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Isolation of lactoferrin from whey by dye-affinity chromatography with Yellow HE-4R attached to chitosan mini-spheres

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ABSTRACT

Novel integrated chromatographic methods need to be developed for specific, low-cost protein purification from raw materials. Here, a process for bovine lactoferrin (Lf) isolation from sweet whey was developed using cross-linked chitosan mini-spheres with immobilised Yellow HE-4R dye as a low-cost ligand. The maximum adsorption capacity was between 51.14 and 58.28 mg Lf g^{-1} matrix. In addition, the mini-spheres adsorbed around 95% of the Lf present in the sweet whey and eluted more than 80% of the adsorbed Lf. A yield of 77% with purity greater than 90% was achieved in only one purification step. The purification process was efficient for three consecutive cycles without regeneration steps.

1. Introduction

Lactoferrin (Lf) is a glycoprotein present in mammalian external secretions with iron-chelating capacity (Tomita, Wakabayashi, Yamauchi, Teraguchi, & Hayasawa, 2002). Its biological properties include regulation of iron adsorption in the gastrointestinal tract, antimicrobial and antifungal activity, and modulation of polymorphonuclear production (Farnaud & Evans, 2003; Na et al., 2004). Hence, this protein is being increasingly investigated, not only for its nutritional value, but also for its potential use in other sectors, such as the dairy, nutraceutical, pharmaceutical and cosmetic industries.

Bovine lactoferrin (Lf) has been isolated from various natural sources by different chromatographic methods, including hydrophobic interaction, ion exchange and affinity with antibodies (Chen & Wang, 1991; Chiu & Etzel, 1997; Kawakami, Shinmoto, Dosako, & Sogo, 1987; Kawakami, Tanimoto, & Dousako, 1992; Pahud & Hilpert, 1976; Tu, Chen, Chang, & Chang, 2002; Yoshida, 1989; Yoshida & Ye, 1991). The method most frequently used for extraction and purification of Lf at industrial level is ion exchange chromatography using concentrated and, in some cases, diafiltered whey. In this process yields between 50 and 96% are obtained and lactoperoxidase – another whey protein with similar isoelectric point – is co-purified. However, when whey without any pretreatment is processed, low yields of Lf are obtained. On the other hand, although the methods of affinity purification using antibodies as ligands show better performance when the protein is found at low concentrations in the source (whey), the production costs increase significantly.

We have previously found that the triazine dye Red HE-3B used as a pseudobiospecific affinity ligand has an adequate chromatographic performance for Lf purification from bovine colostrum and sweet cheese whey (Grasselli & Cascone, 1996). We have also reported the acceptable performance of Yellow HE-4R immobilised on Sepharose 4B for Lf purification from whey (Baieli, Urtasun, Miranda, Cascone, & Wolman, 2014). From an industrial perspective, pseudobioaffinity ligands are of interest as they provide selective purification of proteins with a favourable cost/selectivity ratio. In general, these dye ligands share common characteristics such as low cost and high chemical, thermal and mechanical stability. Triazine dyes are used in the textile industry at tonnes level, are easy to immobilise on different supports and have good capacity and selectivity (Denizli & Piskin, 2001).

On the other hand, chitin is a linear polymer consisting of N-acetyl glucosamine (GlcNAc) units. It is the second most abundant polymer in nature, found mainly in the exoskeleton of crustaceans and insects. Chitosan is obtained by partial de-acetylation of chitin and it is a linear copolymer of randomly distributed D-glucosamine (GlcN) and GlcNAc (Harish Prashanth & Tharanathan, 2007; Kristiansen, Nysæter, Grasdalen, & Vårum, 1999; Kurita, 2001; Roberts, 2008; Terbojevich & Muzzarelli, 2000; Wolman et al.,

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2010). Moreover, chitosan has a structure that exhibits functional groups that promote and facilitate the adsorption and immobilisation of different ligands (Pillai, Paul, & Sharma, 2009). Both chitin and chitosan have the advantage of being highly available, biocompatible, biodegradable and environmentally safe (Harish & Tharanathan, 2007; Terbojevich & Muzzarelli, 2000; Wolman et al., 2010). These features make them ideal support materials to explore new chromatographic possibilities (Osifo et al., 2008).

In a previous work, chitosan mini-spheres were prepared by dropping chitosan on a NaOH solution and, to increase its mechanical strength and to avoid matrix solubilisation at acid pH, mini-spheres were cross-linked using epichlorohydrin (Baieli, Urtasun, Miranda, Cascone, & Wolman, 2012). Because of their large size of about 1.76 mm, these kinds of matrices are very easy to manipulate, thus avoiding the need of centrifugation after each purification step. Thus, mini-spheres can be recovered by simple filtration through a strainer (Baieli et al., 2012; Wolman et al., 2010). This is an important advantage, especially to purify proteins from complex raw materials of high viscosity.

