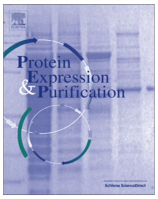
Protein Expression and Purification 93 (2014) 54–62

Contents lists available at ScienceDirect

Protein Expression and Purification

journal homepage: www.elsevier.com/locate/yprep



High-level secretion of recombinant full-length streptavidin in *Pichia pastoris* and its application to enantioselective catalysis

Elisa S. Nogueira [a], Thomas Schleier [b], Marc Dürrenberger [a], Kurt Ballmer-Hofer [b], Thomas R. Ward [a], Rolf Jaussi [b,*]

[a] *Department of Chemistry, University of Basel, Spitalstrasse 51, CH-4056 Basel, Switzerland*
[b] *Laboratory of Biomolecular Research, Paul Scherrer Institut, CH-5232 Villigen-PSI, Switzerland*

ARTICLE INFO

Article history:
Received 14 June 2013
and in revised form 22 August 2013
Available online 31 October 2013

Keywords:
Artificial metalloenzymes
Biotin–streptavidin technology
Pichia pastoris
Imine reductase

ABSTRACT

Artificial metalloenzymes result from the incorporation of a catalytically competent biotinylated organometallic moiety into full-length (*i.e.* mature) streptavidin. With large-scale industrial biotechnology applications in mind, large quantities of recombinant streptavidin are required. Herein we report our efforts to produce wild-type mature and biotin-free streptavidin using the yeast *Pichia pastoris* expression system. The streptavidin gene was inserted into the expression vector pPICZαA in frame with the *Saccharomyces cerevisiae* α-mating factor secretion signal. In a fed-batch fermentation using a minimal medium supplemented with trace amounts of biotin, functional streptavidin was secreted at approximately 650 mg/L of culture supernatant. This yield is approximately threefold higher than that from *Escherichia coli*, and although the overall expression process takes longer (ten days *vs.* two days), the downstream processing is simplified by eliminating denaturing/refolding steps. The purified streptavidin bound ~3.2 molecules of biotin per tetramer. Upon incorporation of a biotinylated piano-stool catalyst, the secreted streptavidin displayed identical properties to streptavidin produced in *E. coli* by showing activity as artificial imine reductase.

Introduction

The general concept of "artificial metalloenzymes", in which a catalytically active transition metal complex is anchored in a host biomolecular scaffold (typically a protein [1,2] or DNA [3,4]), was introduced several decades ago [5–7]. This remarkably versatile strategy takes advantage of the complementarity of both homogeneous- and enzymatic catalysis: the efficiency, robustness and wide scope of reactions of synthetic catalysts, with the high selectivity of enzymes and their activity under mild conditions [8,9].

In natural enzymes, the activity and (enantio)selectivity are caused by second coordination sphere interactions provided by the protein, whereas in metal-catalyzed enantioselective catalysis the activity and selectivity are almost exclusively dictated by the first coordination sphere provided by the ligand [10–12]. Therefore, the introduction of a catalytic metal moiety, which ensures activity in a chiral shell provided by the host biomolecule, is expected to create transition metal complexes with a well-defined second coordination sphere that display enzyme-like activities and selectivities (Fig. 1) [7]. An attractive feature of these hybrid catalysts is that their performance can be improved by "chemogenetic" optimization [13], *i.e.*, by independently modifying the homogeneous catalyst by chemical optimization and the biological scaffold by directed evolution or rational design [14,15].

Streptavidin (Sav)[1] is a ~60 (4 × 15) kDa homotetrameric protein, isolated from the bacterium *Streptomyces avidinii*. Like its namesake avidin from egg-white, streptavidin binds four equivalents of biotin per tetramer, with a virtually unmatched affinity (K_d ~10^{-13} M) [16]. It has been used for a variety of biochemical applications, *e.g.*, immobilization, bioconjugation, cell-surface labeling, or delivery of diagnostic agents [17,18]. Several homologous high-affinity biotin-binding proteins have been identified from a variety of organisms [19–24]. In recent years, mature Sav has been utilized as a host protein for the creation of artificial metalloenzymes. To date eight different reactions have been implemented and optimized using a chemogenetic optimization strategy [5,14,25–29]. From the biological point-of-view, developing new artificial metalloenzymes requires large amounts of purified protein as cell debris tend to "*poison*" the precious metal catalyst [28,30].

