Separation of the Two Major Whey Proteins Using Cation-Exchange Adsorption

Mayyada M.H. El-Sayed, Howard A. Chase

Abstract— **This paper describes the cation-exchange adsorption of the two major whey proteins, alpha-lactalbumin (ALA) and beta-lactoglobulin (BLG) with the purpose of establishing a process for isolating them from cow's milk whey. First, the simultaneous adsorption of a pure binary mixture of 1.5 mg/ml ALA and 3 mg/ml BLG onto a 5-ml column of the cation-exchanger SP Sepharose FF at 20°C using 0.1M acetate buffer of pH 3.7 was studied. The BLG breakthrough curve exhibited an overshoot phenomenon that gave evidence for the presence of a competitive adsorption between the two proteins. Complete separation occurred and it was possible to obtain each of the two proteins in a pure form. The process was then applied to a whey concentrate mixture where incomplete separation took place. However, BLG was produced with 95% purity and a recovery of 80%, while ALA showed an 84% recovery.**

Index Terms— **competitive cation-exchange adsorption, alpha-lactalbumin, beta-lactoglobulin, whey proteins, separation**

I. Introduction

While there are many published studies of the adsorption of single proteins to different adsorbents, fewer studies have been reported using more realistic multicomponent systems which involved more than one adsorbing protein. The work done by Flashner *et al.*, 1983; Nigam *et al.*, 1988; Skidmore and Chase, 1990; Weinbrenner and Etzel, 1993; Martin *et al.*, 2005; and Cano *et al.*, 2007 showed evidence for competitive adsorption of the various components as seen from the characteristics of their breakthrough profiles.

Regarding the ion-exchange chromatographic separation of whey components, Outinen *et al.*, 1996 studied the anion-exchange adsorption of ALA and BLG from sweet whey. Gerberding and Byers, 1998 and Ye *et al.*, 2000 reported a process for separating four protein components from sweet whey using an anion-exchanger followed by a cation-exchanger. The low recoveries and the non-complete separation were the main disadvantages reported. Etzel *et al.*, 1999; Doultani *et al.*, 2004 and Turhan and Etzel, 2004 developed a cation-exchange chromatography process for fractionating proteins from whey. Lozano *et al.*, 2008 described a method for separating BLG from bovine whey based on differential precipitation followed by ion-exchange chromatography. All of the above attempts were based on selective elution rather than selective adsorption which is the focus of our study. The cation-exchange adsorption of a pure binary mixture of ALA and BLG will be studied first so as to lay the foundation for the subsequent study on whey concentrate. To the best of the authors' knowledge, this approach had not been adopted and reported in the previous literature.

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II. Materials

Pure beta-lactoglobulin from bovine milk, approximately 90% (PAGE), chromatographically purified and lyophilized (MW 18 KDa), and pure alpha-lactalbumin from bovine milk, ≥ 90% (GE), calcium depleted (MW 14 KDa) were purchased from Sigma. SP Reagents used for buffer preparation (anhydrous sodium acetate and Tris-HCl, Fluka; glacial acetic-acid, 99%; ethanol 99.8%, Riedel-de Haen; sodium hydroxide, Fisher Scientific; sodium chloride, Riedel-de Haen) were all of analytical-grade. Whey concentrate, MyPro whey protein WPC80 (80 wt% total protein), was purchased online from www.protein.co.uk. It has a composition of 60% BLG, 20% ALA, 8% bovine serum albumin, 10% immunoglobulin, 1%lactoferrin and 1% others.

