THE APPLICATION OF ZEOLITE/FAC COMPOSITES IN PROTEIN SEPARATION

Wang, D.J.[1,2], Zhang, Y.H.[1], Xu, F.[1,3], Shan, W.[1], Zhu, G.B.[1], Yang, P. Y.[1] and Tang, Y.[1*]

[1]Department of Chemistry and Shanghai Key Laboratory of Molecular Catalysis and Innovative Materials, Fudan University, Shanghai 200433, P. R. China. E-mail: yitang@fudan.edu.cn, and pyyang@fudan.edu.cn
[2]Shanghai Research Institute of Petrochemical Technology, Shanghai 201208, P. R. China.
[3]School of Environmental Science and Engineering, Shanghai Jiaotong University, Shanghai, 200240, P. R. China.

ABSTRACT

The aim of this research is to develop a novel zeolite/fly ash cenosphere (FAC) composite as inorganic stationary substrate in immobilized metal ion affinity chromatography (IMAC) for protein separation. After immobilization of metal ions by ion-exchange process, two model proteins, Bovine Serum Albumin (BSA) and Chicken Egg Albumin (CEA), which contain different sequences of histidine residues, could be successfully identified by IMAC method using Zn^{2+}-exchanged ZSM-5/FAC composite as the packing sorbent. It was found that the separation effect of this type of packing substrate depended on the various interactions between the proteins and the immobilized metal ions in the zeolite substrate. Among Cu^{2+}, Zn^{2+} and Co^{2+} ions, Zn^{2+} ion, which possesses the suitable interactions with both the zeolite framework and the guest proteins, exhibited the best separation performance for the two model proteins. Due to its high chemical and mechanical stability, abundant and uniform pores, as well as its ion-exchange ability, this composite, compared with traditional soft gel matrix, is of great promise as a novel and ideal IMAC sorbent for biomolecular separation, especially under high pressure.

Keywords: Zeolite, Fly-ash, Protein, Separation, ZSM-5, Immobilized metal ion affinity chromatography

INTRODUCTION

The rapid development of biological science and biotechnology requires more specific and efficient methods to enrich and/or separate biomolecules from practically complicated systems (e.g., proteins, enzymes or nucleic acids) [1]. Immobilized metal ion affinity chromatography (IMAC) is one of the most powerful separation methods available for protein fractionation. One of the notable features of IMAC process is its specificity on the interactions between special domains in guest proteins and the metal ions on chromatographic support [2]. This forms the basis for its high selectivity and efficiency of protein separation and makes it extensively used in the characterization of natural, recombinant and modified proteins, including identification of amino acid residues, determination of histidine residue surface topography, and analysis of protein-metal ion interaction**.** The traditional stationary phase for IMAC are based on soft gel matrices such as agarose or cross-linked dextran [3], which are deemed as biologically compatible and highly active sorbents. However, the serious drawbacks of weak mechanical strength for this kind of material limit its further applications to some degree, especially under high pressure [4]. To overcome these defects and further apply IMAC under high pressure, some inorganic adsorbents such as silica-based particles have been attempted as stationary phase for high-through output separation because of their excellent mechanical resistance and modifiability [5].

Zeolites are crystalline aluminosilicate inorganic materials with unique intrinsic properties such as high surface area, excellent thermal/hydrothermal stability, high shape-selectivity and superior ion-exchange ability, which form the basis for their traditional applications in catalysis and separation of small molecules [6, 7]. Recently, as an important and novel application of such materials, the purification of nucleic acids or proteins micron-sized zeolite sorbents has been reported on the basis of electrostatic or hydrophobic interaction between the aimed biopolymers and zeolite substrates [8-13]. Therefore, zeolites were expected to be novel chromatographic carriers for biomolecule separation. Nowadays, hierarchical structured zeolitic materials are attracting considerable research enthusiasm in the field of chemistry, biotechnology and advanced materials due to their peculiar hierarchical porous structure, relatively high mechanical strength and external surface area [14-16]. We have found that, after immobilizing transitional metal ions in zeolite

crystals through ion exchange process, the transitional-metal-ion immobilized zeolites would selectively adsorb the histidine-rich domains in the targeted protein molecules, which makes this hierarchical material a promising packing sorbent in IMAC for protein separation [17].

