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# EXPRESSION AND PURIFICATION OF RECOMBINANT PHENYLALANINE 2,3-AMINOMUTASE FROM *PANTOEA AGGLOMERANS*

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**ABSTRACT.** In the present study, the gene of phenylalanine 2,3-aminomutase from *Pantoea agglomerans* (*Pa*PAM) was cloned into pET-19b vector and used for its expression in competent *Escherichia coli* cells. The recombinant plasmid, *Pa*PAM-pET-19b, was transformed into competent *E. coli* strain BL21(DE3)pLysS cells. Overnight culture of the transformed bacteria was induced by the addition of isopropylthio-*β*-D-galactoside (IPTG) to the final concentrations of 0.1, 0.5 and 1 mM. Also, the effects of different temperatures (18, 25 and 30°C) and the incubation time of *Pa*PAM were examined. The fermentation process was scaled up to 10 L fermentor. Affinity purification conditions were analyzed by SDS-PAGE. The $T_m$ and the activity of the purified enzyme was also investigated.

***Keywords:*** *phenylalanine 2,3-aminomutase, Pantoea agglomerans, optimization, protein expression*

## INTRODUCTION

Over the past decades, the preparation of optically pure *α*-amino and *β*-amino acids has received increasing attention because they have

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many applications in their free form and as fundamental building blocks of bioactive natural products[1].

An attractive alternative enzymatic route to obtain enantiomerically pure *α*- and *β*-amino acids involves the use of phenylalanine ammonia-lyases (PALs) and phenylalanine 2,3-aminomutases (PAMs) [2].

Phenylalanine ammonia-lyases and 2,3-aminomutases are emerging as important enzymatic systems, not only in green synthetic routes to chiral amines, but also as potential target for treating diseases such as phenylketonuria and cancer [2].

PAL catalyzes the nonoxidative elimination of ammonia from L-phenylalanine to give *trans*-cinnamic acid, whereas PAM catalyzes the isomerization of *α*- into *β*-phenylalanine [3].

PAMs and PALs are members of the class I lyase-like family that includes tyrosine 2,3-aminomutases (TAMs) [4], tyrosine ammonia-lyases (TALs)[5], and histidine ammonia-lyases (HALs) [6]. All these enzymes rely on a protein-derived cofactor, 4-methylideneimidazol-5-one (MIO), which is generated autocatalytically from three active site residues, Ala-Ser-Gly (Thr-Ser-Gly in *Pa*PAM), forming a MIO signature motif [7,8].

The reactions catalyzed by these enzymes have considerable potential for biotechnological applications. They are used as biocatalysts for the synthesis of (*S*)-*α*-amino acids from arylacrylates (PALs), in kinetic resolution processes for obtaining (*R*)-*α*-amino acids starting from their racemates (PALs), or for the synthesis of (*R*)- or (*S*)-*β*-amino acids (*Tc*PAM and *Pa*PAM respectively) [9,10, 11,12,13].

Further, although *Tc*PAM and *Pa*PAM are both aryl amino acid 2,3-aminomutases and share the same cofactor dependency and similarity (26.7% identity and 42% similarity)[14], they catalyze the *α*-*β*-isomerization with different stereochemistry. *Tc*PAM catalyzes the isomerization of (*S*)-*α*-amino acids to (*R*)-*β*-amino acids, whereas *Pa*PAM converts (*S*)-*α*-amino acids to (*S*)-*β*-amino acids. In this way, both (*R*) and (*S*) enantiomers can be obtained in enantiomerically pure form.

*Tc*PAM has been widely used as biocatalyst for synthetic procedures related to *β*-amino acids but the use of *Pa*PAM as biocatalyst is not so frequently reported. In the consciousness of this fact, the aim of this work is to develop methods for recombinant production of phenylalanine 2,3-aminomutase from *Pantoea agglomerans* (*Pa*PAM) by optimization and scaling up the expression process for high level protein expression, in order to obtain *Pa*PAM efficient biocatalyst for the preparation of enantiomerically pure (*S*)-*β*-amino acids.

