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Modification and Characterization of Phytase for Animal Feed Production

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Abstract: Phytases catalyze the hydrolysis of inorganic phosphate from phytic acid and are able to improve the nutritional quality of phytate rich diet. Monogastric animal such as poultry and fish have lack of significant activity to hydrolyze phytate that contribute to elimination of beneficial nutrient for growth therefore contribute to land pollution, eutrophication of ground water and aquatic environment. Besides, it leads to the negative effect on vitamin utilization that lead to the emaciation, retarded growth and reproductive failure to animal. Due to the importance of, microbial sources for the commercial production of phytases, we have selected waste water bacterium phytase as the subject of interest in this study. *In silico* experiment is used to identify and examine the active site of waste water bacterium phytase. The factors influencing the ligand binding strength in the active site is analyzed and computational site directed mutagenesis experiments were carried out to evaluate the effects of mutations on the binding strength. Multiple mutations of M216R/E219R/H17A, M216R/E219R/F254E and some other multiple mutations showed improvement in the binding strength, primarily due to the addition of hydrogen bond with the adjacent residues. Automated docking based on genetic algorithm is used to dock the phytate in the active site and Partial Mean Force (PMF) scoring is used to calculate the strength of the binding before and after mutation.

Key words: Phytase, *In silico*, site directed mutagenesis, ligand binding

INTRODUCTION

Phytase (EC 3.1.3.8) (myo-inositol hexakisphosphate phosphohydrolase) catalyze phosphate monoester hydrolysis of phytic acid in a step wise manner into the myo-inositol and organic phosphate is a type of histidine acid phosphatase (Kim and Lei, 2008). Phytic acid, which exists as phytate that is complexes with cations such as Ca^{2+} , Zn^{2+} , Fe^{2+} is the major storage form of phosphorous in plant seed and have important roles for nutrition. Animals such as swine and poultry that have lack of significant catalytic activity to hydrolyze phytate which most of the phytate will be excrete to the environment thus polluted the land (Boyce and Walsh, 2007). In addition, phytic acid is negatively charge, therefore it can strongly chelate with cation such as Ca, Mg and Zn, to form insoluble salt and inhibit several enzyme such as amylase, tripsin and acid phosphatase, thus influencing the absorption and digestion of this mineral by animal and reducing the bio-availability (Harland and Moris, 1995). Therefore, to improve the phosphorus absorption and reduce the phosphorus excretion, enzymes are added to

animal diet. Previously, costly inorganic phosphorous had been added to the animal feed to reduce the negative effect of phytate to the environment and animal nutrition (Lim, 1999; Lim *et al.*, 2000). Recent study as reported by Lim (1999) shows that the inclusion of phytase in feedstuff provides an economic means to boost animal production and reduces anti-nutritional effects of phytate that caused the elimination of phosphate supplement as well as reduced of phosphorous content of animal manure. Latest report shows microbial phytase are most promising for a biotechnological application due to some properties such as substrate specificity, resistance to proteolysis and catalytic efficiency.

Bacterial phytase such as *Pseudomonas* sp., *Bacillus* sp., *Raoultella* sp., *Escherichia coli*, *Citrobacter braaki*, *Enterobacter* and anaerobic ruminant bacteria are the best alternative to produce the enzyme (Konietzny and Greiner, 2004). *Escherichia coli*, has the promising value for industrial application due the high phytase specific activity and its resistance to the proteolytic degradation such as pepsin digestion (Greiner *et al.*, 1993). The crystal structure of *E. coli* phytase share a very similar

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overall structure with rat prostatic and *Aspergillus niger* (Lim *et al.*, 2000), which consist two different domain that separated by a large cavity. The lower domain contains a central β -sheet of mixed topology with two α -helices on each side (Lim *et al.*, 2000) and α -domain of *E. coli* phytase define the substrate binding area with a number of helices and loop packed around them consists of two central α -helices as well as a unique β -hairpin structure facing the circumference of the binding pocket opposite the active site (Lim, 1999).

Computational protein design starts with the coordinates of a protein main chain and uses a force field to identify sequences and geometries of amino acids that are optimal for stabilizing the backbone geometry (Trevor, 2001). The development of powerful search algorithms to find optimal solution has provided a major stimulus to the field (Goldberg, 1989). Studies correlating predicted and experimental stability have been used to iteratively improve empirical force field based protein design calculations (Gordon *et al.*, 1999). The combination of predictive force field and fast search algorithm are now being applied to functional enzyme design (Bolon, 2002). The engineering of a completely new function into an inert protein scaffold is likely to require multiple mutations where individual mutation may have no effect (Bolon, 2002). Computational design benefits from the ability to search a large space than possible by purely experimental methods and is well suited to designing novel functions (Bolon, 2002). In the present study, we use computational design approaches to examine and improve the binding strength of phytase isolated from *E. coli*.

