

Production of Recombinant Streptavidin and Optimization of Refolding Conditions for Recovery of Biological Activity

Raoufe Modanloo Jouybari¹, Abdorrahim Sadeghi², Behzad Khansarinejad³,
Shabnam Sadoogh Abbasian⁴, Hamid Abtahi*⁴

Abstract

Background: Streptavidin is a protein produced by *Streptomyces avidinii* with strong biotin-binding ability. The non-covalent, yet strong bond between these two molecules has made it a preferable option in biological detection systems. Due to its extensive use, considerable attention is focused on streptavidin production by recombinant methods.

Methods: In this study, streptavidin was expressed in *Escherichia coli* (*E. coli*) BL21 (DE3) pLysS cells and purified by affinity chromatography. Various dialysis methods were employed to enable the protein to refold to its natural form and create a strong bond with biotin.

Results: Streptavidin was efficiently expressed in *E. coli*. Streptavidin attained its natural form during the dialysis phase and the refolded protein bound biotin. The addition of proline or arginine to the dialysis buffer resulted in a refolded streptavidin with greater affinity for biotin than refolding in dialysis buffer with no added amino acids.

Conclusions: Dialysis of recombinant streptavidin in the presence of arginine or proline resulted in proper refolding of the protein. The recombinant dialyzed streptavidin bound biotin with affinity as great as that of a commercial streptavidin.

Keywords: Biotin, Protein Refolding, Streptavidin, Streptomyces.

Introduction

Streptavidin, a protein with molecular weight of about 60 kDa, is produced by *Streptomyces avidinii*. This tetrameric protein has four binding sites for biotin. The bond between streptavidin and biotin is one of the strongest non-covalent interactions in nature, with a dissociation constant of 10^{-14} mol/l. Each streptavidin subunit binds one biotin molecule with affinity 10^3 - 10^6 times greater than the antigen-antibody bond (1, 2).

The strong bond between streptavidin and biotin has turned this complex into a practical immunological tool used in western blotting,

enzyme-linked immunosorbant assays (ELISA), and immunohistochemistry (3, 4). The streptavidin-biotin system is most used in the purification and identification of biological molecules. This system can also be used to attach various biological molecules to each other and to solid surfaces. Streptavidin has also been used in the developing field of nanobiotechnology, the use of biological molecules such as proteins or lipids to create nanoscale devices/structures. In this context, streptavidin can be used as a building block to link biotinylated DNA

1: Department of Medical Biotechnology, Faculty of Medicine, Arak University of Medical Sciences, Arak, Iran.

2: Endocrinology and Metabolism Research Center, Department of Biochemistry, School of Medicine, Arak University of Medical Sciences, Arak, Iran.

3: Department of Microbiology and Immunology, Faculty of Medicine, Arak University of Medical Sciences, Arak, Iran.

4: Molecular and Medicine Research Center, Department of Microbiology, School of Medicine, Arak University of Medical Sciences, Arak, Iran.

* Corresponding authors: Hamid Abtahi; Tel: +98 863 4173502; Fax: +98 863 4173526; E-mail: abtahi@arakmu.ac.ir.

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molecules to create single-walled carbon nanotube scaffolds (5).

Each streptavidin monomer consists of eight twisted antithetical beta strands, which create the spiral-shaped tertiary β -barrel structure (6). A biotin-binding site includes residues from the interior of the barrel, together with a conserved Trp120 from neighboring subunit. In this way, each subunit is involved in the binding site of its adjacent subunit (7). The main protein contains 159 amino acids, the first 24 of which are the signal peptide (8).

Both ends of the protein are often cleaved to access its core, which increases its biotin-binding affinity (9). The amino acids at the N- and C-termini of streptavidin spatially obstruct biotin binding to the streptavidin active site (10). Recombinant streptavidin expressed in *Escherichia coli* (*E. coli*) in monomeric form migrates with an apparent molecular weight of 16 kDa on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (11). Protein expression systems include mammalian, yeast, plant, and bacterial cells (12, 13).

