Extracellular phytase from *Aspergillus niger* CFR 335: purification and characterization

B. S. Gunashree · G. Venkateswaran

Revised: 1 January 2014 / Accepted: 23 February 2014 / Published online: 6 August 2014
© Association of Food Scientists & Technologists (India) 2014

Abstract Phytase, that is extensively used as a feed additive is capable of hydrolyzing phytic acid, an antinutrient found in about 60–80 % of all the plant commodities. This enzyme improves the bioavailability of essential minerals such as Ca^{2+} , Mg^{2+} , P, Zn^{2+} , Fe^{3+} , that are bound to phytic acid. An extracellular phytase from a local fungal isolate, *Aspergillus niger* CFR 335 was purified to homogeneity through a three-step column chromatography using DEAE-Sephadex anion exchanger. An active fraction of the enzyme was obtained with NaCl gradient of 2.5 M in DEAE Sephadex column. The enzyme was purified up to 16 fold with a yield of 28.5 %. Substrate specificity studies revealed a highest specific activity of 32.6 ± 3.1 U/mg for sodium phytate with the K_m value of 0.08 ± 0.1 mM. The molecular weight of the enzyme was 66 kDa with an optimum temperature of 30 °C and pH 4.5. Up to 80 % of the activity was retained even after storing the enzyme for 6 months at 4 °C.

Keywords Extracellular phytase · Phytic acid · Monogastrics · *Aspergillus niger* CFR 335 · Purification

Introduction

Plant feed ingredients contain considerable number of components that cannot be digested efficiently by animals, and display antinutritional properties (Bedford 2000). Phytic acid (myo-inositol hexakisphosphate) is the main storage form of phosphorus (P) in cereals, legumes and oilseeds. Depending on species, phytic acid constitutes 60–90 % of all phosphorus present in the grains (Selle et al. 2000). Phytic acid covalently

chelates metal ions such as Ca^{2+} , Mg^{2+} , Mn^{2+} , Zn^{2+} , Fe^{3+} and proteins, rendering them insoluble and unavailable for absorption by monogastric animals (Erdman 1979; Erdman and Forbes 1981). The antinutritional effect of phytic acid may lead to poor quality of eggs, meat products and overall metabolism of monogastrics. In biological systems, hydrolysis of phytic acid to myo-inositol and inorganic phosphorus by phytases (myo-inositol hexakisphosphate phosphohydrolases: EC 3.1.3.8 and EC 3.1.3.26) is an important reaction for energy metabolism, signal transduction pathways and metabolic regulations (Vats and Banerjee 2004). Phytase has the potential to reduce the amount of phosphate in poultry and swine wastes by enhancing phosphorus retention by animals (Nagashima et al. 1999).

Pigs and poultry lack phytase enzyme needed to efficiently digest phytic acid in their feed. As a result, they excrete large amounts of phosphorus into environment resulting in pollution. Supplementation of feed with phytase provides an alternative to alleviate deficiencies, effectively. Conversion of phytic acid into an assimilable form of phosphorus has been an object of biotechnological interest for human as well as animal nutrition. Phytases hydrolyze phytic acid, unlike acid phosphatases which are capable of hydrolyzing other organophosphates (Ullah and Gibson 1987). Fungal phytases form the subfamily of histidine acid phosphatases (Mitchell et al. 1997), where, histidine residue is shown to involve in the catalytic mechanism of enzyme (Xiang et al. 2004).

Phytases have attracted considerable attention from scientists in the areas of nutrition, environmental protection and biotechnology. The potential demand for phytase enzyme in swine and poultry feed is around 8,000 tonnes/annum, but the present production level is 250 tonnes/annum (Wodzinski and Ullah 1996). Phytase supplementation can reduce the amount of phosphorus in manure up to approximately 30 % (Pandey et al. 2001). Hence, with increasing poultry sectors worldwide and market demand for phytase enzyme, there is need for

B. S. Gunashree · G. Venkateswaran (✉)
Department of Food Microbiology, Central Food Technological
Research Institute, Mysore 570 020, India
e-mail: venkatcftri@gmail.com

more production of phytases with high specific activity and broad substrate specificity and their use in food and feed formulations. Fungi are highly exploited groups for phytase production due to their easy cultivation and extracellular production. A study on the optimization of media ingredients for phytase production by *Aspergillus niger* CFR 335 through submerged and solid- state fermentation was reported earlier (Gunashree and Venkateswaran 2008). In this work, an extracellular phytase was produced by *Aspergillus niger* CFR 335 through solid- state fermentation. The crude solution obtained was purified to homogeneity by three- step column chromatography through NaCl gradient DEAE- Sephadex column. Purified enzyme was characterized for its thermostability, pH optima and storage stability.

