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Purification and characterization of phytase from *Aspergillus fumigatus* Isolated from African Giant Snail (*Achatina fulica*)

David Morakinyo Sanni[a,*], Olusola Tosin Lawal[a], Victor Ndigwe Enujiugha[b]

[a] Department of Biochemistry, Enzymology Unit, The Federal University of Technology, P. M. B 704, Akure, Nigeria
[b] Department of Food Science and Technology, The Federal University of Technology, P. M. B 704, Akure, Nigeria

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ABSTRACT

Phytase, an enzyme that catalyzes the stepwise hydrolysis of phytate into phosphorous and organophosphate compound and capable of reducing environmental pollution and metal chelating effect of phytate was purified from *Aspergillus fumigatus* isolated from African Giant Snail (*Achatina fulica*). The crude phytase was subjected to ammonium sulphate precipitation, DEAE Sephacel and Sephacryl S-200. Physicochemical parameters of the enzyme were investigated. Approximately 45%- fold purification was achieved with an overall recovery of 15%. The purified phytase had optima temperature and pH activity at 40 °C and pH 6 respectively with a marked activity of 83% and 78% at pH 8.0 and 9.0 respectively. It retained over 80% of its initial activity after 6 h at pH 4.0 − 7.0 with a 48% remaining activity at 50 °C after 1 h incubation time. Vmax and Km were determined to be 35.7μmol/min and 7.2 mM respectively. The phytase activity was enhanced in the presence of Ca^{2+}, Cu^{2+} and Fe^{2+}, but was greatly inhibited by Zn^{2+}, Hg^{2+}, Al^{3+}, sodium dodecyl sulphate (SDS) and urea. The results showed that phytase produced from *A. fumigatus* may contribute significantly to the phytate degrading enzyme system in African giant snail and may serve a useful commercial purpose.

1. Introduction

Plants such as legumes, cereals and oilseeds that are widely cultivated all over the world (Hurrell et al., 2003), serve as major sources of nutrients for human and livestock and contain phytic acid, a storage form of *myo*-inositol – an important growth factor. The salt form, phytate, is an anhydrous storage form of phosphate accounting for more than 80% of the total phosphorus in cereals and legumes. It performs several other important physiological functions in plants (Kumar et al., 2010). Myo-inositol was reported to be involved in seed desiccation, auxin physiology, and biosynthesis of raffinose and galactopinitol oligosaccharides important for osmoregulation and stress responses, in the synthesis of phosphoinositides, in protein anchoring to cellular membranes (Loewus and Murthy, 2000). Phytic acid is very stable because of its structure which has high phosphate content compared to other organo-phosphate compounds (Hurrell et al., 2003). Under normal physiological conditions phytic acid chelates essential minerals such as calcium, magnesium, iron and zinc. It inhibits digestive enzymes by binding to amino acids (Hurrell et al., 2003). Thus, phytic acid is an anti-nutritive component in plant-derived foods and feeds; therefore enzymatic hydrolysis of this compound is imperative for the bioavailability of these essential minerals. Non-hydrolysis of phytate results in the release of phosphorus into the environment by monogastric livestock leading to environmental pollution of land and water bodies (Vohra and Satyanarayana, 2001).

Monophosphoester bonds in various organo-phosphate compounds can be hydrolyzed by phosphatases; diverse class of enzymes catalyzing the cleavage of the bonds. However, these enzymes are virtually unable to hydrolyse the monophosphoester bonds in phytic acid (Vohra and Satyanarayana, 2001). Since the hydrolysis of phytic acid is of great importance, a special class of enzymes hydrolyzing phytic acid has evolved – the phytases. These enzymes (*myo*-inositol hexakisphosphate phosphohydrolases) hydrolyse phytic acid to less phosphorylated *myo*-inositol derivatives, releasing inorganic phosphate. Phytase is widespread in nature, occurring in microorganisms (Suresh and Das, 2014; Kalieva et al., 2017), plants (Gibson and Ullah, 1998), as well as in some animal tissues (Ullah, 1998). Several phytases have been isolated and characterized: fungal phytase from *Aspergillus ficuum* (Coban and Demirci, 2014), bacterial phytase from *Bacillus licheniformis Pfbl-03* (Fasimoye et al., 2014) and a mammalian phytase (Craxton et al., 1997). Phytases can be grouped into different classes depending on pH (acidic or alkaline), catalytic mechanisms (histidine acid-phosphatase-like phytase, purple acid phosphatase-like phytase and β-propeller phytase), and specificity of hydrolysis (3-phytase, 6-phytase and more

* Corresponding author.
E-mail address: dmsanni@futa.edu.ng (D.M. Sanni).