## RESULTS AND DISCUSSION

### Optimization of *Pa*PAM overexpression in *E. coli*

*E. coli* is still the preferred host for recombinant protein expression because it is easy to genetically manipulate, it is inexpensive to culture, and expression occurs fast [15]. pET system also has many advantages that determine us to use it for the expression of our gene. pET is one of the most powerful systems developed for the expression of recombinant proteins in *E. coli* [16]. *E. coli* strain BL21(DE3)pLysS is also the most widely used host for recombinant gene expression.[17] Therefore, *E. coli* was selected as host for expression of *Pa*PAM. The aim of this investigation was to examine the effect of different experimental conditions on the expression of *Pa*PAM in order to obtain high purity and yields for the enzyme which can be used as biocatalyst.

*Inducer concentration optimization*

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]. With some proteins, it is important to induce the transcription of the expression plasmid with lower IPTG concentrations, while others tend to aggregate at high concentrations of IPTG [18].

For the optimization of *Pa*PAM 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-*Pa*PAM recombinant plasmid. The SDS-PAGE bands are similar for 0.5 and 1 mM IPTG concentrations (**Figure 1**, Lane C and D), indicating the same level of expression of the 72 kDa *Pa*PAM, despite the increasing inducer concentration. Only a slight difference can be observed at 0.1 mM IPTG. (**Figure 1**, Lane B), showing the most intensive signal at the 72 kDa band of the recombinant *Pa*PAM. Based on these results, the final concentration of IPTG was set up to 0.1 mM.

*Effect of incubation temperature and time on the overexpression of PaPAM*

The recombinant plasmid pET-19b-*Pa*PAM was overexpressed to produce the target protein with an *N*-terminal $His_{10}$ tag in *E. coli* BL21(DE3)pLysS. Generally, the optimum temperature for the recombinant protein production in *E.coli* is 37°C and several studies reported 37°C as the best temperature for maximum protein production [19]. On the other hand, studies showed that the rate of expression and culture temperature can affect the proper folding of recombinant proteins and formation inclusion bodies.[20] Lowering the expression temperature usually leads to slower growth of bacteria, slower rate of protein production and lower aggregation of target protein and also most proteases are less active at lower temperatures [21,22].

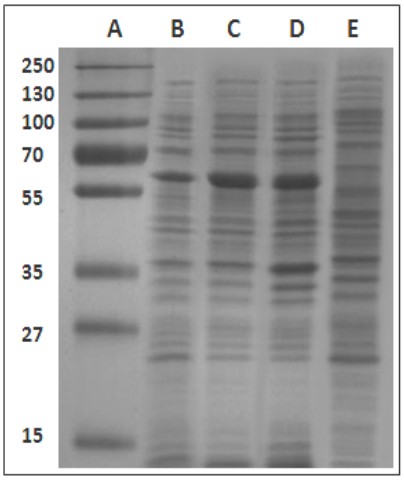
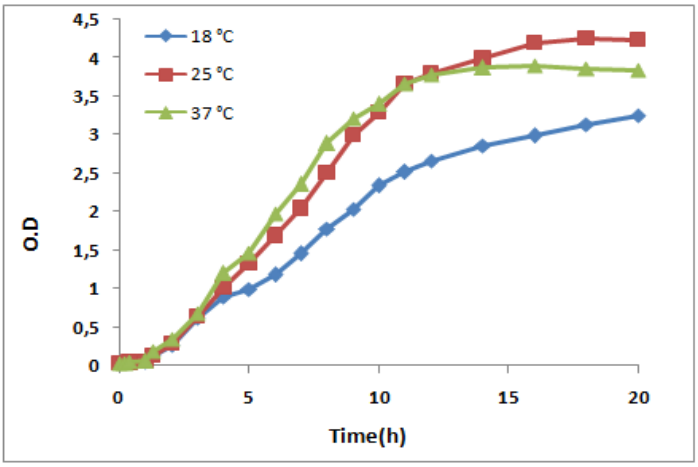



