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ENRICHMENT OF LACTOFERRIN BY A SIMPLE PROCESS BASED ON SOLID PHASE EXTRACTION

ENRIQUECIMIENTO DE LACTOFERRINA MEDIANTE UN PROCESO SIMPLE BASADO EN EXTRACCIÓN EN FASE SÓLIDA

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Abstract

Milk whey (MW) is a by-product of cheese factory, which causes serious environmental problems, due to is poured into the rivers, without treatment. However, MW contains high-value compounds, like lactoferrin (Lf). Several methods have been suggested to separate Lf from MW, including ultrafiltration and chromatography affinity. In this work, extraction of Lf was proposed, which was carried out using a matrix of polyacrylamide (PAM) activated with Cu(II) to improve its affinity this protein. PAMs were prepared at two total percentage concentrations (6 and 10 %T) and were characterized. 10 %T PAM showed higher homogeneity, therefore, it was modified with Cu(II) and used as extraction support. The analysis supported the functionalization of 10 %T PAM; likewise, the Cu(II) role in the extraction procedure. According to SDS-PAGE results, 10 %T PAM-IDA-Cu^{2+}-imidazole enabled to reach a Lf recovery around 82.5%; besides, a Lf-enriched fraction (150 μg per gram of powder) was obtained. The method proposed represents an adequate alternative for the recovery of Lf from MW, which is considered a problematic waste.

Keywords: lactoferrin, bovine whey, PAM matrix, extraction, enriched fraction.

Resumen

El suero lácteo (SL) es un subproducto de la quesería, que causa problemas ambientales graves, ya que es vertido a los ríos, sin tratamiento previo. Sin embargo, el SL contiene compuestos con alto valor agregado, como la lactoferrina (Lf). Se han sugerido diversos métodos para separar la Lf del SL, incluyendo la cromatografía de intercambio por afinidad y la ultrafiltración. En este trabajo, se propuso la extracción de Lf, utilizando una matriz de poliacrilamida (PAM) activada con Cu(II) para mejorar la afinidad a esta proteína. Las PAMs se prepararon a dos diferentes concentraciones de monómeros (6 y 10 %T) y se caracterizaron. La PAM 10 %T presentó mayor homogeneidad, por lo que, se modificó con Cu(II) y se usó como soporte de extracción. Los análisis realizados demostraron la funcionalización de la PAM 10 %T; así como el rol del Cu(II) en el procedimiento de extracción. De acuerdo con los resultados de SDS-PAGE, PAM 10 %T-IDA-Cu^{2+}-imidazol permitió alcanzar una recuperación de Lf alrededor de 82.5%; además, se obtuvo una fracción enriquecida con Lf (150 μg por gramo de polvo). El método propuesto representa una alternativa adecuada para la recuperación de Lf a partir de SL, el cual se considera un residuo problemático.

Palabras clave: Lactoferrina, suero lácteo, matriz de poliacrilamida, extracción, fracción enriquecida.

1 Introduction

Milk whey (MW) is a by-product from cheese manufacture processes. MW is considered a strong organic and saline effluent, and its chemical composition is variable, because is dependent of other factors, such as: raw milk used, the fraction of non-valorized MW, and the amount of cleaning water used (Prazeres *et al*., 2012). MW represents an environmental problem, because only 30% is used as livestock feeding and the remaining is discarded to drain or soils, without treatment (FAO, 2013).

MW has been used to prepare protein concentrates, and as raw material in energy generation process (Prazeres *et al*., 2012). However, MW contains proteins with nutritional and economical high-value,

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like α-lactalbumin, β-lactoglobulin, bovine serum albumin, and lactoferrin (Lf), which can be recovered to use in pharmaceutical and food industries (Kilara and Vaghela, 2004). In fact, MW as a source of compounds of commercial interest, has become an important research topic, in order to avoid its pollution effects (Patel and Murthy, 2011).

Whey proteins have a wide range of chemical, functional, and physicochemical properties (Almecija *et al*., 2007); however, their content in MW is lower (0.01% Lf). Therefore, several separation and purification processes has been developed in order to isolate whey valuable proteins, specially, Lf (Carvalho *et al*.2014; Baieli *et al*. 2014).

Lactoferrin (Lf) is a glycoprotein of transferrins group and is found in mammals' milk in low concentrations (20-200 μg L^{-1}) (Wakabayashi *et al*. 2006). It is constituted by 690 amino acid residues, including all essential amino acids, and has a molecular weight of 80 kDa (da Costa *et al*., 2015). Lf structure (Fig. 1A) (Rose *et al*., 2018) is composed by two lobes (N and C, Figs. 1B and 1C, respectively), which, can be bind a metal ion (as ferric, Fe^{3+}) by protein amino acids (two tyrosine, an aspartate and a histidine) in synergy with carbonate ions (CO_3^{2-}) (Figs. 1B-C) (Alderova *et al*. 2008).

