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Microcalorimetric studies of the interaction mechanisms between proteins and Q-Sepharose at pH near the isoelectric point (p*I*) Effects of NaCl concentration, pH value, and temperature

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Abstract

This study examined the interaction mechanisms of β-lactoglobulins A and B (Lg A, Lg B) with an anion exchanger, Q-Sepharose at pH near the isoelectric point at which the proteins are expected to be electrically neutralized under various NaCl concentrations and temperatures by the equilibrium binding analysis and the adsorption enthalpy directly measured by isothermal titration calorimetry. The data evaluated from isotherms fitted by the Langmuirean model reveal that the addition of NaCl considerably reduced the binding affinities and capacities of both the proteins with Q-Sepharose at pH 5.2, indicating that electrostatic forces are dominant during the adsorption. However, the hydrophobic interaction seems to be involved in adsorption as well at a higher NaCl concentration, and the adsorption enthalpies confirm this suggestion. In addition, the effects of temperature on the equilibrium binding behaviors for Lg A or Lg B with Q-Sepharose were found to be salt concentration-dependent, probably due to their different binding mechanisms at 0.03 *M* and 0.3 *M* NaCl. Where, at 0.3 *M* NaCl, the hydrophobic interaction plays a more pronounced role. This implication was again supported by the adsorption enthalpies. The presented data provide further insight to the interaction mechanisms between proteins and ion exchangers, facilitating the optimization of protein separations. © 2001 Published by Elsevier Science B.V.

Keywords: Calorimetry; Thermodynamic parameters; Isothermal titration calorimetry; Adsorption; Proteins; Lactoglobulins

1. Introduction

Ion-exchange chromatography (IEC) is one of the most commonly used methods for downstream recovery of biomaterials [1–3]. This technique is primary based on the interactions between charged amino acids on the protein surface and electrically charged resins, although other nonspecific interactions such as hydrophobic forces may be involved. For example, Norde and co-workers [4–6] found that the protein was adsorbed onto the surface of the ion exchanger even if they are electrostatically repulsive. This means that, in addition to electrostatic forces, hydrophobic interaction may also play a significant role in adsorption. Notably, a protein with a positive net-charge may have locally negative charge patches on the surface, mediating an electrostatic attraction with an anion exchanger.

The binding strength of the protein with the ion-exchange resin can be affected by numerous factors,

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including pH value, salt concentration, and temperature, etc., and these effects have been widely investigated [7–10]. For instance, the result obtained from equilibrium batch analysis by Finette et al. [10] has demonstrated that the binding capacities of the silica-based ion exchanger, for both human serum albumin and hen egg white lysozyme (HEWL), were reduced as salt concentration increased. Furthermore, the binding capacities and affinities for the adsorption of HEWL onto the cation exchanger were found to be non-linearly dependent on temperature. Moreover, the chromatographic behaviors of proteins in IEC also have been characterized in terms of retention parameters [11–14]. For example, Fang et al. [13] found that the changes in the retention behaviors of the proteins with the cation exchanger (''tentacle-type'' LiChrospher 1000 SO_3^- adsorbent) are not consistent with the retention being controlled by the net charge of the protein. A similar result also has been observed by Yamamoto and Ishihara [12]: the separation of β-lactoglobulins A and B (Lg A, Lg B) became better when the pH approaches the isoelectric point (p*I*) using an anion-exchange (Q-Sepharose HP) column, quite likely due to the electrostatic interaction based molecular recognition, although nonspecific forces such as hydrophobic interactions might be involved. However, there is no further evidence to support this implication.

