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RESEARCH ARTICLE

Purification and Characterization of Extracellular Phytase from *Bacillus licheniformis* Isolated from Fish Gut

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Abstract The phytase producing bacterial strain, *Bacillus licheniformis* ONF2 was isolated from the proximal intestine of the freshwater fish, Nile tilapia, *Oreochromis niloticus*. The bacterial phytase was purified 37.45 fold from the crude supernatant by two step chromatography with an overall yield of 21.3 %. It was a monomeric protein with molecular mass of 40–42 kDa. The enzyme was optimally active at pH 6.5–7.5 and at 50 °C temperature and was quite stable at pH ranging from 5.0 to 9.5. It showed temperature stability range of 20–75 °C. The activity of the enzyme was moderately inhibited by 5 mM Mn^{2+}, Mg^{2+} and K^{+} and largely affected by the metal ions Cu^{2+}, Hg^{2+}, Zn^{2+}, Co^{2+} and EDTA but, in the presence of 1 mM $CaCl_2$, the inhibitory effect was less intense. One unit of purified phytase released 1130.3 ± 40.2 and 720.5 ± 35.2 µg of inorganic phosphate per gram of sesame seed meal and soybean meal respectively. The properties of the presently purified phytase to hydrolyze plant phytate and maintaining stability at high temperature make it suitable for applications in animal feed industry.

Keywords Autochthonous bacteria · Calcium · Fish GI tract · Phytase · Purification

Introduction

Phytate or phytic acid is the storage form of phosphorous in plant seeds, grains and cereals where it may constitute up to two-thirds of the total phosphorous (P) content [1]. This phytate phosphorous is poorly available to mono-gastric and agastric animals because they lack the phytate degrading enzyme in their gastrointestinal tract. This limits the dietary incorporation of phytate rich plant ingredients in aquafeeds as this storage form of phosphorous passes unutilized in the feces [2, 3]. Phytic acid also chelates several metal ions such as Ca^{2+}, Mg^{2+}, Zn^{2+} and Fe^{2+}, which forms complexes with proteins, and thus reduces their digestibility and also inhibits certain digestive enzymes like α-amylase, trypsin, acid phosphatase and tyrosinase [4]. Most of the phytate-P is consequently excreted into water which contributes to the phosphorus pollution problems in areas of intensive livestock production [5, 6].

Phytate degrading enzymes or phytases (*myo*-inositol hexa-kis-phosphate phosphohydrolase; EC 3.1.3.8) catalyze the degradation of phytate to inorganic monophosphate and *myo*-inositol and thus eliminate its anti-nutritional properties. Phytases have significant applicability in animal nutrition because of their ability to hydrolyze the phytate content of the plant ingredients which also improves the bioavailability of protein and minerals and minimizes the need for supplemental inorganic phosphate in the diet [6]. Supplemental phytase has been reported to improve phytate phosphorous bioavailability in different animals like pigs, poultry birds and fish [7–9]. Phytase is already being used in food industry for food processing and in US the annual sales of phytase are currently estimated to be about USD 500 million [10].

Phytases are widespread in nature as they are found in plant and animal tissues and also have been reported in several microorganisms including bacteria, yeast and fungi [11]. The filamentous fungus, *Aspergillus niger* is known as the most potent producer of extracellular phytase and industrial production of phytase currently utilizes *Aspergillus*, on which considerable research has been conducted

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[12]. However, due to several properties, such as substrate specificity, resistance to proteolysis and catalytic efficiency, bacterial phytases have some advantages as compared to the fungal enzymes [13].

Phytases from a number of bacteria have been purified and characterized but phytases with thermostability required for application in animal feed has been lacking [14]. In addition, majority of phytases characterized so far, are acidic and exhibit little enzyme activity at neutral and alkaline pH environment while the environment of the digestive tract in most agastric fish is either neutral or alkaline [15]. Therefore, novel phytases with improved properties are of great interest for their probable future applications in animal nutrition. In the present investigation, the main aim was to purify and characterize the extracellular phytase from *Bacillus licheniformis* ONF2, a fish gastrointestinal isolate [16] and evaluate its efficacy in degradation of plant phytate through in vitro assay.

