STUDIA UBB. CHEMIA, LV, 4, 2010

EXPRESSION OF PHENYLALANINE AMMONIA-LYASES IN *ESCHERICHIA COLI* STRAINS

KLAUDIA KOVÁCS[a],[b], ANDRÁS HOLCZINGER[c], BEÁTA VÉRTESSY[b,c], LÁSZLÓ POPPE[a]*

ABSTRACT. Phenylalanine ammonia-lyase (PAL, EC 4.3.1.5) catalyzes the non-oxidative conversion of L-phenylalanine into (*E*)-cinnamate. PAL can be applied in organic synthesis, and can be considered also for enzyme supplementation cure for genetic disorder phenylketonuria. The aim of this study was to find optimal expression parameters of several previously cloned PAL's (bacterial, plant and a chimera) in pBAD vectors for further functional characterization. Investigation of the expression level of PAL's in *E. coli* hosts with SDS PAGE analysis as well as activity assay of the recombinant PAL enzymes was performed.

Keywords: *phenylalanine ammonia-lyase, heterologous protein expression, biocatalysis, phenylketonuria*

INTRODUCTION

Phenylalanine ammonia-lyase (PAL) catalyzes the non-oxidative conversion of L-phenylalanine into (*E*)-cinnamate (*Figure 1*) [1].

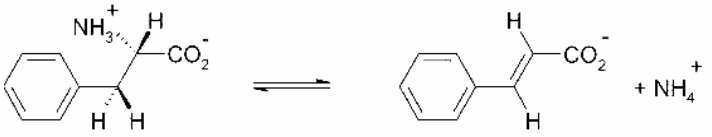

Figure 1. Non-oxidative deamination of phenylalanine

PAL is an important enzyme in both plant development and pathogen defense. In all plants PAL is encoded by a multi-gene family, ranging in copy number from four in *Arabidopsis* to a dozen or more copies in some higher plants [2]. The PAL participates in five metabolic pathways: tyrosine, phenylalanine and nitrogen metabolism, phenylpropanoid and alkaloid biosynthesis. Because of its key role between the primary and secondary metabolism in plants, PAL is a potential target for herbicides.

[a] *Budapest University of Technology and Economics, Department of Organic Chemistry and Technology, and Research Group for Alkaloid Chemistry of HAS, Műegyetem rkp 3, H-1111, Budapest, Hungary, poppe@mail.bme.hu*
[b] *Institute of Enzymology of the Hungarian Academy of Sciences, Karolina út 29, H-1113, Budapest, Hungary*
[c] *Budapest University of Technology and Economics, Department of Applied Biotechnology and Food Science, Műegyetem rkp 3, H-1111, Budapest, Hungary*

By reversing the natural direction of the PAL reaction in the presence of high concentration of ammonia, optically pure L-phenylalanine, which is the precursor molecule of the artificial sweetener aspartame (L-phenylalanyl-L-aspartyl methyl ester) can be produced. Similarly, starting from various (hetero)arylacrylates, further enantiopure L-phenylalanine analogues, such as L-piridil/pirimidil-alanines can be also prepared. Since neither cofactor recycling nor other additives are needed in these asymmetric syntheses, they are potentially interesting as industrial processes as well [3a-e].

In addition to its application in synthetic chemistry, PAL can be applied also in human medicine as treatment to avoid the effects (mental retardation, neurotoxic effects) of the most common congenital metabolic disease, phenylketonuria (PKU). Daily oral administration of micro-encapsulated PAL to PKU rats decreased the systemic toxic phenylalanine level by 75 ± 8% in 7 days ($P < 0.001$) [4]. Because of its many scopes, PAL is an extensively studied enzyme.

Although PAL is an ubiquitous higher-plant enzyme, it has only been encountered in a few bacteria, where cinnamic acid is involved in biosynthesis of several specific bacterial products, such as oligosaccharide antibiotics [5a-c].

