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Site accessibility and the pH dependence of the saturation capacity of a highly cross-linked matrix Immobilized metal affinity chromatography of bovine serum albumin on Chelating Superose

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Abstract

Immobilized metal ion affinity chromatography has shown promise for isolating desired proteins from a mixture based on their affinity for chelated metal ions. Using frontal analysis, the pH dependence of the saturation capacity of chelating Superose matrix for bovine serum albumin (BSA) is examined over a broad pH range. A significant increase in the capacity was observed near the elution pH of BSA (pH 4.5) from a Cu–imminodiacetic acid column. The results of several experiments indicated that this apparently abnormal variation may reflect the low degree of accessibility of a large portion of copper sites inside chelating Superose. In a broader sense, these results suggest that during frontal analysis, the assumption of column saturation based on a plateau in the exit concentration that is almost at the same level as the input concentration could be misleading for highly cross-linked matrices and relatively large sized proteins. That is, the relatively less accessible copper sites may become difficult to be reached due to high levels of protein adsorption in the more accessible regions and thus give the appearance of a plateau in the breakthrough curve prior to complete column saturation. This is likely to be the case at high pH where BSA demonstrates very high affinity for immobilized copper or at high input concentrations where the equilibrium coverage is expected to be high. The results demonstrate that the estimated saturation capacity could be significantly smaller than the actual capacity.

Keywords: Immobilized metal ion affinity chromatography; Adsorption isotherms; Saturation capacity; pH effects; Stationary phases, LC; Proteins; Albumin

1. Introduction

Immobilized metal affinity chromatography (IMAC), introduced by Porath et al. [1], is a powerful tool used in the separation of proteins. The technique consists of passing a mixture of proteins through a column packed with porous beads that are functionalized with metal chelating groups such as imminodiacetic acid (IDA) and nitrilotriacetic acid (NTA). Since the interaction between proteins and chelated metals is pH dependent, elution can be performed by developing a linear, nonlinear or a

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stepwise gradient in pH. Low cost of operation, ease of regeneration of the matrix and the relatively high specificity of protein–metal interactions are among the key advantages of IMAC.

Amongst all the supporting matrices used for IMAC, chelating Superose is one of the most commonly used [2–12]. This matrix has a high degree of cross-linking (12% cross-linked agarose) and approximately 50% more metal loading capacity compared to chelating Sepharose Fast Flow (Pharmacia Biotech, NJ, USA). In this paper, we have evaluated the saturation capacity of the chelating Superose for bovine serum albumin (BSA), as a model protein over a wide range of pH. Although a substantial amount of research has been done on the equilibrium between proteins and immobilized metals [13–18], studies on the pH dependence of the equilibrium parameters, especially the saturation capacity, are rather limited. Hansen and Lindeberg [19] examined the influence of α-amino groups on the pH dependence of the linear retention factors for peptides lacking histidine and cystein residues. Hutchens and Yip [18] obtained the equilibrium parameters of ribonuclease A with immobilized copper at pH 7.0, 5.8 and 4.8 using the chelating Sepharose Fast Flow matrix. The elution of this protein from IDA–Cu column begins at a pH slightly lower than 4.8. The equilibrium parameters below the elution pH of the protein from the column were not measured [18]. Since decreasing pH gradients are frequently employed for protein separation using IMAC, an investigation of the pH dependence of the saturation capacity of chelating Superose and the association constants over a wide pH range is attempted in this investigation.

The results revealed that the observed saturation capacity of the matrix for BSA remains constant at high pH (pH 7 to 5) but increases sharply at low pH (pH 4.5 and 4.0). Since saturation capacity is expected to remain constant, its variation with pH, at first, seems anomalous. The discrepancy, however, can be explained if the immobilized copper sites inside the matrix are divided into two groups, based on their accessibility for the diffusing BSA molecule. Higher affinity of BSA for copper at high pH will increase the coverage of the relatively more accessible copper sites in the matrix. The increased coverage could potentially block or hinder the diffusion of BSA molecules to the relatively less accessible sites. During frontal analysis, this hindered diffusion results in the appearance of an apparent plateau in the exit concentration at a time when a sizable portion of the copper sites are still unoccupied. Experimental results presented in this paper support this hypothesis and suggest that during frontal analysis, the assumption of column saturation based on a plateau in the exit concentration that is almost at the same level as the input concentration could be misleading for highly cross-linked matrices and relatively large sized proteins. That is, the assumption may lead to erroneous values of saturation capacity that are significantly smaller than the actual value.

2. Experimental

2.1. Materials and reagents

In chromatography experiments, 20 m*M* sodium phosphate buffer (0.5 *M* NaCl) was employed from pH 7.0 to pH 6.0 and 0.1 *M* sodium acetate buffer (0.5 *M* NaCl) was used from pH 6.0 to pH 4.0. A solution of 50 m*M* EDTA in 20 m*M* sodium phosphate buffer (0.5 *M* NaCl, pH 7.0) was used to remove copper from the column. A solution of 50 m*M* $CuSO_4$ in 0.1 *M* sodium acetate buffer (0.5 *M* NaCl, pH 4.0) was used for regenerating the column. All the buffers and solutions were prepared in distilled water, degassed and filtered through 0.2 μm filters.

2.2. Protein measurements

The exit protein (BSA) concentration from the column was measured by optical density measurements at a wavelength of 280 nm using an on-line UV detector (Pharmacia LKB Uvicord SII). The output of the UV detector was continuously fed to a chart recorder (Fisher Recordall Series 5000, Fisher Scientific, PA, USA). For selected experiments, protein concentration was also measured using the DC total protein assay kit (BioRad, CA, USA). The measurements were made in the linear range of the calibration curve for the DC protein assay as well as for the optical density.

