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Characterization of enthalpic events in overloaded ion-exchange chromatography

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Abstract

Protein adsorption can be either endothermic or exothermic depending upon the protein, the sorbent and process conditions. In the case of protein adsorption onto ion-exchange surfaces exothermic adsorption heats are usually characterized as representing the electrostatic interaction between two oppositely charged surfaces. Endothermic adsorption heats are typically characterized as representing protein reconfiguration and/or repulsive interactions between adsorbed molecules. In certain segments of the literature surface dehydration and solution non-idealities have been suggested as possible sources of endothermic heats of adsorption. Each of these phenomena was investigated during studies concerning the adsorption of bovine serum albumin and ovalbumin onto an anion-exchange sorbent. The results demonstrated that electrostatic repulsive interactions between adsorbed molecules appears to be a larger contributor to endothermic heats of adsorption than surface dehydration or solution non-idealities. The presence of mobile phase cations can reduce the magnitude of endothermic adsorption heats by screening repulsive interactions between adsorbed molecules. Although water release was not found to be a major contributor to endothermic adsorption heats, it is likely to be a contributor to the entropic driving force associated with the adsorption of bovine serum albumin.

Keywords: Thermodynamic parameters; Ion-exchange chromatography; Heat of adsorption; Proteins; Albumin; Ovalbumin

1. Introduction

Ion-exchange chromatography (IEC) is a very popular methodology that is commonly used in the purification of proteins. IEC is advantageous because separations typically occur in the presence of aqueous buffers and hydrophilic surfaces as opposed to the harsh solvents and hydrophobic stationary phases used in reversed-phase chromatography. As a result the desired protein does not readily denature, thus minimizing the loss of protein activity. Although IEC is commonly used, the mechanisms that establish equilibrium characteristics such as capacity and selectivity are not adequately quantified. A number of models [1–3] have been proposed, with limited success. This is mainly because non-idealities originating from intermolecular interactions are not satisfactorily described under overloaded conditions.

Some researchers have turned to calorimetry as a means to explore the underlying mechanisms of adsorption. The equilibrium capacity of a surface is dictated by the Gibbs-free energy change for adsorption (ΔG_{ads}), which is in turn dependent upon the associated enthalpy change (ΔH_{ads}). Depending on the process conditions the heat of adsorption for proteins can be exothermic or endothermic [4,5]. Exothermic heats of adsorption indicate the dominance of attractive forces between the surface and the adsorbing protein, and/or attractive forces be-

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tween adsorbed molecules on the surface. Endothermic heats of adsorption imply the overall adsorption process is entropically driven, and this is often attributed to protein conformational changes. It is a well-established fact that conformational changes are often accompanied by endothermic heats, however, in IEC and HIC applications, endothermic heats of adsorption have been observed under conditions where conformational changes are usually negligible [4–7]. Although the investigation of entropic forces is important, it is nonetheless equally important that enthalpic events, in particular the sources of endothermic heats of adsorption, be studied.

As previously stated, calorimetry is a valuable tool when investigating intermolecular interactions. Calorimeters are highly sensitive instruments that can sense minute temperature changes. These temperature changes are converted to heats of adsorption by an experimentally determined calibration factor. Exothermic events will produce positive temperature changes and endothermic heats will produce negative temperature changes. An example of a negative temperature drop is shown in Fig. 1. In our laboratory we have measured exothermic heats for the adsorption of bovine serum albumin (BSA) on to C_{18} and C_4 reversed-phase supports [8]. Additionally it has been shown that the adsorption of BSA onto hydrophobic interaction supports can be either endothermic or exothermic [5,7]. In the case of hydrophobic interaction chromatography (HIC) it has been reported that the dehydration of the stationary phase may actually mask the favorable interaction between the protein and the surface by producing endothermic heats of adsorption. In the case of IEC chromatography it has been demonstrated that the selection of stationary phase and process conditions can influence whether protein adsorption is endothermic or exothermic [4]. Endothermic ΔH_{ads} values for the adsorption of BSA onto polyethyleneimine (PEI) have been reported under conditions in which the protein and the surface have opposite net charges [4,6,8]. It has been suggested that dehydration of the sorbent and protein contact surface may in part be responsible for producing endothermic ΔH_{ads} values under conditions where protein conformational changes are not expected [5,10]. Additionally Esquibel-King et al. [7] have reported that repulsive interactions between surface molecules may be a possible contributor to endothermic ΔH_{ads} values accompanying the adsorption of BSA onto an HIC support. Finally, it has also been reported that solution conditions can also significantly affect the heat of adsorption [9].

