International Journal of Biological Macromolecules 80 (2015) 581–587

Contents lists available at ScienceDirect

International Journal of Biological Macromolecules

journal homepage: www.elsevier.com/locate/ijbiomac

Characterization of an extremely salt-tolerant and thermostable phytase from *Bacillus amyloliquefaciens* US573

Ines Boukhris[1], Ameny Farhat-Khemakhem[1], Monia Blibech, Kameleddine Bouchaala, Hichem Chouayekh*

Laboratoire de Microorganismes et de Biomolécules (LMB), Centre de Biotechnologie de Sfax, Université de Sfax, Route de Sidi Mansour Km 6, BP "1177" 3018 Sfax, Tunisia

ARTICLE INFO

Article history:
Received 13 June 2015
Received in revised form 9 July 2015
Accepted 10 July 2015
Available online 16 July 2015

Keywords:
Phytase
Bacillus amyloliquefaciens US573
High thermostability
Animal feed
Extreme salt tolerance
Phosphorus nutrition

ABSTRACT

The extracellular phytase produced by the *Bacillus amyloliquefaciens* US573 strain, isolated from geothermal soil located in Southern Tunisia was purified and characterized. This calcium-dependent and bile-stable enzyme (PHY US573) was optimally active at pH 7.5 and 70 °C. It showed a good stability at pH ranging from 4 to 10, and especially, an exceptional thermostability as it recovered 50 and 62% of activity after heating for 10 min at 100 and 90 °C, respectively. In addition, PHY US573 was found to be extremely salt-tolerant since it preserved 80 and 95% of activity in the presence of 20 g/l of NaCl and LiCl, respectively. The gene corresponding to PHY US573 was cloned. It encodes a 383 amino acids polypeptide exhibiting 99% identity with the highly thermostable phytases from *Bacillus* sp. MD2 and *B. amyloliquefaciens* DS11 (3 and 5 residues difference, respectively), suggesting the existence of common molecular determinants responsible for their remarkable heat stability. Overall, our findings illustrated that in addition to its high potential for application in feed industry, the salt tolerance of the PHY US573 phytase, may represent an exciting new avenue for improvement of phosphorus-use efficiency of salt-tolerant plants in soils with high salt and phytate content.

1. Introduction

Phytic acid (*myo*-inositol 1,2,3,4,5,6-hexakis dihydrogen phosphate) and its salts (phytates) represent the primary storage from of phosphorus (P) in cereal grains, the principal ingredients of the monogastric animals diets [1]. However, the bioavailability of phytate-P (~85% of the P stored in cereal grains) is limited because the digestive enzyme catalyzing the release of P from phytate molecule is not produced in sufficient amount in the digestive tracts of monogastrics [2,3]. Historically, significant quantities of inorganic P were added to monogastric feeds to meet the animal's P requirement for growth, health, and welfare. However, since the spreading out of exogenous microbial phytases, dietary supplementation of inorganic P has been considerably reduced seeing that these enzymes increase the bioavailability of phytate-P [4,5]. The original impetus to use phytase was to minimize diet costs by reducing the requirement for inclusion of costly inorganic P sources, and to improve production performances above that expected from the increase in P availability [5]. On the other hand, nowadays, there are also documented environmental benefits to the inclusion of phytase throughout lowering manure P excretion [5–8].

Today, phytases are extensively used as feed supplement (present in about 70% of global single-stomached feed products and account for 60% of feed enzymes) with a market volume exceeding US$ 350 million annually and an impressive growth rate of 10% per year [9]. Therefore, searching phytases with higher specific activity and thermostability, lower production cost and withstanding the transient high temperatures applied during feed pelleting process (80–85 °C), is of great importance in feed industry.

There are four types of phytases as classified by protein structures and catalytic properties: histidine acid phosphatases (HAP), β-propeller phytases (BPPs), cysteine phytases, and purple acid phosphatases [10]. Among those especially the fungal HAP phytases with main site of action in the acidic upper part of the animal digestive tract were used as animal feed supplements due to their high specific activity. On the other hand, the extracellular BPPs produced essentially by *Bacillus* strains are presently considered as the best alternative owing to their higher natural thermostability, strict substrate specificity, neutral pH profile and resistance to proteolysis [11]. Preferably, BPPs can be incorporated into monogastric diets

* Corresponding author.
E-mail address: hichem.chouayekh@cbs.rnrt.tn (H. Chouayekh).
[1] These authors contributed equally to this work.

http://dx.doi.org/10.1016/j.ijbiomac.2015.07.014
0141-8130/

in combination with HAP phytases for further improving P release form phytate during the transit of the digesta through the entire gastrointestinal tract [12].

Alternatively, transgenic plants secreting microbial phytases could replace additional supplementation of feed with these enzymes, thereby reducing the production and formulation costs in the animal feed industry. The phytase expression in plant roots increased phytase activity in the rhizosphere and promoted P assimilation from soils with high phytate-P content [13–15].

This study reports the purification, characterization and molecular cloning of the extracellular phytase originating from the *Bacillus amyloliquefaciens* US573 strain newly isolated from geothermal soil located in Southern Tunisia. The biochemical properties of the enzyme (PHY US573) were assessed and compared to the commercial phytases "Ronozyme PL" and "Natuphos" predominantly active at acidic pH values. Finally, the suitability of PHY US573 for application in animal feed industry and in promoting phytate-P assimilation especially by salt-tolerant plants was discussed in view of the enzyme features.

2. Materials and methods

2.1. Substrates and chemicals

Wheat bran was acquired from the local company "Nutrisud/Medimix". Phytic acid sodium salt hydrate from rice (P0109) was purchased from Sigma. All other chemicals used in this study were commercially available in analytic grade.

2.2. Bacterial strains, plasmids, and media

Escherichia coli DH5α was used in this study as host strain for molecular cloning. Cultures of recombinant *E. coli* strains were performed at 37 °C in LB medium, supplemented when necessary, with ampicillin at 100 μg/ml, X-gal at 320 μg/ml and isopropyl-β-D-thiogalactopyranoside (IPTG) at 160 μg/ml. The pGEMT-Easy cloning vector (Promega) was used according to the manufacturer's instructions.

