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*Pc*PAL's biocatalytic activity on benzofuran-2-yl-alanines

Norbert-Artur Dima, Alina Filip, Monica Ioana Tosa, Csaba Paizs

Biocatalysis and Biotransformation Research Group, Department of Chemistry, University of Babeş-Bolyai, Cluj-Napoca, Romania

Email: dna31@chem.ubbcluj.ro

1. Introduction

Phenylalanine ammonia-lyase (PAL) is an important enzyme, present in higher plants, some fungi, yeasts and Streptomyces[1], as its product, (*E*)-cinnamic acid is the precursor of lignines, coumarins, and flavonoids. It catalyzes the reversible, non-reductive amination of *trans*-cinnamic acid to L-phenylalanine in the presence of high ammonia concentrations. PAL belongs to the class of enzymes containing the unique superelectrophilic prosthetic group 5-methylene-3,5-dihydroimidazol-4-one (MIO), whose reaction mechanism is at great interest[2]. To allow structure guided rational engineering, a detailed understanding of the enzyme's catalytic mechanism is highly desirable. However, despite considerable efforts, elucidation of the PAL non-oxidative deamination reaction mechanism is still ongoing. At present there are two possible ways in the literature: an E1cB and a Friedel-Crafts like mechanism[3]. We previously proposed that both reaction mechanisms are applicable to the deamination of aromatic amino acids depending whether the enzyme is wild-type or mutated[4]. The substrate range of PAL from *Petroselinum crispum* (*Pc*PAL) is considerable, showing activity toward a range of heteroaromatic phenylalanine derivatives.

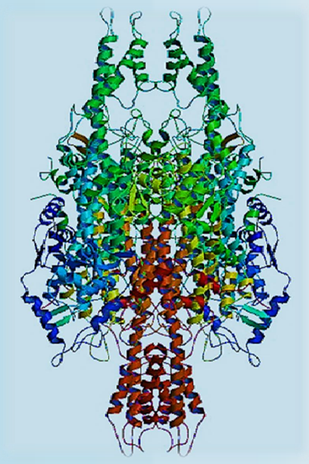
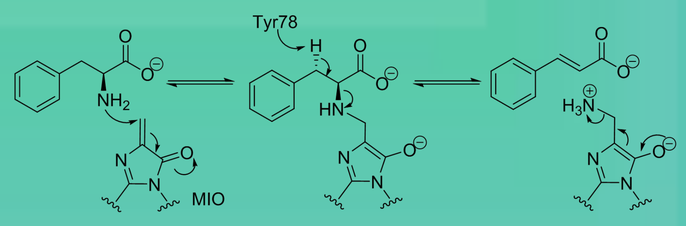
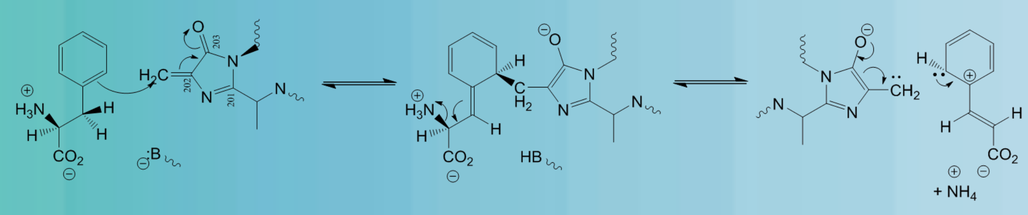


Figure 1. PcPAL's tetramer quaternary molecular structure



Scheme 1. Proposed E_1cB mechanism for PAL mediated L-phenylalanine deamination



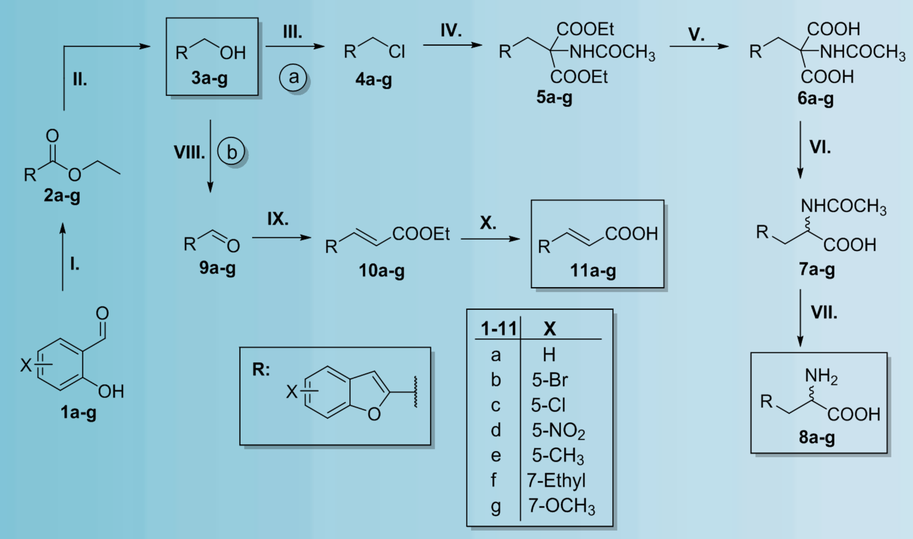
Scheme 2. Friedel-Crafts type mechanism of the deamination process involving the MIO cofactor

