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# Thermodynamic modelling of hydrophobic interaction chromatography of biomolecules in the presence of salt

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ABSTRACT

Hydrophobic interaction chromatography (HIC) is a useful method for isolation and purification of macromolecules. HIC separates proteins on the basis of surface hydrophobicity while generally retaining the activity of proteins. Aqueous mobile phases with high salt concentrations are often used to adsorb the proteins on a mildly hydrophobic support. In this research, the thermodynamic model of Chen and Sun, which predicts the adsorption isotherms of protein in presence of different type of salts, was modified by substitution the protein and salt activities in the mobile phase instead of their concentrations. In addition, model was examined for studying the adsorption of BSA, HSA, α-lactalbumin and Trypsinogen on different sepharose gels. The model parameters of Chen and Sun are adsorption equilibrium constant ($K_P$), protein dehydration equilibrium constant ($K_s$), salt coefficient ($\alpha$) and number of ligand binding ($n$). By substitution activity instead of salt and protein concentration, two other parameters ($c_1$ and $A_s$), which related to the activity coefficients, are added to the model. The parameters of this nonlinear model are calculated by genetic algorithm (GA).

The maximum average absolute percentage deviation (AAD) for the data which are obtained from the adsorption isotherm of BSA on phenyl sepharose gel, in the presence of different concentration of NaCl was 4.8%, while for Chen and Sun model, was 22.0%. Also maximum ADD for HSA, α-lactalbumin, and Trypsinogen adsorption was 7.8, 6.9, and 8.4, respectively. The results indicate that the modified model has adequate accuracy to predict protein HIC behaviour.



## 1. Introduction

Hydrophobic interaction chromatography (HIC) is a useful and powerful chromatographic technique for purification of macromolecules [1–4]. HIC uses high salt concentrations to encourage an interaction between the protein and a weakly hydrophobic matrix. Due to the high salt concentrations (1–3 M) used to induce binding of the solute to the stationary phase, HIC was explained for the first time as "salting-out chromatography" by Tiselius and Shepard [5]. Elution is achieved either isocratically or with a descending salt gradient. The descending salt gradient weakens the hydrophobic interaction and allows the protein to be eluted from the column.

HIC is used for the separation and purification of macromolecules in their native state [6], protein complexes and protein folding [7]. The process shows milder interaction in comparison with ion exchange chromatography and other separation method, so it can be used as an effective method to achieve the highly separation of macromolecules.

The selectivity in this kind of chromatography is strongly affected by changing the intensity of interaction between the hydrophobic adsorbent and hydrophobic zones on the protein surface. So the ability to predict equilibrium data is important in this chromatography processes. Ionic strength has long been recognized as one of the most important factors affecting the equilibrium characteristics of the adsorption of proteins onto chromatography adsorbents [8]. The effect of salt concentration (ionic strength) on the protein adsorption onto chromatography adsorbents is a very complex phenomenon. It could be considered from some aspects as follows: The salt counter ions compete against the protein for binding sites. They can also shield the protein and the active binding sites from each other. The presence of salt ion could be affected the folding and configuration of protein molecules, resulting in variation of the hydrophobic interaction between the protein and the resin matrix. Also, the increase of ionic strength may reduce the porosity of the resins and hence the availability of binding sites. As a result, there is clearly a need to develop a precise model to incorporate the effects of ionic strength on the protein adsorption.

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For a specified protein when different types of salt were uses in the eluent, the protein retention time usually acts based on the Hofmeister series [9]. It suggested that the electrostatic theories [10] could not provide a full interpretation of the observed phenomena. There have been considerable efforts for research about theoretical understanding of the mechanism of protein retention on the hydrophobic chromatography column and surfaces [11–13]. The solvophobic theory and the preferential interaction theory are the main two theories which describe the salt effects in HIC. In the solvophobic theory the retention times are related to the nonpolar contact area and the surface tension of the aqueous salt solution [14]. Fausnaugh and Regnier [15] studied the adsorption of some proteins in the presence of different salt and found that the solvophobic theory alone could not successfully explain retention variances. The presence of salt changes the entropy of solution due to the displacement of structured solvent molecules. On the other side, the preferential interaction theory considers the different interactions of salts with proteins and stationary phases [16]. This theory has been shown to successfully discuss salt type effects and has been applied to study these effects on solute binding and selectivity [21]. Based on this theory the salt binds water and induces the exposure of hydrophobic surface on ligands which causes the interaction of hydrophobic amino acid with the functional group on the stationary phase surface, and making the protein–ligand complex. After the formation of this complex, the water around the complex will redistribute, and due to the reduction of hydrophobic exposed surface area it will be released during the adsorption process [16].

For the first time, Melander [14] presented an adsorption isotherm equation, which considers salt effects term in his equation. Although this model did not has adequate accuracy for prediction of the isotherm equation. Staby and Mollerup [17] presented a model to predict the retention time of solutes on HIC perfusion media based on the Debye–Huckel theory, which was capable of correlating the capacity factor data for a wide range of ionic strength. Chen and Sun [18] shown that the Langmuir equation could not precisely describe the mechanism of protein adsorption in a hydrophobic media due to the salt effect. Therefore, they introduced a two states model for prediction the effect of salt on adsorption isotherm. They assumed that two equilibrium states exist for the protein in a solution phase, which are hydrated and the dehydrated phase.

