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Expression and purification of a recombinant avidin with a lowered isoelectric point in *Pichia pastoris*

Andrea Zocchi,[a,*] Anna Marya Jobé,[b] Jean-Marc Neuhaus,[c] and Thomas R. Ward[a,*]

[a] *Institut de Chimie, Université de Neuchâtel, Av. de Bellevaux 51, CH-2000 Neuchâtel, Switzerland*
[b] *Département de Chimie, Laboratoire de Génie Chimique et Biologique, Ecole Polytechnique Fédérale de Lausanne, CH-1024 Lausanne-Ecublens, Switzerland*
[c] *Laboratoire de Biochimie, Institut de Botanique, Université de Neuchâtel, Rue E. Argand 9, CH-2000 Neuchâtel, Switzerland*

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Abstract

A recombinant glycosylated avidin (recGAvi) with an acidic isoelectric point was expressed and secreted by the methylotrophic yeast *Pichia pastoris*. The coding sequence for recGAvi was de novo synthesized based on the codon usage of *P. pastoris*. RecGAvi is secreted at approximately 330 mg/L of culture supernatant. RecGAvi monomer displays a molecular weight of 16.5 kDa, as assessed by ESI mass spectrometry. N-terminal amino acid sequencing indicates the presence of three additional amino acids (E-A-E), which contribute to further lowering the isoelectric point to 5.4. The data presented here demonstrate that the *P. pastoris* system is suitable for the production of recGAvi and that the recombinant avidin displays biotin-binding properties similar to those of the hen-egg white protein.

Keywords: Biotin–avidin technology; Low isoelectric point avidin; *Pichia pastoris*; High-cell-density fermentation; High-level expression

Avidin, a glycoprotein found in avian, reptilian, and amphibian egg white, was first noticed for its antinutritive effects [1,2]. The active form of hen-avidin is a homotetramer composed of four singly glycosylated subunits. Each monomer can bind a (+)-biotin molecule with an extraordinary affinity ($K_a = \sim 10^{14}\ M^{-1}$) [2]. (+)-Biotin, which is commonly known as Vitamin H, is an essential nutrient for most organisms. Since the growth of various micro-organisms is inhibited by avidin, it is believed that the protein's primary function is as a host defense factor [3]. Recent studies demonstrate that avidin and some avidin-related genes are induced during inflammation [4]. Moreover, the evidence that avidin binds specifically heparin [5] and possibly its natural counterparts, heparan sulfate proteoglycans that are activated and secreted as part of normal defenses to injury, stress or inflammation, reinforces the hypothesis that avidin plays a critical role in the innate immune response.

The primary sequence of avidin (128 amino acids) was determined in 1971 [6]. Kulomaa and co-workers [7,8] documented the cDNA of the chicken oviduct avidin gene and isolated a genomic clone. The first X-ray structure of the biotin–avidin complex reported by Bayer and co-workers [9], allowed to rationalize the strength of the biotin–avidin interaction. The remarkable stability of the biotin–avidin complex can be traced back primarily to the following features:

(i) the depth of the biotin-binding pocket;
(ii) the H-bonding interactions between the polar amino acid side chain residues (N12, S16, Y33, and Y40) and the ureido ring;
(iii) the van-der-Waals contacts between the bicyclic framework and aromatic amino acids of the protein (W70, F72, F79, W97, and W110 from the adjacent monomer) [9,10].

The primary source for the commercial production of avidin is the extraction and purification from chicken egg white. More recently, biologically active recombinant

* Corresponding authors. Fax: +21-32-718-22-01.
E-mail addresses: andrea.zocchi@unine.ch (A. Zocchi), thomas.ward@unine.ch (T.R. Ward).

isoforms have been produced in several expression systems, including *Escherichia coli* [11,12], Baculovirus-infected cells [13] and maize [14].

The biotin–avidin technology has been utilized during the last three decades for various biotechnological applications, ranging from diagnostics to targeting various materials both in vivo and in vitro [15]. However, the high-isoelectric point of avidin ($pI = 10.5$) limits its field of applications. The production of either genetically [16,17] or chemically [18] modified avidin allows one to overcome this problem. For example, the existence of monomeric isoforms [19] makes avidin an ideal partner for the expression of fusion proteins. A non-glycosylated acidic mutant is particularly useful in affinity-based drug targeting [17]. Moreover, avidin clones with a neutral or slightly acidic isoelectric point were reported to be expressed at higher levels in different expression systems [16,20].

Yeast offers an attractive alternative to the previously reported expression systems for avidin. *Saccharomyces cerevisiae* has been used extensively as a host for the expression of foreign genes. This expression system combines the advantages of microbial organisms with those of eukaryotic organisms, namely the formation of disulfide bridges and the glycosylation. However, the frequent low production yields and the difficulties in secreting some proteins have led to the investigation of substitutes, such as the expression system based on *Pichia pastoris* [21].

We are interested in producing artificial metalloenzymes based on the biotin–avidin technology [22]. The possibility, given by *P. pastoris*, to obtain active avidin directly in the medium provides an attractive alternative to other expression systems, thus allowing one to screen directly the culture medium as well as to conveniently perform directed-evolution experiments.

Here, we report on the use of the methylotrophic yeast *P. pastoris* to produce and secrete in its medium an easily purifiable, fully active recombinant avidin.

