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# ROLE OF ASSOCIATION ON PROTEIN ADSORPTION ISOTHERMS

# β-LACTOGLOBULIN A ADSORBED ON A WEAKLY HYDROPHOBIC SURFACE

R. BLANCO[a], A. ARAI, N. GRINBERG[b], D. M. YARMUSH[c] and B. L. KARGER*
*Barnett Institute of Chemical Analysis and Materials Science, and Department of Chemistry, Northeastern University, 341 Mugar Building, Boston, MA 02115 (U.S.A.)*
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## SUMMARY

This paper explores the role of association on the adsorption isotherms of β-lactoglobulin A on a weakly hydrophobic stationary phase at 4°C and mobile phases of 0.85 *M* and 1 *M* ammonium sulfate, pH 4.5. The isotherms, obtained by frontal analysis, show an S-shape and the corresponding Scatchard plots indicate positive cooperativity. The slopes and intercepts of the Scatchard plots at low solute concentration are analyzed in terms of two species —a protomer and a higher order stronger adsorbing species. An explicit equation of the isotherm is developed based on this model, and this expression is shown to reproduce the isotherm shape using the appropriate derived parameters. It is further shown from this equation that a Langmuir-shaped adsorption isotherm can be obtained if the higher order associate or aggregate binds weaker to the support than the protomer. These results indicate that protein–protein interactions and the formation of associates can play a significant role on the shape of the isotherm and ultimately on the behavior of the species in preparative scale chromatography.

## INTRODUCTION

The adsorption of proteins at the liquid–solid interface is under active study for chromatography[1] and in the design of biomaterials[2,3], among other areas. The adsorption process has been studied using both static[4–6] and dynamic methods[7,8]. It has been established that the dynamic measurement of adsorption isotherms, *e.g.*, frontal analysis, provides a good general description of the process[9] and is relevant for chromatography[10].

Recently, the importance of adsorption isotherms as a tool in the prediction of

[a] Present address: Universidad de Costa Rica, Chemistry School, San Pedro 2060, Costa Rica.
[b] Present address: Merck, Sharp and Dohme Research Labs., Rahway, NJ 07065, U.S.A.
[c] Dept. of Chemical and Biochemical Engineering, Rutgers University, Piscataway, NJ 08855, U.S.A.

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elution behavior in preparative scale chromatography of small and large molecules has been emphasized[10,11]. Several important points can be learned from these studies and that of others: (1) highly precise data is required for differentiation between various adsorption mechanisms[12]; (2) the shape of the isotherm near the origin is important for determining the chromatographic elution profile[13]; and, (3) there is a lack of an understanding of the macroscopic distribution processes involved in adsorption under chromatographic conditions.

With respect to proteins, models of adsorption generally use the Langmuir isotherm to describe the non-linear adsorption behavior[14–16]. The Langmuirian isotherm is only an experimental fit of the adsorption data, since the system may be far removed from the actual Langmuir model, *e.g.* fixed homogeneous adsorption sites, no solute lateral interaction on the surface, ideal solution behavior.

In preparative scale liquid chromatography, injection of relatively high concentrations of substances is a common practice[17]. Protein–protein interactions leading to association or aggregation can be an important consideration at high concentrations; however, if precipitation does not occur, association may be neglected as a significant factor on chromatographic behavior, especially when general recommendations for preparative scale separation of proteins are presented[18]. Multiple equilibria between oligomers opens a new dimension of complexity in such systems, and subtle and/or major effects on adsorption behavior can be expected. An understanding and control of such behavior can be important in liquid chromatography, as well as in general protein adsorption phenomena.

A classical example of a protein aggregating system is $\beta$-lactoglobulin A ($\beta$-lact A)[19]. Previously, we have studied this protein at pH 4.5, 4°C, under hydrophobic interaction chromatographic (HIC) conditions, to illustrate the formation of oligomeric species and the resultant chromatographic behavior[20]. From a pure sample, several chromatographic peaks were observed whose relative amount depended on the injected concentration. Using a mass balance mathematical model, it was possible to determine that the first eluting peak was a dodecamer, the second a tetramer and the third an octamer. This stoichiometry was in agreement with molecular weight determinations using low angle laser light scattering (LALLS)[21]. The model that emerged was rapid aggregation of $\beta$-lact A upon injection into the chromatographic column.

