Journal of Chromatography B, 917–918 (2013) 30–35

Contents lists available at SciVerse ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

Purification of recombinant EGFP by fusion with L2 (252–273) from ribosomal protein L2 using magnetic particles

Junhua Li[a,b], Yiting Dong[a,b], Yang Zhang[a,b], Yanjun Yang[a,b,*]

[a] State Key Laboratory of Food Science and Technology, Jiangnan University, Wuxi 214122, China
[b] School of Food Science and Technology, Jiangnan University, Wuxi 214122, China

ARTICLE INFO

Article history:
Received 1 October 2012
Accepted 2 January 2013
Available online 9 January 2013

Keywords:
Ribosomal protein L2
Small ubiquitin-related modifier
Diatomite
magnetic particles
Cation exchangers
Recombinant protein purification

ABSTRACT

A basic polypeptide L2 (252–273) derived from *Escherichia coli* ribosomal protein L2 was used as a purification tag. In order to develop faster, less expensive methods for expression and purification of proteins, the L2 (252–273)-small ubiquitin like modifier (SUMO) fusion expression system was constructed. We comparatively analyzed the adsorption properties of the deleted protein of L2 (L2 (252–273)) on diatomite and superparamagnetic carboxymethyl chitosan nanoparticles. The time required to reach adsorption equilibrium of L2 (252–273) fusion protein on diatomite was shorter than that of L2 (252–273) fusion protein on magnetic particles. The maximum adsorption capacity of L2 (252–273) fusion protein on magnetic particles was about 5 times larger than that of L2 (252–273) fusion protein on diatomite. SUMO was introduced as a specific protease cleavage site between the target protein and the purification tags. The enhanced green fluorescent protein (EGFP) as a model protein was fused with the L2 (252–273)-SUMO fusion protein and purified by a simple method which involves the electrostatic adsorption of L2 (252–273) fusion proteins on superparamagnetic carboxymethyl chitosan nanoparticles and the L2 (252–273)-SUMO fusion partner was removed based on the robust cleavage by the poly lysine tagged SUMO protease. The high purity of tag-free EGFP (>93%) was obtained. Our results preliminary proved that the system was an effective fusion expression system for the production of recombinant proteins in *E. coli*.

1. Introduction

Purification of recombinant proteins by traditional chromatography purification methods constitutes a significant cost of biomanufacturing due to the tedious purification steps. Recent development of affinity purification techniques provides a powerful mean for the high efficient purification of recombinant proteins from crude biological process liquors (e.g. fermentation broths, cell disruptates, plasma and plant extracts) [1]. Ribosomal protein L2, a constituent protein of the 50S large ribosomal subunit from *Escherichia coli*, can be used as Si-tag for immobilizing proteins on a silica surface [2,3]. It contains 273 amino acids and has been successfully applied into the purification of recombinant proteins under non-denaturing and denaturing conditions [4,5]. Recently, we found the C-terminal regions (203–273) of ribosomal protein L2 can be effectively adsorbed by diatomite due to the electrostatic interaction between L2 (203–273) (isoelectric point (pI), 12.28, computed by DNAstar software) and diatomite (pI, 2) [6,7].

If recombinant proteins are expressed as fusion proteins, it is usually necessary to remove the purification tag before subsequent use of the target proteins [8]. The small ubiquitin-like modifier (SUMO) based fusion tag technology has gained increasing use for the expression of recombinant proteins, since SUMO serves not only as a solubility enhancer but also as a protease recognition site [9–12]. Furthermore, SUMO can also be effectively cleaved from the fusion protein by SUMO protease.

The purification media is critical to an affinity purification method. The cost of the purification media can be the main factor for an affinity technique whether can be successfully applied into bioprocessing. Since magnetic particles possess high specific surface areas and can be easily separated with a magnet, it has received considerable attention in recent years [13,14]. In our laboratory, we had successfully purified lysozyme and poly lysine tagged SUMO protease using newly developed magnetic carboxymethyl chitosan nanoparticles [15,16].

In this study, in order to minimize the ribosomal protein L2 purification tag, we exploited whether the size of L2 (203–273) can be further reduced, comparatively studied the adsorption properties of the deletion mutants of L2 (203–273) on the cation exchangers including diatomite and magnetic carboxymethyl chitosan nanoparticles, and verified the feasibility of SUMO mediated tag removal method. A novel affinity purification method was

* Corresponding author at: State Key Laboratory of Food Science and Technology, Jiangnan University, Wuxi 214122, China. Tel.: +86 510 85329080; fax: +86 510 85329080.
E-mail address: jiamaolifang1@163.com (Y. Yang).