Material and Methods

Materials Used

Most of the chemicals and reagents used were of the analytical grade and were purchased from Merck, Bangalore, India unless otherwise stated. Phytic acid, as a dodecasodium salt, was obtained from Himedia, India and DEAE-Sepharose CL-6B and Sephadex G-100 (G100120) from Sigma-Aldrich, India. The acrylamide, bis-acrylamide and the molecular weight marker (Genei PPMWM) were from Genei, Merck Milipore, India. The phytic acid rich plant ingredients i.e. soybean meal and sesame oilseed meal used in the phytate hydrolysis assay were obtained from the local market.

Identification of Bacterial Isolate by 16S rRNA Gene Sequence Analysis

The phytase-producing bacteria were isolated from the proximal part of intestine of the freshwater fish, Nile tilapia, *Oreochromis niloticus* and was identified by partial 16S rRNA gene sequence analysis. In brief, a single chloroform extraction of genomic DNA was done following Roy et al. [17] and polymerase chain reaction (PCR) amplification of the 16S rRNA genes was done using the forward primer 27F 'AGAGTTTGATCMTGGCTCAG' and the reverse primer 1492R 'GGTTACCTTGTTACGACTT' and a high fidelity PCR polymerase. PCR products were sent to Laragen Inc., Culver city, California, USA for sequencing. The sequenced data were aligned using ClustalW software and analyzed to find the closest homolog of the isolate using a combination of National Center for Biotechnology Information (NCBI) GenBank and RDP (Ribosomal Database Project) database. A phylogenetic tree was made in MEGA 6 software using the Neighbor-joining method with Bootstrap analysis to obtain information on molecular phylogeny.

Preparation of Cell Extract

The modified phytase screening medium (MPSM) was used as the production medium for cultivation of the isolate ONF2. The composition of the MPSM medium [18] was as follows (g/L): glucose, 10.0; L-arginine, 0.2; urea, 1.0; citric acid, 3.0; sodium citrate, 2.0; $MgSO_4 \cdot 7H_2O$, 1.0; sodium phytate, 3.0; $FeSO_4 \cdot 7H_2O$, 0.1; agar, 20.0; 1 M Tris buffer (pH 8.0),100 mL/L; biotin, 50 mg/L; thiamine HCl, 20 mg/L. For preparation of the MPSM, 3 g sodium phytate was dissolved in 100 mL sterile deionized water and then mixed with 900 mL of sterilized sodium phytate-free MPSM, the pH of which was adjusted to 7.0 prior to sterilization. 100 mL of this production media was inoculated with 200 µL of starter culture containing 1×10^6 no of viable bacteria per mL of broth. The production medium was incubated at 37 °C for 96 h with vigorous shaking at 120–140 rpm. After incubation, the bacterial culture was centrifuged at 12,000×*g* for 15 min at 4 °C; the supernatant was collected and used for purification of the enzyme.

Purification of Phytase

All the purification steps were carried out at 4 °C. The culture supernatant was first fractionated by ammonium sulphate precipitation from 20 to 80 % saturation. The precipitate was collected after centrifugation at 12,000×*g* for 10 min, dissolved in 20 mL of Buffer A (20 mM Tris-HCl, pH 7.5) and evaluated for phytase activity. The fraction with highest phytase activity was collected and dialyzed against the same buffer overnight with frequent change to remove the residual $(NH_4)_2SO_4$. The dialyzed suspension was again centrifuged to remove any insoluble particulate matter. The solution was then applied to DEAE-Sepharose anion exchange column (2 cm × 10 cm), equilibrated with buffer A and eluted with a NaCl step gradient (0–1.0 M) prepared in the above mentioned buffer. The fractions with high phytase activity were pooled, concentrated and loaded onto a pre-equilibrated Sephadex G-100 column (2 cm × 30 cm). The enzyme was eluted with buffer A and fractions each of 3 mL were collected. At each stage of purification process phytase activity and total protein content of the fractions were determined.

Procedure for Enzyme Assay

Phytase activity of the culture supernatant and purified enzyme was estimated following the method of Heinonen

and Lahti [19] with slight modification. A reaction mixture containing 100 µL of enzyme and 900 µL of substrate solution (4 mM sodium phytate in 0.25 M sodium acetate buffer, pH 5.5) was incubated at 37 °C for 30 min. To this reaction mixture, 2 mL of freshly prepared acetone ammonium molybdate (AAM) reagent [acetone: 5 N H_2SO_4: 10 mM ammonium molybdate (2:1:1 v/v)] was added. After incubation for another 1 min, the reaction was stopped by adding 200 µL of 1 M citric acid. The color developed was read spectrophotometrically at 405 nm. One unit (U) of phytase activity was defined as the amount of enzyme that released 1 µmol of inorganic phosphate from sodium phytate per minute.

