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Lactoperoxidase purification from whey by using dye affinity chromatography

Nicolás Urtasun[a,b], María Fernanda Baieli[a,b], Daniela Belén Hirsch[a,b], María Camila Martínez-Ceron[a,b], Osvaldo Cascone[a,b], Federico Javier Wolman[a,b,*]

[a] Universidad de Buenos Aires, Facultad de Farmacia y Bioquímica, Cátedra de Biotecnología. Junín 956, 1113 Buenos Aires, Argentina

[b] CONICET- Universidad de Buenos Aires, Instituto de Nanobiotecnología (NANOBIOTEC), Junín 956, 1113 Buenos Aires, Argentina

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ABSTRACT

Bovine lactoperoxidase is a glycoprotein present in milk, whey and colostrum, which might be used in dairy, cosmetic, pharmaceutical, veterinary and agricultural applications due to its broad antimicrobial activity. Here, we describe a novel process for bovine lactoperoxidase purification by using dye affinity chromatography.

Eighteen triazine dyes were immobilized on Sepharose 6B and screened for their performance as possible ligands. Five of the dye-Sepharose matrices showed over 90% adsorption of bovine lactoperoxidase directly from whey without any pretreatment using the batch mode, and were thus selected for further adsorption and elution studies. The highest elution degree was obtained using 20 mM acetate buffer, pH 5.0, 2 M NaCl, as the eluent for all the matrices. Whey processed using the Reactive Red 4-Sepharose matrix in batch mode showed the highest bovine lactoperoxidase purification yield (86.5 ± 3.8%), purification factor (46.1 ± 1.1), and a relative purity higher than 80% according to SDS-PAGE gel densitometry. Whey processed using packed-bed column mode showed lower yields and additional whey pretreatments were needed for dynamic processing.

The interaction between bovine lactoperoxidase and Reactive Red 4-Sepharose matrix was characterized using Langmuir isotherm model. The K_d value was 0.21 ± 0.03 mg/mL and the Q_{max} was 32.21 ± 1.24 mg/g. The results presented here suggest the potential application of the Reactive Red 4-Sepharose matrix to one-step purification of bovine lactoperoxidase from whey.

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1. Introduction

Lactoperoxidase (Lp) is a glycoprotein present in the mammary, salivary and lachrymal glands of mammals and their respective secretions (milk, saliva and tears) (Seifua et al., 2005). Lp catalyzes the oxidation of certain molecules (especially SCN^-) by using hydrogen peroxide to generate reactive intermediates and products with a wide antimicrobial activity. Lp plays an important role in protecting the lactating mammary gland and the intestinal tract of newborn infants against pathogenic microorganisms (Boots and Floris, 2006; Sharma et al., 2013). Lp has also been involved in the degradation of various carcinogens and in the protection of animal cells against peroxidative effects. All these functional properties allow Lp to be used in different sectors such as the dairy, cosmetic, pharmaceutical, veterinary and agricultural industries (Boots and Floris, 2006; Jooyandeh et al., 2011; Kussendrager

Abbreviations: Lp, lactoperoxidase; bLp, bovine lactoperoxidase; bLf, bovine lactoferrin.

* *Corresponding author at*: Junín 956, (1113) Buenos Aires, Argentina. Fax: +54 11 4508 3645.
E-mail addresses: nicourtasun@gmail.com (N. Urtasun), fer.baieli@gmail.com (M.F. Baieli), daniela.b.hirsch@gmail.com (D.B. Hirsch), camartinez@ffyb.uba.ar (M.C. Martínez-Ceron), ocasco@ffyb.uba.ar (O. Cascone), federicowolman@gmail.com, fwolman@ffyb.uba.ar (F.J. Wolman).

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and van Hooijdonk, 2000; Seifua et al., 2005; Sharma et al., 2013).

Bovine lactoperoxidase (bLp) has been purified from natural sources such as milk, whey and colostrum by using different methods that include ion exchange and affinity chromatography (Atasever et al., 2013; Fweja et al., 2010; Liang et al., 2011; Ng and Yoshitake, 2010; Pan et al., 2015; Plate et al., 2006; Ye et al., 2000). Particularly, ion exchange chromatography is mainly used in large-scale processes. However, the concentration of bLp is very low and the starting material requires concentration and conditioning to improve the performance of the chromatographic process. Ion exchange chromatography processes reach variable yields of bLp – between 40 and 90% – and is mainly co-purified with significant levels of bovine lactoferrin (bLf), another protein with a similar isoelectric point present in mammalian secretions (Andersson and Mattiasson, 2006; Fweja et al., 2010; Mecitoğlu and Yemenicioğlu, 2007; Pan et al., 2015; Plate et al., 2006).

In contrast, the use of affinity chromatography allows the direct adsorption of a protein at low concentration, but the purification cost increases significantly according to the ligand price. The triazine dyes used in the textile industry are low-cost molecules widely used as ligands for protein purification purposes (Baieli et al., 2014a,b; Iannucci et al., 2003; Urtasun et al., 2013; Wolman et al., 2007; Wolman et al., 2006). They are easily immobilized on different supports, present chemical and thermal stability, and allow the purification of proteins with a favorable cost/selectivity ratio (Denizli and Piskin, 2001; Wolman et al., 2005). All these advantages make them suitable for industrial-scale protein purification. In previous works, bLf has been purified from colostrum and sweet whey by using different triazine dyes as chromatographic ligands (Baieli et al., 2014a,b; Grasselli and Cascone, 1996; Wolman et al., 2007). However, triazine dye ligands for bLp purification have not been reported yet. The aim of the present study was to select and characterize a dye affinity ligand immobilized onto Sepharose 6B for direct bLp recovery and purification from sweet whey.

