Brazilian Journal
of Chemical
Engineering

ISSN 0104-6632
Printed in Brazil
www.abeq.org.br/bjche

Vol. 33, No. 01, pp. 215 - 223, January - March, 2016
dx.doi.org/10.1590/0104-6632.20160331s20140112

STERIC MASS ACTION MODEL FOR LACTOFERRIN ADSORPTION IN CRYOGEL WITH IMMOBILIZED COPPER IONS

B. M. A. Carvalho[1*], W. F. Silva Júnior[2], L. M. Carvalho[3],
L. A. Minim[4] and G. G. P. Carvalho[5]

[1]Institute of Agricultural Sciences, Federal University of Minas Gerais, 39404-547, Montes Claros - MG, Brazil.
Phone: + 55 021 38 21017915
E-mail:brunamara.carvalho@gmail.com
[2]Department of Chemistry, Biotechnology and Bioprocess Engineering, Federal University
of São João Del Rei, 36420-000, Ouro Branco - MG, Brazil.
[3]Department of Veterinary, Federal University of Viçosa, 36570-000, Viçosa - MG, Brazil.
[4]Department of Food Technology, Federal University of Viçosa, 36570-000, Viçosa - MG, Brazil.
[5] Department of Animal Science, Federal University of Bahia, 40110-909, Salvador - BA, Brazil.

(Submitted: October 14, 2014 ; Revised: March 8, 2015 ; Accepted: March 18, 2015)

Abstract - Parameters of equilibrium adsorption obtained from experiments using immobilized metal affinity chromatography (IMAC) were used to evaluate the applicability of the steric mass-action (SMA) model to describe the adsorption of lactoferrin to cryogel resin under different conditions. The adsorption of lactoferrin on continuous supermacroporous cryogel with immobilized Cu^{2+} ions was evaluated in batch adsorption experiments at different pH (6-8) and temperature (293-313 K) values. Estimated values of the equilibrium constant (K) and characteristic number of binding sites (n) showed that these parameters decreased with increasing ionic strength, pH and temperature, while the nonlinear parameter, the steric factor (σ), increased with increasing ionic strength and temperature. Expressions correlating these parameters with pH, ionic strength and temperature were then derived.
Keywords: SMA; IMAC; Cryogel; Protein.

INTRODUCTION

The recent requirements of high purity proteins (both natural and recombinant), and the need of reducing costs of downstream processes have stimulated the development of more efficient and cheaper separation techniques (Frerick *et al*., 2008). It has been shown that Immobilized Metal Affinity Chromatography (IMAC) meets these requirements (Carvalho *et al*., 2014; Wang *et al*., 2008; Cheung *et al*., 2012). This technology is based on the chemical affinity displayed by certain groups of amino acid residues present on the surface of proteins (e.g., the imidazole group of histidine, thiol group of cysteine and tryptophan's indole group) with the metal ions immobilized on chromatographic resins (Gupta *et al*., 2002; Çimen and Denizli, 2012).

IMAC has been reported as a promissing technology for purification of proteins with an "N-terminal metal biding tag" (e.g., histidine tail and NT1A) (Puri *et al*., 2010; Petzold *et al*., 2014; Cheung *et al*., 2012), including on a large scale (Gaberc-Porekar and Menart, 2005), due to its low cost and the high purity of the eluate, even in a single step process (Puri *et al*., 2010; Carvalho *et al*., 2014). However, when approaching the purification of natural pro-

*To whom correspondence should be addressed

teins, IMAC may present some challenges since the amount of histidine on the surface accounts for only 1% of the total number of amino acids (Ueda *et al.*, 2003). Then, optimum conditions of the medium, such as temperature, salt concentration and pH, should be determined to improve the specific adsorption of the target protein. Over the years, acceptance of IMAC as a reliable technique for separation on a laboratory scale has increased. However, its use on an industrial scale has still been only slightly explored.

The main interaction in IMAC is the coordinate bond. However, other interactions, such as electrostatic and hydrophobic, may also occur due to the environmental conditions. Generally, these three types of interplay should be considered. Nonetheless, it is not always possible to determine the relative contribution of each of them. It is known that the prevalence of a particular type of interaction over the others is essentially governed by certain variables, such as the nature of the chelating agent, the composition of the protein's surface and the chemical environment where the interplay occurs, that is, the solution's ionic strength, pH and type of salts are also determinants of the driving forces (Bresolin *et al*., 2009). This makes IMAC less predictable and its optimization a difficult task. Projects for IMAC on a preparative scale require a deep understanding of all the fundamental mechanisms that govern the protein adsorption. The knowledge of these processes provides a rational basis to establish chromatographic conditions for purification and scale-up (Bornhorst and Falke, 2000; Ueda *et al*., 2003).