Therefore, in order to extend our ongoing work on the microcalorimetric studies of biomaterials at a liquid–solid interface [15–21], and along with the attempt to further confirm the results mentioned above, this investigation applied the batch equilibrium binding analysis and directly measured adsorption enthalpies by using isothermal titration calorimetrry (ITC) to probe the interaction mechanisms of the proteins with Q-Sepharose by varying pH values, NaCl concentrations and temperature. The proteins used are Lg A and Lg B, an isoform of β-lactoglobulin, differing only in two amino acids of the sequences, i.e., 64: Asp/Gly and 118: Val/Ala for the A versus the B variant, respectively [22]. That is, Lg A has one more acidic residue than Lg B, leading to a small difference in their p*I* (p*I* values for Lg A and Lg B are 5.1 and 5.2, respectively). These two proteins have been reported to be successfully separated by means of IEC using Q-Sepharose resin [12,23–26]. Q-Sepharose is a strong anion exchanger, containing quaternary amine groups as binding sites, chemically attached to the hydrophilic agarose-based gel via hydroxyl chains (spacer arm).

Since the protein adsorption is the net result of various processes, dividing the overall adsorption process into some sequential subprocesses may, therefore, facilitate the thermodynamic analysis [27–29]. As imitated by our previous studies regarding the microcalorimetric studies of biomaterials at a liquid–solid interface [15–21], the adsorption of Lg A and Lg B with Q-Sepharose can be divided into five sequential subprocesses: (a) water or ion molecules surrounding the protein surface are excluded, i.e., the dehydration or de-ion (removing of the electrical double layer, EDL) process of the protein; (b) water molecules surrounding the ion exchanger are excluded, i.e., the dehydration or de-ion process of the ion exchanger; (c) electrostatic interactions and/or nonspecific interactions such as hydrophobic interactions between the protein and the ion exchanger; (d) the structural conformation of the protein is rearranged upon adsorption; and (e) rearrangement of the excluded water or ion molecules in a bulk solution.

There are, so far, no directly measured adsorption enthalpies available for studying the interaction behaviors of Lg A and Lg B with Q-Sepharose at a pH near the p*I*. The data presented, therefore, provide further thermodynamic information about the binding mechanisms between the proteins and the ion exchanger.

2. Experimental

2.1. Materials

Q-Sepharose Fast Flow gel was purchase from Pharmacia Biotech (Uppsala, Sweden) and β-lactoglobulins A and B were purchased from Sigma (St. Louis, MO, USA). All other chemicals were of analytical grade and purchased from Merck (Darmstadt, Germany). The equilibrium buffer was composed of 10 m*M* acetate, at various pH values and NaCl concentrations.

2.2. Equilibrium binding isotherms

Equilibrium binding isotherms were performed at various pH values and NaCl concentrations at 298 or

313 K, using the procedure developed in this laboratory [30]. First of all, Q-Sepharose was suspended in the equilibrium buffer solution. The ratio of gel volume to total volume was 3:1 for the binding isotherms of Lg A or Lg B. The protein (1.0 ml) solution prepared in an equilibrium buffer solution at various concentrations was added to 0.5 ml of the homogenous gel suspension in a 1.5-ml microcentrifuge tube. The solution in a microcentrifuge tube was equilibrated by incubating at 10 rpm and 298 K or 313 K for 6 h and then spun at 6000 rpm for 3 min. The suspenatant was transferred to a fresh tube, and the concentrations of proteins was determined by UV at 280 nm. The equilibrium concentrations of bound protein on the Q-Sepharose were determined by a simple material balance.

2.3. Heat measurements using isothermal titration calorimetry

The ITC system is a part of the Thermal Activity Monitor (TAM) which is a heat conduction type microcalorimeter (Thermometric, Sweden) and controlled by a computerized software called DIGITAM. For ITC measurements, the microreaction system was employing as an ''insertion'' vessel with a 4-ml stainless steel ampoule which is filled with a suspension of the gel. When thermal equilibrium between the ampoule and the heat sink was reached, the protein solution was titrated into the dispersed gel suspension through a Hamilton syringe fitted with a stainless steel needle driving by a computer-controlled pump at a time interval of 30 min. The output signal was collected as power, *P*, versus time, *t*, and was integrated and quantified by the amount of adsorbed protein to give the enthalpy change of adsorption. Notably, the apparent heat from titration should be corrected by the dilution heat of proteins and adsorbents to obtain the net heat of interaction between proteins and adsorbents, the adsorption enthalpy change (ΔH_{ads}) thus can be calculated by the following equation:

$$Q_{ads} = Vq^*\Delta H_{ads}$$

where Q_{ads} (J) is the net heat attributed to the adsorption between proteins and adsorbents, and it has been corrected by subtracting the dilution heat of proteins and adsorbents measured at the same condition, V (ml) represents the volume of the resin in the ampoule, q^* (mol/ml) is the amount of bound protein, and can be obtained from the isotherms.

