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MIO-enzyme toolbox: cloning, expression and purification of recombinant *Rt*PAL

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SUMMARY. The recent advances in the molecular engineering of aromatic ammonia lyases (ALs) and aminomutases (AMs) attracted increased interest towards their applications in the treatment of phenylketonuria and/or the synthesis of non-natural amino acids. Herein we describe the cloning, isolation and purification of recombinant L-phenylalanine ammonia-lyase from *Rhodosporidium toruloides* for future biocatalytic and therapeutic applications.

Keywords: MIO–enzyme, non-natural amino acid, phenylalanine ammonia-lyase, phenylketonuria

Introduction

The recent advances in the molecular engineering of aromatic ammonia lyases (ALs) and aminomutases (AMs) attracted increased interest towards their applications in the treatment of phenylketonuria (Gamez *et al*., 2005; Babich *et al*., 2013) and/or the synthesis of non-natural amino acids (Turner, 2011). All these enzymes have in common an auto-catalically formed 5-methylene-3,5-dihydro-imidazole-4-one (MIO) electrophilic prosthetic group (Rétey, 2003) and show high structural and sequence similarities (Poppe, 2013; Bánóczi *et al*., 2015). Our research activities focus on the design and isolation of more stable ammonia lyases and their substrate scope extension towards novel valuable substrates, one of the interested MIO-enyzmes being phenylalanine ammonia-lyase from *Rhodosporidium toruloides* (Prosekov *et al*., 2014).

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Materials and methods

LB medium, tryptone, yeast extract, agarose, antibiotics (tetracycline, chloramphenicol, carbenicillin), imidazole, IPTG, HEPES, Tris, PMSF, NaCl, KCl, NaH_2PO_4, Na_2HPO_4, SDS, EDTA, TEMED, glycerol, β-mercaptoethanol, Coomassie Brilliant Blue are reagents used in experiments and were purchased from companies Sigma, Aldrich, Carl Roth GmbH, Poch, Liofilchem, Serva.

Lysozyme, DNAse, RNAse were purchased from the company Carl Roth GmbH and cOmplete Protease Inhibitor Cocktail Tablets from Roche.

Molecular cloning and expression optimization

The synthetic gene encoding for *Rt*PAL was synthesized by Life Technologies according to the sequence of the desired protein and optimized for expression *in E. coli.* (Figure 1). The synthetic gene encoding *Rt*PAL, containing an enterokinase cleavage site at the N-terminus, was cloned in the pET-19b_J906 expression vector using XhoI and Bpu1102I cloning sites. The obtained plasmid was transformed in *E.coli* XL-1 Blue heat competent cells as well as in different *E.coli* host strains (Rosetta (DE3) pLysS, BL21(DE3) pLysS, Origami 2) in order to optimize the expression yields. Different concentrations of IPTG (0.1mM, 0.5mM and 1mM) and different fermentation temperatures (20°C, 30°C and 37°C) were tested during the expression optimization.

Protein production, isolation and purification

The recombinant plasmids, containing the *N*-terminal $(His)_{10}$-tag were produced in *E. coli* Rosetta (DE3) pLysS cells using LB media supplemented with the carbenicilin and chloramphenicol.

First a preculture was prepared by the inoculation of 100 ml of sterile LB medium, containing carbenicillin (50 μg/ml) and chloramphenicol (30 μg/ml) with the bacterial cells from the agarplate, followed by overnight incubation at 37°C and shaking at 200 rpm. 8 × 0.5 L of LB medium (in 2L flasks) was inoculated with 2% (*v/v*) of the preculture and grown at 37°C, 200 rpm until OD_{600} reached 0.7-0.8. Protein expression was induced *via* the addition of 0.1 mM IPTG, and the cell growth was maintained at 25°C for another 8 h, reaching an OD_{600} value of 3.6. Cells were harvested by centrifugation (30 min, 4500 rpm), followed by their resuspension (with vortex and pipetting) in 100 ml lysis buffer (50 mM Tris, 300 mM NaCl, 0.5 mM EDTA; pH 8) supplemented with RNAse (3 mg), Lysozyme (10 mg), PMSF (20 mg/ 1 ml EtOH) and 1 tablet of complete protease inhibitor from Roche. The cells were lysed by sonication (2 sec pulse, 40% intensity, 30 min, $T < 20\ ^0C$) and cell debris, respectively membrane fractions were removed by centrifugation (15000 rpm, 35 min). The supernatant was loaded on Ni-NTA affinity chromatography, using appoximately 2 ml of Ni-NTA superflow resin from Qiagen and the protocol described by the

