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Journal of Chromatography A, 872 (2000) 37–47

JOURNAL OF CHROMATOGRAPHY A

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Microcalorimetric studies of interactions between proteins and hydrophobic ligands in hydrophobic interaction chromatography: effects of ligand chain length, density and the amount of bound protein

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Received 9 August 1999; received in revised form 10 November 1999; accepted 15 November 1999

Abstract

Using isothermal titration calorimetry (ITC), this investigation directly measured the adsorption enthalpies of proteins on various hydrophobic adsorbents. Various amounts of butyl and octyl groups were attached onto CM-Sepharose to form C_4 and C_8, two types of hydrophobic adsorbents. The adsorption enthalpies of both trypsinogen and α-chymotrypsinogen A were measured at 4.0 *M* NaCl and pH 10.0, in which most ionic interaction was suppressed. The adsorption isotherms of both proteins on various adsorbents were also measured, thus allowing us to calculate the Gibbs free energy and entropy of adsorption. Experimental results indicated that the adsorption of both proteins on butyl-containing adsorbents was exothermic, while their adsorption on octyl ones was endothermic. In addition, binding of both proteins with the butyl ligand is basically an adsorption process, while binding with the octyl ligand is adsorption and partition processes. Moreover, on both butyl or octyl, the adsorption enthalpy became increasingly positive as the ligand density increased, while the adsorption entropy became more positive as the alkyl chain length or density of the adsorbent increased. In addition, ITC was used to measure protein–protein interaction. The adsorption enthalpy of both proteins increased as the amount of bound protein increased, and the enthalpy increase of trypsinogen appeared to be higher than that of α-chymotrypsinogen A. This observation implies that protein–protein repulsion was stronger among trypsinogen molecules in the experiments.

Keywords: Isothermal titration calorimetry; Adsorption enthalpy; Protein adsorption; Thermodynamic parameters; Proteins

1. Introduction

Hydrophobicity is a characteristic of constituted biomaterial residues, and plays an important role in the formation of the three-dimensional native structure of proteins in solution. In an aqueous solution, most of the hydrophobic amino acid residues are buried in the interior of the molecules. Hydrophobicity of biomaterial, is therefore determined by the area exposed and the location of hydrophobic pat-

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0021-9673/00/$ – see front matter
PII: S0021-9673(99)01231-5

ches on the surface of the biomaterial. A related study [1] indicated that the hydrophobicity of the protein increased with the area of exposed hydrophobic patches. Protein adsorption by hydrophobic interaction chromatography (HIC) thus strongly relies on the accessible area of hydrophobic patches on the protein surface.

Shepard and Tiselius [2] first reported on HIC, observing the retention of dye in a so called salting-out chromatography where sulfate and phosphate solutions were present. As a follow up study, Shaltiel and Er-el [3,4] used the term hydrophobic chromatography or hydrophobic affinity chromatography, Hofstee [5] described the method as hydrophobic adsorption chromatography, and finally Hjerten [6] called the method hydrophobic interaction chromatography. HIC is an important method for separating and purifying biomaterials. The method is based on the interfacial interaction between biomaterial and hydrophobic ligands which are chemically attached to hydrophilic matrices. Specifically, the exposed hydrophobic groups from accessible patches on the biomaterial's surface can interact with the hydrophobic matrix of a support, the implication of which has been confirmed by Vogel and co-workers [7,8]. The major contribution to the hydrophobic interaction is Van der Waals forces as proposed by Van Oss and co-workers [9,10]. The distance between two interacting surfaces is relatively small and, thus, hydrophobic interaction can be understood in terms of short-range forces. Owing to HIC having a weaker interaction than affinity, ion-exchange or reversed-phase chromatography, the structural damage to the biomaterial of interest is assumed to be minimized, and the biological activity of biomaterial is maintained [11,12]. Therefore, HIC has been widely applied to separate and purify biomaterials [13–20].

The adsorbents of HIC are mainly hydrophilic polysaccharide gels, such as Sepharose, dextran or modified silica gels. The ligands coupling with the hydrophobic compounds, such as butyl, octyl and phenyl groups can be covalently attached to the adsorbents. Proteins can be retained in HIC by utilizing a buffer solution with a high salt concentration as a mobile phase. In doing so, elution is achieved by reducing the hydrophobic interaction by a descending salt gradient.

