



Increasing Performance and Thermostability of D-Phenylglycine Aminotransferase in Miscible Organic Solvents

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Background: D-Phenylglycine aminotransferase (D-PhgAT) is highly beneficial in pharmaceutical biotechnology. Like many other enzymes, D-PhgAT suffers from low stability under harsh processing conditions, poor solubility of substrate, products and occasional microbial contamination. Incorporation of miscible organic solvents into the enzyme's reaction is considered as a solution for these problems; however, native D-PhgAT is not significantly stable in such solvents.

Objective: Halophiles are known to survive and withstand unsavory habitats owing to their proteome bios. In the current study, with an eye on further industrial applications, we examined the performance and thermostability of four halophilic peptides fused D-PhgAT variants in reaction mixtures of various proportions of different miscible organic solvents and various temperatures as well as desiccation.

Materials and Methods: Plasmid constructs from the previous study (Two alpha helices and loops between them from *Halobacterium salinarum* ferredoxin enzyme fused at N-terminus domain of D-PhgAT) expressed in *Escherichia coli* and then D-PhgAT purified. Purified proteins were subjected to various proportions of miscible organic solvents, different temperatures, and desiccation and then performance and thermostability monitored.

Results: Study confirmed increased C_{50} of all halophilic fused D-PhgAT variants, where the highest C_{50} observed for ALAL-D-PhgAT (30.20±2.84 %V/V). Additionally, all halophilic fused variants showed higher thermostability than the wild-type D-PhgAT in the presence of different fractions of acetone, *N,N*-Dimethylformamide and isopropanol in aqueous binary media, while zero activity observed at the presence of methanol.

Conclusion: Our results suggest that applying this new technique could be invaluable for making enzymes durable in discordant industrial conditions.

Keywords: D-Phenylglycine aminotransferase; Halophilic peptide fusion; Miscible organic solvents; Stability

1. Background

Stereo-inverting pyridoxal-5'-phosphate dependent D-Phenylglycine aminotransferase (D-PhgAT; EC 2.6.1.72) is highly beneficial for pharmaceutical and biotechnological utilizations e.g., production of enantiomerically pure synthons and side chain moieties such as D-(-)- α -Phenylglycine and 4-Hydroxy-D-phenylglycine, which are essential for manufacturing highly demanded semi-synthetic antibiotics from cheap L-Glutamate and benzoylformate as substrates

(1, 2).

The biggest obstacle in using enzymes is their low stability under detrimental processing conditions (3) and occasional microbial contaminations (4); which D-PhgAT is not exceptional. Incorporating miscible organic solvents into reaction or storage buffers considered as a prominent solution for these major problems. Additionally, owing to shift the thermodynamic equilibrium, incorporating miscible organic solvents into media prevents unfavorable aqua-

induced reactions (5). Moreover, organic solvents, provide media for solubilization of substrates (6) or products, where poorly soluble or even insoluble components are desired. Despite these invaluable benefits, yet many enzymes might be inactive in organic solvents (7). Various techniques have been investigated to prevail and minimize instability impediments e.g., screening intrinsically stabilized extremozymes (8), enzymes production in genetically modified organisms (9), modification of amino acid signature in mesophilic enzymes (10) and chemical modification of enzymes (11, 12).

Extremozymes from extremophiles are stable and functionally active under precluding conditions where other proteins cannot withstand (13). Among these extremophiles, halophiles are considered as tenacious microorganisms in hypersaline habitats and as natural sources for halophilic enzymes (14). Recovery of 41 million-year-old halophilic bacteria (15) shows their survival capability in desiccation or very minute water in fluid inclusions. Adaptation to low water activity (a_w) and high salt are the survival key in salty dry habitats. Since halophilic proteins are functional at low a_w (16); they supposed to be stable and active in net organic solvents or in a water-organic binary solvent (17).

In our previous study (2), mesophilic D-PhgAT was successfully fused on its N-terminus domain with two highly negatively charged alpha helixes (A_1 and A_2) and a hybrid of them connected with two loops (ALAL) from halophilic ferredoxin of *Halobacterium salinarum* (*Hs Fdx*; PDB: 1E0Z_A). The solubility and activity of these variants (A_1 -D-PhgAT, A_2 -D-PhgAT and ALAL-D-PhgAT) determined in various concentrations of L-glutamate sodium salt as substrate and other additives to the reaction mixture. Results showed the solubility and performance of the ALAL-D-PhgAT variant increased significantly compared with wild-type and other two variants.

In the current study, for the first time, we determine the performance and thermostability of D-PhgAT variants in presence of different fractions of water-miscible organic binary solvents.

2. Objective

Since halophiles are capable to survive their unsavory habitats owing to their proteome bios and with an eye on further industrial applications of D-PhgAT, we investigate the performance and thermostability of halophilic peptide fused D-PhgAT in presence of different proportions of miscible organic solvents, temperature and desiccation.