Materials and methods

Synthesis of the gene

The amino acid sequence of an avidin with an isoelectric point of 7.2, which contained the K3E, K9D, R122A, and R124A mutations [16], was back-translated selecting the preferred codons in *P. pastoris* [23,24] (http://www.kazusa.or.jp/codon). A non-glycosylated variant of this avidin was produced first. In this case, the asparagine of the N-glycosylation site at position 17 [17] was replaced by an isoleucine (N17I mutation). The resulting coding strand (387 bp) was subdivided into six fragments chosen to minimize the formation of secondary structures (Fig. 1). Five shorter oligomers, on the antisense strand, were synthesized across couples of adjacent coding fragments, complementary with at least 15 bases to their ends, except for their 3′-base, to avoid a priming action in the following PCR. All coding fragments, except the first, were 5′-phosphorylated. All fragments, oligomers, and primers were purchased from Microsynth, Switzerland.

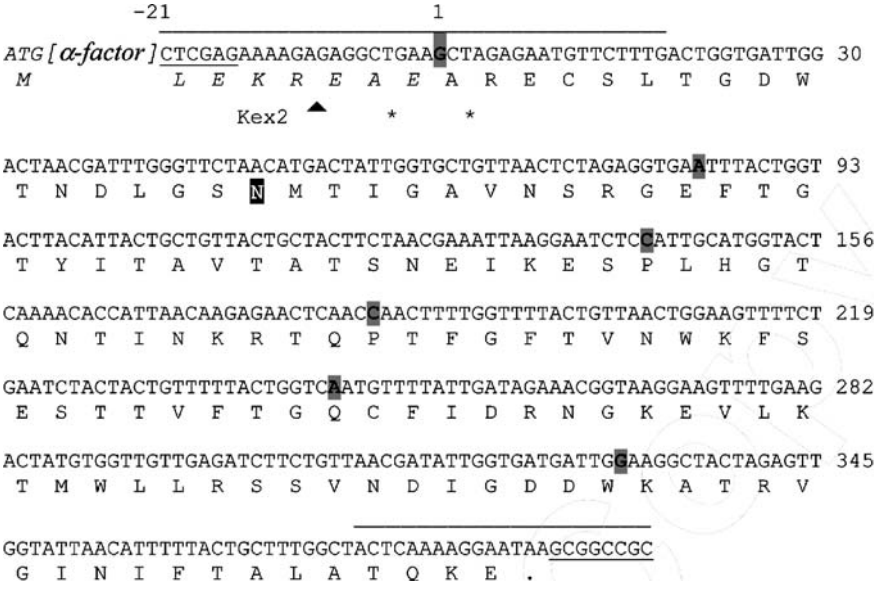

Fig. 1. Sequence of the artificial avidin reading frame (recGAvi) and its translation. The sequences in italic are vector-derived and encode part of the yeast α-factor. The beginning of each synthetic fragment used in the synthesis is highlighted by a dark box. The *Xho*I (CTCGAG) and *Not*I (GCGGCCGC) sites are underlined. The position of the PCR primers is indicated by a line above the DNA sequence. The sequences upstream of the codon 1 and downstream of the stop codon (TAA) have been introduced by the primers. The effective cleavage of *KEX2* site is indicated by an arrow, the ineffective *STE13* sites by asterisks. The asparagine of the N-glycosylation site is highlighted in a black box.

An equimolar solution of fragments and oligomers in standard T4-DNA ligase buffer [25] without adenosine-triphosphate (ATP) and dithiothreitol (DTT) was prepared. The tube containing the solution was heated at 98 °C for 10 min, to denature secondary structures, and gently cooled to room temperature (RT) to anneal coding fragments with antisense oligomers. ATP, DTT, and T4-DNA ligase were subsequently added to perform a ligation [25]. The ligation reaction was carried out for 24 h at RT. A high-fidelity PCR (Expand High Fidelity PCR system; Roche, Rotkreuz, Switzerland), with primers containing *Xho*I and *Not*I restriction sites (underlined in Fig. 1), was performed to obtain enough material for cloning and sequencing. The forward primer (upperlined in Fig. 1) also contained a sequence encoding the *KEX2* and *STE13* proteolytic sites, placed upstream of the mature avidin-coding sequence. The glycosylation site was reintroduced (vide supra) by high-fidelity PCR (Expand High Fidelity PCR system), utilizing a 90 base oligomer as the 5′ primer. This primer included the C-terminal part of the α-factor coding sequence and a part of the mature avidin (bases from −21 to +67, Fig. 1).

Construction of the expression vector and transformation

The DNA fragment encoding the recombinant avidin was inserted in-frame between the *Xho*I site, located in the sequence coding for the α-factor signal peptide, and the *Not*I site of *P. pastoris* expression vector pPIC 9K (Invitrogen, Basel, Switzerland). This cloning step required the use of a *Sac*I site from the vector and a ligation of three DNA fragments in order to leave intact the second *Xho*I site of the plasmid. All enzymes and buffers were purchased from Promega-Catalys, Wallisellen, Switzerland, and the reaction conditions were set as advised by the manufacturer.

Electrocompetent cells of *P. pastoris* GS115 (*his4*) were transformed by electroporation with the expression plasmid pPIC 9K—recGAvi or pPIC 9K—recNGAvi (recombinant non-glycosylated avidin = recNGAvi; recombinant glycosylated avidin = recGAvi), which had been linearized with *Sal*I [21]. Transformants were screened for His^+ Mut^+ phenotype [26,27].

Screening transformants for high-level expression

The filter-based colony immunodetection method, termed ''yeastern blot'' [28], was used as a preliminary screen to semi-quantitatively detect the clones expressing the highest levels of recombinant avidin.

