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Full Length Research Paper

High-yield production of Streptavidin with native C-terminal in *Escherichia coli*

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To increase the production yield of functional recombinant streptavidin in *Escherichia coli*, the effects of host strains and culture conditions on expression of streptavidin with native C terminal (CNSA, amino acid residues 13 to 159) were investigated. Results show that the CNSA, encoded by the *CNSA* gene, was produced by *E. coli* BL21(DE3)pLysS strain in the inclusion body with a high yield up to 46.3% of the total cell protein (about 230 mg/g dry cell weight) after culture condition optimization. The dialysis method was adapted to refold CNSA and the refolding conditions were optimized. More than 90% of inclusion body protein was refolded to mature CNSA under optimized refolding conditions. The purity of the recombinant CNSA achieved 95.0% without using any affinity separation method. Enzyme linked immunosorbent assay (ELISA) analysis indicated that the biotin binding capability of our recombinant CNSA was similar to that of commercial products.

Key words: Streptavidin, *Escherichia coli*, protein refolding, recombinant protein.

INTRODUCTION

Streptavidin (SA) is an approximately 60-kDa protein naturally secreted from bacterium *Streptomyces avidinii*, which can bind up to four molecules of D-biotin (Hunt, 2005). The interaction between streptavidin and biotin is noncovalent but extremely tight, with a dissociation constant of 10^{-15} M, which is about 3-6 orders of magnitude higher than that of a typical antigen–antibody interaction (Scholle et al., 2004). Because of its highly specific and almost irreversible binding to biotin, SA has been widely used as a capturing agent to bind biotinylated molecules for applications in immunology, molecular biology and histochemistry, etc (Peters and Rehm, 2008). The three-dimensional structure of mature SA shows, it is a homotetramer with each subunit folded into an eight-stranded antiparallel β-barrel (Laitinen et al., 2006). Each monomer of the natural full-length SA (NFSA) is composed of 159 amino acids with a molecular weight of 16.5 kDa. However, the natural SA is usually in the form of a mixture of heterogeneous molecules because of cleavages at both N- and C-termini by host proteases and presents a smaller molecular weight than NFSA (Argarana and Kuntz, 1986; Miksch et al., 2008).

In recent years, it has been found that the binding ability to biotins and solubility of the shorter recombinant SA are better than those of NFSA. The main reason for the lower binding capacity of NFSA is that the hydrophobic amino acid patches, at positions 10-12 from the N-terminal and positions 19-21 from the C-terminal, tend to form the steric hindrance due to the mutual cohesion and thus limit the accessibility of the biotin binding motifs of SA to those biotinylated molecules. This may also lead to lower solubility of NFSA (Sano and Cantor, 1995). To obtain highly functional recombinant SA for biomedical applications, SA monomers of different length, including NFSA (Miksch et al., 2008), natural core

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SA (NCSA, amino acid residues 13-139) (Bayer et al., 1989), and core SA [CSA, including Stv-13 (amino acid residues 16-133) and Stv-25 (amino acid residues 14-138)], etc. (Thompson and Weber, 1993; Wu and Wong, 2002), have been expressed in various microbial expression hosts as a soluble protein or as an insoluble form in the inclusion body. However, the yield of SA never made great breakthrough and was generally 70-120 mg per liter of bacterial culture (about 20 to 35% of the total bacterial protein) in soluble form (Takeshi et al., 1995; Thompson and Weber, 1993; Sano and Cantor, 1990), while the yields of SA expressed in insoluble form were about 35% of total cell protein (Wu and Wong, 2002; Sano and Cantor, 1995).

In this study, one SA gene fragment, native C-terminal of SA (CNSA, amino acid residues 13-159), was amplified from *S. avidinii* genome. The polymerase chain reaction (PCR) product was sub cloned into the *Escherichia coli* expression vector plasmid pET11a-T7. The resulting plasmid was introduced into two *E. coli* strains to produce recombinant SA. By the optimization of culture conditions, the yield of recombinant CNSA of *E. coli* BL21(DE3)pLysS strain was higher, up to 46.3% of the total cell protein (about 230 mg/g dry cell weight). Under optimized conditions in the dialysis refolding process, more than 90% of inclusion body protein was refolded into mature streptavidin.

MATERIALS AND METHODS

Bacterial strains, media, and growth conditions

S. avidinii was obtained from China center of industrial culture collection (CCICC) and used for amplification of the SA gene. *E. coli* BL21 (DE3) and BL21 (DE3) *pLysS* were used for expression studies. *E. coli* was cultured at 37°C in ATM medium containing 20 g/L tryptone, 0.65 g/L Na_2HPO_4, 0.5 g/L KH_2PO_4, 4 g/L NaCl and 7.5 g/L yeast extract. The final pH of the medium was adjusted to 7.2 before autoclaving. The plates contained 15 g/L agar. When needed, ampicillin (50 µg/ml) and/or chloromycetin (10 µg/ml) were supplemented to the medium.