2.3. Frontal chromatography

Frontal chromatography experiments were performed on the fast performance liquid chromatography (FPLC) system (Pharmacia Biotech) equipped with the chelating Superose HR-10/2 column. Before each experiment, the column was loaded with copper using six column volumes of 50 m*M* $CuSO_4$ solution (0.5 *M* NaCl, pH 4.0) and washed with an equal volume of 0.1 *M* sodium acetate buffer (0.5 *M* NaCl, pH 4.0). This was followed by an equilibration step consisting of passing six column volumes of the buffer of appropriate pH at which the frontal analysis was to be performed. After the experiment, the column was washed with six column volumes of 50 m*M* EDTA solution (0.5 *M* NaCl, pH 7) followed by reloading of copper. This protocol of regenerating the column was rigorously followed before each experiment in order to ensure uniform copper loading.

An appropriate amount of BSA was dissolved in the equilibration buffer and the pH of the solution was checked. The pH tended to change by small amounts (<0.1 pH units), especially at high protein concentrations. After adjusting the pH, the protein solution was applied to the column continuously at a flow-rate of 0.4 ml/min until saturation. The column was assumed to be saturated when the absorbance at 280 nm reached an approximate plateau level. The bound protein concentration was measured by integrating the area behind the breakthrough front and dividing it by the volume of the solid-phase accessible to the protein molecule. The accessible volume of the column (and the intraparticle diffusivity) was measured by the pulse technique described in Section 2.4 [20].

2.4. Pulse experiments

A small pulse of BSA, dissolved in 20 m*M* sodium phosphate buffer (0.5 *M* NaCl, pH 7.0) was injected in a blank (without copper) column and washed with the same buffer at varying flow-rates. In experiments using blank columns, high concentrations of NaCl and dilute protein solutions were used to ensure minimum interaction with the matrix. The eluted peaks exhibited Gaussian symmetry, allowing the use of simple height equivalent to a theoretical plate (HETP) theory [20] to obtain the accessible volume of the gel and the intraparticle diffusivity of the protein in the gel.

$$t_m = \frac{H}{v}\left\{F_e\left(1 + \frac{\rho_p}{\beta}B_{blank}\right)\right\} + \frac{t_0}{2} \quad (1)$$

$$F_e = \varepsilon + (1-\varepsilon)\beta \quad (2)$$

$$\mathrm{HETP} = \frac{\sigma^2 H}{t_m^2} = \frac{2D_s}{v} + \frac{2(1-\varepsilon)^2\beta^2 R^2 v}{15(1-\varepsilon)[\varepsilon + (1-\varepsilon)\beta]^2} \times\left(\frac{1}{D_e} + \frac{5}{k_f R}\right) \quad (3)$$

The parameters are: σ, the variance of the eluted peak; R, the particle radius; t_m, the retention time of the pulse; t_0, the duration of the pulse; H, the height of the column; v, the superficial velocity; ρ_p, the particle density; β, the particle porosity; ε, the bed porosity; F_e, the fraction of the total volume accessible to the diffusing protein molecule; B_{blank}, the association constant for the interaction of the protein with the blank column; D_s, the axial dispersion coefficient and k_f, the film mass transfer coefficient. D_s/v can be assumed to be constant for liquid chromatography of large molecules [20] and k_f can be obtained from literature correlations [21]. According to Eqs. (1,3), the fraction of the total accessible volume (F_e) and the intraparticle diffusivity (D_e) can be obtained from the slope of the plots of $(t_m - t_0/2)$ vs. H/v_0 and HETP vs. v, in the absence of any interaction (i.e., $B_{blank}=0$), respectively. Negligible interaction of the protein with the column is a valid assumption in this case since blank IDA column is used along with a high enough NaCl concentration (0.5 *M*) to suppress ionic interactions.

3. Results and discussion

3.1. Frontal analysis

Frontal chromatography data is commonly analyzed using the equation described by Nichol et al. [22] based on the Langmuir isotherm [$q = q_m BC/(1+BC)$] [13,14,23].

$$\frac{V_a}{V-V_0}=\frac{1}{Bq_m}+\frac{C}{q_m} \quad (4)$$

The parameters are: B, the equilibrium association constant; q_m, the saturation capacity of the matrix for the protein; q, the adsorbed concentration in equilibrium with the input concentration (C); V, the elution volume where the exit protein concentration reaches half of the input concentration; V_0, the elution volume of the protein without any interaction and V_a, the accessible volume of the column. The value of V_a is estimated to be about 60% of the total column volume (Fig. 1a), using pulse analysis, described in Section 2.4. According to Eq. (4), a plot of $V_a/(V-V_0)$ vs. C should yield a straight line with slope of $1/q_m$ and an intercept of $1/Bq_m$.

Eq. (4) uses the median bisector method of measuring the elution volumes [13,22,24,25]. That is, V and V_0 of Eq. (4) are measured when the exit protein concentration reaches half of the final value. The method is valid only for breakthrough curves whose shapes can be approximated by a trapezoid. The shape of the breakthrough curves obtained in our frontal analysis deviated significantly from a trapezoid, probably due to the large intraparticle diffusional resistance experienced by the diffusing BSA molecule in the chelating Superose gel. In order to eliminate any errors arising from the assumption of a trapezoidal shape, the calculations were performed using the integrated area behind the breakthrough front which is a true measure of the amount of protein bound in the column at any input concentration [26]. Such an analysis leads to an equation analogous to Eq. (4), with elution volume replaced by the actual area behind the eluted front,

$$\frac{C}{A-A_0}=\frac{1}{q_mBV_a}+\frac{C}{q_mV_a} \quad (5)$$

Here, A is the area behind the breakthrough front and A_0 is the area in the absence of any interaction. A plot of $C/(A-A_0)$ vs. C should yield a straight line with a slope of $1/(q_mV_a)$ and intercept of $1/(Bq_mV_a)$.

