One-Step Metal-Affinity Purification of Histidine-Tagged Proteins by Temperature-Triggered Precipitation

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Abstract: The feature of elastin-like proteins (ELPs) to reversibly precipitate above their transition temperature was exploited as a general method for the purification of histidine (His)-tagged proteins. The principle of the single-step metal-affinity method is based on coordinated ligand-bridging between the modified ELPs and the target proteins. ELPs with repeating sequences of $[(VPGVG)_2(VPGKG)(VPGVG)_2]_{21}$ were synthesized and the free amino groups on the lysine residues were modified by reacting with imidazole-2-carboxyaldehyde to incorporate the metal-binding ligands into the ELP biopolymers. Biopolymers charged with Ni^{2+} were able to interact with a His tag on the target proteins based on metal coordination chemistry. Purifications of two His-tagged enzymes, β-D-galactosidase and chloramphenicol acetyltransferase, were used to demonstrate the utility of this general method and over 85% recovery was observed in both cases. The bound enzymes were easily released by addition of either EDTA or imidazole. The recovered ELPs were reused four times with no observable decrease in the purification performance. © 2003 Wiley Periodicals, Inc. *Biotechnol Bioeng* **82**: 605–611, 2003.
Keywords: elastin; protein purification; metal affinity; His-tag

INTRODUCTION

Many systems have been developed in recent years for the rapid purification of recombinant proteins. One of the most efficient methods is based on specific interactions between an affinity tag (usually a short peptide with specific molecular recognition properties such as maltose binding protein (Maina et al., 1988), thioredoxin (Smith et al., 1998), cellulose binding domain (Ong et al., 1989), glutatione S-transferase (Smith and Johnson, 1988), streptag (Skerra and Schmidt, 1999) and polyhistidines (Smith et al., 1988; Hochuli et al., 1988; Kumar et al., 1998)) and an immobilized ligand. Immobilized metal-affinity chromatography (IMAC) is particularly popular and widely used. The principle of IMAC is based on selective interaction between a solid matrix immobilized with either Cu^{2+} or Ni^{2+} and a polyhistidine tag (His tag) fused to either the N- or C-terminal of proteins. Proteins containing a polyhistidine tag are selectively bound to the matrix while other cellular proteins are washed out. Despite the fact that IMAC has been used successfully for protein purification, it still has serious limitations for large-scale processes due to the high cost and frequent occurrence of column fouling (Kumar et al., 1998).

Metal-affinity precipitation is an effective alternative to IMAC, affording selective recovery of the desired proteins by simple environmental triggers, such as pH and temperature (Galaev et al., 1999). The target protein is selectively bound to a stimuli-responsive (tunable) polymer-metal ligand conjugate and removed from other cell extracts by precipitation. Purified proteins are recovered by dissociation from the polymer conjugates, which can be reused for subsequent cycles. Although tunable polymers such as poly(N-isopropylacrylamide) (poly-NIPAM) have been used for this purpose, the ability to tune for network formation and to provide metal-binding functionality is far from straightforward. Typically, controlled copolymerization of NIPAM with either vinylimidazole or IDA-derivatives is required to introduce the necessary ligands for metal coordination (Kumar et al., 1998).

Elastin-like proteins (ELPs) are biopolymers consisting of the repeating pentapeptide, VPGVG. They behave very similar to poly-NIPAM polymers and have been shown to undergo reversible phase transitions within a wide range of conditions (Urry, 1997; Kostal et al., 2001). Unlike the statistical nature of step and chain polymerization reactions, ELP biopolymers are specifically preprogrammed within a synthetic gene template that can be precisely controlled over chain length, composition, and sequence (Urry et al., 1997). We hypothesized that by replacing the valine residue at the 4th position with a lysine in a controlled fashion, metal-binding ligands such as imidazole can be specifically coupled to the free amine group on the lysine residues, creating the required metal coordination chemistry for metal-affinity precipitation.

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In this article, we present a novel metal-affinity purification method for His-tagged proteins based on temperature-triggered precipitation of the chemically modified ELP biopolymers. The utility of this principle was used to demonstrate the successful purification of His-tagged β-galactosidase (β-gal) and chloramphenicol acetyltransferase (CAT) in repeated cycles. To our knowledge this is the first report exploiting the features of ELP for protein purification based on metal-affinity purification.

