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One-step affinity purification of bacterially produced proteins by means of the "Strep tag" and immobilized recombinant core streptavidin

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Abstract

The "Strep tag" is a nine amino acid peptide with intrinsic streptavidin-binding activity. If this sequence is genetically fused to the C-terminus of a polypeptide the recombinant protein can be directly purified by affinity chromatography from the host cell extract on immobilized streptavidin. However, variations were observed in the suitability of different commercial streptavidin–agarose preparations for this purpose. Therefore, the influence of the source of streptavidin, the coupling chemistry, and the nature of the affinity chromatography resin was investigated. A procedure was developed for the production of recombinant core streptavidin in *Escherichia coli*, followed by its efficient refolding and purification with an overall yield of up to 140 mg functional protein per 1 l bacterial culture. When coupled to activated CH-Sepharose 4B this truncated form of streptavidin showed a performance in the affinity chromatography of Strep tag fusion proteins that was superior to all other combinations tested. In contrast to its conventional preparation from *Streptomyces* strains the recombinant core streptavidin was produced without a proteolytic processing step. Thus, deleterious effects during the streptavidin affinity purification of proteins due to residual proteolytic activity in the immobilized streptavidin were avoided. The versatility of the optimized purification system was demonstrated with five different Strep tag fusion proteins.

1. Introduction

The development of generic purification techniques for recombinant proteins has gained recent interest, particularly because the biochemical characteristics of a gene product are often unknown. In this respect, the use of a short peptide tag with defined affinity properties has the advantage that it does not necessarily interfere with the function of the protein and, therefore, its removal is not needed for in vitro applications [1].

Recently, we described the engineering of a C-terminal peptide tag with intrinsic streptavidin-binding activity, which was termed "Strep tag" [2]. This affinity peptide was shown to be suitable for the efficient single-step purification of a bacterially produced, functional antibody F_v fragment on immobilized streptavidin using mild competitive elution with biotin or its analogues. Furthermore, the Strep tag was employed for the direct and specific detection of the

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protein —on a Western blot or in an enzyme-linked immunosorbent assay (ELISA)— using a commercially available streptavidin–enzyme conjugate.

However, during routine use of this protein purification system significant variations in the ability to bind the Strep tag were observed between different preparations of streptavidin–agarose, even among charges from the same manufacturer. Generally, the biochemical activity of streptavidin is defined and quantified according to its capacity for the binding of biotin, which is not necessarily a valid criterion for the binding of the Strep tag. Therefore, a streptavidin affinity matrix had to be developed with optimized properties for this novel application. The parameters that had to be considered were the composition of the solid support, the coupling chemistry for the immobilization of streptavidin, and, most importantly, the preparation of streptavidin itself.

Commercially produced streptavidin consists of a N- and C-terminally shortened form, called core streptavidin [3], comprising the sequence from Ala^{13} or Glu^{14} to Ala^{138} or Ser^{139} of the mature polypeptide. Core streptavidin is produced via an undisclosed proteolytic digestion protocol from the protein that is naturally secreted by *Streptomyces* strains. In contrast to the full length form, it shows high solubility, reduced tendency towards oligomerization and is extremely resistant to further degradation. In addition, Bayer et al. [4] demonstrated that its binding activity for biotinylated proteins —e.g. alkaline phosphatase— is significantly enhanced, probably due to improved accessibility of the ligand-binding pocket.

Remarkably, an affinity matrix produced with commercial core streptavidin led to degradation during purification of a Strep tag fusion protein, most likely as a result of residual proteolytic activity that was still present in the streptavidin preparation. Therefore, the production of recombinant core streptavidin using *Escherichia coli* as expression host was established together with a simple refolding and purification procedure yielding highly pure and active protein. With this material we developed a standardized purification protocol for Strep tag fusion proteins and present here its application in several examples.

2. Experimental

2.1. Materials

Streptomyces avidinii (ATCC 27419) was obtained from the American Type Culture Collection. *E. coli* strain BL21(DE3) carrying a chromosomal copy of the T7 RNA polymerase gene [5] was provided by H. Reiländer (Max-Planck-Institut für Biophysik, Frankfurt/Main, Germany). *E. coli* K12 strain JM83 (*ara*, Δ(*lac-pro*AB), *rps*L, *Φ*80, *lac*ZΔM15) [6], which was used for the expression of Strep tag fusion proteins, was from our own collection. N-Hydroxysuccinimide (NHS)-activated CH-Sepharose 4B, epoxy-activated Sepharose 6B, as well as Q-Sepharose were purchased from Pharmacia (Uppsala, Sweden), Eupergit C and Eupergit C30N were from Röhm Pharma (Weiterstadt, Germany). The plasmid pLysE for constitutive expression of T7 lysozyme, a natural inhibitor of T7 RNA polymerase [5], was from AMS Biotechnology (Lugano, Switzerland). Commercial streptavidin preparations were samples from Società Prodotti Antibiotici (Milan, Italy) and Amresco (Solon, OH, USA).