Although the adsorption process in IMAC is considerably different from conventional methods, previous studies have shown that the interaction between a protein molecule and immobilized metal ions may be explained based on the application of isotherm models of both affinity and ion exchange phenomena (Jiang and Hearn, 1996; Chen *et al*.. 2005). Nonetheless, the universal applicability of a model to elucidate the precise molecular mechanisms by which proteins are retained in resins with chelated metals still must be established.

Brooks and Cramer (1992) presented a new model, steric mass action (SMA), for protein adsorption equilibrium in nonlinear ion exchange systems. This model combines the stoichiometric displacement model (Rounds and Regnier, 1984; Drager and Regnier, 1986) with the concept of steric shielding of macromolecules presented later by Velayudhan (1990). Mainly used in ion exchange chromatography, the model involves three parameters, the number of identical binding sites (n), the equilibrium constant (K) and the steric factor (σ). It is assumed that the adsorption is an exchange process between the free protein molecule and a certain number of ions bound to the resin, which considers explicitly the steric hindrance of the counter ions of the salt on the binding of proteins. Recent studies have used the SMA model to describe protein adsorption equilibrium and the results have shown its efficiency for predicting the non-linear adsorption behavior of proteins (Chen *et al.*, 2006; Barz *et al*., 2010).

Given the high demand for purified proteins, the development of mechanistic models which both predict adsorption with accuracy and provide one with process information (e.g., pH, salt concentration, temperature) is needed to design purification plants. Langmuir and other adsorption models, for example, do not explain the process at the molecular level. Detailed models like the SMA can indicate more clearly the driving force for the adsorption. Thus, in the present study, the applicability of the SMA model and the parametric sensitivity that describes the adsorption equilibrium of bovine lactoferrin in cryogel resins with copper ions immobilized through iminodiacetic acid (IDA-Cu^{+2}-cryogel) was analyzed under different conditions (pH, salt concentration and temperature) as an approach to mechanistic modeling for further industrial process development applications.

MATERIALS AND METHODS

Materials

Lactoferrin (LF) (M.W., 80 kDa), acrylamide (AAm, 99.9% electrophoretic grade), N,N-methylene-bisacrylamide (MBAAm, 99%), ammonium persulphate (APS, 98%), $CuSO_4 \cdot 4H_2O$ (98%), iminodiacetic acid (IDA), N-2-hydroxyethylpiperazine-N'-2-ethanosulfenic acid (HEPES), N,N,N',N'-tetramethyl-ethylenediamine (TEMED, 99%) and allyl glycidylether (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).

Methods

Synthesis of IDA-Cu^{2+} Cryogel Adsorbent Resin

The synthesis of IDA-Cu^{2+}-cryogel resin was carried out according to Kumar *et al*. (2006), as described by Carvalho *et al*. (2013). The cryo-copoly-

merization reaction of solution containing AAm, MBAAm, AGE, TEMED and APS was performed. IDA was used as chelating agent to load copper within the matrix. All experiments were performed in triplicate.

Obtaining Adsorption Isotherms

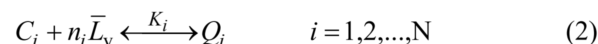
Adsorption isotherm data were obtained by batch experiments, as described by Carvalho *et al*. (2013). 3 mg of dried cryogel matrix were weighed in eppendorf tubes. Initially, the resin was equilibrated with 400 μL of equilibration buffer (20 mmol.L^{-1} HEPES containing 200 mmol.L^{-1} NaCl, pH 7.0). The tubes were left under mild stirring for a period of two hours to condition the resin. After this period, the equilibration buffer was removed and different volumes (0 to 300 μL) of a 10 mg.mL^{-1} solution of lactoferrin were added. Adsorption buffer (20 mmol.L^{-1} HEPES containing 400 mmol.L^{-1}, different pHs) was then added to make the volume up to 1200 μL. The final protein concentrations 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 hours under controlled temperature (101M Mod BOD/3 Eletrolab ®, Brazil), to achieve the equilibrium state. Subsequently, the resin was removed from the eppendorf tubes and the concentration of protein contained in the supernatant was determined spectrophotometrically at 280 nm (Thermo Scientific Model BIOMATE 3). The protein concentration in the solid phase was determined according to Equation (1):

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

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 of protein in the liquid phase and (mg.mL^{-1}) C is the final protein concentration in the liquid phase after the equilibrium state has been established. The experiment was conducted using a factorial design with three temperatures (20, 30 and 40 °C), three different pHs (6.0, 7.0 and 8.0) and a salt concentration of 400 mmol.L^{-1}.

