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Adsorption Equilibrium and Kinetics of Egg-White Proteins on Immobilized Metal Ion Affinity Gels for Designing Fractionation

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Abstract. Designing an Immobilized Metal ion Affinity (IMA) chromatographic process on large scale demands a thorough understanding to be developed regarding the adsorption behaviour of proteins on metal loaded IMA (IMA-M(II)) gels and the characteristic adsorption parameters to be evaluated. This research investigation illustrates the significance of these aspects for the proposed fractionation of chicken egg-white proteins on these gels. Consequently, a systematic investigation of the adsorption characteristics of three chicken egg-white proteins viz., ovalbumin, conalbumin and lysozyme on Cu(II) and Ni(II) loaded IMA gels, iminodiacetate (IDA) and tris(2-aminoethyl)amine (TREN), has been undertaken. These gels differ in their selectivity towards the proteins of interest under the identical sets of experimental conditions. While TREN-Ni(II) was selective only for lysozyme, IDA-Cu(II), IDA-Ni(II) and TREN-Cu(II) showed varying affinities for all the three proteins. The equilibrium and kinetic data were analysed using various theoretical models and adsorption parameters were quantified. On the basis of these investigations, various strategies have been proposed for the efficient large-scale fractionation of chicken egg-white proteins on these gels.

Keywords: adsorption, immobilized metal affinity, IMAC, iminodiacetic acid, tris(2-aminoethyl)amine, protein fractionation

1. Introduction

Fractionation of proteins from their crude extract or culture broth is generally regarded as the most time consuming and expensive part of a research project. As a consequence, the recent years have witnessed continued improvements in the separation techniques for more effective, yet relatively less expensive, downstream processing of biomolecules. Though biospecific affinity techniques still remain the most favoured ones owing to their extreme specificity; "pseudobiospecific" or "group-specific" techniques such as dye-ligand affinity and immobilized metal ion affinity chromatography are also gaining prominence.

Immobilized Metal ion Affinity Chromatography (IMAC) exploits the chemical affinity shown by certain functional groups on the surface of proteins for metal ions immobilized on a support matrix. The recognition mainly occurs due to the surface-exposed histidine, cysteine and tryptophan residues of protein (Porath et al., 1975). Currently, there has been a dramatic increase in the use of IMAC for the separation and purification of proteins at the laboratory scale.

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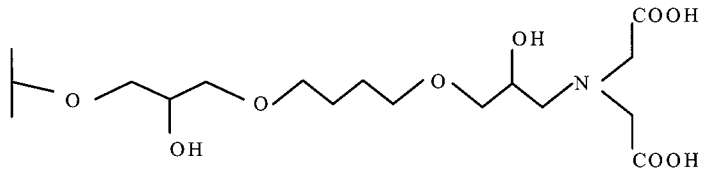
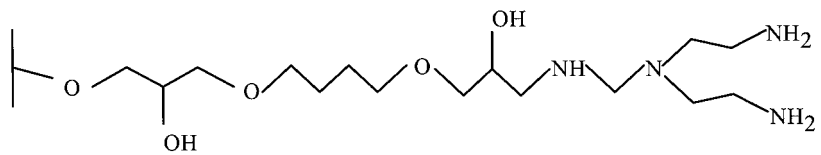
However, its application at the industrial scale is still in its infancy.

In order to design and optimize large-scale IMAC separations, there is a need to develop a thorough understanding of the fundamental mechanisms involved in separation and establish a theory that is able to account for them. As such, adsorption of proteins on immobilized metal ion affinity (IMA) gels is a complex interplay of a large number of variables including the nature of the sorbent matrix, spacer arm, chelating agent, ligand density, method of immobilization, metal ion and solution environment (Sulkowski, 1985; Vijayalakshmi, 1989; Kagedal, 1989; Wong et al., 1991; Arnold, 1991; Porath, 1992). Thus, the adsorption selectivity and specificity of a particular IMA gel for a particular protein can be altered by varying these conditions. This, in turn, can be utilized to achieve chromatographic fractionation of a complex mixture either on the basis of differential affinities of various proteins for the IMA-M(II) gel or by using a number of selected adsorbents arranged sequentially having selectivity for different components of the mixture. The second approach based on the sequential adsorption of proteins on tandem-coupled-columns having different adsorbents is known as Cascade-mode multiaffinity chromatography (CASMAC) (Porath and Hansen, 1991) and has demonstrated high group-resolving power for the fractionation of human serum as a model mixture at laboratory scale. Further, the equilibrium position together with the rate of attainment of that equilibrium also governs the separation performance of a chromatographic process. However, the relevant quantitative information on this aspect that can be utilized for process design is quite limited. Almost all the IMAC separation protocols to date seem to have been selected by hit and trial or accidentally applied and only a few are based on a rational approach to achieve efficient separation (Porath and Hansen, 1991; Anspach et al., 1996). As a consequence, an urgent need to either explore the existing adsorption models/theories or develop a new model/theory to effectively analyze equilibrium and kinetic data in qualitative and quantitative manner has been realized in the recent years (Sharma, 1998; Sharma and Agarwal, 2001). These theoretical models can be utilized for estimating adsorption parameters that can be employed for process optimization with retention of the resolving power.