As a continuation of this work, we have measured adsorption isotherms under weak hydrophobic surface binding conditions in order to study further the role of association or aggregation on protein adsorption. Positive cooperativity has been found, and this behavior has been interpreted in terms of associate formation with the higher order associate binding stronger to the adsorbent than the protomer. Various aspects of the adsorption behavior of $\beta$-lact A have been deduced from Scatchard plots, and an equation of the adsorption isotherm developed. Based on this equation, conditions are explored under which a Langmuir isotherm may be obtained as a result of protein association. Other studies, to be reported separately, reveal a hysteresis loop when the desorption isotherm is measured. It is not surprizing to find complex behavior in the distribution of proteins with solid surfaces.

## EXPERIMENTAL

### *Equipment*

Frontal and elution chromatography were conducted on a Model 334M liquid chromatography system (Beckman Instruments, San Ramon, CA, U.S.A.) equipped with a UV–VIS variable-wavelength detector, Model LC90 B10 (Perkin-Elmer, Norwalk, CT, U.S.A.), and a Beckman 210C sample injection valve. The absorbance signal was output to a recorder (Linear Instruments, Reno, NV, U.S.A.) and simultaneously processed by a data acquisition system (Nelson Analytical, Cupertino, CA, U.S.A.).

The column packing consisted of Vydac silica gel (Separations Group, Hesperia, CA, U.S.A.) bonded with a methyl polyether phase (particle size 5 $\mu$m, pore diameter 300 Å, specific surface area 72 $m^2/g$) and prepared as described elsewhere[22]. The surface coverage was 6.3 $\mu mol/m^2$, as determined by elemental analysis (assuming a stoichiometry of 2 for the binding of the silane to silica). Columns (2.78 cm × 0.29 I.D.) were slurry packed under pressure using a methanol–carbon tetrachloride solution (10:90, v/v). The temperature of the column, injection loop and tubing before the detector was regulated at 4.0°C, by a Neslab Exacal EX-300 water bath (Portsmouth, NH, U.S.A.) with an independent U-Cool Neslab cooling system.

### *Chemicals*

HPLC water, chromatographic grade organic solvents, acetic acid, ammonium hydroxide, and reagent grade ammonium sulfate were purchased from J. T. Baker (Phillipsburg, NJ, U.S.A.). HEPES (N-2-hydroxyethyl-piperazine-N-2-ethane-sulfonic acid) and MES [2-(morpholino)ethane sulfonic acid] were obtained from Research Organics (Cleveland, OH, U.S.A.). Bovine $\beta$-lact A, electrophoretically pure, was purchased from Sigma (St. Louis, MO, U.S.A.) and used without further purification.

### *Procedures*

Mobile phases were prepared by adding the correct weight of salt and buffer (20 m*M* HEPES, 20 m*M* MES, 20 m*M* acetic acid) to a volumetric flask containing HPLC water. The pH was adjusted to 4.5 with either ammonium hydroxide or acetic acid. Solutions were filtered and degassed under vacuum before use. Protein solutions were freshly prepared immediately before injection.

For isotherm measurements, the column was first equilibrated with the mobile phase buffer. The protein solution was then loaded through a temperature controlled loop (400 $\mu$l). When the outlet concentration of the sample reached a plateau in frontal development, the pure mobile phase buffer was switched into the column. Before each run, the column was reequilibrated with the mobile phase for 20 min. In order to detect systematic errors due to poor surface regeneration, a random procedure was followed to define the sequence of protein sample concentrations. The result of this set of experiments did not show any difference in loading (within experimental error) with samples injected in an increasing or decreasing concentration sequence. Protein concentration was monitored at a wavelength of 303 nm or 313 nm (for high concentration samples). Calibration plots were obtained at both wavelengths.

