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Effect of pH changes on water release values in hydrophobic interaction chromatographic systems

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Abstract

The effect on pH on protein binding in HIC systems was investigated. Isocratic experiments were carried out to determine the capacity factors of various proteins as a function of temperature, pH and salt type. This paper presents a framework based on the Maxwell linkage function for estimating the number of released water molecules during the adsorption/desorption process due to a change of buffer pH. This approach also enables one to predict the effect of pH change on the water released values upon binding at any temperature condition. The results indicate that the total number of released water molecules ($\Delta\nu$) for a pH change increased more on aromatic surfaces (phenyl Sepharose) than on aliphatic resins (butyl Sepharose). The results also indicate that the total number of released water molecules ($\Delta\nu$) for a pH change increased with salt concentration and when changing from chaotropic to kosmotropic salts. The ($\Delta\nu$) values also increased as the buffer pH approached the protein's p*I*, and decreased away from its p*I*. This work helps to establish a framework for the investigation of pH effects on protein selectivity in HIC systems.

Keywords: HIC; Retention; Salt type; pH; Water release

1. Introduction

Hydrophobic interaction chromatography (HIC) is an important separation mode for the purification of biomolecules [1–3] and is widely employed for the downstream processing of proteins [4–6]. HIC is based on hydrophobic interactions between the hydrophobic resin and the non-polar groups on the protein surface [1]. Factors affecting protein binding in HIC systems include the stationary phase (ligand type, ligand density, and backbone chemistry), salt type, salt concentration, temperature, buffer pH and additives in the buffer. Among these factors, salts concentration [7–16] and salt type effects [12,17,18] have received the most attention to date.

The effect of temperature on solute binding in HIC systems has also been examined. Vailaya and Horváth [19] studied the retentions of dansyl amino acids in HIC systems, and found that this was an entropically driven process at low temperature and an enthalpically driven process at high temperature. Wu et al. [20] studied the behavior of cytochrome *c* and lysozyme in HIC systems and concluded that protein retention increased at higher temperature due to temperature-induced conformational changes. Lin et al. [16,21,22] used isothermal titration calorimetry to measure the enthalpy and entropy changes during the protein adsorption and desorption process, and proposed a five-step adsorption model. A larger heat of dehydration was obtained when evaluating more hydrophobic proteins [16,23–25]. Huang et al. [26] have examined the binding mechanism of protein–protein and protein–ligand interactions in HIC systems from both experimental and theoretical perspectives. All of this research indicates that changes of water structure, especially the change of entropy due to the hydration/dehydration process, play a key role in determining solute affinity in the HIC systems.

Van't Hoff plot's using linear and nonlinear regressions have been widely employed to obtain thermodynamic parameters from chromatographic experiments. While a linear Van't Hoff plot is associated with a zero heat capacity (ΔC_p) process, a nonlinear Van't Hoff plot is associated with a ΔC_p either greater or less than 0, which is a more realistic scenario

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for protein binding in HIC systems [27]. Nonlinear Van't Hoff plots tend to be either logarithmic or quadratic in nature. The logarithmic Van't Hoff plot assumes that the heat capacity is independent of temperature, while the quadratic form assumes a dependence of heat capacity on temperature. Enthalpy and entropy values obtained from either logarithmic or quadratic forms have shown good agreement [19,28]. In the current work, we employ the logarithmic nonlinear Van't Hoff plot approach to estimate thermodynamic parameters from HIC data.

The effect of mobile phase pH on protein retention in HIC system can be complex [29–33]. Hjertén et al. [29] found that while basic proteins such as lysozyme exhibited high binding when the buffer pH was close to its p*I*, human serum albumin had a lower capacity factor as the pH increased. Sanz et al. [33] studied pH and temperature effects on tripeptidase purification and showed that an optimal pH could be identified to obtain the highest purity and yield. Alberty [34] derived a relationship based on Maxwell's equations for how changes in buffer pH, temperature and salt concentration can induce Gibbs energy changes. His work was the first to link calorimetric measurements to pH effects. The change of Gibbs energy is related to chromatographic affinity, which can be related to the number of water molecules released upon protein binding. Thus, one can connect the calculation of water release values directly with pH and temperature effects in HIC systems.

