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ORIGINAL PAPER

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Biochemical characterisation of extracellular phytase (*myo*-inositol hexakisphosphate phosphohydrolase) from a hyper-producing strain of *Aspergillus niger* van Teighem

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Abstract *Aspergillus niger* van Teighem, isolated in our laboratory from samples of rotten wood logs, produced extracellular phytase having a high specific activity of 22,592 units (mg protein)$^{-1}$. The enzyme was purified to near homogeneity using ion-exchange and gel-filtration chromatography. The molecular properties of the purified enzyme suggested the native phytase to be oligomeric, with a molecular weight of 353 kDa, the monomer being 66 kDa. The purified enzyme exhibited maximum activity at pH 2.5 and 52–55°C. The enzyme retained 97% activity after a 24-h incubation at 55°C in the presence of 10 mM glycine, while 87% activity was retained when no thermoprotectant was added. Phytase activity was not affected by most metal ions, inhibitors and organic solvents. Non-ionic and cationic detergents (0.1–5%) stabilise the enzyme, while the anionic detergent (SDS), even at a 0.1% level, severely inhibited enzyme activity. The chaotropic agents guanidinium hydrochloride, urea, and potassium iodide (0.5–8 M), significantly affected phytase activity. The maximum hydrolysis rate (V_{max}) and apparent Michaelis-Menten constant (K_m) were 1,074 IU/mL and 606 μM, respectively, with a catalytic turnover number of 3×10^5 s^{-1} and catalytic efficiency of 3.69×10^8 M^{-1} s^{-1}.

Keywords *Aspergillus niger* · Phytases · Acid-phosphatases

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Introduction

Phytases (*myo*-inositol hexakisphosphate phosphohydrolase; EC 3.1.3.8 and EC 3.1.3.26) belong to the family of histidine acid phosphatases, a subclass of phosphatases, which catalyse the hydrolysis of phytic acid, the principle storage form of phosphorus in cereals, legumes, oil seeds and nuts. Phytases catalyse hydrolysis of phytic acid in a stepwise manner to inositol phosphates, *myo*-inositol and inorganic phosphate, all utilising a phosphohistidine intermediate in their phosphoryl transfer reaction [17]. Phytases are either absent, or present at very low level, in the gastro-intestinal tract of monogastric animals [10–14, 16, 34]. Consequently, dietary phytate is not digested in the intestine and contributes to phosphorus pollution in areas of intensive livestock production. Phytic acid acts as an anti-nutrient factor, as it forms insoluble complexes with proteins and a variety of metal ions (Ca^{+2}, Zn^{+2}, Mg^{+2}, Fe^{+3}), thus decreasing dietary bioavailability [36]. Hence, supplementation of phytase in fodder is highly desirable to reduce phosphorus excretion and to improve the bioavailability of metal ions [3]. A sizeable number of phytase-producing microorganisms, including bacteria, fungi and yeast, have been reported [5–7, 21, 39] in the literature; however, thermostable and acid stable phytase with broad substrate specificity and high specific activity is still highly desirable for animal nutrition. Although phytases are fairly specific for phytic acid, the substrate specificity may vary due to differences in molecular characteristics [38]. In the present study, we report the purification of phytase produced by *Aspergillus niger* van Teighem, isolated in our laboratory from samples of rotten wood logs [33], and its biochemical and kinetic characterisation. We have previously reported the optimised process parameters for production of phytase by *A. niger* van Teighem in a laboratory-scale fermenter [35].

Materials and methods

Chemicals

Sodium phytate, gel filtration markers, and other biochemicals were obtained from Sigma (St. Louis, Mo.). Gel-filtration and ion exchange matrices were procured from Pharmacia Biotech (Uppsala, Sweden). All other chemicals used were of analytical grade and were obtained from standard sources.

