Scale-Up of Native β -Lactoglobulin Affinity Separation Process

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ABSTRACT

Affinity separation of β -lactoglobulin in its native form with all-trans-retinal immobilized on calcium bio-silicate was scaled up and applied to separate it from industrial sweet whey. Three different methods of mixing the modified calcium bio-silicate and whey for the interaction between all-trans-retinal and β -lactoglobulin were tried at pilot scale. The three methods used were 1) a column packed with calcium bio-silicate, 2) a stirred tank, and 3) a fluidized bed column of calcium bio-silicate particles. Adsorption and desorption of β -lactoglobulin were carried out at pH 5.1 and 7.0, using 0.01 and 0.1 M phosphate buffers, respectively. The phosphate buffer containing desorbed β -lactoglobulin was concentrated 20 times using ultrafiltration and then freeze-dried. The packed column, stirred tank, and fluidized bed column produced β -lactoglobulin with purity of 80, >95, and >95%, and recovery of 0.65, 2.88, and 2.88 g per kilogram of calcium bio-silicate, respectively. The comparative poor purity and recovery of β -lactoglobulin in the case of the packed column was attributed to insufficient contact between the passing fluids and the calcium bio-silicate during adsorption, desorption, and intermittent washing. The fluidized bed column method, with a gentle mixing action, was considered the best suited for further scale up to the industrial level. (**Key words:** β -lactoglobulin, affinity separation, process scale up)

Abbreviation key: FPLC = fast protein liquid chromatography, WPC = whey protein concentrate.

INTRODUCTION

In cow's milk, β -lactoglobulin is one of the two major whey proteins; the other is α -lactalbumin. β -Lactoglobulin exists primarily as a dimer in the pH range from 5.2 to 7.5. The molecular mass of the β -lactoglobulin dimer is 36,720 Da (Whitney, 1988). Besides high nutri-

tional value, this protein has some unique properties that are useful in food processing. It has an important function in heat stability of dairy products (Wong, 1989) and has excellent gelation properties (Hines and Foegeding, 1993). It binds hydrophobic or amphipathic molecules (e.g., free fatty acids and triglycerides), conjugated compounds (e.g., retinol), and macromolecules (e.g., milk and nonmilk proteins) (Hambling et al., 1992). Many reports on the binding of different vitamins (vitamins A, D, E, and K) to β -lactoglobulin are found in the literature (Wang et al., 1997a, 1997b; Allen et al., 1999). Because of its unique vitamin binding properties, β -lactoglobulin may be used as a carrier of these fat-soluble vitamins in low fat or fat-free foods (Anon, 1999).

Different methods for separating β -lactoglobulin from the bovine whey were reported as early as 1957 (Aschaffenburg and Drewry, 1957). Primarily, the lowering of pH of the whey with or without heating has been used to cause precipitation of all whey proteins other than β -lactoglobulin (Aschaffenburg and Drewry, 1957, Pearce, 1983). Alternatively, a combination of FeCl_3 addition and pH reduction has been found to precipitate β -lactoglobulin, leaving α -lactalbumin in the supernatant (Kaneko et al., 1985, Kuwata et al., 1985). Polyphosphates (e.g., sodium hexametaphosphate), commonly used as food ingredient, can be used in place of FeCl_3 so that the antimicrobial activity of lactoferrin in the β -lactoglobulin depleted bovine milk or whey is not adversely affected by the presence of iron (Al-Mashikh and Nakai, 1987). Maillart and Ribadeau-Dumas (1988) reported separation of β -lactoglobulin from ultrafiltration retentates of acid as well as rennet whey by NaCl salting out at low pH. Konrad et al. (2000) recently reported four different methods for purifying β -lactoglobulin from whey or reconstituted whey protein isolates. These methods involved precipitation of all whey proteins other than β -lactoglobulin using TCA or HCl or pepsin. All the methods mentioned above denature either the β -lactoglobulin itself or the other proteins in the system. Significantly, Wang and Swaisgood (1993) using the ability of β -lactoglobulin to retinal developed a laboratory-scale method for affinity purification of native β -lactoglobulin from bovine whey with-

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out denaturing the other proteins present. They used all-trans-retinal immobilized on calcium bio-silicate for their study. De John et al. (2001) also have recently reported a method for separating native β -lactoglobulin by an ion-exchange method.

In the present study, the process of affinity separation of β -lactoglobulin developed by Wang and Swaisgood (1993) has been scaled up and mass balancing performed with an aim of evaluating its applicability at industrial level. Finally, using the results of the present study a process strategy for industrial scale is suggested. The aim of this scale-up process was to obtain β -lactoglobulin from whey in its native form and not necessarily to produce a β -lactoglobulin free product.