2. Chemical synthesis of the substrates

The chemical synthesis of the amino and acrylic acids started from salicylaldehydes **1a-g**. These were transformed using ethyl bromoacetate, followed by condensation into benzofuran-2-yl esters **2a-g**, which were next reduced to **3a-g**.

Further transformations were performed in two ways :

- in the **route a**, the chloromethylene derivatives **4a-g** resulting from the alcohols reacting with thionyl chloride, were converted by a coupling reaction with *N*-protected diethyl aminomalonate into **5a-g**. The workup by basic hydrolysis and decarboxylation yielded the *N*-protected amino acids **7a-g**. In the next step the protecting group was removed and the phenylalanine analogues **8a-g** were isolated by precipitation at their isoelectric point.
- alternatively (**route b**), the alcohols were oxidized to aldehydes **9a-g** by manganese dioxide. These compounds were transformed using Wittig-reagent into the corresponding acrylic acid esters, which were subsequently hydrolyzed to yield the desired cinnamates **11a-g**.



I. Ethyl bromoacetate, K_2CO_3, 18C6, Acetonitrile; **II.** $NaBH_4$, THF, MeOH; **III.** Thionyl chloride, benzotriazole, CH_2Cl_2 dry; **IV.** NaH, diethyl acetamidomalonate, DMF; **V.** NaOH, H_2O; **VI.** Toluene; **VII.** HCl 18%; **VIII.** MnO_2, toluene; **IX.** Ethyl triphenyl-phosphanilidene acetate, toluene; **X.** KOH/Na_2CO_3, H_2O.

Scheme 3. Synthesis of the acrylic and amino acids

3. Protein production and purification

The synthetic genes were cloned into pET-19b. The recombinant plasmids which contain a *N*-terminal $(His)_{10}$-Tag were produced in *E.coli* Rosetta (DE3) pLysS cells using LB media supplemented with the appropriate antibiotic. After cell growth, the pellet was lysed by sonication and the intracellular solution has been subjected to a Ni-NTA-agarose column. The purity of the resulting fractions were verified by SDS-PAGE analysis (Fig.2.) and the purified protein was concentrated by centrifugal ultrafiltration after the dialyzing step. In order to verify the isolated protein's tetramer content, a gel filtration chromatographic method was developed. The chromatogram below shows about 95% of the desired protein.

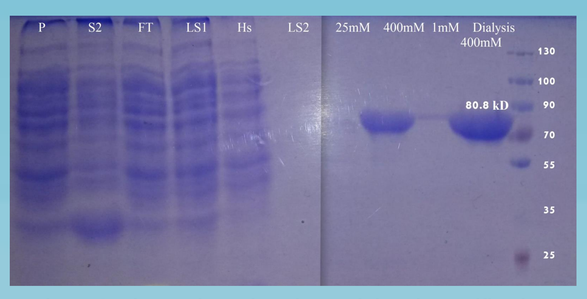


Figure 2. Electrophoresis gel containing the purification steps of PAL

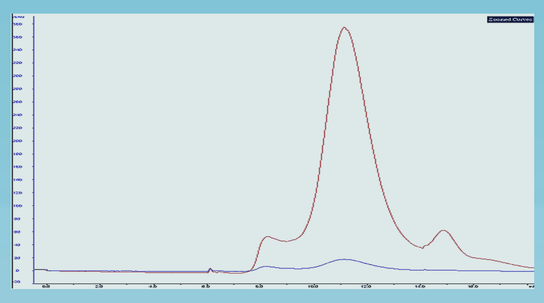


Figure 3. PcPAL's homogeneity on a gel filtration column

4. Determination of the kinetic parameters using *Pc*PAL

The kinetic measurements were based on the spectrophotometrical (UV) determination of the concentration of the formed acrylates. The values obtained for our substrates were compared with the results obtained for the unsubstituted benzofuran-2-yl alanine **8a** (Table 1). It was noticed that in the case of the electron withdrawing groups (EWG) we have an increasing tendency of the velocity, however towards the electron donating groups (EDG) the enzyme didn't show catalytic activity.