MATERIALS AND METHODS

Structure: The *E. coli* phytase crystal structure complex with phytate (Lim *et al.*, 2000) at pH 4.5 was obtained from the database (1DKQ). This structure has mutations at position H17A and A116T of the sequence. Side chain of H17 play a role as the site for nucleophilic attack initiated by hydrogen bonding interaction with the carbonyl oxygen of Gly 18. Therefore, the H17A mutation does not improve the binding of the substrate to the active site (Lim *et al.*, 2000). The wild structure of the enzyme was obtained by reversing the mutation by substituting back the original amino acid residue at the mutated position. Bonding in hetero groups, valences and atom hybridization were checked before the calculation is initiated. The structure is then optimized using Molecular Mechanic (MM3) method until it reached convergence of 0.001 kcal mol⁻¹.

Mutant modeling: Two set of double and three set of triple mutation were introduced in wild type of protein-

ligand complex crystal structure of *E. coli* phytase. Selections of mutation process were done randomly. The selected amino acid residue is replaced with the new amino acid that is expected to increase hydrogen bond interaction of ligand with the active side residue. Each mutated structure will be optimized until it reaches the energy convergence threshold of 0.001 kcal mol⁻¹.

Automatic docking and scoring: It is assumed that bonds are not formed between the ligand and protein because bond formation would cause changes in the atom types and possible substantial changes in the shape of the ligand and active sites. Hence, docking model used in this study assumes that the protein and ligand bind non-covalently. Ligand and active site is set to be both flexible. Automated docking method based on genetic algorithm, is used to dock the phytate in the active site and Partial Mean Force (PMF) scoring is used to calculate the strength of the binding before and after mutation. The docking process is simulated at least three times and at the average score is reported here.

Structural analysis: The mutated models were compared with the wild type structure for various structural analysis. Hydrogen bonding, surface accessibilities and side chain conformation were analyzed, respectively.

RESULTS

Structure: The *E. coli* phytase crystal structure complexed with phytate obtained from the database has mutation at position H17A and A116T of the sequence. The wild type of *E. coli* phytase structure without the bound ligand is available (1DKL). This structure is used in our studies for the checking the structure of wild type enzyme obtained by reverse mutation of H17A and A116T (1DKQ). Active site was defined by selecting the neighboring residue within 7Å radius from the ligand. There are few residues that are in contact with the ligand which are shown in the picture and table below. This is the region that can accommodate the binding of ligand with various non-covalent interactions to form a low energy (stable) enzyme-ligand complex (Fig. 1).

Mutant modeling: In this study, we have investigated the effect of double and triple mutation on the binding strength of the enzyme. Residues in the active site are considered as the leading candidate for the mutation studies. *Escherichia coli* phytase1DKQ structure used has mutation at position 17 and 116 with H17A and A116T, respectively. The wild type structure was obtained by reversing the mutation to the original enzyme sequence and the optimum structure was found. In this

