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A NOVEL PHENYLALANINE AMMONIA-LYASE FROM *KANGIELLA KOREENSIS*

ANDREA VARGA[a], ZSÓFIA BATA[b, d], PÁL CSUKA[b, d], DIANA MONICA BORDEA[a], BEÁTA G. VÉRTESSY[c, d], ADRIANA MARCOVICI[e], FLORIN DAN IRIMIE[a], LÁSZLÓ POPPE[* a, b], LÁSZLÓ CSABA BENCZE[* a]

ABSTRACT. This study describes the cloning of the gene encoding a novel phenylalanine ammonia-lyase from *Kangiella koreensis* (*Kk*PAL) into pET19b expression vector. Optimization of protein expression and purification conditions yielded 15 mg pure soluble protein from one liter of *E. coli* culture. Enzymatic activity measurements of the ammonia elimination reaction from different natural aromatic amino acids proved the protein to be a phenylalanine ammonia-lyase. The isolated protein showed remarkably high, 81.7 °C melting temperature, making it especially suitable for biocatalytic applications.

Keywords: *phenylalanine ammonia-lyase, Kangiella koreensis, protein expression, optimization*

INTRODUCTION

The use of enzymes as biocatalysts for the preparation of chemicals has received steadily increasing attention over the past few years and found significant applications in many areas, especially in the synthesis of

[a] *Babeş-Bolyai University, Faculty of Chemistry and Chemical Engineering, Arany János str. 11, RO-400028, Cluj-Napoca, Romania.*
[b] *Department of Organic Chemistry and Technology, Budapest University of Technology and Economics, Műegyetem rkp. 3, H-1111 Budapest, Hungary.*
[c] *Department of Biotechnology and Food Sciences, Budapest University of Technology and Economics, Szt. Gellért tér 4, H-1111 Budapest, Hungary.*
[d] *Institute of Enzymology, Research Centre for Natural Sciences of Hungarian Academy of Sciences, Magyar tudósok körútja 2, H-1117 Budapest, Hungary.*
[e] *Clinical, Pharmacology and Pharmacokinetics Department, Terapia SA, Fabricii str. 124, RO-400632 Cluj-Napoca, Romania.*
[*] *Corresponding authors: cslbencze@chem.ubbcluj.ro; poppe@mail.bme.hu;*

pharmaceutical and fine chemical targets. [1] Microbial sources received significant attention in the development of enzymes for research or industrial purposes, as microbes can be produced economically in short fermentation time and using inexpensive media. [2]

The natural role of phenylalanine ammonia-lyases (PALs) is the catalysis of non-oxidative ammonia elimination from L-phenylalanine (L-Phe), to form (*E*)-cinnamic acid,[3] as part of the phenylpropanoid synthesis pathway in case of plants, and to form secondary metabolites in fungi and bacteria. [4] Structurally, PALs resemble to phenylalanine 2,3-aminomutases (PAMs),[5] tyrosine 2,3-aminomutases (TAMs),[6] tyrosine ammonia-lyases (TALs),[7] and histidine ammonia-lyases (HALs). [8] All of these enzymes rely on the protein-derived electrophilic prosthetic group, 3,5-dihydro-4-methylidene-5*H*-imidazol-5-one (MIO), that forms autocatalytically from an XSG triade which contains usually Ala-Ser-Gly active site residues. [9]

Synthetic applications are based mostly on the reverse reaction of PAL, as the stereoselective ammonia addition results in the formation of enantiopure unnatural L-amino acids from the corresponding arylacrylates. [10] However, PALs as biocatalysts in these reactions must withstand as high as 6M ammonia concentrations to achieve high conversions. PALs of marine origin – especially PAL from *Idomarina loihiensis* (*Il*PAL) – were capable of catalyzing the ammonia addition with high activity at elevated ammonia and substrate concentrations. [11] Alternatively, enzyme immobilization proved to be a successful strategy for prolonging the lifetime of PALs as biocatalysts. [12]

In frame of our general interest to clone thermotolerant and stable PALs as efficient biocatalysts focusing on enzymes of marine and extremophile origin, herein we describe the molecular cloning, expression and purification of a novel PAL from a marine bacterium *Kangiella koreensis* (*Kk*PAL). *[*13]

RESULTS AND DISCUSSION

Identification of *Kk*PAL

Prokaryotic MIO enzymes are about 150-200 residues shorter than the MIO enzymes from eukaryotes, as the eukaryotic ones contain an additional shielding domain at their C-terminus (**Table 1**). The shorter bacterial enzymes tend to be more stable than the ones of eukaryotic origin.

Table 1. Comparison of six typical MIO enzymes.

	Uniprot code	**Seq. length**	**Seq. identity** [14]
Kangiella koreensis PAL	C7R9W9	516	100%
Idomarina loihiensis PAL	Q5QXE5	515	66%
Anabaena variabilis PAL	Q3M5Z3	567	28%
Petroselinum crispum PAL	P24481	716	22%
Rhodobacter sphaeroides TAL	Q3IWB0	523	29%
Pseudomonas putida HAL	P21310	510	27%

Proteins encoded in extremophile organisms, similarly to their host organisms, are adopted to their living conditions. Thus, enzymes isolated from thermophilic or marine organisms are expected to function efficiently at high temperatures and salt concentrations, respectively. A recently identified PAL from *Idomarina loihiensis* (*Il*PAL) [15] showed promising results in the production of optically active phenylalanine derivatives. [11] A bioinformatics based search using Blastp in the NCBI Non-redundant protein sequence database identified the *Kk*PAL sequence to be 66% identical to *Il*PAL (**Table 1**). Thus, it was expected that *Kk*PAL could be a stable and efficient biocatalyst for the synthesis of optically active phenylalanine analogues.