2.2. Protein characterization

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) was performed using standard slab gel methodology with the buffer system of Fling and Gregerson [7], followed by staining with Coomassie Brilliant Blue R 250. The samples were heated to 95°C for 5 min prior to loading onto the gel.

The molar extinction coefficient of recombinant core streptavidin, ϵ_{280}, was determined as 35600 M^{-1} cm^{-1} from measurements of A_{205} and A_{280} according to Scopes [8] with a Perkin-Elmer Lambda 15 UV–Vis spectrophotometer. The measurements were performed in 10 m*M* potassium phosphate pH 7.6 at 20°C and the

peptide bond absorbance at 205 nm was corrected for the tryptophan and tyrosine content. The value agreed almost with that published for proteolytically prepared core streptavidin [9].

The biotin-binding capacity of the recombinant core streptavidin was determined by measuring the ligand-induced quenching of the protein tryptophan fluorescence [10,11]. A 1 μM protein solution (1.8 ml) in 50 mM Tris · HCl pH 8.0 was placed in a quartz cuvette thermostatted at 22°C and a total of 130 μl 20 μM biotin in the same buffer was added in increments of 5 μl. After thorough mixing the tryptophan fluorescence (excited at 295 nm and detected at 350 nm, averaged over 1 s) was measured at each step with a Perkin-Elmer LS50 fluorimeter. All solutions were initially cleared from particles by sterile filtration.

2.3. Preparation and comparison of streptavidin affinity matrices

NHS-Activated CH-Sepharose 4B, epoxy-activated Sepharose 6B, Eupergit C and Eupergit C30N were coupled with 2 mg streptavidin per 1 ml swollen material under conditions as recommended by the suppliers. The binding of a Strep tag fusion protein was then investigated by means of a batch assay. A 15-μl volume of each affinity matrix was dispersed in 50 μl of periplasmic bacterial cell extract containing the D1.3 F_v fragment with the Strep tag fused to the V_H domain [2]. After centrifugation and removal of the supernatant, the material was washed three times with 50 μl 50 mM Tris · HCl pH 8.0 and then incubated with 50 μl of the same buffer containing 1 mM biotin for release of the bound F_v fragment. Finally, the resin was suspended in gel loading buffer and heated for the recovery of residual adsorbed protein. The supernatant from each step was analyzed by SDS-PAGE.

The matrix which was utilized for affinity purification was prepared as follows. NHS-Activated CH-Sepharose 4B was swollen and washed as recommended by the manufacturer. The supernatant was drained and the gel was mixed with twice its volume of a 2.5 mg/ml solution of streptavidin (for the immobilization of 5 mg streptavidin per ml gel) that had been dialyzed against 100 mM sodium carbonate pH 8.0, 500 mM NaCl. After 2 h of gentle shaking at room temperature coupling was complete. The supernatant was decanted and the gel was mixed with 5 volumes of 100 mM Tris · HCl pH 8.0 for the blocking of residual activated groups (overnight at 4°C).

2.4. Preparation of streptavidin from Streptomyces avidinii

Natural streptavidin was isolated from *S. avidinii* as described by Cazin et al. [12] and Suter et al. [13]. After ammonium sulfate precipitation of the protein from the culture supernatant and dissolution of the precipitate in water the sample was further purified via ion-exchange chromatography on Q-Sepharose in 50 mM sodium borate pH 8.5. Essentially pure streptavidin was eluted with 100 mM sodium phosphate pH 7.0.