Lf has important biological properties: anticarcinogenic, anti-inflammatory, antifungal, antiviral, and immuno-modulatory (Sinha *et al*. 2013). In addition, Lf hydrolysis allows to obtain lactoferricin and lactoferrampin (Zhang *et al*. 2016; Akal, 2017) which also have antimicrobial and bactericide activity in Gram-positive and Gram-negative pathogens (Brock, 2002).

Several methods for Lf separation from MW have been proposed, which include ultrafiltration, use of ion exchange columns, affinity chromatography and solid phase extraction with commercial polymers and resins (Ng and Yoshitake, 2010; Du *et al*. 2013). However, these methods are affected by the complexity of the sample and make them less accessible, because of their high cost. Therefore, the aim of the present work was to the study of Lf enrichment by a solid phase extraction process, using Cu(II)-imidazole immobilized in polyacrylamide gel. The Lf extraction is reached by the exchange/coordination reaction between Fe^{3+} of Lf, and Cu(II)-imidazole. This proposal is an inexpensive and simple alternative separation method for the valorization of MW.

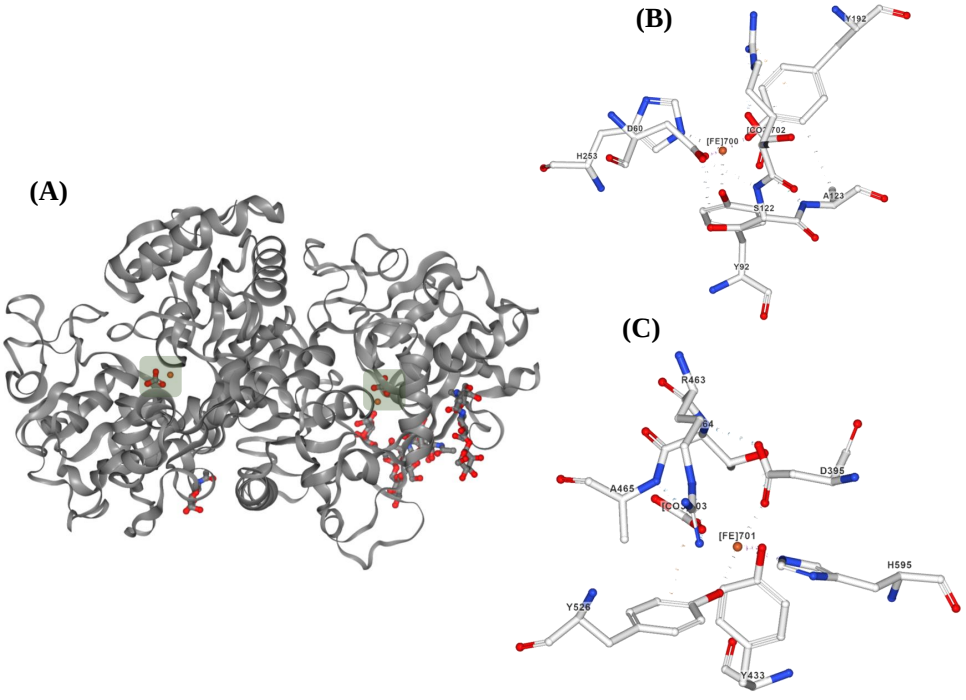


Fig. 1. A) 3D Lactoferrin structure. B) N-lobe and C) C-lobe.

2 Materials and methods

2.1 Reagents

Acrylamide/bisacrylamide 40% solution for proteins (37.5:1, 2.6% crosslinker, electrophoretic grade), ammonium persulphate (APS, 99.9%), Tris hydroxymethyl aminomethane (electrophoretic grade 99.8%) N,N,N',N'-tetramethylethylenediamine (99% TEMED), Comassie Blue G-250, and Broad-Range SDS-PAGE standard were purchased from Bio-Rad (California, USA). Iminodiacetic acid (IDA 98%), and imidazole (ACS reagent ≥ 99%) were supplied by Sigma-Aldrich (St. Louis, MO, USA). $CuSO_4 \bullet 5H_2O$ 98% was acquired from J.T. Baker (Center Valley, PA, USA); and Bioferrin^{TM} 2000 was purchased from Glanbia Nutritionals (Wisconsin, USA).