Our present research effort illustrates the significance of both the aspects mentioned above for the proposed fractionation of chicken egg white proteins using IMAC. Therefore, a systematic study of the adsorption characteristics of the three chicken egg-white proteins viz., ovalbumin, conalbumin and lysozyme on metal loaded IMA gels viz., iminodiacetate (IDA) and tris (2-aminoethyl)amine (TREN) has been conducted (Fig. 1). The choice of the proteins was prompted by the fact that these proteins constitute a real mixture. The adsorption studies were carried out for the two most frequently used metal ions namely, Cu (II) and



IDA-Sepharose 6B



TREN-Sepharose 6B

Figure 1. Structures of IDA-Sepharose 6B and TREN-Sepharose 6B gels.

Ni (II). All the metal loaded gels were compared in terms of their adsorption characteristics for the proteins of interest. Further, in order to determine the adsorption parameters required for designing large-scale fractionation process, the equilibrium and kinetic data has been analysed using various theoretical models and characteristic adsorption parameters have been evaluated. On the basis of these investigations two different strategies have been proposed for the efficient separation of egg-white proteins.

2. Experimental

2.1. Materials

Sepharose 6B was procured from Pharmacia-LKB Biotechnology (Uppsala, Sweden). Tris(2-aminoethyl) amine (22563-0) from Aldrich Chemical Co. (Milwaukee, WI, USA); and iminodiacetic acid (I-5629) and 1,4-butanediol diglycidyl ether (B-7381) from Sigma Chemical Co. (St. Louis, MO, USA). The three chicken egg white proteins viz., ovalbumin (A-5503), conalbumin (C-0755) and lysozyme (L-6876) were brought from Sigma Chemical Co. (St. Louis, MO, USA). All other chemicals and buffer reagents were of analytical grade and procured locally. Sodium acetate, acetic acid and ethylenediaminetetraacetic acid disodium salt (EDTA) were purchased from Qualigens, sodium chloride, sodium dihydrogen phosphate dihydrate, disodium hydrogen phosphate, copper sulphate and nickel sulphate from E. Merck.

2.2. Preparation of Immobolilized Metal Ion Affinity Gels and Different Solutions

The method published by Winzerling et al. (1992) was used to prepare the two IMA gels, Iminodiacetic acid (IDA)- Sepharose 6B and Tris(2-aminoethyl)amine (TREN)- Sepharose 6B. All the buffers and solutions were prepared in Ultrapure water (resistivity 18.2 M ohm-cm) obtained from a Milli-Q unit (Millipore Corporation, Bedford, MA, USA) and prefiltered through a 0.22 μM membrane filter just prior to use to remove any particulate or colloidal matter.

2.3. Metal Loading of the IMA Gel

All the experiments were conducted at room temperature (25°C). The IDA-Sepharose 6B/TREN-Sepharose 6B column (glass, 10 cm $\times$ 10 mm ID, packed bed volume = 1.57 mL) pre-equilibrated with 10 mM sodium acetate of appropriate pH (IDA-Cu(II): 4.0; TREN-Cu(II): 5.0; IDA-Ni(II): 7.0, TREN:Ni(II): 7.0) was charged with metal ions by passing 16 column volumes of copper or nickel sulphate solution (20 mM) followed by washing with 20 column volumes of the same buffer. All the experiments were performed in duplicate at a volumetric flow rate of 1 mL/min. The flow was always in the upward direction.

2.3.1. Determination of the Amount of Immobilized Metal(II) Ions. The metal loaded column was equilibrated with 6–7 column volumes of 20 mM sodium phophate buffer (1 M NaCl, pH 7.0) followed by elution with 50 mM EDTA in the same buffer. The total amount of M(II) ions in the eluate was determined by Atomic Absorption Spectrophotometry (AAS) (Hitachi Ltd., Tokyo, Japan; Model # Z-8100) after proper dilution using EDTA as blank. The selected λ_{max} for Cu(II) and Ni(II) were 324.8 nm and 232 nm, respectively. Using the protocol outlined above, the metal loading capacities of IDA-Sepharose 6B and TREN-Sepharose 6B were: IDA-Cu(II): 45.7; TREN-Cu(II): 32.9; IDA-Ni(II): 36.8; TREN-Ni(II): 26.6 (μmol/mL gel).

2.4. Protein Adsorption Studies

2.4.1. Equilibrium Studies. Adsorption isotherms of proteins were determined through batch experiments. The protein solution was prepared in 10 mM sodium acetate buffer (pH 7.0, 2 M NaCl) filtered through 0.45 μM membrane filter. Equal volumes of the buffered protein solutions of different concentrations were added to a series of flasks each containing an equal volume of 1 : 5 (v/v) suspension of IMA-M(II) gel. The flasks were incubated for 2 hours in a shaking water bath at 25°C (at 120 rpm) to allow for the equilibrium to be established. The solution from each flask was, then, filtered through a 0.45 μm membrane filter. The protein concentration in the supernatant was determined using UV-Spectrophotometry (280 nm) and the amount of bound protein was calculated by mass balance.