2.3. DNA manipulation

General molecular biology techniques were performed as described in [16]. DNA restriction and modification enzymes were used according to supplier's recommendations. PCR amplifications were performed using Phusion high-fidelity DNA polymerase from Thermo Fisher Scientific. Amplified fragments were purified from agarose gels by using the GFX™ PCR DNA and Gel Band Purification Kit (Amersham Bioscience).

2.4. Screening of isolates for phytase activity

Samples taken from geothermal soils located in Southern Tunisia (El Hamma region) were homogenized with sterile NaCl 9 g/l. Successive dilutions were carried out and spread onto Lauria–Bertani (LB) agar to allow isolation of bacterial strains. Colonies with dissimilar morphological aspect were picked up individually, further purified by streaking and then, screened for their ability to produce phytase on LB agar supplemented with phytic acid at 3 mM by using the two step counterstaining treatment as described in [17]. The isolates developing clear phytic acid hydrolysis zones around were further screened on the optimized liquid wheat bran medium as described in [18]. Cultures were carried out in 500 ml flasks containing 100 ml of medium, inoculated at 0.1 OD_{600} from 19 h-old culture grown on LB and incubated at 37 °C for 72 h under shaking conditions of 250 rpm. Cultures samples were taken at regular intervals and the corresponding supernatants were collected by centrifugation and then, assayed for phytase activity (U/ml). Bacterial cell density (CFU/ml) was monitored during growth by preparing serial decimal dilutions and surface plating on LB agar. Plates were incubated overnight at 37 °C and the resulting colony forming units were counted.

2.5. Taxonomic identification of the selected strain

The isolate US573 having the highest extracellular phytase activity was selected for taxonomic identification. PCR amplification of the 16S rRNA gene (PCR 1) and the 16S–23S intergenic region (PCR 2) from the US573 strain were performed using primers corresponding to highly conserved regions within the rRNA operon of *E. coli* (Oligonucleotide l: 5′AGAGTTTGATCCTGGCTCAG3′ and Oligonucleotide 2: 5′AAGGAGGTGATCCAAGCC3′ for PCR 1; Oligonucleotide 3: 5′GGCTTGGATCACCTCCTCCTT3′ and Oligonucleotide 4: 5′ACTTAGATGTTTCAGTTC3′ for PCR 2) [19]. Amplification conditions were 30 cycles of 30 s at 94 °C, 60 s at 65 °C and 90 s at 72 °C for PCR 1 and 30 cycles of 30 s at 94 °C, 60 s at 52 °C and 60 s at 72 °C for PCR 2.

2.6. Cloning and sequencing of phytase gene from Bacillus amyloliquefaciens US573

To amplify the *phy* US573 gene, we designed two primers based on alignment of several phytases from the *Bacillus* genus. The oligonucleotide sequences were Oligonucleotide 5: 5′CACATTTGACAATTTTCACAAA3′ and Oligonucleotide 6: 5′CATGTTTATTTTCCGCTTCT3′. Chromosomal DNA isolated from *B. amyloliquefaciens* US573 was used as a template and Phusion high-fidelity DNA polymerase for amplification. PCR conditions were: 5 cycles of 30 s at 94 °C, 45 s at 55 °C and 110 s at 72 °C; 25 cycles of 30 s at 94 °C, 45 s at 50 °C and 110 s at 72 °C. The PCR product was purified using the GFX™ PCR DNA following the manufacturer's instructions. Then, the resulting 1247 bp PCR fragment was cloned in pGEMT-Easy Vector (Promega) and transformed into *E. coli* DH5α competent cells. Recombinant colonies were selected on LB agar medium containing ampicillin (100 μg/ml), X-gal (320 μg/ml) and IPTG (160 μg/ml). A recombinant *E. coli* DH5α strain harboring the recombinant plasmid (pIB_2) was used for further studies. The complete nucleotide sequence of the insert was determined by an automated 3100 Genetic Analyser (Applied Biosystems) confirming that pIB_2 contained the whole phytase gene. Homology search was performed using the Basic Local Alignment Search Tool (BLAST).

2.7. Amino acid sequence analyses and homology-modeling

Sequence analysis and multiple alignments were performed using the BLAST and CLUSTALW programs [20]. The prediction of the protein secondary structure was performed using the DSSP program [21], while the editing of the alignment, including the superimposition of secondary structures was conducted using the ESPript program [22]. The automated comparative protein structure homology modeling server, SWISS-MODEL (http://www.expasy.org/swissmod) was used to generate the 3D-structure model of PHY US573 using the TS-Phy structure as template (PDB-code 1POO) [23]. PyMOL (http://www.pymol.org) was used to visualize and analyze the generated model structures and to construct graphical presentations and illustrative figures.

2.8. Purification of PHY US573 and electrophoretic analysis

Phytase was produced after cultivation of the *B. amyloliquefaciens* US573 strain in the optimized wheat bran medium as described in [18], for 110 h at 37 °C. The extracellular phytase

activity was recovered by centrifugation (8000 rpm, 20 min). This crude supernatant was heat treated (90 °C for 2 min) followed by centrifugation at 15,000 rpm for 20 min at 4 °C. Proteins were precipitated between 65 and 85% ammonium sulphate, suspended in 0.1 M Tris–HCl buffer pH 7.5 and dialysed for 48 h. The degree of homogeneity of the purified enzyme was judged by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and Coomassie blue staining as described in [24]

2.9. Preparation of commercial enzymes diluted samples and enzyme assay

Diluted preparations of the commercial phytases "Ronozyme PL" produced by *Peniophora lycii* and "Natuphos" produced by *Aspergillus ficuum* were made as follows: 0.05 g of the coated enzyme sample was stirred in 15 ml of buffer at pH optimal with 0.01% Tween 20 for 1 h at 37 °C. 1.5 ml of the sample were centrifuged for 3 min at maximal speed and then diluted to a final concentration of 1.5 U/ml.

Phytase activity was carried out at 70 °C for 30 min as described in [25]. 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. Protein concentration was determined using Bradford's method with bovine serum albumin as the standard [26].