Amplification and expression of the CNSA fragment

S. avidinii genomic DNA was extracted as described by (Saito and Miura, 1963). The fragment was amplified from the genomic template by PCR using the upstream primer CNSA F (5'-TTCGGATCCGCCGAGGCCGGCATCACCG-3', the introduction of a *Bam*HI restriction site was underlined) and the downstream primer CNSA R (5′-ATTCGGCCGCTATTACTAGTGCTGAA-CGGCGTC-3′, the introduction of a *Not*I restriction site was underlined). The PCR product was purified using a QIAquick PCR purification kit (Qiagen, Germany), and cloned into the pET11a vector according to standard procedures. The resulting pET11a-*cnsa* vector was introduced into *E. coli* BL21 (DE3) and *E. coli* BL21 (DE3) *pLysS* by electroporation. Briefly 1.5 ml overnight *E. coli* culture was inoculated into 100 ml of ATM medium in a 250 ml flask and cultivated at 37°C with vigorous shaking. When $OD_{600\ nm}$ of the culture reached 1.8-2.0, isopropyl β-D-thiogalactopyranoside (IPTG) was added to induce the protein expression for up to 13 h. Sodium dodecyl sulfite polyacrylamide gel electrophoresis (SDS-PAGE) and a gel imaging analyzer (Bio-Rad, USA) were employed to evaluate CNSA expression. Denaturing SDS-PAGE was performed with a 12% acrylamide resolving gel with a 5% acrylamide stacking portion after heating the samples at 95°C for 5 min in the presence of 1% SDS. Non-denaturing SDS-PAGE was performed in the same discontinuous system except the heat treatment of samples (Bayer et al., 1996; Mark et al., 2004). To determine the SA concentration, a calibration curve of streptavidin standard (Promega, USA) was generated using Coomassie brilliant blue G250 staining (Bradford, 1976) and the CNSA samples of unknown concentration stained in the same way were compared to the linear region of the standard curve.

Purification and refolding of CNSA

The cells were harvested by centrifugation at 3500 g for 10 min and washed twice with 100 ml of resuspension solution (20 mM Tris-HCl pH 7.4). The cell pellets were resuspended in 40 ml of resuspension solution and frozen at -70°C for 0.5 h. After fully thawed at 40°C, the cells were sonicated for 40 cycles of 3 s at full amplitude with 2 s interval. The inclusion body was collected by centrifugation at 15,000 g for 10 min and washed four times in buffers (pH 7.2) containing 1% Triton X-100, 0.3 mM ethylene-diaminetetraacetic acid (EDTA) and 0.5 mM β-mercaptoethanol and four times in the same buffer without Triton X-100 (Sensen et al., 2003). The inclusion body was dissolved with 8.0 M urea and the denatured protein was refolded and purified by dialysis (Fang and Huang, 2001). The refolding efficiency (RE) was calculated as the following equation:

$$RE = [Cs/ (Cs+ C_P)] \times 100\%$$

Where, C_S is the amount of mature CNSA in the supernatant and C_P is the amount of CNSA in the precipitation.

ELISA assay for determination of the biotin binding capability of CNSA

One hundred microliter (100 µL) of SA standard (5.0 µg/ml) or the mature CNSA (5.0 µg/ml) and their serial dilutions (2, 4, 8, 16 and 64-folds) in 0.01 M phosphate buffered saline (PBS) were coated on micro titer plates (Dingguo Biotechnology, Shanghai, China) by incubation at 37°C for 1 h. After washing three times with PBST (PBS containing 0.5% Tween 20), the wells were blocked with 350 µL of 1% BSA in PBS at 37°C for 1 h and then washed three times with PBST. 0.01 µg of biotinylated HRP in 100 µL PBS (Zodolabs biotechnology, Wuxi, China) was added into each well and incubated at 37°C for 1 h. After final washing, 100 µL of 3, 3, 5, 5-tetramethyl benzidine (TMB) substrate was added and the plate was kept in the dark at 37°C for 15 min. The enzymatic reactions were terminated by the addition of 50 µL of 2 M sulfuric acid, and the absorbance was read at 450 nm using a Multiskan MK3 micro plate reader (Thermo fisher scientific, USA).