The mobile and stationary phases as well as the protein structure all play a role in determining the binding behavior in HIC. Therefore, the studies on effects of the protein binding with hydrophobic adsorbents were widely investigated, including pH values [21–24], salt and concentrations [25–30], ligand chain length and density of adsorbents [31–41], and temperature [42–48]. For instance, one study [22] on the effect of salt and concentrations indicated that ionic groups can be involved in the adsorption of proteins on alkyl derivatives of cyanogen bromide-activated Sepharose, and the electrostatic interaction will be eliminated at a high ionic strength. Furthermore, the retention of α- and β-trypsin increased almost linearly with increasing NaCl concentration. However, studies involving the effects of HIC, focused mainly on the retention and binding affinity of proteins with hydrophobic adsorbents. For example, Hofstee [49] reported that an increment of the ligand chain length and density of the *n*-alkyl chain on the support surface enhanced the binding affinity and retention of proteins in the column. On the other hand, studies adopting from the thermodynamic perspective are limited [50–52], and only a few reports [53,54] have demonstrated that the hydrophobic interaction is an entropy-driven process. However, to our knowledge, no evidence directly supports the statement.

So far, the adsorption enthalpy of HIC has not been directly measured. This study elucidates the effects on the binding mechanism by varying the ligand chain length and density of hydrophobic adsorbents. Also discussed herein are the differences in enthalpy change of two protein, α-chymotrypsinogen A and trypsinogen with two modified hydrophobic adsorbents, CM-butyl-Sepharose and CM-octyl-Sepharose under varying amounts of bound protein.

By following the previous study [55–58] which demonstrated the binding mechanism of imidazole and lysozyme with immobilized Cu(II) and Fe(III) by varying pH values, NaCl concentrations and metal ions in the immobilized metal ion affinity chromatography (IMAC). The discussion of the binding mechanism in HIC can also be divided into five sequential subprocesses: (a) water or ion molecules surrounding hydrophobic patches on protein surface, i.e., the dehydration or deionization (removing the electrical double layer) of the protein; (b) water or ions molecules surrounding the hydrophobic ligand of the adsorbent, i.e., the dehydration or

deionization of the adsorbent; (c) hydrophobic interaction (Van der Waals forces) between proteins and hydrophobic adsorbents; (d) the structural rearrangement of the protein; and (e) rearrangement of the excluded water or ion molecules in a bulk solution. However, in this study, focuses mainly on the dehydration process (i.e., the b process) in considering the differences in the ligand chain length of adsorbents. The differences in ligand density are also considered in relation to the dehydration process (i.e., a and b processes), as well as hydrophobic interaction (i.e., the c process), and structural rearrangement of the protein (i.e., the d process). Finally, for investigating the adsorption enthalpy by varying the amount of bound protein, this study emphasizes the protein–protein interaction and the intrinsic characteristics of both proteins.

Although much can be learned from the early work with HIC in terms of both retention behavior and applications for protein separation, information relating to thermodynamics is still lacking, particularly in terms of directly measured data. Therefore, results presented herein have important implication, both for providing further insight into the binding mechanism of protein adsorption and for improving theoretical approaches to HIC.

2. Experimental

2.1. Materials

CM-Sepharose gel was purchased from Pharmacia Biotech (Uppsala, Sweden). The synthesis of CM-butyl-Sepharose and CM-octyl-Sepharose gels was followed by applying the method reported [59]. Trypsinogen and α-chymotrypsinogen A came from Sigma (USA). All other chemicals were analytical grade and purchased from E. Merck (Germany). The equilibrium buffer was composed of 20 m*M* carbonate, at pH 10.0 and 4.0 *M* NaCl. The high salt concentration, eliminated the electrostatic interaction between proteins and adsorbents.

2.2. Isothermal titration calorimetry measurements

The microcalorimetry system used is a Thermal Activity Monitor (Thermometric, Sweden) controlled by Digitam software. The microreaction system is a titration mode with a 4-ml stainless steel ampoule. An isothermal system provides continuous heat leakage measurements. The heat from adsorption flows through high-sensitivity thermopiles surrounded by a heat sink which is stabilized at $\pm 2 \cdot 10^{-4}$°C. The heat exchange of the thermopile with the heat sink is proportional to the time interval of the voltage signal. The performance of electrical calibration allows the results to be quantified.

