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Fractionation of the major whey proteins and isolation of β-Lactoglobulin variants by anion exchange chromatography

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ABSTRACT

A method for the separation and fractionation of the major whey proteins from a whey protein concentrate (WPC80) by anion-exchange chromatography coupled to a Fast Protein Liquid Chromatography (FPLC) system is proposed. The method is based on the use of an ionic column (Mono Q) and a salt gradient elution by increasing the ionic strength of the elution buffer (Tris–HCl 20 mM plus 0 to 1 M NaCl). The proposed method was found to be suitable to fractionate the major whey proteins from the WPC80 in different fractions, namely one fraction containing all the α-Lactalbumin and immunoglobulins; another fraction containing all the bovine serum albumin; and two distinct fractions each containing a different variant of β-Lactoglobulin. A 60.5% (w/w) recovery of the two main β-Lactoglobulin variants was obtained.

1. Introduction

Whey, a by-product from the cheese and curd manufacturing, was once considered a waste product. However, as stricter environmental laws were approved and more attention was given to its benefits, whey has become a new source of functional ingredients [1]. Whey proteins correspond to about 18–20% of the total milk proteins and its major components are β-Lactoglobulin (β-Lg), α-Lactalbumin (α-La), bovine serum albumin (BSA) and immunoglobulin (Ig), representing, 50%, 20%, 10% and 10% of the whey fraction, respectively [2]. Besides these, whey contains numerous minor proteins, such as lactoferrin (LF), lactoperoxidase (LPO), proteose peptone (PP), osteopontin (OPN), lizozyme (LZ), among others [3]. Table 1 presents the main characteristics and functions of each of the main proteins present in whey.

Usually, whey protein products are available in three major forms: concentrates (WPC), isolates (WPI) and hydrolysates (WPH) [9]. However, the lack of consistency in the gross composition and functionality of these products has limited their acceptance by the food processing industry. Moreover, WPCs can also develop a stale off-flavor due to the presence of lipid and protein impurities [10]. Each whey protein has unique attributes for nutritional, biological and food ingredient applications [1]. Furthermore, purified individual milk proteins exhibit better functionality than in their native protein mixtures [11], thus there is a great interest in developing easier and more efficient methods to recover pure protein fractions [9].

Protein functions have been related to their native structure, which depends on pH, temperature, pressure and solvent effects [12]. Changes in native structure affect functional properties, so there has been a renewed interest in developing efficient separation and purification processes that prevent denaturation and loss of biological activity [13].

The dairy industry has conducted many efforts to develop efficient separation technologies that enable the production of new products, such as precipitation, membranes and chromatography [5]. However, by precipitation and membrane techniques, whey protein is prone to denaturation and these processes are volume-dependent, which makes the fractionation of whey very expensive [1,14]. Additionally, ultrafiltration (UF) is neither sufficient for the complete removal of lactose, nor for the isolation of single pure proteins [15]. Within the last decade there has been increasing interest in liquid chromatographic processes because of the growing biotechnology industry and the special needs of the pharmaceutical and chemical industries [16]. A promising technology that has been used to purify whey proteins is ion exchange membrane chromatography [17,18]. Many advantages over column chromatography have been reported for this technology, such as the very rapid rate of association between target proteins and functional groups; short processing times; ease of scale-up and operation without the need for lengthy column packing procedures; no heat-treatments, extremes of pH, or chemical pretreatment that could compromise protein structure and functionality; among others [19]. Ulber and co-workers [20] using a cation exchange membrane obtained a protein stream of LF and LPO. Moreover, Bhattacharjee et al. [17] using strong anion exchange membranes were able to separate α-La and β-Lg from whey. Despite the promise of ion exchange membrane

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Table 1
Characteristics and functions of the major whey proteins.

