CHROMSYMP. 2837

Histidine-ligand chromatography of proteins

Multiple modes of binding mechanism

Shenggen Hu and Duong D. Do*

Department of Chemical Engineering, University of Queensland, Brisbane 4072 (Australia)

ABSTRACT

The objective of this work is to exploit the versatile applications of matrix-linked histidine in protein chromatography. The negatively charged histidine at alkaline pH acts as a cation exchanger for proteins with high isoelectric points. Because of the hydrophobicity of the imidazole ring in histidine, enzymes, such as yeast alcohol dehydrogenase, are strongly retained by this histidine-ligand column in presence of 1.2 M ammonium sulfate. Due to the high formation constants of complexes between immobilised-histidine and metal ions, this column could be applied to serve as a ligand-exchange chromatography. In this instance, the interaction between proteins and the ligand takes place via the coordination sphere of the complex-forming metal ions. All these binding modes have been elucidated by the investigations of the effects of pH, metal ions, salt type and concentrations on (1) the purification of trypsin and (2) the binding of model proteins. It should be noted that this work differs from the reported applications [M.A. Vijalakshmi, *Trends Biotechnol.* 7 (1989) 71] where the protein binding occurs at low ionic strength and at pH values near or around their isoelectric points.

INTRODUCTION

A number of amino acids have been coupled to matrices for the purification of proteins and enzymes [1]. Phe and Trp were used as hydrophobic ligand for the isolation of serum proteins [2,3], bovine γ-globulin [4] and enzymes [5]. Arg- and Lys-immobilised columns were employed to purify fibronectin and plasminogen [6,7]. It has been also reported that histidine can be immobilised and acts as ligand to adsorb proteins [8–11].

Histidine has a –COOH group, a –NH$_2$ group and an imidazole side chain, all of which could contribute to hydrophobic and electrostatic interactions with protein molecules. There have been some investigations [8–11] showing the possibility of purifying proteins using histidine-ligand adsorbents. The recovery of subclasses of IgG from different sources were demonstrated [10,11]. In order to elucidate the mechanism involved in the interaction between IgG and the immobilised histidine, El-Kak *et al.* [11] carried out a systematic investigation on the effects of coupling methods and chromatographic parameters. The effects of pH, salt and temperature indicated an ion-pairing mechanism, rather than a mechanism based on the net charge of the protein (IgG), but with some localised complementary charge recognising the unprotonated imidazole nitrogen. Although some important studies have been carried out on the application of histidine-ligand, the researches are limited to cases where proteins and peptides are preferentially adsorbed to histidine-ligand columns at low ionic strength and at pH values near or around their isoelectric points [8,9].

This work presents the experimental results of the protein binding onto immobilised-histidine at

---

* Corresponding author.

conditions differing from those reported in literature. The intension of this work is to illuminate the multiple modes of binding mechanism by the investigations of the effects of various factors on the binding of several model proteins.

MATERIALS AND METHODS

The adsorbent used in this work is the histidine-Sepharose CL-4B, which was purchased from Sigma (St. Louis, MO, USA). Egg white lysozyme (L6876), bovine serum albumin (BSA) (A7906), ovalbumin (A5503), yeast alcohol dehydrogenase (ADH) (A7011), and ethanol precipitate of trypsin (T8003) were obtained from Sigma. All other reagents were analytical grade or higher and were purchased from Sigma or Ajax (Auburn, Australia). The proteins and buffer chemicals were used as received.

Protein concentrations were determined by measuring the optical density at 280 nm which could be converted to concentration by reference to calibration data. The measurement of trypsin activity is using α-N-benzoyl-DL-arginine-p-nitroanilide (BAPNA) as substrate. The activity was assayed in 0.75 ml reaction mixtures containing 0.125 ml enzyme sample, 0.375 ml assay buffer (0.2 M Tris–HCl, pH 7.6) and 0.25 ml substrate (0.8 mg BAPNA/ml). After adding the substrate the absorbance at 410 nm was recorded at 15-s intervals for 5–7 min. A unit of enzyme was defined as the change of absorbance at 410 nm in 1 min under above conditions.

