Appl Biochem Biotechnol (2015) 175:3084–3092
DOI 10.1007/s12010-015-1485-6

Replacement P212H Altered the pH–Temperature Profile of Phytase from *Aspergillus niger* NII 08121

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Received: 13 October 2014 / Accepted: 5 January 2015 /
Published online: 17 January 2015

Abstract Microbial phytase, a widely used animal feed enzyme, needs to be active and stable in the acidic milieu for better performance in the monogastric gut. *Aspergillus niger* phytases exhibit an activity dip in the pH range from 3.0 to 3.5. Replacement of amino acids, which changed the pKa of catalytic residues H82 and D362, resulted in alteration of the pH profile of a thermostable phytase from *A. niger* NII 08121. Substitution P212H in the protein loop at 14 Å distance to the active site amended the pH optimum from 2.5 to pH 3.2 nevertheless with a decrease in thermostability than the wild enzyme. This study described the utility of amino acid replacements based on pKa shifts of catalytic acid/base to modulate the pH profile of phytases.

Keywords *Aspergillus niger* · Phytase · pH stability · Thermostability · Site-directed mutagenesis

Introduction

Monogastric diet, based on plant seeds and legumes, consists of phytate, the main form of phosphorus. However, because these animals have low phytase activity in their digestive tract, phytate phosphorous is poorly available to monogastrics. This problem can be resolved by supplementing animal feed with the enzyme phytase, which catalyzes the hydrolysis of phytic acid releasing lower inositol phosphates and inorganic phosphate [1, 2]. For its potential application in animal feed industry, phytases should be thermostable in order to withstand the feed pelleting temperature; it should also perform well in the monogastric gut pH of 3.0–

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3.5 [3]. *A. niger* phytase (phyA), widely used as feed additive, has a characteristic bi-hump pH profile, with two pH optima (5.0 to 5.5 and 2.5) and a trough in activity at pH 3.0 to 3.5 [4, 5]. This dip in activity at acidic pH prohibits it from full function in the stomach of animals. Hence, a relatively high phytase inclusion is required to maximize the phytate–phosphorous utilization, which is uneconomical. Thus, efforts have been made to improve the pH tolerance of phytases or develop the enzymes, which act best at low pH. Augspurger and Baker, in 2004, reported an *Escherichia coli*-derived phytase with higher activity at pH range 2.5 to 3.5, which exhibited more efficiency in phosphorous release than either of the two commercially available fungal phytases from *Peniophora lycci* (with optimal activity at pH 4.0 to 4.5) and *Aspergillus ficuum* (optimal activity at 2.5 and 5.5) evaluated respectively [6].

There are a number of strategies adopted by various research groups to provide the feed sector with re-engineered pH activity profile for a better performance in the monogastric animal gut. These include replacement of ionizable groups directly involved in catalysis by amino acids with different pKa value, or replacement of residues that are in direct contact with substrate-binding residues, or alteration of longer range (indirect) charge–charge interactions on the surface of the enzyme [7–9]. Among these, since enzyme activity is dependent on the ionization state of the catalytic residues, amino acid substitutions to shift the pKa values of the catalytic residues have attained much success. Advances have been made to shift the pH optima of phyA to match the stomach condition by substituting the amino acids in the substrate-binding sites with different charges and polarities [10]. Protein engineering was carried out to shift the pH optimum of a thermostable *Aspergillus fumigatus* phytase to acidic range [9].

The present work aimed to study the impact of amino acid substitutions which alter the pKa values of catalytic residues that can modify the pH activity profile of phytases. Site-directed mutants were constructed and expressed in *Kluyveromyces lactis* cells, and pH activity profile of the mutants and wild proteins were compared. Effect of these substitutions on temperature activity profile of the enzyme was also analyzed. Web-based automated protein stability prediction softwares were employed to evaluate the impact of this substitution on overall protein stability.

Materials and Methods

Microorganisms, Vectors, Chemicals, and Reagents

Phytase gene from *A. niger* NII 08121 cloned in vector pKLAC1 (*phyA*:pKLAC1) was used to generate the site-directed mutants. *K. lactis* GG799 (New England Biolabs, Inc., Ipswich) cells used for the expression of wild and mutant phytases were routinely cultured in YPD medium (1 % yeast extract, 2 % peptone, and 2 % glucose). QIAquick gel extraction kit was obtained from Qiagen, Germany. Restriction enzymes (SacII and DpnI) were purchased from MBI Fermentas, USA, and *pfu* turbo DNA polymerase was purchased from Stratagene, USA. Oligonucleotide primers were synthesized by the Integrated DNA Technology, USA (Table 1). Phytic acid sodium salt was purchased from Sigma-Aldrich. All other molecular biology grade chemicals used in the present study were procured from either Sigma, USA or from Hi-media, India.