2.2 Sample preparation

Fresh cheese was produced by enzymatic curdling and 10 L of MW was recovered. The sample was filtered and sterilized at 90° C for 10 min in an autoclave. MW was freeze stored (-21°C) in 500 mL-bottles until its use.

2.3 Preparation and characterization PAM

2.3.1 Polymerization

Polyacrylamide matrix (PAM) was prepared according to Laemmli (1970) with some modifications. Two acrylamide:bisacrylamide dilutions (100 mL) of 6 and 10 total monomer concentration (%T) were prepared from 40% solution. Polymerization was performed using 10% of APS (as free radical initiator) and 10 μL of TEMED (as catalyzer) for 4 h at room temperature.

The polymerized gel was separated in six fractions and dried at 37°C for five days, until constant weight was reached. The dried polymer was milled and the 50-mesh sieved fraction (297 μm) was used to prepare the column.

2.3.2 Characterization

Scanning electron microscopy (SEM) and swelling analysis were performed in order to characterize the PAM.

From SEM analysis, PAM as powder was placed on an aluminum stub (coated with double-sided carbon tape) and coated with gold. Analysis were carried out using a JSM-5600 field emission scanning electron microscope equipment (JEOL, Japan). Micrographs were acquired at 100 and 200X magnification. The samples were analyzed at 15A and 20 kV.

The texture parameter was measured analyzing the micrographs according to the grayscale obtained using ImageJ freeware v.1.46r (National Institutes Health, Bethesda, MD, USA) as reported by Haralick (1973).

Entropy values were calculated from different random sections of the micrographs (71x71 pixels), and this parameter allowed evaluating the homogeneity of the particle phases (Shih *et al*. 2013).

Swelling degree (Equation 1) was calculated using the gravimetric method reported by Orozco-Guareño *et al*. (2011).

$$Swelling = \frac{W_S - W_d}{W_d} \quad (1)$$

Where W_d is the weight of the dried hydrogel and W_s is the weight of the swollen hydrogel after 24 h. All measurements were performed in triplicate.

2.4 Lactoferrin enrichment process

2.4.1 Column preparation

Column preparation was performed following the method proposed by Carvalho *et al*. (2014) with some modifications. Dry PAM (3 g) was hydrated with 100 mL of deionized water (18 mS cm^{-1}) and transferred to a glass chromatographic column (i.d. x L, 25mm x 46 cm). After that, the following elution steps were carried out to activate the adsorbent: 1) 50 mL of 0.5 mol L^{-1} Na_2CO_3, 2) 30 mL of IDA buffer (0.5 mol L^{-1} IDA in 1 mol L^{-1} Na_2CO_3, pH 10), 3) deionized water until pH of 8.0 was reached. Subsequently, 50 mL of 0.1 mol L^{-1} Cu^{2+} (from $CuSO_4 \bullet H_2O$) were added, and the column was kept at rest for 20 min. The excess of Cu^{2+} was removed with 250 mL of deionized water. Finally, 100 mL of imidazole buffer (2 mmol L^{-1} imidazole, 20 mmol L^{-1}, Tris and 0.2 mol L^{-1} NaCl, pH 7) were added. All elution steps were made by gravity, avoiding air spaces, and the flowrate was controlled at 1.3 mL min^{-1}. The process is showed in Fig. 2A.

2.4.2 Characterization of extraction support

The polymeric modified support (PAM-IDA-Cu^{2+}-imidazole) was analyzed to assess the main changes in the solid, which are related with its functionality. Copper amount (by atomic absorption) (Katime and Rodríguez, 2007), homogeneity (by SEM) (Martinez-Vázquez *et al*., 2007), and functional groups variations