In order to obtain precise protein adsorption loadings, the flow-rate of the

mobile phase (20 μl/min) was measured during each run at 1–2 min intervals using a 1-ml calibrated Corning disposable micropipette, connected to the outlet of the detector. The volume of the stationary phase and the hold-up volume of the column were determined by a standard weight difference procedure, using methanol and carbon tetrachloride.

The amount adsorbed, $Q$, was determined from the area of the shaded portion of the breakthrough curve, see Fig. 1. The precision in $Q$ between three independent injections of the same protein solution was better than 3% in the intermediate concentration region (8–12 mg/ml of protein) and less than 7% in the low concentration region (< 5 mg/ml of protein). The poorer precision at the low concentration end is due to the intrinsic error of the calculation method, in which absolute concentration errors are constant over the range of concentration, and consequently, the relative errors are larger for smaller concentrations. Nevertheless, high overall precision has been obtained for the isotherm measurements.

## RESULTS AND DISCUSSION

### *Isotherms*

Mobile phases of 0.85 *M* and 1.0 *M* ammonium sulfate were selected for frontal analysis isotherm determinations in order to reduce the adsorption coefficients of the oligomers to measurable solution concentration levels. Fig. 1 shows a typical break-through curve for β-lact A at pH 4.5 and 4°C, illustrating a diffuse front boundary. As is well known, a diffuse front is typical of an anti-Langmuir isotherm[23].

It is interesting to note that over the whole concentration range, identical isotherms were obtained with protein solutions freshly prepared and with solutions left for up to one day at 4°C. In previous studies[20], we found that protein precipitate was observed upon standing; however, the antichaotropic salt concentration was 2–3 *M*. Evidently, the salt concentrations in the present work are sufficiently low that any aggregation in solution does not lead to precipitation.

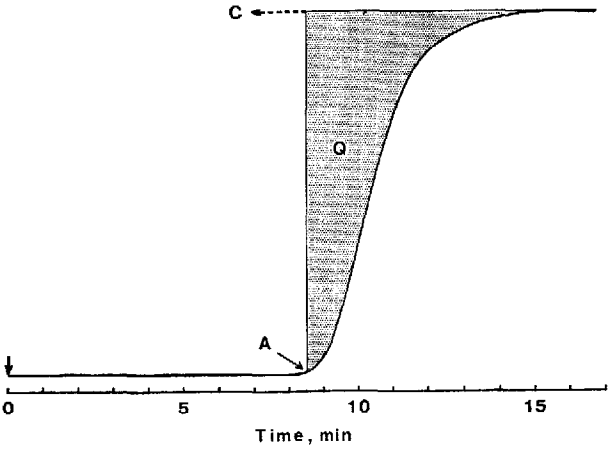


Fig. 1. Typical breakthrough curve using frontal analysis. The arrow at time 0 indicates loading of β-lact A (20 mg/ml) with a flow-rate of 20 μl/min. Point A signifies the characteristic point where the species begins to elute. Conditions: mobile phase: 0.85 *M* ammonium sulfate, 20 m*M* HEPES, 20 m*M* MES, 20 m*M* acetic acid, pH 4.5, 4°C. Column: 2.78 × 0.29 cm I.D.; packing: 5 μm C-1 ether phase.

It should also be pointed out that, from a macroscopic point of view, the information obtained from a breakthrough curve is independent of the mass transfer kinetics of the adsorption process[9]. Indeed, $Q$ was found to be independent of flow-rate over a five-fold range for a given protein solution concentration. Therefore, the values of $Q$ obtained from these experiments are actual measurements of the amount of protein adsorbed on the stationary phase, when the plateau-total protein concentration is $C$ (see Fig. 1), and one can consider that the system reaches a quasi-equilibrium state that can be modeled using a mass balance approach (see below).