III. Methods

A. Packed-bed experiments

The frontal analysis packed-bed column experiments were performed on an 'AKTA Explorer 100' chromatography system (GE Healthcare). Five-ml HiTrap SP Sepharose FF prepacked columns, purchased from the same company, of dimensions 1.6 cm internal diameter and 2.5 cm bed height were used. Buffers (for equilibration, elution and washing) and samples were loaded onto the column in a downflow manner using the system's piston and peristaltic pumps, respectively. All buffers and samples were filtered, through 0.45 μm membranes, and degassed on a glass filter connected to a vacuum pump, in order to mitigate bubble entrainment problems. 0.1M sodium-acetate buffer was used for column equilibration, 0.1M Tris-HCl for elution, 1M NaOH for periodical column cleaning, and 20% ethanol in 0.2M sodium acetate for long-term storage. For the experiments with the pure proteins, the column was loaded with a binary mixture

of BLG and ALA at pH 3.7 in the ratio of 2:1 with concentrations of 3 and 1.5 mg/ml, respectively. It should be noted that the protein concentrations employed were chosen to be those that would normally exist in whey. For whey experiments, on the other hand, whey concentrate of concentration 7.6 mg/ml corresponding to total (ALA+BLG) concentration, C_{AB}, of 4.5 mg/ml (i.e. C_{AB} that was previously used for pure mixtures) was applied to the column. Fractions from the column effluent were collected every 10 minutes in 15-ml tubes using the AKTA Explorer 100 Fraction-Collector 950. Some of these fractions were analysed on an FPLC system using size exclusion (gel-filtration) technique described below.

B. Size-exclusion analysis

Size exclusion experiments were carried out on a Fast Protein Liquid Chromatography (FPLC) system using a Superdex^{TM} 200 column GL, purchased from GE Healthcare, of 1-cm internal diameter, 30-cm height, exclusion limit of $1.3*10^6$ globular protein and a separation range for globular proteins of 10,000 to 600,000. This technique separates BLG and ALA on the basis of the difference in their molecular weights at pH 3.7, BLG being a dimer with an overall molecular mass of 36 KDa (Sawyer, 2000) and ALA a monomer of 14 KDa. A 0.2-ml protein sample is injected onto the column using a 1-ml syringe, while 0.1M sodium acetate buffer of pH 3.7 at flowrate of 0.5 ml/min was passed through the column in order to elute the protein. As expected, BLG was eluted first with a peak retention volume of 17.5 ml, whereas ALA was eluted at 19.5 ml. The protein concentrations were calculated using peak area-concentration calibration curves constructed for binary mixtures of both proteins as reported in a previous paper. The percentage recovery was calculated as follows:

$$\%\text{recovery}=\left(\frac{C_oV_f - CV_c}{C_oV_f}\right)*100 \quad (1)$$

where,
C_o = initial concentration of protein (mg/ml)
C = concentration of protein in the collected peak (mg/ml)
V_f = volume of feed sample loaded onto the column (ml)
V_c = volume of collected peak (ml)

C. Sodium Dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

Fractions were analysed under reducing conditions using Dithiol-threitol (DDT) as a reducing agent. Electrophoresis was run at a potential difference of 200 volts for 35 min, using NuPAGE Novex 4-12% Bis-Tris gel and NuPAGE MES running buffer, purchased from Invitrogen. Samples were heated at 70^{o}C for 10 min prior to electrophoresis. A 7 μL volume of each sample was loaded per well. The protein was fixed for 20 min using 40% methanol and 7% acetic acid solution before it was stained overnight with Coomassie Brilliant Blue R250, and then destained in the same fixing solution. The protein bands were quantified using ImageJ software.

IV. Results and Discussion

Results in this paper will fall into two main sections: the first (A) deals with pure binary mixtures where both ALA and BLG are adsorbed simultaneously onto the column, while the other (B) pertains to adsorption from whey concentrate solutions.

A. Pure binary mixtures

The adsorption profiles pertaining to 1.5 mg/ml ALA and 3 mg/ml BLG when both were adsorbed simultaneously onto the cation-exchanger SP Sepharose FF at pH 3.7 and flowrate 1 ml/min are depicted in Fig. 1. A distinctive breakthrough curve for each protein can be observed; with BLG breaking through first with a $t_{1/2}$ of 190 min, while ALA following with a $t_{1/2}$ of 725 min. It can also be noticed that the breakthrough profiles of the two proteins have different slopes as BLG breakthrough goes steeper than that of ALA; and that the concentration of BLG even overshooted by more than 50% before if fell back to the normal value of unity. This implies that ALA is able to displace and, thereby elute, a certain amount of adsorbed BLG. This might be because the ALA, by virtue of its small size, was able to compete for the finer pores of the adsorbent.