Eq. (4) or Eq. (5) results directly from the Langmuir isotherm [13,22] which assumes a single site interaction between protein and the metal and neglects any site exclusion effects (i.e., the coverage of multiple vacant copper sites due to the adsorption of one protein molecule) [27]. Vunnum et al. [16] have analyzed a case in which the protein molecule binds to IMAC supports via multiple interactions of approximately equal strength. The analysis results in an isotherm that cannot be simplified to Eq. (5). Thus, the use of Langmuir isotherm and hence Eq. (5) would be inappropriate in the case of multiple interactions of approximately equal strength. The BSA–Cu^{2+} interaction, however, is known to be dominated by a single strong binding site involving the sequence Asp–Thr–His at the N-terminus of the protein [28]. Thus, the use of Langmuir isotherm and Eq. (5) to analyze the frontal chromatography data is justified in the case of BSA–immobilized Cu^{2+} interaction.

3.2. pH dependence of the saturation capacity

The results of frontal analysis are shown in Fig. 2a Fig. 2b. The straight lines shown are the best least square fits of Eq. (5) with $r^2 \geq 0.95$. Note that only three observations are used in the regression at pH 4 since the fourth measurement at the lowest input concentration (3.5 mg/ml) deviated significantly from a straight line. Using the values of B and q_m, obtained from Fig. 2, as initial estimates, the parameters are determined using a non-linear least square data fitting subroutine (SAS: Proc. NLIN, Version 6.08) and are plotted in Fig. 3. Fig. 3a shows the effect of pH on the observed saturation capacity of the matrix for BSA. The value of capacity remains approximately constant at high pH (pH 7 to pH 5) but increases sharply in the region where the association constant drops (i.e., near pH 4.5). Since the saturation capacity of the matrix is expected to remain unchanged, independent of pH, the variation in the observed value of q_m is seemingly anomalous.

Using the values of B and q_m given in Fig. 3, Langmuir isotherm curves are plotted for pH 6.0, 4.5 and 4.0 in Fig. 4. The same anomalous trend in the saturation capacity is also evident from the comparison of the Langmuir isotherm curves generated at high and low pH. Since the binding affinity of BSA for immobilized copper is expected to be higher at higher pH, the adsorbed concentration at a particular input concentration should always be higher at pH 6

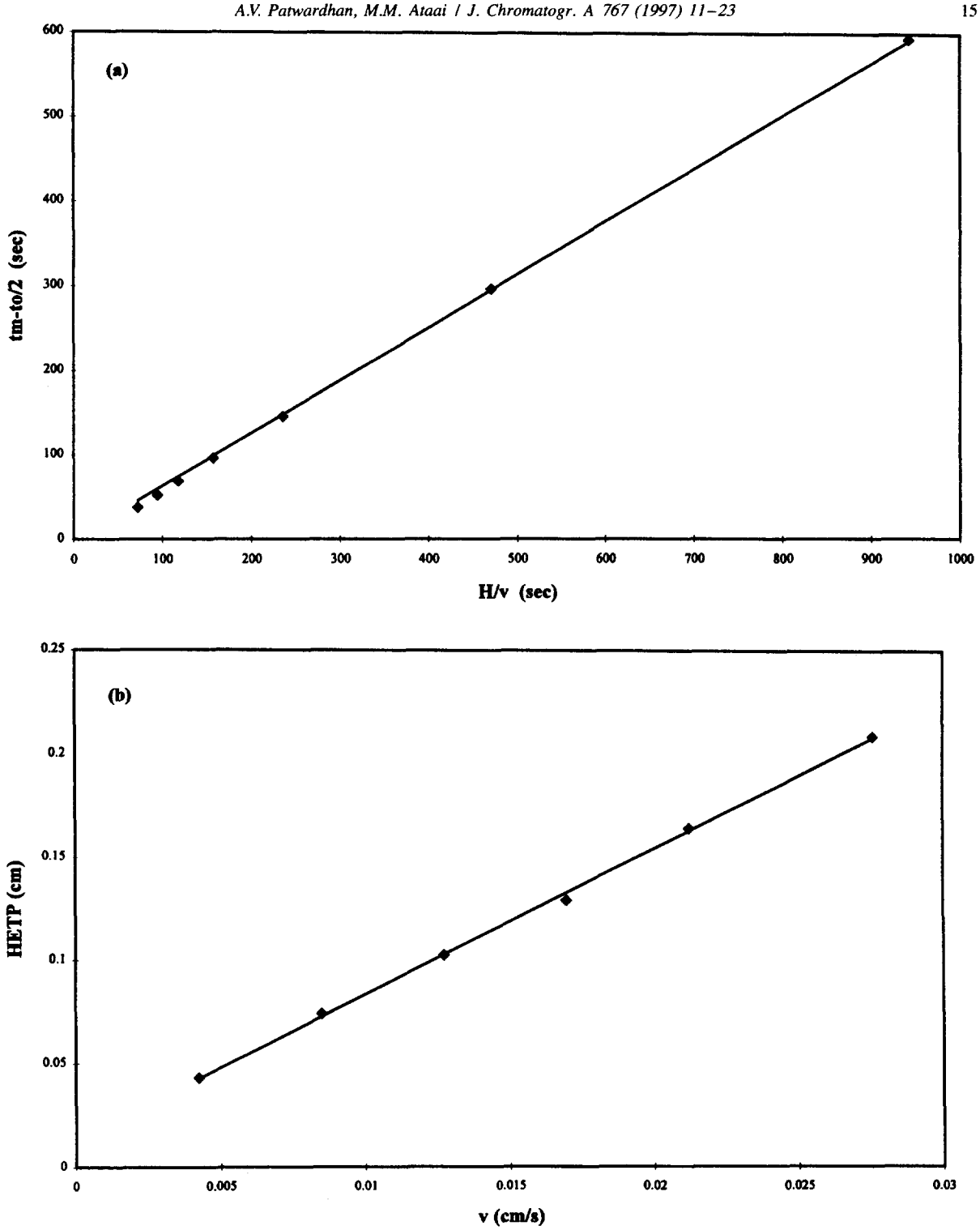

Fig. 1. (a) Estimation of the accessible volume of the column for BSA based on the pulse analysis according to Eq. (1). (b) Plot of HETP vs. velocity for estimating the intraparticle diffusivity of BSA in chelating Superose according to Eq. (3).

