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A novel approach to make homogeneous protease-stable monovalent streptavidin

Min Zhang [a, b], Jinhui Shao [a], Juan Xiao [a], Wenbing Deng [b, *], Hongjun Yu [c, **]

[a] Medical College, Hubei University of Arts and Science, Xiangyang, Hubei, China
[b] Department of Biochemistry and Molecular Medicine, School of Medicine, University of California, Davis, CA, USA
[c] Department of Biology, Brookhaven National Lab, NY, USA

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ABSTRACT

The interaction between the tetramer streptavidin and biotin is recognized as one of the strongest non-covalent associations. Owing to the tight and specific binding, the streptavidin-biotin system has been used widely for bimolecular labeling, purification, immobilization, and even for targeted delivery of therapeutics drugs. Here, we report a novel approach to make homogeneous monovalent tetramer streptavidin. The purified monovalent protein showed both thermal stability and protease stability. Unexpectedly, we found that two proteases, Proteinase K (PK) and Subtilisin (SU), can efficiently remove the His_8-tag from the wild-type subunit without affecting the tetramer architecture of monovalent streptavidin, thus making it more homogeneous. In addition, crystallization was performed to assure the homogeneity of the monovalent protein prepared. Overall, monovalent streptavidin shows increased homogeneity and will likely be valuable for many future applications in a wide range of research areas.

1. Introduction

Streptavidin is a 60-kD tetrameric protein derived from *Streptomyces avidini*. It possesses a tight and specific attraction for biotin with the dissociation constant of 10^{-15} M, and the streptavidin-biotin system is acknowledged as one of the most stable non-covalent interactions [1–3]. This feature has made streptavidin one of the most widely exploited protein tools for applications in the life sciences across a range of biochemical, pharmaceutical, and biophysical areas [4–6]. However, the multiple biotin binding sites of tetrameric streptavidin may cause unwanted close proximity of biotin-labeled targets, leading to their aggregation or affecting their normal functions [6]. This can be overcome by reducing the biotin binding sites [7,8]. Despite the importance of this strategy, little is known about the protease-resistance of the variant streptavidin tetramers, and this information is necessary for their *in vivo* applications. This strategy also needs further improvements such as increasing the homogeneity and removing the unwanted recombinant tag. The recombinant tag (usually His-tag for most streptavidin studies) could interfere with the ligand binding, especially for large macromolecules with labeled biotin. Actually, His6-tag was found to decrease the biotin affinity of wild-type streptavidin [8].

Here, we developed an improved purification method to generate the monovalent streptavidin without affecting its tetramer architecture. We further determined the stability of monovalent streptavidin by the thermal and protease stability assays. Notably, we found that with the optimal incubation time and concentration of the Proteinase K (PK) and Subtilisin (SU), His_8-tag linked to the wild-type subunit of the nature monovalent streptavidin could be efficiently removed. The deletion of N-terminal His_8-tag can make streptavidin more homogeneous, will remove the potential adverse effects of the recombinant tag and will be useful for further applications. In addition, we described the successful crystallization and preliminary analysis of monovalent streptavidin as a first step towards elucidating its structure, which is structurally unknown.

2. Materials and methods

2.1. Preparation of homogeneous monovalent streptavidin

The wild-type streptavidin (W, with His_8-tag) and inactive mutant streptavidins (N23A, S27D, S45A) (M, without His_8-tag) were introduced into NdeI and XhoI site of pET28a, and were then

* Corresponding author.
** Corresponding author.
E-mail addresses: wbdeng@ucdavis.edu (W. Deng), yuhongjun05@gmail.com (H. Yu).