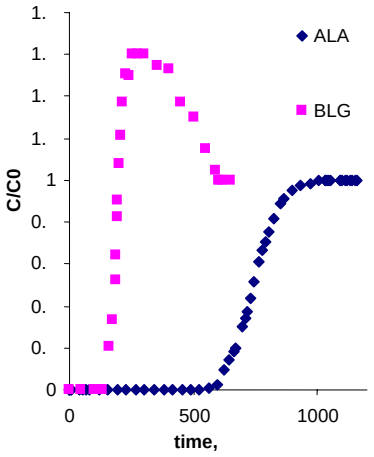

Fig. 1 Breakthrough profiles of ALA and BLG when a mixture of both proteins was applied onto a (5-ml) SP Sepharose FF bed at pH 3.7, flowrate of 1ml/min, and concentrations of 1.5 and 3 mg/ml, respectively.

To quantitatively determine the amount of BLG eluted by ALA, the experiment was performed again but this time was terminated just before the ALA breakthrough point (~500 min). The protein coming off the column was collected up to this point, and was analysed on a size-exclusion chromatography system. The results revealed an overall average concentration of BLG in the collected fractions of 3 mg/ml equivalent to the inlet concentration (Fig. 2) which indicates that ALA had eluted all the BLG off the column. This, in turn, proves that the separation of the two proteins could be successfully achieved under the previously employed conditions. Fig. 3 shows the analysis of the eluted peak as detected on the FPLC using size exclusion.

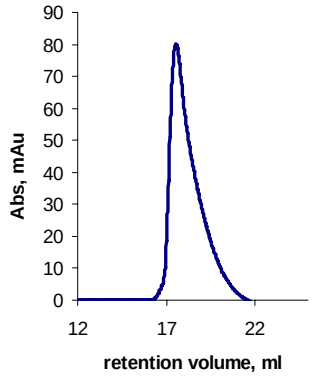

Fig. 2 Analytical size exclusion chromatography on Superdex 200 for the SP Sepharose column effluent produced during the course of the BLG breakthrough shown in the previous figure. A 0.2-ml sample volume was applied to the size-exclusion column and eluted with 0.1M sodium acetate buffer of pH3.7.

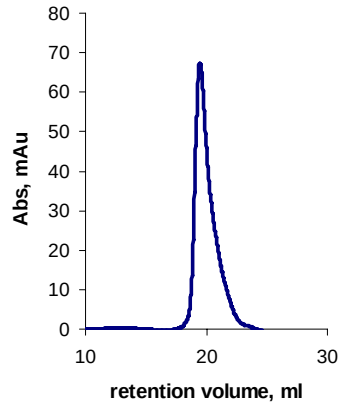

Fig. 3 Size-exclusion analysis of ALA eluted fraction on Superdex 200, obtained when eluting with 0.1M Tris-HCl buffer at pH 9.0. A 0.2-ml sample volume was applied to the size-exclusion column and eluted with 0.1M sodium acetate buffer of pH3.7.

In conclusion, the ability of ALA to displace most of the adsorbed BLG which was subsequently driven off the column indicated the interplay of competitive adsorption between the two proteins. The above finding was used to develop a process for separating ALA and BLG proteins. This process will be further extended to whey mixtures as will be discussed in the following section.

B. Whey concentrate

Analysis of 7.6 mg/ml of whey concentrate on an FPLC chromatography system using size exclusion (Fig. 4) showed that it contains BLG with the higher molecular weight (first peak) and ALA (second peak).