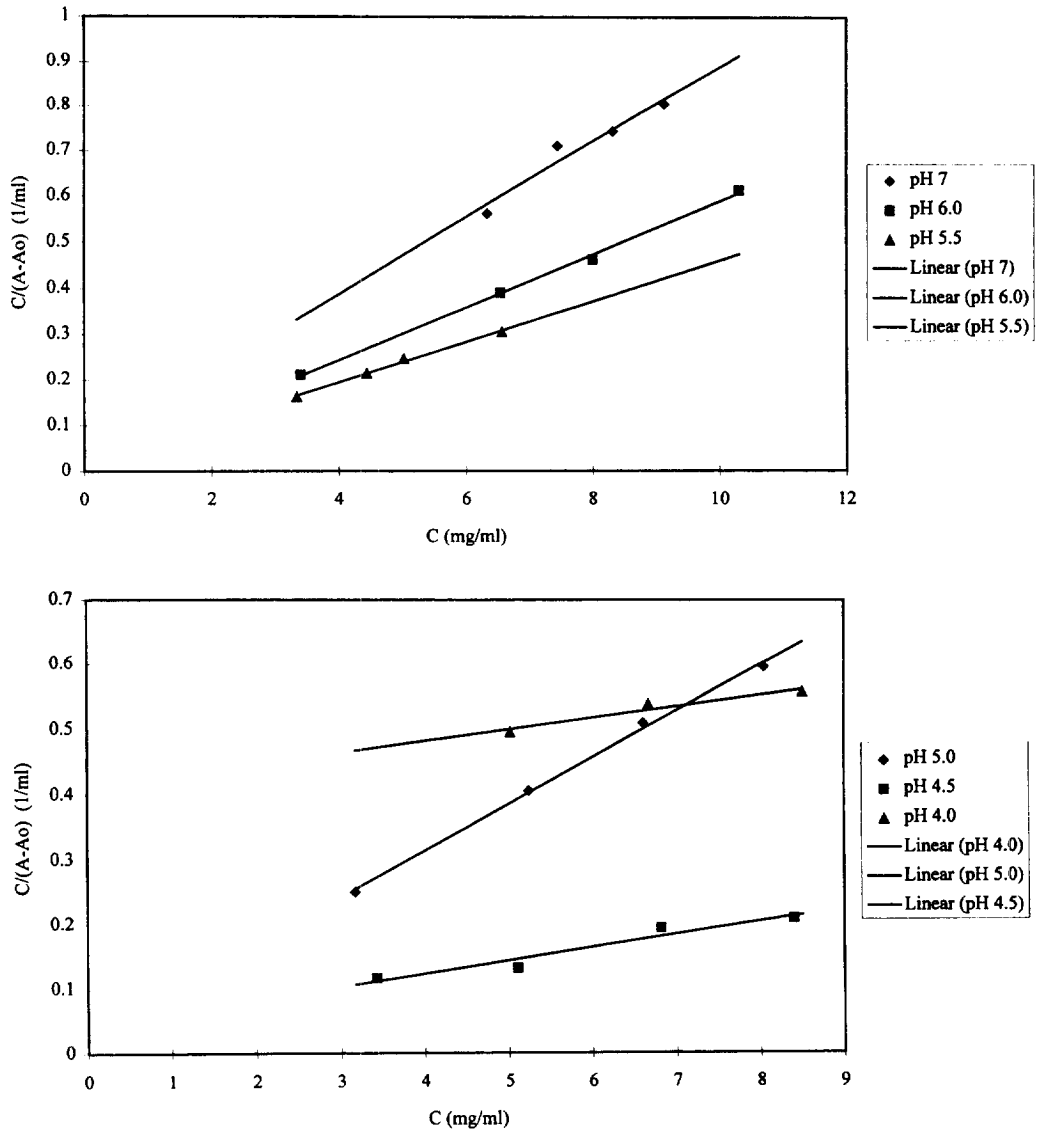

Fig. 2. Frontal analysis of BSA–immobilized Cu^{2+} interaction. The points are the experimental data and the lines are the best least square fits ($r^2 \geq 0.95$) using Eq. (5).

than at pH 4.5 and 4. Fig. 4 shows that for some input concentrations (>1 mg/ml for pH 4.5 and >11 mg/ml for pH 4.0), the adsorbed protein concentration at pH 4.5 and 4 is higher than that at pH 6.0.

We initially thought that the above abnormality is caused by the coverage of several copper sites by a single protein molecule (i.e., the site exclusion effect). Todd et al. [15] have presented a detailed discussion of the site exclusion effects on the parameters of the Langmuir isotherm in IMAC. Their analysis leads to the following isotherm, identical in mathematical form to the Langmuir isotherm but with modified parameters.