3. Materials and Methods

3.1. Expression and Purification of D-PhgAT

Construction of plasmids and expression of different D-PhgAT variants are explained in (2). Briefly, oligonucleotides of two alpha helixes (A_1 and A_2) and two loops (ALAL) synthesized base on amino acid sequences of *Hs Fdx*. These Oligonucleotides ligated to pET-17b possessing *dpgA* gene for coding D-PhgAT. The construct was transfected to *Escherichia coli* XL-10 Gold and checked by colony PCR and Sanger sequencing. D-PhgAT expression performed in *E. coli* tuner (DE3) pLysS at 0.4 mM IPTG. After 16 h expression at 20 °C and 100 rpm, cells were harvested at 13,000 ×g, 4 °C for 40 min by Sorvall® RC 5C Plus centrifuge (Kendro Laboratory Products, USA) and resuspended in lysis buffer (20 mM Tris-HCl pH 7.6, 1 mM EDTA, 0.01% β-mercaptoethanol and 100 μM pyridoxal phosphate). Ultra-sonic cell disruption performed on ice in 20 cycles of 10 s burst and 10 s cooling intervals by Vibra-Cell™ SONICS (Sonics and Materials INC., USA), followed by centrifugation at 17,000 ×g, 4 °C and 20 min and followed by fractional ammonium sulfate precipitation. Protein pellet at 25-45% saturation which contained D-PhgAT (verified by SDS-PAGE and activity assay) was dissolved in TEMP buffer (20 mM Tris-HCl pH 7.6, 1 mM EDTA, 0.01% β-mercaptoethanol and 2.5 μM pyridoxal phosphate) containing 1 M ammonium sulfate. Protein mixture applied onto Phenyl Sepharose® FF Hydrophobic Interaction Chromatography (HIC) column (GE Healthcare Bio-Science, Uppsala, Sweden) pre-equilibrated with the same buffer. The elution of bound proteins achieved by a four-bed volume of linear descending gradient 1-0 M ammonium sulfate at 2 mL.min⁻¹ flow rate. Active fractions containing D-PhgAT then pooled, concentrated and desalted using Amicon® 30 kDa cut off centrifugal device (Merck Millipore Ltd., Cork, Ireland) at 4,000 ×g, 4 °C and 20 min and applied onto a DEAE Sepharose™ FF Ion Exchange Chromatography (IEX) column (GE Healthcare Bio-Science, Uppsala, Sweden) pre-equilibrated with TEMP buffer. The elution of bound proteins was achieved by an eight-bed volume of linear ascending 0-1 M NaCl in TEMP buffer. Active fractions containing D-PhgAT were pooled and desalted as mentioned above. ÄKTA purifier FPLC system (GE Healthcare Bio-Science, Uppsala, Sweden) used in all chromatography steps.

3.2. D-PhgAT Assay

D-PhgAT activity was assayed by monitoring the formation of benzoylformate upon transamination of D-(-)-α-Phenylglycine with 2-oxoglutaric acid at 340

nm for 180 s. One milliliter of the reaction mixture contained: 20 μL of enzyme solution, 10 mM D-(-)- α -Phenylglycine, 10 mM 2-oxoglutaric acid, 5 μM pyridoxal phosphate, 5 μM EDTA and 50 mM CAPSO buffer pH 9.5. The molar absorption of $24 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$ was used to calculate the generated benzoylformate in 60 s. One unit of D-PhgAT was defined as the amount of enzyme used for transamination of 1 μmol of D-(-)- α -Phenylglycine to 1 μmol benzoylformate per 60 s at 37 °C.

3.3. Effect of Different Miscible Organic Solvents on pH Values of D-PhgAT Reaction Buffer

Upon adding miscible organic solvent, the buffer pH may change (18), therefore determining the effect of organic solvents concentration on pH of D-PhgAT reaction buffer is critical. Modified method (19-21) used: 100 μL of 500 mM CAPSO buffer with various pH values, 40 μL of pH indicator solution (0.85 mM thymol blue and 10 mM NaOH), and deionized sterile water mixed to the final concentration of 50 mM CAPSO buffer. Helios Alpha UV-Vis spectrophotometer (Spectronic Unicam, Cambridge, UK) used to determine the absorption at 400 nm to 750 nm range and the maximum absorbance (λ_{max}). The equation (1) was used to calculate the pka of thymol blue pH indicator in different pH values:

$$\text{Log}_{10} [(A - A_{\text{Ind}^-}) / (A_{\text{HInd}} - A)] = \text{pka} - \text{pH} \quad (1)$$

Where A_{HInd} and A_{Ind^-} are λ_{max} of protonated and deprotonated forms of thymol blue in 50 mM CAPSO buffer pH 2 and 12, respectively at 594 nm. Then 100 μL of 500 mM CAPSO pH 9.5, 40 μL of pH indicator, different amounts of absolute miscible organic solvents (DMF, acetone, isopropanol and methanol) and deionized sterile water were mixed to the final concentration of 0, 10, 20, 30 and 40% of miscible organic solvents. Helios Alpha UV-Vis spectrophotometer used to determine the λ_{max} in presence of miscible organic solvents proportions. These λ_{max} values at 594 nm and pka from equation (1) used to calculate pH values of solutions containing miscible organic solvents concentrations. Then pH shift of CAPSO buffer (ΔpH) was obtained and used for the preparation of CAPSO buffer with various pH to minimize the pH shift upon adding solvents.

3.4. D-PhgAT Assay in Various concentrations of Miscible Organic Solvents

The reaction mixture (1 mL) contained 1U enzyme, 10 mM D-(-)- α -Phenylglycine, 10 mM 2-oxoglutaric acid, 5 μM pyridoxal phosphate, 5 μM EDTA, 50 mM CAPSO buffer with appropriate pH and 0, 10, 20, 30

and 40% of organic solvents. The concentration of organic solvents where 50% of activity remained (C_{50}) was calculated for D-PhgAT variants.

3.5. Effects of Absolute Organic Solvents, Desiccation and Temperature on D-PhgAT Performance

One unit of enzyme variants in TEMP storage buffer vacuum centrifuged at 20 °C until completely dried. Then 10 μL of deionized sterile water or 10 μL of absolute organic solvents were added followed by incubation at 25, 40 and 60 °C for 1 h. Samples dried completely by vacuum centrifugation and 10 μL of TEMP buffer was added for rehydration and then incubated on ice for 1h followed by D-PhgAT activity assay.