The selected best clones, after two pre-cultures in YPD (yeast extract 10 g/L, tryptone 20 g/L, and glucose 20 g/L), were cultured in 125 mL of buffered YPD (YPD, 6 mL/L of 1 M K_2HPO_4, 40 mL/L of 1 M KH_2PO_4) medium supplemented with 20 μL of sterile antifoam (SIGMA, Buchs, Switzerland). The cultures were incubated at 30 °C under shaking at 200 rpm. When the cultures reached an $OD_{600} = \sim 30$ (about 24 h), cells were collected, washed twice with sterile distilled water, and resuspended into 125 mL of buffered YPMetOH (containing 10 mL/L methanol instead of glucose). The expression was carried out for 4–5 days. Methanol (1.2 mL) and antifoam were added every 12 h. The clone producing the most avidin, as detected by SDS–PAGE as well as biotin-4-fluorescein titration of binding sites, was utilized for the fermentation.

Laboratory-scale high-cell-density fermentation

The fermentation was carried out in a laboratory reactor (Bioengineering, Wald, Switzerland) of 3.6 L total volume. The dissolved oxygen in the culture medium was kept at least at 20% of its saturation value by adding air and pure oxygen to the inlet gas flow. The total inlet gas flow was kept at 4 L/min. The pH was kept constant by addition of 28% NH_4OH. The temperature and the stirring rate were kept constant throughout the fermentation (30 °C and 1300 rpm, respectively).

All liquid inlet flows were quantified gravimetrically. The feed pump rate was controlled by a proportional integral derivative (PID) controller based on the derivative of the reservoir scale.

The inoculum was prepared through two pre-cultures. Initially, a colony of the best clone was inoculated into 20 mL YPD and incubated at 30 °C for 24 h at 300 rpm. Ten milliliters was then inoculated into 75 mL of fermentation basal salts medium [29], pH 5. Glycerol was present at a concentration of 20 g/L. This second pre-culture was also incubated for 24 h. Sixty milliliters of the second pre-culture were utilized to inoculate the fermentor containing 1 L of fermentation basal salts medium [29], 40 g/L, glycerol supplemented with 4.35 mL/L PTM1 solution [29] and 0.5 mL/L antifoam SAG 471.

After inoculation, the process was run until the glycerol was consumed (~23 h) [29]. During this phase, the pH was maintained at 5. In the following glycerol-fed phase, a continuous feed of 21 mg/h of 50% glycerol (w/v), supplemented with 12 mL/L PTM2 solution (same formulation as the PTM1 solution, but with biotin eplaced by 1 g/L thiamine HCl, 1 g/L pyridoxine HCl, 1 g/L alanine, 0.5 g/L riboflavin, and 0.2 g/L inositol), was provided. This phase lasted 5 h. During the glycerol fed-batch phase, the pH control was stopped, allowing the metabolic activity to lower the pH of the culture to 3. At this point, the pH-control was reactivated and set to 3 for the remainder of the fermentation. In the methanol fed-batch phase (induction phase), the methanol was also supplemented with 12 mL/L of PTM2 solution. The initial feed rate was 3.5 mg/h and was

gradually increased to 28 mg/h. The fermentation was stopped after about 65 h of induction (~100 h total fermentation time). The biomass measurements were performed on the dry cell weight.

Purification of recombinant avidin

The extracellular medium was diafiltrated and concentrated to 50 mL using a Vivaflow 200 system 30'000 MCWO (Sartorius, Sissach, Switzerland). During diafiltration, the medium was exchanged with a buffered solution at pH 9.8 containing 0.05 M $NaHCO_3$ and 0.5 M NaCl. The proteic solution was then centrifuged (47,000*g* for 20 min at 4 °C) and the supernatant was filtered again through a 0.45 μm filter. The resulting clear solution was applied on a 2-iminobiotin–agarose column. The protein was eluted applying 100 mM acetic acid, pH 2.9, [30]. The purified protein was immediately dialyzed against 10 mM Tris/HCl, pH 9.5, followed by two dialyses against distilled water. The protein solution was frozen at −80 °C, lyophilized, and stored at 4 °C.

Analytical methods

SDS–PAGE [31] and Western blot analysis [32–34] were carried out with Bio-Rad equipment (Bio-Rad, Reinach, Switzerland). Rabbit polyclonal anti-avidin antibodies and goat anti-rabbit IgG alkaline phosphatase-conjugated antibodies (both from SIGMA, Buchs, Switzerland) were utilized as primary and secondary antibodies, respectively. Non-denaturing SDS–PAGE was carried out as explained in [18].

The molecular mass of the expressed construct was determined by electrospray ionization mass spectrometry (ESI-MS) (VG platform mass spectrometer, Micromass instruments, Manchester, UK).

The N-terminal amino acid sequence was carried out using Eldman degradation in a pulsed-liquid-phase sequenator 477A from Applied Biosystems. The released amino acids were analyzed on-line by reversed phase high-pressure liquid chromatography (RP-HPLC).

The functionality of the recombinant avidin was checked by quantifying the protein's binding capacity to the biotin-4-fluorescein (Molecular Probe-Juro, Luzern, Switzerland) [35]. This protocol allows us to determine the concentration of the protein's active sites.

Results and discussion

Gene construction and plasmid modification

A gene encoding a recombinant avidin was synthesized by PCR using synthetic oligonucleotides. The resulting 426 bp coding sequence, encoding the proteolytic signals *KEX2* and *STE13* fused to the mature avidin, was obtained (Fig. 1). This fragment was cloned in-frame with the pro-peptide of the *S. cerevisiae* mating factor α, contained in the expression vector pPIC 9K. The insertion of this DNA in pPIC 9K replaced the original C-terminal proteolytic recognition sequence by a shorter one.