RESULTS

Expression of the CNSA fragment in different *E. coli* strains

The *CNSA* fragment was amplified and cloned into pET11a-T7 expression vector. To compare the effects of different host strains on protein expression, two well-known commercially available *E. coli* stains BL21 (DE3)

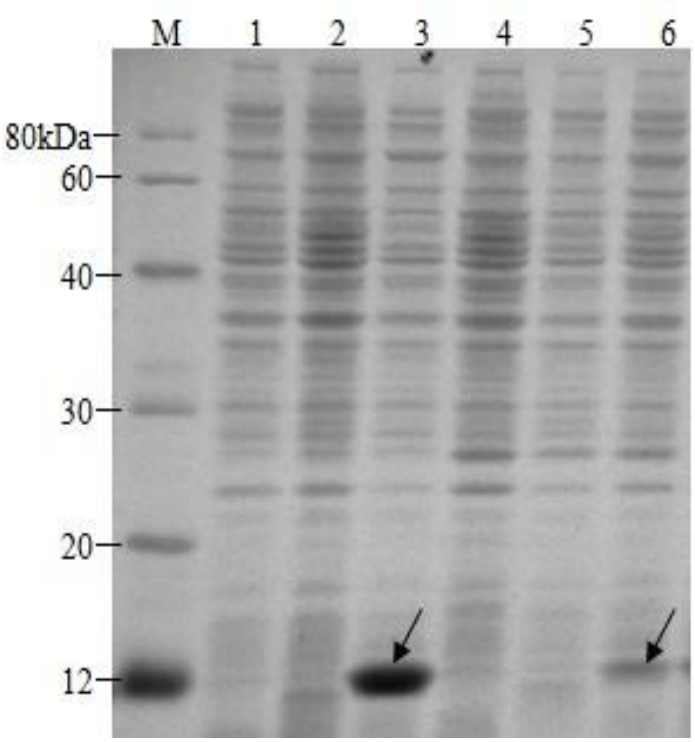

Figure 1. Expression of *CNSA* fragment in different *E. coli* strains. Total proteins from BL21(DE3)*pLysS* cells (lane 1), BL21(DE3)*pLysS* cells transformed with an empty pET11a vector (lane 2), recombinant strain of BL21(DE3)*pLysS* with pET11a-*cnsa* vector (lane 3), BL21(DE3) cells (lane 4), BL21(DE3)cells transformed with an empty pET11a vector (lane 5), and recombinant strain of BL21(DE3)*pLysS* with pET11a-*cnsa* vector (lane 6) were extracted and subjected to SDS-PAGE analysis. Arrows indicate the monomer of CNSA. Lane M, protein molecular weight markers.

and BL21 (DE3) *pLysS* were used for plasmid transformation. As shown in lanes 3 and 6 of Figure 1, the amount of CNSA produced in the BL21(DE3)*pLysS* (pET11a-CNSA) strain was significantly higher than that in the BL21(DE3) (pET11a-CNSA) strain. Sodium dodecyl sulphate-polyacrylamide gel electrophresis (SDS-PAGE) analysis showed that CNSA mostly existed in the inclusion body.

Optimization of culture condition for BL21 (DE3) pLysS (pET11a-cnsa)

The protein expression in BL21 (DE3) *pLysS* (pET11a-CNSA) was induced at different temperature (24, 28, 32 and 37°C) to determine the influence of induction temperatures on CNSA expression. The results shown in Figure 2, indicated that the CNSA expression in the inclusion body increased with the rise of the induction temperature (indicated by an arrow in lane 3 of Figure 2), while there was a small amount of protein expressed in a soluble form at the lower temperature (lane 6 of Figure 2). Analysis of the CNSA expression in BL21 (DE3) *pLysS* induced by different concentration of IPTG (from 0.1 to 1.0 mM) revealed that the effect of IPTG concentration was minimum on the CNSA expression and 0.4 mM of IPTG was chosen as the induction concentration (data not shown). However, the IPTG induction time significantly influenced the production of CNSA during our experiment period (from 1 to 13 h). The CNSA expression increased progressively with the induction time and reached its plateau at 11 h. The maximal expression of CNSA exhibited nearly nine-fold increase than that at 2 h (Figure 3B).

The effect of glucose concentration in the medium on CNSA expression was also investigated. Different concentrations of glucose (from 0.2 to 1.0% (w/v)) were tested. As shown in Figure 4, the expression of CNSA was highest in the ATM medium containing 0.4% of glucose and the yield reached about 230 mg/g dry cell weight (46.3% of the total cell protein). However, when the concentration of glucose was 1.0%, the expression level of CNSA dropped dramatically, about six-fold less than that with optimal concentration of glucose (Figure 4B).

