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Microcalorimetric study of the adsorption of lactoferrin in supermacroporous continuous cryogel with immobilized Cu^{2+} ions

B.M.A. Carvalho[a], L.H.M. Da Silva[b], L.M. Carvalho[c], A.M. Soares[d,e], L.A. Minim[b], S.L. Da Silva[a,*]

[a] Chemistry, Biotechnology and Bioprocess Department, Federal University of São João Del Rei – Ouro Branco, MG 36420-000, Brazil
[b] Food Technology Department, Federal University Federal of Viçosa – Viçosa, MG 36270-000, Brazil
[c] Federal University of Viçosa – Viçosa, MG 36270-000, Brazil
[d] Center for the Study of Biomolecules Applied Health (CEBio), Fundação Oswaldo Cruz Fundation (Fiocruz), Porto Velho, RO 76812-245, Brazil
[e] Department of Medicine, Federal University of Rondônia, Porto Velho, RO 76812-245, Brazil.

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ABSTRACT

The adsorption affinity of lactoferrin from whey in monolithic supermacroporous cryogel was analyzed using equilibrium data adsorptive isothermal titration microcalorimetry to measure thermodynamic information governing the process. Isotherm data was obtained at temperatures of 20, 30 and 40 °C, pH 6, 7 and 8, and ionic strength of 200, 600 and 1000 mmol L^{-1} NaCl. The Langmuir model was fitted to equilibrium data. The binding was tighter at higher temperatures. The adsorption of protein was observed as spontaneous in all cases analyzed. The microcalorimetric study indicated that, in most cases examined, the adsorption of the protein in the matrix was entropy and enthalpy favored and entropy driven. Results provide data to enable the improvement of technical processes for the affinity separation of proteins.

1. Introduction

Lactoferrin (LF), also known as lactotransferrin (LTF), is a multifunctional transferrin family protein. LF is a globular glycoprotein with a molecular mass of approximately 80 kDa and that is widely found in various animal secretions, such as milk, tears, saliva and nasal secretions. Lactoferrin is considered an important defense molecule and has a variety of other physiological functions and biological properties such as antiviral, antibacterial, antioxidant and immunomodulatory activity (UniProt: B9VPZ5) [1,2]. During the last decade, it became clear that oral administration of lactoferrin exert various benefits in human and animal health, including anticancer and anti-inflammatory effects [3].

Adsorption is a phenomenon widely exploited by various chromatographic techniques for protein purification; however its mechanism is not fully understood [4]. Biospecific affinity adsorption exhibits a high selectivity for biological separation and has been widely used in laboratories and industrial applications, although it must be remembered that the stability and elution conditions are the most significant constraints inherent to this type of chromatography [5].

Porath et al. [6] developed the technique of affinity chromatography on immobilized metal (IMAC). IMAC employs a metal ion as the affinity ligand in the separation of proteins and has been widely and successfully applied [7–10]. The type of interaction between the protein with immobilized metal ions, in general, depends on the topography of the surface protein and the chemical and physical conditions of such interactions. According to Bresolin et al. [4], the coordination complex is responsible for these interactions and is formed by the electron-donating group on the accessible surface of the protein and the immobilized metal ions. Among the factors that may influence the adsorption in IMAC are: (1) multiple interactions, including the specific interaction (binding of coordination), (2) non-specific interaction (electrostatic and hydrophobic binding), (3) strength of hydration of ions and immobilized protein [11] and (4) the possible structural rearrangement of the protein [12,13].

Factors that influence the protein interaction with the binder immobilized metallic include: (1) number of electron donor groups on the surface of the protein [14,15], (2) the pH value of interaction [10,11,15], (3) the type and concentration of salt [11,16], (4) temperature [15,16], and (5) the structure and degree of hydration of the protein [11]. These factors appear to be a suitable basis for

* Corresponding author. Tel.: +55 021 31 37413962.
E-mail address: biomol2@hotmail.com (S.L. Da Silva).

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the use of IMAC for the separation of proteins, but are not sufficient to understand the mechanism of the adsorption of metal ions and proteins. Few thermodynamic studies have been devoted to elucidate the mechanism of adsorption in IMAC, and probably the lack of further investigation is due to the complexity of the process of adsorption in IMAC [17].

Thermodynamic parameters provide valuable information for understanding the process of adsorptive proteins, but unfortunately these parameters cannot be obtained by simple measures. Furthermore, a statistical calculation of entropy direct adsorption on IMAC is difficult, because the rearrangement of protein structure and the redistribution of the molecules of solvent and salt are unpredictable. Thus, quantifying the adsorption enthalpy, ΔH°_{ads}, is an important aspect in thermodynamic investigations involved in IMAC.

This study aims to understand some aspects involved in thermodynamic immobilized metal affinity chromatography (IMAC). Adsorption isotherms were obtained under different conditions of pH (6.0–8.0), salt concentration (200–1000 mmol L^{-1} NaCl) and temperature (20–40 °C). Isothermal titration calorimetry (ITC) was employed to obtain these isotherms. ITC is a highly sensitive technique that allows the measurement of the enthalpy change of a protein solution (lactoferrin) when titrated in continuous supermacroporous matrix cryogel with immobilized Cu^{2+} metal ions.

2. Materials and methods

2.1. Materials

Lactoferrin (M.W., 80 kDa), acrylamide (AAm, 99.9% electrophoretic grade), *N*,*N*′-methylenebisacrylamide (MBAAm, 99%), Per-ammonium sulphate (APS, 98%), $CuSO_4{\cdot}4H_2O$ (98%), iminodiacetic acid (IDA), *N*-2-hydroxyethyl piperazine-*N*′-2-ethanosulfenic acid (HEPES), *N*,*N*,*N*′,*N*′-tetramethyl-ethylenediamine (TEMED, 99%) and allyl glycidyl ether (AGE, 99%) were purchased from Sigma–Aldrich (Steinheim, Germany). Imidazole was purchased from Merck (Germany). Ultrapure water was used during all experiments (Milli-Q system, Millipore Inc., USA).