2.10. Effects of temperature and pH

Temperature profiles of PHY US573 and commercial phytases (diluted preparations) were obtained by determining their activity between 37 and 80 °C at pH optimal. To evaluate the thermal stability of PHY US573, the enzyme solution was incubated in 0.1 M Tris–HCl pH 7.5 at 75 and 90 °C in the presence of 5 mM $CaCl_2$ or without added calcium. For comparison, Natuphos and Ronozyme PL were incubated in 0.1 M acetate buffer pH 5.5 and 5, respectively, at the same temperatures. The residual activity was measured at regular time intervals, right after heat treatment.

The effect of pH on PHY US573, Natuphos and Ronozyme PL activity was investigated at 70, 55 and 55 °C, respectively by using the following buffer solutions at 0.1 M: glycine–HCl, pH 3–4; sodium acetate, pH 4–5.5; Tris–maleate, pH 5.5–7.5; Tris–HCl, pH 7.5–9 and glycine–NaOH, pH 9–9.5. The effect of pH on PHY US573 and commercial phytases stability was performed by incubating these enzymes at pH ranging from 2 to 9 for 1 h at 37 °C, followed by measuring their residual activity.

2.11. Metal ion requirement and EDTA effect

The effect of several metallic ions ($LiCl_2$, $CoSO_4$, $ZnSO_4$, $MgSO_4$ $2H_2O$, $CaCl_2$ $6H_2O$, $HgCl_2$, CsCl, $CuSO_4$, $FeSO_4$, $MnSO_4$, $BaCl_2$) at 2 mM on PHY US573 activity was studied under the optimal conditions. The effect of calcium concentration was investigated by measuring PHY US573 activity between 0 and 10 mM $CaCl_2$ at pH 7.5. Activity was defined as the value relative to the activity detected in the presence of 1 mM $CaCl_2$ at pH 7.5. To study the effect of ethylenediaminetetraacetic acid (EDTA) on PHY US573 activity, reactions were carried out with the addition of EDTA at concentrations ranging from 0 to 10 mM.

2.12. Bile bovine effect and salt tolerance

The effect of bile bovine (B3883-Sigma) on PHY US573 stability was performed by incubating the enzyme in the presence of this bile salt at concentrations ranging from 0 to 0.5% for 1 h at 37 °C, followed by measuring its residual activity under the optimal conditions. The effect of salts (NaCl and LiCl) on PHY US573 activity was studied under the optimal conditions at concentrations ranging from 0 to 50 g/l.

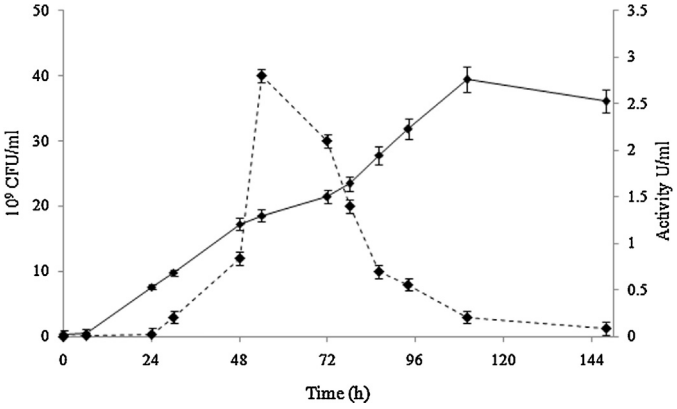

Fig. 1. Extracellular phytase activity (U/ml) (-♦-) and growth (CFU/ml) (-✸-) of the *Bacillus amyloliquefaciens* US573 strain during cultivation on optimized wheat bran medium. Values represent the means of triplicate experiments with comparable results.

2.13. Determination of kinetic parameters

Assays were carried out using a 0.1 M Tris–HCl buffer pH 7.5, 1 mM $CaCl_2$, and 0.1–3 mM substrate (Phytic acid sodium salt hydrate from rice). The enzyme samples were assessed under optimal conditions.

2.14. Substrate specificity

The action of PHY US573 on several phosphorylated substrates was tested in 0.1 M Tris–HCl buffer pH 7.5 supplemented with 1 mM $CaCl_2$ and 3 mM of tested substrate. In addition to sodium phytate, *p*-nitrophenyl phosphate, acetyl phosphate, glucose-1-phosphate, glucose-6-phosphate, ATP, ADP and AMP were also tested as substrates.

3. Results and discussion

3.1. Screening and identification of extracellular phytase producing bacterium

Several bacterial strains newly isolated from geothermal soils located in Southern Tunisia were screened for phytase production on agar medium supplemented with phytic acid. The isolates developing the largest clear zones around were further screened on optimized liquid wheat bran medium [18] to confirm their ability to produce extracellular phytase activity. The selected isolate, namely US573 comes out as the most efficient phytase producer. Kinetics of US573 bacterium growth and extracellular phytase activity (Fig. 1) shows that the exponential growth phase was accompanied with an increase in phytase activity. From 54 h, we note a decline phase in which bacterial growth ceases, but the enzyme secretion continues to increase reaching its maximum value of 2.76 U/ml after a cultivation period of 110 h. The lag observed between maximal growth and highest extracellular phytase production levels can be explained to some extent by the time needed for complete functional recognition and processing of the signal peptide of the phytase precursor by the bacterial secretion machinery as suggested in [18]. The maximal level of phytase production attained by the US573 isolate was comparable to that of *Bacillus laevolacticus* (2.9 U/ml), but higher than the values of 0.64 and 0.40 U/ml corresponding to phytase production by *B. subtilis* US417 and *B. subtilis* VTTE 68013, respectively [25,27,28]. The US573 strain was subjected to genotypic