Prior to each experiment, the ampoule and the stirrer were washed with water and acetone, and were then dried in air. In this investigation, Q-Sepharose gel was suspended in the acetate buffer solution at various pH values and salt concentrations. A 3-ml gel suspension with 0.09 ml gel was placed in the ampoule stirring at 120 rpm. After the thermo-equilibrium had been reached, a 40-μl Lg A or Lg B solution prepared in acetate buffer solutions was titrated into the dispersed gel suspension. All experiments were performed at a temperature of 298 or 313 K.

3. Results and discussion

3.1. Equilibrium binding analysis

3.1.1. Effects of NaCl concentration

The Langmuirean model has been reported to be suitable for simply describing the behaviors of proteins in IEC [31–34]. All the equilibrium binding isotherms presented in this study are thus fitted by the Langmuir equation to evaluate the binding affinity and maximum binding capacity of the proteins onto Q-Sepharose. For the purpose of this study, we are looking for the binding affinity at the low concentrations of protein adsorbed. That is, evaluating the free energy change from the corresponding initial binding equilibrium constant of protein adsorption. In view of this, the Langmuir model equation provides a reasonable formula for calculating the initial slope of the isotherm, although it may not be the best choice for isotherm fitting in all the concentration ranges of the protein adsorbed. The equilibrium binding isotherms for Lg A or Lg B adsorption onto Q-Sepharose at various salt concentrations, at pH 5.2 and 25°C, are shown in Fig. 1. The steeper decrease in adsorption affinities and capacities for both the proteins with this anion exchanger in the presence of increasing concentrations of NaCl, indicate that the major force involved during the adsorption is electrostatic attraction. This charge-based attractive force for Lg A or Lg B with the anion-exchange resin at pH near p*I*, probably resulted from the locally distributed nega-

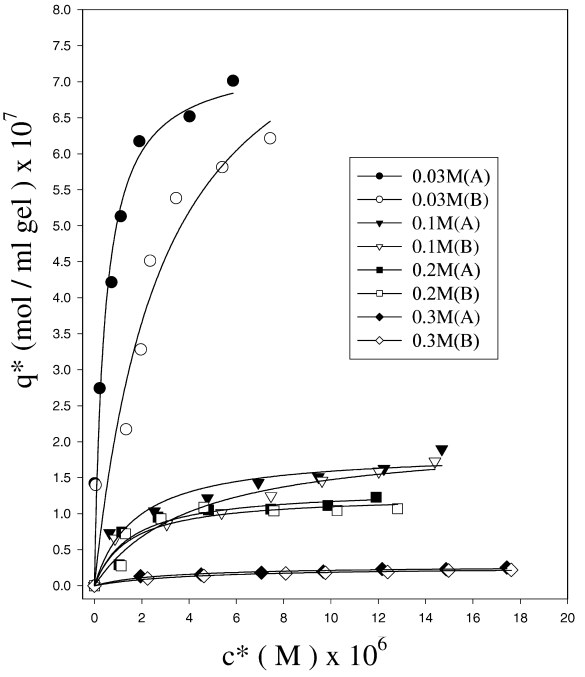

Fig. 1. The equilibrium binding isotherms for β-lactoglobulins A (A) or B (B) adsorption onto Q-Sepharose at various salt concentrations, at pH 5.2 (10 m*M* acetate) and 25°C.