Fly ash cenospheres (FACs) come from the environmental waste produced during the process of coal combustion in power plants, and possess unique spherical hollow structure [18]. Recently, we have successfully prepared a series of zeolite membranes on the surface of FACs by hydrothermally treating the nanozeolite pre-seeded FACs [18], which were expected to be used as novel catalysts, adsorbents and other advanced materials due to their spherical shape, relatively high mechanical strength and the surface zeolitic properties. It has also been noticed in our experiments that their relatively high mechanical strength and surface zeolitic characters, such as ion-exchange ability might make them good candidates for IMAC protein separation. Here, as an example, the zeolite ZSM-5 membrane coated FACs (ZSM-5/FAC) with different immobilized transitional metal ions were prepared and packed into the IMAC columns to separate two model proteins with different histidine-rich domains.

EXPERIMENTAL SECTION

Preparation of the ZSM-5/FAC

The FAC used in this work was provided by Research Centre for the Application of Fly Ash in Henan Province of China. The sample was sieved by a 200-mesh sieve, treated by floatation in boiling water to remove the fragments, and dried at 100°C. To prepare the aimed ZSM-5/FAC composites, the FAC was first seeded with the nanosized silicalite-1 (70-80 nm, prepared as reference 19), which has the same topological structure of MFI framework with zeolite ZSM-5, through electrostatic adsorption with the aid of polyelectrolytes [20-22]. After rinsed in a dilute ammonia solution to remove excess zeolite seeds and dried at 100 °C, the seeded FAC products were then treated in a solution with a molar ratio of 28 Na_2O: 1.5 Al_2O_3: 100 SiO_2: 4000 H_2O [23] at 180 °C for 10-24 h in a closed stainless steel autoclave with PTFE-line. At last, the products were washed with distilled water and dried. The final products were characterized by X-ray powder diffraction (XRD) and scanning electron microscopy (SEM).

Immobilization of transitional metal ions

The Zn^{2+}, Cu^{2+} and Co^{2+} were immobilized into ZSM-5/FAC composites (denoted as Zn-ZSM-5/FAC, Cu-ZSM-5/FAC and Co-ZSM-5/FAC, respectively) by routine ion exchange process in a 0.3 mol L^{-1} aqueous solution of corresponding metal nitrate at room temperature, respectively, followed by washing the products with water [24]. By this method, the transitional metal ions were immobilized at the cationic sites near $(AlO_4)^-$ in the zeolite coating to form the metal ion immobilized ZSM-5/FAC sorbents.

Protein separation process

Separation of proteins was carried out on a self-made IMAC column with an inner diameter of 8 mm and a length of 5 cm using the Zn-ZSM-5/FAC, Cu-ZSM-5/FAC and Co-ZSM-5/FAC as the packing sorbents, respectively (Figure 1). Bovine Serum Albumin (BSA) and Chicken Egg Albumin (CEA) with different histidine-rich domains were used as the model proteins. About 0.5 g of metal ion modified ZSM-5/FACs were slurry-packed into the column and equilibrated with Tris-HCl buffer solution at pH 7.0 (Tris is the abbreviation of hydroxymethyl-imino-methane). Then the mixture of BSA and CEA (1:1, in weight) was dissolved in Tris-HCl buffer solution at pH 7.0 and added onto the IMAC column for protein adsorption. Afterwards, the IMAC column was washed thoroughly by Tris-HCl buffer solution of pH 7.0 to remove the excess and unaffintively absorbed proteins. The protein molecules which affinitively adsorbed on the metal ions in packing sorbents were eluted at a gravitational flow velocity using imidazole solutions with the gradient-concentrations of 20, 40, 60, 80, 100, 200 and 1000 mmol L^{-1}. With the increasing imidazole concentrations, the proteins affinitively adsorbed would be desorbed and eluted out in sequence according to their adsorptive strength to the metal ions. The proteins collected in a series of fraction vials were finally analyzed by Ultraviolet (UV) spectrometry at the wavelength of 280 nm. The separation efficiency was evaluated from the number and the separation degree of the eluting peaks, and the amount of each kind of protein adsorbed on the column could be estimated by the area or height of the corresponding eluting peak. To further confirm the protein separation of this method, the composition of proteins in the eluted fraction vials at the peaks of elution curve was examined by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) method.