MATERIALS AND METHODS

General Methods

All procedures for DNA manipulation were performed according to standard methods (Sambrook et al., 1989). High fidelity *Pfu* DNA polymerase (Stratagene, La Jolla, CA) and *Taq* DNA polymerase (Promega, Madison, WI) were used for PCR reactions with an MJ Research Thermal Cycler—200 (MJ Research, Waltham, MA, USA). Protein electrophoresis was performed using 10% or 12% (w/v) SDS polyacrylamide gels (Laemmli, 1970) and proteins were detected with either silver staining (BioRad, Hercules, CA) or standard coomassie blue staining. The size of ELP was determined using MALDI-TOF mass spectrophotometry with sinapinic acid as a matrix. Protein concentration was determined by the method of Bradford (1976) using bovine serum albumin as a standard.

Synthesis of DNA Monomer

To generate the basic building block for the ELP biopolymers, oligonucleotides (Loma Linda University, CA) 5′CCGAATTCCCAGGTGTTGGCGTACCGGGTGTTGGTGTACCGGGTGTTGGTGCCGGGTAA3′ and 5′GGAATTCACCAACACCTGGGACGCCTACACCCGGAACACCTTTACCCGGCACACCAACACCC3′ at a concentration of 10 μ*M* were denatured for 1 min at 95°C and annealed for 1 min at 80°C using a 21 bp complementary region. The 3′ ends were extended for 5 min at 72°C using *Taq* DNA polymerase to create a synthetic gene coding for the monomeric protein $(VPGVG)_2VPGKG(VPGVG)_2$ flanked by *Eco*RI and *Pfl*MI. The final concentration of $MgCl_2$ and dNTPs in the PCR reaction was 2.5 m*M* and 0.2 m*M,* respectively. The amplified product (102 bp) was excised from a 2.5% (w/v) low melting agarose gel (Fisher, Tustin, CA) and digested with *Eco*RI. The 98 bp fragment was extracted from a 2.5% (w/v) agarose gel and the product was cloned into the pBluscript SK (+/−) phagemid (Stratagene). The inserted fragment (98 bp) was verified by sequencing from both directions (Loma Linda University, CA).

The monomeric gene was amplified with KS and SK universal primers (Stratagene) using *Pfu* DNA polymerase. The PCR product was purified using phenol:chloroform treatment followed by ethanol precipitation. The concentrated product was digested with *Pfl*MI, separated on a 2.5% (w/v) agarose gel (low melting), and the desired band (75 bp) was subsequently excised from the gel (Qiagen, Chatsworth, CA). Self ligation of the monomeric gene was achieved using T4 DNA ligase and the reaction was carried out for 12 h at 16°C. T4 DNA ligase was inactivated at 70°C for 10 min and 1.6 U of *Pfl*MI was used to open the circularized DNA molecule (10 min, 37°C). The resulting concatamers were analyzed using a 1% (w/v) agarose gel and the fragments of the desired size (1600 bp) were extracted from the gel (Qiagen). The fragments were cloned into a dephosphorylated pJAN08 cloning vector (Kostal et al., in prep.) previously digested with *Pfl*M I.

Expression and Purification of ELP Biopolymers

The expression vector for the ELP biopolymers was prepared as follows. Vector pJAN08 was digested with *Bam*HI and *Nde*I and the fragment encoding for ELP was cloned into a T7-based expression vector pET(38+) (Novagen, Madison, WI) previously digested with the same enzymes to generate pELP. The resulting vector was transformed into *E. coli* BLR(DE3) (Novagen), a common strain used for high-level expression from the T7 promoter. Since the highest level of expression was observed without induction, production of ELP was carried out in Terrific Broth (TB) supplemented with 30 μg/mL kanamycin for 24 h. Cells were grown in a BIOFLO 3000 fermentor (New Brunswick Scientific, Edison, NJ) containing 3 L TB. After 24 h cultivation, cells were harvested, resuspended in sterile water, and lysed with a French press. Cell debris was removed by centrifugation for 20 min at 30,000*g*. Purification of ELP by repeated temperature transition was performed by modifying the procedure of McPherson et al. (1996). Cell extracts were mixed in a ratio of 1:1 with 100 m*M* CHES buffer pH 10.0 and NaCl was added to a final concentration of 1.5 *M*. The sample was heated to 37°C and centrifuged at 30,000*g* at the same temperature for 30 min. The pellet was resuspended in cold sterile water and again mixed with CHES buffer and NaCl as described above. The remaining insoluble debris was removed by centrifuging at 30,000*g* at 4°C for 30 min. The temperature transition cycle was repeated three times and the purified ELP was resuspended in sterile water. Protein concentrations were determined by spectrophotometric measurement at 215 nm (ε_{215} = 69.9 $(\mu g/mL)^{-1}$ cm^{-1}), based on previous calibration (Kostal et al., 2001). The purity of ELP was verified by SDS-PAGE electrophoresis, followed by silver staining (BioRad).