Molecular Mass Determination

The molecular mass of the purified protein was determined by Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE). It was carried out according to Laemmli [20] with 12 % poly-acrylamide (w/v) resolving gel. The protein bands were stained and visualized by Coomassie brilliant blue R-250. The molecular weight marker was run in the adjacent lane with the purified protein for molecular mass determination.

Effect of pH and Temperature on Enzyme Activity

The ability of phytase to degrade phytate in the GI tract of animal is determined by its biochemical properties. Therefore, it is necessary to determine the effect of pH (2.0–9.0 with interval of 0.5) and temperature (20–80 °C with interval of 5 °C) on the enzyme's activity and stability [21]. To determine the optimum pH stability of the purified phytase, the following buffers were used in the previously mentioned phytase assay procedure: 100 mM Glycine–HCl for pH 2.0 and 3.0, 100 mM citrate buffer for pH 4.0–6.0, 100 mM Tris-HCl buffer for pH 7.0–9.0, and 100 mM Glycine–NaOH for pH 10.0. The effect of different temperatures on enzyme activity was determined by carrying out the phytase activity assay at 20–80 °C temperature. The temperature stability of the enzyme was examined by incubating at different temperatures for 15 min, cooled rapidly to 4 °C and assayed for residual phytase activity (at optimal temperature). The pH stability of the purified phytase was also determined by incubating with buffers of different pH for 1 h followed by assessment of the residual enzyme activity.

Protein Determination

Total protein concentration of the fractions and purified enzyme were estimated by the coomassie blue G-250 dye-binding assay using bovine serum albumin as a standard [22].

Effect of Cations and Potential Inhibitors on Enzyme Activity

The effect of cations and other potential inhibitors on the activity of the purified phytase was estimated by pre-incubation of the enzyme with equal volumes of $CaCl_2$, $MgCl_2$, $CuCl_2$, $HgCl_2$, $ZnCl_2$, NaCl, KCl, $CoCl_2$, $MnCl_2$ and EDTA (to a final concentration of 5 mM) for 30 min followed by the standard assay for phytase activity.

In Vitro Phytate Hydrolysis Assay

In vitro assay with soybean meal (*Glycine max*) and sesame (*Sesamum indicum*) seed meal at different pH were carried out following the method of Fu et al. [23] to determine the efficiency of the presently purified phytase in hydrolyzing the phytate phosphorous of the oilseed meal. 0.5 g of finely ground oilseed meal was added to 4.5 mL of buffer (0.25 M citrate buffer for pH 5.0, 5.5, 6.0 and 6.5; 0.25 M Tris–HCl buffer for pH 7.0, 7.5 and 8.0.). To this mixture 0.5 mL of purified phytase was added (1 U g^{-1} of seed meal) and incubated with shaking at 50 °C for 1 h followed by the addition of 5 mL of trichloroacetic acid to terminate the reaction. The released inorganic phosphate was measured by the method of Han et al. [24].

Statistical Analysis

The data were subjected to analysis of variance (ANOVA) using Origin 6.1 software. Duncan's multiple range test [25] was employed to test differences among means. The significance of differences was tested at the significance level $P < 0.05$.

Results and Discussion

Identification of the Isolate

On the basis of the partial 16S rRNA sequencing which was followed by nucleotide homology and phylogenetic analysis, the isolate ONF2 (Genbank Accession no. JX912557.1) was identified as *B. licheniformis* [16]. It showed maximum similarity with the bacterial strain *B. licheniformis* LCR32 (Accession no. FJ976541.1). The phylogenetic relationship of ONF2 with its close relatives in the NCBI Genbank is shown in the dendrogram (Fig. 1).

