Phenylalanine, Tyrosine, and DOPA Are bona fide Substrates for Bambusa oldhamii BoPAL4

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Abstract: Phenylalanine ammonia-lyase (PAL) links the plant primary and secondary metabolisms, and its product, trans-cinnamic acid, is derived into thousands of diverse phenylpropanoids. Bambusa oldhamii BoPAL4 has broad substrate specificity using L-phenylalanine, L-tyrosine, and L-3,4-dihydroxyphenylalanine (L-DOPA) as substrates to yield trans-cinnamic acid, p-coumaric acid, and caffeic acid, respectively. The optimum reaction pH of BoPAL4 for three substrates was measured at 9.0, 8.5, and 9.0, respectively. The optimum reaction temperatures of BoPAL4 for three substrates were obtained at 50, 60, and 40 °C, respectively. The Km values of BoPAL4 for three substrates were 2084, 98, and 956 µM, respectively. The kcat values of BoPAL4 for three substrates were 1.44, 0.18, and 0.06 s−1, respectively. The major substrate specificity site mutant, BoPAL4-H123F, showed better affinity toward L-phenylalanine by decreasing its Km value to 640 µM and increasing its kcat value to 1.87 s−1. In comparison to wild-type BoPAL4, the specific activities of BoPAL4-H123F using L-tyrosine and L-DOPA as substrates retained 5.4% and 17.8% residual activities. Therefore, L-phenylalanine, L-tyrosine, and L-DOPA are bona fide substrates for BoPAL4.

Keywords: Bambusa oldhamii; phenylalanine ammonia-lyase; phenylalanine/tyrosine ammonia-lyase; substrate specificity; plant secondary metabolism; phenylpropanoid

1. Introduction

Phenylalanine ammonia-lyase (PAL, EC 4.3.1.24) is the enzyme that catalyzes the first committed step of general phenylpropanoid pathways in plants, catalyzing the conversion of L-phenylalanine (L-Phe) to trans-cinnamic acid via a non-oxidative deamination reaction (Scheme 1) [1–3]. Trans-cinnamic acid served as the start point of secondary metabolism is then hydroxylated by a membrane-bounded cinnamate 4-hydroxylase (C4H, EC 1.14.14.91) to yield p-coumaric acid [4] for the synthesis of thousands of phenylpropanoids [5,6]. Plant hormone, salicylic acid, is also synthesized via the PAL-mediated pathway [7]. PAL activity is elevated by light treatment, maintaining phenolic compound levels in fresh-cut sweet peppers [8]. Trans-cinnamic acid and p-coumaric acid have been shown to exert multifarious health benefits for humans, including anti-diabetic, anti-obesity, anti-oxidant, and anti-microbial activities [9,10]. P-coumaric acid is further hydroxylated by 4-coumarate 3-hydroxylase (4CMH or C3H, EC 1.14.14.9) to yield caffeic acid, an intermediate involved in the early stage of lignin biosynthesis [11]. In humans, caffeic acid provides numerous beneficial biological activities, including anti-oxidant and anti-microbial activities [12–14].
Scheme 1. The enzyme reaction catalyzed by the bamboo BoPAL4 phenyl alanine/tyrosine ammonia-lyase (PTAL). BoPAL4 PTAL catalyzes the non-oxidative deamination of L-phenylalanine to yield trans-cinnamic acid for the synthesis of p-coumaric acid via the enzyme reaction catalyzed by the C4H cinnamate 4-hydroxylase. BoPAL4 PTAL can bypass the BoC4H requirement to produce p-coumaric acid by the deamination of L-tyrosine. In plants, p-coumaric acid is hydroxylated at C-3 position to yield 3,4-dihydroxy trans-cinnamic acid, also known as caffeic acid, by the 4CMH/C3H 4-coumarate 3-hydroxylase. BoPAL4 PTAL can also bypass the 4CMH requirement to produce caffeic acid by the deamination of L-3,4-dihydroxy phenylalanine (L-DOPA).

