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Short Communication

Bovine lactoferrin purification from whey using Yellow HE-4R as the chromatographic affinity ligand

The worldwide production of whey increases by around 186 million tons each year and it is generally considered as a waste, even when several whey proteins have important economic relevance. For its valorization, inexpensive ligands and integrated chromatography methods need to be developed for specific and low-cost protein purification. Here, we describe a novel affinity process with the dye Yellow HE-4R immobilized on Sepharose for bovine lactoferrin purification. This approach based on a low-cost ligand showed an efficient performance for the recovery and purification of bovine lactoferrin directly from whey, with a yield of 71% and a purification factor of 61.

Keywords: Bovine lactoferrin / Dye affinity chromatography / Sweet whey / Triazine dyes
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1 Introduction

Lactoferrin (Lf) is a glycoprotein present in mammalian external secretions with iron-chelating capacity [1]. Its biological properties include regulation of iron adsorption in the gastrointestinal tract, modulation of polymorphonuclear production, and antimicrobial and antifungal activity [2, 3]. All these functional properties imply Lf valorization in different sectors such as the dairy, nutraceutical, pharmaceutical, and cosmetic industries.

Bovine Lf (bLf) has been traditionally purified from natural sources such as milk, whey, and colostrum by different chromatographic methods that include hydrophobic interaction, ion exchange, and affinity with antibodies [4–10]. Particularly, ion-exchange chromatography is mainly used in large-scale processes. However, the concentration of bLf in cheese whey is low, therefore, the starting material requires concentration and conditioning to improve the performance of the chromatographic process. These processes reach variable yields between 50 and 96% and bLf is usually copurified with lactoperoxidase, a whey protein with a similar p*I*. Using commercial sulfonic adsorptive membranes, Chiu et al. [5] copurified lactoperoxidase and bLf from defatted whey with yields of 73 and 50%, respectively. Plate et al. [11] achieved an 84% yield for bLf purification from a sweet whey concentrate using a similar cation-exchange system, however, the final product showed 10% of the initial lactoperoxidase content. On the other hand, the use of affinity chromatography makes possible the direct adsorption of bLf at low concentration, but the purification cost significantly increases according to the ligand cost and stability.

Triazine dyes are pseudobioaffinity ligands widely used for chromatography, which allows the purification of proteins with favorable cost/selectivity ratio [12,13]. Triazine dyes used in the textile industry in tons are low-cost ligands. They are easily immobilized on different supports and are chemically and thermally stable. All these advantages make them suitable for industrial-scale protein purification [14]. Previous research showed that the triazine dye Red HE-3B has an acceptable chromatographic performance for bLf purification from colostrum and sweet whey [15,16]. Recently, we studied the interaction of several triazine dyes, including Red HE-3B, toward lactoferricin B (bLfcin), a 25 amino acid peptide present in the N-terminal region of bLf, using surface plasmon resonance and concluded that Yellow HE-4R had higher affinity for bLfcin than Red HE-3B [17]. Taking this result into account, in the present study we compared the performance of both dyes (Red HE-3B and Yellow HE-4R) for bLf recovery and purification directly from sweet whey.

2 Materials and methods

Red HE-3B dye (C.I. name: reactive red 120) and Yellow HE-4R dye (C.I. name: reactive yellow 84) were from Vilmax S.A. (Buenos Aires, Argentina). Both dyes were immobilized on Sepharose 4B (Sigma–Aldrich, St. Louis, MO, USA) as previously reported [16,18]. These matrices are named hereafter S-R and S-Y, respectively. The dye density in S-R and S-Y was

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Abbreviations: bLf, bovine lactoferrin; **Lf**, lactoferrin

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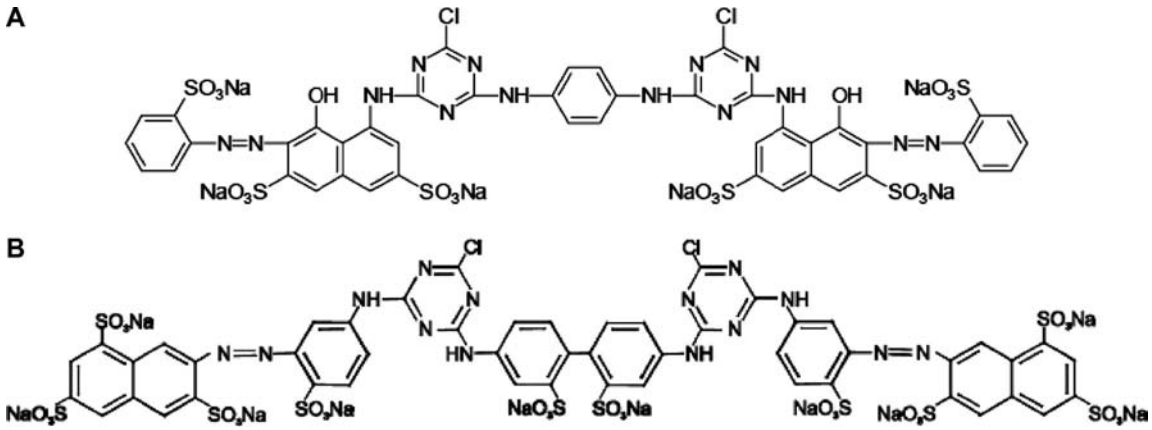