Comparison of the active site residues of *Kk*PAL with *Il*PAL and two further MIO enzymes (**Table 2**) showed that all catalytic residues and residues in the carboxylate binding region of the active site were conserved. However, the aromatic binding region of the active site of *Kk*PAL appeared to be different, as at position 90 in *Kk*PAL, a histidine was found instead of leucine within the mesophilic PALs (*Av*PAL and *Pc*PAL). Automated annotation of the sequence database assigned HAL function to the *Kk*PAL sequence due to the characteristic histidine residue at position 90. However, phenylalanine at position 89 renders the aromatic binding pocket of the active site more “PAL-like” (**Table 2**). Based on sequence similarity, we hypothesized that amongst the natural aromatic amino acids, this enzyme will be the most active in the ammonia elimination reaction from phenylalanine.

Histidine to phenylalanine mutations of the adjacent residue (residue 89 in **Table 2.**) enhanced the PAL activity of the tyrosine *a*mmonia-lyase from *Rhodobacter sphaeroides* (*Rs*TAL), and decreased significantly the TAL activity. [16] The presence of a hydrogen bond forming residue, histidine at the aromatic binding pocket of *Kk*PAL suggested a possible tyrosine ammonia-lyase activity and probable substrate promiscuity towards other natural aromatic amino acids.

Table 2. Sequence alignment of active site residues[a] in six typical MIO enzymes

	60	66	86	90	149	153	202	283	316
*Kk*PAL	IYGVTTGYG	LHLTRF	HGCGL	VGASGDLT	MNGTAV		QDRYSIR	NDNPI	
*Ii*PAL	IYGVTTGYG	IHLTRF	HGCGL	VGASGDLT	MNGTAV		QDRYSIR	NDNPI	
*Av*PAL	IYGVTSGFG	TNLVWF	LKTGA	IGASGDLV	MNGTSV		QDRYSLR	TDNPL	
*Pc*PAL	SYGVTTGFG	KELIRF	LNAGI	ITASGDLV	VNGTAV		QDRYALR	NDNPL	
*Rs*TAL	VYGLTTGFG	ANLVHH	LASGV	VGASGDLT	VNGTSA		QDAYSLR	TDNPV	
*Pp*HAL	AYGINTGFG	RSLVLS	HAAGI	VGASGDLA	LNGTQA		QDPYSLR	SDNPL	

[a] Active sites residues are colored by their locations and roles. Residues directly involved in catalysis are shown in green. Residues forming the hydrophobic binding pocket (binding of the aromatic group) are shown in orange and residues found at the hydrophilic part (carboxylate binding) of the binding pocket are shown in blue.

Molecular cloning of *Kk*PAL

The gene encoding *Kk*PAL was codon optimized for better expression in *E. coli* and synthesized. The synthetic *Kk*PAL gene was cloned in pUC57 production vector and later sub-cloned into the pET19b expression vector. Restriction sites for *NdeI*, *NcoI* and *BamHI* were added to the protein coding sequence allowing directional cloning into the expression vector. Primers detailed in **Table 3** (*Kk*PAL_forward and *Kk*PAL_reverse primers) were used to amplify the synthetic gene from the pUC57 cloning vector, followed by restriction cloning to the expression vector using *NdeI* and *BamHI* enzymes and T4 DNA ligase. The pET19b vector contains an *N*-terminal His_{10}-tag attached through enterokinase cleavage site to the inserted sequence, facilitating protein purification.

Table 3. Primers used for amplification of *Kk*PAL gene and for the colony PCR

Primers	Sequences	T_m (°C)
*Kk*PAL_forward primer	5'CTAGATAATACCATGGGCCATATG3'	62
*Kk*PAL_reverse primer	5'CCGATTATGGATCCTTAGTTAGC3'	62
T7-promoter_forward primer	5'AATACGACTCACTATAGGGGAATTG3'	54
T7-terminator_reverse primer	5'TGCTAGTTATTGCTCAGCGG3'	55

Colony PCR verified the successful insertion of the DNA encoding *Kk*PAL to the pET19b vector. This convenient high-throughput method determines the presence or absence of the inserted DNA sequence by a standard PCR process, from individual colonies obtained after the ligation reaction. Vector specific T7-promoter forward and T7-terminator reverse oligonucleotides (**Table 3**) served as primers for the PCR reactions. Sequencing of plasmid DNA, isolated from the colonies with positive colony PCR reactions, ascertained the cloning results.

Optimization of the overexpression of *Kk*PAL

Variation of the host cell strain, growth temperature and inducer concentration influence the overexpression levels of the target protein. Strategy for optimization and the identified optimal conditions for *Kk*PAL overexpression are detailed in the next section.

E. coli is the most commonly used bacterial host for recombinant protein production. It has become the most popular expression platform, as it is easy to manipulate genetically, inexpensive to culture, and its expression is rapid. [17] *E. coli* strain Rosetta(DE3)pLysS enables low background expression, and expresses T7 lysozyme suppressing basal T7 RNA polymerase expression, reducing translation of the pET recombinants in the absence of inducer. Hence, we choose Rosetta(DE3)pLysS as host strain for expression of *Kk*PAL.