Modeling the Equilibrium Data - The SMA Model

The applicability of the Zhang and Sun (2002) SMA model to describe the adsorption of LF onto IDA-Cu^{+2}-cryogel was evaluated in this work. In this model, the protein (Lactoferrin) would interact with ligands (immobilized Cu^{2+} ions), present at a density L_t (mmol.L^{-1}), according to a "characteristic number of binding sites", n_i. Further, during the protein adsorption, there may be a number of copper ions blocked by the 3D structure of the molecule, which prevents that other proteins coordinate to the ions. The number (average) of shielded cooper ions is given by the steric factor, σ_i. The following interaction balance can be written for each protein of the mixture (i):



$$C_i + n_i \bar{L}_v \xleftrightarrow{K_i} Q_i \qquad i = 1,2,...,N \qquad (2)$$

where C_i and Q_i represent, respectively, the concentrations of protein free in solution and bound to metal ions immobilized in the cryogel, and $\bar{L}_v$ is the amount of immobilized copper free and unblocked to bind to molecules of LF. The equilibrium constant K_i for the metal affinity process is given by:

$$K_i = \frac{Q_i}{\bar{L}_v^{n_i} C_i} \qquad i = 1,2,...,N \qquad (3)$$

Because many ligands may be blocked by bound LF molecules, the equilibrium constant is redefined as:

$$K_i = \frac{Q_i}{\left[L_t - \sum_{i=1}^{N} (\sigma_i + n_i) Q_i \right]^{n_i} C_i} \qquad (4)$$

where the term $\left[L_t - \sum_{i=1}^{N} (\sigma_i + n_i) Q_i \right]$ is a mass balance for the copper ions and is equal to the density of metal available for protein binding. In this study, the equilibrium adsorption of lactoferrin onto IDA-Cu^{2+}-cryogel was studied to examine the usefulness of the SMA model in describing such process. For this system, Equation (4) may be reduced to:

$$C = \frac{Q}{K\left[L_t - (\sigma + n) Q \right]^n} \qquad (5)$$

In a dilute protein solution, the isotherm can be written as:

$$K = \lim_{C \to 0} \frac{Q}{C L_t^n} \qquad (6)$$

from which one can obtain K and n, given the values of Q, C and L_t, which are obtained experimentally. It is important to note that the ratio Q/C indicates the resin's partition coefficient m, so Equation (6) can be re-written as:

$$m = \frac{Q}{C} = KL_t^n \tag{7}$$

The plot of ln m versus ln L_t was used to obtain the SMA model parameters (K, and n), so that the steric factor could be calculated.

RESULTS AND DISCUSSION

Equation (7) can be seen as a kind of Freundlich isotherm model (Carvalho *et al.,* 2014[b]), with capacity factor equal to 1 (first order adsorption process). Thus, one can conclude that the equilibrium constant calculated through the SMA model may not represent the adsorption process accurately for all protein concentrations, since it considers that the adsorption is indefinitely proportional to the concentration of the lactoferrin in the media. Besides, because the derivation of equation (7) assumes lactoferrin concentration close to zero, K values will not be realistic at high lactoferrin concentrations.

To overcome this, the Langmuir isotherm was used to fit the experimental data, since the Langmuir equation (Equation (8)) can fit the adsorption of proteins into affinity adsorbents containing immobilized ions.

$$Q = \frac{Q_m C}{(K_d + C)} \tag{8}$$

where Q_m is the maximum concentration of adsorbed protein and K_d is the dissociation constant. Since Q_m and K_d are the concentration of LF in the adsorbent and supernatant in the equilibrium for different lactoferrin concentrations, respectively, the partition coefficient can be described as:

$$m = \frac{Q_m}{K_d} \tag{9}$$

As can be seen in Figure 1, the plot ln m vs. ln L_t is a straight line at each pH and temperature evaluated. The linear relationship between ln m and ln L_t indicates that the hypothesis of independence of the linear parameters from the ion density is acceptable.

