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Functional polymer affinity matrix for purifying hexahistidine-tagged recombinant protein

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Abstract

A functional polyacrylic acid (PAA) adsorbent has been prepared for metal chelate affinity chromatography. It has been found to chelate nickel ion Ni^{2+} strongly, and was evaluated for the ability to bind proteins containing neighbouring histidine residues. The principle of the technique was illustrated with *Aeromonas hydrophila* outer membrane protein OmpTS. DNA elements coding for adjacent histidines were fused to the *Aeromonas hydrophila* ompTS gene. Subsequent expression in *E. coli* resulted in the production of hybrid protein His_6-OmpTS that could be purified by Ni^{2+}–PAA affinity chromatography. The remarkable specificity found makes it an attractive addition to the range of adsorbents for metal chelate affinity chromatography.

Keywords: Affinity sorbents; Polyacrylic acid sorbents; Proteins; Metal chelates

1. Introduction

Purification of recombinant proteins has been greatly facilitated in recent years by affinity interactions with specific protein sequence recognition ''affinity tags'', genetically engineered into the protein of interest. This allows the expressed-tagged protein to be purified by affinity chromatography techniques, often in a single step. The use of affinity-tags has allowed rapid purification of proteins expressed at both low and high levels in all common expression systems and has dramatically increased the rate at which new proteins can be brought from DNA sequence to purified reagents for screening or evaluation as therapeutic agents themselves and has developed into a cutting edge technology. There have been over 200 examples of purification using affinity-tag systems described in the literature since 1990 [1–10]. One of the most versatile purification methods utilizes affinity tags containing six consecutive histidine residues (His_6) engineered into recombinant proteins, and Ni^{2+} ions immobilized on commercially available nitrilotriacetic acid–agarose (Agarose–NTA–Ni^{2+}) were widely used as affinity matrix [11–14]. However, the preparation procedure of Agarose–NTA–Ni^{2+} is a cumbersome procedure [15], agarose is notoriously mildewy, and the cost of purification is quite expensive. Here we describe the application of a simple, stable and cheaper immobil-

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ized metal affinity chromatography (IMAC) adsorbent, Ni^{2+} chelate polyacrylic acid (PAA). We demonstrate that Ni^{2+}–PAA displayed low non-specific binding of irrelevant proteins and was specific for proteins containing His_6 tags.

2. Experimental

2.1. Material

Acrylic acid was purchased from Merck (Darmstadt, Germany) and used without further purification. *N*,*N*′-Methylenebis(acrylamide) and IPTG (isopropyl-β-D-thiogalactopyranoside) were purchased from Promega (Madison, USA). Plasmid pRSET A was kindly provided by Dr. S.M. Chan (Department of Zoology, Hong Kong University, China). *E. coli* strain XL1 blue was purchased from Clontech Labs. Yeast extract and tryptone were purchased from Unipath (Basingstoke, UK). Guanidine hydrochloride was purchased from Sigma (St. Louis, MO, USA). Ammonium persulfate and other inorganic chemicals were all of analytical grade and used without further purification. A 20-ml polypropylene spin column with snap-off caps at both ends was purchased from Invitrogen (San Diego, CA, USA).

2.2. Preparation of granules of the polymer

From an aqueous solution of a mixture of the monomers acrylic acid and *N*,*N*′-methylenebis(acrylamide) a stiff gel is prepared by a block polymerization procedure. The gel is dehydrated by freeze-drying and pulverized. Uniform granular size is obtained by sieving. Details are as follows: 4 g *N*,*N*′-methylenebis(acrylamide) and 40 g acrylic acid were added to 500 ml water. The solution is filtered using a filter funnel with granulated glass porous plate, and 2 ml 10% ammonium persulfate were added into it. The solution was incubated at 80°C for 4 h, a stiff gel was formed, which was frozen at −20°C. The gel was then submitted to freeze-drying. The freeze-dried polymer was cautiously pulverized and sieved. Particles passing 200- but not 300-mesh sieves were collected.