Phytase activity has been detected previously in a number of bacteria such as, *Aerobacter aerogenes* [26], *Bacillus subtilis* [27], *Bacillus* sp. DS 11 [2],

Bifidobacterium sp. [28], *Citrobacter braakii* [29], *Enterobacter* sp. [30], *Escherichia coli* [31], *Klebsiella pneumoniae* [32], *Lactobacillus sanfranciscensis* [33] *Pseudomonas* sp. [34], *Selenomonas ruminantium* [35] and *Mitsuokella jalaludinii* [36]. Several species of *Bacillus* namely, *B. subtilis*, *Bacillus pumilus*, *Bacillus megaterium*, and *Bacillus coagulans* have been reported to produce the enzyme phytase [21]. The bacterial phytases are generally cell associated in nature with the exception from those of the genera *Bacillus, Enterobacter* and *Lactobacillus amylovorus* [37]. The phytase produced by the presently reported *B. licheniformis* ONF2 was also detected to be extracellular in nature.

Purification and Molecular Properties of the Phytase

The highest phytase production by *B. licheniformis* ONF2 (1.05 U/mg of total protein) was obtained after optimization of nutritional parameters and culture conditions which include incubation for 96 h at 37 °C with MPSM (pH 7.0) as the production medium containing L-arginine as the alternative nitrogen source (Data not shown). The phytase activity of the culture supernatant was low when any medium other than MPSM was used as the production media. The enzyme was first concentrated by $(NH_4)_2SO_4$ fractionation and the target protein was detected in the precipitate after 40 % saturation. After removal of residual $(NH_4)_2SO_4$ by dialysis, the protein was purified by ion-exchange chromatography followed by gel filtration. A summary about the purification scheme is given in Table 1. In each of the purification step the specific activity of the enzyme was increased by several folds. In ion-exchange chromatography the target protein was detected in the fractions after eluting the column with 0.2 M NaCl. Three fractions with highest activity were collected, concentrated and applied to Sephadex G-100 gel filtration chromatography column. The elution profile of the fractions has been represented in the relative chromatograms in Figs. 2A, B. At the end of the three step purification process, the phytate degrading enzyme was purified 37.4 fold with a recovery of 21.3 %. The specific activity was increased up to 39.3 U mg^{-1} of total protein. The purified phytase was determined to be a monomeric protein with molecular mass of 40–42 kDa as it developed a single band after electrophoresis and coomassie blue staining of the polyacrylamide gel (Fig. 2C). Phytases are generally known to be of high molecular mass proteins with their molecular mass ranging from 38 to 700 kDa [38]. Phytases from several species of *Bacillus* such as, *B. subtilis* (37 kDa), *Bacillus amyloliquefaciens* (44 kDa) have been shown to be monomeric proteins with their molecular mass ranging from 38 to 46 kDa [4]. Bacterial phytases are generally slightly smaller than fungal phytases, including those from *Aspergillus fumigatus* (75 kDa), *A. niger* (84 kDa) and *Aspergillus ficuum* (85–100 kDa) [27].

Effect of pH and Temperature on the Activity and Stability of the Purified Phytase

Phytate-degrading enzymes or phytases are special kind of phosphatases which are able to release orthophosphate from phytate and other phosphorylated compounds and

