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Purity and Yield of β-Lactoglobulin Isolated by an *N*-Retinyl-Celite Bioaffinity Column

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A bioaffinity column of *all-trans*-retinal immobilized on Celite was capable of isolating high-purity (94.5%) β-lactoglobulin from bovine acid whey. Conditions for producing a potentially hypoallergenic reduced β-lactoglobulin whey were investigated. Reapplication of pH 5.1 eluate to the column resulted in a final purity of 87% α-lactalbumin. The purity of β-lactoglobulin was slightly lower upon elution with buffers containing <0.4 M sodium phosphate, whereas the yield from desorbing buffers <0.1 M decreased to approximately 40% of that obtained with 0.4 M sodium phosphate. Desorption with low phosphate concentration was improved when pH was increased, suggesting that desorption involves titration of a protophilic group on β-lactoglobulin. These findings suggest that the retinal matrix shows promise in its application for creating hypoallergenic products and the isolation of high-purity β-lactoglobulin with useful functional properties.

Keywords: *β-Lactoglobulin; α-lactalbumin; bioselective adsorption; N-retinyl-Celite*

INTRODUCTION

Bovine β-lactoglobulin (β-LG) comprises approximately 45% of the protein present in whey. A sulfhydryl group buried within the native structure is exposed at temperatures used for processing dairy products (Swaisgood, 1989). Sulfhydryl−disulfide interchange and exposure of hydrophobic residues may lead to possible formation of large complexes with α-lactalbumin (α-LA) and serum albumin and interaction with casein micelles, affecting the heat stability of milk and dairy products. The functional properties of β-LG, including its role in the heat stability of milk products (Wong, 1989) and its gelation properties (Hines and Foegeding, 1993), make β-LG a useful food ingredient.

β-LG is also a primary allergen in bovine milk (Enomoto et al., 1993; Huang et al., 1985; Taylor, 1986). At least four antigenic sites can induce antibody production (Takahashi et al., 1990; Otani et al., 1987). Strategies for reduction of the allergenicity of β-LG have included enzymatic hydrolysis (Nakamura et al., 1993), thermal processing aimed at denaturation of the protein (Baldo, 1984; McLaughlin et al., 1981), and chemical modification of residues (Otani et al., 1987). All of these approaches have shortcomings, so an efficient method for separation of β-LG and α-LA would provide food ingredients enhanced by the excellent nutritional and functional properties of each protein. Ion-exchange columns that are currently used for commercial processing of whey proteins can result in products with some enrichment of β-LG or α-LA, but high-purity products of these proteins for food use are generally not produced on a large scale. Development of a solid-phase matrix material that could selectively bind and later desorb one or more of the major whey proteins could be a feasible method for large scale separation of β-LG and α-LA.

Among the unique properties of β-LG is its ability to bind small hydrophobic molecules such as retinol (Monaco et al., 1987) with high affinity. β-LG exhibits a similar high affinity to immobilized retinal (Jang and Swaisgood, 1990). Recently, Wang and Swaisgood

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(1993) examined conditions for adsorption and desorption of β-LG to immobilized *trans*-retinal and showed that this solid-phase matrix can selectively remove β-LG from mixtures of β-LG and α-LA in phosphate buffer. The present study expands upon the research of Wang and Swaisgood (1993). The objectives were to demonstrate purification of β-LG from acid whey, to establish conditions for maximal extraction of β-LG to produce β-LG of high purity and a reduced β-LG whey product, and to determine the lowest ionic strength conditions that would cause desorption of β-LG from the immobilized *N*-retinyl-Celite bioaffinity column.

MATERIALS AND METHODS

Materials. Celite beads (R648, 50/100 mesh) were a gift from Celite Corp., Lompoc, CA. Reagent grade or higher chemicals (Fisher Scientific, Raleigh, NC) and deionized reverse osmosis water were used throughout the study. Milk for use in this project was provided by the North Carolina State University Process and Applications Laboratory.

Immobilization of *N*-Retinyl-Celite. Celite beads were silanized and prepared according to the procedure previously described (Wang and Swaisgood, 1993). Free amino groups derivatized to the beads were quantified using the method of Janolino and Swaisgood (1992) and were present at a concentration of 8.0 μmol/mL. Thirty grams of silanized beads was treated with *trans*-retinal (426 mg in 750 mL of methanol) for 20 h at room temperature (23 °C), followed by the addition of 942 mg of $NaCNBH_3$ (Aldrich Chemical Co., Milwaukee, WI) for the reduction of the Schiff base. The beads were incubated for 7 h at room temperature with periodic shaking and then washed extensively with methanol, followed by acetone, to remove excess *trans*-retinal and reductant. The beads were then air-dried and stored in the dark. The immobilization procedure and washing and drying steps were conducted under low light intensity to minimize the likelihood of isomerization of the *trans*-retinal.