2.2. Synthesis of the IDA-Cu^{2+} cryogel adsorbent resin

Synthesis of the cryogel-IDA-Cu^{2+} adsorbent resin was performed according to Arvidsson et al. [18], with some adaptations. Monomers of acrylamide and *N*,*N*′-methylenebisacrylamide (AAm/MBAAm (mol/mol) = 30:1) were dissolved in 30 mL of MilliQ ultrapure water and the mixture was degassed using an ultrasound bath for 5 min to remove soluble oxygen. The polymerization of free radicals was initiated by the addition of TEMED and APS. After the addition of TEMED (15 μL) the solution was cooled in an ice-bath (about 5 °C) for 2–3 min. Subsequently, APS (7.5 mg) was added and the mixture stirred for 1 min. Five mL of this mixture was added to 5 mL-plastic syringes. The solutions of the syringes were frozen at −11 °C, and maintained at this temperature for 24 hurs. After this step, the columns were maintained at room temperature to thaw and then washed with 200 mL of MilliQ ultrapure water at a rate of 1 mL min^{-1}. The samples were dried at 60 °C to a constant weight. For activation, Na_2CO_3 solution (50 mL, 0.5 M) was pumped through the cryogel at a rate of 1 mL min^{-1}, followed by 1 mol L^{-1} Na_2CO_3 solution (50 mL) at the same rate. The IDA solution (0.5 mol L^{-1} in 1.0 mol L^{-1} Na_2CO_3, pH 10.0) was applied to the column at a flow rate of 1 mL min^{-1} in order to reflux for 24 h at room temperature. After this, the modified gel was washed with 0.5 mol L^{-1} Na_2CO_3 (100 mL) and then with deionized water to pH 8.0. A solution of 0.1 mol L^{-1} copper sulphate (30 mL dissolved in deionized water) was pumped through the column in order to reflux at a rate of 1 mL min^{-1} for 2 h in the metal matrices. Finally, the cryogel was washed with deionized water to remove unbound metal and then with imidazole buffer (15 mmol L^{-1} HEPES and 0.2 mol L^{-1} NaCl, pH 7.0) to remove weakly-bound metal. The adsorbent cryogel has pores with an average diameter of 10–100 μL; these act as exchangers due to the gel's affinity for ions, enabling coordinated connections with imidazole groups contained in some of the amino acids (histidine, tryptophan) of the lactoferrin protein.

2.3. Obtaining adsorption isotherms

The adsorption isotherms were obtained in each condition, using the static method. Approximately 30 mg of the matrix cryogel were weighed in eppendorf tubes. Initially the resin was equilibrated with 400 μL of equilibration buffer (20 mmol L^{-1} HEPES containing 0.2 mol L^{-1} NaCl, pH 7.0). The tubes were left under mild stirring for a period of 2 h to condition the resin. After this period, different volumes (0–300 μL) of a 10 mg mL^{-1} solution of lactoferrin were added. Equilibration buffer was then added to make the volume up to 1200 μL. Final concentrations of protein in the tubes were 0, 0.1, 0.2, 0.3, 0.4, 0.6, 0.9, 1.2, 1.5, 1.8 and 2.2 mg mL^{-1}. The tubes were kept under constant agitation for 24 h under controlled temperature (101 M Mod BOD/3 Eletrolab®, Brazil), to establish an equilibrium. Subsequently, the resin was removed from the eppendorf tubes and the concentration of protein contained in the supernatant was determined spectrophotometrically at 495 nm (Thermo Scientific Model BIOMATE 3) using the Bradford method [19]. The protein concentration in the solid phase was determined according to Eq. (1):

$$q = \frac{V(C_0 - C)}{M} \quad (1)$$

where q (mg g^{-1}) is the concentration of protein in the solid phase, V (mL) the volume of the liquid phase, M (g) is the mass of the solid phase, C_0 (mg mL^{-1}) is the initial concentration protein C in the liquid phase and (mg mL^{-1}) is the final protein concentration in the liquid phase after equilibrium has been established. The experiment was conducted using a fractional factorial design with three temperatures (20 and 40 °C), three concentrations of salt (200 mmol L^{-1}, 600 mmol L^{-1} and 1000 mmol L^{-1}) and three pH (6.0 and 8.0). The Langmuir isotherm [20] (Eq. (2)) which has been widely used to describe the adsorption of proteins was adjusted to the equilibrium data obtained in this work. For adjust to the equilibrium data was used the program SigmaPlot statistical graph [21].

$$q = \frac{q_m C}{K_d + C} \quad (2)$$

In Eq. (2), K_d is the dissociation constant and q_m is the apparent maximum adsorption capacity.

2.4. Microcalorimetry experiments

The enthalpy changes were measured following the methodology developed by Lira et al. [22], using an isothermal titration microcalorimeter (CSC, ITC model 4200). Measurements were made at two different temperatures (20 °C and 40 °C), two concentrations of NaCl (200 mmol L^{-1} and 1000 mmol L^{-1}) and two pH values (6.0 and 8.0). For ITC measurements, the microreaction system consisted of a stainless steel ampoule (1.8 mL), filled with the adsorbent cryogel balanced in a buffer solution. When the thermal equilibrium between the bulb and the heat sink had been reached, the protein solution was titrated (10 μL) using a Hamilton syringe with a stainless steel needle, driven by an automatic pump at intervals of 30 min. The output signal was collected in power *P*