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* Corresponding author. Address: Laboratory of Biomolecular Research, Paul Scherrer Institut, OFLC/107, CH-5232 Villigen-PSI, Switzerland. Fax: +41 56 310 5288.

E-mail address: rolf.jaussi@psi.ch (R. Jaussi).

[1] *Abbreviations used:* Sav, streptavidin; WT, wild-type; B4F, biotin-4-fluorescein; RP-HPLC, reversed phase high-pressure liquid chromatography; MOPS, 3-morpholinopropane-1-sulfonic acid; T_R, retention time; *ee*, enantiomeric excess; ATHase, artificial transfer hydrogenase; Cp∗-Ir, catalyst precursor [Cp∗Ir(biot-1)Cl].

1046-5928/$ - see front matter © 2013 Published by Elsevier Inc.
http://dx.doi.org/10.1016/j.pep.2013.10.015

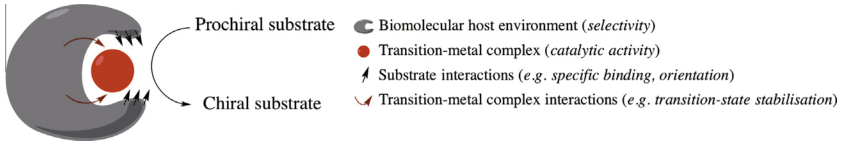

Fig. 1. Concept of artificial metalloenzymes, modified from [7]. Authorized reprint from John Wiley and Sons.

Until the mid-1980s, streptavidin was obtained by fermentation of *S. avidinii* with a yield of 53 mg per liter of culture [31,32]. Since then, both recombinant core- and mature Sav have been produced in different microorganisms. Cantor et al. first efficiently expressed mature and core Sav in the cytoplasm of *Escherichia coli*, with yields of about 50 mg per liter of culture [33–35]. Upon continuous optimization efforts, soluble mature Sav could be produced in the cytoplasm of *E. coli* at higher levels (∼230 mg/L) [36]. Moreover, systems to target the protein to different extra-cytoplasmic compartments have been developed in the past few years: *Bacillus subtilis* was used to secrete Sav, with a reported yield of ∼55 mg of protein per liter of culture [37,38], while periplasmic expression of mature streptavidin in *E. coli* was achieved using a signal peptide from bacterial *ompA*, resulting in low expression levels [38]. Most recently, core streptavidin has been expressed in *Pichia pastoris* by Casteluber et al. at 4 g/L [39].

Thanks to its high expression levels and its well-established extracellular secretion system, we speculated that *P. pastoris* might be a suitable host to produce soluble full-length Sav in its culture medium at high concentration, thus simplifying downstream purification efforts [40,41]. In our hands, full-length Sav is more soluble than core Sav, and the resulting artificial metalloenzymes display better catalytic properties. The performance of an artificial imine reductase using either the *P. pastoris* or *E. coli* produced Sav was compared for the production of salsolidine [28,42], an enantiopure tetrahydoisoquinoline alkaloid.

The incentive for the creation of active and selective artificial metalloenzymes is both practical and theoretical. It has the potential to provide valuable insight in catalytic reaction mechanisms as well as catalyst design.

