Available online at www.sciencedirect.com
ScienceDirect
Journal of Chromatography B, 864 (2008) 116–122
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Purification of recombinant enhanced green fluorescent protein expressed in *Escherichia coli* with new immobilized metal ion affinity magnetic absorbents

Chen-Li Chiang *, Chuh-Yean Chen, Ling-Wei Chang

Department of Chemical and Materials Engineering, Southern Taiwan University, 710 Yung-Kang City, Tainan Hsien, Taiwan

Received 11 October 2007; accepted 29 January 2008
Available online 20 February 2008

Abstract

A new immobilized metal ion affinity (IMA) adsorbent containing superparamagnetic nanoparticles and coated with hydrophilic resins are proposed here to improve the purification of His-tagged proteins. The magnetic chelating resin was prepared by radical polymerization of magnetite (Fe_3O_4), styrene, divinyl benzene (DVB) and glycidyl methacrylate–iminodiacetic acid (GMA–IDA) in ethanol/water medium. IDA is immobilized on magnetite as a ligand and pre-charged Cu^{2+}, Zn^{2+} and Ni^{2+} as metal ions. To identify the GMA–IDA magnetic particles easily, we named these particles MPGI. The MPGI adsorbent was used to test their suitability for the direct recovery of an intracellular, polyhistidine-tagged protein, enhanced green fluorescent protein [EGFP-$(His)_6$], from *Escherichia coli* lysates in a single step. Parameters influencing the purification efficiencies such as pH, ionic strength and imidazole concentration were optimized to achieve improved separation. The optimal selectively was observed in binding buffer (0.2 M NaCl, 0.02 M imidazole), washing buffer (0.4 M NaCl, 0.03 M imidazole) and elution buffer (0.50 M imidazole). The Cu^{2+}-charged MPGI adsorbent had the highest yield and purification factor at 70.4% and 12.3, respectively. The calculated isotherm parameters (Q_m = 53.5 mg/g, K_d = 5.84 mg/mL and Q_m/K_d = 9.2 mL/g) indicated that the MPGI adsorbent could be used as a suitable adsorbent for EGFP from an aqueous solution.

Keywords: Immobilized metal ion affinity; Enhanced green fluorescent protein; Superparamagnetic nanoparticles

1. Introduction

In biotechnology, magnetic separation can be used as a quick and simple method for the efficient capture of selected target species in the presence of other suspended solids. It is possible to separate them directly from complex biological mixtures, like fermentation broth, cell disruptates, blood, and tissues. The purified biomolecules include specific proteins, antibodies, peptides, nucleic acids, enzymes, and even cells and viruses. Magnetic separations eliminate pretreatment, such as centrifugation, or filtration. Magnetic separation offers a gentle, fast, easily automated and scalable alternative. Targets are captured on magnetic particles coated with a target-specific surface, and separated from the sample using a magnetic field. In the recent years, the nanosized magnetic particles receive increasing attention with rapid development of nanostructured materials and nanotechnology in the fields of biotechnology and medicine [1–10]. Due to strong magnetic property and low toxicity, its applications in biotechnology and medicine have gained significant attention. Microbial cell lysates are relatively viscous and may present problems in a column operation. A further advantage of using magnetic particles for bioseparation applications is their utility with variously sized starting samples [11,12]. With most commercially available protein purification systems, the amount of starting material is limited by the fixed size of the purification matrix within plastic columns or centrifugation baskets.

In contrast to the conventional method, immobilized metal affinity chromatography (IMAC) has been shown to be a simple and effective method to purify recombinant proteins [13–17]. To use IMAC, the protein is fused with six or more additional

* Corresponding author. Tel.: +886 6 2533131x6943; fax: +886 6 2425741.
E-mail address: chiang@mail.stut.edu.tw (C.-L. Chiang).

1570-0232/$ – see front matter © 2008 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2008.01.056

histidine residues at the C- or N-terminus. The metal is held by chelation with reactive groups covalently attached to a solid support. Although IMAC is easily adaptable to any protein expression system, it still requires pretreatment to remove cell debris and colloid contaminants. It is also limited by a relatively long operation time, solvent consumption, and protein solubility. To develop a simpler and more versatile platform, we create surface-modified magnetic nanoparticles as the binder, carrier, and anchor for histidine-tagged proteins.

