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Fractionation and recovery of whey proteins by hydrophobic interaction chromatography

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ABSTRACT

A method for the recovery and fractionation of whey proteins from a whey protein concentrate (80%, w/w) by hydrophobic interaction chromatography is proposed. Standard proteins and WPC 80 dissolved in phosphate buffer with ammonium sulfate 1 M were loaded in a HiPrep Octyl Sepharose FF column coupled to a fast protein liquid chromatography (FPLC) system and eluted by decreasing the ionic strength of the buffer using a salt gradient. The results showed that the most hydrophobic protein from whey is α-lactalbumin and the less hydrophobic is lactoferrin. It was possible to recover 45.2% of β-lactoglobulin using the HiPrep Octyl Sepharose FF column from the whey protein concentrate mixture with 99.6% purity on total protein basis.

1. Introduction

Management of dairy whey has often involved the use of the most economical disposal methods, including discharge into water streams and onto fields, or simple processing into low value commodity powders [1]. However, because of its very high BOD (Biological Oxygen Demand), whey was considered as one of the strongest industrial wastewater pollutants of any kind [2]. With increasing restrictions and environmental concerns, as well as the discovery of the nutritional and functional properties of whey, it began to be recognized as a valuable resource and gained increasing interest for its potential use in functional food, nutraceuticals, pharmaceuticals and cosmetics [3]. For these reasons, much effort has been aimed at recovering the proteins from whey [4].

For a long time, milk proteins were considered to provide only nutrition components such as nitrogen and essential amino acids for young mammals but in the last decades, several studies indicated that milk proteins also possess important health benefits, thus renovating the interest in the individual proteins from whey [5]. Health benefits of the individual whey proteins that have been reported in the literature include the transport of retinol, palmitate, fatty acids, vitamin D and cholesterol by β-lactoglobulin (β-Lg) [6]; the induction of apoptosis in tumor cells by α-lactalbumin (α-La) and lactoferrin (Lf) [7,8]; the prevention of cancer by bovine serum albumin (BSA) [9]; the host defense against organisms requiring iron by Lf [9,10]; the antimicrobial and antiviral activity by lactoperoxidase (Lp) [10]; and the antiviral activity against HIV by immunoglobulins (Ig) [11].

Dairy proteins commonly available today are typically concentrates of caseins or whey proteins, being the isolated proteins less common. The isolated protein could be used by individuals with special nutritional needs to tailor their diet, thus improving health [12].

Liquid-chromatography is the most widely used method for protein recovery and purification as it is a robust and efficient technique [13] and hydrophobic interaction chromatography (HIC) is a methodology commonly used in the purification of biomolecules [14]. This technique is based on the hydrophobic interaction between hydrophobic ligands and non-polar regions on the surface of the biomolecules [15]. It is a powerful adsorptive separation technique because of the fast separations achieved with slight product degradation, low solvent requirements and good purification levels [16]. The type of salt and its concentration greatly influences the hydrophobic interactions between proteins with hydrophobic media, and HIC chromatography is often carried out by gradient elution with decreasing salt concentrations [17]. The most widely used ligands for HIC are linear chain alkanes with or without a terminal amino group and constitute a homologous series in a hydrophobicity scale: methyl < ethyl < propyl < butyl < pentyl < hexyl < heptyl < octyl [18]. The hydrophobicity and the strength of interaction increase with the increase of the *n*-alkyl chain length of the ligand used, but the adsorption selectivity may decrease.

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Several works have been conducted with the purpose of recover and purify whey proteins. Among these are Bramanti et al. [19] who studied the separation of caseins in a TSK-Gel Phenyl-5PW column; Sousa et al. [20] who studied the separation of different forms of proteose peptone 3 by hydrophobic interaction chromatography with a dual salt system, in four different adsorbents; Yoshida [21] who isolated whey Lf in a Butyl Toyopearl 650 M column; and Machold et al. [22] who studied the selectivity and separation efficiency of whey proteins onto different HIC sorbents from various manufactures.

The aim of this study was to develop a method to recover and fractionate the proteins from a whey protein concentrate (WPC 80) by hydrophobic interaction chromatography using a HiPrep Octyl Sepharose FF 16/10 column.

2. Materials and methods

2.1. Materials

2.1.1. Hydrophobic interaction chromatography column

The column used for the fractionation and recovery of proteins from the whey concentrate (WPC80) was a 20 mL HiPrep Octyl Sepharose FF 16/10 (GEHealthcare, Pittsburgh, PA). This column consists of a 4% highly cross-linked spherical agarose matrix with an average particle size of 90 μm, and a 5 μmol/mL medium density of the hydrophobic ligand (octyl).