Automated DNA sequencing confirmed the presence of a single open reading frame coding for a translation product of 216 amino acids, consisting of the modified α-factor yeast secretion signal and the mature avidin.

Any sequence cloned in-frame with the C-terminal α-factor coding sequence, contained in pPIC 9K is expressed with additional (from 3 to 9) amino acids at the N-terminal (manufacturer's reference book). These amino acids derive from the proteolytic processing of the α-factor fused with the recombinant protein. We shortened the α-factor sequence in order to reduce the number of additional amino acids. The removal of this sequence eliminates both the *KEX2* and the *STE13* protease sites, which are required for the maturation of the recombinant protein. We thus reintroduced both the proteolytic sites *KEX2* (K-R) and *STE13* (E-A-E) upstream of the N-terminal region of mature recombinant avidin.

Expression and detection of recombinant avidin

After obtaining the artificial avidin sequence, it was introduced into two types of plasmids for extracellular expression (pPIC 9K and pHIL S1) following the strategy suggested by the manufacturer. The two recombinant plasmids were then electroporated into two *Pichia* strains (GS115 and SMD1168). The expression levels were estimated by "yeastern" and Western blots. The expression vector pPIC 9K in combination with *Pichia* strain GS115 (phenotype Mut^+) was selected for further use, as it afforded the highest yields.

The immunodetection on filters ("yeastern blot") was used to confirm the presence of the expression cassette in transformants displaying a His^+ Mut^+ phenotype. As shown in Fig. 2, the colonies expressing the recombinant avidin leave a dark spot on the nitrocellulose membrane. The colonies which reveal as the darkest stains were cultured in shaking flasks. The culture of the recombinant cells in shaking flasks was not only used to further screen for high-level expression clones, but also allowed a first characterization of the recombinant avidin.

The non-glycosylated avidin was produced first. However, the SDS–PAGE of aliquots of culture medium did not show the presence of any predominant protein, when stained with Coomassie brilliant blue. In addition, Western blotting (Fig. 3) suggested the presence of a truncated recombinant avidin smaller than 14.5 kDa, the computed molecular weight of the non-glycosylated amino acid sequence of a monomer. The

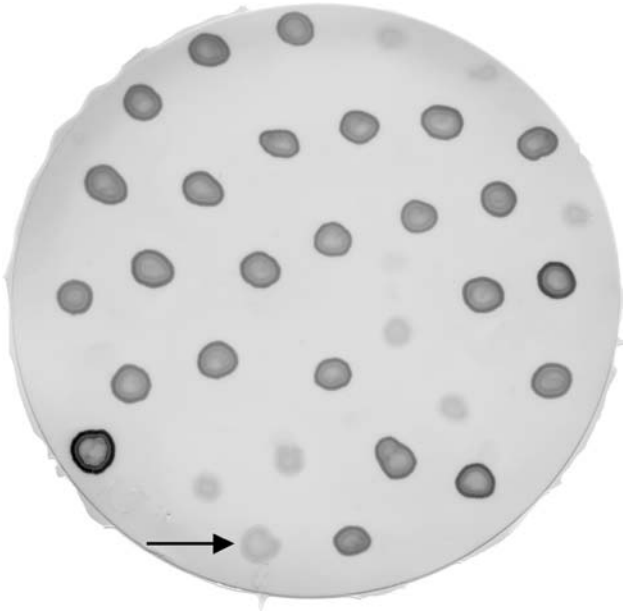

Fig. 2. Example of a "yeastern" colony blot screen. Darker spots indicate colonies expressing higher levels of recombinant avidin. The arrow indicates the stain of a colony of which was transformed with an empty pPIC 9K (negative control).

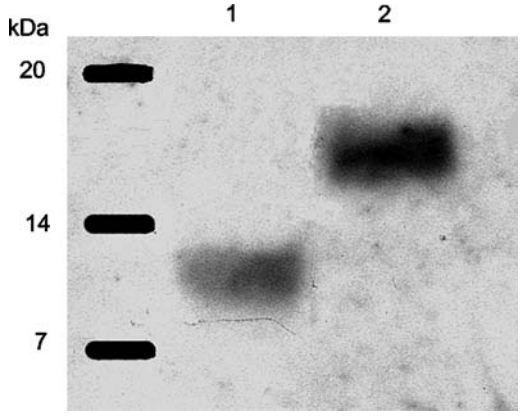

Fig. 3. Immunoblot analysis of the medium of a baffled Erlenmeyer flask cell-culture expressing the non-glycosylated recombinant avidin (after 105 h induction); (1) culture supernatant (20 μL); (2) wild-type avidin (10 ng).

large hydrophobic isoleucine at position 17, replacing the highly hydrophilic glycosylated asparagine, may cause misfolding and expose the N-terminal part of the protein to proteolytic degradation.