manufacturer. The *Rt*PAL protein with the *N*-terminal His-tag eluted with the 400 mM imidazole fraction. The protein was 4 × fold concentrated through amicons with 10kDa cut-off, followed by their further purification with size-exclusion chromatography, using SEC200 10/300 GL column and 20 mM Tris and 150 mM NaCl, pH 7.5 as eluent. The homotetrameric protein eluted at 10.5-12 ml retention volumes. The protein was stored until further use at -20^{0}C, with 10% glycerol. The purity of the isolated protein was determined through SDS-PAGE, using 12% Tris-glycine Laemmli gels.

Enzyme activity measurements

Activity of *Rt*PAL was determied spectrophotometrically, by monitoring the production of trans-cinnamic acid at 290 nm, using Quartz cuvettes of 1,4 ml and an UV-VIS Cary 50, Varian spectrophotometer.

Results and discussion

Based on the sequence of *Rt*PAL (PDB code:1Y2M) (Figure 1a) we designed a gene sequence with an enterokinase cleavage site for directional cloning into the pET19b(+) vector, using XhoI and BpuI102 cloning sites. The designed gene sequence was obtained through gene synthesis services, and was cloned succesfully into the expression vector, obtaining the novel recombinant plasmid encoding *Rt*PAL (Figure 1b) with an *N*-terminal His(10)-tag and an enterokinase cleavage site, serving for the His-tag removal ulterior to protein purification. The recombinant plasmid was tranformed through heat-shock into different *E.coli* competent cells in order to optimize the expression yields. During expression optimization, besides the influence of the host cells (*E.coli* Rossetta, BL21, C41 all with (DE3), pLysS modifications) we investigated the influence of inducer (IPTG) concetration and of the temperature upon the expression yields. The optimal conditions were found to be *E.coli* Rosetta (DE3) pLysS as host strain, 0.1 mM IPTG and 25 ^{o}C fermentation temperature.

Using the optimal conditions we performed the expression and purification of the *Rt*PAL enzyme. The enzyme containing the *N*-terminal His_{10}-tag was purified with Ni-affinity chromatography followed by size-exclusion chromatography. The purity of the protein, verified by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis proved to be > 90%, while its tetrametric, native fold was determined through its molecular weight estimated from its elution volume from the SEC200 10/300 GL column and the calibration curve (molecular weight as function of retention volume) of the column. (Figure 2).

1a.

MGHHHHHHHHHHSSGHIDDDDKHMLEMAPSLDSISHSFANGV
ASAKQAVNGASTNLAVAGSHLPTTQVTQVDIVEKMLAAPTDS
TLELDGYSLNLGDVVSAARKGRPVRVKDSDEIRSKIDKSVEFLR
SQLSMSVYGVTTGFGGSADTRTEDAISLQKALLEHQLCGVLPSS
FDSFRLGRGLENSLPLEVVRGAMTIRVNSLTRGHSAVRLVVLEA
LTNFLNHGITPIVPLRGTISASGDLSPLSYIAAAISGHPDSKVHVV
HEGKEKILYAREAMALFNLEPVVLGPKEGLGLVNGTAVSASMA
TLALHDAHMLSLLSQSLTAMTVEAMVGHAGSFHPFLHDVTRPH
PTQIEVAGNIRKLLEGSRFAVHHEEEVKVKDDEGILRQDRYPLR
TSPQWLGPLVSDLIHAHAVLTIEAGQSTTDNPLIDVENKTSHHG
GNFQAAAVANTMEKTRLGLAQIGKLNFTQLTEMLNAGMNRG
LPSCLAAEDPSLSYHCKGLDIAAAAYTSELGHLANPVTTHVQPA
EMANQAVNSLALISARRTTESNDVLSLLLATHLYCVLQAIDLRA
IEFEFKKQFGPAIVSLIDQHFGSAMTGSNLRDELVEKVNKTLAKR
LEQTNSYDLVPRWHDAFSFAAGTVVEVLSSTSLSLAAVNAWKVA
AAESAISLTRQVRETFWSAASTSSPALSYLSPRTQILYAFVREELG
VKARRGDVFLGKQEVTIGSNVSKIYEAIKSGRINNVLLKMLA