Histidine ammonia-lyase (HAL, EC 4.3.1.3) [15], PAL, and tyrosine ammonia-lyase (TAL, EC 4.3.1.24) share the conserved active site motif, Ala-Ser-Gly, which can spontaneously merge into a 4-methylidene-imidazol-5-one (MIO) group [16,17]. PAL is one of the highly post-translational modified enzymes in plant secondary metabolism [18]. The degradation of PAL is mediated by ubiquitin proteasome system [19,20]. PAL is a phosphoprotein [21–23], and the phosphorylation site identified in French bean is the Thr-545 residue in a conserved R/K-X-X-S/T motif [24]. PAL proteins generally function as tetrameric enzymes [25–27].

In dicot plants, PAL exclusively uses L-Phe as substrate [28–30]; however, several PAL isoforms discovered in monocot plants exhibit dual functions using both L-Phe and L-Tyr as substrates for the synthesis of trans-cinnamic acid and p-coumaric acid, respectively (Scheme 1) [27,31,32]. Therefore, the bifunctional PAL is renamed as phenylalanine/tyrosine ammonia-lyase (PTAL, EC 4.3.1.25) [32–34]. Unlike PALs, specific tyrosine ammonia-lyase (TAL, EC 4.3.1.24) solely occurs in some microorganisms [35–39]. Hence, the detectable TAL activity in monocot plants is contributed by the PTAL bifunctional enzyme [29,40], accounting for half lignin biosynthesis in grass plants [33,41].

Rhodobacter sphaeroides TAL (RsTAL) enzyme is firstly utilized as a model to investigate the substrate specificity between L-Phe and L-Tyr; two independent studies reveal that His-89 of RsTAL is the major substrate specificity switch site [36,42]. Bambusa oldhamii BoPAL1 is only specific to L-Phe but not to L-Tyr; site-directed mutagenesis studies have demonstrated that TAL activities are detectable in the BoPAL1-F133H and BoPAL1-V197I mutants [27,32]. Sorghum bicolor SbPAL1 is also a PTAL enzyme, utilizing L-Phe and L-Tyr.
as substrates (Scheme 1) [34]. In addition, SbPAL1 exhibits L-3,4-dihydroxy phenylalanine (L-DOPA) ammonia-lyase (DAL) activity to convert L-DOPA to caffeic acid [34]. TAL activities are completely eliminated in RsTAL-H89F [36] and SbPAL1-H123F mutants [34]. Taken together, Phe or His residue at the substrate specificity switch site is critical for PAL or TAL activity, respectively.

Bamboo PAL proteins are encoded by a multi-gene family, namely, BoPAL1 [43], BoPAL2 [26], BoPAL3, and BoPAL4 [27]. The substrate specificity site of BoPAL1, BoPAL2, or BoPAL3 is a Phe residue, whereas a His residue is present in the BoPAL4 protein (Figure 1A) [27]. BoPAL4 exhibits both PAL and TAL activities [27], making it a good candidate for studying substrate specificity. In the present study, a site-directed mutagenesis combined with PAL/TAL/DAL activities analysis toward diverse substrates, L-Phe/L-Tyr/L-DOPA, was carried out to better understand the catalytic functions in the BoPAL4. Furthermore, the optimum reaction conditions of BoPAL4 using three substrates were examined.

![Diagram of BoPAL4](image)

**Figure 1.** Expression and purification of recombinant BoPAL4 in *Escherichia coli*. (A) The primary structure of the BoPAL4 is illustrated. BoPAL4 is a 701-a.a protein with the conserved Ala-Ser-Gly catalytic motif as well as the substrate specificity site at His-123. (B) Recombinant BoPAL4 protein was purified using Ni-NTA affinity chromatography and separated using 10% SDS-PAGE and then stained with Coomassie Blue. Mr, molecular weight SDS-PAGE marker; lane 1, crude extract; lane 2, unbound protein; lane 3, flow-through; lane 4, 125 mM imidazole-buffer-eluted BoPAL4.

2. Results

2.1. Expression and Purification of Recombinant BoPAL4 in *Escherichia coli*

BoPAL4 contained a 2106 bp open-reading frame (ORF) and encoded a 701- amino acids polypeptide (Figure 1A). The Ala-Ser-Gly catalytic motifs of BoPAL4 and SbPAL1 [34] are from 190 to 192 and from 189 to 191, respectively. Coincidentally, the substrate specificity
switch site of both proteins is located at His-123 (Figure 1A). To better understand the catalytic function of BoPAL4, the coding region of BoPAL4 was inserted into pTrcHisA plasmid and expressed heterologously in the E. coli Top10 strain (Table 1). N-terminal His6-tag was fused to the recombinant BoPAL4 protein for facilitating affinity purification by the Ni-NTA resin (Figure 1B). The recombinant BoPAL4 protein was highly expressed and mainly eluted at 125 mM imidazole buffer (Figure 1B, lane 4). On the SDS-gel, a nearly homogeneous protein band with a molecular mass of 75 kDa corresponded to the predicted molecular mass of BoPAL4 protein. Thus, E. coli expression system was useful for producing recombinant BoPAL4 protein.