Before each experiment, the ampoule and the propeller stirrer were washed with water and acetone, then dried in the air. This investigation, suspended CM-butyl- or CM-octyl-Sepharose gel in the equilibrium buffer solution. A 3-ml gel suspension, containing 0.1 ml gel, was placed in the ampoule, and stirred at a rate of 100 rpm. When thermo-equilibrium between ampoule and the heat sink was achieved, a 50-μl trypsinogen or 40-μl α-chymotrypsinogen A solution prepared in an equilibrium buffer solution was titrated into the dispersion gel suspension by the Hamilton microliter syringe at 25 min intervals. All experiments were performed at a temperature of 298 K. Fig. 1 displays typical microcalorimetric thermograms of titration of α-chymotrypsinogen A or trypsinogen into the dispersed CM-octyl-Sepharose gel at pH 10.0 and 4.0 *M* NaCl, respectively,

Before adsorption enthalpy from titration is calculated, the heat of titration should be corrected to take account of the following terms to obtain the net heat of interaction between proteins and the adsorbents: (a) dilution heat of proteins, and (b) dilution heat of gels. In this study, measured adsorption enthalpy for α-chymotrypsinogen A and trypsinogen with CM-butyl- or CM-octyl-Sepharose gel in various binding conditions. The enthalpy of adsorption (ΔH_{ads}) is calculated by the following equation:

$$Q_{ads} = V \times q^* \times \Delta H_{ads}$$

where V represents the volume of the gel in the ampoule, q^* denotes the amount of protein adsorbed on the unit volume of gels, and ΔH_{ads} is the enthalpy of adsorption. q^* can be obtained from the isotherm [60].

The data obtained by calorimetric measurements and adsorption isotherms, allows us to calculate the adsorption enthalpies of proteins onto the gels containing hydrophobic ligands, and this allows the

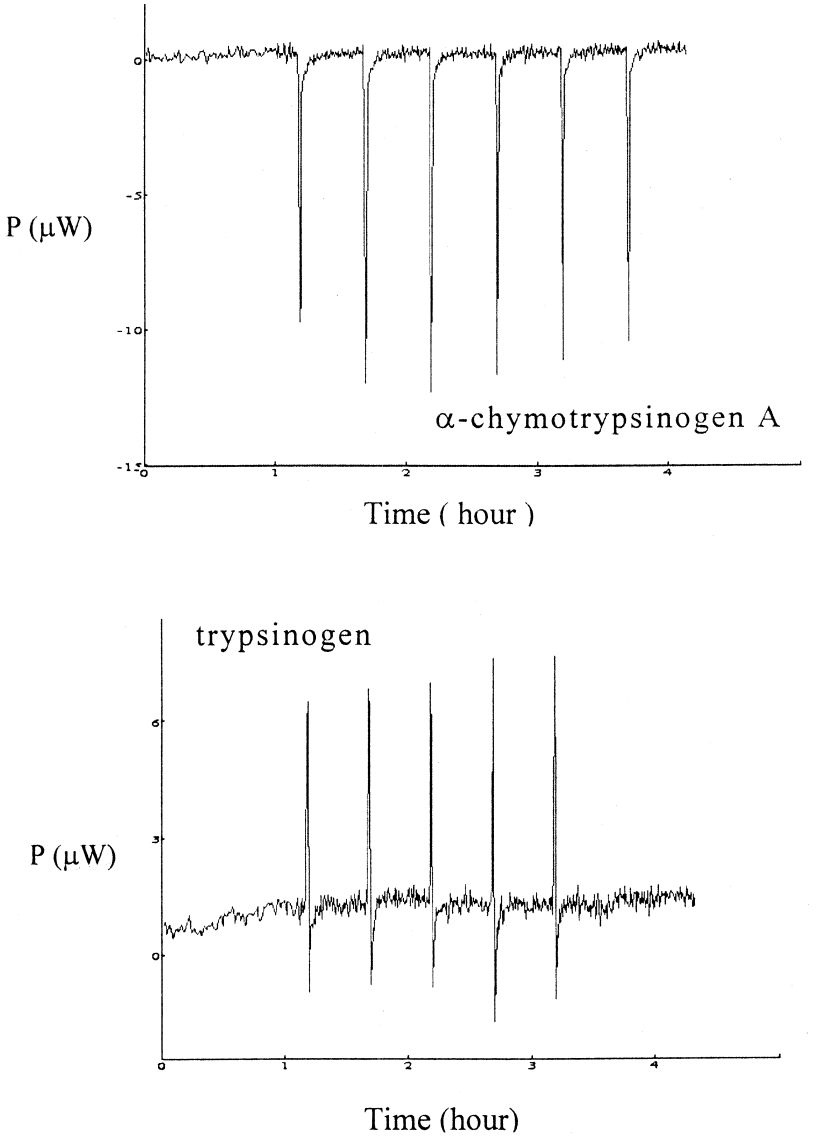

Fig. 1. The typical microcalorimetric thermograms of titration of α-chymotrypsinogen A or trypsinogen into dispersed CM-octyl-Sepharose at 4.0 *M* NaCl and pH 10.0, respectively.

adsorption behavior to be interpreted from a thermodynamic perspective.