In this manuscript, this theoretical framework is employed to compare the pH induced water release values on various HIC resins in the presence of different salt types. Isocratic experiments were carried out to obtain the capacity factors of proteins as a function of temperature at various pH in the presence of various salts on two HIC resins. The proteins' thermodynamic parameters were then calculated from the chromatographic data using a logarithmic nonlinear Van't Hoff plot. The total numbers of released water molecules were then calculated from the thermodynamic parameters to evaluate the effect of buffer pH on protein affinity in HIC systems in the presence of various salts. The work presented in this paper helps to increase our understanding of pH effects in HIC systems.

2. Theory

2.1. Change of water molecules numbers upon pH

Alberty [34] first linked calorimetric measurements with pH and salt concentration and obtained the following relationship:

$$\left(\frac{\partial \Delta H}{\partial \Delta \mathrm{pH}}\right)_{T,\mathrm{salt}} = 2.303RT^2\left(\frac{\partial \Delta \nu}{\partial T}\right)_{\mathrm{pH,salt}} \tag{1}$$

where ΔH is the change of enthalpy and $\Delta\nu$ is the number of released protons. In HIC systems, the proton change can be taken as the number of released water molecules due to the pH change. If we assume that $\Delta\nu$ is independent of T, then the Maxwell linkage function requires that ΔH is independent of pH. This then enables one to separate temperature and pH effects in Eq. (1) as [35,36]:

$$\Delta\nu = \frac{\Delta H}{2.303RT^2}\left(\frac{\partial T}{\partial \Delta \mathrm{pH}}\right)_{\mathrm{salt}} \tag{2}$$

The dependence of a solute's capacity factor on the temperature is defined as:

$$\ln k' = \ln\phi - \frac{\Delta H^\circ}{RT} + \frac{\Delta S^\circ}{R} \tag{3}$$

where ΔH° and ΔS° are the standard enthalpy and entropy change when solute transfers from the mobile phase to the stationary phase and ϕ is the column phase ratio. If ΔH°, ΔS°, and ϕ are all independent of temperature, the plot of $\ln k'$ versus $1/T$ results in a linear Van't Hoff plot. However, ΔH°, ΔS° are both dependent on temperature in most HIC systems. In a nonlinear Van't Hoff plot, both enthalpy and entropy are a function of heat capacity and temperature. The simplified relationships are as follows:

$$\Delta H^\circ = \Delta C_\mathrm{p}(T - T_\mathrm{H}) \tag{4}$$

$$\Delta S^\circ = \Delta C_\mathrm{p} \ln\left(\frac{T}{T_\mathrm{S}}\right) \tag{5}$$

where T_H and T_S are the temperatures at which the standard enthalpy and entropy are 0. A nonlinear logarithmic Van't Hoff plot [19] is then derived from Eqs. (3)–(5) as:

$$\ln k' = \ln\phi + \frac{\Delta C_\mathrm{p}}{R}\left(\frac{T_\mathrm{H}}{T} - \ln\frac{T_\mathrm{S}}{T} - 1\right) \tag{6}$$

where ΔC_p, T_H, and T_S are calculated by a least square fit from the nonlinear Van't Hoff plot. ΔC_p is independent of temperature, but dependent on buffer pH. Both T_H and T_S are dependent on pH. Therefore, we can link the free solution relationship of Eq. (2) with the solute capacity factor in chromatography as [23]:

$$\int_{\mathrm{pH}_1}^{\mathrm{pH}_2} \Delta\nu\,\mathrm{dpH} = \int_{T_\mathrm{H}(\mathrm{pH}_1)}^{T_\mathrm{H}(\mathrm{pH}_2)} \Delta C_\mathrm{p}\frac{T_\mathrm{R} - T_\mathrm{H}}{2.3RT_\mathrm{H}^2}\,\mathrm{d}T_\mathrm{H} \tag{7}$$