Figure 1. Structures of Red HE-3B (A) and Yellow HE-4R (B).

determined by the hydrolytic procedure reported by Ruckenstein and Zeng [19].

Adsorption isotherms were performed with commercial bLf from Glanbia Nutritionals (Fitchburg, WI, USA). To study the interactions, 50 mg matrices (S-R and S-Y) were soaked with 1 mL of bLf solutions (0.063–10 mg/mL) in adsorption buffer (20 mM sodium phosphate buffer, pH 7.0) for 16 h at 25°C. The concentration of commercial bLf in the supernatants was determined spectrophotometrically at 280 nm considering an absorption coefficient of 1.51 mL/mg·cm [20]. The equilibrium concentration of bLf bound to the matrix per unit of total amount of matrix was calculated by difference between the concentration of bLf 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 as described by Chase and analyzed according to the Langmuir model [21]. Five elution solutions were tested: 2 M NaCl, pH 7.0; 0.5 M NaSCN (sodium thiocyanate), pH 7.0; 25% ethylene glycol, pH 7.0; 25% ethylene glycol + 0.5 M NaSCN, pH 7.0; and 25% ethylene glycol + 2 M NaCl, pH 7.0.

Bovine whey was donated by Cooperativa Tambera Gualeguaychú (Gualeguaychú, Entre Ríos, Argentina). The removal of residual fat from whey was carried out by thermocalcic precipitation according to Maubois et al. [22]. For the purification of bLf, 25 mg matrices (S-R and S-Y) previously equilibrated with 20 mM sodium phosphate buffer, pH 7.0, were soaked with 1 mL of defatted whey and incubated for 16 h with gentle stirring at 25°C. The matrices were washed four times with 0.5 M NaCl in 20 mM sodium phosphate buffer, pH 7.0, for 1 h each, and the elution was performed using 0.5 mL of 25% ethylene glycol + 2 M NaCl for 12 h at 25°C. The process was repeated for three cycles without regeneration of the matrices. Each step was monitored by measurement of bLf concentrations with the Bovine Lactoferrin ELISA Quantification kit (Bethyl Laboratories, USA) and 15% SDS-PAGE with Coomassie Blue staining [23]. Whey, supernatants, and eluates were concentrated four times by centrifugal filtration (Amicon Ultrafree MC with MWCO 10000, Millipore, MA, USA) before SDS-PAGE. Total protein was determined by the Bradford method [24]. All experiments were carried out in triplicate and results are expressed as the average ± SD.

3 Results and discussion

The use of triazine dyes as ligands for bLf purification makes its direct isolation possible from whey without previous conditioning [16]. These ligands allow protein adsorption even from sources with moderate ionic strength. These advantages might reduce the time and costs associated with bLf purification.

The Structures of Red HE-3B and Yellow HE-4R dyes are shown in Fig. 1. In order to characterize the synthesized matrices, adsorption isotherms were performed. Figure 2 shows the adsorption isotherms of S-R and S-Y matrices. Results were analyzed according to the Langmuir model, and the thermodynamic parameters calculated are shown in Table 1. Both matrices showed affinity for bLf with similar K_d. As the bLf concentration in whey was 0.08–0.10 mg/mL, the K_d values obtained indicates that these matrices might ensure an efficient bLf adsorption from whey. The Q_{max} for S-Y was

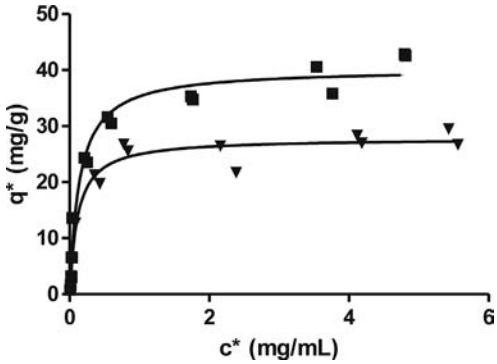

Figure 2. Pure bLf adsorption isotherms using S-R matrix (▼) and S-Y matrix (■). c*, equilibrium concentration of bLf in the supernatant; q*, equilibrium concentration of bLf bound to the matrix per unit of total matrix amount.