3.6. Statistical Analysis

Experiments performed triplicated and analyzed by ANOVA. The $p < 0.05$ considered as significant and used to construe data. Means and standard deviations shown on graphs.

4. Results

4.1. Purification of D-PhgAT

The results of plasmids construction and protein expression are reported previously (2). In purification phase, protein pellets from 25-45% fractional ammonium sulfate precipitation were loaded onto Phenyl Sepharose® FF column. **Figure 1A** shows the chromatogram and SDS-PAGE analysis of D-PhgAT peak eluted fractions of Phenyl Sepharose. Active fractions were loaded onto DEAE Sepharose™ FF column. **Figure 1B** shows the chromatogram and SDS-PAGE analysis of D-PhgAT peak eluted fractions of DEAE Sepharose. **Table 1** shows the summary of this three-step purification strategy.

4.2. Effect of Different Miscible Organic Solvents on pH Values of D-PhgAT Reaction Buffer

Figure 2 shows spectrogram and maximum absorbance peaks (λ_{Max}) of 50 mM CAPSO buffer (various pH) at 595 nm which used to plot reference curve and spectrograms of proportions of acetone, DMF, isopropanol in CAPSO buffer pH 9.5. Upon adding an organic solvent, the pH value of CAPSO buffer changed drastically. **Figure 2A** shows λ_{max} of 50 mM CAPSO buffer with various pH at 594 nm and used to calculate pka values (equation 1). These pka values and λ_{max} at 594 nm upon adding miscible organic solvents to CAPSO buffer pH 9.5 (**Figs. 2B, 2C and 2D**) where used to estimate pH shift (ΔpH) of CAPSO buffer. The buffer pH was reduced as

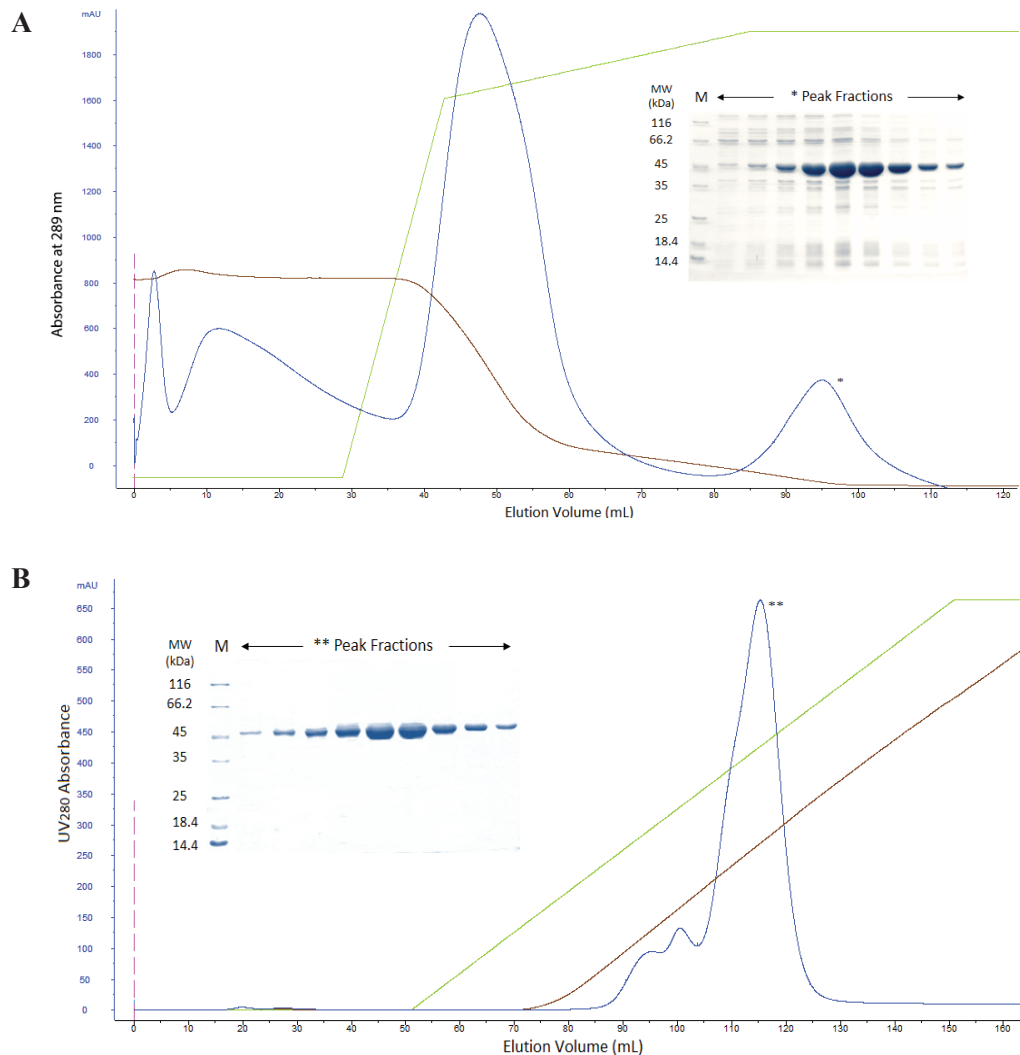


Figure 1. Purification chromatograms D-PhgAT. **(A)** The chromatogram of Phenyl Sepharose 6 FF hydrophobic interaction chromatography. Peak fractions (*) containing D-PhgAT analyzed with SDS-PAGE. **(B)** The chromatogram of DEAE Sepharose FF ion exchange chromatography. Peak fractions (**) containing D-PhgAT analyzed with SDS-PAGE. The pink line (injection into the column), the blue graph (UV280 absorbance graph), the green line (Concentration gradient of elution buffer) and the brown graph (Conductivity).

proportions of organic solvents increased. To minimize unfavorable pH change and effects on D-PhgAT performance, CAPSO buffer with various pH used to maintain pH at 9.5.