In this paper we investigate the effects of surface dehydration, repulsive interactions and solutions conditions on the heat of adsorption of ovalbumin and BSA onto a weak anion support (PEI). The effects of surface dehydration were investigated with the method developed by Perkins et al. [11], and by correlating these data to heat of adsorption measurements obtained with flow microcalorimetery. Repulsive interactions were investigated by correlating microcalorimetry results with isotherm measurements. Finally, the effects of mobile phase conditions were examined by estimating the magnitude of protein solution activity coefficients.

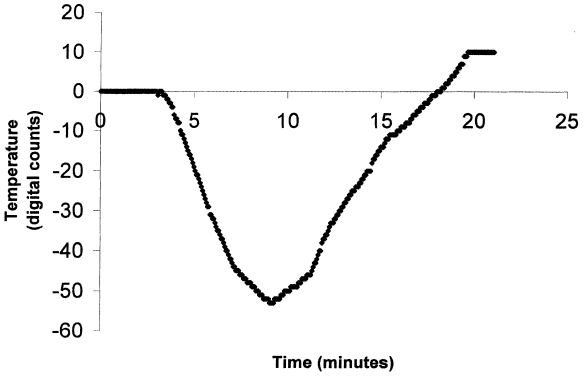

Fig. 1. Typical endothermic BSA-PEI temperature profile.

2. Experimental

2.1. Materials and apparatus

BSA and ovalbumin (OVA) grade VII were purchased from Sigma (St. Louis, MO, USA) and used without further purification. The ovalbumin was denatured prior to use, and the denaturation was verified with UV spectroscopy and flow microcalorimetry. The IEC supports used (PEI-1000-10) were siliceous-based particles with an average diameter of 10 μm and an average pore size of 1000 Å. The surface contained cross-linked polyethylene-

imine ligands. The IEC support was purchased from Millipore (Bedford, MA, USA).

The carrier fluid for the IEC experiments was 10 m*M* piperazine (pH 6.2). Sodium chloride, potassium chloride and lithium chloride were used as modulators for all IEC experiments. The NaCl, KCl, LiCl and were purchased from Fisher Scientific Company (Hanover Park, IL, USA). Piperazine was purchased from the Eastman (Kingsport, TN, USA).

2.2. Flow microcalorimetry

The flow microcalorimeter (FMC; Gilson Instruments, Westerville, OH, USA) is operated similar to a liquid chromatograph. The column or cell volume is 0.171 ml. The flow-rate through the cell is controlled by precision syringe micropumps. Interfaced with the cell are two highly sensitive thermisters. These instruments are capable of detecting small temperature changes within the cell that are associated with the adsorption of an analyte onto the surface of a particular adsorbent. A block heater is used to monitor and control the cell temperature. As in a chromatograph, the FMC is equipped with a configurable injection loop to accommodate different injection volumes. The effluent was collected and analyzed with a UV spectrophotometer (Milton Roy, Rochester, NY, USA).

