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(RECOMMENDED CITATION)

EXPRESSION AND PURIFICATION OF RECOMBINANT PHENYLALANINE AMMONIA-LYASE FROM *PETROSELINUM CRISPUM*

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ABSTRACT. In the present study the molecular cloning, expression and purification of recombinant *Pc*PAL, with a cleavable *N*-terminal His-tag is described. The *Pc*PAL gene was cloned into pET-19b vector and transformed to different *E.coli* host cells. The optimization of expression and purification processes provided recombinant protein with high purity in its native, tetrameric fold with a yield of 7-8 mg protein / 1 L culture. The activity of the recombinant protein was tested towards its natural substrate L-Phe, the K_M, and k_{cat} values suggesting excellent catalytic properties of the recombinant enzyme.

Keywords: *phenylalanine ammonia-lyase, Petroselinum crispum, molecular cloning, expression and expression optimization*

INTRODUCTION

In higher plants, phenylalanine ammonia-lyase catalyses the conversion of L-phenylalanine to *trans*-cinnamic acid, the first step of the phenylpropanoid pathway. This process produces many secondary metabolites that fulfill many essential functions for plants growth and development, such as mechanical

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support, pigment and flavonoid production [1,2]. PAL, the first enzyme involved in the metabolism of phenylpropanoid derivatives, has been extensively studied for its crucial function as a branch point between primary and secondary metabolism [3,4].

The currently known aromatic ammonia-lyases use an auto-catalytically formed 5-methylene-3,5-dihydro-imidazole-4-one (MIO) electrophilic prosthetic group, and show high structural and sequence similarities [5].

The recent advances in improving the functional properties of these enzymes increased both their biocatalytic and therapeutic applications. Sufficiently stable phenyalanine ammonia-lyases (PALs) are used in enzyme replacement therapy [6] of phenylketonuria (PKU) [7], or as a potential therapeutic enzyme in cancer treatment [8]. DSM company incorporated a recombinant PAL into the chemoenzymatic synthesis of (*S*)-2-indolinecarboxylic acid [9], a key intermediate for hypertension pharmaceuticals; while BASF (Germany) recently patented [10] hydroamination reactions catalyzed by recombinant PALs originating from *Petroselinum crispum* (*Pc*PAL) and *Rhodoturula glutinis* (*Rg*PAL). In the academic area the substrate scope of *Pc*PAL was expanded to several aryl-, heteroaryl alanines [11,12,13] and even to the non-aromatic propargylglycine [14]. Furthermore the immobilization of *Pc*PAL on single-walled carbon nanotubes [15] and magnetic nanoparticles [14,16] provided their successful employment for the synthesis of non-natural amino acid in continuous-flow microreactors. Therefore the importance of the large-scale production of recombinant *Pc*PAL gained increased attention. Herein we describe the molecular cloning, expression, purification and large scale production of *Pc*PAL. The purity, oligomerization state and the activity of the isolated enzyme suggest its correct, native-like tetrameric folding [17].

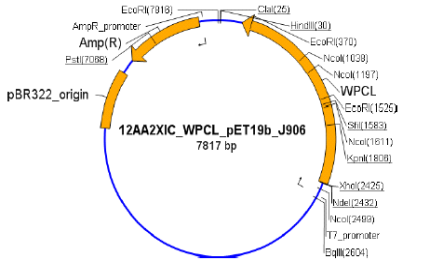
RESULTS AND DISCUSSION

Molecular cloning of recombinant *Pc*PAL

Based on the sequence of *Pc*PAL (PDB code: 1W27) we designed a codon optimized gene sequence for expression in *E.coli* and for directional cloning into the pET-19b vector, using *XhoI* and *BpuI1102I* cloning sites.

The designed gene sequence was obtained through gene synthesis services, and was cloned successfully into the expression vector, obtaining the novel recombinant plasmid encoding *Pc*PAL (**Figure 1a**) 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 transformed through

heat-shock into different *E.coli* competent cells in order to optimize the expression yields. The successful outcome of the cloning and transformation step was determined by agarose gel electrophoresis (**Figure 2**).