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transformed into *Escherichia coli* strain BL21 (DE3), respectively. At OD_{600} = 0.8, protein synthesis was induced with 1 mM β-D-1-thiogalactopyranoside (IPTG) for 5 h at 37 °C. Both proteins were expressed as inclusion bodies. The collected cells were disrupted by sonication and the inclusion bodies were isolated and washed twice with washing buffer (20 mM Tris–HCl, pH 8.0, 0.3 M NaCl, 2 M urea). The purified inclusion bodies of wild-type and mutant streptavidin were both solubilized in solubilization buffer (20 mM Tris–HCl, pH 8.0, 0.3 M NaCl, 8 M urea) and were mixed in 1:3 M ratio (concentration was measured used NanoDrop 2000 at OD_{280}). Refolding of streptavidin tetramer was achieved through fast dilution into PBS buffer as previously described [8,9]. The refolded protein was purified through Ni-NTA affinity chromatography and eluted using a step gradient method from buffer Ni-A (20 mM Tris–HCl, pH 8.0, 0.3 M NaCl) to Ni–B (20 mM Tris–HCl, pH 8.0, 0.3 M NaCl, 0.5 M imidazole). The stepwise elution products were collected by every 0.5 ml fraction. The samples of each fraction were loaded without boiling on to 8% SDS-PAGE, and their counterparts were then denatured for 10 min in boiled water and loaded on to 17% SDS-PAGE. The final purification was achieved by size-exclusion chromatography with a Superdex 75 column equilibrated with Tris-sodium buffer (20 mM Tris–HCl, pH 8.0, 0.15 M NaCl). The elution proteins were collected by every 0.5 ml fraction and the tetramer purity was determined by 15% SDS-PAGE.

2.2. Thermal stability assay

Either streptavidin W0M4 (zero wild-type subunit with 4 triple-mutant subunits) or monovalent streptavidin W1M3 (one wild-type subunit with 3 triple-mutant subunits) at 1 mg/ml in Tris-sodium buffer was pretreated at the temperature of 37 °C, 50 °C, 70 °C and 100 °C for 10 min in a PTC-200 PCR machine and then immediately placed on ice. Samples (5 μl) from each PCR tube were mixed with loading buffer and loaded onto a 15% SDS-PAGE.

2.3. Protease stability assay

The monovalent streptavidin was subjected to a protease stability assay. Streptavidin was treated with proteases (with target: protease weight ratio of 100:1) at 37 °C for 5 h. Panels of six proteases were analyzed, including the protease Pepsin (PE), Proteinase K (PK), Trypsin (TR), Thermolysin (TH), Alpha Chymotrypsin (α–C) and Subtilisin (SU). Both the samples without heat treatment and their denatured counterparts boiled in loading buffer were subjected to a 15% SDS-PAGE, followed by Coomassie Blue staining.

2.4. Crystallization

The proteinase K (PK) protease-cleaved monovalent streptavidin (10 mg/ml) was then used for crystallization trials. Initial crystals were found using commercial screens (Hampton Research) by the hanging drop vapor diffusion in 96-well plates. Crystals of monovalent proteins were grown at 20 °C when 1 μl of the well solution was mixed with 1 μl protein sample (10 mg/ml, 20 mM Tris–HCl, pH 8.0, 0.15 M NaCl). For the verification of crystal formation, the specific Izit crystal dye was used (Hampton Research). Crystals were then mounted and flash-frozen in liquid nitrogen. Preliminary diffraction data of the frozen crystals were obtained at SSRF.

3. Results

3.1. The preparation of monovalent streptavidin

To produce the monovalent streptavidin (W1M3, one wild-type subunit with 3 triple-mutant subunits), we made construction of wild-type streptavidin with N-terminal His_8-tag (W subunit), and another construction of mutated streptavidin (with N23A/S27D/S45A triple mutations) without recombinant tag (M subunit) which is unable to bind biotin [8]. Inclusion bodies of these two constructs were purified. Then different streptavidin tetramers were refolded and were further separated based on their different affinities to Ni-NTA column, resulting from varying numbers of recombinant tags between them. Step-gradient imidazole elution of bound streptavidin produced multiple elution peaks (Fig. 1A). Considering that small molecular weight differences exist among different tetramers due to different numbers of His_8-tags on them and streptavidin tetramer is stable in RT even in the SDS-loading buffer [10,11], 8% SDS-PAGE analysis was used to identify the composition of each elution peak (Fig. 1B). As streptavidin can be denatured at high temperature, the identity of each peak was further confirmed by 17% SDS-PAGE analysis of boiled sample (Fig. 1C). Based on these analyses, we concluded that W0M4 (4 triple-mutant subunits) in 2% and 10% Ni–B elution peak, W1M3 (one wild-type subunit and 3 triple-mutant subunits) in 14% and 20% Ni–B elution peak and W2M2 (2 wild-type subunits and 2 triple-mutant subunits) in 100% Ni–B elution peak were the three major streptavidin variants obtained. There was a tiny amount of W3M1 (3 wild-type subunits and 1 triple-mutant subunit) mixed with W2M2 in the 100% Ni–B elution peak, and refined imidazole gradient might be needed to further separate them. Notably, each aliquot from the 100 mM (20% Ni–B) elution peak contained pure monovalent streptavidin W1M3.

