Computational Evaluation of Designed Phosphatase from Conserved Sequence Scratch for Diverse Substrate Specificity

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Abstract: Background: *The ability to design efficient enzymes for a broad class of different reactions would be of tremendous practical interest in both science and industry. Computer-assisted designing is a novel approach to generating industrial enzymes for biotechnological applications.*
Objectives: *The main aim of this study was to design an enzyme construct with diverse substrate-binding specificity based on the evolutionary conservation of archaeal vanadium-dependent phosphatases.*
Materials and methods: *A rational 3D structural model of enzyme construct was developed from conserved sequence scratch encompassing a vanadium-binding site and functional domain. Substrate-binding specificity of the designed enzyme was computed with different myo-inositol polyphosphate analogous by a molecular docking program.*
Results: *A designed enzyme has shown more substrate-binding specificity with 1D-myo-inositol 3, 4, 5, 6-tetrakisphosphate. Its catalytic function closely resembled myo-inositol polyphosphate-5-phosphatase and multiple inositol polyphosphate phosphatases. Moreover, the enzyme construct was energetically stable with a low degree of conformational changes upon substrate-binding.*
Conclusion: *Substrate specificity and catalytic competence of designed enzymes were computationally evaluated for further biotechnological applications.*

Keywords: *Molecular docking, Phosphatase, Archaea, Phytase, Molecular evolution, Enzyme design.*

Introduction

Computer-assisted enzyme designing has become a subject of major interest and activity in recent years [6, 48]. Several strategies have been employed to design artificial enzymes [1, 44]. Molecular dynamics simulation provides a means to sample configuration phase space, thereby generating an ensemble of structures from which thermodynamic averages may be accumulated [4, 27, 29, 47]. Molecular dynamics simulation of proteins has been instrumental in demonstrating that proteins flux and undergo complex internal motions, which in some cases are directly related to function [32]. It is widely used for the generation of ensembles necessary to calculate protein-ligand relative binding free energy [2, 40], salvation-free energy [22], and activation free energy for enzyme-catalyzed reactions [5, 11, 12, 51]. It is also used to describe a connection between the internal dynamics of enzymes and

their mechanism of catalysis [15, 37, 42]. A protein motion is vital to many enzymes as it helps in binding on substrates and releasing products. However, it is not yet known whether a protein movement accelerates chemical steps in biotransformation processes.

A site-directed mutagenesis is a prominent tool used for enzyme designing and engineering. A point mutation can affect the catalytic rate of many enzymes that have been suggested as an effective measure for enzyme designing [18, 49]. It was used to design a histidine-bearing catalyst for the hydrolysis of *p*-nitrophenyl acetate into *p*-nitrophenol [3]. Triosephosphate isomerase from ribose binding protein scaffold [33] and *de novo* O_2-dependent phenol oxidase from four-helix bundle fold [25] have been designed and developed earlier. An *in silico* benchmark for computational enzyme design has been developed from the recapitulation of locations and structures of native enzyme active sites in a set of naturally occurring enzymatic scaffolds [52]. Besides, the molecular evolution-directed approach is a great concern for designing metalloenzymes of archaebacteria due to the conservation of the metal-binding domain determining the catalytic functions of many archaeal enzymes [24]. A similar approach has been previously applied to design some archaeal metalloenzyme constructs such as β-methylaspartate mutase [7], formyltetrahydrofolate ligase [8], urease [10], sirohydrocholine cobalt chelatase, and coenzyme F_{420} non-reducing hydrogenase [9].

Phosphatase is an enzyme that removes a phosphate group from its substrate. It hydrolyzes phosphoric acid monoesters into a phosphate ion and a molecule with a free hydroxyl group [14]. The catalytic activity of this enzyme depends on the concentration of vanadium ions in archaea [16]. Vanadium-dependent phosphatases can co-ordinate two catalytically essential metal ions within their active sites. A hydroxyl ion bridging two metal ions takes part in the nucleophilic attack on phosphorus ions [16]. The phosphatidic acid phosphatase (PAP2) is a superfamily of phosphatases and haloperoxidases, which may act as a membrane-associated phosphatidic acid phosphatase in eukaryotes. The PAP2 domain is highly conserved within eukaryotes with >70-80% amino acid homology in mammals and plant origins [28, 41]. However, the metal-ligating site is low conserved between plant and mammal PAPs [17]. The sequence-structure-function relationships of many metallophosphatases are evolutionarily related to the metal-binding site of vanadium-dependent phosphatases [50]. Therefore, a vanadium-binding site in the phosphatases has gained attention to design an enzyme construct with diverse substrate specificity.

