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Molecular Dynamics Study of the Effect of Calcium Ions on The Thermostability of *Bacillus Amyloliquefaciens* Phytase

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Abstract: Phytase is added to animal feeds to improve phosphorus absorption and reduce phosphorus excretion of swine and poultry. It hydrolyses phytate, which is a major form of storage of cereal grains and legumes in commercial animal feeds, into *myo*-inositol and inorganic phosphate. Adequate thermostability of phytase is necessary for field application as diets for swine and poultry are normally pelleted at high temperatures (60-80°C). In this study, Molecular Dynamics simulation (MD) was used to study the effect of calcium metal ions on the thermostability of *Bacillus amyloliquefacience* phytase. MD simulations of the enzyme in the calcium-loaded and calcium-free states were performed at 60°C (333 K) and 80°C (353 K) in water as the solvent medium, for duration of 4 ns. Root mean square deviations (RMSD) of calcium-bound residues, backbone atoms, and other secondary structures were found to be lower in the presence of calcium ions at both temperatures. In addition, calcium-loaded enzyme was found to have fewer numbers of hydrogen bonds and salt bridges at both temperatures, yet calcium-loaded enzyme remains more thermostable due to network of electrostatic interactions induced by calcium binding. The binding effect of calcium ions becomes weaker at 80°C and the small increase of binding effect offered by the calcium ions at higher temperatures in not sufficient enough to maintain the activity at this temperature. It is proposed that suitable mutations at the coil region would lead to increase in the stability of the enzyme at high temperatures.

Key words: *Bacillus amyloliquefaciens* phytase, Molecular Dynamics simulation, calcium ions, thermostability, RMSD, hydrogen bonds, salt bridges.

INTRODUCTION

An increasing number of enzymes are being used in industrial applications where most of these enzymes require high thermal tolerance. Understanding the factors contributing to the thermostability of enzymes help design thermostable enzymes from mesophilic enzymes, and further improve the thermal tolerance of thermostable enzymes (Purmonen, 2007). Proteins thermostability is decided by their ability to resist heat denaturation, similar to proteins derived from hyperthermophilic organisms, and/or their ability to refold properly into their native and active conformation after heat denaturation (Lei, 2003). However, thermostability studies often use the term 'thermostability' or 'thermostable proteins' to represent an increase in melting/unfolding temperature (Lehmann, 2000; Williams, 1999; Lehmann, 2000; Lehmann, 2002; Kim, 2008; Zhang, 2007), higher optimum temperature (Lehmann, 2000), and/or retain greater activity at high temperatures (Kim, 2008; Zhang, 2007; Zhang, 2008). As the Molecular Dynamics (MD) simulation (Noorbatcha, 2010) is capable of providing a detailed description of the motion of enzymes at different temperatures, such information can be used to investigate the factors affecting the thermostability of enzymes by following their conformational changes as a function of time.

Phytases catalyze the stepwise hydrolysis of phytic acid (phytate) into myo-inositol and inorganic phosphate (Anis Shobirin, 2009). Phytase is added to animal feeds to improve phosphorus absorption and reduce phosphorus excretion of poultry and swine as they do not produce phytase, hence they are unable to degrade phytate (Tran, 2010). The excreted unhydrolyzed phosphorus from phytate creates ecological problems (eutrophication) when enters into rivers (Vats, 2004). It is crucial for phytases to have adequate thermostability in order to withstand the high temperature generated during the pelleting process of animal diets which ranges from 60-80°C(Lehmann, 2000). The presence of calcium ions has been reported to contribute to the thermostability of the β propeller *Bacillus amyloliquefacience* phytase (Shin, 2001). In this study, we investigate the effect calcium ions on the thermostability of *B. amyloliquefacience* phytase by performing MD simulation of this enzyme with and without calcium ions in water as the solvent medium at two different temperatures.