Table 1. Bacterial strain and plasmids used for recombinant protein expressions.

| Strain         | Relevant Characteristics                                                                 | Source or Ref. |
|----------------|------------------------------------------------------------------------------------------|----------------|
| E. coli Top10  | F− mcrA Δ(mrr-hsdRMS-merBC) ϕ80lacZΔM15 ΔlacX74 recA1 araD139 Δ ara-leu7697 galU galK X− rpsL(StrR) endA1 mupG | Invitrogen     |

Plasmid | Relevant Characteristics | Source or Ref. |
|---------|--------------------------|----------------|
| pTrcHisA | E. coli expression vector with N-terminal His6-tag fusion | Invitrogen |
| pTrcHisA-BoPAL4 | BoPAL4 coding sequence inserted into pTrcHisA | [27] |
| pTrcHisA-BoPAL4-H123F | Point mutation H123F derivative of pTrcHisA-BoPAL4 | This study |

2.2. Optimum pH and Temperature for PAL, TAL and DAL Activities of BoPAL4

To examine if BoPAL4 can use L-Phe, L-Tyr, and L-DOPA as substrates, PAL, TAL, and DAL activities were performed with 12.1 mM L-Phe, 1.9 mM L-Tyr, and 10 mM L-DOPA in a range of temperatures between 25 and 80 °C (Figure 2A). The maximum/ optimum PAL, TAL, and DAL activities of BoPAL4 were measured at 50, 60, and 40 °C, similar to the optimum temperatures of BoPAL1 and BoPAL2 (Table 2) [26,43]. Accordingly, PAL, TAL, and DAL activities were performed with standard assay conditions in a range of pH between 5 and 11 (Figure 2B). The maximum/ optimum PAL, TAL, and DAL activities of BoPAL4 were measured at pH 9.0, 8.5, and 9.0 °C, also similar to the optimum temperatures of BoPAL1 and BoPAL2 (Table 2) [26,43]. Therefore, L-Phe, L-Tyr, and L-DOPA were bona fide substrates for BoPAL4 PTAL.

Figure 2. Optimum pH and temperature of PAL, TAL, and DAL activities for BoPAL4 PTAL. (A) Optimum temperatures of BoPAL4 using L-Phe (●), L-Tyr (♦), or L-DOPA (▲) as substrate. Activities were measured under standard assay conditions in a range of temperatures from 25 to 80 °C. (B) Optimum pH of BoPAL4 using L-Phe (●), L-Tyr (♦), and L-DOPA (▲) as substrates. Activities were measured under standard assay conditions in a range of pH from 5 to 11. All experiments were performed in triplicate and expressed as average ± standard deviation (S.D., error bars).
Table 2. Comparison of biochemical properties and kinetic parameters of E. coli expressed BoPAL proteins.

| Protein   | Substrate 1 | Optimum pH | Optimum Temp (°C) | $k_{\text{cat}}$ (s$^{-1}$) | $K_m$ (μM) | $k_{\text{cat}}/K_m$ (s$^{-1}$ μM$^{-1}$) |
|-----------|-------------|------------|-------------------|----------------------------|------------|-------------------------------------|
| BoPAL4    | L-Phe       | 9.0        | 50                | 1.44                       | 2084       | 6.9 × 10$^{-4}$                     |
|           | L-Tyr       | 8.5        | 60                | 0.18                       | 98         | 18.4 × 10$^{-4}$                    |
|           | L-DOPA      | 9.0        | 40                | 0.06                       | 956        | 0.6 × 10$^{-4}$                     |
| BoPAL4-H123F | L-Phe     | 9.0        | 50                | 1.87                       | 640        | 29.2 × 10$^{-4}$                    |