Optimization of the inducer concentration

Induction of the *lac* promoter expresses the target genes in pET systems. Naturally, this promoter is induced by the lactose metabolite allolactose. However, in practice the non-degradable IPTG (isopropyl β-D-1-thiogalactopyranoside) is employed, instead of lactose. Varying the concentration of IPTG regulates the expression of the target protein. Lowering the IPTG concentration may reduce the protein expression level, thereby increasing the solubility of aggregation prone protein. Although at the cost of increased expression time and lower protein yield. [18]

Protein expression levels of recombinant *Kk*PAL as a function of various inducer concentrations were evaluated in liquid cultures. Upon achieving exponential growth phase (OD at 600 nm ~ 0.6), cultures were induced with five different concentrations of IPTG between 0 mM and 0.5 mM and expression proceeded for 3 h at 37 °C (**Figure 1**)**.** *Kk*PAL expression occurred already at 0.1 mM IPTG (60 kDa band Lane **3**), and inducer concentration above 0.1 mM enhanced *Kk*PAL production (Lanes **4-6**). However, further increase of the IPTG concentration above 0.2 mM (Lane **4**) did not increase *Kk*PAL expression (Lanes **5,6**). As later experiments showed, the protein expressed in a soluble form, therefore the IPTG concentration was set to 0.2 mM.

Effect of expression temperature on the overexpression of KkPAL

The optimum temperature for *E. coli* growth is 37 °C, and several studies reported 37 °C as the best temperature for maximum protein production as well. [19] On the other hand, other studies showed that not

only the rate of expression, but the culturing temperature affects the proper folding of recombinant proteins. [20] Lowering the expression temperature leads to slower growth of bacteria and reduces the rate of protein production, hence decreasing the risk of aggregation of target protein. In addition, most of the proteases express much less activity at lower temperatures. Thus, degradation of the target proteins at lower temperatures are much less pronounced. [21,22]

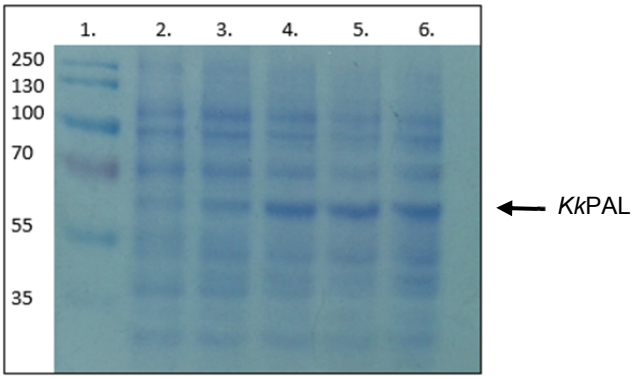

Figure 1. SDS-PAGE showing the effect of various IPTG concentrations on the expression of *Kk*PAL (after 4 h at 37 °C). Samples in the lanes: **1**: protein ladder, **2**: control (0 mM IPTG), **3**: induction with 0.1 mM IPTG, **4**: induction with 0.2 mM IPTG, **5**: induction with 0.3 mM IPTG, **6:** induction with 0.5 mM IPTG.

Intermittent optical densitometry (OD) measurements at 600 nm evaluated the effect of growth temperature on the expression of *Kk*PAL after induction. Initially, the cell cultures were incubated at 37 °C. After the density of cells reached OD_{600} ~ 0.4 (approx. 2-3 h), the temperature was reduced (to 20, 25 or 30 °C). To allow cultures to adjust to the temperature change, protein production was only induced 30 minutes after decreasing the temperatures. The density of the cells was monitored as a function of time (**Figure 2**).

Due to the reduced incubation temperature, the protein synthesis rate was slower at 20 °C than at 25 or 30 °C and longer induction times were necessary for cells growth. At higher expression temperatures (25 or 30 °C), the protein synthesis was faster and the stationary phase was reached after 8 h, compared to 14 h at 20 °C. After post-induction, the cells were harvested by centrifugation, followed by sonication and the crude protein mixture in the lysate was purified by metal affinity chromatography on Ni-NTA resin. The maximum yield, 15 mg L^{-1} purified enzyme, was obtained at 25 °C expression temperature. The optimal post-induction time on the expression of *Kk*PAL was 12-14 h.

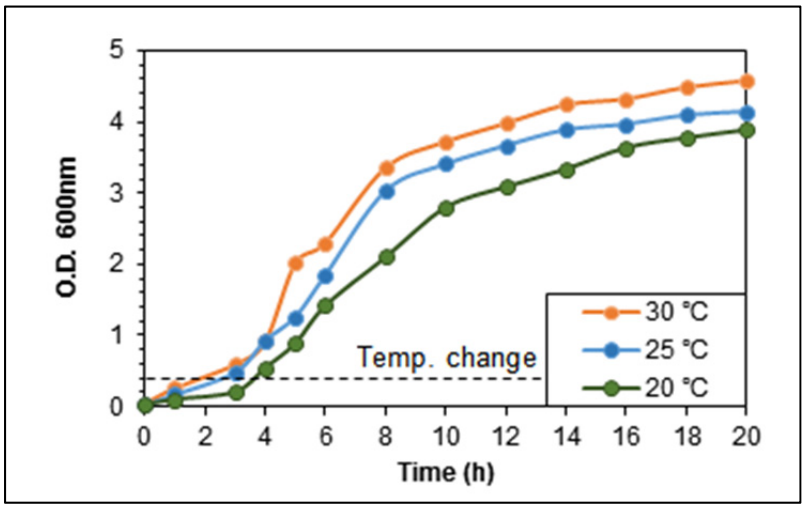

Figure 2. Growth curves of *E. coli* Rosetta(DE3)pLysS containing the pET19b-*Kk*PAL plasmid, at 20, 25 and 30 °C in LB medium.

