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Purification and characterization of two distinct acidic phytases with broad pH stability from *Aspergillus niger* NCIM 563

S. K. Soni · A. Magdum · J. M. Khire

Abstract *Aspergillus niger* NCIM 563 produced two different extracellular phytases (Phy I and Phy II) under submerged fermentation conditions at 30°C in medium containing dextrin-glucose-sodium nitrate-salts. Both the enzymes were purified to homogeneity using Rotavapor concentration, Phenyl-Sepharose column chromatography and Sephacryl S-200 gel filtration. The molecular mass of Phy I and II as determined by SDS–PAGE and gel filtration were 66, 264, 150 and 148 kDa respectively, indicating that Phy I consists of four identical subunits and Phy II is a monomer. The *pI* values of Phy I and II were 3.55 and 3.91, respectively. Phy I was highly acidic with optimum pH of 2.5 and was stable over a broad pH range (1.5–9.0) while Phy II showed a pH optimum of 5.0 with stability in the range of pH 3.5–9.0. Phy I exhibited very broad substrate specificity while Phy II was more specific for sodium phytate. Similarly Phy II was strongly inhibited by Ag^+, Hg^{2+} (1 mM) metal ions and Phy I was partially inhibited. Peptide analysis by Mass Spectrometry (MS) MALDI-TOF also indicated that both the proteins were totally different. The K_m for Phy I and II for sodium phytate was 2.01 and 0.145 mM while V_{max} was 5,018 and 1,671 μmol min^{-1} mg^{-1}, respectively. The N-terminal amino acid sequences of Phy I and Phy II were *FSYGAAIPQQ* and *GVDERFPYTG*, respectively. Phy II showed no homology with Phy I and any other known phytases from the literature suggesting its unique nature. This, according to us, is the first report of two distinct novel phytases from *Aspergillus niger*.

Introduction

Phytase, a phosphomonoesterase, is an enzyme capable of hydrolysing phytate [*myo*-inositol (1,2,3,4,5,6) hexakis-phosphate], the major storage form of phosphorus in cereals and legumes, representing 18–88% of total phosphorus content (Konietzny and Greiner 2002). Phytases (EC 3.1.3.8 and EC 3.1.3.26) belong to the family of histidine acid phosphatases which hydrolyses phytate to liberate inositol and inorganic phosphate (Mullaney et al. 2000). Phytate phosphorus is biologically unavailable to non-ruminants as they lack or secrete a very low level of phytase activity in the intestine. Consequently, the phytate in animal feeds is discharged in the feces of these animals into rivers and seas, resulting in severe pollution of water resources (Mullaney et al. 2000). Similarly, phytic acid is an anti-nutrient, which due to its strong chelating potential can bind essential minerals such as calcium, zinc and copper, rendering them unavailable or poorly available for absorption (Singh 2008). To overcome this difficulty the feed has to be supplemented with inorganic phosphate to meet the nutritional requirements of the animals (Selle and Ravindran 2007). Supplementation of feed with phytases in contrast increases the bioavailability of phytic acid-bound phosphate, facilitating a reduction in the addition of inorganic phosphate to the feed and a decrease in phosphorus excretion in areas of intensive animal husbandry.

Although a large number of bacteria, yeasts and fungi are reported to produce phytase (Vohra and Satyanarayana 2003; Vats and Banerjee 2004; Kaur et al. 2007) fungal phytases are preferred in animal feed due to their high yield

S. K. Soni · A. Magdum · J. M. Khire (✉)
Division of Biochemical Sciences, National Chemical Laboratory, Pune 411 008, India
e-mail: jmkhire@yahoo.com

and more acid tolerance as compared to phytases from bacteria (Kim et al. 1998). Similarly the pH in poultry gut varies from 2.5 to 6.0, thus phytase active and stable in acidic environment is highly preferred (Radcliffe et al. 1998). Among fungi many Aspergilli (Ullah 1988; Vohra and Satyanarayana 2003; Vats and Banerjee 2004; Vats et al. 2004) are known to be active phytase producers. As *Aspergillus niger* is Generally Recognised as Safe (GRAS) it is frequently used in food and feed applications. Earlier we have reported phytase production by *Aspergillus niger* NCIM 563 under submerged fermentation (Soni and Khire 2007; Bhavsar et al. 2008; Shah et al. 2009) which includes production and partial characterization of two types of phytase from *Aspergillus niger* NCIM 563. The present communication reports purification and characterization of two novel phytases (Phy I and Phy II), which according to us, is the first report of two distinct forms of extracellular acidic phytases produced simultaneously under submerged fermentation.

Materials and methods

Chemicals

Phytic acid sodium salt was purchased from Sigma Chemical Company, St Louise, MO, USA. All other chemicals used were of analytical grade and obtained from leading manufacturers including BDH, Sigma and Glaxo. SDS–PAGE and gel filtration markers, Coomassie Brilliant Blue R-250 and Bromophenol Blue were purchased from Sigma Chemical Company, USA Sephacryl S-300, Phenyl-Sepharose CL-4B were obtained from Sigma.