2.4.1.1. Analysis of Equilibrium Data. Several investigators have shown that the equilibrium relationship between a free and bound adsorbate can be described by a Langmuir type isotherm (Langmuir, 1918). Chase (1984) described a mass-transfer rate equation that is consistent with the Langmuir isotherm. The mass transfer of the adsorbate to the immobilized phase can be

described by:

$$\frac{dq}{dt} = k_1 C\left(q_{m(L)} - q\right) - k_2 q \tag{1}$$

where C is the concentration of the free adsorbate, q is the concentration of the bound adsorbate, $q_{m(L)}$ is the maximum binding capacity of the adsorbate and t is the time. The rate constants, k_1 and k_2, are "lumped" parameters that represent rate of adsorption and desorption of the adsorbate to the immobilized ligand as well as contributions from mass transfer limitations. With the assumptions that all binding sites have equal energy, are independent in nature and single-site interaction occurs between protein and ligand, at equilibrium, Eq. (1) can be reduced to the adsorption isotherm:

$$q^* = \frac{q_{m(L)} C^*}{(K_d + C^*)} \tag{2}$$

As the protein-ligand interactions are often characterized by the participation of non independent binding sites, it is frequently observed that Langmuir model is unable to describe the shape of the experimental isotherm satisfactorily. Another well known isotherm which is frequently used to describe the adsorption behaviour is Freundlich isotherm (Freundlich, 1907). It relates the adsorbed concentration as the power function of solute concentration:

$$q^* = KC^{\eta} \tag{3}$$

where K and η are Freundlich equilibrium constant and power term of Freundlich isotherm respectively. K and η are constants for a particular protein system and can be determined experimentally. Although usually applied in a strictly empirical sense, this can be of theoretical interest in terms of adsorption on energetically heterogeneous surface. According to the Freundlich isotherm, the amount adsorbed increases indefinitely with the concentration in solution.

The Langmuir and Freundlich isotherm have often been found to be inadequate to describe the actual adsorption behaviour of proteins on affinity sorbents. To overcome this inadequacy the two models may be combined to give the composite Langmuir-Freundlich equation (Andrade, 1985):

$$q^* = \frac{q_{m(LF)} \cdot (C^*)^n}{K_d^* + (C^*)^n} \tag{4}$$

where K_d^* is the apparent dissociation constant that includes contributions from ligand binding to monomer, monomer-dimer and more highly associated forms of proteins, $q_{m(LF)}$ is the maximum binding capacity and n is the Langmuir-Freundlich coefficient number. As the equation has three fitting terms, it is much better for approximating adsorption of heterogeneous nature and explaining adsorption cooperativity. For purely independent, non-interacting sites, the value of $n = 1$. When $n > 1$ positive cooperativity is suggested, while when $0 < n < 1$ negative cooperativity in the binding process is indicated. The value of n can, thus, be employed as an empirical coefficient, representing the type and extent of cooperativity present in the binding interactions.

2.4.2. Kinetic Studies. A known volume of the prefiltered protein solution of known concentration (in 10 mM sodium acetate; 2 M NaCl; pH 7.0) was added to an equal volume of 1 : 5 (v/v) suspension of IMA-M(II) gel in a flask which was continuously agitated in a shaking water bath (120 rpm) maintained at 25°C. Total volume of the protein solution with gel suspension was 100 mL (for 30 min kinetics experiment). This solution was properly mixed before taking the samples to ensure that the ratio of the gel suspension to the total solution volume (1 : 10) is maintained constant throughout the experiment. Aliquots of 3 mL were withdrawn at different time intervals, filtered through 0.45 μm filter and analyzed for the protein concentration in the supernatant.

2.4.2.1. Analysis of the Kinetic Data. The kinetic rate constant model based on a single "lumped" adsorption rate constant was used to analyze the kinetic data (Horstmann et al., 1986). The model takes an empirical approach to the adsorption process and assumes that all the rate limiting processes can be represented by kinetic rate constants. In such an approach, the rate of mass transfer of the protein to the adsorbent is assumed to be described by Eq. (1) above. For batch adsorption in a stirred tank, the protein concentration in solution at time t is given by the analytical solution of Eq. (1), namely:

$$C = C_0 - \phi\left[\frac{(y + x)(1 - \exp\{-2x\phi k_1 t\})}{((y + x)/(y - x)) - \exp\{-2x\phi k_1 t\}}\right] \tag{5}$$

where

$$x^2 = y^2 - C_0 \phi q_m$$
$$y = 0.5(C_0/\phi) + q_m + (K_d/\phi)$$

C_0 is the initial liquid phase concentration and ϕ is the volume fraction of the settled adsorbent.

3. Results and Discussion

The protein adsorption studies on IMA-M(II) gels were conducted using batch method due to the rapidity, flexibility of choosing experimental conditions and low protein requirements associated with batch approach as compared to frontal chromatography. The adsorption of proteins on IMA-M(II) gels was very fast. In all the cases, 90% of the reaction was complete within five minutes of the addition of protein solution. While the influence of protein type on the rate of adsorption was insignificant (Fig. 2(a)), reasonable variations were observed for different IMA-M(II) gels (Fig. 2(b)). Figure 2(b) shows how the rates of lysozyme adsorption vary with the IMA-M(II) gel selected. Faster adsorption kinetics were usually observed for IDA-Cu(II) as compared to the other three IMA-M(II) gels, viz., TREN-Cu(II), IDA-Ni(II) and TREN-Ni(II).