3. Results and discussion

3.1. Effects of ligand chain length

Figs. 2 and 3 present adsorption enthalpies (ΔH_{ads}) of trypsinogen or α-chymotrypsinogen A adsorption onto CM-butyl-Sepharose or CM-octyl-Sepharose with variable ligand density at 4.0 *M* NaCl and pH 10.0, respectively. According to those figures, the ΔH_{ads} values of both proteins with CM-octyl-Sepharose at constant ligand density were higher than with CM-butyl-Sepharose and the difference in value was 7~8 kJ/mol. Since the adsorption process can be divided into five sequential subprocesses, as men-

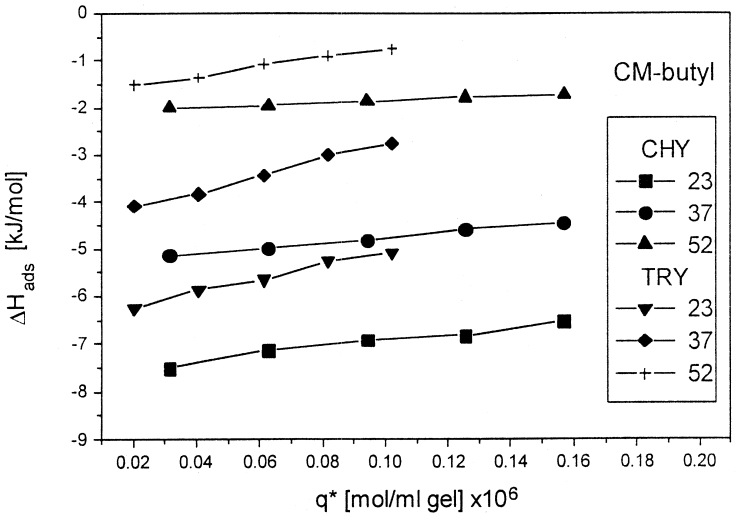

Fig. 2. Adsorption enthalpies (ΔH_{ads}) of α-chymotrypsinogen A (CHY) or trypsinogen (TRY) onto CM-butyl-Sepharose at various ligand densities, 4.0 *M* NaCl, and pH 10.0. (The number in the key of the figure represents the ligand density of the adsorbent, mol/ml gel).

tioned earlier, the analysis of ΔH_{ads}, for studying the effect of variable ligand chain length of the adsorbent can be similarly achieved. Specifically, the dehydration of hydrophobic ligand of the adsorbent and the hydrophobic interaction between proteins and hydrophobic ligands (i.e., b and c processes) should be considered mainly for the difference. The major contributor to the hydrophobic interaction is Van der Waals forces as suggested by Van Oss et al. [9,10]. The ligand chain length of the adsorbent should not significantly affect the heat generated by Van der Waals forces from both proteins' adsorption on CM-butyl-Sepharose and CM-octyl-Sepharose at a constant ligand density. (i.e., the c process). Therefore, the difference Figs. 2 and 3 shown in the value of ΔH_{ads} for CM-butyl-Sepharose and CM-octyl-Sepharose implies that the heat required for the dehydration (i.e., the b process) differs. The reported

