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Crucial role of Pro 257 in the thermostability of *Bacillus* phytases: Biochemical and structural investigation

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ABSTRACT

We have previously cloned and characterized the thermostable phytase (PHY US417) from *Bacillus subtilis* US417. It differs with PhyC from *B. subtilis* VTTE-68013 by the R257P substitution. PHY US417 was shown to be more thermostable than PhyC. To elucidate the mechanism of how the Pro 257 changes the thermostability of *Bacillus* phytases, this residue was mutated to Arg and Ala. The experimental results revealed that the thermostability of the P257A mutants and especially P257R was significantly decreased. The P257R and P257A mutants recovered, respectively, 64.4 and 81.5% of the wild-type activity after incubation at 75 °C for 30 min in the presence of 5 mM $CaCl_2$. The P257R mutation also led to a severe reduction in the specific activity and catalytic efficiency of the enzyme. Structural investigation, by molecular modeling of PHY US417 and PhyC focused on the region of the 257 residue, revealed that this residue was present in a surface loop connecting two of the six characteristic β sheets. The P257 residue is presumed to reduce the local thermal flexibility of the loop, thus generating a higher thermostability.

1. Introduction

Phytases catalyze the sequential hydrolysis of phytate, the principal storage form of phosphorus (P) present in cereal grains into inorganic phosphate (Pi) and a series of myo-inositol phosphate intermediates, and thus release digestible P and vital minerals [1]. They are important enzymes used as feed additives for monogastric animals such as poultry, pig and fish. In addition to improving phytate P utilization, the supplementation of monogastric feed with phytases removes or reduces the need for supplemental Pi, which not only decreases the cost of diets, but also contributes significantly to environmental protection by minimizing manure P excretion, especially in areas of extensive animal production [2–5].

A large number of phytases have been identified, cloned and characterized from several plant and microbial sources [6–19]. Based on their structures and catalytic mechanisms, phytases can be classified into four major classes: (i) histidine acid phosphatases (HAPs); (ii) cysteine phytases; (iii) purple acid phosphatases (PAPs) which are acid phytases; and (iv) β-propeller phytases (BPPs) also known as alkaline phytases [1]. Most of the phytases that have been used as a commercial feed supplement are HAPs derived primarily from *Aspergillus niger*, *Peniophora lycii* and *Escherichia coli*. They possess high catalytic activity at the pH values ranging from 2.5 to 6 [20,21], but their major drawbacks are low thermostability and poor specificity. On the other hand, bacterial BPPs characterized mainly from the *Bacillus* genus are currently considered as the best alternative because of their natural high thermal stability, high specificity, neutral optimum pH and proteolysis resistance [22]. The beta-propeller phytases are the major phytate-degrading enzyme in nature [23]. Alkaline phytase has a β-propeller folding that consists of six blades composed of four to five antiparallel beta-strands. The active site of the enzyme is located on the top of the β-propeller and is composed mainly of negatively charged amino acids [24,25]. Recently, Zeng et al. [26] have demonstrated that there are both direct and indirect interactions between the BPP and its substrate. The direct interactions are formed *via* the side chains of K76, K77, R122 and K179 residues, while the indirect interactions involve five active site-associated metal ions (Ca-4 to Ca-8), which are chelated by amino acid residues including D52, D55, Y159, E211, E227, D228, D258, E260, and D314. These findings are supported by previous site-specific mutagenesis experiments, which revealed that the calcium-binding amino acid residues D55, Y159, E211, E227, D258, E260 and D314, in addition to the substrate binding residues K76 and R122 were critical for the enzyme catalytic activity [27]. Although the catalytic mechanism of BPPs was elucidated, hitherto little is known about the molecular determinants responsible for their natural thermostability.

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It is widely recognized that the thermostability of proteins can be influenced by many structural factors, such as core and side-chain packing, oligomerization, proline substitutions, helical content, polar surface area, hydrogen bonds and salt bridges [28]. Few reports have been published concerning the thermostability of *Bacillus* phytases. Among them, a recent structural analysis has suggested that the high thermostability of these enzymes may be due to a large number of hydrogen bonds connections, particularly the hydrogen bond between the T37 and K361 residues, and the presence of D336 residue [29]. Furthermore, the presence of a proline residue in the 257 position situated in a surface loop has previously been suggested to contribute to the enhanced thermostability of the PHY US417 phytase from *Bacillus subtilis* US417 [13]. Indeed, although it displays only one substitution (R257P) with the PhyC phytase from *B. subtilis* VTTE-68013 [10], PHY US417 was shown to be more thermostable than PhyC, particularly in the absence of calcium [13].

In this study, site-directed mutagenesis and structural analysis, focused on the 257 position, were applied to probe its role in the activity and thermostability of *Bacillus* phytases using PHY US417 as a model. Subsequently, the importance of the proline residue in this position was demonstrated experimentally and theoretically.