The FMC is initially filled with a specified volume of adsorbent. The next step (although not always necessary) is the evacuation of the cell. The evacuation process to a vacuum pressure of 30 in.Hg usually requires 24 h (1 in.Hg=338.638 Pa). The purpose of this step is to remove all air from the resin surface. Once the cell has been successfully evacuated, the contents are 'wetted' with the carrier fluid. Following wetting the syringe pumps are turned on and the adsorbent is equilibrated with the carrier solution at a flow-rate of 3.3 ml/h. Once the system has reached thermal equilibrium, the sample (20 mg/ml of protein dissolved in the carrier fluid) is loaded into a 1-ml injection loop, and introduced into the cell by switching a multiport valve. The adsorption of the sample onto an adsorbent surface causes a change in cell temperature, which is converted to a heat signal by the FMC through an experimentally determined calibration factor. (The calibration factor was obtained using the 50-μm PEI-1000 particles.) Once the mass in the effluent is quantified with the spectrophotometer, a simple mass balance is performed to determine the quantity of sample adsorbed. From these data the specific heat of adsorption is calculated.

2.3. Isocratic elutions

Retention factor data were collected on an HP1100 chromatograph unit with a 5.0×.21-cm I.D. column at 25°C at pH 6.2. The column was equilibrated with buffer with different concentrations of salt at a flow-rate of 0.5 ml/min. Elution times were obtained by injecting 3 μl of 2.0 mg/ml of protein. The response was monitored with a UV detector at 280 nm.

2.4. Isotherms

BSA and ovalbumin adsorption isotherms were measured at selected modulator concentrations in 10 m*M* piperazine buffer at 25°C (pH 6.2) by the batch method. PEI-1000-10 was first weighed into test tubes, and protein solution of a known concentration was pipetted into the tube. The test tubes were sealed with Parafilm, placed in a shaker, and agitated at 200 rpm for 24 h at 25°C. Preliminary experiments have established that equilibrium can be reached in 8–10 h. After equilibration, the slurry solution was allowed to stand for 1 h before filtering with 0.45-μm filter. The absorbance of the filtrate was measured at 280 nm to obtain the equilibrium solution concentration. The equilibrium distribution was calculated from a mass balance

3. Results and discussion

The equation developed by Perkins et al. [11] was utilized to calculate the water released from the sorbent surface and the contact surface of the sorbent. This equation relates the quantities of water molecules released and ion molecules released to the chromatographic retention factor. The final form of this equation is shown as Eq. (1); the complete derivation of this equation can be found elsewhere [11]:

$$\ln k' = c + \frac{(\Delta\nu_+ + \Delta\nu_-)}{g}\ln m_3 - \frac{n\Delta\nu_1}{m_1 g}\cdot m_3 \qquad (1)$$

where c is a constant of integration, $\Delta\nu_1$ is the net displacement of water molecules from the sorbent surface and the protein contact surface per molecule of protein adsorbed ($\Delta\nu_+ + \Delta\nu_-$) is the net change in modulator ions released from the sorbent surface and the protein contact surface per molecule of protein adsorbed, m_3 is the molal modulator concentration, m_1 is the molal water concentration, n is the total number of cations and anions per formula unit of the modulator, and g is the ratio of $(\partial\ln(m_3)/\partial\ln(a_3))_{T,P}$, where a is the activity of the modulator (a discussion on calculating g can be found in the original Ref. [11]). Although this model was initially developed for calculating water release for HIC applications, it is a general model that is applicable to other forms of chromatography such as IEC and RPC. In this case $\ln k'$ is plotted as function of the $\ln m_3$ and the water-release and counter-ion release parameters are determined from a non-linear regression analysis. The regression analysis was performed by Curve Expert Software which employs the Leventhal–Marquardt method.

Eq. (1) was used to calculate the water release for both denatured OVA and BSA in the presence of three different modulators at 25°C and pH 6.2. The salt concentration range for ovalbumin was 0.2–0.6 molal. The salt concentration range for BSA was 0.2–1.0 molal. The modulators selected for these experiments were NaCl, KCl and LiCl. Since the counter-ion in each application is chloride, the effect of the cation on retention and water release can be investigated.

The results from Eq. (1) are shown in Table 1. In the case of BSA the water release is greatest with potassium and smallest with lithium. This is also consistent with the retention order (KCl>NaCl>LiCl), and can be explained on the basis of the chaotropic nature of each modulator. Of the three salts potassium chloride possesses the greatest ability to disrupt the structure of water. Lithium chloride enhances the orderly structure of water. In the presence of potassium chloride the water structure around the protein and the surface may be unstable, thus accounting for larger amounts of water release. In the case of lithium chloride the structure of water may be more stable, thus accounting for smaller amounts of water release.