Selection of Residues for Site-Directed Mutagenesis

A thermostable phytase A from *A. niger* NII 08121 (accession number JN196454.1) was previously been cloned and expressed in *K. lactis* [5], and its protein sequence was nearly

Table 1 List of oligonucleotide primers used for mutagenesis

Primers	Sequences
P212H forward	5′-CAACACTCTCGACCATGGCACCTGCACTG-3′
P212H reverse	5′-CAGTGCAGGTGCCATGGTCGAGAGTGTTG-3′
S238D forward	5′-CGCCACGTTTGTCCCCGATATTCGTCAACGTCTG-3′
S238D reverse	5′-CAGACGTTGACGAATATCGGGGACAAACGTGGCG-3′
T255E forward	5′-GTGACTCTCACAGACGAAGATGTGACCTACC-3′
T255E reverse	5′-GGTAGGTCACATCTTCGTCTGTGAGAGTCAC-3′
G377T forward	5′-CTTTGGGTCTGTACAACACCACTAAGCCGCTGTCTAC-3′
G377T reverse	5′-GTAGACAGCGGCTTAGTGGTGTTGTACAGACCCAAAG-3′
D461N forward	5′-CTAGATCTGGGGGTAATTGGGCGGAGTG-3′
D461D reverse	5′-CACTCCGCCCAATTACCCCCAGATCTAG-3′

identical (98 % similarity) to previously reported *A. niger* phytase (GenBank accession M94550.1). Multiple alignments of phytases from different microbial sources with known pH profile were aligned using Clustal W [11] to identify the positions of considerable variability. Solvent accessibility of these residues was determined by Netsurf P server of Expasy [12]. A number of residues were selected for the substitution, which on replacement imparted a change in pKa of the catalytic residues His82 and Asp362. Positions chosen in this way were Pro 212 (replaced with His), Ser 238 (replaced with Asp), Thr 255 (replaced with Glu), Gly 377 (replaced with Thr) and Asp 461 (replaced with Asn). The web server PROPKa [13] was used to predict the effects of the mutations on the pKa of the catalytic acid and base.

Oligo nucleotide primers were generated using the web-based server, PrimerX (http://bioinformatics.org/primerx/). Models of the mutant and wild enzymes were generated using Swiss model workspace (http://swissmodel.expasy.org//SWISS-MODEL.html) and visualized using the program Swiss-PDB viewer DeepView [14].

Construction of phyA Mutants

PhyA mutants in pKLAC1 were constructed according to Quick Change site-directed mutagenesis protocol (Stratagene, La Jolla, CA, USA). Five single mutants were generated by the site-directed mutagenesis. PCR amplifications consisted of 1 cycle of denaturation (92 °C, 2 min), followed by 18 cycles of amplification that included denaturation (92 °C, 10 s), annealing (52–62 °C, 30 s), primer extension (68 °C, 23 min) and final extension (68 °C, 10 min). The PCR product was treated with DpnI at 37 °C for 1 h to remove the methylated and hemimethylated parental DNA template and then transformed into *E. coli* DH5α cells. The desired mutations in the selected transformants were confirmed by DNA sequencing.

Expression and Purification

The pKLAC1 plasmid containing *phyA* mutant and wild genes were linearized by the restriction enzyme SacII and was transformed into *K. lactis* cells using the electroporation as described by Lin-Cereghino et al. [15]. Transformants were selected on YCB agar plates supplemented with 5 mM acetamide. Wild and mutant strains were grown in YP galactose

expression medium with aeration (200 rpm) at 22 °C for 72 h. Culture supernatant was collected by centrifugation at 8000*g* for 10 min at 4 °C, concentrated by lyophilization and purified sequentially by using Q sepharose column [50 mM Tris acetate buffer, pH 6.3, linear NaCl gradient] and phenyl sepharose hydrophobic interaction column [10 mM Tris acetate buffer, pH 6.3, linear gradient of 2.0 to 0.5 M $(NH_4)_2SO_4$] chromatography.