MGHHHHHHHHHHSSGHIDDDDKHMLEMENGNGATTNGHVNGNGMDFCMKT
EDPLYWGIAAEAMTGSHLDEVKKMVAEYRKPVVKLGGETLTISQVAAISA
RDGSGVTVELSEAARAGVKASSDWVMDSMNKGTDSYGVTTGFGATSHRRT
KQGGALQKELIRFLNAGIFGNGSDNTLPHSATRAAMLVRINTLLQGYSGI
RFEILEAITKFLNQNITPCLPLRGTITASGDLVPLSYIAGLLTGRPNSKA
VGPTGVILSPEEAFKLAGVEGGFFELQPKEGLALVNGTAVGSGMASMVLF
EANILAVLAEVMSAIFAEVMQGKPEFTDHLTHKLKHHPGQIEAAAIMEHI
LDGSAYVKAAQKLHEMDPLQKPKQDRYALRTSPQWLGPQIEVIRSSTKMI
EREINSVNDNPLIDVSRNKAIHGGNFQGTPIGVSMDNTRLAIAAIGKLMF
AQFSELVNDFYNNGLPSNLSGGRNPSLDYGFKGAEIAMASYCSELQFLAN
PVTNHVQSAEQHNQDVNSLGLISSRKTSEAVEILKLMSTTFLVGLCQAID
LRHLEENLKSTVKNTVSSVAKRVLTMGVNGELHPSRFCEKDLLRVVDREY
IFAYIDDPCSATYPLMQKLRQTLVEHALKNGDNERNLSTSIFQKIATFED
ELKALLPKEVESARAALESGNPAIPNRIEECRSYPLYKFVRKELGTEYLT
GEKVTSPGEEFEKVFIAMSKGEIIDPLLESLESWNGAPLPIS

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

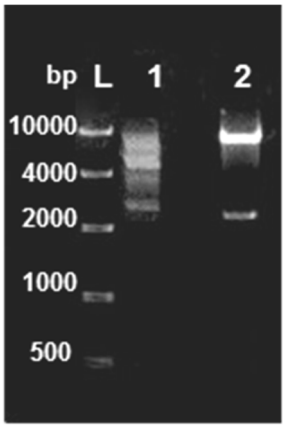


Figure 2. Agarose gel electrophoresis. L: DNA Ladder, 1. circular plasmid DNA, extracted from colonies obatined after transformation in Rosetta (DE3) pLysS cells, 2. Plasmid DNA from 1, digested with restriction enzymes *XhoI* and *Bpu1102I*.

Optimization of *Pc*PAL overexpression in *E.coli*

Further we performed the optimization of overexpression in *E.coli*, studying the influence of the nature of host cells (*E.coli* Rossetta, BL21, C41 all with (DE3), pLysS modifications), of the inducer (IPTG) concentration and of the temperature upon the expression levels of the *Pc*PAL. Varying the concentration of IPTG, expression of proteins can be regulated at different levels, lower level expression can increase the solubility and activity of the target proteins [18] For the optimization of *Pc*PAL expression, the effect of different concentrations of inducer (0.1, 0.5 and 1 mM IPTG) was tested on the growing culture of BL21(DE3)pLysS containing the pET-19b-*Pc*PAL recombinant plasmid and the cells before and after induction were analyzed by SDS-PAGE. No significant difference was observed in the *Pc*PAL protein expression levels of the differently induced cells, therefore the lowest 0.1 mM IPTG concentration was used in further experiments.

The effect of the nature of host *E.coli* cells proved to be crucial, in BL21 and C41 the yields of the isolated protein were low, while in Rosetta 20-30 mg *Pc*PAL / liter ferment was obtained. This might be due to the fact that Rosetta encodes for a larger number of rare-codons than BL21 and C41 [19], providing successful translation of the recombinant gene.

To evaluate the effect of growth temperature on the expression of *Pc*PAL after induction, the cultures were incubated at different temperatures (18, 25, and 37°C). Initially the cell cultures were incubated at 37°C. After the density of cells reached OD_{600} ~0.6 (approx. 4 h) the temperature was reduced (to 18 or 25°C) and the cultures were induced with 0.1 mM IPTG. The density of the cells was monitored in time (**Figure 3**), the maximum yield of enzyme being obtained in case of 25°C incubation temperature, with an optimal post-induction time of 12-14 h, where the cell growth reaches the stationary phase.

Therefore the optimal overexpression conditions *E.coli* Rosetta (DE3)pLysS as host strain, 0.1 mM IPTG and 25°C fermentation temperature were further used for the large scale (10 L) production of the enzyme.

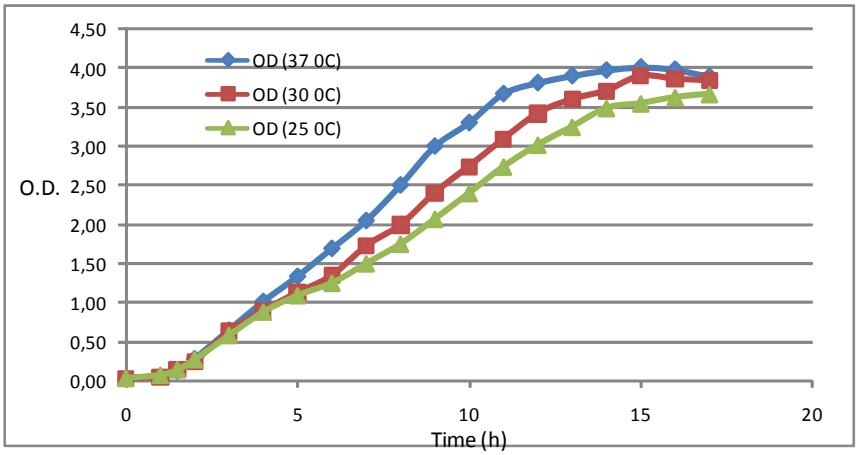


Figure 3. Effect of growth temperature on the expression of *Pc*PAL.