Organism and culture conditions

The strain used throughout the present work was *Aspergillus niger* NCIM 563 (Soni and Khire 2007). It was maintained on Potato Dextrose Agar (PDA) slants. The fungus was grown in modified fermentation medium containing (per 100 ml): Dextrin 5 g; Glucose 2.5 g; $NaNO_3$ 0.86 g; KH_2PO_4 0.004 g; KCl 0.05 g; $MgSO_4 \cdot 7H_2O$ 0.05 g; $FeSO_4 \cdot 7H_2O$ 0.01 g. pH 5.5 before sterilization. Fermentation medium (100 ml in 250-ml Erlenmeyer flasks) was inoculated with 1% (v/v) of spore suspension (5×10^7 spores per ml) prepared by suspending the spores from 7-day-old sporulated slants of *Aspergillus niger* NCIM 563 grown on PDA in 10 ml of sterile distilled water containing 0.01% (v/v) Tween 80 and incubated at 30°C at 200 rev/min. Samples were removed after every 24 h and checked for pH, growth, total residual reducing sugar, extracellular protein and phytase activity.

Assay of phytase and protein

Phytase activity was measured at 55°C as described earlier (Soni and Khire 2007). The reaction for Phy I and Phy II was carried out at pH 2.5 (100 mM Glycine-HCl buffer) and pH 5.0 (100 mM acetate buffer) at 55°C for 30 min, respectively. The liberated inorganic phosphate was measured by a modification of the ammonium molybdate method (Heinohen and Lathi 1981). A freshly prepared 4 ml solution of acetone:2.5 M H_2SO_4:10 mM ammonium molybdate (2:1:1 v/v/v) and 400 μl of 1 M citric acid were added to the assay mixture. Absorbance was measured at 370 nm. One unit of phytase activity (IU) was expressed as the amount of enzyme that liberates 1 μmol phosphorus per minute under standard assay conditions.

Each experiment was carried out in triplicate and the values reported are the mean of three such experiments in which a maximum of 3–5% variability was observed.

Protein concentration in the fermentation broth and in the purified enzyme preparation was determined by the Lowry method as well as measurement of absorbance at 280 nm using BSA as a standard.

Purification of phytase

After fermentation, mycelium was separated by filtration followed by centrifugation at $10,000 \times g$ for 30 min and the clear supernatant was collected. It was further concentrated (50%) by Rotavapor rotary evaporation at 40°C under vacuum and subjected to hydrophobic column chromatography using Phenyl-Sepharose CL-4B (30 ml bed volume), previously equilibrated with 30% ammonium sulphate in 20 mM acetate buffer, pH 2.5. The column was washed thoroughly with 20 bed volumes of the above buffer and eluted with a 120 ml linear decreasing gradient of ammonium sulphate (30–0%) with a flow rate of 20 ml per hour and approximately 3.0 ml fractions were collected. Fractions showing activity at pH 2.5 (Phy I) and pH 5.0 (Phy II) were pooled separately, concentrated by rota vapor and loaded on a Sephacryl S-300 gel filtration column with a flow rate of 12 ml h^{-1} and 2 ml fractions were collected. Unless otherwise mentioned, all the purification procedures were carried out at 4°C.

Determination of molecular mass (M_r)

The molecular mass of purified enzyme was estimated by gel filtration, according to Andrews (1964). The gel filtration column of Sephacryl S-300 (1×150 cm) column equilibrated with 50 mM glycine-HCl buffer, pH 2.5 was calibrated using gel filtration standard molecular mass markers; cytochrome *c* (12.4 kDa), bovine serum albumin (66 kDa),

alcohol dehydrogenase (150 kDa), β-amylase (200 kDa), apoferritin (443 kDa and thyroglobulin (669 kDa).

Electrophoretic techniques, mass spectrometry and N-terminal amino acid sequences analysis

Native PAGE (8%) was performed in a vertical gel apparatus at pH 7.5. Samples containing approximately 5–10 μg of protein were applied to the gel and electrophoresis was carried out at 200 V for 3–4 h. Protein bands were visualized by silver staining (0.4% w/v)(Deutscher 1990).

The subunit molecular mass was determined by SDS–PAGE in a vertical slab gel apparatus at pH 8.3 (Laemmli 1970) using Sigma high molecular mass markers. Isoelectric Focusing Polyacrylamide Gel Electrophoresis (IEF–PAGE) was performed in a tube gel (7.5%) using wide range Ampholytes (pH, 3–10) using a mini scale density gradient isoelectric focusing unit (Chinnatambi et al. 1994). Protein bands were detected either by Coomassie Brilliant Blue R-250 (0.2% w/v) or by silver staining (0.2% w/v) and phytase band was revealed by sodium phytate as a substrate.