1 L-Phe, phenylalanine; L-Tyr, tyrosine; and L-DOPA, 3,4-dihydroxy-phenylalanine.

2.3. Kinetic Parameters for PAL, TAL, and DAL Activities of BoPAL4

The kinetic parameters of BoPAL4 using L-Phe as substrate were measured with its PAL activity. Hyperbolic saturation curve (Figure 3A) and double reciprocal plot (Figure 3B) were obtained to calculate the kinetic parameters. The $K_m$ value of BoPAL4 for L-Phe was estimated as 2084 μM, higher than the values of BoPAL1 (230 μM) [43], BoPAL2 (333 μM) [27], and SbPAL1 (340 μM) [34]. The kinetic parameters of BoPAL4 using L-Tyr as substrate were measured with its TAL activity. Hyperbolic saturation curve (Figure 3C) and double reciprocal plot (Figure 3D) were obtained to calculate the kinetic parameters. The $K_m$ and $k_{\text{cat}}$ values of BoPAL4 for L-Tyr were estimated as 98 μM and 0.18 s$^{-1}$, respectively. The kinetic parameters of BoPAL4 using L-DOPA as substrate were measured with its DAL activity. Hyperbolic saturation curve (Figure 3E) and double reciprocal plot (Figure 3F) were obtained to calculate the kinetic parameters. The $K_m$ value of BoPAL4 for L-DOPA was estimated as 956 μM, which was 2.4-fold higher than SbPAL1 (0.40 mM) [34]. Taken together, BoPAL proteins were highly active at about 50 °C in alkaline reaction conditions.

Figure 3. Kinetic parameters of BoPAL4 PTAL. To determine kinetic parameters using L-Phe as substrate, substrate saturation curve (A) and Lineweaver–Burk double reciprocal plot (B) of the initial rate result of BoPAL4 were used. To determine kinetic parameters using L-Tyr as substrate, substrate saturation curve (C) and Lineweaver–Burk double reciprocal plot (D) of the initial rate result of BoPAL4 were used. To determine kinetic parameters using L-DOPA as substrate, substrate saturation curve (E) and Lineweaver–Burk double reciprocal plot (F) of the initial rate result of BoPAL4 were used. All experiments were performed in triplicate and expressed as average ± standard deviation (S.D., error bars).
2.4. Kinetic Parameters for PAL Activity of BoPAL4-H123F

His-123 is the predicted substrate specificity switch site in the BoPAL4 (Figure 1A). Accordingly, site-directed mutant protein BoPAL4-H123F (Table 1) was also expressed and purified in the E. coli Top10 strain under the same procedure of BoPAL4 expression. After affinity purification, the purities of the 125 mM imidazole-buffer-eluted wild-type (WT) BoPAL4 and BoPAL4-H123F proteins were migrated at the same position on the SDS-gel (Figure 4A). The expression level of BoPAL4-H123F was comparable to that of BoPAL4, indicating that E. coli expression system is also useful for producing BoPAL4-H123F protein.

![Image of SDS-PAGE gel](https://example.com/sds-page.png)

**Figure 4.** Kinetic parameters of BoPAL4-H123F. (A) BoPAL4-WT and BoPAL4-H123F were expressed and purified in E. coli. The 125 mM imidazole eluted fractions were separated using 10% SDS–PAGE and then stained with Coomassie Blue. Mr, molecular weight SDS–PAGE marker. To determine kinetic parameters using L-Phe as substrate, substrate saturation curve (B) and Lineweaver–Burk double reciprocal plot (C) of the initial rate result of BoPAL4-H123F were used. All experiments were performed in triplicate and expressed as average ± standard deviation (S.D., error bars).

The kinetic parameters of BoPAL4-H123F using L-Phe as substrate were measured its PAL activity. Hyperbolic saturation curve (Figure 4B) and double reciprocal plot (Figure 4C) were obtained to calculate the kinetic parameters. The $K_m$ and $k_{cat}$ values of BoPAL4-H123F for L-Phe were estimated as 640 μM and 1.87 s$^{-1}$, respectively (Table 2). The overall catalytic properties ($k_{cat}/K_m$ value) of BoPAL4-H123F using L-Phe as substrate were 4.2-fold higher than that of wild-type BoPAL4.

TAL and DAL activities were significantly decreased in the BoPAL4-H123F. By using L-Tyr and L-DOPA as substrates, kinetic parameters of BoPAL4-H123F are not readily obtained, presumably due to their miniature TAL and DAL activities. Therefore, PAL-, TAL-, and DAL-specific activities were compared instead between WT and mutant proteins.