Protein	Molecular weight (MW) (kDa) [4]	Isoelectric point (pI) [5]	Functions	References
β-Lg	18.3	5.35–5.49	Binding and transport of retinol, vitamin D and palmitic acid Enzymic synthesis of prostaglandins Olfactation, opiodergic, cryptic coloration Anti-hypertensive, anti-cancer, hypocholesterolemic	[6]
α-La	14.2	4.2–4.5	Binding of calcium, absorption Lactose synthesis Tumor cells apoptosis	[7]
BSA	66.4	5.13	Transport, metabolism and distribution of ligands Protection from free radicals Contribution to osmotic pressure of blood	[4]
Ig	<150	5.5–8.3	Immunological protection against microbial pathogens and toxins Protect mammary gland against infections	[8]

chromatography it should be emphasized that the binding capacities of membranes currently available are lower than the ones achieved with packed columns with similar geometries, due to a greater surface are obtained when using beads. Overall, ion-exchange packed column chromatography (IEC) is an advantageous method due to its high capacity, relatively low cost and ability to survive severe cleaning regimes [21].

IEC is an efficient separation method for proteins in which electrostatic interaction play an important role [22]. IEC is based on the binding of charged molecules to the oppositely charged groups attached to an insoluble matrix. Therefore, proteins will bind to the resin whenever their net charge is opposite to the resin one [23].

Proteins are usually characterized by their isoelectric points (pI), the pH value at which they have a net charge equal to zero [24]. This net charge has been used to predict the proteins behavior on IEC processes based on the assumptions that the proteins will not be retained at their pI; will be retained by anion resins at pH above their pI; or by cation resins below their pI [22].

The aim of this study was to develop a method to fractionate and recover the four major whey proteins, β-Lg, α-La, BSA and Ig, from a WPC with 80% (w/w) of total proteins (WPC80) by anion-exchange chromatography (Mono Q column) coupled to a Fast Protein Liquid Chromatography (FPLC) system.

2. Materials and methods

2.1. Materials

2.1.1. Anion-exchange chromatography column

The column used for the fractionation and recovery of proteins from the WPC80 was a Mono Q 5/50 GL (GE Healthcare, Pittsburgh, PA). No significant degradation in the column performance could be observed over 500 runs. All the prevention and routine cleaning procedures have been conducted according to the manufacturer's guidelines to assure reproducibility and integrity of the column over the timeline of the experiments.

2.1.2. Standard pure proteins and whey protein concentrate

Standard pure proteins, namely α-La, β-Lg and BSA, used to determine their retention times, were all purchased from Sigma (Sigma–Aldrich Co., St. Louis, MO).

A WPC80 supplied by the Arve Nutriclyn Ltd. (Brazil) and named "Maximus Whey Protein" was used. This concentrate is composed of 80% w/w protein, 5% w/w lactose, 8% w/w fat and 1.8% w/w salts.

2.2. Methods

2.2.1. Proteins recovery and fractionation

Protein solutions were prepared in Tris–HCl buffer with different final concentrations depending on the experiment: for the experiments with the single standard proteins, 1 g/L; for the experiments with the mixture of the standard proteins, 3 g/L; and for the experiments with WPC80, 5 g/L. Before loading the column, all the standard proteins and WPC solutions were filtered through a 0.45 μm membrane. Two buffers were prepared to run the experiments, namely the equilibration (buffer A) and elution (buffer B) buffer. Equilibration buffer consists of Tris–HCl 20 mM pH 6.3; and elution buffer consists of Tris–HCl 20 mM pH 6.3 with NaCl 1 M. Buffers were prepared with Millipore water, filtered under vacuum through a 0.45 μm membrane and degasified by ultrasound. Afterwards, the buffers were sterilized at 121 °C for 20 min.