The common structural features can determine the thermostability of engineered *Escherichia coli* phytase [20, 21, 36, 46]. The site-directed mutagenesis has been used to develop a multiple inositol polyphosphate phosphatase (MuIPPP) from the active site of phytase for utilization of a substrate analogue, *scyllo*-InsP6 (*scyllo*-1D-myo-inositol hexakisphosphate) [13, 38]. It suggests the possibility of designing similar catalysts from small molecular mimics of the enzyme active site. Therefore, the present work was aimed to design an enzyme construct with diverse substrate-binding specificity from archaeal vanadium-dependent phosphatases based on evolutionary conservation of sequences at the active site in the vanadium-binding region.

Materials and methods

Evolutionary conservation analysis

Amino acid sequences of archaeal phosphatase were retrieved from the GenPept of the National Centre for Biotechnology Information (NCBI). Conserved domains architecture in the retrieved sequences was searched by using the NCBI-conserved domain search tool [34]. Metal-binding sites were identified from similar sites present in crystallographic structures

available in the Protein Data Bank (PDB). The selected sequences were aligned and manually inspected for multiple substitutions using the ClustalX 2.0 software [45]. Aligned sequences were iterated at each alignment step and a low-scoring sequence was deleted manually. The phylogenetic tree was constructed with 1000 bootstraps values using MEGA 6.0 software [43] using the Neighbor-joining algorithm.

Molecular modeling and construct designing

ModWeb is an automatic comparative protein modeling server used to build the model [19]. It was used to generate homology models of selected protein sequences based on the structural templates. All side-chains of the resulted models were optimized by ModPipe that consisting of a set of non-redundant chains extracted from crystallographic structures. Functional residues in the protein sequences were predicted with the ProFunc server [30]. The likely biochemical functions of protein models were identified from crystallographic structures whose catalytic domains are similar to the functional regions. Amino acid residues in a metal-binding site that is not covered in the active site and substrate-binding site were omitted from the atomic coordinates of a protein model. Amino acid residues corresponding to selected atomic coordinates were further used to build a homology model by using the Prime program in the Maestro software package (Schrödinger Inc.). The structural quality and accuracy of the final homology model of each enzyme construct were evaluated with Structural Analysis and Verification Server (SAVS), http://nihserver.mbi.ucla.edu/SAVES/. Amino acid residues that are deviated were identified by structural superimposition using the DaliLite Pairwise Alignment Tool, http://ekhidna.biocenter.helsinki.fi/dali_lite/start.

Molecular dynamics simulation of enzyme constructs

A standard dynamic simulation cascade module in the Discovery Studio software (Accelys, Inc) was used to obtain structural conformers of the enzyme constructs. The CHARMM force field and steepest descent as well as an adopted basis Newton-Raphson algorithms were used for molecular dynamic simulations. A distance constraint was assigned between N-terminal to the C-terminal of each model. A dihedral restraint was started from C to Cα (Φ) of first amino acid residue and Cα to N (ψ) of second amino acid residue until the last amino acid residue in a molecular dynamic ensemble. After molecular dynamic simulations, 30 structural conformers were generated and the top 5 lower energy conformers were selected for further molecular docking studies.

Molecular docking studies

The structures of MIP (1D-*myo*-Inositol 1-phosphate)-derived substrates were retrieved from the KEGG database using the SIMCOM software (www.genome.jp/tools/simcomp). All structures were obtained in mol2 files and then converted to the pdb format. The genetic algorithm and AMBER force field were selected to perform docking simulation of each substrate into enzyme construct using Autodock software 4.2 [35]. A construct was fixed as rigid while the substrate was set as flexible in the docking process. The Ligand Fit program was used to predict the binding pocket of the enzyme construct. An energy grid was selected around the binding cavity of the construct. After obtaining good docked models, minimization was performed with a smart energy minimization algorithm to refine the orientation of the substrate in the binding site of the enzyme construct.

