

Understanding the Effect of Ionic Liquid–Mediated Solvent Engineering on the Kinetics and Thermodynamic Stability of Phenylalanine Ammonia-Lyase

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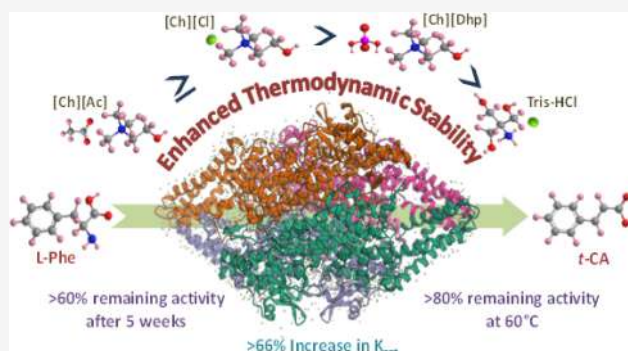
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ABSTRACT: Phenylalanine ammonia-lyase (PAL) plays a central role in the phenylpropanoid pathway and in the treatment of phenylketonuria. However, the integration of PAL into sustainable industrial biocatalysis is hampered by its instability under harsh conditions. This study demonstrates that ionic liquid (IL)–assisted solvent (Tris-HCl buffer) engineering enables improvement of the reaction kinetics and thermodynamic stability of *Rhodotorula glutinis* PAL (RgPAL) under various stresses. Under optimized conditions, a 66.2% higher K_{cat} value, >60% remaining activity after 5 weeks of storage at room temperature, and >80% activity of RgPAL after incubation at 60 °C for 1 h were obtained in the [Ch][Ac]-blended Tris-HCl solvent compared to pristine Tris-HCl. The spectroscopic and molecular docking results suggest that the higher extent of hydration and the soft interactions complemented by the ILs with the D-chain residues of RgPAL jointly contributed to achieving more stable and active conformations of RgPAL. The enzyme showed a higher melting temperature (T_m) in ILs+Tris-HCl compared to that in pristine Tris-HCl, with less change in enthalpy (ΔH_{fu}) and entropy (ΔS_{fu}) of unfolding. Overall, IL-mediated solvent engineering alters the microenvironment of RgPAL and allows the development of a robust PAL-based biocatalytic system.



1. INTRODUCTION

Phenylalanine ammonia-lyase (PAL) is found across living organisms and is known to catalyze the conversion of L-phenylalanine (L-Phe) to *trans*-cinnamic acid.¹ The enzyme also exhibits bisubstrate specificity for the conversion of L-tyrosine together with its physiological substrate L-Phe and plays a key role in protein synthesis, neurotransmission, and cognitive processes and serves as a precursor for pharmaceutically important compounds.^{2,3} PAL initiates the phenylpropanoid pathway through which various secondary metabolites are biosynthesized in plants for defense against biotic and abiotic stressors.^{4–9} Due to its innate ability to reverse the direction of the deamination reaction by varying physiological and substrate conditions, the use of PAL has been extended to the large-scale chemoenzymatic synthesis of enantiomerically pure L-Phe and a calorie-free artificial sweetener, aspartame.¹⁰ In medicine, PAL is administered orally to treat an autosomal recessive disorder, phenylketonuria, a condition in which the enzyme phenylalanine hydroxylase loses its ability to convert L-Phe to tyrosine.^{11,12}

Despite its significant benefits, the enzyme exhibits lower specific activity and instability under harsh conditions, prompting researchers to improve the efficacy of PAL.^{12–18}

Several avenues have been explored in this direction, such as directed evolution,¹³ the permeabilization process,¹⁴ the use of elicitors (such as amino acids, β -cyclodextrins, and glucuronidase),¹⁵ γ radiation-mediated targeted site mutagenesis,¹⁶ the cross-linked enzyme aggregation approach,¹⁷ immobilization on functional biopolymers,¹⁸ and conjugation with silk protein.¹² With the exception of PAL from *Anabaena variabilis* (AvPAL), which was developed by directed evolution,¹³ most existing methods focused on increasing PAL concentration in biological systems rather than manipulating the microenvironment of the enzyme to achieve a higher turnover. In addition to biological activity, the stability of PAL is also important for its function under physiological stress conditions over a prolonged period. The stability strategies described so far are limited to directed evolution under extreme conditions.