2.3. Preparation of Ni^{2+} chelate polyacrylic acid (Ni^{2+}–PAA) column

Five grams of sieved powder of the polymer was slowly added under vigorous stirring to a beaker containing 300 ml of distilled water. The powder sedimenting most slowly were eliminated by decantation. The beaker was allowed to stand for 1 h to ensure the thorough swelling of polyacrylic acid. The swelling powder was then incubated with 200 ml 0.2 *M* $NiCl_2$ for 20 min under vigorous stirring at room temperature. Ni^{2+} charged media were rinsed thoroughly with 0.2 *M* acetic acid and distilled water. Four ml of the charged media were packed into a 20-ml polypropylene spin column. The media in the column was pre-equilibrated with 7 ml denaturing binding buffer (see Section 2.5.1) twice. Ni^{2+}–PAA affinity chromatography matrix made by this method can be stored at room temperature for years prior to use.

2.4. Expression of hexahistidine-tagged recombinant proteins

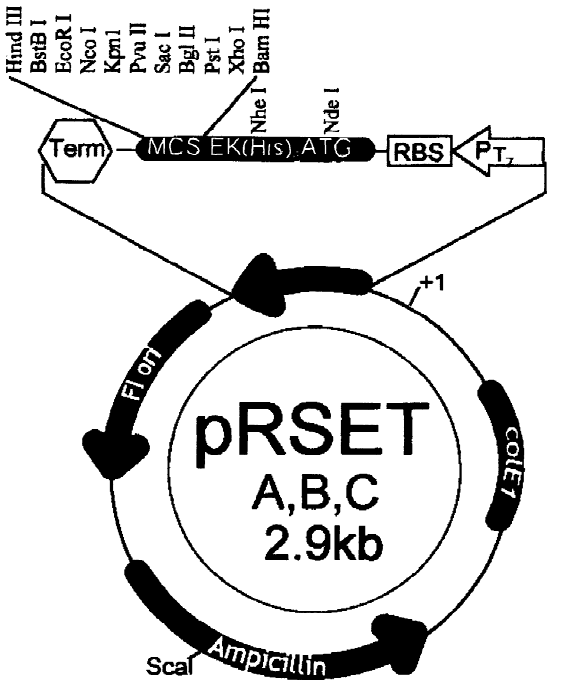
2.4.1. Plasmid vector and bacterial strain

All DNA manipulations were performed using the plasmid pRSET A (Fig. 1) with the *E. coli* strain XL1blue. A target fragment from *Aeromonas hydrophila* ompTS gene, which codes for *A. hydrophila* outer membrane protein OmpTS, was inserted into the linearized plasmid vector pRSET A.

2.4.2. Culture media

SOB medium was prepared by dissolving 20 g tryptone, 5 g yeast extract, 0.5 g NaCl and 186 mg KCl in 950 ml deionized water. The pH was adjusted to 7.0 with 5 *M* NaOH. The volume was adjusted to 1000 ml and sterilized by autoclaving. Once autoclaved, 10 ml of either sterile 1 *M* $MgCl_2$ or sterile 1 *M* $MgSO_4$ were added.

Luria–Bertani (LB) medium was made as follows: 10 g tryptone, 5 g yeast extract, 10 g NaCl with 950 ml deionized water were combined. The solution was mixed until dissolved. The pH was adjusted to 7.0 with 5 *M* NaOH, the volume to 1 l with water. Sterilization was by autoclaving.



pRSET A

RBS — Polyhistidine region

2870 AAGAAGGAGA TATACAT ATG CGG GGT TCT CAT CAT CAT CAT CAT CAT GGT ATG
Met Arg Gly Ser His His His His His His Gly Met

Nhe 1 — T7 gene 10 leader — Anti-Xpress™ Antibody Epitope

2817 GCT AGC ATG ACT GGT GGA CAG CAA ATG GGT CGG GAT CTG TAC GAC GAT
Ala Ser Met Thr Gly Gly Gln Gln Met Gly Arg Asp Leu Tyr Asp Asp