The aim of this work was to develop a new affinity matrix with high adsorption capacity and optimal mechanical resistance to purify Lf from whey. To this end, we used mini-spheres of chitosan with Red HE-3B or Yellow HE-4R triazine dyes as the immobilised ligands. In addition, for comparison purposes, both dyes were immobilised on commercial Sepharose 4B.

2. Materials and methods

2.1. Materials

Chitosan (medium molecular weight) and Red HE-3B dye (C.I. name: reactive red 120) were from Sigma–Aldrich (St. Louis, MO, USA). Yellow HE-4R dye (C.I. name: reactive yellow 84) was from Vilmax (Buenos Aires, Argentina). Sepharose 4B and the Low Molecular Weight calibration kit for SDS-PAGE were from GE Healthcare Bio-Sciences (Little Chalfont, UK). Epichlorohydrin was from Fluka Analytical (Buchs SG, Switzerland) and bovine lactoferrin (Lf) was from Glanbia Nutritionals, Inc. (Fitchburg, WI, USA). The Quick Start ™ Bradford reactive for total protein determination was from BioRad (Hercules, CA, USA). The Bovine Lactoferrin ELISA Quantification kit was from Bethyl Laboratories Inc. (Montgomery, TX, USA). Bovine cheese whey was kindly donated by Cooperativa Lechera Gualeguaychú (Gualeguaychú, Entre Ríos, Argentina). All other reagents were analytical reagent grade.

2.2. Matrix preparation

Chitosan-based matrices were obtained as described in a previous work (Baieli et al., 2012). Briefly, a 2% chitosan solution in 2% acetic acid was dripped on 2 M NaOH. After 10 h, they were washed with distilled water until neutrality (Guo et al., 2005). Then, the beads were cross-linked with 250 or 500 mM epichlorohydrin solutions. The matrices cross-linked with 250 mM epichlorohydrin are named hereafter C250, and those cross-linked with 500 mM epichlorohydrin, C500.

Red HE-3B and Yellow HE-4R dyes were coupled to the matrices according to Baieli et al. (2014). C250 and C500 matrices coupled to Red HE-3B are named hereafter C250-R and C500-R, respectively, whereas those coupled to Yellow HE-4R are named hereafter C250-Y and C500-Y, respectively.

For comparison purposes, Red HE-3B and Yellow HE-4R dyes were also immobilised on Sepharose 4B: 2 mL of Sepharose 4B suspension (50% in water) was added to 50 mg dye in 2 mL distilled water and 1 mL 4 M NaCl. After 60 min agitation, 100 μL of 10 M NaOH was added and the suspension was made up to a final volume of 10 mL with water. After 10 h of agitation, temperature was raised to 40 °C and maintained for 4 h (Stellwagen, 1990; Wolman, González Maglio, Grasselli, & Cascone, 2007). Finally, the matrix was washed with water, methanol, 2 M NaCl and 1 M NH_4Cl. Sepharose 4B with immobilised Red HE-3B and Sepharose 4B with immobilised Yellow HE-4R are named hereafter S-R and S-Y, respectively.

2.3. Matrix characterisation

2.3.1. Matrix size, density and porosity

The size of the generated matrix, before and after the cross-linking and dye coupling, was measured in quintuplicate with a Vernier sensitive to 0.01 mm, and the density and porosity of the matrix were calculated according to Roh and Kwon (2002) using the liquid displacement technique with freeze-dried matrices. The matrix (250 mg = W_C) was immersed in a graduated tube containing 5 mL isopropanol (V_I) for 5 min. The total volume of isopropanol and that of the isopropanol-impregnated matrix were measured and recorded as V_T. Then, the isopropanol-impregnated matrix was removed from the tube and the volume of the residual isopropanol recorded as V_R. The density of the matrix (d) was calculated as:

$$d\left(\mathrm{g\ mL^{-1}}\right) = W_C/(V_T - V_R) \quad (1)$$

and the porosity of the matrix (ε) was calculated as:

$$\varepsilon(\%) = [(V_I - V_R)/(V_T - V_R)] \times 100 \quad (2)$$

where $(V_T - V_R)$ is the matrix volume (matrix shape volume) and $(V_I - V_R)$ is the volume of isopropanol held in the matrix pores (void volume of the matrix).

2.3.2. Scanning electron microscopy

Scanning electron microscopy (SEM, Zeiss Supra 40 microscope, Jena, Germany) was used to analyse the morphology of chitosan matrices before and after cross-linking and dye coupling. The matrices were lyophilised and metalised with gold (gold sputtering) prior to the analysis.

