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Study of the adsorption of self-associating proteins on an anion exchanger

Application to the chromatography of β-lactoglobulin B

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ABSTRACT

The stoichiometric displacement model (SDM) for the adsorption of proteins on an ion exchanger was extended to describe the adsorption of self-associated molecules. The model based on the mass-balance equations assumes a monomer–dimer equilibrium with a rapid interconversion rate. The role of protein self-association in solution and in the adsorbed state is discussed in terms of adsorption isotherm shapes and of zonal elution chromatographic behaviours. The influence of the parameter Z, defined as the ratio of the protein valency to the displacing counter-ion valency, is demonstrated. The model was applied to analyse the zonal elution behaviour of β-lactoglobulin B (β-lact B) on a polymeric anion-exchange stationary phase. The influence of the counter-ion valency was studied by adding NaCl or Na_2SO_4 to the eluent. The adsorption isotherm of β-lact B on the anion exchanger was determined at pH 7.5 from the non-linear zonal elution profiles observed with increasing sample size. Various aspects of the adsorption behaviour of β-lact B, such as positive cooperativity, were deduced from the isotherm shape and the corresponding Scatchard plot. The column capacity, the association constant in solution and the parameter defining the association in the adsorbed phase were determined from the best fit of the theoretical model to the experimental profiles. In agreement with the model, these parameters can be used to describe the experiments performed with a monovalent or a divalent counter ion. It is shown that protein–protein interactions exist in the adsorbed state and lead to an increase in the protein self-association.

INTRODUCTION

Anion-exchange chromatography is routinely used for the separations of acidic proteins [1]. The process results from the interaction between the protein and the ion exchanger and is described by the stoichiometric displacement model (SDM). It relates the retention volume at low concentration to the displacing salt concentration [2,3]. On the basis of this simple equilibrium model, the adsorption isotherm expression can be derived from the mass balance equations [4,5]. In a previous study [6], we used this SDM adsorption isotherm model to simulate the non-linear chromatographic behaviour of proteins in the isocratic and gradient zonal elution mode.

It has been shown, however, that the SDM model is only a rough representation of the adsorption behaviour of proteins. Several other mechanisms may interfere, such as the hydrophobic interactions [7] or those more complex than the simple electrostatic interactions based on a Coulomb law [8,9]. Another limitation is that the isotherm expression based on the SDM model cannot be applied to predict the elution behaviour of self-associating proteins.

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Milk proteins are often separated using anion-exchange chromatography [10,11] and it is important to understand better the adsorption behaviours of these proteins on ion exchangers. Some whey proteins, such as the β-lactoglobulins (β-lact) A and B, undergo self-associations, which occur in three separate pH ranges [12]. At pH 3.5 and below and at pH 7.5 and above, the monomer is in rapid equilibrium with its dimer. The tendency for dissociation at pH 7.5 and 20°C is in the order A > B. For the pH region 3.5–5.4, β-lact A forms higher order aggregates beyond the dimer such as octamers. In the same pH range, the B variant can form mixed tetramers with the A variant, but B by itself only octamerizes very weakly.

Size-exclusion chromatography has been widely used to study protein self-associations. Quantitative analysis in these experiments is based on a linear partition isotherm model for each of the eluted species. The analytical model [13] for interpreting the frontal elution experiments assumes that the equilibrium between the interacting macromolecules is reached. Numerical simulations are needed to analyse the zonal elution experiments [14,15]. Several algorithms were described that assume rapid [16] or slow kinetic exchanges [17] between the interacting macromolecules.

The frontal elution behaviour of self-associating proteins was studied in subunit-exchange affinity chromatography by Chiancone and co-workers [18–20]. The experiments were analysed on the basis of an equilibrium isotherm expression describing the adsorption behaviour of a self-associating protein on a support where the monomer is immobilized. This experimental approach was applied to extract selectively β-lact A and B from whey [21].

Protein aggregation of β-lact A has been widely studied by Karger and co-workers [22–24] in hydrophobic interaction chromatography. At 4°C, pH 4.5 and high ammonium sulphate concentrations, the chromatograms revealed three peaks which were identified as tetramer, octamer and dodecamer species [22]. These stoichiometries were confirmed in a separate study by on-line low-angle laser light scattering [25]. The study at pH 4.5 of the adsorption isotherm of β-lact A on a methyl ether bonded phase by frontal analysis yielded an S-shape isotherm and the adsorption–desorption isotherms revealed hysteresis loops [23,24]. The role of association on the shape of the adsorption isotherm was demonstrated by Blanco *et al.* [23] and an adsorption isotherm expression is given, valid in the region of low protein concentration, where a linear Henry law can be applied to describe the adsorption behaviour of each individual macromolecule.

A model for protein aggregation was developed by Whitley *et al.* [26] on the basis of a multicomponent Langmuir isotherm accounting for axial dispersion, film mass transfer and intraparticle diffusion. The kinetic interconversion rate for a dimerized system was considered. It was shown that, with a slow reaction rate, the monomer and dimer forms are eluted as two separated species. When the reaction rate increases the chromatogram goes from two well resolved peaks to one broad peak. The model was applied to simulate, in the low concentration range, the elution profile of self-associating proteins and it agrees well with the experiments of Karger and Blanco [27].