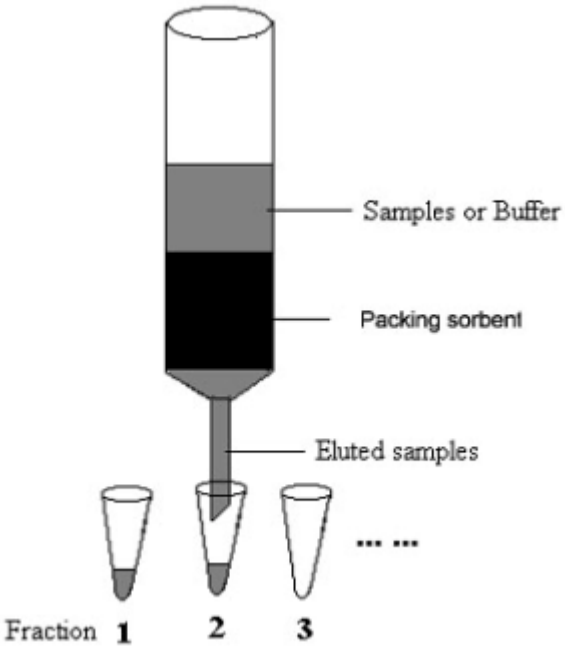

Figure 1. The scheme of self-made protein separation in imac mode.

RESULTS AND DISCUSSION

Preparation and characterization of ZSM-5/FAC composites

Because silicalite-1 possesses the same topological framework as zeolite ZSM-5, we used silicalite-1 nanocrystals as the seeds for the formation of ZSM-5 coating.

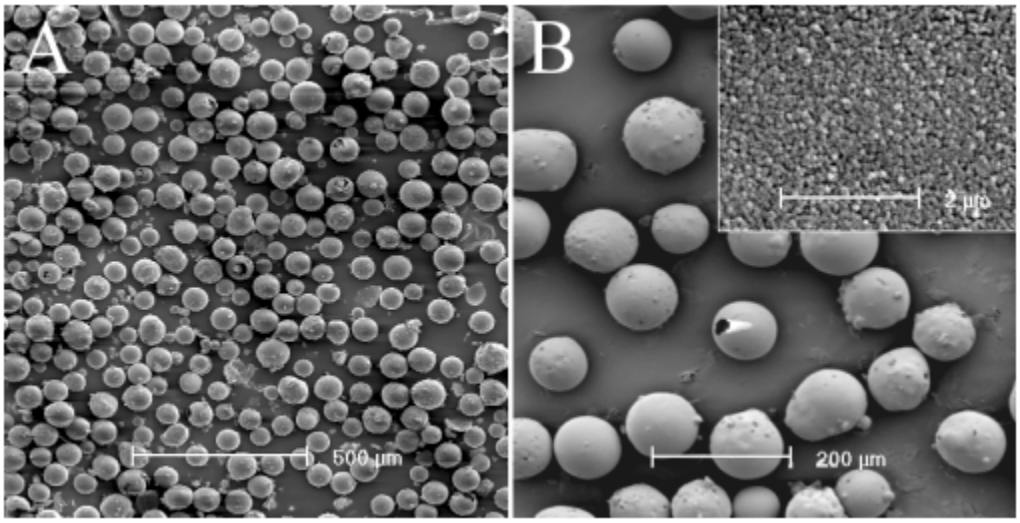

Figure 2. SEM images of the FACs used in this work. The inset shows the silicalite-1 seeded FAC.