Materials and methods

Strain used *Aspergillus niger* CFR 335 was isolated through routine mycological procedures from soil sample collected from a local livestock area of Bannur Road, Mysore, India. The fungus was grown in Czapek Dox Agar (CDA) medium [(g/L): sucrose, 30; sodium nitrate, 3; dipotassium hydrogen phosphate, 1; magnesium sulphate, 0.5; potassium chloride, 0.5; ferrous sulphate, 0.01, pH 7.3±0.2] slants and maintained at 4 °C.

All the reagent chemicals were of analytical grade procured from Sigma Chemicals, St. Louis, Missouri, USA, Hi-media laboratories, Pvt. Ltd, Mumbai, India, Qualigens Fine Chemicals, Lucknow, India, Merck Specialities, Pvt. Ltd, New Delhi, India, Sisco Research Laboratory, Pvt. Ltd, Mumbai, India.

Production and extraction of crude phytase Crude enzyme production and extraction through solid-state fermentation of *Aspergillus niger* CFR 335 was carried out by the method of Gunashree and Venkateswaran 2008.

Purification of native phytase All purification steps were carried out at 4 °C unless otherwise stated. The lyophilized

liquid fraction was dissolved in 1:10 w/v 0.2 M Na- acetate buffer (pH 4.5) and then solid ammonium sulfate was added to enzyme solution to give 80 % saturation. The solution was stored overnight for precipitation in cold condition. Precipitate was collected by centrifugation at 5000 × g for 30 min. The protein pellet was suspended in 0.2 M Na- acetate buffer (pH 4.5) and dialyzed against same buffer for overnight. The contents of dialysis bag were centrifuged at 5000 × g for 20 min and the supernatant was collected. This fractionation was subjected to further purification by three step DEAE- Sephadex column chromatography.

The dialyzed fraction from ammonium sulphate fractionation was serially subjected for chromatography on to DEAE- Sephadex G-250, G-150 and G-50 columns (2.5×30 cm, Pharmacia, Biotech), that were washed thoroughly with 0.2 M Na-acetate buffer (pH 4.5). The adsorbed fractions were eluted from the column with a linear gradient of 0 to 2.5 M NaCl with a flow rate of 0.5 ml/min. The eluted fractions were dialysed to remove NaCl before loading to the next ion-exchange column. Active fractions were pooled, lyophilized and used for molecular mass determination using authenticated Sigma standards. HPLC was also performed for these samples to confirm their homogeneity.

Phytase assay Enzyme activity was determined at each purification step by the method described earlier (Heinonen and Lahti 1981). The assay was initiated by mixing 1 ml of diluted (1:10) crude enzyme solution with 0.5 ml of sodium acetate (0.2 M) buffer of pH 4.5 and 0.5 ml of sodium phytate (15 mM) (Sigma chemicals, St. Louis, Missouri, USA). The reaction mixture was incubated at 40°C in a water bath for 45 min. The reaction was terminated by adding 2 ml of 15 % trichloroacetic acid. The assay mixture (0.5 ml) was then mixed with 4 ml of 2:1:1 v/v of acetone, 10 mM ammonium molybdate and 5 N sulphuric acid (AAM solution) and 0.4 ml of 1 M citric acid. The amount of free phosphate released was determined spectrophotometrically at 355 nm. A standard graph was plotted using potassium dihydrogen phosphate with working concentration ranging from 30 to 360 µmol. One Unit is the activity of the enzyme to release 1 µM of

Table 1 Purification scheme of *Aspergillus niger* CFR 335 phytase

Step	Total unit activity of phytase	Protein (mg)	Specific activity (U/mg)	Yield (%)	Purification fold
Crude extract	135.12±14.9	67.30±8.7	2.00±0.84	100	1
Ammonium sulphate fractionation	97.10±6.3	18.17±4.4	5.34±1.52	71.9	2.7
Dialysis	90.20±8.6	10.95±3.6	8.23±1.45	66.8	4.1
DEAE- Sephadex G-250 column chromatography	59.50±9.3	4.96±1.8	12.0±1.63	44.0	6.0
DEAE- Sephadex G-150 column chromatography	47.60±7.2	2.58±1.2	18.4±2.26	35.2	9.2
DEAE- Sephadex G-50 column chromatography	38.50±3.4	1.20±0.8	32.1±2.85	28.5	16.1

Data are given as ± SEM, n=3

Fig. 1 Elution profile of phytase in DEAE- Sephadex G-50 column

inorganic phosphorus from the substrate in 1 min at 40°C. Soluble protein were quantified by the method described earlier (Bradford 1976), using bovine serum albumin, as standard.