2. Materials and methods

2.1. Dye-Sepharose matrices synthesis

The dyes (1) Reactive Black 5, (2) Reactive Brown 10, (4) Reactive Yellow 2, (5) Reactive Yellow 86, (6) Reactive Blue 5, (7) Reactive Blue 4, (8) Remazol Brilliant Violet 5R (C.I. name: Reactive Violet 5), (9) Reactive Blue 15, (12) Reactive Red 4, (16) Reactive Green 19 and (18) Cibacron Blue 3GA were from Sigma–Aldrich (St. Louis, MO, USA), whereas the dyes (3) Red 7B-HE (C.I. name: Reactive Red 141), (10) Scarlet G-A (C.I. name: Reactive Red 8), (11) Yellow FR (C.I. name: Mordant Yellow 8), (13) Orange R-HE (C.I. name: Reactive Orange 84), (14) Blue R-HE (C.I. name: Reactive blue 171), (15) Yellow HE-4R (C.I. name: Reactive Yellow 84) and (17) Red HE-3B (C.I. name: Reactive Red 120) were from Vilmafix[R] (Vilmax S.A., Buenos Aires, Argentina).

Dyes were immobilized on Sepharose 6B (GE Healthcare, Little Chalfont, UK), as previously reported (Stellwagen, 1990; Wolman et al., 2007). Briefly, 2 mL of Sepharose 6B 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. Finally, the matrix was washed with water, methanol, 2 M NaCl, 1 M NH_4Cl and water again.

2.2. Sweet whey preparation

Milk was kindly donated by Perassolo y Cia. S.A. (Rojas, Buenos Aires, Argentina). Sweet whey was prepared according to Taylor and Woonton (2009), with minor modifications. Briefly, when 1 L of milk reached 37 °C, 0.075 g of chymosin (Sigma–Aldrich) was added. The milk-enzyme solution was incubated until formation of a gel (30–60 min). Then, the bath temperature was raised to 55 °C for 15 min for chymosin inactivation. Finally, whey was centrifuged at 3000 × *g* for 30 min at 4 °C to remove casein precipitate and residual fat.

2.3. bLp quantification and analysis

bLp purification was monitored by measuring bLp activity using ABTS substrate (Sigma–Aldrich) (Putter and Becker, 1983) and 15% SDS-PAGE with Coomassie Blue staining (Laemmli, 1970). To measure bLp activity, samples (5 μL) were placed in a 96-well plate and then 100 μL of reaction solution was added. The reaction solution was ABTS substrate (5 mg/mL in 100 mM sodium phosphate buffer, pH 5.0 and 0.3% of H_2O_2). The reaction was stopped after 45 s by adding 100 μL of 1% SDS and absorbance was determined at 405 nm by using an EZ Read 400 microplate reader (Biochrom Ltd., Cambridge, UK). bLp activity was quantified using a commercial bLp (Sigma–Aldrich) as standard. Linearity was in the range of 0–25 μg/mL. Samples were diluted in 40 mM sodium phosphate buffer, pH 6.8, 0.25% bovine serum albumin, 0.5% Triton X-100.

For SDS-PAGE whey, supernatants and eluates were concentrated four times by centrifugal filtration (Amicon Ultrafree MC with MWCO 10,000, Millipore, MA, USA). PageRuler[TM] Prestained Protein Ladder was used as molecular weight calibration (Thermo Scientific, MA, USA). Presence of bLf in the eluates was determined using a Bovine Lactoferrin ELISA Quantification kit (Bethyl Laboratories Inc., USA).

Total protein was determined by the Bradford method (Bradford, 1976). The purification factor was calculated as a ratio between the amount of bLp relative to the total protein content in the eluate after the purification step, and that of the initial sweet whey.

2.4. bLp batch purification processes

For purification of bLp using the batch mode, 100 mg of each matrix was previously equilibrated with three alternative buffer solutions: (a) 20 mM sodium phosphate buffer, pH 7.0; (b) 20 mM sodium phosphate buffer, pH 7.0, 0.15 M NaCl, or (c) 20 mM sodium phosphate buffer, pH 7.0, 0.5 M NaCl. Then each matrix was soaked with 1 mL of whey and incubated for 4 h with gentle stirring at 25 °C. After bLp adsorption, the matrices were washed four times with 0.15 M NaCl in 20 mM sodium phosphate buffer, pH 7.0, for 1 h each. After adsorption and washing, nine elution solutions were tested: (A) 20 mM acetate buffer, pH 5.0, 25% propylene glycol; (B) 20 mM acetate buffer, pH 5.0, 2 M NaCl; (C) 20 mM acetate buffer, pH 5.0, 2 M NaCl and 25% propylene glycol; (D) 20 mM sodium phosphate buffer, pH 7.0, 25% propylene glycol; (E) 20 mM sodium phosphate buffer, pH 7.0, 2 M NaCl; (F) 20 mM sodium phosphate buffer, pH 7.0, 2 M NaCl and 25% propylene glycol; (G) 20 mM carbonate buffer, pH 9.0, 25% propylene glycol; (H) 20 mM carbonate buffer, pH 9.0, 2 M NaCl or (I) 20 mM carbonate buffer, pH 9.0, 2 M NaCl and 25% propylene glycol. Elutions were performed using 0.5 mL of each solution for 16 h at 25 °C.

For adsorption kinetics of bLp using the batch mode, 100 mg of Reactive Red 4-Sepharose matrix was incubated with 1 mL of bLp pure solution (0.03 mg/mL, in adsorption buffer) or with 1 mL of bLp from whey, at room temperature with gentle agitation. Samples were taken at 0, 30, 60, 90, 120, 150, 180, 210 and 240 min. Adsorbed bLp concentration was calculated as

the difference between the amounts of bLp activity in the supernatant at a determinate time and time zero.

All experiments were carried out in triplicate and results are expressed as the average ± standard deviation.