It is interesting to note that the desorption step (not shown) is diffuse as well. This means that the desorption isotherm is Langmuirian in shape and that there is a hysteresis loop in the adsorption–desorption isotherm. Hysteresis is a well-known phenomenon in protein adsorption where, for example, it has been shown that conformational changes on an adsorbent surface can result in altered species which bind to the surface differently (usually stronger) than the species which initially is

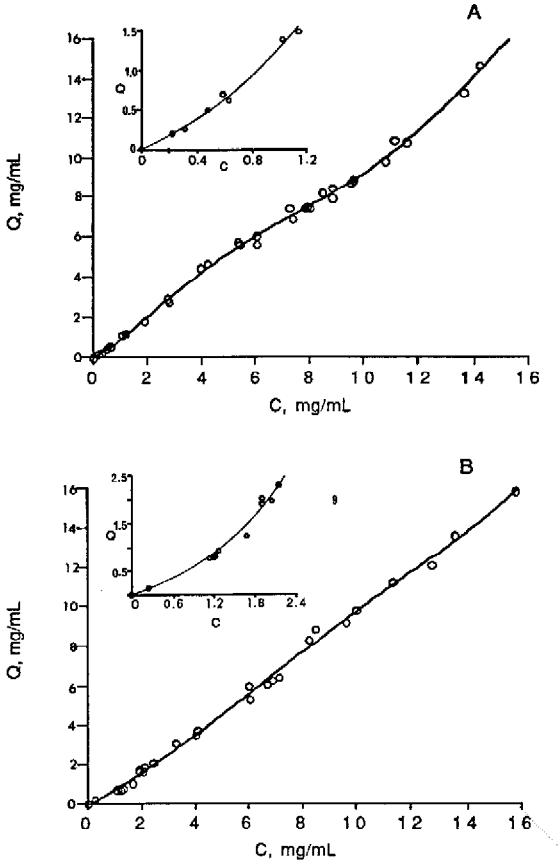


Fig. 2. (A) Adsorption isotherm of β-lact A at 0.85 $M$ ammonium sulfate, other conditions as in Fig. 2. Inset: The low concentration region of the isotherm to emphasize the S-shape. Q = adsorbed amount of protein, C = solution concentration of protein. (B) Adsorption isotherm of β-lact A at 1.0 $M$ ammonium sulfate, other conditions as in Fig. 1. Inset: The low concentration region of the isotherm to emphasize the S-shape.

adsorbed[24,25]. Another mechanism of hysteresis could be loss of reversible association–dissociation of aggregates on the adsorbent surface. A later paper will deal with the desorption loop and the mechanism of hysteresis for β-lact A; in this paper, we shall focus on the adsorption step. As noted, we shall model the adsorption step as a quasi-equilibrium process, as others have done when hysteresis is involved[24,25].

Fig. 2A and B present the adsorption isotherms obtained at 4.0°C, pH 4.5 at the two salt concentrations. These isotherms are S-shaped, *i.e.* proportionately greater adsorption as the sample solution concentration is increased. The S-shape is suggestive of positive cooperativity in which association occurs between molecules either by a side-by-side association on the surface[26], or by association in solution, in which one or more higher order oligomers formed at higher solution concentration adsorb more strongly than the protomer. It is to be noted that the isotherm shape in Fig. 2 is unusual in protein adsorption[27]; as already mentioned, Langmuirian shape is typically observed.

Cooperativity of biopolymers interacting with ligands has been studied using various mathematical models and plotting procedures[28–30]. The Scatchard plot is one of the most widely used approaches to obtain a macroscopic indication of the type of cooperativity in the binding reaction. This approach has also been employed in the study of adsorption of proteins on solid supports[31,32]. In order to apply this approach