Bam HI — Xho I Sac I Bgl II — Pst I Pvu II — Kpn I Nco I

2769 GAC GAT AAG GAT CGA TGG GGA TCC GAG CTC GAG ATC TGC AGC TGG TAC
Asp Asp Lys Asp Arg Trp Gly Ser Glu Leu Glu Ile Cys Ser Trp Tyr

EK recognition sequence — EK cleavage site

EcoR I — BstB I — Hind III

2721 CAT GGA ATT CGA AGC TTG ATC CGG CTG CTA ACA AAG CCC GAA AGG AAG
His Gly Ile Arg Ser Leu Ile Arg Leu Leu Thr Lys Pro Glu Arg Lys

pRSET reverse priming site

2673 CTG AGT TGG CTG CTG CCA CCG CTG AGC AAT AAC TAG
Leu Ser Trp Leu Leu Pro Pro Leu Ser Asn Asn ...

Fig. 1. Physical map of expression vector pRSET A and its multiple cloning site reading frame [20].

2.4.3. Expression of His_6-OmpTS

Inoculate 2 ml of SOB+ampicillin (50 μg/ml) with a single pRSET A-ompTS transformed *E.coli* XL Blue colony. Grow overnight at 37°C with vigorous shaking to an $A_{600\ nm}$=0.3. The next day, inoculate 500 ml of LB+ampicillin (50 μg/ml) with 3 ml of the overnight culture. Grow the culture at 37°C with vigorous shaking (about 3 h) to an $A_{600\ nm}$=0.3. Add IPTG to a final concentration of 1 m*M* (5 ml of 100 m*M* IPTG in 500 ml of stock culture). Grow in the presence of IPTG for an additional 4 h.

2.5. Purification of hexahistidine-tagged recombinant proteins

2.5.1. Buffer preparation

Stock solution A (10×): 200 m*M* monobasic sodium phosphate (NaH_2PO_4), 5 *M* NaCl. Prepare by dissolving 27.6 g of monobasic phosphate and 292.9 g of NaCl in 1000 ml of deionized water.

Stock solution B (10×): 200 m*M* dibasic sodium phosphate (Na_2HPO_4), 5 *M* NaCl. Prepare by dissolving 28.4 g of dibasic sodium phosphate and 292.9 g of NaCl in 1000 ml of deionized water.

Guanidinium lysis buffer: combine 0.58 ml of stock A (10×) with 9.42 ml of stock Solution B (10×). Add 57.3 g guanidine hydrochloride and water to 90 ml. Stir the solution until completely dissolved. Adjust the pH to 7.8 using 1 *M* NaOH or 1 *M* HCl. Bring the volume to 100 ml and filter sterilize the buffer using a 0.45-μm filter.

Denaturing binding buffer: combine 0.58 ml of stock solution A (10×)with 9.42 ml of stock solution B (10×). Add 48.1 g of urea and water to 90 ml. Stir the solution with gentle heating (50–60°C maximum temperature) until complete dissolved. When cooled to room temperature, adjust the pH to 7.8 using 1 *M* NaOH or 1 *M* HCl. Bring the volume to 100 ml and filter sterilize the buffer using a 0.45-μm filter.

Denaturing wash buffer 6.0: combine 7.38 ml of stock solution A (10×)with 2.62 ml of stock solution B (10×). Add 48.1 g of urea and water to 90 ml. Stir the solution with gentle heating (50–60°C maximum temperature) until complete dissolved. Adjust the pH to 6.0 using 1 *M* NaOH or 1 *M* HCl. Bring the volume to 100 ml and filter sterilize the buffer using a 0.45-μm filter.