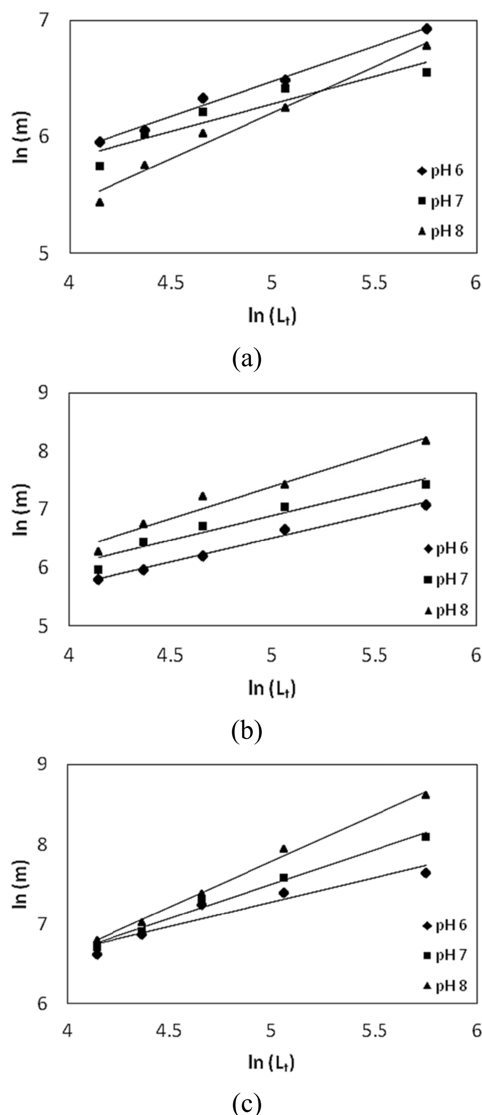


Figure 1: Plots of the natural logarithm of the partition coefficient *vs.* natural logarithm of the immobilized copper ion concentration for lactoferrin adsorption on IDA-Cu^{2+}- cryogel in HEPES buffer 20 mmol.L^{-1}, 400 mmol.L^{-1} NaCl, at different pHs and temperatures: (a) 293 K, (b) 303 K and (c) 313 K. The coefficient of determination were, at 20 °C: pH 6.0, R^2= 0.9897, pH 7.0, R^2= 0.8888, pH 8.0, R^2= 0.9791, at 30 °C: pH 6.0, R^2= 0.9879, pH 7.0, R^2= 0.9343, pH 8.0, R^2= 0.9609; at 40 °C: pH 6.0, R^2= 0.9013, pH 7.0, R^2= 0.9813, pH 8.0, R^2 = 0.9954.

Table 1 shows the values of two linear parameters of the SMA model at different values of temperature and pH. The number of binding sites, n, increased with the increase of pH, which most likely is due to deprotonation of electron donor amino acids found on the surface of lactoferrin (histidine pKa = 6 and cysteine pKa = 8.18). With a higher density of electron

donor residues exposed in its structure, each molecule may coordinate to more metal ion ligands.

The parameter K (except for pH 6) increased with increasing temperature. Since the pI of lactoferrin ranges from 8.0 to 8.5 (González-Chávez *et al.*, 2009), the protein is positively charged at pH equal to 6, possibly leading to electrostatic repulsion between LF and Cu^{2+} ions (electrostatic interaction is then the driving force). At higher values of pH, most of the Lewis base residues are deprotoned (Carvalho *et al.*, 2013), which implies that the coordinate bonding is the prevalent interaction. Since the adsorption of lactoferrin onto IDA-Cu^{2+}-cryogel is entropically favorable (Carvalho *et al.*, 2013), the increase in temperature is bound to increase the ΔG of adsorption when the pH and salt concentration are kept constant.

Table 1: Linear parameters of the SMA model obtained from experiments on adsorption of lactoferrin on cryogel-IDA-Cu^{2+}.

Temperature	pH	K	n
293K	6	370.184	0.602
	7	345.848	0.472
	8	238.651	0.791
303K	6	310.443	0.826
	7	446.304	0.849
	8	577.668	1.110
313K	6	822.213	0.611
	7	810.783	0.859
	8	823.036	1.155

The model's linear parameters for the ion exchange system, determined from a plot of log K vs. log C_s are generally considered to be constant and independent of salt concentration (Brooks and Cramer, 1992; Gadam *et al*., 1993). However, Zhang and Sun (2002) showed that K and n fall exponentially with the increase in ionic strength. Huang *et al*. (2010) studied the adsorption of bovine serum albumin (BSA) on Sorbent DEAE Sepharose FF containing ligand densities of 0.020 to 0.183 $mmol.mL^{-1}$. The SMA model was used to analyze the adsorption behavior of BSA. The results found by the authors showed that the equilibrium constant, characteristic number of binding sites and steric factor gradually increased with ligand density. Thus, the authors concluded that it is possible to adjust the multipoint adsorption of BSA by modulation of ligand density on the ion exchange adsorbent.

Since K and n were determined, the steric factor could be estimated by minimizing the sum of the squares of the deviations obtained with the SMA isotherm equation (5). In the estimation procedure the steric factor was calculated based on the residues between the experimental values of protein concentration and the protein concentration estimated by Equation (5). Then, the least squares method provided a better steric factor value. The values of estimated steric factors are shown in Table 2 and the plots of SMA isotherms for lactoferrin adsorption onto the IDA-Cu^{+2}-cryogel system are shown in Figure 2.