Mass spectral analysis was performed on a Voyager-De-STR (Applied Biosystems) MALDI-TOF. A nitrogen laser (337 nm) was used for desorption and ionization. Spectra were acquired in the range of 10–100 kDa, in linear mode with delayed ion extraction and with an accelerating voltage of 25 kV. The low mass ion gate was set at 4,500 Da. All the analyses were performed in four replications. The instrument was calibrated with myoglobulin and bovine serum albumin. In-gel tryptic digestion and analysis of peptides was done according to Bar et al (2007).

For N-terminal sequence analysis, purified protein from the SDS–PAGE was electrotransfered to polyvinylidene difluoride membrane (PVDF, Bio-Rad, Richmond, CA, USA) using a semi-dry blotting system (Nova Blot, Pharmacia Biotech, Sweden) under a constant current of 52 mA for 30 min and the protein band was visualized by Coomassie Blue R-250 stain. The N-terminal amino acid sequence of the first 10 residues of the enzyme was determined by Edman degradation on an Applied Biosystems Procise sequencer at Commonwealth Biotechnologies, Inc., Richmond, Virginia, USA. Reported sequences of phytase from various sources were obtained from the GenBank and SwissProt Databases and a sequence alignment were performed using a Clustal-W sequence alignment program (Clustal W).

pH and temperature effect

The influence of pH on enzyme activity was determined by assaying phytase activity over the pH range 1.5–9.0, using 50 mM glycine-HCl buffer (pH, 1.5–4.5), acetate buffer

(pH, 4.0–6.5) and Tris–HCl buffer (pH, 6.5–9.0) by the standard assay method. The pH stability was also determined in a same pH range by pre-incubating enzyme samples in above buffers at room temperature for a period of 12 h, aliquots were removed and immediately assayed for residual enzyme activity by the standard assay method.

The optimum temperature of phytase was determined by performing assays at temperatures 30–70°C. The temperature stability was also determined by incubating enzyme samples over a same temperature range for the period of 60 min. Aliquots (100 μl) were withdrawn after suitable time interval and residual enzyme activity was determined by the standard assay method.

Effect of various metal ions, sugars and inhibitor reagents

A suitably diluted enzyme (100 μl) in 50 mM Tris–HCl buffer, pH 7.0, was pre-incubated with various metal salts, sugars and inhibitor reagents at room temperature for 10 min and subsequently residual activity of the samples was determined by standard assay method.

Substrate specificity and kinetic studies

The relative substrate specificity of phytase towards various substrates was determined by varying the substrate concentrations from 0.1 to 10 mM. Assays were carried out at 55°C under standard assay conditions. The kinetic rate constants, K_m, V_{max}, K_{cat}, and K_{cat}/K_m were determined graphically from Lineweaver and Burk plots (1934).

Amino acid analysis

The amino acid composition of purified Phy I and II was determined on a liquid phase automated amino acid analyser (Hewlett Packard Ti series 1050, equipped with a fluorescence detector) after hydrolysis in 6 M HCl for 20 h at 110°C. Total cysteine content was determined by the method of Habeeb (1972) and total tryptophan was determined according to Spande and Witkop (1967).

Results and discussion

Purification of phytases

The purification of Phy I and Phy II was carried out as summarized in Tables 1 and 2. The crude culture supernatant was first concentrated (50%) on a Rotavapor and purified by adsorption and elution ion-exchange chromatography on Phenyl-Sepharose CL-4B, followed by gel filtration on Sephacryl S-300. Major activities were eluted

in two peaks with a reverse gradient of ammonium sulphate (30–0%) in 20 mM acetate buffer, pH 2.5. The first peak was obtained at 15% ammonium sulphate with higher enzyme activity at pH 5.0 (Phy II), while the second peak was obtained at 5% ammonium sulphate concentration in 20 mM acetate buffer with higher enzyme activity at pH 2.5 (Phy I). These fractions were dialysed, concentrated and applied to a gel filtration column. The purified Phy I and II had a specific activity of 3436.4 and 1215.3 Umg^{-1} with 16.7- and 14.3-fold purification, respectively. It is reported in the literature (Ullah and Cummins 1987, 1988; Ullah and Gibson 1987) that *A. niger* NRRL 3135 phytases, viz., phyA, phyB, and pH 6.0 optimum acid phosphatase were secreted in relatively higher amounts under phosphate starvation conditions in starch medium, and only 5- to 25-fold purification was required to achieve near homogeneity using ion-exchange chromatography and chromatofocussing.

