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RESEARCH PAPER

Enzymatic Synthesis of D-pipecolic Acid by Engineering the Substrate Specificity of *Trypanosoma cruzi* Proline Racemase and Its Molecular Docking Study

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Abstract Pipecolic acid is an unnatural amino acid mostly used for pharmaceutical purposes. Pipecolic acid has two types of enantiomers with different roles in the synthesis of drugs. The development of efficient catalytic methods for the production of enantiopure pipecolic acid is currently a crucial topic of research. Few chemo- or biosynthetic methods have been proposed for the synthesis of pure enantiomers; however, enzymatic conversion of the chirality of pipecolic acid has not been demonstrated because no pipecolic acid racemase has been reported yet. In this work, we attempted to engineer pipecolic acid racemase activity into *Trypanosoma cruzi* proline racemase (TcPRAC) for the enzymatic synthesis of D-pipecolic acid from L-pipecolic acid. For the binding of pipecolic acid (C6 ring) into the active site of TcPRAC, which was optimized for the original substrate proline (C5 ring), four bulky aromatic residues (Phe102, Phe120, Phe220, and Phe 290) of TcPRAC were mutated to smaller hydrophobic residues. Among the mutants, six single-point mutants (F102A, F102I, F102L, F102V, F290L, and F290V) exhibited significant racemase activity against L-pipecolic acid. The most efficient variant, F102V, showed 74% racemization. Molecular docking simulations revealed that lowering the binding energy of L-pipecolic acid to the active site was important for achieving high racemization activity of TcPRAC mutant proteins.

Keywords: D-pipecolic acid, enzymatic synthesis, substrate specificity engineering, *Trypanosoma cruzi* proline racemase, racemization

1. Introduction

The production of industrial chemicals, biofuels and high-value pharmaceuticals by enzymatic methods has grown in the past decades. The use of enzymatic methods has many advantages such as fast reaction, high selectivity, and low side reactions, which makes them superior to chemical catalyst methods [1]. However, the use of natural enzymes is often limited by their catalytic repertoire [2]. The modulation of enzymes has occurred to overcome this limit, especially by engineering enzyme substrate specificity [3-5,34,35]. Protein engineering methods could break the barrier of enzyme catalytic ability and broaden enzyme usage. Among protein engineering methods, structural guide design approaches have become favorable and efficient because of their increasing PDB(Protein Data Bank) and mechanism information [6].

Recently, pipecolic acid (piperidine-2-carboxylic acid, homoproline), which is a cyclic α-amino acid, has been widely regarded as an attractive synthetic chemical [7]. Pipecolic acid is mostly used in pharmaceutical products such as immunosuppressants [8], anesthetic bupivacaine [9], anticancer agents [10], oxytocin antagonists [11], and antifungal antibiotics [12], etc. [13-15]. Pipecolic acid has

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two types of enantiomers, which have different roles in the synthesis of drugs. The importance of pipecolic acid enantiomers has forced researchers to develop various synthetic and conversion methods such as pathway investigation of microorganisms [16,17], alkylation of chiral glycine enolates [18,19], derivatization of natural amino acids [20], and chemical enantioselective reactions [21,22]. In addition to chemical methods, enzymatic methods have also been developed. Chemoenzymatic methods by Nazabadioko *et al* enabled the synthesis of L-pipecolic acid using (R)-oxynitrilase from almonds [23]. L-pipecolic acid has been synthesized by *Aspergillus* sp. acylase I (AA-I) [24] and lysine cyclodeaminase from *Streptomyces* sp. [25]. Watanabe *et al*. efficiently synthesized both L- and D-pipecolic acid from acetyl-DL-2-amino-6-bromohexanoic acid by using D-aminoacylase [26]. However, there is still a demand for the enzymatic synthesis of pipecolic acid. Despite multiple reports about various amino acid racemases, there are no studies that convert the chirality of pipecolic acid using both industrial synthesis and a biosynthetic method. This fact implies that there is still no enzyme that causes the stereoinversion of pipecolic acids. Thus, enzyme engineering is required to fulfill this demand.