Results and discussion

Analysis of molecular conservation of PAP2

We conducted a simple text mining approach for the identification of metal-dependent archaeal phosphatases entries in the GenPept of NCBI. It indicates that there are several sequence entries found for metal-dependent archaeal phosphatases, but no crystallographic structures are available for them (Table 1). We identified the conserved domain of all entries and then constructed a phylogenetic tree as shown in Fig. 1. It shows that the archaeal PAPs domains selected for the designed enzyme construct are related to the PAPs domains of *Danio rerio* and *Homo sapiens*. It has also shown a domain similarity to phospholipid phosphatases of eukaryotes with a significant bootstrapping value of 993.

Table 1. List of archaeal vanadium-dependent phosphatases selected for designing enzyme constructs

Construct	Molecular function	Organism	Length	Accession
1	Membrane-associated phosphatase	*Thermococcus onnurineus* NA1	216	YP_002307194
2	Phospholipid phosphatase	*Cenarchaeum symbiosum* A	208	YP_876319
3	Phospholipid phosphatase	*Methanopyrus kandleri* AV19	201	NP_614474
4	Dolicholpyrophosphatase	*Sulfolobus acidocaldarius* DSM 639	206	P80143
5	Undecaprenyl-diphosphatase	*Metallosphaera sedula* DSM 5348	203	YP_001190526
6	PAP2 family phosphatase	*Thermococcus kodakarensis* KOD1	213	YP_183101
7	Glucose-6-phosphatase	*Haloarcula marismortui* ATCC 43049	186	YP_137675
8	Type II phosphatidic acid phosphatase	*Pyrococcus furiosus* DSM 3638	191	NP_577769

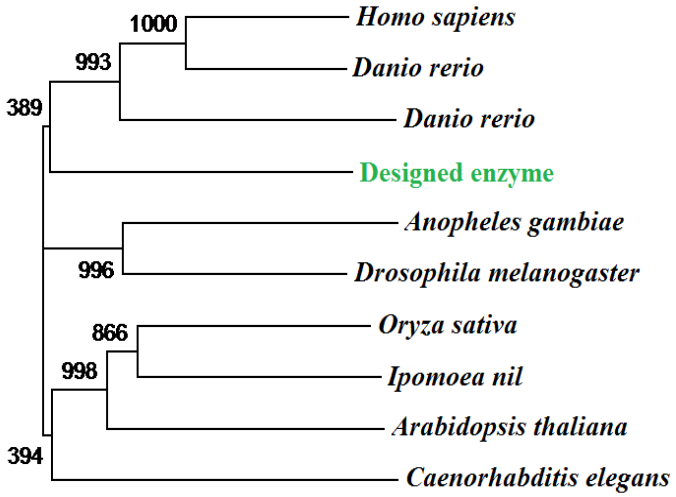


Fig. 1 Phylogenetic inference of designed enzyme based on the PAPs domain sequences of distantly related organisms

Sequence similarity analysis demonstrates that archaeal phosphatases show a 33-42% sequence identity to the template structures in which *Thermococcus onnurineus* NA1 phosphatase has shown the shortest metal-binding site encompassing functional site (Table 2).

Table 2. Homology modeling for predicting
the 3D structure of archaeal vanadium-dependent phosphatases

Construct	Structural template	Sequence identity, (%)	Modeled position	Modeling score	MPQS	Z-Dope
1	1D2T	42	121-183	0.19	0.30	2.32
2	2IPB	41	125-189	0.16	0.37	1.50
3	2IPB	40	32-81	0.03	0.33	2.35
4	1QI9	41	100-192	0.31	0.29	1.69
5	1D2T	40	121-178	0.22	0.58	1.13
6	2E77	49	170-208	0.05	0.22	2.82
7	1D2T	33	26-161	0.26	0.77	1.16
8	2IPB	33	119-185	0.24	0.55	0.79

As shown in Fig. 2, the enzyme construct comprises a metal-binding domain at the positions of 90-180 amino acids. Conserved domain similarity analysis shows that it has a functional domain similar to the PAP2_like superfamily (CD03391). Ala123, Asp124, and Tyr125 are active sites identified similar to those found in crystallographic structure (PDB ID: 1D2T) with a conservation score of 5.578 (Table 3). A model showing low structural quality and functional sites are located beyond the selected metal-binding domain that has been neglected in this study.