Chemical Modification of ELP

The free γ-amino groups of the lysine residues were modified by reacting with 2-imidazolecarboxaldehyde (Aldrich Chemical Co., Madison, WI). Twenty milligrams of ELP was first washed with sterile water three times to remove all residual amino groups from the CHES buffer. Three different pHs were tested for the degree of reductive amination.

Reactions were carried out in the presence of 0.1 *M* 2-imidazolecarboxaldehyde on ice for 24 h either in 0.1 *M* sodium phosphate buffer (pH 6.0 or 7.0) supplemented with 0.15 *M* sodium chloride or in 0.1 *M* sodium citrate, 0.05 *M* sodium carbonate buffer (pH 10.0). The unstable Shiff-base formed was reduced with 40 μL of 5 *M* sodium cyanoborohydrate in 0.1 *M* NaOH (Hermanson et al., 1992) at room temperature for 24 h. The unreacted 2-imidazolecarboxaldehyde and sodium cyanoborohydrate were removed from the modified ELP (ELP-IM) by repeating the temperature transition cycle three times in the presence of 1 *M* NaCl.

Characterization of ELP and ELP-IM

The transition temperatures of ELP and modified ELP were measured in 96-well microplates at 655 nm using a microplate reader (BioRad 3550-UV). The volume of the sample in each well was 200 μL and the protein concentration was 2.5 mg/mL. Measurements were performed between 20–68°C by increasing the temperature every 5 min in 2°C increments. The transition temperature was determined as the temperature where the optical density reached half of the maximum (McPherson et al., 1996).

Metal-binding experiments were performed in 100 μL of 50 m*M* Tris buffer, pH 8.0, and 250 μg of ELP-IM. Nickel was added in excess (1.2 m*M* per reaction) in the form of nickel sulfate. After 1 h binding on ice, the biopolymers were precipitated by addition of 1 *M* NaCl at 37°C and centrifuged for 4 min at 14,000*g* at 37°C. The pellet was redissolved in 100 μL of 50 m*M* Tris buffer, pH 8.0, and precipitated as described above. Proteins were incubated overnight with 100 μL of concentrated HNO_3 to extract all the bound metals. Prior to measurements, 1 mL of water was added to each sample. The amount of bound Ni^{2+} was analyzed by flame atomic absorption spectrometry (Shimadzu AA6701).

The correct molecular weight of the biopolymer was verified by MALDI-TOF mass spectrometry. Briefly, 1 mg of biopolymer was dissolved in 0.1% (v/v) trifluoroacetic acid and 50% (v/v) acetonitrile. Samples were then mixed in a ratio of 1:9 with a matrix solution and air-dried before analyzed by a Voyager-DE STR BioSpectrometry Workstation, PerSeptive Biosystem.

Expression of β-D-Galactosidase (β-Gal) and Chloramphenicol Acetyltransferase (CAT)

For the expression of His-tagged CAT under control of a *trc* promoter, *E. coli* strain BL21 (DE3) containing *p*TrcHis/CAT (Invitrogen Life Technologies, Carlsbad, CA) was used. For the expression of His-tagged β-gal, *E. coli* induction control A (Novagen) was used. This cell line carried a pET14b derivative and expression of β-gal is under control of a T7 promoter. Cultures were inoculated into 25 mL of LB media supplemented with 100 μg/mL of ampicillin and incubated at 37°C. Induction was performed at an OD_{600} of 0.75 with isopropyl α-thiogalactopyranoside (1 m*M* final concentration). Cultures were incubated for an additional 4 h and harvested by centrifugation (10,000*g*, 15 min, 4°C), washed with 50 m*M* Tris buffer, pH 8.0, and resuspended in 5 mL of the same buffer. Cells were lysed by passing through a French press three times and centrifuged for 15 min at 30,000*g* to remove cell debris. Cell extracts were aliquoted and stored at −20°C prior to use.