To obtain a desirable enzyme, a rational design of enzyme substrate specificity was applied to the *Trypanosoma cruzi* proline racemase (TcPRAC, EC 5.1.1.4, PDB ID: 1W61) [27]. TcPRAC is an attractive candidate for chemotherapeutic intervention. Over-expression of TcPRAC increases parasite differentiation into infective forms and subsequent penetration into host cells. TcPRAC is also characterized as a potent host B-cell mitogen that sustains parasite evasion of specific host immune responses. B-cell proliferation and polyclonal antibody activation constitute a widespread mechanism of immune evasion shared by many pathogens [28]. This fact provides strong evidence that TcPRAC represents a promising target for therapies that may more efficiently combat Chagas disease [29]. TcPRAC originally catalyzes the reversible stereoinversion of the chiral center in D- and L-proline. TcPRAC is a suitable candidate for engineering the activity toward D- and L- pipecolic acid because TcPRAC has a stereoinversion reaction and a size similar to the substrate. According to the reaction mechanism and PDB structure, two cysteine (Cys) residues (Cys130 and Cys300) in the active site are responsible for the inversion of the chiral center. The original substrate proline is tightly bound to the closed active site pocket and maintains its plane position between the two Cys residues. Pipecolic acid, our target chemical, consists of a 6-carbon ring, and it is larger than the original substrate. To convert the pipecolic acid, we focused on the binding pocket of TcPRAC. An approach was followed in which proper space was made by mutating the binding pocket residues without hampering the key residues. Here, we report the successful *in vitro* stereoinversion of L-pipecolic acid to D-pipecolic acid by mutated TcPRAC.

2. Materials and Methods

2.1. Materials

The TcPRAC gene and primers were commercially synthesized from Bioneer (Daejeon, Korea). The competent cells *E. coli* Top 10 and *E. coli* BL21 (DE3) were purchased from Invitrogen (Carlsbad, CA, USA) and Novagen (Madison, WI, USA), respectively. L,D-proline and L,D-pipecolic acid were purchased from Sigma (St. Louis, MO, USA).

2.2. Gene cloning, expression, and purification of TcPRAC variants

The synthesized gene was cloned into a pET22b (+) plasmid with *NdeI* and *EcoRI* restriction enzyme cutting sites. Site-directed mutagenesis of the TcPRAC gene was performed according to the QuickChange™ protocol. All primers used for mutagenesis are listed in Table 1. Three hundred mL of LB medium with ampicillin (final concentration 100 μg/ml) was inoculated with 30 mL of precultured *E. coli* BL 21 containing the recombinant plasmid. The culture was incubated at 20°C with shaking until the OD_{600} reached 0.5-0.7. Isopropyl-β-thiogalactopyranoside (IPTG) was then added to induce the expression of the recombinant enzyme to a final concentration of 0.1 mM, and the culture was incubated at 20°C for 16 h. The transformed cells were then harvested by centrifugation and suspended in

Table 1. Mutation primers of the TcPRAC variants

Enzymes		Mutation primers
F102V	Forward	5’ CGATATGGTGGGAGCCTTTC 3’
	Reverse	3’ GAAAGGCTCCCACCATATCG 5’
F102A	Forward	5’ CGATATGGCGGGAGCCTTTC 3’
	Reverse	3’ GAAAGGCTCCCGCCATATCG 5’
F102I	Forward	5’ CGATATGATAGGAGCCTTTC 3’
	Reverse	3’ GAAAGGCTCCTATCATATCG 5’
F102L	Forward	5’ CGATATGCTCGGAGCCTTTC 3’
	Reverse	3’ GAAAGGCTCCGAGCATATCG 5’
F220V	Forward	5’ GGTAACTTTGTGGCGATAGTC 3’
	Reverse	3’ GACTATCGCCACAAAGTTACC 5’
F220A	Forward	5’ GGTAACTTTGCGGCGATAGTC 3’
	Reverse	3’ GACTATCGCCGCAAAGTTACC 5’
F220I	Forward	5’ GGTAACTTTATAGCGATAGTC 3’
	Reverse	3’ GACTATCGCTATAAAGTTACC 5’
F220L	Forward	5’ GGTAACTTTCTCGCGATAGTC 3’
	Reverse	3’ GACTATCGCGAGAAAGTTACC 5’

BugBuster® Protein Extraction Reagent (Novagen). The cell debris was removed by centrifugation, and the supernatant containing soluble recombinant TcPRAC was obtained. Then, 1 mL of Ni-NTA agarose beads (Qiagen, Hilden, Germany) was added to the supernatant, followed by gentle mixing at 4°C for 1 h. After washing eight times with 20 mL of wash buffer (50 mM NaH_2PO_4, 300 mM NaCl, and 20 mM imidazole; pH 8.0), the bound proteins were eluted with 1 mL of elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, and 250 mM imidazole; pH 8.0). The purified enzymes were confirmed by 12% SDS-PAGE, and the enzyme concentration was determined using a modified Bradford assay (Sigma-Aldrich, MO, USA) following the manufacturer's instructions.