Continuous Elution Chromatography of β-Lactoglobulin. Thirty grams of *N*-retinyl-Celite beads was packed into a 2- × 34-cm glass column that was incorporated into a low-pressure chromatography system. The same preparation of packing material was used for the entire study. (Bioselective affinity columns of *N*-retinyl-Celite have been used repeatedly in our laboratory for more than 1 year with no apparent loss of ligand or activity.) The column was equilibrated with approximately 4 column volumes of 0.05 M sodium phosphate buffer (ICN Biochemicals, Aurora, OH), pH 5.1, before the application of acid whey, pH 5.1. Acid whey was prepared by adjusting the pH of pasteurized, homogenized skim milk to 4.6, followed by centrifugation at 3000*g* for 15 min. The pH of the whey was then raised to 5.1 for optimal adsorption of β-LG to the *N*-retinyl-Celite moiety (Wang and Swaisgood, 1993). A single preparation of acid whey was subdivided for loading onto the column in repeated trials. Data shown are from a single trial representative of additional but less complete studies.

To separate β-LG from acid whey, the column was equilibrated with 0.05 M sodium phosphate buffer, pH 5.1, prior to the addition of approximately 200 mL of acid whey, pH 5.1. Up to 200 mL of the acid whey fractions exiting the column was pooled and saved for reapplication. After loading of the whey was complete, desorption of β-LG was accomplished using 0.4 M sodium phosphate buffer (ICN Biochemicals), pH 7.0. The efficiency of removal of β-LG from acid whey was examined by reapplication of pH 5.1 whey eluant collected from the column between initiation of loading and the return to baseline on a 280 nm UV detector (Econo UV Monitor, Bio-Rad, Hercules, CA) monitoring column eluant. The pH 5.1 eluant was reapplied a total of six times, with reequilibration and desorption of β-LG between each reapplication step.

To examine the purity and yield of β-LG eluted from the column at lower ionic strengths, 200 mL of acid whey, pH 5.1, was loaded onto the column. The column was then washed with 0.05 M sodium phosphate, pH 5.1, until a baseline was established on the UV monitor. This was followed by elution with 0.02, 0.04, 0.06, 0.08, 0.1, 0.2, 0.4, 0.6, 0.8, and 1.0 M sodium phosphate buffers (pH 7.0). Buffers and acid whey were sterilized by passing through 0.2 μm filters (Gelman Sciences, Ann Arbor, MI) prior to their application to the column. The column was washed with the appropriate buffer following each elution until a baseline absorption at 280 nm was reached. A flow rate of 1.0 mL/min was used for loading and elution of β-LG from the column, with absorbance monitored at 280 nm.

Gel Filtration Fast Performance Liquid Chromatography (FPLC) Analysis. Fractions collected from the *N*-retinyl-Celite column were analyzed by gel filtration FPLC using a Superose 12-HR 10/30 FPLC column (Pharmacia LKB Biotechnology, Piscataway, NJ) incorporated into a Pharmacia HPLC system with a 50 μL injection loop, Pharmacia P-500 pump for solvent delivery, and a UV-1 single-path UV monitor reading absorption at 280 nm. Samples were analyzed in 20 mM Tris/0.1 M NaCl, pH 7.0, with 0.02% NaN_3 added as an antimicrobial, at a flow rate of 0.5 mL/min. Standards of β-LG and α-LA (Sigma Chemical Co., St. Louis, MO) were obtained for the purpose of calculating the purity and yield of eluates from the bioaffinity column. Calculations of purity did not include contributions from proteose peptones, bovine γ-globulins, or bovine serum albumin because concentrations were not significant.