2.5. *bLp packed bed column purification processes*

The removal of residual fat from whey was carried out by thermocalcic precipitation according to Maubois et al. (1988), with a minor modification (whey was warmed up to 50 °C only for 5 min instead of 8 min). Then, whey was centrifuged for 10 min at 10.000 × *g* at 4 °C and the supernatant was sequential filtered using a 0.45 μm and then a 0.22 μm nominal pore filter (GE Healthcare). For bLp purification using the packed-bed column mode, 1 mL of Reactive Red 4-Sepharose matrix was packed in a 0.5 cm diameter column. The purification was performed using ÄKTA purifier equipment (GE Healthcare). The purification cycle involved equilibration using 20 mM phosphate buffer, pH 7.0, 0.15 M NaCl; loading of 10 mL of whey; washing with 20 mM phosphate buffer, 0.15 M NaCl and elution using 20 mM acetate buffer, pH 5.0, 2 M NaCl. Flow rate during equilibration, washing and elution were performed at 1 mL/min. Flow rate during whey loading was performed at 0.4 mL/min. The process was monitored by measurement of the absorbance at 215 nm, 280 nm and 403 nm. bLp was quantified by enzymatic activity and total protein was determined by the Bradford method.

2.6. *Matrix characterization*

Dye density was determined by the hydrolytic procedure reported by Ruckenstein and Zeng (1998). Adsorption isotherm was performed with commercial bLp (Sigma–Aldrich). To study the parameters of bLp-matrix interaction, 5 mg of Reactive Red 4-Sepharose matrix were soaked with 100 μL of bLp pure solutions (0.004–5 mg/mL) in adsorption buffer (20 mM sodium phosphate buffer, pH 7.0) in a 96-well plate for 16 h at 25 °C with gentle agitation. The activity of bLp in the supernatants was determined using ABTS substrate (Sigma–Aldrich) (Putter and Becker, 1983). The equilibrium concentration of bLp bound to the matrix per unit of total amount of matrix was calculated by the difference between the concentration of bLp at the beginning of the experiment and that remaining in the soluble phase after adsorption. Maximum capacities (Q_{max}) and dissociation constants (K_d) were calculated according to Langmuir single-solute isotherm model (Chase, 1984; Suen, 1996). All determinations were carried out in triplicate and the results are expressed as the average ± standard deviation.

3. Results and discussion

3.1. *Dye-Sepharose matrix selection*

The specific and selective recognition of the target protein by an appropriate affinity ligand allows its adsorption to the chromatographic matrix even at low concentrations and in a complex media. The low concentration of bLp in natural sources (0.03–0.06 mg/mL) makes affinity chromatography an interesting option for its purification (Etzel, 2004; Santos et al., 2012). The use of triazine dyes as ligands allows protein adsorption even from sources with moderate ionic strength or solute load, making the direct isolation of bLp from sweet whey possible. Fig. 1 shows the adsorption of bLp to 18 different dye-Sepharose matrices directly from whey without any conditioning using the batch mode. Five of the matrices showed over 90% adsorption and were thus selected for further studies. The ligands in these selected matrices were the triazine dyes (12) Reactive Red 4, (13) Orange R-HE, (14) Blue R-HE, (15) Yellow HE-4R and (17) Red HE-3B (Fig. 1).

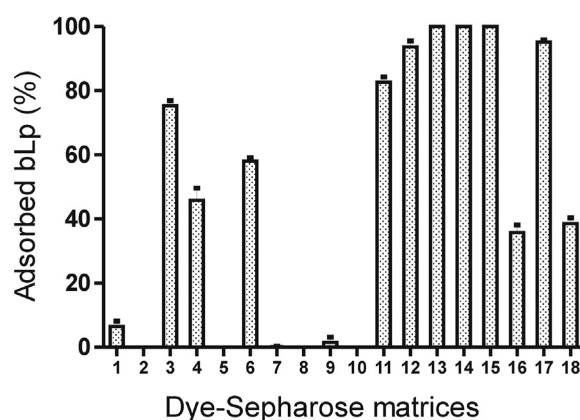

Fig. 1 – Eighteen dye-Sepharose matrices were tested for lactoperoxidase (bLp) adsorption directly from whey without any conditioning. Dyes immobilized onto Sepharose were: (1) Reactive Black 5, (2) Reactive Brown 10, (3) Red 7B-HE, (4) Reactive Yellow 2, (5) Reactive Yellow 86, (6) Reactive Blue 5, (7) Reactive Blue 4, (8) Remazol Brilliant Violet 5R, (9) Reactive Blue 15, (10) Scarlet G-A, (11) Yellow FR, (12) Reactive Red 4, (13) Orange R-HE, (14) Blue R-HE, (15) Yellow HE-4R, (16) Reactive Green 19, (17) Red HE-3B and (18) Cibacron Blue 3GA. Results are expressed as a percentage of the initial bLp activity.

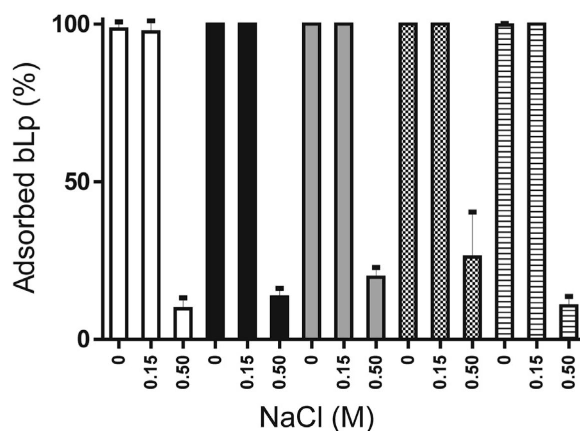

Fig. 2 – Adsorption of bovine lactoperoxidase (bLp) to dye-Sepharose matrices using different NaCl concentrations for matrix equilibration (20 mM sodium phosphate buffer, pH 7.0; 20 mM sodium phosphate buffer, pH 7.0, 0.15 M NaCl or 20 mM sodium phosphate buffer, pH 7.0, 0.5 M NaCl). White bars correspond to the Reactive Red 4-Sepharose matrix, black bars correspond to the Orange R-HE-Sepharose matrix, gray bars correspond to the Blue R-HE-Sepharose matrix, dotted bars correspond to the Yellow HE-4R-Sepharose matrix, and striped bars correspond to the Red HE-3B-Sepharose matrix.