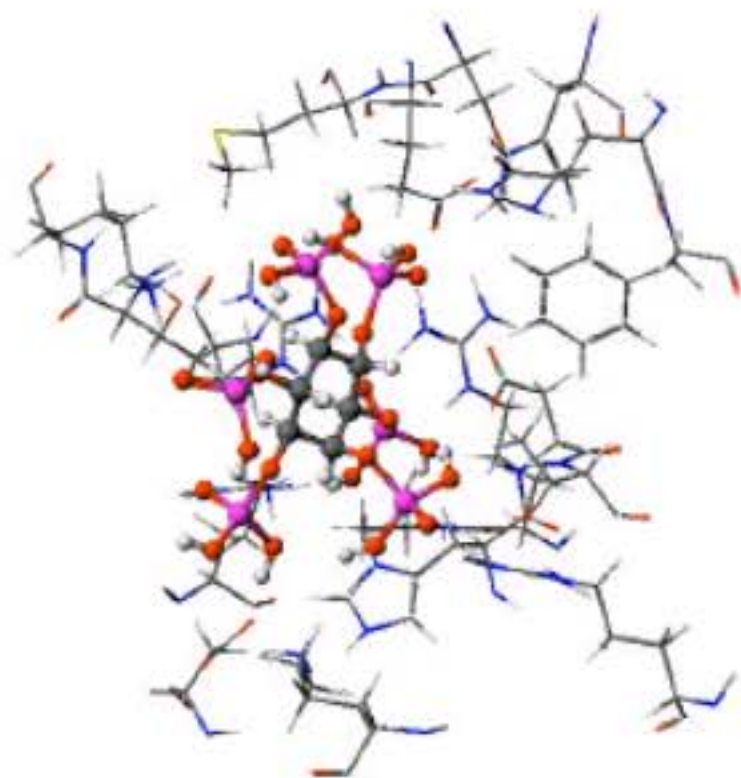


Fig. 1: Ligand and residue in contact at the active site

study, we select few point mutations that give better binding strength of ligand from our previous single point mutation study (Noorbacha *et al.*, 2008). From this study, mutation at residue number of 216 from methionine to arginine and at position 219 from glutamic acid to arginine decreases the docking score by about $-20.00 \text{ kcal mol}^{-1}$. The score indicates the favorability of the ligand to bind to the active site region (Table 1). More negative score implies better binding. These results suggest that the structure with the mutation is better than the original structure. The scores suggest that the ligand bind stronger to the structure with the mutation compared to the structure before mutation. Thus, we conclude that according to the score, all of the introduced mutated structures M216R/E219R/H17A, M216R/E219R/F254E, K43E/K75M/S187G, E219R/M216R and E219R/F254R have improved in term of the binding and suggest that the ligand interact better with the residues in the active site region. In this study, we combine the mutation that are expected to give good binding strength by enhancing hydrogen binding, accessible surface and interactive with the crevice surface due to mutual hydrophobic/hydrophilic interactions. Below in the Table 1 that list three set of triple mutations, double mutations and the wild type structure so that improvement in the binding score and the additional and reduction of the number of hydrogen bond can be compared.

From the result in Table 2, it shows that the binding score for mutation at M216R and E219R that are expected to give good result and the new mutation also being introduce in the combination with the tested mutation such as mutation at H17A and F254E that give good binding score with decreasing in the binding score approximately $-20 \text{ kcal mol}^{-1}$. All of the mutations were

Table 1: Residues in contact with ligand and its distance

Residue No.	Residue name	Distance (Å)
16A	ARG*	2.7
17A	ALA*	4.7
20A	ARG*	2.6
23A	THR*	2.5
24A	LYS*	3.0
90A	ASP*	3.5
92A	ARG*	2.7
129A	LYS*	4.4
215A	SER*	5.1
216A	MET*	2.9
219A	GLU*	3.5
250A	HIS*	3.4
253A	GLN*	5.4
254A	PHE*	3.4
267A	ARG*	3.1
303A	HIS*	2.6
304A	ASP*	2.6
305A	THR*	2.6

*Indicates residue contact with ligand by their side chain

Table 2: Binding score and number of hydrogen bond between ligand and contact residue

Structures	Hydrogen bond	Score (kcal mol^{-1})
Wild type	57	-202.611 ± 4.73
M216R/E219R/H17A	59	-236.373 ± 9.65
M216R/E219R/F254E	61	-220.681 ± 11.33
K43E/K75M/S187G	57	-218.601 ± 5.92
E219R/M216R	60	-234.950 ± 9.27
E219R/F254R	57	-238.874 ± 14.55

Table 3: Accessible surface of structure before and after mutation

Structures	Wild type (Å) ²	Mutated (Å) ²
M216R/E219R/H17A	723.354	804.568
M216R/E219R/F254E	809.201	893.854
E219R/M216R	684.494	704.183
E219R/F254R	688.642	719.938
K43E/K75M/S187G	9313.828	9223.466

done in the active site region except for the mutation at position K43E/K75M/S187G which the residue is not in the active site region. According to the study done by Kim and Lei (2008), which they tested these mutation experimentally in the lab, they found that mutation at position K43E/K75M/S187G give increment in catalytic activity and approximately 20% improvement in thermostability at 80°C for 10 min. Theoretically, the design mutation gives slightly increase in binding score which is from -202.611 to $-207.117 \text{ kcal mol}^{-1}$. Besides docking score as the indicator to the ligand binding strength, the number of additional of hydrogen bond between the ligand and residues before and after mutation also can be analyze as one of the factor that contribute to the better ligand binding. Figure 2 shows the hydrogen bond for mutation at position M216R/E219R/F254E before and after mutation (Table 3).