1570-0232/$ – see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.jchromb.2013.01.002

developed by combining the adsorption properties of the deletion mutants of L2 (203–273) on diatomite or magnetic carboxymethyl chitosan nanoparticles with SUMO mediated tag removal method. The enhanced green fluorescent protein (EGFP) as a model protein was fused with the purification tag. This affinity purification protocol involves three steps: adsorption of fusion protein on affinity matrix, centrifugation (for diatomite) and magnetic separation (for magnetic nanoparticles) and release of recombinant protein from the fusion protein by the lysine-tagged SUMO protease cleavage at the C-terminal of SUMO.

2. Materials and methods

2.1. Chemicals, strains, and plasmids

All chemicals were reagent grade, purchased from Sinopharm Chemical Reagent Co., Ltd (Shanghai, P.R. China), unless otherwise noted. Diatomite filter aid STD and cation exchange resin (Amberlite Cobalamion) were respectively purchased from Celite China (Beijing, P.R. China) and Rohm and Haas (Philadelphia, PA, USA). The magnetic carboxymethyl chitosan nanoparticles, which was designated as Fe_3O_4 (PEG + CM-CTS), was prepared in our laboratory according to the description [15]. Poly lysine tagged SUMO protease was prepared by cation exchangers according to the description [16]. *E. coli* BL21 (DE3) (Novagen, Madison, WI, USA) was used as the cloning and expression host cells. The plasmid pET28a-L2 (203–273)-SUMO-EGFP coding for the L2 (203–273)-SUMO-EGFP was preserved in our laboratory.

2.2. Plasmid construction

For the pET28a-L2 (252–273)-SUMO-EGFP and pET28a-L2 (237–273)-SUMO-EGFP construction, L2 (252–273) and L2 (237–273) DNA fragments were respectively amplified by PCR amplification by the primers of L2252for (5′-CGTGCCATGGCAAAA-ACCAAAGGTAAGAAGACCCG-3′) and L2237for (5′-CGTGCCATGG-CAAAAGGTCGTAACTTTGGTAAGCAC-3′) and L2rev (5′-CAAGGATC-CATTTGCTACGGCGACGTACGA-3′) using the plasmid pET28a-L2 (203–273)-SUMO-EGFP as a template, followed by double digestion by *Nco*I/*BamH*I, were ligated into the pET28a-L2 (203–273)-SUMO-EGFP to replace the corresponding fragment L2 (203–273).

2.3. Expression of recombinant proteins

The *E. coli* strain BL21 (DE3) harboring pET28a-L2 (237–273)-SUMO-EGFP or pET28a-L2 (252–273)-SUMO-EGFP was used for the production of L2 (237–273)-SUMO-EGFP or L2 (252–273)-SUMO-EGFP by auto-induction [17]. Briefly, cells were cultivated in 50 mL of auto induction medium supplemented with 50 μg/mL kanamycin sulfate in 250 mL flasks at 37 °C with vigorous shaking (200 rpm) for 3–4 h and at 20 °C for another 12 h. Harvested cells were suspended in 5 mL of 20 mM sodium phosphate (pH 6.0) and subjected to disruption using ultrasonication. After removing the cell debris via centrifugation, the supernatant was applied to either the adsorption studies of fusion protein on diatomite and magnetic carboxymethyl chitosan nanoparticles or affinity purification of recombinant protein.

2.4. Adsorption properties

0.8 mL of cell lysate supernatant containing L2 (252–273)-SUMO-EGFP (20 mM phosphate buffer, pH 6.0) was mixed with 0.2 g of diatomite (dry weight) or 40 mg of magnetic carboxymethyl chitosan nanoparticles (dry weight). The mixed suspension was shaken in a thermostated shaker (150 rpm) at room temperature for 75 min to reach equilibrium. Samples were withdrawn at suitable time intervals and subjected to determine the EGFP fluorescence of unbound target protein. The amounts of L2 (252–273)-SUMO-EGFP adsorbed onto the diatomite or magnetic particles were calculated using the following equation:

$$q = \frac{V(C_o - C_e)}{m} \quad (1)$$

where q is the amount (EGFP fluorescence arbitrary unit, AU) of L2 (252–273)-SUMO-EGFP adsorbed on the diatomite or magnetic particles (AU/g adsorbent), C_o and C_e are the EGFP fluorescence of L2 (252–273)-SUMO-EGFP in the initial solution and the supernatant phase after adsorption, respectively (AU/mL); V is the volume of the cell lysate supernatant (mL); m is the mass of the diatomite filter aid STD and magnetic nanoparticles (g).