Purification

Purification using Ni-NTA chromatography

Ni–NTA chromatography is a rapid and easy purification technique for recombinant proteins carrying a His-tag at either the *N*- or *C*-terminus. The N atoms of the imidazole rings of the His-tag residues form complexes with the unoccupied coordination sites of the immobilized nickel ions. [23]

In the pET19b vector, a His_{10}-tag at the *N*-terminus is fused to the target proteins, which is longer than the usual His_6-tag. Lengthening the His-tag increases the affinity of the enzyme to the Ni-NTA resin. Consequently, higher imidazole concentrations were required to elute the bounded enzyme from the resin (from 250 mM up to 500 mM). [24] We observed, that *Kk*PAL activity decreased after elution from the Ni-NTA column probably due to prolonged exposure to high imidazole concentration. In order to eliminate this effect, we tested 250, 350, 450, 500 mM imidazole concentrations for protein elution. The best result was obtained by elution with 350 mM imidazole, resulting in a protein solution which gave a single band on the SDS-PAGE (**Figure 3**, Lane **2**), indicating high purity of the target enzyme.

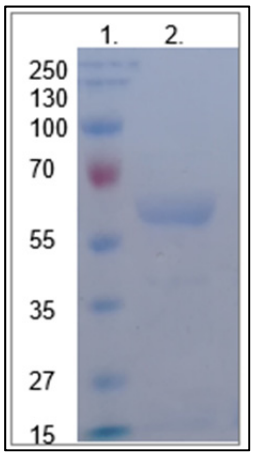

Figure 3. Purification of *Kk*PAL with Ni-NTA chromatography. Lane **1**: protein ladder, Lane **2**: fraction eluted from Ni-NTA with 350 mM imidazole. Samples were prepared as described in the experimental section.

Characterization of *Kk*PAL

Enzyme activity measurements

PAL activity was assayed both in the ammonia elimination and in the ammonia addition reactions. The enzyme activity in the ammonia elimination reaction was determined spectrophotometrically by monitoring the formation of (*E*)-cinnamic acid. Conversions after 16 h obtained by HPLC analysis characterize the enzyme activity in the ammonia addition reaction. [25] In **Table 4** the specific activity and the conversion of the reaction catalyzed by *Kk*PAL were compared with the corresponding properties of the well-studied *Pc*PAL. Contrary to our expectations, *Pc*PAL had better catalytic activity in the addition as well as in the elimination reactions.

Table 4. Specific activities in the ammonia elimination and the conversion in the addition reactions measured for *Kk*PAL and *Pc*PAL.

Enzyme	Elimination reaction	Addition reaction
	Specific activity [a] [μmol min^{-1} mg^{-1}]	**Conversion** [b] [%]
***Kk*PAL**	0.063	3.9
***Pc*PAL**	1.08	77.2

[a] Specific activity measured at 30 °C, with 5 mM L-phenylalanie at pH 8.5 in 100 mM TRIS buffer.
[b] Conversion measured after 16 h at 30°C, with 5 mM (*E*)-cinnamic acid and 6 M ammonium-carbonate, pH 10.

*Kk*PAL catalyzed ammonia elimination also from L-tyrosine, however at a slower rate compared to phenylalanine. The spectrophotometric assays could not detect ammonia elimination from histidine and tryptophan, corroborating with the sequence based annotation of the protein as phenylalanine ammonia-lyase.

Thermal stability

The nanoDSF differential scanning fluorimetry technique is able to analyze the conformational stability and colloidal stability (aggregation behavior) of proteins under different thermal and chemical conditions. The conformational stability of a protein is described by the unfolding transition midpoint T_m (°C), which is the point at which half of the protein is unfolded. The truly label-free nanoDSF method monitors the intrinsic fluorescence of tryptophans in proteins, which relies on the close surrounding of the given tryptophan and changes upon thermal unfolding. Maximum values in the change of the first derivate of the fluorescent signal define the melting temperature of the protein.

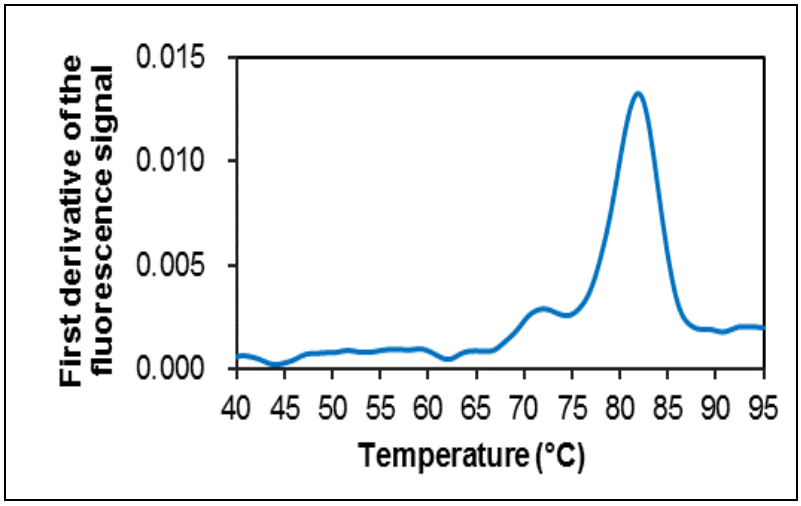

Figure 4. Thermal unfolding curve of *Kk*PAL obtained by nanoDSF measurement

The *Kk*PAL exhibited outstanding thermal stability, as its melting temperature was 81.7 °C (**Figure 4**). This melting temperature is 10 °C higher than that of the eukaryotic *Pc*PAL, 71 °C.