2.5. Construction of the expression vector pSA1 for the production of recombinant core streptavidin in E. coli

The structural gene encoding core streptavidin was amplified by polymerase chain reaction (PCR) from chromosomal DNA of *S. avidinii* ATCC 27419 using *Pfu* DNA polymerase (Stratagene, Heidelberg, Germany) and the oligodeoxynucleotides 5′-GAT ATA *CAT ATG* GAA GCA GGT ATC ACC GGC ACC TGG TAC and 5′-CGG ATC *AAG CTT* ATT AGG AGG CGG CGG ACG GCT TCA C with phosphorothioate bonds at their 3′ termini according to Skerra [14]. The italicized *Nde*I and *Hin*dIII restriction sites were used for direct insertion of the gene fragment into the appropriately cut T7 expression vector pRSET5a [15] following standard methodology [16]. The resulting plasmid pSA1 is depicted in Fig. 1 together with the amino acid sequence of the gene product. The three codons following the start methionine codon —which was positioned in front of the core streptavidin sequence— were modified at their wobble positions for optimal

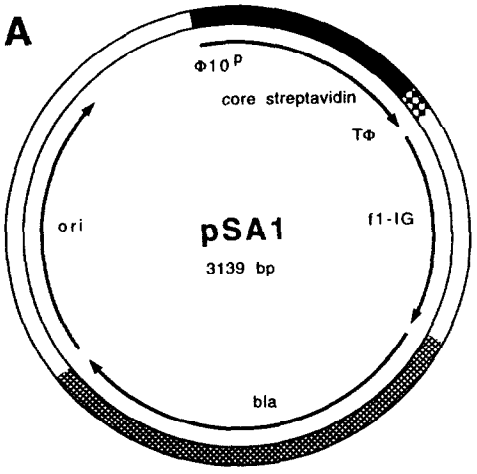

Fig. 1. (A) The expression plasmid pSA1. The structural gene encoding core streptavidin was placed under transcriptional control of the gene 10 promoter of the bacteriophage T7 and ends upstream of the TΦ terminator [5]. Additional vector elements comprise the origin of replication (ori), the ampicillin resistance gene (bla), and the f1 origin of replication for the preparation of single-stranded plasmid DNA (f1-IG) [15]. (B) Amino acid sequence of pre-streptavidin. The signal sequence, which directs secretion of streptavidin in the original host, *S. avidinii*, is underlined [18]. Numbering of the mature sequence starts at 1. The sequence of core streptavidin encoded on pSA1 begins with Glu^{14} and ends with Ser^{139} and is printed in bold. It has an N-terminal fMet residue added as indicated.

initiation of translation [17]. Otherwise the cloned nucleotide sequence was identical to that published by Argaraña et al. [18] as reconfirmed by dideoxy sequencing [19] of the whole insert.

2.6. E. coli expression, refolding and purification of core streptavidin

A single colony of *E. coli* BL21(DE3) freshly transformed with the plasmids pLysE and pSA1 was used for inoculating 20 ml Luria-Bertani (LB)-broth containing 30 μg/ml chloramphenicol and 100 μg/ml ampicillin. After incubation overnight at 37°C the culture was transferred to 1 l of the same medium in a shaking flask prewarmed at 37°C. At an A_{550} of 0.6 expression was induced by the addition of 0.5 m*M* isopropyl-β-D-thiogalactopyranoside (IPTG) (final concentration) and shaking was continued for 16 h. Cells were harvested by centrifugation (4200 *g*, 15 min, 4°C) and washed with 25 ml ice-cold 50 m*M* Tris·HCl pH 8.0, 500 m*M* sucrose. After centrifugation (20 000 *g*, 30 min, 4°C) the cells were resuspended in 15 ml ice-cold 50 m*M* Tris·HCl pH 8.0, 1 m*M* EDTA and passed three times through a French pressure cell at 16 000 p.s.i. (1 p.s.i. = 6894.76 Pa). The homogenate was centrifuged (20 000 *g*, 30 min, 4°C) in order to sediment the streptavidin inclusion bodies. After washing with 10 ml ice-cold 50 m*M* Tris·HCl pH 8.0, 1 m*M* EDTA the inclusion bodies were dissolved in 8 ml 6 *M* guanidine·HCl (practical grade, Sigma, Deisenhofen, Germany) pH 1.5. The solution was dialyzed twice for 4 h in the cold against 100 ml 6 *M* guanidine·HCl pH 1.5 in order to remove traces of biotin [20]. Refolding was then accomplished by rapid dilution into 250 ml of phosphate-buffered saline (PBS) (4 m*M* KH_2PO_4, 16 m*M* Na_2HPO_4, 115 m*M* NaCl) at 4°C. The mixture was incubated for a minimum of 3 h at 4°C and cleared by centrifugation (20 000 *g*, 30 min, 4°C). Solid ammonium sulfate was slowly added in the cold to a saturation of 40% (62.7 g). After incubation for 3 h at 4°C precipitated contaminating proteins and monomeric, incompletely refolded core streptavidin were removed by centrifugation (20 000 *g*, 30 min, 4°C). Then, the ammonium sulfate saturation of the supernatant was raised to 70% (adding 59 g). After overnight incubation at 4°C, the precipitated tetrameric core streptavidin was recovered (20 000 *g*, 60 min, 4°C) and resuspended in 10 ml PBS-buffered 2.2 *M* ammonium sulfate for removal of residual impurities, which became soluble under these conditions. After centrifugation (20 000 *g*, 30 min, 4°C) the recombinant core streptavidin was dissolved in PBS buffer. The solution was finally cleared by centrifugation (30 000 *g*, 30 min, 4°C) and stored at 4°C. Routinely, between 1.2 and 1.4 g purified core