Figure 2 shows the scanning electron microscopy (SEM) images of the original FACs and the inset of Figure 2B shows the surface of the silicalite-1 seeded FACs. As shown in the previous papers [20, 25], the silicalite-1 nanoparticles can be homogeneously adsorbed on the surface of FACs with the aid of polyelectrolytes, which makes it possible to form zeolite ZSM-5 membrane with seed-film method. After the hydrothermal treatment, almost all of the products (Figure 3A) well retained the initial spherical morphology of FAC (Figure 2A), and the zeolite seeds pre-deposited on the surface of FAC have intergrown into a dense membrane composed of cubic crystals with the sizes of 400-600 nm (Figure 3B). The X-ray powder diffraction (XRD) pattern of the product (Figure 4B) displays the clear characteristic diffraction peaks of

zeolite with the MFI framework besides those originally existed peaks in the XRD pattern of FAC (Figure 4A), whereas the XPS analysis indicated that the aluminium in the synthesis system had incorporated into the surface zeolite coating, further proving the formation of the zeolite ZSM-5. The SiO_2/Al_2O_3 calculated from the peak areas of Al_{2p} and Si_{2p} was about 30. The Brunauer-Emmet-Teller (BET) specific surface area of the ZSM-5/FAC composite product was also increased to 161 $m^2\ g^{-1}$ (pore volume was 0.075 $cm^3\ g^{-1}$) from lower than 1.0 $m^2\ g^{-1}$ of the original FACs because of the formation of the ZSM-5 coating on the outer shell of FACs.

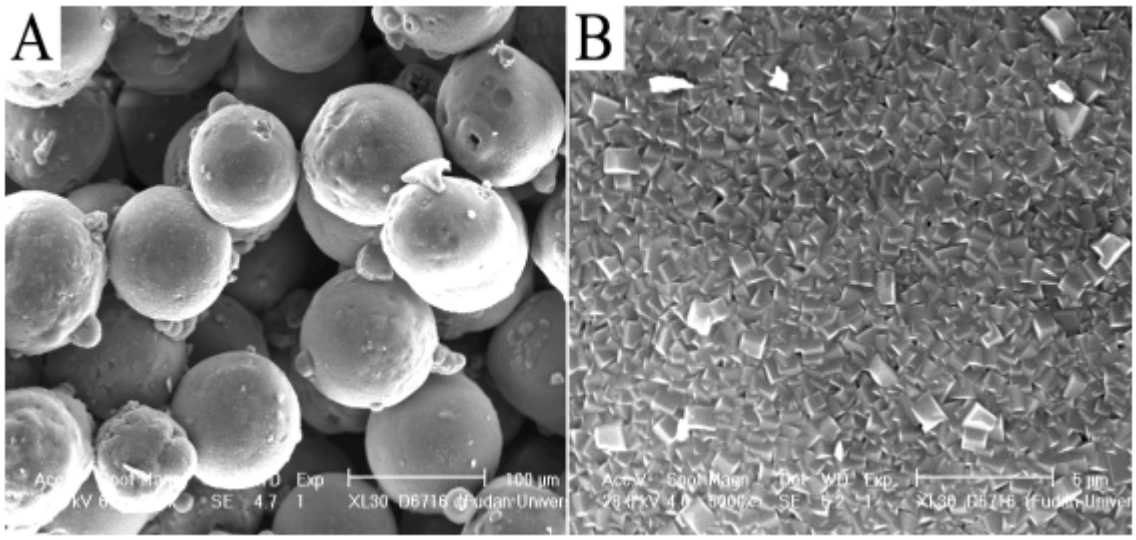

Figure 3. SEM images of the ZSM-5/FAC (A) and the typical surface (B).