The integration of Eq. (7) yields:

$$\begin{aligned}\int_{\mathrm{pH}_1}^{\mathrm{pH}_2} \Delta\nu\,\mathrm{dpH} = {} & \frac{T_\mathrm{R}}{2.303R}\left(\frac{\Delta C_\mathrm{p}(\mathrm{pH}_2)}{T_\mathrm{H}(\mathrm{pH}_2)} - \frac{\Delta C_\mathrm{p}(\mathrm{pH}_1)}{T_\mathrm{H}(\mathrm{pH}_1)}\right) \\ & + \frac{1}{2.303R}[\Delta C_\mathrm{p}(\mathrm{pH}_2)\ln(T_\mathrm{H}(\mathrm{pH}_2)) \\ & - \Delta C_\mathrm{p}(\mathrm{pH}_1)\ln(T_\mathrm{H}(\mathrm{pH}_1))]\end{aligned} \tag{8}$$

where $T_\mathrm{H}(\mathrm{pH}_1)$ and $T_\mathrm{H}(\mathrm{pH}_2)$ are the enthalpy temperature (defined as in Eq. (6)) at any buffer pH 1 and 2, respectively. T_R is the reference temperature where the $\ln k'$ experiment is conducted when the buffer pH is changed. Thus, a plot of the right hand side of Eq. (8) (defined as I) versus pH shows the effect of buffer pH changes on ($\Delta\nu$). Since the heat capacity

is also a function of buffer pH, the total number of released water molecules upon pH change can be calculated from the slope of the *I* versus buffer pH plot (note: the total number of released water molecules calculated from Eq. (8) represents only those induced solely by the change in pH).

3. Experimental and methods

3.1. Materials

Lysozyme (hen egg white), α-amyloglucosidase (*Aspergillus niger*), trypsinogen, sodium phosphate (monobasic), sodium phosphate (dibasic), sodium sulfate, sodium chloride and sodium thiocyanate were purchased from Sigma (St. Louis, MO, USA). Phenyl Sepharose, 6FF (high sub) and Butyl Sepharose, 4FF bulk resin (average particle size is 90 μm) were donated by Amersham Biosciences (Uppsala, Sweden). Then the bulk resin was slurry packed in lab into 5 mm × 100 mm dimension columns.

3.2. Apparatus

All analytical scale isocratic experiments were conducted using a chromatographic system from Waters (Milford, MA), which consisted a 600E Multi-solvent Delivery System, a 484 UV–vis detector and a 712 WISP auto sampler with a cooling module. A Brinkman RM20 water bath (Brinkman Instruments, Westbury, NY) was used to maintain constant temperature.

3.3. Procedures

3.3.1. Isocratic experiments

Isocratic experiments were carried out to obtain retention data of the proteins lysozyme, α-amyloglucosidase and trypsinogen in the presence of various salts at various temperatures and pH. Same types of buffers were used, and pH was adjusted by adding acid/base to the buffer solution. The salts used in these experiments were 1 M Na_2SO_4, 2 M NaCl, or 2 M NaSCN and the temperature range varied from 10 to 50 °C with intervals of 10 °C. The column effluent was monitored using a UV–vis detector at 215 nm for $(NH_4)_2SO_4$ and NaCl, and at 280 nm for NaSCN. The flow rate was maintained at 0.5 ml/min (153 cm/h).

4. Results and discussions

In this manuscript, the theoretical framework described in the theory section was employed to compare the pH induced water release values on various HIC resins in the presence of different salt types. The proteins' thermodynamic parameters were calculated from chromatographic retention data using a logarithmic nonlinear Van't Hoff plot. The total numbers of released water molecules were then obtained from the thermodynamic parameters to evaluate the effect of buffer pH on protein affinity in HIC systems under a variety of conditions. Importantly, this approach enables one to predict the effect of pH change on the water-released values upon binding at any temperature condition.

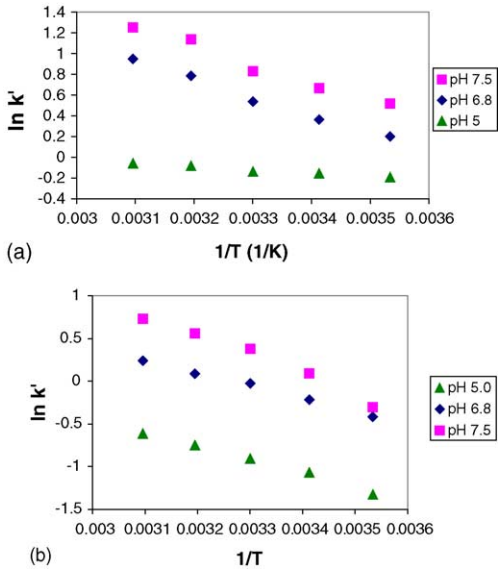

Fig. 1. Change of lysozyme capacity factor as a function of temperature at various pH on phenyl and butyl Sepharose column. (a) Phenyl Sepharose resin; (b) butyl Sepharose resin. (■) pH 7.5; (♦) pH 6.8; (▲) pH 5.0. Salt: sodium sulfate.