The standard proteins retention time, as well as the separation of the proteins from WPC80 was determined using a FPLC system (GE Healthcare, Pittsburgh, PA). Detection of proteins was conducted at 280 nm using an UV detector. Three separation procedures using different salt gradients were used depending on the mixture to be separated, namely for the single standard proteins – procedure#1; for the mixture of standard proteins – procedure#2; and for the WPC80 – procedure#3. The salt gradients used in each situation were settled based on literature data and previous runs using several elution profiles (*data not shown*) that were performed to determine the maximum ionic strength which permitted binding the proteins of interest and the minimum ionic strength required for complete elution of the proteins in the WPC80. In all separation procedures, after equilibrating the column by running 5 or 10 column volumes (CV) of buffer A (10 CV for procedure#1 and 5 CV for procedures #2 and #3), 500 μl of sample were loaded, and elution was conducted at a specific salt gradient. For procedure#1, a linear salt gradient from 0% to 50% was performed between 10 and 30 CV; followed by a second linear salt gradient from 50% to 100% between 30 and 40 CV; followed by a washing step with buffer B (100% salt) using 10 CV. For procedure#2, a linear salt gradient from 0% to 50% was conducted between 5 and 25 CV; followed by a washing step at 50% salt during 5 CV. For procedure#3, a linear salt gradient from 0% to 35% was conducted between 5 and 45 CV; followed by a step gradient from 35% to 50% during 5 CV. It is important to mention that the washing step with buffer B was included in all separation procedures to remove proteins that were still bound to the matrix. During these procedures, samples were collected for further quantification of total soluble protein content using the Bradford Method as described elsewhere [25]. All the experiments were conducted in triplicate and the variation between runs was estimated to be less than 2%.

2.2.2. Electrophoresis

The fractions collected during the runs were concentrated using an ultrafiltration cell, model 8010 Amicon with a 10 kDa membrane (Millipore, Bilerica, MA). The identification of the proteins collected in the several fractions was done by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) using a Mini-PROTEAN system (BioRad). Electrophoresis was conducted at a constant

current 20 mA using a 12% separating gel and a 4% stacking gel [26]. Each fraction (16 μl) was loaded in a different lane, as well as 4 μl of proteins standard (Precision Plus Protein Standards, BioRad) to enable identification of individual proteins according to their molecular masses. After running the electrophoresis, the gels were stained with Coomassie Brilliant Blue R-250. Distaining was carried out in a solution containing methanol, acetic acid and water.

3. Results and discussion

In order to explore the suitable conditions for a good separation and isolation of the major whey proteins, several elution profiles were tested, namely several pHs (8.0, 7.0 and 6.3) and elution modes (isocratic, linear gradient, step gradient and combination of them). It was found that the best operational conditions were a pH 6.3 together with a linear salt gradient. These conditions are supported by the studies reported by other authors [27–29]. Shen and Frey [27] reported that the optimal resolution between two proteins when using IEC with a salt gradient elution is often achieved when the fluid phase pH is near the protein pI. Other authors [28,29] also reported that the linear gradient is the optimum elution mode to purify proteins. Additionally, the salt concentration used in the proteins' elution should not be higher than 1 M, because as the solution ionic strength increases, molecules can move closer to each other due to a reduction in the electrical double layer thickness [30], thus avoiding a good separation.

To determine the standard proteins retention time, the elution conditions used were: 0–5 CV – isocratic elution with buffer B at 0%; 5–15 CV – linear gradient elution with buffer B from 0% to 50%; 15–20 CV – linear gradient elution with buffer B from 50% to 100%; 20–25 CV – isocratic elution with buffer B at 100%. Elution was carried during 50 min at a flow rate of 0.5 ml/min. Results are illustrated in Fig. 1.

As expected, the sequence of elution of the proteins was α-La, BSA and β-Lg. Comparing the three proteins, α-La has the minor pI, so was the first eluted from the column (with approximately 25% of salt). The next protein to be eluted was BSA (with approximately 27% of salt) and the last was β-Lg (with approximately 30% of salt), since this is the protein whose pI is closer to the pH of the buffer solution.