*E. coli*s generally used to express recombinant streptavidin due to its relatively high production (14). Recombinant protein expression in bacteria often results in protein aggregates, known as inclusion bodies. In these cases, the expressed protein is often non-functional. Normal protein activity can often be obtained through proper refolding, which can often be achieved with dialysis and the addition of amino acids (15).

In this study, we aimed to produce recombinant streptavidin, identify the best buffer for refolding the protein, and examine its biotin binding after dialysis.

Materials and Methods

Codon Optimization

After studying the characteristics of the streptavidin gene in the NCBI (AC: A00743) and investigating the protein's properties in Uniprot (P22629), the desired gene sequence was determined. Codons were optimized using Vector NTI software and the gene was synthesized by Biomatik Corporation (Canada). *BamHI* and *XhoI* sequences were added to the 5' and 3' ends of the gene, respectively.

Cloning of streptavidin in pET32a

First, the streptavidin coding sequence and pET32a (Stratagene, USA) were cut with *BamH I* (Fermentase, USA) and *Xho I* (Fermentase, USA) and ligated with *T4 DNA ligase* (Fermentase, USA) at 16 °C overnight. The recombinant plasmid was named pET32a-stv. *E. coli DH5 α* (Stratagene, USA) and *E. coli BL21 (DE3) pLysS* (Pharmacia, Sweden) competent cells were prepared by the calcium chloride method and transformed with pET32a-stv. The transformed bacteria were selected by screening the colonies on media containing ampicillin (16).

Production and purification of the recombinant protein

To produce streptavidin protein, *E. coli BL21 (DE3) pLysS* cells were transformed with pET32a-stv. Transformed cells were incubated for 18 h at 37 °C in nutrient broth containing 100 mg/ml ampicillin and 34 mg/ml chloramphenicol (17). Cultures were then transferred to 50 ml of induction medium containing 0.5 g yeast extract (Merck, Germany), 1 g Bacto Peptone, (Merck, Germany), 0.1 g glucose (Merck, Germany), 0.5 g NaCl (Roche, Germany), 0.05 g KCl (Roche, Germany), 0.025 g MgCl₂.6H₂O (Merck, Germany), 0.025 g CaCl₂ (Merck, Germany), 0.25 g nutrient broth (Merck, Germany), 14 μ l ampicillin, and 14 μ l chloramphenicol and shaken at 220 rpm. When the OD₆₀₀ of the bacterial culture reached 0.6, protein production was induced by adding isopropyl- β -D-thiogalactopyranoside (IPTG) (Merck, Germany) to a final concentration of 1 mM. Samples were taken two and four hours after the addition of IPTG and analyzed by SDS-PAGE (18). Due to the presence of the 6-His tag on the N-terminus of the recombinant protein, the expressed protein was purified with Ni-NTA agarose resin (Qiagen, USA). The protein concentration was determined using the Bradford protein assay (19) and the purified protein was analyzed by SDS-PAGE.

Refolding procedure

To induce refolding, the purified protein was dialyzed in various buffers. The standard buffers were 20 mM phosphate-buffered saline (PBS) and 50 mM Tris-HCl (11, 20, 21). In all experiments, the buffer pH was 8.0, the temperature was 4 °C,

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and the dialysis duration was 24 hours. In addition, for all experiments, the buffer was replaced after two hours and the buffer volume was maintained at 1 liter (22).

Protein dialysis with urea concentration gradients

The purified protein was dialyzed against refolding buffer that contained 50 mM Tris-HCl (Merck, Germany), 150 mM NaCl (Merck, Germany), 10 mM EDTA (Merck, Germany), 0.5% Triton X-100 (Merck, Germany), 0.2 M ammonium sulfate (Merck, Germany), 0.3 M glycine (Merck, Germany), and 0.2

M glucose as a stabilizer (Merck, Germany).

Direct dialysis

For dialysis, two ml of a 1 mg/ml solution of the purified protein was slowly added to a dialysis bag with 14 kDa pores (Sigma). The bag was then placed in an Erlenmeyer flask containing 1000 ml of dialysis buffer. To examine the effects of different reagents on refolding, 0.3 M arginine, proline, cysteine, and glycine (all from Merck, Germany) were added to the dialysis buffer (Table 1).