For denatured ovalbumin the water-release values are essentially identical for all three salts. Based on the argument above it is expected that denatured OVA should give the same trend as BSA. It is possible that the method used for quantifying water release is not sensitive enough to account for smaller differences in water release or that the degree of hydration for the denatured form of ovalbumin is independent of salt concentration.

In order to determine if water release (Table 1) is a major contributor to measured endothermic heats of adsorption, flow microcalorimetry experiments were performed. Microcalorimetry was performed at pH of 6.2 and 27°C under overloaded conditions. The temperature is slightly elevated above room temperature to improve accuracy through tighter temperature control. For purposes of this work a 2°C temperature difference between elution and calorimeter data should not produce a significant difference in adsorption behavior. In order to obtain definable heat of adsorption measurements in the presence of a modulator, all experiments were conducted with a

Table 1
Water release data for denatured OVA and BSA (25°C, pH 6.2)

Protein	Salt	$(n\Delta\nu_1)/(m_1/g)$ (kg/mol)	$(\Delta\nu_+ + \Delta\nu_-)/g$ (mol/mol)	'g' values	Regression correlation coefficient	$\Delta\nu_1$ (mol/mol)	$\Delta\nu_+ + \Delta\nu_-$ (mol/mol)
BSA	NaCl	−3.76	−4.38	1.0757	0.9958	112	5
	LiCl	−2.16	−3.63	0.9862	0.9957	59	4
	KCL	−4.77	−4.70	1.1215	0.9969	149	5
Denatured OVA	NaCl	−6.39	−4.98	1.0881	0.9991	191	5
	LiCl	−6.85	−4.90	1.0185	0.9999	192	5
	KCL	−6.269	−4.92	1.1234	0.9999	194	6

modulator concentration of 0.1 *M*. This is because at higher modulator concentrations protein adsorption is suppressed, and the temperature signal of the FMC is below the detection limit. At higher modulator concentrations the heat of adsorption could be quite different. However, since protein uptake measurements under overloaded conditions at modulator concentrations of 0.1 *M* have show the same retention order as at higher salt concentrations, it is assumed that relative behavior of each protein with respect to each modulator will be the same.

Heat of adsorption measurements are shown in Fig. 2. Note that in the absence of any modulating ions the adsorption of denatured ovalbumin produces a strong exothermic peak and the adsorption of BSA produces a strong endothermic peak. In the presence of NaCl and LiCl the denatured ovalbumin ΔH_{ads} values become slightly more exothermic. The BSA heats are still endothermic; however, the trend is moving in the exothermic direction. The overall movements of ΔH_{ads} values in the exothermic direction in the presence of NaCl and LiCl is most likely related to presence of the cation in the mobile phase. This behavior suggests that the cations may be screening intermolecular interactions between protein molecules in solution and on the surface. The source of these repulsive interactions may be interactions between species of the same charge. Studies have shown that at this pH the approximate charge on BSA is about −11 [12]. If this is truly indicative of the events that are occurring during adsorption, these data suggest that dehydration may not be the major source of endothermic heats produced in these experiments. Rather intermolecular interactions are the root cause of the endothermic heat. In the presence of KCl the adsorption of denatured ovalbumin becomes more exothermic and the adsorption of BSA appears to be slightly less endothermic. The fact that the strongest exothermic signal observed for denatured ovalbumin and weakest endothermic signal for BSA occur in the presence of KCl is further evidence that the cation has significantly greater influence on the heat of adsorption than water release. A possible explanation for this is that the potassium ions are more efficient at neutralizing repulsive interactions between negatively charged proteins molecules. Potassium, sodium and lithium all have the same charge; however, potassium has smallest hydrated radius [13]. A small hydrated radius could mean that the positive charge on the potassium is less shielded and therefore more effective at neutralizing intermolecular interactions between negatively charged proteins in solution and on the surface.