Purification of β-Gal and CAT Using ELP-IM

The imidazole-modified ELP biopolymers were first precharged with Ni^{2+}. To 50 μL of 50 m*M* Tris buffer, pH 8.0, containing 5 mg/mL of ELP-IM, 10 m*M* $NiSO_4$ was added from a range of 2–9 μL in order to explore the optimal concentration of nickel required for protein purification. After incubation for 10 min on ice, 20 μL of cell extract containing either β-gal or CAT was mixed with the precharged ELP-IM. The solution was gently mixed for 1 h before NaCl was added to a final concentration of 1 *M*. The ELP-IM-protein complex was precipitated by incubation at 37°C for 2 min and centrifuged at the same temperature for 4 min at 10,000*g*. The recovered β-gal was released by resuspending the pellet in 50 m*M* Tris buffer pH 7.0 supplemented with 1.5 m*M* EDTA and the bound CAT was eluted in a solution containing 50 m*M* Tris buffer pH 8.0 and 250 m*M* imidazole. ELP-IM was recovered by precipitation with the addition of 1 *M* NaCl, followed by centrifugation at 37°C for 2 min at 14,000*g*. The activities of β-gal and CAT were monitored in each step as described below. The purity of enzymes was analyzed by SDS-PAGE electrophoresis followed by coomassie blue staining.

Recycling of ELP-IM

ELP-IM was recycled by resuspending the biopolymers in 50 m*M* acetate buffer, pH 4.0. After overnight incubation the impurities were removed by centrifugation. ELP-IM was then precipitated and recovered by centrifugation. The pellet was resuspended in 50 m*M* Tris buffer pH 8.0 and reused for another cycle of purification.

Enzyme Assays

β-Gal activity was determined spectrophotometrically according to Sambrook and Russell (2001) using o-nitrophenyl-β-D-galactopyranoside (ONPG) as the substrate. The product of hydrolysis (o-nitrophenol) was monitored at 420 nm. CAT activity was measured spectrophotometrically at 412 nm using 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) as the substrate (Rodriguez and Tait, 1983).

RESULTS

Design and Synthesis of Chemically Modified ELP Biopolymers

ELPs serve as a suitable starting material for metal-affinity purification, not only for the ability to reversibly precipitate by environmental triggers but also for the possibility to precisely control the amino acid sequence at the genetic level. The basic design of ELP biopolymers was based on

21 repeating units of $(VPGVG)_2VPGKG(VPGVG)_2$. For every five elastin repeats, a valine residue at the 4th position was substituted with lysine, enabling the subsequently controlled chemical modifications. A synthetic gene coding for $(VPGVG)_2VPGKG(VPGVG)_2$ was used as the building block. Polymerization of the synthetic gene was carried out through the compatible cohesive ends generated by the restriction endonuclease P*fl*MI, followed by subsequent ligation to form the multimeric genes coding for $[(VPGVG)_2VPGKG(VPGVG)_2]_{21}$.

Production of the biopolymers was easily achieved in *E. coli* BLR (DE3) using the pET expression system. Purification of biopolymers was based on the temperature-induced aggregation as described by McPherson et al. (1996). Typically, 350 mg of biopolymer was obtained from 3 L of culture. The purity of the biopolymers was verified by SDS-PAGE (Fig. 1A). The apparent molecular weight of the biopolymers was slightly higher than the calculated size, a common phenomenon observed with other elastin-based proteins (McPherson et al., 1996). The actual molecular weight of the purified biopolymers was verified by MALDI-TOF mass spectrometry. A peak centered at 44,465 Da (Fig. 1B), corresponding to the calculated molecular weight of the biopolymers, was observed.