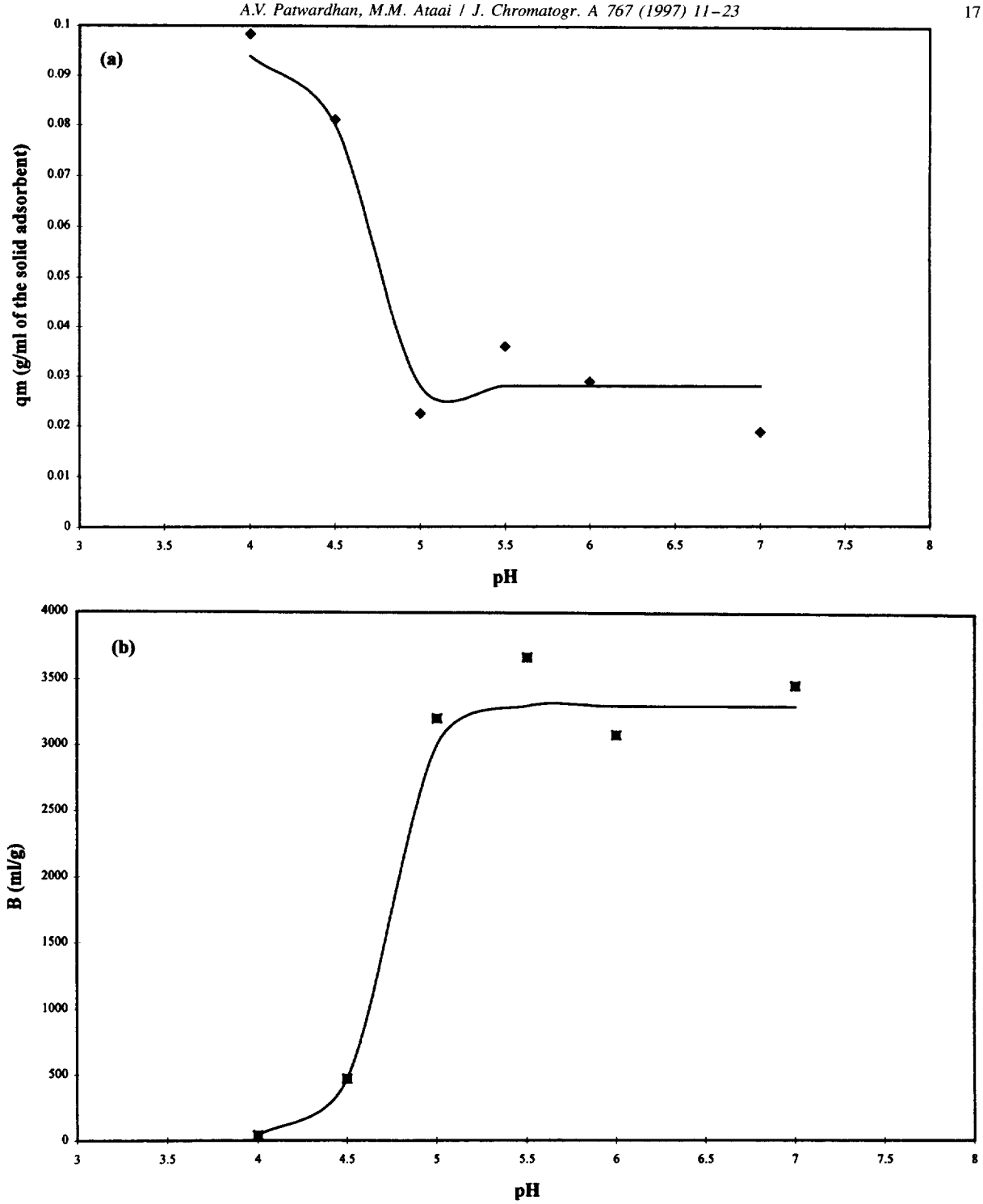

Fig. 3. (a) pH dependence of the saturation capacity of the chelating Superose matrix for BSA. The average error of the estimates reported by the nonlinear least square subroutine is ±12.3%. (b) pH dependence of the association constants of BSA–immobilized Cu^{2+} interaction. The lines represent visual fits.

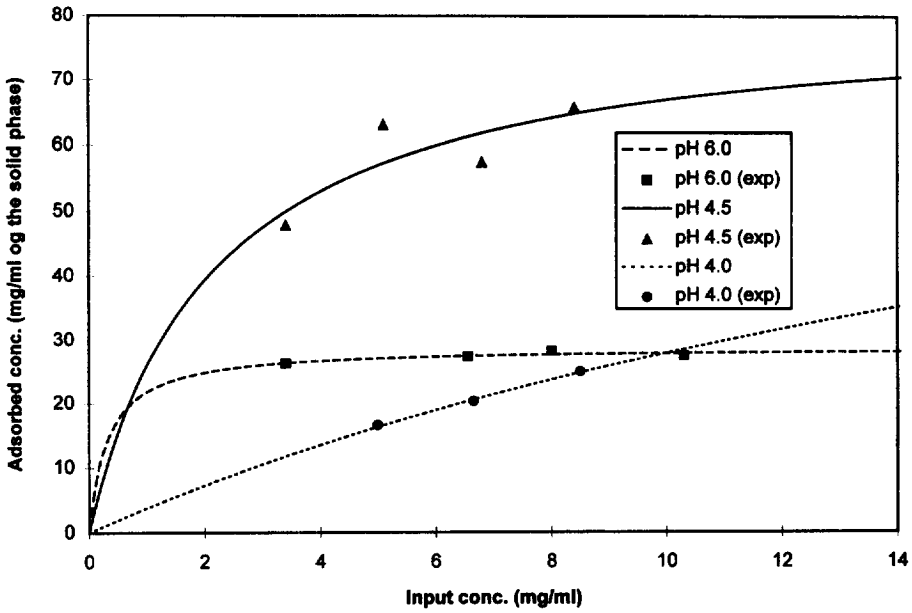

Fig. 4. Langmuir isotherms based on the values in Fig. 3.