Comparison of pH Profile of Wild and Mutants

Activity assays were performed according to the protocol described by Heinonen and Lathi [16]. Enzyme assay was done in 40 μl volume at 37 °C for 30 min in 100 mM sodium acetate buffer containing 1 mM sodium phytate. The reaction was interrupted by adding 160 μl of freshly prepared acetone/5 N H_2SO_4/10 mM ammonium molybdate (2:1:1, *v*/*v*). Released inorganic phosphate was quantified spectrophotometrically by measuring the absorbance at 355 nm. Phytase activity unit (1 IU) is defined as the amount of enzyme that catalyzes the release of 1 μM of inorganic phosphorus from sodium phytate per minute.

Activity profile of the wild type and mutants were determined at different pH. The following buffers were used for the indicated pH range: glycine-HCl, pH 1.5–3.5; Na acetate–acetic acid, pH 3.5–6.0; Tris acetic acid, pH 6.0–6.5; and Tris-HCl, pH 7.0–8.0. Enzyme activities of one of the mutants, which showed alteration in desired pH range, were compared with those of the wild-type PhyA using 0.2 M glycine-HCl for pH 2.5, 3.0, 3.1, 3.2, 3.3, 3.4, 3.5, 3.6, 3.8, 3.9 and 4.0. The stability of wild and mutant enzymes at different pH was determined by incubating the purified phytase in buffers of acidic pH (pH 1.5 to 5.0) at 37 °C for 24 h, and residual activity was determined by regarding the activity of untreated enzyme as 100 %.

Thermal Properties

The optimum temperature of wild and mutant phytases was compared by incubating the assay mixture at different temperatures in the range of 35 to 70 °C for 30 min in sodium acetate buffer of pH 5.5. In order to analyze the thermal stability, purified proteins were subjected to 90 and 100 °C for various time periods (10, 20, 30, 45 and 60 min); then, the samples were placed on ice for 30 min, and phytase activity was measured at 37 °C and pH 5.5. Relative enzyme activity was determined by considering the activity of untreated enzyme as 100 %.

Structural Changes on Replacement

Modelled structure of wild and mutant enzymes were analyzed in SWISS PDB viewer to determine the location of residue 212 and its distance from the catalytic residues H81 and D362. Effect of amino acid substitution on formation of hydrogen bonds between residue 212 and surrounding amino acids was also determined. Effect of single site mutation on stability change was predicted by different programmes, including MUPro [17] (http://www.ics.uci.edu/~baldig/mutation.html), I-Mutant2.0 [18] and ERIS [19].

Results and Discussion

Amino Acid Replacements

Phytase from *A. niger* NII 08121, which exhibited superior thermostability property to the commercial enzyme, was already been cloned and over-expressed in *K. lactis* [5, 20]. In view

of the demand of the feed industry for phytase which has activity and stability at acid milieu coupled with thermostability, efforts were made to tailor the former quality by amino acid substitutions. *A. niger* phytase belongs to histidine acid phosphatase family, which possesses two conserved active site motifs RH(G/N)XRXP—at the N-terminal and a downstream HD motif for substrate binding/product cleaving. The histidine (His 82) N-terminal region acts as a nucleophile and forms a covalent bond with the leaving phosphate group, forming phosphohistidine intermediate. The unique pH profile exhibited by *A. niger* phytase was due to the electrostatic environment created by the residues in the substrate binding site (K91, K94, E228, D262, K300 and K301) located in the α-domain of PhyA and acidic (K91, K94, K300, K301) and basic (R81, H82, R85, K165 and H361) amino acids which surround the active site [21]. Almost all the amino acid residues in phytase, which are involved in the catalysis and substrate binding, have already been altered by means of site-directed mutagenesis, and their effect on change in pH profile has been studied.

In the present study, multiple alignments of phytase sequences (Fig. 1) with different pH profile were used to determine the position of residues to be replaced. Phytase from *A. oryzae* shows activity optimum at pH 5.5 [22], and *A. fumigatus* phytase exhibits two pH optima but in the less acidic range: At pH 4.0 and pH 6.0, *A. niger* also exhibited two pH optima (pH 2.5 and 5.5). Phytases from *E. coli* and *Peniophora lycii* showed pH optima in the acidic range of pH 4.0 [23–25]. Five amino acids selected in the present study (212, 255, 238, 377 and 461) showed variability among these phytases. Residue 377 was conserved in all the species, except *E. coli* phytase.

In this study, amino acid substitutions were made to change the pKa value of the catalytic residues. The three-dimensional structure of phytase and mutants (P212H, T255E, S238D, G377T and D461N) generated by Swiss model workspace was used to determine the change in pKa value of catalytic residues. Table 2 represented the relative burial, pKa change and hydrogen bonding in created mutants.