Table 1. Dye density of S-R and S-Y matrices and thermodynamic parameters for bLf adsorption

Matrices	Dye density (μmol/g)	Langmuir constants	
		Q_{max} (mg/g)	K_d (mg/mL)
S-R	1.46 ± 0.02	27.82 ± 0.70	0.11 ± 0.02
S-Y	2.27 ± 0.14	40.24 ± 1.27	0.14 ± 0.02

higher than that of S-R. This difference in Q_{max} correlates with a higher dye density for S-Y (Table 1).

Different eluents were tested for bLf desorption from S-R and S-Y. The best elution performance for both matrices was achieved with 25% ethylene glycol with 2 M NaCl or 0.5 M NaSCN at pH 7.0. This was consistent with previous results described for S-R [16]. The use of a salt combined with a solvent for bLf desorption suggests that the affinity of bLf to S-Y is a result of both electrostatic and hydrophobic interactions.

For bLf purification from whey, the sample volume (mL)/matrix amount (mg) ratio for the adsorption process was optimized (data not shown). Also, the washing was optimized by using 0.5 M NaCl in 20 mM sodium phosphate buffer, pH 7.0, to remove contaminants. The eluent selected was 25% of ethylene glycol + 2 M NaCl at pH 7.0. The use of NaSCN was eliminated due to its high cost for scale-up applications.

The performance for bLf recovery and purification from whey was studied using both dye matrices. Table 2 shows the results of three consecutive cycles without matrices regeneration. During these purification processes, both matrices showed similar adsorption values, whereas the desorption values slightly increased in the third cycle. This fact might be attributed to an incomplete desorption from the previous purification cycle. Nevertheless, S-Y showed higher desorption values than S-R, resulting in a higher yield. The purification factor was similar along the three cycles for both matrices. The analysis of the eluates by SDS-PAGE (Fig. 3, lanes 6–8) showed the same pattern of contaminants without any significant quantitative difference. The purity of the recovered bLf was higher than 90% using gel density quantification for both matrices. In a previous study, more than 90% purity was achieved by using the Red HE-3B dye attached to polysulfone hollow-fiber membranes [16]. In this work, Sepharose 4B was used as the chromatographic support and the purification degree of bLf obtained with Yellow HE-4R dye attached was higher than that obtained with Red HE-3B.

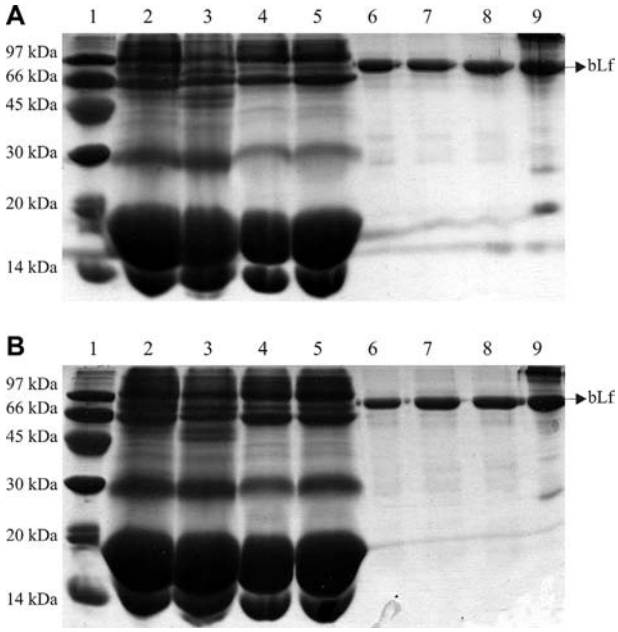

Figure 3. SDS-PAGE analysis of the purification process during three consecutive cycles using S-R (A) and S-Y (B) matrices. Lane 1, molecular weight markers; lane 2, bovine whey; lane 3, supernatant after adsorption from S-R or S-Y in cycle 1; lane 4, supernatant after adsorption from S-R or S-Y in cycle 2; lane 5, supernatant after adsorption from S-R or S-Y in cycle 3; lane 6, eluate S-R or S-Y in cycle 1; lane 7, eluate from S-R or S-Y in cycle 2; lane 8, eluate from S-R or S-Y in cycle 3; lane 9, standard bLf (2 mg/mL).