Denaturing wash buffer 5.3: combine 9.17 ml of stock solution A (10×)with 0.83 ml of Stock solution B (10×). Add 48.1 g of Urea and H_2O to 90 ml. Stir the solution with gentle heating (50–60°C maximum temperature) until complete dissolved. Adjust the pH to 5.3 using 1 *M* NaOH or 1 *M* HCl. Bring the volume to 100 ml and filter sterilize the buffer using a 0.45-μm filter.

Denaturing elution buffer: combine 10 ml of stock solution A (10×)with 48.1 g of urea, and water to 90 ml. Stir the solution with gentle heating (50–60°C maximum temperature) until complete dissolved. Adjust the pH to 4.0 using 1 *M* NaOH or 1 *M* HCl. Bring the volume to 100 ml and filter sterilize the buffer using a 0.45-μm filter.

2.5.2. Preparation of denatured E.coli cell lysate, pH 7.8

Equilibrate the guanidinium lysis buffer to 37°C. Harvest the *E.coli* from 500 ml LB culture by centrifugation (6000 rpm for 10 min). Resuspend the bacteria pellet in 25 ml of guanidium lysis buffer, pH 7.8. Slowly rock the bacteria for 5–10 min at room temperature to assure thorough cell lysis. Sonicate for 5–10 min at room temperature to assure thorough cell lysis. Sonicate the lysate on ice with three 5-s pulses at a high intensity setting to shear the DNA and RNA. Remove insoluble debris by centrifugation at 3000 *g* for 15 min. Transfer the sheared lysate to a fresh tube. Store the lysate on ice or at −20°C until use.

2.5.3. Purification of His_6-OmpTS under denaturing conditions

Batch bind the protein to be purified by resuspending the pre-equilibrated Ni^{2+}–PAA column with 5 ml lysate aliquots. Gently rock the column for 10 min to keep the resin resuspended and allow the His_6-OmpTS to fully bind. Settle the resin by gravity and carefully aspirate the supernatant. Repeat with a second 5 ml aliquot (fractions 1 and 2).

Wash the column twice with 4 ml of denaturing binding buffer by resuspending the resin, rocking for 2 min and then separating the resin from the supernatant by gravity (fractions 3 and 4). Wash the column with 4 ml of denaturing wash buffer 6.0 by resuspending the resin, rocking for 2 min and then separating the resin from the supernatant by gravity.

Repeat this wash procedure once more for a total of two pH 6.0 washes (fractions 5 and 6). Wash the column with 4 ml of denaturing wash buffer 5.3 by resuspending the resin, rocking for 2 min and then separating the resin from the supernatant by gravity. Repeat this wash procedure once more for a total of two pH 6.0 washes. Clamp the column in a vertical position and snap off the cap on the lower end (fractions 7 and 8).

Elute the protein by applying 4 ml of denaturing elution buffer. Repeat this elution procedure twice more for a total of three elutions (fractions 9, 10 and 11). Collect all of the elution and flow through fractions, monitor by taking absorbance reading of the fractions through a Pharmacia Ultrospec 2000 UV–Vis spectrophotometer. Fractions for analysis on sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS–PAGE) were dialyzed against 10 n*M* Tris, pH 8.0, 0.1% Triton X-100 overnight at 4°C to remove the urea. Concentrate the dialyzed material by any standard method (i.e., vacuum concentration units).

2.6. SDS–PAGE electrophoresis

Analytical gel electrophoresis under denaturing conditions (0.1% SDS) were performed on acrylamide slab gels according to the method of Laemmli [16]. The separating and stocking gels were 12 and 5% in acrylamide, respectively. After SDS–PAGE the proteins on the gels were visualized by Coomassie brilliant blue staining.

2.7. IR spectrum

The IR spectrum was recorded on a Bruker Equinox 55 Fourier transform infrared spectrometer. The Spectrum was taken with a resolution of 2 cm^{-1} and was averaged over 120 scans. Sample was thoroughly ground with exhaustively dried KBr and disc was prepared by compression under vacuum.