The major difference between eukaryotic and prokaryotic PAL's is a ca. 150-residue long C-terminal extension of the eukaryotic PAL's (per monomeric unit) which is not present in the prokaryotic PAL's (*Figure 2*) [6]. In plant and

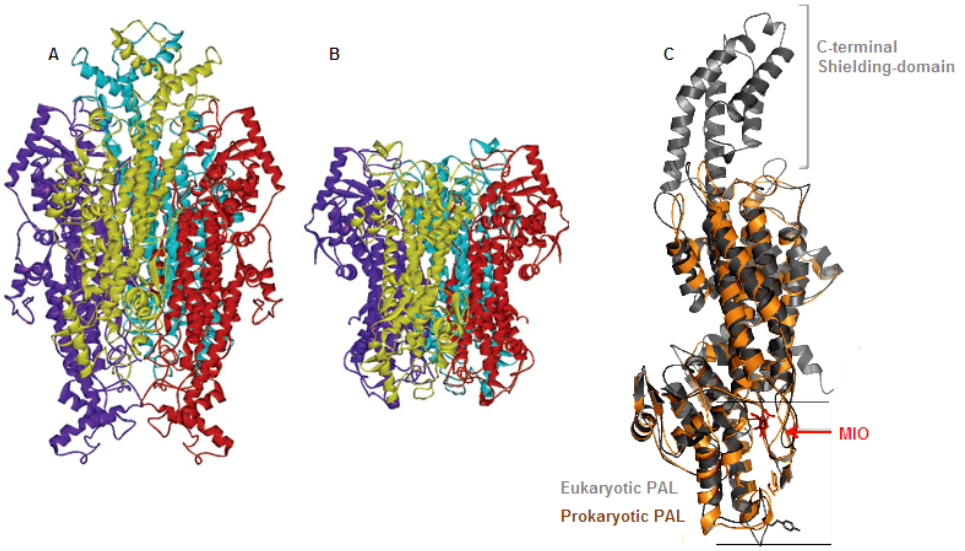

Figure 2. Tetrameric structures of PALs

(A) Homology model of *P. crispum* PAL [7]; (B) Homology model of *P. luminescens* PAL [6]; (C) Difference between eukaryotic and prokaryotic PAL monomers

fungal PAL's,the additional C-terminal domain forms an arch over the active site and has been proposed to function as a shielding domain by restricting substrate entry and product egress. Alternatively, this domain may influence the conformation of an active-site lid loop and thereby affect the stability and catalytic activity of the holoenzyme. Molecular dynamics studies confirmed the hypothesis that the C-terminal extension decreases the lifetime of eukaryotic PAL by destabilization, which might be important for the rapid responses in the regulation of phenylpropanoid biosynthesis [6].

One of the PAL enzymes expressed in this study was an artificial chimera [L. Poppe, A. Holczinger, *unpublished*] composed of the C-terminal domain of a bacterial PAL from *Photorhabdus luminescens* and of the catalytic N-terminal domain of the plant PAL from *Petroselinum crispum*. The main goal with the expression and thermostability investigations of this chimera PAL is to prove the hypothesis on the destabilizing role of the C-terminal extension of the eukaryotic PAL's.

RESULTS AND DISCUSSION

Expressions of *Petroselinum crispum* PAL and the *Photorhabdus luminescens* PAL were used as references. The catalytic N-terminal segment of the *Photorhabdus luminescens* PAL was expressed also, to compare the activity of this segment to native enzyme. In addition to the *Photorhabdus luminescens* PAL, an other PAL (hereafter HA1) gene of a bacterium growing at relatively high temperature was expressed as well.

To express the PAL genes from different organisms (bacteria, plant and a chimera), pBAD-24 and pBAD-HisB vector systems were investigated in two *E. coli* strains (Rosetta (DE3) and TOP 10) as hosts (*Table 1.*).

Table 1. The expressed genes and the applied vectors

Gene	Vector	Main objectives
Petroselinum crispum PAL (*Pc*PAL); ~2150 bp	pT7-7 (2470 bp)	Eukaryotic reference; optimized system
N-terminal segment of *Photorhabdus luminescens* PAL (PhN); ~1420 bp	pBAD-24 (4542 bp)	Study the catalytic activity of the N-terminal segment.
Chimera (CHI): N-terminal of *Pc*PAL and C-terminal of *Photorhabdus luminescens* PAL; ~1650 bp	pBAD-24 (4542 bp)	Activity and thermostability of the artificial enzyme
Photorhabdus luminescens PAL (Phl6); ~ 1600 bp	pBAD-24 (4542 bp)	Bacterial reference; known, characterized bacterial PAL
PAL from a thermophilic bacterium (HA1); ~ 1700 bp	pBAD-HisB (4092 bp.)	Activity and thermostability of the PAL from a thermophile.

The pBAD vectors containing the PAL genes with relatively weak promoter were used for expression work. The advantage of the the *AraC*- pBAD expression system is that in the presence of L-arabinose the expression from the promoter is turned on, while in the absence of L-arabinose very low level of transcription from pBAD promoter can occur. The uninduced level is repressed

even further by growth in the presence of glucose. By varying the concentration of L-arabinose, protein expression levels can be optimized to ensure maximum expression of soluble protein. In addition, the tight regulation of pBAD by AraC is really effective to minimize the leakiness of the promoter.