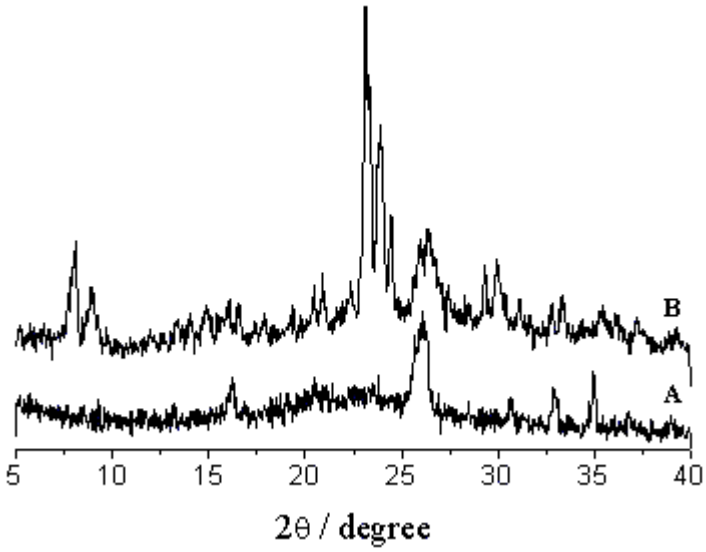

Figure 4. XRD patterns of original FAC (A) and ZSM-5/FAC (B).

Three transitional metal ions of Co^{2+}, Zn^{2+} and Cu^{2+} were employed to study the ion immobilization ability of the ZSM-5 coating in the composite. After exchanging the transitional metal ions by immersing ZSM-5/FAC in the corresponding aqueous solution of metal nitrate and rinsing them with enough amount of water to remove all the excess ions, 0.5g of ion-exchanged ZSM-5/FAC was packed in a glass column with the inner diameter of 8 mm and then flushed with 15 mL of 0.05 mol L^{-1} EDTA solution to elute fully the immobilized metal ions. The amount of the ions extracted by EDTA was quantitatively measured via inductively coupled plasma atomic emission spectrometry (ICP-AES) analysis after making-up the eluted solution in metric flask. Table 1 lists the Zn^{2+}, Co^{2+} and Cu^{2+} ion content eluted from the ZSM-5/FAC, respectively, which indicates that the ion immobilization ability of this novel material is equivalent to the traditional IMAC matrix reported previously [26].

Table 1. The metal ion content in ion-exchanged ZSM-5/FAC composite.

Metal ions	Zn^{2+}	Co^{2+}	Cu^{2+}
Adsorption Capacity (μg g^{-1})	200.0	63.0	210.0

Protein separation

To explore the feasibility of metal-ion-modified ZSM-5/FACs for protein separation, the Cu^{2+}, Co^{2+} and Zn^{2+} exchanged samples were used as the packing materials of IMAC to separate the two model proteins, BSA and CEA, respectively. The protein molecules were first fixed on the metal ions by passing a mixed protein solution through the column followed by eluting all unaffinitively adsorbed proteins by a large quantity of buffer solution. And then the proteins affinitivly adsorbed on the surface of metal ions were desorbed using a series of imidazole solutions with gradiently increasing concentrations. It is very interesting that the separation efficiencies of various metal ions modified zeolites are quite different.