**Figure 1.** Induction of the expression of *Pa*PAM by different concentrations of IPTG in *E.coli* BL21(DE3)pLysS cells, after 4h. Lane **A**: protein ladder, Lane **B**: induction with 0.1 mM IPTG, Lane **C**: induction with 0.5 mM IPTG, Lane **D**: induction with 1 mM IPTG, Lane **E**: control (0 mM IPTG). The samples were prepared as described in experimental section.

To evaluate the effect of growth temperature on the expression of *Pa*PAM 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 (**Graphic 1.**).




**Graphic 1.** Effect of growth temperature on the expression of *Pa*PAM

Due to the reduced incubation temperature the protein synthesis rate is slower at 18°C than at 25 or 37°C, longer induction times are necessary for cells growing. At higher incubation temperature (25 or 37°C), the protein synthesis is faster and the stationary phase is reached sooner than at lower temperatures. After 20 h the cells were harvested by centrifugation, followed by sonication and the protein was purified by metal affinity chromatography on Ni-NTA resin. The maximum yield of enzyme was obtained in case of 25°C incubation temperature. The optimal post-induction time on the expression of *Pa*PAM was 15-16 hours. The determined optimal conditions were also been used for the large-scale fermentation of *E. coli* BL21(DE3)pLysS.

*Purification using Ni-NTA chromatography*

Ni–NTA chromatography system is a rapid and easy purification technique. Proteins fused with His-tag at either ends (*N*- or *C*-terminus) bind tightly with high affinity on immobilized nickel ions. The strong binding between His-tag and matrix allows easy washing and efficient elution of bounded His-tagged protein by competition with imidazole [23].

In the pET-19b vector a $His_{10}$-tag at the *N*-terminus is included 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 are required to elute all the bounded enzyme from the resin (from 250 mM up to 500 mM) [24]. Accordingly, as the SDS-PAGE gel analysis revealed, only small amount of protein remained in the flow through (**Figure 2**, Lane D) and in the washing buffer solutions (**Figure 2**, Lane E-G).

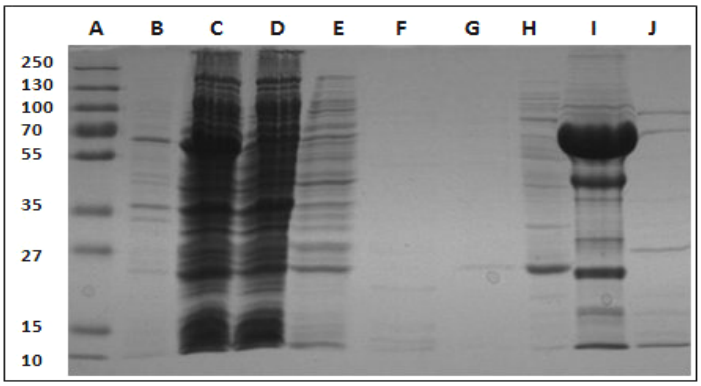



**Figure 2.** Purification of *Pa*PAM with Ni-NTA, in absence of protease inhibitor cocktail. Lane **A**: protein ladder, Lane **B**: supernatant, Lane **C**:pellet, Lane **D**: flow through Lane **E**: LS1, Lane **F**: HS, Lane **G**:LS2, Lane **H**: 20 mM Imidazole, Lane **I**: 500 mM Imidazole, Lane **J**: 1 mM Imidazole. The samples were prepared as described in experimental section.