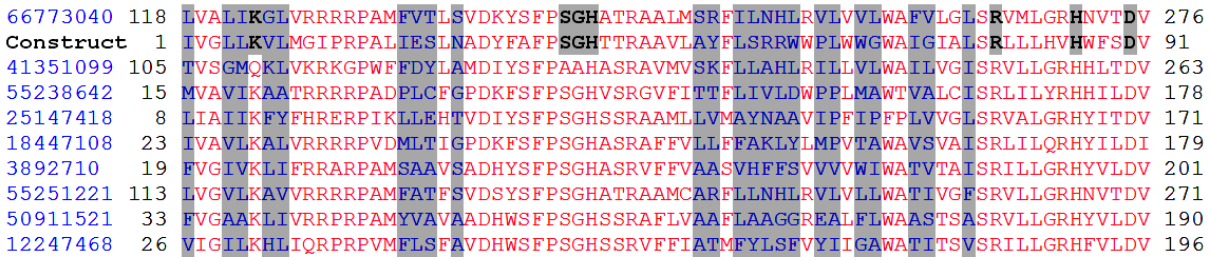


Fig. 2 Functional features of a designed enzyme identified by multiple sequence alignment of closely related domains sequences (shaded regions are showing the highly variable amino acids and bolded regions are functional amino acids)

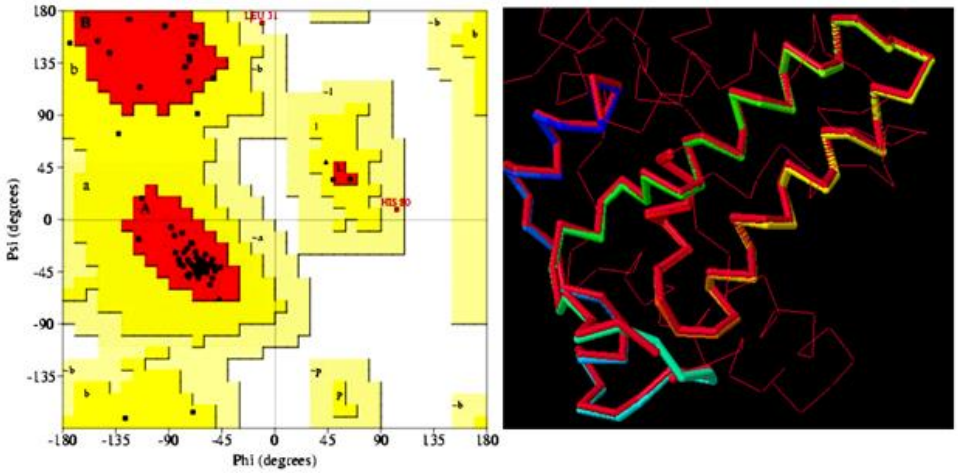
Structural quality and accuracy of the enzyme construct

We selected acid phosphatase (PDB ID: 1D2T: A; 1.90A) as a suitable structural template for modeling the 3D structure of a construct from its sequence. The structural quality and accuracy of the modeled enzyme construct were assessed by several programs (Fig. 3). We predicted the molecular energy and root mean square deviation (RMSD) of modeled structure is -2369.21 KJ/mol and 0.4, respectively. The ERRAT program computed its structural quality with 80% accuracy. The PROVE program calculated its structural accuracy as Z-score RMS 1.454. Verify3d program also proved that 16.85% of the residues show an averaged 3D-1D score greater than 0.2. Ramachandran plot represents 7.9% allowed and 2.6% generally allowed regions found in modeled enzyme construct. We found a good

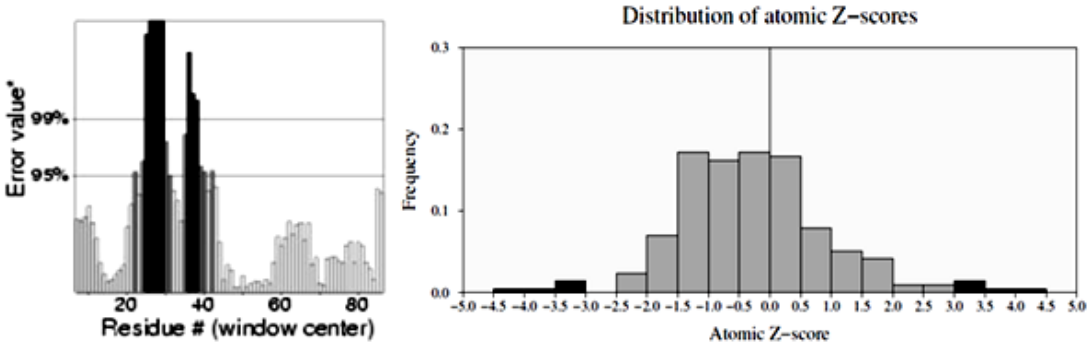
structural alignment between construct and a crystal structure (PDB ID: 1D2T). The modeled enzyme construct consists of 29 H-bonds donors, 3 helixes, 70 turns, and no strands.