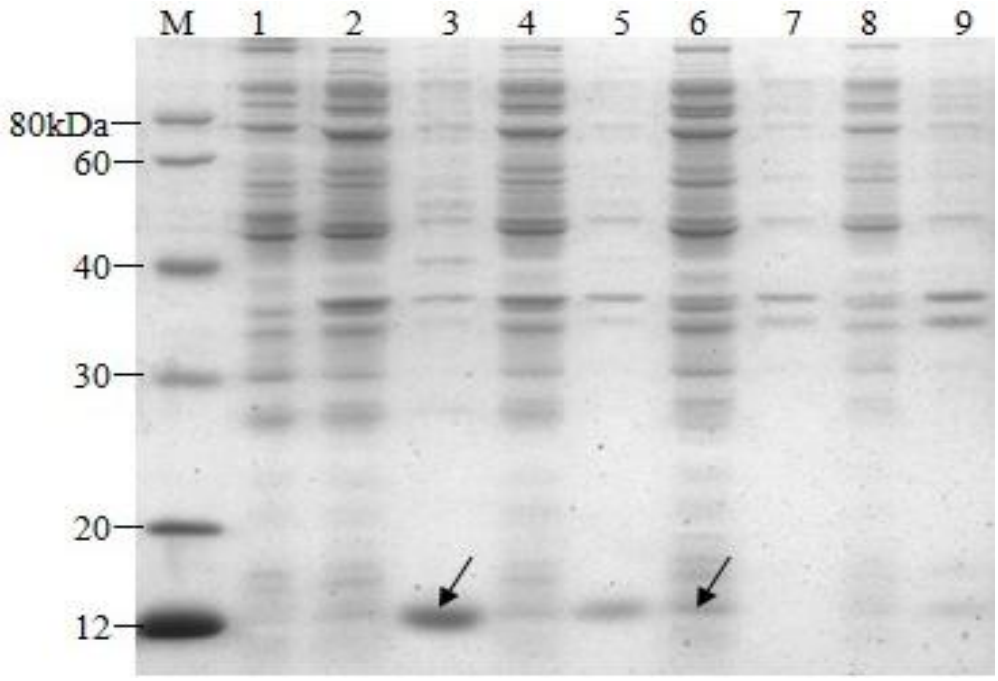

Figure 2. CNSA expression at different induction temperature. Total protein of BL21(DE3) *pLysS* cells transformed with an empty pET11a vector (lane 1); soluble protein from cells expressing CNSA at 37, 32, 28 and 24°C, respectively (lanes 2, 4, 6 and 8), and insoluble fraction from cells expressing CNSA at 37, 32, 28 and 24°C, respectively (lanes 3, 5, 7 and 9) were extracted and subjected to SDS-PAGE analysis. Arrows indicate the monomer of CNSA. Lane M, protein molecular weight markers.