Enzyme production and purification

A. niger van Teighem was grown in a medium with glucose and starch as principle carbon sources at 30°C (200 rpm). Extracellular phytase was isolated by centrifuging the fermentation broth at 10,000 *g* for 20 min. The supernatant was clarified by passing it through a 0.45 μ membrane filter. The concentrated enzyme obtained after ultrafiltration (30 kDa) was subjected to ion exchange and gel filtration chromatography. All active fractions were pooled, desalted and concentrated using 10 kDa cut-off membrane. Standard gel-filtration markers were included, along with blue dextran, to estimate the elution profile and approximate molecular weight of the native protein.

Polyacrylamide gel electrophoresis

Denaturing sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed on 12% polyacrylamide slab gels with 0.1% SDS as described by Laemmli [15]. The gel was allowed to run at 10 mA until the dye front passed through the stacking gel and entered the separating phase, after which it was run at 20 mA for 1 h in an electrophoresis chamber (Sigma Techware, St. Louis, Mo.) filled with Tris-glycine buffer (pH 8.8). Protein bands were visualised by staining with Coomassie Brilliant Blue R-250.

Phytase assay

Phytase activity was estimated colorimetrically by monitoring the release of inorganic phosphorus from phytic acid using the modified method of Heinonen and Lahti [9]. One unit of phytase activity was expressed as nanomoles of phosphorus liberated per millilitre per second (nkat mL^{-1}).

Protein content

Extracellular protein concentration was estimated by Bradford's dye binding assay [1] using bovine serum albumin (1 mg mL^{-1}) as standard. All assays were carried out in triplicate along with appropriate buffer and reagent controls. Absorbance at 595 nm was monitored.

Temperature and pH dependence studies

To determine the optimum temperature of the purified protein, phytase activity was estimated over the temperature range 30–70°C (pH 2.5). The activation-deactivation energy was calculated from an Arrhenius plot. To ascertain the pH optimum of the enzyme, its activity was measured at different pH values using 0.1 M buffer, HCl-KCl for the pH range 1–2; glycine-HCl for pH range 2.5–3.5, and acetate buffer for pH range 4.5–5.5.

pH and thermal stability

The thermostability of phytase activity was checked by incubating the enzyme over the temperature range 4–65°C in glycine-HCl buffer (0.1 M, pH 2.5) in the presence and absence of additives (10 mM $CaCl_2$ and 10 mM glycine). Samples were withdrawn at regular intervals and residual phytase activity was assayed at 55°C (pH 2.5). Similarly, pH stability was investigated over the pH range 2–7.

Effect of potential modulators on enzymatic activity

Various metal ions, sulfhydryl compounds, chelating agents, detergents, chaotropic agents, solvents and other potential inhibitors were tested for their effect on phytase activity. The enzyme was incubated with various concentrations of the above-mentioned chemicals at room temperature (25°C) and residual phytase activity was measured at regular time intervals.

Enzyme kinetics

Phytase activity was estimated over a range of substrate concentration (0.075–1.8 mM) using 10 μL purified enzyme at 55°C (pH 2.5), and kinetic constants (K_m and V_{max}) were calculated from a Lineweaver-Burk plot. To determine the kinetic linearity, catalytic turnover number (K_{cat}), and catalytic efficiency of purified phytase, activity assays were carried out at various concentrations of enzyme protein (12–120 ng), while keeping the substrate concentration fixed.

Results and discussion

Phytase purification

The enzyme was purified 1.8-fold over the culture supernatant with a yield of 76%. After concentration and gel permeation chromatography, a single sharp activity peak was obtained (data not shown). It is reported in the literature [26, 27, 29] 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. Nagashima et al. [18] purified phytase from *A. niger* SK-57 to homogeneity in four steps by using ion-exchange chromatography, gel filtration chromatography, and chromatofocussing. An extracellular phytase from *Bacillus subtilis* (natto) –77 [23] was purified to homogeneity by ultra-filtration and a combination of Sephadex G-100 and DEAE-Sepharose CL-6B column chromatography. Several phytases from *Klebsiella* species have been purified using ion exchange and gel filtration chromatography [7, 22, 25].