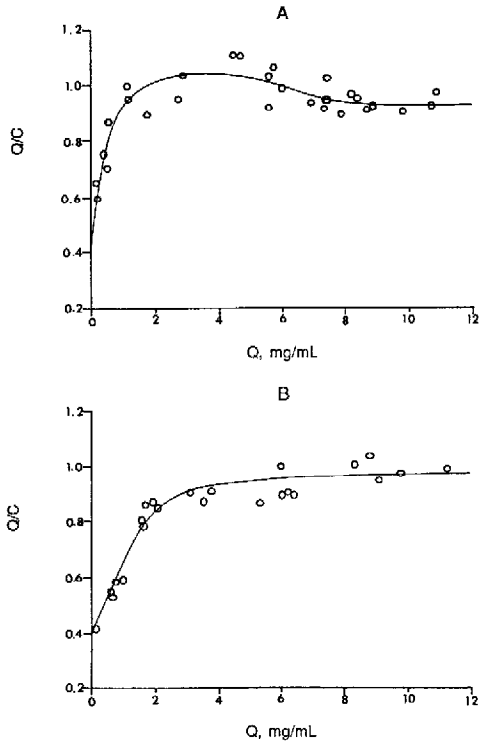


Fig. 3. (A) Scatchard plot of β-lact A derived from the data in Fig. 2A, 0.85 *M* $(NH_4)_2SO_4$. (B) Scatchard plot of β-lact A derived from data in Fig. 2B, 1.0 *M* $(NH_4)_2SO_4$.

to adsorption studies, the concentration of the ligand is maintained constant (*i.e.*, the adsorbing surface), while the concentration of protein is varied.

Fig. 3A and B present Scatchard plots derived from the isotherms in Fig. 2A and B, respectively. $Q$ is the adsorbed amount of protein in mg/ml of adsorbent phase, and $C$ is the plateau total protein solution concentration, in mg/ml. In agreement with the S-shaped isotherms, a positive initial slope, characteristic of positive cooperativity in the low protein concentration region[29], is observed in the two plots. Typical Scatchard plots obtained for systems of positive cooperativity generally present a well-pronounced maximum[33]. In Fig. 3A, a weakly-pronounced maximum is observed at approximately 5–6 mg/ml protein concentration, whereas no maximum can be discerned in Fig. 3B. A plateau is observed in the region beyond 6 mg/ml in both cases. One interpretation of such a plateau is the appearance at higher protein concentration of an associated species with a weaker binding affinity for the surface than the other species existing at low concentration[34]. Since a weighted average distribution coefficient is measured, changes in the relative amount of species with different affinities as a function of overall protein concentration will have an obvious effect on the measured extent of adsorption. It is interesting to note that in the previous HIC experiments[20], it was found that the highest order aggregate adsorbed the weakest.

*Mass balance model*

Based on the literature of $\beta$-lact A at pH 4.5 and 4°C[19] and the HIC results from our previous paper[20], it is reasonable to assume that the protein forms associates or aggregates in solution as a function of concentration. Accordingly, as previously[20], the behavior described in Fig. 3A and B has been analyzed using a mass balance model. We will focus on the low protein concentration region and assume the simplest case that two species predominate in this region of positive cooperativity of the Scatchard plot (below 5.0 mg/ml), with the higher order aggregate adsorbing more strongly than the protomer.

Consider a protomer A that can reversibly form in solution an associate, B, with $n$ protomeric units:

$$n\mathrm{A} \rightleftharpoons \mathrm{B} \qquad [\mathrm{B}] = K_e\,[\mathrm{A}]^n \tag{1}$$

where $n > 1$ and $K_e$ is the association constant in molar concentration units ($M^{1-n}$) for the reaction. The activity coefficients of the species cancel in the concentration ratio of $K_e$, based on the Adams-Fujita assumption[20,35].

Since a fraction of protomer A and associate B can bind to the available surface, $S$, each species with a variety of orientations, the adsorption process can be visualized as one of multiple steps. Each adsorption step can be defined by the number of binding sites used for each fraction of a protein species with a determined orientation. If $q$ is identified as the mean number of binding sites of species A, and $p$ as the mean number of adsorption sites of species B, adsorption can be represented for A as

$$\mathrm{A} + q\mathrm{S} \rightleftharpoons \mathrm{AS}_q \tag{2}$$

$$k_{q,\mathrm{A}} = [\mathrm{AS}_q]/[\mathrm{A}]\,[\mathrm{S}]^q \tag{3}$$

and for B as

$$B + pS \rightleftharpoons BS_p \tag{4}$$

$$k_{p,B} = [BS_p]/[B]\,[S]^p \tag{5}$$

where $p \leqslant nq$ and $k_{i,j}$ is the adsorption coefficient for species $j$, which binds the surface with a mean value of $i$ binding sites. The value of $p$ can be less than $nq$ if some of the binding sites are used to form the associate.