Although the effect of association on the frontal elution behaviour of proteins has been widely studied, there are few examples showing the corresponding zonal elution behaviour in mass overload conditions. The importance of the adsorption isotherm model on the shape of the peak resulting from a pulse injection is already well known [28]. Numerical simulations have been used to predict elution profiles in non-linear zonal elution in ion-exchange chromatography [6,29–33]. The models, however, are generally based on a Langmuir [29–31] or a bi-Langmuir type of isotherm [32,33].

Recently, we applied the rigorous SDM adsorption isotherm expression to simulate the peak profiles of bovine serum albumin (BSA) eluted from an anion-exchange column [6], packed with silica coated with a copolymer of polyvinylpyrrolidone (PVP) and polyvinylimidazole (PVI). The elution behaviour of the protein at column saturation was well predicted. This work is a continuation of the previous one and we shall consider the adsorption of a self-associating protein, β-lact B, on the same anion exchanger.

The aim of this work was to study the non-linear zonal elution behaviour of β-lact B on an anion-exchange support. At pH 7.5 where the experiments were performed, one can assume a monomer–dimer equilibrium. The SDM model for ion-exchange adsorption was extended to describe the adsorption

behaviour of proteins with a rapid monomer–dimer kinetic exchange.

THEORY

Ion-exchange isotherm for a monomer–dimer equilibrium

Let us assume that solute P^{m-} and counter ions A^{n-} are in equilibrium with the dimer P_2^{2m-} and an anion exchanger X^+. The equilibria involved may be described by the following equations:

(a) For the liquid phase:

$$2P^{m-} \rightleftharpoons P_2^{2m-} \tag{1}$$

(b) For the exchange between P^{m-} and A^{n-}:

$$nP^{m-} + mX_nA \rightleftharpoons nX_mP + mA^{n-} \tag{2}$$

(c) For the exchange between P_2^{2m-} and A^{n-}:

$$nP_2^{2m-} + 2mX_nA \rightleftharpoons nX_{2m}P_2 + 2mA^{n-} \tag{3}$$

This set of equilibria is ruled by the following three equations:

$$\frac{[P_2^{2m-}]}{[P^{m-}]^2} = K_a \tag{4}$$

$$\frac{[A^{n-}]^Z \{X_mP\}}{\{X_nA\}^Z [P^{m-}]} = K_1 \tag{5}$$

$$\frac{[A^{n-}]^{2Z} \{X_{2m}P_2\}}{\{X_nA\}^{2Z} [P_2^{2m-}]} = K_2 \tag{6}$$

where $Z = m/n$. The brackets [ ] are used for molar concentrations in the mobile phase; the braces { } are used for molar pseudo-concentrations in the exchanger, *i.e.*, the amount of fixed anion per units of a reference volume V', which may be the volume of the mobile phase V_0 accessible to the protein.

The material balance gives for the global molar concentration C in moiety P

$$C = [P^{m-}] + 2[P_2^{2m-}] \tag{7}$$

and for the total amount Q_x of X^+ in the exchanger

$$\frac{Q_x}{V'} = n\{X_nA\} + m\{X_mP\} + 2m\{X_{2m}P_2\} \tag{8}$$

where the counter-ion concentration is assumed to be in large excess compared with that of the protein. The total amount Q of moieties P fixed on the exchanger is

$$\frac{Q}{V'} = \{X_mP\} + 2\{X_{2m}P_2\} \tag{9}$$

Using eqns. 7–9 enables $\{X_nA\}$ to be expressed as a function of Q:

$$\{X_nA\} = \frac{(Q_x - mQ)}{nV'} \tag{10}$$

Using the expressions for $\{X_mP\}$ and $\{X_{2m}P_2\}$ in eqns. 5 and 6 gives for Q

$$\frac{Q}{V'} = K_1 x + 2K_a K_2 x^2 \tag{11}$$

where

$$x = \left(\frac{\{X_nA\}}{[A^{n-}]}\right)^Z \cdot [P^{m-}] \tag{12}$$

The positive root of eqn. 11 can be written in the form

$$x = \frac{2Q/(K_1V')}{1 + \left(1 + \frac{8K_aK_2}{K_1^2V'} \cdot Q\right)^{\frac{1}{2}}} \tag{13}$$