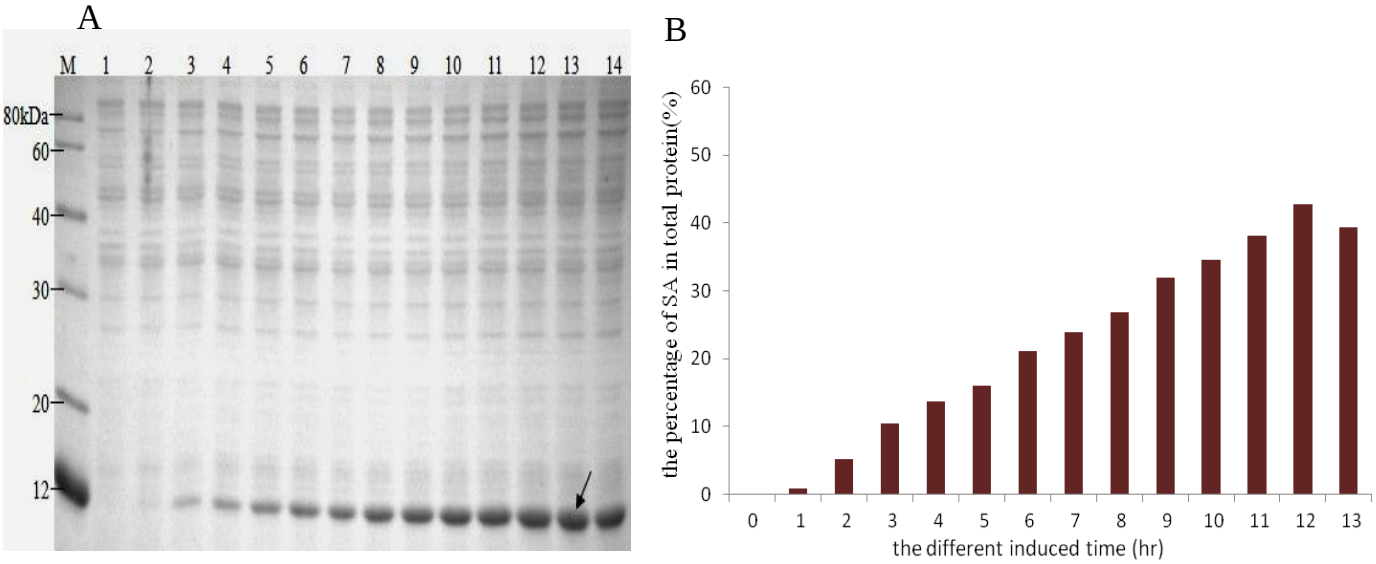

Figure 3. CNSA expression at the IPTG-induced time. Total proteins of recombinant strain without induced by IPTG (lane 1), and induced from 1 h to 13 h in steps of 1 h (lanes 2 to 14) were extracted and subjected to SDS-PAGE analysis (shown in Figure 3A, arrow indicates the monomer of CNSA). The expression level of CNSA at different induced time was shown in Figure 3B.

Optimization of CNSA refolding

Refolding is a critical step to obtain fully functional recombinant protein, especially those insoluble proteins accumulated in the inclusion body. In our study, the refolding temperature, refolding buffer composition and

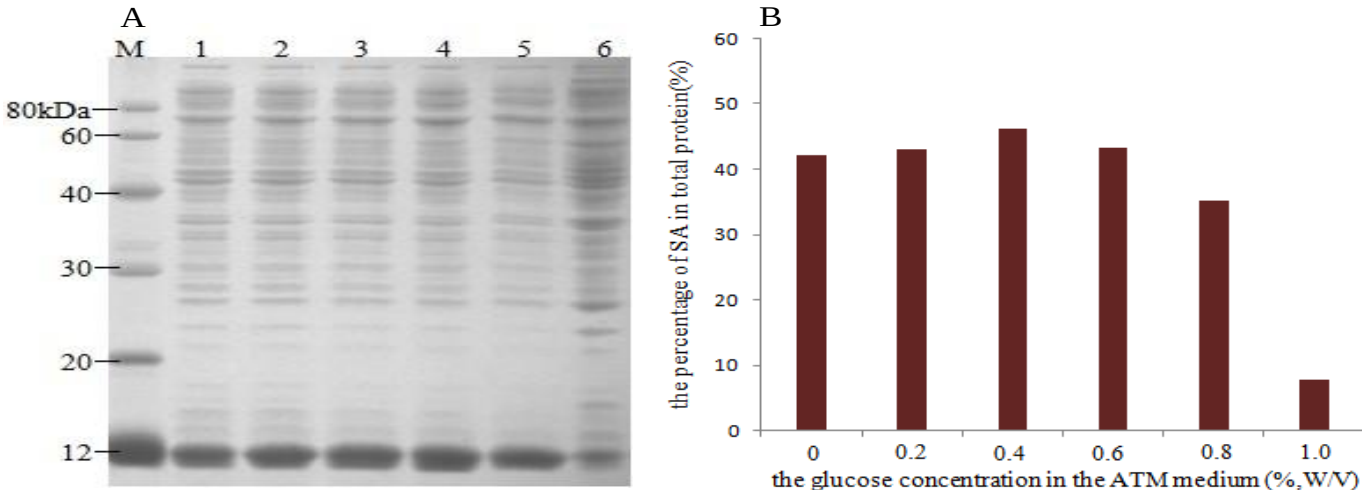

Figure 4. The effect of glucose concentration in the ATM medium on CNSA expression. Total proteins of recombinant strain cultured without glucose (lane 1), and with different concentration of glucose from 0.2% to 1.0% in steps of o.2% were extracted and analyzed with SDS-PAGE (shown in Figure 4A). The expression level of CNSA at different glucose concentration was shown in Figure 4B.

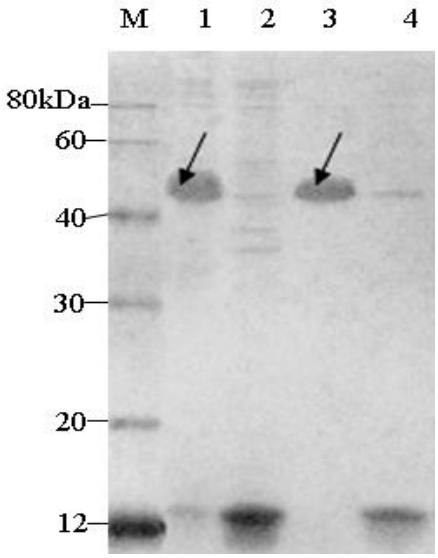

Figure 5. SDS-PAGE analysis of SA refolded in different buffer. Inclusion body protein dialysis with gradient dilution of urea buffer or PBS buffer was subjected to denaturing (lane 1 or 3, respectively) and non-denaturing (lane 2 or 4, respectively) SDS-PAGE analysis. Lane M, molecular weight markers. Arrow indicates the mature CNSA.