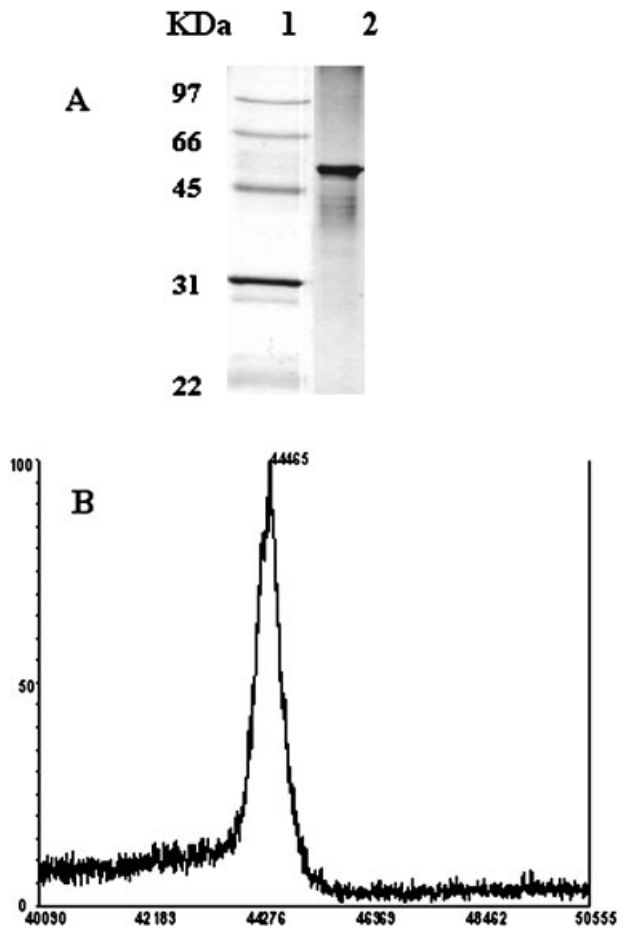

Figure 1. **A:** SDS-PAGE analysis of purified ELP biopolymers. Lane 1: Protein markers and Lane 2: Purified biopolymers. **B:** Analysis of purified ELP biopolymers by MALDI-TOF mass spectrometry.

The reactive amino groups on the lysine residues can be selectively crosslinked with 2-imidazolecarboxyaldehyde to generate biopolymers that can be subsequently charged with Ni^{2+} ions for metal-affinity precipitation. The degree of reductive amination is the most efficient under alkaline pH (~10), whereas lowering the pH reduces the efficiency significantly (Hermanson et al., 1992). By controlling the pH of the reaction, biopolymer-conjugates with an average of 5, 12, and 21 imidazole groups were generated as estimated by MALDI-TOF mass spectrometry (data not shown). The number of imidazole groups incorporated was shown to affect the solubility of modified biopolymers by self-crosslinking between the imidazole groups via nickel ion coordination (Stiborova et al., in prep.). Only the biopolymer (ELP-IM) containing 5 imidazoles remained soluble under room temperature and was chosen for subsequent metal-affinity purification.

Transition Properties of the Biopolymers

Due to the proton-transfer equilibrium of the lysine residues, the transition temperature (T_t) of the biopolymers was highly dependent on pH (Urry et al., 1997). While a transition temperature higher than 98°C was observed at pH 8.0, a condition favoring the ε-amino group on the lysine residues in the charged state, the transition temperature decreased to 37°C in 0.1 N NaOH when the charged ε-amino groups were eliminated (McMillan et al., 1999).

The chemically modified ELP-IM retained the ability to reversibly aggregate above the transition temperature. However, crosslinking with 2-imidazolecarboxyaldehyde lowered the transition temperature by decreasing the number of free amino groups (Fig. 2). While the transition temperature of the original ELP was higher than 98°C, the T_t of ELP-IM dropped to 58°C. By increasing the ionic strength and decreasing the charge repulsion, the addition of NaCl up to 1

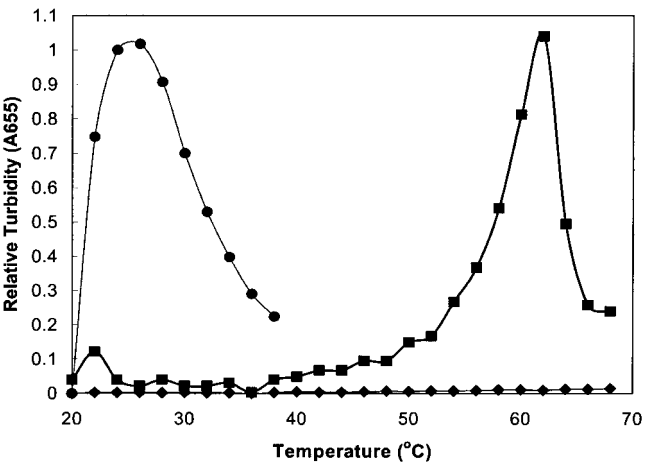

Figure 2. The transition temperature of the ELP-IM biopolymers. The turbidity profiles were measured at 655 nm in 50 m*M* Tris pH 8.0 in the absence of NaCl (■) or presence of 1 *M* NaCl (●). The concentration of proteins was 2.5 μg/μL and the temperature was increased at 2°C per 5 min. The profile for unmodified ELP biopolymer (♦) is also shown for comparison.