2.3. Enzyme reaction conditions

We followed the racemization protocols previously reported [30]. The D,L-pipecolic acid conversion reaction was proceeded in 5 ml of 300 mM of sodium acetate buffer (pH 6.0). The final concentrations of L,D-pipecolic acid and the TcPRAC variants were 10 mM and 0.5 mg/ml, respectively. The reaction was executed for 7 days and then deactivated. The D-proline conversion reaction proceeded in 100 mM of sodium acetate buffer (pH 6.0). The final concentrations of L-proline and TcPRAC were 40 mM and 0.071 mg/ml, respectively.

2.4. Detection

2.4.1. L,D-proline detection condition

The HPLC system used was from Agilent(Santa Clara, CA, US), and the column was a CHIROBIOTIC T column (25 cm × 4.6 mm, 5 μm). The temperature of the column was 30°C, and the flow rate was 1.2 ml/min of a mixture of acetonitrile (95%) and water (5%). The proline was detected by a photodiode array detector at a wavelength of 210 nm. The retention times of the enantiomers were 3.5 and 4.2 min for L-proline and D-proline, respectively.

2.4.2. L,D-pipecolic acid detection

The same system and column as those for proline detection were used. The temperature of the column was 25°C, and the flow rate was 1 ml/min of a mixture of acetonitrile (50%) and water (50%). The pipecolic acid was detected by a photodiode array detector at a wavelength of 220 nm. The retention times of the enantiomers were 4.5 and 5.5 min for L-pipecolic acid and D-pipecolic acid, respectively.

2.5. *In silico* molecular docking study

The molecular docking simulation was performed using a SYBYL-X package (Tripos, ver. 2.1). The crystal structure of TcPRAC (PDB code: 1W61) was used to determine the best docking positions of proline and pipecolic acid at the binding site. The proline and pipecolic acid structures were drawn and optimized using the Discovery Studio Program (Accelrys, CA, USA). The protein and ligand were minimized by using the Tripos force field with the Gasteiger-Hückel charge until the RMS gradient was <0.05. Surflex docking was performed with the options selected as follows: hydrogen and heavy atoms were incorporated to allow for protein movement, the covalent force field weighting was set at full for the "Ligand" option and 0.3 for the "Protein" value, the maximum number of poses per ligand was set to 30, and the minimum RMSD between the final poses was set to 0.05. The Surflex-Dock scoring function was applied to generate the docking results, and the final complex structures that matched as closely as possible to the productive bound form of the ligand were selected (the complex with highest matching score). The pocket size of the mutant was calculated using the Discovery Studio 2.5 Package.

3. Results and Discussion

3.1. Selection and design of the target mutation site

Stereoinversion of the chiral center in proline racemization involves the abstraction of a proton from the carbon atom and reprotonation of the resulting intermediate in the opposite stereo-chemical sense. In the crystal structure of 1W61, the C_2 atom of the inhibitor pyrrole-2-carboxylate (equivalent to the C_α atom position of proline) is in contact with the thiol groups of the two cysteine residues, Cys130 and Cys300 (Fig. 1). The atoms optimally located in the planar conformation in the inhibitor, which followed the basic mechanisms proposed for the PLP-independent racemase reaction. The binding pocket of the holo-form of

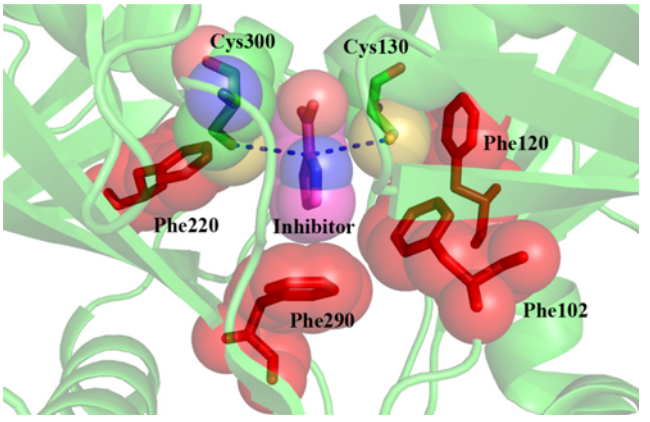

Fig. 1. Active site binding pocket and mutation target residue of TcPRAC. Red sticks are the mutation targets F102, F120, F220, and F290.