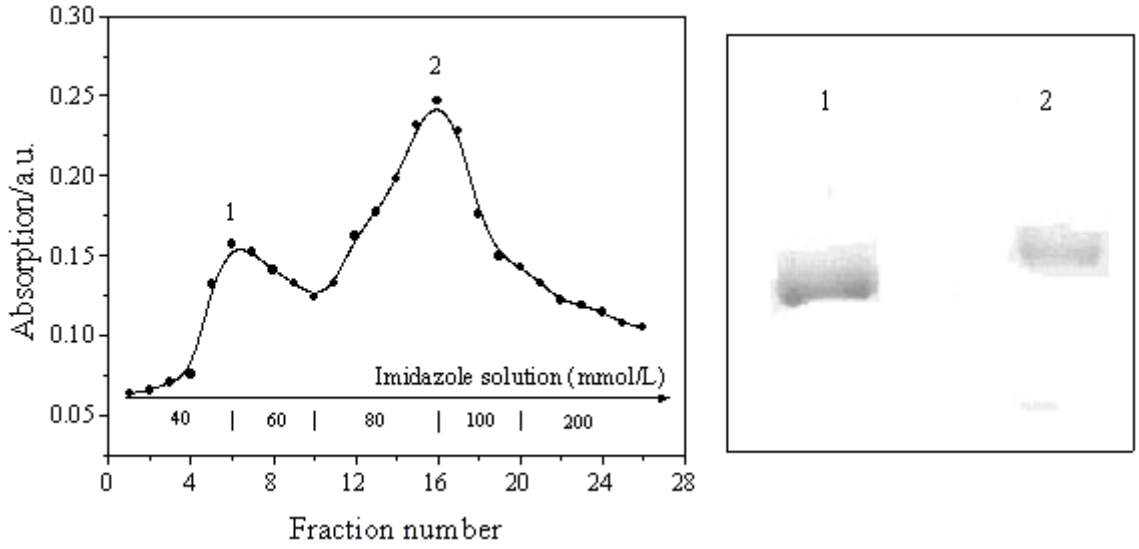

Figure 5. The elution curve of BSA and CEA on Zn-ZSM-5/FAC (a) and the SDS of the elution fraction of peak 1 and 2.

When Zn^{2+} ions exchanged ZSM-5/FAC were used, the BSA and CEA proteins can be effectively separated. Its elution curve clearly displays the two protein eluting peaks (Figure 5a), and SDS-PAGE result (Figure 5b) indicated that the Peak 1 correspond to CEA, while Peak 2 to BSA. This outflow sequence could be explained by the different number of histidine residues on the surface of proteins, whose imidazolyl could chelate with metal ions immobilized in the zeolite on surface of FACs (Figure 6). There are more histidine residues on the BSA protein surface structures than CEA protein surface structures [2,27]. Therefore, BSA has stronger interaction with Zn^{2+} ions than CEA. Consequently, the affinitively adsorbed CEA could be eluted out by imidazole solutions with lower concentrations while BSA could only be eluted out by solutions with higher imidazole concentrations.

However, when Zn-ZSM-5/FAC was replaced with Co-ZSM-5/FAC as the packing materials, the proteins that had been pre-adsorbed by the Co^{2+} ions on the surface were eluted out almost at the same time, and only one wide peak appeared in its elution curve. The SDS-PAGE indicated that the collected samples at elution peak were the mixture of two proteins. On the other hand, when Cu^{2+} was exchanged into ZSM-5/FAC, it is unexpected that the proteins could not be adsorbed on the surface of the ZSM-5/FAC. The proteins were all flushed out even during the proteins adsorbing process and the Cu^{2+} ions pre-immobilized on the column were also flushed off accompanied by the outflow of the proteins, turning the eluting solution to blue. These phenomena could be explained by the different chelation strength between protein and Co^{2+}, Cu^{2+} and Zn^{2+} [28]. The Cu^{2+} ions have very strong chelating ability to histidine residues in protein, probably stronger than that to zeolite, so that when protein was added in the column, the Cu^{2+} ions were pulled out by the protein as the blue Cu^{2+}-protein complex even in the protein adsorption process. On the contrary, when Co-ZSM-5/FAC was used as packing sorbent, because of the relatively weak interaction of Co^{2+} ions with

protein molecules, they can stay stably on zeolite surface. However this weak interaction cannot identify the affinity differences of histidine residual structures between BSA and CEA, both of which desorbed and eluted out at the same imidazole concentration. Only the Zn^{2+} ions, which have the appropriate chelating ability to protein, can both stay on the surface of the substrate and recognize the difference of histidine residues structure between BSA and CEA, leading to a good separation effect as shown in Figure 5.