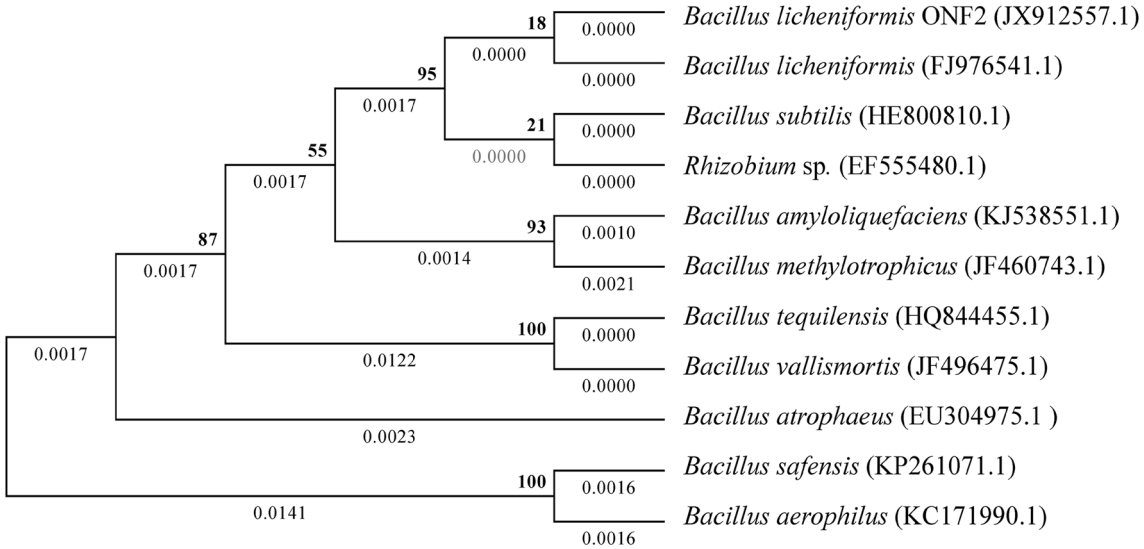

Fig. 1 Dendogram showing phylogenetic relationships of *Bacillus licheniformis* ONF2 with other close homologs based on 16S rRNA gene sequences. The horizontal bars in the phylogenetic tree represent the branch length; similarity and homology of the neighbouring sequences are indicated by the bootstrap values

Table 1 Summary of purification steps of phytase from *Bacillus licheniformis* ONF2

Purification step	Total protein (mg)	Total phytase activity (U[a])	Specific activity (U/mg)	Purification fold	Yield (%)
Culture supernatant	228	240	1.05	1.0	100
$(NH_4)_2SO_4$ precipitation	48.5	168.96	3.48	3.31	70.41
DEAE-Sepharose	7.32	86.2	11.77	11.21	35.92
Sephadex G-100	1.3	51.12	39.32	37.45	21.3

U^a the amount of enzyme required to liberate 1 µmol of inorganic phosphate from sodium phytate per minute

depending on the source of origin they may have discrete catalytic properties. In comparison to plant enzymes, microbial phytases are much more pH and thermo stable as they are active at pH below 3.0 and above of 8.0 [39]. The purified phytase in the present study has an optimal pH of 6.5–7.5 and the enzyme was stable at pH values ranging from 5.0 to 9.5 (Fig. 3). The enzyme retained about 65 % of its activity after incubation for 1 h at pH 9.0. Most of the microbial phytases characterized so far have pH optima in the acidic region (4.5–6.0), but the phytases from *Bacillus* genera are generally seen to have optimum pH of 6.5–8.0 [40]. The purified phytase from *B. licheniformis* ONF2 retained 50 % of its activity after incubation at pH 4.5 for 1 h. Carps generally do not have an acid secreting stomach and have neutral to alkaline pH environment in their GI tract [41]. Therefore, phytases to be used as feed additive for carps, should have higher activities in the neutral to alkaline pH [15] which complied with the pH optima (pH 6.5–7.5) of the presently purified phytase.

For being suitable for incorporation into feed, thermal stability of the enzyme is very important as commercial aquaculture feeds are subjected to pelleting which involves high temperature (60–80 °C) and steam [21]. The presently purified phytase was fairly stable at temperatures from 20 to 75 °C and had maximum activity at 50 °C at neutral pH. Results of enzyme activity at varying temperatures are presented in Fig. 4. Residual phytase activity of 85 % was detected after incubation of the enzyme at 60 °C for 15 min. The purified phytase in the present study required 1 mM $CaCl_2$ for its activity and in its absence the enzyme activity and stability was decreased at high temperature. Similar temperature stability studies have been carried out with phytase from other species of *Bacillus*. The phytase from *Bacillus* sp. DS11 had an optimum temperature at 70 °C and optimal pH of 7, and retained about 50 % of its activity after incubation at 90 °C for 10 min in the presence of 5 mM $CaCl_2$ [2]. Gulati et al. [42] also showed that the partially purified phytase from *Bacillus laevolacticus* with an optimal activity temperature of 70 °C retained 80 % of its residual activity after 3 h of incubation at 60 °C in the presence or absence of $CaCl_2$. The presently purified phytase from *B. licheniformis* exhibited 50 % of residual activity at 80 °C after 15 min of incubation.