In our previous study, we have prepared a chelating vinyl monomer, glycidyl methacrylate–iminodiacetic acid (GMA–IDA), via an epoxy group reaction of GMA with IDA [18]. This monomer has been copolymerized with styrene in an aqueous medium to form a surfactant-free emulsion polymerization. The stability constants of the latex chelated with some metal ions have been found to be close to 10^{11}. These results indicate that the chelating polymers can form stable polymer–metal ions complexes in an aqueous solution. A new magnetic absorbent was tried to synthesize by the polymerization of GMA–IDA, divinyl benzene (DVB) and styrene in the presence of magnetic Fe_3O_4. To identify the GMA–IDA magnetic particles easily, we named these particles MPGI. The aim of this study was to demonstrate the applications of MPGI absorbents as carriers for purification of His-tagged fusion protein directly from bacterial lysates. The MPGI absorbents can be utilized with magnets to provide extremely rapid and efficient purification, and the surface characteristics minimize nonspecific adsorption.

The DNA fragment encoding enhanced green fluorescent protein (EGFP) with T7 promoter was amplified and cloned into the vector pRSETB to obtain a C-terminus His-tagged fusion expression plasmid. The MPGI adsorbent was employed for the direct extraction of EGFP-$(His)_6$ from *E. coli* lysates as a model system. This fusion protein was utilized as a marker for evaluating the properties of metal ion charged MPGI adsorbent as a matrix in protein purification. The experiments were designed to investigate the effect of different operating variables, such as pH, ionic strength and imidazole concentration, on the target protein adsorption performance of MPGI absorbents. The experiments were also designed to determine the most appropriate chelated metal ion among the series Cu^{2+}, Ni^{2+}, or Zn^{2+}, and quantitatively the efficiency of EGFP purification.

2. Experimental

2.1. Materials

Ferric chlorides, 6-hydrate was purchased from J. T. Baker (Phillipsburg, NJ, USA). Ferrous chloride tetrahydrate was purchased from Fluka (Buchs, Switzerland). PEI and lauric acid were supplied from Sigma Chemical Co. (St. Louis, MO, USA). Agarose L (low electroendosmois) was from Amersham Biosciences (Uppsala, Sweden). Supercoiled DNA ladder was obtained from Invitrogen Co. (Carlsbad, CA, USA). Reagents for use in DNA isolation and analysis were of molecular biology grade. Ribonuclease A was obtained from Sigma. All other chemicals and solvents used were of analytical grade and used without further purification. The water used throughout this work was the reagent-grade water produced by Milli-Q SP Ultra-Pure-Water Purification System of Nihon Millipore Ltd. (Tokyo, Japan). All solutions were freshly prepared.

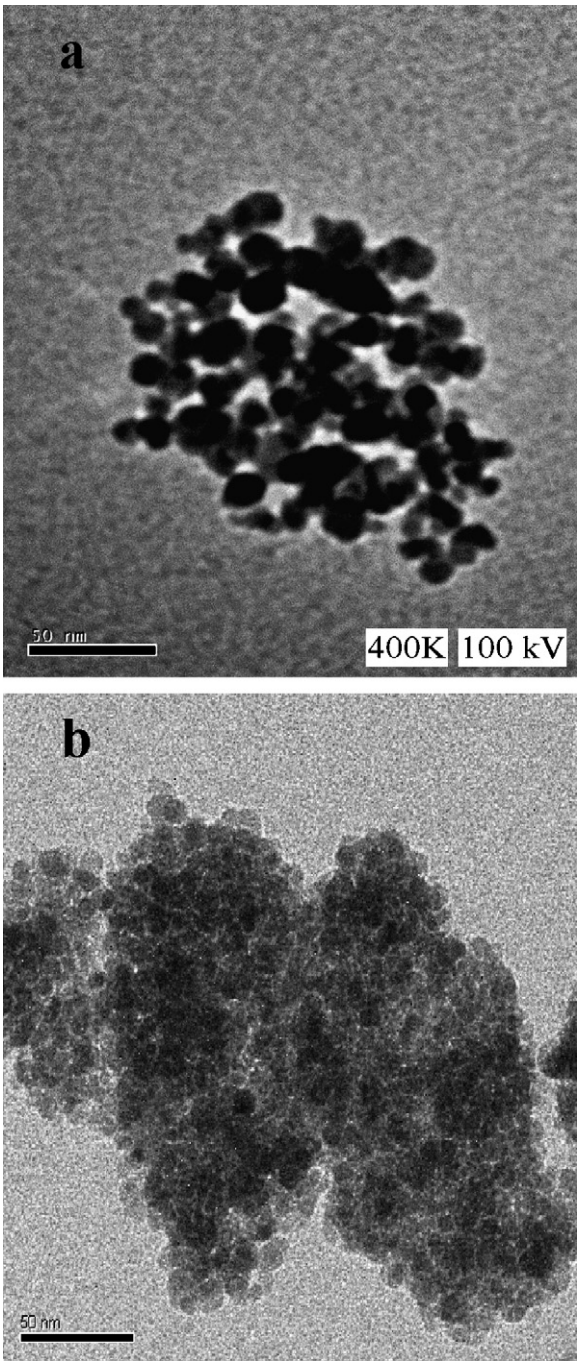

Fig. 1. Transmission electron micrographs of magnetic Fe_3O_4 (a) and MPGI particles (b).

