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Enthalpy contributions to adsorption of highly charged lysozyme onto a cation-exchanger under linear and overloaded conditions

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ABSTRACT

An investigation of the adsorption mechanism of lysozyme onto carboxymethyl cellulose (CMC) was conducted using flow calorimetry and adsorption isotherm measurements. This study was undertaken to provide additional insight into the underlying mechanisms involved in protein adsorption that traditional approaches such isotherm measurements or van't Hoff analysis can't always provide, particularly when protein adsorption occurs under overloaded conditions. Lysozyme and CMC were selected for this study because the characteristics of the protein and the adsorbent are well known, hence, allowing the focus of this work to be on the driving forces influencing adsorption. Calorimetry results have showed that lysozyme adsorption onto CMC produced both exothermic and endothermic heats of adsorption. More specifically flow calorimetry data coupled with peak deconvolution methods illustrated a series of chronological events that included dilution, primary protein adsorption, rearrangement of surface proteins and a secondary adsorption of lysozyme molecules. The observations and conclusions derived from the experimental work presented in our figures and tables were developed within the mechanistic framework proposed by Lin *et al.*, J. Chromatogr. A. 912 (2001) 281.

1. Introduction

It is well known that the conditions under which protein adsorption occurs has a decisive influence on adsorption behavior [2,3]. In the simplest case, it can be said that binding capacity increases linearly with the concentration of protein in solution until a certain point. When the adsorbent's binding sites become saturated with protein, the adsorption of additional molecules is not necessarily ideal [2]. Several empirical and semi-empirical equilibrium models have been used to try to understand protein adsorption [2–4]. The Langmuir isotherm is the simplest theoretical model used to describe adsorption [5]. It is derived from a basic reversible interaction between the adsorbate and the adsorbent. A key assumption in the model framework is that the absorbate molecules are adsorbed on a fixed number of surface sites, each of which only accepts one molecule. Moreover it is assumed that every adsorption site is energetically equivalent and that there is no interaction between adsorbed molecules [2,5]. At low concentrations, the molecules are well distributed on the adsorbent surface, resulting in a linear shaped curve between adsorbed mass and mobile phase concentration. At higher concentrations, the adsorption sites become saturated, leading to a curvature of the isotherm to an asymptote [2,5]. Conversely the principle of the Langmuir theory may not be applicable to the adsorption of biological molecules in every case. The Langmuir model does not take into account solute–solute interactions and does not consider protein steric hindrance as well as the rearrangement of the ligands on the support surface [2,6,7]. Also, frequently observed phenomena during adsorption, like protein structural rearrangements, cooperative adsorption, or protein aggregation are not considered in the majority of proposed models [2,3,6,7].

Despite considerable progress in the adsorption field, there are still widely different and even contradictory opinions on how to explain adsorptive behavior that is not compliant with classical theory. Ion-exchange chromatography (IEC) is no exception. Traditional ion-exchange models used for small molecules do not necessarily capture the non-ideal effects associated with the adsorption of large charged biological molecules onto oppositely charged surfaces [6,8,9]. Consequently those using ion-exchange models to predict the adsorptive behavior of proteins often need

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to include a compensation factor to simulate the equilibrium [6]. Thermodynamic analyses of biomolecule adsorption have helped to elucidate complex adsorption mechanisms in liquid chromatography [10]. Information about the thermodynamic quantities associated with liquid chromatographic processes may be obtained from batch equilibrium experiments, by analyses of data presented as van't Hoff plots or from microcalorimetric measurements [2,10]. However, the resolution with which variations in thermodynamic behavior, as a function of protein loading and temperature, can be detected by batch equilibrium experiments is limited, and the indirect method of van't Hoff analysis may be complicated by the presence of multiple sub-processes associated with adsorption. Furthermore the results may not be representative of what occurs under overloaded conditions [2,10,11]. Calorimetric methods such as isothermal titration calorimetry have been reported, to provide valuable insight about the underlying mechanisms of adsorption [1,8,11–14]. In this technique, a heat signal is generated in a fed-batch cell, as a target solute solution is added to a buffer solution with the adsorbent suspended [10]. ΔH measurements via ITC, represents the total enthalpy change in the adsorption process, which includes the heat of dilution, the heat of interaction, the heat associated with the desolvation of the protein and adsorbent, the heat associated with the release of ions from the protein and the adsorbent, and the heat associated with rearrangement of biological molecules on the surface of the adsorbent [1]. Flow microcalorimetry (FMC) is a more dynamic means of heat signal measurement because the heat signals produced from this technique corresponds to adsorption and desorption events occurring in a small column filled with the adsorbent, thus permitting an improved understanding of the driving forces and mechanisms involved in the interaction [9,15–22]. A flow microcalorimeter is operated in a manner that is analogous to the operation mode of a chromatograph and hence the results are expected to be representative of what happens in an actual chromatography column. This technique has been used, with good results, to study the adsorption of biomolecules, such as proteins, under overloaded conditions [15–20].