2.5. Purification of recombinant EGFP

The cell lysate supernatant was incubated with magnetic particles incubated for 60 min at room temperature. The magnetic particles was collected, washed three times with 20 mM phosphate buffer (pH 8.0) containing 150 mM NaCl, and further suspended in 20 mM phosphate buffer (pH 8.0). The poly lysine tagged SUMO protease (the weight ratio of lysine-tagged SUMO protease to the fusion protein was approximately 1: 400) was added into the above suspension. After 3-hour incubation at room temperature, the supernatant containing the cleaved EGFP protein was collected and analyzed by SDS-PAGE. The protein concentration was calculated by the Bradford method [18] and the EGFP fluorescence was measured by fluorescence spectrophotometer.

2.6. Protein characterization

The purification process of the EGFP was analyzed by SDS-PAGE (12%) and Coomassie brilliant blue staining, followed by densitometric analysis using Quality One software (Bio-Rad Laboratories, USA). The EGFP fluorescence was measured using a fluorescence spectrophotometer (Hitachi Model F-4500, Tokyo, Japan). The fluorescence intensity in arbitrary units (AU) was determined with excitation and emission set at 488 nm and 507 nm, respectively.

3. Results

3.1. Selection of the truncated polypeptides as purification tag

The reason we choose the L2 (252–273) as a purification tag is that the region possesses high ratio of basic amino acid in the whole C-terminal region (203–273) of ribosomal protein L2. According to the analysis by the DNAstar software (Fig. 1), the region (252–273) with a high probability of exposure to the surface of the fusion

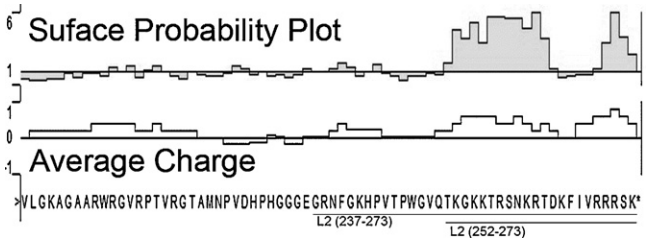

Fig. 1. Surface probability and average charge plots of C-terminal regions (203–273) of ribosomal protein L2. The resulting outputs of Protean prediction programs in DNAstar software, Emini algorithm for the prediction of L2 (203–273) surface probability.

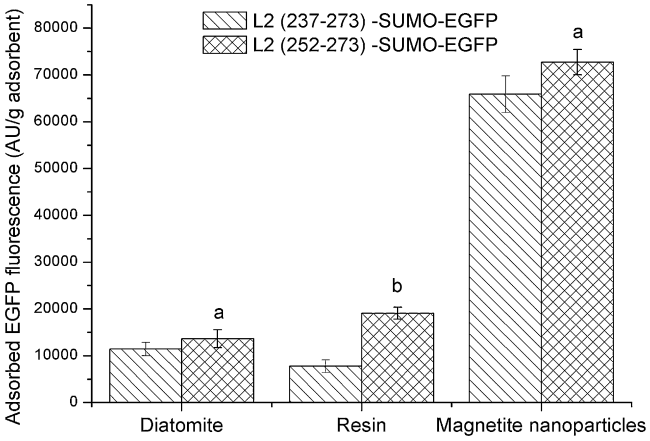

Fig. 2. Comparative analysis of the adsorption capacity of L2 (237–273) and L2 (252–273) fusion protein on diatomite filter aid STD, cation exchange resin (Amberlite Cobalamion) and magnetic particles. Each value represents the mean ± S.D. of three independent measurements. Statistical significance versus L2 (237–273) control: (a) $P < 0.05$, (b) $P < 0.01$.

protein is more easily to interact with adsorbents by electrostatic force. Tosaka et al. also reported that the residues 252–273 of L2 are one of the major binding sites to a silica surface [19]. In order to verify whether the extra N-terminal amino aicd residues of L2 (252–273) has an adverse effect on its probability of exposure to the surface and electrostatic interaction with adsorbents, we choose L2 (237–273) as another purification tag. In our following study, the results show L2 (237–273) and L2 (252–273) tagged proteins have excellent expression level. Fig. 2 shows L2 (252–273)-SUMO-EGFP has the higher adsorption capacity on three adsorbents than that of L2 (237–273)-SUMO-EGFP, this result indicates that extra amino acid residues at the termini of L2 (252–273) can have a significant effect on the electrostatic interaction with adsorbents and the final adsorption capacity.