streptavidin were obtained from 10 l *E. coli* culture in this way.

2.7. *E. coli* expression of Strep tag fusion proteins

In order to demonstrate their purification via streptavidin affinity chromatography five different Strep tag fusion proteins were produced: the antibody D1.3 F_v fragment with the Strep tag fused to the V_H domain [2], retinol-binding protein (RBP) [21], chicken egg-white cystatin [22], *Pseudomonas aeruginosa* azurin [23] and *Escherichia coli* cytochrome b_{562} [24]. The structural genes were introduced into the bacterial expression vector pASK60-Strep [2] except for the F_v fragment, whose production was accomplished using the plasmid pASK68-D1.3 (an optimized version of pASK46-p111 [2] with improved control elements that will be described elsewhere). All proteins became thus fused to the *Omp*A signal peptide —directing secretion to the periplasm of *E. coli*— at their N-terminus and to the Strep tag —comprising the amino acid sequence Ser–Ala–Trp–Arg–His–Pro–Gln–Phe–Gly–Gly— at their C-terminus (cf. the cloning strategies previously outlined [2]). The resulting mature polypeptide sequences were as follows: RBP: Glu^{1} to Cys^{174} plus two intervening Pro residues and the Strep tag; cystatin: Gly^{9} to Gln^{116} plus the Strep tag; azurin: Ala^{1} to Lys^{128} plus the Strep tag; cytochrome b_{562}: Ala^{1} to Arg^{106} plus two intervening Pro residues and the Strep tag. For protein production, a 100-ml culture of *E. coli* JM83 harbouring the appropriate expression plasmid was grown at 22°C (37°C for cytochrome b_{562}) in LB medium containing 100 μg/ml ampicillin (with the addition of 50 μg/ml hemin (Sigma) in the case of cytochrome b_{562}). At an A_{550} of 0.5 IPTG was added to a final concentration of 0.5 m*M* (expression conditions for RBP were modified due to the presence of a second plasmid, pASK61, as described by Müller and Skerra [21]). After 3 h of induction, cells were collected (4200 *g*, 15 min, 4°C) and the pellet was resuspended in 1 ml ice-cold 100 m*M* Tris · HCl pH 8.0, 500 m*M* sucrose, 1 m*M* EDTA and kept on ice for 30 min. The spheroplasts were removed by centrifugation (microfuge, 4°C, 15 min) and the supernatant was recovered as the periplasmic cell fraction.

2.8. Streptavidin affinity chromatography of Strep tag fusion proteins

A column containing 0.5 ml CH-Sepharose 4B coupled with 5 mg/ml recombinant core streptavidin was equilibrated with 5 ml 100 m*M* Tris · HCl pH 8.0, 1 m*M* EDTA. Then the periplasmic cell fraction (1 ml) containing the Strep tag fusion protein was applied and the column was washed with up to 5 ml 100 m*M* Tris · HCl pH 8.0, 1 m*M* EDTA (no EDTA in the case of the metallo-proteins azurin and cytochrome b_{562}). The bound recombinant protein was specifically eluted with 2 ml 1 m*M* biotin dissolved in the same buffer. The Strep tag fusion protein was almost quantitatively recovered in a volume of 1 ml under these conditions. All steps were performed at 4°C using gravity flow. Alternatively, a solution of 5 m*M* diaminobiotin (Sigma) was used for elution, permitting regeneration of the affinity column by washing with 10 ml 100 m*M* Tris · HCl pH 8.0.