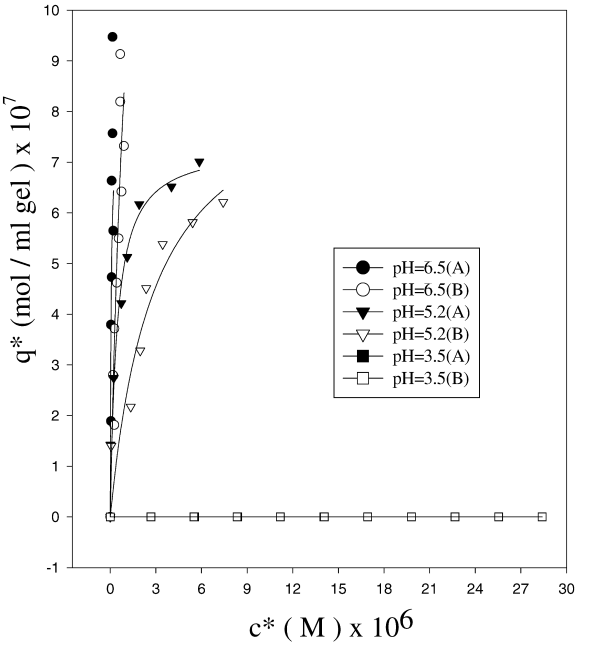

Fig. 2. The equilibrium binding isotherms for β-lactoglobulins A (A) or B (B) adsorption onto Q-Sepharose at various pH values (10 m*M* acetate), at 0.3 *M* NaCl and 25°C.

tive charges on surfaces of the proteins, since on the surface of the protein molecule, there are patches of local excess of positive or negative charge, irrespective of the overall charge as suggested by Arai and Norde [6]. Furthermore, Yamamoto and Ishihara [12] also found that Lg A and Lg B were retained on the anion- or cation-exchange column near the p*I* (pH 5.2), although this is not expected on the basis of a simple protein net charge behavior. Instead, it is quite likely to be due to electrostatic interaction on molecular recognition. Notably, hydrophobic interactions between proteins and Q-Sepharose may also partly contribute to the ion-exchange chromatographic behaviors, particularly at a higher salt concentration (0.3 *M*). This is based on the fact that higher salt concentrations may enhance the nonpolar interactions and at the same time quench the electrostatic effects. These findings are discussed in the following section.

3.1.2. Effects of pH value

The adsorption isotherms for Lg A or Lg B binding to Q-Sepharose at various pH values, at 0.3 *M* NaCl and 25°C, are shown in Fig. 2. The result demonstrates that at pH 3.5, both proteins and Q-Sepharose are all positively charged, however, spontaneous adsorption still occurred. This means that in addition to the normal electrostatic interactions expected in IEC, proteins may interact with the adsorbent surfaces by hydrophobic forces. This observation was similar to the studies of Norde and co-workers [4–6]. They found that proteins of much lower structural stability, adsorb even under the seemingly unfavorable conditions of electrostatically repelling surfaces.

Furthermore, as also shown in Fig. 2, the higher binding affinities and capacities of both the proteins with the anion exchanger were at a higher pH value. This can be explained by the fact that the electrostatic attractive forces between the protein and Q-Sepharose were enhanced with the increment of pH values. The binding sites of Lg A and Lg B with Q-Sepharose derived from the retention parameters by Yamamoto and Ishihara [12] also showed that these binding sites were increased with pH. Specifically, when pH values increased from 5.2 to 6.0, binding sites for Lg A and Lg B were increased from

5 to 6 and 4 to 5, respectively. Notably, the rapidly increasing trend in the binding capacities of both the proteins with Q-Sepharose at pH 6.5 in comparison to other pH values, seems not to reach a well defined plateau, likely resulted form the interactions between surfaces of bound proteins, leading to aggregation and/or precipitation. However, the binding capacities of the proteins obtained in this study are still in a reasonable range, since the dynamic capacities of human serum albumin for Q-Sepharose are 120 mg/ml (ca. 1.74 μmol/ml gel) as listed in the instruction book for Q-Sepharose Fast Flow.

