Expression and Purification of Various Types of PAL and PAM

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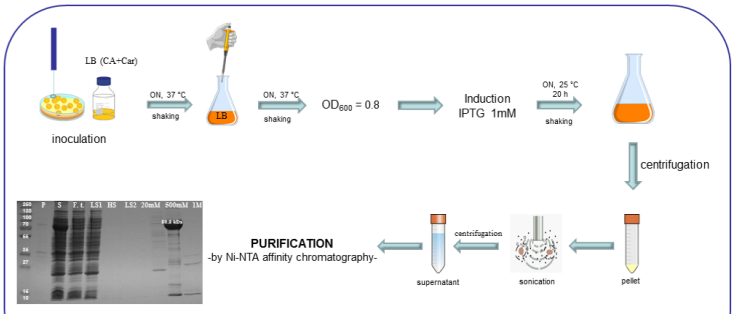
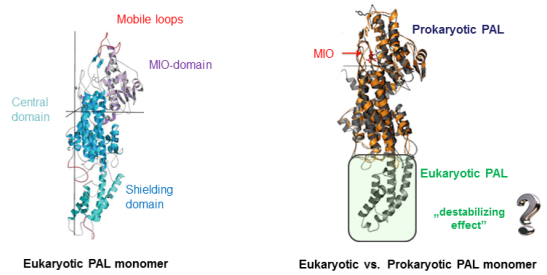
INTRODUCTION

PAL and PAM belong to ammonia lyase class. These enzymes represent a point of interest not only for their unique mode of catalyzing the reversible deamination of amino acids but also for their potential applications. Based on the considerations regarding the differences between eukaryotic and prokaryotic PAL stability, several modified PAL constructs containing the catalytic domain of the eukaryotic PAL and the C-terminal domain of a bacterial PAL were assembled. Different wild types and mutant PAL: **WPCL** – wt from *Petroselinum cripsum;* **WRTL**– wt from *Rhodosporidium toruloides* yeast and **WAVL** – wt from *Anabaena variabilis* bacteria, respectively PAM: **WPAM** – wt from *Pantoea agglomerans* bacteria mutase, from various mesophiles and thermophiles were cloned into the most proper hosts, followed by their expression and purification. In order to determine the general stability, activity and optimal conditions to achieve the highest reaction rate, multiple substrates were tested. The kinetic constants, *Km* and *Vmax*, were determined and evaluated using unsubstituted derivatives (5-phenylthiophenyl- and 5,7-benzofuranyl- alanines) as model compounds.

RESULTS AND DISCUSSIONS

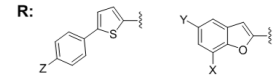


Expression and purification of PAL's and PAM enzymes



Eukaryotic PAL monomer — **Eukaryotic vs. Prokaryotic PAL monomer**

PAL enzyme structure



	X	Y
a	H	H
b	H	Cl
c	H	Br
d	H	CH_3
e	H	NO_2
f	C_2H_5	H
g	CH_3O	H

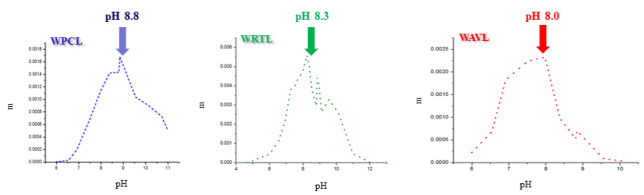
Z: H-, CH_3O, NO_2-

substrate	T (°C)	Km (μM)	V_{max} (μM/s)	$V_{max}/V_{max\,5}$
H-FT	30	97.0	0.0033	1.00
	40	36.0	0.0033	1.00
	45	221.0	0.0100	3.03
	50	423.0	0.0100	3.03
	55	85.9	0.0160	4.85
	60	61.0	0.0500	15.15
$4NO_2$-FT	30	181.0	0.1000	1.00
	40	150.0	0.1400	1.40
	45	170.0	0.2000	2.00
	50	195.0	0.1600	1.60
	55	134.0	0.2500	2.50
	60	97.0	0.3300	3.30
4MeO-FT	60	303.0	0.0010	1.00

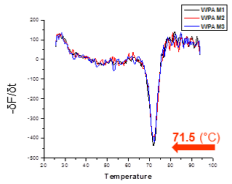
T (°C)	Km (μM)	V_{max} (μM/s)	$V_{max}/V_{max\,L\text{-}Phe}$
30	49.5	6.83E-02	1.000
45	119.0	6.83E-02	1.000
50	179.0	9.11E-02	1.330
55	193.0	9.11E-02	1.330
60	243.0	1.37E-01	2.000

enzyme	substrate	K_M (μM)	V_{max}(μM/s/μg)	$V_{max}/V_{max\,BF}$
WPCL	H-BF	248	1.61E-02	1.000
	7Et-BF	8000	2.90E-03	0.181
	7MeO-BF	1770	2.96E-04	0.018
	5Me-BF	133	1.48E-04	0.009
	5Cl-BF	903	1.74E-04	0.011
	5Br-BF	882	2.92E-03	0.182
	$5NO_2$-BF	3240	5.19E-03	0.323
WRTL	H-Br	2670	8.00E-03	1.000
	5Br-BF	123	8.90E-04	0.111
	7Et-BF	22200	8.00E-04	0.100
WRTL	H-Br	101	7.60E-03	1.000
	5Br-BF	389	3.00E-02	3.947

Kinetic constants for PAL'S mediated reaction



Activity profiles of various PAL enzymes – at different pH conditions



enzyme	T (°C)	T' (°C)
WPCL	75.50	77.71
WRTL	71.27	72.36
WAVL	46.08	45.57
WPAM	71.50	71.97

T-temperature values for samples without *(E)*- cinamic acid
T'-temperature values for samples containing *(E)*- *cinamic acid*

Thermofluor results

CONCLUSIONS

- The expression and purification of PAL's and PAM enzymes were successful. The purity degree of the protein WPCL was high as shown in the figure with the gel electrophoresis. We observed a similar purity degree for the others proteins that we had studied.
- Thermofluor measurements indicate that WPCL, WRTL and WPAM present good stability around 71-75 °C, while the Tm for WAVL is around 46 °C.
- The results of the kinetic studies revealed that the binding pocket of *Petroselinum crispum* enzyme is more selective for the benzofuran-alanine substituted in the 5th position compared with the 7th position, due to steric obstacles. The lowest value of K_M was obtained for 5-methylbenzofuran-alanine, lower than the unsubstituted benzofuran-alanine too. In what concerns V_{max}, it can be seen that 5-methylbenzofuran-alnine is very slowly transformed, even if its affinity is high, while 5-nitro and 7- etilbenzofurane have a high value for the reaction rate. The kinetic studies regarding *Rhodosporidium toruloides* and *Anabaena variabilis* present similar affinity for substrate 5-benzofuran-alanine.
- Due to poor solubility of phenylthiophene alanine derivatives at 30 °C we varied the temperature in order to determine the WPCL affinity for the substrate. Consequently we have noticed a significant increase of the affinity and velocity of the reaction.

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