Table 3. Vanadium-binding and active site similarity regions identified in archaeal vanadium-dependent phosphatases

Construct	Structural template	Metal-binding site	Active site	Score
1	1D2T	90-180	Ala123, Asp124, Tyr125	5.578
2	2IPB	90-180	Leu172, Val173, Glu174,His175	1.677
3	2IPB	90-180	Leu162, Val163, Glu164,His165	4.872
4	1QI9	95-175	Val166, Gly167, Val168, His169	4.822
5	1D2T	95-175	Val163, Gly164, Val165, His166	2.938
6	2E77	85-170	Leu176, Gly177, Ser178	2.507
7	1D2T	105-155	Thr104, Ser105, Phe106	4.439
8	2IPB	85-170	Leu160, His161, Val162,His163	1.651



a) Ramachandran plot b) structural superimposition



c) Errat2 d) PROVE

e) sequence alignment

Fig. 3 Structure validation and quality analysis of the designed enzyme construct

The conformational stability of this construct was further evaluated by molecular dynamic simulation (Table 4). It shows that the top-five conformations of this construct are stable in torsion and total molecular energies (-1126 kcal/mol). Overall structural quality assessments implied that the enzyme construct derived from *T. onnurineus* NA1 is structurally stable with low conformational flexibility.

Table 4. Molecular dynamic simulation data for top-five lower energy conformers of enzyme construct (All of the molecular energies are expressed as kcal/mol)

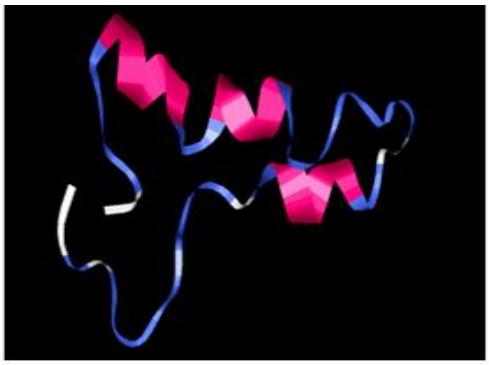
Conformer	Total energy	van der Waals energy	Electrostatic energy	Torsion energy	Temperature, K
1	-1126.66	-311.69	-1303.54	151.23	303.87
2	-1126.40	-294.53	-1283.63	139.21	302.05
3	-1126.28	-319.58	-1282.69	151.70	304.11
4	-1126.17	-315.37	-1358.73	154.05	301.93
5	-1125.97	-286.04	-1282.31	141.42	303.16

The structure of the enzyme construct is more reliable than the near-native conformation. The stability of its conformation may be consecutively maintained with molecular motions by acting van der Waals interactions and electrostatic forces. Moreover, internal molecular forces could not distort the stability of the construct-substrate complex as a strong molecular interaction measured at the functional site. Significant stability of its complex and catalytic function may be retained by aromatic stacking similar to earlier investigations on xylanase [32] and α-amylase [2].

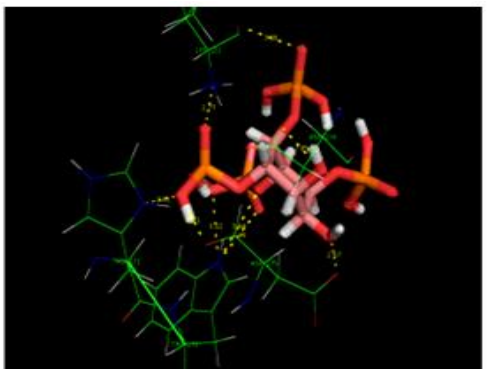
Binding site mapping and interaction analysis