2.3.3. Ligand density

The dye density of the matrices was determined by the procedure reported by Ruckenstein and Zeng (1998). Briefly, the matrices were hydrolysed in a 10 M HCl aqueous solution at 80 °C for 30 min. The solution was then diluted 2-fold with distilled water and the pH was adjusted to 7.0 with 6 M NaOH. The dye concentration was determined spectrophotometrically at its maximum absorbance wavelength obtained by spectrophotometric scanning. The maximum absorbance for Red HE-3B and Yellow HE-4R dyes was at 511 and 400 nm, respectively, using an Ultrospec 2000 spectrophotometer (GE HealthCare, Little Chalfont, UK).

2.4. Pure lactoferrin adsorption isotherms

Adsorption isotherms were determined at pH 7.0. To this end, 50 mg of each matrix (C250-R, C500-R, C250-Y and C500-Y) were soaked with 1 mL Lf solutions (0.063–10 mg mL^{-1} in adsorption buffer). The adsorption buffer consisted of 20 mM sodium phosphate buffer, pH 7.0. All suspensions were gently shaken overnight at room temperature to allow the system to reach its equilibrium. The concentration of Lf in the supernatants at the beginning of the experiment and after adsorption, was measured spectrophotometrically at 280 nm considering an extinction coefficient of

1.51 mL mg^{-1} cm^{-1} (Andersson & Mattiasson, 2006). Maximum capacity (Q_{max}) and dissociation constant (K_d) were calculated and analysed according to the Langmuir model as described by Chase (1984).

The experiments were carried out in triplicate. Results are expressed as the average ± standard deviation.

2.5. Elution studies

Elution studies were performed according to Baieli et al. (2012) with minor modifications. Briefly, 50 mg of each matrix (C250-R, C500-R, C250-Y and C500-Y) were saturated overnight with 1 mL of 10 mg mL^{-1} Lf in the adsorption buffer. After three washings, ten elution solutions were tested: (i) 2 M NaCl, pH 7.0, (ii) 2 M NaCl, pH 9.0, (iii) 0.5 M NaSCN, pH 7.0, (iv) 0.5 M NaSCN, pH 9.0, (v) 25% ethylene glycol, pH 7.0, (vi) 25% ethylene glycol, pH 9.0, (vii) 25% ethylene glycol + 0.5 M NaSCN, pH 7.0, (viii) 25% ethylene glycol + 0.5 M NaSCN, pH 9.0, (ix) 25% ethylene glycol + 2 M NaCl, pH 7.0 and (x) 25% ethylene glycol + 2 M NaCl, pH 9.0. For pH 9.0 eluents, 20 mM carbonate buffer was used.

The experiments were carried out in triplicate. Results are expressed as the average ± standard deviation.

2.6. Lactoferrin purification from whey

2.6.1. Whey clarification

For a comparative study with Sepharose, a whey clarification step was required (Wolman et al., 2007). The residual fat was removed from whey by thermocalcic precipitation according to Maubois, Pierre, Fauquant, and Piot (1988). Briefly, 0.12 g of Ca^{2+}, as $CaCl_2$, was added to 100 mL whey with gentle stirring at 2 °C and the pH was increased to 7.4 with 2 M NaOH. The mixture was incubated for 8 min at 50 °C and centrifuged at 5000 × *g* for 10 min at 4 °C.

2.6.2. Optimisation of the sample-volume/matrix-amount ratio for the purification process

Aliquots of 0.5, 1.0, 2.5, 5.0 and 10.0 mL of clarified whey were incubated with 25 mg of the four matrices (C250-R, C500-R, C250-Y and C500-Y) overnight with stirring at room temperature. The process was monitored by measuring total protein by the Bradford method (Bradford, 1976) and Lf concentrations with the Bovine Lactoferrin ELISA Quantification kit.

All determinations were performed in triplicate and results are expressed as the average ± standard deviation.

2.6.3. Purification process

The adsorption was performed in batch by incubating 25 mg of matrices (C250-R, C500-R, S-R, C250-Y, C500-Y and S-Y) with 1 mL of clarified whey overnight with stirring at room temperature. After four washing steps with 20 mM sodium phosphate buffer, 0.5 M NaCl, pH 7.0, the Lf was eluted with 0.5 mL of 25% ethylene glycol + 2 M NaCl, pH 7.0 overnight with gentle stirring. Finally, all samples (whey, supernatants and eluates) were monitored by measurement of total protein by the Bradford method, and Lf concentration with the Bovine Lactoferrin ELISA Quantification kit.

All determinations were performed in triplicate and results are expressed as the average ± standard deviation.

2.7. Matrix reutilisation

The C500-Y matrix was used for two new adsorptive cycles to assess the matrix reuse. No regeneration steps were applied between cycles. The matrix was equilibrated between cycles by two washing steps with 4 mL 20 mM phosphate buffer, 0.5 M NaCl, pH 7.0, for 2 h each. Finally, all samples were monitored by measurement of total protein by the Bradford method and Lf concentration with the Bovine Lactoferrin ELISA Quantification kit and SDS-PAGE.

All determinations were performed in triplicate and results are expressed as the average ± standard deviation.