2.5. Comparison of Specific Activities in Wild-Type BoPAL4 and BoPAL4-H123F Mutants

The $K_m$ value of BoPAL4-H123F using L-Phe (640 μM) was lower than that of BoPAL4 (2084 μM), indicating that BoPAL4-H123F mutant protein increases the binding affinity toward its substrate L-Phe. Likewise, the specific PAL activity of BoPAL4-H123F was slightly higher (1.3-fold) than that of BoPAL4 (Figure 5A). Unlike this, the specific TAL and DAL activities only retained 7.5% (Figure 5B) and 17.8% (Figure 5C) in comparison with wild-type BoPAL4. Taken together, His-123 of BoPAL4 is the major specificity switch site for using L-Tyr and L-DOPA as substrates.
Green bamboo BoPAL4 is a multifunctional enzyme, catalyzing the nonoxidative deamination of L-Phe, L-Tyr, and L-DOPA to yield trans-cinnamic acid, p-coumaric acid, and caffeic acid, respectively. The $K_m$ value of L-Phe in the BoPAL4-H123F significantly decreases, indicating that His-123 is the major substrate specificity switch site. Phe-123 to His mutation of BoPAL4 exhibits higher L-Phe binding affinity than wild-type BoPAL4 as well as an elevated $k_{cat}$ value of 1.87 s$^{-1}$. On the contrary, the TAL and DAL activities are significantly reduced in BoPAL4-H123F, which further confirms that the His-123 of BoPAL4 is essential for utilizing L-Tyr and L-DOPA as substrates. Two studies have shown that TAL activities are completely abolished in both RsTAL-H89F and SbPAL1-H123F mutants [34,36]. To our knowledge, L-DOPA utilization has never been studied in those mutants. Our results show that BoPAL4-H123F still can monitor TAL and DAL activities to some degree. Site-directed mutagenesis study can be performed in the BoPAL4-H123F to further identify more substrate specificity site(s).

Trans-cinnamic acid derivatives are shown to have useful effects in human health, such as anti-obesity and anti-microbial activities [9,10]. Recently, several studies have successfully immobilized PAL proteins on different carriers [44–47]. Electrospun nanofibers combined with dextran polyaldehyde crosslinker are suitable for BoPAL1 and BoPAL2 immobilization [47]. In this study, we showed that BoPAL4 had broad substrate specificities for synthesizing at least three products. In the meantime, other putative aromatic substrates are being tested to see if they can be utilized by the BoPAL4 enzyme. Furthermore, immobilization of BoPAL4 on electrospun nanofibers has great potential for the synthesis of aromatic compounds.

4. Materials and Methods

4.1. Reagents

Bio-Rad protein assay dye reagent concentrate [48], Nuvia™ IMAC resin, and general chemicals for protein electrophoresis were purchased from Bio-Rad, Hercules, CA, USA. Restriction endonuclease enzymes, DNA proofreading polymerase, PrimeStar, and T4 DNA ligase for molecular biology manipulations were obtained from Takara, Kusatsu, Shiga, Japan. Plasmid mini-preparation kit and gel extraction/PCR cleanup kit were from Geneaid Biotech, New Taipei City, Taiwan. Oligonucleotide synthesis and DNA sequencing were serviced by Tri-I Biotech, New Taipei City, Taiwan. L-phenylalanine, L-tyrosine, L-DOPA, trans-cinnamic acid, p-coumaric acid, and caffeic acid were purchased.
from MilliporeSigma, Burlington, MA, USA. All other chemicals and reagents were ACS grade or higher.

4.2. Bacterial Strains, Plasmids Construction, Site-Directed Mutagenesis, and Bacterial Growth Conditions

E. coli strain and plasmids used in this study are listed in Table 1. Top10 strain was utilized for propagation of plasmids and for the induction of recombinant proteins. Plasmid pTrcHisA-BoPAL4 and pTrcHisA-BoPAL4-H123F directed the expression of N-terminal His$_6$-tagged wild-type BoPAL4 [27] and BoPAL4-H123F mutant protein, respectively. The pTrcHisA-BoPAL4-H123F plasmid containing an His to Phe point mutation was generated by QuikChange® site-directed mutagenesis (Strategene, Agilent, Santa Clara, CA, USA) as described by Hsieh et al. 2020 [32]. PCR product was obtained by PrimeStar DNA polymerase, and mixture was digested by DpnI restriction endonuclease overnight and transformed chemically into Top10 component cells followed by DNA sequencing.