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recently 5-phytase) (Mullaney and Ullah, 2003).

African giant land snail, *Achatina fulica,* is a species of large, air breathing land snail, a terrestrial snail pulmonate gastropod mollusc in the family Achatinidae (Ahmed and Nabil, 2012). African giant snail grows in the wild and feeds on wide range of plant materials; leaves, fruits and vegetables (Ahmed and Nabil, 2012) which therefore suggests that the Snail must possess a well developed phytate degrading enzyme system that enhances the bioavailability of phosphorus, calcium and other essential minerals required for growth and shell formation. Microorganisms in the guts of African giant snail therefore, become a primary suspect as the major source of phytate degrading enzyme since snail has long been reported to harbour different diversity of micro-flora which are metabolically active (Pawar et al., 2012). These microorganisms produce enzymes that enable the breakdown of macromolecules (Ekperigin, 2006; Pawar et al., 2012; Oyeleke et al., 2012). However, phytase has not been produced from African giant snail or from microorganisms living in the intestine. Thus, this study sought to isolate, purify and characterise phytase from *Aspergillus fumigatus* isolated from the gut of African giant snail.

2. Materials and methods

2.1. Materials

2.1.1. Sample collection and preparation of seed culture

Pure strain of *Aspergillus fumigatus* isolated from the gut of African Giant Snail, was obtained from the Department of Microbiology, Federal University of Technology, Akure, Nigeria. The organism was cultured in petri-dish containing potatoe dextrose agar (PDA). The seed culture was done by suspending the fungus in potatoe dextrose broth (PDB) using water bath shaker for 72 h in order to make the organism active prior to its being inoculated in phytate screening medium.

2.2. Method

2.2.1. Screening of Aspergillus fumigatus for phytase production

Aspergillus fumigatus spores were transferred from freshly prepared seed culture into sterile phytate screening medium containing 1.5% glucose, 0.5% $(NH_4)_2SO_4$, 0.05% KCl, 0.01% $MgSO_4{\cdot}7H_2O$, 0.01% NaCl, 0.001% $FeSO_4$, 0.001% $MnSO_4$ and 0.5% Sodium phytate at pH 7 and incubated for 72 h using water bath shaker. The fraction of 10 ml was collected at every 12th hour into sterile container and phytase activity of each collection was determined using phytase standard assay procedure.

2.2.2. Isolation of phytase from Aspergillus fumigatus

Broth medium consisted of 1.5% glucose, 0.5% $(NH_4)_2SO_4$, 0.05% KCl, 0.01% $MgSO_4{\cdot}7H_2O$, 0.01% NaCl, 0.001% $FeSO_4$, 0.001% $MnSO_4$ and 0.5% Sodium phytate at pH 7 according to Kerovuo et al. (1998) which is consistent with Lee et al. (2005) and Gaind and SeighSingh (2015) with little modification. The broth was scaled up to 1 l and autoclaved for 15 min at 121 °C. The broth was allowed to cool to room temperature while about 1×10^7/ml of fungal spores were transferred from a 5-day old seed culture into the broth medium under aseptic conditions. The broth was thereafter incubated for 24 h at the agitation rate of 125 rpm at 37 °C using water bath shaker. The broth was filtered to remove fungal cells and then centrifuged at 14,000 rpm (21,952 g) for 20 min using refrigerated centrifuge. The supernatant obtained after centrifugation was stored as extracted crude phytase for further purification and characterization.