Considering these observations, the glycosylation site was reintroduced (Fig. 1). After production and screening of *P. pastoris* clones expressing the glycosylated avidin, the sample collected during induction indeed contained a protein with the expected size. Fig. 4 displays the SDS–PAGE analysis of the culture supernatant of the best clone, after staining with Coomassie brilliant blue. Immunoblot analysis revealed a single

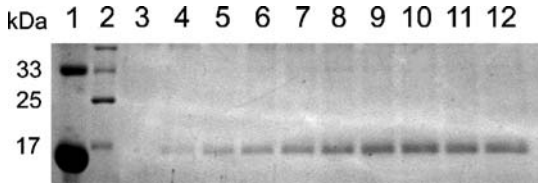

Fig. 4. SDS–PAGE of the medium of a baffled flask culture expressing the glycosylated recombinant avidin. Commercially available avidin was utilized as standard and 15 μL of culture supernatant was loaded at different induction times. (1) Wild-type avidin (40 μg): the high-molecular weight band corresponds to a dimeric form of avidin; (2) molecular mass markers; (3) sample before induction; (4) 12 h induction; (5) 24 h induction; (6) 36 h induction; (7) 48 h induction; (8) 60 h induction; (9) 72 h induction; (10) 84 h induction; (11) 96 h induction; and (12) 105 h induction.

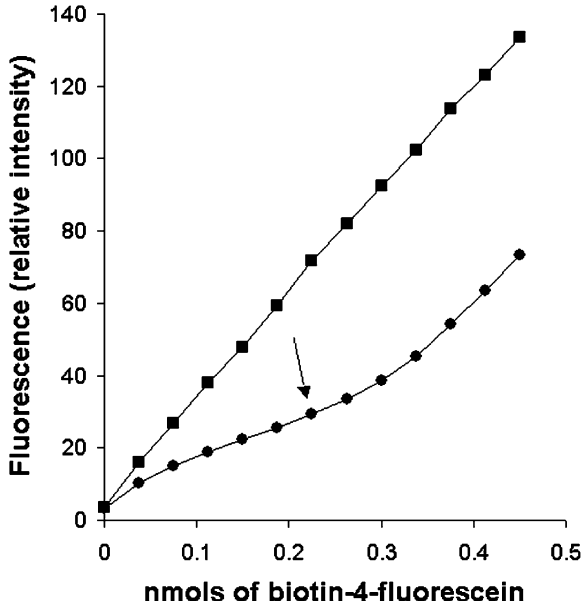

Fig. 5. Biotin-binding activity test for recombinant avidin in the medium of shaking flask cultures. Binding activity is detected by fluorescence quenching; the maximal quenching occurs at saturation of the tetravalent acceptor [35]. (■) Titration of 100 μl of culture supernatant the cells of which were transformed with empty pPIC 9K. (●) Titration of 100 μl of culture medium of one of the best clones. Both cultures were induced in 105 h. The arrow indicates the equivalence point (0.225 nmol of biotin-4-fluorescein, corresponding to 0.056 nmol of tetrameric avidin).

avidin band corresponding to the band in the SDS–PAGE.

The biotin-binding activity was confirmed by titrating the culture medium of *Pichia* cells bearing pPIC 9K—recGAvi with biotin-4-fluorescein. Fig. 5 shows the titration of the culture supernatant of the best clone. The equivalence point, observed at 0.225 nmols of biotin-4-fluorescein added to 100 μL of the culture medium, corresponds to 0.56 μM avidin (~37 mg/L), assuming a tetrameric form of the recombinant avidin, with four active-binding sites.

High-cell-density fermentation

To obtain a large amount of active protein, the most productive clone of the shaking flask cultures was selected for a fed-batch high-cell-density fermentation.

In contrast to standard *Pichia* fermentations, we supplemented the carbon source of both the glycerol fed- and the methanol fed-batch phases with PTM2 salts instead of PTM1 salts. The PTM2 salts did not contain biotin because its presence would severely hinder the subsequent affinity purification of avidin by blocking the binding sites. During glycerol fed- and methanol fed-batch phases, the pH of the culture was gradually reduced and maintained at pH 3. Such a low pH is known to inhibit neutral proteases secreted by *Pichia* [36].

During the initial glycerol-batch phase, the biomass increased exponentially to 21 g/L (Fig. 6), with a maximal specific growth rate (μ_{max}) of 0.24 h^{-1}. Under these conditions, the initial glycerol content was depleted within ~23 h. In the following phases, the biomass concentration increased linearly to attain 44.5 g/L at the end of the glycerol fed-batch phase and 160 g/L at the end of the fermentation (Fig. 6). After 65 h of induction (~100 h of total culture time), the fermentation was terminated and the medium was harvested. Indeed, even in the presence of small amounts of methanol and adding pure oxygen, the pO_2 remained under 20%, suggesting that the culture was too dense for an efficient oxygen transfer.

The biotin-binding activity of the culture medium was first determined 12 h after induction (~42 h of total culture time, 0.38 μM binding sites equivalent to ~6 mg/L of tetrameric avidin). Fig. 6 suggests that the biotin-4-fluorescein-binding response increases linearly throughout the induction phase to reach ~21 μM (corresponding to ~5.2 μM of tetrameric avidin or ~340 mg/L).

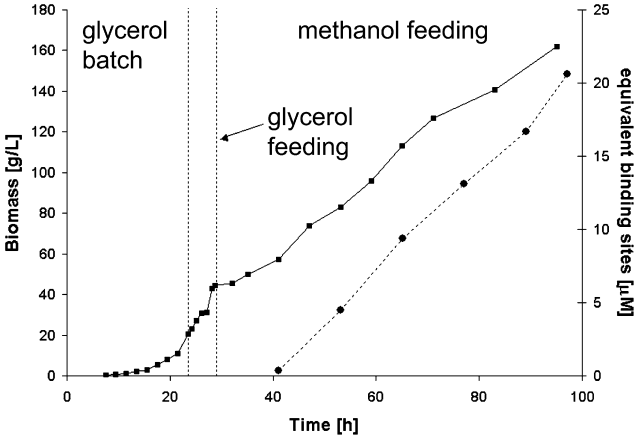

Fig. 6. High-cell-density fermentation of a clone expressing glycosylated recombinant avidin. (■) Dry-cell weight per liter of culture broth. (●) Concentration of biotin-binding sites in the medium (binding activity).