2.8. Kinetic studies

Adsorption was performed in batch by incubating 250 or 2500 mg of C500-Y with 10 mL of clarified whey for 24 h with stirring at room temperature. Supernatant aliquots were taken at different times of incubation (0.5–24 h) to measure their Lf content.

2.9. Sodium dodecylsulphate polyacrylamide gel electrophoresis

Whey, supernatants and eluates were concentrated four times using centrifugal filtration (Amicon Ultrafree MC, 10 kDa cut-off; Millipore, Billerica, MA, USA) before 15% SDS-PAGE. Eluates were subjected to buffer exchange to 20 mM sodium phosphate buffer, pH 7.0, using this centrifugal filtration. Gels were stained with Coomassie Blue under standard conditions.

3. Results and discussion

3.1. Matrix characterisation

Before the cross-linking and coupling of the dyes to the matrix, its size, density and porosity were measured. The generated matrix had a size of 1.760 ± 0.120 mm. After freeze-drying, the matrix showed a density of 0.138 ± 0.009 g mL^{-1} and a porosity of 40.0 ± 2.4%. The freeze-dried matrix was characterised by SEM. The aspect of the matrix is shown in the SEM image of Fig. 1. The image evidenced the homogeneity of the material, the absence of agglomerations and a macroporous structure. The pictures of the cross-linked matrices and those with immobilised dyes showed the same porous and homogeneous structure, thus evidencing that the macroporous structure was not affected (data not shown).

Each matrix was then cross-linked with two different epichlorohydrin concentrations and either Red HE-3B or Yellow HE-4R was immobilised on them. These matrices showed an average size of 1.649 ± 0.038 mm, i.e., slightly smaller than that of the unmodified

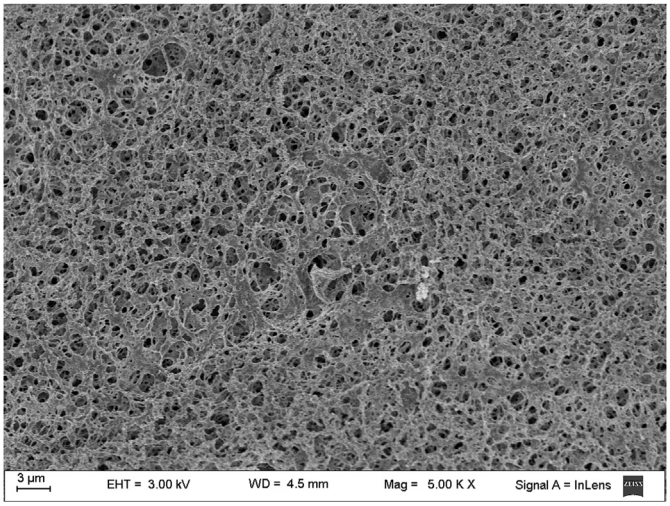

Fig. 1. Scanning electron microscopy image of chitosan matrix before cross-linking and dye coupling.

matrix. The dye density of C250-R, C500-R, C250-Y and C500-Y matrices was 8.44 ± 1.07, 7.27 ± 0.72, 4.16 ± 0.09 and 2.91 ± 0.13 μmol g^{-1}, respectively. Chitosan matrices showed a greater density of Red HE-3B than of Yellow HE-4R under both cross-linking conditions. In addition, chitosan matrices showed a higher density of Red HE-3B than the Sepharose matrix (1.46 ± 0.02 μmol g^{-1}). No differences were observed between Yellow HE-4R on the Sepharose matrix (2.27 ± 0.14 μmol g^{-1}) (Baieli et al., 2014) and chitosan cross-linked with epichlorohydrin (500 mM).

Ligand density increased as the cross-linking degree for both dyes decreased. A possible explanation for the higher ligand density on the C250 series is the higher number of reactive nucleophilic primary amino groups present for reaction with the dyes in C250. Osifo et al. (2008) studied the influence of the cross-linking degree on the adsorption of Cu^{2+} by chitosan beads and found that the maximum capacity decreased as the cross-linking degree increased. In the present work, the dye coupling to each of the different cross-linked matrices was significant different.

3.2. Pure lactoferrin adsorption isotherms

Adsorption isotherms were determined in order to characterise the matrices synthesised (Fig. 2). The thermodynamic parameters calculated from the isotherms are shown in Table 1.

The maximum adsorption capacity for Lf obtained with the C250-R and C500-R matrices was 40.17 and 51.04 mg g^{-1}, respectively, whereas that with the C250-Y and C500-Y matrices was 58.28 and 51.14 mg g^{-1}, respectively. These results show that the maximum adsorption capacity of chitosan matrices was higher than that previously reported for Sepharose matrices (27.82 for Red HE-3B and 40.24 mg g^{-1} for Yellow HE-4R) (Baieli et al., 2014). C250-Y showed higher maximum adsorption capacity than C250-R. Matrices of the C500 series showed similar values for both dyes, thus evidencing that increased ligand density not always corresponds to a higher adsorption capacity of a protein (Wolman, Smolko, Cascone, & Grasselli, 2005).