Determination of molecular mass, isoelectric point and glycosylation

The subunit molecular masses of purified Phy I and II were determined to be 66 and 150–160 kDa, respectively, by SDS–PAGE analysis (Fig. 1a), while that of native enzyme was found to be 264 and 150–160 kDa, respectively, by gel filtration on Sephacryl S-300 (Table 3). This suggests that Phy I is a tetramer and Phy II is a monomer. Dimeric Phy B has been reported from *Aspergillus niger* NRRL 3135 which has a molecular mass of 68 kDa (Ullah and Sethumadhavan 1988). The purity of phytases was further confirmed by IEF which showed a single band with p*I* value of ∼3.65 and 3.95 for Phy I and Phy II, respectively (Fig. 1b). However MALDI data revealed the molecular weight of Phy I and II to be 66 and 74 kDa (Fig. 2). Thus
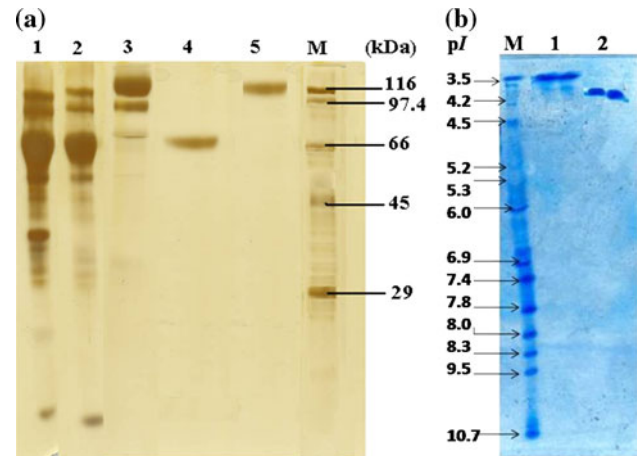

Fig. 1 a SDS–PAGE of purified Phy I and Phy II from *Aspergillus niger* NCIM 563. *Lane M*: Molecular weight markers. *Lane 1*: Crude filtrate (after rota vapour conc.). *Lane 2*: Phenyl Sepharose elute (with 5% w/v Ammonium sulphate). *Lane 3*: Phenyl Sepharose elute (with 15% w/v Ammonium sulphate). *Lane 4*: Purified Phy I. *Lane 5*: Purified Phy II. **b** Iso-electric focusing of purified Phy I and Phy II. *Lane M*: Standard p*I* markers (Pharmacia). *Lane 1*: Purified Phy I. *Lane 2*: Purified Phy II

the molecular weight of Phy II by MALDI-TOF was half the molecular weight shown by SDS gel electrophoresis. This could be because of the doubly charged species of Phy II during ionization process of MALDI-TOF spectrometry (Korwar et al. 2008).

Effect of pH and temperature on phytases

Table 3 indicates that the optimum temperature of Phy I and II was 55°C but optimum pH was 2.5 and 5.0, respectively (Data not shown). Purified phytase from *Aspergillus niger* van Teighem also exhibited maximum activity at pH 2.5 and 52–55°C (Vats and Banerjee 2004).

Table 1 Summary of purification of Phy I from *A. niger* NCIM 563

Purification steps	Total protein (mg)	Total activity (Units)	Specific activity (Units/mg)	Purification (Fold)	Recovery (%)
Culture filtrate	242	50,000	206	1	100
Rotavapor concentration	169	41,130	243.3	1.18	82.20
Phenyl-Sepharose CL-4B (5%)	17.6	31,240	1,775	8.61	62.48
Sephacryl S-200	4.4	15,120	3436.36	16.67	30.24

Table 2 Summary of purifications steps for Phy II from *Aspergillus niger* NCIM 563

Purification steps	Total protein (mg)	Total activity (Units)	Specific activity (Units/mg)	Purification (Fold)	Recovery (%)
Culture filtrate	242	20,600	85.12	1	100
Rotavapor concentration	169	16,312	96.5	1.13	79.10
Phenyl-Sepharose CL-4B (5%)	14.0	10,520	751	8.83	51.06
Sephacryl S-200	4.5	5,469	1215.33	14.29	26.55

Table 3 Some molecular and biochemical properties of purified Phy I and Phy II from *Aspergillus niger* NCIM 563

Properties	Values	
	Phy I	Phy II
pH		
Optimum	2.5	5.0
Stability	2.0–9.0	4.0–8.5
Temperature		
Optimum	55°C	55°C
Stability	$t_{1/2}$ at 55°C, 6–8 h	$t_{1/2}$ at 55°C, 10–12 h
Isoelectric point	3.65	3.95
Molecular mass (kDa)		
Native	264	150–160
Subunit (SDS)	66	150
MALDI	66	74
Glycoprotein nature	Glycosylated	Non-glycosylated
Substrate specificity	Broad	Specific for Na phytate

However phytase produced from *Aspergillus niger* CFR335 either under solid state or submerged fermentation showed phytase activity at pH 4.5 only (Gunashree and Venkateswaran 2008). Similarly Phy I had a half life of 6–8 h at 55°C while that of Phy II was 10–12 h. Several other microbial phytases (Konietzny and Greiner 2002) and various commercial phytases display an optimum temperature range of 45–55°C (Boyce and Walsh 2006). Phy I showed broad pH stability (pH 2–9) while Phy II was stable between pH 4.0 to 8.5 (Data not shown). After 65°C for 1.5 h, Phy I retained 40% activity while Phy II retained 55% of its original activity. However crude phytase from *Aspergillus niger* NCIM 563 was found to retain 100% of the original enzyme activity in the presence of 12% skim milk after treatment at 70°C for 3 min (Shah et al. 2009).