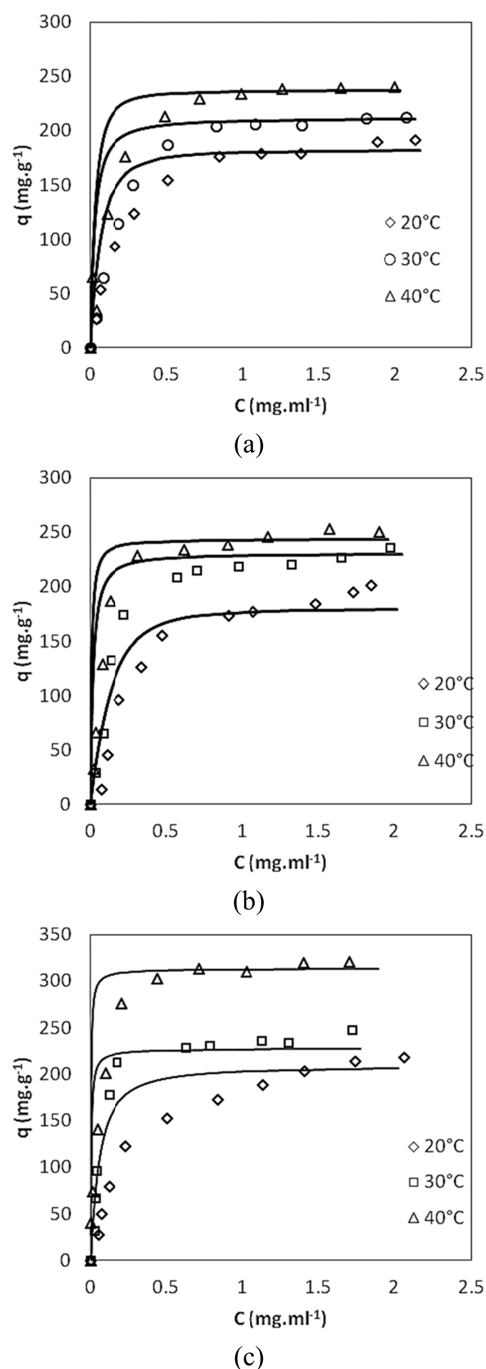


Figure 2: Adsorption isotherms of lactoferrin in 400 $mmol.L^{-1}$ NaCl: (a) pH 6.0, (b) pH 7.0 and (c) pH 8.0. Solid lines were calculated from the SMA model.

As shown in Table 2, for the majority of the cases studied, the steric factor value decreases with increasing temperature and, in some cases, it passes through a minimum at a temperature equal to 303 K. Indeed, as seen in Figure 2, the temperature increase leads to an increase of adsorption capacity of lactoferrin on IDA-Cu^{+2}-cryogel. When the temperature rise occurs in the adsorption process, there is a decrease in the energy of protein-water interaction, which results in increasing interactions between protein-copper ion. Thus, the increase of temperature is sufficient to break down the diffuse region of the electrical double layer, and thus the protein molecules interact more strongly with the resin, favoring the adsorptive phenomenon.

Table 2: Steric factor of the lactoferrin- IDA-Cu^{2+} cryogel system at different pHs and temperatures.

pH	Steric Factor		
	Temperature/293 K	Temperature/ 303 K	Temperature/ 313 K
6	24.40	20.74	21.01
7	25.01	22.06	22.27
8	17.67	22.12	17.74

Regarding the behavior of the steric factor values with respect to ionic strength and pH, it was observed that the steric factor decreased with increasing ionic strength, except for the conditions of pH 8, 293 K and 303 K. With regard to the pH, it is noted that, in some cases, the steric factor decreased with increasing pH, except at pH 7.0, where there was a peak in its value. Reasons for such behavior may be due to the fact that, in IMAC, the adsorption capacity and selectivity depend not only on the metal chelates immobilized on the chromatographic matrix, but also on the composition of the mobile phase. The protein retention in IMAC adsorbents occurs due to the contribution of various physico-chemical interactions, which may be enhanced or minimized depending on the composition of the mobile phase. When IMAC is operated at high salt concentration (0.5 to 1.0 $mol.L^{-1}$ NaCl, for example), the predominant interaction is the coordinate bond between the immobilized metal ions and amino acid residues accessible on the surface proteins, while the electrostatic interactions occur with a lesser intensity. The electrostatic effects are more intense when employing a mobile phase with low ionic strength. These effects occur between charged proteins and the positive charges of the metal ions or negative charges remaining on the surface of the matrix (unreacted functional groups remaining from the activation and coupling of the chelator, or residual carboxylic groups of chelating agents due to incomplete chelation of metal ions) (Winzerling *et al*., 1992; Guitiérrez *et al*., 2007).