3.2. Effect of pH on the adsorption of L2 (252–273)-SUMO-EGFP by diatomite and magnetic particles

The isoelectric point (pI) value of L2 (252–273) and L2 (252–273)-SUMO-EGFP is respectively 12.29 and 6.95 (computed by DNAstar software), while the pI of diatomite is 2 [6] and magnetic carboxymethyl chitosan nanoparticles is 5.1 [15]. It is reasonable to believe that the diatomite and magnetic carboxymethyl chitosan nanoparticles will adsorb the L2 (252–273)-SUMO-EGFP by electrostatic interaction at an appropriate pH. Using 150 mg of diatomite and 20 mg of magnetic particles and 0.5 mL of soluble cell lysate containing L2 (252–273)-SUMO-EGFP, the effect of pH on the adsorption of fusion protein on the diatomite and magnetic particles was shown in Fig. 3. The maximum protein adsorption was obtained at pH 6.0 for both adsorbents, with a clear increase with pH from high to low. These results indicate that the buffer pH has a crucial effect on the interaction between L2 (252–273)-SUMO-EGFP and absorbents.

3.3. Adsorption kinetics

To ascertain the time required to reach adsorption equilibrium of the L2 (252–273)-SUMO-EGFP, binding experiments were performed using 0.2 g of diatomite or 40 mg of magnetic particles and 0.8 mL of soluble cell lysate containing L2 (252–273)-SUMO-EGFP (20 mM phosphate buffer with pH 6.0) at room temperature. The adsorption kinetics were studied as shown in Fig. 4(A).

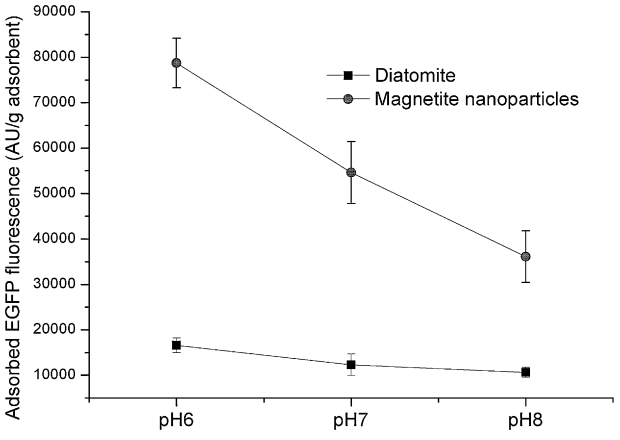

Fig. 3. Effect of pH on the adsorption capacity between L2 (252–273)-SUMO-EGFP and diatomite filter aid STD and magnetic particles.

Fig. 4(A) shows that 90% of the L2 (252–273)-SUMO-EGFP had been adsorbed on the diatomite during the initial 5 min incubation, while 90% of the L2 (252–273)-SUMO-EGFP adsorbed by magnetic particles took 45 min. The time necessary to achieve the adsorption equilibrium was around 30 min on diatomite and 60 min on magnetic carboxymethyl chitosan nanoparticles. The