4.3. Effects of Various Fractions of Miscible Organic Solvents on D-PhgAT

The D-PhgAT performance determined in reaction mixtures contained 0, 10, 20, 30 and 40% of miscible organic solvents (**Fig. 3**). The organic solvent concentrations, where 50% of enzyme activity (C_{50}) retained are shown in **Table 2**. The performance of all D-PhgAT variants decreased as the concentration of

solvents increased, especially in presence of DMF and acetone. No activity observed for methanol.

4.4. D-PhgAT Performance and Absolute Organic Solvents

D-PhgAT performance determined prior and after desiccation in TEMP buffer and in organic solvents at various temperatures (**Fig. 4**). After desiccation in TEMP buffer, absolute acetone and isopropanol and rehydration, residual activity of all D-PhgAT variants decreased in a temperature-dependent manner. The highest residual activity detected for ALAL-D-PhgAT at all temperatures. In case of DMF, the highest residual

Table 1. Purification table of different variants of D-PhgAT.

Purification steps	Protein variants	Total protein (mg)	Total activity (U)	Specific activity (U.mg ⁻¹)	Purification fold	Yield (%)
Clarified cell lysate	WT-D-PhgAT	92.56	474.20	5.12	1	100
	A ₁ -D-PhgAT	89.30	446.50	5.00	1	100
	A ₂ -D-PhgAT	91.20	460.56	5.05	1	100
	ALAL-D-PhgAT	93.12	485.15	5.21	1	100
(NH ₄) ₂ SO ₄ precipitation	WT-D-PhgAT	29.85	285.60	9.57	1.86	60.22
	A ₁ -D-PhgAT	30.58	302.74	9.90	1.98	67.80
	A ₂ -D-PhgAT	30.70	297.48	9.69	1.91	64.57
	ALAL-D-PhgAT	32.33	321.68	9.95	1.90	66.30
Phenyl Sepharose 6 FF (HIC)	WT-D-PhgAT	5.53	187.85	33.97	6.63	39.61
	A ₁ -D-PhgAT	5.56	198.15	35.64	7.12	44.37
	A ₂ -D-PhgAT	5.59	191.73	34.30	6.79	41.62
	ALAL-D-PhgAT	5.63	201.66	35.82	6.87	41.56
DEAE Sepharose FF (IEX)	WT-D-PhgAT	1.58	136.24	86.23	16.84	28.73
	A ₁ -D-PhgAT	1.64	142.15	86.98	17.39	31.83
	A ₂ -D-PhgAT	1.61	155.70	86.71	17.17	33.80
	ALAL-D-PhgAT	1.68	146.78	87.37	16.76	30.25

The specific activity of enzyme variants in each step is divided by the specific activity of clarified cell lysate to calculate purification fold. The total activity of enzyme variants in each step is divided by the total activity of clarified cell lysate to calculate purification yield in percent.