Electrophoresis of Eluted Fractions. Fractions eluted from the bioaffinity column were analyzed using discontinuous sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS−PAGE). Stacking gels contained 1.25 M Tris·HCl, pH 6.8, 5.0% polyacrylamide, and 1.0% SDS, while resolving gels contained 1.875 M Tris·HCl, pH 8.8, 15% polyacrylamide, and 1.0% SDS. The electrode buffer contained 0.02 M Tris·HCl, 0.2 M glycine, and 0.1% SDS, pH 8.3. Samples and purified protein standards (0.3 mg/mL) were each mixed with loading buffer (4:1 v/v) containing 0.12 M Tris·HCl, 4% (w/v) SDS, 0.7 M β-mercaptoethanol, 30% (v/v) glycerol, and 0.02% (w/v) bromophenol blue. Each lane was loaded with 15 μL of sample in loading buffer. Electrophoresis equipment consisted of a dual minivertical gel electrophoresis unit (E-5889 mini, Sigma-Aldrich Techware, St. Louis, MO) run at 25 mA for approximately 1.5 h. Gels were stained with Coomassie Brilliant Blue (CB-R250, Bio-Rad Laboratories, Richmond, CA).

RESULTS

Reduction of β-Lactoglobulin Concentration in Acid Whey. The reapplication of acid whey eluant to the *N*-retinyl-Celite column resulted in a 2.3-fold increase (from 37.7 to 87.0%) in the purity of α-LA, as represented by the fractional concentration, α-LA/(α-LA + β-LG) (Table 1). Applying the pH 5.1 fraction to the column the seventh time did not result in an appreciable increase in the ratio of α-LA to total protein (data not shown). As the concentration of β-LG in the fractions decreased, the range of values of the purity of β-LG also decreased from an initial median value of 94.5% to a final value of 30.5%. This indicates that while the *N*-retinyl moiety has a strong affinity for β-LG due primarily to hydrophobic interactions, the binding is not entirely specific. The interaction also appears to be subject to mass action effects, in which α-LA outcompetes β-LG for binding sites at higher α-LA to β-LG ratios. It is also likely that the β-LG that did not bind after repeated passage through the column had the hydrophobic binding site already occupied by retinol or other hydrophobic molecules. The highest purity of β-LG was found in the eluate from the first application of whey to the column, in which the median purity was 94.5%, with a yield of 164 mg of β-LG collected in a total of 50 mL, as determined by gel filtration FPLC. The percentage of total protein recovered within each application or reapplication ranged from 71 to 98%.

Table 1. Reduction of the Concentration of β-Lactoglobulin in Acid Whey, pH 5.1, by *N*-Retinyl-Celite Bioaffinity Chromatography

			pH 5.1 fractions			pH 7.0 fractions			protein
treatment	initial total protein[a] (mg)	initial[b] [α]/([α] + [β])	range of [α]/([α] + [β])	yield of α (mg)	yield of β (mg)	range of [β]/([β] + [α])	yield of α (mg)	yield of β (mg)	yield (%)
acid whey, pH 5.1	1140	0.377	0.15−0.68 (0.385)[c]	239	400	0.90−0.97 (0.945)[d]	8.2	164.4	71[e]
pH 5.1 eluate, 2nd appln	467	0.570	0.42−1.00 (0.680)	197	95.8	0.83−0.93 (0.930)	8.3	106.3	87
pH 5.1 eluate, 3rd appln	241	0.643	0.44−0.74 (0.710)	110	44.6	0.68−0.76 (0.730)	5.1	14.1	72
pH 5.1 eluate, 4th appln	139	0.712	0.50−0.83 (0.690)	77.7	30.4	0.20−0.76 (0.300)	7.4	5.6	87
pH 5.1 eluate, 5th appln	89	0.730	0.40−0.82 (0.665)	55.6	26.9	0.25−0.43 (0.300)	4.6	2.2	93
pH 5.1 eluate, 6th appln	53	0.870	0.52−1.00 (0.620)	28.5	18.0	0.20−0.40 (0.305)	3.7	1.5	98

[a] Total protein recovery for each trial was 87.1 ± 11.1% (median value), as determined by gel filtration chromatography. [b] α is α-lactalbumin; β is β-lactoglobulin. All values of protein purity and yield were determined using a Superose 12-HR 10/30 FPLC gel filtration column (Pharmacia LKB Biotechnology). [c] Values in parentheses represent the median value of α-LA purity (approximately 12 fractions analyzed for each determination). For determination of α-LA purity, one 5 mL fraction was analyzed for every 25 mL collected. [d] Values in parentheses represent the median value of β-LG purity (approximately 9 fractions analyzed for each determination). For determination of β-LG purity, every 5 mL fraction collected was analyzed. [e] Protein yield represents sum of the proteins recovered relative to the quantity of protein applied.