Our ongoing interest focuses on the illustration of the complexity of protein adsorption in an ion exchange application and on showing the important role of nonspecific effects in the establishment of the adsorptive process. To this end, traditional systems are studied under different working conditions. In this paper, we studied lysozyme adsorption onto carboxymethyl cellulose (CMC) at pH 5 (the pH at which the protein is fully charged) in absence and presence of 50 mM salt. This study is part of a comprehensive study where the results obtained at pH 5 are compared with the ones obtain at pH 8 (separate paper). For this purpose adsorption isotherms measurements and flow microcalorimetric data (heats of adsorption and thermogram patterns) were used to study the potential underlying adsorptive mechanisms driving adsorption.

2. Experimental methods

2.1. Adsorption isotherm measurements

Two lysozyme (Sigma-Aldrich) solutions were prepared in 20 mM of piperazine buffer (Sigma-Aldrich) at pH 5. One solution contained 50 mM of sodium chloride (NaCl, Sigma-Aldrich) and the second was void of any salt. Carboxymethyl cellulose, purchased form the Whatman Company, was weighted into individual test tubes and then transferred with 1 mL of protein solution to a multi-well plate. The plates were sealed with parafilm and were left to shake for 24 h at 230 rpm and room temperature. Preliminary experiments have established that equilibrium is effectively reached in 24 h. After equilibration, the slurry was transferred to Eppendorf tubes and left to settle for 30 min. The supernatant was separated from the adsorbent with a 0.22 μm syringe and the absorbance of each solution was measured at 280 nm with a UV spectrophotometer (Amersham Biosciences, Uppsala, Sweden), to obtain the equilibrium solution concentration. Control experiments were also performed to verify that lysozyme adsorption was not occurring on the container walls. Finally, in order to know the amount of protein bound to the adsorbent, a mass balance was applied.

2.2. Flow microcalorimetry

Lysozyme adsorption thermograms were generated using a flow microcalorimeter (FMC) (Microscal FMC 4 Vi, Microscal Limited, London, UK). This microcalorimeter can also be operated in the titration mode. In the flow mode, the FMC is operated similar to a liquid chromatograph. The 171 μL cell (or column) located internally in the FMC is interfaced with two highly sensitive thermistors capable of detecting small temperature changes produced during the adsorption process. The flow rate through the cell is controlled by precision syringe pumps. A block heater is used to monitor and control the cell temperature, and as in a chromatographic system, the FMC is equipped with a configurable injection loop to accommodate different injection volumes.

The column was packed with approximately 15 mg of CMC and the adsorbent was wetted with the mobile phase buffer. Following wetting, the syringe pumps were turned on and the adsorbent equilibrated with the carrier fluid (piperazine 20 mM at pH 5, in the absence or in presence of 50 mM NaCl) at a constant flow rate of 1.5 mL/h. When the system reached thermal equilibrium, the sample (lysozyme prepared in the respective carrier fluid) was loaded into either a 30 μL or 230 μL injection loop, and introduced into the cell by switching a multiport valve. The adsorption of the sample onto the adsorbent surface causes a change in cell temperature, which is converted to a heat signal by the FMC electronics through an experimentally determined calibration factor (the calibration factor was obtained using an electrical impulse of 0.030 J). The effluent was collected and analyzed by the UV spectrophotometer and a mass balance was done to calculate the adsorbed amount of protein. Three replicates were done at each experimental condition, giving reproducible results. Piperazine 20 mM at pH 5 with 1 M NaCl was used as washing solution between each lysozyme injection.

Caldos 4 software (Microscal Limited, London, UK) was used to acquire, store, calibrate, process and present enthalpy data. Peak deconvolution was performed by the PEAKFIT software package (version 4.12, Seasolve Software Inc., San Jose, USA) using asymmetric Gaussian peaks. The integral heat of adsorption was calculated from the area of the de-convoluted peaks. This approach facilitates the elucidation of obscure enthalpic events in the thermogram.

3. Results and discussion

Results for the static binding capacity of lysozyme adsorption onto CMC at pH 5 are displayed in Fig. 1. By analysing the isotherm profile, it can be seen that, for both conditions (in presence and absence of salt), with increasing protein equilibrium liquid concentrations an increase from zero capacity to a plateau region is observed. This plateau is followed by a region of increasing capacity, that may indicate the formation of multiple layers of lysozyme on the surface or a reorientation of the adsorbed protein to accommodate more molecules. The structural rearrangement of adsorbed lysozyme was assumed to be negigible since this protein has been reported to have a high degree of structural stability [23]. It has also been reported by some authors that lysozyme suffers dimerization in a pH range 5 to 9 [24]. However, having its isoelectric point at