In order to study the interaction between bLp and each selected dye-Sepharose matrix, different solutions with increasing ionic strength were used for matrix equilibration. Fig. 2 shows the adsorption of bLp to the selected dye-Sepharose matrices using different ionic strength solu-

Table 1 – Yield and purification factor obtained using 2 M NaCl as the eluent at different pH values for the dye-Sepharose matrices selected.

Dye-Sepharose matrix	Adsorption (%)	Eluent	Elution (%)	Yield (%)	Purification factor[a]
Reactive Red 4	99.2 ± 0.2	20 mM acetate buffer, pH 5.0, 2 M NaCl	91.8 ± 3.5	88.2 ± 3.4	49.1 ± 1.1
Orange R-HE	97.7 ± 0.3		78.4 ± 8.1	74.5 ± 7.8	29.0 ± 1.8
Blue R-HE	98.4 ± 0.1		81.6 ± 2.2	78.7 ± 2.1	39.1 ± 3.4
Yellow HE-4R	98.0 ± 0.1		87.9 ± 0.8	84.1 ± 0.8	32.0 ± 0.9
Red HE-3B	99.4 ± 0.2		78.3 ± 1.1	77.1 ± 1.1	32.4 ± 4.0
Reactive Red 4	99.2 ± 0.2	20 mM sodium phosphate buffer, pH 7.0, 2 M NaCl	72.9 ± 2.6	70.0 ± 2.5	36.1 ± 0.5
Orange R-HE	97.7 ± 0.3		73.8 ± 6.5	70.1 ± 6.1	17.5 ± 1.7
Blue R-HE	98.4 ± 0.1		86.9 ± 4.0	83.8 ± 3.8	25.9 ± 3.8
Yellow HE-4R	98.0 ± 0.1		87.7 ± 4.5	83.9 ± 4.3	21.4 ± 0.6
Red HE-3B	99.4 ± 0.2		77.3 ± 8.4	76.1 ± 8.3	20.9 ± 0.1
Reactive Red 4	99.2 ± 0.2	20 mM carbonate buffer, pH 9.0, 2 M NaCl	70.4 ± 5.2	67.6 ± 6.2	30.7 ± 3.5
Orange R-HE	97.7 ± 0.3		68.4 ± 6.6	65.0 ± 6.3	15.7 ± 1.1
Blue R-HE	98.4 ± 0.1		78.1 ± 1.9	75.3 ± 1.8	20.0 ± 1.9
Yellow HE-4R	98.0 ± 0.1		79.5 ± 4.4	76.2 ± 4.2	15.1 ± 0.1
Red HE-3B	99.4 ± 0.2		70.0 ± 6.3	68.9 ± 6.7	15.7 ± 0.4

[a] The purification factor was calculated as a ratio between the amount of bLp relative to the total protein content in the eluate after the purification step, and that of the initial sweet whey.

tions for matrix equilibration prior to bLp batch adsorption directly from whey. The interaction between bLp and the different dye-Sepharose matrices showed the same behavior since equilibration with 20 mM sodium phosphate buffer, pH 7.0 with or without the addition of 0.15 M NaCl provided the highest levels of bLp adsorption. Equilibration with 20 mM sodium phosphate buffer, pH 7.0, 0.5 M NaCl showed a lower level of bLp adsorption (Fig. 2). These results revealed the importance of both electrostatic and hydrophobic interactions between bLp and each selected dye-Sepharose matrix.

Possibly, the sulfonic groups of the triazine dyes (Fig. 3) might interact with the positive surface charges present in the bLp structure at pH 7.0 (pI of bLp ~9.6). Unlike ion exchange chromatography, where adsorption in the presence of 0.15–0.20 M NaCl is not possible (Plate et al., 2006; Ye et al., 2000), dye affinity chromatography allows bLp adsorption and even astringent matrix washes using 0.15 M NaCl without bLp desorption. Therefore, hydrophobic and hydrogen bonding might be also involved in the interaction between the dye-Sepharose matrix and bLp. These multiple types of interactions resemble those of multimodal or mixed-mode chromatography (Wolfe et al., 2014; Yang and Geng, 2011).

When a protein adsorbs onto the dye-Sepharose matrix through multiple interactions, the elution of the protein might become a difficult step. Therefore, looking for an efficient eluent is an essential step for the development of a dye affinity chromatographic process.

Fig. 4 shows the elution of bLp, previously adsorbed onto the selected dye-Sepharose matrix using the batch mode. Different eluents were tested for bLp desorption using buffer solutions at different pH values (5.0, 7.0 or 9.0). Elution of bLp increased when the pH value decreased, especially in the presence of 2 M NaCl for all the matrices (Fig. 4, bars B, E, H) with the exception of Blue R-HE-Sepharose matrix. Possibly, desorption of bLp from the dye-Sepharose matrices occurred when electrostatic attractions were impaired due to high salt concentration. In addition, hydrophobic interactions were possibly disrupted at low pH values far from the pI of bLp (Lienqueo et al., 2007; Xia et al., 2005). For all the matrices, the highest elution degree was obtained using 2 M NaCl, especially at pH 5.0 (Fig. 4, bars B).

Table 1 shows the yield and the purification factor obtained using 2 M NaCl as the eluent, at different pH values, for the five dye-Sepharose matrices selected. The Reactive Red 4-Sepharose matrix showed the highest yield (88.2% ± 3.4%) and purification factor (49.1 ± 1.1) using 20 mM acetate buffer, pH 5.0, 2 M NaCl as the eluent.

In previous reports, when bLp was purified using ion exchange chromatography, it was mainly co-purified with significant levels of bLf. Plate et al. (2006), purified bLp and bLf from whey using cation-exchange membrane systems. bLp eluted with 0.1 M NaCl co-purified with 1% of bLf. Andersson and Mattiasson (2006), purified bLp and bLf from concentrate whey using SP-Streamline resin and a significant amount of bLf was detected in the bLp fraction. In this work, when bLp was purified using dye affinity chromatography under the conditions stated before, low levels of bLf co-purified with bLp. Less than 0.5% of bLf was detected by ELISA in the eluent (0.0007 mg/mL) respect to the original bLf concentration in whey (0.16 mg/mL).