3. Results and discussion

3.1. Production of functional core streptavidin using *E. coli* as expression host

For expression in *E. coli* the structural gene encoding residues Glu^{14} to Ser^{139} of streptavidin was PCR-amplified from *S. avidinii* chromosomal DNA and inserted into the T7 promoter vector pRSET5a [15] yielding pSA1 (Fig. 1). Expression conditions were essentially as described earlier by Sano and Cantor [20] for a genetic fusion between the non-truncated streptavidin and the N-terminal part of the T7 gene 10 protein. For the preparation of the correctly folded, tetrameric core streptavidin from the inclusion bodies an optimized refolding and purification procedure was developed (Fig. 2A). The method is simple, without a chromatograph-

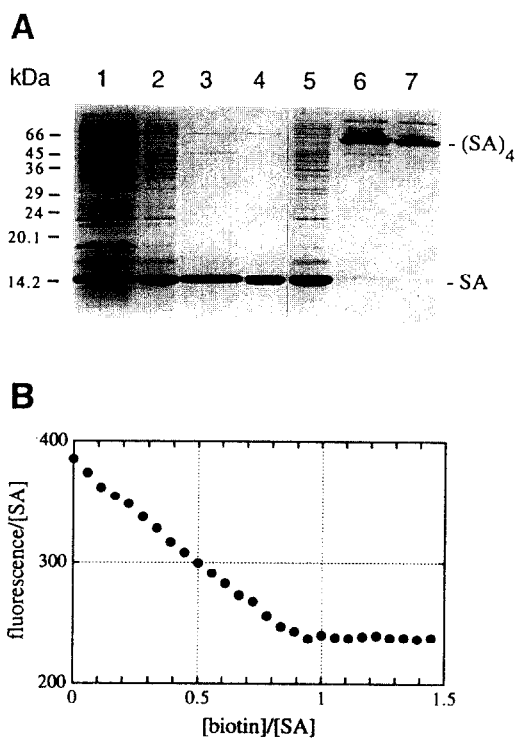

Fig. 2. (A) Purification and refolding of the recombinant core streptavidin. A 15% SDS-PAGE is shown with samples (equivalent portions) from different stages during the preparation. Lanes: 1 = total cell protein of *E. coli* BL21(DE3)(pLysE)(pSA1) 16 h after induction of expression; 2 = sedimented inclusion bodies; 3 = protein solution after renaturation; 4 = purified core streptavidin after fractionated ammonium sulfate precipitation; 5–7 = same samples as in 2–4 without heat treatment prior to SDS-PAGE. Under these conditions the core streptavidin tetramer remains intact [26]. Thus the correctly folded state of the recombinant protein in the final preparation was confirmed (lane 7) whereas small amounts of monomeric streptavidin were still present after refolding (lane 6). kDa = kilodalton, SA = streptavidin. (B) Biotin binding assay with the recombinant core streptavidin. The tryptophan fluorescence is plotted versus the relative amount of biotin added. The two almost linear segments of the curve were fitted separately and from the intercept a ratio of 0.88 bound biotin molecules per core streptavidin monomer was calculated.

ic step, can be easily scaled up and leads to pure and highly soluble core streptavidin, which can be directly used for coupling to, e.g., NHS-activated CH-Sepharose 4B. The final yield of the functional core streptavidin was up to 140 mg per 1 l *E. coli* culture.

Sequencing of the four N-terminal residues revealed that the start methionine residue was not removed by the Met-aminopeptidase as could have been expected from the presence of glutamic acid as the penultimate residue [25]. In order to assess the biochemical activity of the *E. coli*-produced core streptavidin its biotin-binding capacity was determined by fluorescence titration. Reproducibly a value of 0.88 bound biotin molecules per subunit was found (Fig. 2B).

3.2. Choice of the activated resin for the coupling of streptavidin

Four different activated chromatography supports were tested: (i) NHS-activated CH-Sepharose 4B, (ii) epoxy-activated Sepharose 6B, (iii) Eupergit C (epoxy-activated) and (iv) Eupergit C30N (epoxy-activated). After coupling with recombinant core streptavidin the affinity matrices were compared in a batch assay for the specific binding of a bacterially expressed F_v fragment with the Strep tag fused to the C-terminus of the V_H domain [2] from periplasmic protein extract. It was found that the epoxy-activated Sepharose 6B did not bind the Strep tag fusion protein at all whereas the remaining affinity matrices bound the protein equally well (data not shown). However, the two Eupergit supports gave rise to a substantial background of non-specifically bound host cell proteins that were slowly released throughout the assay. A similar effect was not observed for the NHS-activated CH-Sepharose 4B, which thus appeared to be the support of choice for the streptavidin affinity chromatography of Strep tag fusion proteins.