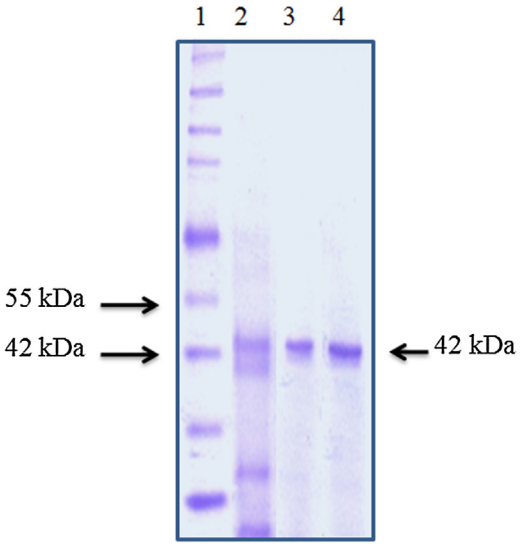

Fig. 2. SDS-PAGE of the purified PHY US573. Lane 1, protein markers (molecular weights in kDa); lane 2, crude supernatant (40 μg); lane 3, sample from lane 2 after heat treatment 10 min at 90 °C (30 μg); lane 4, sample from lane 3 after ammonium sulfate precipitation (30 μg).

identification. Based on the analysis of the determined nucleotide sequences corresponding to the 16S rRNA gene (EMBL accession no. LN864508) and the 16S–23S intergenic spacer (EMBL accession no. LN864509), the US573 bacterium was identified as *B. amyloliquefaciens* US573. Indeed, the DNA sequence of the US573 16S rRNA gene showed 99% identity to that of *B. amyloliquefaciens* FZB42 (accession no. NR075005) and *B. amyloliquefaciens* LSC04 (accession no. EF176772). Pertaining to the 16S–23S intergenic region, it exhibited 96% identity with the intergenic spacer from *B. amyloliquefaciens* FZB42 (accession no. CP000560.1) and *B. amyloliquefaciens* strain DSM7 (accession no. FN597644.1).

3.2. Purification of the mature PHY US573 phytase

The purification steps of the mature PHY US573 phytase were summarized in Table 1. The enzyme was purified at about 6-fold with a yield of 52%. The specific activity of PHY US573 was 27 U/mg, which is comparable to that of TS-Phy of *B. amyloliquefaciens* DS11 and PHY US417 of *B. subtilis* US417 [24,28]. SDS-PAGE showed that the molecular mass of the mature PHY US573 was of 42 kDa (Fig. 2). Previous studies have reported that the molecular mass of phytases purified from *Bacillus* species is ranging from 38 to 47 kDa [25,27,29,30].

3.3. Effect of temperature and pH on PHY US573 activity

The optimum temperature of the native PHY US573 enzyme was 70 °C (Fig. 3A). This temperature was superior to that of the phytases purified from *B. subtilis* VTTE 68013 [27] and *B. subtilis* US417 [25], but comparable to those produced by *B. amyloliquefaciens* DS11 and *Bacillus* sp. MD2 which have been reported to be optimally active at 70 °C and 67–73 °C, respectively [29,31]. Study of the effect of temperature on the activity of Ronozyme PL and Natuphos showed that the optimum of these commercial enzymes was 55 °C (Fig. 3A).

Investigation of the effect of pH on PHY US573 activity illustrated that similar to the majority of the β-propeller phytases from *Bacillus* [25], this enzyme was optimally active at neutral pH range (6–7.5) with the highest activity at pH 7.5, and inactive at pH values (below 4), contrary to Ronozyme PL and Natuphos, which showed optimal activity at pH 5 and 5.5, respectively (Fig. 3B).

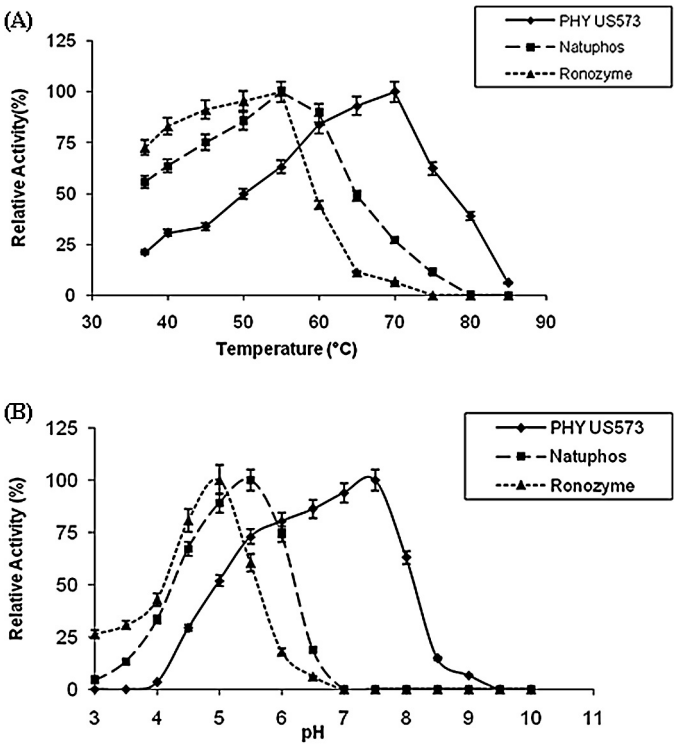

Fig. 3. (A) Temperature profiles of PHY US573, Natuphos and Ronozyme were determined under standard conditions at temperatures ranging from 37 to 80 °C; (B) Effect of pH on the activity of PHY US573, Natuphos and Ronozyme. The results are means of three different assays. Error bars represent the standard deviation.

3.4. Calcium requirement for PHY US573 activity

Similar to the phytases previously characterized from *Bacillus* species [29,31–33], the PHY US573 showed dependence toward calcium for its catalytic activity. Increasing the concentration of this metal ion enhanced the enzyme activity, which attains its maximal level in the presence of 1 mM $CaCl_2$. Calcium ions are presumed to reduce the negative charge around the active site cleft to enable easier fitting of phytic acid as suggested in [34].

3.5. Effect of temperature and pH on PHY US573 stability

The thermostability of PHY US573 was proved to be notably enhanced in the presence of 5 mM calcium. In fact, under such conditions, the enzyme recovered 93, 62.8 and 50% of activity after incubation for 10 min at 75 °C (Fig. 4A), 90 (Fig. 4B) and 100 °C (data not shown), respectively. For comparison, TS-Phy and the recombinant phytase from *Bacillus* sp. MD2 (expressed in *Escherichia coli*), which are considered as the highest thermostable *Bacillus* phytases so far characterized, retained 50 and 40% of activity following incubation for 10 min in the presence of 5 mM $CaCl_2$ at 90 and 100 °C, respectively [29,31]. Pertaining to the commercial enzymes, Ronozyme PL recovered 45 and 33%, while Natuphos retained 53 and 16.04% subsequent to heat treatment for 10 min at 75 and 90 °C, respectively. This may explain why these phytases require to be coated to withstand the high temperatures applied during feed pelleting process.