Previously, we have reported the adsorption of bLf to Red HE-3B-Sepharose and Yellow HE-4R-Sepharose matrices (Baieli et al., 2014a,b; Grasselli and Cascone, 1996; Wolman et al., 2007). In the present study, bLf adsorption was also detected using Reactive Red 4-Sepharose matrix, Orange R-HE-Sepharose matrix and Blue R-HE-Sepharose matrix (data not shown). Nevertheless, the use of the Reactive Red 4-Sepharose matrix allowed the specific elution of bLp by using 20 mM acetate buffer, pH 5.0, 2 M NaCl without significant bLf elution.

Since Reactive Red 4-Sepharose matrix showed the highest performance for bLp purification direct from whey using the batch mode, this matrix was selected for its characterization and was tested using packed-bed column chromatographic conditions.

3.2. *Reactive Red 4-Sepharose matrix characterization*

Adsorption isotherm was performed to characterize the Reactive Red 4-Sepharose matrix and its interaction with pure standard bLp solution (Fig. 5). Table 2 shows the parameters calculated from the data adjustment of Fig. 5 to Langmuir single-solute isotherm model. Also, ligand density was calculated. According to Langmuir isotherm, the maximum

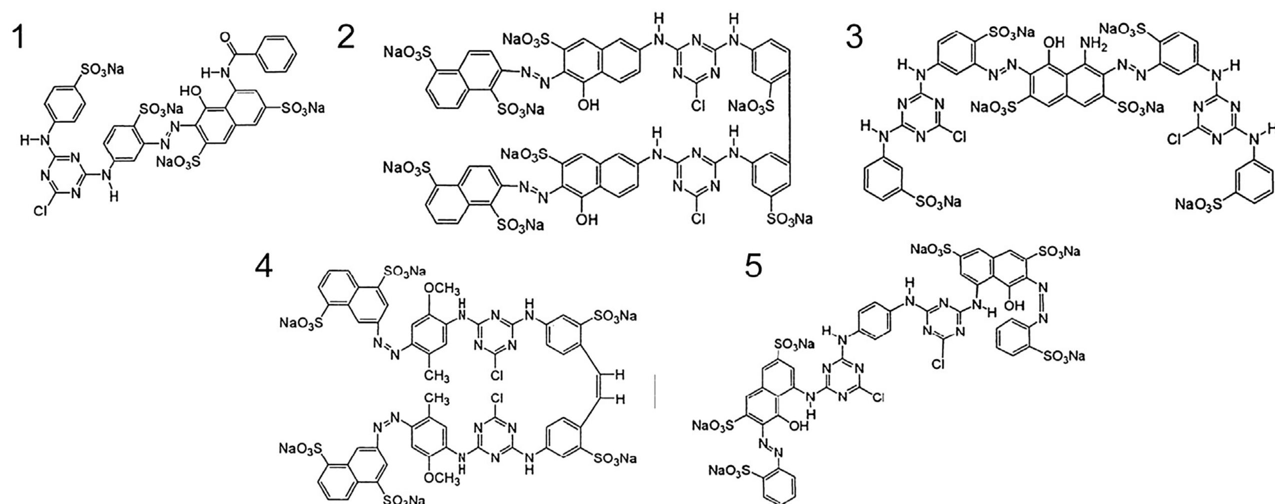

Fig. 3 – Structures of triazine dyes. Reactive Red 4 (1), Orange R-HE (2), Blue R-HE (3), Yellow HE-4R (4) and Red HE-3B (5).

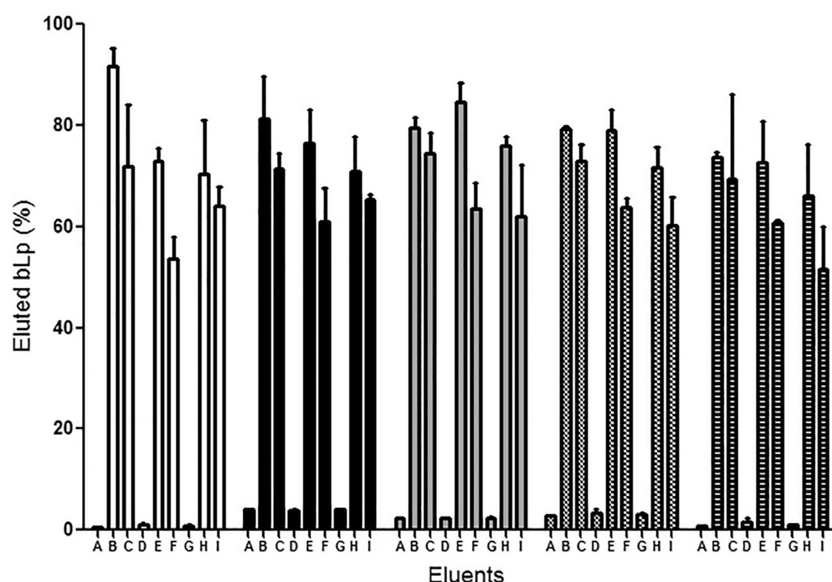

Fig. 4 – Elution of bovine lactoperoxidase (bLp) from dye-Sepharose matrices using different eluents: (A) 20 mM acetate buffer, pH 5.0, 25% propylene glycol; (B) 20 mM acetate buffer, pH 5.0, 2 M NaCl; (C) 20 mM acetate buffer, pH 5.0, 2 M NaCl and 25% propylene glycol; (D) 20 mM sodium phosphate buffer, pH 7.0, 25% propylene glycol; (E) 20 mM sodium phosphate buffer, pH 7.0, 2 M NaCl; (F) 20 mM sodium phosphate buffer, pH 7.0, 2 M NaCl and 25% propylene glycol; (G) 20 mM carbonate buffer, pH 9.0, 25% propylene glycol; (H) 20 mM carbonate buffer, pH 9.0, 2 M NaCl; (I) 20 mM carbonate buffer, pH 9.0, 2 M NaCl and 25% propylene glycol. White bars correspond to the Reactive Red 4-Sepharose matrix, black bars correspond to the Orange R-HE-Sepharose matrix, gray bars correspond to the Blue R-HE-Sepharose matrix, dotted bars correspond to the Yellow HE-4R-Sepharose matrix and stripped bars correspond to the Red HE-3B-Sepharose matrix.