MATERIALS AND METHODS

Materials

All-trans-retinal covalently immobilized on calcium bio-silicate (Celite Corporation, Lompoc, CA) was prepared by the Wang and Swaisgood method (Wang and Swaisgood, 1993). Industrial cheese whey was obtained from the pilot plant of the Dairy Products Technology Center, California Polytechnic State University. The whey was clarified in a centrifugal separator (model 340, De Laval Separator Co., New York) in the pilot plant. The pH of the whey was then adjusted to 5.1 with 1 N HCl. NaH_2PO_4 and NaHPO_4 (Fischer Scientific, Pittsburgh, PA) and deionized water were used to prepare the 0.01 M (at pH 5.1) and 0.1 M (at pH 7.0) phosphate buffers used for adsorption and desorption of β -lactoglobulin, respectively. Standard β -lactoglobulin for the comparison purpose was obtained from Sigma (St. Louis, MO).

Methods

The general steps followed for the β -lactoglobulin separation process at laboratory as well as pilot scale are shown in Figure 1. The period for each step was optimized using the results of the preliminary experiments. The last step of rinsing of the column and storing in 20% ethanol was not followed when the column was to be used for further experiments on the same day. In the case of the subsequent experimental runs done on the same day as the postdesorption rinsing step also served as the first step of activating the calcium bio-silicate particles, and, therefore, the process was reduced to four steps that were completed in 60 min.

Laboratory-Scale Operation

The laboratory-scale process reported by Wang and Swaisgood (1993) was repeated using fast protein liquid

chromatography (FPLC; Bio-Rad, Richmond, CA). A fiberglass cylindrical 10-ml column (MT10, BIORAD) was packed with the treated calcium bio-silicate and used to separate β -lactoglobulin from 40 ml of clarified whey. The whey was passed (single pass) through the column at 2 ml/min flow rate. Three different designs were used in the scale-up study as shown in Figure 2.

Pilot-Scale Operation

In the case of the first design, a packed column, a stainless steel cylindrical column (length = 29 cm, diameter = 15.5 cm) was packed with 2.5 kg of retinal-treated calcium bio-silicate particles. The feed entered from the top and exited after passing through the column at the bottom. In the second design, the stirred tank, 10% of the volume of the stainless steel tank was filled with the treated calcium bio-silicate particles (1.85 kg). A stainless steel agitator operated at ~45 rpm to keep the calcium bio-silicate particles well suspended in the fluid. In the third design, the fluidized bed column, two-third volume (66%) of fiber glass column (length = 28 cm, diameter = 7.2 cm) was filled with retinal treated calcium bio-silicate (0.345 kg), and the feed entered from the bottom and left at the top end of the column. The circulation flow was so adjusted that the calcium bio-silicate particles remained suspended in the fluid. The fluid in the case of both the packed column and the fluidized bed column was circulated with help of a variable speed twin lobe rotary pump (160M, Waukesha, WI).

Product Purification and Analyses

The resultant phosphate buffer at pH 7.0 containing desorbed β -lactoglobulin was then concentrated (20 times volume reduction) by ultrafiltration with a 10-kDa polysulfone membrane at 100-kPa transmembrane pressure. Diafiltration was carried out using six lots of DI water, each equal to original volume of the phosphate buffer during the concentration step. The concentrated and purified β -lactoglobulin was then dried using a freeze dryer (Lyphlock 4.5, Labconco Corp., MO). The finished product was analyzed for total solids and ash contents by the standard gravimetric method, for total protein using the standard Kjeldahl's method and for purity using 15% SDS-PAGE (Laemmli, 1970; Kim and Jimenez-Flores, 1995). Brilliant blue R (Sigma Chemical Co.) was used to stain the gels. The cheese whey was analyzed for the TS, ash, protein, and fat (Babcock method) content, and for β -lactoglobulin content using 15% SDS-PAGE. The treated calcium bio-silicate was analyzed for the residual proteins by Kjeldahl's method and 15% SDS-PAGE after postdesorption rinsing with

Figure 1. Flowchart of the β -lactoglobulin separation process used in the present study.

Figure 2. Schematic presentation of the three methods (a) packed column and (b) stirred tank for batch operation and (c) fluidized bed column for continuous operation used for the experiments.

phosphate buffer. Each experimental run was carried out at room temperature (22 to 25°C) in triplicate, and the chemical analyses for each run were done in duplicate and the results averaged.

Mass Balance

Fluidized bed column was used for the mass balance study. Four liters of clarified whey was circulated through the column and four cycles of the adsorption-desorption process were carried out using the same whey. More than one process cycle and this small volume of whey were chosen for accuracy in mass balance determination. The amounts of total protein and β -lactoglobulin present in the whey, treated whey, and finished product were calculated based on the total protein and β -lactoglobulin contents in these samples determined by Kjeldahl's method and SDS-PAGE as mentioned earlier.