Table 1. Dialysis condition and Optical densities of the dialysis samples in Tris-HCl buffer and PBS buffer in the presence of various additives and compared Molar ratio of biotin to streptavidin with standard protein.

Dialysis condition						OD of the dialysis sample in absence and presence of biotin		Molar ratio of biotin to streptavidin
Combination of glucose with Arg/Pro/Cys/Gly	Combination of NaCl and Ammonium sulfate	Triton X100	Urea	EDTA	Dialysis buffer	OD1	OD2	
Arg (0.3 M) + Glu (0.2 M)	Ams (0.2 M) + NaCl (150mM)	Triton X100 (0.5%)	6M	EDTA (10 mM)	-HCl (50 mM) Tris	0.257	0.236	0.15
Pro (0.3 M) + Glu (0.2 M)	Ams (0.2 M) + NaCl (150mM)	Triton X100 (0.5%)	4M	EDTA (10 mM)	-HCl (50 mM) Tris	0.246	0.228	0.09
Cys (0.3 M) + Glu (0.2 M)	Ams (0.2 M) + NaCl (150mM)	Triton X100 (0.5%)	2M	EDTA (10 mM)	-HCl (50 mM) Tris	0.248	0.224	0.11
Gly (0.3 M) + Glu (0.2 M)	Ams (0.2 M) + NaCl (150 mM)	Triton X100 (0.5%)	1M	EDTA (10 mM)	-HCl (50 mM) Tris	0.295	0.317	-0.03
-	-	-	-	-	PBS (20 mM)	0.307	0.311	-0.2
Gly (0.3M) + Glu (0.2 M)	Ams (0.2M)	Triton X100 (0.5%)	-	-	PBS (20 mM)	0.273	0.249	0.7
Arg (0.3M) + Glu (0.2 M)	Ams (0.2M)	Triton X100 (0.5%)	-	-	PBS (20 mM)	0.615	0.540	1.5
Pro (0.3M) + Glu (0.2 M)	Ams (0.2M)	Triton X100 (0.5%)	-	-	PBS (20 mM)	0.801	0.692	1.3
Cys (0.3M) + Glu (0.2 M)	Ams (0.2M)	Triton X100 (0.5%)	-	-	PBS (20 mM)	0.226	0.191	0.9
Commercial streptavidin (Sigma)						0.397	0.283	1.3

Ammonium sulfate= Ams, Glucose= Glu, Glycerol= Glyc, Arginine= Arg, Glycine= Gly, Proline = Pro.

Row 1: 6 M urea, Row 2: 4M urea, Row 3:2M urea, Row 4:1M urea, and Row 5-9: without urea and in PBS buffer.

OD1 = OD500 of the streptavidin - HABA reagent in the absence of biotin.

OD2 =OD500 of the streptavidin-HABA reagent after the addition of biotin.

Measurement of protein concentration and SDS-PAGE

After dialysis the purified protein was analyzed for its presence and stability by Bradford assay and SDS-PAGE (19).

Assessment of biotinylation of the dialyzed streptavidin

To investigate the effects of dialysis conditions on the structure and function of the produced streptavidin, the strength of the streptavidin-biotin bond was

evaluated after each dialysis. For this, 4'-hydroxyazobenzene-2-carboxylic acid (HABA), (Sigma, Germany) was used (2). HABA creates a yellow complex when bound to streptavidin. The binding ability of streptavidin produced in this design was expressed relative to that of commercial streptavidin (Sigma, USA).

When added, biotin replaces HABA, resulting in an OD₅₀₀ absorbance reduction (1). Streptavidin biotinylation was assessed based on reduced absorbance and the Green method (2). The biotin-binding affinity of streptavidin was measured, and the molar ratio of biotin to streptavidin was quantified and compared with commercial streptavidin (Sigma).

The following formula was used to obtain absorption reduction:

$$\Delta A = (0.9 \times OD_1) + OD_3 - OD_2$$

OD1 = OD₅₀₀ of the streptavidin - HABA reagent in

the absence of biotin.

OD2 = OD₅₀₀ of the streptavidin-HABA reagent after the addition of biotin.

OD3 = Blank (streptavidin, HABA, and water).