Molecular mass determination The concentrated enzyme sample eluted through three chromatography steps was subjected to SDS-PAGE on 10 % gel and the molecular mass was determined according to the method described earlier (Laemmli 1970). The samples were mixed with equal volume of 2X sample buffer [50 mM Tris- HCl buffer, pH 6.8, 2 % SDS, 10 % glycerol (v/v), 5 % β -mercaptoethanol (v/v) and 0.1 % bromophenol blue (w/v)] and kept in boiling water bath for 5 mins prior to loading. Protein molecular weight marker (10 mg/ml) (St. Louis, Missouri, USA) used was a mixture constituting, aprotinin (MW 6,500), lysozyme (MW 14,200), trypsin inhibitor (MW 20,100), carbonic anhydrase (MW 29,000), lactate dehydrogenase (MW 36,000), glutamic dehydrogenase (MW 55,000), bovine serum albumin (MW 66,000), phosphorylase b (MW 97,400), β - galactosidase (MW 116,000) and myosin (MW 205,000). Each well was loaded with 15 μ g of purified protein and electrophoresis was carried out at room temperature for 6 h at 60 V. The gel was stained by silver nitrate method and de-stained by repeatedly washing with 10 % acetic acid and stored in 5 % acetic acid. The molecular weight of phytase was determined using Kodak 1D, Eastman Kodak, Rochester, NY, USA software.

HPLC analysis of purified phytase enzyme The active fractions obtained through ion- exchange chromatography were then subjected to reverse phase HPLC to prove protein's homogeneity. This was conducted on a Shimadzu LC- 10A VP system equipped with a UV detector. A reverse phase C-18 column (Water Radialpak) was equilibrated with a linear gradient of mobile phase comprised of 0.1 % trifluoroacetic

acid in water (solvent A) and 95 % acetonitrile (solvent B). The solvent gradation started with 0 % and rose to 100 % acetonitrile containing 0.1 % trifluoroacetic acid in 20 min. The elution profile was monitored at 280 nm with a flow rate of 1 ml/min.

Characterization of purified enzyme The purified enzyme was characterized for its temperature and pH optima. The temperature studies were carried out by incubating the enzyme reaction mixture at various temperatures ranging from 5 to 60 °C at an interval of 5 °C. All other assay steps were similar to the method discussed earlier. Thermostability profile (80 °C

Fig. 2 SDS-PAGE: Lane 2: Crude enzyme sample, Lane 3: Partially purified enzyme (dialysed sample), Lane 4: Purified enzyme, Lane 7: Standard protein marker

over a 5 min time course up to 60 mins in 0.2 M Na-acetate, pH 4.5) was determined as described earlier (Ullah and Gibson 1987). The optimum pH was determined using borate-phosphate-citrate buffer (g/L boric acid, 5.15; disodium phosphate heptahydrate, 23.09; sodium citrate dehydrate, 30.35) with pH ranging from 2.0 to 8.5 by varying the concentration of boric acid and sodium phosphate. The enzyme samples were diluted and prepared with the corresponding pH buffer. In order to keep the pH stable in the reaction, sodium phytate solutions were also prepared with respective pH buffers. Effect of metal ions on the purified phytase was also studied by incorporating 5 mM concentration of various metal ions such as Al^{3+} , Ca^{2+} , Cu^{2+} , Fe^{3+} , Mg^{2+} , Mn^{2+} , and Zn^{2+} .

Kinetic studies Specific activity and Michaelis-Menten constant (K_m) of phytase was determined using Lineweaver-Burk

plot. Various inorganic phosphates such as sodium phytate, *p*-nitrophenyl phosphate, potassium dihydrogen phosphate, sodium dihydrogen phosphate, ammonium phosphate and phosphoric acid were used at 15 mM concentration in the present study.