It has been observed that *Pa*PAM activity descreased after elution from the Ni-NTA column probably due to prolonged exposure to very high imidazole concentration and we also observed that the obtained enzyme has some unspecific bands on the SDS-PAGE gel (**Figure 2**, Lane I) due to the protease activity for the enzyme. In order to eliminate these facts we added protease inhibitor cocktail to the lysis buffer and we reduced de imidazole concentration, testing 250, 350, 450, 500 mM imidazole solutions for protein elution. The best result was obtained by elution with 350 mM imidazole, resulting in protein solution appearing as single band on the SDS-PAGE (**Figure 3**, Lane I), indicating a highly purified enzyme.

*Thermal stability*

Thermal shift (ThermoFluor) assays offer a rapid and simple technique for assessing the thermal stability of proteins and to investigate factors affecting this stability. An environmentally sensitive fluorescent dye is used to monitor protein unfolding with respect to temperature [25]. In the ideal case, no fluorescence is observed at low temperature because the protein is completely and correctly folded and no hydrophobic areas are exposed. Upon an increase in temperature the protein starts to unfold and hydrophobic areas become exposed and the fluorescent dye can bind to these areas and fluorescence occurs.

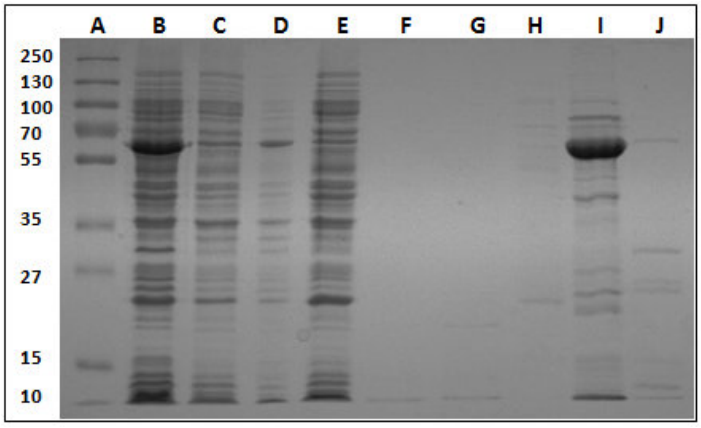



**Figure 3.** Purification of *Pa*PAM with Ni-NTA, in presence of protease inhibitor cocktail. Lane **A**: protein ladder, Lane **B**: supernatant, Lane **C**: flow through, Lane **D**: pellet, Lane **E**: LS1, Lane **F**: HS, Lane **G**:LS2, Lane **H**: 20 mM Imidazole, Lane **I**: 350 mM Imidazole, Lane **J**: 1 mM Imidazole. The samples were prepared as described in experimental section.

The *Pa*PAM enzyme presents a good thermal stability, it can be observed in **Figure 4** that its maximum melting temperature is approximately 73°C.

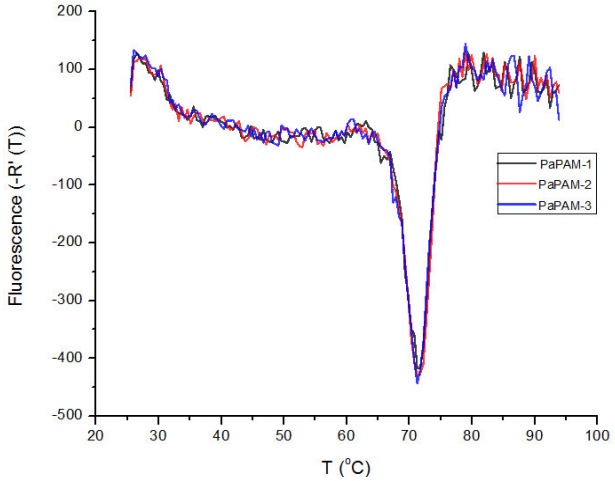



**Figure 4.** The first derivative of the melt curves.

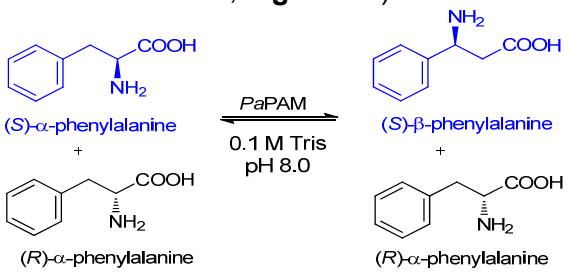
We also investigated if the substrate of the enzyme has a stabilizing effect. Therefore following measurements were performed in TRIS buffer solution, pH 8.5 in the presence of L-phenylalanine.