Lf concentration in whey was 0.08–0.10 mg mL^{-1} and the K_d values obtained from all the isotherms (Table 1) were in the same order, indicating that these matrices ensure an efficient Lf removal from whey.

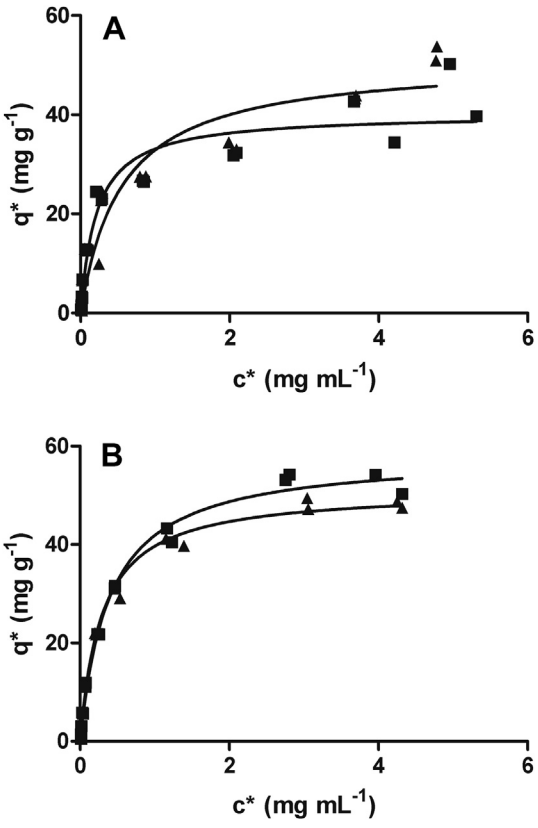

Fig. 2. Pure lactoferrin (Lf) adsorption isotherms at pH 7.0 with Red HE-3B matrices (A) and Yellow HE-4R matrices (B): chitosan matrix cross-linked with epichlorohydrin 250 mM, coupled to Red HE-3B (C250-R) or Yellow HE-4R (C250-Y) (■), and chitosan matrix cross-linked with epichlorohydrin 500 mM coupled to Red HE-3B (C500-R) or Yellow HE-4R (C500-Y) (▲); c*, equilibrium concentration of Lf in the supernatant; q*, equilibrium concentration of Lf bound to the matrix per unit of total amount of matrix.

Table 1
Thermodynamic parameters for lactoferrin adsorption of chitosan matrices calculated from the isotherms.[a]

Langmuir constants	Matrices			
	C250-R	C500-R	C250-Y	C500-Y
Q_{max} (mg g^{-1})	40.17 ± 2.05	51.04 ± 3.63	58.28 ± 1.29	51.14 ± 1.00
K_d (mg mL^{-1})	0.21 ± 0.05	0.55 ± 0.15	0.39 ± 0.03	0.29 ± 0.02

[a] Chitosan matrix cross-linked with 250 mM epichlorohydrin, coupled to Red HE-3B (C250-R) or Yellow HE-4R (C250-Y), and chitosan matrix cross-linked with 500 mM epichlorohydrin, coupled to Red HE-3B (C500-R) or Yellow HE-4R (C500-Y), were characterised. The maximum capacity (Q_{max}) and dissociation constant (K_d) were calculated according to the Langmuir model. Results are expressed as the average ± standard deviation.

3.3. Assay of different mobile phases for lactoferrin elution

The efficiency of the eluents tested is shown in Table 2. It can be observed that 25% ethylene glycol + 0.5 M NaSCN and 25% ethylene glycol + 2 M NaCl, at pH 7.0 or 9.0, were the best eluents for Lf desorption. However, it is preferable to select the latter as eluent since NaSCN is not allowed in the food industry and has a higher cost. Since we found no difference between the eluent pH (7.0 or 9.0), pH 7.0 was chosen to avoid pH modification. Therefore, the eluent selected for further purification processes was 25% ethylene glycol, 2 M NaCl, pH 7.0.