Production, purification and characterization of recombinant *Pc*PAL

After overexpression optimization, the production and purification of the recombinant *Pc*PAL protein was performed. The *N*-terminal $(His)_{10}$-tag of the protein allows affinity purification with Ni-NTA resin. Ni–NTA affinity chromatography is a rapid and easy purification technique for proteins fused with His-tag at either ends (*N*- or *C*-terminus). The strong binding between

His-tag and the immobilized nickel ions allows easy washing and efficient elution of bounded His-tagged protein by competition with imidazole [20].

Performing the Ni-NTA purification, the protein fraction eluted with 250 mM imidazole, besides the *Pc*PAL protein (81 kDa), contains additional proteins with lower molecular weight (**Figure 4**, lane 6). Despite our optimization efforts (varying the imidazole concentration of the elution buffer, introducing intermediate washing steps with high and low salt concentration buffers, according to the manufacturer description [21]) the protein purity, determined by SDS-PAGE remained below 80%.

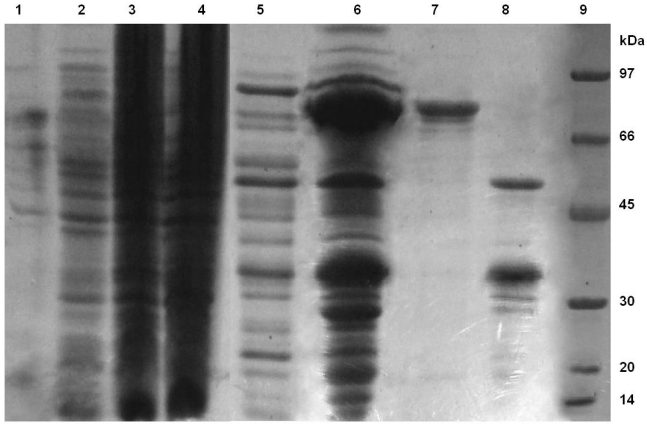
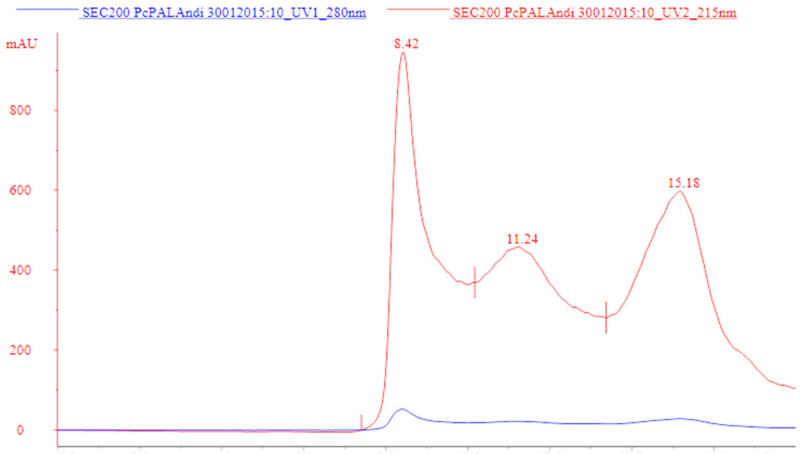


Figure 4. SDS-PAGE gel containing samples from the purification steps of *Pc*PAL: 1: before induction; 2: after induction; 3: supernatant after centrifugation of the lysate; 4: flow through after application; 8: low salt; 5: 25mM fraction of imidazole; 6: 400mM fraction of imidazole; 7: fraction eluted at 10-12 mL from the column; 8: fraction eluted at 14-16 mL from the column; 9: marker. Samples were prepared as described in the experimental section.

In order to increase the purity of the isolated *Pc*PAL we introduced additional chromatographic purification steps. Due to the large difference in the molecular weights of the target protein and of the other, unwanted protein impurities we opted for size-exclusion chromatography, using a Superdex 200 10/300 GL. This chromatography step also allows the determination of the oligomerization state of the protein, and thus the homogeneity of the isolated protein [22]. This is crucial since different oligomerization states of the same protein (including aggregated forms of the protein) on SDS-PAGE cannot be distinguished, they migrate at the same molecular weight corresponding to the monomer however their enzyme activity can significantly differ. Thus, besides the separation of the protein impurities, the SEC method will provide additional results related to the correct, native-like oligomerization state of the isolated protein.

Performing the SEC purification of the fraction eluted with 250 mM imidazole from the Ni-NTA column, several protein peaks were eluted (**Figure 5**). The identity of the protein peaks eluted was determined by SDS-PAGE (**Figure 4**, lane 7-8). These results show that the protein fractions eluted at 8.4 and 11.2 mL retention volumes are pure *Pc*PAL proteins, presumably having different oligomerization states. In order to determine the molecular weight determination by SEC the comparison of an elution volume parameter, such as K_{av} of the protein of interest, with the values obtained for several known calibration standards (**Table 1**) should be done [23].



