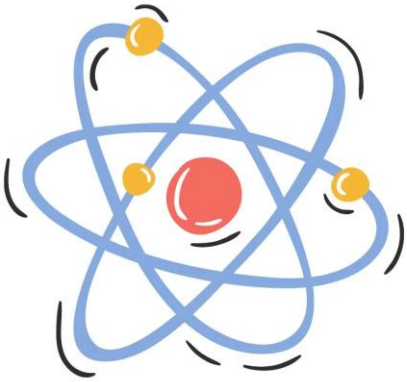

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EVOLUTIONARY DYNAMICS OF PROLIDASE SUBSTRATE SPECIFICITY ACROSS BIOLOGICAL KINGDOMS: A COMPUTATIONAL STRUCTURAL AND DOCKING ANALYSIS

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EVOLUTIONARY DYNAMICS OF PROLIDASE SUBSTRATE SPECIFICITY ACROSS BIOLOGICAL KINGDOMS: A COMPUTATIONAL STRUCTURAL AND DOCKING ANALYSIS

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Abstract: *Prolidase is a metalloprotease which functions primarily by catalysing the hydrolysis of Xaa-Proline (Xaa:any hydrophobic amino acid) and Xaa-Hydroxyproline(Hyp) dipeptides. This enzyme is ubiquitously present across the three biological domains, spanning all kingdoms of life. In the context of prokaryotic organisms, there has been a hypothesis suggesting that prolidase plays a role in nutrient acquisition and functions as a defence mechanism against toxins. However, the precise physiological function of prolidase in unicellular organisms remains yet to be definitively determined. Human prolidase is known to be involved in terminal steps of collagen metabolism. The present investigation was carried for in-silico evaluation of substrate specificity of Prolidase in selected representatives across genera of living systems. The study involved phylogenetic analysis, molecular modelling, structural analysis followed by molecular docking of Prolidase enzyme across genera of biological systems. Literature reports suggest that Glycine-Proline dipeptide is the preferred substrate for Prolidase. We compared the binding efficiency of three ligands viz. Glycine-Proline, Alanine-Proline and Methionine-Proline against Prolidase enzyme from different genera. Molecular docking results revealed in humans the substrate Alanine-Proline and Glycine-Proline had more or less comparable binding energies. Also, it was observed that as we move along the hierarchal system of classification the prolidase of most of the higher organisms were seen to prefer Methionine-Proline as substrate rather than Glycine-Proline. This data may provide a platform to discover role of prolidase in variety of organisms depending upon their binding specificity towards different substrates[1].*

Keywords: *Prolidase, Metalloproteinase, Proline, Protein structure, Phylogenetic tree, Substrate specificity*

1. Introduction

Prolidases, alternatively referred to as Xaa-Proline(Pro) dipeptidases, are a type of metalloproteases responsible for facilitating the enzymatic breakdown of dipeptides. These dipeptides consist of two amino acids, with the C-terminal end specifically containing a Proline residue. Prolidases exert their catalytic activity by facilitating the hydrolysis of these dipeptides. In the realm of proteases, a wide range of enzymes are found, but only a limited number possess the capability to cleave peptide bonds located before a Proline residue [22]. Within the set of 20 standard amino acids, Proline (Pro) possesses a distinctive characteristic. Unlike other amino acids, the side chain of Proline folds back