Characterization of recombinant avidin

The secreted recombinant avidin was purified from the high-density-cell culture by affinity chromatography on a Sepharose–iminobiotin column to yield ~330 mg/L (~5.1 μM) avidin, which appears as a white fluffy material.

The purified recombinant avidin (recGAvi) was dissolved in water and back-titrated with biotin-4-fluorescein [37] to determine its specific activity: ~13.5 U/mg. This specific activity is comparable to the activities of most common commercial avidins (10–15 U/mg).

The N-terminal amino acid sequence of the recombinant avidin was determined as E-A-E-A-R-E-C-S. From this sequence, it appears that the first three amino acids (underlined), part of the proteolytic site *STE13* (E-A-E-A), are retained. Several cases of retention of this sequence have been reported [38–40]. This suggests that either the protein fold imposed by the sequence is not suitable to recognition and proteolytic cleavage by *STE13* or that the amount of the protein produced exceeds the catalytic capacity of *STE13* protease, preventing the effective cleavage of the fusion protein formed by the α-factor signal and the recombinant avidin [38–40]. Considering this N-terminal modification in combination with the primary sequence containing the K3E, K9D, R122A, and R124A mutations [16], the isoelectric point of the recombinant avidin is estimated at pI = 5.4 [41].

A "non-denaturing" SDS–PAGE [18] was performed on purified recombinant avidin, redissolved in water, to

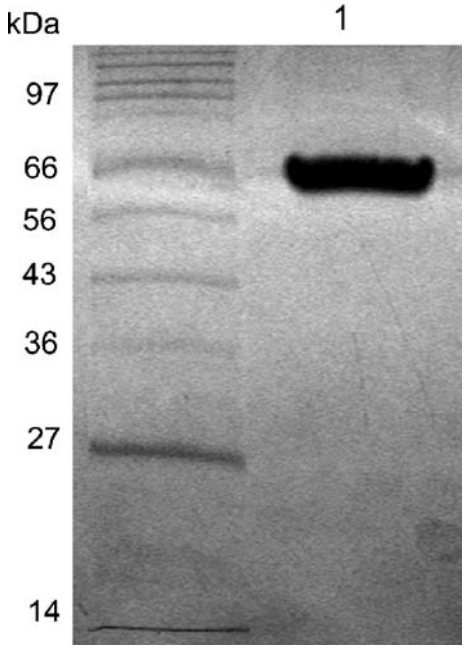

Fig. 7. Non-denaturing SDS–PAGE of purified recombinant avidin. Under these conditions the protein (lane 1) migrates in the tetrameric form.

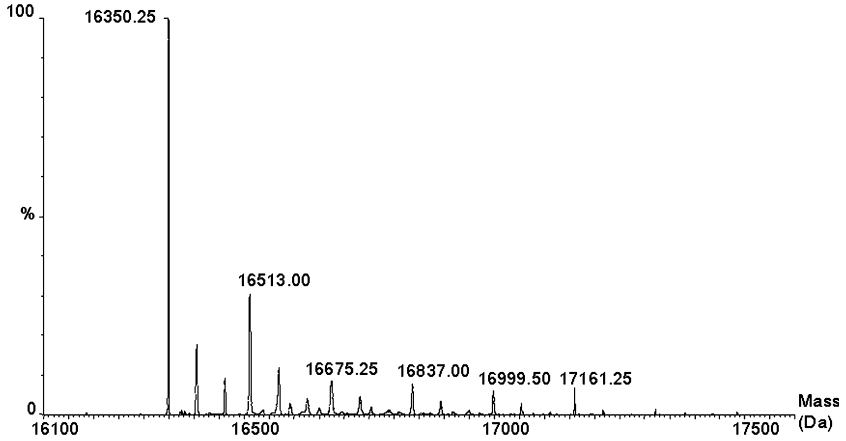

Fig. 8. Positive ESI-MS of recGAvi (calculated mass of the main peak: 16350.3 Da) dissolved in water/acetonitrile (1:1 v/v) containing 0.5% formic acid.

assess its quaternary state. The results unambiguously show that the recombinant protein is tetrameric and migrates as such (Fig. 7). Moreover, the fact that the protein is able to enter the separating gel confirms that the p*I* of the recGAvi is significantly lower than the p*I* (10.5) of wild-type hen-egg white avidin [17,18].

ESI-MS of the purified recombinant avidin displayed a main molecular peak centered at 16350.3 Da, accompanied by periodic minor peaks (Fig. 8). The most intense peak is attributed to monomeric avidin with a single *N*-glycan (Man_9-$GlcNAc_2$, Man = mannose, GlcNAc = *N*-acetyl-glucosamine) typical of *P. pastoris* [42]. The minor peaks of higher molecular weight are attributed to monoglycosylated monomeric avidin containing additional mannose (Man_{9+n}-$GlcNAc_2$, $n = 1$–5).

Conclusion

In this study, we have demonstrated that a glycosylated recombinant avidin (recGAvi) can be produced at 330 mg/L in the culture medium of the methylotrophic yeast *P. pastoris*. This protocol allows a straightforward purification of the recombinant protein. Despite a lowered p*I* = 5.4, caused by K3E, K9D, R122A, and R124A mutations and the additional N-terminal amino acids (E-A-E) as well as a glycosylation pattern differing from the one of hen egg-white avidin, the recGAvi produced in *P. pastoris* displays a specific activity very similar to that of the commercial avidin.