Accessible surface: Accessible surface is the surface of a protein that could be touched by a 1.4 Å spheres which is approximately the size of a single water molecule. In this

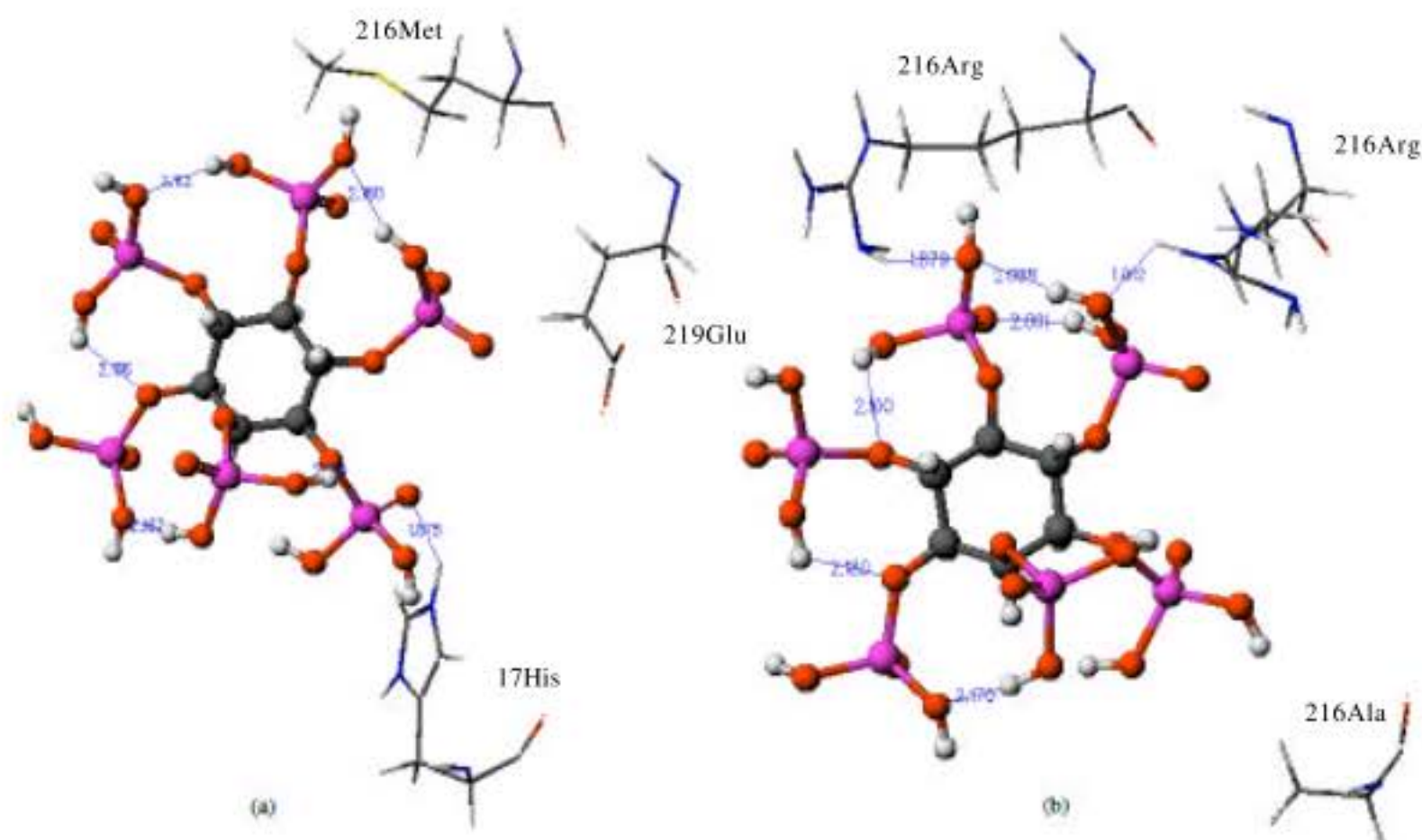


Fig. 2: Hydrogen bond (a) before mutation and (b) after mutation at M216R/E219R/H17A