3.3. Comparison of differently prepared streptavidin samples

Initially it was attempted to use natural streptavidin produced by *S. avidinii* in order to prepare affinity matrices that could be used for the purification of Strep tag fusion proteins. The protein was isolated from the culture supernatant of this Gram-positive bacterium and coupled to NHS-activated CH-Sepharose 4B. However,

when the periplasmic *E. coli* extract containing the F_v fragment with the Strep tag was applied to the affinity column only modest retardation was observed with respect to other periplasmic proteins.

This finding was in contrast to earlier results obtained with streptavidin–agarose purchased from Biomol (Hamburg, Germany) under otherwise unchanged conditions, where almost all of the recombinant F_v fragment bound specifically to the column and was then eluted in pure form with 2-iminobiotin [2]. The diminished affinity for the Strep tag observed here was thus attributed to the presence of N- and C-terminal extensions in the natural streptavidin compared to commercial preparations, which consist of the proteolytically truncated core streptavidin [3,4,26]. The improved sterical accessibility of the biotin-binding site in core streptavidin [4] might also be important for the binding of the Strep tag, which is likely to occupy the same pocket on the streptavidin surface [27].

Consequently, two commercial preparations (SPA and Amresco) of core streptavidin were used for coupling to NHS-activated CH-Sepharose 4B. In both cases satisfactory binding activity was observed in purification experiments with several different Strep tag fusion proteins. Unexpectedly however, when a larger amount of immobilized streptavidin was used (5 mg streptavidin per ml gel, SPA or Amresco) significant proteolytic degradation of the Strep tag fusion protein was observed (Fig. 3A). The proteolytic activity, that was obviously present in the affinity matrix (and not in the protein extract), could be inhibited by treatment of the streptavidin Sepharose with phenylmethanesulfonyl fluoride (PMSF) prior to use. Most likely, a contamination with proteases from the preparation process for core streptavidin was responsible for this effect.

In contrast, no protein degradation was detected during the purification of several different Strep tag fusion proteins (see below) when the *E. coli*-derived recombinant core streptavidin was employed (Fig. 3B). Thus, the combination of recombinant core streptavidin with NHS-activated CH-Sepharose 4B as support (cf. above)

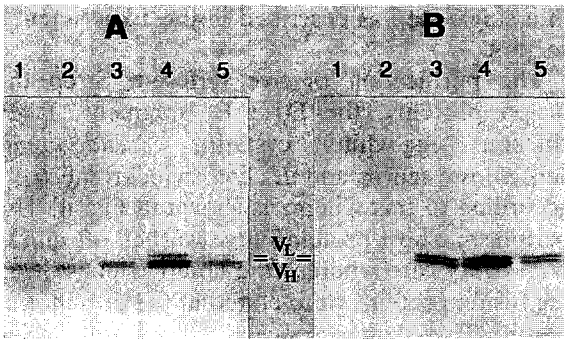

Fig. 3. Proteolytic degradation during purification of a Strep tag fusion protein using immobilized commercial core streptavidin (A) compared to recombinant core streptavidin (B). Affinity chromatography was performed with a periplasmic *E. coli* cell extract containing the D1.3 F_v fragment with the Strep tag at the C-terminus of the V_H domain. The streptavidin was coupled to NHS-activated CH-Sepharose 4B at 5 mg protein per ml gel in each case. Both chromatography experiments were performed in parallel under identical conditions (essentially similar as in the optimized protocol described in the Experimental section). Aliquots from five consecutive fractions collected during elution of the bound F_v fragment were subjected to 15% SDS-PAGE (lanes 1–5). Only small quantities of the intact V_L domain could be detected in (A) (lane 4) and a likely degradation product eluted almost constantly over the range investigated. In contrast elution of stoichiometric amounts of the two immunoglobulin domains V_H and V_L, which make up the F_v fragment, was observed in (B).

led to optimal performance in the streptavidin affinity chromatography of Strep tag fusion proteins.

3.4. A standardized protocol for the purification of Strep tag fusion proteins

In order to permit the rapid purification of different Strep tag fusion proteins on a small scale a standardized procedure was established. For cloning and expression the previously described vector pASK60-Strep [2] was used. pASK60-Strep was designed for secretion of the gene product into the periplasm of *E. coli* and carries a cassette for insertion of the corresponding structural gene permitting concomitant C-terminal fusion with the plasmid-encoded Strep tag. After induction of expression and preparation of the periplasmic cell extract streptavidin affinity chromatography was performed

in a single step as described in the Experimental section.