Acknowledgments

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References

[1] P. Gyorgy, C.S. Rose, K. Hofmann, D.B. Melville, V. Du Vigneaud, A further note on the identity of vitamin H with biotin, Science 92 (1940) 609.
[2] N.M. Green, Avidin, Adv. Prot. Chem. 29 (1975) 85–133.
[3] J. Korpela, Avidin, a high affinity biotin binding protein, as a tool and subject of biological research, Med. Biol. 62 (1984) 5–26.
[4] M.K. Ahlroth, A. Grapputo, O.H. Laitinen, M.S. Kulomaa, Sequence features and evolutionary mechanisms in the chicken avidin gene family, Biochem. Biophys. Res. Commun. 285 (2001) 734–741.
[5] W.C. Kett, R.I.W. Osmond, L. Moe, S.E. Skett, B.F. Kinner, D.R. Coombe, Avidin is a heparin-binding protein. Affinity, specificity and structural analysis, Biochim. Biophys. Acta 1620 (2003) 225–234.
[6] R.J. De Lange, T.S. Huang, Egg white avidin. III. Sequence of the 75-residue middle cyanogen bromide peptide. Complete amino acid sequence of the protein subunit, J. Biol. Chem. 246 (1971) 698–709.
[7] M.L. Gope, R.A. Keinanen, P.A. Kristo, O.M. Conneely, W.G. Beattie, T. Zarucki-Schulz, B.W. O'Malley, M.S. Kulomaa, Molecular cloning of the chicken avidin cDNA, Nucleic Acids Res. 15 (1987) 3595–3606.