2.2. Preparation of magnetic Fe_3O_4

The magnetic Fe_3O_4 was prepared by co-precipitating Fe^{2+} and Fe^{3+} ions in a NaOH solution and treating under hydrothermal conditions. A solution of 100 ml, 1.0 M NaOH was added to a 100 ml aqueous solution containing $FeCl_3$ (0.128 mol) and $FeCl_2$ (0.064 mol) under vigorous stirring at 60 °C for 2 h. Then

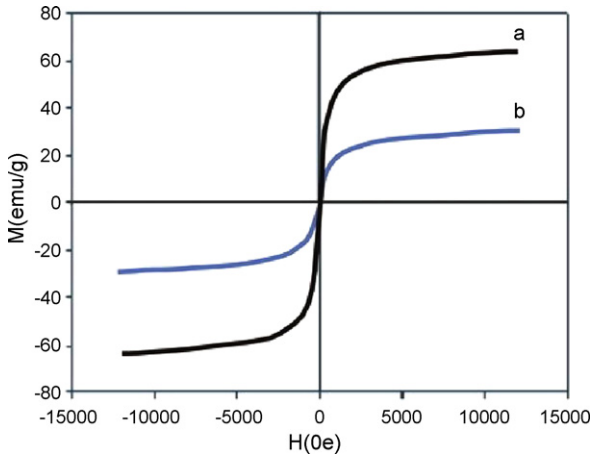

Fig. 2. Magnetization vs. magnetic field for the magnetic Fe_3O_4 (a) and MPGI particles (b).

a solution of 1.5 M lauric acid at pH 10 was added to this solution and stirred for 2 h. The products were collected in a magnetic field, repeatedly washed with distilled water and vacuumed at 60 °C.

2.3. Preparation of magnetic chelating resin MPGI

A mixture of magnetic Fe_3O_4 (0.4 g), styrene (9 g), DVB (1 g) and ethanol (70 ml) was taken in a four necked round bottom flask and stirred in ultrasonic apparatus for 5 min. Then the flask was placed in a water bath of 75 °C and stirred with mechanical agitator. Finally, another mixture of 30 ml, 30 wt% GMA–IDA aqueous solution and 0.4 g KPS (initiator) was added to the flask with continuous stirring at 75 °C for 12 h. The magnetic chelating beads obtained by the polymerization were separated and collected by magnetic control and washed thoroughly with distilled water and ethanol. To identify the magnetic chelating resin easily, we named this resin MPGI absorbent. To charge MPGI absorbent with metal ions, a salt of the appropriate cation (e.g. 500 ppm $CuCl_2$, $NiCl_2$ and $ZnCl_2$) is dissolved in water. Metal charged media is rinsed thoroughly with water and equilibrated in 0.2 M NaCl, 0.02 M imidazole prior to use in equilibrium binding studies or protein isolation experiments.

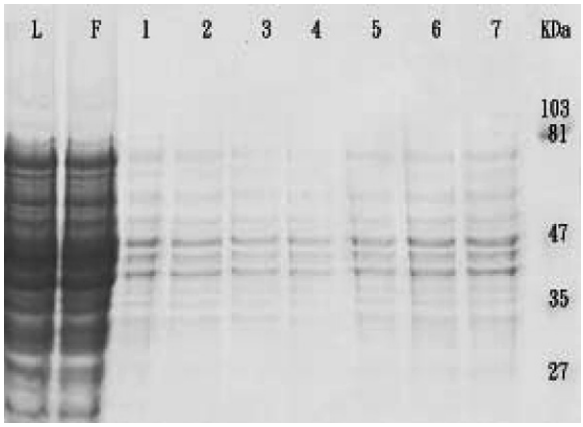

Fig. 4. The influence of the NaCl concentration on the elution of nonspecific bound proteins other than EGFP used in washing buffer. Lanes L, protein lysate loaded into MPGI absorbent solution; F, flow through; 1, 0 M NaCl; 2, 0.10 M NaCl; 3, 0.20 M NaCl; 4, 0.30 M NaCl; 5, 0.40 M NaCl; 6, 0.60 M NaCl. Purification of EGFP using Cu^{2+}-charged MPGI.