Table 2 – Chromatographic characterization of the Reactive Red 4-Sepharose matrix.

Matrix	Ligand density	Langmuir constants
Reactive Red 4-Sepharose 6B	5.87 ± 0.32 μmol/g	Q_{max} = 32.21 ± 1.24 mg/g K_d = 0.21 ± 0.03 mg/mL R^2 = 0.98

capacity of the matrix (Q_{max}) was in the same order as other protein-dye Sepharose matrices (Baieli et al., 2014a; Iannucci et al., 2003; Wolman et al., 2007). The K_d value (0.21 ± 0.03 mg/mL) was higher than the bLp concentration in whey (0.03–0.06 mg/mL). However, besides this high K_d value, adsorption of bLp from whey was almost complete (Fig. 2, white bars). Single-solute isotherms were made using pure standard bLp and not necessarily reflect the behavior for a multi-solute system like whey. The behavior of each species in this kind of complex systems depends strongly on the number and properties of other species present, the pH, and the physical, chemical and specific properties of both the chromatographic matrix and the protein to be purified. Multi-solute adsorption behavior includes both interaction and competition effects, which decide the shape and constants of the isotherms and the real K_d value (Suen, 1996). Usually, ligands are screened using pure protein solutions and the selected ligand might not necessarily work appropriately with real samples. In our case, the screening was performed by using whey as starting material.

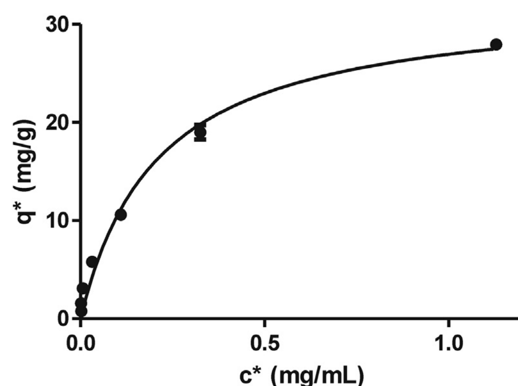

Fig. 5 – Pure bovine lactoperoxidase (bLp) adsorption isotherm at pH 7.0 using Reactive Red 4-Sepharose matrix. c*: equilibrium concentration of bLp in the supernatant; q*: equilibrium concentration of bLp bound to the matrix per unit of total matrix amount. Line represents the adjustment of the isotherm to Langmuir single-solute isotherm model.

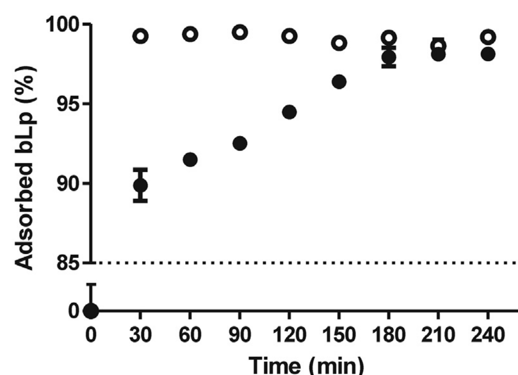

Fig. 6 – Adsorption kinetics of pure bovine lactoperoxidase (bLp) (○) and bLp from whey (●) using batch operated conditions. About 100 mg of Reactive Red 4-Sepharose matrix was incubated with 1 mL of bLp pure solution (0.03 mg/mL, in adsorption buffer) or with 1 mL of bLp from whey, at room temperature with agitation. Samples were taken at 0, 30, 60, 90, 120, 150, 180, 210 and 240 min. Adsorbed bLp concentration was calculated as the difference between the amounts of bLp in the supernatant at a determinate time and time zero.

3.3. *Batch and packed-bed column purification processes*

In order to optimize the minimum contact time for maximum bLp depletion from whey in the batch mode, adsorption kinetics using Reactive Red 4-Sepharose matrix were developed. Fig. 6 shows the bLp adsorption kinetics on the Reactive Red 4-Sepharose matrix from a pure solution and from whey using the batch mode. The concentration of bLp was 0.03 mg/mL. Using a pure solution, total bLp adsorption to Reactive Red 4-Sepharose matrix was complete after 30 min of contact. On the other hand, adsorption of bLp from whey was slower and reached ~90% after 30 min of contact. Maximum adsorption of bLp from whey (~98%) was achieved only after 180 min of contact. Noteworthy, bLp is a minor protein present in whey and its concentration is 10–100 times lower than the concentration of the major whey proteins (α-lactalbumin, β-lactoglobulin, inmunoglobulins, bovine serum albumin or caseinmacropeptide) (Etzel, 2004; Santos et al., 2012). Therefore, adsorption kinetics of bLp from whey to Reactive Red 4-Sepharose matrix

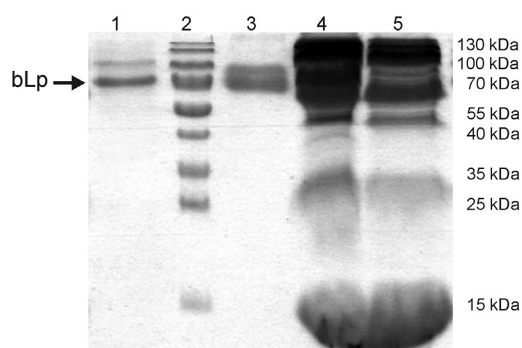

Fig. 7 – SDS-PAGE of bovine lactoperoxidase (bLp) purification process. Lanes: (1) eluate from the Reactive Red 4-Sepharose matrix, (2) PageRuler^{TM} prestained protein ladder (thermo scientific), (3) commercial bLp from Sigma–Aldrich, (4) bovine whey, (5) whey after bLp adsorption.

was evidently modified with the competitive mass effect of other major whey proteins.