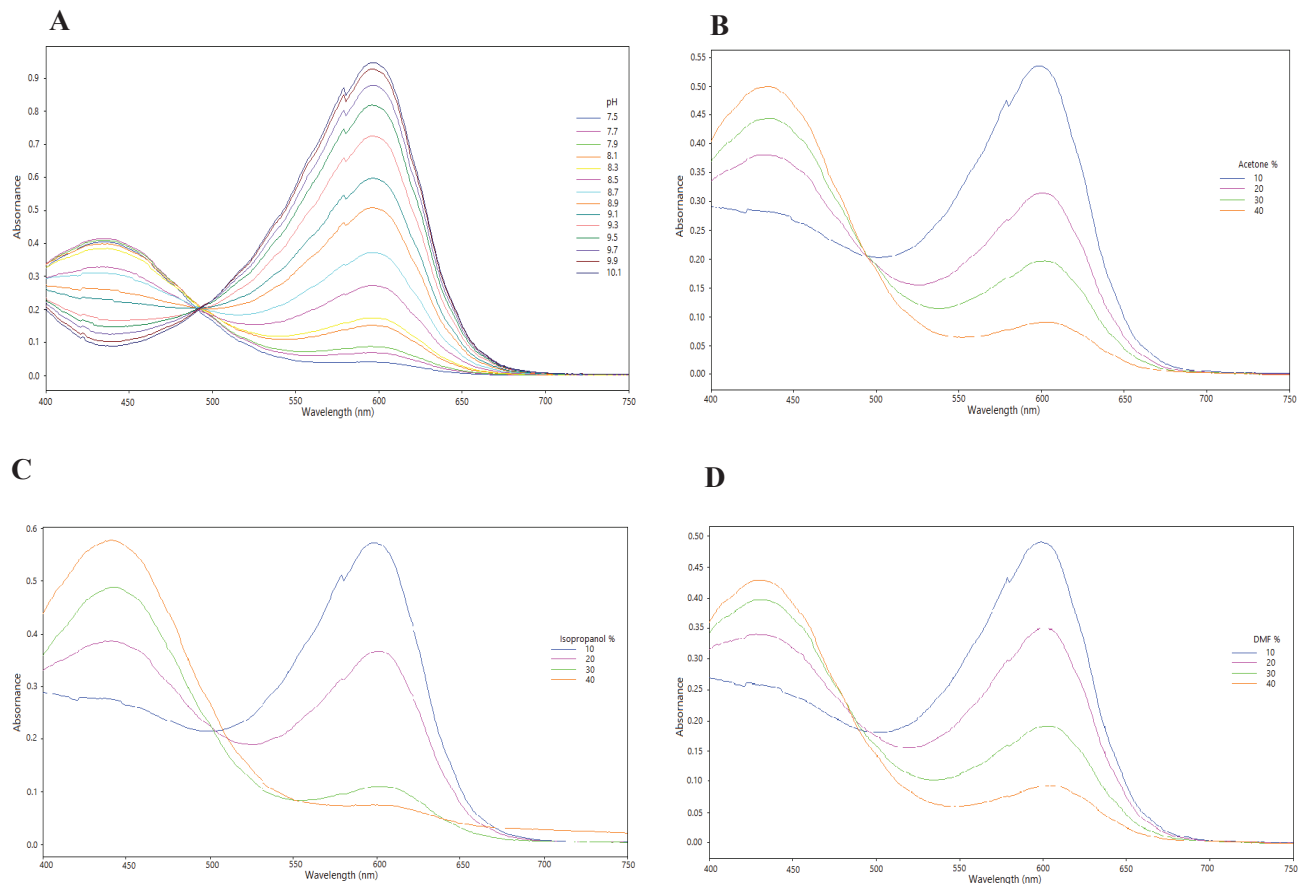


Figure 2. The spectrogram at 400 nm – 750 nm of (A) 50 mM CAPSO buffer + thymol blue with various pH values, and different fractions of acetone (B), isopropanol (C) and DMF (D), respectively, in 50 mM CAPSO buffer pH 9.5 + thymol blue.

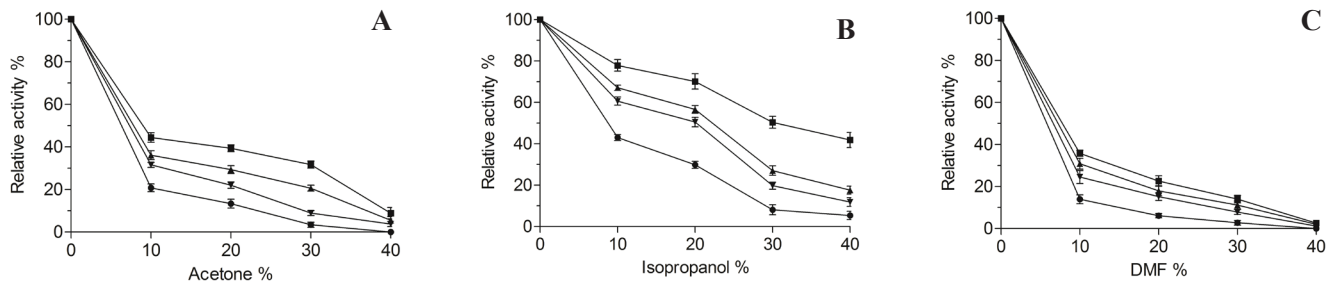


Figure 3. Shows effects of various fractions of (A) acetone, (B) isopropanol and (C) DMF water-miscible organic binary solvents on of D-PhgAT variants. WT-D-PhgAT (●), A₁-D-PhgAT (▲), A₂-D-PhgAT (▼) and ALAL-D-PhgAT (■). Bars represent the standard deviation (n = 3)

activity observed at 40 °C for all variants while no activity observed at 60 °C.

5. Discussion

D-PhgAT Purification achieved in three steps. Firstly, at a specific concentration of ammonium sulfate, protein surficial charges are neutralized and the tendency of a protein to overcome aggregation is nullified. For D-PhgAT, 25-45% saturation was executed with 60-70% yield and 1.8-1.9fold purification. Secondly, protein

pellets from fractional precipitation dissolved in TEMP buffer containing 1 M ammonium sulfate and applied on the Phenyl Sepharose. Pre-equilibration with 1 M ammonium sulfate eliminates free water molecules in microenvironments around hydrophobic phenyl ligands that is essential for binding of hydrophobic proteins via their surficial hydrophobic patches. After elution of bound proteins by descending gradient of ammonium sulfate, 39-44 % yield and 6-7 fold purification achieved. Lastly, active fractions desalted completely