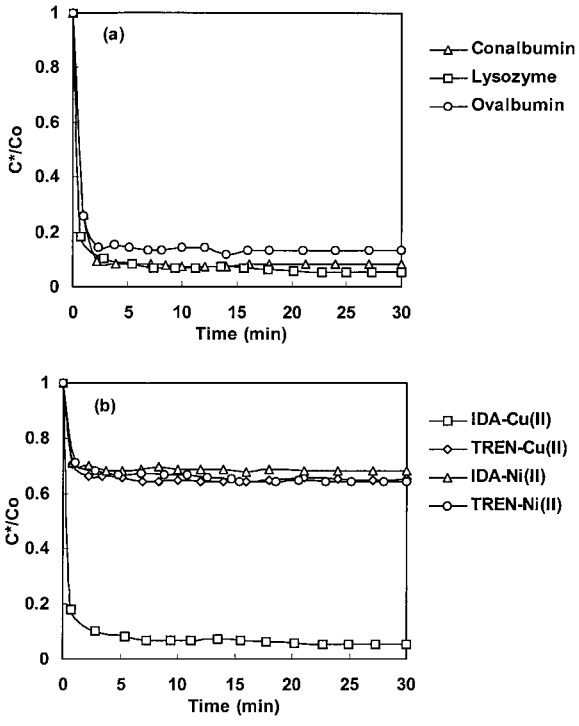


Figure 2. The kinetics of protein adsorption on IMA-M(II) gels (10 mM sodium acetate; pH 7.0; 2 M NaCl; $C_0 = 0.2$ mg/mL). (a) Three egg-white proteins on IDA-Cu(II) gel; (b) Lysozyme adsorption on different IMA-M(II) gels.

Nevertheless, all the flasks were kept for two hours in a shaking water bath so as to give sufficient time for the equlibrium to be established. Initially, the equilibrium adsorption studies were conducted in the low concentration range. The idea was to determine the selectivity of the metal loaded IMA gels for the proteins under investigation.

A scanning of the four metal loaded IMA gels viz., IDA-Cu(II), IDA-Ni(II), TREN-Cu(II) and TREN-Ni(II), for adsorption of three egg-white proteins revealed that all the proteins adsorbed on both the copper loaded gels but with differential affinities (Fig. 3(a)–(d)). Protein binding capacities were in the following order: Ovalbumin < Conalbumin < Lysozyme. In contrast, both the nickel loaded gels differed in their selectivity towards the three egg-white proteins. While all the three proteins were adsorbed on IDA-Ni(II) but with varying capacities in the following order (Fig. 3(c)): Ovalbumin < Lysozyme < Conalbumin, TREN-Ni(II) was selective for lysozyme only (Fig. 3(d)). Other two proteins did not have quantitative sorption on this gel. These observations indicated higher selectivity of TREN-Ni(II) gel for lysozyme over other IMA-M(II) gels under the identical experimental conditions. These differences in the selectivity and capacity of the gels can be explained on the basis of the nature of the chelating agent, metal ion and structure of the metal chelate. Tridentate chelators such as IDA are on the borderline as suitable metal binding groups. It forms a double five-membered ring chelate with tetra- and hexa-coordinate metal ions. TREN, a tetradentate chelator with four nitrogen atoms three of which are primary in nature and the fourth one is tertiary, may give rise to three five-membered ring metal chelates. While only two "free" sites are likely to be available on a hexacoordinate metal ion bonded to the TREN gel, there are at least two, usually three, free coordination sites on an IDA gel. In the later case, chelate structure involving more than two ligands on the proteins may be formed. Moreover, chelator IDA offers less steric hindrance to the proximal contact and interactions than does the TREN ligand. These circumstances can explain the higher selectivity in some cases for the TREN gel and in other cases for the IDA gels.

In order to determine the protein adsorption parameters for design purposes, equilibrium studies were performed on the metal loaded gels at higher concentrations as well. The model parameters were evaluated by performing non-linear regression using Maquardt method (1963). The coefficient of correlation was

greater than 0.98 for all the cases except those reported in the tables.

Initially, the Langmuir model was used to analyse the equilibrium data and adsorption parameters viz., the dissociation constant, K_d and maximum binding capacity, q_m, were quantified (Table 1). However, it was realised that Langmuir isotherm does not describe the adsorption behaviour satisfactorily. This is evident from the poor correlation between the experimental data and theoretical profiles based on Langmuir isotherm (Fig. 4(a)–(d)). The theoretical profile is more like a straight line for most of the systems disregarding the extreme points. As a result, negative or unrealistically high values of adsorption parameters were obtained for many systems. In fact, the extreme points have significant contribution towards determining the maximum sorption capacity (q_m) of the gel; and throw light on binding affinities and the mechanism of adsorption (Sharma and Agarwal, 2001). When we attempted to compute adsorption parameters using data points in the higher concentration range (especially for

Table 1. Langmuir adsorption parameters for various egg-white proteins on IMA-M(II) gel (10 mM sodium acetate; pH 7.0, 2 M NaCl).

IMA-M(II)	Protein	K_d (μM)	q_m (μ moles/mL gel)
IDA-Cu(II)	Lysozyme	25.194	13.288
	Conalbumin[a]	18.972	7.435
	Ovalbumin	nr	nr
TREN-Cu(II)	Lysozyme	79.821	0.482
	Conalbumin	58.167	0.169
	Ovalbumin	nr	nr
IDA-Ni(II)	Lysozyme	23.662	0.133
	Conalbumin	20.859	1.357
	Ovalbumin	nr	nr
TREN-Ni(II)	Lysozyme	595.827	3.766
	Conalbumin[b]	–	–
	Ovalbumin[b]	–	–

nr: not reported as the values of adsorption parameters were unrealistically high.
[a]Coefficient of correlation = 0.965.
[b]No quantitative adsorption of these proteins.