2.1.2. Standard proteins and whey protein concentrate

Standard pure proteins, namely α-lactalbumin (α-La), β-lactoglobulin (β-Lg), lactoferrin (Lf) and bovine serum albumin (BSA), were used to determine their retention times. These proteins were purchased from Sigma (Sigma–Aldrich Co., St. Louis, MO) and their main characteristics, such as molecular weight (kDa) and purity (%), are: α-La (14.2, 85), β-Lg (18.4, 80), Lf (79, 98) and BSA (69, 90). The GRAVY (GRand AVerage of hydropathicitY) index indicates the solubility of the proteins, which is calculated by the ratio between the sum of the hydrophobicity value of all amino acids that constitute each protein and the total number of residues in the sequence. A positive GRAVY value indicates that the protein is hydrophobic and a negative value that it is hydrophilic [23]. Using the Expasy Tools program (http://www.expasy.org/tools/) the following GRAVY indexes were retrieved for the studied proteins: α-La (−0.151), β-Lg (−0.006), Lf (−0.293) and BSA (−0.433).

A whey protein concentrate (WPC 80) 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. The protein composition of the WPC 80 is 25% α-La, 50% β-Lg, 1% Lf and 10% BSA.

2.2. Methods

2.2.1. Proteins recovery and fractionation

Protein solutions were prepared in phosphate buffer with ammonium sulfate (1 M) with different final concentrations depending on the experiment: for the experiments with each of the standard proteins, 1 g/L; for the experiments with the mixture of the four standard proteins, 4 g/L; and for the experiments with WPC80, 30 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 sodium phosphate 0.05 M with ammonium sulfate 1 M, pH 7.0; and elution buffer consists of sodium phosphate 0.05 M, pH 7.0. Buffers were prepared with Millipore water, filtered under vacuum with 0.45 μm filter and degasified by ultrasounds. Afterwards, the buffers were sterilized at 121 °C for 20 min.

The retention times of each standard protein were determined, as well as the separation of the proteins from whey, using a Fast Protein Liquid Chromatography (FPLC) system (GEHealthcare, Pittsburgh, PA). Detection of proteins was conducted at 280 nm using an UV detector. After equilibrating the column by running 5 column volumes (CV) of buffer A, 500 μL of sample were loaded, and elution was conducted at a salt gradient from 100% to 0%, using 20 CV. Afterwards, a washing step with buffer B (0% salt) was included to remove proteins that were still bound to the matrix using 13 CV. During this process, samples were collected for further quantification of total soluble protein content using the Bradford Method as described elsewhere [24].

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 [25]. A 16 μL volume of each fraction and WPC80 (20 μg protein) was loaded in a different lane, as well as 4 μL of a mixture of the four standard proteins (S) (20 μg protein) and 4 μL of Precision Plus Protein Standards, BioRad (M) (5 μg protein) 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

Currently, whey is generally accepted as a functional food with several health benefits, despite it was once considered a waste and a relevant environmental issue. Many proteins from whey have been reported to possess important nutritional and biological properties (e.g. antioxidant, antihypertensive, antitumor, antiviral, and antibacterial, among others) [10]. Consequently, there is a growing interest by the dairy industry and other food industries to design and formulate products that incorporate specific bioactive components derived from whey, namely its isolated proteins that could be used for special nutrition needs or tailored diets [12]. Generally, the commercially available dairy proteins are concentrates of the caseins or whey proteins, being the isolated proteins less frequent. Therefore, several researchers have developed techniques and methods to recover and purify the isolated whey proteins [19–22]. However, comparing to the method hereby proposed, the above mentioned methods are more complex, involving two step processes [21], dual salt systems [20] and other hydrophobic matrices [19,22]. Also, these authors focused their studies in an individual protein from whey, for example caseins [19], proteose peptone 3 [20] or Lf [21], but none addressed the recovery of βLg that can be obtained with a very high purity using the method proposed in the current work. Despite the success of some of these proposals, much effort is still required in order to find more economical solutions and optimize recovery yields. Based on this discussion, a method for the recovery and fractionation of the proteins from a whey protein concentrate by hydrophobic interaction chromatography was proposed.