1b.

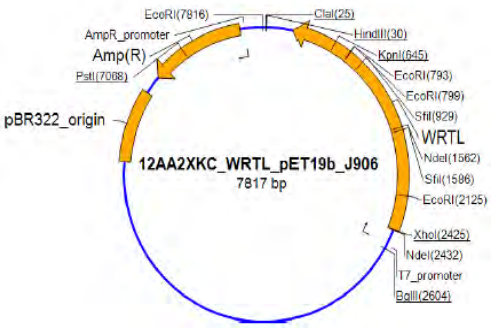

Figure 1. 1a. The amino acid sequence of the designed recombinant *Rt*PAL and **1b.** The genetic map of the created recombinant plasmid containing the gene encoding *Rt*PAL

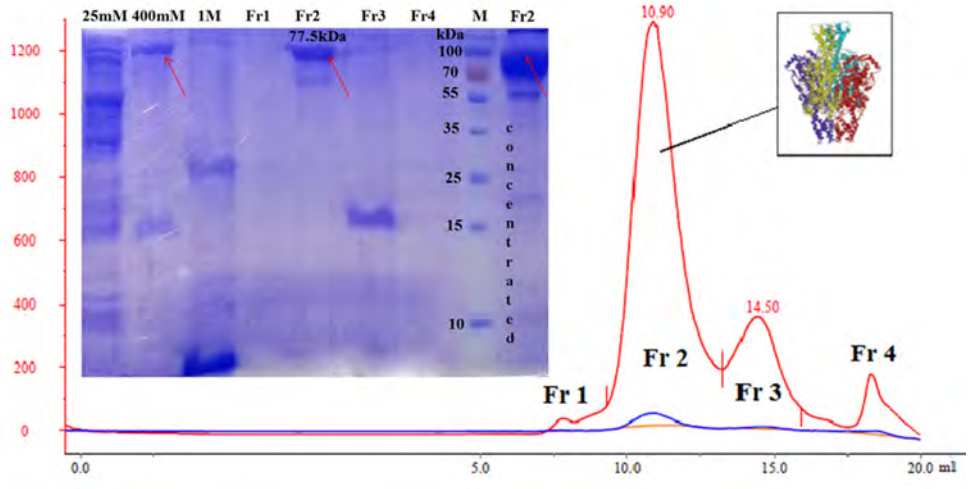

Figure 2. Purification of *Rt*PAL on SEC200 size-exclusion column and the SDS-PAGE gel containing samples from the purification steps of *Rt*PAL

The use of protease inhibitors during all of the isolation and purification steps proved to be crucial, in order maintain the integrity of the enzyme.

The enzyme activity and the kinetic parameters of the purified enzymes was determined towards the natural substrate *L*-phenylalanine. The values of the maximal velocity (v_{max}) and of the Michaelis-Menten constant *(Km)*: v_{max} = 0.47 $\mu M \times s^{-1}$

K_m=648 *mM*, k_{cat}=3.8 s^{-1} are in accordance with the values from literature (Babich *et al*., 2013), proving the success of the cloning, production, isolation and purification processes.

Conclusions

The succesfull cloning, expression, isolation and purification of phenylalanine ammonia lyase from *Rhodosporidium toruloides* was successfully achieved, obtaining enzyme with >90% purity and enzymatic activity similar with values reported in the literature.

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