Assessment of PHY US573 pH stability was measured at pH ranging from 2 to 9 for 1 h at 37 °C. The observed profile (Fig. 4C) illustrated that similar to the Natuphos phytase, PHY US573 exhibited good stability at pH value ranging from 3 to 9, but less stable at pH 2. This indicates the ability of PHY US573 to overcome the acid pH conditions encountered during the transit through the upper part of the animal digestive tract prior to reaching the intestine.

Table 1
Purification of the mature phytase from *Bacillus amyloliquefaciens* US573.

Purification step	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Purification (fold)	Yield (%)
Crude supernatant	1822	405	4.5	1	100
Heat treatment	1718	230	7.4	1.6	94.2
Ammonium sulphate precipitation	945	35	27	6	52

Note: Value represents the arithmetic of two measurements.

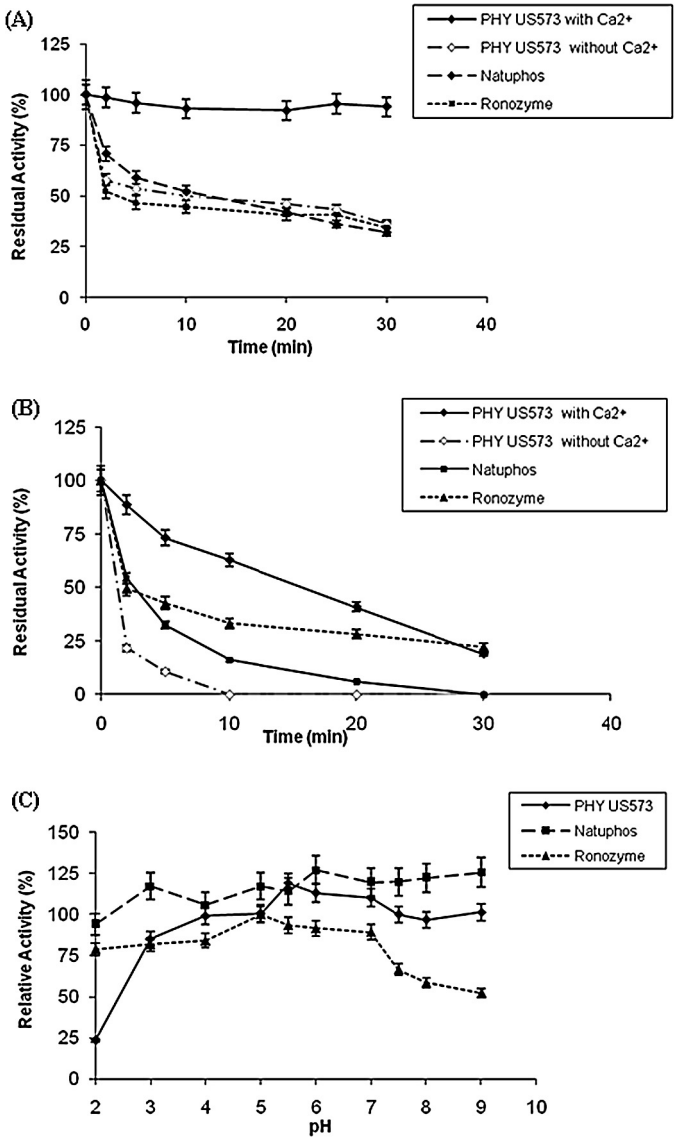

Fig. 4. (A) Thermostability of PHY US573 (in the presence and absence of 5 mM $CaCl_2$ respectively), Natuphos and Ronozyme at 75 °C and (B) at 90 °C, respectively. (C) pH stability of PHY US573, Natuphos and Ronozyme at pH ranging from 2 to 9. Residual activities were measured under standard conditions. The results are means of three different assays. Error bars represent the standard deviation.

The Ronozyme PL enzyme showed also important stability toward the pH values tested, with some limitation at alkaline pH values.

3.6. Substrate specificity and kinetic studies of PHY US573

The ability of PHY US573 to hydrolyze several phosphorylated compounds was tested. In contrast to the histidine acid phytases known for their broad substrate specificity, the PHY US573 enzyme showed high specificity for phytic acid (apparent K_m 1.125 mM). In reality, it was also able to dephosphorylate ATP and ADP (95 and 29% of the activity with phytic acid, respectively)

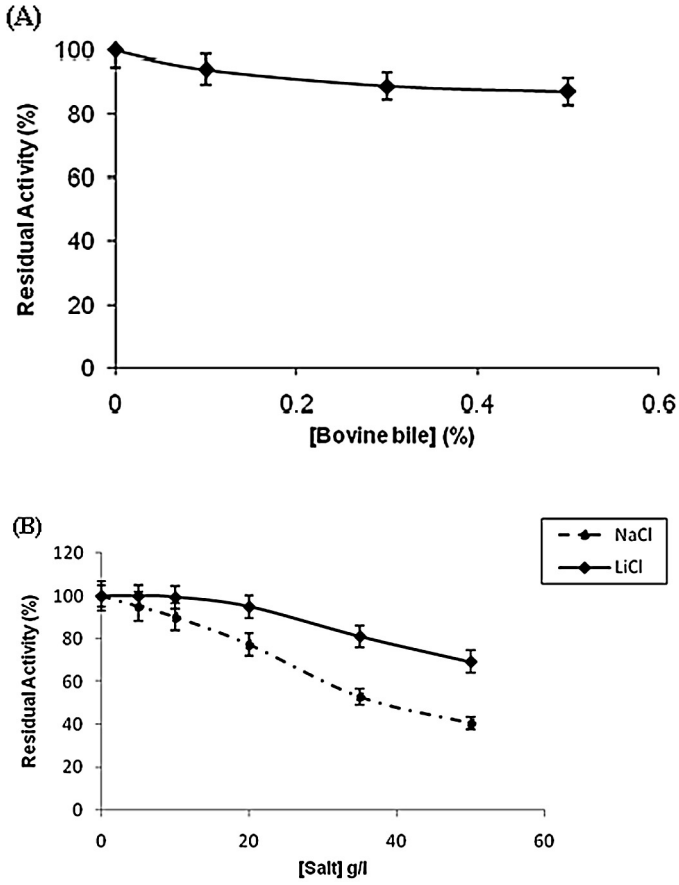

Fig. 5. (A) Effect of bile bovine on the activity of PHY US573. (B) Effect of salts LiCl (-♦-) and NaCl (-■-) on the activity of PHY US573. Activities measured without salt represent the 100%. The results are means of three different assays. Error bars represent the standard deviation.