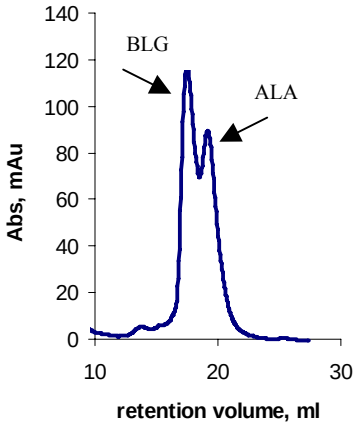

Fig. 4 Analysis of 7.6 mg/ml of whey concentrate using size-exclusion chromatography.

Table I shows the whey compositions according to the manufacturer's specifications, the FPLC analyses and the SDS estimates.

The adsorption profiles of BLG and ALA pertaining to a 7.6 mg/ml concentration of whey concentrate and flowrate 1ml/min are depicted in Fig. 5.

Table I Whey concentrate composition

	Manufacturer's specifications	SDS estimates	Gel filtration analyses
total proteins	80%	—	—
BLG*	60%	54%	53%
ALA*	20%	21%	20%

*BLG, ALA percentages are taken on the basis of total proteins in the whey mixture.

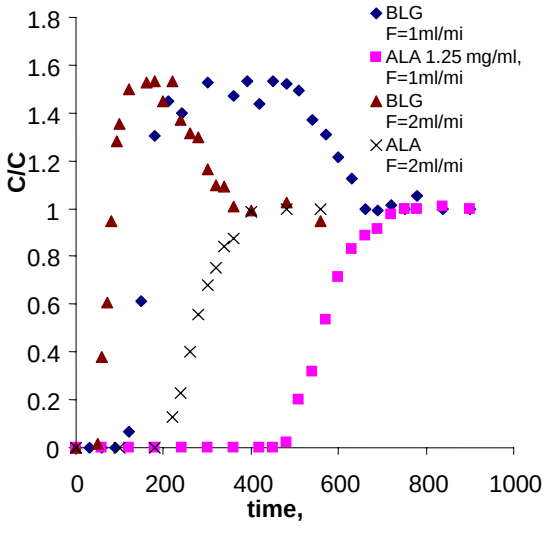

Fig. 5 Packed-bed adsorption profiles for BLG and ALA when 7.6 mg/ml whey concentrate was applied onto an SP Sepharose FF column at pH 3.7 and flowrates of 1 and 2ml/min.

As in the pure binary mixture, studied earlier, the BLG breakthrough started before that of ALA and its maximum outlet concentration exceeded approximately 1.5 that of its feedstock concentration. This indicates the ability of ALA to elute some of the BLG off the column and hence the presence of competitive adsorption between both proteins. In spite of having the total concentrations of ALA and BLG, C_{AB}, and flowrate comparable to those used in the experiments with mixtures of pure proteins, no complete separation was achieved as the ALA breakthrough began when the BLG concentration was still at its maximum and hence overlapping between the two breakthrough curves occurred. It is to be recalled that for binary mixtures, the ALA breakthrough occurred immediately after the BLG concentration had fallen back to its feedstock value. This difference in behaviour could be due to the difference in the ratio (BLG to ALA) in the binary mixtures (3:1.5) and that found in whey (3.25:1.25), as well as the complex nature of whey. A loss in protein recovery and purity would thus be expected, as a result of this overlapping of the ALA and BLG breakthrough curves.

Furthermore, doubling the flowrate to 2 ml/min did not further decrease the separation of the two proteins as no change occurred to the position of ALA breakthrough point with respect to that of BLG (Fig. 5). Therefore in order to reduce the operational time, the experiment was performed at the higher flowrate of 2 ml/min which will be adopted in the remainder of the study.