SEC200 PcPALAndi 30012015:10_UV2_215nm@02,PEAK1

No	Ret ml	Area mAU*ml	Height mAU
1	-2.02	0.0928	10.989
2	-1.86	37.0035	38.647
3	8.42	1152.3995	946.250
4	11.24	1191.1324	459.374
5	15.18	1512.1388	598.230
6	18.45	87.8232	148.133

Figure 5. Purification of *Pc*PAL on Superdex 200 10/300 GL size-exclusion column.

Thus the molecular weight of an unknown protein can be determined from the calibration curve (**Figure 6**, plot of K_{av} versus the logarithm of molecular weight) once its K_{av} value is calculated from its measured elution volume [23].

Accordingly the molecular weight of the *Pc*PAL protein fractions eluted were estimated to be 355 kDa for the peak eluted at 11,2 mL and > 600 kDa for the fraction eluted at 8,4 mL, corresponding to the presumed native tetrameric and higher oligomerization states (octameric or even higher aggregated forms) of the protein, respectively.

Table 1. Elution volume parameters K_{av} of standard globular proteins used for the calibration curve set-up

Protein standard	Mol. weight (Da)	Volume of elution (V_e) (mL)	K_{av}*	Log_{MW}
Thyroglobulin	669000	8,26	0,074	5,83
Ferritin	440000	10,56	0,209	5,64
Aldolase	158000	12,38	0,316	5,20
BSA	67000	13,9	0,406	4,83
Ovalbumin	45000	15,08	0,475	4,65
Chimotrypsinogen A	25000	17,21	0,601	4,40

- **$K_{av} = (V_e-V_o)/(V_c-V_o)$; V_c = 24 mL (column volume); V_o = 8.1 mL (column void volume)**

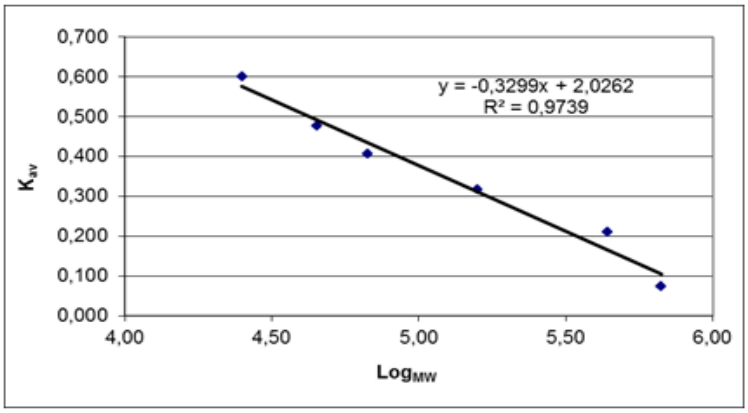


Figure 6. Calibration curve for Superdex 200 10/300 GL column. Resolution range of the column is 10-600 kDa.

Furthermore we compared the enzyme activities of the presumed largely aggregated and tetrameric forms of *Pc*PAL, separated by size-exclusion chromatography, towards the natural substrate L-Phe. Performing the experiment at 1 mM substrate concentration, we observed three times higher activity for the presumed native, tetrameric form of the protein.

$$\frac{v_{tetramer}}{v_{aggregate}} = \frac{3{,}409 * 10^{-2}[\frac{\mu M}{s}]}{1{,}149 * 10^{-2}[\frac{\mu M}{s}]} \cong 3$$

These results directed us to optimize the isolation and purification procedure in order to achieve high yields for the production of the *Pc*PAL tetramer. The presence of higher oligomerization states of the protein might be the results of aggregation processes, caused by the presence of free thiol

groups, able to form intermolecular disulphide bridges, or hydrophobic aggregation processes, caused by high protein or salt concentration in the solution [24]. Furthermore the removal of the 35 kDa protein impurity during isolation steps is also preferable for yield and purity increasements. This protein band coelutes with *Pc*PAL at the Ni-affinity purification step, furthermore during longer incubation times (>4 hours) its amount increases in the protein sample (checked by SDS-PAGE), suggesting that it is *Pc*PAL fragment, containing the *N*-terminal His_{10}-tag, obtained by proteolytic degradation.

Therefore in order to alleviate these problems several modification were introduced in the isolation and purification protocol: *i*) the imidazole concentration used for the elution at the Ni-affinity chromatography was decreased, *ii*) the concentration steps of the solutions with high imidazole content were avoided, performing the protein concentration step after the removal of the imidazole with SEC chromatography or dialysis, *iii*) the use of protease inhibitor cocktail (mini-tablets from Roche) during all isolation and purification steps. These modifications provided an optimal isolation and purification procedure for the recombinant *Pc*PAL, resulting in protein with high purity (> 95% on SDS-PAGE, **Figure 8**) and presumable in its native, tetrameric fold (**Figure 7**).