TcPRAC was completely buried; thus, no solvents or solvent channel were observed. This structural property suggested the conformational movement of substrate access/release and that the reaction occurred at buried circumstance. Moreover, according to previous molecular dynamics simulations [31], TcPRAC showed large-scale motions of functional relevance. This result supported the justification of the holo-form as a good starting point and suitable for *in silico* simulations. Following the structural properties, we built a mutation strategy based on targeting the inside of the binding pocket rather than the entrance of the active site to engineer the substrate specificity without hampering the original reaction mechanism.

Pipecolic acid needs more space than proline to locate in its favorable conformation with key residues. Therefore, the strategy of engineering TcPRAC was to enlarge the active site binding pocket. An efficient engineering method for enlarging the pocket is replacing the large hydrophobic residue with a smaller one. Phenylalanine (Phe), which is a large hydrophobic residue, is the preferred target for *in vitro* mutagenesis. In the active site of TcPRAC, 4 potential target residues (Phe102, Phe120, Phe220, and Phe290) exist. Phe102, Phe120, and Phe220 are located at the backside of the catalytic residues, Cys130 and Cys300. Modulation of these residues could enlarge the space between the Cys residues. In addition, the Phe102 residue offers a hydrophobic restriction area to the pocket occupancy, restraining the accessibility of OH-Pro compared with proline epimerase [32]. Phe290 shows cation-pi interactions with the inhibitor. Phe290 supports the ring side of the substrate to maintain the conformation with the Cys residues. Substitutions of these residues into smaller hydrophobic residues such as leucine, isoleucine, valine, and alanine could enlarge the space of binding pocket, which might occur for the binding pocket to properly accept 6-carbon chemicals. Furthermore, this substitution could result in a positive binding position with pipecolic acid. We designed sixteen *in vitro* mutants, which were leucine, isoleucine, valine, and alanine mutations in four Phe residues, to synthesize D-pipecolic acid.

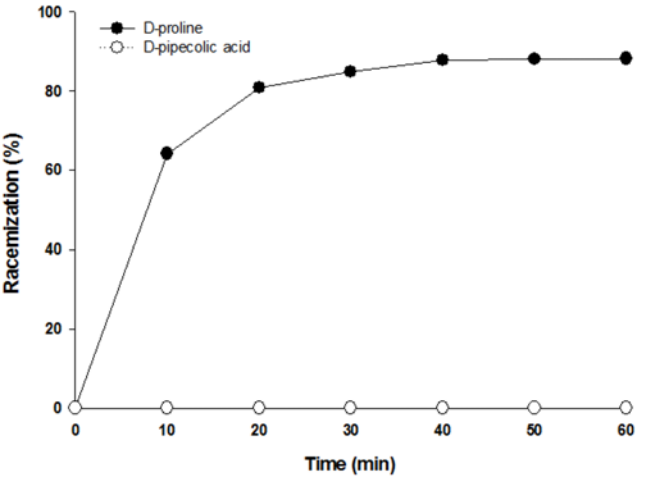

Fig. 2. D-proline (black circle) and D-pipecolic acid (white circle) racemization by wild-type TcPRAC.

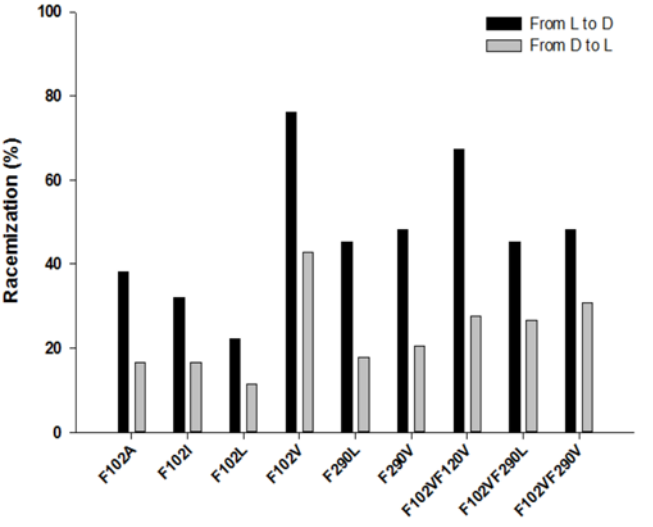

Fig. 3. Racemization percentage of the TcPRAC variants from the D-form to the L-form (Grey bar) and from the L-from to the D-form (black bar). The D-proline conversion reaction proceeded in 100 mM of sodium acetate buffer (pH 6.0) and analyzed after 7 days.