E. coli Top10 cells carrying BoPAL4 wild-type and mutant plasmids were grown at 37 °C in Luria-Bertani medium (1% tryptone, 0.5% yeast extract, 1% NaCl, and pH 7.0) supplemented with 100 mg/mL ampicillin. The expression of BoPAL4 proteins was induced at 30 °C with 1 mM isopropyl-β-D-thiogalactoside (IPTG) for 6–8 h [27]. Cells were centrifuged at 6000 × g for 10 min, and pellets were stored at −20 °C freezer before use.

4.3. Preparations of BoPAL4 Enzymes

E. coli cells that expressed His$_6$-tagged BoPAL proteins were freshly collected or resuspended from −20 °C freezer, disrupting by sonication using a Branson cell disruptor [32,49,50]. Proteins were purified from cell lysates by affinity chromatography with Nuvia™ Ni-NTA resin. Samples were fractioned and eluted by varied concentrations of imidazole. Protein content of each step of the preparation was measured at A$_{595}$ nm using a microplate reader (BioTek 800TS, Winooski, VT, USA), and bovine serum albumin (BSA) was served as reference. All purification procedures were conducted at 4 °C cold room.

4.4. Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis

Purified BoPAL4 proteins were analyzed by polyacrylamide gel electrophoresis (PAGE) using a Mini-PROTEAN Tetra Cell system (Bio-Rad, Hercules, CA, USA). Proteins were stained by Coomassie Brilliant Blue R-250 and destained by 20% methanol solution. Gel image was obtained by a Gel Doc XR+ Imaging System (Bio-Rad, Hercules, CA, USA).

4.5. PAL Activity Assay

Phenylalanine ammonia-lyase activity was measured by following the formation of trans-cinnamic acid as the absorbance varied at 290 nm [32] using trans-cinnamic acid as standard. The reaction mixture for PAL contained 50 mM Tris-HCl (pH 8.5), 12.1 mM L-Phe, and an aliquot amount of BoPAL4 enzyme in a total volume of 1.0 mL. The reaction was incubated at 37 °C for 30 min and ceased by adding 100 µL 6N HCl. PAL activity was defined as nkat (nanomole of trans-cinnamic acid formed per second).

4.6. TAL Activity Assay

Tyrosine ammonia-lyase activity was measured by following the formation of p-coumaric acid as the absorbance varied at 310 nm [32] using p-coumaric acid as standard. The reaction mixture for TAL contained 50 mM Tris-HCl (pH 8.5), 1.9 mM L-Tyr, and an aliquot amount of BoPAL enzyme in a total volume of 1.0 mL. The reaction was incubated at 37 °C for 30 min and ceased when 100 µL 6N HCl was added. TAL activity was defined as nkat (nanomole of p-coumaric acid formed per second).

4.7. DAL Activity Assay

DOPA ammonia-lyase activity was measured by following the formation of caffeic acid as the absorbance varied at 350 nm [34] using caffeic acid as standard. The reaction
mixture for DAL contained 50 mM Tris-HCl (pH 8.5), 10 mM L-DOPA, and an aliquot amount of BoPAL enzyme in a total volume of 1.0 mL. The reaction was incubated at 37 °C for 30 min and ceased when 100 µL 6N HCl was added. DAL activity was defined as nkat (nanomole of caffeic acid formed per second).

4.8. Biochemical Properties and Enzyme Kinetic

To determine the optimum reaction pH, activity assays were performed at standard reaction mixture using various pH universal buffers. To obtain the optimum reaction temperature, activity assays were carried out at standard reaction mixture using in various temperatures.

To determine the kinetic parameter for PAL, TAL, and DAL, a range of concentration of L-Phe, L-Tyr, and L-DOPA was varied from 0 to 12.1 mM, from 0 to 1.28 mM, and from 0 to 10 mM, respectively. Substrate saturation curves were carried out after 10 min incubation [32,51] based on Michaelis–Menten equation [52], adapting to double reciprocal plot [53] for the calculation of the $K_m$ and $k_{cat}$ values.

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