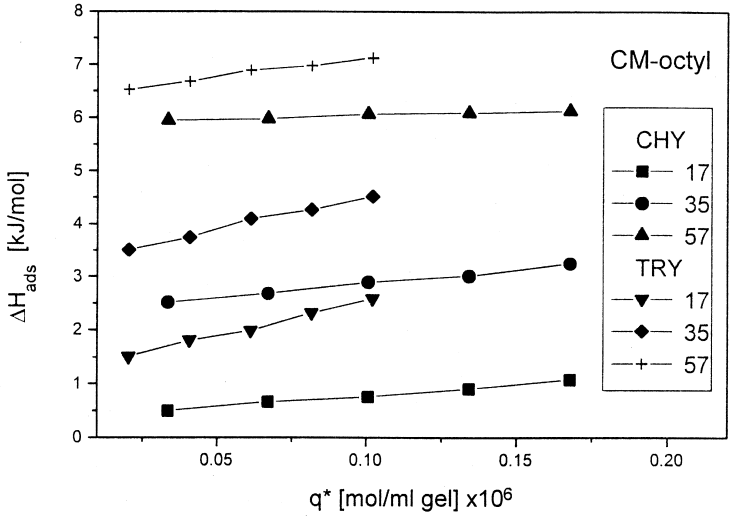

Fig. 3. Adsorption enthalpies (ΔH_{ads}) of α-chymotrypsinogen A (CHY) or trypsinogen (TRY) onto CM-octyl-Sepharose at various ligand densities, 4.0 *M* NaCl, and pH 10.0. (The number in the key of the figure represents the ligand density of the adsorbent, mol/ml gel).

data [61] also supported this finding indicating that the heat required to dehydrate the *n*-octyl chain was 10 kJ/mol higher than that required to dehydrate the *n*-butyl chain. This result also agrees with the study of Haidacher et al. [62]. They used derived quadratic and logarithmic equations to calculate the adsorption enthalpy values of amino acids with gels containing hydrophobic ligands and found the results were within a range of 2~3 kJ/(per carbon of the ligand chain length). From the above findings, it seems that the values of ΔH_{ads} obtained by ITC measurements correlate well with the values calculated by the retention data in the literature. Notably, the structural rearrangement of proteins with CM-butyl-Sepharose may differ from that with CM-octyl-Sepharose, because it may be affected by the ligand chain length of the adsorbent [63]. However, the contribution of structural rearrangement of proteins to the difference in ΔH_{ads} values was assumed to be minimized due to the relatively weaker interactions involved in HIC.

In addition, the ΔH_{ads} values of CM-octyl-Sepharose with both proteins were positive implying the driven force of interactions for CM-octyl-Sepharose was mostly by entropy. Furthermore, as Figs. 4 and 5 demonstrate, the adsorption entropies (ΔS_{ads}) determined by fitting the equilibrium binding isotherms (Ref. [60] reports the data of the isotherms) of both proteins with CM-octyl-Sepharose at the constant ligand density were higher than that with CM-butyl-Sepharose and the difference in values was about 25 J/mol K. This result was also in agreement with the study of Horváth et al. [62]. Since adsorption per mole of proteins onto CM-octyl-Sepharose required more energies to the dehydration than that of CM-butyl-Sepharose as discussed, we believed that the higher entropy-gain of proteins with CM-octyl-Sepharose as compared with CM-butyl-Sepharose derives mainly from the dehydration process. Therefore, with the fact that the interaction of proteins with CM-octyl-Sepharose was mostly driven by entropy, and the heat required for the dehydration was higher than that for CM-butyl-Sepharose. We further conclude that the interaction mechanism of both proteins with CM-octyl-Sepharose is more like partitioning, while with the CM-butyl-Sepharose it is an adsorption dominated process. This conclusion is also supported by Dorsey and Dill's summary [64]. The conclusions of their studies on the molecular mechanism of retention in reversed-phase liquid chromatography revealed that the mechanism of solutes with short chains of the adsorbents dominated the adsorption, whereas that with longer chains dominated the partition mechanism.

3.2. Effects of ligand density

Figs. 2 and 3 show the ΔH_{ads} of α-chymotrypsinogen A or trypsinogen adsorption onto CM-butyl-

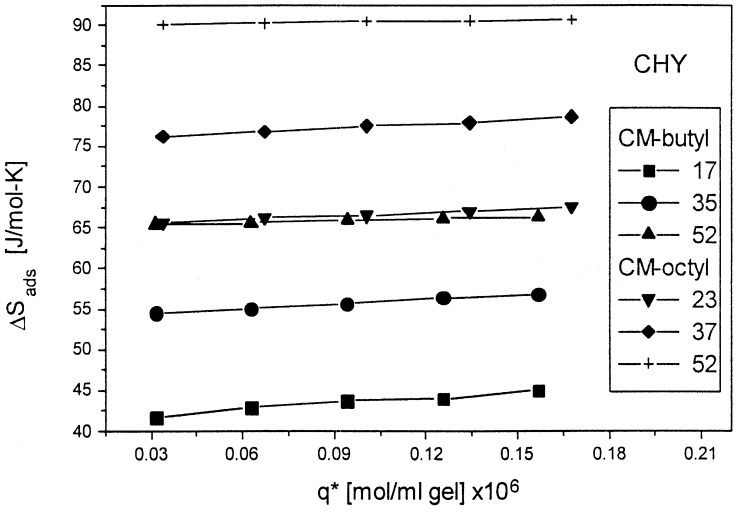

Fig. 4. Adsorption entropies (ΔS_{ads}) of α-chymotrypsinogen A (CHY) onto CM-butyl- and CM-octyl-Sepharose at various ligand densities, 4.0 *M* NaCl, and pH 10.0. (The number in the key of the figure represents the ligand density of the adsorbent, mol/ml gel).