We evaluated the possible molecular interactions of this enzyme construct with substrates. We used different substrates for docking studies. They are MI6P (1D-*myo*-Inositol 1,2,3,4,5,6-hexakisphosphate or phytic acid), MI4P (1D-*myo*-inositol 3,4,5,6-tetrakisphosphate) and MI3P (1D-*myo*-inositol 1,3, 4-trisphosphate). The best docking models were selected based on the molecular interaction types and binding affinity of the substrate to construct. Molecular docking studies indicate that the binding energy of the construct-substrate complex ranges from -5.73 to -1.28 kcal/mol. We obtained a suitable docked model when MI4P was docked into the construct. It has 5.09 kcal/mol intermolecular energy and -4.03 kcal/mol internal energy. There are nine H-bonds contributed between the carbonyl group of MI4P and side chains of Lys121, Asp124, His171, Trp172, and Asp175 of the construct. All possible interaction sites and distances between individual atoms of MI4P and binding residues of the construct are O6-Lys121 (3.28Å), O14-Lys121 (2.77Å), H10-Asp124 (2.85 Å), O16-His171 (2.81 Å), O17-Asp175 (2.67 Å), H8-Asp175 (2.61Å), O4-Trp172 (3.02Å), O3-Trp172 (3.46Å) and O1-Trp172 (3.44Å).

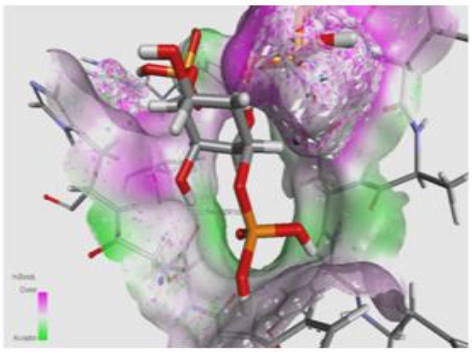
Fig. 4 presents different molecular graphical representations. Fig. 4a shows the enzyme construct in ribbon view, Fig. 4b – the enzyme interactive view with MI4P, Fig. 4c – the electrostatic potential view with MI4P, while Fig. 4d – a hydrogen-bonding pattern. Fig. 4e demonstrates the predicted binding energies of different substrates (MI6P, MI4P and MI63) into the enzyme construct.



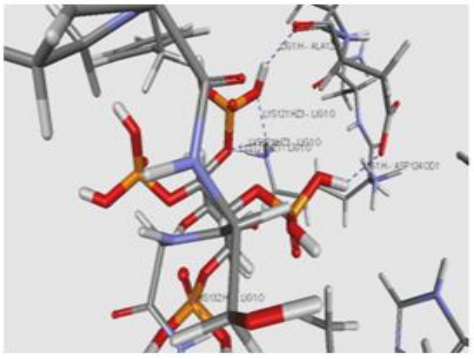
a) molecular view



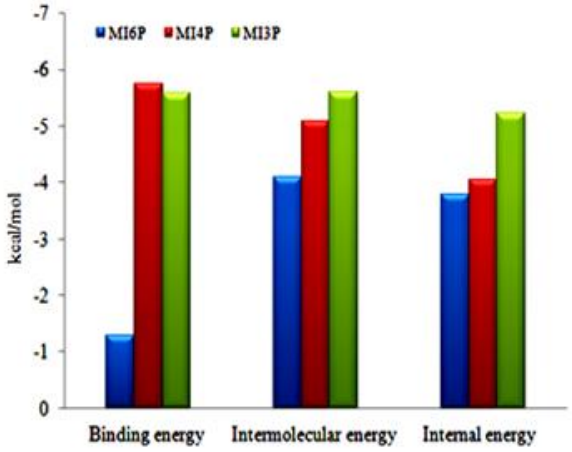
b) docking view with MI4P



c) electrostatic potential view with MI4P



d) H-bonding pattern



e) binding energies prediction

Fig. 4 Molecular graphical representations.
The yellow/blue dot lines denote the hydrogen bonds.
All the amino acid residues are shown inline drawing and colored
by residue types in which hydrogen is colored white,
carbon green, oxygen red, nitrogen blue, and sulfur orange.
Ligand is shown in a stick in which carbon is colored tints,
hydrogen gray, nitrogen blue, and sulfur orange.
All the interaction distances are represented as RMSD and expressed in Å.