3.2. Preparation of more homogeneous monovalent streptavidin

Refolding experiments may produce a misfolded protein as well as a correctly folded one [12]. The misfolded protein cannot be removed by metal affinity chromatography only, but may complicate the analysis of refolding conditions and further applications. Thus, we combined all the aliquots of 100 mM (20% Ni–B elution peak, Fig. 1) and further utilized liquid chromatography gel filtration to analyze the refolding efficiency of this monovalent streptavidin. As shown in Fig. 2A, the sole symmetrical peak appeared at the 11.3 ml elution volume, demonstrating high homogeneity of the monovalent tetramer. The calibration of Superdex 75 gel filtration column by molecular weight standard (43 kD, 29 kD and 13.7 kD) indicated that monovalent streptavidin elute at a volume slightly larger than two subunits, which is reasonable considering the relative compact tetramer architecture of streptavidin. Samples from different aliquots of elution peak were further analyzed via the denatured polyacrylamide gel electrophoresis (Fig. 2B lane 2–4) and were confirmed to be tetramer because they all migrated as about 60 kD. Boiled sample from combined elution peak (Fig. 2B, Lane 1) also showed the subunit ratio of 1:3 (W:M), which is in accordance with the result in Fig. 1C.

3.3. Thermal stability and proteases sensitivity of homogeneous monovalent streptavidin

It has been reported that the multivalent streptavidin tetramers may be applied in the construction of streptavidin-based conjugates with a defined number of binding sites for proteins fused to streptavidin-binding peptides [1]. This application of monovalent tetramer may require it being stable in high temperatures, and the *in vivo* application may require resistance to multiple proteases. Therefore, the thermal stability of streptavidin tetramers was carried out to determine whether streptavidin tetramer was intact at indicated temperature [11]. As shown in Fig. 3A, the W1M3 and the W0M4 tetramers were significantly thermos-stable up to 70 °C. After incubation at the indicated temperature (37 °C, 50 °C or