The purification of five different Strep tag fusion proteins, the D1.3 F_v fragment, RBP, chicken egg-white cystatin, *Pseudomonas aeruginosa* azurin and *E. coli* cytochrome b_{562}, according to this scheme is shown in Fig. 4. The periplasmic host proteins were washed off quickly in every case whereas the homogeneous Strep tag fusion proteins were eluted in the presence of biotin. This demonstrates the low non-specific binding of the affinity matrix. For cytochrome b_{562} weaker retardation on the column was observed but this protein could still be specifically eluted in concentrated form when the amount of washing buffer was reduced (see Fig. 4E). Due to the intense red colour of the holo-protein it was possible to follow visibly its binding and elution so that the cytochrome can be used for the quick control of newly prepared batches of the streptavidin Sepharose.

The streptavidin affinity chromatography was performed under native buffer conditions and a solution of 1 m*M* biotin was usually used for the elution of the bound Strep tag fusion protein. However the extremely high affinity of this ligand for streptavidin prevented re-use of the column. This could be avoided when a solution of 5 m*M* diaminobiotin, a much weaker-binding chemical analogue of biotin [28], was used instead. With this compound elution of the Strep tag fusion protein was only slightly less efficient (Fig. 5) and the column could be regenerated several times without loss of activity. The use of diaminobiotin turned out to be a clear improvement compared to 2-iminobiotin [2] or lipoic acid described before in the case of the purification of the bilin-binding protein [29].

In conclusion all components necessary for the Strep tag purification methodology were optimized and its successful application was demonstrated in a number of cases. Both the recombinant production of core streptavidin in *E. coli* and the use of diaminobiotin for elution in the chromatography make an important contribution to the economic aspect of this method, permitting its use even on a larger scale.

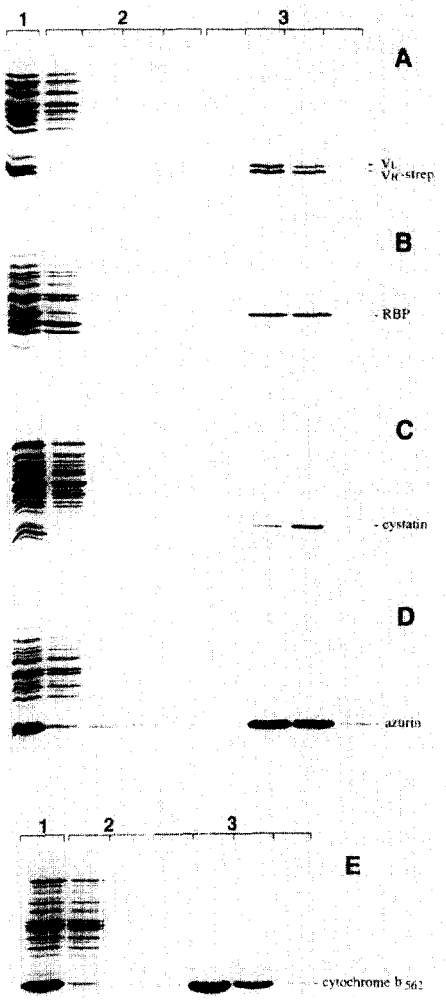

Fig. 4. The one-step purification of various Strep tag fusion proteins via streptavidin affinity chromatography: the D1.3 F_v fragment (A), retinol-binding protein (B), chicken egg-white cystatin (C), *Pseudomonas aeruginosa* azurin (D) and *E. coli* cytochrome b_{562} (E). Chromatography was performed under standardized conditions as described in the Experimental section. After application of the periplasmic *E. coli* cell extract containing the Strep tag fusion protein the column was washed with 5 ml buffer and the effluent was collected in four fractions of 1.25 ml. Then the bound protein was eluted with 1 m*M* biotin and four fractions of 0.5 ml were collected. 20-μl samples of each fraction were subjected to 15% SDS-PAGE. Lanes: 1 = periplasmic protein solution; 2 = washing fractions; 3 = elution fractions. In the case of the cytochrome (E) only 2 ml buffer were used for washing and the effluent was collected in two fractions of 1 ml. Then the bound protein was eluted as above.