To obtain the amount of biotin (μmole/ml) of reaction mixture (C), $C = \Delta A / 34$ was used. To measure the amount of biotin (B) (mmole/ml per ml of sample), $B = C \times 10$ was used. B/P (P = Streptavidin concentration in molarity) was used to determine the ratio of biotin to streptavidin (1, 2, 23).

Results

Gene preparation and transferring the plasmid with gene fragment to DH5a bacteria

To optimize biotinylation, the first 24 amino acids comprising the streptavidin signal peptide were removed, reducing the apparent molecular weight from approximately 18.8 to 16.6 kDa (Fig. 1).

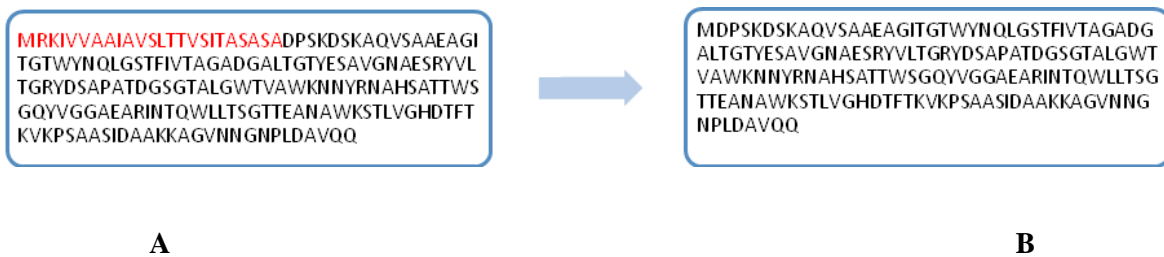


Fig. 1. Amino acid sequence of streptavidin. The first 24 amino acids that comprise the signal peptide and inhibit biotin binding were removed. A: The full-length protein sequence. B: The protein sequence minus the signal peptide.

Production and purification of the recombinant streptavidin

Protein production was induced with IPTG. Samples were collected two and four hours after induction, cells were pelleted and lysed, and then lysates were analyzed by SDS-PAGE (Fig. 2). The calculated molecular weight of the recombinant streptavidin with the 6-His tag was 17.6 kDa; however, the protein migrated with an apparent molecular weight of approximately 36 kDa on SDS-PAGE. The expressed protein in pET32a contains several extra amino acids linked to the C or N terminal extension of protein. These additional amino acids increased the size of expressed protein by approximately 20 kDa, (Fig. 3) (24).

Protein dialysis with urea gradient

Purified streptavidin samples were dialyzed sequentially in the urea gradient refolding buffers overnight at 4 °C with stirring. Table 1 and Figure 4 show the optical densities and binding strengths

of biotin to streptavidin in the dialyzed samples in refolding buffer containing 6, 4, 2, 1, 0.5, and 0 M urea and PBS buffer.

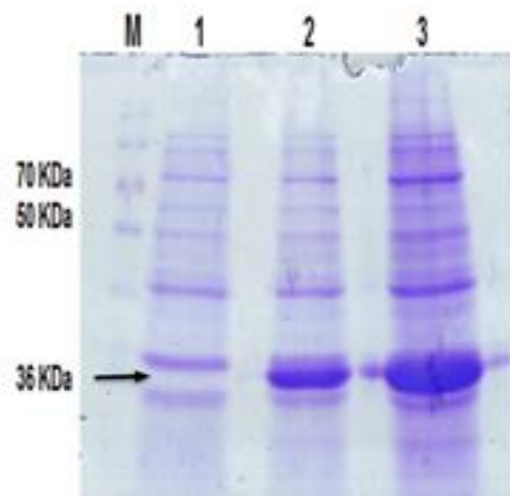


Fig. 2. SDS-PAGE of pET32a-stv-transformed cell lysates. Lane M, protein marker; Lane 1, cell lysates before induction; Lanes 2 and 3, cell lysates 2 and 4 h after induction, respectively. Recombinant streptavidin appears as a 36-kDa band in lanes 2 and 3.