3.1.3. Effects of temperature

The effects of temperature on the interaction behaviors for Lg A or Lg B onto Q-Sepharose at different NaCl concentrations and at pH 5.2, presented in terms of equilibrium binding isotherms, are shown in Fig. 3. The result reveals that the effects of temperature on interaction behaviors of Lg A or Lg B with Q-Sepharose were salt concentration-dependent. For instance, at a low NaCl concentration (0.03 *M*), no markedly change in the binding affinities and capacities of both the proteins with this anion exchanger were observed when the temperature increased from 25 to 40°C, in comparison to that at 0.3 *M* NaCl. This result further implies that the interaction mechanisms of Lg A or Lg B with Q-Sepharose at 0.03 *M* and 0.3 *M* are quite different. As discussed, the electrostatic attraction was dominated for the interactions of both the proteins with Q-Sepharose at 0.03 *M* NaCl, while at 0.3 *M* NaCl, the hydrophobic interaction was strengthen and played a more pronounced role in adsorption. In view of this, the result obtained can be explained by the following reasons: first, for the hydrophobic interaction dominated adsorption (0.3 *M* NaCl), it may show a linear correlation with temperature as compared to charges-based interactions (at 0.03 *M* NaCl). This suggestion also agrees with the study of Finette et al. [10] that the profound increasing trend in binding capacities between the protein and Cibacron Blue F3GA, a dye affinity resin which displayed a bi-functional ligand that contains both charged and hydrophobic groups was increased with temperatures mainly due to the contribution of hydrophobic interactions. Second, the temperature induced structural rearrangements of the proteins may facilitate the adsorption and enhance the hydrophobic interactions by the increment of exposed hydrophobic patches on surfaces of the proteins. A similar conclusion also has been reached by Fang et al. [13]: hydrophobic interactions were shown to significantly contribute to the interaction of horse heart cytochrome *c* with the cation exchanger at a higher temperature (65°C). Also, Finette et al. [10] reported that states of protein conformation at a higher temperature may lead to accessibility of a larger number of hydrophobic patches on protein surfaces that can participate in the adsorption. The discussion above was further confirmed by the measured enthalpies, in the portion of microcalorimetric measurements

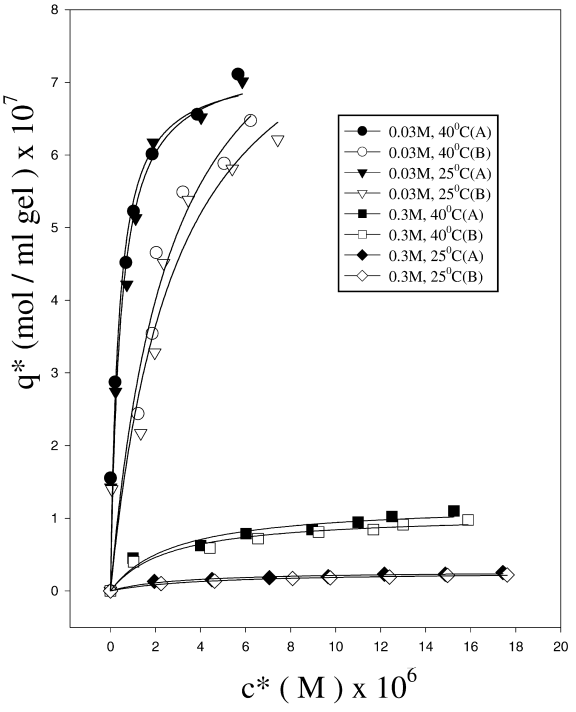

Fig. 3. The equilibrium binding isotherms for β-lactoglobulins A (A) or B (B) adsorption onto Q-Sepharose at various NaCl concentrations and temperature, at pH 5.2 (10 m*M* acetate).