as it was observed for PhyC from *B. subtilis* VTTE-68013 and PHY US417 [25,27]. On the other substrates tested (AMP, *p*-nitrophenyl phosphate, Glucose-1-phosphate, Glucose-6-phosphate and acetyl phosphate), the enzyme activity was very low (data not shown).

The kinetic parameters of the native PHY US573 for phytic acid were determined at optimal temperature and pH conditions (70 °C; pH 7.5). The apparent K_m, V_{max}, k_{cat} and catalytic efficiency k_{cat}/K_m were 1.125 mM, 27.71 U/mg, 1687.37 min^{-1} and 1499.88 mM^{-1} min^{-1} respectively. The catalytic efficiency of PHY US573 was higher than other phytases characterized from the genus *Bacillus* [35–37] and other bacterial genera like *Erwinia carotovora*, *Pedobacter nyackensis* MJ11 CGMCC 2503, *Klebsiella pneumoniae* and *E. coli* [38,39].

3.7. Bile bovine effect and salt tolerance of PHY US573

The investigation of bile bovine effect on PHY US573 stability revealed that this enzyme exhibits excellent stability when pre-incubated in the presence of this bile salt (Fig. 5A). Indeed, PHY US573 was able to maintain 90% of activity in the presence of 0.5%

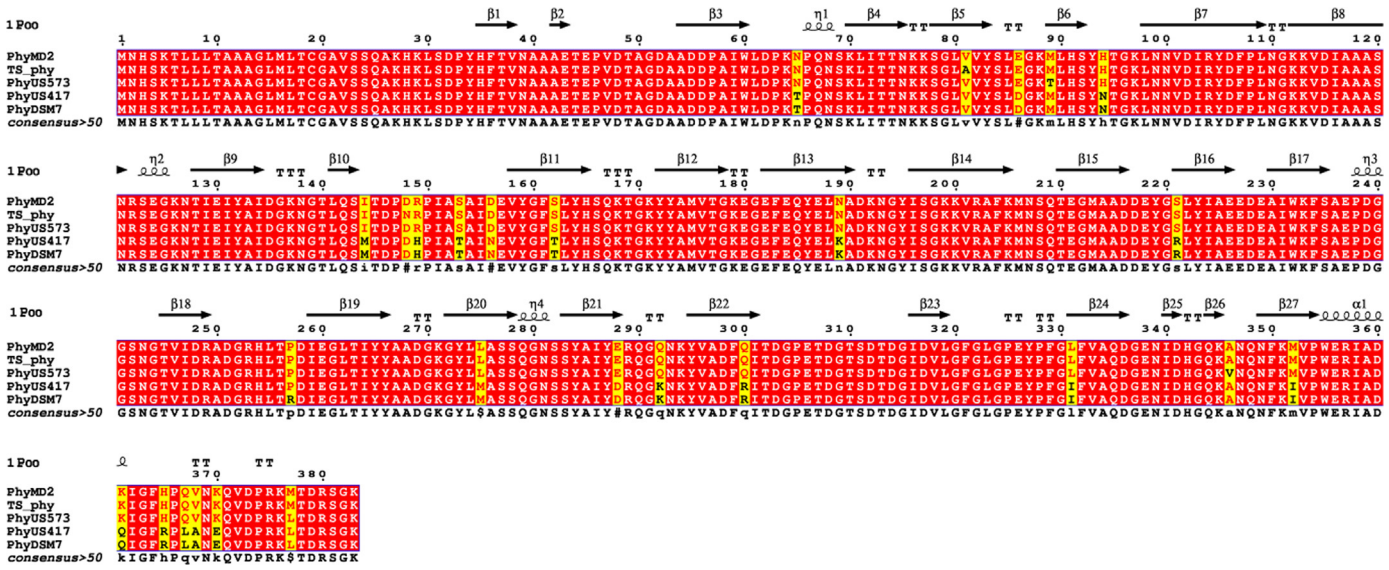

Fig. 6. Structure-based multiple sequence alignment of PHY US573 with other *Bacillus* phytases (PHY US417: phytase from *Bacillus subtilis* US417; Phy DSM7: phytase from *Bacillus amyloliquefaciens* DSM7; TS-Phy: phytase from *Bacillus amyloliquefaciens* DS11; Phy MD2: phytase from *Bacillus sp.* MD2). Residues invariable among sequences are typed in white on a red background; residues conserved within each group are typed in red on a yellow background. Secondary structure elements from known *Bacillus* phytase structure are indicated at the top of the alignment with TS-Phy (PDB-code: 1POO) (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.).

bile bovine (concentration obviously superior to the physiological conditions), while the phytase PHY US417 has been reported to retain only 41% of activity in such conditions [25]. This result illustrate the great ability of PHY US573 to tolerate exposure to bile salts for the duration of transit through the upper part of the animal digestive tract prior to reach the intestinal compartment where it is presumed to be predominantly active.

In addition to the assessment of the PHY US573 potential for application in feed industry, we explored the salt tolerance of this phytase with the aim to evaluate the possibility of using the enzyme (either expressed by transgenic crops or secreted by the bacterium US573 applied as bio-inoculant) for improvement of P-use efficiency by salt-tolerant plants in soils containing high salt and phytate-P contents. Indeed, recent report has estimated that soil salinity is claiming about three hectares of arable land from conventional crop farming every minute. At the same time, the challenge of feeding 9.3 billion people by 2050 is forcing agricultural production into marginal areas, and providing sufficient food for this growing population cannot be achieved without a major breakthrough in crop breeding for salinity tolerance [40]. As illustrated in Fig. 5B, PHY US573 was found to be extremely salt-tolerant since it preserved 80 and 95% of activity in the presence of 20 g/l of NaCl and LiCl, respectively. When LiCl and NaCl were added at 50 g/l the enzymes retained 70 and 35% of its activity (data not shown). This remarkable salt tolerance represents an exciting new avenue for phytase application in agriculture.