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MATERIALS AND METHODS

Crystal structure of *Bacillus amyloliquefacience* phytase obtained from the Protein Data Bank (Berman, 2000) under PDB code 1H6L (Shin, 2001) with a resolution of 1.80Å was used. The structure contains 353 amino acid residues, and in complex with two phosphate (PO_4) and seven calcium (Ca) ions. Prior to preparing the input files for simulation, the two phosphate ions and all crystallographic water molecules were removed from the structure. The structure was then solvated by placing it in a 15Å water box of TIP3P explicit solvent model. Following the solvation step, the system was neutralized by replacing 31 water molecules with 17 sodium (Na) and 14 chloride (Cl) ions. Afterward, the structure was minimized using the conjugate gradient method (Fletcher, 1964) and then heated to 333K (60°C) and 353K (80°C) using the velocity reassign function prior to the equilibration step which was done by a rescaling thermostat to equilibrate kinetic and potential energies. The equilibrated structure was subjected to molecular dynamics simulation for 4 ns. The molecular dynamics simulations were carried out using NAMD software package (Phillips, 2005) with CHARMM force field. The simulation results were analyzed using VMD (Humphrey, 1996) and VEGA ZZ (Pedretti, 2004).

RESULTS AND DISCUSSIONS

Calcium ions play a critical role for the activity and thermostability of *Bacillus amyloliquefaciens* phytase. The enzyme has a six-bladed β propeller fold with six calcium binding sites (Fig. 1). Binding of three calcium ions (Ca1, Ca2, and Ca3) to high-affinity calcium binding sites causes the enzyme thermostability to increase remarkably as a result of joining loop segments adjacent in the structure but remote in the amino acid sequence. Binding of three more calcium ions (Ca4, Ca5, and Ca6) to low-affinity calcium binding sites at the top of the molecule's shaft activates the enzyme by converting the highly negatively charged region into a favourable environment for the binding of substrate (phytate) (Konietzny, 2004). Binding of phosphate induces the binding of an additional calcium ion (Ca7) by providing a coordination arm. Electron density analysis of the active site region shows that calcium-bound structure displayed tight binding of calcium ions with active site residues compared to calcium-free molecule (Shin, 2001).

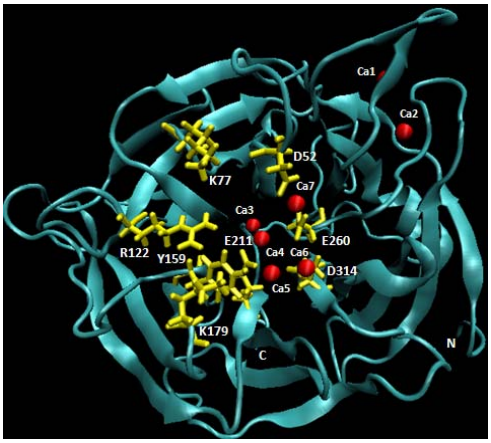

Fig. 1: Cartoon diagram of *B. amyloliquefaciens* phytase viewed down the propeller shaft. Calcium ions are highlighted in spheres, and active site residues are shown in licorice representation.

In the fully calcium-loaded molecule there are around nineteen residues bound or interacting with the seven calcium ions. It can be noticed that most calcium-bound amino acid residues are negatively charged, which interacts strongly with the positively charged calcium ions. Root Mean Square Deviation (RMSD) of these residues in both calcium-loaded and calcium-free states at 333 K and 353 K has been calculated (Table 1). These residues are found to show a large increase in RMSD compared to calcium-loaded enzyme at 333 K, while their RMSD values showed a significantly small change at higher temperature. This is due to the strong electrostatic binding network offered by calcium ions, which holds bound residues at similar positions at both temperatures, whereas in the absence of calcium ions, these residues are relatively free to move and their RMSD increases as temperature is increased. This effect is consistent with other studies, which revealed that thermophilic enzymes exhibit lower RMSD values compared to their mesophilic counterparts (Purmonen, 2007; Merkley, 2010). Accordingly, we can see that the presence of calcium ions in the more thermostable calcium-loaded enzyme contributed positively to its thermostability by producing lower fluctuations for calcium-bound residues compared to calcium-free enzyme.