The chromatography experiments were performed on a Beckman System Gold (San Ramon, CA, USA) equipped with a dual-pump programmable solvent module 126, a rapid scanning dual-wavelength monitory UV detector module 167, a Model 210A sample injection value and an analogue module 406. A Beckman serial interface card was used to provide the communication between the computer and the modules.

All column experiments were carried out in a stainless-steel column having a diameter of 4.6 mm and a length of 100 mm. Adsorbent (1.66 ml) was packed from a slurry into the column at a flow-rate 2 ml/min. The particle size (volume averaged) is 89 μm, determined from a MAL-

VERN sizer. The void fraction of the packed bed is 0.42, as measured by exclusion of Blue Dextran (Sigma-D5751). A sample loop of 250 μl was used. The flow-rate for the elution experiments is 0.25 ml/min.

RESULTS AND DISCUSSION

A filtered solution, prepared from the ethanol precipitate of trypsin, was chromatographed at 10°C on a histidine-Sepharose CL-4B column. The elution profile is shown in Fig. 1. The separation of trypsin and impurity proteins could be achieved by stepwise elution. The trypsin activity in elution fractions was detected and presented in symbols in the figure. As shown in the figure, the early eluted peaks of both cases mainly contain the impurities (indicated by low trypsin activity). Before introducing buffer B, the elution profiles of the two cases were nearly overlapped because of the same elution buffer (0.05 M). The minor difference on the heights of early eluted peaks is due to the slight difference in feed concentrations. After the introduction of buffer B, a concentrated peak was generated. The results revealed that the later-eluted peak is rich in trypsin, indicating that the purification of trypsin was obtained. At pH 7.6, trypsin is positively charged. The matrix-linked histidine
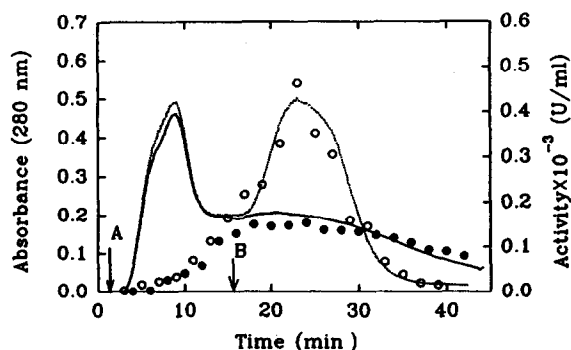

Fig. 1. Purification of crude trypsin from ethanol precipitate of bovine pancreas on histidine-ligand column. Lines, UV absorbance; symbols, enzyme activity of trypsin. Stepwise elution (dotted line and open symbols): (A) 0.05 M NaCl; (B) 0.25 M NaCl. Isocratic elution (solid line and filled symbols): 0.05 M NaCl. Other conditions: 20 mM Tris–HCl (pH 7.6), 10°C. Injected sample concentration (mass of trypsin precipitate/volume of solvent), 10 mg/ml.

should be negatively charged at alkaline pH, although it may have different ionisation from that in bulk solution. The opposite net charge suggests that the retention of trypsin on the column is based on electrostatic interaction. This is further evidence by the fact that the retention of trypsin is reduced with an increase in the concentration of NaCl.

Fig. 2 shows the elution profiles of lysozyme and ovalbumin. As in the case of trypsin, the separation of lysozyme and ovalbumin could be achieved on the column. Egg white lysozyme has a pI of 11.1, and possess net positive charge under the experimental conditions. Consequently, the binding of lysozyme on the column is also attributed to the electrostatic interaction.