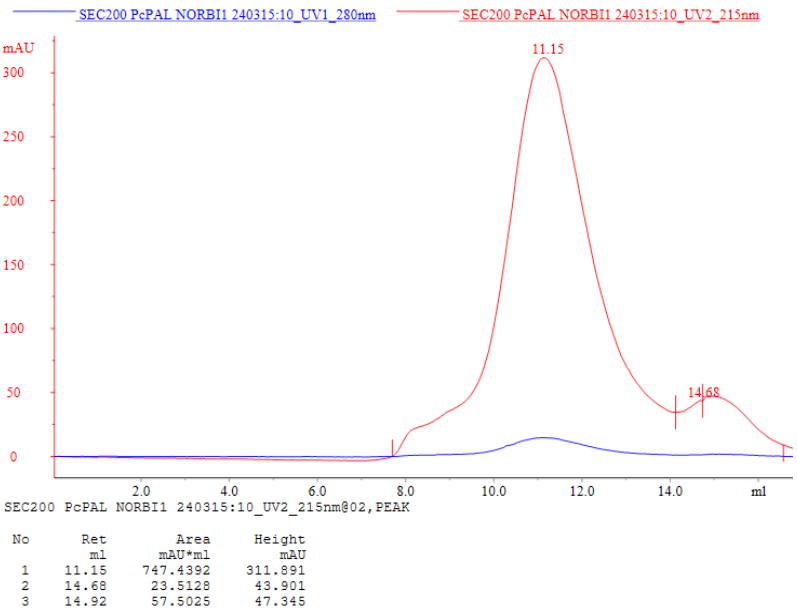


Figure 7. FPLC chromatogram of the pure tetramer obtained with the optimized procedure.

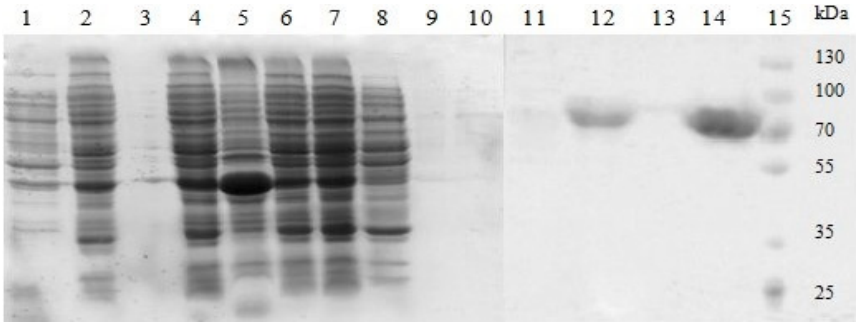


Figure 8. SDS-PAGE gel containing samples from the purification steps of *Pc*PAL: 1: before induction; 2: after induction; 3: supernatant of cells; 4: cell lysate 5: pellet of the cell lysate; 6: supernatant after centrifugation of the lysate; 7: flow through after application; 8: low salt; 9: high salt; 10: low salt; 11: 25mM fraction of imidazole; 12: 400mM fraction of imidazole (pure elute); 13: 1M fraction of imidazole; 14: fraction eluted at 10-12 mL from the column and 15: marker. Samples were prepared as described in the experimental section.

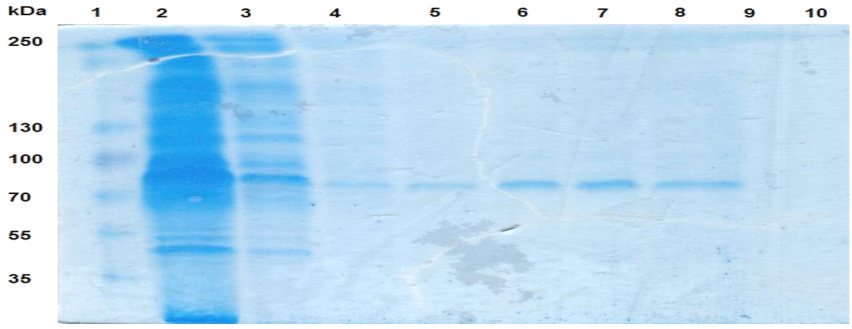


Figure 9. SDS-PAGE gel containing samples from the purification steps of *Pc*PAL with the support Dv1000-EDTA-La: 1: marker; 2: flow through after application; 3: low salt; 4: high salt; 5: low salt; 6: 5 mM fraction of EDTA 7: 25 mM fraction of EDTA; 8: 50 mM fraction of EDTA; 9: 100 mM fraction of EDTA; 10: empty. Samples were prepared as described in the experimental section.