Effect of Metal Ions on the Phytase Activity

The effects of various chemicals on enzyme activity were tested in the presence or absence of 1 mM $CaCl_2$. The phytase activity was determined with sodium phytate as substrate. The enzyme activity was observed to be greatly inhibited by EDTA in the absence of $CaCl_2$. The phytase activity was also largely inhibited by the metal ions Cu^{2+}, Hg^{2+}, Zn^{2+}, Co^{2+} and moderately by Mn^{2+}, Mg^{2+} and K^+ at 5 mM concentration. The residual phytase activities of the purified enzyme after incubation with metal ions are given in Table 2. The inhibitory effects might be because of the fact that phytate forms insoluble complexes with metal ions, which may decrease the active concentration of the substrate in the phytase assay [30]. It was also noticed that the enzyme activity was increased by the presence of 1 mM $CaCl_2$ and in its absence the inhibitory effect of the tested chemicals on purified phytase was more intense. Wang et al. [15] suggested that besides stabilizing effect on phytase Ca^{2+} also may play an important role as substrate activator. Ca^{2+} dependencies on enzyme activity and stability of phytases also have been reported from other species of *Bacillus* such as *B. subtilis* and *B. amyloliquefaciens* [43]. This is quite dissimilar to the properties of acid phytases from *Yersinia kristeensenii* and *Citrobacter braakii* which were neither stimulated by calcium ions, nor EDTA have any inhibitory effect on their activity [23, 29] and in fact, the enzyme activity of phytase from *K. pneumoniae* 9-3B was slightly stimulated by EDTA [32].

In Vitro Phytate Hydrolysis by the Purified Phytase

Two phytate rich plant oilseed meals, sesame and soybean were used for the in vitro hydrolysis assay with the purified phytase from *B. licheniformis* strain ONF2. The assay was carried out at pH from 5.0 to 8.0 as the purified phytase was maximally active at the pH of 7.0 with sodium phytate as substrate. The highest release of inorganic phosphate