Several elution profiles were tested and fractions were collected at different time points in order to determine the retention times of both the standard proteins and the isolated fractions from WPC80. To determine the retention times of each standard protein eluted from the Octyl Sepharose FF 16/10 column, by a gradient from 100% to 0% of phosphate buffer with ammonium sulfate 1 M, 500 μL of

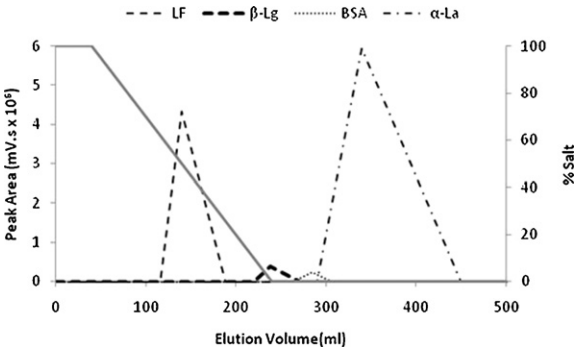

Fig. 1. Chromatographic profile of the standard proteins (1 g/L of each protein: LF, βLg, BSA and αLa) on a HiPrep Octyl Sepharose FF 16/10 in a salt system ($(NH_4)_2SO_4$) with a linear salt gradient from 100 to 0% (full grey line).

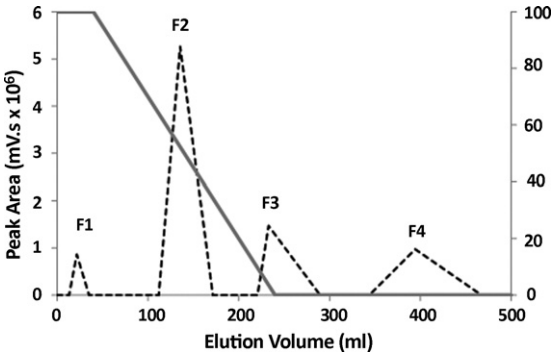

Fig. 2. Chromatographic profile of a mixture of the standard proteins (concentration of the mixture loaded 4 g/L) on a HiPrep Octyl Sepharose FF 16/10 in a salt system ($(NH_4)_2SO_4$) with a linear salt gradient from 100 to 0% (full grey line).

each sample was loaded. According to the GRAVY values for each protein, it was expected that proteins would elute in the following order: BSA < Lf < αLa < βLg. However, from Fig. 1 a different elution profile was observed since the proteins eluted in a different order: Lf < βLg < BSA < αLa. These results can be explained by the different conformations that BSA and βLg can assume for distinct pH values. Also, it is important to notice that the GRAVY values represent the hydrophobic character of each amino acid in each protein sequence, not taking into account their rearrangement as a function of pH. The GRAVY value presented for βLg corresponds to the protein in its monomeric form. Nevertheless, at pH 7.0 this protein occurs in the dimeric form [26]. βLg possesses two hydrophobic binding sites, one in its interior, referred as the calyx (a large hydrophobic invagination) [27]; and the other located in its external surface [28]. Between pH 2 and 9, βLg presents various reversible conformations and the most important is called the Tanford transition, occurring from pH 6.5 to 8.5 [29]. This transition involves the displacement of the loop EF (residues 85–90 in the βLg structure) that acts as a lid which closes the protein interior/binding site below pH 7.3, and opens it at higher pH values [30]. Consequently, as the experiments were carried out at pH 7.0, the calyx of βLg was closed, not allowing its binding to the ligand octyl and therefore the decrease in hydrophobicity was observed. This result is in good agreement with the conclusions of Gao and Dubin [31], who compared the hydrophobicity of βLg at pH 6.3 and 8.7 in an octyl ligand, confirming a tenfold increase in hydrophobicity with increasing pH values.

BSA and αLa eluted from the column almost at the same time, or at least at the same salt concentration, meaning that they were eluted when the percentage of salt reached 0%. Again, according to the GRAVY values, BSA should elute before αLa. However, it was found that they co-eluted. These results are supported by Bigelow's work [32]. This author studied the relation of proteins' structure with their hydrophobicity, and concluded that BSA and αLa have identical hydrophobicity values and that both proteins are more hydrophobic than Lf. Therefore, the results presented in Fig. 1 are in agreement with previous studies reported in the literature.

