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Electrophoretic behavior of streptavidin complexed to a biotinylated probe: A functional screening assay for biotin-binding proteins

The biotin-binding protein streptavidin exhibits a high stability against thermal denaturation, especially when complexed to biotin. Herein we show that, in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), streptavidin is stabilized at high temperature in the presence of biotinylated fluorescent probes, such as biotin-4-fluorescein, which is incorporated within the binding pocket. In nondenaturing SDS-PAGE, streptavidin is detectable when complexed with biotin-4-fluorescein using a UV-transilluminator. Using biotin-4-fluorescein, the detection limit of streptavidin lies in the same range as with Coomassie blue staining. The functionality of streptavidin mutants can readily be assessed from crude bacterial extracts using biotin-4-fluorescein as a probe in nondenaturing SDS-PAGE.

Keywords: Biotin-4-fluorescein / Functional screening / Nondenaturing sodium dodecyl sulfate-polyacrylamide gel electrophoresis / Streptavidin

1 Introduction

The biotin-(strept)avidin technology ((strept)avidin refers to either avidin or streptavidin), commonly referred to as molecular velcro, relies on the extraordinary affinity of biotin for either avidin or streptavidin ($K_a \sim 10^{14}\ M^{-1}$) [1]. This remarkable affinity has been exploited for more than 20 years in analytical biology, immunolabelling, affinity targeting, and biomolecular purification [2–5]. More recently, this technology has been applied in bioengineering and biomedicine, notably in drug targeting [4].

Inspired by Whitesides' work, our group has produced artificial metalloenzymes for enantioselective catalysis by anchoring an organometallic biotinylated catalyst inside (strept)avidin [6–9]. The biotin-(strept)avidin technology relies on the fact that derivatization of the valeric acid side chain of biotin does not interfere too significantly with the biotin-(strept)avidin affinity [1]. With the aim of performing random mutations of the (strept)avidin gene to produce selective artificial metalloenzymes, we require a convenient method to rapidly identify the functional (strept)avidin mutants from the crude cell extracts. Neither antibodies nor Coomassie blue staining fulfill these requirements.

Bayer and Wilchek [10, 11] reported that biotin stabilizes the tetrameric structure of (strept)avidin. This stabilization effect is observed at elevated temperatures and in SDS-PAGE [11]. This thermal stabilization is currently exploited as a tool for (strept)avidin characterization [12]. In the present study, we show that this stabilization effect can be extended to a biotinylated molecule which can be readily detected by optical methods.

Biotin-4-fluorescein is a convenient, commercially available probe used to quantify the number of active sites of purified biotin-binding proteins or to estimate their concentration in complex biofluids [13]. The quantification principle of this assay is based on the (partial) fluorescence quenching of biotin-4-fluorescein upon incorporation in (strept)avidin. In this work, we determine the thermal stabilization extent of the tetrameric structure of streptavidin, complexed with biotin-4-fluorescein. The biotin-4-fluorescein streptavidin adduct is detectable using a UV transilluminator and a comparison with Coomassie blue staining is made to determine the detection limits of both techniques. We apply the biotin-4-fluorescein on bacterial extracts of *Escherichia coli* expressing different streptavidin mutants and analyze these extracts in nondenaturing SDS-PAGE. We show that the presence of a fluorescent band in the gel, exposed to UV light, is correlated with the functionality of the mutants.

2 Materials and methods

2.1 Materials

Chemicals and media were purchased from Acros-Brunschwig (Basel, Switzerland), Sigma, Fluka (Buchs, Switzerland), or Gibco-Invitrogen (Basel, Switzerland). The 2-iminobiotin-agarose was provided by Affiland (Ans-Liège, Belgium). SDS-PAGE analysis was carried out with

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Abbreviations: IPTG, isopropyl β-D-1-thiogalactopyranoside; **LB**, Luria broth; **RT**, room temperature

Bio-Rad equipment (Bio-Rad, Reinach, Switzerland) [14]. The molecular mass of the expressed streptavidin mutants was determined by electrospray ionization mass spectrometry (ESI-MS) (VG platform mass spectrometer, Micromass instruments, Manchester, UK). The functionality of the recombinants proteins was assessed by fluorimetric titration using biotin-4-fluorescein (Molecular Probes – Juro, Luzern, Switzerland) [13].