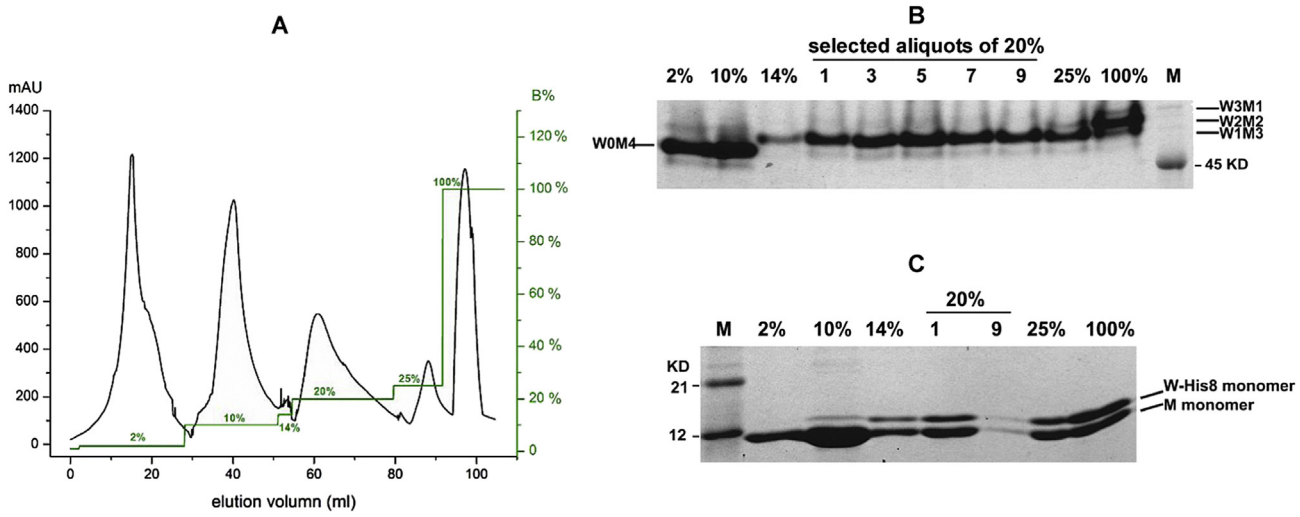

Fig. 1. Separation of streptavidin variants. A: After refolding, stepwise elution from NTA column to separate different streptavidin variants. The gradient concentrations of imidazole used here were 10 mM (2%Ni–B), 50 mM (10%Ni–B), 70 mM (14%Ni–B), 100 mM (20%Ni–B), 125 mM (25%Ni–B) and 500 mM (100%Ni–B). The black line showed the spectrophotometric absorbance at 280 nm, and the cyan line showed the imidazole's concentration. B: The fractions from each gradient of imidazole were loaded without boiling on to 8% SDS-PAGE. C: The counterparts of streptavidin were denatured 10 min in boiled water before loaded on to 17% SDS-PAGE. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

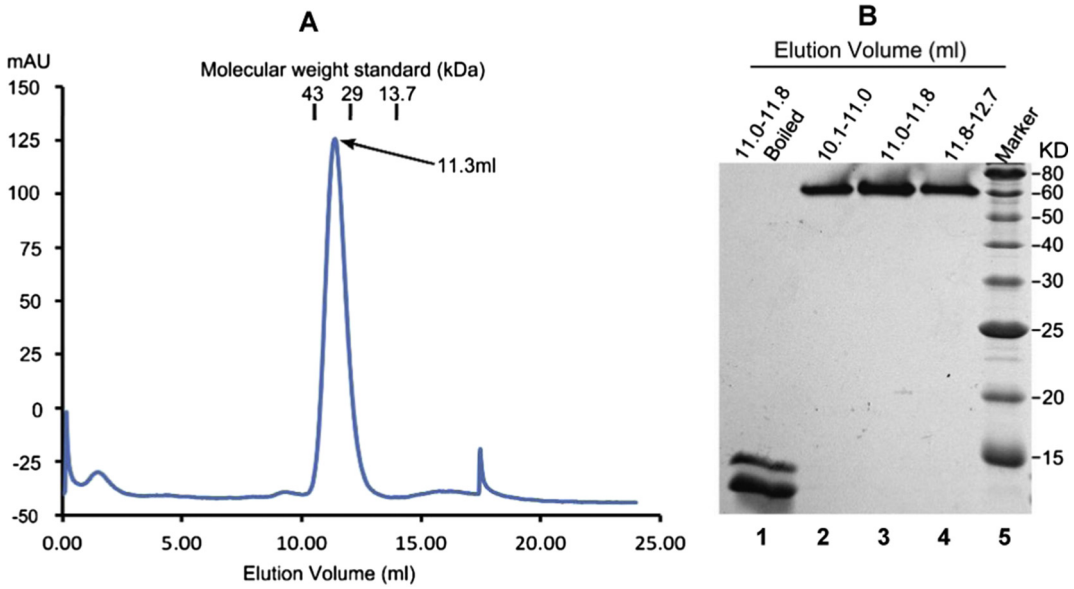

Fig. 2. Gel filtration analysis of homogeneous chimeric monovalent streptavidin (W1M3). A: The monovalent streptavidin W1M3 (aliquots from 100 mM imidazole seen in Fig. 1) was further purified by size-exclusion chromatography with a Superdex 75 column. The blue line showed the spectrophotometric absorbance of fractions at 280 nm. The calibration results using standard protein marker were shown on the top. B: The elution products on 15% SDS-PAGE. Lane 2–4, Samples from different aliquots of the elution peak were loaded on SDS-PAGE without heating. Lane 1, the combined peak was denatured in boiling water before loading. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