Fig. 3 shows the effect of pH on the elution profiles of trypsin and lysozyme, respectively. It should be noted that the ethanol precipitate of trypsin (not the purified trypsin) was used in the experiments for Fig. 3a. Because of the impurities, multiple peaks were observed. The impurities were not or very weakly retained by the column, probably due to their low values of pI. The trypsin (pI = 10.5) and lysozyme are always positively charged over the range pH 6.6–8.6. However, histidine is negatively charged only at alkaline pH. The data in the figure indicate that the retention of the two

Fig. 3. Effect of pH on the elution profiles of trypsin and lysozyme. (a) Trypsin: 0.05 M NaCl, 20°C; injected sample concentration (mass of trypsin precipitate/volume of solvent), 10 mg/ml. (b) Lysozyme: 0.1 M NaCl, 25°C; injected sample concentration (mass of lysozyme/volume of solvent), 5 mg/ml.
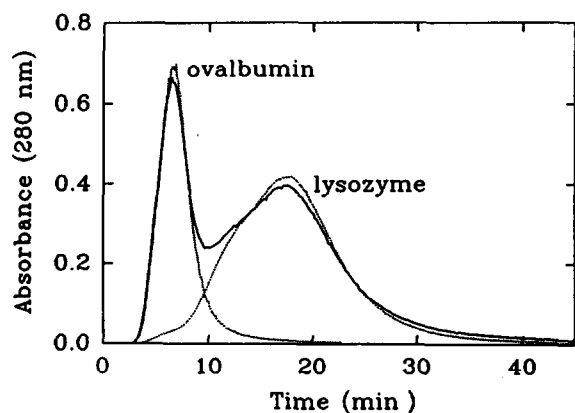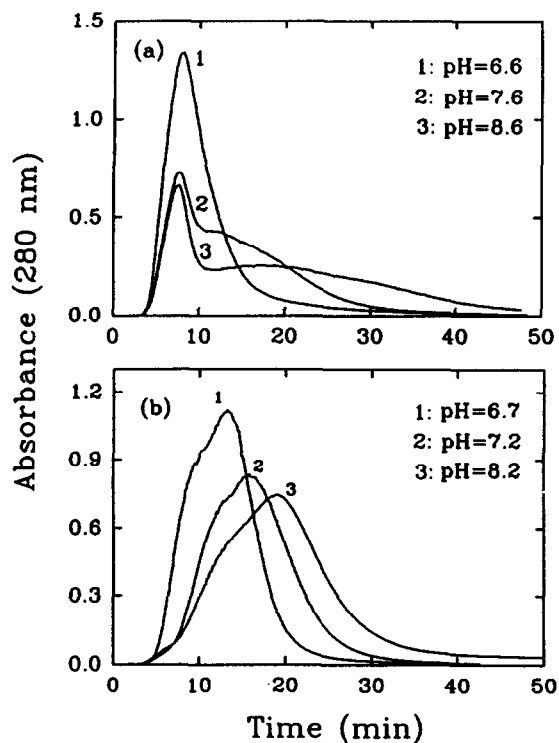

Fig. 2. Elution profiles of lysozyme and ovalbumin on histidine-Sepharose CL-4B column. Solid line, binary mixture, dotted line, single proteins. Conditions: 0.1 M NaCl–20 mM Tris–HCl (pH 7.2), 25°C. Injected sample concentration (mass of protein crystal/volume of solvent): lysozyme, 2.5 mg/ml; ovalbumin, 5 mg/ml.

proteins depends on the ionisation of matrix-linked histidine. Therefore, the negatively charged histidine at alkaline pH acts as a cation exchanger for proteins with high isoelectric points.