Effect of metal ions and other reagents on phytase activity

The effect of various metal ions (Fig. 3) and other reagents (Table 4) on Phy I and II were investigated. It was observed that Phy II activity was completely inhibited by Hg^{2+} at 1 mM concentrations, while Phy I retained 71% of its original activity. Fe^{3+} showed a negative effect (85%inhibition) on Phy I activity at 1 mM concentrations. Phy II was strongly inhibited by Cu^{2+} (retained 11%). The phytase from *Saccharomyces cerevisiae* was totally inhibited by Hg^{2+} and strongly inhibited by Fe^{2+} at 5 mM concentrations (In et al. 2009). The presence of EDTA stimulated Phy I activity, while showing slight inhibition of Phy II activity. Phytase from the marine yeast *Kodamaea ohmeri* BG3 also showed slight activation in phytase activity at concentrations up to 5 mM (Xiaoyu et al. 2008).

However SDS showed negative effect on both Phy I and II. Oxalic acid and pCMB also showed a stimulating effect on Phy I activity (Table 4). Phytase from *Aspergillus niger* van Teighem, however, was not affected by most metal ions, inhibitors and organic solvents (Vats and Banerjee 2004).

Substrate specificity

The hydrolysis of several phosphate esters (3 mM) by purified Phy I and II was examined by measuring the release of free phosphate. The relative rate of hydrolysis of various substrates is summarized in Table 5. Phy I showed considerably broader substrate specificity and was noticeably active with other phosphate esters including *p*-nitrophenyl phosphate, a general substrate for acidic phosphatase. Similar results were also recorded for the phytase from *Saccharomyces cerevisiae* CY strain (In et al. 2009) and *Mucor hiemalis* (Boyce and Walsh 2007). Phytases derived from *Aspergillus niger*, namely Allzyme™ and Natuphos® also displayed broad substrate specificity (Martin et al. 2006). Many other fungal phytases also exhibit broad substrate specificity, which is desirable in terms of animal feed application (Wyss et al. 1999).

Kinetics

Results of the kinetic studies (Table 6) indicated that K_m values for sodium phytate (2.01 and 0.145 mM, respectively) were lower than calcium phytate (37.27 mM & 1.14 mM, respectively) indicating that Phy I and II showed more affinity towards sodium phytate. However Phy I and ll showed higher V_{max} (26,674 and 2,205 $\mu mol\ min^{-1}\ mg^{-1}$ respectively) for calcium phosphate. Lower K_m values (0.04–0.44 mM) for purified phytases from *Aspergillus niger* 92 and *Aspergillus niger* NRRL 3135 (Ullah and Gibson 1987) have been reported. Similarly among all substrates for phytase activity the turnover number (K_{cat}) values of both phytases (Phy I and II) was higher with calcium phytate as substrate. Furthermore the ratio of K_{cat}/K_m i.e. catalytic efficiency, showed that Phy II degraded sodium phytate and calcium phytate, 2.62- and 1.53-fold more efficiently than Phy I. The estimated K_{cat} of the phytase I and II as computed from the kinetic linearity experiment was 2.2×10^4 and $4.2 \times 10^3\ s^{-1}$ with a catalytic efficiency of 1.1×10^4 and $2.8 \times 10^4\ M^{-1}s^{-1}$ respectively. Despite the fact that the phytase I studied here showed a pH optimum similar to that of phyB, it showed marked differences in catalytic constants from the previously reported phytase (Ullah and Sethumadhavan 1998), which was found to possess a comparatively high affinity for phytate, with a K_m of 0.103 mM and catalytic turnover of $628\ s^{-1}$. However, the phytase studied here, although