In addition to the metal ion immobilized sites on the large external surface of submicron-sized zeolite coating on zeolite/FAC samples, which is a crucial factor for the protein separation, the unique characters of this material also provide some other advantages for IMAC process. For examples, the abundant macropores in the array of FAC spheres fit for biomolecule to pass through, and the rigidity of such materials provide possibility to be used under high pressure, which is expected to be significant for rapid or high-through output biomolecule separation on a large scale [29].

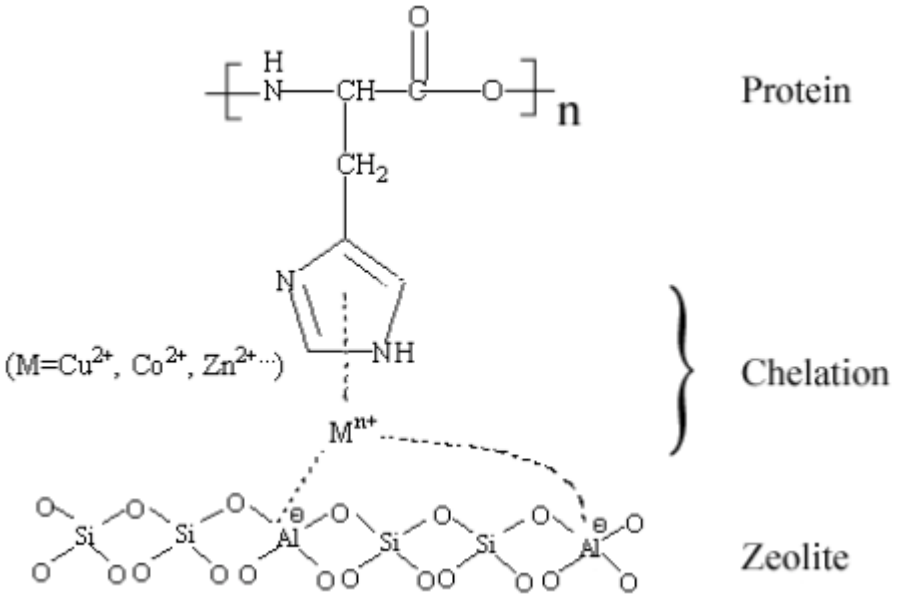

Figure 6. Diagrams of the interaction between the protein and zeolite coating by the chelation of imidazolyl in histidine with metal ions immobilized in zeolite.

CONCLUSION

A novel inorganic material of metal-ion-modified zeolite/FAC composite has been developed as stationary phase for IMAC protein separation. Two model proteins, BSA and CEA, could be successfully separated by IMAC method with Zn^{2+} ion exchanged ZSM-5/FAC composite as the packing sorbent. The outflow sequence of the proteins depends on the number of histidine residues on the surface of proteins, and the interaction of ions with guest proteins and zeolites determines the separation effect of this kind of material. Besides, in comparison with traditional soft gel matrix, such composite is expected to be an ideal IMAC sorbent for biomolecular separation because of their high chemical and mechanical stability, abundant and uniform pores, and their ion-exchange ability.

ACKNOWLEDGEMENTS

The financial supports from the NSFC (20233030, 20273016, 20303003), Shanghai Science Committee (0249nm028, 03DJ14004), National Basic Research Priorities Programme (2001CB510202) and National HighTec Research Developing Programme (2001AA233031) are gratefully acknowledged.