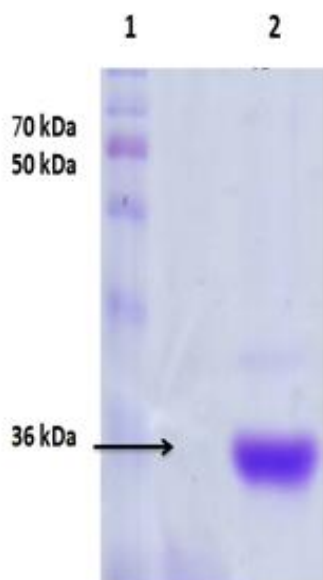


Fig. 3. SDS-PAGE of Ni-NTA-purified recombinant streptavidin. Lane 1, protein marker; Lane 2, Ni-NTA-purified streptavidin.

Molar ratio of biotin to streptavidin

The biotin/streptavidin molar ratios with recombinant streptavidin dialyzed in PBS buffer containing either 0.3 M arginine or 0.3M proline were 1.5 and 1.3, respectively, greater than and equal to that of the commercial streptavidin standard (Table 3).

Discussion

Production of recombinant proteins eliminates the need to purify proteins from natural biological sources. Unfortunately, protein production in prokaryotic systems often results in inclusion bodies. It is often necessary for the protein to gain its natural form to maintain its function. Streptavidin is an important protein used in many biological systems, including immunological assessment reactions (25).

The production of streptavidin via recombinant methods has been well studied. The major aim of this study was to produce streptavidin with high biotin binding affinity. The first step in this study was cutting the signal sequence from the protein. It was previously shown that deleting this part of the protein increases its structural stability and access to biotin (10). Indeed, the signal peptide inhibits streptavidin-biotin binding by blocking streptavidin's biotin binding site.

Recombinant protein expression in *E. coli* often results in protein accumulation and deposition in inclusion bodies (30). To destroy the inclusion body and open the protein strands, high concentrations of urea or guanidinium hydrochloride were used (31). These chemical compounds lead to a flexible and disordered structure, and may inhibit protein function. Consequently, correct protein refolding is

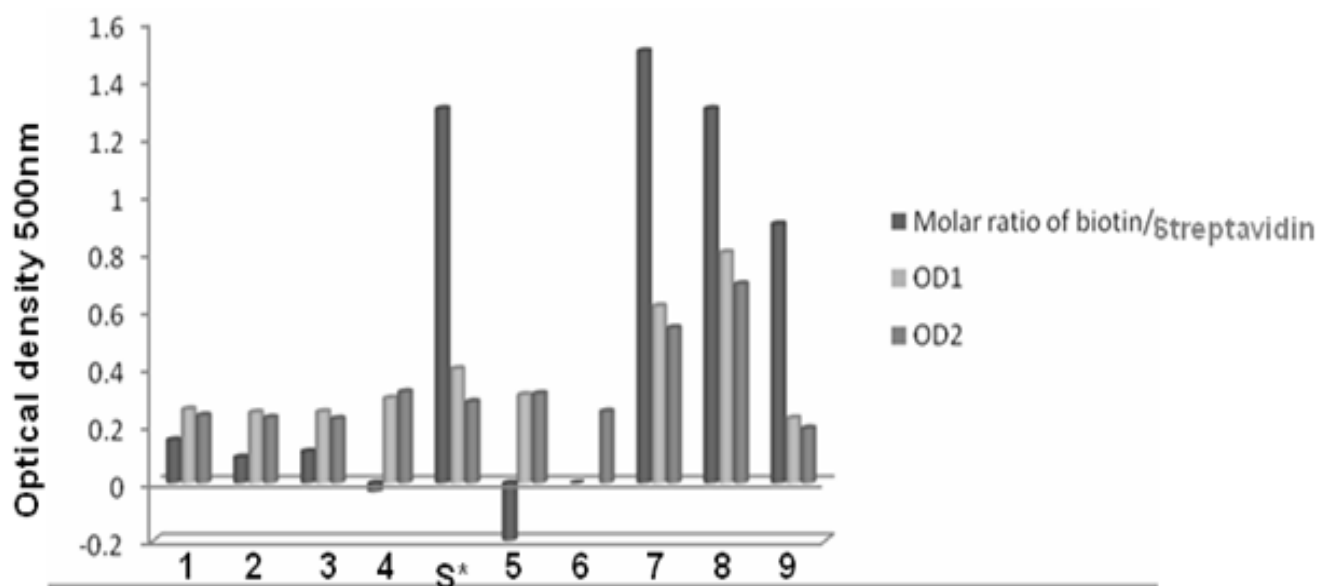


Fig. 4. Comparison of reading the optical density and Molar ratio of biotin to streptavidin in different condition of dialysis, according to table 1. S*: commercial streptavidin (Sigma)