Isocratic experiments were conducted to obtain the capacity factor of lysozyme at different temperatures on both a phenyl and butyl Sepharose column. As seen in Fig. 1, lysozyme's capacity factor increased with temperature in the pH range of 5–7.5. At relatively high temperatures (e.g. $1/T = 0.0031$), lysozyme's capacity factor changed significantly with buffer pH; while at a lower temperatures (e.g. $1/T = 0.00353$), this effect was less pronounced. As shown in Fig. 1b, the protein's $\ln k'$ versus $1/T$ behavior on the butyl Sepharose column was similar to that on the phenyl Sepharose column at pH 7.5 and 6.8, but was more temperature dependent at pH 5.

Eq. (6) was employed to calculate the thermodynamic parameters at each buffer pH value. The CONSTR function in Matlab was used to determine the optimum fit of the parameters ΔC_p, T_H and T_S. The resulting parameters are listed in Table 1 for both stationary phases. As seen in the table, the heat capacity values were negative for all of the pH conditions, and decreased with increasing pH. This general trend is what one would expect since a more negative heat capacity at higher pH corresponds to a more negative enthalpy, which in turn corresponds to higher binding affinity. This exothermic adsorption is consistent with the higher retention observed for lysozyme at pH 7.5 as compared to pH 5. The T_H values decreased with increasing buffer pH for the butyl Sepharose. However, it was difficult to obtain clear trends in the T_H data for phenyl Sepharose as well as the T_S data.

Table 1
Thermodynamic parameters associated with the retention of lysozyme on phenyl and butyl Sepharose columns at various buffer pH in the presence of 1 M Na_2SO_4

Stationary phase	pH	ΔC_p (J mol^{-1} K^{-1})	T_H (K)	T_S (K)
Phenyl Sepharose	5.0	−28.26	393.59	467.08
	6.8	−98.24	449.93	504.57
	7.5	−199.03	375.11	491.44
Butyl Sepharose	5.0	−109.91	423.497	467.64
	6.8	−114.17	410.23	476.11
	7.5	−137.61	346.55	361.67

Parameters were evaluated by fitting Eq. (6) to experimental data under each pH conditions.

Once the thermodynamic parameters are obtained from the chromatographic retention data, Eq. (8) was employed to calculate the total number of released water molecules ($\Delta\nu$). The right hand side of Eq. (8) (defined as I) was computed as a function of pH on phenyl and butyl Sepharose column and the results are shown in Fig. 2. The symbols denote the results calculated using Eq. (8). The ($\Delta\nu$) values from one pH to another pH are also presented in the figures. As can be seen in the figure, mobile phase pH has a measurable effect on the change in the number of water molecules released upon binding ($\Delta\nu$). As the pH increased, the number of water molecules released increased for lysozyme, which is what one would expect as the pH moves towards the pI. This behavior was observed on both the phenyl and butyl columns. The total released water molecules on the phenyl Sepharose column were 14 and 49 when the buffer changed from 5 to 6.8 and 6.8 to 7.5, respectively. The total released water molecules on the butyl Sepharose column were 4 and 12 when buffer changed from 5 to 6.8 and 6.8 to 7.5, respectively. Clearly, higher $\Delta\nu$ values were observed on the phenyl Sepharose column,

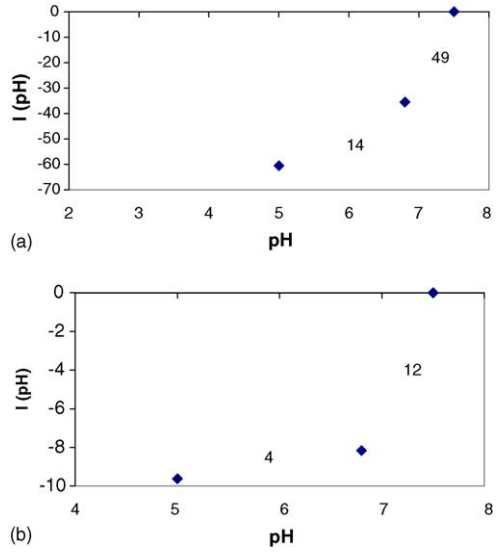

Fig. 2. Plot of the right hand side of equation (8), I(pH), as a function of pH for lysozyme on phenyl and butyl Sepharose column in the presence of sodium sulfate. (a) Phenyl Sepharose resin; (b) butyl Sepharose resin. (♦) Calculated from Eq. (8). Reference temperature is 323 K.