In IMAC, regarding the pH, coordinate bonds are favored when ionizable groups of electron donors amino acid residues present in biomolecules are partially deprotonated, i.e., when the pH of the solution is above the pKa of the ionizable groups (Bresolin *et al.* 2009). Moreover, since the coordinate bonds with the immobilized metal ions may occur simultaneously with electrostatic interactions, protein adsorption on IMAC is pH dependent. However, the effects of each interaction on the protein adsorption are difficult parameters to be determined.

On the one hand, high ionic strength leads to strong binding of salt ions to the IDA-Cu^{2+}-cryogel resin, reducing the number of accessible binding sites for the protein (Wrzosek *et al*., 2009). On the other hand, there is a greater selectivity of coordinate binding, which is favored by high salt concentrations. The decreased resin adsorption capacity is a direct result of the binding of salt ions to metal ligands. It is shown in the SMA model as an apparent increase in σ.

Figure 2 shows a comparison between the simulations of the SMA model and equilibrium affinity data for the lactoferrin- IDA-Cu^{2+}-cryogel complex at 400 $mmol.L^{-1}$ NaCl, temperatures equal to 293 K, 303 K and 313 K, and pH 6.0, 7.0 and 8.0. It was found that the predicted adsorption capacities had higher values than the corresponding experimental ones, in most of the analyzed cases. At pHs near the lactoferrin isoelectric point, the accuracy of the model decreased further. It is known that the net charge and three-dimensional structure of proteins are influenced by the pH of the buffer solution. Because lactoferrin has nine histidine residues (responsible for protein retention on the IDA-Cu^{2+}-cryogel complex) having pKa = 6.5, in conditions above this value the medium is deprotonated, favoring other types of binding, not only the affinity. Thus, under these conditions, the SMA model failed to predict the change in behavior.

Figure 3 shows a comparison between experimental and predicted protein concentration values. As can be seen, there was a reasonable agreement between predicted concentrations of lactoferrin and those measured experimentally through variation of ionic strength, pH and temperature. Osberghaus *et al.* (2012) have determined parameters for the steric mass action model through two methods; approach I was based on frontal and gradient experiments, while approach II relied on the "application of an inverse method for parameter estimation". The SMA parameter obtained from this last method led to the best accuracy of "a mechanistic model" developed for column

chromatography when theoretical and experimental chromatographic data were compared. Ribonuclease A, cytochrome *c* and lysozyme were used in the study of Osberghaus et al. (2012). The authors concluded that "the inverse method based on a mechanistic model for column chromatography is the most comfortable way to establish highly predictive SMA parameters" (Osberghaus *et al*., 2012).

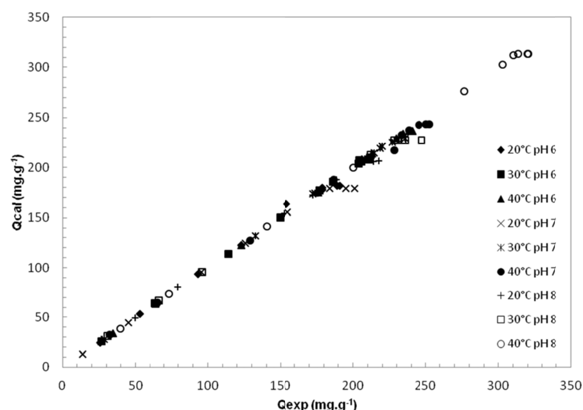


Figure 3: Plots of the experimental and concentrations predicted by the SMA model for lactoferrin bovine serum under conditions of about 400 mmol.L^{-1} at different pHs (6.0, 7.0 and 8.0) and temperatures (293, 303 and 313 K).

CONCLUSION

The SMA model was applied to the adsorption equilibrium affinity of bovine lactoferrin on monolithic columns of cryogel with immobilized copper ions. The model parameters were estimated by adsorption equilibrium experiments performed in batch. The analysis of the dependence of these parameters provided valuable information about the mechanism of interaction between metal ions, process parameters and protein. The simulations showed that, at high temperature and pH near the isoelectric point of the protein, accuracy of the model decreased due to interactions other than coordination. The SMA model was able to predict adsorption isotherms of lactoferrin in IDA-Cu^{2+}-cryogel. Further improvement of this model would take into account the influence of factors in the protein structure.