Statistical analysis Experimental data were expressed as standard error means ($\pm$ SEM). The data were analyzed using the ANOVA procedures of Statistical Analysis Systems Institute

Fig. 3 a HPLC profile of standard phytase b HPLC profile of partially purified phytase from *Aspergillus niger* CFR 335 c HPLC profile of purified phytase from *Aspergillus niger* CFR 335 (Peak numbered 1 is phytase)

Fig. 4 a Effect of temperature on the activity of purified phytase from *Aspergillus niger* CFR 335 b Thermostability profile (80 °C over 60 min) of purified phytase from *Aspergillus niger* CFR 335 c Storage stability of purified phytase from *Aspergillus niger* CFR 335

(SASI), 1992. The linear regression analyses were used to establish the regression models for estimating phytase activity based on the values of temperature and pH, while the non-linear regression analyses were used to estimate the amount of phosphorus released from different inorganic phosphates. Differences were considered statistically significant at $p < 0.05$.

Results and discussion

Purification studies The various stages of purification are summarized in Table 1. The crude enzyme extract obtained through solid-state fermentation exhibited lowest specific activity of 2.0 U/mg with enzyme activity of 135.12 U/ml. The protein precipitate obtained after 80 % ammonium sulphate fractionation showed 97.1 U/ml enzyme activity with a relatively higher specific activity of 5.34 U/mg than crude sample. The dialyzed sample showed 90.2 U/ml of enzyme activity with a significantly higher specific activity of 8.23 U/mg.

Purified phytase in the present study showed 16-fold higher activity than the crude extract. In the third step, phytase enzyme was eluted through a series of NaCl gradient DEAE-Sephadex column chromatography, where the activities and specific activities obtained in each step (DEAE-Sephadex G-250, G-150 and G-50 column) were 59.5, 47.6, 38.5 U/ml and 12.0, 18.4, 32.1 U/mg respectively. About 50 fractions were eluted rapidly after three chromatography step and the elution pattern is given in Fig. 1. The purified phytase obtained from a thermophilic strain, *Thermomyces lanuginosus* had a specific activity of 24.94 U/mg (Wodzinski and Ullah 1996; Judith et al. 2006). The specific activity and enzyme yield obtained in the present study was significantly higher than earlier reports.

Fig. 5 Effect of pH on the activity of purified phytase from *Aspergillus niger* CFR 335

Table 2 Effect of different metal ions on the purified phytase activity

Metal ions (5 mM)	% Relative phytase activity
Ca ²⁺	122.7±3.8
Cu ²⁺	108.6±3.2
K ⁺	116.2±2.9
Co ²⁺	87.30±2.7
Zn ²⁺	106.4±1.9
Mn ²⁺	82.40±3.1
Mg ²⁺	112.8±3.5
Al ³⁺	73.26±2.6
Fe ³⁺	55.72±2.2

Data are given as ± SEM, $n=3$. The phytase activity in the absence of metal ions was control and regarded as 100 %

The active fractions pooled after last chromatography step was used for molecular mass determination through SDS-PAGE. Silver nitrate staining of the gel showed the extract was pure with a single discrete band (Fig. 2). This is also evident from HPLC chromatograms, showing a single major peak (Fig. 3). Retention time of the peak was compared with authenticated standard phytase obtained from Sigma Chemicals St. Louis, Missouri, USA. The molecular mass of *A. niger* CFR 335 phytase was also found to be 66 kDa. Molecular mass of *Aspergillus niger* CFR 335 phytase is within the range of 60–100 kDa, that is one of the characteristics of phytases from filamentous fungi (Wodzinski and Ullah 1996; Judith et al. 2006). The molecular masses of *A. oryzae* phytase and acid phosphatase were found to be 60 and 70 kDa respectively (Shimizu 1993).

Characterization of purified phytase for temperature The purified phytase showed 100 % relative activity at 30 °C which is an optimum temperature and retention of 50 % activity at 50 °C. Beyond this temperature, activity declined and found <40 % at 60 °C (Fig. 4a). Thermostability studies showed 49 % retention of enzyme activity after subjecting to 80 °C for 20 mins. Above this thermal treatment period, enzyme activity diminished and there was >80 % loss at 60 mins (Fig. 4b). The enzyme obtained from *Aspergillus niger* CFR 335 retained >80 % activity when stored for more than

Table 3 Specific activities and K_m values of purified *Aspergillus niger* CFR 335 phytase for various phosphate compounds

Phosphate compound	Specific activity (U/mg)	K_m (mM)
Sodium Phytate	32.6±3.1	0.08±0.01
Potassium dihydrogen phosphate	18.6±2.6	0.24±0.04
Sodium dihydrogen phosphate	15.7±2.8	0.34±0.04
Ammonium phosphate	9.60±1.72	0.42±0.09
<i>p</i> -Nitrophenyl phosphate	8.30±1.3	0.45±0.08
Phosphoric acid	2.87±1.14	0.82±0.10