2.4. Plasmid construction

An intact enhanced green fluorescent gene (EGFP) was amplified from pEGFP-N1 (BD Bioscience, Franklin Lakes, NJ, USA) using polymerase chain reaction. Upstream and downstream primers (5′-cgggatccACCGGTCGCCAC-3′ and 5′-cggaattcGCGGCCGCTTTACTTGTACAGC-3′) that were engineered to contain *BamHI* and *EcoRI* cutting sites at their 5′-end respectively were located at the start and stop codons of EGFP gene respectively. PCRs were performed in 100 μl volume using a minicycler™ (MJ Research). Each reaction contained 50 mM KCl, 10 mM Tris–HCl pH 8.3, 1.5 mM $MgCl_2$, 0.001% gelatin (W/V), 5.0 pmol of each primer, 200 μM of

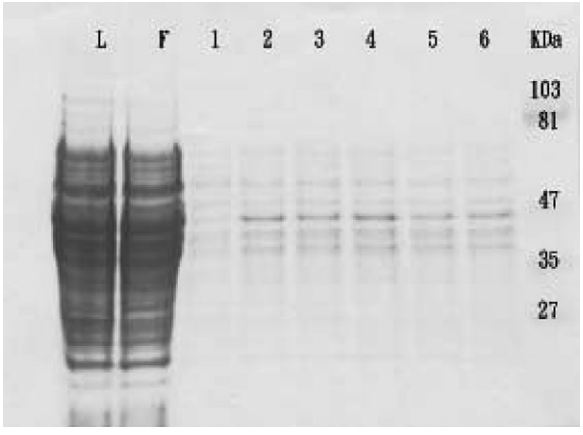

Fig. 3. The influence of the imidazole concentration on the elution of nonspecific bound proteins other than EGFP used in washing buffer. Lanes L, protein lysate loaded into MPGI absorbent solution; F, flow through; 1, 0 M imidazole; 2, 0.01 M imidazole; 3, 0.02 M imidazole; 4, 0.03 M imidazole; 5, 0.04 M imidazole; 6, 0.05 M imidazole. Purification of EGFP using Cu^{2+}-charged MPGI.

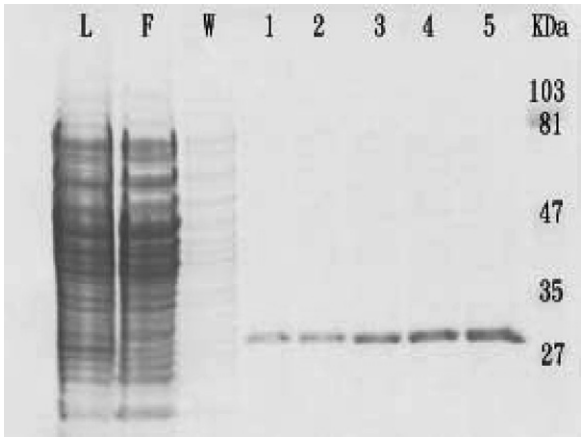

Fig. 5. The influence of the imidazole concentration on the elution of EGFP-$(His)_6$ from MPGI adsorbent. Lanes L, protein lysate loaded into MPGI absorbent solution; F, flow through; W, washing; 1, 0.1 M imidazole; 2, 0.2 M imidazole. 3, 0.3 M imidazole; 4, 0.4 M imidazole; 5, 0.5 M imidazole. Purification of EGFP using Cu^{2+}-charged MPGI.

each deoxyribonucleoside triphosphate (dNTP), 2.5 U Ex Tag (Takara) and 0.1 μg pEGFP-N1. Cycling conditions were 20 cycles of 95 °C for 1 min; 60 °C for 1 min and 72 °C for 1 min. Amplified PCR fragments were linearized with *BamHI* and *EcoRI* and then purified from the 1% agarose gel using GFX™ PCR DNA and Gel Band Purification Kit (Amersham Bioscience) following the manufacture's protocol. Restricted PCR fragments were ligated with the *BamHI*/*EcoRI*-digested pRSET B (Invitrogen Life Technologies Ltd.) in frame with the $(His)_6$ tag driven under the T7 promoter to result in the pRSET-EGFP.

2.5. Bacteria transformation

BL21 (DE3) *E. coli*. (Invitrogen Life Technologies Ltd.) containing a T7 RNA polymerase gene under the control of IPTG-induced Lac promoter was thawed on ice and then 1 ng of pRSET-EGFP was gently mixed with *E. coli*. After incubation on ice for 30 min, *E. coli* was incubated at 37 °C for 30 s and then kept on ice for 2 min. After heat shock, *E. coli* was cultured in LB broth for recovery at 37 °C and lasted 1 h. Then 100 μl *E. coli* suspension was spread on the LB agar containing 60 μg/ml ampicillin and incubated at 37 °C overnight for the colony formation.