NOMENCLATURE

AAm	Acrylamide
AGE	Allyl glycidylether
APS	Ammonium persulphate
BSA	Bovine serum albumin
β	Column phase ratio
C	Final protein concentration in the supernatant (mg.mL^{-1})
C_0	Initial concentration of protein in the liquid phase (mg.mL^{-1})
ΔG	Gibbs free energy of adsorption
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanosulfenic acid
IDA	Iminodiacetic acid
IMAC	Immobilized Metal Affinity Chromatography
K	Equilibrium constant (mmol L^{-1})^{n_i}
LF	Lactoferrin
L_t	Density of ligand immobilized on the resin's surface (mmol.L^{-1})
$\bar{L}_v$	Density of copper accessible for the adsorption of proteins (mmol.L^{-1})
m	Partition coefficient
M	Mass of the solid phase (g)
MBAAm	N,N-methylenebisacrylamide
n	Characteristic number of binding sites
σ	Steric factor
q	Concentration of protein in the solid phase (mg.g^{-1})
Q_i	Concentration of protein bound to metal ion (mg.mL^{-1})
Q_m	Maximum concentration of adsorbed protein (mg.mL^{-1})
SMA	Steric Mass Action
TEMED	N,N,N',N'-tetramethyl-ethylenediamine
t_0	Retention time of protein (s)
t_R	Retention time of the liquid phase (s)
V	Volume of the liquid phase (mL)

REFERENCES

Akgol, S., Turkmen, D. and Denizli, A., Cu(II)-incorporated, histidine-containing, magnetic-metal-complexing beads as specific sorbents for the metal chelate affinity of albumin. J. Appl. Polym. Sci., 93, 2669 (2004).

Barz, T., Loffler, V., Arellano-Garcia, H. and Wozny, G., Optimal determination of steric mass action model parameters for β-lactoglobulin using static batch experiments. J. Chromatogr., A, 1217, 4267 (2010).

Bornhorst, J. A. and Falke, J. J., Purification of proteins using polyhistidine affinity tags. Methods Enzymol., 326, 245 (2000).

Bresolin, I. T. L., Miranda, E. A. and Bueno, S. M. A., Cromatografia de afinidade por íons metálicos imobilizados (IMAC) de biomoléculas: Aspectos

fundamentais e aplicações. Quím. Nova, 32, 1288 (2009). (In Portuguese).

Brooks, C. A. and S. M. Cramer., Steric mass-action ion exchange: Displacement profiles and induced salt gradients. AIChE J., 38, 1969 (1992).

Brooks, C. A. and Cramer, S. M., Solute affinity in ion-exchange displacement chromatography. Chem. Eng. Sci., 51, 3847 (1996).

Carvalho, B. M. A., Carvalho, L. M., Silva Jr., W. F., Minim, L. A., Soares, A. M., Carvalho, G. G. P. and da Silva, S. L., Direct capture of lactoferrin from cheese whey on supermacroporous column of polyacrylamide cryogel with copper ions. Food Chemistry, 154, 308 (2014a).

Carvalho, B. M. A., Da Silva, L. H. M., Carvalho, L. M., Soares, A. M., Minim, L. A. and Da Silva, S. L., Microcalorimetric study of the adsorption of lactoferrin insupermacroporous continuous cryogel with immobilized Cu^{2+} ions. J. Chromatogr., A, 1312, 1 (2013).

Carvalho, B. M. A., Carvalho, L. M., Silva Jr., W. F., Minim, L. A. and Carvalho, G. G. P., Interaction of whey lactoferrin with copper immobilized in polyacrylamide cryogel. J. Bioproces. Biotech., 4, 7 (2014b).

Chen, W. -D., Hu, H. -H. and Wang, Y. -D., Analysis of steric mass-action model for protein adsorption equilibrium onto porous anion-exchange adsorbent. Chemical Engineering Science, 61, 7068 (2006).

Chen, W., Wang, Y. and Su, Z., Suitability of steric mass-action model for ion-exchange equilibrium of micromolecule. Chin. J. Chem. Eng., 13, 18 (2005).

Cheung, R. C. F., Wong, J. H. and Ng, T. B., Immobilized metal ion affinity chromatography: A review on its applications. Appl. Microbiol. Biotechnol., 96, 1411 (2012).

Cimen, D. and Denizli, A., Immobilized metal affinity monolithic cryogels for cytochrome C purification. Colloids Surf., B, Biointerfaces, 93, 29 (2012).

Drager, R. R. and Regnier, F. E., Application of the stoichiometric displacement model of retention to anion-exchange chromatography of nucleic acid. J. Chromatogr., A, 359, 147 (1986).