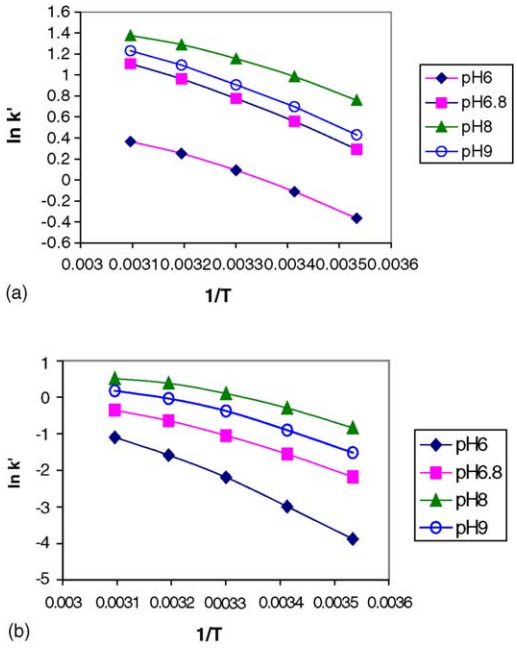

Fig. 3. Change of α-amyloglucosidase capacity factor as a function of temperature at various pH on phenyl and butyl Sepharose column in the presence of sodium sulfate. (a) Phenyl Sepharose resin; (b) butyl Sepharose resin. (♦) pH 5.0; (■) pH 6.8; (▲) pH 8.0; (◯) pH 9.0. Reference temperature is 323 K. Salt: sodium sulfate.

which is consistent with lysozyme's higher affinity on this HIC material as compared to the butyl Sepharose stationary phase. It is worth noting that since this theoretical approach does not explicitly account for conformational changes of the proteins during the adsorption process, the values obtained from this analysis are overall changes in water release values and could very well include some conformational effects.

A similar study was also carried out with α-amlyloglucosidase on both phenyl and butyl Sepharose columns. The α-amlyloglucosidase's capacity factor change as a function of temperature at various pH are shown in Fig. 3. Fig. 3a and b present the change of $\ln k'$ on the phenyl Sepharose and butyl Sepharose column, respectively. The salt used in these experiments was 1 M sodium sulfate. As can be seen in the figures, α-amlyloglucosidase exhibited very similar trends at all pH values on both resins. The capacity factor increased as the temperature increased at all pH in the range of 6–9. As expected, the protein exhibited the highest binding at pH 8, which corresponds to the pI of α-amlyloglucosidase. When the pH is close to the protein's pI, the protein has 0 net charge, resulting in predominantly hydrophobic interactions with the resin. As the pH moves away from the pI, the charge of the protein increases, which can reduce the protein's hydrophobic interactions with the resins?

Table 2 lists the thermodynamic parameters calculated from Eq. (8). As can be seen in the table, the heat capacity values exhibited a minimum when the pH was at the pI for both resins and the values decreased as the pH approached the pI and increased as the pH passed the pI. Fig. 4 presents

Table 2
Thermodynamic parameters associated with the retention of α-amyloglucosidase on phenyl and butyl Sepharose columns at various buffer pH in the presence of 1 M Na_2SO_4 and 2 M NaCl

Stationary phase	pH	ΔC_p (J mol^{-1} K^{-1})	T_H (K)	T_S (K)
Phenyl Sepharose (Na_2SO_4)	6.0	−290.1	394.1	422.4
	6.8	−320.3	410.8	435.9
	8.0	−346.2	455.7	491.4
	9.0	−325.6	420.1	448.7
Butyl Sepharose (Na_2SO_4)	6.0	−201.1	360.0	405.7
	6.8	−216.6	374.5	417.3
	8.0	−248.3	357.2	380.5
	9.0	−229.8	377.2	411.9
Phenyl Sepharose (NaCl)	6.0	−312.9	405.8	420.5
	6.8	−339.4	415.9	431.5
	8.0	−361.7	411.6	424.4