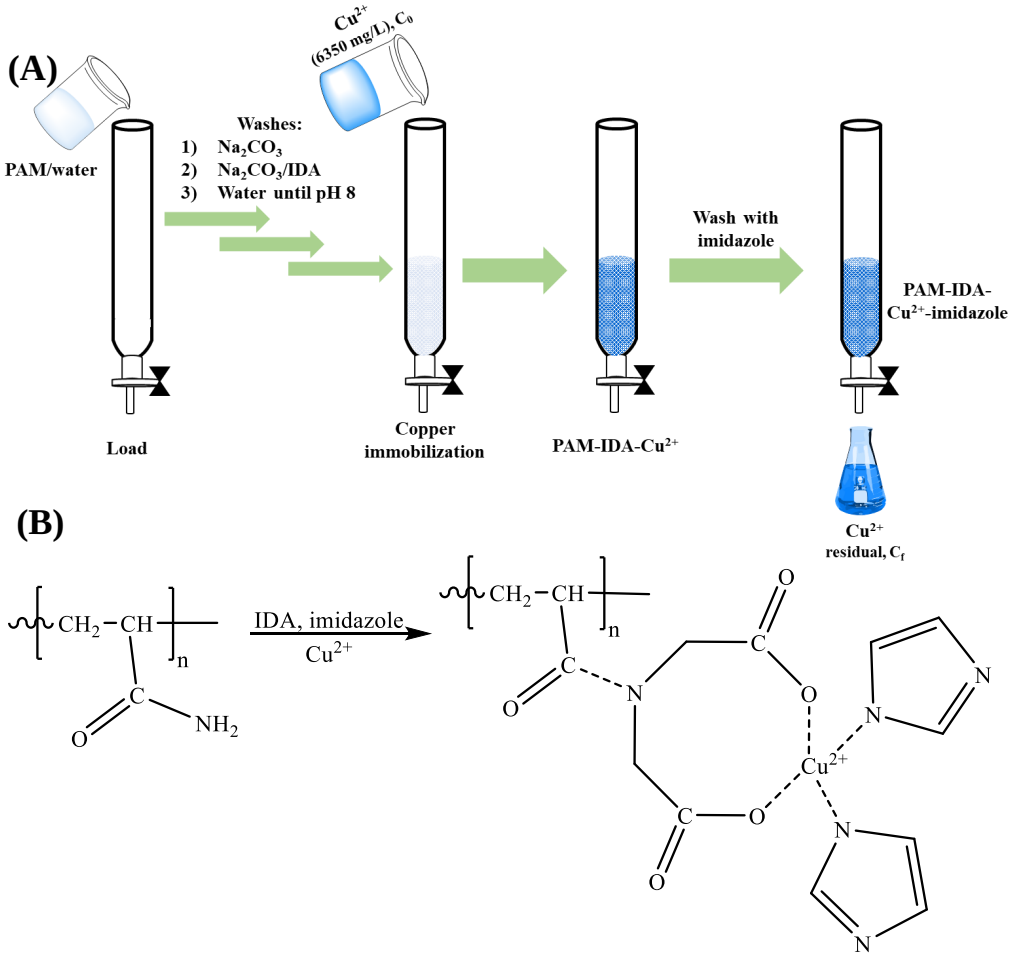


Fig. 2. Column preparation process (A) and mechanism of copper immobilization (B).

(by FT-IR) (Nascimento *et al*., 2019) from polymeric support were determined during the stages of activation and copper immobilization.

Copper contain was measured by flame atomic absorption spectrometry using a SpectrAA 880 equipment from Varian (Australia) at 224.4 nm and air-acetylene flame. A set of standard copper solution was prepared (2-10 mg/L) from stock solution (1000 mg/L) in a nitric matrix (5% HNO_3). An aliquot (100 μL) of each copper solutions at C and C_0 concentrations (Fig. 2A) were taken and transferred to 10 mL volumetric flask; then, 500 μL of nitric acid was added and filled with deionized water. The procedure was done by triplicate.

SEM analysis was carried out using the same method described in section 2.3.2. The FT-IR spectra of polymeric support during the stages of activation and copper immobilization, preparing a KBr pellet with the solid (1:100). A GX FT-IR System from Perkin-Elmer (Massachusetts, EE.UU.) was used to record the spectra in the range of 4000-370 cm^{-1}.

2.4.3 *Elution process*

A 0.1% Lf standard solution (100 mL) was prepared, which was loaded into the packed column with the polymeric modified support (PAM-IDA-Cu^{2+}-imidazole). The non-retained sample was removed; after, 100 mL of imidazole buffer (pH 7.0) were added to elute the retained proteins. The recovered fractions (non-retained and retained fractions) were kept cooled at -70°C and freeze dried at -40°C and 133×10^{-3} bar. The powders were kept in Eppendorf vial until analysis. All elution processes were repeated four times to evaluate the reproducibility. This elution process was used to assess Lf retention into the polymeric support.

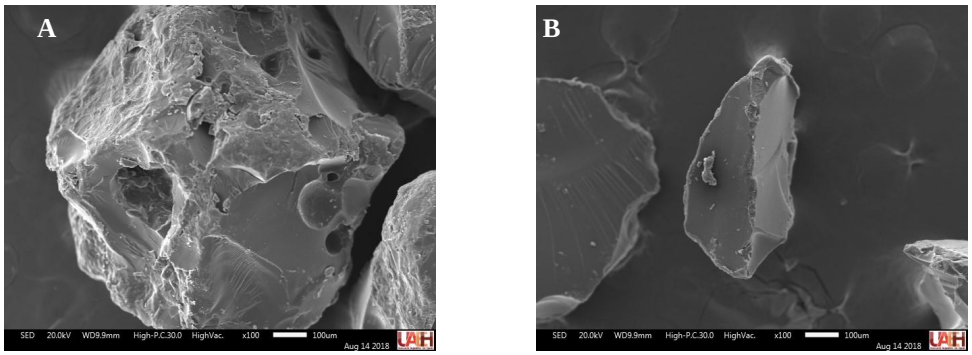


Fig. 3. SEM micrographs of polyacrylamide matrices: 6%T (A) and 10%T (B) at 100x magnifications.