In 2003, the preferential interaction model with a quadratic nonlinear isotherm was developed by Perkins et al. [19] which described the adsorption behaviour at high solute concentrations. This model was examined for its ability to describe the protein adsorption behaviour under both linear and nonlinear conditions over the wide range of salt concentrations in HIC systems. Xia et al. [20,21] developed a model for predicting solute behaviour under both linear and nonlinear conditions over a wide salt range. According to their model the effect of salts on protein retention was explained by the number of released water molecules induced by different types of salt. Thus, selectivity of a certain salt type in HIC was interpreted as differences in their ability to reject water molecules from a protein surface and a resin surface.

In addition, Mollerup [22–24] studied the effect of salt concentration, ligand density, mobile phase and protein loading. He suggested that the change in adsorption strength is depend on the protein species, salt concentration and salt type, while it is independent on property of resin and experimental evidences confirm this result [17], however this suggestion was not applied to be confirmed by other HIC data [19,25]. Ueberbacher et al. [26] investigate the effect of conformational change of protein on their adsorption in HIC. In addition, they compare van't Hoff analysis and isothermal titration calorimetry (ITC) results with regard to thermodynamic analysis of protein adsorption. The effect of surface coverage, temperature and $(NH_4)_2SO_4$ concentration on the thermodynamic of adsorption is studied in the context of protein conformational changes.

In 2009, Deitcher and Rome [27] suggested a thermodynamic model, which describes both pulse-response and loading behaviour of proteins in HIC. The model describes the adsorption in terms of protein, solvent activities, protein species, ligand type and water displacement from hydrophobic interfaces.

In this work, the thermodynamic model of Chen and Sun will be modified by substitution the protein and salt activities, instead of their concentrations in the mobile phase. Finally, model was examined for studying the adsorption of BSA, HSA, α-lactalbumin and Trypsinogen on phenyl sepharose gels and also HSA and Trypsinogen on butyl sepharose gel. The results show that the modified model can describe the adsorption isotherm better than previous models.

## 2. Thermodynamic modelling

A system involving the hydrophobic ligand, hydrophobic substrate, protein, and certain salts was considered. In deriving the relationships describing the behaviour of protein adsorption, we follow closely the approach recently demonstrated by Chen and Sun [18].

Based on their proposed model, the following equation is expressed for the adsorption isotherm:

$$\frac{Q}{C_P} = \frac{K_S K_P C_S^{\alpha} [\Lambda - (\sigma + n)Q]^n}{1 + K_S C_S^{\alpha}} \quad (1)$$

where $K_s$ and $K_p$ stand for the protein dehydration and protein adsorption equilibrium constants and $n$ represents the number of ligands that can interact with one protein molecule. Also $Q$, $C_p$, $C_s$, $\Lambda$, $\alpha$ and $\sigma$ are the adsorbed protein concentration on adsorbent ($mol\,l^{-1}$), protein concentration in liquid phase ($mol\,l^{-1}$), salt concentration in liquid phase ($mol\,l^{-1}$), density of ligand ($mol\,l^{-1}$), salt coefficient and strict factor, respectively. The total concentration of the ligand on the adsorbent phase, $\Lambda$, is expressed by the following equation according to mass balance:

$$\Lambda = l_v + (n + \sigma)Q \quad (2)$$

where $l_v$ *is* the accessible ligand concentration ($mol\,l^{-1}$).

Based on the assumed proposed model the ion-exchange effect in adsorption of protein on the hydrophobic substrate is very small, so this effect can be disregarded. Also, the salt effect on the hydrophobic ligand groups is insignificant. Solid-phases, such as hydrophobic ligand and adsorbed protein in the substrate phase, are thermodynamically ideal with $\gamma = 1$, so their concentrations could be used instead of activities. The binding of protein to the hydrophobic adsorbent surface may cause steric hindrance of the hydrophobic ligands and the number of blocked sites is proportional to the adsorbed protein concentration. The multipoint nature of protein binding can be represented by a characteristic number of binding sites "$n$" for each dehydrated-state protein molecule.

It was assumed that adsorption equilibrium between the hydrophobic ligand and the protein is reversible. In solution, consists of protein and water, the water molecules hydrates protein molecules, like the shell, so this shell inhibits the interaction of protein and hydrophobic ligand [18]. Although, water molecules have higher tendency for hydration of the salt molecules relative to the macromolecules and consequently the presence of the salt can improve the interaction between protein and hydrophobic ligand. Therefore, higher salt concentration will reduce the number of water molecules that are surrounding the proteins.

According to the model of Chen and Sun, for protein molecules in aqueous solution there are two hydrated and dehydrated states. Only the dehydrated proteins can interact with the hydrophobic

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