Fig. 2 MALDI-TOF analysis of peptides after in gel tryptic digestion of Phy I and Phy II. **a** Peptides of Phy I [Molecular weight of peptides (Da) 1563.3011, 2273.9826, 2304.9520, 2611.8672, 2928.8716, 3226.8563]. *Inset*: Molecular weight of Phy I by MALDI-TOF. **b** Peptides of Phy II [Molecular weight of peptides (Da) 2176.9194, 2214.9406, 2464.9820, 2693.8450, 3494.7825] *Inset*: Molecular weight of Phy II by MALDI-TOF
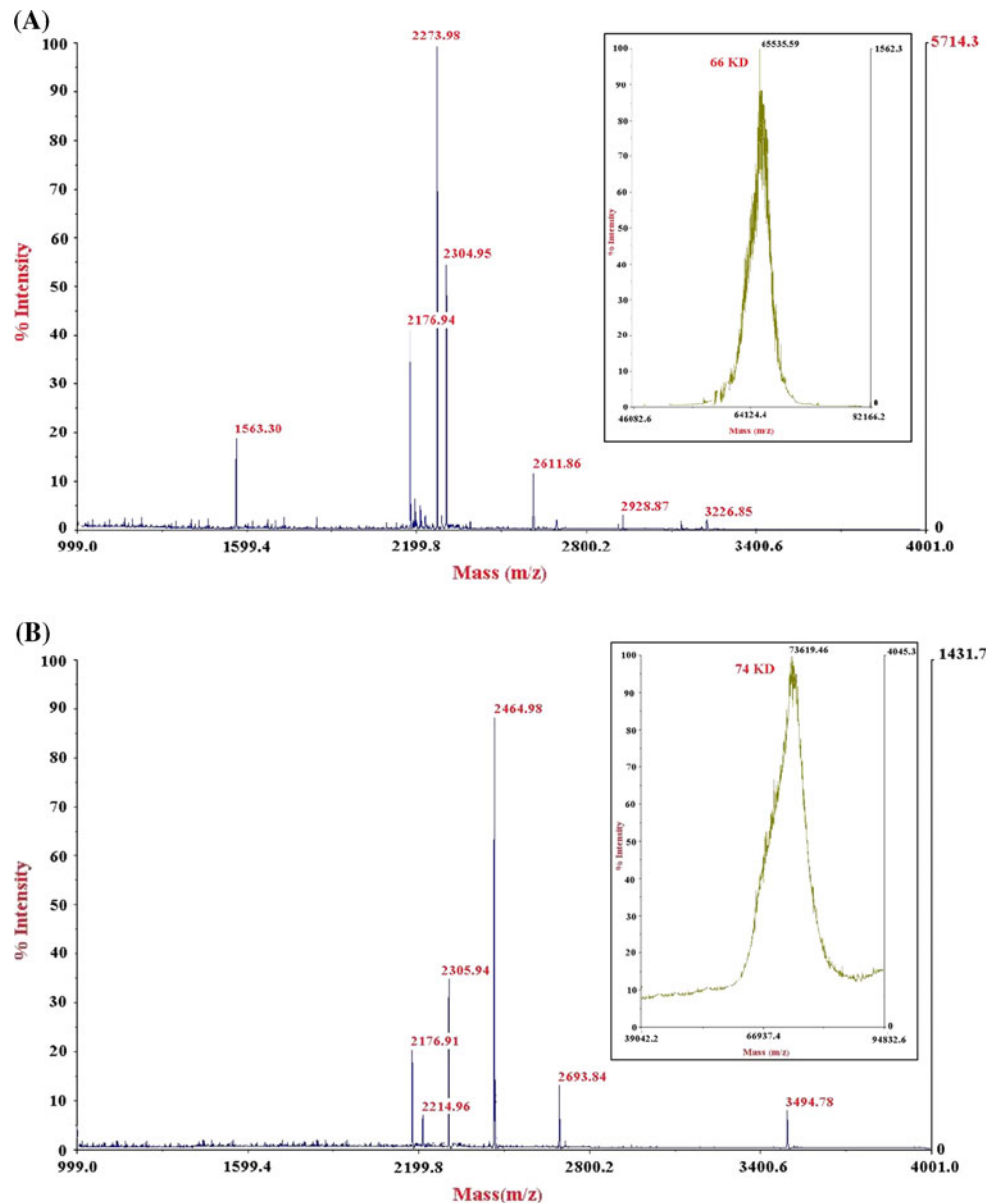
displaying a higher K_m of 0.145 mM, was much more efficient in hydrolysing phytate, with a higher catalytic turnover and approximately 80% greater catalytic efficiency (6.1×10^6 $M^{-1}s^{-1}$) than phyB (Ullah and Sethumadhavan 1988). Phytase from *Aspergillus niger* van Teighem reported by Vats and Banerjee (2005) showed V_{max} and K_m 1,074 IU/ml and .606 mM, respectively, with a K_{cat} 3×10^5 s^{-1} and catalytic efficiency of 3.69×10^8 $M^{-1}s^{-1}$.

N-terminal sequence

The N-terminal amino acid sequences of the first 10 amino acid residues of purified Phy I and II were found to be *FSYGAAIPQQ* and *GVDERFPYTG,* respectively.

The comparison of the N-terminal amino acid sequence of Phy I and Phy II from other Aspergilli with other selected examples available in the literature is given in Table 7. The sequence of Phy II was different from known phytases. However, Phy I showed strong homology with the N-terminal sequence of *Aspergillus ficuum* pH 2.5 optimum acid phosphatase, which was later referred to as *phyB* phytase (Ehrlich et al. 1993; Vats and Banerjee 2006).