A proper transition state stabilization is a key challenge to designing an enzyme with native activity [12, 22]. Transition state mimics of our construct may be induced by forming covalent bonds between vanadium ions and a variety of ligands [32]. Moreover, our approach was simply based on the evolutionary conservation of its vanadium-binding center, suggesting

the probability of transition state stabilization during catalytic reactions. Several molecular modeling and docking studies have been conducted to investigate the structure, function, and catalytic mechanisms of some hypothetical proteins from plant and pathogenic bacteria [23, 26, 31, 37, 39]. Molecular docking studies of this work indicated that the construct was catalytically suitable to convert MI3P and MI6P. Asp124, Trp172, and Asp175 are the primary covalent attachment site for MI6P and MI4P. The construct was more suited for MI4P than MI3P and MI6P due to a strong binding affinity calculated for MI4P. Hence, molecular interaction data is supported to describe its catalytic efficiency on different substrates as similar to earlier work [5].

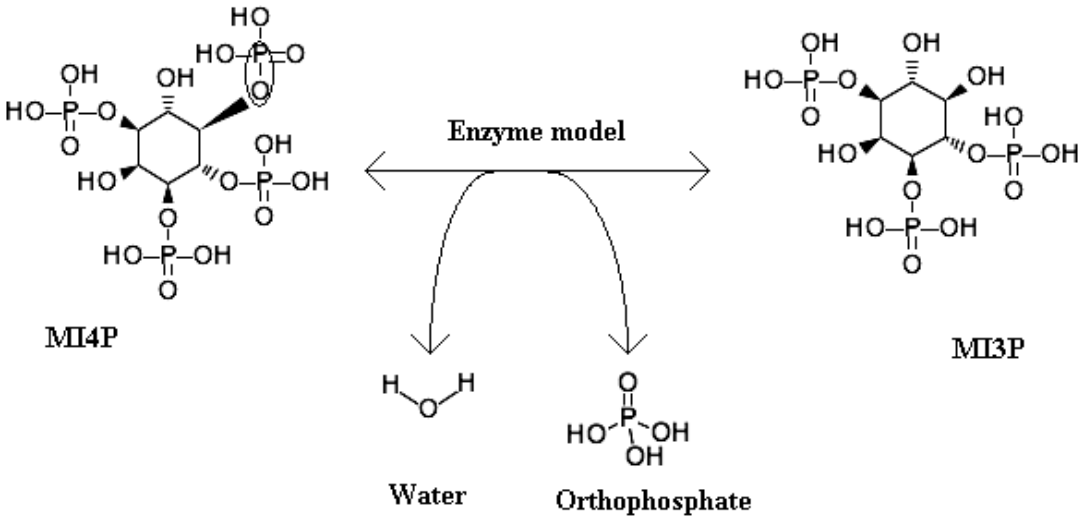


Fig. 5 Proposed chemical reaction catalyzed by a designed phytase construct

Our enzyme construct may remove orthophosphates from MI6P, MI4P, and MI3P and finally produce MIP similar to the catalytic function of MIPPP (*myo*-inositol polyphosphate-5-phosphatase; EC 3.1.3.56) and MuIPPP (EC 3.1.3.62). As shown in Fig. 5, a proposed chemical reaction catalyzed by a designed phytase construct is represented.

The proposed enzyme construct has diverse substrate-binding specificity, which is closely related to *myo*-inositol polyphosphate-5-phosphatase and multiple inositol polyphosphate phosphatases. It could act on MI4P and then convert to MI3P by removing the phosphate group. The analogous enzymes are present in the inositol polyphosphate pathway [21], and the active structure of phytase (MI6Pase) is evolutionarily conserved [15, 42, 51]. Enzymes from thermophilic archaea are more conserved than other archaeal enzymes even at the sequence level [6]. Since, the designed enzyme is a good candidate for catalytic function on a wide range of substrates by earlier works [2, 12, 15, 18, 22, 37, 42, 49].

Conclusion

The molecular evolution-directed approach is a reliable one for designing enzyme construct with diverse substrate-binding specificity, particularly with MI4P. Enzyme construct derived from sequences of vanadium-dependent phosphatases closely resembles *myo*-inositol polyphosphate-5-phosphatase and multiple inositol polyphosphate phosphatases. Considering its substrate specificity and catalytic competence, the designed enzyme would replace native phytase and MIPPP enzymes in the agriculture and food industry and treat environmental hazards. A degree of evolutionary conservation in the catalytic domain is a very decisive discernment for the catalytic competence and substrate-specificity of any designed industrial

enzymes. The present approach would provide an insight to design further experimental set-up for industrial scale-up of synthetic enzymes using enzyme engineering.

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