It is important to mention that a synergistic effect of the elution is observed by adding ethylene glycol to salts. The ethylene glycol alone does not produce elution of Lf; however, its addition to salt

Table 2
Performance of different eluents for lactoferrin (Lf) elution.[a]

Eluents	Elution (%)			
	C250-R	C500-R	C250-Y	C500-Y
2 M NaCl, pH 7.0	63.11 ± 4.71	62.95 ± 3.06	63.42 ± 1.25	59.26 ± 2.92
0.5 M NaSCN, pH 7.0	60.42 ± 2.84	63.39 ± 1.24	65.49 ± 1.68	66.74 ± 0.25
25% ethylene glycol, pH 7.0	1.77 ± 0.11	0.92 ± 0.07	1.77 ± 0.11	2.18 ± 0.45
25% ethylene glycol + 0.5 M NaSCN, pH 7.0	79.56 ± 6.68	69.29 ± 5.01	80.86 ± 1.19	75.29 ± 2.23
25% ethylene glycol + 2 M NaCl, pH 7.0	75.06 ± 3.35	73.41 ± 3.07	76.94 ± 3.81	78.96 ± 3.50
2 M NaCl, pH 9.0	57.54 ± 1.71	54.65 ± 2.77	62.87 ± 3.22	63.07 ± 3.00
0.5 M NaSCN, pH 9.0	59.34 ± 1.31	63.63 ± 3.02	64.73 ± 1.39	68.56 ± 3.50
25% ethylene glycol, pH 9.0	0.67 ± 0.23	0.75 ± 0.06	3.26 ± 0.98	2.75 ± 0.38
25% ethylene glycol + 0.5 M NaSCN, pH 9.0	71.80 ± 2.73	68.11 ± 3.58	71.67 ± 1.12	68.84 ± 2.00
25% ethylene glycol + 2 M NaCl, pH 9.0	70.12 ± 6.80	66.44 ± 0.53	68.40 ± 2.97	70.19 ± 1.98

[a] Chitosan matrix cross-linked with 250 mM epichlorohydrin, coupled to Red HE-3B (C250-R) or Yellow HE-4R (C250-Y), and chitosan matrix cross-linked with 500 mM epichlorohydrin, coupled to Red HE-3B (C500-R) or Yellow HE-4R (C500-Y), were saturated with commercial Lf for testing several elution solutions. Results are expressed as the average ± standard deviation.

solutions increases Lf elution, thus evidencing the simultaneous existence of electrostatic and hydrophobic interactions.

3.4. Optimisation of the sample-volume/matrix-amount ratio for the isolation of lactoferrin

The sample-volume (mL) to matrix-amount (mg) ratio was optimised to ensure maximum Lf adsorption. Fig. 3 shows the results of this optimisation with different matrices. A ratio of 1.0/25, with high adsorption percentage, was optimal for all matrices; a ratio of 0.5/25 did not bring about a significant improvement in the adsorption percentage. Ratios of 2.5/25, 5.0/25 and 10/25 showed a significant decrease in Lf adsorption. With a ratio of 1.0/25, all matrices had a similar adsorption percentage; therefore, this ratio was chosen for the purification process.

3.5. Isolation of lactoferrin from whey

After the sample-volume/matrix-amount ratio was optimised, purification processes were performed with all the matrices developed (C250-R, C500-R, C250-Y and C500-Y) and with Sepharose (S-R and S-Y) to compare and choose the best matrix for Lf purification. Table 3 shows that chitosan matrices with Red HE-3B dye had lower adsorption percentages that those with Yellow HE-4R dye. The chitosan matrices with Yellow HE-4R dye showed higher elution percentage, which led to a purification process with high yield.

When comparing chitosan matrices with Sepharose matrices, we observed that the latter showed 100% adsorption but that the elution was less than that obtained for the chitosan matrices, leading to a purification yield lower than that of the chitosan matrices. All the matrices developed showed a better purification performance than Sepharose matrices.

The C250-Y and C500-Y matrices showed no significant differences in Lf adsorption and elution. Since cross-linking degree had a low impact in the Lf purification, the higher cross-linked matrix was chosen due to its higher mechanical strength.

Taking these results into account, C500-Y was the best matrix to purify Lf from whey, so this matrix was selected for further experiments.

3.6. Process for lactoferrin isolation and reutilisation of the chromatographic matrix

The matrices reutilisation was studied over three consecutive purification cycles without regeneration steps between them.

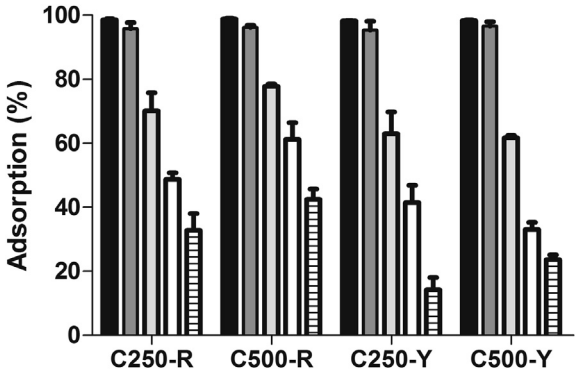

Fig. 3. Optimisation of the sample-volume/matrix-amount ratio. Chitosan matrix cross-linked with 250 mM epichlorohydrin, coupled to Red HE-3B (C250-R) or Yellow HE-4R (C250-Y), and chitosan matrix cross-linked with 500 mM epichlorohydrin coupled to Red HE-3B (C500-R) or Yellow HE-4R (C500-Y) were tested. In this optimisation, 0.5, 1.0, 2.5, 5.0 and 10.0 mL of whey were incubated with 25 mg of the four matrices, generating a 0.5/25 (■), 1.0/25 (■), 2.5/25 (■), 5.0/25 (□) and 10.0/25 (⊟) ratio, respectively.