Molecular mass estimation

The molecular mass and homogeneity of purified phytase was estimated by SDS-PAGE (12%), where a very prominent diffuse band of 66 kDa was observed (Fig. 1). This was similar to phytase B secreted by *A. niger* NRRL 3135, which has a molecular mass of 68 kDa and is dimeric in nature [30, 32]. Shimizu [24] purified an extracellular phytase with a molecular mass of 60 kDa from *Aspergillus oryzae* and reported it to be dimeric.

pH and temperature dependence studies

The optimum pH for phytase activity was in the range pH 2–2.5 (Fig. 2), with a sharp decline in activity as the pH moves towards the neutral range, reaching negligible activity at pH 5–5.5. At pH 1.5, the purified protein retained 49% of maximum phytase activity (384 nkat/mL). This is similar to values reported for pH 2.5 optimum acid phosphatase—later referred to as phytase B (*phyB*)—from *A. ficuum* NRRL 3135, which shows maximum phosphohydrolase activity at pH 2.5 with negligible catalytic activity at pH 5.5 [4, 26]. *phyB* lacks phytate-degrading activity at pH 5, while at pH 2.5 it efficiently hydrolyses phytate with a turnover number of 628 s^{-1} [31]. However, the pH optimum of most of reported fungal phytases lies between 2.5 and 7. A commercial *A. niger* phytase (Natuphos) exhibited dual pH optima at 2.5 and 5.5, retaining 60 and 97% of maximal activity at these pH values, respectively [37, 38]. Generally, bacterial and yeast phytases are optimally active in neutral to alkaline pH, while in fungi, maximum activity is displayed in the pH range 2.5–6. This wide range of pH optima could be due to variations in molecular conformation or stereo specificity of the enzyme protein molecule [11, 22, 23, 39].

It is evident from Fig. 3 that the activity of purified enzyme increases as the temperature increases, and was highest at 55°C; activity declined very sharply at higher temperatures. Sano et al. [20] reported the optimum temperature of phytase activity from *Arxula adeninivorans* to be 75°C (pH 4.5–5) while several yeast strains (*Pichia*, *Candida*, *Kluyveromyces*, *Torulaspora*, *Schwanniomyces* sp.) are reported to have optimum temperatures in the range of 60–80°C [19]. The effect of temperature on hydrolysis of sodium phytate was also investigated, and the activation and deactivation energy, as calculated from an Arrhenius plot, were found to be 384 and 1,438 cal mol^{-1}, respectively (data not shown).

Temperature- and pH-stability

Absolutely no loss of enzyme activity was observed, even after 24 h incubation, at 30, 37 and 45°C in the absence of any additive (data not shown). The enzyme retained

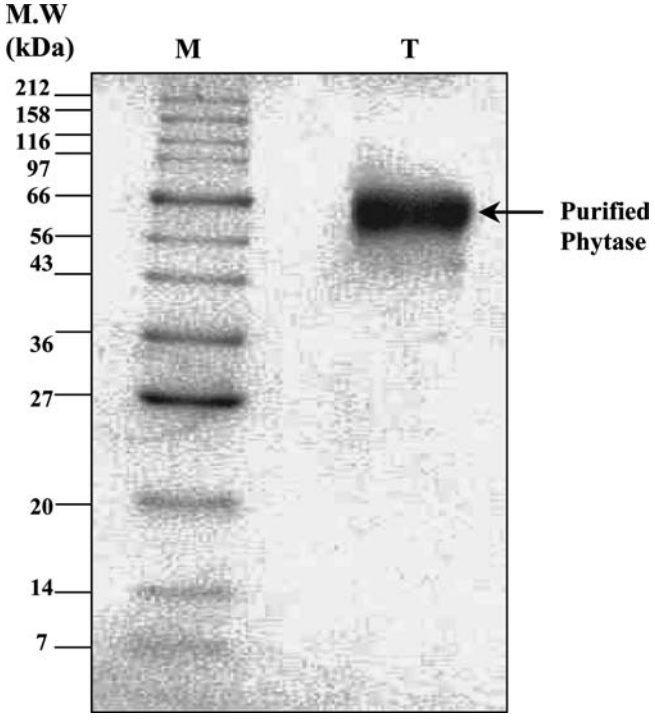

Fig. 1 SDS-polyacrylamide gel electrophoresis of phytase. Lanes: *M* Molecular weight markers, *T* purified phytase