Amino acid analysis

The amino acid composition(Table 8) of the purified phytases I and II revealed the enzymes to have 2,471, 1,448 amino acid residues and a calculated molecular weight of about 271.81 and 159.29 kDa respectively. The content of
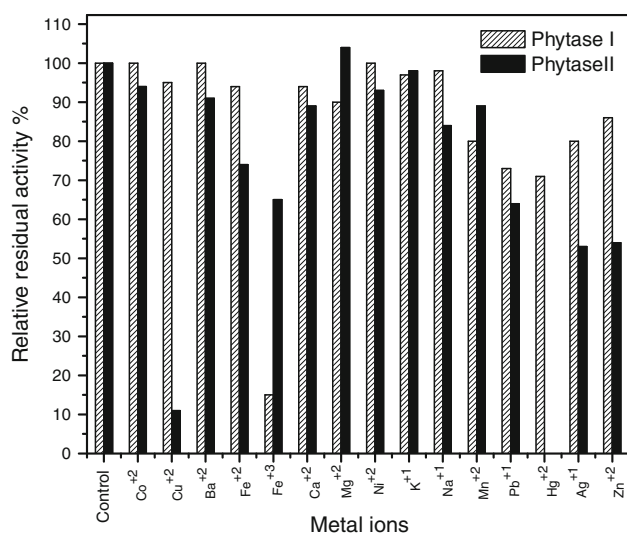
Fig. 3 Effect of metal ions on Phy I and Phy II activity

Table 4 Effect of various reagents on phytase activity

Solvents and reagents	Residual relative activity (%)	
	Phy I	Phy II
None (control)	100	100
Acetic acid (0.1%)	95	92
Oxalic acid (1 mM)	194	91
DTT (1 mM)	101	97
EDTA (1 mM)	181	88
Sodium azide (1 mM)	101	97
SDS (1 mM)	4	4
pCMB (1 mM)	129	77
Triton X-100 (0.1%)	101	93
Tween 80 (0.1%)	113	89
Tween 20 (0.1%)	114	94
Glycerol (0.1%)	97	95
Toluene (0.1%)	105	91
β-Mercaptoethanol (0.1%)	105	93
Ethylene glycol (0.1%)	104	92

A suitably diluted enzyme was assayed with different solvents (0.1%) and reagents (1 mM) in reaction mixture. Activities are expressed as percentage of activity compare to the control sample

hydrophobic or apolar amino acids (leucine, isoleucine, valine, methionine, proline, alanine, phenylalanine, tryptophan) of Phy I and II is about 40.3 and 38.4% (residues/mol). Polar amino acids (Gly, Ser, Thr, Cys, Asx, Glx, Tyr) content is 52.6 and 53.3%. The Asx and Glx content is about 22.5 and 19.8% and outnumbered the basic amino acids (Lys, Arg, His) about 7.1 and 8.3%. The enzymes had higher proline content (6.0 and 6.2%). The deduced amino-acid sequence is currently available for three *phyB* genes: *phyB* from *A. niger* NRRL 3135 (Ehrlich et al.[1993]), *aph*

Table 5 Substrate specificity of purified phytases

Substrates (3 mM)	Relative phytase activity (%)	
	Phy I	Phy II
Sodium phytate	100	100
Calcium phytate	72.98	92.49
p-Nitrophenyl phosphate	229.00	126.99
Sodium phenyl phosphate	224.41	62.99
α-D-Glucose 1-phosphate	137.77	–
D-Glucose 6-phosphate	166.36	–
1-Naphthyl phosphate	196.39	24.49
2-Naphthyl phosphate	195.39	64.66
ATP	262.91	46.16
ADP	168.95	51.66
AMP	20.97	48.33
β-NADP	215.50	–
Sodium pyrophosphate	293.37	31.83
Glycerol-1-phosphate	28.44	–
Phenyl phosphate	132.32	31.83

The hydrolysis rate of sodium phytate (3 mM) was taken as 100% for comparison

from *A. niger* var. *Awamori* ATCC 38854 (Piddington et al. [1993]), and pH-2.5 acid phosphatase *A. niger* T213 (Kostrewa et al. [1999]).

Conclusion

Earlier Fujita et al. ([2003]) reported two types of phytase from *Aspergillus oryzae* under solid state fermentation conditions which showed the same pH optima (5.0), thermostability (100% stable up to 50°C) but differed in their temperature optima (65 and 55°C, respectively) and molecular weight (120 and 116 kDa, respectively). Extracellular phytase with two pH optima (2.5 and 5.0) has been reported from *Aspergillus niger* (Ullah [1988]) and commercial *Aspergillus niger phytase* (Natuphos) (Wyss el al. 1999). We have earlier reported production and partial characterization of two types of phytase from *Aspergillus niger* NCIM 563 under submerged fermentation (Soni and Khire [2007]). In the present communication we were able to purify both phytases (Phy I active at pH 2.5 and Phy II active at pH 5.0) from *Aspergillus niger* NCIM 563 produced under submerged fermentation to homogeneity and found that they differed from each other in molecular and biochemical properties including pH optima, temperature and pH stability, effect of various metal ions and other reagents, MALDI-TOF analysis, affinity towards various substrates and N-terminal amino acid analysis. This, according to our knowledge, is the first report of two