Fig. 4 shows the effects of Zn^{2+} on the binding of proteins onto matrix-linked histidine. The effects of Zn^{2+} on the binding were investigated by adding 1 mM Zn^{2+} into the equilibrating buffer, the loaded sample and the elution buffer. In the absence of Zn^{2+}, proteins display low affinity for the adsorbent. Addition of 1 mM Zn^{2+} produced a marked improvement in the protein binding. Particularly dramatic was the effect of Zn^{2+} on the binding of lysozyme, where the concentration of NaCl is as high as 0.5 M. The effect of the concentration of Zn^{2+} was studied and it has been shown that the effect is
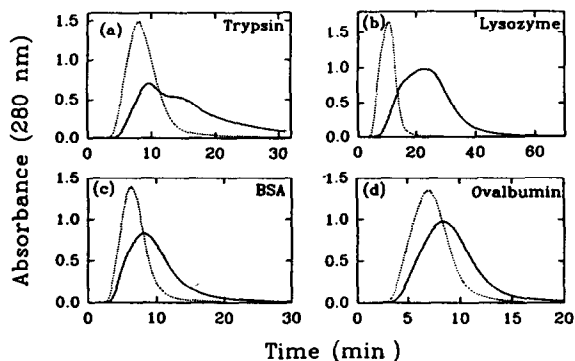
Fig. 4. Zn^{2+}-promoted binding of proteins onto immobilised-histidine adsorbent. Solid lines, addition of 1 mM Zn^{2+}; dotted lines, nil Zn^{2+}. (a) Trypsin: 0.25 M NaCl–20 mM Tris–HCl (pH 7.6), 20°C, injected sample concentration (mass of trypsin precipitate/volume of solvent), 10 mg/ml. (b) Lysozyme: 0.5 M NaCl–20 mM Tris–HCl (pH 7.2), 25°C; injected sample concentration (mass of lysozyme crystal/volume of solvent), 5 mg/ml. (c) BSA: 0.25 M NaCl, 20 mM Tris–HCl (pH 7.0), 25°C; injected sample concentration (mass of BSA crystal/volume of solvent), 10 mg/ml. (d) Ovalbumin: 0.25 M NaCl–20 mM Tris–HCl (pH 7.2), 25°C; injected sample concentration (mass of ovalbumin crystal/ volume of solvent), 10 mg/ml.

not significant if the concentration of Zn^{2+} is higher than 0.5 mM. In order to mask the effects of electrostatic interaction, high concentrations of NaCl were employed in the experiments for Fig. 4. However, it should be pointed out that the effect of metal ions on the protein binding was insensitive to the salt type and concentration when the concentration is higher than 0.1 M. Other metal ions, such as Co^{2+}, Ni^{2+} and Cu^{2+}, were also tested and similar but less enhanced binding was observed (data not shown). The effect of Zn^{2+} on the binding of proteins to immobilised histidine was pH-dependent with a more pronounced Zn^{2+}-promoted binding effect being observed at alkaline pH. At pH lower than 6.5, the addition of Zn^{2+} has no effect because the formation of histidine complex with Zn^{2+} is unfavourable. The metal ion promoted binding of proteins on histidine-Sepharose CL-4B column is apparently attributed to the effect of ligand exchange. In such a case, the interaction between proteins and matrix-linked histidine takes place via the coordination sphere of the complex-forming metal ions. As shown in Fig. 4, the extent of the enhanced adsorption varies for

different proteins. This may result from the different number of histidine and cysteine residues in proteins. Precipitation of test proteins was not observed at the selected conditions. The Zn^{2+}-induced aggregation was also negligible. Otherwise, multiple peaks would be generated due to the different size of aggregation.