CONCLUSIONS

Different experimental conditions were examined for the expression and purification of *Kk*PAL in order to obtain the enzyme in high yield and high purity. After optimization of IPTG concentration, post-induction temperature on the expression and the imidazole concentration in the purification steps, 15 mg L^{-1} of high purity protein was obtained. For production of *Kk*PAL the induction level with an IPTG concentration of 0.2 mM was sufficient, followed by 12-14 h post-induction incubation at 25 °C. During the purification of the His_{10}-tagged protein on Ni-NTA column, a reduction of the imidazole concentration from 500 mM to 350 mM improved the stability of the resulted enzyme.

Activity measurements showed that the newly cloned *Kk*PAL was less active in the ammonia elimination and addition reactions than the most frequently used *Pc*PAL, but the melting temperature of this novel PAL from a marine bacterium exceeded that of the eukaryotic protein by about 10 C.

The hydrophobic binding pocket *Kk*PAL, similarly to *Il*PAL is a hybrid between the typical motifs found in TALs and HALs. In agreement with our sequence-based annotation, *Kk*PAL showed the highest activity towards phenylalanine amongst the aromatic amino acids. Nevertheless, residue patterns at the hydrophobic region of the binding pocket and the recently reported F137V *Pc*PAL with expanded substrate range,[26] suggest that site directed mutagenesis could enhance activity towards other aromatic amino acids.

EXPERIMENTAL SECTION

All reagents were purchased from Sigma-Aldrich, unless otherwise specified.

Synthesis and cloning of *Kk*PAL gene

The gene of the *Kangiella koreensis* PAL (Uniprot code: C7R9W9, encoding 735 AA – **Table 5**) was optimized to the codone usage of *E. coli*. The 1538 bp long synthetic gene was produced by Genscript in pUC57 vector. At the end of the gene, restriction sites of *NdeI*, *NcoI* and *BamHI* restriction enzymes were introduced for directional cloning into the pET19b expression vector (**Figure 5**).

Table 5. Amino acid and DNA sequences of recombinant *wt-Kk*PAL

Amino acid sequence of *Kk*PAL	Nucleotide sequence of the gene
MTDTKTNITFGHSSLTIEQICQLAKG	5′ AATACCATGGGCCATATGATGACCGACACCAAAACCAACATCACCTTCGGTCACTCTTCTCTGACCA
NATAKLNSAPEFKHKIDQGADFIKEL	TCGAACAGATCTGCCAGCTGGCTAAAGGTAACGCTACCGCTAAACTGAACTCTGCTCCGGAATTTAAAC
LREDGVIYGVTTGYGDSVTTPVPVQD	ACAAAATCGACCAGGGTGCTGACTTCATCAAAGAACTGCTGCGTGAAGACGGTGTTATCTACGGTGTTA
THELPLHLTRFHGCGLGSIFSAEHTR	CCACCGGTTACGGTGACTCTGTTACCACCCCGGTTCCGGTTCAGGACACCCACGAACTGCCGCTGCACC
AILATRLASLSQGYSGVSWSLLQQLE	TGACCCGTTTCCACGGTTGCGGTCTGGGTTCTATCTTCTCTGCTGAACACACCCGTGCTATCCTGGCTA
LLLQKDILPRIPEEGSVGASGDLTPL	CCCGTCTGGCTTCTCTGTCTCAGGGTTACTCTGGTGTTTCTTGGTCTCTGCTGCAGCAGCTGGAACTGC
SYVAAALIGEREVLYKGQTQPTEQVF	TGCTGCAGAAAGACATCCTGCCGCGTATCCCGGAAGAAGGTTCTGTTGGTGCTTCTGGTGACCTGACCC
KSLGIKPITLQPKEGLAIMNGTAVMT	CGCTGTCTTACGTTGCTGCTGCTCTGATCGGTGAACGTGAAGTTCTGTACAAAGGTCAGACCCAGCCGA
ALACLAFQRADYLTQLCSRITSLCSI	CCGAACAGGTTTTCAAATCTCTGGGTATCAAACCGATCACCCTGCAGCCGAAAGAAGGTCTGGCTATCA
ALQGNSAHFDELLFSVKPHPGQNQVA	TGAACGGTACGGCTGTTATGACCGCTCTGGCTTGCCTGGCTTTCCAGCGTGCTGACTACCTGACCCAGC
AWIRDDLNHYKHPRNSDRLQDRYSIR	TGTGCTCTCGTATCACCTCTCTGTGCTCTATCGCTCTGCAGGGTAACTCTGCTCACTTCGACGAACTGC
CAPHIIGALKDAMPWMRQTIETELNS	TGTTCTCTGTTAAACCGCACCCGGGTCAGAACCAGGTTGCTGCTTGGATTCGTGACGACCTGAACCACT
ANDNPIIDGAGQHVLHGGHFYGGHIA	ACAAACACCCGCGTAACTCTGACCGTCTGCAGGACCGTTACTCTATCCGTTGCGCTCCGCACATCATCG
MVMDSMKTGIANLADLMDRQMALLVD	GTGCTCTGAAAGACGCTATGCCGTGGATGCGTCAGACCATCGAAACCGAACTGAACTCTGCTAACGACA
SKFNNGLPNNLSAASEQRRPLNHGFK	ACCCGATCATCGACGGTGCTGGTCAGCACGTTCTGCACGGTGGTCACTTCTACGGTGGTCACATCGCTA
AVQIGVSAWTAEALKLTMPASVFSRS	TGGTTATGGACTCTATGAAAACCGGTATCGCTAACCTGGCTGACCTGATGGACCGTCAGATGGCTCTGC
TECHNQDKVSMGTIAARDCLRILDLT	TGGTTGACTCTAAATTCAACAACGGTCTGCCGAACAACCTGTCTGCTGCTTCTGAACAGCGTCGTCCGC
EQVAAASLMAATQAVTLRIKQSQLDK	TGAACCACGGTTTCAAAGCTGTTCAGATCGGTGTTTCTGCTTGGACCGCTGAAGCTCTGAAACTGACCA
SSLSDGVLSTLEQVFEHFELVSEDRP	TGCCGGCTTCTGTTTTCTCTCGTTCTACCGAATGCCACAACCAGGACAAAGTTTCTATGGGTACGATCG
LEHELRHFVALIQEQHWSTYAN	CTGCTCGTGACTGCCTGCGTATCCTGGACCTGACCGAACAGGTTGCTGCTGCTTCTCTGATGGCTGCTA
	CCCAGGCTGTTACCCTGCGTATCAAACAGTCTCAGCTGGACAAATCTTCTCTGTCTGACGGTGTTCTGT
	CTACCCTGGAACAGGTTTTCGAACACTTCGAACTGGTTTCTGAAGACCGTCCGCTGGAACACGAACTGC
	GTCACTTCGTTGCTCTGATCCAGGAACAGCACTGGTCTACCTACGCTAACTAAGGATCCATA 3′