Subsequently, two "clean-up" support processes were done with: 1) 50 mL of imidazole buffer (pH 7), and 2) 150 mL of deionized water. A reactivation support was carried out, using the same procedure detailed previously (section 2.4.1). Afterwards, an aliquot of MW (500 mL) was taken and loaded into the packed column and the same elution process used to Lf standard was performed.

2.4.4 *Lactoferrin identification by SDS-PAGE*

Eluate solutions were prepared using 1 g of freeze-dried samples (Lf standard, MW, non-retained or retained fractions) dissolved in 5 mL of deionized water. The sample preparation and the separations were performed according to Laemmli (1970) with some modifications.

Polyacrylamide gels were prepared at 15 %T (for separation), and at 4 %T (for concentration), and separation conditions were: all runs were performed at 200 V for 80 min in a Mini PROTEAN II (Bio-Rad), with spacer plates of 1 mm. Gels staining were performed with Comassie Blue G-250 solution. The gels were analyzed in Gel Doc^{TM} EZ Imager from Bio-Rad (California, USA).

2.4.5 *Protein quantification*

Bradford method (1976) was used to determine the protein content in the initial sample of MW, retained, and non-retained fractions. The retained protein was calculated by difference.

3 Results and discussion

3.1 *Characterization of the polyacrylamide matrix*

Fig. 3 shows the micrographs of PAM with 6 and 10 %T at 100x magnification. Entropy values were obtained in different regions of the particles (6 and 10 %T PAMs), which were 7.23±0.71 from 6 %T PAM (Fig. 3A), and 5.14±0.99 from 10 %T PAM (Fig. 3B). The entropy values obtained by image analysis represent randomness of gray-level distribution and are directly related with the polymer surface (Shih *et al*., 2013). Therefore, high entropy values correspond to heterogeneous surface.

The entropy values of an image are directly related to the degree of homogeneity of a particle matrix; so, more homogeneous particle structures have the smaller entropy value (Haralick, 1973). According with entropy values found, 10 %T PAM is the most homogeneous support; hence, it was used for elution lactoferrin experiments.

On the other hand, the swelling degree of PAM (6 and 10 %T) were 15.99 and 10.23 g H_2O per g of gel, respectively. Swelling capacity is related to the degree of crosslinking of the matrices. These values agree with other reports, which have been found in the range of 3-15 g g^{-1} for other polymeric gels (Bereli *et al*., 2012; Cimen and Denizli, 2012; Uygun *et al*., 2012).

According with the homogeneity observed (entropies values), all experiments described from the following sections were carried out using 10 %T PAM.

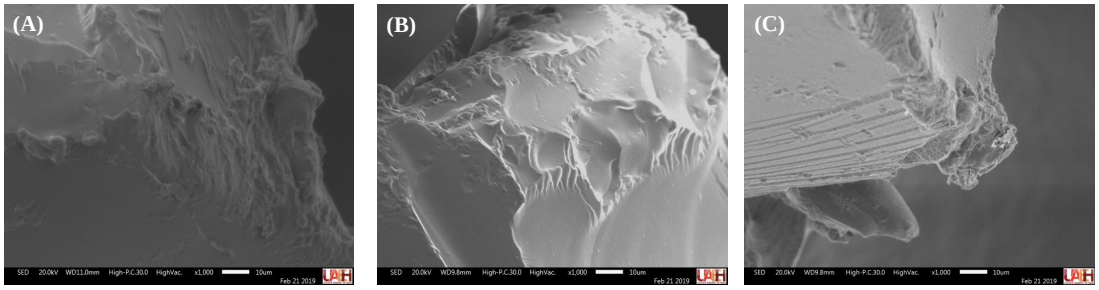


Fig. 4. SEM micrographs of PAM at 1000X magnifications of: (A) PAM at 10%T, (B) PAM-IDA-Cu^{2+}, and (C) PAM-IDA-Cu^{2+}-imidazole.