The melting temperature was read from the negative curve of the first derivative of the experimental curve. By comparing the results of the measurements obtained in presence and in absence of the substrate, it can be observed that the modification of melting temperature in presence of L-phenylalanine is minor (from 73°C to 74°C), suggesting no stability increase of the enzyme upon substrate binding.

*Activity assay*

In nature, phenylalanine 2,3-aminonutase from *Pantoea agglomerans* is an (*S*)-selective enzyme, transforming the (*S*)-*α*-phenylalanine in (*S*)-*β*-phenylalanine [8].

The activity and the selectivity of *Pa*PAM was tested using *rac*-*α*-phenylalanine as substrate (**Scheme 1**). HPLC analysis of the reaction supports the formations of the (S)-β-phenylalanine (**Figure 5B**) with maximum selectivity (compared to the racemic mixture, **Figure 5A**).




**Scheme 1.** Transformation of the *rac*-*α*-phenylalanine by *Pa*PAM.

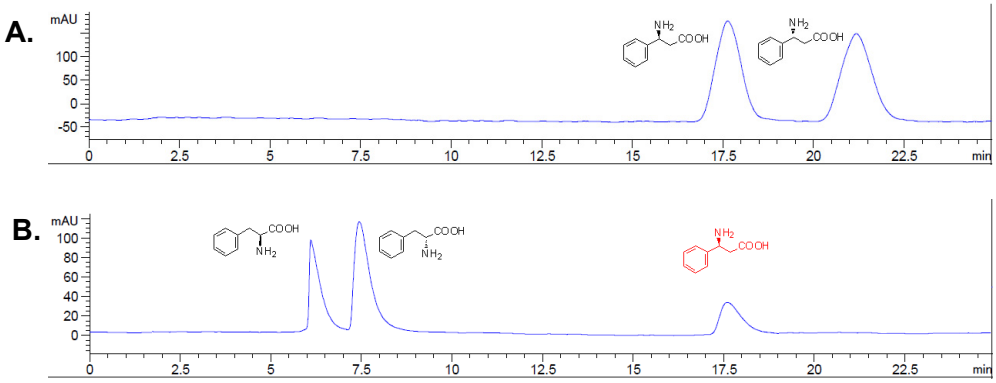



**Figure 5.** HPLC chromatograms for: **A.** *rac*-*β*-phenylalanine as control. **B.** Transformation of *rac*-*α*-phenylalanine by *Pa*PAM.

## CONCLUSIONS

We examined different experimental conditions regarding the expression and purification of *Pa*PAM in order to obtain high purity and yield for the enzyme. IPTG concentrations, various post-induction temperatures on the expression, the imidazole concentration in the purification steps, the thermal stability and the activity of the enzyme were also examined.

The results showed that induction with 0.1 M IPTG was sufficient to induce the expression of *Pa*PAM, which is 10 times less than normally used IPTG concentration. Moreover, 15 hour post-induction incubation at 25°C was found to be optimal for producing a higher level of *Pa*PAM. Reducing the imidazole concentration to 350 mM and adding protease inhibitor cocktail improved the stability of the yielding enzyme. Furthermore, electrophoretically pure recombinant *Pa*PAM enzyme preparation was obtained using Ni affinity chromatography.

The detemined optimized parameters were also successfully applied for large scale fermentation of *Pa*PAM, obtaining high levels of protein.