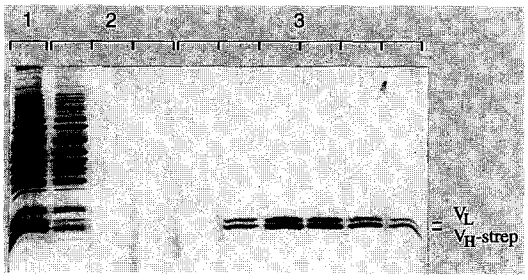

Fig. 5. Use of diaminobiotin for elution of the bound Strep tag fusion protein. The streptavidin affinity chromatography of the bacterial periplasmic cell extract containing the D1.3 F_v fragment was performed as in Fig. 4A except that 3 ml 5 m*M* diaminobiotin was used for elution. Samples (20 μl) of each fraction were subjected to 15% SDS-PAGE. Lanes: 1 = periplasmic protein solution; 2 = washing fractions (fractions 2 and 3 were pooled); 3 = elution fractions.

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References

[1] H.M. Sassenfeld, *Trends Biotechnol.*, 8 (1990) 88.
[2] T.G.M. Schmidt and A. Skerra, *Prot. Eng.*, 6 (1993) 109.
[3] A. Pähler, W.A. Hendrickson, M.A. Gawinowicz Kolks, C.E. Argaraña and C.R. Cantor, *J. Biol. Chem.*, 262 (1987) 13933.
[4] E.A. Bayer, H. Ben-Hur, Y. Hiller and M. Wilchek, *Biochem. J.*, 259 (1989) 369.
[5] F.W. Studier, A.H. Rosenberg, J.J. Dunn and J.W. Dubendorff, *Methods Enzymol.*, 185 (1990) 60.
[6] C. Yanisch-Perron, J. Vieira and J. Messing, *Gene*, 33 (1985) 103.
[7] S.P. Fling and D.S. Gregerson, *Anal. Biochem.*, 155 (1986) 83.
[8] R.K. Scopes, *Anal. Biochem.*, 59 (1974) 277.
[9] N.M. Green, *Methods Enzymol.*, 184 (1990) 51.
[10] N.M. Green, *Biochem. J.*, 90 (1964) 564.
[11] C.F. Chignell, D.K. Starkweather and B.K. Sinha, *J. Biol. Chem.*, 250 (1975) 5622.
[12] J. Cazin, Jr., M. Suter and J.E. Butler, *J. Immunol. Methods*, 113 (1988) 75.
[13] M. Suter, J. Cazin, Jr., J.E. Butler and D.M. Mock, *J. Immunol. Methods*, 113 (1988) 83.
[14] A. Skerra, *Nucleic Acids Res.*, 20 (1992) 3551.
[15] R. Schoepfer, *Gene*, 124 (1993) 83.
[16] J. Sambrook, E.F. Fritsch and T. Maniatis, *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 2nd ed., 1989.
[17] T.D. Schneider, G.D. Stormo, L. Gold and A. Ehrenfeucht, *J. Mol. Biol.*, 188 (1986) 415.
[18] C.E. Argaraña, I.D. Kuntz, S. Birken, R. Axel and C.R. Cantor, *Nucleic Acids Res.*, 14 (1986) 1871.
[19] S. Tabor and C.C. Richardson, *Proc. Natl. Acad. Sci. U.S.A.*, 84 (1987) 4767.
[20] T. Sano and C.R. Cantor, *Proc. Natl. Acad. Sci. U.S.A.*, 87 (1990) 142.
[21] H.N. Müller and A. Skerra, *J. Mol. Biol.*, 230 (1993) 725.
[22] E.A. Auerswald, G. Genenger, I. Assfalg-Machleidt, J. Kos and W. Bode, *FEBS Lett.*, 243 (1989) 186.
[23] G.W. Canters, *FEBS Lett.*, 212 (1987) 168.
[24] H. Nikkila, R.B. Gennis and S.G. Sligar, *Eur. J. Biochem.*, 202 (1991) 309.
[25] P.-H. Hirel, J.-M. Schmitter, P. Dessen, G. Fayat and S. Blanquet, *Proc. Natl. Acad. Sci. U.S.A.*, 86 (1989) 8247.
[26] E.A. Bayer, H. Ben-Hur and M. Wilchek, *Methods Enzymol.*, 184 (1990) 80.
[27] P.C. Weber, M.W. Pantoliano and L.D. Thompson, *Biochemistry*, 31 (1992) 9350.
[28] N.M. Green, *Adv. Protein Chem.*, 29 (1975) 85.
[29] F.S. Schmidt and A. Skerra, *Eur. J. Biochem.*, 219 (1994) 855.