The expression of the relationship between the concentration of the protein in solution, C, and the amount adsorbed, Q, is described by the following set of equations:

$$x = \frac{2Q}{K_1V'\left[1 + \left(1 + 8 \cdot \frac{K_aK_2}{V'K_1^2} \cdot Q\right)^{\frac{1}{2}}\right]} \tag{14}$$

$$[P^{m-}] = x\left(\frac{nV'[A^{n-}]}{Q_x - mQ}\right)^Z \tag{15}$$

$$C = [P^{m-}] + 2K_a[P^{m-}]^2 \tag{16}$$

One often uses the experimentally measurable quantity k', called the limit capacity factor:

$$k' = \frac{1}{V_0}\left(\frac{dQ}{dC}\right)_{C=0} \tag{17}$$

A more proper way of calculating it is given by the expression:

$$k' = \frac{1}{V_0\left(\frac{dC}{dp}\right)_{p=0}\left(\frac{dp}{dQ}\right)_{Q=0}} \tag{18}$$

where $p = [P^{m-}]$. The intermediate expressions are

$$\left(\frac{dp}{dQ}\right)_{Q=0} = \left(\frac{nV'[A^{n-}]}{Q_x}\right)^Z \left(\frac{dx}{dQ}\right)_{Q=0} \tag{19}$$

Since $(dx/dQ)_{Q=0} = 1/K_1V'$ and $(dC/dp)_{p=0} = 1$, we obtain finally for k'

$$k' = K_1 \cdot \frac{V'}{V_0}\left(\frac{Q_x}{n[A^{n-}]V'}\right)^z \tag{20}$$

The equilibrium isotherm as a function of k' reduces to

$$[P^{m-}] = \frac{2Q}{k'V_0}\left(1 - \frac{mQ}{Q_x}\right)^{-z}\left[1 + \left(1 + 8 \cdot \frac{K_aK_2}{V'K_1^2} \cdot Q\right)^{\frac{1}{2}}\right]^{-1}$$

$$C = [P^{m-}] + 2K_a[P^{m-}]^2 \tag{21}$$

This set of equations describing the equilibrium isotherm can be conveniently written as a function of the protein "surface coverage", $\theta = mQ/Q_x$, and in terms of reduced variables:

$$p' = [P^{m-}] \cdot \frac{mV_0}{Q_x} = \frac{2\theta}{k'}(1 - \theta)^{-z}[1 + (1 + 8 \cdot K_a'\theta)^{\frac{1}{2}}]^{-1}$$

$$C \cdot \frac{mV_0}{Q_x} = p' + 2K_a'p'^2 \tag{22}$$

where

$$K_a' = K_a \cdot \frac{Q_x}{mV_0} \text{ and } \beta = \frac{K_2V_0}{K_1^2V'}$$

Influence of the parameters on the shape of the adsorption isotherm

With given values of k', Z and β, Fig. 1a shows the influence of the parameter K_a' on the shape of the adsorption isotherm. Reduced coordinates were used for the graph and the surface coverage θ was plotted as a function of the total amount of protein in solution (CV_0), divided by the maximum amount that can be adsorbed (Q_x/m). For non-zero values of the association constant in solution, K_a, an S-shape is observed. The corresponding Scatchard plot (Fig. 1b) presents a maximum, which indicates a positive cooperativity.

When $K_a = 0$ (dashed line), the adsorption isotherm is that of the SDM model and the resulting Scatchard plot is a non-linear decreasing function. The observation of this hyperbolic-shaped diagram often suggests a heterogeneous nature of the adsorbent surface. As already emphasized, this conclusion is wrong with an ion exchanger if $Z \neq 1$. For comparison, a Langmuir-type isotherm ($Z = 1$, dotted line) and the corresponding linear Scatchard plot are also shown.

The influence of the parameter β on the shape of the equilibrium isotherm is shown in Fig. 2 for given k', Z and β values. The parameter β characterizes the role of the support in the protein aggregation process. Combining eqns. 5 and 6 leads to another expression for β:

$$\beta = \frac{K_2V_0}{K_1^2V'} = \frac{\{X_{2m}P_2\}}{\{X_mP_2\}} \cdot \frac{V_0}{K_aV'} \tag{23}$$

Therefore, β is proportional to the degree of association induced by the support relative to that in solution, the ratio $\{X_{2m}P_2\}/\{X_mP\}$ being considered as a pseudo-association constant for the adsorbed species in the reference volume V'. The protein self-association is increased with increasing β values (Fig. 2a) and larger amounts of protein are ad-

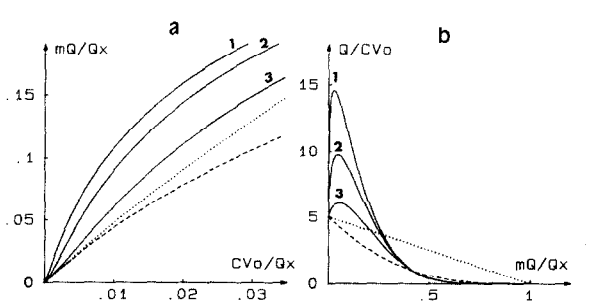

Fig. 1. Influence of the association constant on the shape of the adsorption isotherm of a self-associating protein. (a) Isotherm in reduced coordinates; (b) Scatchard plot. $k' = 5$; $K_a' = K_aQ_x/mV_0$; $Z = 3$. Solid lines, $Z = 3$, (1) $K_a' = 1000$, (2) $K_a' = 100$, (3) $K_a' = 10$; dashed lines, $Z = 3$, $K_a' = 0$; dotted lines, $Z = 1$, $K_a' = 0$.