For cloning and transformation, a *rec*A, *end*A, *ara*BADC- and *ara*EFGH+ TOP10 strain was used, which is capable of transporting L-arabinose, but not metabolizing it, and Rosetta (DE3), which doesn't have the *ara*BADC- mutation to prevent arabinose degradation. To the latter strain, the same amount of inductor was re-added at 4 h after the first induction to maintain full induction.

Our aim was to optimize the conditions of the expression with the pBAD vector constructions in the two *E. coli* hosts to achieve expression levels of the PAL enzymes which are satifactory for further biochemical and biocatalytic characterizations. The effects of the expression conditions (temperature, expression time, inductor concentration) on the protein expression level and the activity and thermostability of the enzymes have been studied. Thus, the temperature of expression was varied between 18 ºC to 37 ºC in 3-5 ºC steps, the time of expression was changed between 8-22 h in 2-4 h steps. The inductor concentration was increased gradually from 0,002 to 0,02 %.

After cell disruption by sonication, the PAL activity of the crude extract was determined by measurement PAL by monitoring the formation of (*E*)-cinnamate at 290 nm (ε_{290}= 10^4 L M^{-1} cm^{-1} [3e]). At 290 nm, absorbance of aromatic amino acid residues of the proteins, nucleic acids and denaturation of proteins can influence the measurement, therefore the absorbance values were always corrected with the blind values from determinationd with same amount of substrate-free buffer and supernatant without substrate. The PAL content of the crude extract was confirmed also by SDS-PAGE investigation of samples from various fractions (supernatant, pellet etc.).

Increasing the expression temperature and shortening the expression time had favorable effect on expression levels of all bacterial expressions. The highest expression levels were found at the maximum concentration (0.02 %) of the arabinose inductor. In all cases, expressions with TOP 10 strain (*Figure 3a*) resulted higher level expression than with Rosetta (DE3) strain (*Figure 3b*). Unfortunately, no expression was detected with the chimera PAL in the pBAD systems in our hands (*Figure 3a*).

The optimal parameters of the expressions were determined by considering the SDS-PAGE and activity (U_s: Units/L of crude extract) results together, compared to the expression level and the activity of the *Petroselinum crispum* PAL expression as reference (*Table 2.*). Although the bacterial PAL's were expressed at low level according to the SDS-PAGE, the activites of the expressed bacterial PAL's in the crude extracts (3.3−7.5 U L^{-1}) were comparable to the PAL activity of the crude extract of *Pc*PAL (8.5 U L^{-1}) (*Table 2*). As the purification method for *Pc*PAL at this level of expression is well established [3e,7], expression of the bacterial PAL's at comparable levels followed by further purification steps will be enough to investigate the thermostability (*Figure 3a*) and biotransformation properties of the prokariotic PAL's.

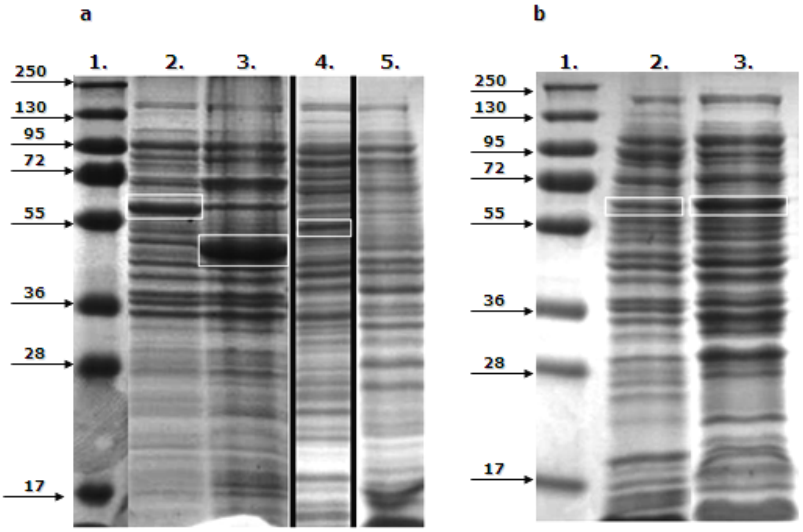

Figure 3. SDS-PAGE on PAL expressions in E. coli TOP 10 and Rosetta strains

(a) SDS-PAGE of the PAL expression in *E. coli* TOP 10 [*Lane 1*: molecular mass markers (250, 130, 95, 72, 55, 36, 28,17 kDa); *Lane 2*: supernatant after cell lysis of the expression of HA1 (58,4 kDa); *Lane 3*: supernatant after cell lysis of the expression of phN; (51,17 kDa); *Lane 4*: supernatant after cell lysis of the expression of Phl6; (57,57 kDa); *Lane 5*: supernatant after cell lysis of the expression of Chi (~58 kDa)];