From Fig. 1, it can be observed that all the proteins exhibit two peaks. This occurrence is related with the variants or conformations that proteins can assume depending on the pH of the solution, among others factors. α-La standard was found to possess the two variants, A and B. Variant A is the first to elute, because it has a slightly lower pI compared to variant B [31]. BSA exists in solution as a dimer–monomer equilibrium [30]. At a pH near to its pI, the ratio monomer/dimeric form is 60/40; however, as far as the pH value is from the pI, the monomeric form becomes predominant, reaching beyond 85% [32]. Therefore, since the experiments were conducted at pH 6.3, the BSA monomeric form is expected to be present in a larger amount than the dimeric form. Association of BSA monomers to form dimers occurs through a reversible path that involves specific interactions between the protein molecules [33]. The further the pI is from the solution pH, the greater is the increase in the molecules net charge and the greater is the increase in the repulsive electrostatic effects. Therefore, the probability of dimer formation is reduced.

For BSA, it can be observed that the dimeric form binds more strongly to the resin than the monomeric form, since the dimer elutes later than the monomer. This result is in accordance with the study reported by Hunter and Weinbrenner [34]. This situation suggests that even apparently single-protein systems may display multicomponent competitive behavior [35].

Additionally, in Fig. 1 two peaks for β-Lg can be observed, representing the two main variants in milk [36]: β-Lg B (first peak) and β-Lg A (second peak). These two variants have a slight difference in the amino acid composition [37], resulting in a difference of 0.3 unit in their pI [38]. As a consequence, β-Lg B is the variant being eluted first as compared to β-Lg A.

Besides the determination of the proteins' retention time, these results enabled the optimization of the elution conditions. It was found that at a percentage of salt above 50% no proteins were eluted. Thus, subsequent experiments were conducted with a NaCl concentration varying between 0 and 0.5 M (0–50% of salt).

In order to predict the behavior of the major proteins in WPC80, an initial chromatographic run was conducted using an equimolar mixture of the standard proteins. The elution condition used was: 0–5 CV – isocratic elution with buffer B at 0%; 5–25 CV – linear gradient elution with buffer B from 0% to 50%; 25–30 CV – isocratic