To determine the recovery and purity of the produced ALA and BLG fractions, another adsorption experiment was conducted but this time loading of the feedstock was terminated at the ALA breakthrough point (also the BLG maximum), i.e. 200 min. Analysing the column effluent collected up to this point using size exclusion revealed that this fraction contained mostly BLG with a recovery of 80% of the amount of BLG that had been fed to the column (Fig. 6). The column was then eluted with 0.1M Tris-HCl buffer at pH 9.0. The eluted fraction was analysed using gel filtration and was found to contain mainly ALA with a recovery of 84% of that in the feedstock, along with some BLG.

The purity of the produced ALA and BLG eluted fractions was determined using SDS-PAGE gel electrophoresis. Fig.7 shows the electrophoretic patterns pertaining to whey concentrate. Lanes 1, 2, 3 and 4 from the left present the molecular mass marker, whey concentrate starting material, flow through-fraction and eluted fraction. The purity of BLG in the obtained flow through-fraction was 95%, while the composition of the eluted fraction was 32% ALA and 38% BLG. The higher molecular-weight bands also present are those of bovine serum albumin (BSA) and immunoglobulin (Ig).

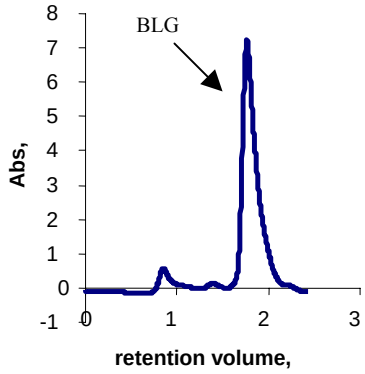

Fig. 6 Analytical size-exclusion chromatography on Superdex 200 for the flow-through fraction, collected up to the start of the ALA breakthrough, for 7.6 mg/ml whey concentrate.

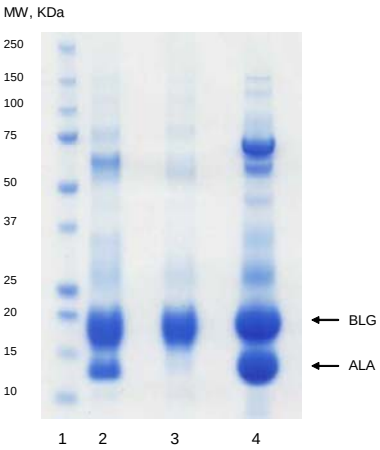

Fig. 7 Electrophoretic patterns pertaining to 7.6 mg/ml whey concentrate, lane 1: molecular mass marker; lane 2: whey concentrate starting material; lane 3: BLG-fraction; lane 4: ALA-eluted fraction.

In light of the above study, it can be deduced that:

- the purity of ALA in the eluted fraction drops as a result of the overlapping that starts at the ALA breakthrough point, corresponding to BLG maximum, and ends when BLG breakthrough reaches a plateau at its inlet concentration (i.e. at $C/C_o = 1.0$).
- overlapping also affects the recovery of BLG in the flow-through fraction as some of it (20%) was not yet been eluted from the column by the competitive adsorption of ALA, at the point of terminating the application of feedstock.
- in spite of the similarity in the shapes of the ALA and BLG breakthrough curves between pure and whey mixtures, it was not possible to attain the same degree of separation obtained in the mixtures of pure proteins due to the complex nature of whey as well as its different BLG to ALA ratio.

V. Conclusion

A process was established to separate ALA and BLG from whey concentrate mixtures. A 95% purity BLG was produced. Recoveries of 84% and 80% of ALA and BLG were obtained respectively. The current work looks into further modifying this process so as to obtain ALA with higher purity. This is achieved by prolonging the time of application of feedstock up to the point where BLG reaches its inlet concentration (i.e. at $C/C_o=1$). Two fractions are then collected throughout the loading period; a fraction containing mostly BLG and is collected up to the start of the ALA breakthrough, and another fraction containing both ALA and BLG and this is recycled and mixed with the feedstock for a second-stage step. The column, which now has almost no BLG, is eluted to obtain ALA.

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