Cleavage sites of the restriction enzymes: *NcoI* :CCATGG, *NdeI* :CATATG, *BamHI*: GGATCC

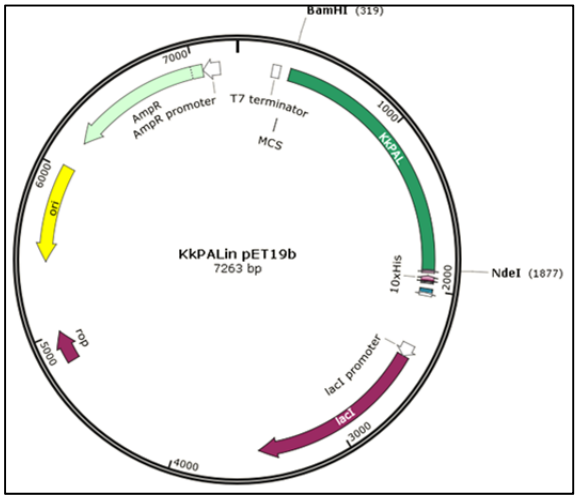

Figure 5. Vector map of the pET19b-*Kk*PAL construct.

PCR reaction for amplification of the gene

The PCR reactions with a total volume of 50 µL consisted of 90 ng of DNA template (plasmid containing the gene of *Kk*PAL), 1 µM of each of the primers, 200 µM dNTP (ThermoFischer) and 2.5 units of *Phu Hot-Start DNA polymerase* (ThermoFischer). The PCR cycles were initiated at 95 °C for 3 min to denature the template DNA, followed by 35 amplification cycles (95 °C for 30 s, 57 °C for 30 s and 72 °C for 3 min). The PCR cycles were finished with a final extension step at 72 °C for 15 min.

The PCR products were further purified, using the *DNA Clean & Concentrator™-25 Kit,* by Zymo research. The purified PCR products and the recipient circular pET19b vector were digested with *NdeI* and *BamHI* restriction enzymes (ThermoFischer), at 37 °C for 1 h and then 40 µL (approx. 2 µg) of each digested DNA was purified by agarose gel electrophoresis. The DNA bands were cut out from the agarose gel. The recipient plasmid and insert at a molar ratio 1:3 were co-extracted using *Gen Elute Gel Extraction Kit* (Genomed) and afterwards ligated in presence of T4 DNA ligase (ThermoFischer) at 22 °C for 1 h.

Transformation in *E. coli* cells

For the transformation of plasmid DNA into *E. coli* XL1-Blue (for plasmid amplification) and Rosetta(DE3)pLysS (for expression) the heat shock method was used. Thawing the chemically competent bacterial cells (50 µL) on ice for 15 min, 1-2 µL of plasmid DNA was added followed by incubation for 20 min on ice. The heat shock was performed by incubating the sample at 42°C for 45 s, and on ice for further 2 min. 400 µL LB media was added and the cells were grown at 37 C for 1 h. In case of XL1-Blue transformation the transformed bacteria were plated on LB agar-plates containing tetracycline (30 µg mL^{-1}) and carbenicilin (50 µg mL^{-1}). In case of Rosetta(DE3)pLysS transformation carbenicillin (50 µg mL^{-1}) and chloramphenicol (30 µg mL^{-1}) were used. pET19b encodes the resistance gene for ampicillin, however carbenicillin was used for selection, due to its higher stability. Agar plates were incubated overnight at 37°C, forming single colonies of bacteria bearing the plasmid encoding the recombinant protein.