M concentration markedly decreased the T_t to 21°C (Fig. 2), a temperature range suitable for precipitation of functional proteins.

In addition to transition temperatures, the efficiency of precipitation varied as a function of salt concentration. Although turbidity was visible at 37°C in the presence of 0.5 *M* NaCl, incomplete recovery (less than 60%) of ELP-IM was observed. However, in the presence of 1 *M* NaCl, 100% recovery was obtained. This condition (37°C and 1 *M* NaCl) was thus chosen for the maximum recovery of aggregated biopolymer–protein complexes.

Nickel Binding Properties of the Biopolymers

Since the basic principle of metal-affinity precipitation of His-tagged proteins depends on metal coordinated bridging between the imidazole groups on ELP-IM and the polyhistidine tag on the target protein, the ability of ELP-IM to bind metal ions (Ni^{2+}) was determined using an absorption flame spectrometer. While the original ELP without any imidazole did not bind Ni^{2+} ions, the modified ELP-IM biopolymers bound Ni^{2+} ions at a ratio of 3.5 imidazoles per Ni^{2+}. This is lower than the theoretical value of 6 imidazoles per Ni^{2+} (Smith and Martell, 1989), probably due to the spatial separation of the 5 imidazoles randomly incorporated in ELP-IM that prevents the proper orientation to form a complex with Ni^{2+} ions at the preferred ratio. However, this is the exact requirement for the bound Ni^{2+} ions to associate further with additional imidazole ligands on the polyhistidine tag of the target proteins.

Purification of His-Tagged Enzymes

The utility of ELP-based metal-affinity purification was demonstrated with two model His-tagged enzymes, β-gal and CAT. Since these enzymes differ in both their sizes and the multimeric conformations (β-gal is a tetrameric enzyme of MW 464,000 Da (Clemmit and Chase, 2000; Cazorla et al., 2001) and CAT is a trimer, approximately 6 times smaller (Shaw and Leslie, 1991)), their successful purification will demonstrate the broad application of this method to a wide range of targets.

For the recovery of His-tagged β-gal or CAT from cell extracts, the feature of ELP-IM to reversibly precipitate above the transition temperature was utilized. His-tagged enzymes associated with ELP-IM through Ni^{2+} complexation were coprecipitated at 37°C in the presence of 1 *M* NaCl and separated from the supernatant. The percentage of bound β-gal and CAT was calculated from the activities in the supernatant and the pellet.

The effect of Ni^{2+} concentration on the binding efficiency of His-tagged enzymes was first investigated. The ELP-IM biopolymers were charged with different concentrations of Ni^{2+}, and the amount of enzymes recovered after precipitation was measured. As shown in Figure 3, the binding profiles for both enzymes were strongly dependent on Ni^{2+} concentration and exhibited a similar trend in the tested concentration range (0.15–0.9 μ*M*). In both cases, 95% recovery was achieved at a Ni^{2+} concentration of 0.9 μ*M*. As a control, ELP biopolymers lacking the ability to bind Ni^{2+} ions were used in a similar fashion with virtually no recovery.