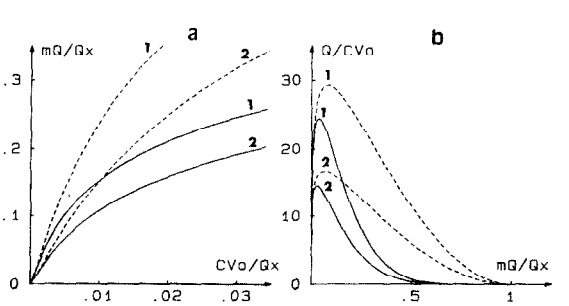

Fig. 2. Influence of the self-association in the adsorbed phase on the shape of the adsorption isotherm of a self-associating protein. (a) Isotherm in reduced coordinates; (b) Scatchard plot. $k' = 5$; $K_a' = 1000$; (1) $\beta = 2$; (2) $\beta = 1$. Solid lines, $Z = 3$; dashed lines, $Z = 1$.

sorbed. Similarly, the Scatchard plot indicates an increased cooperativity, with a higher maximum. The influence of β on the shape of the isotherm differs from that of K'_a in the high concentration range: an increase in β leads to a more convex shape for the decreasing part of the Scatchard plot, while the apparent saturation level is increased.

Fig. 2 shows the isotherm generated with the same parameters (dotted line), except that $Z = 1$. Previously [6], we studied the effect of increasing the ratio Z on the adsorption behaviour of non-associating proteins on an ion exchanger. It was shown that a lower apparent saturation level is reached for larger Z values. This is true also for a self-associating protein as shown in Fig. 2, where the isotherms with $Z = 3$ (solid line) and $Z = 1$ (dotted line) are compared.

Numerical simulations

On the basis of the isotherm model developed for the adsorption on an ion-exchange support of self-associating proteins undergoing a monomer–dimer equilibrium (eqn. 21), we used the numerical simulation methods to generate the theoretical zonal elution profiles.

The numerical method used to solve differential equations describing the propagation of the solute through the column has already been explained [6]. The method ensures mass conservation all through the simulated chromatographic process. The column is divided into an arbitrary number of discrete slices in which the equilibrium is assumed to be reached. The thickness of the slices was chosen so as to give a numerical dispersion equal to the "plate height" of the elution peaks [28].

The parameters defining the equilibrium isotherm are V_0, k', Q_x, m, Z, K_a and β (eqns. 21 and 23). The parameters m and Z can be determined experimentally from independent measurements according to the SDM model: one studies the variation of the retention volume of the solute *versus* the displacing salt concentration, measured at infinite dilution. The parameters k', Q_x, K_a and β are to be optimized by curve fitting of the theoretical model to the experimental profiles observed in mass overload conditions, for various amounts of protein injected.

The influence of the parameters K'_a and β on peak shapes was studied for a retention capacity factor k' equal to 5 (Fig. 3). The elution profiles are given in

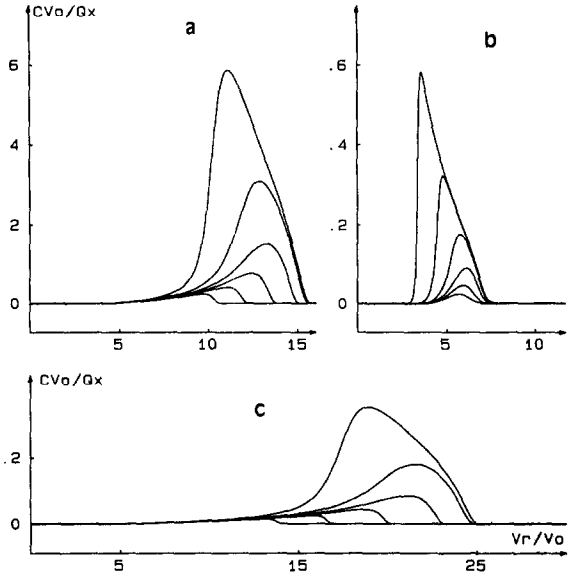

Fig. 3. Chromatographic simulations in isocratic elution on the basis of the isotherms in Figs. 1 and 2. $k' = 5$; $K'_a = K_aQ_x/mV_0$; $Z = 3$. (a) $K'_a = 1000, \beta = 1$; (b) $K'_a = 10, \beta = 1$; (c) $K'_a = 1000$, $\beta = 2$.

reduced coordinates and were simulated on the basis of the isotherms of Figs. 1 and 2. The influence of the association constant is illustrated in Fig. 3a and b, where the profiles are generated for two different values of the association constant, given in reduced units ($K'_a = 1000$ and $K'_a = 10$), but the same β parameter ($\beta = 1$). In agreement with the diagram in Fig. 1, the isotherm with a more convex shape leads to more diffuse fronting peaks, while a shock is observed at the end of the peak. An increase in β, while keeping K'_a constant (Fig. 3a and b), results in an increase in the front tailing at low concentrations and a marked second shock at the peak end.