initiated by removing the denaturants (32). One method to remove these factors is protein dialysis using semi-permeable bags. The usual method of protein dialysis is systematic reduction of the denaturant concentration. By reducing the denaturant concentration, cross-bonds between protein strands are generated and the protein gains its natural form. Addition of monosaccharides (32, 33) and amino acids (34) to dialysis buffers have been shown to aid in with proper protein refolding during dialysis.

In this study two Tris-HCl and PBS buffers were used for dialysis (Table 1). To enhance refolding efficiency ammonium sulfate, glucose, and amino acids were employed. Despite the findings of other studies on the effects of urea on protein refolding, in our study, urea in Tris-HCl did not affect streptavidin refolding or biotin binding. Additionally, various combinations such as amino acids with urea are not influential in biotin binding. In other studies, it was demonstrated that 4.6 or 7 M urea had no effect on streptavidin tetrameric structure (35).

In the present study, the effect of ammonium sulfate on protein refolding was also investigated. Ammonium sulfate stabilizes protein structure through electrostatic interactions. In this study we also examined the effects on refolding of the amino acids arginine, glycine, proline, and cysteine. Arginine plays a significant role in the refolding process by promoting protein solubility and inhibition of protein aggregation. The amine and carboxyl groups in arginine create a weak bond with hydrogen, thus preventing protein aggregation (36).

Proline also prevents protein aggregation by removing the factors that aggregate protein and helps proper protein folding (34). Glucose, another compound used in this study, stabilizes protein structure (33, 37). The effects of these amino acids and other compounds on protein refolding and function were investigated. These additives bind to protein folding intermediates or hydrophobic chains and provide a suitable condition to reduce protein aggregation and allow proper protein refolding (38-40).

The recombinant streptavidin refolded more correctly in PBS than in any other base buffer. Also, addition of arginine or proline resulted in

streptavidin/biotin ratios greater than and equal to those achieved with the commercial streptavidin standard, indicating their refoldings were similar to the natural form, and for cysteine and glycine, ratios were close to those of the streptavidin standard. In a similar study by Sorensen *et al.*, it was observed that intermittent changing of dialysis buffer, as well as adding various additives (41).

In the current study, streptavidin-biotin binding was also investigated with spectrophotometry using HABA. HABA binds to streptavidin and creates a yellowish compound. When biotin is added, it replaces HABA and reduces absorbance. This reduction in absorption is a criterion for assessing streptavidin activity and its biotin-binding affinity (8). HABA has been employed in many studies to evaluate streptavidin activity (42).

The absorbance reduction of the streptavidin standard in the presence of biotin was 0.1, therefore its molar ratio to biotin was 1.3. These amounts are also observed in recombinant protein dialyzed in PBS containing arginine and proline. The main feature of streptavidin is binding to biotin. In this study, the biotin binding to recombinant streptavidin was investigated. Our results show that the addition amino acids increased recombinant streptavidin-biotin affinity considering the wide range of applications of streptavidin in laboratory and diagnostic fields, production of this protein can help improve the process of diagnosis and speed up laboratory studies.

In conclusion, analysis of the recombinant streptavidin in PBS with amino acids improved the protein refolding process. Based on the Green protocol, adding biotin to the sample reduced absorbance by 0.1. This level of reduction is observed in natural streptavidin. Therefore, increased binding affinity of recombinant streptavidin to biotin similar to that of the streptavidin standard was observed with the addition of arginine or proline to the PBS dialysis buffer. While the addition glycine or cysteine resulted in biotin binding less than that of the streptavidin standard.

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