2.2 Production of recombinant streptavidin

Heat-shock competent BL21(DE3)pLysS *E. coli* strain (Novagen-Juro, Luzern, Switzerland) were transformed by pET11b-SAV plasmid containing a gene encoding for recombinant streptavidin fused with the T7-tag [15–18]. Transformed bacteria were plated on Luria broth (LB) dishes [19] containing ampicilline (60 μg/mL), chloramphenicol (34 μg/mL) and glucose (1% w/v), and further incubated overnight at 37°C. The culture medium (150 mL total, 20 g/L bactotryptone, 2 g/L Na_2HPO_4, 1 g/L KH_2PO_4, 8 g/L NaCl, 15 g/L bacto yeast extract) containing ampicilline (60 μg/mL), chloramphenicol (34 μg/mL), and glucose (1% w/v) were inoculated with a single colony. This preculture was incubated overnight on an orbital shaker (37°C, 230 rpm). High-cell-density fermentation was performed in a 15 L fermentor. The culture medium (10 L) containing ampicilline (60 μg/mL), chloramphenicol (34 μg/mL), and glucose (0.4% w/v), was inoculated with the whole preculture. The cells were grown at 37°C until an OD_{600} ~1.4–1.6 was reached. Then, isopropyl β-D-1-thiogalactopyranoside (IPTG) was added (final concentration, 0.4 mM). After 3 h induction, the cells were harvested (centrifugation at 3600 × *g*, 10 min, 4°C). The pellet was washed with Tris-suspension buffer (50 mL, 20 mM Tris-HCl, pH 7.4, 0.02% w/v sodium azide, 10 mM $MgCl_2$) and recentrifuged (3600 × *g*, 10 min, 4°C). The supernatant was discarded and the pellet was frozen at −80°C until purification.

2.3 Protein purification

The pellet was thawed and resuspended in Tris-suspension buffer (50 mL). Lyophilized DNase I (5 mg; Roche, Rotkreuz, Switzerland) was added and incubated at 25°C until total degradation of nucleic acids. The volume of resuspension was completed with 6 M guanidium hydrochloride pH 1.5 (final volume, 400 mL) and the sample was dialyzed against the same solution to fully denature the protein (24 h, room temperature (RT)). Two consecutive dialyses were performed (20 mM Tris-HCl, pH 7.4, followed by 50 mM Na_2CO_3, pH 9.8, 0.5 M NaCl) to renature the streptavidin and to prepare the proteic extract for affinity chromatography. The proteic extract was then centrifuged (20 000 × *g*, 30 min, 4°C), the pellet was washed with the above carbonate buffer (100 mL) and recentrifuged in the same conditions. The supernatant was filtered (0.45 μm filter) and applied on a 2-iminobiotin agarose column equilibrated at pH 9.8 (50 mM Na_2CO_3, 0.5 M NaCl). Pure streptavidin was eluted with acetic acid (0.1 M pH 2.9), immediately dialyzed against the Tris-buffer (10 mM Tris-HCl, pH 7.4, 24 h, 4°C), against distilled water and finally against bidistilled water. Following a final filtration (0.22 μm filter), the purified protein was frozen at −80°C, lyophilized, and stored at 4°C until use. The molecular weight of the purified protein was assessed by ESI-MS.

2.4 Site-directed mutagenesis

The streptavidin mutants' genes were obtained using the QuickChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA). The PCR reaction was performed in the presence of DMSO (5%). The cycle conditions were: initial denaturation (94°C, 5 min), followed by 16 cycles of 1 min at 94°C; 1 min at 65°C; 15 min at 68°C. The final extension was performed at 68°C for 1 h.

After analysis, the template (nonmutated plasmid) was digested by *Dpn*I (1 h at 37°C). An aliquot was used to transform the XL1-blue *E. coli* strain. Plasmids were extracted from bacteria using Wizard®Plus Miniprep System (Promega-Catalys, Wallisellen, Switzerland) and were sequenced by Synergene (Schlieren, Switzerland).