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engineered buffer systems, indicating that RgPAL in buffer has a higher tendency to transition to a denatured state than the ILs+Tris-HCl systems. Among the ILs, ΔH_{fu} and ΔS_{fu} were found in the order of $[Ch][Dhp]$ (20.72 kcal mol⁻¹ and 0.061 kcal K⁻¹) > $[Ch][Ac]$ (20.28 kcal mol⁻¹ and 0.059 kcal K⁻¹) > $[Ch][Cl]$ (19.07 kcal mol⁻¹ and 0.056 kcal K⁻¹). In general, the thermodynamic parameters indicate that the thermal stability of RgPAL in different solvent media follows the order $[Ch][Ac] \gtrsim [Ch][Cl] > [Ch][Dhp] > \text{Tris-HCl buffer}$.

4. CONCLUSION

In summary, detailed kinetic and thermodynamic studies using various spectroscopic and in silico approaches were discussed to understand the suitability of IL-mediated solvent engineering strategy for packaging of RgPAL, envisaging sustainable development of a robust industrial PAL-based biocatalysis. The best IL was $[Ch][Ac]$, which showed a 66% higher Kcat of RgPAL than Tris-HCl at a concentration of 50 mM. Although $[Ch][Dhp]$ is known to increase the efficacy of many proteins, including heme proteins, it had a detrimental effect on the turnover number of RgPAL. Overall, the relative activity followed the trend $[Ch][Ac] > [Ch][Cl] > \text{Tris-HCl} > [Ch][Dhp]$. UV-vis and DLS studies show that all ILs affect the hydration layer of RgPAL and the changes in the microenvironment of the enzyme. However, no unfolding was observed in all IL systems, as confirmed by the d^2A_{Tyr}/d^2A_{Trp} values and the concentration-dependent CD spectra. Due to the higher extent of hydration and the additional soft interactions that the ILs make mainly with chain D (~residues 450–650) of RgPAL, the enzyme achieves more stable and active conformations. Although the activity of RgPAL was relatively lower in $[Ch][Dhp]$, the stability was higher than that in the buffer system. In addition, all ILs showed improved thermodynamic stability of RgPAL with higher T_m , lower ΔH_{fu} and less ΔS_{fu} compared to the buffer. Overall, solvent engineering by ILs offers an ecofriendly and sustainable biocatalytic media for RgPAL with improved kinetics and increased thermodynamic stability.

■ ASSOCIATED CONTENT

SI Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acs.jpcb.4c04272>.

Figures S1–S4 and Tables S1–S5 is available. Figure S1 represent UV-vis spectra of RgPAL activity assay. Figure S2 shows docking poses of RgPAL with various systems. Figure S3 and S4 show UV-vis and hydrodynamic radius of RgPAL, respectively. Table S1–S4 show docking score of RgPAL with various solvents and Figure S5 shows the data of entropy and enthalpy of unfolding (PDF)

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Notes

The authors declare no competing financial interest.

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■ LIST OF ABBREVIATION:

RgPAL, *Rhodotorula glutinis* Phenylalanine ammonia lyase; L-Phe, L-phenylalanine; t-CA, trans-cinnamic acid; IL, Ionic liquid; Tris-HCl, Tris(hydroxymethyl)-aminomethan-hydrochloride; $[Ch]^+$, Choline; $[Cl]^-$, Chloride; $[Ac]^-$, Acetate; $[Dhp]^-$, Dihydrogen phosphate; Tyr, Tyrosine; Trp, Tryptophan; CD, Circular dichroism; UV-vis, Ultraviolet-visible

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