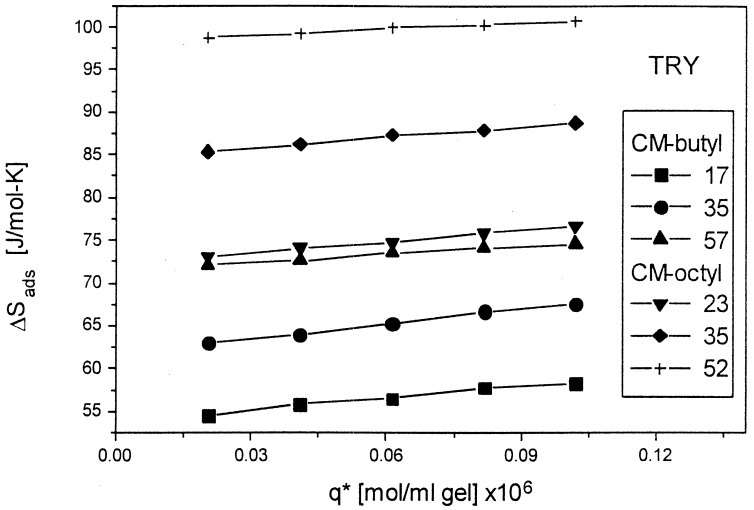

Fig. 5. Adsorption entropies (ΔS_{ads}) of trypsinogen (TRY) onto CM-butyl- and CM-octyl-Sepharose at various ligand densities, 4.0 *M* NaCl, and pH 10.0. (The number in the key of the figure represents the ligand density of the adsorbent, mol/ml gel).

Sepharose or CM-octyl-Sepharose with various ligand densities at 4.0 *M* NaCl and pH 10.0, respectively. The adsorption enthalpies of both proteins increased with ligand density. Possible explanations are (i) a reduction in the endothermic amount of the dehydration (i.e., a and b processes) at a lower ligand density. The heat required for the dehydration of the adsorbent per binding process may be enhanced as ligand density is raised; (ii) an increase in the exothermic amount of the hydrophobic interaction (i.e., the c process). As from the binding isotherms determined by the equilibrium batch condition as dictated in Ref. [60]. The results show that the adsorption affinities of both proteins with CM-butyl-Sepharose or CM-octyl-Sepharose increased with ligand density, indicating that the hydrophobic interaction of both proteins with CM-butyl-Sepharose or CM-octyl-Sepharose was enhanced as ligand density increased. Therefore, the heat generated by the hydrophobic interaction increased with the ligand density; (iii) a reduction in the endothermic amount for structural rearrangement of the protein (i.e., the d process) at a higher ligand density of the adsorbents. The binding sites of the adsorbent enlarged with an increase of the ligand density, and enhanced the accessibility for protein adsorption. The degree of structural rearrangement of the protein needed to enlarge the exposed hydrophobic patches buried in the interior thus is reduced, and this minimizes the heat required for structural rearrangement. Since the ΔH_{ads} value increased with ligand density, the hydrophobic bonding as well as the structural rearrangement of the protein may not play important roles in the increase of the ligand density. Therefore, the major contributor to the result was the heat required for the dehydration. Furthermore, the adsorption entropies of both proteins increased with ligand density, as shown in Figs. 4 and 5, implying the contribution of the dehydration process to the entropy-gain increased with ligand density. This result agrees with the study of Cole and Dorsey [65]. They found that the enthalpy and entropy values of benzene with C_{18} ligands calculated by Van 't Hoff plots, both increased with ligand density.