2.5 Minicultures of streptavidin mutants

Heat-shock competent BL21(DE3)pLysS *E. coli* strain (Novagen – Juro) was transformed by wild-type, empty or mutant plasmids and plated on LB dishes containing ampicilline (60 μg/mL), chloramphenicol (34 μg/mL), and glucose (1% w/v), and further incubated overnight (37°C). The culture medium (1 mL) containing the antibiotics and glucose in the same proportions was inoculated with a single colony and incubated overnight at 37°C. This preculture (80 μL) was used to inoculate the culture medium (4 mL) containing antibiotics in same proportions and glucose (0.4% w/v) in 50 mL falcon tubes. After 3 h of incubation at 37°C under orbital shaking, IPTG was added (final concentration, 0.4 mM). The bacteria were grown for 2 h and centrifuged (5 min, 5000 × *g*). The culture medium was removed and the pellet was stored at −20°C until use.

2.6 Nondenaturing and denaturing SDS-PAGE

Each experiment was performed in 16% acrylamide-bisacrylamide separating gels. The stacking gels contained 5% of acrylamide-bisacrylamide. The denatur-

ing conditions refer to heating the samples in the presence of a denaturing loading buffer (50 mM Tris-HCl, pH 6.8, 1% w/v SDS, 2% v/v β-mercaptoethanol, 10% w/v sucrose, 0.006% w/v bromophenol blue). The nondenaturing conditions refer to heating the samples without the loading buffer. Following this treatment, the nondenaturing loading buffer (same formulation as above except for SDS and β-mercaptoethanol) was added prior to loading the samples in the gels.

2.7 Heat stability experiments

The purified streptavidin (12 μg) was combined with biotin-4-fluorescein (1 μL of a 0.6 mM stock solution) and incubated under gentle stirring (15 min, RT) to ensure complete binding of the biotinylated compound. The samples were then incubated at the selected temperature for 15 min and were then loaded in SDS-PAGE. Following the migration, the gel was stained by Coomassie Brilliant Blue [19].

2.8 Determination of the detection limit of streptavidin with biotin-4-fluorescein

A solution of purified wild-type streptavidin (10 mg/mL) was combined with biotin-4-fluorescein (1 μL of a 0.6 mM stock solution), incubated 15 min at RT under gentle stirring, and successively diluted to obtain a concentration ranging from 10 μg to 20 ng (in 15 μL). The nondenaturing loading buffer was added and the protein was loaded on SDS-PAGE. After migration, the gel was immediately analyzed by UV exposition in a transilluminator. For comparison, the gel was stained with Coomassie Brilliant Blue.

2.9 Analysis of the minicultures

The cell pellets were thawed and resuspended in the Tris-suspension buffer (600 μL) containing DNase I (1 μL of a stock solution at 1 mg/mL), and incubated until the nucleic acids were thoroughly digested. The samples were centrifuged (5 min, 16 000 × *g*, RT) and the supernatants were transferred in to a new tube. The supernatants (12 μL) were combined with biotin-4-fluorescein (1 μL of 0.6 mM) and equilibrated under gentle stirring (15 min, RT). The nondenaturing loading buffer was then added and the samples were loaded in SDS-PAGE. Revealing was first performed by UV exposition and then by Coomassie Brilliant Blue staining.

3 Results and discussion

3.1 Production and mutation of recombinant streptavidin

Using the protocol described above, we routenely produce wild-type and mutant streptavidin at high concentration levels (up to 200 mg of protein per liter cell culture) in *Escherichia coli*. The DNA sequencing and mass spectral analysis of the protein unambiguously confirm the gene sequence and the molecular weight of the corresponding purified protein [16].

3.2 Stabilization of streptavidin by biotin-4-fluorescein in denaturing and nondenaturing SDS-PAGE

As biotin is not readily detectable under UV-visible illumination, we sought for biotinylated molecules which can be revealed by this simple procedure [11]. For this purpose, we tested the biotinylated chromophore biotin-4-fluorescein (Fig. 1). Figure 2A depicts the behavior of wild-type streptavidin in nondenaturing SDS-PAGE. The protein appears as a tetramer with a small quantity of oligotetramers at low temperatures. At 72°C, the oligotetramers start to disappear with the concomitant appearance of monomers (and dimers to a small extent). The presence of SDS during the heat-denaturation contributes to decrease the denaturation temperature to 63°C (Fig. 2B).