EXPERIMENTAL

Equipment

Isocratic elution chromatography was conducted on a system consisting of a pump (HPLC PUMP 420; Kontron Instruments, Zürich, Switzerland) and a variable-wavelength UV detector (Spectra-100, Spectra-Physics, San Jose, CA, USA) operating at 280 nm. A sample injector (Model 7125; Rheodyne, Berkeley, CA, USA) with a 0.02-ml loop was used for injecting proteins in the low chromato-

graphic concentration range and a 0.5-ml loop was used for mass-overload experiments. The absorbance output was connected to a digital voltmeter (Model 3497; Hewlett-Packard, Palo Alto, CA, USA). The data stored on floppy disk were processed with a personal computer (Compaq Deskpro, Model 386/20e) equipped with an arithmetic coprocessor.

The column packing was the anion exchanger LiChrospher silica (particle size 10 μm and pore diameter 300 Å) (Merck, Darmstadt, Germany) coated with the copolymer PVP–PVI (75:25), cross-linked with butane-1,4-diol diglycidyl ether and prepared as described by Sébille and co-workers [34,35]. The ion-exchange capacity per unit volume was 0.15 mequiv./ml as determined by frontal analysis [6]. The chromatographic column (100 × 4.6 mm I.D.) was slurry-packed under pressure. The temperature of the column was regulated at 25°C by a thermostated water-bath.

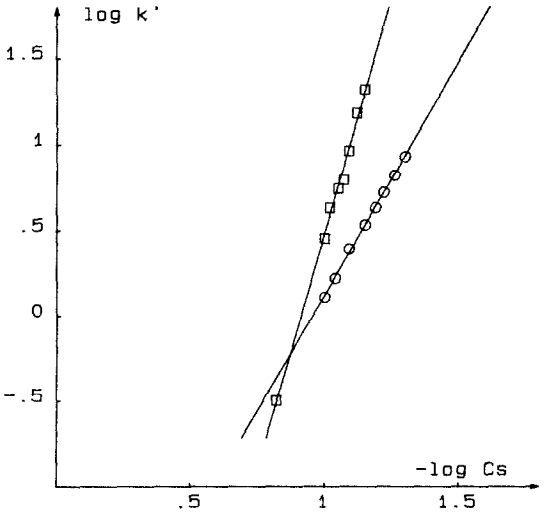

Fig. 4. Dependence of log k' on log(salt concentration) for β-lact B on an anion exchanger, PVP–PVI on silica. Flow-rate, 1 ml/min; temperature, 25°C; eluent, 0.020 *M* Tris buffer (pH 7.5). □ = NaCl; ○ = Na_2SO_4.

Chemicals

Bovine β-lact B was purchased from Sigma (St. Louis, MO, USA). The Tris buffer was obtained from Aldrich-Chemie (Steinheim, Germany).

Procedures

For isocratic elution, the eluent was 20 m*M* Tris buffer (pH 7.5) and the ionic strength was imposed by a monovalent salt (NaCl) or a divalent salt (Na_2SO_4). The protein solutions were prepared in the same eluent. The mobile phase volume accessible to the protein, V_0, was measured from the elution volume of α-chymotrypsinogen A, a protein that is not retained by the anion exchanger.

RESULTS

Retention studies of β-lactoglobulin B at infinite dilution

The value of Z was determined from the retention studies of the protein at infinite dilution according to the SDM model. The capacity factor k' corresponding to zero sample size is related to the displacing counter-ion concentration (A^{n-}) according to the equation

$$\log k' = a - Z\log(A^{n-}) \quad (24)$$

Fig. 4 illustrates the variations of k' with the concentration of two different displacing counter anions, Cl^- and SO_4^{2-}. This was achieved in the isocratic elution mode by adding various concentrations of NaCl and Na_2SO_4 to the eluent and measuring the retention times at the peak maximum, extrapolated to zero sample size. The Z value is 5.7 ± 0.3 with NaCl as the displacing salt and 2.7 ± 0.1 with Na_2SO_4. These values were calculated by a linear regression analysis and the errors are given for a 95% confidence interval. Within experimental errors, the result is in agreement with theory, *i.e.*, Z measured in the presence of a monovalent displacing ion is twice as large as that measured with a divalent ion. The value of the effective charge of the protein, m, is close to 6, a value selected for curve-fitting simulations, with $Z = 6$ when NaCl is used as the displacing salt and $Z = 3$ with Na_2SO_4.