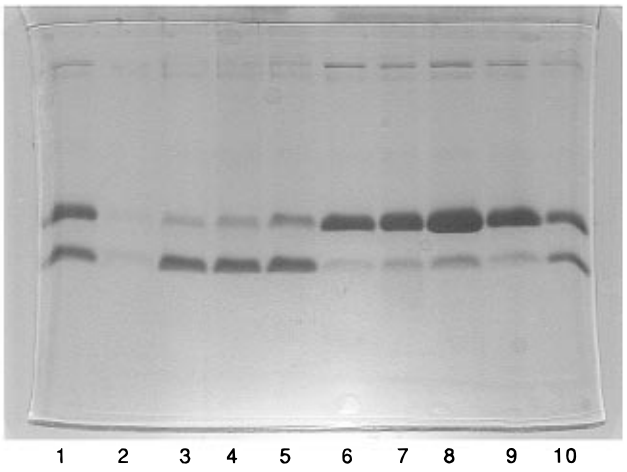

Figure 1. SDS−PAGE patterns of fractions eluted from an *N*-retinyl-Celite chromatographic column after the addition of acid whey and elution using 0.05 M sodium phosphate buffer, pH 5.1, for fractions in lanes 2−5, and 0.4 M sodium phosphate buffer, pH 7.0, for fractions in lanes 6−10. Results shown are for the second of six applications of acid whey, pH 5.1, onto the retinal column. (Lane 1) acid whey, pH 5.1; (lane 2) 25 mL eluted (1.0); (lane 3) 100 mL eluted (0.83); (lane 4) 125 mL eluted (0.73); (lane 5) 175 mL eluted (0.68); (lane 6) 315 mL eluted (0.94); (lane 7) 325 mL eluted (0.93); (lane 8) 330 mL eluted (0.93); (lane 9) 350 mL eluted (0.93); (lane 10) acid whey, pH 5.1. Numbers in parentheses indicate the ratio of α/(α + β) in lanes 2−5 and the ratio of β/(α + β) in lanes 6−9, as determined by gel filtration chromatography. On the basis of previous gels of purified proteins run under the same conditions, the upper band is β-LG and the lower band is α-LA.

Fractions within each large peak that eluted with either 0.05 M sodium phosphate, pH 5.1, or 0.4 M sodium phosphate, pH 7.0, were characterized by SDS−PAGE (Figure 1). On the second of the six reapplications of pH 5.1 eluate, the purity of α-LA was greatest [median α-LA/(α-LA + β-LG)] with a value of 0.78 ± 0.11 in the initial 125 mL of pH 5.1 buffer eluted and gradually decreased over time. This trend was similar for all trials. When the binding sites became saturated with β-LG, more β-LG appeared in the eluant. Typically, the purity of β-LG in the pH 7.0 fractions was greatest at the beginning of the elution peak and decreased over time. However, less variation in the purity of β-LG was observed in pH 7.0 eluant than was observed in α-LA values for pH 5.1 eluant (Figure 1). This result was expected, because at pH 5.1 adsorption of β-LG is optimal (Wang and Swaisgood, 1993), and thus upon elution with pH 7.0 buffer, the protein released from immobilized retinal should be primarily β-LG.

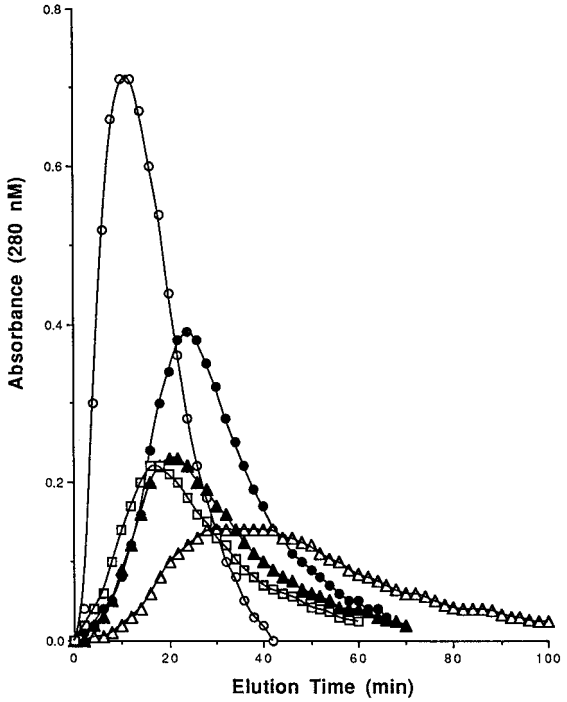

Figure 2. Elution profiles of acid whey protein desorption from an *N*-retinyl-Celite affinity column. Sodium phosphate buffer concentrations of 0.40 (○) and 0.10 (●) at pH 7.0 and 0.04 M at pH 7.0 (△), 8.0 (▲), and 9.0 (□) were evaluated for their effect on purity and recovery of β-lactoglobulin. Elution with the buffers was after initial loading of the column with 200 mL of acid whey, pH 5.1, and washing with 0.05 M sodium phosphate, pH 5.1, until a baseline was achieved. Flow rate was 1.0 mL/min at 25 °C.