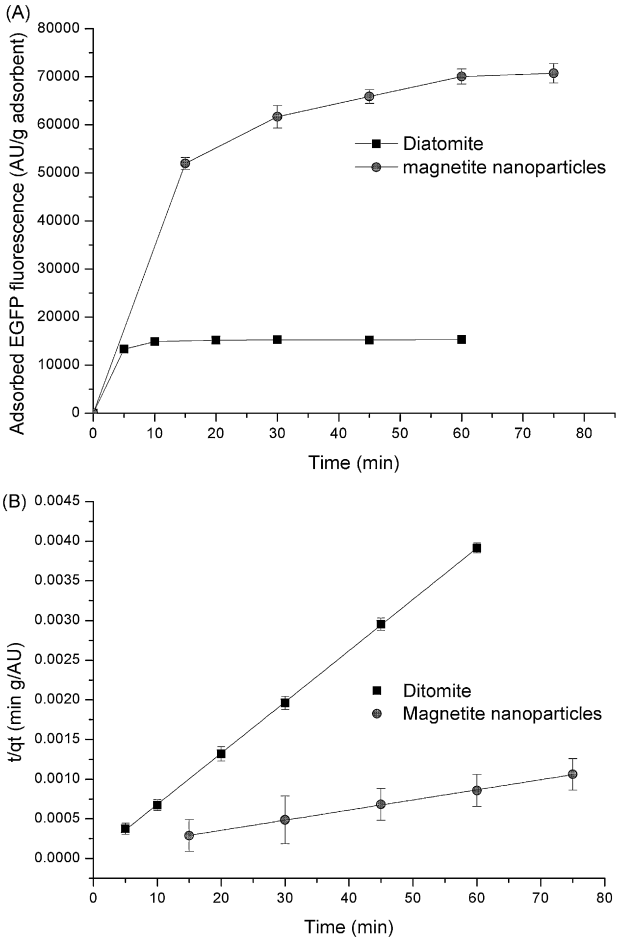

Fig. 4. Adsorption kinetic properties. (A) Kinetic curves of adsorption of L2 (252–273)-SUMO-EGFP by diatomite filter aid STD and magnetite nanoparticles. (B) Pseudo-second order kinetic plots of adsorption of L2 (252–273)-SUMO-EGFP by diatomite filter aid STD and magnetite nanoparticles (pH 6.0, temperature: 25 °C).

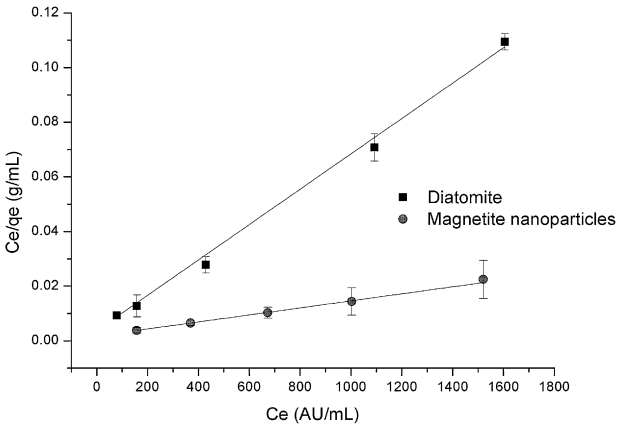

Fig. 5. Adsorption isotherms. Langmuir adsorption isotherms for the L2 (252–273)-SUMO-EGFP adsorption on diatomite filter aid STD and magnetite nanoparticles (pH 6.0, temperature: 25 °C).

controlling mechanism of adsorption process (mass transfer or chemical reaction) can be described by using kinetic models. The pseudo-second-order model usually describes the adsorption kinetics well and is expressed as shown in Eq. (2):

$$\frac{t}{q_t} = \frac{1}{k_2 q_e^2} + \left(\frac{1}{q_e}\right) t \quad (2)$$

where k_2 is the pseudo-second-order rate constant (min^{-1}) of adsorption; q_e is the maximum adsorption capacity for pseudo-second-order (AU/g); q_t is the amounts of L2 (252–273)-SUMO-EGFP adsorbed on adsorbents at time t (min) (AU/g).

Fig. 4(B) was obtained by plotting t/q_t versus t according to pseudo-second-order model. The adsorption kinetics were expressed as follows: $y = 0.00006465x + 0.00003372$, $q_e = 15467.90$ AU/g diatomite, $k_2 = 0.000124$, $R^2 = 0.999$; $y = 0.00001276x + 0.0001009$, $q_e = 78398.17$ AU/g magnetic particles, $k_2 = 0.00000161$, $R^2 = 0.999$. From the above results we can see that the theoretical q_e value was close to the experimental q_e value and the correlation coefficient for the linear plots of t/q_t against t was more than 0.99 for both two adsorbents. The pseudo-second-order equation fitted well with the experimental data and can be used to describe the adsorption kinetics of L2 (252–273)-SUMO-EGFP on diatomite and magnetic particles by electrostatic interaction.

3.4. Adsorption isotherms

The adsorption model was studied by using 0.2 g of diatomite or 40 mg of magnetic particles and different initial volume of the cell lysate supernatant containing L2 (252–273)-SUMO-EGFP. The adsorption behaviors of L2 (252–273)–SUMO-EGFP on diatomite and magnetic particles were described using the Langmuir model which equation can be expressed as follows:

$$\frac{C_e}{q_e} = \frac{C_e}{q_m} + \frac{1}{qm^k} \quad (3)$$

where C_e (AU/mL) and q_e (AU/g absorbent) are the fluorescence intensity of L2 (252–273)-SUMO-EGFP in the supernatant solution and fluorescence intensity of the absorbed target protein on the absorbent at equilibrium, respectively; q_m is the maximum adsorption capacity and k is the adsorption constant (mL/AU).