2.8. Determination of the Ni^{2+} chelate content in the adsorbent,

For the determination of the Ni^{2+} chelate concentration in the adsorbent, a sample of the metal chelate gel was eluted with 0.1 *M* EDTA. The eluate was collected and its metal content measured by an IRIS Advantage (HR) plasma atomic emission spectroscope (Thermo Jarrell-Ash Co., USA). The density of the metal chelate in mg/g dry resin was calculated as the amount of Ni^{2+} eluted divided by the dry mass of polyacrylic acid powder.

3. Result and discussion

3.1. IR spectrum

The IR spectrum for the obtained polymer resin is shown in Fig. 2. It exhibits the characteristic absorption band of polyacrylic acid at 1714 cm^{-1} due to the C=O stretch vibration of the carboxylic groups, and at 1250–1165 cm^{-1} due to the C–O stretch vibration. The strong and broad absorption at 3431–2500 cm^{-1} is attributed to O–H and N–H stretch vibration [17]. The band at 1454–1401 cm^{-1} due to O–H deformation appears within the CH_2 bending bands. The peak at 1538 cm^{-1} is ascribed to the hydrogen of N–H in the *trans* position to carbonyl. The absorption at 2940 cm^{-1} is due to C–H stretch band. The peaks at 798 and 611 cm^{-1} are attributed to N–H wag vibration. The IR spectrum confirm that *N*,*N*′-methylenebis(acrylamide) form cross-linked structure in polyacrylic acid.

3.2. The polymer matrix adsorbent

Commercial Agarose–NTA–Ni^{2+} is based on agarose, a natural polysaccharide. It is well known that agarose is the natural culture media for bacteria and microorganisms. This means that Agarose–NTA–Ni^{2+} had to be stored under 20% ethanol and at 4°C. Even in this way Agarose–NTA–Ni^{2+} is notoriously mildewy; macroscopic mould often arose from it. On the contrary, Ni^{2+}–PAA based on *N*,*N*′-methylenebis(acrylamide) cross-linked polyacrylic acid soft gel, a synthesized polymer, is not easily contaminated by mould. So Ni^{2+}–PAA can be stored at room temperature for years prior to use.

The amount of Ni^{2+} retained by the resin after washing with 0.2 *M* acetic acid and distilled water is 170 mg/g. Ni^{2+} has a coordination number of six. In most of the cases, polyacrylic acid occupies four coordination sites of Ni^{2+}, remaining two sites are free for interactions with biopolymers (Fig. 3). This