Data are given as ± SEM, $n=3$

3 months at room temperature (Fig. 4c). Most fungal phytases characterized till date exhibit temperature optima in the range of 50–60 °C. Extracellular enzymes produced by mesophilic fungi generally display temperature optima below 60 °C although a few of them display higher temperature optima (Manzanares et al. 1997; Chien et al. 2002). This is also evident in the present study. The *Rhizopus*-derived phytase retained 68 % activity when assayed at 39 °C, which is the physiological temperature of pig (Casey and Walsh 2004). The result obtained is in contrast to earlier report (Ullah and Gibson 1987), where, about 40 % of the activity was retained after being subjected to 68 °C for 10 mins. The exposure time in the present study is double, with higher temperatures. This thermostability may be attributed to the extent of glycosylation (Han and Lei 1999). Significantly highest residual phytase activity was shown by one of the phytases at 80 °C, among the five kinds of phytases tested for their thermostability, while the relative activity of other phytases decreased significantly when temperature was above 60 °C (Yin et al. 2007). The optimum temperature of purified *Aspergillus niger* phytase was found to be 50°C (Nagashima et al. 1999). Recombinant *Aspergillus fumigatus* phytase was resistant to heat denaturation and displayed maximal activity at 60°C and above (Judith et al. 2006).

Characterization of purified phytase for optimum pH In the present investigation, the purified phytase enzyme displayed maximum activity at pH 4.5 (Fig. 5). Recombinant *Aspergillus fumigatus* phytase has been shown to have highest activity at a broad pH range (2.5–7.5), with maximal activity between pH 4.0 and 7.0 (Judith et al. 2006). The *Mucor hiemalis* enzyme exhibited maximum activity at pH 5.0–5.5 with 50–100 % in the pH range of 3.5–5.0, which is the characteristic pH of stomach after initial ingestion of feed (Boyce and Walsh 2007).

Effect of metal ions on the purified phytase The results on the effect of various metal ions on purified phytase is summarized in Table 2. The results indicated that the enzyme was activated by $\text{Cu}^{2+} > \text{Ca}^{2+} > \text{Mg}^{2+} > \text{Zn}^{2+}$ while inhibited by $\text{Mn}^{2+} > \text{Fe}^{3+} > \text{Al}^{3+}$. An extracellular phytase from *A. niger* was shown to have molecular weight ~100 kDa with pH and temperature optima of 5.0 and 50 °C respectively. This enzyme was inhibited by Cu^{2+} , Zn^{2+} , Hg^{2+} , Sn^{2+} and Cd^{2+} ions and activated by Ca^{2+} , Mg^{2+} and Mn^{2+} ions (Dvorakova et al. 1997). It is evident from the present study that phytase enzyme is activated by Ca^{2+} , Mg^{2+} .

Kinetic parameters Kinetic studies of enzyme showed that the specific activities with various phosphate compounds as substrates ranged from 2.87 ± 1.14 to 32.61 ± 3.1 and the highest value being for sodium phytate. The order of preference for substrate specificity was sodium phytate > potassium

dihydrogen phosphate > sodium dihydrogen phosphate > ammonium phosphate > *p*-nitrophenyl phosphate > phosphoric acid. The results are summarized in Table 3. This was also confirmed by determining Michaelis-Menten constant (K_m) which showed that the purified phytase was more specific to sodium phytate with a specific activity of 32.6 ± 3.1 which was significantly higher than any other phosphate compounds used in the study. This is in agreement with earlier reports (Wyss et al. 1999).

Conclusions

From the present study it is concluded that, unlike other fungal strains *Aspergillus niger* CFR 335 produces only phytase with pH and temperature optima of 4.5 and 30°C respectively. The purified enzyme also exhibited highest specificity towards sodium phytate than other inorganic phosphates. The enzyme retained up to 80 % activity even after storing for more than 6 months at 4 °C and 2–3 months at room temperature. It has become a challenging issue to obtain an enzyme with broader temperature and pH range with high specific activity and broad substrate specificity which is better suited for animal nutrition and bioavailability of phosphate and other minerals bound in the heterogeneous feed formulations.