Table 3
Comparison of lactoferrin (Lf) purification using different matrices.[a]

Matrix	Adsorption (%)	Elution (%)	Yield (%)	Purification factor
C250-R	89.14 ± 4.55	66.83 ± 0.12	59.57 ± 2.02	43.06 ± 5.24
C500-R	91.10 ± 3.50	70.59 ± 0.87	64.31 ± 2.05	32.65 ± 0.90
S-R	98.90 ± 1.55	74.42 ± 1.17	73.60 ± 4.01	48.41 ± 13.50
C250-Y	96.21 ± 5.37	80.31 ± 0.75	77.27 ± 2.04	42.09 ± 3.48
C500-Y	93.30 ± 1.59	83.42 ± 0.87	77.83 ± 1.55	45.21 ± 6.40
S-Y	99.11 ± 1.26	72.99 ± 1.30	72.34 ± 6.22	36.81 ± 11.93

[a] Chitosan matrix cross-linked with 250 mM epichlorohydrin, coupled to Red HE-3B (C250-R) or Yellow HE-4R (C250-Y), and chitosan matrix cross-linked with 500 mM epichlorohydrin, coupled to Red HE-3B (C500-R) or Yellow HE-4R (C500-Y), were tested for Lf isolation from whey (Lf concentration, 0.09 mg mL^{-1}). For comparison purpose, Red HE-3B Sepharose 4B matrix (S-R) and Yellow HE-4R Sepharose 4B matrix (S-Y) were also tested. Results are expressed as the average ± standard deviation.

Table 4 shows the results for C500-Y. The Lf adsorption capacity decreased slightly after three cycles of use, whereas the elution percentages increased. However, the overall yield of the purification process increased in successive cycles of reuse.

An important feature of the Lf purification process developed is the purity of the final product obtained after only one step. Fig. 4 shows the SDS-PAGE analysis of the process using the C500-Y matrix during the three purification cycles and the pattern of commercial Lf. Also, the SDS-PAGE showed Lf depletion from the whey as judged by the absence of the band corresponding to Lf after adsorption. The faint band present after adsorption may be attributed to denatured or non-active Lf present in the whey as it is not measurable by ELISA. Moreover, the interaction specificity between Lf and the matrix is evidenced, as there were no differences in the SDS-PAGE pattern of the other proteins present in the whey after Lf removal (Fig. 4, lanes 2–5). The purity of the final product was analysed by gel densitometry, obtaining a purity higher than that of the 90% commercial Lf (Fig. 4, lanes 6–9).

The integrity of the chitosan matrix was not affected during the purification cycles. Another interesting feature of the developed matrix is its easy recovery without any loss of material using a strainer after the adsorption, washing and elution steps. Moreover, chitosan is a low cost polymer and it is easy to manipulate to generate beads with an adequate mechanical resistance for Lf isolation directly from a dairy waste as whey. Considering these facts and the purification parameters obtained (Table 4), we conclude that the C500-Y matrix is a good matrix to purify Lf from whey.

After three purification cycles, matrices were regenerated by overnight incubation with 50 mM NaOH. Then, a new purification process was done and the parameters of the first cycle were reached (data not shown).

For a comparative study with Sepharose, a whey clarification step was required. However, one advantage of the matrices developed is that they can be used without whey conditioning (i.e., ion strength modification, diafiltration). This fact was confirmed using non-clarified whey (data not shown). Another advantage is the purification degree (>90%) obtained in only one step. In addition, due to the large size and density of hydrated mini-spheres, they are

Table 4
Matrix reutilisation tested over three cycles of lactoferrin purification from whey.[a]

Cycle	Adsorption (%)	Elution (%)	Yield (%)	Purification factor
1	92.18 ± 0.27	75.80 ± 1.05	69.87 ± 0.85	50.73 ± 1.78
2	87.16 ± 1.96	75.26 ± 2.74	65.60 ± 1.50	45.10 ± 1.15
3	80.83 ± 1.69	84.86 ± 3.13	68.60 ± 6.57	55.18 ± 3.06

[a] Chitosan matrix cross-linked with 500 mM epichlorohydrin coupled to Yellow HE-4R (C500-Y); lactoferrin concentration, 0.09 mg mL^{-1}. Results are expressed as the average ± standard deviation.