Effect of Substitutions on the pH Profile

The wild enzyme exhibited enhanced activities at pH 2.5 and 5.5. Mutants D461N, G377T, T255E and S238D retained bi-peak pH profiles, but there was an observed enhancement in the activity at pH 6.0 instead at pH 5.5. Mutant P212H exhibited enhancement in the activity in the pH range pH 3.0 to 3.5 compared to the wild enzyme (Fig. 2a).

One of pH optimum peak of wild enzyme lied at pH 2.5, and its activity was reduced as pH increased to 3.0. Mutant P212H showed an increased activity at pH 3.1, 3.2, 3.3 and 3.4 than its activity at pH 2.5. Optimum pH of mutant P212H shifted to pH 3.2 from pH 2.5 (Fig. 2b). Similarly, stability studies at acidic pH range showed that the mutant exhibited higher activity retention than the wild enzyme except at pH 2.5 and 4.0 (Fig. 2c). Previously, a mutant K300E

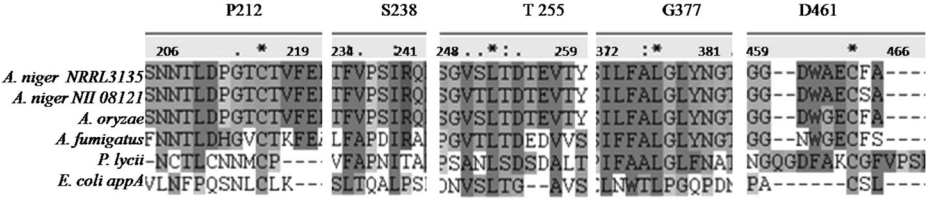

Fig. 1 Multiple alignments of phytases with different pH profiles. Amino acid sequences from microbial phytases including *A. niger* (pH optimum at 2.5 and 5.5), *A. oryzae* (pH 5.5), *A. fumigates* (pH 4.0 and 6.0), *P. lycii* and *E. coli* (pH 4.0 to 4.5) showing the targeted positions

Table 2 Residue replacements and characteristics of each substitution

Residue change	Wild enzyme	P212H	S238D	T255E	G377T	D461N
pKa value of H82	0.61	0.64	0.62	0.62	0.62	0.62
pKa value of D362	−0.14	−0.12	−0.14	−0.14	−0.13	−0.14
Formation of hydrogen bond		Yes	Yes	Yes	Yes	Yes
Location in secondary structure		Coil	Alpha helix	Alpha helix	Coil	Coil
Absolute surface accessibility (%)		67.70	58.83	90.00	34.68	56.89
pH optimum	2.5, 5.5	3.2, 5.5	2.5, 6.0	2.0, 6.0	2.5, 6.0	2.5, 6.0

generated by the site-directed mutagenesis resulted in enhanced activity at pH range 3.5–5.0 [26]. Substitutions G277K and Y282H gave rise to a second pH optimum, pH 2.8–3.4, in *A. fumigatus* phytase [9]. In 2006, Kim et al. [10] designed 17 mutants which either lost one pH optimum or shifted the pH optimum from pH 5.5 to the more acidic side. The mutant E228K exhibited the best overall changes, with a shift of pH optimum to 3.8.

Effect of Mutation on Thermal Properties

Optimum temperature of both wild and mutant enzyme was observed at 55 °C; however, there was a sharp decrease in the activity of mutant P212H beyond 55 °C (Fig. 3a). In addition,