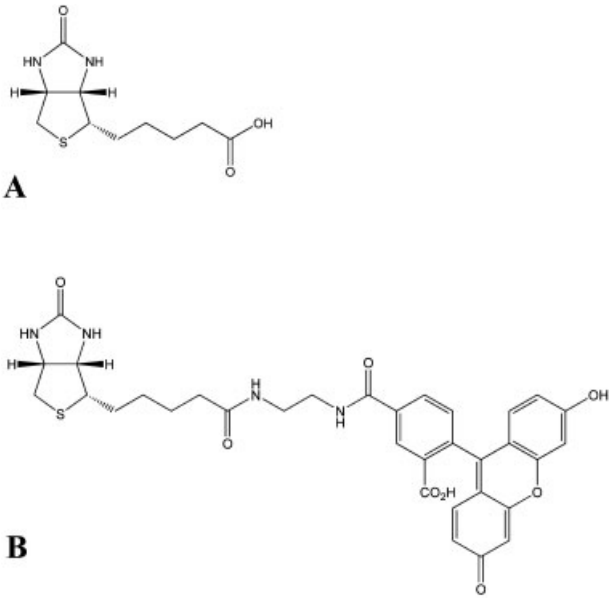

Figure 1. Structure of (A) (+)-biotin and (B) biotin-4-fluorescein.

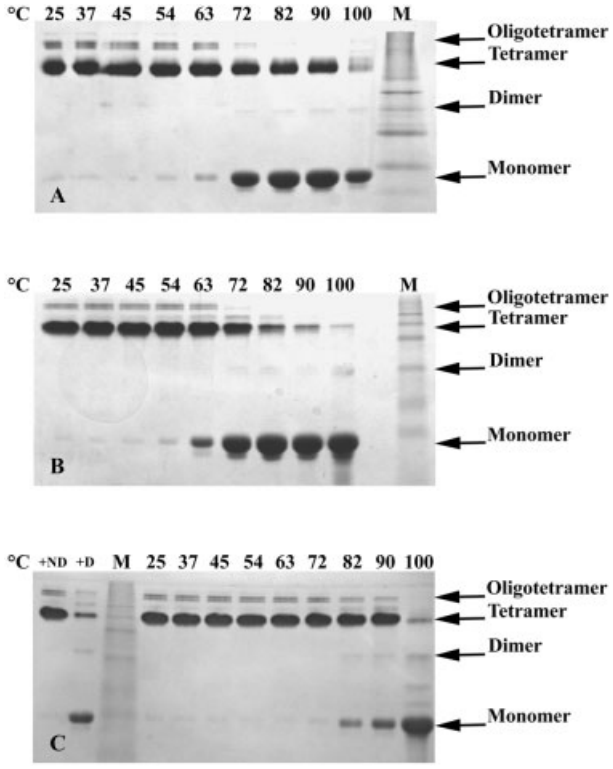

Figure 2. SDS-PAGE of (A) apo-streptavidin under nondenaturing conditions, (B) apo-streptavidin under denaturing conditions, and (C) streptavidin complexed with biotin-4-fluorescein under nondenaturing conditions exposed to increasing temperatures. The proteins were incubated 15 min at RT with or without biotin-4-fluorescein and heated at the indicated temperature for 15 min. M, BioRad Prestained Protein Marker Broad Range; +ND, nondenatured apostreptavidin; +D, denatured apostreptavidin. The gels were stained with Coomassie Brilliant Blue.

As shown by Wilchek and co-workers, the presence of biotin stabilizes the tetrameric form of streptavidin [11]: the tetramer denaturation occurs above 100°C. The same trend is observed with biotin-4-fluorescein (Fig. 2C), but the tetramer to monomer transition appears at lower temperatures (*i.e.*, 72°C in the presence of SDS and 82°C under nondenaturing conditions).

3.3 Comparison of the detection limit of the protein by Coomassie blue staining and by biotin-4-fluorescein under nondenaturing conditions

In order to detect streptavidin in SDS-PAGE using an UV transilluminator, we exploit the biotin-4-fluorescein fluorescent properties (λ_{ex} = 485 nm, λ_{em} = 525 nm). Figure 3 presents a comparison between the fluorescence detect-