Elution time was also optimized using 20 mM acetate buffer, pH 5.0, 2 M NaCl as eluent. Whey bLp adsorbed onto Reactive Red 4-Sepharose matrix showed maximum elution before 180 min (data not shown). Fig. 7 shows the bLp batch purification process monitored by SDS-PAGE. Gel density quantification showed that the relative purity for the recovered bLp (Fig. 7, lane 1) was higher than 80%. Also, the eluate obtainedfrom Reactive Red 4-Sepharose matrix showed an absorbance ratio 412 nm/280 nm of 0.7. This result was in concordance with absorbance ratio of pure commercial standard of bLp (absorbance ratio 412 nm/280 nm of 0.7–0.85).

In order to compare yields, the bLp purification process from whey was also performed using the packed-bed column mode. For packed-bed column process, whey was previously treated by thermocalcic precipitation to remove residual fat to prevent column clogging. Then, whey was centrifuged and the supernatant was sequential filtered using a 0.45 μm and 0.22 μm filter pore. Despite these pretreatments, backpressure raised during whey inlet to the column. Table 3 shows comparative yields between batch and packed-bed column processes.

In packed-bed column processes, a part of the bLp adsorbed (~38%) was not tightly bounded to the matrix and was removed during the column washes. This might be due to the presence of other major whey proteins, which bind to the Reactive Red 4-Sepharose matrix nonspecifically and might sterically affect bLp adsorption.

The main drawback of protein purification from a raw material like whey is its processability. Pretreatment of whey is critical for reducing fouling in subsequent chromatographic steps especially using a packed-bed column format. Also, whey requires concentration and conditioning to improve the performance of the chromatographic process, even more when the target protein is in low concentration.

In this work, the application of dye affinity chromatography allowed direct bLp capture and purification from whey without any pretreatment, using batch operated conditions. When the packed-bed column process was performed using dye affinity ligand attached to Sepharose 6B (with a particle diameter between 45–165 μm) several whey pretreatments were necessary to avoid excessive backpressure rise of the packed-bed column. Higher yields and purity were obtained using the batch mode in comparison with packed-bed col-

Table 3 – Comparison of bovine lactoperoxidase purification processes using batch or packed-bed column mode.

	Whey (mL)	Total bLp (mg)	Adsorbed (mg)	Elution (mg)	Yield	Purification factor
Batch	10	0.411 ± 0.004	0.399 ± 0.005 (97.9 ± 0.3%)	0.356 ± 0.010 (89.2 ± 3.7%)	86.5 ± 3.8%	46
Column	10	0.368 ± 0.003	0.229 ± 0.002 (62.1 ± 1.0%)	0.231 ± 0.006 (99.2 ± 2.4%)	64.3 ± 1.1%	29

umn process. Possibly, in the conditions studied, a longer time contact between the whey and the matrix in batch operations allowed the strong interaction of bLp and the displacement of the contaminants present in whey in higher concentrations.

It would be interesting to perform further dynamic purification studies in order to develop and optimize this chromatographic process using Reactive Red 4 dye ligand for bLp purification. Packed-bed column process might be performed without whey pretreatments using the dye attached onto chromatographic particles with bigger diameter avoiding backpressure rise and blockage of the column (Fee and Chand, 2006; Liang et al., 2011). Also, dye affinity membrane chromatography will be explore for direct bLp purification from whey increasing the productivity of the process (Wolman et al., 2007).

4. Conclusions

Eighteen triazine dyes immobilized onto Sepharose 6B were tested for bLp purification directly from whey without any conditioning using batch operated conditions. Dye-Sepharose matrices allowed equilibration and even astringent washing steps in the presence of 0.15 M NaCl. Whey processed using the Reactive Red 4-Sepharose matrix using the batch mode showed the highest bovine lactoperoxidase purification yield (86.5 ± 3.8%), purification factor (46.1 ± 1.1), and a relative purity greater than 80% according to SDS-PAGE gel densitometry. Whey processed using the packed- bed column mode showed lower yields and additional whey pretreatments were needed for dynamic processing. These results suggest the potential application of the dye affinity chromatography for the recovery and purification of bLp direct from whey using different chromatographic supports.

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References

Andersson, J., Mattiasson, B., 2006. Simulated moving bed technology with a simplified approach for protein purification: separation of lactoperoxidase and lactoferrin from whey protein concentrate. J. Chromatogr. A 1107, 88–95.

Atasever, A., Ozdemir, H., Gulcin, I., Irfan Kufrevioglu, O., 2013. One-step purification of lactoperoxidase from bovine milk by affinity chromatography. Food Chem. 136, 864–870.

Baieli, M.F., Urtasun, N., Miranda, M.V., Cascone, O., Wolman, F.J., 2014a. Bovine lactoferrin purification from whey using Yellow HE-4R as the chromatographic affinity ligand. J. Sep. Sci. 37, 484–487.

Baieli, M.F., Urtasun, N., Miranda, M.V., Cascone, O., Wolman, F.J., 2014b. Isolation of lactoferrin from whey by dye-affinity chromatography with Yellow HE-4R attached to chitosan mini-spheres. Int. Dairy J. 39.

Boots, J.-W., Floris, R., 2006. Lactoperoxidase: from catalytic mechanism to practical applications. Int. Dairy J. 16, 1272–1276.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254.

Chase, H.A., 1984. Prediction of the performance of preparative affinity chromatography. J. Chromatogr. 297, 179–202.

Denizli, A., Piskin, E., 2001. Dye-ligand affinity systems. J. Biochem. Biophys. Methods 49, 391–416.

Etzel, M.R., 2004. Manufacture and use of dairy protein fractions. J. Nutr. 134, 996S–1002S.

Fee, C.J., Chand, A., 2006. Capture of lactoferrin and lactoperoxidase from raw whole milk by cation exchange chromatography. Sep. Purif. Technol. 48, 143–149.

Fweja, L.W.T., Lewis, M.J., Grandison, A.S., 2010. Isolation of lactoperoxidase using different cation exchange resins by batch and column procedures. J. Dairy Res. 77, 357–367.