3.2. Microcalorimetric measurements

3.2.1. Effects of NaCl concentration

The effects of NaCl concentration on the adsorption enthalpy (ΔH_{ads}) of Lg A or Lg B with Q-Sepharose at pH 5.2 and 25°C are presented in Fig. 4. These values are of similar orders of mag-

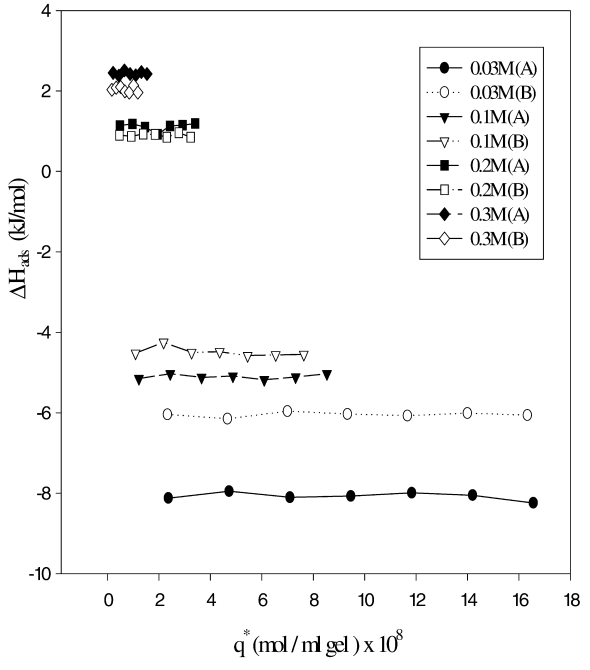

Fig. 4. The adsorption enthalpies (ΔH_{ads}) of β-lactoglobulins A (A) or B (B) with Q-Sepharose at various NaCl concentrations, at pH 5.2 (10 m*M* acetate) and 25°C.

nitude to those obtained by directly microcalorimetric measurements or derived from Van 't Hoff analysis of protein adsorption in IEC [10,33–36]. The results show that the values of ΔH_{ads} for both the proteins increase with concentrations of NaCl. This fact is due to: (i) an reduction in the degree of endothermic amount of the dehydration and the removing of electrical double layer (EDL) processes (i.e., processes a and b) at higher salt concentrations; (ii) the electrostatic attractive force was increasingly screened by an increment of the salt concentration, leading to a decrease in the exothermic amount of heat (i.e., process c). In contrast, the hydrophobic interactions may be enhanced at a higher salt concentration; (iii) the heat required for structural rearrangement of the protein decreased with salt concentrations (i.e., process d). However, this contribution to the result cannot be clearly evaluated, although Bowen and Hughes [34] have suggested that for the protein adsorption onto the ion exchangers, the contribution of substantially structural changes of proteins to the ΔH_{ads} values are not markedly observed. Also, Norde and co-workers [6,37,38] reported that the conformational alteration of the protein upon adsorption in IEC majorly contributed to the entropy gain rather than to the enthalpy. Furthermore, effects of NaCl concentrations on the dehydration and the removing of EDL processes (i.e., processes a and b) were also not obviously in this study, owing to the ΔH_{ads} values of both the proteins were increased with NaCl concentrations.

As a consequence, the exothermic values of ΔH_{ads} for Lg A or Lg B adsorption onto Q-Sepharose at lower NaCl concentrations (0.03 *M* and 0.1 *M*) at pH 5.2 and 25°C, was attributed mostly to the electrostatic attraction. While at higher NaCl concentrations (0.2 *M* and 0.3 *M*), adsorption was dominated by the hydrophobic interactions and the exothermic contribution from the electrostatic attraction became smaller, hence leading to higher positive values of ΔH_{ads}. Moreover, the data obtained also evidenced that the ion-exchange chromatographic behaviors of Lg A and Lg B at pH near p*I* were partly entropy-driven, implying that the hydrophobic interactions play a more pronounced role under this condition.