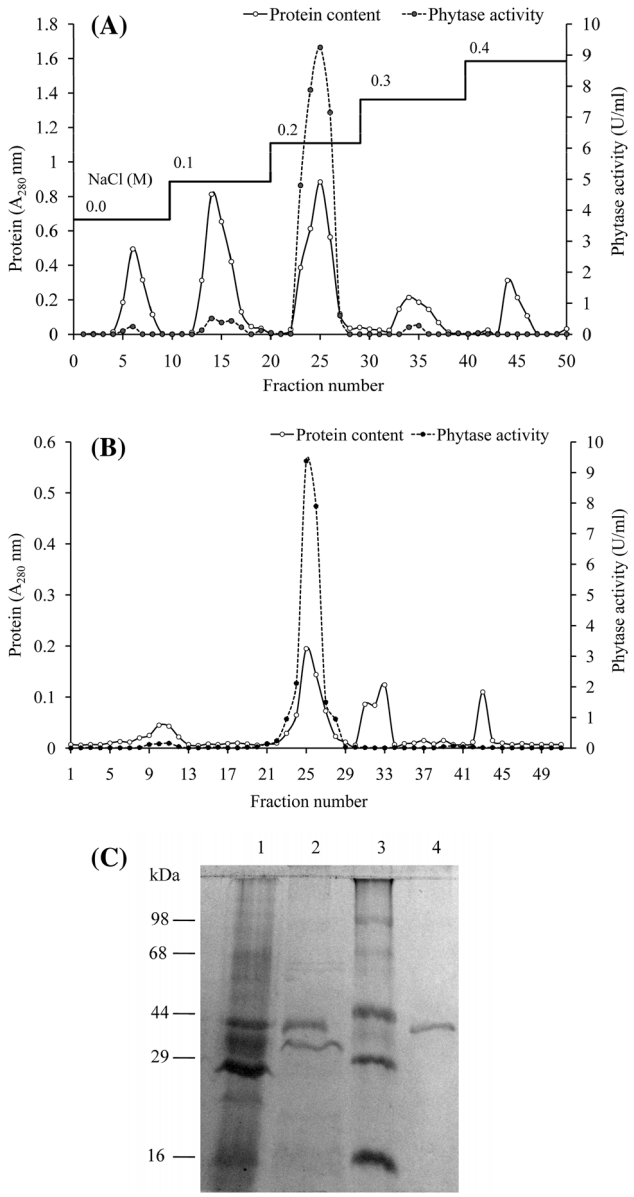

Fig. 2 **A** Ion-exchange chromatography of the purified phytase by DEAE-Sepharose column. The column was pre equilibrated with 20 mM Tris Hcl (pH 7.5) and was eluted with a stepwise gradient of 0–1 M NaCl at a flow rate of 0.8 mL/min. No protein was eluted after 0.4 M NaCl. [U = The amount of enzyme required to liberate 1 µmol of inorganic phosphate per minute]. **B** Purification of phytase by gel filtration chromatography (Sephadex G-100). The column was equilibrated with 20 mM Tris Hcl (pH 7.5) and fractions of 3 mL each were collected with a flow rate of 0.4 mL/min. **C** SDS-PAGE analysis of the phytase from ONF2. *Lane 1* crude supernatant (20 µg), *lane 2* sample after ion-exchange chromatography (8 µg), *lane 3* protein markers (molecular masses in kDa), *lane 4* purified phytase (5 µg)

(1130.3 ± 40.2 µg g^{-1}) was detected at pH 7.0 for sesame seed meal whereas with soybean meal 720.5 ± 35.2 µg of inorganic P was released with 1 U of phytase per gram of seed meal (Fig. 5). Being protein rich the soybean meal has

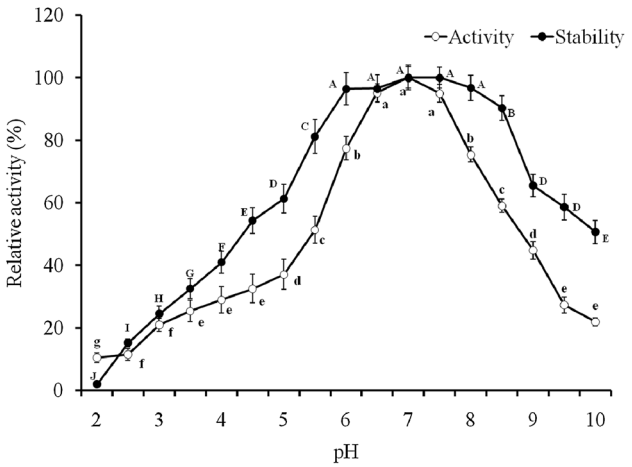

Fig. 3 Effects of pH on the activity and stability of the purified phytase. The enzyme was assayed at various pH from 2 to 10. The highest phytase activity was considered as 100 %. Data (mean ± SE) with different letters are significantly different (n = 3) ($P < 0.05$)

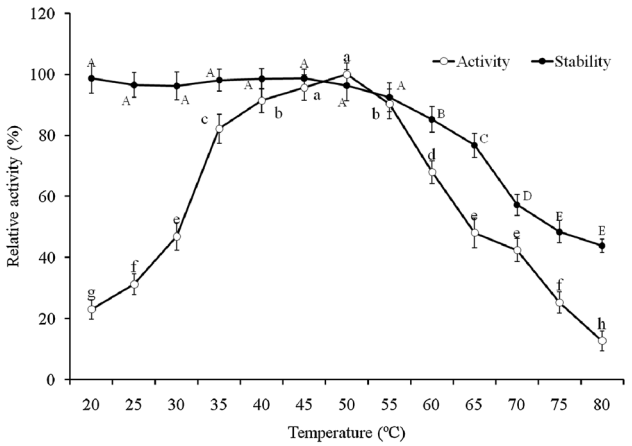

Fig. 4 Effects of temperature on the activity and stability of the purified phytase. The enzyme was assayed at various temperatures (20–80 °C). The highest phytase activity was considered as 100 %. Data (mean ± SE) with different letters are significantly different (n = 3) ($P < 0.05$)

high prospect because of its ability to partially replace the fish meal in aquafeed [44]. The sesame oilseed meal is rich source of phosphorous but most of its P is present in the form of phytic acid which is not digestible and thus unavailable to non-ruminants [9].