the initial protein concentration were optimized. The results of temperature effect on refolding of inclusion body indicated that there was no significant difference between refolding at room temperature and at 4°C (data not shown). In consideration of low temperature being helpful in stabilizing the protein, 4°C was selected as the refolding temperature. The effects of refolding buffer constituents on CNSA refolding were studied by comparing the series dialysis against urea solutions of decreasing concentrations with the direct dialysis against 0.01 M PBS (pH 7.2). CNSA did not show any precipitation when refolded under the former conditions and most of the target protein in the supernatant was in the form of tetramer (lane 1 of Figure 5). However, this

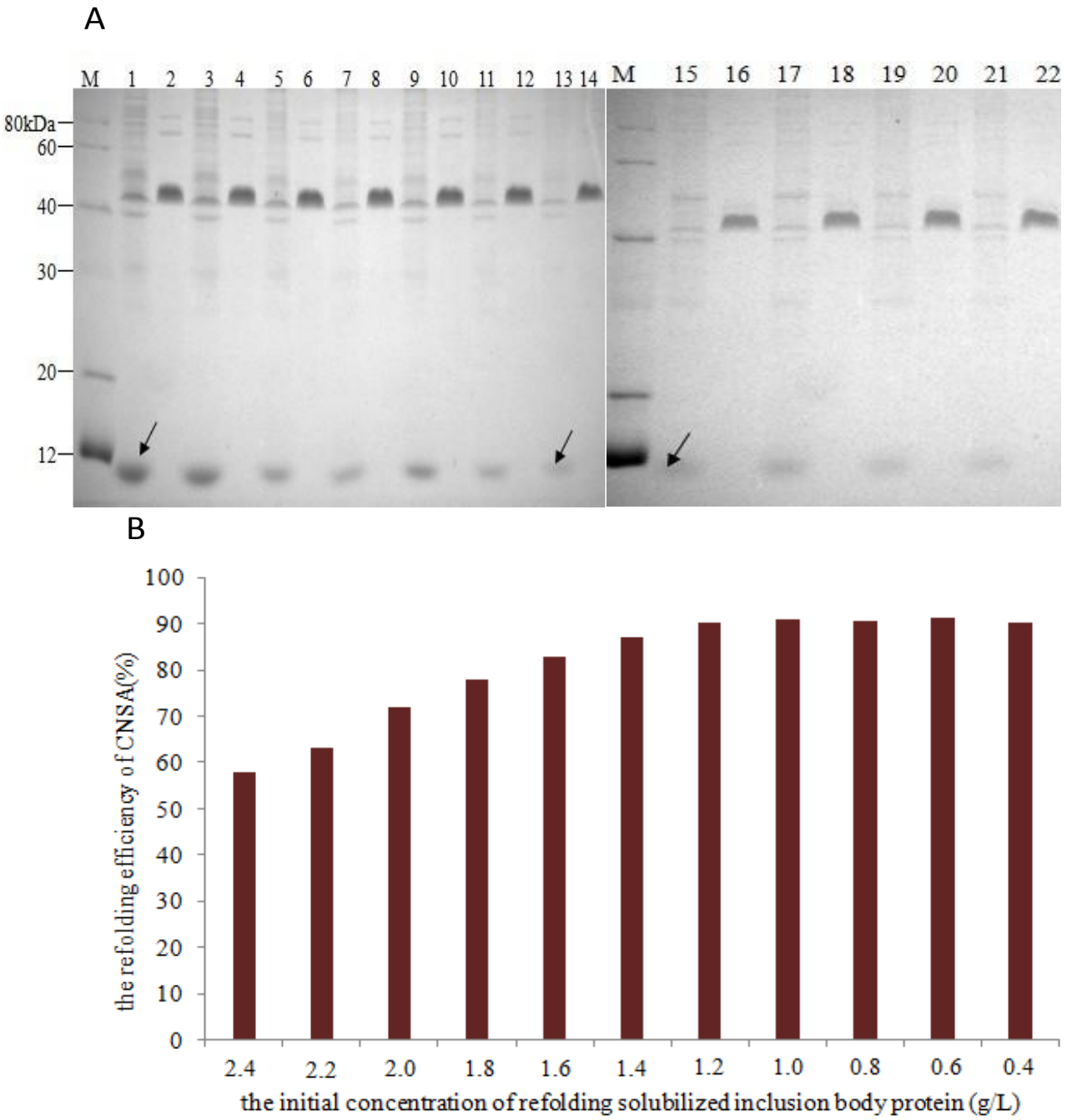

Figure 6. The refolding efficiency of SA at different initial protein concentration. The inclusion body protein was dialyzed with PBS buffer at different initial protein concentration and then centrifugated at 15 000 g for 10 min. The supernatant and precipitation were subjected to denaturing (lanes 1, 3, 5, 7, 9, 11, 13, 15, 17, 19 and 21, the initial refolding protein concentration decreasing from the range of 0.4 g/L to 2.4 g/L in steps of 0.2 g/L) and non-denaturing (lanes 2 , 4, 6, 8, 10, 12, 14, 16, 18, 20 and 22, the initial refolding protein concentration decreasing from the range of 0.4 g/L to 2.4 g/L in steps of 0.2 g/L)) SDS-PAGE analysis, respectively (Figure 6A).The refolding efficiency of SA at different initial protein concentration was shown in Figure 6B.