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onto its own backbone amino group, resulting in the formation of a relatively rigid pyrrolidine ring that is challenging to break down hydrolytically. Moreover, in peptide molecules, Proline exhibits the ability to shield the peptide bond preceding it
from undergoing hydrolysis [4]. Prolidase stands out as the sole identified metalloenzyme found in eukaryotes that facilitate the hydrolysis of dipeptides consisting of a Xaa-Pro sequence (Xaa representing any hydrophobic amino acid). In the case of human prolidase, it exhibits a preference for the dipeptide substrate GlyPro [11]. Nevertheless, it is also capable of utilizing other Xaa-Pro dipeptides, such as those where Xaa is Ala, Met, Phe, Val, or Leu[2,11]. Although the exact physiological function of prolidase in bacteria is yet to be fully understood, the enzyme has been recognized for its protective activity against toxic organophosphates[13, 17, 20]. In addition, it is speculated that the *E. coli* variant of prolidase may serve a similar role to its human counterpart, participating in the breakdown of dipeptides derived from protein catabolism. It is also possible that the *E. coli* prolidase has an additional regulatory function[9]. Supporting this hypothesis, Xaa-Pro peptidases have been identified in *Mycoplasma* species [6,7,8]. These bacteria have evolved to retain only the essential cellular functions required for their parasitic lifestyle, relying on the import of amino acids and lipids from the host cell. The presence of an enzyme capable of cleaving Xaa-Pro bonds in *Mycoplasma* indicates that prolyl peptide catabolism likely plays a broad and significant physiological role. Prolidase in humans primarily functions in the hydrolysis of dipeptides containing Proline (Pro) and hydroxyproline residues. This enzymatic activity occurs during the final stage of collagen breakdown, as well as in the degradation of other proteins derived from both endogenous sources and dietary intake [5]. Prolidases also hold significance in the field of biotechnology. They are commonly employed in the cheese ripening process to mitigate the development of a bitter taste resulting from the presence of dipeptides containing Proline residues[1,3]. Prolidase deficiency (PD) is a rare inherited disorder found in humans, characterized by either the absence or reduced activity of the prolidase enzyme. This condition is considered an orphan disease, with an estimated incidence of 1-2 cases per million births[5,15]. Unfortunately, orphan diseases like PD often attract limited attention from the pharmaceutical industry. The initial elucidation of the crystal structure of prolidase was accomplished using the hyperthermophilic microorganism *Pyrococcus furiosus*[12]. Through structural analysis, it was determined that prolidase shares a similar protein fold to other proteins such as aminopeptidase P (APP), creatinase, and methionine aminopeptidase (MetAP). This fold, known as the pita-bread fold, is characterized by the presence of two structurally analogous domains comprising two α helices and an antiparallel β-sheet. These structural elements combine to form a deep binding cavity resembling the layers of a pita-bread.Within the deep binding cavity of the pita-bread fold, a bimetallic active centre is situated. Notably, the metal-binding site in all the aforementioned proteins displays remarkable similarity, indicating a shared catalytic strategy among them [9]. The present investigation was carried for *in-silico* evaluation of substrate specificity of Prolidase in selected representatives across genera of living systems.

2. Material and methods

2.1 Protein sequence retrieval

A total of 25 prolidase protein sequences from different genera were obtained from protein database of NCBI. These sequences were downloaded in FASTA format for further studies. List of selected organisms with their accession number is given in table 1.

Table 1 Prolidase Protein Identifiers across Diverse Biological Organisms

Organism	Protein ID
Homo sapiens	NP_000276.2
Danio rerio	NP_944594.1

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Thamnophis elegans	XP_032086406.1
Rana temporaria	XP_040185373.1
Callorhinchus milli	XP_007906104.1
Drosophila simulans	XP_016034983.1
Trichinella spiralis	KRY36933.1
Trifolium pratense	PNY11433.1
Oryza brachyantha	XP_006648470.1
Selaginella moellendorffii	XP_024542263.1
Brassica rapa	XP_009128635.1
Thermococcus barophilus	ALM74333.1
Sulfolobus acidocaldarius Ron12/I	AGE73671.1
Methanobacterium formicicum	CEA13130.1
Pyrococcus furiosus	WP_011012489.1
Yasminevirus	VBB18021.1
Lactococcus cremoris	BBC75739.1
Alteromonas	WP_061997083.1
Yersinia	WP_050076893.1
Escherichia coli	EFU60029.1
Mucor ambiguous	GAN05580.1
Alternaria burnsii	XP_038791328.1
Aspergillus tubingensis	XP_035352887.1
Suillus spraguei	KAG2366004.1
Mycoplasma mycoides	BCU84174.1

2.2 Multiple sequence alignment & Phylogeny tree construction

All of the selected prolidase sequences were subjected to multiple sequence alignment using PRALINE [31] in order to obtain information about the conserved residues. A phylogeny tree was constructed using Neighbor-joining method in MEGA11[24].