Analyzing the kinetic parameters (Table 1), we can see a major decrease of the V_{max} for the substituted arylalanines compared to the unsubstituted **8a**, thus in the specific activity of the enzyme. The halogenated benzofuranyl alanines differs in the maximum velocity, K_M values approx. 8-10 times, showing that the enzyme has much more affinity for the 5-bromo derivative **8b** than for the 5-chloro derivative **8c.**

Afterwards, we investigated the interaction of the nonsubstrate amino acids with PAL, by kinetic measurements on the parent reaction, monitoring the formation of cinnamate at 290 nm. The results obtained confirmed that these compounds **8e-g** are weak competitive inhibitors. The kinetic constants are shown in Table 2.

Table 1. Kinetic constants of the substrates-PcPAL interactions

Substrates	ε ($cm^{-1}\times\mu M^{-1}$)	λ (nm)	K_M (μM)	V_{MAX} ($\mu M\times s^{-1}$)	K_{CAT} (s^{-1})	S.A. ($\mu mol\times min^{-1}\times mg^{-1}$)
L-Phe	8800	290	119	25E-02	200E-02	150E-02
8a	19567	295	165	10E-02	80E-02	60E-02
8b	18951	311	215	0.3E-02	2.4E-02	1.8E-02
8c	33821	308	1695	3E-02	24E-02	18E-02
8d	22199	245	103	2E-02	16E-02	12E-02

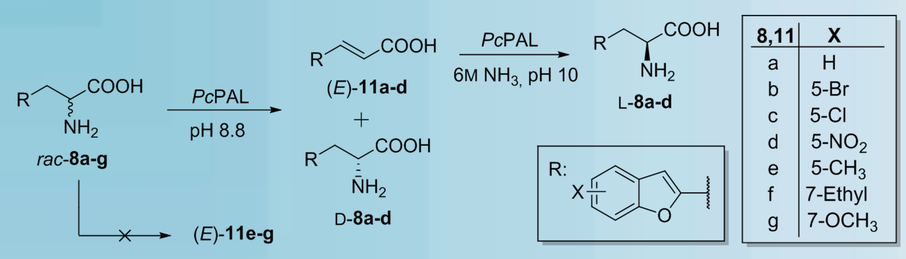
Table 2. Kinetic constants of the inhibitors-PcPAL interactions

Inhibitors	[a]$K_{M\ OBS}$ (μM)	K_i (μM)	$V_{MAX}/V_{MAX\ L\text{-}Phe}$	K_{CAT} (s^{-1})
8e	239	202		
8f	238	217	1	2
8g	237	204		

[a]calculated for 0.2 mM inhibitor concentration

5. Preparative scale biotransformations

The preparative scale biotransformations were performed for both the forward and the reverse process. The starting conditions and the substrate:enzyme ratio was similar to those at the kinetic measurements. The addition step took place in 6M $(NH_4)_2CO_3$ pH 10.2 solution, while the elimination step was performed in Tris buffer pH 8.8 at r.t. The reactions were monitorized by HPLC method on a chiral column. The optical rotation of the isolated L-amino acids were measured by polarimeter.



Scheme 4. PcPAL mediated biotransformations

Table 3. Enantiomeric excesses and opticals rotations of the L-aminoacids

Substrate	Product	ee (%)	$[a]_D^{20}$ ($10mg\times mL^{-1}$)
(*E*)-**11a**	L-**8a**	>99	-14,5
(*E*)-**11b**	L-**8b**	>99	-30,8
(*E*)-**11c**	L-**8c**	>99	-29,1
(*E*)-**11d**	L-**8d**	>99	-21,3

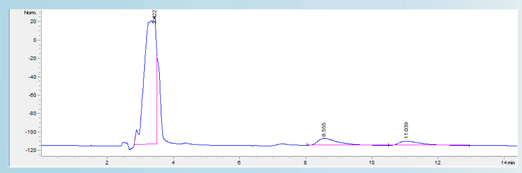


Figure 5. (E)-11d and rac-8d

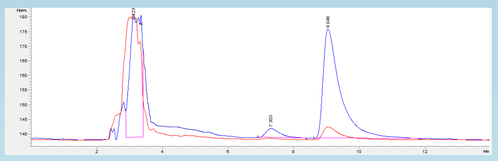


Figure 4. NH_3 elimination of rac-8b: blue – after 4 days; red – after 7 days

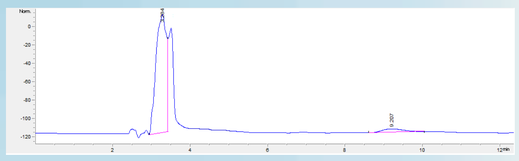


Figure 6. NH_3 addition of (E)-11d after 7 days

6. Conclusion

In this work, the biocatalytic activity of PAL from parsley was tested with several heteroaryl alanines and acrylic acids. The success of the forward and reverse reactions were moderate, depending on the nature of the phenyl group's substituents. Due to the kinetic measurements we found 3 weak inhibitors and 3 substrates, which were compared to the natural substrate and the unsubstituted benzofuranyl alanine, respectively.

References

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Acknowledgements

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