method had several disadvantages which include large urea consumption, time consuming and especially high impurities (lane 2 of Figure 5).

It was reported that direct dialysis against PBS overcame these disadvantages when relatively low initial concentration of protein was used (Fang and Huang, 2001). The effect of initial protein concentration was studied in the refolding setup with PBS only. As shown in Figure 6, the refolding efficiency increased with the reduction of the protein concentration from 2.4 to 1.2 g/L,

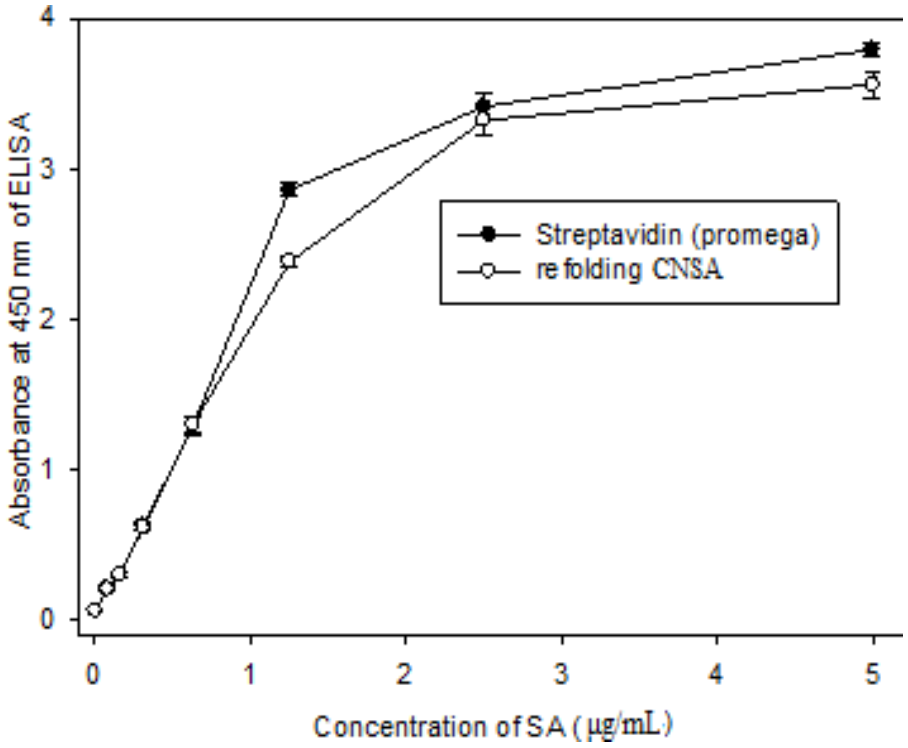

Figure 7. Determination of activity of recombinant CNSA binding biotin by direct ELISA.

whereas the efficiency remained flat from 1.2 to 0.4 g/L. The refolding efficiency was more than 90% at the optimal protein concentration and the purity achieved 95.0%. Therefore, 1.2 g/L was optimal as the initial protein concentration in the refolding dialysis. Taken together, the yield of refolded CNSA from 1L ATM medium by optimal culture conditions and optimal refolding conditions can reach about 370 mg.

ELISA assay for the activity of refolded CNSA

The binding activity of mature CNSA was determined by direct ELISA. The result indicates that the ability of the mature protein to bind biotinylated molecules had only a subtle difference from commercially available recombinant SA (Figure 7).