Table 1: RMSD values of calcium-bound residues in calcium-loaded *B. amyloliquefaciens* phytase (BA) and calcium-free enzyme (BA-noCa) at 333 K and 353 K. Plus sign denotes an increase in RMSD in calcium-free enzyme compared to calcium-loaded enzyme, and minus sign denotes a decrease.

Residue	BA-333 K	BA-noCa-333 K	Effect*	BA-353 K	BA-noCa-353 K	Effect*
E43	1.79562	1.14173	--	1.15037	1.25178	O
D52	1.07753	1.99594	++	1.70199	2.15980	+
D55	1.01036	2.55724	++	1.20634	1.63887	+
D56	1.26812	1.48180	+	0.62492	1.10343	+
P57	1.07494	1.30808	+	0.75685	0.58355	o
V101	1.30163	0.63673	--	0.83180	1.42807	+
Y159	0.91781	1.28504	+	1.62137	1.95849	+
E211	0.68669	1.53791	+	1.09102	1.48329	+
E227	0.65408	2.09769	++	1.05509	1.68291	+
E260	0.59965	2.16433	++	1.34196	1.18341	o
Q279	1.22555	2.38285	++	1.83112	2.25591	+
D308	2.06742	1.32095	--	1.85798	1.52621	-
G309	0.91193	0.70313	-	1.35037	0.77757	-
D314	0.62907	1.42328	++	1.11093	1.24606	+
N336	1.22663	1.34629	+	1.09528	1.22800	+
E338	1.57428	1.89231	+	2.00357	2.23853	+
N339	0.93295	1.39725	+	1.45486	1.39200	o
I340	2.33404	1.91755	-	1.87913	1.64013	-
D341	2.89666	2.39512	-	2.10832	2.23845	o

*O denotes RMSD difference of <0.2
+ and - denote RMSD difference in the range of 0.2-0.6
++ and -- denote RMSD difference of >0.6

RMSD of backbone (Cα) atoms of the enzyme in both calcium states and temperatures is displayed in Fig 2. Similar to the effects described above, the RMSD of backbone atoms are lower when in the calcium-loaded state compared to the calcium-free state at 333 K (Fig. 2A). However, the enzyme showed no significant difference in RMSD values between both calcium states at 353 K (Fig. 2B). It can be noted that backbone RMSD for both calcium-loaded enzyme and calcium-free enzyme increases as the temperatures is increased (Fig. 2A & 2B), even though this increase is small in the case of calcium-loaded enzyme. The calcium-loaded enzyme was able to maintain the conformation necessary for enzyme activity at lower temperature, but at higher temperature, a small increase in the RMSD of backbone lead to deviations from the preferred conformation leading to loss of activity at higher temperature even for calcium-loaded enzyme. Hence, if calcium-loaded enzyme is modified by appropriate mutations so as to maintain the conformation similar to that of lower temperature, then it would be possible to maintain the activity at higher temperatures.

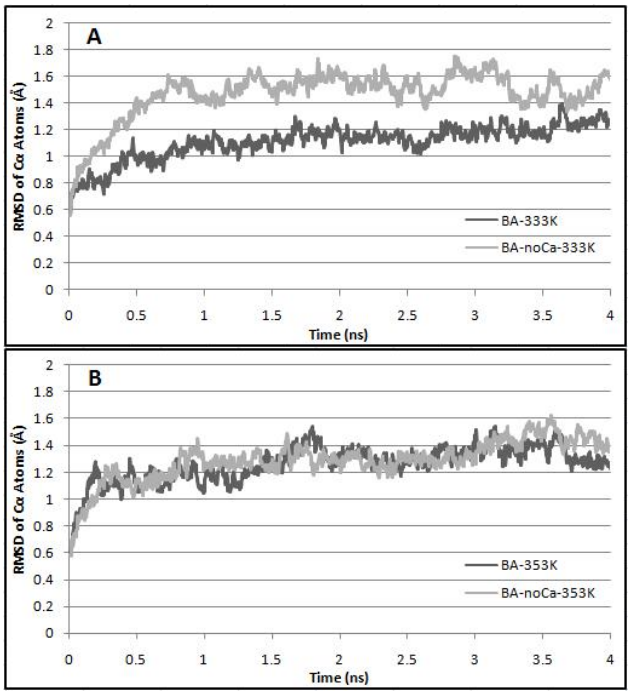

Fig. 2: RMSD of Cα atoms of calcium-loaded *B. amyloliquefaciens* phytase (BA) and calcium-free enzyme (BA-noCa) at (A) 333 K and (B) 353 K.