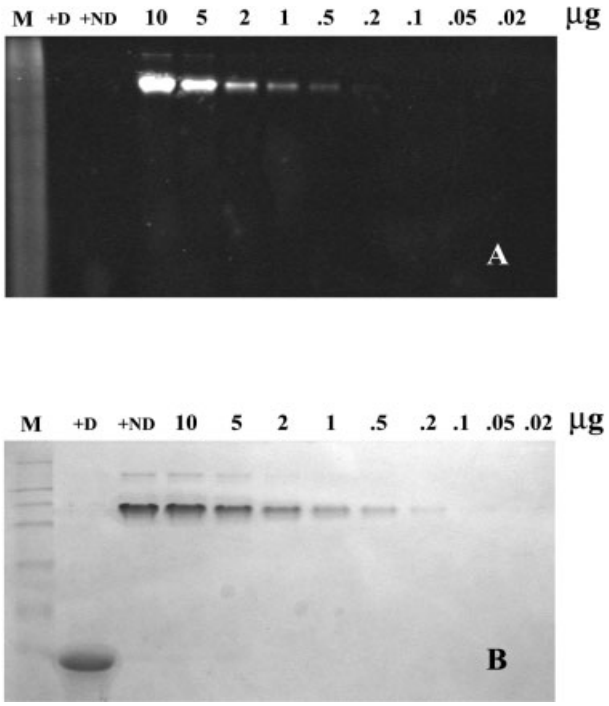

Figure 3. Comparison of the detection limit of streptavidin complexed with biotin-4-fluorescein in nondenaturing SDS-PAGE, by fluorescence and Coomassie blue staining. Decreasing amounts of the biotin-4-fluorescein streptavidin adduct were incubated 15 min at RT and loaded in the SDS-PAGE. The same gel was analyzed in UV transilluminator (A) and then stained with Coomassie Blue (B).

ed upon UV transillumination (Fig. 3A) and Coomassie blue staining (Fig. 3B) of samples with decreasing quantities of purified streptavidin containing biotin-4-fluorescein, loaded in an SDS-PAGE. In both cases, the streptavidin appears as a tetramer with small amounts of oligotetramer. The presence of a fluorescence signal under the UV transilluminator clearly shows that biotin-4-fluorescein remains bound to streptavidin during migration on the gel. As can be judged from Fig. 3, the detection limit for both methods is comparable.

Upon incorporation in (strept)avidin, the fluorescence of biotin-4-fluorescein is partially quenched [13]. However, sufficient fluorescence is maintained to allow the detection of the biotin-4-fluorescein streptavidin adduct in the gel without any further time-consuming manipulation after electrophoresis. In contrast with Coomassie blue staining or Western blotting, this detection provides a functional screen for the biotin-binding activity. These results suggest that the incorporation of biotin-4-fluorescein does not induce any significant shift in the position of the bands corresponding to the tetramer (Fig. 3B, lanes +ND and 10 µg). As molecular weight markers cannot be detected using UV-transillumination, we rely on the use of

purified functional wild-type streptavidin, complexed with biotin-4-fluorescein as positive size marker (Fig. 3B, lanes +ND and 10 μg).

3.4 Detection of functional streptavidin mutants in bacterial extract

The biotin-binding activity of streptavidin present in complex biofluids is detectable exploiting the fluorescence of biotin-4-fluorescein [13]. We adapted this protocol to SDS-PAGE conditions as it can readily be fitted to high-throughput screening of DNA-shuffled mutants. For this purpose, biotin-4-fluorescein was added to bacterial extracts expressing four different streptavidin isoforms: wild-type, V47G, K80G, and N81P. Each mutant displays different characteristics compared to the wild-type protein. The V47G mutant is a functional protein which proved difficult to quantify in a cuvette using the biotin-4-fluorescein protocol (Fig. 4) [13]. However, it is readily purified by affinity chromatography on iminobiotin, suggesting that this mutant indeed has a strong affinity for (imino)biotin. The residue V47 is located in loop 3,4 close to the biotin-binding site. Its backbone amide has an H-bonding interaction with S45 which is in contact with biotin [20]. The production of N81P proved problematic as it was expressed exclusively in the form of inclusion bodies. Residue N81 is located in loop 5,6 close to the biotin binding site. Substitution of an amino acid by a proline is known to have a profound effect on loop conformation [21]. The mutant K80G was expressed mainly as inclusion bodies but, in contrast to N81P, it can be purified by affinity chromatography and quantified using the biotin-4-fluorescein titration protocol. The K80G mutation was designed to suppress a positive charge in loop 5,6 and to introduce flexibility, thus possibly influencing the adjacent W79 which is involved in biotin-streptavidin interaction [22].