(b) SDS-PAGE: the different between the expression of the two E. coli strains [*Lane 1:* molecular mass markers (250, 130, 95, 72, 55, 36, 28,17 kDa); *Lane 2:* expression of HA1 (58,4 kDa) in Rosetta (DE3) strain; *Lane 3:* expression of HA1 (58,4 kDa) in TOP 10 strain].

Table 2. The optimal expression conditions of PALs in pBAD vectors and activity of the crude extracts

Expressed enzyme	Inductor	Temperature (°C)	Time (h)	U_s (U/L)
P. crispum PAL [8]	1 mM IPTG	20	20	8.5
P. luminescens PAL	0,2 % arabinose	30	12	7.5
N-terminal segment of *P. luminescens* PAL	0,2 % arabinose	25	18	6.2
HA1 bacterial PAL	0,2 % arabinose	25	16	3.3

To achieve higher PAL yield, coexpression with pREP4-groESL chaperon plasmid was investigated as well (*Figure 4.*).

Analysis of the expression level of the bacterial PAL's with this coexpression system proved to be difficult, because the size of groEL chaperon (~65 kDa) is similar to the bacterial enzymes (~60 kDa). Thus, appraise the level of PAL expression by SDS-PAGE failed (*Figure 4.*). Because the level of chaperon expression seems to exceed the level of PAL expression, construction of a vector system carrying the PAL and chaperon genes under the contol of the same promoter is considered.

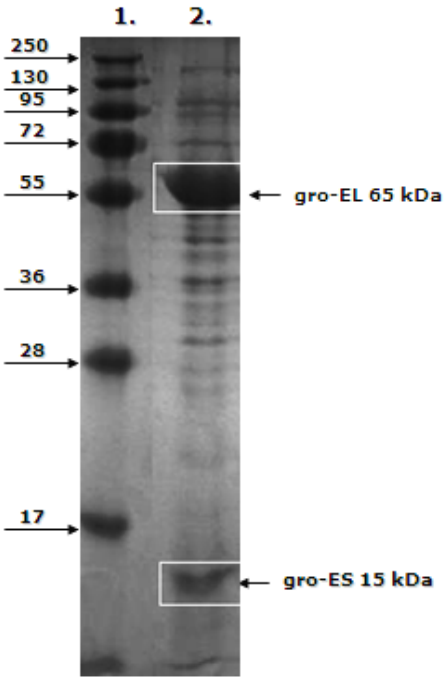

Figure 4. The gro-EL chaperone coexpression with bacterial PAL's (~60 kDa)
Lane 1: molecular mass markers (250, 130, 95, 72, 55, 36, 28, 17 kDa);
Lane 2: supernatant after cell lysis of the reported expression of HA1 (58,4 kDa) in *E. coli* TOP 10 (coexpression of the pREP4-groESL chaperon plasmid)

CONCLUSIONS

In this study, expression of several bacterial phenylalanine ammonia-lyases in *Eschericia coli* TOP 10 using pBAD vactor was investigated. The activity assay of the expressed bacterial PAL's using the supernatant of the *E. coli* lysate indicated catalytic activity and thus the presence of active soluble enzymes. The expression levels of the bacterial PAL's were adequate for further investigations. The highest protein expression level by SDS-PAGE was found in the expression of *N*-terminal segment of *Photorhabdus luminescens* PAL, whereas the highest crude supernatant activity was achieved with the native *Photorhabdus luminescens* PAL. Due to the non-synchronized expression and overproduction of the chaperon protein in the pBAD-PAL / pREP4-groESL chaperon coexpression system, cloning of the PAL genes without and with groESL chaperon into pET vector is considered.