The experimental data were analyzed using the Langmuir model; the related results were shown in Fig. 5. In this work the Langmuir equations for two adsorbents have the expression as follows: $C_e/q_e = 0.00006411C_e + 0.00173$, $q_m = 15598.19$ AU/g diatomite, $R^2 = 0.990$; $C_e/q_e = 0.00001357Ce + 0.00141$, $q_m = 73691.96$ AU/g magnetic particles, $R^2 = 0.995$. High R^2 values indicate that the model describe well the adsorption behavior. The maximum equilibrium adsorption capacity for both adsorbents was close to maximum adsorption capacity which calculated from the previous pseudo-second-order kinetic models.

3.5. Purification of recombinant protein EGFP

The recombinant EGFP can be simple and efficiently purified from soluble cell lysate by using magnetic nanoparticles as an absorbent and poly lysine tagged SUMO protease as tag removal method. The purification process was determined by SDS-PAGE (Fig. 6(A)). Fig. 6(A) shows our developed affinity purification method can be used to prepare high purity protein when L2 (237–273) or L2 (252–273) acts as an affinity tag. Fig. 6(B) and Fig. 6(C) shows our developed purification protocol has high recovery and yield of target protein using EGFP as a model protein. The purity of the released EGFP was higher than 93%. The average yield of the recombinant EGFP was respectively 5.98 ± 0.14 and 6.96 ± 0.68 mg per 50 mL culture for L2 (237–273)-SUMO-EGFP and L2 (252–273)-SUMO-EGFP. Since the L2 (252–273) tagged protein had a larger adsorption capacity on adsorbents than that of the L2 (237–273) tagged protein on adsorbents as shown in Fig. 2, L2 (252–273) led to the higher recovery of target protein than that of L2 (237–273) when using the equal quantity adsorbents.

4. Discussion

Affinity tags are now widely used to purify recombinant proteins. However, many fusion systems share the common drawback of the high cost of purification media. A fusion tag which can be purified based on electrostatic interaction may provide a cost effective strategy over other affinity fusion tags due to the cheap ion-exchangers. The traditional poly basic amino acid tags can be purified by a cation exchange resin. These tags can effectively purify the most of recombinant proteins. Poly basic amino acids tag can usually be placed at C-terminal or the linker region between two proteins [20,21]. The L2 (252–273) tag has the 6 lysine and 5 arginine. Since the L2 (252–273) basic polypeptide has several basic amino acids that resembles the poly lysine/arginine tag, L2 (252–273) could be also used as a purification tag when cation exchange resin was chosen as affinity matrix. In our laboratory, the study about the 10 poly lysine tag as the N terminal fusion partner had been conducted, but unfortunately the expression level of fusion protein (K10-SUMO-EGFP) was significantly decreased (unpublished data). The relatively discrete mono basic amino acid of L2 (252–273) tag may help the fusion protein enhance the initial translation efficiency and obtain high expression level. In this study, we attempted to combine L2 (252–273) basic polypeptide tag with the SUMO tag removal method to construct an effective tag-free recombinant protein purification protocol. The L2 (252–273)-SUMO-EGFP have the lower adsorption capacity on the magnetic carboxymethyl chitosan nanoparticles than Ulp1-K10 [16]. This result may be ascribed to the low isoelectric point and large fusion protein of L2 (252–273)-SUMO-EGFP. The difference between L2 (252–273) and poly lysine/arginine tag still need to be further studied. Although L2 (237–273) has more basic amino acids, L2 (252–273) is favorable for the electrostatic interaction with adsorbents as a result of the higher intensity of positive charge and high probability of exposure at the surface. The adsorption mechanism between L2 (252–273) and diatomite or magnetic