2.6. Expression in E. coli and preparation of bacterial lysates

Cultures were grown at the appropriate temperature (37 °C.) in Luria-Bertani (LB) media containing 100 μg/ml ampicillin to an OD_{600} of 0.6 and induced with 1 mM isopropyl-beta-D-thiogalactopyranoside (IPTG) for 4 h. 100 mL cell cultures were harvested by centrifugation for 15 min at 3000 × *g* at 4 °C and resuspended in 50 mM NaH_2PO_4 (pH 7.0), 300 mM NaCl. Lysozyme was added to a final concentration of 0.75 mg/ml and the resuspended cells were incubated at room temperature for 30 min. The samples were then disrupted in an ice bath using a sonicator (Dr. Hielscher Ultrasonification, UP50H, Germany). Disruption was carried out for a total of 5 min, using 30 s pulses with 1 min in between to allow the disrupt ate to cool. The sample was sonicated 6 × 20 s on ice at the minimum power setting using an ultrasonic cell disruptor equipped with a microprobe (Dr. Hielscher Ultrasonification, UP50H, Germany). The lysate was centrifuged for 20 min at 12,000 × *g* at 4 °C and soluble EGFP-fusion protein in the supernatant was purified by MPGI adsorbent.

2.7. Immobilized metal ion affinity procedures

The procedure to use MPGI adsorbent for protein separation consists of three simple steps: (1) aliquots of bacterial lysate were loaded into 3.5 mg MPGI adsorbent solution chelated with Cu^{2+}, Ni^{2+}, or Zn^{2+} and shaking for 10 min, (2) using a small magnet to attract the MPGI adsorbents to the wall of the vial and washing them with washing buffer to remove the residual protein solution, and (3) using elution buffer to wash the MPGI adsorbents to yield pure proteins. After releasing the proteins and being washed sequentially by EDTA, buffer, and Cu^{2+} solution, MPGI adsorbent can be recovered and reused.

2.8. Analytical procedures

2.8.1. Protein assay

Protein was determined using the Micro BCA Protein Assay Reagent (Pierce Chemical, Rockford, IL) with bovine serum albumin or purified recombinant EGFP protein (Clontech) as the standard. Total protein in bacterial lysates was quantified using a protein assay reagent (Bio-Rad, Hercules, CA) with bovine serum albumin as the standard.

2.8.2. Protein gel electrophoresis

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) using the mini-Protean II apparatus and a Tris–glycine buffer system was used to monitor purification during chromatography. Protein samples were denatured in SDS sample buffer containing 2-mercaptoethanol for 3 min at 100 °C and resolved on a 10% or 12% SDS–polyacrylamide gel. The BenchMark Protein Ladder (Invitrogen) was loaded for molecular weight determination. Gels were stained with Coomassie brilliant blue.

2.8.3. Fluorometric assays

EGFP fluorescence was measured using a fluorescence spectrophotometer (Hitachi Model F-4500, Tokyo, Japan) with purified recombinant EGFP protein (Clontech) as the standard. Wavelength scans were performed to determine excitation and emission peaks. The fluorescent intensity per unit protein was determined with the EGFP samples diluted in buffer with excitation and emission set at 488 and 507 nm, respectively.

2.9. Characterization of MPGI

The mole content of GMA–IDA within MPGI was determined by potentiometric titration of carboxylic acids. The weight of magnetic Fe_3O_4 in MPGI was examined by thermogravimetric analyzer (TGA, PerkinElmer TGA-Q50, USA) in N_2 stream to remove the polymers. The concentrations of $CuCl_2$, $NiCl_2$ and $ZnCl_2$ solutions were determined by atomic absorption spectrophotometer (PerkinElmer, AAnalyst100, USA). The magnetic measurement was done using a superconducting quantum interference device (SQUID) (Quantum Design, MPMS7 magnetometer, USA).

3. Results and discussion

3.1. Characterization of magnetic particles

Size and morphology of magnetic Fe_3O_4 and MPGI particles were characterized by TEM. A typical TEM micrograph of magnetic particles is shown in Fig. 1. The superparamagnetic properties of the magnetic particles were verified by the magnetization curve measured by SQUID. A typical plot of magnetization versus applied magnetic field (M–H loop) at 298 K is