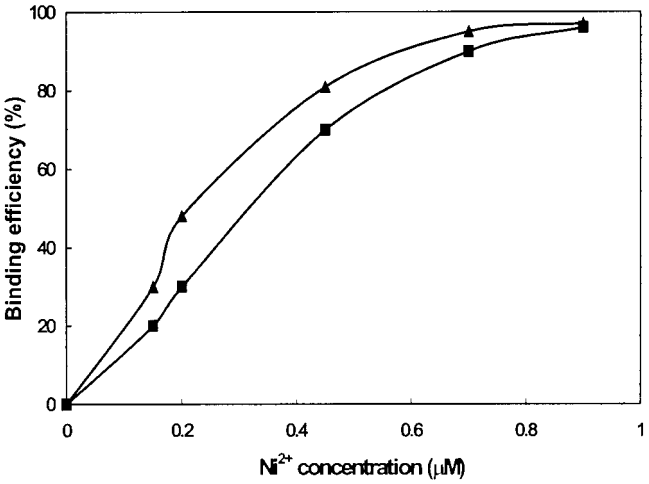

Figure 3. Binding of β-D-galactosidase (▲) and chloramphenicol acetyltransferase (■) to the ELP-IM biopolymers in different concentration of Ni^{2+} ions. Binding was performed for 1 h on ice in 50 m*M* Tris buffer pH 8.0. Enzymes were coprecipitated with ELP-IM in the presence of 1 *M* NaCl at 37°C. Percentage removal was calculated from the residual activity left in supernatant.

Using the optimal precipitation conditions (37°C, 1 *M* NaCl, and 0.9 μ*M* Ni^{2+}), the His-tagged enzymes were recovered with the biopolymers from the total cell extracts as a pellet after centrifugation. To release the desired enzyme from the ELP-IM-enzyme conjugate, the recovered pellet was dissolved in a cold stripping buffer, containing either 1.5 m*M* EDTA or 220 m*M* imidazole. Both enzymes were easily released from the biopolymer aggregates and the biopolymers were separated from the released enzymes by the addition of 1 *M* NaCl; only purified enzymes remained soluble in the supernatant after centrifugation. Typical results for a complete purification cycle are shown in Figure 4. As demonstrated, purification by metal-affinity precipitation is highly efficient, as precipitation of the target enzymes from the cell lysates was essentially complete. Virtually pure β-gal and CAT were recovered in a single precipitation and stripping step as indicated by the SDS-PAGE gel. Over 85% of the total activity was recovered in both cases, a result in line with the initial precipitation efficiency.

The significant advantage of the proposed purification method is the feasibility of recycling the ELP-IM biopolymers for continual reuse. To demonstrate this property, the recovered ELP-IM biopolymers from the previous affinity precipitation cycle were subjected to the same conditions for purification of His-tagged CAT. Since the stripping buffer was expected to remove a fraction of the Ni^{2+} ions from the biopolymers, the recovered ELP-IM was recharged with Ni^{2+} ions before the next purification cycle. The purification cycles were repeated four times and the results are

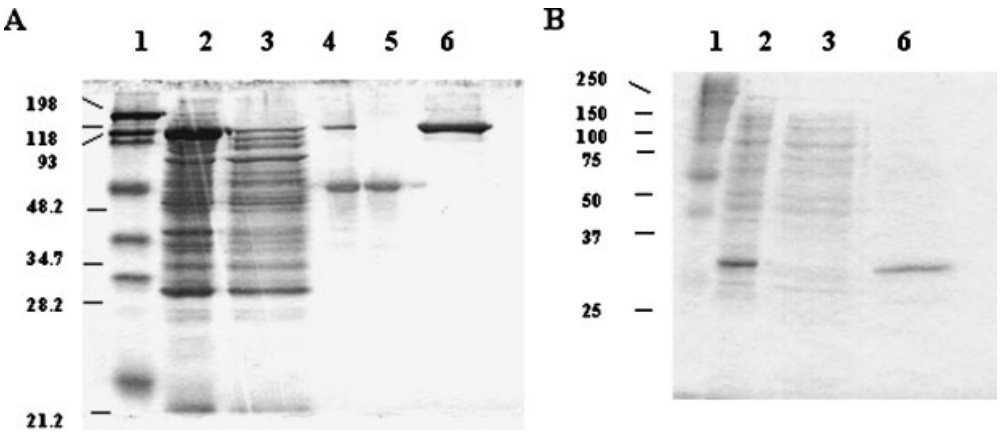

Figure 4. Purification of His-tag proteins by Ni^{2+}-charged ELP-IM biopolymers. SDS-PAGE analysis of each stage of purification for **(A)** β-D-galactosidase (116 kDa) and **(B)** chloramphenicol acetyltransferase (30 kDa). Lane 1, broad range prestained marker (BioRad); lane 2, total soluble cell extract of *E. coli* expressing **(A)** β-D-galactosidase and **(B)** chloramphenicol acetyltransferase; lane 3, supernatant containing proteins that did not bind to ELP-IM; lane 4, ELP-IM with bound β-D-galactosidase; lane 5, ELP-IM after releasing β-D-galactosidase; lane 6, purified **(A)** β-D-galactosidase and **(B)** chloramphenicol acetyltransferase.

summarized in Table I. As illustrated by the recovery of total protein and enzyme activity, the capacity of ELP-IM for purification did not change even after repeated usage for four cycles, demonstrating good capacity and recycling efficiency of the ELP-IM biopolymers.