2.3 Amino acid propensity

Amino acid of composition of all the protein sequences of Prolidase enzyme from different living systems were analyzed using PROTPARAM [25] web server to reveal the distribution of amino acids in different Prolidase.

2.4 Homology modelling, model evaluation and Structural comparison

3D structures of the selectedProlidase proteins were constructed using homology modelling method due to its unavailability at Protein Data Bank. Swiss Model server was used to build 3D structures of the proteins in auto-template mode. Stereochemical quality of predicted protein models was checked using SAVES v6.0 andProSAweb server[33-38]. RMSD of the 3D predicted protein models was calculated by aligning the structures in UCSF-Chimera 1.16 [23]. The binding pockets of the 3D proteins models were predicted using CastP[26] server to compare the difference in binding pocket size throughout evolution of the enzyme.

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2.5 Ligand construction

3D structures of three ligands viz. Glycine-Proline, Alanine-Proline, Methionine-Proline were constructed and structurally optimized using ACD/ChemSketch 2022 [27] 1.0. UCSF-Chimera 1.16 [23]was used to dock –prep the three ligands.

2.6 Molecular docking

The modelled 3D protein structures were minimized using YASARA [28] energy minimization server. Theenergy minimized 3D structures were docked against the ligands viz. Glycine-Proline, Alanine-Proline, Methionine-Proline using AutoDockTools-.1.5.7 [32] to analyse the substrate specificity of different ligands towards prolidase from various organisms. Prior to docking, all water molecules were deleted and polar hydrogens were added. Kollman and Gastegier charges were assigned to the receptors. A total of 100 independent genetic algorithm (GA) runs per ligand with population size 300 were set as fixed parameters fordocking parameter file preparation. The binding energies, inhibition constant, hydrogen-bonding residues and hydrophobic residues of the respective ligands were noted. The 2D interaction maps of docked complexes were generated using LigPlot^{+} v2.2 [29] to determine interacting residues between the ligand and receptor.

3. Results and Discussion

3.1 Multiple sequence alignment and phylogeny tree analysis

A total of 21 residues viz. R295, K298, E302, E354, H390, D432, G434, D443, T445, H536, G539, H540, G543, H457, E590, P591, G592, Y594, G629, R631 and E633 were found to be conserved throughout the life kingdoms. The phylogenetic analysis revealed two major clades in which one clade constituted of archaebacteria alongwith gram +ve bacteria which may indicate their parallel evolution and second clade further bifurcated into two sub-clades (Figure 1). This may be due to high isoleucine content in their protein sequences as revealed by PROTPARAM [25](supplementary sheet 3). One of the two sub-clade consisted of gram-negative bacteria which had low glycine and valine contentand the second sub-clade extended further into two groups. One of the groups contained fungi and the second group had two out-groups (*Yasminevirus sp.*, *Trichinella spiralis)*.Prolidase of all the selected fungi were found to have a common ancestor. Prolidase of *Yasminevirus* and *Trichinella spiralis* were found in between the prolidase of plants and fungi, also they both had low alanine and glycine content. This may be due to host parasite relationship of viruses and nematodes with plants as well as fungi. The second groupcontained two monophyletic groups and two out-groups. One of the monophyletic groups contained *Homo sapiens* and *Rana tempraria* together.While the other monophyletic group contained *Thamnophis elegans* and *Callorhincus* which may be due to their common aquatic habitat. The monophyletic group of animals consisted of two out-groups (*Danio rerio*, *Drosophila simulans)*.Both *Danio rerio* and *Drosophila simulans* were found to have low alanine content as compared to other organisms.