In addition, the difference in ΔH_{ads} values of Lg A and Lg B under the same experimental conditions were all smaller, and Lg A has a lower values about 0.5~2 kJ/mol as compared with Lg B. This is probably because Lg A has an one more negatively charged amino acid than Lg B [22] and results in an reduction in the ΔH_{ads} values which was attributed mostly to the electrostatic attraction. The result obtained by Yamamoto and Ishihara [12] also supported this implication. They found that Lg B has less binding sites (derived from the retention parameters of IEC) than Lg A when adsorption onto Q-Sepharose, and the difference in binding sites was about one. Notably, the heat required for the dehydration and the removing of EDL processes (i.e., processes a and b) and the structural rearrangement of Lg A (i.e., process d) may be larger than that of Lg B. However, the more exothermic contribution from the electrostatic interactions (i.e., process c) for Lg A compensated these enthalpic unfavorable approaches. Finally, the adsorption enthalpies were found to be varied insignificantly with the amount of bound protein, indicating that the interactions between surfaces of bound proteins can be ignored, likely due to the lower amount of bound protein used

Table 1
The averaged thermodynamic parameters obtained in this study under various pH values

	pH 3.5		pH 5.2		pH 6.5	
	Lg A	Lg B	Lg A	Lg B	Lg A	Lg B
ΔG_{ads}	−4.05	−5.87	−7.76	−3.05	−12.83	−11.05
ΔH_{ads}	8.54	9.28	−8.15	−6.04	−13.25	−12.11
$T\Delta S_{ads}$	12.59	15.15	−0.39	−2.99	−0.42	−1.06

Note: (1) Lg A and Lg B represent β-lactoglobulins A and B, respectively. (2) The unit of the thermodynamic parameter is kJ/mol.

to quantify ΔH_{ads} values in this study. Also, as suggested by Norde and co-workers [27,29] that in the isoelectric region of the protein, the lateral interactions between proteins are enthalpically favored, but they becomes unfavorable when the protein molecule attains a net positive or negative charge. Therefore, at a pH near the p*I*, the repulsions between bound protein molecules are expected to be small.

3.2.2. Effects of pH value

For simply discussing pH effects (and temperature effects) on the interaction mechanisms between Lg A or Lg B and Q-Sepharose thermodynamically, the averaged values of ΔH_{ads} and ΔS_{ads}, calculated from the initial binding of the isotherms, for preventing the lateral interactions between the adsorbed protein molecules as at the higher binding capacity, are listed in Tables 1 and 2, respectively. Values of ΔS_{ads} are calculated by the equilibrium binding isotherms and the measured adsorption enthalpies. As can be seen in Table 1, the ΔH_{ads} values decreased with the increment of pH, probably owing to the negative charges on the proteins' surfaces increased with pH so that enhanced the electrostatic attraction between Lg A or Lg B and Q-Sepharose. Interestingly, at pH 3.5, the electrostatic forces between both the proteins and the anion exchanger are repulsive, however, adsorption still occurred spontaneously as has been found in the equilibrium binding isotherms, indicating that the hydrophobic interactions play a pronounced role under this condition as discussed. The endothermic ΔH_{ads} values measured in this study were further confirmed this implication. The unfavorable electrostatic repulsion was found to be partly compensated by the large entropy gain, as can be seen in Table 1, mostly resulting from the dehydration and removal of EDL processes [27,29].

Furthermore, at pH values above 5.2, the electrostatic attractive force was enhanced and caused a reduction in ΔH_{ads} values. Specifically, at pH 5.2, the electrostatic and hydrophobic forces collectively controlled the interaction mechanisms between Lg A or Lg B and Q-Sepharose. Nevertheless, at pH 6.5, the adsorption seems majorly governed by the electrostatic attractions, since the ΔH_{ads} values become more exothermic. It should be noted that partial denaturation of the protein may expose more hydrophobic patches and results in stronger hydrophobic interactions. However, the experimental conditions operated in this study are not that extreme to cause protein partial denauration, therefore, this effect is expected to be minor.