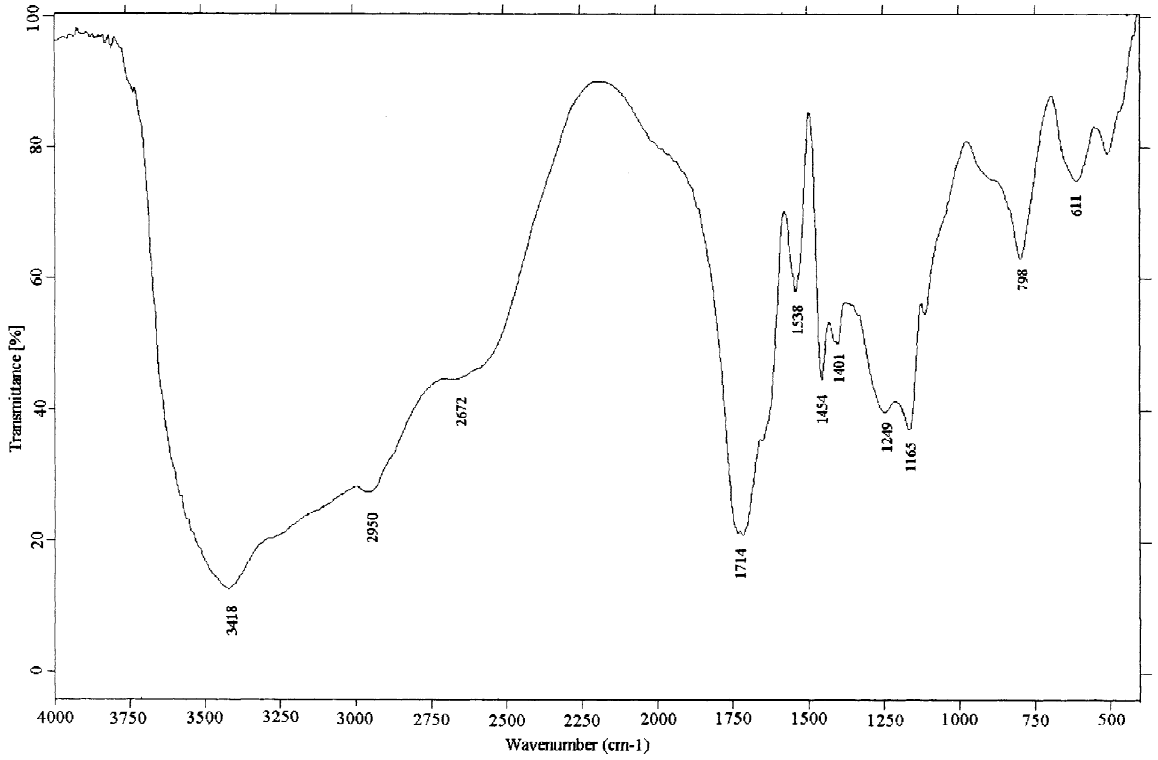


Fig. 2. Infrared spectrum for the obtained *N,N′*-methylenebis(acrylamide) cross-linked PAA polymer resin.

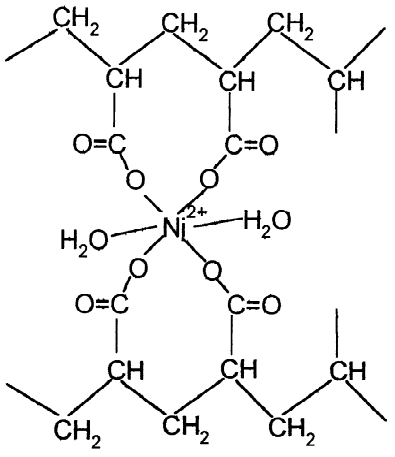


Fig. 3. Schematic structure of Ni^{2+} chelate polyacrylic acid (Ni^{2+}–PAA), two valences are occupied by water and remain for biopolymer interaction.

makes Ni^{2+}–polyacrylic acid an ideal chelate resin for the purification of biopolymers. On the one hand polyacrylic acid strongly complexes Ni^{2+}, and on the other hand it permits reversible interactions between Ni^{2+} and proteins.

3.3. Expression and purification of His_6-OmpTS from E. coli

3.3.1. Expression of the recombinant protein

The pRSET vector is a pUC-derived expression vector designed for high-level protein expression and purification from cloned genes in *E. coli*. High levels of expression of DNA sequence cloned into the pRSET vectors are made possible by the presence of the T7 promoter. In addition, DNA inserts are positioned downstream and in frame with a sequence that encodes an N-terminal fusion peptide. This sequence includes (in 5′–3′ order from N-terminal to C-terminal), an ATG translation initiation codon, a series of six histidine residues that function as a