Fig. 3 shows average RMSD values for different secondary structures. Except for α-helix, RMSD of all secondary structures were lower for the enzyme in the calcium-loaded state at 333 K (Fig. 3A), compared to calcium-free enzyme. There is very large lowering of RMSD values of coil regions of calcium-free enzyme compared to other regions. As a result, the presence of calcium ions was able to significantly reduce the mobility of coil regions in the enzyme, thus preserving the conformation necessary for enzyme activity. However, at 353 K, the heat inactivation temperature of the enzyme [23], no significant difference in average RMSD of secondary structures (except for α-helix) between both states of the enzyme was observed (Fig. 3B), and there is no enzyme activity at both the temperatures.

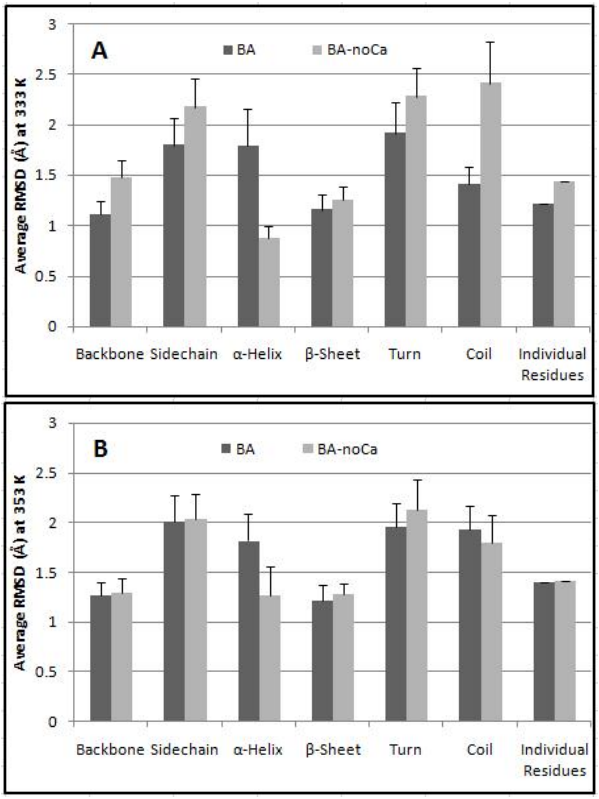

Fig. 3: Average RMSD of various secondary structures of calcium-loaded *B. amyloliquefaciens* phytase (BA) and calcium-free enzyme (BA-noCa) at (A) 333 K and (B) 353 K. Error bars denote one standard deviation.

An analysis of number of hydrogen bonds and salt bridges revealed that calcium-free enzyme showed more hydrogen bonds and salt bridges compared to calcium-loaded enzyme at both temperatures (Fig. 4A, 4B, 5A, and 5B). This effect is different compared to the role of hydrogen bonding and salt bridges reported by other workers [9, 24-29]. The present observation can be attributed to the free conformational space created in the absence of calcium ions allowing calcium-free residues to form hydrogen bonds and salt bridges in the absence of calcium ions.