RESULTS AND DISCUSSION

The composition of the clarified cheese whey used for β -lactoglobulin purification based on the results of the analyses is shown in Table 1. Laboratory-scale operation using FPLC was at a pressure of ~400 kPa. In comparison, the pilot-scale operations were at lower pressure. The packed column operated at 50 kPa, and the operations using stirred tank and the fluidized bed

Table 1. Composition range of the clarified cheese whey used for β -lactoglobulin purification.

Constituent	Content (%)
Total solids (TS)	6.0–6.3
Fat	0.15–0.2
Protein	0.7–0.75
Ash	0.55–0.62
pH	6.1–6.2

column were at or nearly at atmospheric pressure. The presence of β -lactoglobulin along with some α -lactalbumin was detected in the finished products prepared at the laboratory scale as well as at pilot scale when they were analyzed using SDS-PAGE. No changes in the SDS-PAGE protein profiles were observed in the protein profile of the whey following a process cycle of adsorption and desorption after process scale runs. However, when four cycles of the process were carried out using the same whey, while operating with fluidized bed column, a clear decrease in the concentration of β -lactoglobulin could be noticed. However, the concentration of α -lactalbumin also decreased, although to a lesser extent. The protein profiles of the whey as well as the finished product after each cycle are shown in Figure 3.

The concentration of β -lactoglobulin in the finished product resulting from each cycle was similar. A very small amount of α -lactalbumin was present in these finished products. However, other high molecular weight proteins and some peptides present in the whey did not appear in the finished product. The change in the ratio of β -lactoglobulin to α -lactalbumin with each

process cycle calculated from the SDS-PAGE results is shown in Figure 4. A preferential removal of β -lactoglobulin during this process is clearly evident from the decrease in this whey proteins ratio with each process cycle.

The gross compositions of the freeze-dried finished products obtained by three different pilot-scale designs when concentrated without diafiltration are shown in Figure 5. Products from both stirred tank and fluidized bed column were very similar in gross composition with ~55% total proteins and ~43% ash. But the product from the packed column had very low (~14%) total protein and high ash content, suggesting poor recovery of β -lactoglobulin. The presence of ash in all the samples was due to the phosphates in the buffer used for desorption of β -lactoglobulin. The moisture content in all the products was about 2%. The ash content in the products could be reduced to <10% when diafiltration was done during concentration by ultrafiltration. This in turn increased the total protein content to ~90% in the finished products. The effect of diafiltration on the gross composition of the finished product in the case of stirred tank and fluidized bed column is shown in Figure 6. Due to the low protein recovery, studying the effect of diafiltration was not considered in the case of the product obtained by packed column.

The levels of purity (in terms of % β -lactoglobulin of total proteins) of the final products obtained at laboratory scale as well as in the case of the three pilot-scale designs are shown in Figure 7. The products obtained from laboratory experiments as well as the stirred tank and fluidized bed column were >95% pure. However, the purity of the product obtained from the packed col-

Figure 3. SDS-PAGE showing 1) standard β -lactoglobulin, 2–5) reduction in protein concentration of whey with number of β -lactoglobulin recovery attempts from 0 to 3 using fluidized bed column, 6–8) β -lactoglobulin recovered.

Figure 4. Change in the ratio of β -lactoglobulin/ α -lactalbumin in the whey using fluidized bed column.

umn was ~80%, i.e., about 15% lower than that obtained from other designs. The recovery of β -lactoglobulin (in terms of g of protein/kg of calcium bio-silicate particles) for different pilot- and laboratory-scale experiments is shown in Figure 8. The recovery was 2.88 g/kg in the case of stirred tank (± 0.085) and fluidized bed column (± 0.032), very similar to that obtained at laboratory scale. However, once again, the performance of packed column was lowest with recovery of 0.65 (± 0.062) g/kg.

Lower purity and recovery clearly suggests that although successfully used at the laboratory scale, packed column is not a suitable design for the larger scale operation. Poor performance is attributed to the insufficient contact between the passing fluids and the immo-

Figure 5. Gross composition of the freeze-dried final products obtained without diafiltration using three pilot-scale designs.

Figure 6. Gross composition of the freeze-dried final products obtained with diafiltration using two of the pilot-scale designs.

bilized retinal resulting from the improper distribution of the entering fluid over the cross section of the packed column and tight packing of the calcium bio-silicate particles (Perry and Green, 1997). These problems were not encountered in the case of the laboratory-scale operation with the packed column using FPLC, probably because of comparatively very small size of the column and the higher pressure of operation (~400 kPa compared with ~50 kPa at pilot scale). The performances of the stirred tank and the fluidized bed column in terms of purity and recovery of β -lactoglobulin were almost identical and significantly also at par with the performance of laboratory-scale operation. However, some breakage of the calcium bio-silicate particles was observed during the stirred tank operation likely to be

Figure 7. Purity of the finished products (% β -lactoglobulin of total proteins) for different methods used in pilot- and laboratory-scale operations.