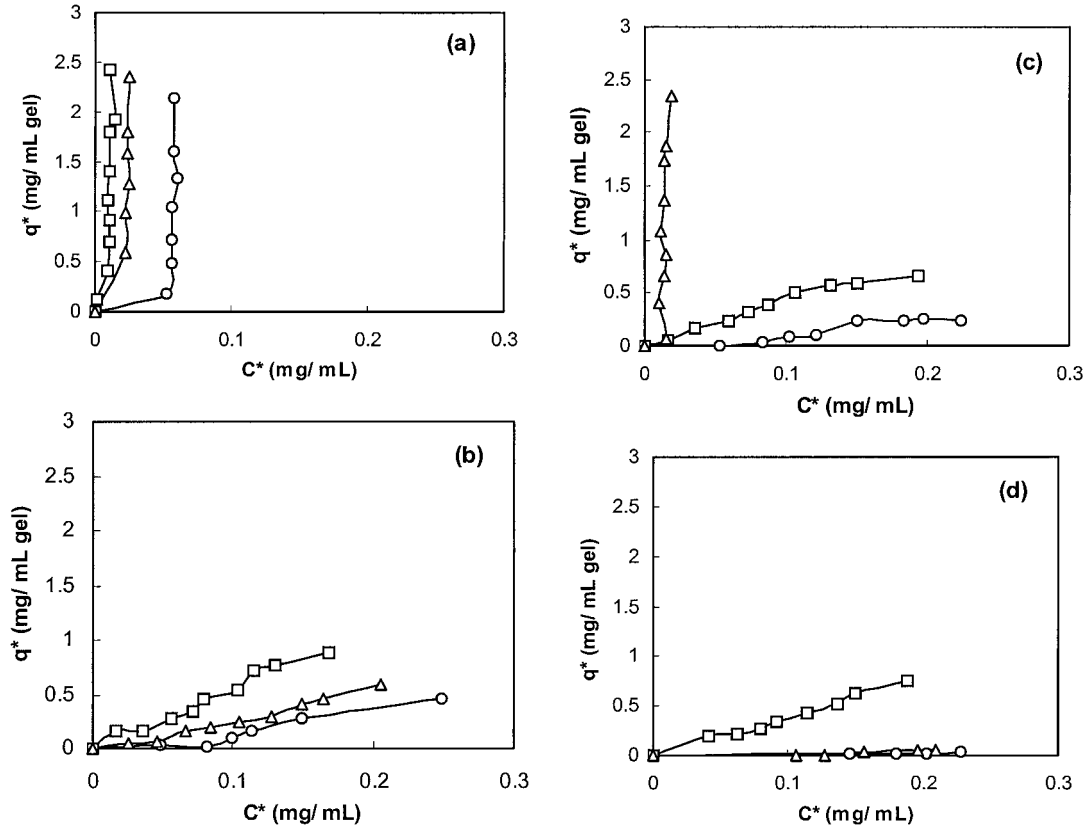


Figure 3. Comparative adsorption studies of egg-white proteins on IMA-M(II) gels (10 mM sodium acetate; pH 7.0; 2 M NaCl; C_0 = 0–0.25 mg/mL.). (△) Conalbumin; (□) Lysozyme; (○) Ovalbumin. (a) IDA-Cu(II); (b) TREN-Cu(II); (c) IDA-Ni(II); (d) TREN-Ni(II).

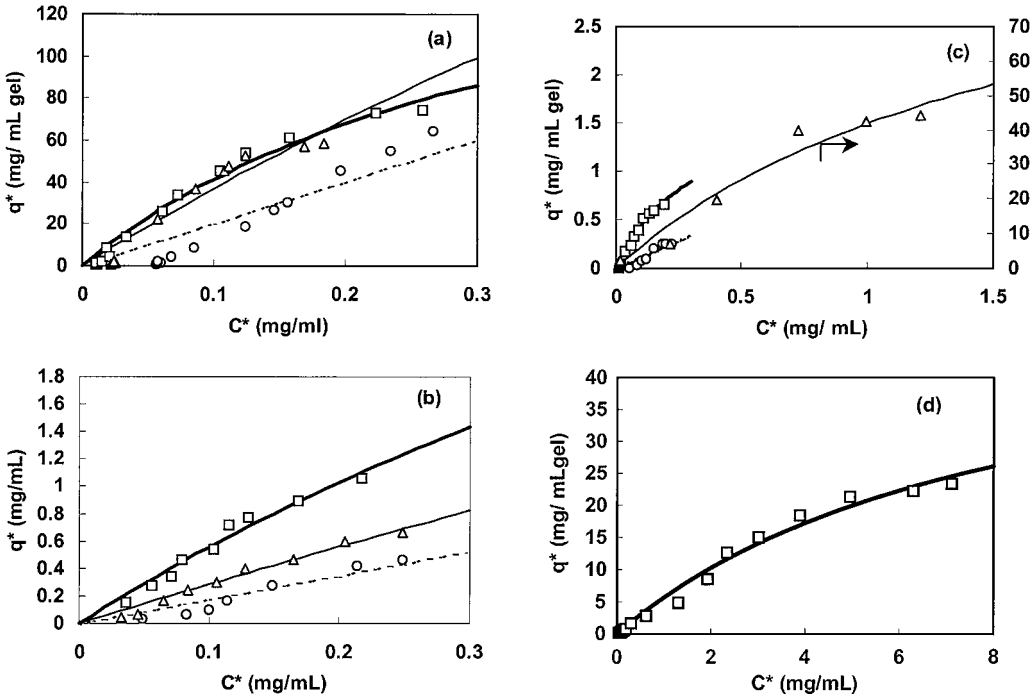


Figure 4. Experimental and theoretical profiles for the adsorption of egg-white proteins on IMA-M(II) gels based on Langmuir model (10 mM sodium acetate; pH 7.0, 2 M NaCl). Experimental data: (△) Conalbumin; (□) Lysozyme; (○) Ovalbumin. Theoretical profile (—) Conalbumin; (—) Lysozyme; (---) Ovalbumin. (a) IDA-Cu(II); (b) TREN-Cu(II); (c) IDA-Ni(II); (Δ-Y-axis: 0–70); (d) TREN-Ni(II).

the systems with negative or unrealistically high values of adsorption parameters), we found the values of these parameters relatively less and acceptable to some extent (e.g. IDA-Ni(II)-Ovalbumin: $K_d = 0.007$ M; $q_m =$ 0.014 μmol/mL gel). Still, such an approach was not applicable to all the systems showing deviation from Langmuirean behaviour.