In addition, *B. amyloliquefaciens* phytase was found to have higher number of hydrogen bonds at 60°C (333 K) compared to 80°C (353 K) in both calcium states (Fig. 4C and 4D). This is due to the increase in backbone fluctuation of the enzyme at higher temperature, and consistent with the fact that the enzyme is much more active at 60°C than at 80°C where it is heat inactivated. Interestingly, the enzyme showed different behavior in number of salt bridges of both calcium states when compared, separately, at both temperatures. Whereas, calcium-free enzyme displayed no significant difference in number of salt bridges at 60°C and 80°C (Fig. 5D), calcium-loaded enzyme showed a higher number of salt bridges at 80°C compared to 60°C (Fig. 5C). This clearly shows that, unlike the calcium-free enzyme, the presence of calcium ions did affect the number of salt bridges. The fact that the number increased at higher temperature suggest that some or all of calcium ions may have lost their conformational position as a result of the heat inactivation of the enzyme at 80°C which allowed residues in close proximity to calcium ions to form salt bridges as a result of the new available space

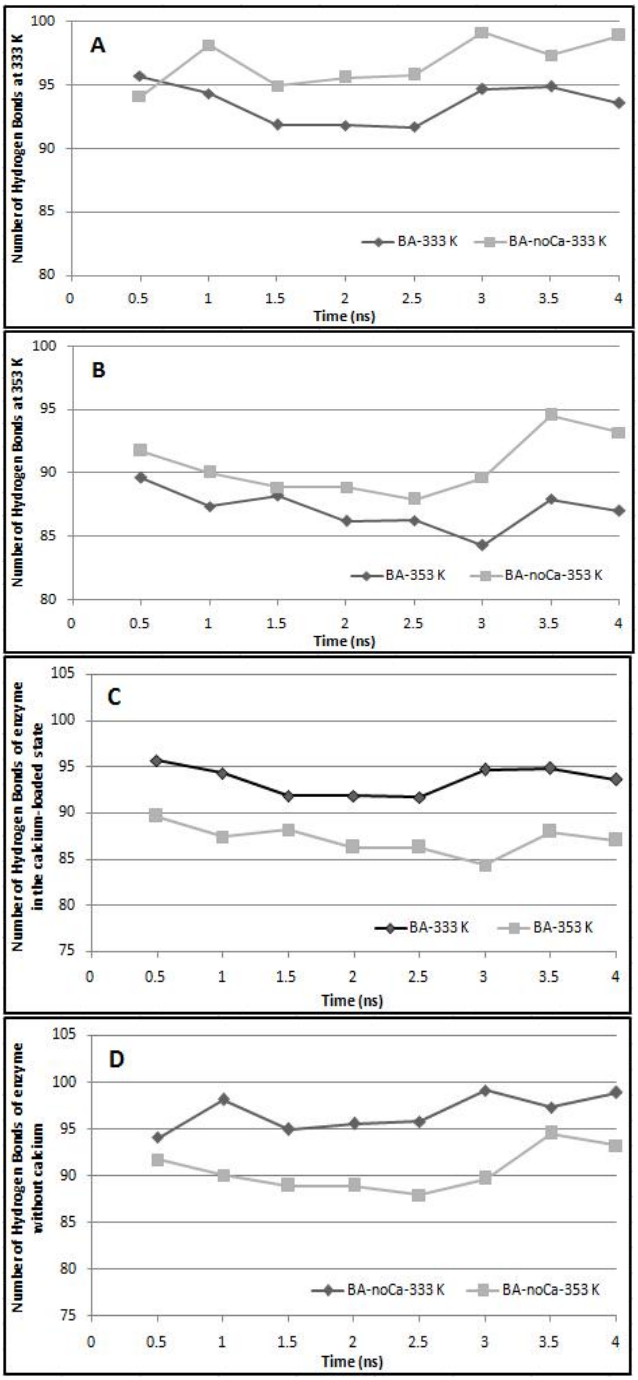

Fig. 4: Number of hydrogen bonds of the calcium-loaded *B. amyloliquefaciens* phytase (BA) and calcium-free enzyme (BA-noCa) at (A) 333 K and (B) 353 K, and number of hydrogen bonds of the enzyme in (C) calcium-loaded state and (D) calcium-free state, at both temperatures.