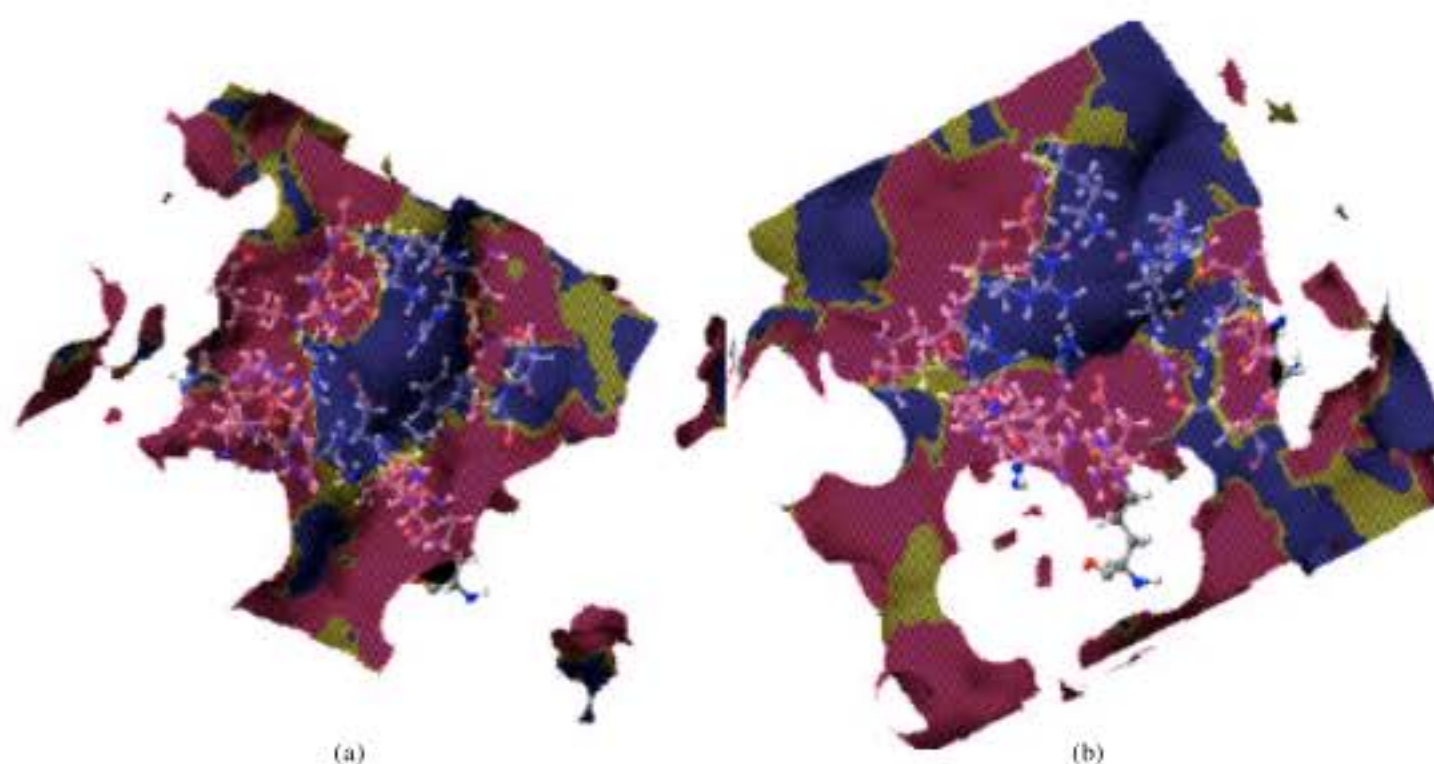


Fig. 3: Accessible surface of M216R/E219R/F254E mutation (a) before and (b) after mutation

study, the accessible surface is study in the region of mutation. From the result, most of mutation gives improvement in the surface area in the region of mutation (Table 3).

As an example, the accessible surface area of M216R/E219R/F254E mutation is shown in the Fig. 3. There are several regions that are shown in Fig. 3, the hydrophilic area that acts as hydrogen acceptor is shown in purple, whereas a blue color indicates a hydrophilic region that act as hydrogen donors and on the other hand a cream colored area represents the hydrophobic region. The increase in the accessible surface area after mutation can be clearly seen from Fig. 3. The enlargement of the

blue shaded area shows that hydrogen acceptors are increasing. The increase in the total accessible surface of the mutated enzyme suggest that interaction between ligand and active site in the mutated enzyme will be stronger, because it offers more possibilities for the stronger interaction to take place.