Consequently, Q-Sepharose, although an anion

Table 2
The averaged thermodynamic parameters obtained in this study under various salt concentrations and temperature

	0.03 *M* NaCl				0.3 *M* NaCl			
	25°C		40°C		25°C		40°C	
	Lg A	Lg B	Lg A	Lg B	Lg A	Lg B	Lg A	Lg B
ΔG_{ads}	−7.76	−3.05	−8.27	−3.28	−3.01	−2.12	−3.15	−3.35
ΔH_{ads}	−8.15	−6.04	−7.58	−6.23	2.45	2.03	0.51	0.25
$T\Delta S_{ads}$	−0.39	−2.99	0.69	−2.95	5.46	4.15	3.66	3.6

Note: (1) Lg A and Lg B represent β-lactoglobulins A and B, respectively. (2) The unit of the thermodynamic parameter is kJ/mol.

exchanger, can be used as a bi-functional-like adsorbent based on the electrostatic and hydrophobic interactions (which has been confirmed in this study) for protein separations at a pH near the p*I*. Moreover, the combination of electrostatic and hydrophobic forces involved during the adsorption process for both the proteins with Q-Sepharose is not surprising, taking into account the hydrophobic patches on protein surfaces and the ability of quaternary ammonium chemically attached to the agarose through a long hydroxyl chain (acting as bi-functional ligands) to form Q-Sepharose. The optimization for purifying protein using this bi-functional-like adsorbent can be thus achieved by choosing properly experimental conditions. For instance, Yamamoto and Ishihara [12] has reported that the separation (resolution) of Lg A and Lg B became better when the pH approached the p*I* in Q-Sepharose columns, although the electrostatic attraction can be enhanced with increasing pH, the resolution was poor.

3.2.3. Effects of temperature

Since the dependence of the interaction behaviors between Lg A or Lg B and Q-Sepharose with temperature indicates the more contribution of the hydrophobic interactions to adsorption process as mentioned above. The effects of temperature on ΔH_{ads} and ΔS_{ads} of Lg A or Lg B adsorption onto Q-Sepharose at various NaCl concentrations and at pH 5.2, were presented to confirm this implication. As can be seen in Table 2, the result was found to be dependent on concentrations of NaCl. For example, the ΔH_{ads} values measured do not appear to correlate well with temperature at 0.03 *M* NaCl, that is, the ΔH_{ads} of Lg A increased with temperature, while that of Lg B showed the opposite trend. Since adsorption was dominated by the electrostatic attraction under this condition as discussed, these findings quite likely resulted from the nature of the charge-based interactions which were expected to decrease with temperature. A result reported by Hutchens and Yip [39] also supported this implication that the decrease in the strength of electrostatic forces at a higher temperature upon protein adsorption could be owing to a reduction in the ionization degree of the amino acid residues associated with the binding sites. In the contrast, at a higher NaCl concentration (0.3 *M*), the values of ΔH_{ads} for both the proteins were decreased with temperature, probably because adsorption was major governed by the hydrophobic interactions. This suggestion was further confirmed by the study of Finette et al. [10], they observed that for proteins adsorption onto an cation exchanger, the change in affinity and binding capacity could be due to the interplay of both electrostatic and hydrophobic forces, and the electrostatic effect will prevail at low temperature, whilst the hydrophobic effect becomes more significant at a higher temperature. Moreover, this observation can be explained in detail as follows: (i) the heat required for dehydration and the removal of EDL process of the protein and the adsorbent were diminished as temperature raised (i.e., processes a and b); (ii) the hydrophobic forces were enhanced with temperature (i.e., process c). Notably, the hydrophobic patches buried in the interior of the protein molecules were tend to be exposed when temperature increases, leading to the enhancement of hydrophobic forces as well. (iii) The heat required for structural rearrangements of the proteins decreased with the increment of temperature.

In conclusion, the presented ΔH_{ads} and ΔS_{ads} values at various NaCl concentrations and temperature have confirmed that the effects of temperature on equilibrium binding behaviors of Lg A and Lg B with Q-Sepharose were salt concentration-dependent with pH near the p*I*, due to their different interaction mechanisms at 0.03 *M* and 0.3 *M* NaCl. That is, at a lower salt concentration (0.03 *M* NaCl), adsorption was dominated by the electrostatic attraction, while at 0.3 *M* NaCl, adsorption was mostly attributed to the hydrophobic interaction.

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