Besides the commercially available Qiagen Ni-NTA affinity chromatography resin another silica based support was investigated with the optimized purification procedure of $(His)_{10}$-tagged *Pc*PAL. This product allows purification of recombinant proteins through its high surface area (>100 m^2/g) and mesoporous structure (100 nm) in addition strong binding capability between the His-tag and EDTA-immobilized lanthanum ions. The purification method was performed in batch mode by shaken the suspension of support (Dv1000-EDTA-La) and supernatant of the centrifuged cell lysate. Thereafter the nonspecific proteins were desorbed by buffers (**Figure 9**,

lane 3-4) and the target protein, *Pc*PAL was eluted by gradient elution with different concentration of EDTA solutions and given in high purity (> 95% on SDS-PAGE, **Figure 9**, lane 6-8). The purity and yields of the isolated protein were in similar range as those obtained from the Ni-NTA affinity purification (7-8 mg protein / 1 L culture).

Further we determined the activity of the recombinant *Pc*PAL towards its natural substrate L-Phe and compared the values with those obtained in the literature [6,7,8,14].

The activity measurements were performed by UV-monitoring the production of acrylic acid at 290 nm. The obtained K_M value of 116 ± 4 and k_{cat} value of 1±0.05 were highly reproducible and suggested better catalytic properties of the enzyme than those described in the literature [11,12,25]. The Michaelis-Menten plot of the measurement using the purified enzyme is shown at **Figure 10**. The K_M, v_{max} and k_{cat} values were determined using the Lineweaver-Burk plot (**Figure 11**).

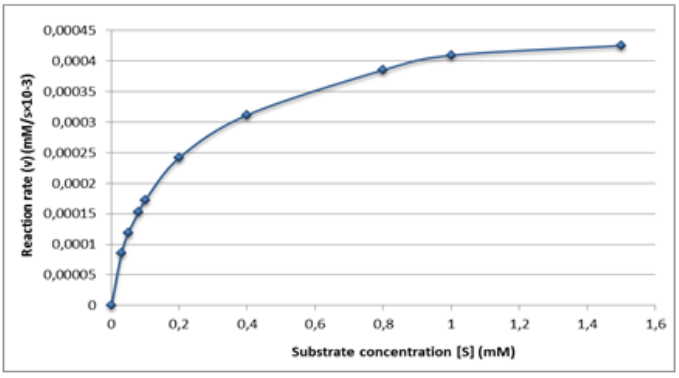


Figure 10. Michaelis-Menten curve on different concentrations of L-Phe.

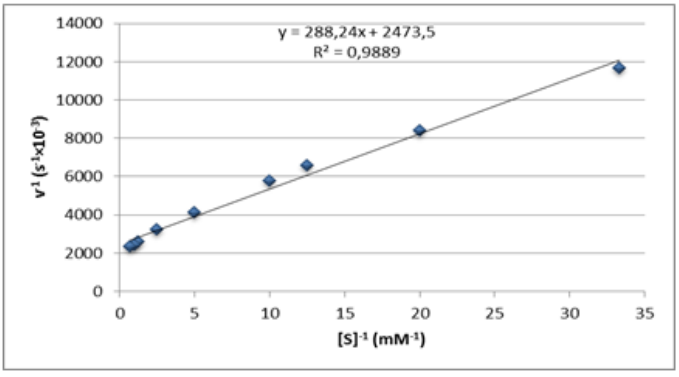


Figure 11. Lineweaver-Burk plot of the kinetic measurement.

CONCLUSIONS

During this study we report the successful cloning, expression and isolation of recombinant *Pc*PAL with a removable *N*-terminal His_{10}-tag. The oligomerization state of the expressed protein was determined with size-exclusion chromatography and suggested that the tetrameric *Pc*PAL is the most active oligomerization form. Optimization of the isolation and purification protocol provided pure, homogeneous *Pc*PAL tetramer, with excellent and reproducible catalytic activities.

EXPERIMENTAL SECTION

Materials and methods

IPTG, tetracycline, chloramphenicol, carbenicillin, imidazole, Tris,HCl, phenylalanine, LB medium, tryptone, yeast extract, NaCl, KCl, NaH_2PO_4, Na_2HPO_4, EDTA, SDS, TEMED, glycerol, β-mercaptoethanol, Coomassie Brilliant Blue, FastRuler™ High Range DNA Ladder, Lysozyme, DNAse, RNAse are reagents used in experiments and were purchased from companies Sigma, Aldrich, Carl Roth GmbH, Poch, Liofilchem or ThermoFischer. The gel filtration calibration protein kit were purchased from GE Healthcare, while the Complete Protease Inhibitor Cocktail Tablets from Roche. *E. coli* host strains, XL1-Blue, BL21(DE3)pLysS, Rosetta (DE3)pLysS and C41(DE3)pLysS were purchased from Novagen.

The kinetic measurements were performed on a Varian Cary 50 and on an Agilent 8453 UV-Vis Spectrophotometer. The gel filtration purifications were made on a Äkta purifier fast protein liquid chromatograph with autofractioner.