Parameters were evaluated by fitting Eq. (6) to experimental data under each pH conditions.

plots of I(pH) as a function of buffer pH on the phenyl and butyl Sepharose columns. The ($\Delta\nu$) values from one pH to another pH are also presented in the figures. As was observed for the lysozyme results (Fig. 2) mobile phase pH had a measurable effect on the change in the number of water molecules released upon binding ($\Delta\nu$). As seen in the figures, ($\Delta\nu$) values are positive when pH changes from 6 to 8, which indicates higher binding at pH 8. However, the ($\Delta\nu$) values were observed to decrease when going from pH 8 to 9, which is indicative of less binding at pH 9. In addition, the ($\Delta\nu$) values were higher on the phenyl Sepharose than on the butyl Sepharose resin, which is also consistent with the proteins' higher affinity on the phenyl Sepharose material.

A study was also carried out to examine how protein capacity factors change with buffer pH in the presence of various

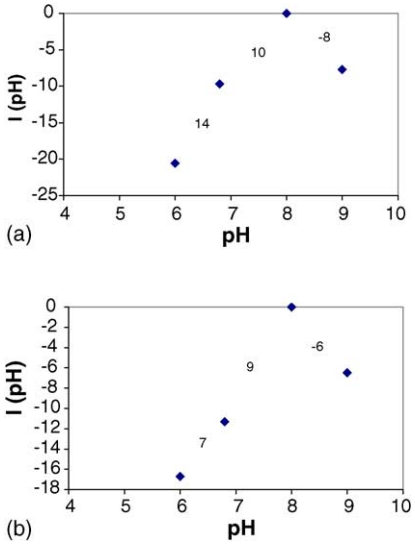

Fig. 4. Plot of the right hand side of Eq. (8), I(pH), as a function of pH for α-amyloglucosidase on phenyl and butyl Sepharose column in the presence of sodium sulfate. (a) Phenyl Sepharose resin; (b) butyl Sepharose resin. (♦) Calculated from Eq. (8). Reference temperature is 323 K. Salt: sodium sulfate.

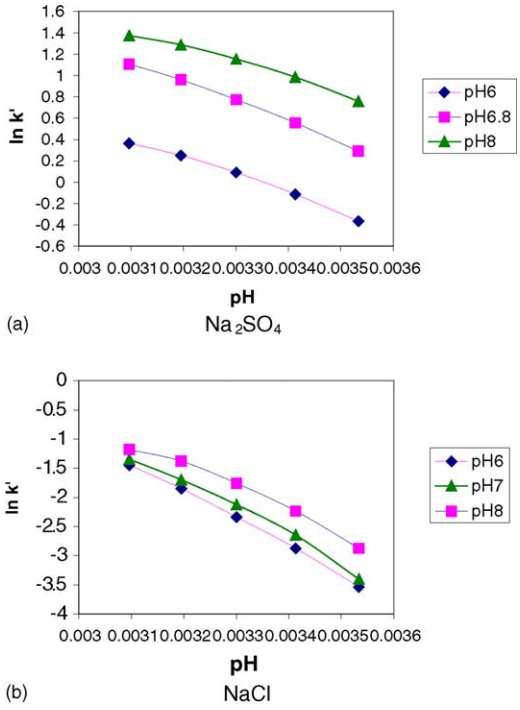

Fig. 5. Change of α-amyloglucosidase capacity factor as a function of temperature at various pH on phenyl Sepharose column in the presence of various salts. (a) In the presence of 1 M Na_2SO_4; (b) in the presence of 2 M NaCl. (♦) pH 6.0; (▲) pH 6.8; (■) pH 8.0.

salts. Three types of salts were used: sodium sulfate (kosmotrope), sodium chloride (neutral), and sodium thiocyanate (chaotrope). α-Amlyloglucosidase was used in the presence of neutral and kosmotropic salt ion effects. Trypsinogen was selected as the model proteins due to its relatively high pI value, as well as the moderate affinity when the salt ionic strength is between 2 and 3 M.