3.3. Effects of the amount of bound protein

In this study, adsorption enthalpies (ΔH_{ads}) of both proteins increased with the amount of bound protein at a constant ligand chain length and density of the adsorbent at 4.0 *M* NaCl and pH 10.0 as Figs. 2 and 3 show. The results can be explained by (i) the protein–protein interaction being enhanced by an increase in the amount of bound protein. The adsorbed protein molecules adjacent on the adsorbent interact with each other due to the distance between the adsorbed molecules reduced as the amount of bound protein enlarged (e.g., through electrostatic

repulsion or the steric hinderance of the dimensional structure of the protein molecules). Therefore, additional heat will be required to overcome this unfavorable approach; (ii) the hydrophobic bonding of the protein with the adsorbent (i.e., the c process) minimized by an increase in the bound protein. The increase in the bound protein will reduce the binding sites on the surface of the adsorbent. This will promote steric hinderance between the protein and the adsorbent, and the hydrophobic bonding will become less favorable; (iii) the heat required for the dehydration of the adsorbent (i.e., the b process) reducing as the amount of bound protein increases. One possible reason for this is as follows. Water molecules partitioning around the hydrophobic surface of the adsorbent were initially stabilized by the hydrogen bonding. However, as the dehydration process destroyed the hydrogen bonding, the structure of water molecules surrounding the surface of the adsorbent were destabilized, minimizing the heat required for the dehydration of the adsorbent as the amount of bound protein grew. But since the ΔH_{ads} values increased with the amount of bound protein, the above mentioned processes (i.e., b and c processes) may not be significant in this study. This study, thus believes that protein–protein interaction was the major contributor to the results obtained.

In addition, the variation in the adsorption enthalpies of α-chymotrypsinogen A with the two adsorbents was greater than that of trypsinogen as the amount of bound protein enlarged, implying that the protein–protein interaction and intrinsic characteristics of proteins, are different for both proteins. The dilution heat of the protein obtained by ITC measurements, shown in Fig. 6, demonstrate that the dilution heat of α-chymotrypsinogen A was endothermal, while that of trypsinogen was exothermal. This result implies that protein–protein repulsion among trypsinogen molecules is stronger than in α-chymotrypsinogen A molecules. Consequently, the heat required to overcome the repulsive interaction of trypsinogen molecules became greater that for α-chymotrypsinogen A molecules as the amount of bound protein increased.

Adsorption enthalpies (ΔH_{ads}) of α-chymotrypsinogen A with two adsorbents were all lower than those of trypsinogen, as Figs. 2 and 3 show. The possible explanations are (i) a higher exothermic amount of hydrophobic interaction of α-chymotrypsinogen A with adsorbents than that of trypsinogen (i.e., the c process). From the data reported by Katti et al. [1] revealed that the hydrophobicity of the protein increased as the area of hydrophobic patches increased, and α-chymotrypsinogen A had a higher exposed hydrophobic area than trypsinogen did. Therefore, there was a more reduction in the exothermic amount of the hydrophobic bonding for trypsinogen than for α-chymotrypsinogen A; (ii) a lower endothermic amount for the dehydration process of trypsinogen than that of α-chymotrypsinogen A (i.e., the b process). Since trypsinogen had a lower exposed hydrophobic area than α-chymotrypsinogen A, the endothermic amount of the dehydration for trypsinogen also reduced; (iii) a reduction in the endothermic amount for structural rearrangement of α-chymotrypsinogen A (i.e., the d process). The accessibility of the protein with the adsorbent grew with the area of exposed hydrophobic patches. The degree of structural rearrangement of the protein needed to enlarge the exposed hydrophobic patches buried in the interior thus is reduced, and this minimizes the heat required for structural rearrangement. As a result, the ΔH_{ads} values of α-chymotrypsinogen A with two adsorbents were all lower than those of trypsinogen. Therefore, this study, concludes that the major contribution to the different values of adsorption enthalpy was the hydrophobic interaction and the structural rearrangement of the protein.

4. Conclusions

The adsorption enthalpies of both proteins on octyl-containing adsorbents were all positive, varying between 0 and 8 kJ/mol, which indicated that the adsorption on C_8 adsorbents were mostly entropy driven. However, the adsorption enthalpies on butyl-containing adsorbents were all negative, ranging from −8 to 0 kJ/mol. On low butyl content, 23 μmol/ml gel, the adsorption enthalpies contributed more than one third of the negativity of the adsorption Gibbs free energy. Therefore, the adsorption on low density C_4 adsorbents should have been partially enthalpy driven. As the butyl density increased, the adsorption enthalpy became more posi-