Zonal elution profiles in the isocratic mode

Fig. 5a and b illustrate the elution peaks of β-lact B observed for increasing sample sizes with Na_2SO_4 and NaCl as the displacing salt, respectively (dashed lines). These experiments were performed in the range of low concentrations for the output signal, by using a 20-μl sample loop. The salt concentrations were chosen so as to obtain comparable k' values. Nevertheless, this was only partially achieved be-

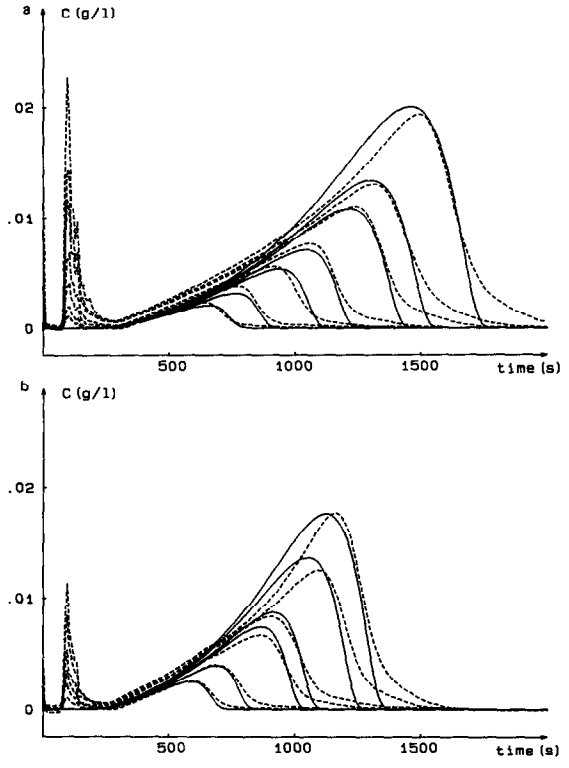

Fig. 5. Experimental and simulated elution profiles of β-lact B eluted from an anion-exchange support for small sample sizes. Sample loop, 0.020 ml; flow-rate, 1 ml/min; V_0 = 1.2 ml; column length, 10 cm; temperature, 25°C; eluent, 0.020 *M* Tris buffer (pH 7.5). Dashed lines, experiment; solid lines = theoretical model with Q_x, K_a and β parameters from Table I. (a) k' = 4, 0.035 *M* Na_2SO_4, sample size 0.005–0.2 mg; (b) k' = 3.6, 0.075 *M* NaCl, sample size 0.005–0.15 mg.

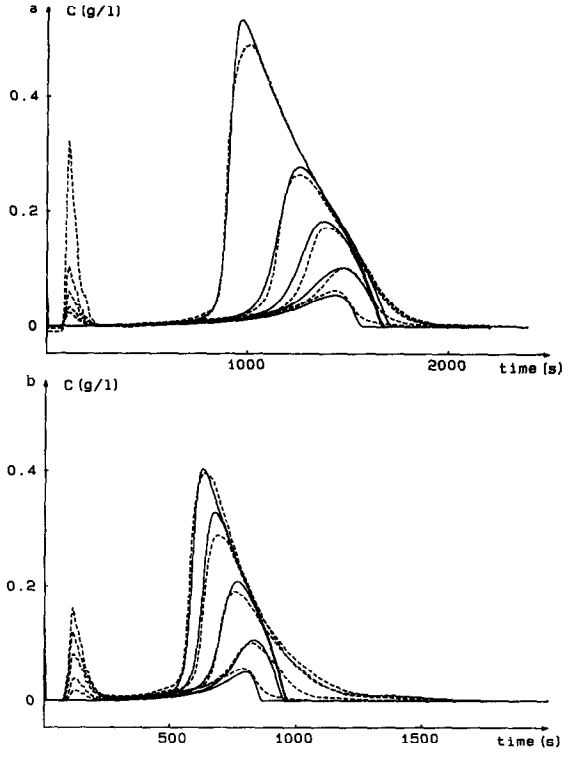

Fig. 6. Experimental and simulated elution profiles of β-lact B eluted from an anion-exchange support for large sample size. Sample loop, 0.5 ml; flow-rate, 1 ml/min; V_0 = 1.2 ml; column length, 10 cm; temperature, 25°C; eluent, 0.020 *M* Tris buffer (pH 7.5). Dashed lines, experimental values; solid lines, best fit of the simulated peak. (a) k' = 3.6, 0.045 *M* Na_2SO_4, sample size, 0.05–5 mg; (b) k' = 2.56, 0.085 *M* NaCl, sample size, 0.05–4 mg.

cause of the large retention time differences observed with small variations of the displacing salt concentration (eqn. 24).

When increasing amounts of β-lact B are injected into the column, the elution peaks begin to deviate from the original symmetric shape by exhibiting a diffuse front. This front tailing is enhanced and the retention time at peak maximum increases. This behaviour reveals a convave adsorption isotherm in the lower protein concentration range.

When increased mass overload conditions, Fig. 6a and b show that the chromatographic behaviour of β-lact B is totally modified. Larger amounts of protein were injected with a 0.5-ml sample loop, 40 times as large as that used in the experiments in Fig. 2. The diffuse front disappears, but a tailing at the rear edge of the peak is observed for large sample sizes and the retention time at the peak maximum decreases with increasing amounts of protein injected.

Curve fitting of theoretical peak profiles to experiments

The profiles for β-lact B observed with increasing sample sizes can be predicted by numerical simulations based on the isotherm model developed for the adsorption on an ion exchanger of a protein undergoing a monomer–dimer equilibrium. The parameters of the adsorption isotherm (eqn. 21) can be determined from the best fit of the theoretical peaks

TABLE I

ISOTHERM PARAMETERS OF β-LACTOGLOBULIN B IN MASS OVERLOAD CONDITIONS

Sample loop: 0.5 ml.