DISCUSSION

Over expression of recombinant proteins toxic to the host is often problematic, leading to either low production or insoluble proteins accumulated in inclusion bodies. SA is of no exception because biotin is necessary for the growth of cells. It was inferred, by analyzing the expression of different length SA, that the main function of those hydrophobic amino acid residues at both ends of NFSA was to make NFSA condense and lose the capability to bind biotin *in vivo* to avoid self-damaging by SA. SA obtained its biotin binding ability only after NFSA was secreted out of cells and the hydrophobic termini were cut by proteases (Liu et al., 2005). Up till now, SA expressed by pET system in *E. coil* was different in the amino acid sequence at either or both ends, resulting to different yields (Gallizia et al., 1998). In our study, CNSA with C-terminal hydrophobic amino acid residues was successfully expressed in inclusion bodies at 37°C. At lower culture temperature, the toxic SA was expressed in soluble form (lane 6 of Figure 2), but the SA expression was quite low. The solubility of CNSA was higher than that of NFSA due to the lack of the N-terminal 12 hydrophobic amino acid residues in CNSA, which also leads to cell toxicity. For protection, the host cells had to impel CNSA to inclusion bodies when CNSA was expressed in cells in a large amount.

The gene encoding SA from *S. avidinii* has high GC content in its DNA sequence (69%), which leads to some less frequently used codons in the NFSA gene (Humbert et al., 2008). *E. coli* generally shows a strong preference for codon usage. Replacement of unfavorable codons with highly utilized codons has been suggested to account for the increased production. In recent years, some host *E. coli* strains have been modified to overcome biased codon usage, such as B strains BL21 (DE3) and BL21 (DE3) *pLysS*. The expression vector pET11b and BL21(DE3) as the host stain were used to successfully express SA (amino acid residues 15-159),

and the expression yield of soluble SA was 70 mg/L under optimized conditions (Gallizia et al., 1998). In another report, three recombinant polypeptides of SA, the NFSA with a signal peptide, NFSA and NCSA (amino acid residues 13-139), were also successfully expressed in *E. coli* strain BL21 (DE3) with the expression vector pET28a. The expression yield of the three recombinant proteins was about 80 mg/g dry cell weight and NCSA was expressed mainly as soluble native protein. However, the signal peptide and extra amino acid residues in NFSA made it difficult to fold into functional proteins (Liu et al, 2005).

In our study, CNSA expression was compared in BL21 (DE3) and BL21 (DE3) *pLysS* host strains. Significantly more CNSA was produced in BL21 (DE3) *pLysS* than in BL21 (DE3) and expressed mainly in inclusion bodies. The result suggests that BL21 (DE3) *pLysS* was more suitable for expression of toxic recombinant CNSA. It was reported that the SA yield increased from 70 to 120 mg/L by adding 0.4% of glucose in culture medium (Humbert et al., 2008). In our study, less than 0.8% of glucose in culture medium had little impact on the CNSA expression while more than 0.8% of glucose made the CNSA expression suddenly decline. The reason remained unclear. Optimization of critical parameters, mainly including culture temperature, induced time, led to up to 46.3% protein expressed in cells was CNSA (about 230 mg/g dry cell weight).

The presented CNSA expression yield was higher than any other assays developed thus far, suggesting that an optimal growth of bacteria be required to obtain a maximal protein yield. It was difficult for insoluble proteins in inclusion bodies to refold to active and mature form (Gallizia et al., 1998). By optimizing the refolding temperature, the refolding buffer and especially the initial protein concentration, the refolding efficiency was more than 90% and the purity can achieve 95.0% without any affinity chromatography. Our method to refold SA was simple and yet highly efficient, which was first used for SA expressed in inclusion body form. SA expressed in a soluble form was purified without refolding, but the biotin binding capability was lower than that of the proteins refolded from denatured inclusion bodies, implying that some of the active sites of soluble SA were occupied by intracellular biotin before purification (Liu et al., 2005). In our study, the active sites of SA present in the inclusion body was not subjected to biotin binding, thus the ELISA result showed that the ability of the mature CNSA to bind biotin was similar to that of commercially available recombinant SA.

Conclusion

The active CNSA from *E. coli* inclusion bodies was successfully obtained. In order to enhance the yield, the conditions of CNSA expression, including host strains, temperature of culture, the induction time of IPTG and the concentration of glucose in the culture medium were optimized. The results show that the CNSA, induced by 0.4 M IPTG at 37°C for 12 h, was produced by *E. coli* BL21(DE3)pLysS strain in the inclusion bodies with a high yield up to 46.3% of the total cell protein (about 230 mg/g dry cell weight) in the ATM medium containing 0.4% of glucose.

In order to enhance the efficiency of refolding of the inclusion bodies, the refolding temperature, refolding buffer composition and the initial protein concentration were optimized. The results indicated that the effect of the initial protein concentration on the refolding efficiency was the most significant. More than 90% of inclusion body protein was refolded to mature CNSA under 1.2 g/L of the initial protein concentration with the dialysis against 0.01 M PBS (pH 7.2), and the purity of the recombinant CNSA achieved 95.0% without using any affinity separation method. ELISA analysis indicated that the biotin binding capability of our recombinant CNSA was similar to that of commercial products.

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