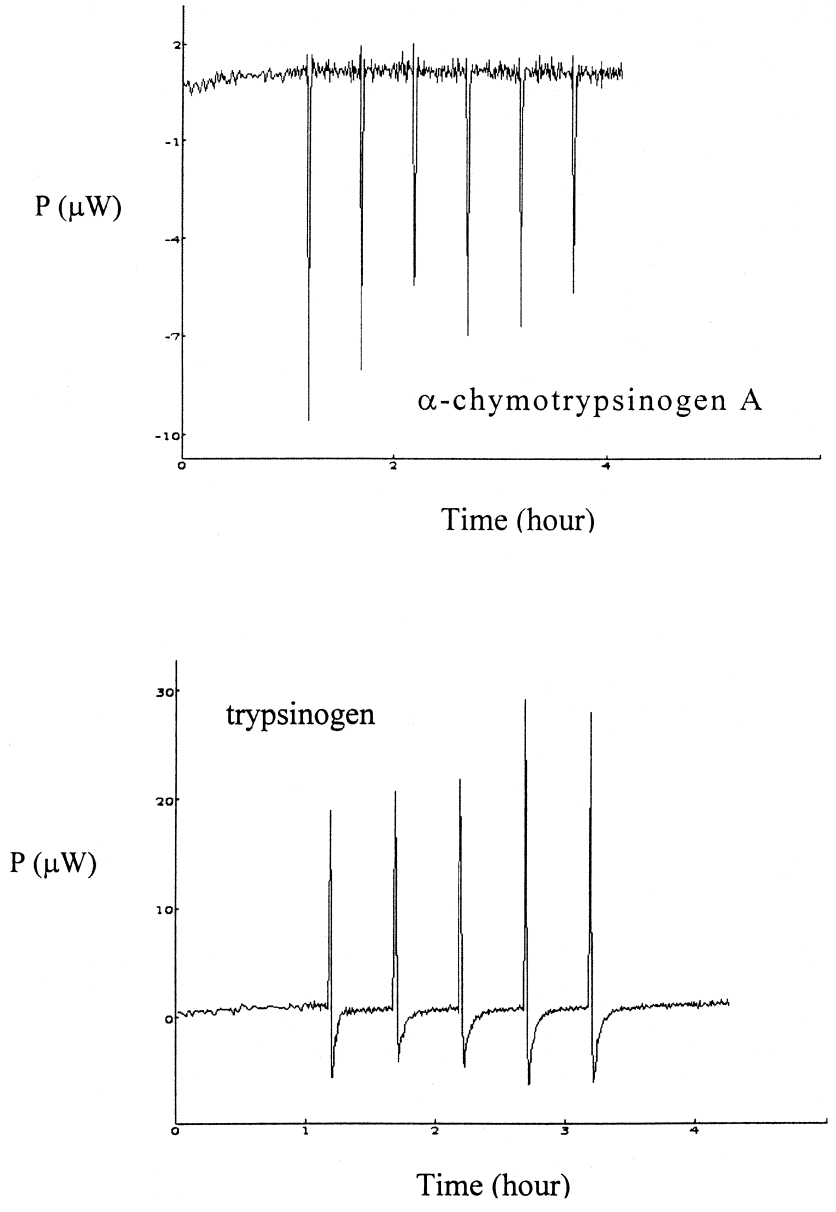

Fig. 6. The microcalorimetric thermograms of titration of α-chymotrypsinogen A or trypsinogen into carbonate buffer solution at 4.0 *M* NaCl and pH 10.0, respectively.

tive. The adsorption process became entropy driven again. The above results indicate that increase resin hydrophobicity, by increasing either alkyl length or density, increased the adsorption enthalpy and entropy. Thus the positivity of adsorption enthalpy, directly measurable by ITC, apparently could serve as an index for hydrophobic interaction.

Furthermore, the adsorption enthalpy of both proteins increased as the amount of bound protein increased, but trypsinogen apparently had a higher

increment of enthalpy than α-chymotrypsinogen A did. This result implies that protein–protein repulsion was strong among trypsinogen molecules. Dilution heat measurement confirmed this fact.

The results presented in this paper have provided further information about the binding mechanism of protein adsorption and advanced theoretical understanding of HIC.

5. Symbols

Q_{ads}	Means Heat of adsorption of the protein onto the gel (J)
V	Means The volume of the gel in ampoule (ml)
q^*	Means The amount of protein adsorbed on the unit volume of the gel (mol/ml)
ΔH_{ads}	Means Enthalpy of adsorption of the protein onto the gel per mole adsorbed protein (kJ/mol)
ΔS_{ads}	Means Entropy of adsorption of the protein onto the gel per mole adsorbed protein (J/mol K)
P	Means Power (μW)

Acknowledgements

The authors would like to thank the National Science Council of the Republic of China for the financial supporting this research under Contract No. NSC-87-2214-E-008-019.

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