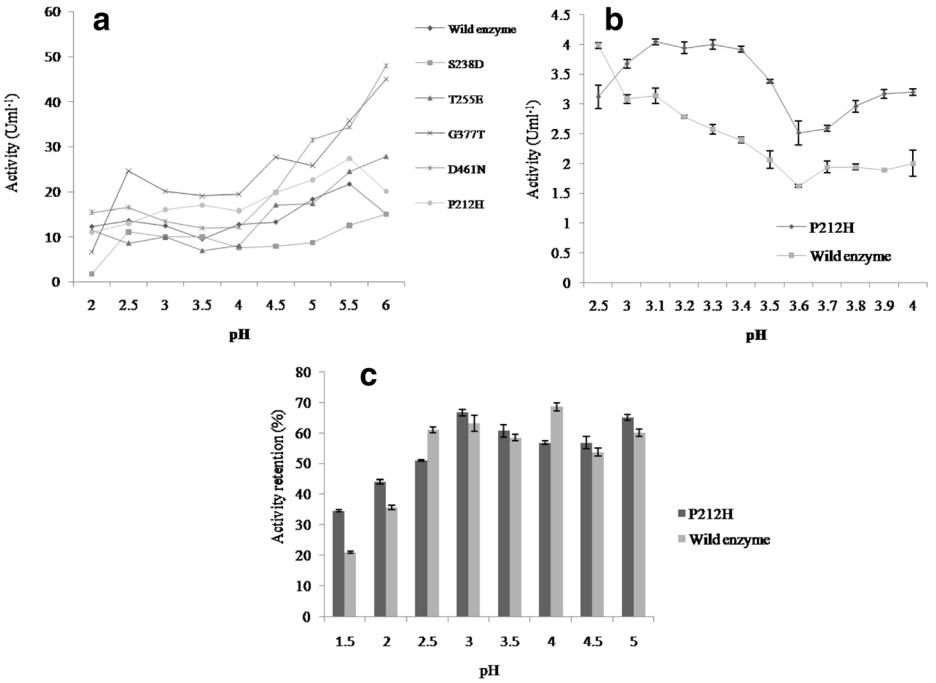

Fig. 2 Comparison of pH activity profile of wild and mutants. **a** Analysis of activity of wild and mutant P212H at narrow pH range. **b** Effect of different pH on the stability of wild and mutant P212H. **c** Effect of pH on phytase activity was determined according to Heinonen and Lathi in the presence of 1 mM sodium phytate at 37 °C using buffers glycine-HCl, pH 1.5–3.5; Na acetate–acetic acid, pH 3.5–6.0; Tris acetic acid, pH 6.0–6.5; and Tris-HCl, pH 7.0–8.0

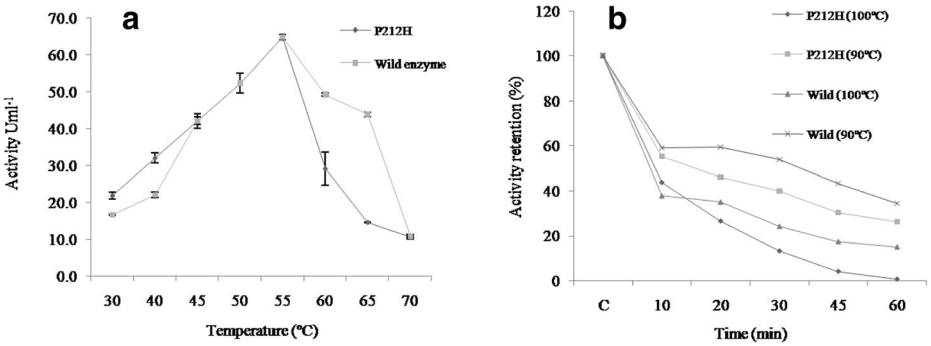

Fig. 3 Effect of temperature on activity (**a**) and stability (**b**) of wild enzyme and mutant P212H. Phytase activity was evaluated at pH 5.5. Residual activities were determined after pre-incubation of enzyme at temperatures 90 and 100 °C

temperature stability was also affected by the substitution at residue 212. While wild enzyme retained 17 and 15 % activity retention after a pre-incubation at 100 °C for 45 and 60 min, respectively, mutant lost most of the activity at similar condition (Fig. 3b). This again confirmed the role of this residue in thermostability of PhyA.

Structural analysis

Since the substitution of histidine at position 212 had remarkable effect on pH and temperature properties of the enzyme, distance of residue 212 with respect to the catalytic residues H82 and

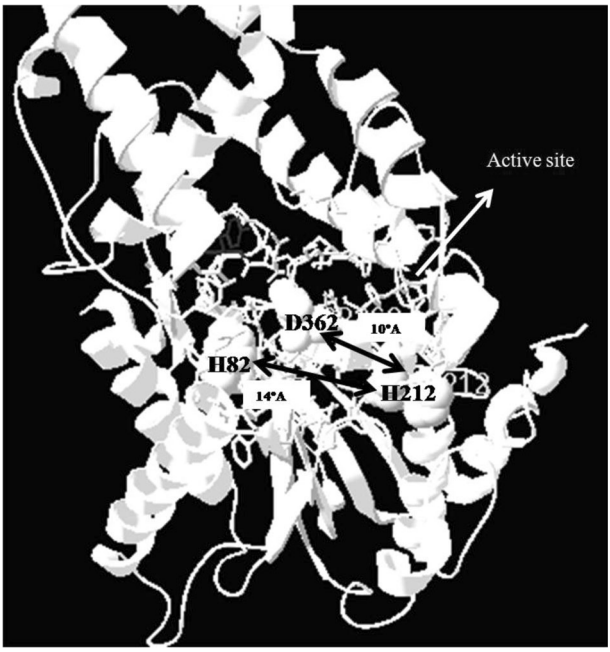

Fig. 4 Modelled structure of mutant P212H. Active site and distance between substituted residue His 212 and catalytic residues H82 and D362 are labelled