EXPERIMENTAL SECTION

Transformation and expression of PAL genes

E. coli TOP 10 and Rosetta (DE3) strains were transformed with different plasmids containing PAL genes (see *Table 1*.) and grown in 50 mL low salt LB broth/medium with 100 μg/mL ampicillin [and 35 μg/mL chlorampehnicol only for Rosetta (DE3) strain] overnight 37°C with shaking (220 rpm) until OD600= ~1.5. From the resulting culture, 800 μL volume was added to 50 mL LB (containing 100 μg/mL ampicillin and 35 μg/mL chlorampehnicol). The resulting cultures were grown at 37°C with vigorous shaking until OD600 = ~0.6. At this OD, the temperature was changed to the induction temperature (in a range of 18 ºC – 37 ºC; 4 ºC steps for screening) and the cells were induced with arabinose (in a range of 0,02-0,2 %; 0.05 % steps for screening). At 4 h after the first induction, the same amount of inductor was added to Rosetta cell cultures. After addition of the inducer, the cultures were further cultivated for 12 h and harvested by centrifugation. All subsequent operations were carried out at 4 ºC.

Cell disruption and activity measurement

The pellet was resuspended in 5 mL of lysis buffer (150 mM NaCl, 50 mM TRIS pH 8.0, 10 mM BME Protease inhibitor cocktail, 2 mM PMSF, 5 mM BA) and sonicated in ice bath at amplitude 40 % and pulsation 60 % using a Bandelin Sonopuls HD 2070 instrument. The sonication was performed until the viscosity of the suspension was significantly lowered. After centrifugation (30 min at 5000 x g), PAL activity was determined in the crude extract by monitoring the formation of (*E*)-cinnamate at 290 nm (ε_{290} =10^4 L M^{-1} cm^{-1}). The assay contained performed at room temperature by addition of 100 μL of the supernatant from the crude extract to 1000 μL of 0.1 M Tris-HCl, pH 8.8, containing 20 mM L-phenylalanine and recording the absorbance at 290 nm for 10 min. The PAL content of the crude extract, supernatant and pellet were analyzed by SDS-PAGE as well.

ACKNOWLEDGMENTS

This work was supported by the Hungarian National Office for Research and Technology (NKFP-07-A2 FLOWREAC), by the Hungarian National Science Foundation (OTKA T-048854, K68229 and CK78646) and by Howard Hughes Medical Institutes #55000342. This work is also related to the scientific program of "*Development of quality-oriented and harmonized R+D+I strategy and functional model at BME*" project (TÁMOP-4.2.1/B-09/1/KMR-2010-0002), supported by the New Hungary Development Plan. The authors thank Prof. János Rétey (Institute of Organic Chemistry, Karlsruhe Institute of Technology, Germany), Prof. G. E. Schulz and M. Baedeker (University of Freiburg, Germany) for providing us with the *Petroselinum crispum* PAL expression system,) and also Dr M. Stieger (Hoffmann-La Roche, Basel, Switzerland) for the vector pREP4-GroESL carrying the HSP-60 system.

REFERENCES

1. H. Ritter, G.E. Schulz, *Plant Cell*, **2004**, *16*(12), 3426.
2. A. Chang, M.H. Lim, S.W. Lee, E.J. Robb, R.N. Nazar, *J. Biol. Chem.*, **2008**, *283*(48), 33591.
3. (a) S. Yamada, K. Nabe, N. Izuo, K. Nakamichi, I. Chibata, *Appl. Environ. Microbiol.*, **1981**, *42*, 773. (b) C.T. Evans, K. Hanna, C. Payne, D. Conrad, M. Misawa, *Enz. Microb. Tech.,***1987**, *9*, 417. (c) M. Yanaka, D. Ura, A. Takahashi, N. Fukuhara, *Japan Patent,* **1994**, 06-113 870. (d) A. Gloge, B. Langer, L. Poppe, J. Rétey, *Arch. Biochem. Biophys.,***1998**, *359*, 1. (e) A. Gloge, J. Zon, Á. Kővári, L. Poppe, J. Rétey, *Chem. - Eur. J.*, **2000**, *6*, 3386.
4. L. Bourget, T.M. Chang, *FEBS Lett.*, **1985**, *180*, 5.
5. (a) A.V. Emes, L.C. Vining, *Can. J. Biochem.,* **1970**, *48*(5), 613. (b) L. Xiang, B.S. Moore, *J. Bact.*, **2005**, *187*(12), 4286. (c) J.S. Williams, M. Thomas, D.J. Clarke, *Microbiol.*, **2005**, *151*, 2543.
6. S. Pilbák, A. Tomin, J. Rétey, L. Poppe, *FEBS J.*, **2006**, *273*(6), 1004.
7. D. Röther, L. Poppe, G. Morlock, S. Viergutz, J. Rétey, *Eur. J. Biochem.*, **2002**, *269*(12), 3065.
8. M. Baedeker, G.E. Schulz, *FEBS Lett.*, **1999**, *457*, 57.