## EXPERIMENTAL SECTION

*PaPAM gene synthesis and cloning*

The gene of the *Pantoea agglomerans* PAM (Uniprot code: Q84FL5, PBD code: 3UNV, encoding 623 AA – **Figure 6**) was optimized to the codone usage of *E. coli*. The 1639 bps long synthetic gene was produced by *Life*

*Tehnologies*. The gene was cloned into pET-19b host vector using *XhoI* and *Bpu1102I* cloning site (**Figure 7**). The pET-19b vector in which the *Pa*PAM gene was cloned contains a $His_{10}$-tag *N*-terminal sequence, an enterokinase cleavage site, a gene resistant to ampicillin and the T7*lac* promoter sequence. In the supplementation of the growth medium ampicillin was replaced by carbenicillin, due to the highest stability of later.

**w*t-Pa*PAM**

MSIVNESGSQPVVSRDETLSQIERTSFHISSGKDISLEEIARAARDHQPV
TLHDEVVNRVTRSRSILESMVSDERVIYGVNTSMGGFVNYIVPIAKASEL
QNNLINAVATNVGKYFDDTTVRATMLARIVSLSRGNSAISIVNFKKLIEI
YNQGIVPCIPEKGSLGTSGDLGPLAAIALVCTGQWKARYQGEQMSGAMAL
EKAGISPMELSFKEGLALINGTSAMVGLGVLLYDEVKRLFDTYLTVTSLS
IEGLHGKTKPFEPAVHRMKPHQGQLEVATTIWETLADSSLAVNEHEVEKL
IAEEMDGLVKASNHQIEDAYSIRCTPQILGPVADTLKNIKQTLTNELNSS
NDNPLIDQTTEEVFHNGHFHGQYVSMAMDHLNIALVTMMNLANRRIDRFM
DKSNSNGLPPFLCAENAGLRLGLMGGQFMTASITAESRASCMPMSIQSLS
TTGDFQDIVSFGLVAARRVREQLKNLKYVFSFELLCACQAVDIRGTAGLS
KRTRALYDKTRTLVPYLEEDKTISDYIESIAQTVLTKNSDI

**Figure 6.** Amino acids sequence of recombinant *wt-Pa*PAM.


**Figure 7.** pET-19b-*Pa*PAM vector map.
The vector map was generated using Snapgene.

*Transformation in E.coli host cells*

Transformation of plasmid DNA into *E.coli* XL1-Blue (for plasmid amplification) and BL21(DE3)pLysS (for expression) was performed using the heat shock method, inserting the foreign plasmid into bacteria. After 20 min

incubation on ice, the mixture of 100 µL chemically competent bacterial cells and 2 µL of plasmid DNA was incubated at 42°C for 45 seconds (heat shock) and then placed back on ice for 25 min. 200 µL SOC media was added and the transformed cells were incubated 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) and carbenicilin (50 µg/mL). In case of BL21(DE3)pLysS transformation, 50 µg/mL carbenicillin and 30 µg/mL chloramphenicol were used. Both cultures were then incubated overnight at 37°C, forming single colonies of bacteria bearing the plasmid encoding the recombinant protein.

*Expression and production of the recombinant PaPAM*

The recombinant *Pa*PAM carrying *N*-terminal $(His)_{10}$-tag was overexpressed in *E.coli* host cells (BL21(DE3)pLysS. For the expression step, a colony of the transformed plasmid was grown overnight at 37°C in 5 mL of Luria-Bertani (LB) medium containing carbenicillin (50 µg/mL) and chloramphenicol (30 µg/mL). A 0.5 L of LB medium was inoculated with 1% (v/v) of the overnight culture in an Erlenmeyer flask and grown until the measured optical density at 600 nm ($OD_{600}$) reached 0.6-0.7 at 37°C (optimal temperature for *E. coli* growth) and only at the induction phase the temperature was reduced to 18, 25, 37°C and the cells were induced by the addition of (0.1, 0.5, 1 mM) IPTG. The culture was shaken at 200 rpm for 16 h.