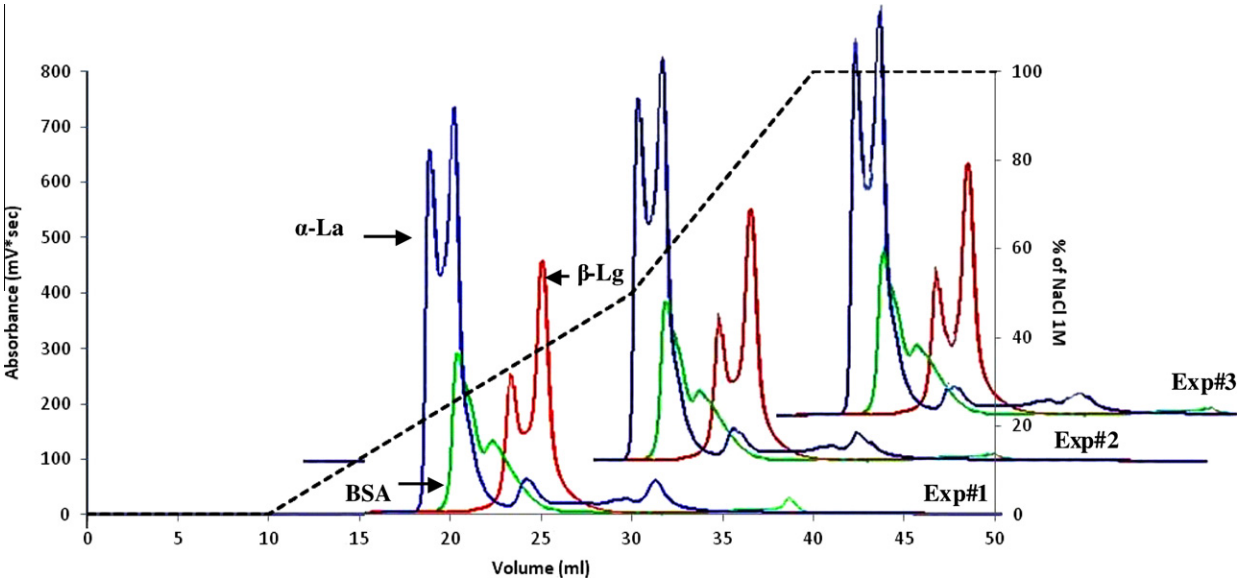

Fig. 1. Elution profile for each standard protein (1 g/l), α-La (in blue), β-Lg (in red) and BSA (in green), eluted with a salt gradient ranging from 0 to 1 M NaCl (black dashed line). Three independent experiments (Exp #1, Exp #2 and Exp #3) have been conducted. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

elution with buffer B at 50%. Elution was carried out during 30 min, at a flow rate of 1 ml/min. Results are illustrated in Fig. 2.

As expected, the elution order of the standard proteins in a multicomponent solution (Fig. 2) was the same as the standard proteins in a single-component solution (Fig. 1). However, since in a mixture the proteins can interact with each other, some slight deviations could be observed in their retention times.

Fig. 2 shows a single peak for α-La and BSA, and two peaks for β-Lg, corresponding to its two variants, B and A, respectively. The retention times were changed due the competitive adsorption of the proteins, which is a typical behavior of the proteins in a mixture when fractionated by IEC [39,40]. Aboudzadeh et al. [41] reported that, in multicomponent solutions, factors like molecular size and protein interactions can be significant in the adsorption onto ion-exchange resins. Comparing to their chromatographic profiles in single-component experiments (Fig. 1), α-La and BSA when in a mixture were found to elute earlier, with 16.3% and 19.8% of salt, respectively. This may be explained due to the competition displayed by β-Lg, whose affinity for the column is higher compared with the other two proteins. Between α-La and BSA, a better separation could be observed (Fig. 2) compared to the single-component experiment (Fig. 1). This result is in good agreement with Weinbrenner and Etzel [35] that studied the competitive adsorption of these two proteins in anion exchange resins, and found that α-La was completely displaced by the more strongly binding of BSA. For BSA, this one peak could be related to the fact that, onto highly activated anion exchange resins, BSA mainly keeps its monomeric form (97%) [42]. Regarding β-Lg, a better separation of its two variants was found as compared to the single-component experiment. β-Lg B eluted slightly later than in a single-component experiment (with 27.3% of salt). The results suggest that the variant A competes for the adsorption sites, thus displacing the β-Lg B.

In an attempt to isolate the main proteins from a whey concentrate, a solution of 5 mg/ml of WPC80 was injected onto the anion-exchange column. The elution conditions were: 0–5 CV: – isocratic elution with buffer B at 0% salt; 5–45 CV – linear gradient elution with buffer B from 0% to 35% salt; 45–50 CV – isocratic elution with buffer B at 50%. The elution was carried out during 50 min, at a flow rate of 1 ml/min (Fig. 3). Furthermore, fractions collected during elution process (F1–F7) were analyzed by SDS–PAGE as can be seen in Fig. 3.

Fractions F1 and F2 correspond to the proteins that present a pI higher than the pH value used (pH 6.3), so they were eluted in the void volume. This means that these proteins had an excess of OH^- ions because they were in a basic environment, and thus they do not adsorb onto the anion-exchange resin. From the SDS PAGE gel (Fig. 3), no bands could be visualized in these fractions. This was expected since these proteins (basic proteins) altogether constitute about 5% of the total whey proteins, thus an extremely small amount to be detected by SDS–PAGE (detection limit of proteins stained with Coomassie brilliant blue is near 50 ng).