Molecular cloning and expression optimization

The gene encoding phenyalanine ammonia-lyase from *Petroselinum crispum* has been codon optimized for bacterial expression in *E. coli*. (**Figure 2**). The synthetic gene produced by Life Technologies was introduced into the pET-19b vector using the *XhoI* and *Bpu1102I* cloning sites, for expression with an *N*-terminal $(His)_{10}$-tag and an enterokinase cleavage site, for His-tag removal.

The obtained plasmid was transformed in *E.coli* XL1-Blue competent cells by heat shock as well as in different *E.coli* host strains (Rosetta (DE3)pLysS, BL21(DE3)pLysS, C41(DE3)pLysS) in order to optimize the expression yields.

Different concentrations of IPTG (0.1 mM, 0.5 mM and 1 mM) and different fermentation temperatures (25°C, 30°C and 37°C) were tested during the expression optimization.

Expression and production of the recombinant *Pc*PAL – optimized procedure

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

Batch mode fermentation: 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 agar plate, followed by overnight incubation at 37°C and shaking at 200 rpm. 8 × 0.5 L of LB medium (in 2 L 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 12 h, reaching an OD_{600} value of 3.4-3.6

Large scale fermentation of the recombinant PcPAL: ten litres of Terrific broth (TB) medium were sterilized at 121°C, 1.2 bar for 25 min. After sterilization, the media was cooled down to 37°C and ampicillin was added to the fermentation broth for a final concentration of 100 µg/mL. The fermentation media was inoculated with 100 mL of the overnight seed culture of the *E. coli* producer strain. The following fermentation parameters were set up: temperature at 37°C, agitation 300 rpm, overpressure 0.2-0.5 bar and bottom air inlet 5 L/min. The pH value of the fermentation broth was controlled at pH 7.1±0.1. The dissolved oxygen (DO) value was set to a minimum of 30% and was controlled by the stirring speed. When the OD_{600} of the culture reached 0.8±0.1, the temperature was set to 25°C and the culture was induced with IPTG (0.1 mM final concentration). The fermentation broth was harvested after 21 h, when the culture reached the stationary growth phase.

*Purification of the recombinant Pc*PAL – optimized procedure

Cells were harvested by centrifugation (30 min, 2000 × g, 4500 rpm); followed by their resuspension (with vortex and pipetting) in 100 mL lysis buffer (50 mM Tris.HCl, 300 mM NaCl, 0.5 mM EDTA; pH 8) supplemented with RNAse (3 mg), Lysozyme (10 mg), PMSF (20 mg/ 1 mL EtOH) and one tablet from the protease inhibitor cocktaile of Roche. The cells were lysed by sonication (2 sec pulse, 40% intensity, 30 min, T < 20 ^{0}C) and the cell debris, respectively membrane fractions were removed by centrifugation (24000 × g, 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 using the protocol described by the manufacturer. The *Pc*PAL protein with the *N*-terminal His-tag eluted with the 400 mM imidazole fraction. The protein was 4 × fold concentrated through amicons with 10 kDa cut-off, followed by their further purification with size-exclusion chromatography, using Superdex 200 10/300 GL column and 20 mM Tris,150 mM NaCl, pH 7.5 buffer 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.

***Purification with Dv1000-EDTA-La support* – alternative procedure**

The Dv1000-EDTA-La [26] support (100 mg) was dispersed in 1 mL lysis buffer (50 mM Tris.HCl, 300 mM NaCl, 0.5 mM EDTA, pH 8.0) and the suspension was shaken (10 min, 350 rpm). Thereafter 1 mL of centrifuged supernatant of cell lysate was added and shaken further (30 min, 350 rpm). This suspension was centrifuged (3 min, 3500 rpm) and the supernatant was removed. Then the support was shaken (10 min, 350 rpm) with the following solutions and between two fractions the supernatant was removed after centrifugation: low salt buffer (50 mM HEPES, 30 mM KCl, pH 7.5), high salt buffer (50 mM HEPES, 300 mM KCl, pH 7.5), low salt buffer and four different concentration ethylenediaminetetraacetic acid disodium dihydrate solutions (5, 25, 50, 100 mM of disodium salt of EDTA and 50 mM HEPES, 30 mM KCl, pH 8.0). The fractions were investigated by SDS-PAGE.

SDS-PAGE electrophoresis was performed according to the Laemmli-protocol [27] using acrylamide gels.

Samples were taken for every step of the expression and purification stage and prepared by denaturation at 95°C for 5-10 min with addition of reducing 3x sample buffer.

Enzyme activity measurements: Activity of *Pc*PAL was determined spectrophotometrically by monitoring the production of *trans*-cinnamic acid at 290 nm for 10 min, using Quartz cuvettes of 1.4 mL. The measurements were performed at 37°C for 10 min, in presence of 30 µg of enzyme, varying the substrate concentration between 0.03-1.5 mM. Kinetic constants (K_M and v_{max}) were obtained by determining the initial rate of reactions at different substrate concentrations. The extinction coefficient (ε) was determined in 0.1 M Tris-buffer (pH 8.8) at 290 nm: ε_{290}=8.8 $mM^{-1}\times s^{-1}$.