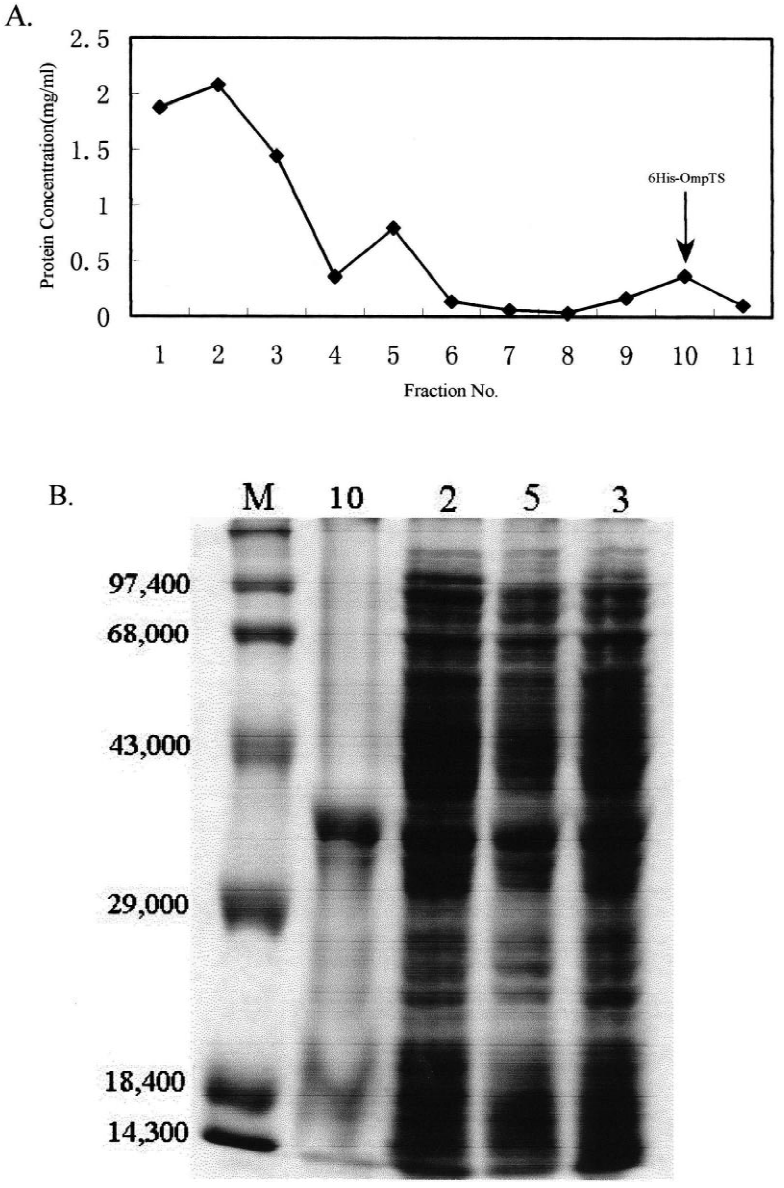


Fig. 4. Purification of the recombinant protein. (A) Ni^{2+}–polyacrylic acid chromatography. The guanidinium lysate fraction was obtained from XL Blue *E. coli* transformed with pRSET A. The lysate fractions were pooled and mixed with Ni^{2+}–polyacrylic acid. The following column binding, washing and elution were processed under denaturing conditions. For more details see Section 2. (B) SDS–PAGE analysis of the purification of the His_6-tagged protein. XL Blue strain contain pRSET A was grown, induced with IPTG, and the cell lysate was subject to Ni^{2+}–polyacrylic acid affinity chromatography as described in Section 2. Aliquots of dialyzed and condensed samples from appropriate fractions were boiled for 5 min in the presence of 1× electrophoresis sample buffer and analyzed by SDS–PAGE gels stained with Coomassie Blue R250. Lanes: (M) molecular mass standard proteins, the position of molecular mass standards are indicated on the left; (10,2,5,3) proteins from Ni^{2+}–polyacrylic acid column fractions: 10 (10 μg), 2 (40 μg), 5 (25 μg) and 3 (30 μg), respectively.

metal binding domain in the translated protein, a transcript stabilizing sequence from gene 10 of phage T7, and the enterokinase cleavage recognition sequence.