Colony PCR reaction

The PCR reactions with a total volume of 20 µL consisted of 10 µL *Dream Tag Green Master Mix* (ThermoFischer), 1 µM each of the primers

(**Table 3**), one colony of DNA template and 8 µL of ddH_2O. The PCR cycles were initiated at 95°C for 3 min to denature the template DNA, followed by 40 amplification cycles. Each amplification cycle consisted of 95°C for 3 min, 57°C for 30 s and 72 C for 1 min 30 sec. The PCR cycles were finished with a final extension step at 72°C for 15 min. The PCR reactions were analyzed by agarose gel electrophoresis. Presence of the amplified ~1500 bp product indicated colonies where insertion of the gene was successful.

Expression of the recombinant *Kk*PAL

The recombinant *Kk*PAL carrying *N*-terminal $(His)_{10}$-tag was overexpressed in *E. coli* host cells Rosetta(DE3)pLysS. For the expression step, a colony of the transformed plasmid was grown overnight at 37°C in 50 mL of Luria-Bertani (LB) medium containing carbenicillin (50 µg mL^{-1}) and chloramphenicol (30 µg mL^{-1}). A 0.5 L of LB medium was inoculated with 1 v/v% of the overnight culture and grown at 37°C until the optical density at 600 nm (OD_{600}) reached 0.4, after which the temperature was reduced to 20, 25 and 30°C. To induce protein production varying concentrations of IPTG was added to the cells at OD_{600} of 0.6-0.7. In the expression phase the culture was shaken at 180 rpm for 16 h.

Purification of *Kk*PAL

All protein purification steps were performed at 4 C. Cells were harvested by centrifugation (25 min, 5000×g) and resuspended in 50 mL lysis buffer (150 mM NaCl, 50 mM TRIS (2-amino-2-(hydroxymethyl)propane-1,3-diol) pH 7.5) supplemented with DNAse, RNAse, Lysosyme and EDTA-free protease-inhibitor cocktail. Further, the cells were lysed by sonication and cell debris was removed by centrifugation (12000×g, 30 min).

The His-tagged *Kk*PAL was separated from other proteins in the supernatant by Ni-NTA-agarose column. After loading the sample, the column was washed with low salt buffer, (50 mM HEPES (4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid), 30 mM KCl pH 7.5 4V; V= resin volume) high salt buffer (50 mM HEPES, 300 mM KCl pH 7.5, 2V), and low salt buffer (2-4V) again. The low salt buffer supplemented with 25 mM imidazole removed the non-specifically bound contaminating proteins. Low salt buffer supplemented with varying amounts (between 250-500 mM) imidazole eluted the *Kk*PAL from the column.

The resulting eluate was dialyzed against 100 mM TRIS-buffer (pH 8.0) for 5 h at 4 C. The purity of the resulting fractions was verified by SDS-

PAGE analysis on a 12% SDS-PAGE. After dialysis the fractions containing purified protein were concentrated by centrifugal ultrafiltration with Amicon filter units. The concentration of the purified protein was determined by the Bradford method.

Enzyme activity measurements

Elimination reactions. Activity of *Kk*PAL in the ammonia elimination reaction was determined spectrophotometrically by monitoring the formation of the conjugated acrylic acid product. The measurements were performed at 30 C for 5 min with 5 mM L-phenylalanine, in the presence of 0.3 μM enzyme in 0.1 M TRIS-buffer (pH 8.5). Phenylalanine ammonia-lyase activity was determined by measuring the formation of (*E*)-cinnamic acid at 290 nm for 10 min, using quartz cuvettes of 1 mL. Histidine ammonia-lyase activity was determined as the rate of urocanate formation measured spectrophotometrically at 277 nm. The conversion of L-tyrosine to *p*-coumarate followed at 310 nm, determined the tyrosine ammonia-lyase acitvity. Tryptophan ammonia-lyase activity was measured by the rate of indole 3-acrylic acid formation at 315 nm.

Addition reactions. Into the solution of (*E*)-cinnamic acid (5 mM) in 6 M NH_3, pH 10 (adjusted with CO_2), *Kk*PAL or *Pc*PAL (0.6 μM) was added and the reaction mixtures were shaken at 300 rpm, at 30°C. After 16 h samples (50 μL) were taken from the enzymatic reaction mixtures, quenched by adding an equal volume of MeOH, vortexed and centrifuged (13000 rpm, 2 min). The supernatant was filtered through a 0.22 μm membrane filter and used directly for HPLC analysis.

Conversions were determined on *Phenomenex Gemini* NX-C-18 column, using as mobile phase: NH_4OH buffer (0.1 M, pH 8.5): MeOH 90:10 to 61:39 in 12 min and 1 mL min^{-1} flow rate. Conversions were calculated from peak area integrations with use of appropriate response factors. [25]

Thermal stability assay

The thermal stability of the enzyme was determined by nanoDSF. The capillaries were filled with the 0.125 mg mL^{-1} (2 μM) *Kk*PAL in 100 mM TRIS-buffer pH 8.5 and placed onto the capillary tray of the *Prometheus NT.48, NanoTemper Technologies.* Melting curves were measured by heating the samples by 1°C min^{-1} increment from 20°C to 95°C.