All these observations therefore firmly establish that all the assumptions i.e. all binding sites have equal energy, adsorption of one protein molecule occurs on each binding site and binding sites are independent in nature, cardinal to the derivation of Langmuir model are not valid for the systems under investigation. Such deviations from Langmuirean behaviour have earlier been reported (Todd et al., 1994; Johnson and Arnold, 1995; Jiang and Hearn, 1996; Sharma, 1998; Sharma and Agarwal, 2000). Consequently, various modifications of the Langmuir model have been explored for explaining the adsorption behaviour of proteins on immobilized metal ion affinity gels (Todd et al., 1994; Johnson and Arnold, 1995; Jiang and Hearn, 1996; Sharma, 1998; Sharma and Agarwal, 2000). The adsorption behaviour of chicken egg-white proteins on these IMA gels was better explained by Langmuir-Freundlich model (Andrade, 1985) (Fig. 5 (a)–(d)). The model was able to account for all the data points including that at the extremes and the values of various adsorption parameters viz., apparent dissociation constant (K_d^*), maximum adsorption capacity ($q_{m(LF)}$) and Langmuir-Freundlich coefficient number (n) were evaluated (Table 2).

The binding affinity (inverse of apparent dissociation constant, K_d^*) of egg-white proteins on IDA-Cu(II) was found to decrease in the following order (with amino acid composition): Conalbumin (6 His, 6 Trp, 31 Cys) > Ovalbumin (7 His, 3 Trp, 6 Cys) > Lysozyme (1 His, 6 Trp, 9 Cys). However, interpretation of protein adsorption on immobilized metal ions solely on the basis of amino acid composition and histidine topography (Hemdan et al., 1989) is difficult. It is well established that three-dimensional structure and surface characteristics of proteins are altered under the influence of chemical environment (such as pH, ionic strength and buffer salts) and temperature resulting in modified adsorption behaviour. It is also believed that certain other groups at the surface of proteins such as deprotonated amines (e.g. lysine and amino-terminus) and aromatic side chains (Trp, Phe, Tyr) also have a

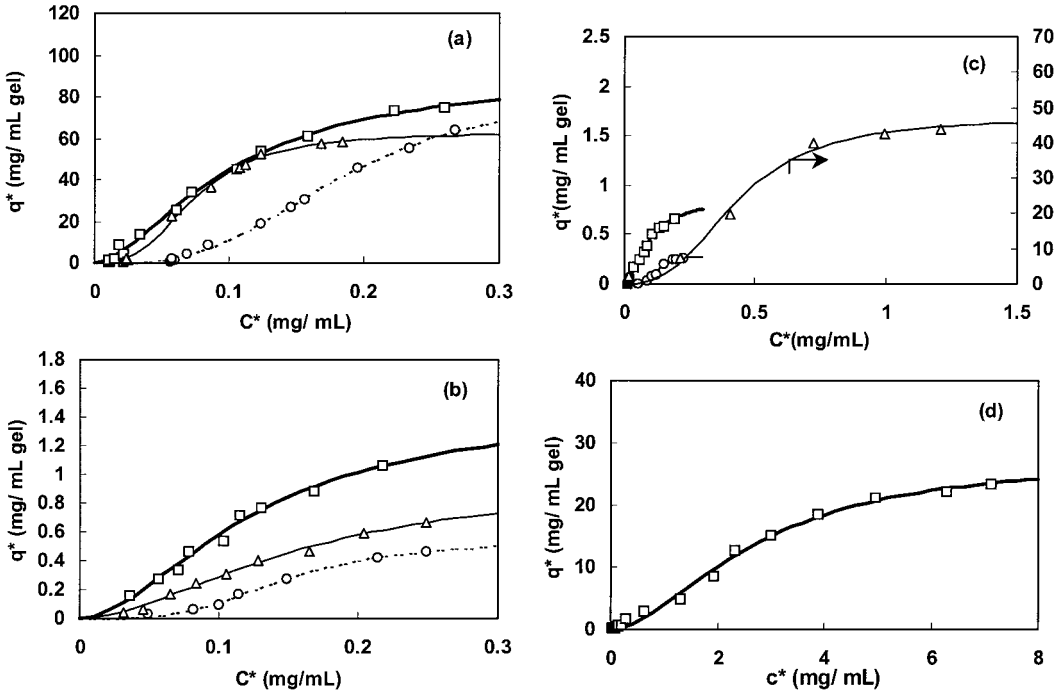


Figure 5. Experimental and theoretical profiles for the adsorption of egg-white proteins on IMA-M(II) gels based on Langmuir-Freundlich model (10 mM sodium acetate; pH 7.0, 2 M NaCl). Experimental data: (△) Conalbumin; (□) Lysozyme; (○) Ovalbumin. Theoretical profile (—) Conalbumin; (—) Lysozyme; (---) Ovalbumin. (a) IDA-Cu(II); (b) TREN-Cu(II); (c) IDA-Ni(II) (Δ-Y-axis: 0–70); (d) TREN-Ni(II).