Phytate is considered as an important anti-nutritional factor in plant material because of its low bioavailability and when combined with other nutrients, without phytase, it leads to reduced utilization efficiency by monogastric or agastric aquatic animals [45]. Therefore, the phytate content of plant material needs to be hydrolyzed before its incorporation in the diet of aquatic animals. Fu et al. [23] reported inorganic P release from soybean meal by the phytase from *Y. kristeensenii* employing the pH range of

Table 2 Effect of metal ions on the phytase activity from *Bacillus licheniformis* ONF2

Chemicals (5 mM)	Relative activity (%)	
	Without $CaCl_2$	With $CaCl_2$ (1 mM)
Control	85 ± 2.5[b]	100 ± 2.5[a]
$CaCl_2$	98 ± 3.7[a]	96 ± 3.2[b]
$MgCl_2$	75 ± 2.4[c]	92 ± 2.1[c]
$CuCl_2$	34 ± 2.1[e]	76 ± 2.4[e]
$HgCl_2$	35 ± 2.4[e]	58 ± 1.8[g]
$ZnCl_2$	41 ± 1.8[d]	66 ± 2.2[f]
NaCl	89 ± 3.4[b]	103 ± 2.9[a]
KCl	74 ± 3.2[c]	98 ± 3.1[b]
$CoCl_2$	12 ± 0.8[f]	43 ± 1.4[h]
$MnCl_2$	63 ± 1.4[d]	85 ± 2.2[d]
EDTA	11 ± 0.5[f]	78 ± 2.4[e]

The activity at temperature 50 °C and pH 7 in the presence of 1 mM $CaCl_2$ was used as 100 %. Data are mean ± SD of three determinations. Means in the same column with different superscripts are significantly different ($P < 0.05$)

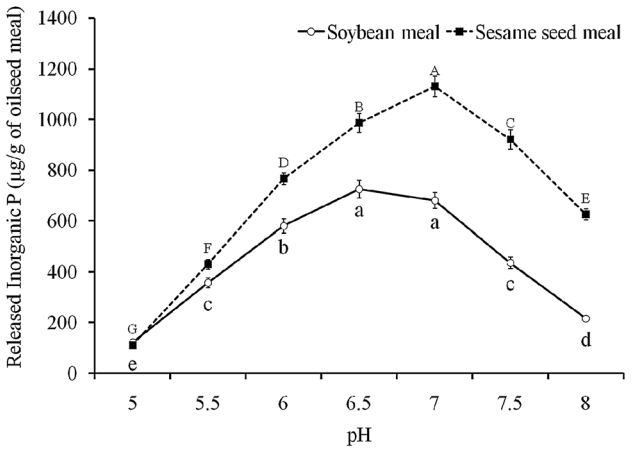

Fig. 5 Phytate hydrolyzing ability of the purified phytase at different pH (5–8.0) with soybean and sesame seed meal as substrate. The reaction was carried out at the optimal temperature of 50 °C. Hydrolysis activity was measured as the amount (µg) of inorganic P released per gram of oilseed meal. Data (mean ± SE) with different letters are significantly different ($n = 3$) ($P < 0.05$)

1.5–5.5 to simulate the conditions of the digestive tract of monogastric animals. The maximal inorganic phosphorus release was obtained at pH 4.5 when either 0.25 or 1 U of enzyme was added per gram of soybean meal. In the present study, the plant phytate hydrolysis activity was carried out at pH 5, 5.5, 6, 6.5, 7, 7.5 which is similar to the pH conditions in the digestive tract of most agastric fish like carps. The efficiency of the presently purified phytase in releasing inorganic P indicates that it might be useful in degrading the phytate compounds of these plant ingredients resulting in improved nutritional value.

Conclusion

In the present study, high activity of the purified phytase from *B. licheniformis* in the pH range of 6.5–7.5 makes it an interesting and suitable candidate for application in animal feed industry. The results of the in vitro hydrolysis test indicated that the enzyme was able to release inorganic P from phytate rich sesame and soybean oilseed meal at pH 6.5–7.0. The enzyme was also seen to have high stability spanning broad temperature range from 20 to 75 °C with activity maxima at 50 °C. These properties of the presently purified phytase indicate that this enzyme also might have probable applications in fish feed industry for improving nutritional value of phytate rich plant feedstuffs. To the authors' knowledge this is the first report regarding purification and characterization of phytase from a fish gastrointestinal isolate. Several commercial phytases like Natuphos, Ronozyme P, Biophos-TS etc. are being used as animal feed additives but the phytase producing bacteria offer some advantages as they can be utilized as live probiotic supplement in feed. The isolate used in the present study was isolated from the intestine of Nile tilapia and is able to survive and colonize in the GI tract environment of the fish. If used as probiotic, they offer permanent solution to the regular application of phytase feed additives. Further research should be conducted to evaluate the potential of the phytase from fish GI tract bacteria in improving the growth and health of commonly cultured freshwater fish.

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