3.2. Biotransformation of L-pipecolic acid by protein engineered proline racemase

The production of D-proline and D-pipecolic acid was measured with a wild-type enzyme for the initial study. The conversion rate was measured every 10 min during an hour. As shown in Fig. 2, wild-type TcPRAC well converted L-proline to D-proline. However, wild-type TcPRAC did not convert any L-pipecolic acid to D-pipecolic acid. After *in vitro* mutagenesis of TcPRAC, 16 mutant variants were tested for L-pipecolic acid conversion. Among them, 6 variants (F102A, F102I, F102L, F102V, F290L and F290V) exhibited activity toward L-pipecolic acid (Fig. 3). The percentages of racemization were 38.3, 32.1, 22.3, 76.1, 45.2, and 48.3%, respectively. Interestingly, all of Phe102 mutants showed catalytic activity toward L-pipecolic acid and we demonstrated that Phe102 was the crucial residue for engineering TcPRAC to obtain pipecolic acid activity. Among the Phe290 mutants, F290V and F290L converted L-pipecolic acid. None of the F120 and F220 mutants showed activity toward L-pipecolic acid. The reverse reaction (from the D-form to the L-form) was also measured. The L-pipecolic acid racemization extent for F102V, F290L and F290V were 42.9, 17.8, and 20.5%, respectively. Although

the mutated variants showed activity toward D-pipecolic acid, the racemization extents were much lower than the L-form activity. The racemization reaction is often reversible; however, it was interesting that the mutants showed better activity toward L-pipecolic acid.

To improve the D-pipecolic acid conversion, a double point mutation was suggested. Because F102V showed the highest racemization extent, it was the beginning point. The D-pipecolic acid and L-pipecolic acid racemization results were 67.3% and 27.6% for F102V/F120V, 45.2% and 26.6% for F102V/F290L, and 48.3% and 30.8% for F102V/F290V. The final conversion was lower than that for the single mutant F102V; however, the initial conversion after 24 h was higher than that for the single mutant and the initial racemization for double mutants were 26.9, 23.4, and 13.9%, whereas F102V was 12.4%.

3.3. Molecular docking simulation results

To understand the difference between TcPRAC and its variants, molecular docking simulations were proposed. The wild-type TcPRAC and 5 variants that exhibited activity toward pipecolic acid were simulated. The binding site of the TcPRAC variants was selected approximately 10 Å from the inhibitor. The residue numbers of the binding sites were 52, 53, 55~57, 59, 101~103, 120, 122, 126~136, 186, 216~218, 220, 221, 269~272, 286~291, 294~304, and 306. The docking position was selected based on the positive binding position that was similar to the inhibitor and suggested by SYBYL. The average distance from the Cα center of proline to catalytic residues of TcPRAC was 3.55, which was the proper distance for the reaction. The binding energy (ΔG) was -6.65 Kcal/mol, and the binding pocket volume was 57.88 $Å^3$. The TcPRAC variants showed positive average distances (Table 2), which were in the criteria of the reaction. Fig. 4 shows the docking pose of L-proline for the wild type and L-pipecolic acid for the F102V mutant. His132, Gly301, Thr302, and Gly131 formed hydrogen bonds with the carboxylate group. Asp 296 also interacted with nitrogen at the ring structure of the substrates. The molecular docking poses of the wild type and mutant variants showed reasonable binding poses. The pocket volumes of F102A, F102I, F102L, and F102V were 86.00, 66.75, 74.13, and 77.25 $Å^3$, respectively. The binding pocket volume increased as the amino acid size decreased. However, the largest volume did not show the highest racemization extent. The binding pocket volume of the F102V mutant seemed to be the proper volume and could preserve the positive position for pipecolic acid racemization.