The variables in the Scatchard plot ($Q$ and $C$) can be obtained from eqns. 3 and 5. The total amount of protein on the surface, $Q$, in mg/ml of packing, can be defined as

$$Q = M_A([AS_q] + n[BS_p]) \tag{6}$$

or

$$Q = M_A([A][S]^q\, k_{q,A} + n[B][S]^p\, k_{p,B}) \tag{7}$$

where $M_A$ is the molecular weight of the protomer. If we assume that the available surface, $S$, is much larger than the adsorbed amount of protein in the region of low protein concentration, then $S$ can be considered to be a constant. Therefore, eqn. 7 can be simplified to

$$Q = M_A([A]\, k_A + n[B]\, k_B) \tag{8}$$

where $k_A = [S]^q\, k_{q,A}$ and $k_B = [S]^p\, k_{p,B}$. Combining eqns. 1 and 8, we obtain

$$Q = M_A(k_A\,[A] + nK_e k_B\,[A]^n) \tag{9}$$

and with the conservation of mass in the mobile phase and eqn. 1, we can write that

$$C = M_A([A] + nK_e\,[A]^n) \tag{10}$$

where $C$ is the solution concentration of $\beta$-lact A in mg/ml. The ratio $Q/C$, used in the Scatchard plot, can then be written as

$$Q/C = (k_A + nK_e k_B\,[A]^{n-1})/(1 + nK_e\,[A]^{n-1}) \tag{11}$$

Eqn. 11 provides an explicit expression of an experimental quantity, $Q/C$, in terms of the protomer solution concentration in the mobile phase [A]. This latter parameter is not directly accessible but follows the general trend of $C$ in the low concentration region. Since $[A] \rightarrow 0$ as $C \rightarrow 0$, from eqn. 11, the value of $Q/C$ at zero concentration of protein reduces to $k_A$, the adsorption coefficient of A. The value of $k_A$ can thus be obtained from the intercept of the Scatchard plot. The results from such an extrapolation were found to be: $k_A = 0.34 \pm 0.09$ and $0.43 \pm 0.04$, at 0.85 and 1.0 $M$ ammonium sulfate, respectively. These adsorption coefficient values are reasonable given the relatively low antichaotropic salt concentration employed.

In order to obtain $k_B$ and $K_e$, the initial slopes of the Scatchard plots ($Q/C$ *vs.* $Q$) can be used along with the slopes of an alternative plotting procedure of $Q/C$ *vs.* $C$. Defining $R = Q/C$, we have

$$dR/dQ = (dR/d[A])\,(d[A]/dQ) \tag{12}$$

and

$$dR/dC = (dR/d[A])\,(d[A]/dC) \tag{13}$$

Explicit relationships for each term in eqns. 12 and 13 can thus be derived by taking the derivatives of eqns. 9–11 with respect to [A]. These expressions evaluated in the limit when $C \rightarrow 0$ ([A] $\rightarrow$ 0) yield values of the limiting slope in each plot:

$$\lim_{C\rightarrow 0}\,(dR/dQ) = 2K_e\,(k_B - k_A)/k_B \tag{14}$$

$$\lim_{C\rightarrow 0}\,(dR/dC) = 2K_e\,(k_B - k_A) \tag{15}$$

From eqns. 14 and 15, the ratio of the two limiting slopes directly yields the value of $k_B$. The results obtained for $k_B$ were $1.2 \pm 0.2$ and $1.5 \pm 0.3$ at 0.85 $M$ and 1.0 $M$ salt concentration, respectively. These values are higher than the corresponding $k_A$ values and reflect the increase in binding for the higher order aggregate relative to the protomer. It is noted from eqn. 14 that an initial positive slope in the Scatchard plot will always be observed if $k_B > k_A$, a condition that is fulfilled by this system. The value of $K_e$ can be determined from $k_A$ and $k_B$ in eqns. 14 and 15 as: $K_e = (0.90 \pm 0.50) \cdot 10^4$ $M^{1-n}$ and $(2.7 \pm 0.8) \cdot 10^4$ $M^{1-n}$ at 0.85 $M$ and 1.0 $M$ salt concentration, respectively.