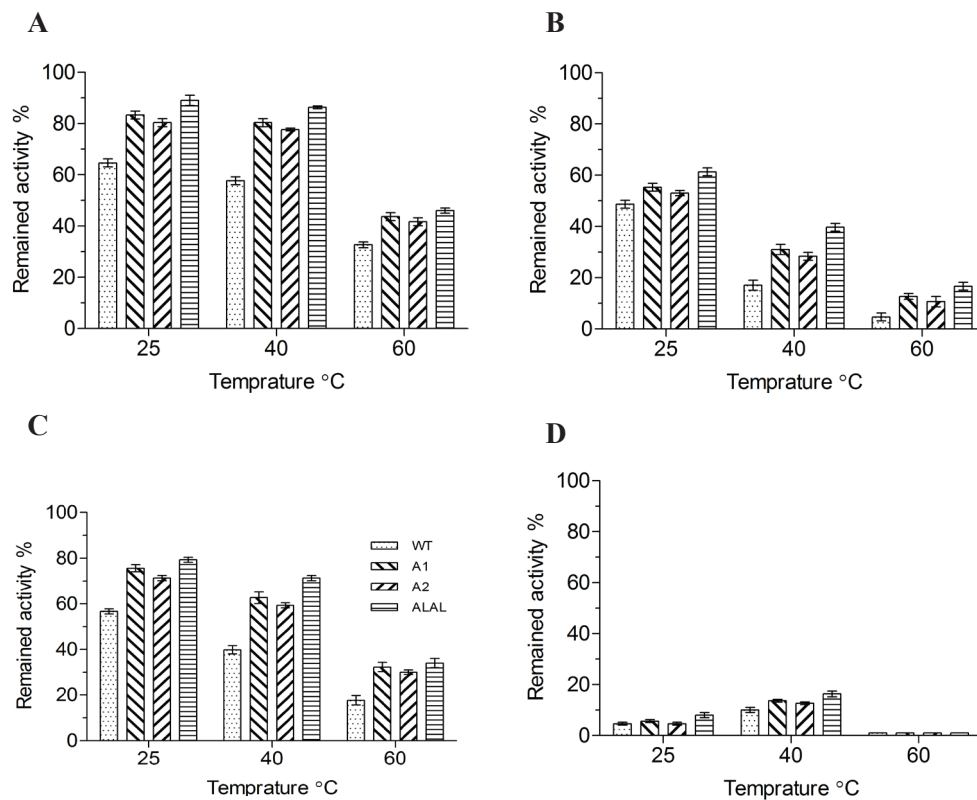


Figure 4. Remained activity of D-PhgAT variants at (A) TEMP buffer pH 7.6 as control and absolute miscible organic solvents (B) acetone, (C) isopropanol and (D) DMF; after 1 h incubation at different temperatures (25, 40 and 60 °C). Bars represent the standard deviation (n = 3)

Table 2. C_{50} values of D-PhgAT variants in different organic solvents

Protein variants	C_{50} (% V/V)		
	DMF	Acetone	Isopropanol
WT-D-PhgAT	5.82 ± 0.86	6.27 ± 0.72	8.70 ± 0.47
A ₁ -D-PhgAT	7.22 ± 0.35	7.80 ± 0.49	22.30 ± 1.52
A ₂ -D-PhgAT	6.60 ± 0.48	7.20 ± 0.53	20.10 ± 2.30
ALAL-D-PhgAT	7.80 ± 0.62	9.00 ± 0.97	30.20 ± 2.84

Different variants of D-PhgAT examined against fractions of the selected organic solvents in reaction media. The organic solvent where 50% of enzyme activity remained (C_{50}) was used to compare the stability of variants in presence miscible organic solvents. The experiments done in triplicated (mean ± SD, n = 3)

and injected into DEAE Sepharose which was pre-equilibrated with TEMP buffer. In contrast with HIC; the IEX column separates proteins based on charge differences and isoelectric pH (pI). Different variants of D-PhgAT have pI range from 5.28 to 6.2 (1, 2), thus in TEMP buffer, D-PhgAT will be negatively charged and binds to positively charged diethylaminoethyl ligands. Elution of bound proteins in this step happened with ascending gradient of NaCl, in which Cl substituted with D-PhgAT. In this step 28-30% yield and 16-17 fold purification achieved.

In aqueous solutions, pH value largely influences solubility, performance and stability of enzymes (18, 22). Addition of organic solvents into a buffer shifts pH value drastically (23) with plausible indecent effects on protein. Aiming to verify the effects of organic solvents on D-PhgAT, interrogation of their effects on pH value of CAPSO buffer pH 9.5 is irrevocable. Thymol blue with the second transition, yellow-blue at pH 8.0-9.6 and average pka 8.9 used as an indicator. Owing to pka shift in different pH, pka of each was calculated. Upon adding solvents to an aqueous buffered solution, the pka of the acid participant and the solvents autoprotolysis constant change and as a result, pH jumps (23, 24). To maintain pH at 9.5 and minimize unfavorable pH change on D-PhgAT performance, CAPSO buffer with various pH used.

Upon increasing proportion of organic solvents up to 40%, the performance of D-PhgAT variants diminished, which at least partly could be due to the denaturation capacity of solvents (21, 25). The log p value (octanol/water partition coefficient) is -1.01 for DMF and -0.24 for acetone (26). Both have low hydrophobicity, thus are highly potent to strip water molecules off the hydration shell at the expense of proteins flexibility (27) therefore promote denaturation and inactivation of D-PhgAT.

The fusion of halophilic peptides provides eminent numbers of negatively charged residues which serve as extra sites for water molecules binding, therefore

maintaining protein hydration shell and partial activity preservation. Methanol has log p value -0.81 (26) seems to strip more water molecules off the protein hydration shell at expense of flexibility. Moreover, structural studies have shown that aqueous solution of methanol stabilizes α -helices and simultaneously denatures other structures or accumulates on protein surface (28). All these reasons participate to nullify D-PhgAT performance in methanol solutions. Despite large performance decrease in DMF, acetone and methanol solutions, D-PhgAT showed higher performance in isopropanol (log p 0.05) solution (26) which indicate relatively higher hydrophobicity of isopropanol compared with other used solvents. This isopropanol characteristic results in stripping fewer water molecules off the hydration shell of proteins and thereby results in more flexibility and less denaturation. In all cases, halophilic fused variants manifest higher C_{50} value compared with wild-type (Table 2), where the highest C_{50} observed for ALAL-D-PhgAT in isopropanol solutions (C_{50} : 30.20 ± 2.84 (%V/V)).