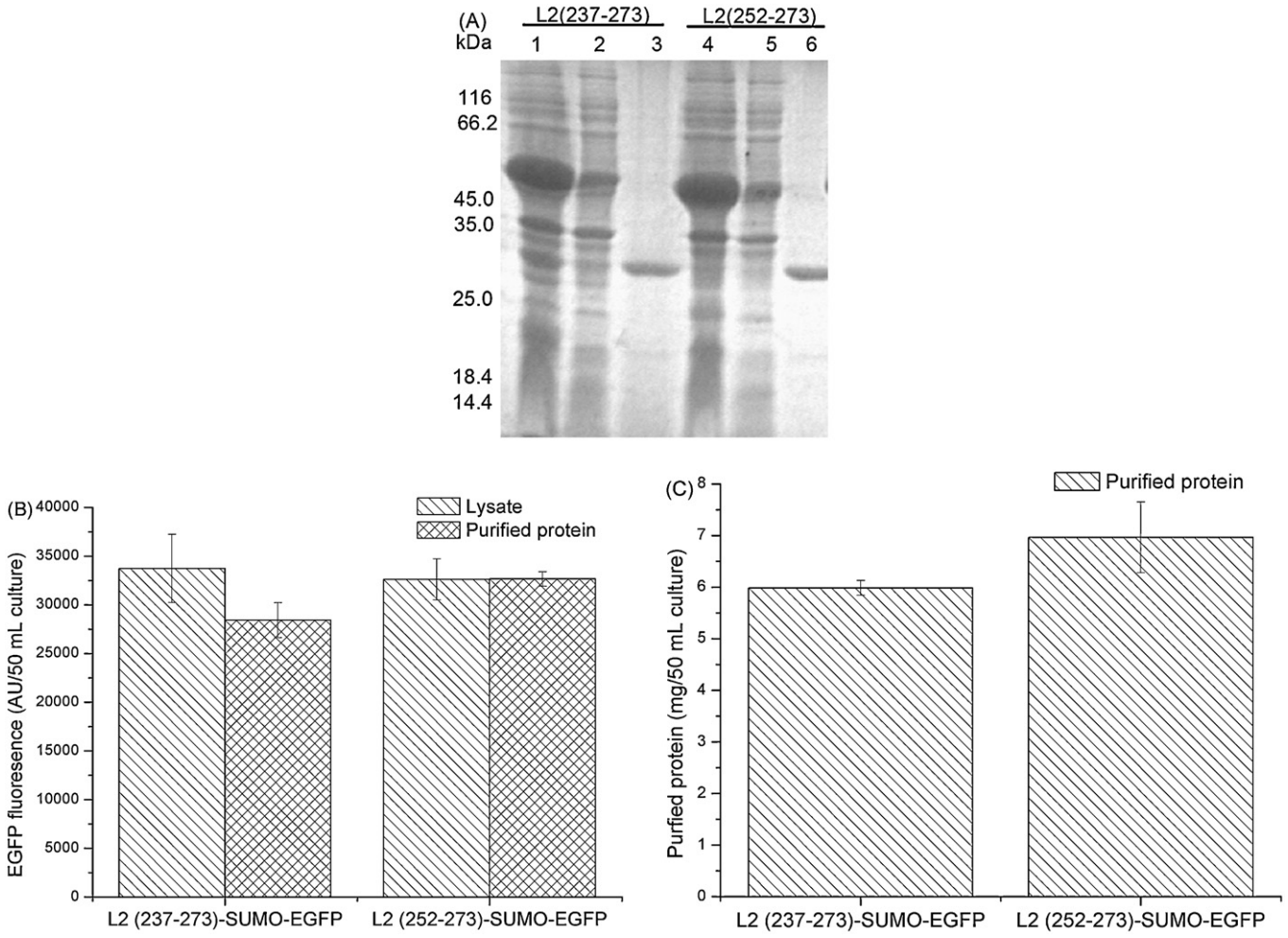

Fig. 6. Comparative analysis of the purification performance of L2 (237–273) and L2 (252–273). (A) SDS-PAGE analysis (12%) following the EGFP purification of L2 (237–273)-SUMO-EGFP and L2 (252–273)-SUMO-EGFP. Lanes 1 and 4, soluble cell lysate; lanes 2 and 5, the supernatant after adsorption by magnetic particles; lanes 3 and 6, the released EGFP from L2 (237–273)-SUMO-EGFP and L2 (252–273)-SUMO-EGFP. (B) Quantitative analysis of protein purification recovery by magnetic particles in triplicate. (C) EGFP yields obtained after purification of each fusion protein in triplicate.