70 °C), the streptavidin still kept the intact tetramer structure. Only at the temperature of 100 °C, the tetramers were denatured into four subunits. The results also confirmed that the W1M3 tetramer has two kinds of subunits (W subunit and M subunit) and the W0M4 tetramer with only one kind of subunit (Fig. 3A).

Streptavidin was shown to be susceptible to proteinase K digestion [13]. Therefore, we tested the protease sensitivity of monovalent streptavidin. Monovalent streptavidin was treated with a panel of six different proteases (at the weight ratio of protein: protease = 100:1, 37 °C for 5 h), and then both unheated and boiled samples were analyzed by SDS-PAGE, respectively (Fig. 3). It was observed that the W1M3 monovalent tetramer was stable with all the proteases tested. Notably, while keeping the intact tetramer architecture, Proteinase K (PK) and Subtilisin (SU) can eliminate the difference between wild-type and triple-mutant subunits under our assay conditions. Therefore, we suspected the His_8-tag from wild-type subunit can be removed by these two proteases, which was confirmed by western blot analysis (Fig. 3C). This is a novel approach to remove unwanted recombinant tag from streptavidin

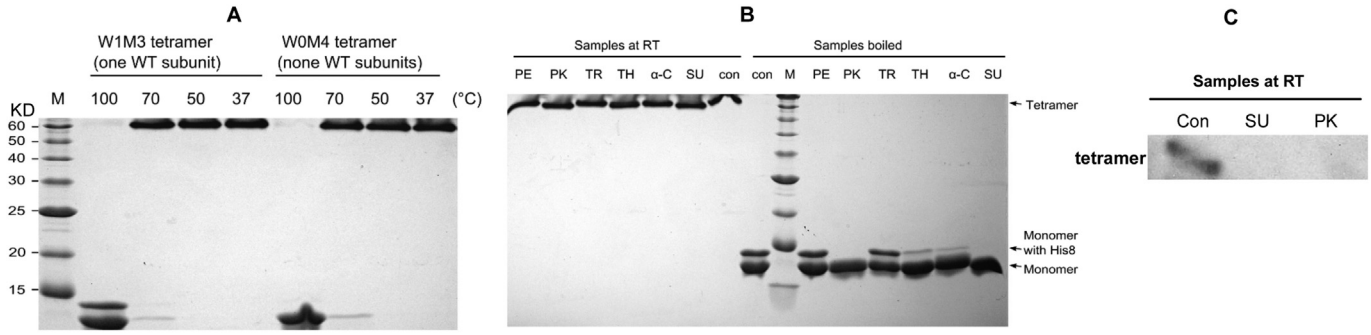

Fig. 3. Thermal and protease stability of tetramer streptavidin. A: Thermal stability of streptavidin. The homozygotic W0M4 or chimeric W1M3 streptavidin was incubated at the temperature of 37 °C, 50 °C, 70 °C and 100 °C for 10 min. Samples were subjected to a 15% SDS-PAGE. B: Protease stability of chimeric W1M3 tetramer. Streptavidin was treated with protease Pepsin (PE), Proteinase K (PK), Trypsin (TR), Thermolysin (TH), Alpha Chymotrypsin (α–C) and Subtilisin (SU) at 37 °C for 5 h. Treated samples were then analyzed by SDS-PAGE as indicated. C: The western blot assay to determine to removal of His_8-tag.