2. Materials and methods

2.1. Materials

The oligonucleotides used in site-directed mutagenesis experiments (Table 1) were synthesized from PROLIGO (Paris, France). PCR amplifications were carried out using the Phusion high-fidelity DNA polymerase from Thermo Fisher Scientific. Phytic acid sodium salt hydrate from rice (P0109) was purchased from Sigma and wheat bran was obtained from the local company "Nutrisud/Medimix". All other chemicals used in this study are commercially available in analytic grade.

2.2. Site-directed mutagenesis

To overproduce PHY US417 in *B. subtilis* 168, the corresponding *phy* US417 gene (with its native promoter) was previously cloned in the pMSP3535 vector yielding the phytase overexpression plasmid pAF3 [30]. The single mutants of the *phy* US417 gene used in this study were generated through the method of site-directed mutagenesis by overlap extension [31] using pAF3 as template and two overlapping complementary oligonucleotides containing the desired nucleotide changes specific for each mutation (P257R; P257A) (Table 1). Both mutant *phy* US417 genes were cloned into the pMSP3535 vector following standard procedures [32] and overexpressed in *B. subtilis* 168, as described by Farhat-Khemakhem et al. [30] for the wild-type *phy* US417 gene. The production, purification, identification and SDS-PAGE analysis of the mutant phytases were carried out in the same way as the wild-type rPHY US417 protein [30].

Table 1
Mutagenic primers used to construct the mutant phytases. Nucleotides in bold are mutated sites.

Primer	Sequence
P5′p	CACATTTGACAATTTTCACAAA
P3′	CATGTTTATTTTCCGCTTCT
FwdP257R	GGCATTTAACT**CGT**GATATTGAAAGG
RevP257R	CCTTCAATATC**ACG**AGTTAAATGCC
FwdP257A	GGCATTTAACT**GCT**GATATTGAAAGG
RevP257A	CCTTCAATATC**AGC**AGTTAAATGCC

2.3. Amino acid sequence analyses and homology modeling

Sequence analysis and multiple alignments were performed using the BLAST and CLUSTALW programs [33]. The prediction of the protein secondary structure was performed using the DSSP program [34] while the editing of the alignment including the superimposition of secondary structures was conducted using the ESPript program [35]. The automated comparative protein structure homology modeling server, SWISS-MODEL (http://www.expasy.org/swissmod) was used to generate the 3D-structure models of PHY US417 and its derivative mutants as well as PhyC using the TS-Phy structure as template (PDB-code 1POO) [36]. PyMOL (http://www.pymol.org) was used to visualize and analyze the generated model structures and to construct the graphical presentations and illustrative figures.

2.4. Enzyme assay

Phytase activity was assayed as described by Farhat et al. [13]. One phytase unit (U) was defined as the amount of enzyme capable of releasing 1 μmol of Pi/min (from phytic acid) under the optimal conditions.

2.5. Effect of pH and temperature on the activity and stability of wild-type and mutant phytases

The effect of the temperature on the activity of the two mutant phytases rPHY US417/P257R (P257R) and rPHY US417/P257A (P257A) was investigated by determining their activities between 37 and 80 °C at pH 7.5. Thermostability was checked by incubating the mutant enzymes up to 1 h at 75 °C in 0.1 M Tris–HCl buffer pH 7.5 in the presence and absence of 5 mM $CaCl_2$. The effect of pH (from 3 to 9.5) on the activity of the P257R and P257A mutants was investigated at 60 °C and 65 °C, respectively, using the same buffer solutions reported by Farhat et al. [13]. The effect of pH on the stability of the P257R and P257A mutants was performed by incubating the enzymes at pH ranging from 2 to 9 for 1 h at 37 °C, followed by measuring their residual activity under optimal conditions. For comparison, similar assays were performed with the wild-type rPHY US417 under the enzyme optimal conditions as described by Farhat-Khemakhem et al. [30].

2.6. Calcium requirement

The effect of calcium concentration on the activity of rPHY US417 as well as the P257R and P257A mutants was investigated by measuring phytase activity between 0 and 2 mM $CaCl_2$ at pH 7.5 at the enzymes optimal temperatures. For control experiments (without addition of calcium), the enzymes solutions were dialyzed against 0.1 M Tris–HCl buffer pH 7.5 and assays were performed in this buffer in the presence of 2 mM ethylenediaminetetraacetic acid (EDTA).

2.7. Determination of kinetic parameters

Assays were carried out using a 0.1 M Tris–HCl buffer (pH 7.5), 1 mM $CaCl_2$, and 0.1–5 mM substrate (phytic acid). The enzyme samples were assessed under optimal conditions.

3. Results and discussion

3.1. Design of mutant phytases

A thorough comparison of the primary and secondary structures of the PHY US417 phytase from *B. subtilis* US417 was carried out with those of previously reported homologous *Bacillus* phytases