DISCUSSION

Compared with the wild type structure, all five introduced mutations showed enhancement in the docking score which will result in improvement in binding strength and may also improve the catalytic activity of the

mutated enzyme. The idea of introducing the mutation to the active site residues is to obtain a significant change in the binding interactions as it is well known that the residues in the active site play significant role in ligand binding and/or catalysis.

The M216R/E219R/H17A mutations showed enhancement in ligand binding by improvement of $-33.763 \text{ kcal mol}^{-1}$ binding energy. Methionine, which is at position 216 has a nonpolar amino acid side chain with four carbons and one sulfur atom, has been substituted with arginine which has a basic side group. The positive charge and polar properties of arginine make it very hydrophilic, contributing to hydrophilic region in the accessible surface (colored blue in the Fig. 3). This hydrophilic region has hydrogen donor characteristic due to the polar amide group. The positive charge makes the residue susceptible to interact with the ligand which is negatively charged due to the electrostatic interaction. From the structure analysis, it is shown that the hydrogen bond is formed between the amide group of arginine and one of the phosphorous atom in the ligand.

Another mutation is at position 219 where glutamic acid is substituted with arginine. In this substitution, both amino acid residues are polar but the original glutamate is negatively charged. Before mutation there is no hydrogen bond formed with any of the ligand atoms. After E219R mutation, the presence of a positively charged functional group in arginine introduces one hydrogen bond which binds to one of the negatively charged phytate atoms. Therefore the effect might be the same as the mutation of M126R, provided there is no major effect on the conformation due to the slightly bulky side chain of arginine compared to glutamic acid which provides less conformational flexibility toward substrate binding. Mutation from histidine at position 17 to alanine, is reported by Lim *et al.* (2000). Mutation at position 17 will lower the catalytic activity because histidine acts as a nucleophile in the phytase reaction mechanism. Substitution of the histidine to alanine also gives more conformational flexibility towards substrate binding due to the removal of the bulky side chain of histidine, which may thereby improve catalytic activity. The combined effects of the three mutations resulted in an increase in the ligand binding which will result in the improvement in catalytic activity of the enzyme.

Mutation at M216R/E219R/F254E is just the repetition of the two previous mutations with one newly introduced mutant residue which is at position 254 of the structure. At this position, phenylalanine is substituted with glutamate. This transition is from a non-polar residue to a negatively charged polar residue. This will result in the increase in the hydrophilic region in the accessible

surface area as shown in Fig. 3, which offers better possibilities of interaction between the active site residues and the ligand. Besides, glutamate also reduces the bulky conformation from the benzene ring of the phenylalanine side chain that increases the area of

Mutations at M216R/E219R and E219R/F254E are the combined version of the triple mutation, so that the role of the interaction and the catalytic activity is the same.

Kim and Lei (2008) has reported that the mutations K65E/K97M/S219G resulted in an increase of phytase activity. The data obtained from the current computational studies is consistent with this observation. It can be seen from Table 2, the docking score increased by about 16 kcal mol^{-1} for this mutated enzyme, suggesting the possibility of better catalytic activity of the newly introduced mutation. This also can be used as validation to our study, since this mutated structure had been tested experimentally and the result can be supported by a theoretical-computational study. Mutation at K65E adds three new hydrogen bonds to the neighboring residues and the removal of a bulky side chain in K65E provides more conformational flexibility towards substrate binding (Kim and Lei, 2008). The other two mutations might improve interaction with neighboring residues by addition of hydrogen bonding. However, the most significant aspect of the study by Kim and Lei (2008) is the enhancement of the thermostability of the enzyme. Hence a combination of the mutations shown in Table 2, will result in a more efficient enzyme with enhanced thermostability as well as increased phytase activity. Further work in this direction is currently under progress in our laboratory.

CONCLUSION

The effects of different mutations on the binding strengths of phytate in waste water bacterium phytase have been analyzed using computer simulations. The data obtained from the simulations are in good agreement with the available experimental results. The mutations M216R/E219R/H17A, M216R/E219R/F254E, K43E/K75M/S187G, E219R/M216R and E219R/F254R are found to improve the binding strength which will lead to improvement in the catalytic activity of the enzyme. This improvement in the binding is attributed to the possibilities of additional hydrogen bonding, hydrophilic or hydrophobic interactions and the accessible surface area available for the enzyme. Addition of hydrogen bonding has been reported to have resulted in the improvement in the thermostability of this enzyme. On the basis of the experimental observation of concurrent improvement in the thermostability of the mutated

enzyme, we suggest that the computation simulations can be successfully used to design new thermostable and more active enzymes.

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