The vector with the inserted DNA is propagated in an *E. coli* strain which does not contain the T7 polymerase necessary for expression. When expression is desired, the *E. coli* are infected with an M13 phage that contains the T7 RNA polymerase gene driven by the *E. coli* lac promotor. Infection is carried out in the presence of IPTG which includes the lac promotor and therefore induces T7 RNA polymerase expression from the M13/T7 phage. In the presence of IPTG and M13/T7 phage, the cells now contain high levels of T7 RNA polymerase, and expression of the T7 promotor-driven recombinant protein from the pRSET vector is possible. High levels of recombinant protein are produced within a short period of time following infection with the M13/T7 and addition of IPTG to the *E. coli* growth medium.

3.3.2. Purification of His_6-OmpTS

In the protocols described above, loading and washing are performed in ''batch mode'' from N^{2+}–PAA columns. This means that the processes are completed in the columns provided with the snap-off cap at the bottom of the column still intact. Only the elution steps are performed by gravity flow column chromatography. Elution can also be accomplished in ''batch mode'' by collecting the eluent off the top of the column. Alternatively, the whole protocol could be adapted successfully to gravity flow column chromatography.

Since His_6-OmpTS was expressed with an affinity tag consisting of six consecutive histidine residues, metal ion affinity chromatography was applied as a key step for its purification. It is based on the affinity of the hexahistidine recombinant protein for divalent metal ions such as Ni^{2+}, Cu^{2+}, etc. [18], a feature that is not so common for bacterial protein [19]. This ensures the low nonspecific binding of irrelevant *E. coli* proteins. The His_6-OmpTS containing *E. coli* lysate was subjected to affinity chromatography on a Ni^{2+}–PAA column. His_6-OmpTS is completely retarded by the column (Fig. 4B, lane 10) and remains bound to it during the washing procedure until eluted with pH 4.0 denaturing elution buffer (Table 1 and Fig. 4A) to a purity about 90%. In this case, between 800 and 1000 μg of homogeneous His_6-OmpTS could be obtained according to one batch mode procedure described in Section 2 after being dialyzed.

4. Conclusions

Here, we have proposed a simplified and econ-

Table 1
Protein concentration of fractions from Ni^{2+}–PAA column, determined by taking an absorbance reading using a Pharmacia Ultrospec 2000 UV–Vis spectrophotometer

Fractions	No.	A_{260nm}	A_{280nm}	A_{320nm}	Protein conc. (mg/ml)
Guanidinium lysis buffer	1	3.558	3.427	0.921	1.879
flow through fractions	2	3.481	3.282	0.460	2.079
Denaturing binding buffer	3	3.935	3.805	1.853	1.443
flow through fractions	4	2.080	1.328	0.148	0.360
Denaturing wash buffer 6.0	5	3.153	2.165	0.209	0.795
flow through fractions	6	1.062	0.662	0.107	0.134
Denaturing wash buffer 5.3	7	0.483	0.302	0.051	0.061
flow through fractions	8	0.268	0.173	0.036	0.036
Denaturing elution buffer 4.0	9	1.106	0.681	0.059	0.169
elute fractions	10	2.437	1.567	0.265	0.368
	11	0.474	0.380	0.164	0.100

omical method for purifying His_6-tagged recombinant protein with Ni^{2+}–PAA. We have shown that His_6-OmpTS fusion protein can be specifically and efficiently purified on the novel Ni^{2+} chelate polymer matrix under denaturing washing and elution conditions. This indicates that Ni^{2+}–PAA is selective for proteins and peptides which have neighbouring histidine structural elements. The simpler preparation procedure and higher mildew-resistance of Ni^{2+}–PAA compared to that of the Agarose–NTA–Ni^{2+} adsorbent make the new resin an attractive addition to the range of sorbents for metal chelate chromatography.

Acknowledgements

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