The respective binding energies of the Phe102 mutants F102A, F102I, and F102L, and F102V were -7.96, -7.75, -7.56, and -9.17 Kcal/mol. When correlated with the racemization extent, we found a perfect linear relationship between the two properties (R^2 = 0.9956) (Fig. 5). In the order of F102L, F102I, F102A, and F102V, when the binding energy decreased, the racemization extent increased. This result indicated that the binding energy was also important with the binding site pocket volume. In addition to this TcPRAC engineering case, other research showed the importance of the binding energy for engineering substrate specificity [6,33]. Molecular docking also proceeded for the double mutants. The binding energies of the three variants F102V/F120V, F102V/F290L, and F102V/F290V were -9.47, -6.46, and -6.23, respectively. Linear results were not found for the single mutants; however, the binding energy revealed significant similarity (R^2 = 0.8836) when plotted with F102V. The binding pocket volumes of the mutants were 78.13, 81.50, and 108.75. The F102V/F120V variant preserved a similar volume to F02V. The volumes of the other two mutations were higher than the F102 mutant, which could explain the decrease in the racemization extent. These molecular docking simulations revealed that lowering the binding energy of L-pipecolic acid to the active site was also important for high racemization activity of the TcPRAC mutant proteins. Engineering TcPRAC with proper binding pocket sizes and lower

Table 2. Binding energies and structural properties of the docking simulation results of the TcPRAC variants

Receptor	Ligand	Binding energy (Kcal/mol)	Distance from Cα center (Å)			Binding pocket volume ($Å^3$)
			130CYS	300CYS	Average	
1W61	L-Proline	-6.65	.3	3.8	3.55	57.88
F102A	L-Pipecolic acid	-7.96	3.4	3.7	3.55	86.00
F102I	L-Pipecolic acid	-7.75	3.9	3.1	3.5	66.75
F102L	L-Pipecolic acid	-7.56	4.1	3.4	3.75	74.13
F102V	L-Pipecolic acid	-9.17	3.4	3.7	3.55	77.25
F102V/F120V	L-Pipecolic acid	-9.47	3.5	3.6	3.55	78.13
F102V/F290L	L-Pipecolic acid	-6.46	3.6	3.5	3.55	81.50
F102V/F290V	L-Pipecolic acid	-6.23	3.1	3.9	3.5	108.75