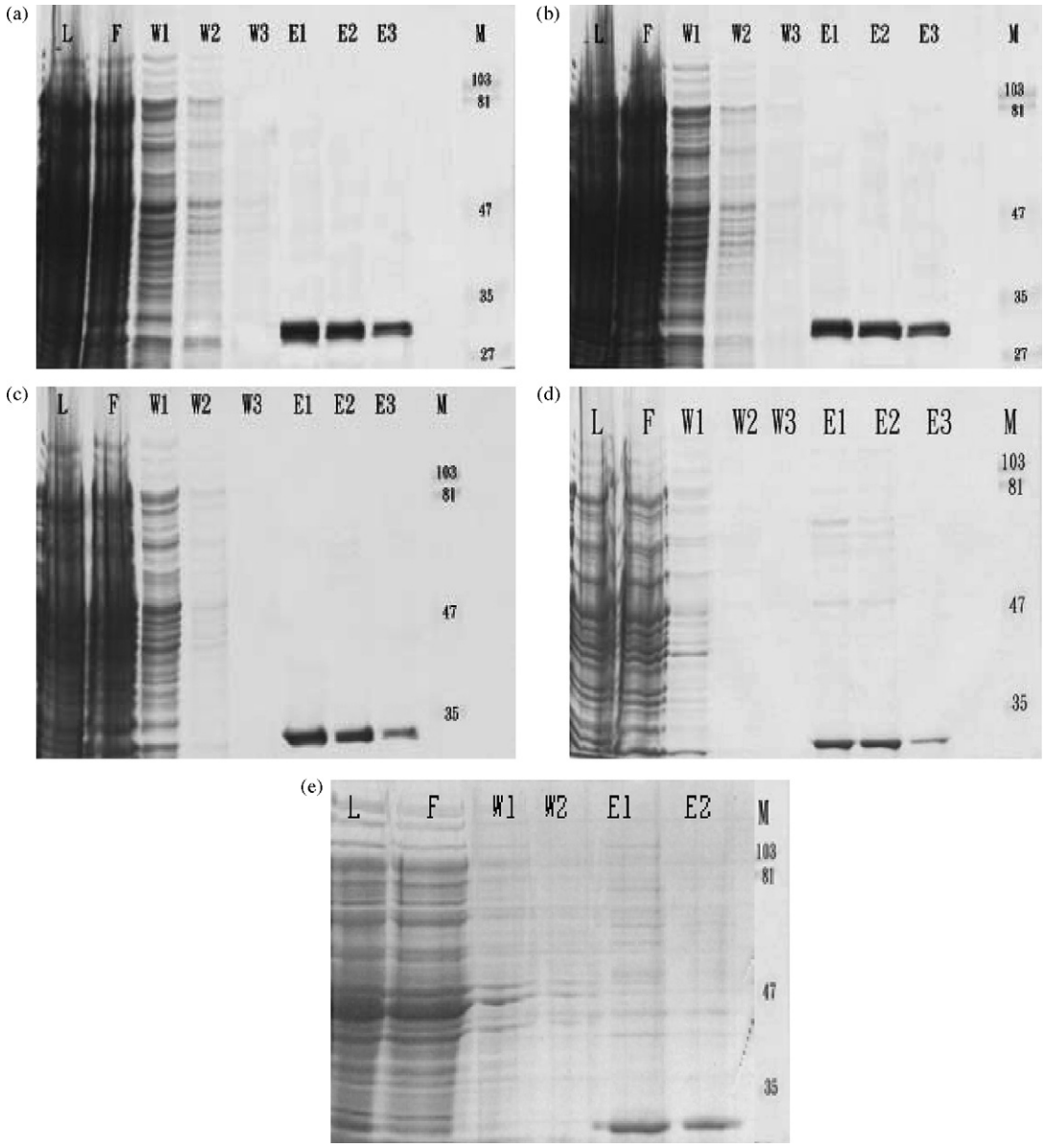

Fig. 6. SDS–PAGE analysis of purified EGFP from *E. coli* lysate by IMA using different chelated metal ion. (a) Cu^{2+}; (b) Ni^{2+}; (c) Zn^{2+}; (d) Qiagen Ni-NTA Spin Kit; (e) Amersham His MicroSpin Purification Module. Lanes L, protein lysate loaded into MPGI absorbent solution; F, flow through; W1, 1st washing; W2, 2nd washing; W3, 3rd washing; E1, 1st elution; E2, 2nd elution; E3, 3rd elution; M, molecular mass standard.

shown in Fig. 2. The saturation magnetization of the obtained Fe_3O_4 magnetic and MPGI particles is 64 and 26 emu/g. This large saturation magnetization of magnetic particles makes them very susceptible to magnetic fields, and therefore makes the solid and liquid phases separate easily. The very weak hysteresis revealed the resultant magnetic nanoparticles were nearly superparamagnetic. Table 1 presents the compositions of MPGI by TGA and potentiometric titration of carboxylic acids. The weight fraction of GMA–IDA in MPGI is 28.8%.