Grasselli, M., Cascone, O., 1996. Separation of lactoferrin from bovine whey by dye affinity chromatography. Neth. Milk Dairy J. 50, 551–561.

Iannucci, N.B., Navarro del Canizo, A.A., Cascone, O., 2003. Purification of neutral protease by dye affinity chromatography. Appl. Biochem. Biotech. 104, 173–184.

Jooyandeh, H., Aberoumand, A., Nasehi, B., 2011. Application of lactoperoxidase system in fish and food products: a review. Am. Eurasian J. Agric. Environ. Sci. 10, 89–96.

Kussendrager, K.D., van Hooijdonk, A.C., 2000. Lactoperoxidase: physico-chemical properties, occurrence, mechanism of action and applications. Brit. J. Nutr. 84 (Suppl. 1), S19–25.

Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680–685.

Liang, Y., Wang, X., Wu, M., Zhu, W., 2011. Simultaneous isolation of lactoferrin and lactoperoxidase from bovine colostrum by SPEC 70 SLS cation exchange resin. Int. J. Environ. Res. Public Health 8, 3764–3776.

Lienqueo, M.E., Mahn, A., Salgado, J.C., Asenjo, J.A., 2007. Current insights on protein behaviour in hydrophobic interaction chromatography. J. Chromatogr. B Anal. Technol. Biomed. Life Sci. 849, 53–68.

Maubois, J.L., Pierre, A., Fauquant, M., 1988. Industrial fractionation of main whey proteins. IDF Bull. 212, 154.

Mecitoğlu, Ç., Yemenicioğlu, A., 2007. Partial purification and preparation of bovine lactoperoxidase and characterization of kinetic properties of its immobilized form incorporated into cross-linked alginate films. Food Chem. 104, 726–733.

Ng, P.K., Yoshitake, T., 2010. Purification of lactoferrin using hydroxyapatite. J. Chromatogr. B 878, 976–980.

Pan, M., Shen, S., Chen, L., Dai, B., Xu, L., Yun, J., Yao, K., Lin, D.-Q., Yao, S.-J., 2015. Separation of lactoperoxidase from bovine whey milk by cation exchange composite cryogel embedded macroporous cellulose beads. Sep. Purif. Technol. 147, 132–138.

Plate, K., Beutel, S., Buchholz, H., Demmer, W., Fischer-Fruhholz, S., Reif, O., Ulber, R., Scheper, T., 2006. Isolation of bovine lactoferrin, lactoperoxidase and enzymatically prepared lactoferricin from proteolytic digestion of bovine lactoferrin using adsorptive membrane chromatography. J. Chromatogr. A 1117, 81–86.

Putter, J., Becker, R., 1983. In: Verlag Chemie (Ed.), Methods in Enzymatic Analysis. Verlag Chemie, Weinheim.

Ruckenstein, E., Zeng, X., 1998. Albumin separation with Cibacron Blue carrying macroporous chitosan and chitin affinity membranes. J. Membr. Sci. 142, 13–26.
Santos, M.J., Teixeira, J.A., Rodrigues, L.R., 2012. Fractionation of the major whey proteins and isolation of b-lactoglobulin variants by anion exchange chromatography. Sep. Purif. Technol. 90, 133–139.
Seifua, E., Buysb, E.M., Donkinc, E.F., 2005. Significance of the lactoperoxidase system in the dairy industry and its potential applications: a review. Trends Food Sci. Technol. 16, 137–154.
Sharma, S., Singh, A.K., Kaushik, S., Sinha, M., Singh, R.P., Sharma, P., Sirohi, H., Kaur, P., Singh, T.P., 2013. Lactoperoxidase: structural insights into the function,ligand binding and inhibition. Int. J. Biochem. Mol. Biol. 4, 108–128.
Stellwagen, E., 1990. Chromatography on immobilized reactive dyes. Methods Enzymol. 182, 343–357.
Suen, S.-Y., 1996. A comparison of isotherm and kinetic models for binary-solute adsorption to affinity membranes. J. Chem. Technol. Biotechnol. 65, 249–257.
Taylor, C.M., Woonton, B.W., 2009. Quantity and carbohydrate content of glycomacropeptide fractions isolated from raw and heat-treated milk. Int. Dairy J. 19, 709–714.
Urtasun, N., Baieli, M.F., Romasanta, P.N., Fernandez, M.M., Malchiodi, E.L., Cascone, O., Wolman, F.J., Miranda, M.V., 2013. Triazinic dye ligand selection by surface plasmon resonance for recombinant lactoferricin purification. Process Biochem. 48, 1972–1979.
Wolfe, L.S., Barringer, C.P., Mostafa, S.S., Shukla, A.A., 2014. Multimodal chromatography: characterization of protein binding and selectivity enhancement through mobile phase modulators. J. Chromatogr. A 1340, 151–156.
Wolman, F.J., González Maglio, D., Grasselli, M., Cascone, O., 2007. One-step lactoferrin purification from bovine whey and colostrum by affinity membrane chromatography. J. Membr. Sci. 288, 132–138.
Wolman, F.J., Grasselli, M., Cascone, O., 2006. Rapid neutral protease purification by dye-affinity membrane chromatography. Process Biochem. 41, 356–361.
Wolman, F.J., Smolko, E.E., Cascone, O., Grasselli, M., 2005. Improved hollow-fibre membranes for dye-affinity chromatography. J. Sep. Sci. 28, 45–51.
Xia, F., Nagrath, D., Cramer, S.M., 2005. Effect of pH changes on water release values in hydrophobic interaction chromatographic systems. J. Chromatogr. A 1079, 229–235.
Yang, Y., Geng, X., 2011. Mixed-mode chromatography and its applications to biopolymers. J. Chromatogr. A 1218, 8813–8825.
Ye, X., Yoshida, S., Ng, T.B., 2000. Isolation of lactoperoxidase, lactoferrin, alpha-lactalbumin, beta-lactoglobulin B and beta-lactoglobulin A from bovine rennet whey using ion exchange chromatography. Int. J. Biochem. Cell B 32, 1143–1150.