Salt	k'	Z	Q_x/m (mg)	K_a (l/mol)	β
Na_2SO_4 (45 mM)	3.6	3	150	$3.7 \cdot 10^5$	1.6
NaCl (85 mM)	2.7	6	150	$3.7 \cdot 10^5$	1.6

to the experimental ones obtained for large amounts injected (Fig. 6). This was achieved by fixing the three parameters measured at infinite dilution (k', Z, m) and determining Q_x, K_a and β from the least-squares fit of the theoretical profiles to the experimental values (full and dashed lines, respectively, in Fig. 6).

In Table I are listed the parameters of the isotherm determined from the best fit of the model to the experimental profiles observed in isocratic elution when a monovalent or a divalent counter ion is added to the eluent. The concentration of the displacing salt was selected so as to obtain reasonable k' values for performing the experiments. Fig. 7 shows the corresponding adsorption isotherm, in the presence of sodium sulphate (solid line, $Z = 3$). In agreement with the model, the parameters determined for large amounts injected (Table I) predict well the experiments in Fig. 5 performed at a different displacing salt concentration.

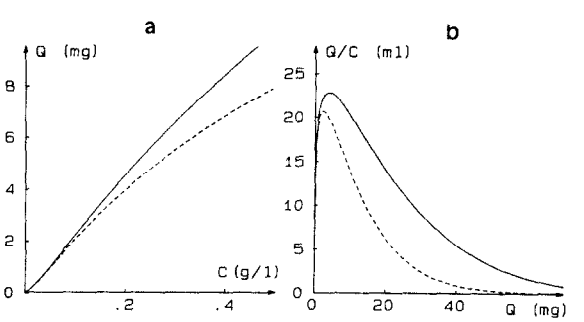

Fig. 7. Equilibrium isotherm of β-lact B adsorbed on the PVP–PVI anion-exchange support. (a) Adsorption isotherm; (b) Scatchard plot. Solid lines, model corresponding to the experiments in Fig. 6a, with $k' = 3.6$, $m = 6$, $Z = 3$, $Q_x = 900$ mg, $K_a = 3.7 \cdot 10^5$ l/mol, $\beta = 1.6$; dashed lines, model with the same parameters, except $m = Z = 6$.

DISCUSSION

The adsorption isotherm on an ion exchanger accounting for solute self-association exhibits an inflection point, with a concave shape towards the abscissa axis in the low concentration range and a convex shape near saturation (Fig. 7). This determines the increase in the retention volumes in the low sample size range and generates a rear shock with a shape more or less dispersed according to diffusion effects or slow mass-transfer kinetics. For larger amounts injected, a second shock appears at the front of the peak.

It is only possible to observe a tailing front in the low sample size range. This effect may not be noticed if large sample loops are employed with heavily loaded columns. For example, in work outlining the advantages of using displacement chromatography [11] for separating β-lact A and B on an anion exchanger, a tailing at the rear edge of the peaks is observed in isocratic elution, probably because of the large sample loop used and therefore the large sample sizes injected.

Simulations of the experimental profiles based on the isotherm assuming a monomer–dimer equilibrium describe well the elution profiles observed in mass-overload conditions. The same association constants K_a and the same maximum loading capacity for the protein are found with monovalent and the divalent counter ions. This result is in good agreement with theory and demonstrates the validity of the isotherm model that assumes protein self-association for describing the elution behaviour of β-lact B on the anion-exchanger support.

The maximum loading capacity of protein is $Q_x/m = 150$ mg, but as with the SDM model without association, an apparent saturation is reached for a smaller amount adsorbed (Fig. 7a). Comparison with the equilibrium isotherm (dashed line) generated with the same values of k' and Q_x/m, but with $Z = 6$ (monovalent displacing ion), indicates that the apparent saturation capacity decreases with increasing Z.

Fig. 7b presents the corresponding Scatchard plots. The S-shaped isotherm induces a positive cooperativity that indicates protein self-association. When there is no association, as previously shown for BSA adsorbed on the same anion exchanger [6], the isotherm has a convex shape and the correspond-

ing Scatchard plot deviates from linearity, indicating that the equilibrium isotherm is not of the Langmuir type. The plot has a hyperbolic shape located lower than the straight line generated with a Langmuirian isotherm ($Z = 1$).

The best fit of the theoretical model to the experimental peaks yields an association constant of $3.7 \cdot 10^5$ l/mol, which is of the same order as that measured in solution. The dimer–monomer equilibrium constant in solution of β-lact B near neutral pH was measured by several methods. The values of association constant obtained at 20°C and pH 7–7.5 by sedimentation equilibrium [36] and light-scattering measurements [37] are $1.26 \cdot 10^5$ and $1.42 \cdot 10^5$ l/mol, respectively.

The difference between the constant determined in this work and that reported in the literature may be due to the different experimental conditions used in this work (higher ionic strength of the solvent). Other reasons are inherent to the theoretical model applied, which is based on simplifying assumptions.