4 Concluding remarks

The S-Y matrix showed a better performance than S-R, given mainly by a higher elution and consequently a higher yield process. Moreover, both matrices were used for three consecutive cycles without any regeneration treatment between

Table 2. Purification process of bLf from sweet whey

Matrix	Cycle	Adsorption (%)	Elution (%)	Yield (%)	Purification factor
S-R	1	97.8 ± 0.1	44.9 ± 5.3	43.9 ± 5.2	35.4 ± 4.2
	2	97.2 ± 0.2	50.6 ± 7.0	49.2 ± 6.9	36.7 ± 3.7
	3	98.5 ± 1.2	63.3 ± 5.8	62.3 ± 4.9	52.3 ± 4.1
S-Y	1	98.2 ± 0.2	64.6 ± 0.2	63.4 ± 0.1	33.2 ± 3.3
	2	98.4 ± 0.2	60.2 ± 0.6	59.3 ± 0.4	38.7 ± 2.9
	3	99.6 ± 0.5	71.7 ± 5.6	71.4 ± 5.2	61.0 ± 4.3

The matrices were used during three purification cycles without regeneration steps between cycles.
Total whey protein = 5.90 mg/mL and cheese whey bLf = 0.09 mg/mL.

purification cycles. These results demonstrated the potential use of S-Y matrix to recover bLf directly from sweet whey.

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The authors have declared no conflict of interest.

5 References

[1] Tomita, M., Wakabayashi, H., Yamauchi, K., Teraguchi, S., Hayasawa, H., *Biochem. Cell Biol.* 2002, *80*, 109–112.

[2] Farnaud, S., Evans, R. W., *Mol. Immunol.* 2003, *40*, 395–405.

[3] Na, Y. J., Han, S. B., Kang, J. S., Yoon, Y. D., Park, S. K., Kim, H. M., Yang, K. H., Joe, C. O., *Int. Immunopharmacol.* 2004, *4*, 1187–1199.

[4] Chen, J., Wang, C., *J. Food Sci.* 1991, *56*, 701–706.

[5] Chiu, C. K., Etzel, M. R., *J. Food Sci.* 1997, *62*, 996–1000.

[6] Kawakami, H., Shinmoto, H., Dosako, S., Sogo, Y., *J. Dairy Sci.* 1987, *70*, 752–759.

[7] Pahud, J. J., Hilpert, H., *Protides Biol. Fluids* 1976, *23*, 571–574.

[8] Tu, Y. Y., Chen, C. C., Chang, J. H., Chang, H. M., *J. Food Sci.* 2002, *67*, 996–1001.

[9] Yoshida, S., *J. Dairy Sci.* 1989, *72*, 1446–1450.

[10] Yoshida, S., Xiujun, Y., *J. Dairy Sci.* 1991, *74*, 1439–1444.

[11] Plate, K., Beutel, S., Buchholz, H., Demmer, W., Fischer-Fruhholz, S., Reif, O., Ulber, R., Scheper, T., *J. Chromatogr. A* 2006, *1117*, 81–86.

[12] Denizli, A., Piskin, E., *J. Biochem. Biophys. Methods* 2001, *49*, 391–416.

[13] Wolman, F. J., Smolko, E. E., Cascone, O., Grasselli, M., *J. Sep. Sci.* 2005, *28*, 45–51.

[14] Boyer, P. M., Hsu, J. T., *Adv. Biochem. Eng. Biotechnol.* 1993, *49*, 1–44.

[15] Grasselli, M., Cascone, O., *Neth. Milk Dairy J.* 1996, *50*, 551–561.

[16] Wolman, F. J., González Maglio, D., Grasselli, M., Cascone, O., *J. Membr. Sci.* 2007, *288*, 132–138.

[17] Urtasun, N., Baieli, M. F., Romasanta, P. N., Fernandez, M. M., Malchiodi, E. L., Cascone, O., Wolman, F. J., Miranda, M. V., *Process Biochem.* 2013, *48*, 1972–1979.

[18] Stellwagen, E., *Methods Enzymol.* 1990, *182*, 343–357.

[19] Ruckenstein, E., Zeng, X., *J. Membr. Sci.* 1998, *142*, 13–26.

[20] Andersson, J., Mattiasson, B., *J. Chromatogr. A* 2006, *1107*, 88–95.

[21] Chase, H. A., *J. Chromatogr.* 1984, *297*, 179–202.

[22] Maubois, J. L., Pierre, A., Fauquant, J., Piot, M., *Bull. Int. Dairy Fed.* 1988, *212*, 154–159.

[23] Laemmli, U. K., *Nature* 1970, *227*, 680–685.

[24] Bradford, M. M., *Anal. Biochem.* 1976, *72*, 248–254.