In presence of absolute organic solvents, the interval dehydration and raised temperature increased denaturation propensity. Owing to decreased water as a molecular lubricant (29) and insufficient flexibility for denaturation (30) protein rigidity increased, so protection against temperature stress developed. Halophilic proteins, owing to their charged residues, manifest interesting interactions with salts in aqueous or organic solutions which maintain their active structures in dry conditions (31). Therefore, it is feasible to assume fusion of halophilic peptides preserves the active structure of D-PhgAT in dehydrated conditions and elevates the thermostability. Compared with aqueous solutions, thermostability of D-PhgAT variants in presence of absolute organic solvents increased as the log p of the organic solvents increased. DMF (log p : -1.01) has highest adverse effect compared with acetone (log p : -0.24) and isopropanol (log p : 0.05). The

rigidity and eliminated water participation in hydrogen bonds after dehydration, reduced dielectric constant and increased strong intra-protein interactions, reduce remained activity in such absolute non-aqueous media. Surprisingly, in case of DMF, the residual activity at 40 °C of all D-PhgAT variants was higher than 25 °C and was zero at 60 °C. This unusual behavior of D-PhgAT in presence of absolute DMF is yet to be fully understood but might be due to the nature and position of amino acid residues in A₂ alpha helix, yet it cannot explain the higher remained activity of WT-D-PhgAT at 40 °C than 25 °C.

Removing solvents and then rehydration elevates flexibility and partially restores D-PhgAT performance. Dehydration-rehydration cycles also lead to irreversible protein damages (32, 33) and diminish performance (which is highly pH dependent) as an outcome of side chains ionization and salt bridge alternation. Some organic solvents e.g., glycerol and ethylene glycol mimic the water effect owing to multiple hydrogen bonds formation. Crown ethers can bind to cationic groups e.g., amine groups and stabilize molecular interactions and protein structure and cyclodextrins by the formation of stable supramolecular complexes and clasp hydrophobic amino acid residues in their inner cavities (34).

Our results, hereby, confirmed that practiced halophilic peptide fusion can increase thermostability of a mesophilic protein, both in aqueous and non-aqueous media.

6. Conclusion

Despite many benefits of utilizing enzymes in biotechnological processing, they might largely suffer from denaturation and performance reduction. Additionally, insolubility of substrates or products and occasional microbial contamination are problematic. A suggested strategy to partially eliminate these drawbacks is applying miscible organic solvents in enzyme media. Nevertheless, miscible organic solvents might have detrimental effects. Extremophiles, particularly halophiles, have extraordinary properties to withstand unsavory habitats, making them invaluable for industrial applications. In this study, for the first time, we practiced the effects of fusion of two alpha helices and loops between them from a halophilic enzyme at N-terminus domain of mesophilic D-PhgAT, as an industrial model enzyme in presence of miscible organic solvents at various temperatures. Our results suggest that applying this new technique could be an invaluable pragmatic strategy for engineering durable enzymes for precluding industrial applications.

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References

1. Wiyakrutta S, Meevootisom V. A stereo-inverting D-phenylglycine aminotransferase from *Pseudomonas stutzeri* ST-201: purification, characterization and application for D-phenylglycine synthesis. *J Biotechnol.* 1997;**55**(3):193-203. doi: 10.1016/S0168-1656(97)00075-8
2. Javid H, Jomrit J, Chantarasiri A, Isarangkul D, Meevootisom V, Wiyakrutta S. Effects of halophilic peptide fusion on solubility, stability, and catalytic performance of D-phenylglycine aminotransferase. *J Microbiol Biotechnol.* 2014;**24**(5):597-604. doi: 10.4014/jmb.1312.12040
3. Iyer PV, Ananthanarayan L. Enzyme stability and stabilization- Aqueous and non-aqueous environment. *Proc Biochem.* 2008;**43**(10):1019-1032. doi : 10.1016/j.procbio.2008.06.004
4. Shultz MJ. Liquid interfaces in chemical, biological, and pharmaceutical applications. *J Am Chem Soc.* 2001;**123**(41):10143-10143. doi: 10.1021/ja015249a
5. Klibanov AM. Improving enzymes by using them in organic solvents. *Nature.* 2001;**409**(6817):241-246. doi: 10.1038/35051719
6. Zaks A, Klibanov AM. Enzymatic catalysis in nonaqueous solvents. *J Biol Chem.* 1988;**263**(7):3194-3201.
7. Kieboom APG. Enzymes that do not work in organic solvents. In: Abramowicz DA, editor. *Biocatalysis*. Dordrecht: Springer Netherlands; 1990;p:357-364. doi: 10.1007/978-94-010-9124-4_17
8. Raddadi N, Cherif A, Daffonchio D, Neifar M, Fava F. Biotechnological applications of extremophiles, extremozymes and extremolytes. *Appl Microbiol Biotechnol.* 2015;**99**(19):7907-7913. doi: 10.1007/s00253-015-6874-9
9. Adrio J-L, Demain AL. Recombinant organisms for production of industrial products. *Bioeng Bugs.* 2010;**1**(2):116-131. doi: 10.4161/bbug.1.2.10484
10. Sugathan S, Pradeep NS, Abdulhameed S. *Bioresources and Bioprocess in Biotechnology*. Singapore: Springer Singapore; 2017;p:151-68. doi: 10.1007/978-981-10-4284-3
11. DeSantis G, Jones JB. Chemical modification of enzymes for enhanced functionality. *Curr Opin Biotechnol.* 1999;**10**(4):324-330. doi: 10.1016/S0958-1669(99)80059-7
12. Nwagu TN, Okolo B, Aoyagi H, Yoshida S. Chemical modification with phthalic anhydride and chitosan: Viable options for the stabilization of raw starch digesting amylase from *Aspergillus carbonarius*. *Int J Biol Macromol.* 2017;**99**:641-647. doi: 10.1016/j.ijbiomac.2017.03.022
13. D'Amico S, Marx JC, Gerday C, Feller G. Activity-stability relationships in extremophilic enzymes. *J Biol Chem.* 2003;**278**(10):7891-7896. doi: 10.1074/jbc.M212508200
14. Kunte HJ, Trüper HG, Stan-Lotter H. Halophilic microorganisms. Astrobiology In: *The quest for the conditions of life*. Berlin,