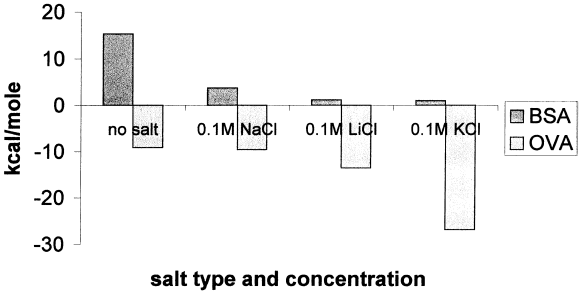

Fig. 2. Heat of adsorption data; 27°C, pH 6.2, PEI-1000-10.

Intermolecular interactions between adsorbed molecules of net like charge are usually repulsive in nature. A number of calorimetry experiments show the existence of repulsive interactions between adsorbed molecules [4–6,8]. Repulsive interactions are usually present at high surface concentrations. As surface concentration increases, the heat of adsorption moves in the endothermic direction. Clear examples of this are shown in the adsorption of BSA onto a C_{18} support [8] and the adsorption of BSA onto a PEI support [9].

In order to determine if repulsive interactions produced the temperature drop shown in Fig. 1, an examination of how BSA molecules in solution interact may provide some insight as to how BSA molecules interact on the surface. Osmotic pressure studies have shown [14] that repulsive interactions between BSA molecules in solution can be very strong, particularly if the solution pH is greater than the isoelectric point (p*I*) of the protein. Solution non-idealities are typically represented by activity coefficients. Protein activity coefficients are usually assumed to be unity because infinite dilution in solution is assumed. However, results reported by Vilker et al. [14] and recent developments [15] have demonstrated that his assumption may be erroneous. The second and third viral coefficients for BSA have been reported to be positive in the presence of 0.15 *M* NaCl [14] and 0.0667 *M* phosphate [14]. Positive values for the second and third viral coefficients are

usually indicative of repulsive interactions between solution molecules. If the second and third viral coefficients are known for a given protein, the activity coefficients can be calculated from the following equations [15]:

$$\ln \gamma = 2C_2 m + \frac{3}{2} \cdot C_3 m^2 \quad (2)$$

$$C_2 = (B_2 - \bar{v}_p M_p)\rho_{mp} \quad (3)$$

$$C_2 = (B_3 - 2B_2 \bar{v}_p M_p + \bar{v}_p^2 M_p^2)\rho_{mp}^2 \quad (4)$$

where B_2 and B_3 are the second and third viral coefficient, M_p is the protein molecular mass, v_p is the protein-specific volume and ρ_{mp} is the density of the solvent. The second and third viral coefficients for BSA have been calculated by Vilker et al. [14] at 25°C in 0.15 *M* NaCl at pH 7.4. Based on these data the activity coefficients for BSA at 0.1 *M* NaCl and 0.2 *M* NaCl were calculated with Eqs. (2)–(3). The results are shown in Fig. 3. Activity coefficients greater than unity are indicative of strong repulsive interactions between BSA molecules. At 0.2 *M* NaCl the activity coefficients are smaller thus indicating a more favorable environment for BSA. The smaller activity coefficients indicate that the additional salt is neutralizing the surface charges on the protein surface. Finally, in calorimetry studies reported by Bowen and Hughes [4] the heat of dilution for BSA in the absence of salt was reported to be exothermic. Exothermic heats of dilution are indicative of repulsive interactions between BSA molecules. If BSA molecules exhibit strong repulsive interactions at low salt concentrations in solution then perhaps it is reasonable to assume that they will exhibit strong repulsive interactions on the surface. These repulsive interactions actually mask the heat released due to favorable interactions between the net negatively charged BSA and the positively charged PEI surface. The heat of adsorption data shown in Fig. 2 supports this hypothesis. In the absence of salt, where the repulsive interactions between BSA molecules is the strongest, the endothermic heat of adsorption is the largest. However, at 0.1 *M* NaCl the magnitude of the endothermic heat of adsorption is significantly reduced. In the case for ovalbumin the heat of adsorption in the absence of salt and in the presence of 0.1 *M* NaCl is not significantly different. This suggests that the repulsive interactions between ovalbumin molecules are negligible, and the heat released from the interaction of ovalbumin and the PEI surface is not masked. Thus in the case of ovalbumin exothermic adsorption heats are observed.