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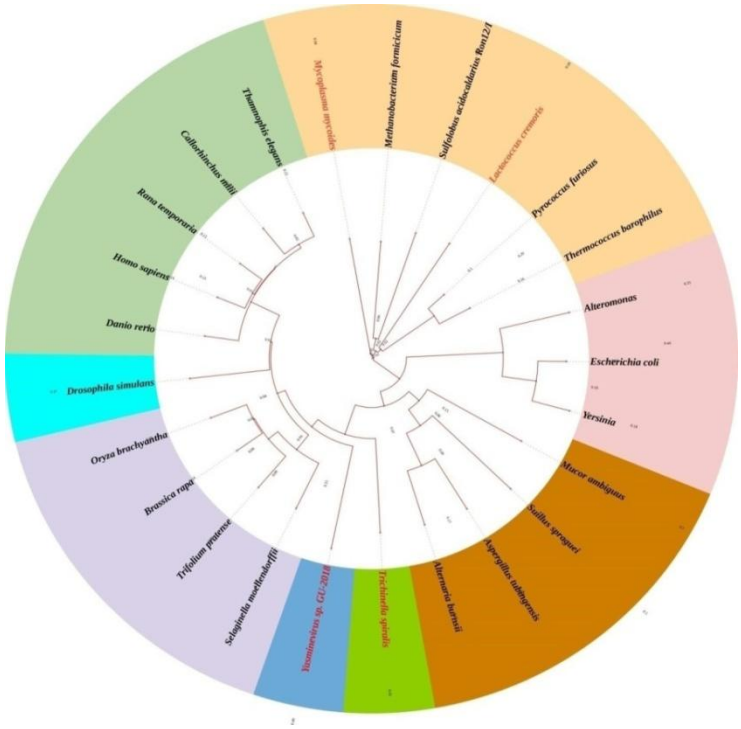

Figure 1: Phylogenetic Tree of Prolidase Enzymes: Evolutionary Relationships across Biological Domains