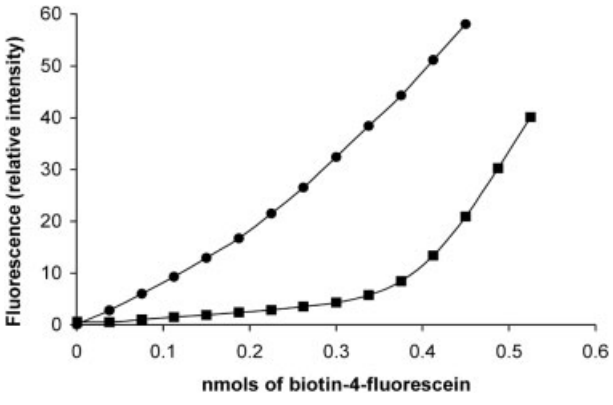

Figure 4. Biotin-binding activity assay for (■) purified recombinant wild-type streptavidin and (●) V47G mutant. The titration was performed with 50 μL of a 2 μM protein solution.

Figure 5 displays the SDS-PAGE as revealed by UV transilluminator, containing different mutants treated with biotin-4-fluorescein prior to loading. In lane 4, the wild-type streptavidin appears as a fluorescent band in the top of the gel, suggesting the presence of a tetrameric protein. As a blank, lane 5 displays the bacterial extract (treated with biotin-4-fluorescein) from a clone devoid of the streptavidin gene. No fluorescence is observed, thus demonstrating that biotin-4-fluorescein does not interact with other proteins or bacterial fragments. The streptavidin mutant V47G displays the same pattern as wild-type streptavidin (lane 6), suggesting that indeed the mutant is functional. This observation, combined with the fact that the titration protocol is not applicable to this mutant, suggests that biotin-4-fluorescein is indeed incorporated within V47G but its fluorescence is not quenched (Fig. 4). The inability of N81P to bind biotin derivatives is confirmed as no band appears in lane 7. This observation suggests that the inactivity of the mutant is due to misfolding. Although not functional, this mutant is revealed by Western blot. In lane 8, the mutant K80G exhibits a retarded migration compared to the other proteins with similar molecular weight. This migration pattern was further confirmed using purified K80G (lane 9), thus excluding an effect due to bacterial fragments. Biotin-4-fluorescein applied to a protein extract from *E. coli* expressing streptavidin mutants confirms that this fluorophore is a useful tool that allows to quickly asses the biotin-binding properties of streptavidin mutants prior to large-scale production. Moreover, no interference with other proteins or other cellular fragments is observed.

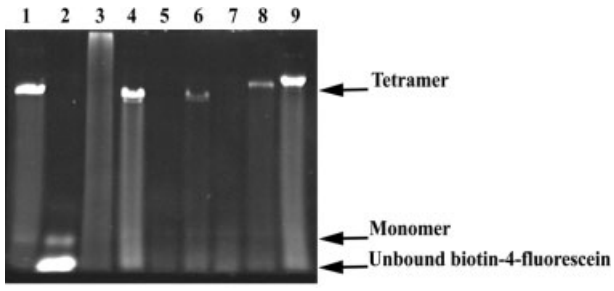

Figure 5. UV revealing of biotin-4-fluorescein streptavidin-mutants adducts present in bacterial extracts. Biotin-4-fluorescein (1 μL of a 0.6 mM solution) was applied to the *E. coli* soluble extract. Samples were equilibrated with gentle stirring during 15 min and loaded in SDS-PAGE (nondenaturing conditions). Lanes 1 and 2, non-denatured and denatured purified wild-type streptavidin; lane 3, BioRad Prestained Protein Marker Broad Range; lanes 4–8, bacterial extracts of *E. coli* transformed with wild-type plasmid (4), empty plasmid (5), V47G mutant (6), N81P (7), and K80G (8); lane 9, purified K80G mutant.