- Heidelberg. Springer Berlin Heidelberg; 2002;p:185-200. doi: 10.1007/978-3-642-59381-9
15. Jaakkola ST, Zerulla K, Guo Q, Liu Y, Ma H, Yang C, *et al.* Halophilic archaea cultivated from surface sterilized middle-late eocene rock salt are polyploid. *PLoS One*. 2014;**9**(10):e110533. doi: 10.1371/journal.pone.0110533
 16. Karan R, Capes MD, DasSarma S. Function and biotechnology of extremophilic enzymes in low water activity. *Aquat Biosyst*. 2012;**8**(1):4. doi: 10.1186/2046-9063-8-4
 17. Gupta S, Sharma P, Dev K, Sourirajan A. Halophilic bacteria of lunsu produce an array of industrially important enzymes with salt tolerant activity. *Biochem Res Int*. 2016;**2016**:10. doi: 10.1155/2016/9237418
 18. Shubhada S, Sundaram PV. The role of pH change caused by the addition of water-miscible organic solvents in the destabilization of an enzyme. *Enz Micro Technol*. 1995;**17**(4):330-335. doi: 10.1016/0141-0229(94)00028-X
 19. L. E. Vidal Salgado CV-H. Spectrophotometric determination of the pka, isosbestic point and equation of absorbance vs. ph for a universal ph indicator. *American J Anal Chem*. 2014;**5**(17):1290-1301. doi: 10.4236/ajac.2014.517135
 20. Patterson GS. A simplified method for finding the pka of an acid-base indicator by spectrophotometry. *J Chem Ed* . 1999;**76**(3):395.
 21. Miroliaei M, Nemat-Gorgani M. Effect of organic solvents on stability and activity of two related alcohol dehydrogenases: a comparative study. *Int J Biochem Cell Biol*. 2002;**34**(2):169-175. doi: 10.1016/S1357-2725(01)00109-1
 22. Klibanov AM. Why are enzymes less active in organic solvents than in water? *Trends Biotechnol*. 1997;**15**. doi: 10.1016/S0167-7799(97)01013-5
 23. Subirats X, Rosés M, Bosch E. On the effect of organic solvent composition on the ph of buffered HPLC mobile phases and the pk a of analytes-a review. *Sep Pur Rev*. 2007;**36**(3):231-255. doi: 10.1080/15422110701539129
 24. Kiliç E, Aslan N. Determination of autoprotolysis constants of water-organic solvent mixtures by potentiometry. *Microchimica Acta*. 2005;**151**(1):89-92. doi: 10.1007/s00604-005-0380-1
 25. Khmelnitsky YL, Mozhaev VV, Belova AB, Sergeeva MV, Martinek K. Denaturation capacity: a new quantitative criterion for selection of organic solvents as reaction media in biocatalysis. *Eur J Biochem*. 1991;**198**(1):31-41. doi: 10.1111/j.1432-1033.1991.tb15983.x
 26. Sangster J. Octanol-water partition coefficients of simple organic compounds. *J Phys Chem Ref Data*. 1989;**18**(3):1111-1229. doi: 10.1063/1.555833
 27. Hartsough DS, Merz KM. Protein flexibility in aqueous and nonaqueous solutions. *J American Chem Soci*. 1992;**114**(26):10113-10116. doi: 10.1021/ja00052a004
 28. Hwang S, Shao Q, Williams H, Hilty C, Gao YQ. Methanol strengthens hydrogen bonds and weakens hydrophobic interactions in proteins - a combined molecular dynamics and NMR study. *J Phys Chem B*. 2011;**115**(20):6653-6660. doi: 10.1021/jp11448a
 29. Stepankova V, Bidmanova S, Koudelakova T, Prokop Z, Chaloupkova R, Damborsky J. Strategies for stabilization of enzymes in organic solvents. *ACS Catal*. 2013;**3**(12):2823-2836. doi: 10.1021/cs400684x
 30. Mukherjee J, Gupta MN. Increasing importance of protein flexibility in designing biocatalytic processes. *Biotechnol Rep*. 2015;**6**:119-123. doi: 10.1016/j.btre.2015.04.001
 31. Patel S, Saraf M. Perspectives and application of halophilic enzymes. In: *Halophiles: Biodiversity and Sustainable Exploitation*, edited by Maheshwari DK, Saraf M, Springer International Publishing; 2015;p:403-419. doi: 10.1007/978-3-319-14595-2
 32. Qvist J, Ortega G, Tadeo X, Millet O, Halle B. Hydration dynamics of a halophilic protein in folded and unfolded states. *J Phys Chem B*. 2012;**116**(10):3436-3444. doi: 10.1021/jp3000569
 33. Noinville S, Revault M, Quiquampoix H, Baron MH. Structural effects of drying and rehydration for enzymes in soils: a kinetics-FTIR analysis of alpha-chymotrypsin adsorbed on montmorillonite. *J Colloid Interface Sci*. 2004;**273**(2):414-425. doi: 10.1016/j.jcis.2004.01.067
 34. Lee C-C, Maestre-Reyna M, Hsu K-C, Wang H-C, Liu C-I, Jeng W-Y, *et al.* Crowning proteins: modulating the protein surface properties using crown ethers. *Angew Chem Int Ed Engl*. 2014;**53**(48):13054-13058. doi: 10.1002/anie.201405664