In conclusion, calcium-loaded *B. amyloliquefaciens* was found to have lower RMSD values for calcium-bound residues and all secondary structures (except α-helix) at 60°C compared to 80°C. Despite the fact that calcium-free phytase showed higher number of hydrogen bonds and salt bridges at 60°C, calcium-loaded enzyme remains more thermostable as a result of the tight bindings of propeller shaft residues (including active site resides) induced by the binding of calcium ions. Hence, the stronger binding offered by the calcium ions plays much more significant role compared to the increased possibility of hydrogen bonds and salt bridges among amino acid residues in calcium-free enzyme to retain the conformation necessary for the enzyme

activity. However, both enzymes lose their activity due to increased fluctuations of backbone atoms. If calcium-loaded enzyme, particularly the coil regions, is modified by appropriate mutations so that it maintains the conformation similar to that of lower temperature, then it would be possible to maintain the enzyme activity at higher temperatures.

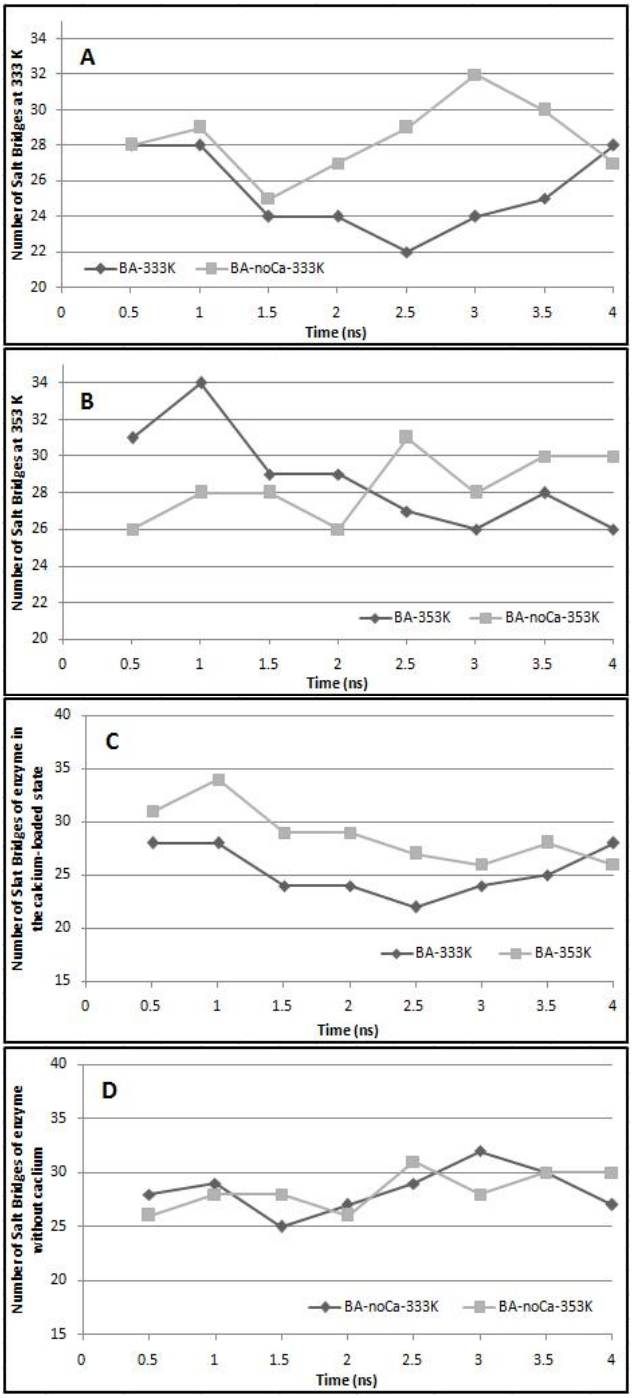

Fig. 5: Number of salt bridges of the calcium-loaded *B. amyloliquefaciens* phytase (BA) and calcium-free enzyme (BA-noCa) at (A) 333 K and (B) 353 K, and number of salt bridges of the enzyme in (C) calcium-loaded state and (D) calcium-free state, at both temperatures.

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