Effect of Buffer Concentration and pH on the Purity and Yield of β-LG. Lower concentration phosphate buffers (<0.04 M) were ineffective at desorbing β-LG from the column matrix. This was likely due to the poor buffering capacity of the solutions, which apparently resulted in the inability to weaken electrostatic interactions involved in the structural conformation of β-LG that affect the adsorption of protein to *N*-retinyl-Celite. Sodium phosphate concentrations ≥0.04 M were necessary for appreciable desorption of protein from the column (Figure 2). A sodium phosphate concentration of 0.04 M was the minimum necessary for achieving yields of at least 0.1 g with a purity of 87% or greater (Table 2). Of the buffer concentrations examined, 0.4 M sodium phosphate yielded the highest purity (94.5 ± 2.3%) and quantity of β-LG, approximately double the amounts recovered at 0.06 and 0.04

Table 2. Effect of Buffer Concentration and pH on the Purity and Quantity of β-Lactoglobulin Eluted from an *N*-Retinyl-Celite Bioaffinity Column[a]

eluant	area under elution curve[b]	range of [β]/([α] + [β])[c]	yield of α (mg)	yield of β (mg)
0.40 M sodium phosphate, pH 7.0	664	0.90−0.97 (0.945 ± 0.023)[d]	8.2	164.4
0.10 M sodium phosphate, pH 7.0	523	0.81−0.90 (0.870 ± 0.028)	19.4	120.2
0.06 M sodium phosphate, pH 7.0	260	0.81−0.90 (0.870 ± 0.029)	9.0	63.3
0.04 M sodium phosphate, pH 7.0	376	0.78−0.93 (0.880 ± 0.036)	9.0	69.6
0.04 M sodium phosphate, pH 8.0	346	0.84−0.94 (0.900 ± 0.035)	10.3	89.8
0.04 M sodium phosphate, pH 9.0	308	0.82−0.91 (0.890 ± 0.035)	7.2	61.5

[a] 200 mL of acid whey, pH 5.1, was loaded onto the column and nonbinding protein eluted off with 0.05 M sodium phosphate, pH 5.1. Once a baseline was established, the eluant buffers listed above were applied to the column. For all trials, the whey loaded was all from the same original preparation. All values of protein purity and yield were determined using a Superose 12-HR 10/30 FPLC gel filtration column (Pharmacia LKB Biotechnology). For determination of β-LG purity, every 5 mL fraction collected was analyzed. [b] Integration of the area under the elution curve was calculated by using Simpson's rule (Mizrahi and Sullivan, 1982). [c] α is α-lactalbumin; β is β-lactoglobulin. [d] Values in parentheses represent the median value ± standard deviation of β-LG purity.

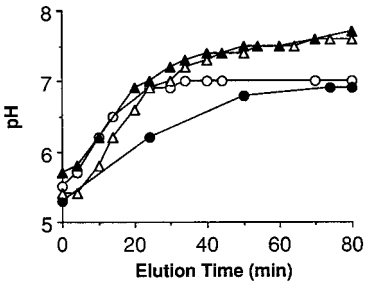

Figure 3. pH of fractions eluting at various times from an *N*-retinyl-Celite affinity column upon elution of adsorbed whey proteins with 0.06 M sodium phosphate, pH 7.0 (○), and 0.04 M sodium phosphate, pH 7.0 (●), 8.0 (△), and 9.0 (▲) buffers. The column was eluted with the buffers after initial loading with 200 mL of acid whey, pH 5.1, and washing with 0.05 M sodium phosphate, pH 5.1, until a baseline was achieved. Flow rate was 1.0 mL/min at 25 °C.

