considerable equipment cost savings, as well as a greater rates of production of the protein fractions; on the other hand, we found that the purity of the products was enhanced with longer residence times at certain operating conditions, such as C = 10% WPI, T_R = 60 °C, and pH 4.6 (rows 8−11), where recovery of α-LA in the precipitate increased consistently with time and increased more rapidly than the total recovery of β-LG, resulting in progressive improvement of the purities of both fractions with time (ratios α/β = up to 1.65 in the α fraction and ratio α/β = as low as 0.14 in the β fraction).

Because of the high sensitivity of the kinetics of aggregation of α-LA and β-LG to time, T_R, C, and pH(C,P_R), optimization of the SCO_2 process will be complex due to the various conflicting effects on the purities and yields of the products and on the efficiency and economy of the process. A model for the aggregation kinetics of α-LA and β-LG versus C, P_R, and T_R, coupled with modeling and cost estimation of the SCO_2 fractionation process, is under study in our laboratory and will be necessary to mathematically optimize the operating conditions according to the desired yields and purities of the protein products and design a scaled-up or continuous version of the process.

Our new, clean whey protein fractions enriched up to 7 times with α-LA or up to 5 times with β-LG are ready-to-use in various health-promoting food applications, or can be post-treated further with washing and ultrafiltration to obtain purified α-LA, β-LG, and GMP.

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Notes

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