$$q = \frac{\frac{Cu_{acc}}{n}(nB)C}{1 + (nB)C} \quad (6)$$

The association constant and the saturation capacity of the conventional Langmuir isotherm are replaced by nB and Cu_{acc}/n, respectively. Cu_{acc} is the concentration of the accessible copper sites and n is the number of copper sites covered by a single protein molecule. Since n is a constant, independent of pH, the site exclusion effect will not explain the variation in the capacity. However, if there are variations in Cu_{acc}, it will lead to changes in Cu_{acc}/n and thus the value of the saturation capacity. The results presented next, indicate that Cu_{acc} can indeed change with changes in pH if the binding sites are classified into two groups, based on their accessibility. Higher affinity of BSA for copper at high pH will increase the coverage of the relatively more accessible copper sites. This increased coverage could potentially block or hinder the diffusion of BSA to the less accessible sites and thus in principle, explain the apparent low capacity observed at high pH.

If the blockage of some of the sites in the matrix leads to an apparent low capacity at high pH, the diffusivity of BSA in chelating Superose will be expected to be low. The diffusivity was measured using the pulse technique described in Section 2.4 (see Fig. 1b). The measured value of $3.41 \cdot 10^{-9}$ cm^2/s is significantly lower than the diffusivity of BSA in some of the other commonly used chelating gels such as Sepharose CL-4B ($9.6 \cdot 10^{-8}$ cm^2/s) which has 4% cross-linked agarose [20] and Sepharose CL-6B ($5.6 \cdot 10^{-8}$ cm^2/s) which has 6% cross-linked agarose [29].

Moreover, if higher equilibrium coverage leads to lower observed capacities, frontal chromatography experiments at low input concentrations should eliminate or at least, decrease this effect. Figs. 5a and 5b compare the breakthrough curves at input concentration of 1 mg/ml and 9 mg/ml. In both cases the exit BSA concentration reached an apparent plateau level at about 95% of the input value. In accordance with the hypothesis based on the heterogeneity of the binding sites, the adsorbed amount of BSA, as measured by the area behind the breakthrough front (Fig. 5), was significantly (two-fold) higher at input concentration of 1 mg/ml than 9 mg/ml. In order to confirm this observation, the experiment was repeated. This time, the bound protein in each case was eluted by a step change in pH from 7.0 to 4.0 and the amount of eluted protein

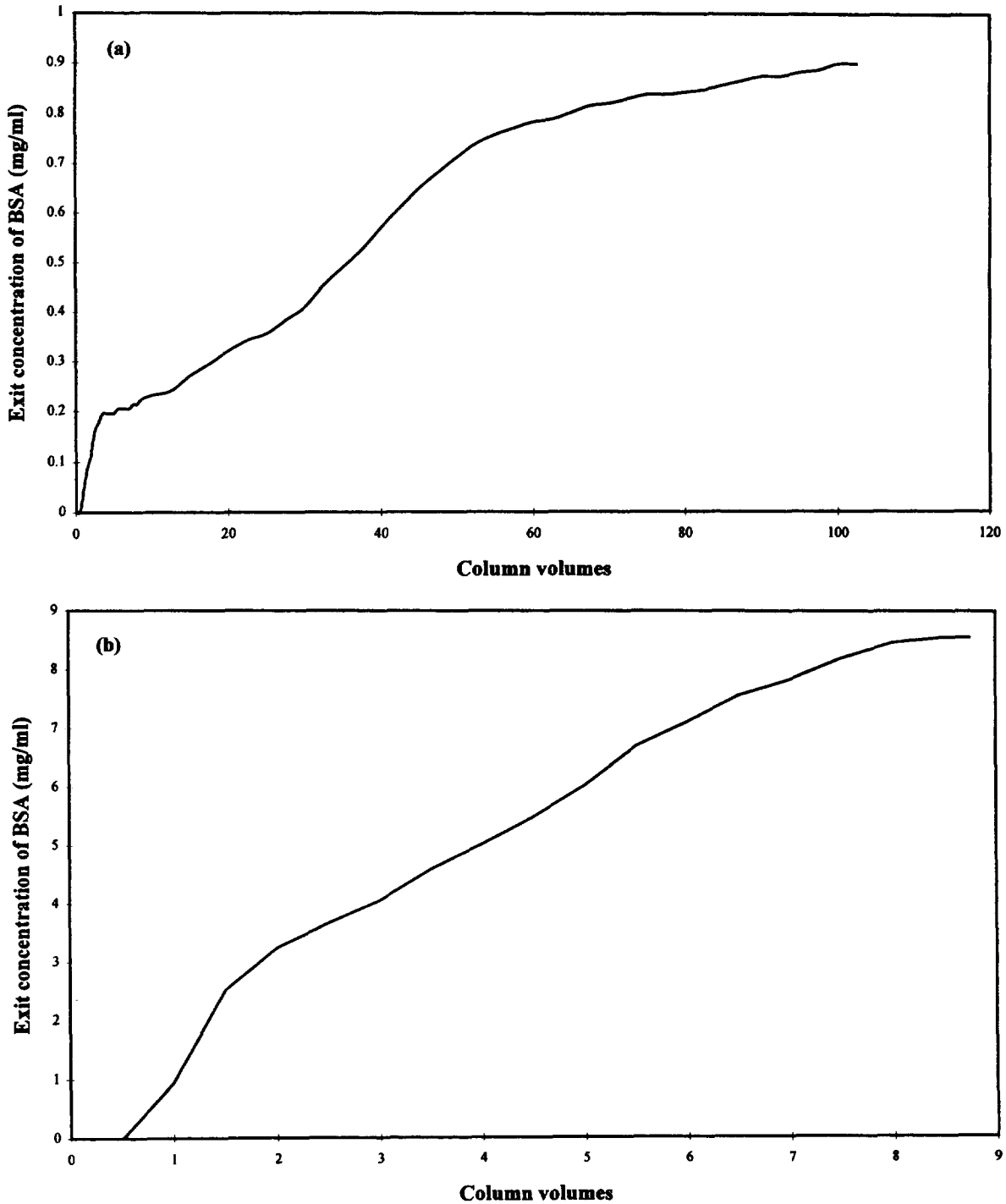

Fig. 5. Breakthrough behavior of BSA from the chelating Superose column at input concentrations of 1 mg/ml (a) and 9 mg/ml (b).

was measured by the DC total protein assay (Bio-Rad). The results of the protein assay were also consistent with the area measurements as the amount of protein bound at lower input concentration was about two-fold higher than the value at higher input concentration.