Fraction F3 contains all immunoglobulins (first band at the top in the gel) and all α-La (last band in the gel) present in the WPC80. It also contains some β-Lg. It is important to notice that Ig and α-La can be further separated using gel filtration (or size exclusion chromatography), because α-La has a very small molecular weight compared to Ig. According to Kunz et al. [43], Ig and α-La can be separated in a Superose 12 size exclusion column. From Fig. 3, only one peak for α-La can be observed. This is because, contrarily to what happens when using single α-La for which two variants are present, in whey just variant B is normally present [44]. Fraction F4 contains all the BSA and some residual amount of β-Lg (Fig. 3). Fractions F5 and F6 were found to contain only β-Lg, variant B and A, respectively. A greater amount of β-Lg A was recovered compared to β-Lg B since variant B is usually present in lower concentrations [11]. Fraction F7 corresponds to the washing step and it can be observed that no protein was eluted at this point, i.e. all the proteins initially present in the WPC80 were adsorbed and desorbed during the gradient elution.

With this work, it was possible to recover in a single fraction (F3) all the α-La and Ig, present in the WPC80. Also, the single fraction (F4) containing all the BSA and residual amounts of β-Lg could be further processed in order to purify the BSA. An appropriate method could be gel filtration, thus exploiting their many conformations at different pHs. Changing the pH values, BSA can acquire different conformations such as monomers, dimers and octamers [37] that differ, among other properties, in their molecular size. Gel filtration has been reported by some authors as an efficient way to separate β-Lg from BSA [45,46]. Alternatively BSA could be isolated from β-Lg by hydrophobic interaction chromatography as previously reported [47]. Finally, in the current work it was

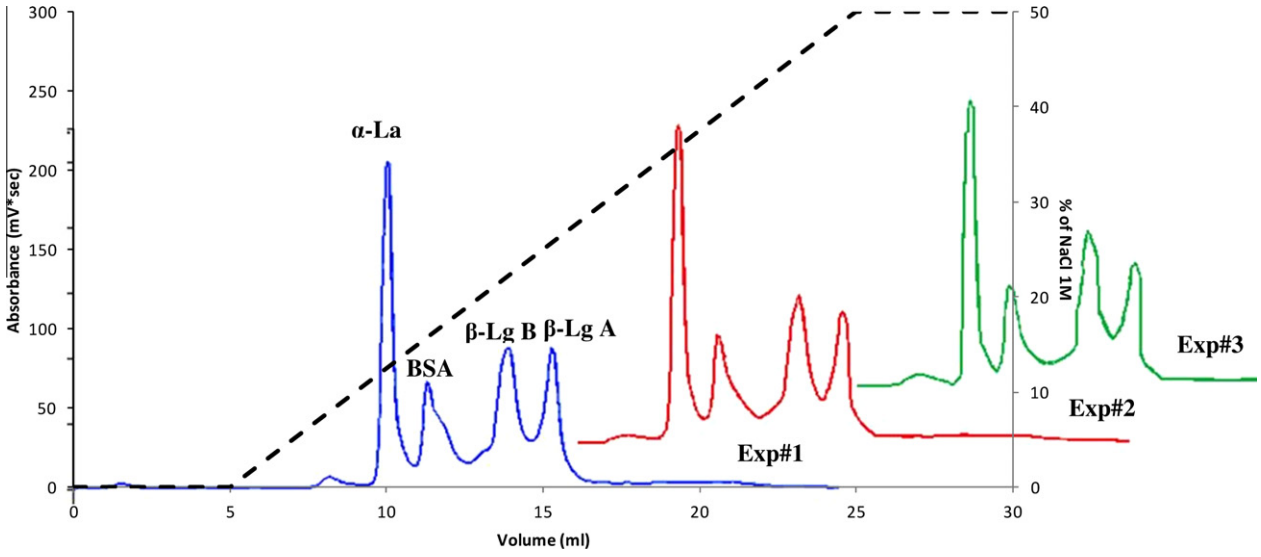

Fig. 2. Elution profile of a mixture of standard proteins (3 g/l), α-La, β-Lg and BSA, eluted with a salt gradient ranging from 0 to 0.5 M NaCl (black dashed line). Three independent experiments, Exp #1 (in blue), Exp #2 (in red) and Exp #3 (in green) have been conducted. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