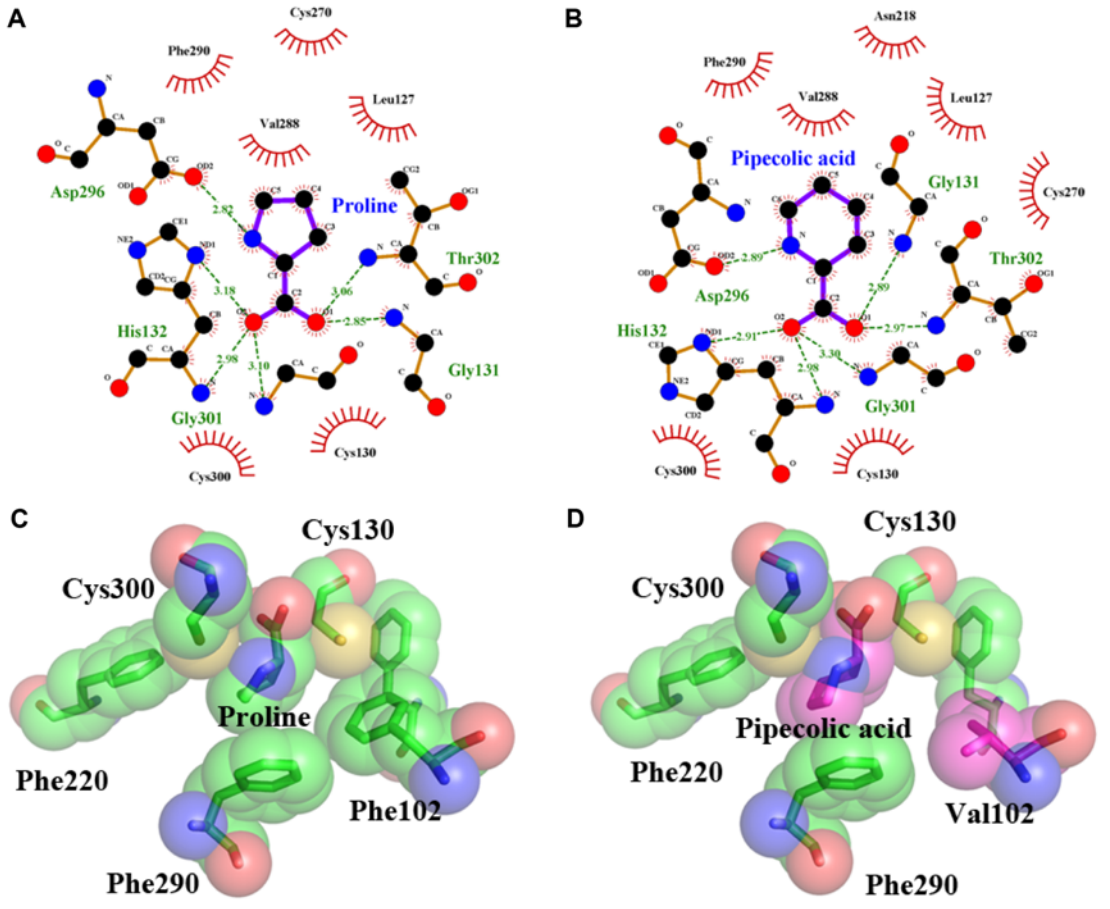

Fig. 4. *In silico* molecular docking simulation results: (A) Interaction between L-proline and TcPRAC by LigPlot. (B) Interaction between L-pipecolic acid and F102V by LigPlot. (C) Binding mode of L-proline at TcPRAC. (D) Binding mode of L-pipecolic acid at F102V.

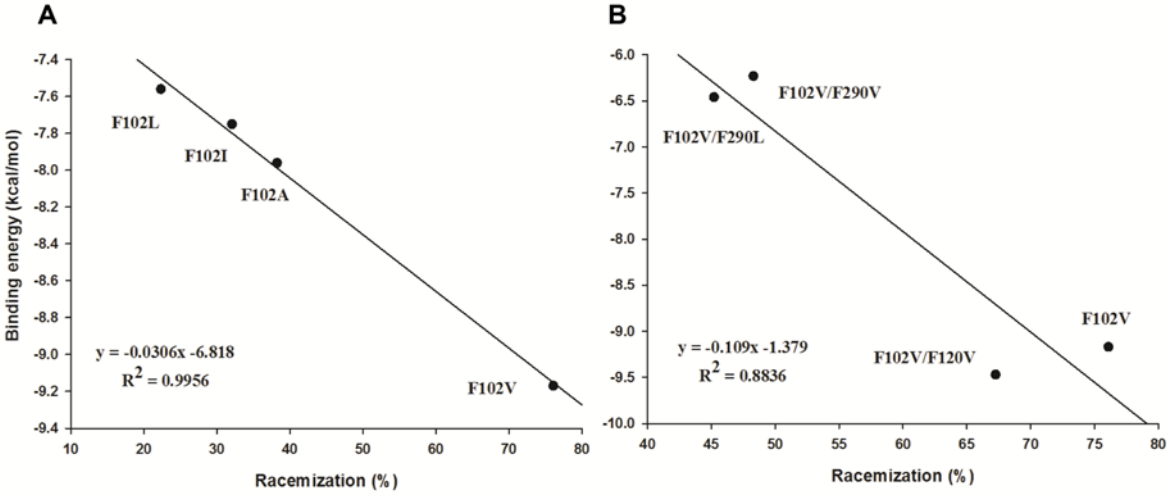

Fig. 5. The correlation between the binding energy and percent racemization of the (A) single mutants and (B) double mutants of the TcPRAC variants.

binding energies for a target substrate could be a good rational strategy. Furthermore, this docking simulation analysis could be used to rationalize engineering other proline racemase family enzymes.

4. Conclusion

In this study, we engineered the substrate specificity of *Trypanosoma cruzi* proline racemase to synthesize D-pipecolic acid from L-pipecolic acid. The research was focused on modulating the space of the binding pocket of TcPRAC by *in vitro* mutagenesis without hampering the key residues and reaction mechanism. Among the mutants, the most efficient variant, F102V, showed 74% racemization. Every F102 mutation showed activity toward L-pipecolic acid and confirmed this residue as a hot spot. Additionally, these mutations showed definite linear relationships between the binding energy and racemization extent. This result indicated that, during the substrate engineering of TcPRAC, the binding energy is also important with proper binding pocket volume. This finding could be a good beginning for pipecolic acid racemization and the engineering of a proline racemase family.

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