DISCUSSION

Inclusion of a polyhistidine tag to either the N- or C-terminus of a recombinant protein is a common strategy used for convenient and rapid purification. Purified proteins are usually eluted from the binding matrix either by displacing the ligands (Smith et al., 1988; Hochuli et al., 1988; Kumar et al., 1998) or by enzymatic cleavage (Hefti et al., 2001). Metal-affinity precipitation is an emerging technique that allows simple and rapid purification of His-tagged proteins (Van Dam et al., 1989). Although metal-affinity precipitation has been reported with metal-chelating polymers (Galaev and Mattiasson, 1993), it is based on interaction between surface-exposed histidine residues and Cu^{2+}-charged polymers. This results in relatively weak binding and nonspecific interactions with other cellular proteins containing histidine residues (Johnson and Arnold, 1995; Todd et al., 1994; Anspach, 1994).

Table I. Recycling of the ELP-IM biopolymer.

No. of use	Protein (mg)[a]	Specific activity (U/mg)[b]	Recovery (%)
Cell extract	0.550	2.8	N.A.
1	0.082	15.5	82.7
2	0.087	15.2	86
3	0.074	16.6	80
4	0.09	15.3	90

Purification of His-tag chloramphenicol acetyltransferase was performed for four cycles by recharged biopolymers.

[a]Protein concentrations were determined by the Bradford assay using BSA as a standard.

[b]One unit of CAT activity is defined as the conversion of 1 mmol of chloramphenicol per minute.

In this study, we have taken advantage of the reversible phase transition property of the ELP biopolymers (Urry, 1997) that are chemically modified with imidazoles to develop a novel purification method for His-tagged proteins based on metal-affinity precipitation that is simple, fast, and universal. Unlike commercially available chelating supports that are based on immobilized ligands, metal-affinity precipitation is a homogenous method that offers a high degree of freedom for the ligands and thereby facilitates multipoint attachments. In the case of ELP-IM biopolymers, the chelating imidazole groups are randomly attached to the lysine residues throughout a flexible biopolymer backbone, resulting in multivalent interactions with the protein of interest. By controlling the number of imidazole attached and the nature of bridging metal, selective binding of the polyhistidine tag allows the direct recovery of proteins after complexation without any substantial washing. The versatility of the method was successfully demonstrated with the purification of two enzymes of different sizes and multimeric conformations. As tested with two His-tagged enzymes, the proposed bridging mechanism provides good accessibility to the histidine tag, enabling the recovery of highly purified enzymes in a single precipitation step.

One major advantage of using ELP biopolymers in metal-affinity precipitation is the recycling of the materials for repeated usages. As demonstrated, the efficiency of purification and the purity of enzymes remained unaffected even after four cycles of reuse, making this method attractive for large-scale industrial applications. The feature to reversibly aggregate ELP above the transition temperature has already been exploited for the purification of recombinant proteins (Meyer and Chilkoti, 1999; Meyer et al., 2001: Shimazu et al., 2003). However, in those studies purification was

based on fusion with ELP and enzymatic cleavage was required to obtain pure enzymes. Unlike the ELP tag, which is around 20–50 kDa, the hexahistidine tag is much smaller (around 0.8 kDa). Removal of the His tag is often unnecessary because it is uncharged at physiological pH, rarely alters or contributes to protein immunogenicity, and rarely interferes with protein structure/function or secretion.

The ELP-based metal-affinity precipitation method presented here is very specific, easy to manipulate, and fast, with only a few short centrifugation steps followed by resolubilization of purified proteins. The separation of purified protein is very convenient, requiring only mild changes in either the ionic strength or temperature. The capability of modulating purification conditions by simple temperature triggers and their low cost of preparation make the ELP-based metal-affinity precipitation a useful method not only for protein purification but also for diverse applications in bioseparation such as DNA purification and environmental remediation (Kostal et al., 2001).

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