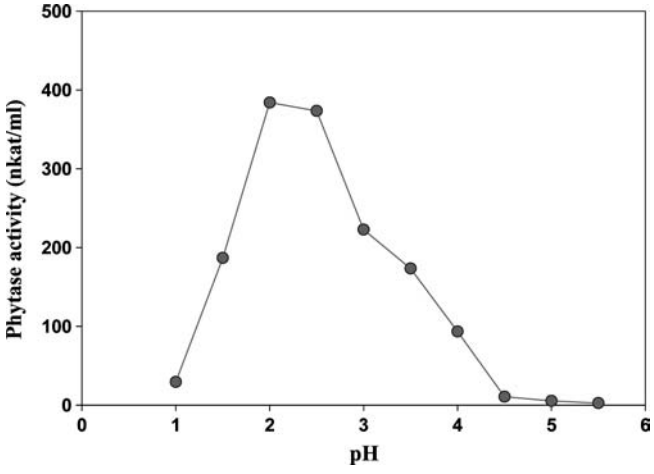

Fig. 2 Effect of pH on the catalytic activity of phytase from *Aspergillus niger* van Teighem

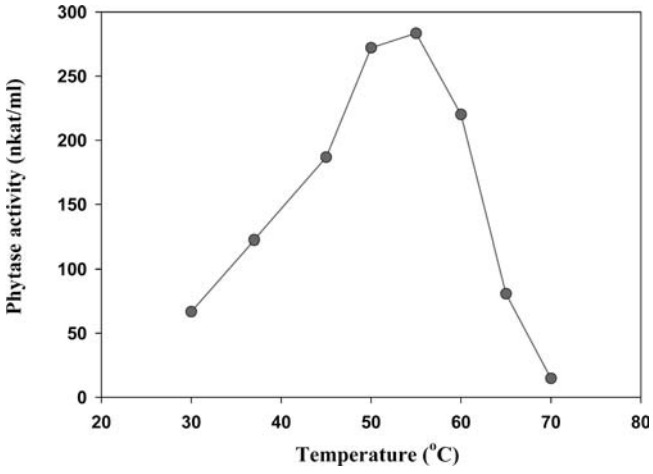

Fig. 3 Effect of temperature on the catalytic activity of phytase from *A. niger* van Teighem

89% activity in the absence of any stabilising agent, while 93 and 97% activity were retained in the presence of 10 mM $CaCl_2$ and 10 mM glycine, respectively, after a 24-h incubation at 55°C (Fig. 4a). At 65°C, the half-life of phytase was reduced to 30 min in the absence of any stabilising agent, with 25% phytase activity being retained after 4 h. The half-life of enzyme activity increased significantly upon addition of thermo-protectants like glycine and $CaCl_2$. The half-life of phytase at 65°C increased to 6 h in the presence of glycine (10 mM) and to 2 h in the presence of $CaCl_2$ (10 mM), suggesting that glycine is more thermoprotectant than calcium (Fig. 4b). It has been reported [28] that phytase prevents protease degradation of peptide bonds. Han and Lei [8] observed the functional expression of phytase in *Pichia pastoris* and found that glycosylation was vital to enzyme thermostability. Calcium has been reported to contribute towards heat tolerance of phytases from many microbes [13]. Chen et al. [2] reported the stabilising effect of sorghum liquor waste on phytase activity, where 90% activity was retained at 70°C. Similarly, phytase stability was tested at various pHs. Almost complete phytase activity was retained over a pH range of 2–7 after 12 h incubation, and no significant loss in phytase activity was observed even after 24 h (data not shown).