Figure 8. Recovery of protein per kilogram of calcium bio-silicate particles for different methods used in pilot- and laboratory-scale operations.

due to the moving agitator used to keep the calcium bio-silicate particles in the suspension. In comparison, the action of the fluidized bed column was very gentle. That the stirred tank is a batch operation, whereas the fluidized bed column is a continuous operation, should also be considered. Therefore, the fluidized bed column is considered the most suitable out of the three designs studied for the scale up of the affinity separation process.

Considering the fluidized bed column as the most suitable for the scale up, mass balancing was carried out for the protein using the results obtained from the study of four process cycles using the same 4 L whey for this design. The results of mass balance are shown in Table 2. Out of the total proteins in the whey, 13.7% was recovered and 83% was detected in the treated whey. But a deficit of ~3.3% (~1 g) protein was noticed for all the replications. This puzzle was solved when the calcium bio-silicate particles were analyzed for total N after the postdesorption rinsing by Kjeldahl's method and the identity of the protein residue by SDS-PAGE. Mainly, α -lactalbumin was attached to the calcium bio-silicate particles. The attached α -lactalbumin was removed from the particles by thorough rinsing with phosphate buffer and 20% methanol. About 46% of the total β -lactoglobulin was recovered in 4 L of whey after four cycles of the process. The rest of this protein remained in the treated whey.

Figure 9. Schematic of the suggested process design for the industrial-scale operation (A = adsorption buffer tank, C₁ to C₆ = fluidized bed columns, D = desorption buffer tank and W = whey tank).

De John et al. (2001) have recently reported a method for separating native β -lactoglobulin from whey protein fraction (acid casein whey at pH 4.4 to 4.5) by binding at pH 7.2 using diethylaminoethyl Sepharose and eluting at high ionic strength. However, the binding of the protein in their method requires overnight stirring at 4°C. This may present some limitations for industrial-scale operations. Also the protein eluted using 0.25 M NaCl solution is expected to contain very high NaCl when dried despite the concentration by ultrafiltration unless also extensively diafiltered. In comparison, in the Wang and Swaisgood method, the binding step lasts for only 20 min, and a much lower ionic strength (0.1 M) phosphate buffer is used for eluting the protein.

DESIGN RECOMMENDATIONS FOR FURTHER SCALE UP

We suggest a design comprising a battery of four columns connected in parallel for the industrial-scale operation. The schematic of the suggested design is shown in Figure 9. This suggested design is based on the fact that a brief contact between the whey and calcium bio-silicate particles, as in the case of passing the whey only once through the fluidized bed column,

Table 2. Mass balance of the protein for fluidized bed column operation.

Component	In 4 L whey (g)	Recovered in the finished product (g)	In the treated whey (g)
Total proteins	29.00 ± 0.96	3.98 ± 0.023	24.07 ± 0.92
β -lactoglobulin	8.25 ± 0.34	3.80 ± 0.022	4.45 ± 0.385

removes a very small quantity of β -lactoglobulin. Note that only native β -lactoglobulin binds to the media and although this reaction is fast, the efficiency of adsorption can be regulated by the flux through the column. Therefore, the effect of this treatment on the protein profile of the whey is not significant. Using this fact, the whey stored in a tank after its production can be passed through a fluidized bed column once, and then the treated whey can be sent for the regular whey processing, i.e., manufacture of whey powder or WPC or whey protein isolate, milk calcium or minerals and lactose. Whey flows through first three columns one after another for 20 min each (i.e., adsorption step). Once adsorption is over for the first column and the whey is diverted to the second and then to the third column, the remaining three steps of the process (see Materials and Methods) i.e., 1) rinsing for 10 min, 2) desorption for 20 min, and 3) rinsing for 10 min can follow for the first column. Thus, with a three-column system, a continuous isolation of native β -lactoglobulin as well as an uninterrupted flow of whey to the subsequent whey processing can be ensured. The fourth column works as a standby.

CONCLUSIONS

The process of affinity purification isolates native β -lactoglobulin (>95% pure) without precipitating it or causing any structural damage to other whey proteins. Only the native β -lactoglobulin is adsorbed during this process. Some α -lactalbumin is also removed from the whey during the process, most of which attaches to the surface of calcium bio-silicate particles, and a very small amount is desorbed along with β -lactoglobulin. Out of the three methods studied at pilot scale, the fluidized bed column method is considered best suited for industrial-scale operations.

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