Table 1
The chemical compositions of MPGI (1 g) by TGA and potentiometric titration of carboxylic acids

Polymer	MPGI
Chemical compositions (g/g MPGI)	
GMA–IDA	0.288 g (0.903 mmol)
Styrene + DVB	0.686 g
Fe_3O_4	0.026 g

3.2. The optimum conditions for the IMA

The MPGI adsorbents were used for the purification of EGFP and different parameters were varied to compare the efficiency of the adsorbents. The solution and environment conditions were optimized to give the maximum yield and purity of recombinant EGFP. After loading the crude protein lysates into MPGI adsorbent solution, few proteins other than EGFP also bind to the MPGI adsorbents as some of the crude proteins also have other surface accessible amino acids like cysteine, tryptophan, or due

Table 2
Comparative study of yield and purification factor with different chelated metal ion

Metal ion	Yield of EGFP (%)	Purification factor
Cu^{2+}	70.4	12.3
Ni^{2+}	66.2	7.6
Zn^{2+}	63.7	8.8
Qiagen	49.9	4.9
Amersham	53.6	5.3

to ionic interactions. In order to determine the effect of ionic strength and imidazole on the adsorption of EGFP onto MPGI adsorbent and nonspecific ionic interactions between the metal and charged feedstock components, binding buffer was studied at several NaCl concentrations (0–0.5 M) and imidazole concentrations (0–0.05 M). The proteins binding to the absorbent through ionic interactions were suppressed by increasing the salt concentration. Meanwhile, these ionic interactions helped in reducing many contaminating proteins other than EGFP in the eluted solution. We can find that 0.2 M NaCl was present to suppress weak nonspecific ionic interactions between the metal and charged feedstock components (data not shown). A low level of imidazole (0.02 M) was provided to suppress the retention of other metal-binding proteins present in the feed, while not preventing binding through the hexa-histidine tail of EGFP-$(His)_6$. In order to wash out nonspecific bound proteins other than EGFP, different concentrations of imidazole (0–0.05 M) and NaCl (0–0.60 M) were investigated in the washing buffer. As shown in Figs. 3 and 4, it was found that bound proteins other than EGFP eluted out with a low concentration of 0.03 M imidazole and 0.40 M NaCl. The influence of the imidazole concentration on the elution of EGFP-$(His)_6$ from MPGI adsorbent was investigated between 0.10 and 0.50 M. As shown in Fig. 5, the major part of the bound EGFP was eluted with the 0.50 M imidazole concentration in the elution buffer.

Experiments were performed to compare the elution of EGFP-$(His)_6$ from MPGI adsorbent charged with Cu^{2+}, Ni^{2+}, Zn^{2+}, Amersham His MicroSpin Purification Module, and Qiagen Ni-NTA Spin Kit. The results from these experiments are shown in Fig. 6 and Table 2 in terms of the EGFP-$(His)_6$ yield and purification factor. Comparing these results, the Cu^{2+}-charged MPGI adsorbent had the highest yield and the purification factor at 70.4% and 12.3, respectively. According to the above results, the yield and the purification factor of EGFP were found to be maximum using 0.2 M NaCl, 0.02 M imidazole in binding buffer, 0.4 M NaCl, 0.03 M imidazole in washing buffer, and 0.50 M imidazole in elution buffer.

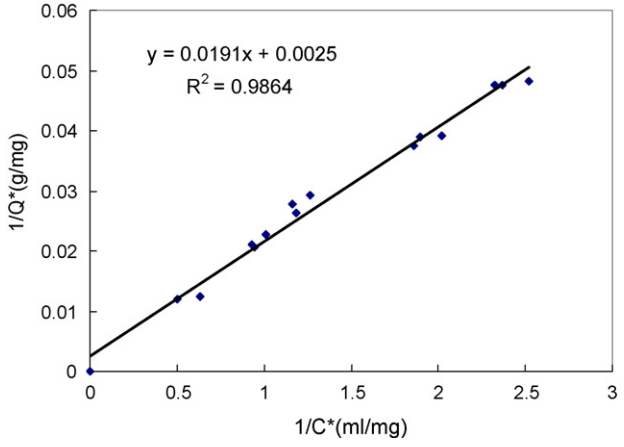

Fig. 7. Langmuir isotherm for EGFP by the MPGI adsorbent.