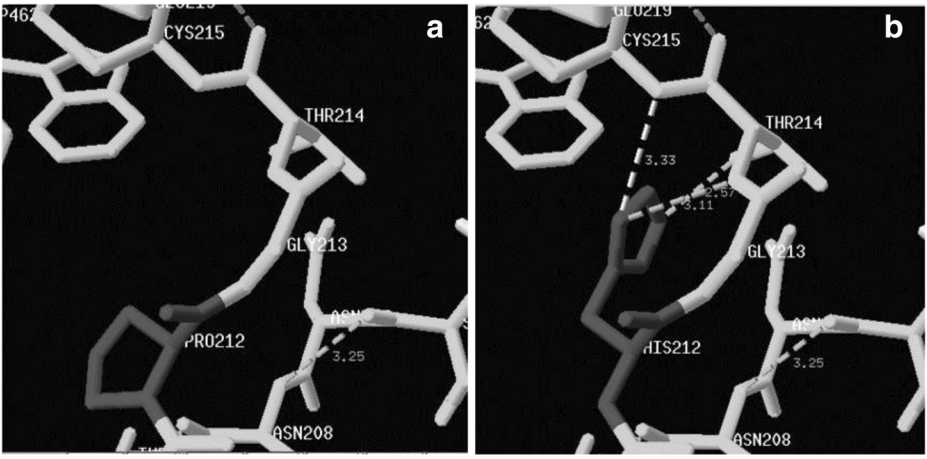

Fig. 5 Hydrogen bonding in wild (**a**) and mutant enzyme (**b**). Substitution P212H introduced additional hydrogen bonds

D362 were determined using the Swiss PDB viewer. As shown in Fig. 4, residue 212 was positioned at 10 and 14 Å distance to the catalytic residues D362 and H82, respectively. Since residue 212 was closer to the catalytic residues, its impact on enzymatic properties would be more. Replacement of histidine in place of proline resulted in the formation of two additional hydrogen bonds in the structure of the enzyme (Fig. 5). Addition of hydrogen bonds generally enhances the protein stability; however, here, although there was an improvement in the pH stability as mentioned above, thermal properties were negatively affected by this substitution.

Residue 212 is situated in the protein loop region, and since proline residues in loop restricts the backbone bond rotation because of their pyrrolidine rings, it thereby decreases the entropy during protein unfolding [27]. Deletion of this residue could have reduced the structural stability of protein. We used different web-based programmes (I-Mutant2.0, MU_{PRO} and Eris) to predict the effect of single site mutation on the stability. The stability of a protein is generally represented by the change in the Gibbs free energy upon folding (ΔG), where an increasingly negative number represents greater stability. The substitution of a single amino acid in a protein sequence can result in a significant change in the protein's stability (ΔΔG), where a positive ΔΔG represents a destabilizing mutation and a negative value represents a stabilizing mutation [28]. All the three programmes consistently gave the output that was destabilization on replacement P212H.

Conclusions

Results of this study clearly showed that the substitution of amino acid residues which changed the pKa value of the catalytic residues altered the pH profile of the enzyme. The introduction of histidine at position 212 close to the active site altered the activity optimum peak to pH 3.2 from 2.5 and improved the activity profile at pH 3.1 to 3.4, which was a desirable property for its feed application. Since this replacement was close to the active site, enzyme activity at elevated temperature was negatively affected; however, the impact was not deleterious. Hence, the substitution of titrable residues in the vicinity of the active site of the phytase by predicting

the pKa value of catalytic residues could help to explore the role of amino acids in the three-dimensional structure in determining the pH profile.

Acknowledgments One of the authors (MVU) thank the Indian Council of Medical Research, New Delhi for the award of a Senior Research Fellowship to her. Financial support from the Department of Biotechnology, New Delhi (Grant No. GAP 1410) is gratefully acknowledged.

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