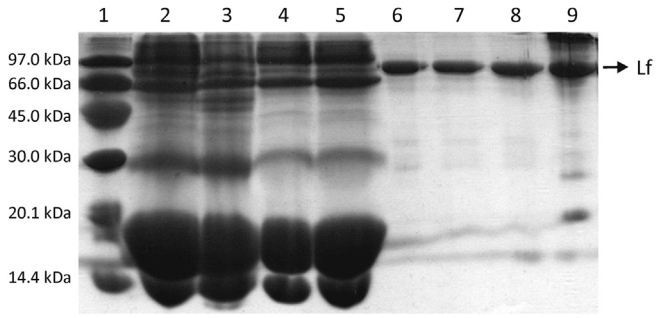

Fig. 4. SDS-PAGE of the lactoferrin (Lf) purification process using a chitosan matrix cross-linked with 500 mM epichlorohydrin coupled to Yellow HE-4R (C500-Y) during three purification cycles: lane 1, protein ladder; lane 2, whey; lane 3, supernatant after adsorption from cycle 1; lane 4, supernatant after adsorption from cycle 2; lane 5, supernatant after adsorption from cycle 3; lane 6, eluate from cycle 1; lane 7, eluate from cycle 2; lane 8, eluate from cycle 3; lane 9, commercial Lf (2 mg mL^{-1}).

very easy to manipulate, thus avoiding the need of centrifugation after each purification step. Thus, mini-spheres can be recovered by simple filtration through a strainer. These facts are of industrial interest and thus impact in the process economics.

Fee and Chand (2006) developed a purification process for Lf and lactoperoxidase from raw milk using SP-Sepharose Big Beads™. These authors suggested that changing liquid/solid state of the major fatty acids through changes in the working temperature prevents the column occlusion under dynamic conditions. In the whey, fats are not particulate, so the mini-spheres developed in the present work have higher size than the Sepharose Big Beads ™ (300 μm) and the processes were performed in batch mode.

In a previous work (Wolman et al., 2007), we immobilised Red HE-3B in hollow-fibre membranes for lactoferrin purification from bovine whey. The aim was to decrease the duration of the purification process by using a convective chromatographic support. We found a ten-fold reduction compared with the use of a diffusive Sepharose matrix. Adsorptive hollow-fibre membranes are of high cost and are not profitable if they are used for a halfway value added molecule. Due to the large volume of whey production, the processing rate is an economic issue to be considered (Peters, 2005).

3.7. Time process optimisation, kinetic studies

To study the optimisation of the purification process in terms of productivity and time consumption, we also studied the adsorption kinetics. Taking into account the low cost of the matrix, it was interesting to reduce the process time by increasing the amount of matrix. This study was performed using the sample volume/matrix amount ratio previously determined and a 10-fold increase in the amount of matrix.

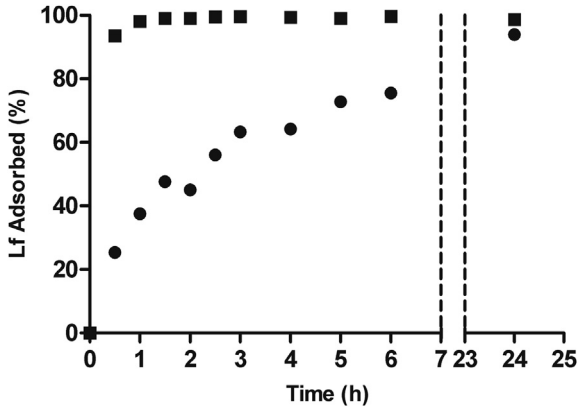

Fig. 5. Adsorption kinetics of bovine lactoferrin (Lf) from whey. Either 250 or 2500 mg of a chitosan matrix cross-linked with 500 mM epichlorohydrin coupled to Yellow HE-4R (C500-Y) was incubated with 10 mL clarified whey for 24 h at room temperature with gentle agitation. Samples were taken at 0.5–24 h. 250 mg C500-Y (●) and 2500 mg C500-Y (■).

Fig. 5 shows the adsorption kinetics of Lf from whey on the C500-Y matrix. A plateau was reached at approximately 24 h for 250 mg of C500-Y and after 1 h for 2500 mg of C500-Y. The longer time to reach the plateau with 250 mg matrix can be explained taking into account the high viscosity of the whey, which limits the protein molecular diffusion and, therefore, slows the adsorptive process. Also, the porous nature of the matrix probably contributes for this slow adsorption. From Fig. 5, it is evident that 1 h is an adequate time for adsorption of approximately 96% of the Lf present in the cheese whey using 2500 mg of C500-Y.

4. Conclusions

Matrices with Yellow HE-4R dye showed better performance than matrices with Red HE-3B dye as the ligand for Lf purification from whey. The adsorptive affinity matrix finally proposed (C500-Y) was able to bind Lf from whey selectively, recovering Lf with a high purity degree (>90%) in a single step. Therefore, this matrix is presented as an attractive alternative for a low-cost purification process of Lf from whey.

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