Additionally, experiments with a mixture of the four standard proteins were conducted and the elution profile is illustrated in Fig. 2. During elution, four fractions (F1, F2, F3 and F4) were collected for further analysis by SDS-PAGE. Also, a fifth fraction (F5) was collected in the washing step with water (not shown on Fig. 2). Fig. 3 shows the SDS-PAGE gel obtained for all of the above mentioned collected fractions. Fraction F1 was collected in the first CV and corresponds to the proteins that were not adsorbed onto the matrix. Although a peak corresponding to F1 was observed in Fig. 2, in the SDS-PAGE gel (Fig. 3) no protein band could be found, probably due to the fact that a very small amount of protein was adsorbed onto the column and therefore could not be detected by SDS-PAGE.

Furthermore, as none of the four standard proteins (βLg, αLa, Lf and BSA) was found in F1 (void volume) it was possible to conclude that all were bound to the hydrophobic octyl ligand. Fractions F2 and F3 were collected during the salt gradient and fraction F4 was collected after the gradient ended (salt concentration 0%). Elution of the proteins solution followed the same behavior as the one previously observed for the isolated standard proteins. However, some contaminations from the other proteins could be observed in all the fractions, for example BSA, even in small amounts, was present in all the collected fractions. Lf was totally eluted in F2 and the majority of βLg was eluted in F3, although small amounts were also seen in F4. The F4 fraction contained all the αLa from the initial mixture and a great amount of BSA. As described above, the fraction F5 was collected during the column washing step with water. In this step, all proteins that are still bound to the matrix should be removed. From Fig. 3, it was possible to observe that none of the proteins from the initial mixture was found in F5, meaning that all proteins were adsorbed and desorbed from the column using the proposed elution salt gradient.

Furthermore, a 30 g/L solution of WPC80 was loaded onto the Octyl column and the same elution salt gradient was used (Fig. 4).

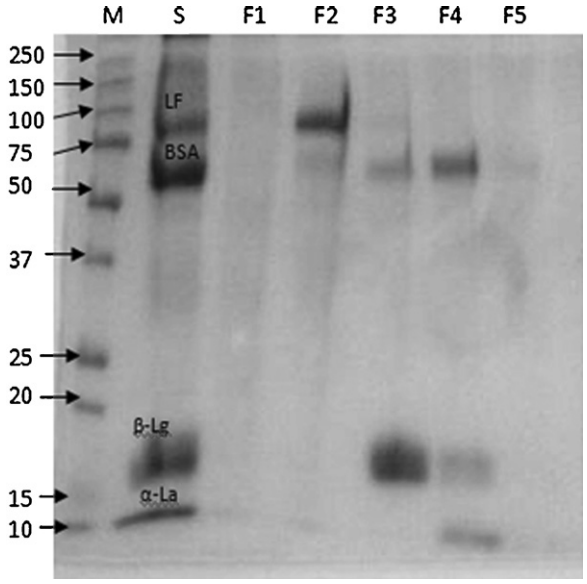

Fig. 3. SDS-PAGE of the fractions collected during elution of a mixture of the four standard proteins from the HiPrep Octyl Sepharose FF 16/10 column. The samples analyzed were: M, Bio-Rad marker (molecular weights in kDa); S, mixture of the four standard proteins (LF, βLg, BSA and αLa); F1 to F5, fractions eluted from the column with linear salt gradient from 100 to 0%.

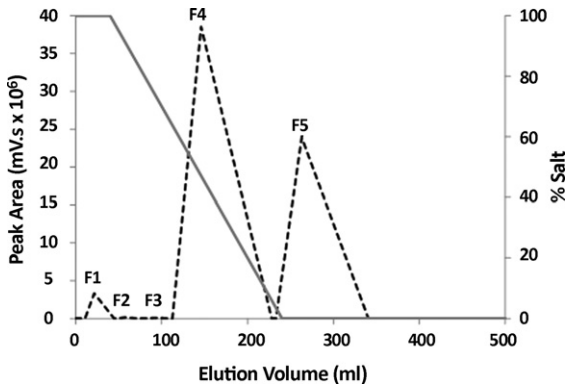

Fig. 4. Chromatographic profile of the WPC80 (concentration loaded 30 g/L) on a HiPrep Octyl Sepharose FF 16/10 in a salt system ($(NH_4)_2SO_4$) with a linear salt gradient from 100 to 0% (full grey line).