The final endorsement of our hypothesis came from the shapes of the eluted peaks of BSA loaded at 1 mg/ml and 9 mg/ml (Fig. 6). The eluted peak for the case of 1 mg/ml exhibits a larger tailing effect as compared to the elution peak of 9 mg/ml. As mentioned, larger number of BSA molecules are bound in the relatively less accessible regions of the matrix for input concentration of 1 mg/ml than 9 mg/ml. These molecules, upon unbinding, face a greater mass transfer resistance for transport out of the pores which results in a large tail for the eluted peak.

We would like to emphasize at this point that the hypothesis of the heterogeneity of binding sites accounts for variations in the observed capacity of the matrix. The actual saturation capacity of the gel should be a constant and independent of the pH. The value of the saturation capacity observed at low pH (pH 4.5 and 4; see Fig. 3a) is closer to the actual saturation capacity of the matrix than that at high pH (pH 7 to 5). The difference between the actual and the observed values, particularly at high pH, arises mainly because of the shape of the breakthrough in frontal chromatography experiments. That is, the exit

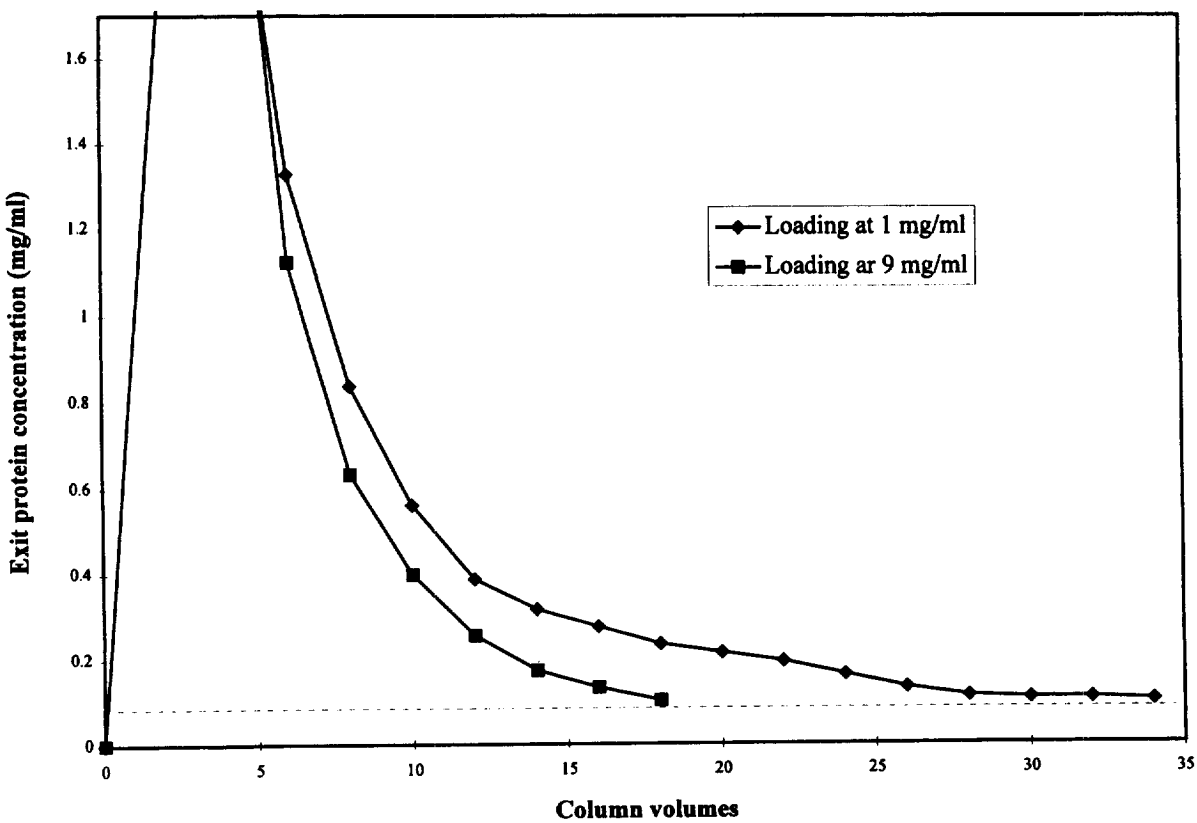

Fig. 6. Elution behavior of BSA loaded at 1 mg/ml and 9 mg/ml. Elution is accomplished by a step in pH from pH 7 to 4. The experiment was terminated when A_{280} reached a value corresponding to 0.1 mg/ml (dotted line). Concentrations above 1.7 mg/ml were outside the detection range of the on-line UV detector.

concentration reaches an apparent plateau level due to the saturation of the more accessible sites (Fig. 5b). After this point, further diffusion of the protein to the less accessible sites would occur at an extremely slow rate until full saturation is attained. However, the changes in the exit concentration will be difficult to discern due to insignificant variations over a long period of time.