3.2 Characterization polyacrylamide matrix modified with copper immobilized

3.2.1 Copper immobilization

Protein-metal complexes have been explained based on interactions as Cu(II)-His, Ni(II)-His and Co(II)-His. Therefore, cupric ion was used in PAM, because its facility to interact with amino acids (Da Silva and Williams, 2001). In fact, the addition of IDA can allow to improve the efficiency of Lf separation, because increases the affinity between cupric ion with histidine of Lf in amino acid position 595 (Kagedal, 2011; Carvalho *et al*. 2014).

Due to the copper role in the proposed method, the amount of this metal in the polymeric matrix was determined. For this, initial and residual copper concentrations (C_0 and C_f in Fig. 2A) were analyzed by atomic absorption spectrometry (AAS) analysis; and the amount of copper linked to PAM was obtained by difference.

The C_0 was 635 mg/L; while C_f was 605 mg/L. Considering the matrix weight (3 g), and the respective volumes, the amount of copper adsorbed into the PAM was 0.16 mmol/g of matrix. Value found in the present work is lower than the reported by Kasgöz *et al*. (2006), however, the amount of copper adsorbed was enough to interact with whey proteins. On the other hand, the adsorption metal capacity of polymeric materials is dependent of several factors, such as: polymer functionalization, crosslinking grade, and the thermodynamic constant values of metal-polymer complexes (Mathew and Pillai, 1993).

3.2.2 Homogeneity of modified matrix

SEM micrographs of 10 %T PAM at 1000x magnifications were displayed in Fig. 4 PAM with copper immobilized showed higher brightness (Figs. 4B and 4C) caused by the high atomic number of the metal. According with the micrographs, the morphology of the polymeric support did not change with the copper immobilization; besides, three entropy values were similar (5.74±0.94, 6.74±0.63, and 6.28±1.10), therefore, the matrix homogeneity was not modified.

3.2.3 FT-IR spectroscopy

FT-IR analysis were performed to identify the changes of polymeric matrix by addition of IDA, cupric salt, and imidazole, spectra are showed in Fig. 5. The FT-IR spectrum of PAM is represented in Fig. 5A, in which, different characteristic adsorption bands were observed, such as: 3440 and 3200 cm^{-1} (N-H stretching), 2923, 2850, and 2765 cm^{-1} (C-H stretching), 1659 cm^{-1} (C=O stretching), 1446 cm^{-1} (C-H bending), and 1120 cm^{-1} (N-H stretching). These signals were similar to those reported by Dweik *et al*., (2008) and Nascimento *et al*., (2019).

The FT-IR spectrum of PAM with IDA added was displayed in Fig. 5B. Signals found from PAM did not changed, except the double bands at 1735 and 1644 cm^{-1}, which can be associated to two different carbonyl groups (one of the poly-acrylamide and the other from IDA). A strong absorption band was observed between 2500-2250 cm^{-1}, which correspond to O=C=O found in carbonate buffer. Additional adsorption bands at 1533 and 1423 cm^{-1} were assigned to carboxylate ion of IDA.

The copper salt addition only caused changes in the absorption bands related with carboxylate ion (Fig. 5C), which were shifted at 1419 and 1316 cm^{-1}, respectively. These modifications can be given for the coordination equilibria between IDA and cupric ion (Sharma and Agarwal, 2001).

Imidazole has been reported as a good ligand, especially from cupric ion (Drolet *et al*.1988).