In agreement with the model, the constant K_a and the ratio β are the same with both displacing salts. The parameter β is characteristic of the degree of association of the protein by the support compared with that in solution (eqn. 23). Its value, larger than 1, shows that the adsorption process on the ion exchanger increases the protein self-association. However, the self-association constant of the protein in the adsorbed phase cannot be determined from the β value: this parameter is a function of the reference volume used to define the concentrations of the adsorbed species. When dealing with porous materials, V' may be the pore volume accessible to the protein.

The adsorption isotherm model (eqn. 21) is based on several assumptions and probably the equilibrium constant for associations in solution has to be considered as an apparent value. The model assumes that the charge number of the monomer remains unchanged after association. In fact, the association may mask some charges of the protein and therefore reduce the number of charges of the subunit when associated. Further, the model assumes that the surfaces available for adsorption of the monomer and dimer are equal. This may not be the case, as the Stokes radius of the monomer (2.0 nm) is significantly lower than that of the dimer (2.68 nm) [38].

On the other hand, the desorption step might be slower owing to slow mass exchange with the support. Probably slow mass-transfer kinetics from the bulk phase to the support are the main contribution to the overall kinetics. They are the main source of band broadening for proteins when eluted in isocratic elution [35]. This effect may be accounted for in peak simulations by using a global dispersive term [28].

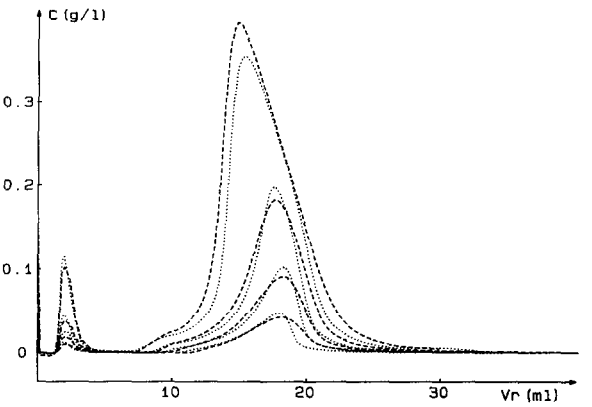

Fig. 8. Study at different flow-rates. Comparison of the experimental profiles of β-lact B eluted from a PVP–PVI anion-exchange column. Sample loop, 0.5 ml; sample size, 0.05–2.5 mg; column length, 10 cm; V_0 = 1.2 ml; temperature, 25°C; eluent, 0.020 M Tris buffer (pH 7.5), 0.04 M Na_2SO_4. Dotted lines, 0.5 ml/min; dashed lines, 1.5 ml/min.

Another limitation of the model is that it neglects the kinetic effects due to the slow dimer–monomer exchange: it assumes that the equilibrium is reached for the protein association. Kinetic effects can be revealed by performing the isocratic elution of β-lact B under mass-overload conditions at various flow-rates. The corresponding chromatograms are shown in Fig. 8, where peak profiles are given as a function of the retention volumes for two flow-rates.

For a given mass injected, the general peak shapes observed at 0.5 and 1.5 ml/min are almost the same, except for the diffusive part. This diffusion and also peak tailing become more important with increasing flow-rate. This observation qualitatively shows that kinetic effects are not negligible and do have an influence on peak shapes.

These effects may be due to the slow kinetics of the dimerization of the β-lact B. Indeed, in this study, the rate of the monomer–dimer interconversion during the reversible association has been assumed to be so high that the association equilibrium is

established almost instantaneously. In previous studies of the dimerization of β-lact B near neutral pH, the equilibrium was found to be established immediately on mixing the solution [37,38].

It is difficult to evaluate the relative contribution between mass-transfer kinetics and the slow monomer–dimer kinetic exchanges. Moreover, the chromatograms in Fig. 8 show that the profiles at the two different flow-rates superimpose well enough to be described by a single theoretical profile assuming instantaneous chemical equilibrium.

CONCLUSIONS

This study has shown that the rigourous adsorption isotherm derived from the SDM could be extended to establish the adsorption isotherm expression of self-associating proteins in ion-exchange chromatography. The comparison of the elution profiles predicted on the basis of the SDM theory and the experimental profiles provided information on the nature of the adsorption process on ion exchangers. Using the appropriate parameters derived from the numerical simulations, it has been demonstrated that the β-lact B isotherm is S-shaped and the protein displays a positive cooperativity on adsorption on the ion-exchange surface as indicated by the corresponding Scatchard plot. These results described well the adsorption behaviour of a protein undergoing monomer–dimer equilibrium and predicted well the zonal elution behavior of β-lact B at pH 7.5.

As was shown in the previous work on the adsorption isotherm of a non-associating protein (BSA) in anion-exchange chromatography [6], the non-linear effects in zonal elution due to column overloading could be reduced by using a counter ion with higher valency.

These results indicate that protein aggregation and the value of the ratio Z play a significant role in protein adsorption isotherms in ion-exchange chromatography and should be considered for optimizing protein separations on a preparative scale.

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