Table 6 Kinetic parameters of the hydrolysis of different substrates by Phytase I and II

Substrates	K_m^a (mM)		V_{max}^a µmol min^{-1} mg^{-1}		K_{cat}^a s^{-1}		K_{cat}/K_m^a mM^{-1} s^{-1}	
	I	II	I	II	I	II	I	II
Sodium phytate	2.01	0.145	5,018	1,675	2.2×10^4	4.2×10^3	1.1×10^4	2.8×10^4
Calcium phytate	37.27	1.14	26,674	2,205	1.17×10^5	5.5×10^3	3.14×10^3	4.83×10^3
p-Nitrophenyl phosphate	4.07	1.18	15,029	2,169	6.6×10^4	5.4×10^3	1.6×10^4	4.59×10^3
Sodium phenyl phosphate	5.34	0.69	18,463	1,056	8.1×10^4	2.6×10^3	1.5×10^4	3.82×10^3
α-D-Glucose 1- phosphate[b]	3.65	NH	8,704	NH	3.8×10^4	NH	1.05×10^4	NH
D-Glucose-6- phosphate[b]	5.5	NH	14,338	NH	6.3×10^4	NH	1.1×10^4	NH
Glycerol-1- phosphate[b]	5.71	NH	2,890	NH	1.27×10^4	NH	2.2×10^3	NH
Phenyl phosphate	8.32	1.51	15,470	1,436	6.8×10^4	3.6×10^3	8.18×10^3	2.37×10^3

[a] Determined at 55°C under standard assay conditions, incubation time was 30 min. Units of the kinetic parameters are K_m = mM, V_{max} = µmol min^{-1} mg^{-1}, K_{cat} = s^{-1}, K_{cat}/K_m = mM^{-1} s^{-1}

[b] D-Glucose 1-Phosphate and D-glucose 6-phosphate, Glycerol-1-phosphate were not hydrolysed by Phy II (pH 5.0 Active)

Table 7 Comparision of N-terminal amino acid sequence of phytases from various sources

Source and gene name	N-terminal sequence
Aspergillus niger NCIM 563 *Phy I	FSYGAAIPQQ
Aspergillus niger NCIM 563 *Phy II	GVDERFPYTG
Aspergillus niger Acid phosphatase aph, 3-phytase phy-B[a]	FSYGAAIPQQ
Aspergillus niger CBS 513.88 (Phosphate-repressible acid phosphatase precursor)[a]	-VDERFPYTG
Aspergillus fumigatus Af293 (acid phosphatase)[a]	MVRNTPVIMA
Aspergillus niger T213 phyB[b]	FSYGAAIPQSTAEKQF
A. niger var awamori (phyB)[b]	FSYGAAIPQSTAEKQF
Aspergillus ficuum (natuphos)[b]	PASRXQSSCDTV
A. niger CB[b]	ASRXQSTXDTV
Aspergillus terreus CBS[b]	SKSXDTVDLGY
Aspergillus terreus 9A1[b]	SDXNSVDHGY
Aspergillus terreus CBS[b]	TALGPXGXHSDX
E. coli[c]	SE(P)ELKL(E)AVV
Bacillus sp. DS11[c]	SDPYHFTVNAAXET
A. niger van Teighem[d]	FYYGAALPQS

* Present work

[a] NCBI Protein Blast Data Base

[b] Wyss et al. (1999)

[c] Kim et al. (1998)

[d] Vats and Banerjee (2006)

Table 8 Amino acid composition of purified phytases (I & II) from *Aspergillus niger* NCIM 563

Amino acids[a]	Phytase I	Phytase II
As(X)	324	174
Gl(X)	232	112
Ser	184	120
His	36	40
Gly	236	188
Thr	176	126
Ala	236	116
Arg	76	32
Tyr	148	52
Cys[b]	0*	0*
Val	120	90
Met	32	18
Trp[c]	55*	32*
Phe	108	58
Ile	100	60
Leu	196	92
Lys	64	48
Pro	148	90
Total	2,471	1,448
Mol. wt(kDa)[d]	271.8	159.3

* Determined by spectrophotometrically

[a] Values are given in residues/mol

[b] Habeeb (1972)

[c] Spande and Witkop (1967)

[d] Calculated molecular weight

distinct phytases from *Aspergillus niger* produced under submerged fermentation conditions.

The novelty of Phy I with highly acidic pH optimum (2.5), stability under wide pH range (1.5 to 9.0) and broad substrate specificity suggests its application in poultry feed. However, the further work needed to improve its thermostability by immobilization and/or micro encapsulation along with site directed mutagenesis for its application in pelleted poultry feed is in progress.

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