*Large scale fermentation of the recombinant PaPAM*

Ten litres of Luria-Bertani (LB) medium were sterilized at 121°C, 1.2 bar for 25 min. After sterilization, the media was cooled down to 37°C and ampicillin sodium salt 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 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 0.7±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 16 h when the culture reached the stationary growth phase.

*Purification of the recombinant PaPAM*

The cells were harvested by centrifugation (25 min, 5000×g) and resuspended in 50 mL lysis buffer (150 mM NaCl, 50 mM TRIS pH 7.5,)

supplemented with DNAse, RNAse, Lysosyme, 2 mM PMSF and EDTA-free protease-inhibitor cocktail. Further, the cells were lysed by sonication and cell debris was removed by centrifugation (10000 × g, 30 min).

The proteins were further purified using a Ni-NTA-agarose column and washed with different buffer solutions.

The column was washed: LS (low salt) buffer, pH 7.5 (4-5V; V=resin volume), HS (high salt) buffer, pH 7.5 (2V), LS (2-4V), LS with 20 mM imidazole, LS with (250, 350, 450, 500 mM) imidazole (protein elution), LS with 1 M imidazole (4V), $ddH_2O$ (7V) and 20% ethanol (1-2V), the resin was stored in 20% ethanol at 4°C.

The resulting eluate was dialyzed in 50 mM PBS, 4-5h at 4°C. The purity of the resulting fractions was verified by SDS-PAGE analysis. The samples were boiled for 5 min in Laemmli buffer and were loaded on a 12% SDS-PAGE. After dialysis the fractions containing purified protein were concentrated by centrifugal ultrafiltration. The concentration of the purified protein was determined by Bradford method.

*Optimization parameters*

To determine the *optimal IPTG concentration* on the recombinant protein production, the recombinant clone culture was grown to $OD_{600}$ of about 0.6-0.7, and induced by adding IPTG to final concentrations of 0.1, 0.5 and 1 mM. After 4 hours of induction, 1 mL samples were colected, centrifuged and the pellet resuspended in 0.5 mL $dH_2O$. The samples were lysed by boiled for 5 min in Laemmli buffer, and were loaded on a 12% SDS-PAGE.

To examine the *effect of temperature*, recombinant protein expression was induced by addition of 0.1 mM IPTG at different temperatures (18, 25 and 37°C). The $OD_{600}$ was monitored by time, after 20 hours of induction, cells were harvested by centrifugation, followed by sonication and the protein was purified by Ni-NTA.

*Thermostability assay*

The thermal stability of the enzyme was determined by thermofluor measurements using real-time PCR as described earlier [26].

The assay was performed using a 96-well iCycler iQ Real Time PCR plate. The total reaction volume was 25 µL and the plate was set up on ice. 20 µL of 0.1M TRIS buffer solution was pipetted into the wells of the plate. A 5000 × SYPRO Orange stock solution in DMSO was diluted 1:100 in water and 1 µL was added to the 20 µL well solution which contained 1.5 mg/mL *Pa*PAM protein. The plates were sealed with Optical Quality Sealing Tape and centrifuged at 2000 rpm for 1 min. The plate was heated from 20 to 90°C in 1°C increments in of iCycler iQ Real Time PCR Detection System.

*Activity assay*

*Pa*PAM activity was determined by the conversion of (*S*)-*α*-Phe to (*S*)-*β*-Phe. Into the solution of *rac*-*α*-phenylalanine (2 mg) in TRIS buffer (100 mM, pH 8.0, 2 mL), wt-*Pa*PAM (0.8 mg) was added and the reaction mixtures was stirred at room temperature. A 100 µL sample was taken and the reaction was stopped by heating for 10 min at 90°C in the presence of a small amount of activated charcoal. After filtration, the sample was analyzed with high performance liquid chromatography (HPLC), using a Chiralpak ZWIX(+) column, and MeOH (50 mM formic acid and 100 mM diethyl amine): ACN:$H_2O$ 49:49:2 (v/v/v) as eluent, respectively.

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