4 Concluding remarks

Biotin has long been known to favor the folding of (strept)avidin under various denaturing conditions provided by (i) extreme pH values, (ii) high concentrations of urea or of guanidium hydrochloride, (iii) detergents, and (iv) high temperatures [23, 24]. The results presented herein shed light on the stabilizing effect of a biotinylated chromophore towards thermal- and detergent-assisted denaturation of streptavidin as reflected by the electrophoretic behavior of the streptavidin in SDS-PAGE. Most importantly, the biotin-4-fluorescein assay presented here allows to assess the biotin-binding properties and to roughly estimate the amount of functional streptavidin in a bacterial extract without any interference caused by the presence of cellular fragments. The behavior of four representative streptavidin isoforms is presented, but this technique was applied to over 20 mutants. The semiquantitative results, concerning the state of aggregation and the activity, are complementary to those obtained with other time-consuming detection protocols, such as Western blot and titration of biotin-binding sites using a spectrofluorimeter. We are currently using this protocol towards the development of DNA-shuffled streptavidin genes.

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5 References

[1] Wilchek, M., Bayer, E. A., *Methods Enzymol.* 1990, *184*, 5–13.

[2] Graf, R., Friedl, P., *Anal. Biochem.* 1999, *273*, 291–297.

[3] Nakamura, M., Tsumoto, K., Ishimura, K., Kumagai, I., *Anal. Biochem.* 2002, *304*, 231–235.

[4] Wilchek, M., Bayer, E. A., *Biomol. Eng.* 1999, *16*, 1–4.

[5] Wilchek, M., Bayer, E. A., *Anal. Biochem.* 1988, *171*, 1–32.

[6] Wilson, M. E., Whitesides, G. M., *J. Am. Chem. Soc.* 1978, *100*, 306–307.

[7] Lin, C. C., Lin, C. W., Chan, A. C. S., *Tetrahedron Asym.* 1999, *10*, 1887–1893.

[8] Collot, J., Gradinaru, J., Humbert, N., Skander, M., Zocchi, A., Ward, T. R., *J. Am. Chem. Soc.* 2003, *125*, 9030–9031.

[9] Skander, M., Humbert, N., Collot, J., Gradinaru, J., Klein, G., Loosli, A., Sauser, J., Zocchi, A., Gilardoni, F., Ward, T. W., *J. Am. Chem. Soc.* 2004, *126*, 14411–14418.

[10] Williams, G. J., Domann, S., Nelson, A., Berry, A., *Proc. Natl. Acad. Sci. USA* 2003, *100*, 3143–3148.

[11] Bayer, E. A., Ehrlich-Rogozinski, S., Wilchek, M., *Electrophoresis* 1996, *17*, 1319–1324.

[12] Hytonen, V. P., Laitinen, O. H., Grapputo, A., Kettunen, A., Savolainen, J., Kalkkinen, N., Marttila, A. T., Nordlund, H. R., Nyholm, T. K., Paganelli, G., Kulomaa, M. S., *Biochem. J.* 2003, *372*, 219–225.

[13] Kada, G., Falk, H., Gruber, H. J., *Biochim. Biophys. Acta* 1999, *1427*, 33–43.

[14] Laemmli, U. K., *Nature* 1970, *227*, 680–685.

[15] Zocchi, A., Humbert, N., Berta, T., Ward, T. R., *Chimia* 2003, *57*, 589–592.

[16] Gallizia, A., de Lalla, C., Nardone, E., Santambrogio, P., Brandazza, A., Sidoli, A., Arosio, P., *Prot. Expr. Purif.* 1998, *14*, 192–196.

[17] Sano, T., Cantor, C. R., *Proc. Natl. Acad. Sci. USA* 1990, *87*, 142–146.

[18] Zocchi, A., Jobe, A. M., Neuhaus, J. M., Ward, T. R., *Prot. Expr. Purif.* 2003, *32*, 167–174.

[19] Sambrook, J., Russel, D. W., *Molecular Cloning. A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Plainview, NY 2001.

[20] Klumb, L. A., Chu, V., Stayton, P. S., *Biochemistry* 1998, *37*, 7657–7663.

[21] Chou, P. Y., Fasman, G. D., *Biochemistry* 1974, *13*, 222–245.

[22] Freitag, S., Le Trong, I., Chilkoti, A., Klumb, L. A., Stayton, P. S., Stenkamp, R. E., *J. Mol. Biol.* 1998, *279*, 211–221.

[23] Gonzalez, M., Argarana, C. E., Fidelio, G. D., *Biomol. Eng.* 1999, *16*, 67–72.

[24] Green, N. M., *Adv. Prot. Chem.* 1975, *29*, 85–133.