[8] R.A. Keinanen, M.-L. Laukkanen, M.S. Kulomaa, Molecular cloning of three structurally related genes for chicken avidin, J. Ster. Biochem. Mol. Biol. 30 (1988) 17–21.
[9] O. Livnah, E.A. Bayer, M. Wilchek, J.L. Sussman, Three-dimensional structure of avidin and the avidin–biotin complex, Proc. Natl. Acad. Sci. USA 90 (1993) 5076–5080.
[10] L. Pugliese, A. Coda, M. Malcovati, M. Bolognesi, Three-dimensional structure of the tetragonal crystal form of egg-white avidin and its functional complex with biotin at 2.7 Å resolution, J. Mol. Biol. 231 (1993) 698–710.
[11] K.J. Airenne, P. Sarkkinen, E.-L. Punnonen, M.S. Kulomaa, Production of recombinant avidin in *Escherichia coli*, Gene 144 (1994) 75–80.
[12] E. Nardone, C. Rosano, P. Santambrogio, F. Curnis, A. Corti, F. Magni, A.G. Siccardi, G. Paganelli, R. Losso, B. Apreda, M. Bolognesi, A. Sidoli, P. Arosio, Biochemical characterization and crystal structure of a recombinant hen avidin and its acidic mutant expressed in *Escherichia coli*, Eur. J. Biochem. 256 (1998) 453–460.
[13] K.J. Airenne, C. Oker-Blom, V.S. Marjomäki, E.A. Bayer, M. Wilchek, M.S. Kulomaa, Production of biologically active recombinant avidin in Baculovirus-infected insect cells, Protein Express. Purif. 9 (1997) 100–108.
[14] E.E. Hood, D.R. Witcher, S. Maddock, T. Meyer, C. Baszczynski, M. Bailei, P. Flynn, J. Register, L. Marshall, D. Bond, E. Kulisek, A. Kusnadi, R. Evangelista, Z. Nikolov, C. Wooge, R.J. Mehigh, R. Hernan, W.K. Kappel, D. Ritland, C.P. Li, J.A. Howard, Commercial production of avidin from transgenic maize: characterization of transformants, production, processing, extraction and purification, Mol. Breeding 3 (1997) 291–306.
[15] M. Wilchek, E.A. Bayer, Foreword and introduction to the book (strep)avidin–biotin system, Biomol. Eng. 16 (1999) 1–4.
[16] A.T. Marttila, K.J. Airenne, O.H. Laitinen, T. Kulik, E.A. Bayer, M. Wilchek, M.S. Kulomaa, Engineering of chicken avidin: a progressive series of reduced charge mutants, FEBS Lett. 441 (1998) 313–317.
[17] A.T. Marttila, O.H. Laitinen, K.J. Airenne, T. Kulik, E.A. Bayer, M. Wilchek, M.S. Kulomaa, Recombinant NeutraLite avidin: a non-glycosylated, acidic mutant of chicken avidin that exhibits high affinity for biotin and low non-specific binding properties, FEBS Lett. 467 (2000) 31–36.
[18] E.A. Bayer, S. Ehrlich-Rogozinski, M. Wilchek, Sodium dodecyl sulfate–polyacrylamide gel electrophoretic method for assessing the quaternary state and comparative thermostability of avidin and streptavidin, Electrophoresis 17 (1996) 1319–1324.
[19] O.H. Laitinen, H.R. Nordlund, V.P. Hytönen, S.T.H. Uotila, A.T. Marttila, J. Savolainen, O. Livnah, E.A. Bayer, M. Wilchek, M.S. Kulomaa, Rational design of an active avidin monomer, J. Biol. Chem. 278 (2003) 4010–4014.
[20] A. Gallizia, C. de Lalla, E. Nardone, P. Santambrogio, A. Brandazza, A. Sidoli, P. Arosio, Production of a soluble and functional recombinant streptavidin in *Escherichia coli*, Protein Express. Purif. 14 (1998) 192–196.
[21] C.A. Scorer, J.J. Clare, W.R. McCombie, M.A. Romanos, K. Sreekrishna, Rapid selection using G418 of high copy number transformants of *Pichia pastoris* for high-level foreign gene expression, Biotechnology 12 (1994) 181–184.
[22] J. Collot, J. Gradinaru, N. Humbert, M. Skander, A. Zocchi, T. Ward, Artificial metalloenzymes for enantioselective catalysis based on biotin–avidin, J. Am. Chem. Soc. 125 (2003) 9030–9031.
[23] N.S. Outchkourov, W.J. Stiekema, M.A. Jongsma, Optimization of the expression of equistatin in *Pichia pastoris*, Protein Express. Purif. 24 (2002) 18–24.
[24] G. Sinclair, F.Y.M. Choy, Synonymous codon usage bias and the expression of human glucocerebrosidase in the methylotrophic yeast *Pichia pastoris*, Protein Express. Purif. 26 (2002) 96–105.
[25] J. Sambrook, D.W. Russel, Molecular Cloning. A laboratory manual, third ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, USA, 2001.
[26] P.J. Koutz, G.R. Davis, C.A. Stillman, K.J. Barringer, J.M. Cregg, G. Thill, Structural comparison of the *Pichia pastoris* alcohol oxidase genes, Yeast 5 (1989) 167–177.
[27] J.M. Cregg, K.R. Madden, K.J. Barringer, G. Thill, C.A. Stillman, Functional characterization of the two alcohol oxidase genes from the yeast, *Pichia pastoris*, Mol. Cell. Biol. 9 (1989) 1316–1323.
[28] P. Baumgartner, R. Raemaekers, A. Duriex, A. Gatehouse, H. Davies, M. Taylor, Large-scale production, purification, and characterisation of recombinant *Phaseolus vulgaris* phytohemagglutinin E-form expressed in the methylotrophic yeast *Pichia pastoris*, Protein Express. Purif. 26 (2002) 394–405.
[29] J. Stratton, V. Chiruvolu, M.M. Meagher, High cell-density fermentation, in: D.R. Higgins, J.M. Cregg (Eds.), Methods in Molecular Biology, Humana Press, Totowa, NJ, 1998, pp. 107–120.
[30] N.M. Green, Avidin and streptavidin, in: M. Wilchek, E.A. Bayer (Eds.), Avidin–Biotin Technology, Academic Press, San Diego, 1990, pp. 51–67.
[31] U.K. Laemmli, Cleavage of structural proteins during the assembly of the head of bacteriophage T4, Nature 227 (1970) 680–685.
[32] H. Towbin, T. Staehelin, J. Gordon, Electrophoretic transfer of protein from polyacrylamide gels to nitrocellulose sheet: procedure and some application, Proc. Natl. Acad. Sci. USA 76 (1979) 4350–4354.
[33] W.N. Burnette, "Western blotting": electrophoretic transfer of protein from sodium dodecyl sulfate–polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A, Anal. Biochem. 112 (1981) 195–203.
[34] H. Towbin, J. Gordon, Immunoblotting and dot immunoblotting—current status and outlook, J. Immunol. Methods. 72 (1984) 313–340.
[35] H.J. Gruber, G. Kada, M. Marek, K. Kaiser, Accurate titration of avidin and streptavidin with biotin–fluorophore conjugates in complex, colored biofluids, Biochim. Biophys. Acta 1381 (1998) 203–212.
[36] R.A. Brierley, Secretion of recombinant human insulin-like growth factor (IGF-I), in: D.R. Higgins, J.M. Cregg (Eds.), Methods in Molecular Biology, Humana Press, Totowa, NJ, 1998, pp. 149–177.
[37] G. Kada, H. Falk, H.J. Gruber, Accurate measurement of avidin and streptavidin in crude biofluids with a new, optimized biotin–fluorescein conjugate, Biochim. Biophys. Acta 1427 (1999) 33–43.
[38] T.R. Kim, N. Goto, N. Hirota, K. Kuwata, H. Denton, S.-Y. Wu, L. Sawyer, C.A. Batt, High-level expression of bovine β-lactoglobulin in *Pichia pastoris* and characterization of its physical properties, Protein Eng. 10 (1997) 1339–1345.
[39] L.H. Villarate, J. Campos, A. Chung, C.P. Liu, Comparison of prepro α-factor and PHO1 signal peptides in the efficient high yield secretion of ovine INFt by *Pichia pastoris*, in: Current Topics in Gene Expression Systems. March 28–31, 1999. P53, San Diego, CA, USA.
[40] M.S. Almeida, K.M.S. Cabral, L.N. de Medeiros, A.P. Valente, F.C.L. Almeida, E. Kurtenbach, cDNA cloning and heterologous expression of functional cysteine-rich antifungal protein *Psd1* in the yeast *Pichia pastoris*, Arch. Biochem. Biophys. 395 (2001) 199–207.
[41] B. Bjellqvist, G.J. Hughes, C. Pasquali, N. Paquet, F. Ravier, J.-C. Sanchez, S. Frutiger, D.F. Hochstrasser, The focusing positions of polypeptides in immobilized pH gradients can be predicted from their amino acid sequences, Electrophoresis 14 (1993) 1023–1031.
[42] G.P.L. Cereghino, J.L. Cereghino, C. Ilgen, J.M. Cregg, Production of recombinant proteins in fermenter cultures of yeast *Pichia pastoris*, Curr. Opin. Biotechnol. 13 (2002) 329–332.