Effect of metal ions on catalytic activity

Phytase activity was severely inhibited by Al^{+3} at 0.5 mM. In general, all metal ions tested above 1 mM were found to be inhibitory except Ca^{+2} and K^{+}, where 90% phytase activity was retained even at 15–20 mM (Table 1). Several authors have shown the effect of metal ions on phytase activity [6, 7, 21, 39]. EDTA, Zn^{+2}, Cd^{+2}, Ba^{+2}, Cu^{+2}, Fe^{+2} and Al^{+3} inhibited phytase activity from *B. subtilis* (natto) –77 [23]. Yoon et al. [39] reported severe inhibition of phytase activity from *Enterobacter* sp. 4 by Zn^{+2}, Ba^{+2}, Cu^{+2}, Al^{+3} and

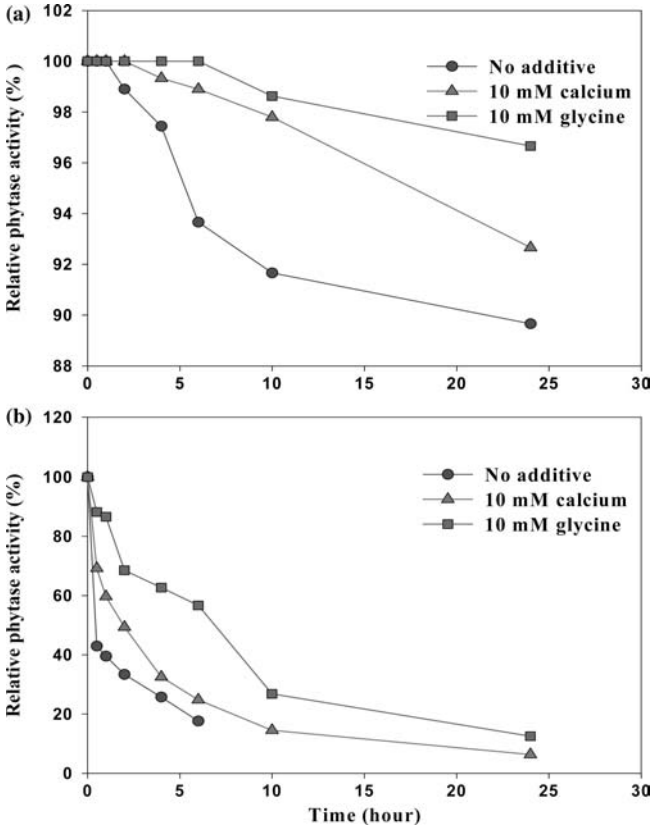

Fig. 4 Thermostability profile of purified phytase in the presence of glycine and calcium chloride at **a** 55°C, **b** 65°C

EDTA. Kerovuo et al. [12] studied the metal-ion requirement of *B. subtilis* phytase and found complete inactivation of the enzyme upon removal of metal ions by EDTA.

Effect of inhibitors, solvents and denaturing agents

Various chelating agents (EDTA, oxalate, citrate, tartrate) and sulfhydryl inhibitors (β-mercaptoethanol and iodoacetate), serine and cysteine specific modifying agents like PMSF, para-hydroxymercurybenzoic acid (pHMB) and phosphomycin were used at varying concentrations to investigate their effect on the catalytic properties of phytase. It was noticed that none of these compounds acted as an inhibitor up to 2 mM; in contrast, they slightly increased phytase activity (Table 2). The increase in activity at lower levels of metal ion concentration (0.1 mM) may indicate inhibition of proteolytic enzymes. The enzyme was insensitive to the serine-specific reagent PMSF up to 0.5 mM, above which a drastic fall in enzyme activity was seen. Ullah and Cummins [26] observed that phosphomycin, a competitive inhibitor of pH 6-optimum acid phosphatase from *A. ficuum*, did not affect enzyme activity.