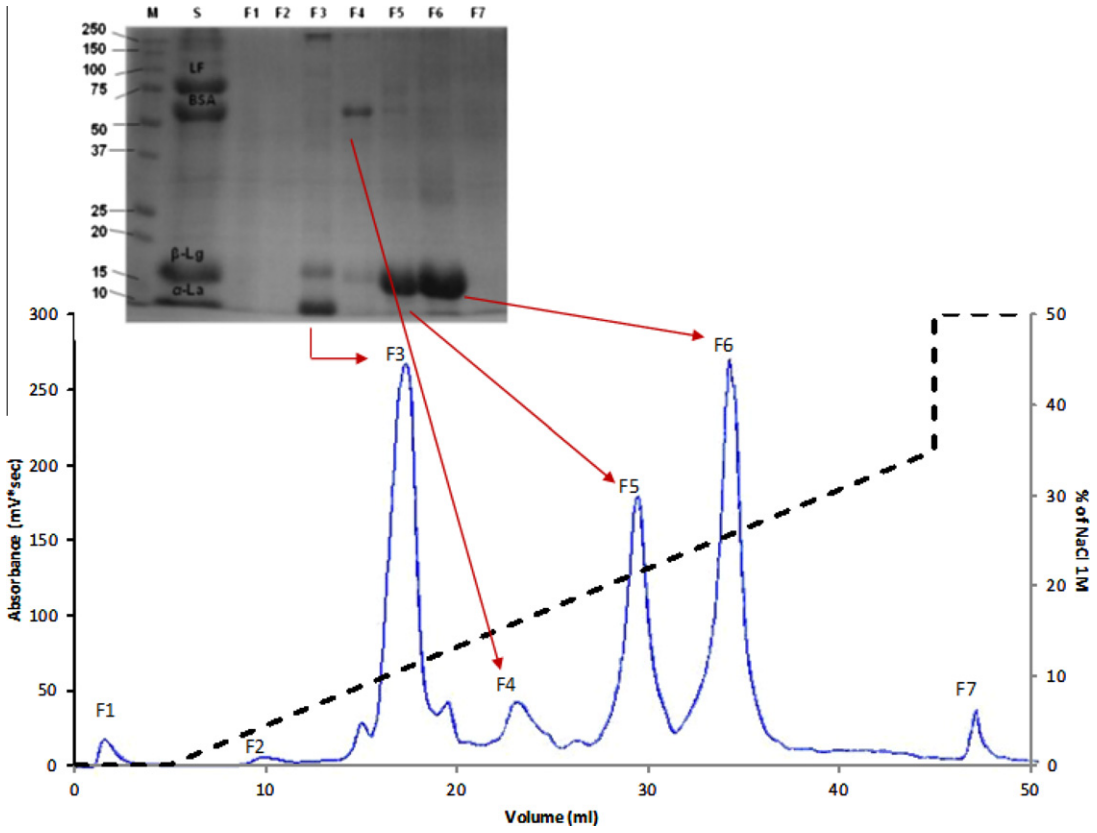

Fig. 3. Elution profile of the WPC80 (5 g/l) eluted with a salt gradient ranging from 0 to 0.5 M NaCl (black dashed line) and SDS–PAGE gel of the fractions collected during elution. Variation in elution times of the several fractions between three independent experiments was estimated to be less than M – Bio-Rad marker (molecular weights in kDa); S – sample of four proteins; F1–F7 – fractions collected during elution.

possible to recover β-Lg A (F5) and β-Lg B (F6) (about 60.5% (w/w) collectively) without any other contaminant proteins.