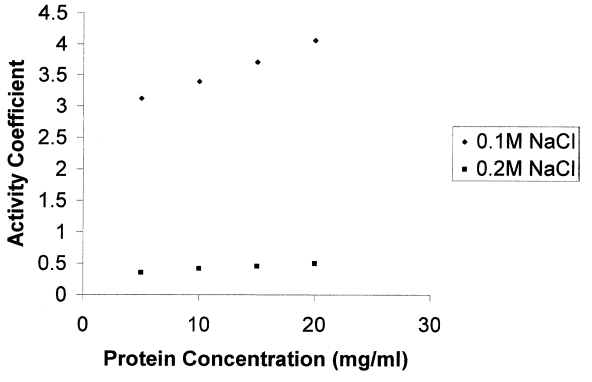

Fig. 3. BSA solution activity coefficients as a function of BSA solution concentration.

The heat of adsorption data for BSA and ovalbumin in the absence of salt suggest that the isotherms under the same process conditions would be significantly different. In fact the single component isotherms for each protein measured under these conditions are not significantly different. In Fig. 4a the single component adsorption isotherms for BSA and ovalbumin are presented with the surface concentrations in units of mg protein/g support. In Fig. 4b we have presented the data with the surface concentrations in terms of molecules protein/g support. In this figure the number of molecules of ovalbumin on the surface is slightly greater than the number of molecules of BSA, but both are of the same order of magnitude. The fact that the heat of adsorption is significantly different for each protein and the fact that the number of surface molecules for each protein are of the same order of magnitude suggest that the adsorptive driving forces are different. An additional evaluation was done by calculating the approximate surface area occupied by the molecules on the surface. BSA is an ellipsoid shaped molecule with dimensions of 140×40×40 Å [16]. Ovalbumin in its natural state is also described as an ellipsoid with a major axis length of