To investigate the effect of organic solvents on phytase activity, enzyme was incubated with various solvents (10% v/v) viz., hexane, butanol, dimethylsulphoxide

Table 1 Effect of metal ions on the catalytic activity of phytase. Activity is expressed as a percentage of the activity level in the absence of metal ions. The enzyme was pre-incubated with metal ions (55°C, 5 min). Separate blanks with individual metal ions were prepared

Relative phytase activity (%)					
	Concentration (mM)				
Metal ions[a]	1	5	10	15	20
Cu^{+2}	90	30	10	5	3
Mg^{+2}	106	39	15	8	5
Mn^{+2}	107	27	7	3	2
Ba^{+2}	153	139	149	145	146
Fe^{+2}	113	41	41	41	–
Ni^{+2}	85	92	86	81	56
Hg^{+}	113	52	34	19	13
Co^{+2}	135	92	64	61	40
Zn^{+2}	113	33	11	5	4
Al^{+3}	2	1	0.6	0.6	0.37
Ca^{+2}	145	105	98	93	–
K^{+}	140	106	89	86	84

[a]Ba^{+2} and Fe^{+2} interfere with the colorimetric assay

(DMSO), dimethylfluoride (DMF), or benzyl alcohol, at room temperature (25°C) for 2 h, and assayed at 55°C (pH 2.5). As seen in Table 2, the enzyme retained complete activity in the presence of hexane, DMSO and DMF, while in the presence of benzyl alcohol and butanol, 83 and 88% activity was retained, respectively. These results suggest that hydrophobic residues may not be involved in the catalytic properties of this phytase. Of the various detergents tested, increasing concentrations (0.1–5%) of non-ionic detergents like Tween-20, Tween-80, Triton-X-100 and cationic detergent like CTAB stabilised enzyme activity, while anionic detergent (SDS) severely inhibited the enzyme, even at 0.1% concentration, resulting in the loss of 92% phytase activity. Chaotropic agents like guanidinium hydrochloride, urea and potassium iodide (0.5–8 M) also significantly affected phytase activity (Table 2).

Kinetics studies

Phytase activity increased linearly with increasing substrate concentration up to 0.6–0.75 mM (Fig. 5). The kinetic constants K_m and V_{max}, were 606 μM and 1,074 IU/mL, respectively. Phytase activity was observed to be linear from 12 to 120 ng protein (Fig. 6). The estimated K_{cat} of the enzyme as computed from the kinetic linearity experiment was 3×10^5 s^{-1}, with a catalytic efficiency of 3.69×10^8 M^{-1} s^{-1}. Despite the fact that the phytase studied here showed a pH optimum similar to that of *phyB*, it showed marked differences in catalytic constants from the previously reported phytase [32], which was found to possess a comparatively high affinity for phytate, with a K_m of 103 μM and catalytic turnover of 628 s^{-1}. However, the phytase studied here, although displaying a higher K_m of 606 μM, was much more efficient in hydrolysing phytate, with a significantly

Table 2 Effect of various chemical agents on the catalytic activity of phytase. Activity is expressed as a percentage of the activity level in the absence of any chemicals; the enzyme was pre-incubated with the individual chemical (55°C, 5 min). Separate blanks with individual chemical ions were prepared

Organic solvent (10%, v/v)	Relative phytase activity (%)			
Control	100			
Hexane	112			
Dimethylsulphoxide (DMSO)	110			
Dimethylfluoride (DMF)	109			
Benzyl alcohol	83			
Butanol	88			
Inhibitor	0.1 mM	0.5 mM	1.0 mM	2.0 mM
EDTA	154	152	147	141
PMSF	163	103	32	20
L-Tartaric acid	134	154	161	–
Oxalate	152	154	141	121
Citrate	145	142	136	125
Sodium azide	137	137	136	132
β-Mercaptoethanol	159	152	126	123
Phosphomycin	137	121	118	114
p-Hydroxymercuric benzoate	122	110	98	92
Iodoacetic acid	128	98	96	95
Detergent	0.1%	0.5%	1%	5%
Triton-X-100	139	145	167	175
Tween-20	144	154	180	–[a]
Tween-80	152	171	177	–[a]
SDS	8	7	6	6
CTAB	154	162	180	*
Additive	0.5 M	2 M	6 M	8 M
Guanidium-HCl	90	59	8	–[a]
Urea	90	65	45	35
Potassium iodide	77	28	5	–[a]