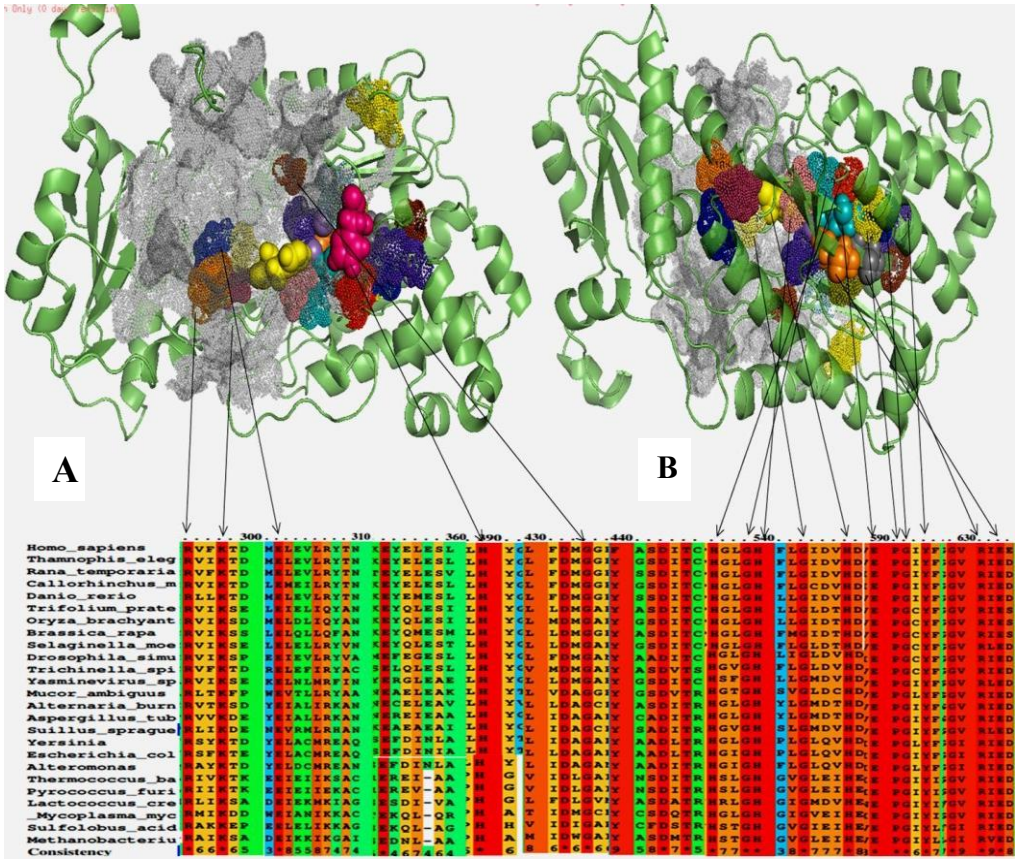

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Figure 2 Conserved Amino Acid Residues shown in Human Prolidase; A-front view, B-back view. Grey surface- Binding pocket,Colourful dots represent the Conserved residues, Colourful spheres represent the Conserved Residues present in Binding Pocket

3.2 Homology modelling and model evaluation

The 3D structures of proteins were modelled using Swiss Model Server. The selected models had sequence identity higher than 30%. The 3D models of prolidase protein were validated using SAVES v6.0 [33-38] and were found to be of good quality for further investigation. The Ramachandran plot showed values within range of 91.0%-93.0% and 0.0% to 0.3% for the most favored and disallowed regions(supplementary sheet 1). The RMSD gave information about the structure deviation of different modelled prolidases. The average RMSD of modelled prolidase was calculated to be 0.8274 Å(supplementary sheet 4). CastP results revealed that the size of binding pocket of Prolidase has increased over the time from Archaea to Eukarya(supplementary sheet 1).Some of the conserved amino acid residues of the enzyme Prolidase were also found in its binding pocket as revealed by CastP (Figure 2). Largest binding pocket was found to be of *Trifolium pretense* whereas smallest binding pocket was found to be of *Mycoplasma mycoides.*

3.3 Molecular docking

Docking studies were carried out for Prolidase protein from different life kingdoms against three ligands viz. Glycine-Proline, Alanine-Proline and Methionine-Proline. The binding energies, and inhibition constant of Prolidase protein against the three selected ligands is given in Table 2 and Table 3. The hydrogen bonding and hydrophobic interacting residues of the docked complexes are given in supplementary sheet 2. The binding energy of prolidase with Glycine-Proline, Alanine-Proline, Methionine-Proline ranged from -4.72kcal/mol to -10.05 kcal/mol,-5.03 kcal/mol to -10.09 kcal/mol and -5.32 kcal/mol to -10.22 kcal/mol respectively. From this data it can be inferred that although Glycine-Proline dipeptide is considered to be the preferred substrate for prolidase but *in-silico* prediction of binding energies revealed that majority of higher organisms have greater binding affinity towards Methionine-Proline rather than Glycine-Proline. This may be due to increase in binding pocket size and difference in interacting residues of Prolidase protein as we move above in the life kingdoms i.e. Archaea to Eukarya.

4. Conclusion

Our research explored the prolidase enzyme across different forms of life using advanced computational methods. By examining the protein sequences and molecular structures of prolidase from various organisms, we gained valuable insights into how this important enzyme works in different biological systems. We found that while the basic structure of prolidase remains consistent across life forms, there are interesting variations in how the enzyme interacts with different substrates.One of the most interesting findings was the way prolidase binds to different amino acid combinations. In humans, we observed that Alanine-Proline and Glycine-Proline can both effectively interact with the enzyme. Interestingly, as we looked at organisms from simpler to more complex life forms, we noticed a shift in substrate preferences. Many higher-level organisms showed a stronger tendency to work with Methionine-Proline, while simpler organisms like archaebacteria and Mycoplasma seemed to work more efficiently with Glycine/Alanine-Proline.Our study identified 21 specific amino acid residues that remain consistent across different life kingdoms, highlighting the fundamental importance of this enzyme. These findings provide a better understanding of how prolidase functions in different organisms and suggest that the enzyme may play various roles depending on the specific biological context. While previous research has primarily focused on role of prolidase in human collagen metabolism, our computational analysis suggests that the enzyme might have diverse functions across different types of life. This research

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opens up new possibilities for understanding enzyme evolution and could potentially guide future investigations into the specific roles of prolidase in different biological systems.