Fig. 5 shows the change of α-amlyloglucosidase's capacity factor with temperature in the pH range of 6–8 in the presence of Na_2SO_4 and NaCl. Although the protein binding was significantly higher in the presence of Na_2SO_4, the qualitative trend with respect to pH was similar. Fig. 6 presents plots of I(pH) as well as the ($\Delta\nu$) values in the presence of the two salts when going from one pH to another. The results indicate that while the trends are quite similar, a decrease of the ($\Delta\nu$) values was observed when the salt changes from sodium sulfate to sodium chloride. This indicates that there are more water molecules released when going from one pH to another in the presence of kosmotropic salts as compared with a neutral salt, which is consistent with our previous work [17].

Fig. 7 shows the change of trypsinogen's capacity factor with temperature at various pH in the presence of various types of salts. A kosmotropic salt (sodium sulfate), neutral salt (sodium chloride) and chaotropic salt (sodium thiocyanate) were employed in the experiments resulting in Fig. 7a–c, respectively. Interestingly, as can be seen in the figures, this protein's capacity factor decreased as the temperature increased, which is in sharp contrast to the trends

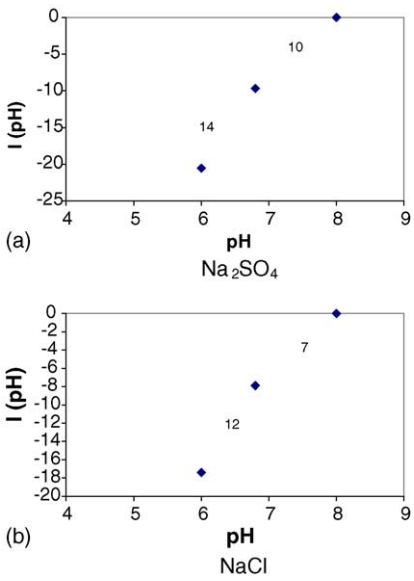

Fig. 6. Plot of the right hand side of Eq. (8), I(pH), as a function of pH for α-amyloglucosidase on phenyl Sepharose column in the presence of various salts on phenyl Sepharose column. (a) In the presence of 1 M Na_2SO_4; (b) in the presence of 2 M NaCl. (♦) Calculated from Eq. (8). Reference temperature is 323 K. Phenyl Sepharose column.

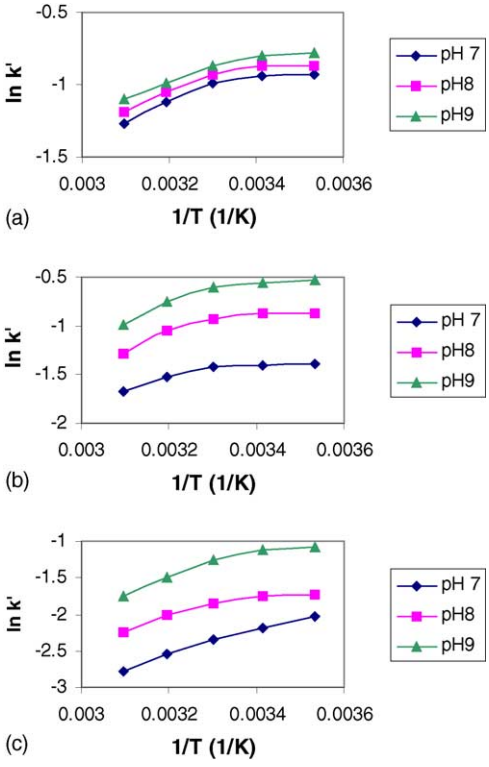

Fig. 7. Change of trypsinogen's capacity factor as a function of temperature at various pH in the presence of various salts on phenyl Sepharose column. (a) Na_2SO_4; (b) NaCl; (c) NaSCN. (♦) pH 7.0; (■) pH 8.0; (▲) pH 9.0. Phenyl Sepharose resin.

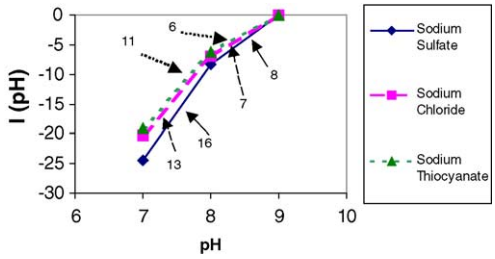

Fig. 8. Plot of the right hand side of Eq. (8), I(pH), as a function of pH for trypsinogen in the presence of various types of salts. (♦) Sodium sulfate; (■) sodium chloride; (▲) sodium thiocyanate. Phenyl Sepharose column is used. Reference temperature is 323 K (note: the numbers in this figure indicate the change in the number of released water molecules associated with pH changes).

observed with lysozyme and α-amyloglucosidase. Clearly, there is a very different mechanism of binding between this protein and the other proteins in HIC systems. The reason for this is under investigation in our laboratory and will be the subject of a future report [19,37].