3.3. pH dependence of the association constants

Fig. 3b shows the variation in the association constant with pH. The data points at pH values between pH 7 and 5 fall in the plateau region of the Langmuir isotherm (e.g., pH 6.0; Fig. 4) while those at low pH fall in the nonlinear region (e.g., pH 4.5 and 4.0; Fig. 4). Since the observations in the plateau region of the Langmuir isotherm are largely influenced by the saturation capacity of the matrix as opposed to the combination of saturation capacity and association constants, the error in the estimated values of B is expected to be higher at high pH (pH 7 to 5) than that at low pH (pH 4.5 and 4). Belew et al. [14] reported a value of 3400 ml/g for association constant of BSA with immobilized Cu^{2+} at pH 7.0. The value of the same parameter estimated in this study is 3461 ml/g. Furthermore, the associations constants remain approximately constant at high pH (pH 7 to 5) and then drop rapidly near pH 4.5 which is the elution pH of BSA from the column (Fig. 7). Hutchens and Yip [18] observed similar trends in the association constants between ribonuclease A and immobilized Cu. They found a slight decrease in values of the association constants from 1872 ml/g at pH 7 to 1622 ml/g at pH 5.8 followed by a sharp drop from 1622 ml/g (pH 5.8) to 456 ml/g (pH 4.8). Ribonuclease A elutes from the column at a pH slightly lower than pH 4.8; thus the drop in association constants between pH 5.8 and a pH lower than 4.8 could have been even more drastic, as was obtained in our experiments.

The following equation can be used to explain the

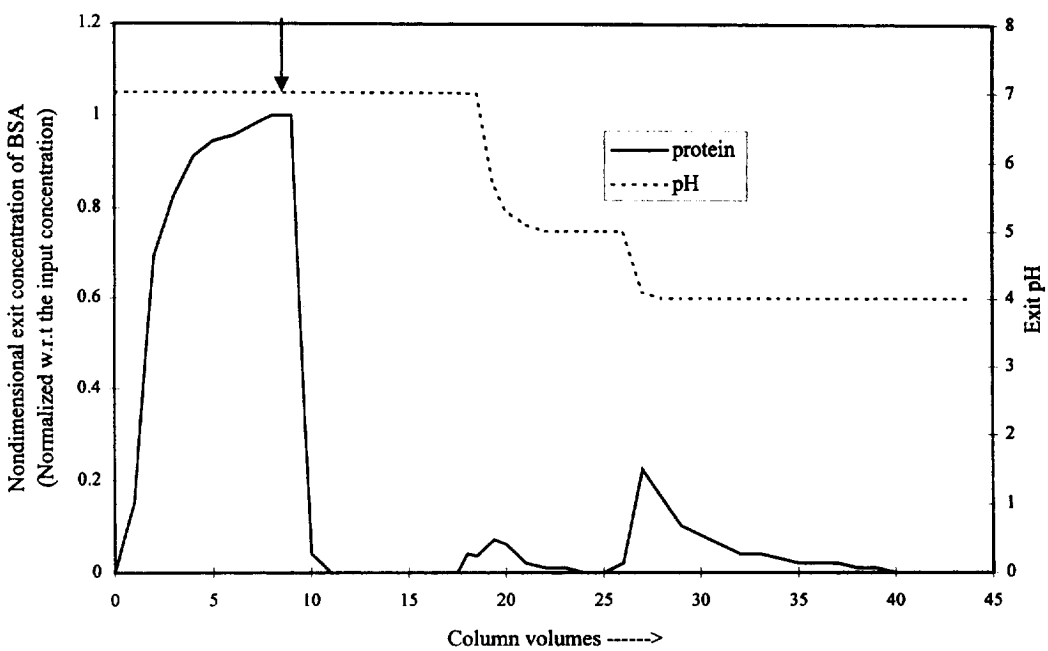

Fig. 7. Experimental adsorption and elution behavior of BSA from a Cu–IDA column. The arrow represents the end of loading and the beginning of elution. Elution is accomplished by a step gradient from pH 7 to 4. The peak at pH 6 may represent impurities or multimerically associated forms of BSA.

sharp drop in the binding constants in the vicinity of the elution pH [30].

$$B = \frac{K_m}{1 + K_h[H^+]} \quad (7)$$

Eq. (7) considers the competition between the metal ions and the H^+ ions for a single binding site on the protein molecule. The parameters K_m and K_h are the intrinsic association constants for the interaction of the metal binding site of the protein with the metal and the H^+ ions, respectively. Although, this equation has been derived for protein–metal complexes in solution [30], it can be applied to protein–immobilized metal interactions, characterized by a single predominant binding site (i.e., constant values of K_m and K_h). According to Eq. (7), the value of B would largely remain constant, at a value close to K_m for low H^+ ion concentration (i.e., high pH) but would rapidly fall off in the vicinity of the region where the H^+ ion concentration equals the reciprocal of K_h.

4. Conclusions

In summary, a significant variation in the capacity of the chelating Superose matrix near the elution pH of BSA from a Cu–IDA column was observed. The results exhibited an apparent plateau in the exit protein concentration, during frontal analysis, prior to the saturation of all the copper sites in the column. The assumption of column saturation based on such a plateau led to erroneous values of the saturation capacity which are much smaller than the actual capacity. This apparently abnormal variation may reflect the existence of two types of copper sites inside the matrix, differing from each other in their accessibility to the diffusing BSA molecule. The relatively less accessible copper sites may become difficult to be reached due to high levels of protein adsorption in the more accessible regions. This is likely to be the case at high pH (corresponding to high affinity of BSA for immobilized copper) or at high input concentrations where the equilibrium coverage is expected to be high. At low pH and/or low input concentrations, the decreased coverage would allow a greater access to the sites located in the less accessible regions and reduce the difference between the actual and the observed capacities.

Acknowledgments

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