2.2.3. Determination of phytase activity

Phytase activity was determined according to Fiske and Subbarow (1925) using ammonium molybdate by measuring the rate of phosphorus release, as indicated by an increase in absorbance at 660 nm. The sample test tubes contained 1 ml of enzyme solution, 2 ml of substrate solution (5% phytic acid in 0.1 M sodium acetate, pH 5.5) incubated at 37 °C for 30 min using regulated thermostatic water bath cabinet. The reaction was stopped with 1 ml of trichloroacetic acid (TCA) (5% w/v) and colour development was achieved by addition of 1 ml colour reagent (3.66 g of $FeSO_4{\cdot}7H_2O$, 0.5 g of $(NH_4)_6MO_7O_{24}{\cdot}4H_2O$ and 3.2 ml of concentrated H_2SO_4 in 100 ml of distilled water). The blank was set up without the substrate and treated in the same condition with the sample test tubes. The enzyme activity was calculated by drawing a calibration curve in the range of 100–1500 μg/ml orthophosphate. One unit of the enzyme activity was defined as the amount of the enzyme able to hydrolyse phytate resulting in 1 μmol of inorganic phosphorus per minute under the assay condition, and expressed in international unit (U).

2.2.4. Determination of protein concentration of each step of purification

The Protein concentration was routinely determined according to Bradford (1976) using Bovine serum albumin standard and the absorbance was measured at 595 nm with a spectrophotometer.

2.2.5. Enzyme purification

Enzyme purification was done according to the method described by Garchow et al. (2006) with modification, and as follows:

2.2.5.1. Ammonium sulphate precipitation. Crude extract was brought to 30–75% saturation with solid ammonium sulphate at 4 °C by gentle and continuous stirring and allowed to stand overnight. The resulting solution was centrifuged at 22,000 g for 20 min to obtain the precipitated protein in the form of pellet. The pellet obtained was dissolved in 10 ml of 20 mM Tris buffer (pH 7.0) and dialysed for 24 h with four changes of the same buffer.

2.2.5.2. Ion exchange chromatography. The dialysed protein solution was loaded onto DEAE-Sephacel column (1.25 × 15 cm; flow rate: 50 ml/h). The column was equilibrated with 20 mM Tris-HCl buffer, pH 7.0 before the sample application. After eluting the unbound inactive protein from the column with the starting buffer, a linear gradient of 1.0 M NaCl in 20 mM Tris-HCl buffer, pH 7.0 was used to elute the bound protein. The presence of protein in the eluent was measured at 280 nm while the phytase activity was assayed according to standard assay procedure. The pooled fractions of the purified phytase were concentrated using 4 M Sucrose.

2.2.5.3. Gel filtration. Gel filtration of the concentrated pooled fractions from ion exchange column was carried out on sephacryl S-200 (2.5 × 70 cm; flow rate: 25 ml/h) with 20 mM Tris-buffer (pH 7.0). Five millilitres (5 ml) fractions were collected and the protein concentrations were measured at 280 nm while the phytase activities were determined according to the standard procedure. The fractions containing phytase activity were pooled while the protein concentration and phytase activity were determined. The pooled fractions were used for further investigations.

2.2.6. Physicochemical properties of purified enzyme

2.2.6.1. Effect of pH on the activity of the purified phytase. The effect of pH on the activity phytase from *Aspergillus fumigatus* was carried out using 0.05 M glycine/HCl (pH, 2.0–3.0), sodium acetate (pH, 4.0, 5.0 and 6) and Tris/HCl (pH, 7.0, 8.0 and 9.0). The substrate solutions were prepared by dissolving sodium phytate in each of the buffer solutions while the phytase activity at each pH was determined using standard assay procedure.

2.2.6.2. Effect of temperature on the activity of purified phytase. The effect of temperature on the activity of the purified phytase from *A. fumigatus* was determined by varying the temperature condition of the reaction mixture which consisted of 1 ml purified enzyme and 2 ml sodium phytate solution. The reaction mixture was incubated for

37 min at different temperatures ranging between 30 and 80 °C at an interval of 10 °C. The enzyme activity was measured according to standard assay procedure.

2.2.6.3. Thermal stability of phytase from Aspergillus fumigatus. The thermal stability of the purified enzyme was investigated by measuring residual activity of the phytase relative to initial activity. The temperature stability was investigated by incubating the purified phytase at different temperatures ranging from 30° to 80°C intervals for 1 h. One millilitre (1 ml) aliquot of the purified enzyme was withdrawn at 10 min interval for one hour. The enzyme activity was determined according to standard assay procedure while the percentage of residual activity was calculated relative to the initial activity at 0 h.