References

- Bedford MR (2000) Exogenous enzymes in monogastric nutrition- their current value and future benefits. *Anim Feed Sci Technol* 86:1–13
- Boyce A, Walsh G (2007) Purification and characterisation of an acid phosphatase with Bedford phytase activity from *Mucor hiemalis* Wehmer. *J Biotechnol* 132:82–87
- Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principal of protein dye binding. *Anal Biochem* 72:248–254
- Casey A, Walsh G (2004) Identification and characterization of a phytase of potential commercial interest. *J Biotechnol* 110:313–322
- Chien HCR, Lin LL, Chao SH, Chen CC, Wang WC (2002) Purification, characterization and genetic analysis of a leucine aminopeptidases from *Aspergillus sojae*. *Biochim Biophys Acta Gene Struct Exp* 1576:119–126
- Dvorakova J, Volfova O, Kopecky J (1997) Characterization of phytase produced by *Aspergillus niger*. *Folia Microbiol* 42:349–352
- Erdman JWJ (1979) Oilseed phytates: nutritional implications. *J Am Oil Chem Soc* 56:736–741
- Erdman JWJ, Forbes RM (1981) Effects of soy protein on mineral availability. *J Am Oil Chem Soc* 58:489
- Gunashree BS, Venkateswaran G (2008) Effect of different cultural conditions for phytase production by *Aspergillus niger* CFR 335 in submerged and solid-state fermentation. *J Ind Microbiol Biotechnol* 35:1587–1596

- Han YH, Lei XG (1999) Role of glycosylation in the functional expression of an *Aspergillus niger* phytase (phyA) in *Pichia pastoris*. Arch Biochem Biophys 364:83–90
- Heinonen JK, Lahti RJ (1981) A new and convenient colorimetric determination of inorganic orthophosphate and its application to the assay of inorganic pyrophosphatase. Anal Biochem 113:313–317
- Judith AM, Richard AM, Ronan FGP (2006) Purification and physicochemical characterisation of genetically modified phytases expressed in *Aspergillus awamori*. Bioresour Technol 97:1703–1708
- Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685
- Manzanares P, De Graaff LH, Visser J (1997) Purification and characterization of an α -L-rhamnosidase from *Aspergillus niger*. FEMS Microbiol Lett 157:279–283
- Mitchell DB, Vogel K, Weimann BJ, Pasamontes L, van Loon APGM (1997) Cloning of the phytases from *Emericella nidulans* and the thermophilic fungus *Talaromyces thermophilus*. Biochem Biophys Acta 1353:217–223
- Nagashima T, Tange T, Anazawa H (1999) Dephosphorylation of phytate by using the *Aspergillus niger* phytase with a high affinity for phytate. Appl Environ Microbiol 65(10):4682–4684
- Pandey A, George S, Soccol CR, Rodriguez-Leon JA, Soccol VT (2001) Production, purification and properties of microbial phytases. Bioresour Technol 77:203–214
- Selle PH, Ravindran V, Caldwell RA, Bryden WL (2000) Phytate and phytase: consequences for protein utilisation. Nutr Res Rev 13:255–278
- Shimizu M (1993) Purification and characterization of phytase and acid phosphatase produced by *Aspergillus oryzae* K1. Biosci Biotech Biochem 57:1364–1365
- Ullah AHJ, Gibson DM (1987) Extracellular phytase (E.C. 3.1.3.8) from *Aspergillus ficuum* NRRL 3135: purification and characterization. Prep Biochem 17:63–91
- Vats P, Banerjee UC (2004) Production studies and catalytic properties of phytases (myo-inositol hexakisphosphate phosphohydrolases): an overview. Enzym Microbiol Technol 35:3–14
- Wodzinski RJ, Ullah AHJ (1996) Phytase. Adv Appl Microbiol 42:263–303
- Wyss M, Brugger R, Kronenberger A, Rémy R, Fimbel R, Oesterhelt G, Lehmann M, van Loon APGM (1999) Biochemical characterization of fungal phytases (myo-inositol hexakisphosphate phosphohydrolases): catalytic properties. Appl Environ Microbiol 65(2):367–373
- Xiang T, Liu Q, Deacon AM, Koshy M, Kriksunov IA, Lei XG, Hao Q, Thiel DJ (2004) Crystal structure of a heat-resilient phytase from *Aspergillus fumigatus*, carrying a phosphorylated histidine. J Mol Biol 339:437–445
- Yin QQ, Zheng QH, Kang XT (2007) Biochemical characteristics of phytases from fungi and the transformed microorganism. Anim Feed Sci Technol 132:341–350