Based on these results, we next explored the numerical simulation of the data (*i.e.*, the isotherms in Fig. 3) in order to examine how well the model can predict experimental behavior. The adsorption isotherms can be reconstructed using eqns. 9 and 10, which relate experimental concentrations with the concentration of protomer [A]. Selecting the simplest case of $n = 2$ in eqn. 10, the resulting quadratic equation can be resolved for the solution concentration of ($A$)

$$[A] = [(1 + 8K_e^*C)^{1/2} - 1]/4K_e \tag{16}$$

where $K_e^*$ is the apparent association constant in concentration units of mg/ml. Eqn. 16 can then be substituted into eqn. 9 to yield

$$Q = \{(k_B - k_A)\,[1 - (1 + 8K_e^*C)^{1/2}] + 4k_BK_e^*C\}/4K_e^* \tag{17}$$

Eqn. 17 provides an explicit expression for the adsorption isotherm. Fig. 4 displays the calculated isotherm at pH 4.5, 4°C and 1.0 $M$ ammonium sulfate. There is reasonable agreement between the calculated and the experimental isotherms. Fig. 5 shows the corresponding calculated and experimental Scatchard plots, where the most significant features observed experimentally, *i.e.* the positive slope and the plateau at higher protein concentration are reproduced. At high $Q$ values, the estimated $Q/C$ values appear to be greater than the experimental values; however, a good fit appears in the

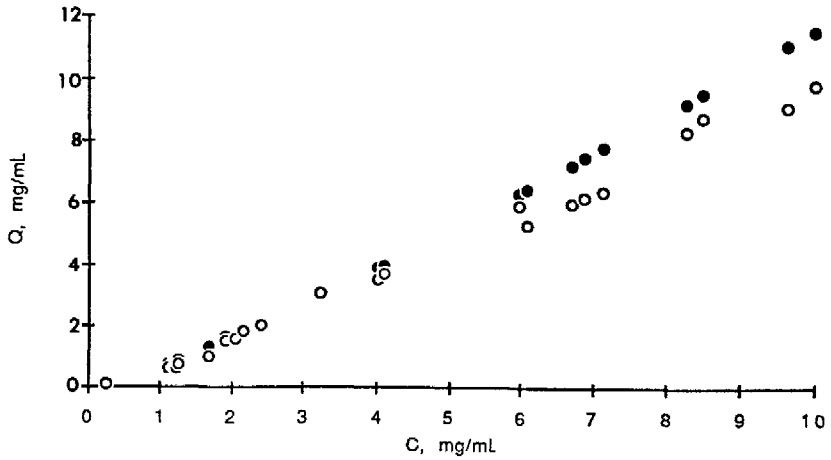


Fig. 4. Comparison of experimental (○) and calculated (●) isotherms of β-lact A. Calculation based on eqn. 17 using parameters $k_A = 0.80$, $k_B = 3.0$, and $K_e = 1.0 \cdot 10^4\ M^{-1}$ obtained from the model. Salt concentration, 1 $M$ $(NH_4)_2SO_4$. Other conditions as in Fig. 1.

low to moderate $Q$ range. The poorer fit at higher protein concentration may be related to the formation of a larger aggregate with weaker strength than the protomer. We briefly examined the adsorption isotherm assuming the formation of a third species ($n = 3$) with weaker binding than the protomer. If the third component is considered in the calculation of $Q$, as in eqn. 17, the numerical result is qualitatively similar to Figs. 5 and 6; however, a cubic equation results and the precision of the parameters unfortunately does not allow a good estimation of the fit of the experimental data. Nevertheless, the appearance of a third component could be the cause of the poorer fit at high $Q$ values in Fig. 5 (as well as the plateau in Fig. 3).