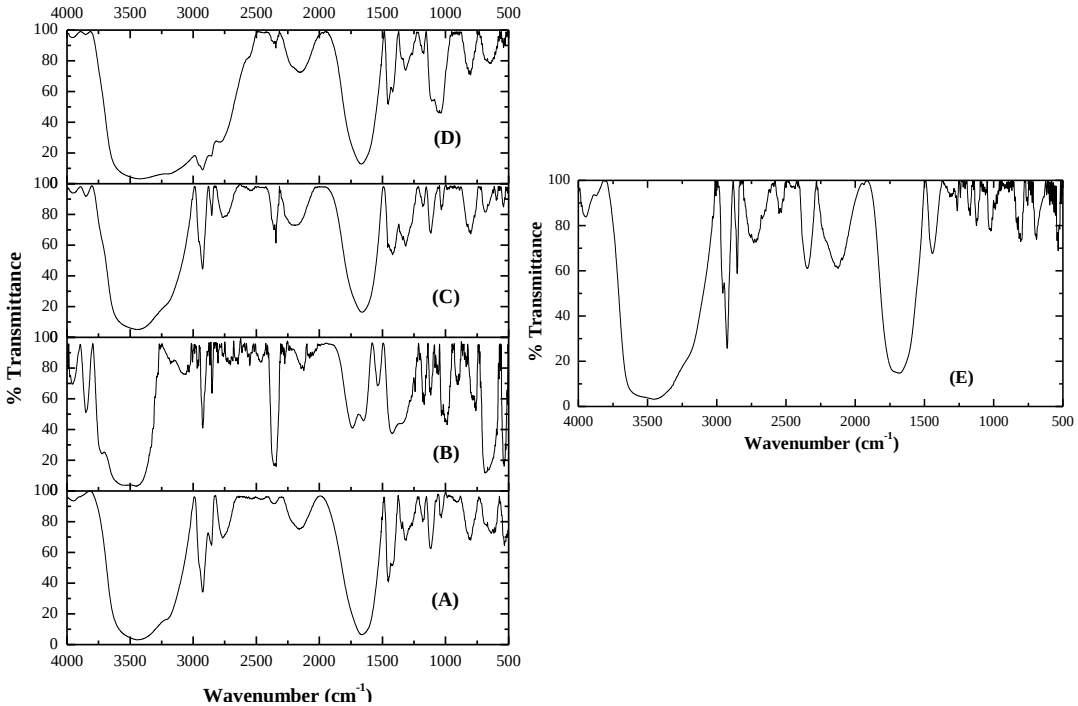


Fig. 5. FT-IR spectra of PAM 10 %T (A), PAM-IDA (B), PAM-IDA-Cu^{2+} (C), PAM-IDA-Cu^{2+}-imidazole (D) and PAM-IDA-Cu^{2+}-imidazole after proteins desorption (E).

The main different signal observed in this FT-IR spectrum (Fig. 5D) was at 1052 cm^{-1}, that was related with the system =C-H of the imidazole ring. Wide absorption band between 3800-2500 cm^{-1} overlapped with several stretching vibrations, for example, C-H, N-H (for imidazole), =C-H (for imidazole ring), which are medium-intensity bands.

The main signals found in FT-IR spectra (Figs. 5A-D) allowed to suggest a mechanism of PAM with copper immobilized (Fig. 2B); in which, cupric ion is coordinated with IDA and imidazole.

3.3 Lactoferrin separation by SDS PAGE

3.3.1 Lactoferrin standard analysis

SDS-PAGE from lactoferrin standard is showed in Fig. 6A. Lane 1 is protein standard, lane 2 is Lf standard, and lane 3 is enriched Lf fraction.

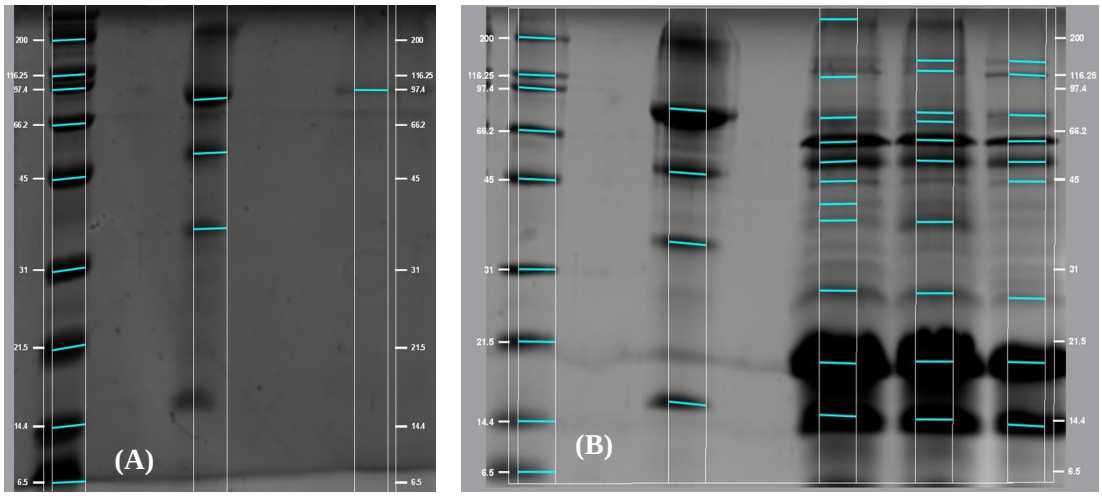
This analysis was carried out to evaluate the percentage of protein extracted for the methodology proposed; besides, R_f (relative front) of Lf was calculated in order to compare WW and enriched Lf fractions.

Band in lane 3 (Fig. 6A) corresponding to molecular weight of Lf (around 85.333 kDa), and R_f was 0.187±0.01. Solid phase extraction methods have advantages compared to other methods as liquid-liquid extraction, because provides low solvent consumption, low intrinsic costs and reduction of processing time. Moreover, it is possible to automated whole process (Zygler *et al*., 2010).