The presence of the imidazole ring in histidine makes it have the capacity for hydrophobic interaction with proteins. Since the interaction is relatively weak, the high concentrations of sulfate or phosphate salts are required to enhance the hydrophobic interaction of protein with the ligand. Fig. 5 shows that the binding of enzyme ADH occurs at 1.2 M $(NH_4)_2SO_4$ and elution
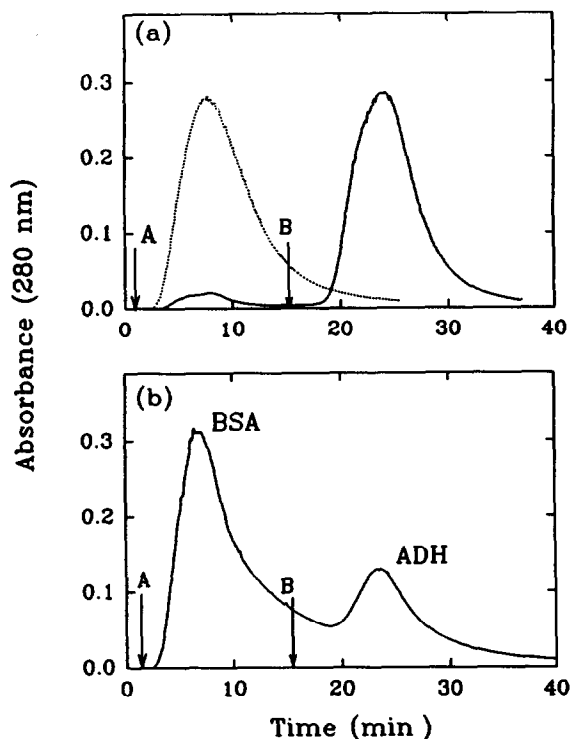

Fig. 5. Salt-mediated binding of ADH on the histidine-ligand column. (a) Dotted line: isocratic elution with 0.6 M $(NH_4)_2SO_4$; solid line: stepwise elution from (A) 1.2 M $(NH_4)_2SO_4$ to (B) 0.6 M $(NH_4)_2SO_4$; other conditions: 20 mM Tris–HCl (pH 7.0), 30°C; injected sample concentration (mass of ADH crystal/volume of solvent), 2 mg/ml. (b) Stepwise elution of binary mixture (ADH + BSA). (A) 1.2 M $(NH_4)_2SO_4$ to (B) 0.5 M $(NH_4)_2SO_4$; other conditions: 20 mM Tris–HCl (pH 7.4), 25°C; injected sample concentrations (mass of protein/volume of solvent), 1 mg ADH/ml + 2 mg BSA/ml.

occurs as the salt concentration is reduced. The binding of BSA does not take place at the same conditions. It was also found that the ADH was not retained on the blank Sepharose CL-4B at 1.2 M $(NH_4)_2SO_4$. Because it is the salt that mediates the degree of interaction of proteins with the histidine-ligand, this kind of technique has been named salt-mediated hydrophobic chromatography to distinguish it from hydrophobic chromatography using ligands of varying hydrophobicity [3].

CONCLUSION

Investigations of binding modes of proteins onto immobilised histidine have been carried out. The experimental results show that the histidine-ligand column can be used as cation-exchange, ligand-exchange and salt-mediated hydrophobic chromatography, depending on operating conditions.

REFERENCES

1 C.R. Lowe, *An Introduction to Affinity Chromatography*, Elsevier, Amsterdam, 1979.
2 B.H.J. Hofstee, *Biochem. Biophys. Res. Commun.*, 63 (1975) 618.
3 G.J. Doellgast and A.G. Plaut, *Immunochemistry*, 13 (1976) 135.
4 M. Kim, K. Saito and S. Furusaki, *J. Chromatogr.*, 585 (1991) 45.
5 M.H. Gething, B.E. Davidson and T.A.A. Dopheide, *Eur. J. Biochem.*, 71 (1976) 317.
6 M. Vuento and A. Vaheri, *Biochem. J.*, 183 (1979) 331.
7 L. Suamri, F. Spitz and L. Arzadon, *J. Biol. Chem.*, 251 (1976) 3693.
8 S. Kanoun, L. Amourache, S. Krishnan and M.A. Vijayalakshmi, *J. Chromatogr.*, 376 (1986) 259.
9 L. Amourache and M.A. Vijayalakshmi, *J. Chromatogr.*, 303 (1984) 285.
10 A. El-Kak and M.A. Vijayalakshmi, *J. Chromatogr.*, 570 (1991) 29.
11 A. El-Kak, S. Manjini and M.A. Vijayalakshmi, *J. Chromatogr.*, 604 (1992) 29.