3.8. Cloning and structural analysis of PHY US573

The gene corresponding to the phytase of *B. amyloliquefaciens* US573 (*phy* US573) was cloned using sequence information from data based *Bacillus* phytase genes. The specific oligonucleotides 5 and 6 (see Section 2) were designed for the 5′ and 3′ ends. A 1244 bp PCR fragment corresponding to *phy* US573 was amplified using the chromosomal DNA from US573 as template, cloned and the complete DNA sequence was determined from 3 independent clones.

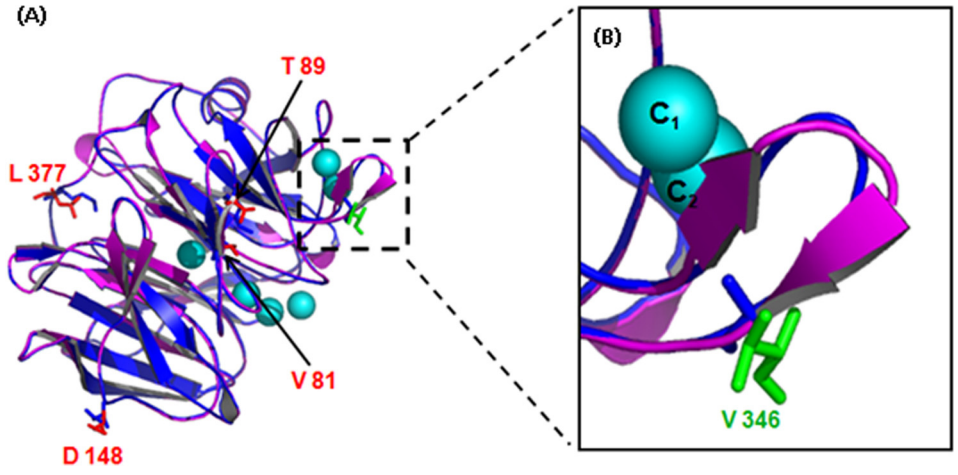

Fig. 7. (A) Superposition of structural models of TS-Phy (blue) and PHY US573 (magenta). The calcium ions are represented as turquoise balls; the V346 residue is shown in green at the close of β sheet calcium ions involved in the thermostability; the residue L377 is presented in red to the surface at a loop; the D148 residue is shown in red on the surface. (B) Position of V 346 in PHY US573. The valine residue is shown in green at a β sheet (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.).

The analysis of the nucleotide sequence obtained (EMBL accession no. LN864510), revealed an ORF encoding a polypeptide of 383 amino acids with a theoretical molecular mass of 42 kDa. The primary structure of the PHY US573 showed significant identities with *Bacillus* phytases (Fig. 6). The highest identity of 99% was observed with the phytases from *B. amyloliquefaciens* DS11 and *B.* sp. MD2 (3 and 5 residues difference, respectively) [29,31].

In this study, through physicochemical characterization we have revealed the higher thermostability of PHY US573 compared to the phytases from *B.* sp. MD2 and *B. amyloliquefaciens* DS11 (TS-Phy), which are up till now considered the most thermostable *Bacillus* phytases [28,30]. Indeed, PHY US573 recovered 62.8 and 50% of activity after incubation for 10 min at 90 and 100 °C in the presence of 5 mM $CaCl_2$ while TS-Phy and the recombinant phytase from *Bacillus* sp. MD2 retained 50 and 40% of activity following incubation for 10 min at 90 and 100 °C, respectively. Given that both enzymes PHY US573 and TS-Phy differ only by five residues (V81, T89, D148, V346 and L377 in PHY US573 and A81, M89, N148, A346 and M377 in TS-Phy) (Fig. 6), we explored the structural features determining this enhanced stability of PHY US573. Indeed, model of PHY US573 was performed on the basis of the crystal structure of the TS-Phy phytase (PDB-code 1POO) [29]. The superposition of these two enzymes showed that 3 of the 5 different residues could probably be responsible for the observed difference in thermostability such as D148, L377 and V346 (Fig. 7).

Comparative structural study of models of both enzymes TS-Phy and PHY US573 (Fig. 7) shows that the residue at position 148 (D/N) was exposed to the surface and generates interactions with water molecules. Also, the residues L/M at position 377 were exposed to the surface and both are hydrophobic amino acids having the same length and the same structural side chain properties. So, these two substitutions N148D and M337L probably did not have a significant impact on enzymes thermostability. Therefore, we thought to explore the effect of the substitution A346V. Indeed, the residue V346 is present in PHY US573 in a loop near the calcium ions binding sites (high affinity). These calcium Ca1 and Ca2 are involved in the thermal stability [33,34]. The presence of V instead of the A in this position could reduce the flexibility of the loop (by decreasing the effect of the side chain in the thermal agitation) and therefore, improves the thermal stability of the enzyme especially at high temperatures as observed in the case of PHY US573 (Fig. 7).

Acknowledgments

This research was funded by the Tunisian Government "Contract Program LMB-CBS" and in part, by the CMCU project (2011–2014) no. 11G0924 "CHOUAYEKH/VIROLLE".