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REFERENCES

[1] B. M. Nestl, S. C. Hammer, B. A. Nebel, B. Hauer, *Angewandte Chemie, International Edition* **2014**, *53*, 3070–3095.
[2] J. L. Barredo, *Microbial Enzymes and Biotransformations.* **2005,** Humana Press, Totowa.
[3] D. S. Hodgins, *Journal of Biological Chemistry* **1971**, *246*, 2977–2985.
[4] M. Petersen, J. Hans, U. Matern in *Annual Plant Reviews*, 2nd ed., Vol. 40, *Biochemistry of Plant Secondary Metabolism* (Ed.: M. Wink), Wiley-Blackwell, **2010**, 182–257.
[5] L. Feng, U. Wanninayake, S. Strom, J. Geiger, K. D. Walker, *Biochemistry* **2011**, *50*, 2919–2930.
[6] a) S. D. Christenson, W. Liu, M. D. Toney, B. Shen, *Journal of the American Chemical Society* **2003**, *125*, 6062-6063; b) C. V. Christianson, T. J. Montavon, S. G. Van Lanen, B. Shen, S. D. Bruner, *Biochemistry* **2007**, *46*, 7205–7214.
[7] J. A. Kyndt, T. E. Meyer, M. A. Cusanovich, J. J. Van Beeumen, *FEBS Lett*ers **2002**, *512*, 240–244.
[8] a) L. Givot, T. A. Smith, R. H. Abeles, *Journal of Biological Chemistry* **1969**, *244*, 6341–6353; b) R. B. Wickner, *Journal of Biological Chemistry* **1969**, *244*, 6550–6552.
[9] T. F. Schwede, J. Rétey, G. E. Schulz, *Biochemistry* **1999**, *38*, 5355–5361.
[10] L. Poppe, Cs. Paizs, K. Kovács, F.D. Irimie, B.G. Vértessy in *Methods in Molecular Biology,* Vol. 794 (Eds.: L. Pollegioni, S. Servi), **2012**, 3–19. Humana Press, Totowa.
[11] F.B.J. van Assema, N. Sereinig (DSM0). WO 2008/031578, **2008** and PCT/EP 2007/007945, **2007**.
[12] a) 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* , 2257–2402; b.) J. H. Bartha-Vári, M. I. Tosa, F.D. Irimie, D. Weiser, Z.

Boros, B. G. Vértessy, Cs. Paizs, L. Poppe, *ChemCatChem* **2015**, *7*, 1122–1128; c.) F. Ender, D. Weiser, B. Nagy, L.C. Bencze, C. Paizs, P. Pálovics, L. Poppe, *Journal of Flow Chemistry* **2016** , *6,* 43–52.

[13] C. Han, J. Sikorski, A. Lapidus, M. Nolan, T. Glavina Del Rio, H. Tice,J. F. Cheng, S. Lucas, F. Chen, A. Copeland, N. Ivanova, K. Mavromatis, G. Ovchinnikova, A. Pati, D. Bruce, L. Goodwin, S. Pitluck, A. Chen, K. Palaniappan, M. Land, L. Hauser, Y. J. Chang, C. D. Jeffries, P. Chain, E. Saunders, T. Brettin, M. Göker, B. J. Tindall, J. Bristow, J. A. Eisen, V. Markowitz, P. Hugenholtz, N. C. Kyrpides, H. P. Klenk, J. C. Detter, *Standards in Genomic Sciences*, **2009**, *1,* 226–233.

[14] Söding J, *Bioinformatics,* **2005**, *21,* 951–960.

[15] S. Hou, J. H. Saw, K. S. Lee, T. A. Freitas, C. Belisle, Y. Kawarabayasi, S. P. Donachie, A. Pikina, M. Y. Galperin, E. V. Koonin, K.S. Makarova, M. V. Omelchenko, A. Sorokin, Y. I. Wolf, Q. X. Li, Y. S. Keum, S. Campbell, J. Denery, S. Aizawa, S. Shibata, A. Malahoff, M. Alam, *Proceedings of the National Academy of Sciences USA*, **2004,** *101*, 18036–18041.

[16] G. V. Louie, M.E. Bowman, M. C. Moffitt, T. J. Baiga, B. S. Moore, J. P. Noel, *Chemistry & Biology* **2006,** *13*, 1327–1338.

[17] F. Baneyx, *Current Opinion in Biotechnology*, **1999**, *10*, 411–421.

[18] J. Sambrook, D. W. Russell, *Molecular Cloning a Laboratory Manual. 3rd Edition,* New York: Cold Spring Harbor Laboratory Press; **2001**.

[19] H. M. Sadeghi, M. Rabbani, E. Rismani, F. Moazen, F. Khodabakhsh, K. Dormiani, Y. Khazaei, *Research in the Pharmaceutical Science,* **2011**, *6*, 87–92.

[20] R. Y. Li, C. Y. Cheng. *Journal Bioscience and Bioengineering,* **2009**, *107,* 512–515.

[21] A. Vera, N. Gonzalez-Montalban, A. Aris, A. Villaverde, *Biotechnology and Bioengineering,* **2007,** *96,* 1101–1106.

[22] J. A. Vasina, F. Baneyx, *Protein Expression and Purif*ication **1997,** *9,* 211–218.

[23] J. Crowe, H. Dobeli, R. Gentz, E. Hochuli, D. Stiber, K. Hence, *Methods in Molecular Biol*ogy, **1994**, *31*, 371–387.

[24] T. Tanaka, M. Kubota, K. Samizo, Y. Nakajima, M. Hoshino, T. Kohno, E. Wakamatsu, *Protein Expression and Purification,* **1999,** *1,* 207–212.

[25] A. Varga, G. Bánóczi, B. Nagy, L. C. Bencze, M. I. Toșa, Á. Gellért, F. D. Irimie, J. Rétey, L. Poppe C. Paizs, *RSC Advances*, **2016**, *6*, 56412–56420.

[26] L. C. Bencze, A. Filip, G. Bánóczi, M. I. Toșa, F. D. Irimie, Á. Gellért, L. Poppe, C. Paizs, *Organic & Biomolecular Chemistry,* **2017**, *15*, 3717–3727.