Several analytical methods have been reported from the separation and purification of bioactive compounds (Du *et al*., 2013), such as affinity-exchange chromatography with immobilized metals (IMAC), polyacrylamide-Cu (II) cryogels and ion exchange, as well as the use of solid phase extraction. These methods have been used in purifications of protein (Cheeks, 2009; Yavuz *et al*., 2006), antigens (Oshima *et al*., 2015), microbial cells, and other macromolecules (Karakus *et al*., 2015).

The gels analysis by Gel Doc^{TM} EZ Imager allowed to obtain the areas for each fraction. The recovery of lactoferrin was calculated by comparison of areas (enrichment Lf fraction vs Lf standard). According with the results, the recovery of lactoferrin was 82.5%.

Lf recovery rate has been achieved over 85% using cryogels monoliths as support (Billakanti and Fee, 2009), which was not described chemically.



SDS-PAGE	Label	Mw, kDa	R_f	% Band	Lane
	Phosphorylase b	97.400	0.173	6.78	1
A	Lf)	87.493	0.194	40.58	2
	Lf eluated	85.333	0.187	100.00	3
	Lf standard	85.583	0.185	36.77	2
B	Lf MW	87.587	0.192	0.10	3
	Lf enriched	86.608	0.196	3.12	5

Fig. 6. (A) SDS-PAGE from lactoferrin standard, lanes: (1) Protein standard, (2) Lf standard and (3) enriched Lf fraction. (B) SDS-PAGE analysis from milk whey, lanes: (1) Protein standard, (2) Lf standard, (3) MW, (4) is non-retained fraction of MW and (5) is enriched Lf fraction of MW.

This separation was based on cation exchange chromatography. On the other hand, Baieli *et al*. (2014) proposed a versatile method of dye-affinity chromatography. Lf recovery in this study was about 80%, the sample was sweet whey, using chitosan minispheres with Yellow HE 4R as support.

The method proposed in the present work allows to reach similar Lf recoveries, and the main advantage is the simplicity in the support (PAM-IDA-Cu^{2+}-imidazole) preparation.

3.3.2 Recovery and enrichment of Lf from bovine whey

Lactoferrin enriched fraction was eluted from the column (PAM-IDA-Cu^{2+}-imidazole) and the support was analyzed by FT-IR (Fig. 5E). This spectrum showed the same peaks of Fig. 5C, so that, the mechanism proposed in Fig. 2B can be demonstrated, in which, imidazole interacts with cupric ion.

SDS-PAGE analysis from bovine whey are showed in Fig. 6B, in which, lane 1 is protein standard, lane 2 is Lf standard, lane 3 is MW, lane 4 is non-retained fraction of MW, and lane 5 is enriched Lf fraction of MW.

Enriched Lf fraction (Fig. 6B, lane 5) showed a similar R_f that Lf of MW without enrichment (Fig. 6B, lane 3) (0.196 vs 0.192, respectively).

Likewise, SDS-PAGE analysis allows to calculate the amount of Lf recovered. The contain of Lf in the analyzed sample (powder from Lf enriched fraction) was above of 155 μg of Lf per gram; while, the MW powder had 44 μg of Lf per gram.

Currently, PAM functionalization has been reported as a good alternative for proteins separation (Lee *et al*., 2016), because has advantages over commercial resins, such as: low cost and optional monomer percentage to increase the specificity. Additionally, Lf separation has been improved using PAM cryogels modified with cupric ion (Carvalho *et al*., 2014).

Several methods have been described to Lf separation (Baieli *et al*., 2014; Carvalho *et al*., 2014; Billakanti and Fee, 2009), however, they have some disadvantages like: the polymeric support preparation and the sample pretreatment use to be complicated, and the separation involves many steps.

The proposed method in this work can be

considered as feasible for different reasons, like 1) minimal sample pretreatments, 2) use of high MW volumes (500 mL), 3) low cost, and 4) the support can be reusable.

Conclusions

Copper content into the polymeric matrix was enough to interact with histidine present in some whey proteins, such as, lactoferrin. SEM micrographs were used to calculate the matrix entropy, this value was related with the matrix homogeneity. 10%T PAM and its modification were more homogeneous than 6%T PAM, due to the monomer concentration. The extraction through PAM-IDA-Cu^{2+}-imidazole matrix allows to obtain a rich-lactoferrin fraction from milk whey. The proposed method has some advantages as: practical synthesis and modification of polymeric matrix, minimal sample pretreatment, high volumes of bovine whey, low cost, mainly.

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