The changes of I(pH) with buffer pH in the presence of these three salts are presented in Fig. 8. The calculated ($\Delta\nu$) values are also listed in the figure. As can be seen in the figure, the number of released water molecules decreased when salt change from the kosmotropic salt to the chaotropic salt. Again, this result is consistent with previous results from our laboratory [17]. It is very interesting that we observed the same results on NaSCN since it is chaotropic salt.

The effect of salt concentration on the pH induced water release values were also evaluated. Experiments were carried out at both 1 and 1.5 M sodium sulfate. As expected, trypsinogen's affinity increased with salt concentration at all buffer pHs (Fig. 9). Furthermore, as shown in Fig. 10, the ($\Delta\nu$) values in 1.5 M salt were 74 and 23 when pH changed from 7 to 8

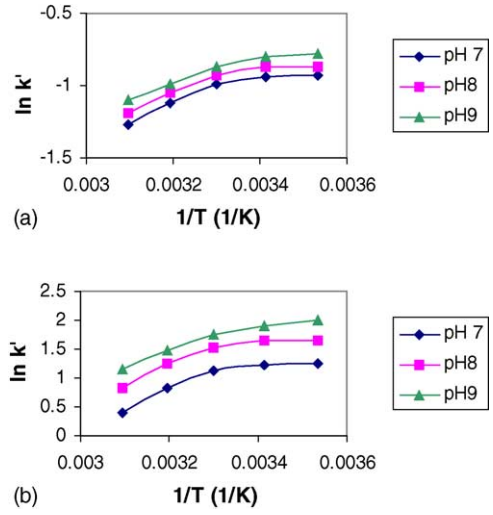

Fig. 9. Change of trypsinogen's capacity factor vs. temperature at various pH in the presence of various salt concentrations on phenyl Sepharose column. (a) 1000 mM salt; (b) 1500 mM salt. (♦) pH 7.0; (■) pH 8.0; (▲) pH 9.0. Phenyl Sepharose resin.

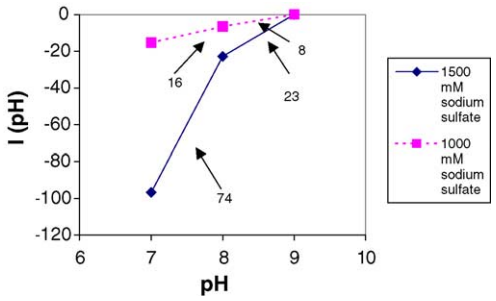

Fig. 10. Plot of the right hand side of Eq. (8), I(pH), as a function of pH for trypsinogen on phenyl Sepharose column in the presence of various concentrations of sodium sulfate. (■) 1000 mm; (♦) 1500 mM. Reference temperature is 323 K.

and 8 to 9, respectively. At 1 M salt, the values were 16 and 8 under the same pH change. Thus, the calculated ($\Delta\nu$) values increased dramatically at the higher salt concentration, which supports the hypothesis that there are more water molecules being released upon pH change at higher binding conditions.

5. Conclusions

In this paper, isocratic experiments were conducted to determine the capacity factors of various proteins as a function of temperature, pH and salt type. The number of released water molecules during the adsorption/desorption process due to the change of buffer pH was then determined using a framework based on the Maxwell linkage function. The results indicate that the total number of released water molecules ($\Delta\nu$) for a pH change increased more on a phenyl Sepharose column than on a butyl Sepharose column. The ($\Delta\nu$) values also increased as the buffer pH approached the protein's pI, and decreased away from its pI. The results also indicate that the total number of released water molecules ($\Delta\nu$) for a pH change increased with salt concentration and when changing from chaotropic to kosmotropic salts. Importantly, this work sets the stage for the investigation of pH effects on protein selectivity in the HIC system. A more detailed analysis of a wide variety of proteins is currently being carried out in our laboratory to examine the generality of this approach and the observed trends.

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