significant influence on the recognition of immobilized metal ions (Arnold, 1991; Andersson and Sulkowski, 1992). For TREN-Cu(II), the protein adsorption capacities as well as binding affinities (except for ovalbumin) were much lower as compared to those for IDA-Cu(II). However, lysozyme and ovalbumin showed greater affinity for IDA-Ni(II) compared to IDA-Cu(II) and TREN-Cu(II). Values of $n > 1$ in all the cases indicate positive cooperativity in binding and heterogeneous nature of adsorption.

Table 2. Langmuir-Freundlich adsorption parameters for various egg-white proteins on IMA-M(II) gels (10 mM sodium acetate buffer, pH 7.0, 2 M NaCl).

IMA-M(II)	Protein	K_d^* (μM)	$q_{m(LF)}$ (μ moles/mL gel)	n
IDA-Cu(II)	Lysozyme	1.486	6.367	1.68
	Conalbumin	0.007	0.729	2.89
	Ovalbumin	0.131	1.946	3.08
TREN-Cu(II)	Lysozyme	1.656	0.102	1.81
	Conalbumin	0.460	0.011	1.78
	Ovalbumin	0.033	0.013	3.44
IDA-Ni(II)	Lysozyme	1.261	0.060	1.70
	Conalbumin	1.162	0.548	2.75
	Ovalbumin	0.003	0.007	4.45
TREN-Ni(II)	Lysozyme	436.038	1.942	1.81
	Conalbumin[a]	–	–	–
	Ovalbumin[a]	–	–	–

[a]No quantitative adsorption of these proteins.

As mentioned previously, kinetics of the adsorption of proteins on IMA gels is very fast. To our knowledge, there are no published reports on the evaluation of kinetic parameters of adsorption of proteins on IMA gels. Kinetic rate constant model by Horstmann et al. (1986) was used for the analysis of the kinetic data. Although it is recognised that this model is a gross oversimplification of the actual adsorption processes occurring at the surface of affinity sorbents and avoids diffusional resistances due to film and pore diffusion, the model has been widely accepted for the analysis of the kinetic data of various affinity and ion-exchange sorbents (Horstmann et al., 1986; Skidmore et al., 1990). Most of the times, it is adopted as a first approach towards quantitative understanding of the kinetics of an adsorption process.

The model takes an empirical approach to the adsorption process and assumes all of the rate limiting processes can be represented by kinetic rate constants.

Table 3. Rate constants for the adsorption of various egg-white proteins on IMA-M(II) gels (Co = 0.2 mg/mL).

IMA-M(II)	Protein	k_1 (mL/mg · s)	k_2 (s^{-1})
IDA-Cu(II)	Lysozyme[a]	0.002	7.216×10^{-4}
	Conalbumin	0.001	1.635×10^{-3}
	Ovalbumin	nr	nr
TREN-Cu(II)	Lysozyme	0.020	2.286×10^{-2}
	Conalbumin	0.001	5.013×10^{-3}
	Ovalbumin	nr	nr
IDA-Ni(II)	Lysozyme	1.769	5.994×10^{-1}
	Conalbumin	0.001	1.798×10^{-3}
	Ovalbumin	nr	nr
TREN-Ni(II)	Lysozyme	0.001	8.532×10^{-3}
	Conalbumin	nd	nd
	Ovalbumin	nd	nd

nd: not determined as there was no quantitative adsorption of these proteins; nr: not reported as standard error was greater than 20%.
[a]Standard error: 15%.

In such an approach, the rate of mass transfer of the protein to the adsorbent is assumed to be described by Eq. (5). All the parameters in the Eq. (5) except the forward rate constant, k_1 are known. In order to evaluate k_1, a program with iterative scheme was written and various simulation curves were drawn for a given system. The value of k_1 with minimum standard deviation was taken. The reverse rate constant, k_2, was simply determined by multiplying K_d with k_1 since the ratio of the reverse to the forward rate constant is the dissociation constant.

The model was moderately sufficient to describe the adsorption kinetics of egg-white proteins on the IMA-M(II) systems investigated and the forward rate constant were computed with reasonable accuracy for most of them (Table 3). For instance, Fig. 6 shows the experimental and theoretical profiles for the kinetics of lysozyme adsorption on IDA-Ni(II). However, the accuracy in measurement of kinetic parameters was quite low for ovalbumin on most of the IMA-M(II) gels (Standard error >20%). This may either be due to the insufficient number of data points in the initial steep zone or heterogeneous nature of adsorption resulting in low accuracy in measurement of the equilibrium parameters. As the rate of adsorption is very fast, more data points need to be taken in the initial region for which it is desirable to follow a more sensitive protocol. Furthermore, the derivation of this model is based on Langmuir adsorption isotherm, which is not followed strictly by our systems, resulting in higher chances of