References

[1] M. Tahir, M.Y. Shim, N.E. Ward, C. Smith, E. Foster, A.C. Guney, G.M. Pesti, Poult. Sci. 91 (2012) 928–935.
[2] T.J. Applegate, D.M. Webel, X.G. Lei, Poult. Sci. 82 (2003) 1726–1732.
[3] K. Baruah, A.K. Pal, N.P. Sahu, K.K. Jain, J.S.C. Mukher, D. Debnath, Aquacult. Res. 36 (2005) 803–812.
[4] P.C.M. Simons, H.A.J. Versteegh, A.W. Jongbloed, P.A. Kemme, P. Slump, K.D. Bos, M.G.E. Wolters, R.F. Beudeker, G.J. Verschoor, Br. J. Nutr. 64 (1990) 525–541.
[5] P. Selle, V. Ravindran, Anim. Feed. Sci. Technol. 135 (2007) 1–41.
[6] O.A. Oluskosi, J.S. Sands, O. Adeola, J. Anim. Sci. 85 (2007) 1702–1711.
[7] O. Adeola, A.J. Cowieson, J. Anim. Sci. 89 (2011) 3189–3218.
[8] E. Kebreab, A.V. Hansen, A.B. Strathe, Curr. Opin. Biotechnol. 23 (2012) 872–877.
[9] A. Cowieson, R. Cooper, Introduction to the Event and Overview of the Phytase Market, International Phytase Summit, Washington, DC, 2010.
[10] X.G. Lei, J.M. Porres, E.J. Mullaney, H. Brinch-Pedersen, Phytase: source, structure and application, in: J. Polaina, A.P. MacCabe (Eds.), Industrial Enzymes, Springer, Netherlands, 2007, pp. 505–529.
[11] S. Fu, J. Sun, L. Qian, Z. Li, Appl. Biochem. Biotechnol. 15 (2008) 1–8.
[12] E.A. Elkhalil, K. Manner, R. Borriss, O. Simon, Br. Poult. Sci. 48 (2007) 64–70.
[13] X. Li, Z. Liu, Z. Chi, J. Li, X. Wang, Mycol. Res. 113 (2009) 24–32.
[14] J.F. Liu, X.F. Wang, Q.L. Li, X. Li, G.Y. Zhang, M.G. Li, Z.Y. Ma, Plant Cell Tiss. Organ Cult. 106 (2011) 207–214.
[15] Y. Wang, H. Xu, J.J. Kou, L. Shi, C.Y. Zhang, Plant Soil 362 (2013) 231–246.
[16] J. Sambrook, E.F. Fritsch, T. Maniatis, Molecular Cloning: A Laboratory Manual, 2nd ed., Cold Spring Harbor Laboratory Press, 1989.
[17] H.D. Bae, L.J. Yanke, K.J. Cheng, L.B. Selinger, J. Microbiol. Methods 39 (1999) 17–22.
[18] A. Farhat-Khemakhem, M. Ben Farhat, I. Boukris, W. Bejar, K. Bouchaala, R. Kammoun, E. Maguin, S. Bejar, H. Chouayekh, AMB Express 2 (2012) 10, http://dx.doi.org/10.1186/2191-0855-2-10
[19] V.V. Gurtler, A. Stanisich, Microbiology 142 (1996) 3–16.
[20] J.D. Thompson, D.G. Higgins, T.J. Gibson, Nucleic. Acids. Res. 22 (1994) 4673–4680.
[21] W. Kabsch, C. Sander, Biopolymers 22 (1983) 2577–2637.
[22] P. Gouet, E. Courcelle, D.I. Stuart, F. Metoz, Bioinformatics 15 (1999) 305–308.
[23] S. Shin, N.C. Ha, B.C. Oh, T.K. Oh, B.H. Oh, Structure 9 (2001) 851–858.
[24] M. Lehmann, C. Loch, A. Middendorf, D. Studer, S.F. Lassen, L. Pasamontes, A.P. Van Loon, M. Wyss, Protein Eng. 15 (2002) 403–411.
[25] A. Farhat, H. Chouayekh, M. Ben Farhat, K. Bouchaala, S. Bejar, Mol. Biotechnol. 40 (2008) 127–135.
[26] M.M. Bradford, Anal. Biochem. 72 (1976) 248–254.
[27] J. Kerovuo, M. Lauraeus, P. Nurminen, N. Kalkkinen, J. Apajalahti, Appl. Environ. Microbiol. 64 (1998) 2079–2085.
[28] H.K. Gulati, B.S. Chadha, H.S. Saini, J. Ind. Microbiol. Biotechnol. 34 (2007) 91–98.
[29] Y.O. Kim, H.K. Kim, K.S. Bae, J.H. Yu, T.K. Oh, Enzyme. Microb. Technol. 22 (1998) 2–7.
[30] A.J. Tye, F.K.Y. Siu, T.Y.C. Leung, B.L. Lim, Appl. Microb. Biotechnol. 59 (2002) 190–197.
[31] T.T. Tran, G. Mamo, B. Mattiasson, R. Hatti-Kaul, J. Ind. Microbiol. Biotechnol. 37 (2010) 279–287.
[32] D.E. Rao, K.V. Rao, V.D. Reddy, J. Appl. Microbiol. 105 (2008) 1128–1137.
[33] Y.f. Zeng, T.P. Ko, H.L. Lai, Y.S. Cheng, T.H. Wu, Y. Ma, C.C. Chen, C.H. Yang, K.J. Cheng, C.H. Huang, R.T. Guo, J.R. Liu, J. Mol. Biol. 409 (2011) 214–224.
[34] N.C. Ha, B.C. Oh, S. Shin, H.J. Kim, T.K. Oh, Y.O. Kim, K.Y. Choi, B.H. Oh, Nat. Struct. Biol. 7 (2000) 147–153.
[35] Y.M. Choi, H.J. Suh, J.M. Kim, J. Protein. Chem. 20 (2001) 287–292.
[36] B.C. Oh, B.S. Chang, K.H. Park, N.C. Ha, H. Kim, B.H. Oh, T.K. Oh, Biochemistry 40 (2001) 9669–9676.
[37] M.A. Borgi, S. Boudebbouze, N. Aghajari, F. Szukala, N. Pons, E. Maguin, M. Rhimi, Appl. Microbiol. Biotechnol. 98 (2014) 5937–5947.
[38] H. Huang, H. Luo, Y. Wang, D. Fu, N. Shao, P. Yang, K. Meng, B. Yao, J. Microbiol. Biotechnol. 19 (2009) 1085–1091.
[39] H. Huang, N. Shao, Y. Wang, H. Luo, P. Yang, Z. Zhou, Z. Zhan, B. Yao, Appl. Microbiol. Biotechnol. 83 (2009) 249–259.
[40] S. Shabala, J. Bose, R. Hedrich, Trends Plant Sci. 19 (11) (2014) 687–691.