REFERENCES

1. Ruedi, A., Cravatt, B. F., Trends Biotechnol., 20 (2002), S1-S2.
2. Porath, J., Carlsson, J., Olsson, I., Belfrage, G., Nature, 258 (1975), 598-599.
3. Hemdan, E. S., Porath, J., J. Chromatogr., 323(1985), 247-257.
4. Gaberc-Porekar, V., Menart, V., J. Biochem. Biophys. Methods., 49 (2001), 335-360.
5. Groman, E.V., Wilchek, M., Trends Biotechnol., 5 (1987) 220-224.
6. Breck, D. W., Zeolite Molecular Sieves, Wiley, New York.
7. Ozin, G. A., Kuperman, A., Stein, A., Angew. Chem. Int. Ed. Engl., 28 (1989), 359-363.
8. Nygaard S, Wendelbo, R., Brown, S., Adv. Mater., 14 (2002), 1853-1856.
9. Matsui, M., Kiyozumi, Y., Yamamoto, T., Mizushina, Y., Mizukami, F., Sakaguchi, K., Chem. Eur. J., 7 (2001), 1555-1560.
10. Klint, D., Karlsson, G., Bovin, J. O., Angew. Chem. Int. Edit., 38 (1999), 2560-2562.
11. Corma, A., Fornes, V., Rey, F., Adv. Mater., 14 (2002), 71-74.
12. Corma, A., Fornes, V., Jorda, J. L., Rey, F., Fernandez-Lafuente, R., Guisan, J. M., Mateo, C., Chem. Commun., (2001), 419-420.
13. Fisher, K., Huddersman, K., Chem. Commun., (2001), 1978-1979.
14. Holland, B. T., Abrams, L., Stein, A., J. Am. Chem. Soc., 121 (1999), 4308-4309.
15. Huang, L. M., Wang, Z. B., Sun, J. Y., Miao, L., Li, Q. Z., Yan, Y. S., Zhao, D. Y., J. Am. Chem. Soc., 122 (2000), 3530-3531.
16. Stein, A., Adv. Mater., 15 (2003), 763-775.
17. Xu, F., Wang, Y.J., Wang, X. D., Zhang, Y. H., Tang, Y., Yang, P. Y., Adv. Mater., 15 (2003), 1751-1753.
18. Wang, D. J., Zhang, Y. H., Dong, A. G., Tang, Y., Wang, Y. J., Xia, J. C., Ren, N., Adv. Funct. Mater., 13 (2003), 563-567.
19. Persson, A. E., Schoman, B. J., Sterte, J., Ottesstedt, J. E., Zeolites, 14 (1994), 557-567.
20. Wang, X. D., Yang, W. L., Tang, Y., Wang, Y. J., Fu, S. K., Gao, Z., Chem. Commun., (2000), 2161-2162.
21. Decher, G., Science, 277 (1997), 1232-1237.
22. Caruso, F., Caruso, R. A., Mohwald, H., Science, 282 (1998), 1111-1114.
23. Balkus, Jr. K.J., Scott, A.S., Microporous Mesoporous. Mater., 34 (2000), 31-42.
24. Bortnovsky, O., Sobalik, Z., Wichterlova, B., Microporous Mesoporous Mater., 2001, 46, 265-275.
25. Wang, Y. J., Tang, Y., Dong, A. G., Wang, X. D., Ren, N., Gao, Z., J. Mater. Chem., 12 (2002), 1812-1818.
26. Arnold, F.H., Biotechnology, 9 (1991) 151-156.
27. Fazal, Md. A., Roy, B. C., Sun, S., Mallik, S., Rodgers, K. R., J. Am. Chem. Soc., 123 (2001), 6283-6290.
28. Ueda, E.K.M., Gout, P.W., Morganti, L., J. Chromatogr. A, 988 (2003) 1-23.
29. Belew, M., Porath, J., J. Chromatogr., 516 (1990) 333-352.