Materials and methods

Materials

P. pastoris strain X-33, *E. coli* strains XL-1 Blue and Mach1, plasmid pPICZαA, antibiotic Zeocin and yeast nitrogen base with ammonium sulfate and without amino acids were purchased from Invitrogen (Karlsruhe, Germany). *P. pastoris* KAI-3 strain [43] was a generous gift from Prof. Callewaert (Univ. of Ghent, Belgium). The oligonucleotides were obtained from Microsynth (Balgach, Switzerland), and the restriction enzymes from Fermentas GmbH (St. Leon-Rot, Germany). The MinElute Reaction Cleanup kit was from Qiagen (Valencia, CA, USA), the CloneEZ kit from GenScript (Piscataway, NJ, USA) and GenElute HP Plasmid Midiprep kit from Sigma–Aldrich (Buchs, Switzerland). Yeast extract, bactotryptone and peptone for media preparations were obtained from Applichem (Darmstadt, Germany) and Becton Dickinson AG (Basel, Switzerland). Iminobiotin-Sepharose was purchased from Affiland (Liège, Belgium), and biotin-4-fluorescein (B4F) from ANAWA Trading SA (Zurich, Switzerland). Suppliers of more specific reagents are referred throughout the text. Reagents and solvents were of the highest commercially available grade and used without further purification. Solutions were prepared in deionized water, unless otherwise indicated.

Media for Pichia pastoris and Escherichia coli

E. coli cells carrying plasmids with Zeocin resistance were grown in LB low salt medium. The medium contained 5% (w/v) bacto yeast extract, 10% (w/v) bacto tryptone, 5% (w/v) NaCl and 25 μg/mL Zeocin.

One drawback with heterologous expression systems secreting (strept)avidin lies in the fact that biotin, which is an important cofactor for most microorganisms, binds strongly to the produced recombinant (strept)avidin. Thus, production of streptavidin can be lethal to *P. pastoris* (a biotin auxotrophic microorganism), if the amount of biotin present in standard media is not sufficient to saturate all biotin-binding sites of the produced Sav. In such a situation, Sav yield would be very low.

For the creation of artificial metalloenzymes, keeping the biotin-binding sites free is a prerequisite for subsequent incorporation of the catalyst moiety [28,31]. The yeast growth media used was therefore modified to overcome this challenge.

The *P. pastoris* strain was grown on complex (shake flask cultivation) or on minimal media (fermentation). *P. pastoris* growth medium (buffered glycerol medium, BMGY) and expression media (buffered methanol medium, BMMY, and basal salts medium, BSM) were prepared as described by Invitrogen (Karlsruhe, Germany), with two modifications for shake flask expression: a final concentration of 0.2% (w/v) biotin was used in the growth media, and cells were washed twice with sterile deionized water prior to resuspension in expression medium, which did not contain biotin. For fermentation, trace salts solution (PTM1, containing biotin) was omitted in the second phase of the fed-batch culture.

Cloning of Sav into pPICZαA

Primers were designed using Vector NTI software (Invitrogen, Carlsbad CA, USA). Two stop codons were introduced at the 3′-end to express the protein. The streptavidin insert was obtained by PCR using the following sense: 5′-GAGAGGCTGAAGCTCGGGATCAGGCCGGCATC-3′ and antisense 5′-CTAAGGCTACAAACTCACTACTGCTGAACGGCGTCG-3′ primers, and a plasmid template of Sav [34,36,44]. The PCR conditions were: 2 min at 94 °C followed by 20 cycles of 15 s at 98 °C, stepdown cycle of 30 s at 60 °C (first cycle) – 50 °C (last cycle), and 30 s at 72 °C; and by 20 cycles of 15 s at 98 °C, 30 s at 55 °C, and 30 s at 72 °C, with a final extension of 1 min at 72 °C. The vector was amplified using the sense: 5′-TGAGTTTGTAGCCTTAGACATGACTG-3′ and antisense: 5′-AGCTTCAGCCTCTCTTTTCTCGAGAG-3′ primers and pPICZαA (Invitrogen) as the template.

The crude PCR products were treated with 20 U *DpnI* restriction enzyme for 18 h at 37 °C. The DNA insert and pPICZαA vector were purified using the MinElute Reaction Cleanup kit from Qiagen. The Sav fragment (478 bp) was joined with the amplified vector pPICZαA by the CloneEZ reaction, following the instructions of the supplier.

Chemically competent *E. coli* strain One Shot Mach1-T1 cells were used for transformation with the CloneEZ reaction mixture and selected on low salt LB agar containing 25 μg/mL Zeocin and saturating concentration of biotin (0.2% w/v). Single colonies were selected and the sequence of the isolated plasmids was analyzed