3.3. Adsorption isotherm

Adsorption isotherm of EGFP on MPGI adsorbent was similar to the Langmuir adsorption isotherm. Data shown in Fig. 7 were fitted to the linear form of the Langmuir equation as follow:

$$\frac{1}{Q^*} = \frac{1}{C^*}\frac{K_d}{Q_m} + \frac{1}{Q_m}$$

where C^* and Q^* represent equilibrium concentration of protein and the amount of adsorbed protein per unit weight of MPGI adsorbent, respectively; Q_m is the maximum amount of protein adsorbed on per gram weight of MPGI adsorbent; and K_d corresponds to the dissociation constant of the binary ligand-target molecule complex. Hence, K_d is a direct measure of the stability of this complex. The smaller the value of K_d, the more stable the complex. The Q_m and K_d could be calculated from the slope and the intercept of the straight line by plotting $1/Q^*$ against $1/C^*$, respectively. As a result, an adsorbent capacity Q_m of approximately 53.5 mg/g and K_d constant of 5.84 mg/mL were obtained. With respect to the applicability in downstream processing, both parameters are important. The initial slope of the binding isotherm ($Q_m/K_d = 9.2$ mL/g), which reflects tightness of binding, was obtained. The calculated isotherm parameters indicated that the MPGI adsorbent could be used as a suitable adsorbent for EGFP from aqueous solution.

4. Conclusions

We demonstrated the synthesis of GMA–IDA-coated magnetic Fe_3O_4 (MPGI adsorbent) and their successful application to the magnetic separation of His-tagged proteins. The MPGI adsorbent was employed for the direct extraction of EGFP-$(His)_6$ from *E. coli* lysates as a model system. The optimal selectively was observed in binding buffer (0.2 M NaCl, 0.02 M imidazole), washing buffer (0.4 M NaCl, 0.03 M imidazole), elution buffer (0.50 M imidazole). The Cu^{2+}-charged MPGI adsorbent had the highest yield and purification factor at 70.4% and 12.3, respectively. The calculated isotherm parameters indicated that the MPGI adsorbent could be used as a suitable adsorbent for EGFP from aqueous solution. Results proved that this new protein purification adsorbent provides a fast and efficient method for purifying His-tagged proteins with high yield and low background.

Acknowledgments

This work was performed under the auspices of the National Science Council of the Republic of China, under contract num-

ber NSC 96-2214-E218-028, to which the authors wish to express their thanks.

References

[1] U. Hafeli, W. Schutt, J. Teller, M. Zborowski (Eds.), Scientific and Clinical Applications of Magnetic Carriers, Plenum Press, New York, 1997.
[2] A.H. Lu, E.L. Salabas, F. Schuth, Angew. Chem. Int. Ed. 46 (2007) 1222.
[3] D. Horak, M. Babic, H. Mackova, M.J. Benes, J. Sep. Sci. 30 (2007) 1751.
[4] A.K.A. Silva, E.L. Silva, A.S. Carrico, E.S.T. Egito, Current Pharm. Des. 13 (2007) 1179.
[5] D.L. Ma, Chem. Mater. 18 (2006) 1920.
[6] A.K. Gupta, M. Gupta, Biomaterials 26 (2005) 3995.
[7] C.-L. Chiang, C.-S. Sung, T.-F. Wu, C.-Y. Chen, C.-Y. Hsu, J. Chromatogr. B 822 (2005) 54.
[8] A. Hütten, D. Sudfeld, I. Ennen, G. Reiss, W. Hachmann, U. Heinzmann, K. Wojczykowski, P. Jutzi, W. Saikaly, G. Thomas, J. Biotechnol. 112 (2004) 47.
[9] C. Berry, A. Curtis, J. Phys. D Appl. Phys. 36 (2003) R198.
[10] P. Tartaj, M. Morales, P.T. González-Carreño, S. Veintemillas-Verdaguer, C.J. Serna, J. Magn. Magn. Mater. 290/291 (2005) 28.
[11] J.I. Taylor, C.D. Hurst, M.J. Davies, N. Sachsinger, I.J. Bruce, J. Chromatogr. A 890 (2000) 159.
[12] S. Buack, D.A. Jones, P.E. Laibinis, T.A. Hatton, Biotechnol. Prog. 19 (2003) 477.
[13] R. Gutierrez, E.M.M. del Valle, M.A. Galan, Sep. Purif. Rev. 36 (2007) 71.
[14] T. Abudiab, R.R. Beitle, J. Chromatogr. A 795 (1998) 211.
[15] E.K.M. Ueda, P.W. Gout, L. Morganti, J. Chromatogr. A 988 (2003) 1.
[16] J. Porath, J. Carlsson, I. Olsson, G. Belfrage, Nature 258 (1975) 598.
[17] G.S. Chaga, J. Biochem. Biophys. Methods 49 (2001) 313.
[18] C.-Y. Chen, C.-L. Chiang, C.-R. Chen, Sep. Purif. Techn. 54 (2007) 396.