During elution, five fractions (fractions F1, F2, F3, F4 and F5) were collected for further analysis by SDS-PAGE (Fig. 5). As in the previous experiments, an extra fraction (fraction F6) was collected during the washing step with water, which is not shown in Fig. 4. The percentage of salt (ammonium sulfate) at which each fraction was eluted from the column was: F1, 100% (v/v); F2, 91.1% (v/v); F3, 71.2% (v/v); F4, 47.0% (v/v); F5, 0% (v/v). According to Fig. 4, five peaks probably corresponding to proteins were detected. However, from the SDS-PAGE gel (Fig. 5), no protein bands were found in fractions F1, F2 and F3. A possible reason could be that other WPC80 components also detected at 280 nm were eluted in the void volume and in the beginning of the salt gradient. As these components are not proteins they are not detected in the SDS-PAGE gel. On the other hand, it could also happen that if any proteins were contained in those fractions (F1, F2 and F3), their amounts were probably too small to be detected in the SDS-PAGE gel. Indeed, these fractions are supposedly constituted by the hydrophilic proteins, namely Lp and lysozyme, and these proteins exist in very small amounts in whey as compared to BSA, αLa and βLg. Fraction F4 was found to be composed only by βLg since a single defined band was observed in the SDS-PAGE gel (Fig. 4). βLg recovery in fraction F4 (45.2% on total protein basis) was determined based on the protein concentration loaded in the column and the protein concentration eluted, as determined by the Bradford method and the relative percentage of the protein on the WPC80. βLg purity in fraction F4 was determined based on band intensity analysis, results showed that βLg was recovered from the WPC80 mixture with 99.6% purity. Although only about half the βLg amount present in the whey concentrate is recovered, its purity is very high compared to other studies that have been reported in the literature using alternative methods [33,34]. Furthermore, the method proposed in the current work is easy, inexpensive and requires a simple set-up. Fraction F5 was constituted by BSA and αLa, although with some contamination of βLg. As described above, fraction F6 was collected during the column washing step and it could be confirmed that no protein was eluted in this step. The last lane in the SDS-PAGE gel (Fig. 5) corresponds to a sample of the WPC80. It was found that only the main proteins in whey were detected, not being visible Igs, lysozyme, Lf and Lp.

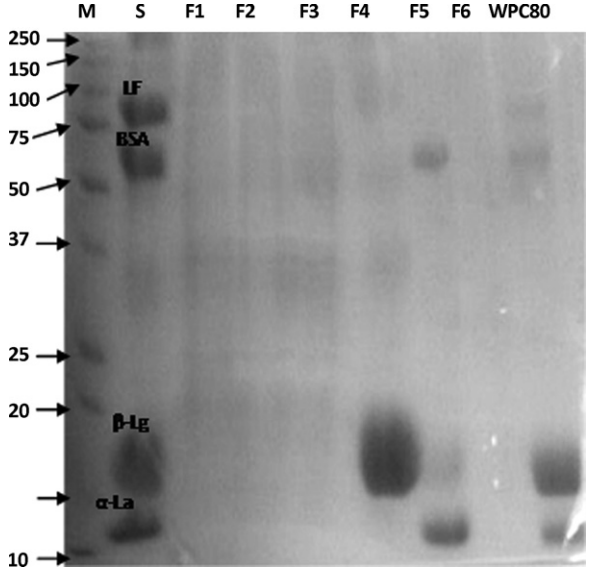

Fig. 5. SDS-PAGE of the fractions collected during elution of the WPC80 from the HiPrep Octyl Sepharose FF 16/10 column. The samples analyzed were: M, Bio-Rad marker (molecular weights in kDa); S, mixture of the four standard proteins (LF, βLg, BSA and αLa); F1 to F6, fractions eluted from the column with linear salt gradient from 100 to 0%.

Finally, it is important to notice that if a full separation of the four main proteins in whey is desired, further separation of BSA from αLa in fraction F5 can be proposed in the future. The use of other gradients and different initial ammonium sulfate concentrations was attempted, but no further improvement on the separation could be observed (data not shown). Nevertheless, dual salt systems could be explored, as well as other chromatographic methods, such as size exclusion since the molecular weights of these two proteins are much different. Therefore, optimization and improvement of the separation methods in order to develop an integrated process for the recovery of the isolated proteins from whey could be further explored.

4. Conclusions

The use of a HiPrep Octyl Sepharose FF 16/10 column with a salt gradient elution method demonstrated that hydrophobic interaction chromatography is a suitable technique to recover 45.2% (on total protein basis) of the βLg present in WPC80 in a pure form (99.6% purity). The proposed method for the recovery of βLg is easy, inexpensive and requires a simple set-up. Furthermore, a preliminary fractionation of the remaining proteins could also be obtained with the proposed method, thus indicating that HIC can be used as a first step of an integrated process for the full recovery and purification of whey proteins.

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