Table 2 Comparative Binding Energies of Dipeptide Substrates to Prolidase across Different Organisms

Organism	Glycine-Proline(kcal/mol)	Alanine-Proline(kcal/mol)	Methionine-Proline(kcal/mol)
Homo sapiens	-7.57	-8.12	-5.88
Danio rerio	-9.39	-9.44	-10.22
Thamnophis elegans	-7.09	-7.7	-7.76
Rana temporaria	-7.86	-8.09	-8.01
Callorhinchus milli	-10.05	-9.81	-10.22
Drosophila simulans	-9.67	-9.61	-9.95
Trichinella spiralis	-5.43	-6.77	-10.02
Trifolium pratense	-9.42	-9.74	-10.02
Oryza brachyantha	-6.77	-7.23	-7.19
Selaginella moellendorffii	-5.74	-6.01	-5.72
Brassica rapa	-9.45	-10.09	-9.9
Mucor ambiguous	-9.4	-9.33	-9.83
Alternaria burnsii	-9.41	-9.41	-8.12
Aspergillus tubingensis	-8.56	-9.34	-8.86
Suillus spraguei	-6.38	-6.54	-7.05
Lactococcus cremoris	-6.28	-5.93	-5.84
Alteromonas	-6.62	-6.61	-6.83
Yersinia	-6.68	-7.12	-6.83
Escherichia coli	-6.13	-6.22	-5.98
Thermococcus barophilus	-6.38	-6.36	-6.28
Sulfolobusacidocaldarius Ron12/I	-8.31	-8.39	-7.94
Methanobacteriumformicicum	-7.16	-6.21	-5.47
Pyrococcus furiosus	-8.99	-9.3	-9.24
Yasminevirus	-6.86	-7.34	-7.51
Mycoplasma mycoides	-4.72	-5.06	-5.32

Table 3 Inhibition Constants of Dipeptide Substrates for Prolidase Enzymes in Various Organisms

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Organism	Glycine-Proline (μM)	Alanine-Proline (μM)	Methionine-Proline (μM)
Homo sapiens	2.82	1.11	48.92
Danio rerio	0.13	0.051	0.032
Thamnophis elegans	6.31	2.27	2.04
Rana temporaria	1.73	1.17	1.35
Callorhinchus milli	0.04266	0.063	0.032
Drosophila simulans	0.08168	0.089	0.051
Trichinella spiralis	104.35	10.83	91.22
Trifolium pratense	0.124	0.072	0.045
Oryza brachyantha	10.93	4.99	5.36
Selaginella moellendorffii	62.41	63.13	64.09
Brassica rapa	0.11896	0.0404	0.055
Mucor ambiguous	0.129	0.144	0.061
Alternaria burnsii	0.127	0.126	1.11
Aspergillus tubingensis	0.533	0.1415	0.322
Suillus spraguei	21.03	16.19	6.74
Lactococcus cremoris	25.08	45.09	52.02
Alteromonas	13.96	14.18	9.83
Yersinia	12.59	6.07	3.34
Escherichia coli	32.33	27.8	41.34
Thermococcus barophilus	21.00	21.64	24.74
Sulfolobusacidocaldarius Ron12/I	0.815	0.709	1.52
Methanobacterium Formicicum	5.64	27.91	98.32
Pyrococcus furiosus	0.255	0.152	0.168
Yasminevirus	9.41	4.19	3.13
Mycoplasma mycoides	345.97	194.96	126.46

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