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REFERENCES

[1] M. WenLi, W. Min, R. ZhuMei, Z. Yang, *Plant Cell Reports*, **2013**, *32*, 1179.
[2] G. Jihai, et al., *Molecular Biology Reports*, **2012**, *39*, 3443.
[3] T. Vogt, *Molecular Plant*, **2010**, *3*, 2.
[4] C.M. Fraser, C. Chapple, *The Arabidopsis Book*, **2011**, e0152.
[5] L. Poppe, J. Rétey, *Current Organic Chemistry*, **2003**, *7*, 1297.
[6] C. W. Abell, D. S. Hodgins, W. J. Stith, *Cancer Research*, **1973**, *33*, 2529.
[7] A. Belanger-Quintana, A. Burlina, C.O. Harding, A.C. Muntau, *Molecular Genetics and Metabolism,* **2011**, *104*(Suppl), S19.
[8] O.O. Babich, V.S. Pokrovsky, N.Y. Anisimova, N.N. Sokolov, A.Y. Prosekov, *Biotechnology and Applied Biochemistry*, **2013**, *60*, 316.
[9] B. de Lange, D.J. Hyett, P.J.D. Maas, D. Mink, F.B.J. van Assema, et al., *ChemCatChem,* **2011**, *3*, 289.
[10] B. Hauer, N. Schneider, D. Drew, K. Ditrich, N. Turner, B.M. Nestl, *US patent*, **2012**, US2012/0123155A1.
[11] A. Gloge, J. Zon, A. Kővári, L. Poppe, J. Rétey, *Chemistry - A European Journal,* **2000**, *6*, 3386.
[12] C. Paizs, A. Katona, J. Rétey, *Chemistry - A European Journal,* **2006**, *12*, 2739.
[13] C. Paizs, A. Katona, J. Rétey, *European Journal of Organic Chemistry,* **2006**, 1113.
[14] D. Weiser, L.C. Bencze, G. Bánóczi, F. Ender, R. Kiss, E. Kókai, A. Szilágyi, B.G. Vértessy, O. Farkas, C. Paizs, L. Poppe, *ChemBioChem*, **2015**, *16*, 2257.
[15] J.H. Bartha-Vári, M.I. Toşa, F.D. Irimie, D. Weiser, Z. Boros, B.G. Vértessy, C. Paizs, L. Poppe, *ChemCatChem*, **2015**, *7*, 1122.
[16] F. Ender, D. Weiser, B. Nagy, L.C. Bencze, C. Paizs, P. Pálovics, L. Poppe, *Journal of Flow Chemistry*, **2016**, *6(1)*, 43–52.
[17] H. Ritter, G.E. Schultz, *Plant Cell*, **2004**, *16*, 3426.
[18] J. Sambrook, D.W. Russell, Molecular Cloning a Laboratory Manual. 3rd Edition, New York: Cold Spring Harbor Laboratory Press, **2001**.
[19] Novagen, User Protocol TB009 Rev. F 0104, 1.
[20] J. Crowe, H. Dobeli, R. Gentz, E. Hochuli, D. Stiber, and K. Hence, *Methods in Molecular Biol*ogy, **1994**, *31*, 371.
[21] Ni-NTA Agarose Purification of 6xHis-tagged Proteins from *E. coli* under Native Conditions, Quick-Start Protocol, Qiagen, **2011.**
[22] E.R.S. Kunji, M. Harding, P.J.G. Butler, P. Akamine, *Methods*, **2008,** *46*, 62.
[23] GE Healthcare - *Gel Filtration Calibration Kits*, Product booklet.
[24] M. Lebendiker, T. Danieli, *FEBS Letters,* **2014**, *588*, 236.
[25] a) D. Röther, L. Poppe, G. Morlock, S. Viergutz, J. Rétey, *European Journal of Biochemistry*, **2002**, *269*, 3065; b) L. Poppe, J. Rétey, *Angewandte Chemie International Edition*, **2005**, *44*, 3668; c) S. Bartsch, U.T. Bornscheuer, *Protein Engineering, Design & Selection*, **2010**, 1; d) M.I. Toşa, J. Brem, A. Mantu, F.D. Irimie, C. Paizs, J. Rétey, *ChemCatChem*, **2013**, *5*, 779; e) S.L. Lovelock, N.J. Turner, *Bioorganic & Medicinal Chemistry*, **2014**, *22*, 5555.
[26] L. Poppe, Z. Boros, B.G. Vértessy, K. Kovács, A. Tóth, G. Hornyánszky, J. Nagy, B. Erdélyi, V.E. Bódai, P. Sátorhelyi., *WO* 179072 A1, **2013**.
[27] U.K. Laemmli, *Nature*, **1970**, *227*, 680.