[a]Interferes with colorimetric assay

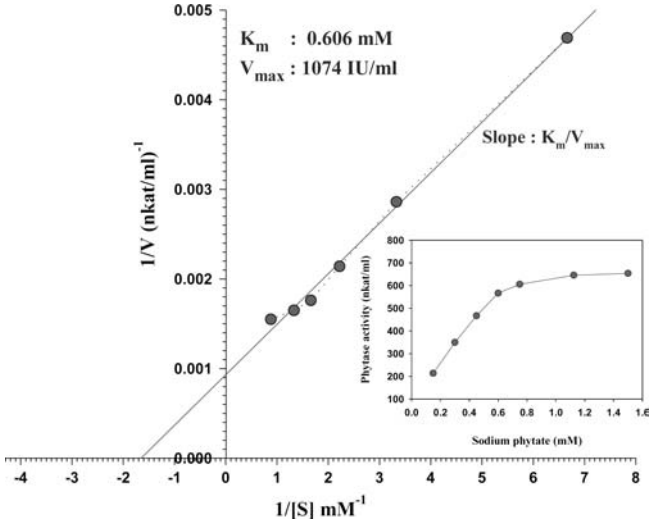

Fig. 5 Lineweaver-Burk plot used to estimate kinetic constants of purified phytase

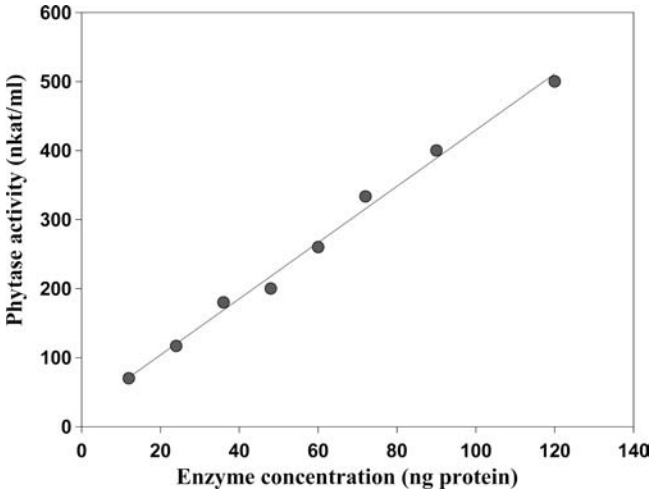

Fig. 6 Estimation of kinetic linearity and catalytic turnover number (K_{cat}) of purified phytase

higher catalytic turnover and approximately 60 times greater catalytic efficiency (6.1×10^6 M^{-1} s^{-1}) than *phyB* [32].

Conclusions

The purified enzyme showed maximum phytase activity in the temperature range 50–55°C and at a pH of 2.0–2.5. It retained 90% of activity in the absence of any stabilising agent, even after 24 h at 55°C. The results of inhibition studies showed that the isolated enzyme remained active in the presence of most of the chelating agents tested. The sulfhydryl inhibitor experiment suggests the lack of participation, or absence, of free, accessible sulfhydryl groups in the active site of the enzyme. Cysteine-specific modifying agents also did not result in a significant decline in phytase activity. On the basis of substrate specificity, two classes of phytases have been reported: phytases with broad substrate specificity (*A. fumigatus*) and phytases that are rather specific for phytic acid (*A. niger, A. terreus* 9A1, *A. terreus* CBS). However, it has been observed that phytases with broad substrate specificity had inherently low specific activities but were better suited for animal nutrition purposes. Thus, the present enzyme has a potential application in animal nutrition.

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