carboxymethyl chitosan nanoparticles is mainly through electrostatic interactions. But the L2 (252–273)-SUMO-EGFP adsorbed on diatomite cannot be eluted with phosphate buffer (pH 8.0) containing 2 M NaCl, only more than 0.1 M NaOH can completely elute the target protein and other strongly binding contaminants from diatomite. This phenomenon can be explained by the binding mechanism of the Si tag as reported by Ikeda and co-researchers [22]. The cumulative interactions between the positively charged side chains and ionized surface silanol groups and between the apolar side chains and hydrophobic surface siloxane sites may result in the strong binding between L2 (252–273)-SUMO-EGFP and diatomite. It is feasible to take L2 (252–273) as a protein fusion tag for immobilization of enzyme since L2 (252–273) has strong adsorption on diatomite while magnetic carboxymethyl chitosan nanoparticles was more suitable as an affinity matrix for the purification of L2 (252–273) tagged proteins due to the higher adsorption load and simple separation with a magnet. This truncated L2 mediated protein purification method has gained high purity and yield protein from soluble cell lysate using EGFP as a model protein. But further studies on this truncated L2 mediated protein purification method still need to conduct about whether the other proteins can be effectively purified by the L2 (252–273) mediated protein purification method.

Acknowledgments

This work was supported by grants from the Chinese National High Technology Research and Development Program 863 (2013AA102207).

References

[1] J. Arnau, C. Lauritzen, G.E. Petersen, J. Pedersen, Protein Expres. Purif. 48 (2006) 1.
[2] T. Ikeda, Y. Hata, K.-i. Ninomiya, Y. Ikura, K. Takeguchi, S. Aoyagi, R. Hirota, A. Kuroda, Anal. Biochem. 385 (2009) 132.
[3] K. Taniguchi, K. Nomura, Y. Hata, T. Nishimura, Y. Ksami, A. Kuroda, Biotechnol. Bioeng. 96 (2007) 1023.
[4] T. Ikeda, K. Motomura, Y. Agou, T. Ishida, R. Hirota, A. Kuroda, Protein Expres. Purif. 77 (2011) 173.
[5] T. Ikeda, K.-i. Ninomiya, R. Hirota, A. Kuroda, Protein Expres. Purif. 71 (2010) 91.
[6] B. Gao, P. Jiang, F. An, S. Zhao, Z. Ge, Appl. Surf. Sci. 250 (2005) 273.
[7] J. Li, Y. Zhang, Y. Yang, Appl. Microbiol. Biol. (2012), http://dx.doi.org/10.1007/s00253-012-4367-7.
[8] A.P.U. Araújo, G. Oliva, F. Henrique-Silva, R.C. Garratt, O. Cáceres, L.M. Beltramini, Biochem. Biophys. Res. Commun. 272 (2000) 480.
[9] T.R. Butt, S.C. Edavettal, J.P. Hall, M.R. Mattern, Protein Expres. Purif. 43 (2005) 1.
[10] J.G. Marblestone, Protein Sci. 15 (2006) 182.
[11] C.-D. Lee, H.-C. Sun, S.-M. Hu, C.-F. Chiu, A. Homhuan, S.-M. Liang, C.-H. Leng, T.-F. Wang, Protein Sci. 17 (2008) 1241.

[12] M.P. Malakhov, M.R. Mattern, O.A. Malakhova, M. Drinker, S.D. Weeks, T.R. Butt, J. Struct. Funct. Genomics 75 (2004).
[13] S. Berensmeier, Appl. Microbiol. Biol. 73 (2006) 495.
[14] M. Franzreb, M. Siemann-Herzberg, T.J. Hobley, O.R.T. Thomas, Appl. Microbiol. Biol. 70 (2006) 505.
[15] J. Sun, Y. Su, S. Rao, Y. Yang, J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. 879 (2011) 2194.
[16] J. Li, Y. Zhang, F. Shen, Y. Yang, J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. 907 (2012) 159.
[17] F.W. Studier, Protein Expres. Purif. 41 (2005) 207.
[18] M.M. Bradford, Anal. Biochem. 72 (1976) 248.
[19] R. Tosaka, H. Yamamoto, I. Ohdomari, T. Watanabe, Langmuir 26 (2010) 9950–9955.
[20] D.-H. Kweon, S.-G. Kim, N.S. Han, J.H. Lee, K.M. Chung, J.-H. Seo, Enzyme Microb. Technol. 36 (2005) 571.
[21] G. Levin, F. Mendive, H.M. Targovnik, O. Cascone, M.V. Miranda, J. Biotechnol. 118 (2005) 363.
[22] T. Ikeda, A. Kuroda, Colloids Surf. B 86 (2011) 359.