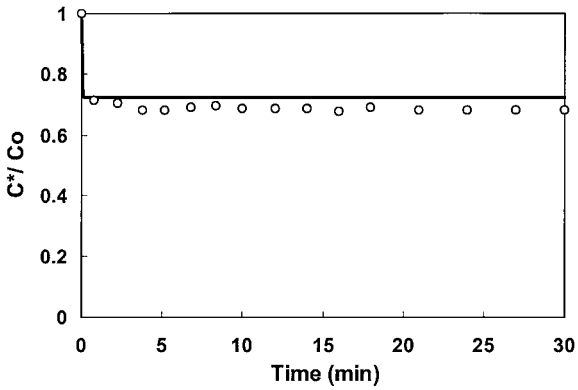


Figure 6. Experimental and theoretical profiles for the kinetics of lysozyme adsorption on IDA-Ni(II) (10 mM sodium acetate; pH 7.0; 2 M NaCl; C_0 = 0.2 mg/mL). (○) Experimental data; (—) Kinetic rate constant model.

error. Also, it was noticed that the error in k_1 estimation was more for the systems showing greater deviation from Langmuirean behaviour and having very low binding capacity (e.g. ovalbumin on IDA-Cu(II), TREN-Cu(II) and IDA-Ni(II)). Hence, in order to have a deeper insight into the kinetics of protein adsorption on IMA-M(II) gels, it is desirable to keep all these constraints in mind and perform kinetic analysis using more complex models including diffusional resistances due to film and pore diffusion (Horstmann and Chase, 1989). Overall, the faster adsorption kinetics on IMA gels ensures their suitability for industrial scale separations.

On the basis of these investigations, we conclude that the three egg-white proteins can either be separated on IDA-Cu(II) or TREN-Cu(II) or IDA-Ni(II)

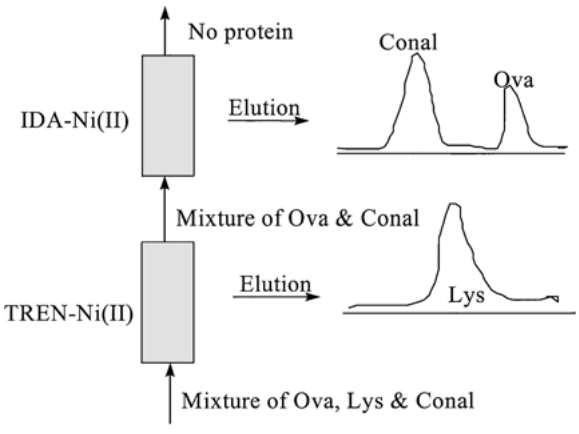


Figure 7. Proposed cascade mode multiaffinity column arrangement for efficient separation of three egg-white proteins.

using the first approach based on the differential affinities of various proteins for an IMA gel or by using a separation strategy based on CASMAC with columns of TREN-Ni(II) and IDA-Cu(II) or TREN-Cu(II) or IDA-Ni(II) arranged sequentially. As only lysozyme binds to TREN-Ni(II), the effluent stream will have other two proteins which can, then, be separated due to their differential affinities for IDA-Cu(II) or TREN-Cu(II) or IDA-Ni(II) gels. However, better resolutions are expected when IDA-Ni(II) is the second column due to vast differences in its affinity for ovalbumin and conalbumin (Fig. 7). The separation performance can be improved further by employing displacement, gradient and affinity elution methods.

4. Conclusions

The present research effort focussed on a thorough analysis of the adsorption behaviour of three egg-white proteins namely, ovalbumin, conalbumin and lysozyme on four IMA-M(II) gels viz., IDA-Cu(II), TREN-Cu(II), IDA-Ni(II) and TREN:Ni(II), with the intent to develop an improved understanding of these interactions for designing large scale IMA separations. The equilibrium adsorption data was analyzed using Langmuir and Langmuir-Freundlich models and the characteristic adsorption parameters were evaluated. The kinetic data was analysed using kinetic rate constant model. On the basis of these comparative adsorption studies two strategies were proposed for designing efficient fractionation of egg-white proteins using IMAC at large scale. The first approach was based on the differential affinities of three egg-white proteins for a particular IMA gel and the second approach highlighted the significance of CASMAC scheme. Despite being specific for egg-white proteins, this study has important general implications on the design of any large-scale IMA separation process. The study illustrated that the characteristic adsorption parameters required for designing an IMA based chromatographic separation process on preparative scale can be estimated on the basis of systematically conducted small scale batch experiments. Also, the information derived from this study can be utilized for the process development using hybrid bioseparation techniques such as metal chelate displacement chromatography and immobilized metal ion-membrane filtration. The efficacy of these parameters in predicting the performance of an actual separation process is currently under investigation.

Nomenclature

List of Symbols

C	Concentration of the free adsorbate (mg/ml)
C_0	Concentration of the adsorbate in the mixture before adsorption begins (mg/ml)
K	Freundlich equilibrium constant (L/mL gel)
k_1	Forward rate constant (mL/mg.s)
k_2	Reverse rate constant (s^{-1})
K_d^*	Apparent dissociation constant (M)
K_d	Dissociation constant ($=k_2/k_1$) (M)
n	Power term of Langmuir-Freundlich isotherm
q	Concentration of the bound adsorbate (μ moles/mL gel)
q_m	Maximum binding capacity of the adsorbate (μ moles/mL gel)
t	Time (s)
ϕ	Volume fraction of the settled adsorbent
η	Power term of Freundlich isotherm

Abbreviations Used

AAS	Atomic absorption spectrophotometry
CASMAC	Cascade-mode multiaffinity chromatography
IDA	Iminodiacetate
IMA	Immobilized metal ion affinity
IMAC	Immobilized metal ion affinity chromatography
TREN	tris(2-aminoethyl)amine

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