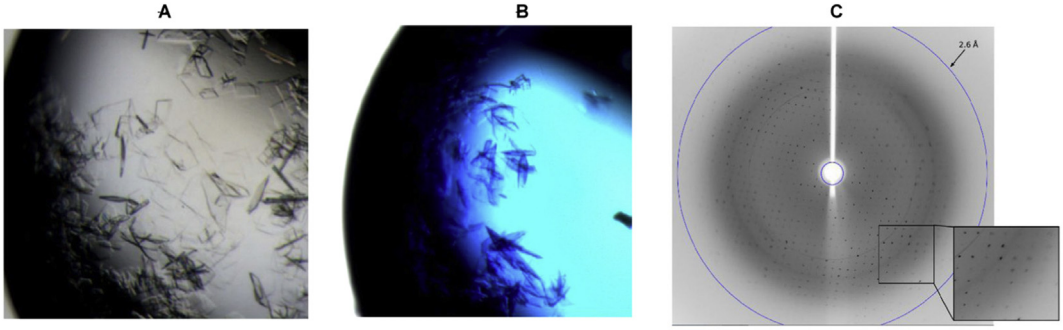

Fig. 4. Crystallization study of monovalent streptavidin. A: The monovalent streptavidin in 100 mM Hepes (pH 7.8) and 58% MPD at 293 K. B: The staining of the crystals. C: Diffraction image of monovalent streptavidin crystal. The outer blue circle corresponds to the resolution of 2.6 Å. The inset shows a zoomed view of local diffraction spots. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

that may hinder its applications, producing the more homogeneous monovalent streptavidin.

3.4. Crystallization of the homogeneous monovalent streptavidin

As indicated above, by our combined preparation method, we have obtained the super-pure monovalent streptavidin without the recombinant tag. Because the availability of the monovalent streptavidin structure will be valuable for the further design and improvement of streptavidin, and also in order to confirm the homogeneity of the prepared sample, we tested crystallization of the sample. The Proteinase K-cleaved monovalent streptavidin (10 mg/ml) was purified through gel filtration to remove the protease and was used for crystallization screen. Thin plate crystals appeared and grew within a few days of setup, which could be stained by specific Izit protein crystal dye (Fig. 4 A and B). The thin crystals tended to be fragile and easily cracked when flash-frozen. Also, there were always ice rings when several cryoprotectants were screened. Fig. 4C showed a typical diffraction image. Crystals seemed to be twinned with high mosaicity. After indexing, it was found that the crystals possessed space group P1, with unit-cell parameters a = 44.357, b = 56.738, c = 57.435 Å, α = 87.536°, β = 85.892°, γ = 67.988°. Efforts are underway to improve the crystal and flash-cooling conditions to get suitable data for structural determination.

4. Discussion

The streptavidin-biotin system is widely used in biotechnology and biological sciences [1,4,14]. However, its applications have limitations. For example, to detect biotinylated ligands, the multiple high affinity binding sites of streptavidin can cause target aggregation. This may restrict the possible exciting applications of *in vivo* drug delivery based on the interaction between streptavidin and biotin, since functions of active molecules can change due to their clustering [8]. To overcome this liability caused by multivalent binding, extensive studies have been carried out to reduce the biotin binding sites without perturbing the tetramer structure [7,8].

Here we describe an improved approach to prepare highly homogeneous monovalent streptavidin with single biotin binding site. The prepared monovalent streptavidin was found stable in both thermal and protease stability assays. Notably, we identified a novel method (protease treatment with Proteinase K and Subtilisin) to efficiently remove the recombinant tag from the streptavidin, which may hinder its further applications. In addition, no structural information has been obtained for the monovalent streptavidin, which would be valuable for the further structure-based design and modification. Therefore, we carried out crystallization studies of monovalent streptavidin. It was found that the removal of recombinant tag by PK was necessary to produce crystals. Further efforts are needed to elucidate the three-dimensional structure of this important monovalent streptavidin.

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Author contributions

M.Z., J.S., J.X., and H.Y. carried out the experiments. H.Y. and W.D. co-supervised the project. M.Z., H.Y. and W.D. designed experiments and wrote the paper.

Competing financial interests

The authors declare that there are no conflicts of interest.

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