Many studies based on anion-exchange chromatography have been undertaken to separate and isolate whey proteins. Kunz and Lönnerdal [43] and Manji et al. [48] reported separations of these proteins using the same column as in the current study (Mono Q column). Nevertheless, Kunz and Lönnerdal [43] used an ethanolamine buffer, which is a quite toxic buffer and not adequate for the food industry, therefore the method proposed in the current work is more advantageous since the buffers used do not present this drawback [49]. On the other hand, Manji et al. used the same buffer and pH solution, however the separation obtained for the β-Lg variants was not satisfactory unlike with the method proposed in the current work [48]. Perhaps, these differences are due to the distinct elution mode used by the authors, namely they used a step gradient combined with inversed linear gradient, at specific intervals. Using DEAE-C anion-exchange chromatography, Neyestani and co-workers [46] could fractionate β-Lg, α-La and BSA. In this study α-La and BSA were found to co-elute. Furthermore, to separate β-Lg into its two variants the authors reported the need to perform a second chromatographic step, which could be achieved in a single step in the current work. Rossano and collaborators [15] attempted to isolate the major whey proteins using a hydroxyapatite column. However, the authors failed to accomplish their goals in a single step, and included a second gel filtration column to produce pure protein fractions. Also, the authors used a pH gradient instead of an ionic strength gradient, which is an easier and more feasible elution mode. It is important to mention that the goal of the current work was to fractionate the main whey proteins and isolate the β-Lg variants. In this view, the results obtained were promising, thus additional purification steps will only be required if purer proteins are envisaged. These additional steps may include, as previously mentioned, a gel filtration or a hydrophobic chromatography column, among others. Kim and co-workers [50] investigated the separation by anion-exchange membrane, which was effective for α-La, BSA and β-Lg, but not for the separation of the β-Lg variants. The same problem was reported by Ye et al. [51] using a QAE-TP column. Finally, El-Hatmi et al. [52] reported the use of a Protein Pack SP 5 PW column (cation-exchange chromatography) coupled to a FPLC system and a salt gradient elution (0–1 M NaCl). However, these authors found that all the collected fractions were contaminated with lactoferrin.

The great majority of studies developed to isolate whey proteins by IEC do not take into account the separation of proteins variants. On the other hand, the studies that address the isolation of the variants usually ignore the possibility to separate in the same run the other proteins that are also present in whey. The method proposed in the current work was successful for the separation and isolation of the two β-Lg variants using a single chromatographic step. This result is very important to subsequently study the different functionalities of the two variants. Additionally, although the other target proteins (α-La, Ig and BSA) could not be isolated as pure proteins, they were completely recovered from the WPC80. Furthermore, the results obtained in the current work are very relevant for future large scale applications. During a method development a small particle size must be used to improve resolution and for optimization purposes. Therefore, Mono Beads (matrices made from polystyrene with divinyl benzene) with 10 μm particle size were used. However, small particles can also result in increased back pressure and this factor may become restrictive when scal-

ing-up. Thus, to scale up the current method moving to a larger particle size, preferably using the same medium, to take advantage of lower back pressure and higher flow rates, should be considered. Source 15 can be used instead of Mono Beads since the matrix is the same but the particle size is 15 μm, and it allows flow rates up to 1800 cm/h, thus is ideal for large scale applications. When scaling-up, the salt concentrations at which peaks elute may decrease with increased sample loads. As sample is applied to the column, components with a low net charge will be displaced by components with a higher net charge. Molecules will elute in the same order, but at a different point in the elution profile. In summary, the results herein obtained are scalable if some operational requirements are fulfilled such as maintain bed height, sample concentration and ratio of sample volume to volume of medium; increase the column volume by increasing the cross-sectional area (diameter) of the column; and run the separation using the same salt profile as used at the lab scale with the same ratio of gradient volume to column volume.

4. Conclusion

The use of a Mono Q 5/50 GL column with a salt gradient elution method demonstrated that anion-exchange chromatography is a suitable technique to recover 60.5% (on total protein basis) of the β-Lg in a pure form, from the WPC80. The proposed method is easy, inexpensive and enables the recovery of β-Lg from WPC80 in a single step. A subsequent chromatographic step, for example gel filtration which is quite simple, could be used to fractionate the α-La, Ig and BSA since these proteins have very distinct molecular weights. Results obtained herein are very relevant for future large scale applications.

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