M. An explanation for the extended elution curve observed when 0.06 and 0.04 M sodium phosphate buffers were used was found by examining the pH of fractions eluting from the column (Figure 3). At concentrations of <0.04 M sodium phosphate (adjusted to pH 7.0), the elution buffer was not capable of altering the effective pH within the column to values necessary for desorption of β-LG from retinal. However, the elution time could be shortened without decrease of purity or yield by adjusting the pH of 0.04 M sodium phosphate to 8 or 9. Of the 0.04 M concentration buffers, the greatest purity and yield were observed at pH 8.0. The results of contrasting absorbance values (280 nm) vs the pH of fractions eluting from the column suggest that maximal desorption occurred at a minimum pH value of 6.5. This is supported by the finding of Wang and Swaisgood (1993) that the dissociation constant of β-LG bound to *N*-retinyl-Celite was 44-fold greater at pH 7.0 than at pH 5.1.

DISCUSSION

In the present study, the purity of α-LA was increased from 38% in acid whey to 87% in the process stream after β-LG removal, slightly exceeding the purity of commercially available preparations of α-LA. Future work should focus on modification of the properties of the *N*-retinyl-Celite column to improve the purity and yield of the downstream reduced β-LG whey, especially if this product is to be used as an ingredient in formulas for reduced allergenicity.

The median purity of β-LG isolated by bioselective chromatography under normal elution conditions (0.4 M sodium phosphate, pH 7.0) was approximately 95% and decreased when buffers of lower concentration and greater pH were employed. Depending upon the application of the downstream product, phosphate could impart undesirable properties, including increased water-holding capacity of gels, chelation (with reduced availability of minerals), and emulsification. While elution with buffers of lower concentration resulted in a minor decrease in β-LG purity, the yield was approximately half that of normal elution conditions. Wang and Swaisgood (1993) suggested that the observed dependence on pH may be a result of an electrostatic interaction due to protonation of a protophilic group in the *N*-retinyl moiety binding site. The pH of fractions eluting after application of lower buffer concentrations implies that the buffering capacity of lower concentrations was not sufficient to allow the extent of desorption observed with 0.4 M sodium phosphate.

Due to dilution with eluting buffers and the use of a fixed volume of the pH 5.1 whey, not all protein recovered in a run was used in the subsequent reapplication. However, even within each reapplication, the protein recovered as α-LA + β-LG was <100%. The gradual increase in the percentage recovery suggests that there may have been some nonspecific binding to the bioaffinity matrix. However, that has not been our experience with previous and subsequent use of this bioselective adsorption material.

Use of bioaffinity chromatography provides several advantages over classical and more recent approaches to the isolation and purification of β-LG and α-LA. Under the conditions outlined in this study, median purity of β-LG was 94.5%, with a yield of 164.4 mg of β-LG collected in a total eluant volume of 50 mL. The greatest purity of α-LA obtained presently was approximately 87%. This improves upon classical methods of precipitation with high salt concentrations, which require large amounts of reagents and extended dialysis times. The method of Fox et al. (1967) to prepare gram quantities of β-LG yields a product contaminated with approximately 15% α-LA. Imafidon and Ng-Kwai-Hang (1992) improved upon this by passing the supernatant from whey treated according to the Fox et al. (1967) protocol through a QAE ZetaPrep 250 anion-exchange cartridge, resulting in electrophoretically pure β-LG and recovery of 73% of the theoretical yield, exceeding that found by Yoshida (1990) using conventional anion-exchange chromatography. Monaco et al. (1987) processed whey using a DEAE-cellulose anion-exchange column, followed by gel filtration, yielding 73% pure α-LA. The use of ultrafiltration and demineralization of whey for obtaining purified β-LG was reported to increase the purity of β-LG from 67% to 80% (w/w) and from 59% to 69% for acid and sweet whey retentates, respectively (Slack et al., 1986). In a process designed

for large scale fractionation of whey proteins, Mailliart and Ribadeau-Dumas (1988) obtained 95% pure β-LG by precipitating whey proteins other than β-LG by holding whey for 1 h at pH 3.0 in 5.0% NaCl. Approximately 84% of the original β-LG content of whey was recovered, with α-LA-enriched fractions found to have <0.5 mg of β-LG/286 mg of total protein.

Although methods employing ion-exchange chromatography are highly effective at isolating high-purity β-LG and α-LA, they often require an additional gel filtration step or higher salt concentrations than is necessary with *N*-retinyl-Celite affinity chromatography. Ultrafiltration and demineralization of whey require several additional steps, compared to bioaffinity chromatography, and are not well suited for the preparation of high-purity α-LA (Slack, 1986). Bioaffinity chromatography shows promise for isolation and purification of β-LG and α-LA and offers the advantages of lower salt concentrations present in the final product, no risk of heat or acid denaturation that could alter functional properties, and a procedure requiring fewer steps than other methods.

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