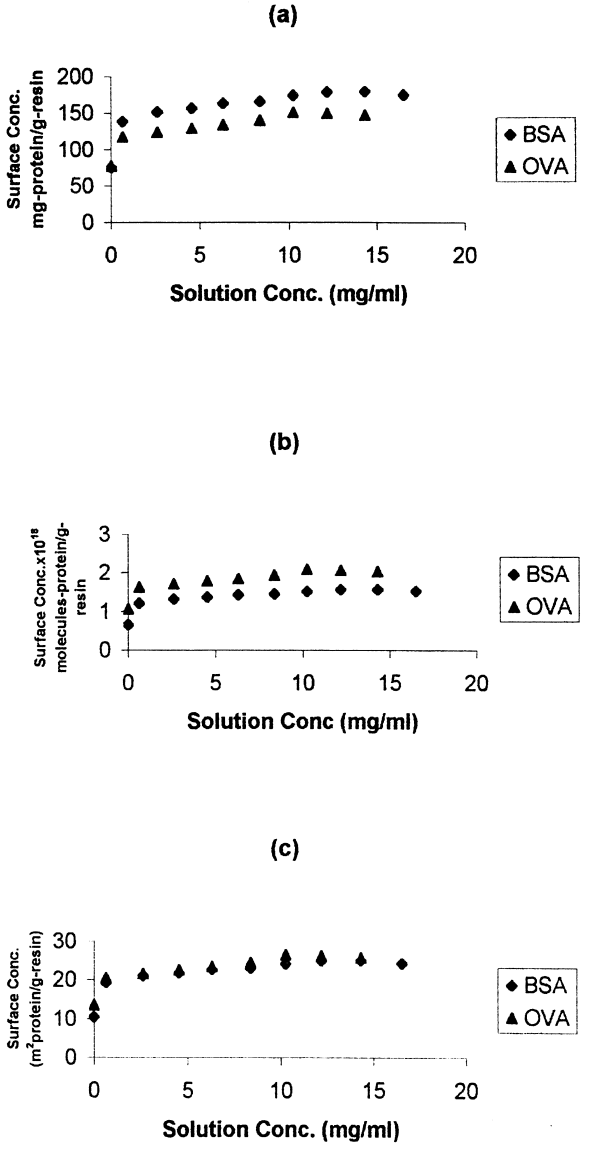

Fig. 4. Single component isotherms for BSA and denatured OVA at 25°C, pH 6.2, no salt. (a) Mass; (b) molecular; and (c) protein surface area.

50.4 Å and minor axis length of 25.2 Å [17]. By conservatively assuming BSA is an ellipsoid that has adsorbed to the surface ''end on'' with a rectangular footprint with dimensions 40×40 Å and the denatured form of ovalbumin also has a rectangular footprint but of 50.4×25.2 Å size (the denatured form is most likely larger), a comparison of the surface occupied by these proteins at equilibrium capacity can be made (Fig. 4c). It is clearly seen in Fig. 4c that BSA and ovalbumin are occupying approximately the same total surface area at equilibrium. Even if the size of the denatured form of ovalbumin is somewhat larger, the difference in surface area occupied by each protein would still be negligible as the sorbent's available surface area reported by the manufacturer is 30 m^2/g. This similarity in occupied surface area is further evidence that the adsorptive driving forces are different for the two proteins. It is well known that the Gibbs-free energy has an enthalpic contribution and an entropic contribution. In the case of BSA the enthalpic contribution is not favorable, therefore, the driving force behind the adsorption of BSA has to be entropic in nature. A possible source for this entropic driving force is the release of water molecules from the sorbent surface and the contact surface of the protein. The increase in entropy associated with the additional release of water for BSA may be the main underlying entropic force that compensates for the unfavorable enthalpic contribution associated with adsorption. Additionally the dilution of BSA molecules in the mobile phase due to adsorption and/or possibly the rearrangement of water molecules in the mobile phase could be a source of entropy. In the experiments conducted for this work the largest protein concentration used was 20 mg/ml. Although this may not appear to be large, it is however important to recall that the solution activity coefficient in the absence of salt is very large at this concentration (Fig. 3). This means that the thermodynamic equivalent of the real concentration (activity) is much larger in magnitude than the actual concentration and, therefore, the entropy increase associated with the dilution of the activity could be significant.

4. Conclusions

We have investigated the source of endothermic heats of adsorption. In the case of BSA the endothermic heat of adsorption appears to be the result of repulsive interactions between the adsorbed surface

molecules. The cations in the mobile phase were found to have a significant influence on the heat of adsorption. Mobile phase cations appear to shield repulsive interactions between negatively charged protein molecules. The thickness of the hydrated shell around the cation may influence this shielding effect.

For the specific experiments presented in this work, water release and solution non-idealities are not major contributors to endothermic heats of adsorption observed. However, water release, in all likelihood, is a major contributor to the entropic driving forces.

5. Nomenclature

B_2	second viral coefficient (m^3/mol)
B_3	third viral coefficient (m^3/mol)
G	$(\partial \ln m_3/\partial \ln a_3)_{T,P}$
m	molal protein composition
m_1	molal concentration of water (55.51 mol/kg)
m_3	molal modulator concentration (mol/kg)
n	number of atoms comprising salt
v_p	protein-specific volume (cm^3/g)
ρ_{mp}	density of the solvent (g/cm^3)
M_p	protein molecular mass
γ	protein activity coefficient
$(\Delta v + \Delta v)$	net release of ions from the contact surface of the protein and the contact surface of the sorbent per molecule of adsorbed protein
Δv_1	net release of water molecules from the contact surface of the protein and the contact surface of the sorbent per molecule of adsorbed protein.

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