Frerick, C., Kreis, P., Górak, A., Tappe, A. and Melzner, D., Simulation of a human serum albumin downstream process incorporating ion-exchange membrane adsorbers. Chem. Eng. and Process., 47, 1128 (2008).

Gadam, S. D., Jayaraman, G. and Cramer, S. M., A predictive model for salt effects on the dye-ligand affinity adsorption equilibrium of protein. J. Chromatogr., A, 630, 37 (1993).

Gaberc-Porekar, V. and Menart, V., Potential for using histidine tags in purification of proteins at large scale. Chem. Eng. Technol., 28(11), 1306 (2005).

González-Chávez, S. A., Arévalo-Gallegos, S. and Rascón-Cruz, Q., Lactoferrin: Structure, function and applications. International Journal of Antimicrobial Agents, 33, 301.e1 (2009).

Gupta, M. N., Jain, S. and Roy, I., Immobilized metal affinity chromatography without chelating ligands - Purification of soybean trypsin inhibitor on zinc alginate beads. Biotechnol. Prog., 18, 78 (2002).

Gutiérrez, R., Martín del Valle, E. M. and Galán, M. A., immobilized metal-ion affinity chromatography: Status and trends. Sep. Purif. Rev., 36, 71 (2007).

Hage, D. S., Anguizola, J. A., Bi, C., Li, R., Matsuda, R., Papastavros, E., Pfaunmiller, E., Vargas, J. and Zheng, X., Pharmaceutical and biomedical applications of affinity chromatography: Recent trends and developments. Pharm. Biomed. Anal., 69, 93 (2012).

Huang, Y., Bi, J., Zhao, L., Ma, G. and Su, Z., Regulation of protein multipoint adsorption on ion-exchange adsorbent and its application to the purification of macromolecules. Protein Expression and Purif., 74, 257 (2010).

Jiang, W. and Hearn, M. T. W., Protein interaction with immobilized metal ion affinity ligands under high ionic strength conditions. Anal. Biochem., 242, 45 (1996).

Kumar, A., V. Bansal, K. S., Nandakumar, I. Y., Galaev, P. K., Roychoudhury, R., Holmdahl, and Mattiasson, B., Integrated bioprocess for the production and isolation of urokinase from animal cell culture using supermacroporous cryogel matrices. Biotechnol. Bioeng., 93, 636 (2006).

Osberghaus, A., Hepbildikler, S., Nath, S., Haindl, M., Von Lieres, E. and Hubbuch, J., Optimizing a chromatographic three component separation: A comparison of mechanistic and empiric modeling approaches. J. Chromatogr., A, 1237, 86 (2012).

Petzold, M., Coghlan, C. J., Hearn, M. T. W., Studies with an immobilized metal affinity chromatography cassette system involving binuclear triazacyclononane-derived ligands: Automation of batch adsorption measurements with tagged recombinant proteins. Journal of Chromatography A, 1351, 61 (2014).

Puri, M., Kaur, A., Singh, R. S., Schwarz, W. H., Kaur, A., One-step purification and immobilization of His-tagged rhamnosidase for naringin hydrolysis. Process Biochemistry, 45, Issue 4, 451 (2010).

Rounds, M. A. and Regnier, F. E., Evaluation of a retention model for high-performance ion-exchange chromatography using two different displacing salts. J. Chromatogr., A, 283, 37 (1984).

Shukla, A. A., Bae, S. S., Moore, J. A., Barnthouse, K. A. and Cramer, S. M., Synthesis and characterization of low molecular mass displacers for cation exchange chromatography. Ind. Eng. Chem. Res., 37, 4090 (1998).

Ueda, E. K. M., Gout, P. W. and Morganti, L., Current and prospective applications of metal ion–protein binding. Journal of Chromatography A, 988, 1 (2003).

Velayudhan, A., Studies in nonlinear chromatography. PhD Thesis, Yale University (1990).

Wanga, L., Shenb, S., Heb, X., Yunb, J., Yaob, K. and Yao, S., Adsorption and elution behaviors of bovine serum albumin in metal-chelated affinity cryogel beds. Biochemical Engineering Journal, 42, 237 (2008).

Wankat, P. C., Rate Controlled Separations. Elsevier, New York (1990).

Winzerling, J. J., Berna, P. and Porath, J., Methods: A companion to Methods in Enzymology. Academic Press, New York (1992).

Wrzosek, K., Gramblicka, M. and Polakovic, M., Influence of ligand density on antibody binding capacity of cation-exchange adsorbents. J. Chromatogr., A, 1216, 5039 (2009).

Zhang, S., Sun, Y., Steric mass-action model for dye-ligand affinity adsorption of protein. J. Chromatogr., A, 957, 89 (2002).