Eqn. 17 can be further used to simulate the possible shape of isotherms in which the values of the adsorption coefficients and association constants are different from

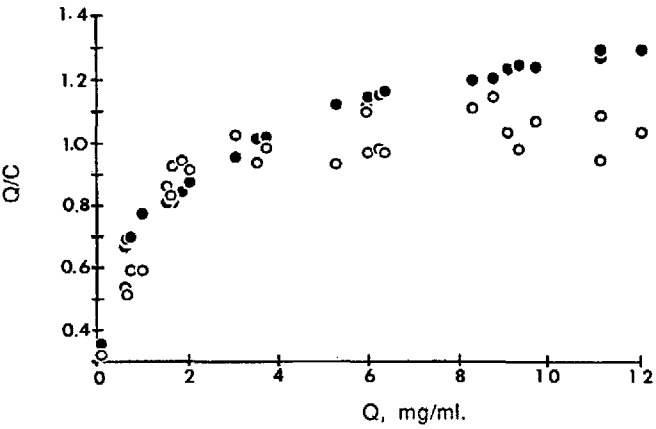


Fig. 5. Scatchard plots derived from the comparison of experimental (○) and calculated (●) isotherms of β-lact A in Fig. 4.

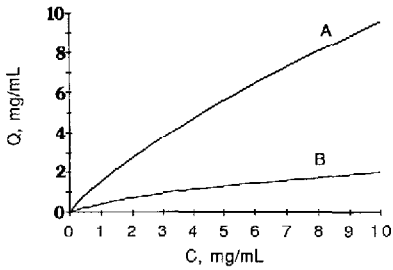


Fig. 6. Calculated isotherms from eqn. 17 for different set of values of adsorption coefficients and equilibrium constants. (A) Protomer (A) more retained than aggregated species (B): $k_A = 3k_B$, $K_e = 1 \cdot 10^4$ $M^{-1}$. (B) No retention of aggregated species: $k_A = 1.0$, $k_B = 0$, $K_e = 1 \cdot 10^4$ $M^{-1}$. The protomer and aggregated species are assumed to be in the stoichiometric ratio of 1:2, respectively.

those obtained in this system. For example, curve A in Fig. 6 corresponds to a case in which the association constant has the same value as that observed for $\beta$-lact A ($K_c^* = 0.75$ ml/mg, equivalent to $K_e = 1 \cdot 10^4\ M^{-1}$) but the protomer A is more adsorbed than the associate B ($k_A = 3k_B$). This behavior corresponds to negative cooperativity, and, interestingly, the isotherm has a Langmuirian shape. Curve B represents the case of zero adsorption of the associate B ($k_B = 0$), calculated with the same association constant as curve A. A Langmuirian shape is also found. From Fig. 6, it can be concluded that associating systems can show adsorption isotherms that follow Langmuirian mathematical behavior in spite of the fact that the system may be far removed from the Langmuir adsorption model.

## CONCLUSIONS

It has been demonstrated that a self-associating protein, $\beta$-lactoglobulin A, displays positive cooperativity upon adsorption under HIC conditions. This behavior is consistent with the presence in the mobile phase of several aggregated species with different adsorption coefficients. The formation of associates can produce an S-shaped isotherm as a result of the increase with total protein concentration of the relative amount of an associate with a larger adsorption coefficient than the protomer. The observed isotherm can be modeled as a mixed competitive one. This behavior is characteristic of positive cooperativity, where an increase in the analyte concentration results in a greater than linear increase in the amount of protein adsorbed. As already noted, the desorption step does not follow the S-shaped adsorption isotherm, creating a hysteresis loop. Desorption and hysteresis will be discussed in a separate paper.

A conclusion of this work is that protein–protein interaction and the formation of aggregates in preparative scale operation must be considered, even if the protein remains in solution over the whole concentration range of the isotherm. The use of LALLS[21] may prove beneficial in the elucidation of the actual factors causing non-linearity of a peptide–protein adsorption isotherm.

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