2.2.6.4. Effect of pH stability on activity of phytase. The pH stability of the purified enzyme was determined by using various buffers at pH ranging from 2.0 to 9.0 M in 0.05 M glycine/HCl (pH, 2.0–3.0), sodium acetate (pH, 4.0, 5.0 and 6.0) and Tris/HCl (pH, 7.0, 8.0 and 9.0). The enzyme was incubated in the various buffer solutions for 6 h and the enzyme activity was determined according to the standard assay procedure. The percentage residual activities were determined relative to the initial activity obtained at 0 h.

2.2.6.5. Effect of different metal ions on the activity of the purified phytase. The effect of different metal ions on the activity of the purified enzyme at 5, 10 and 20 mM concentrations were determined using $MnSO_4$, $MgSO_4$, $FeSO_4$, $CuSO_4$, $CaSO_4$, Na_2SO_4, K_2SO_4, $ZnSO_4$ and $HgSO_4$. The assay mixtures contained the specified concentrations of the metal ions under investigation while the enzymatic activities were determined according to the standard assay procedure.

2.2.6.6. Effect of different inhibitors and activators on purified phytase. The effect of inhibitors on the activity of the purified enzyme at different concentrations of 5, 10 and 20 mM was determined by using EDTA, ascorbic acid, urea and SDS. The assay mixtures containing the specified concentrations of inhibitors under investigation were incubated under the standard assay conditions while the enzyme activities were determined at 660 nm.

2.2.6.7. Measurement of kinetic constants. The kinetic constants, K_m and V_{max}, of the purified enzyme were determined using the Line weaver-Burk plot of the concentration of sodium phytate at different concentrations (0.6–2.4 mM) in 0.2 M sodium acetate buffer (pH 5.5). The enzyme activity was determined according to the standard assay procedure.

2.2.6.8. Molecular weight determination. The subunit molecular weight of the purified enzyme was determined by SDS-PAGE using 10% gel according to Laemmli (1970) with standard marker proteins of molecular weight 11–180 kDa and staining of protein band was carried out with Coomasie brilliant blue.

3. Results

3.1. Growth and phytase production pattern by Aspergillus fumigatus

The growth pattern of *Aspergillus fumigatus* on phytase screening medium is shown in Fig. 1. The isolated enzyme demonstrated an optimum phytase activity of 23 U/ml after 24 h incubation time while optimum microbial growth was achieved at 36 h incubation time. However, phytase production showed decrease in activity after 24 h. Meanwhile, the optimum growth of *A. fumigatus* was maintained until 36 h following its decrease after 36–72 h. Therefore, phytase production was carried out at 24 h.

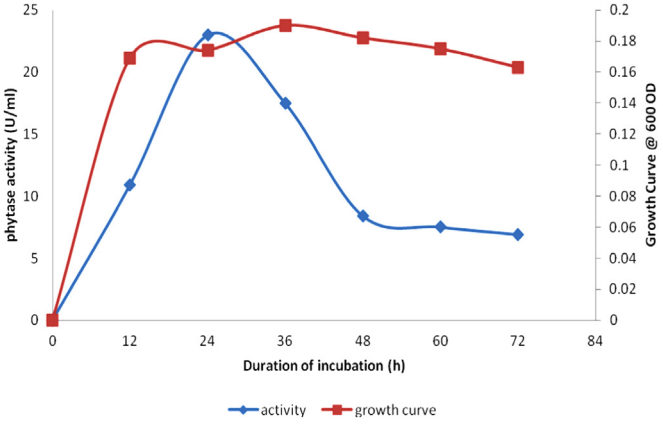

Fig. 1. Growth and phytase production pattern by *Aspergillus fumigatus*.

3.2. Isolation and purification of Phytase from Aspergillus fumigatus

The phytase activity and the specific activity of the crude extract were found to be 0.95 U/ml and 0.23 U/mg, respectively. The results of purified phytase from *A. fumigatus* isolated from African giant snail are summarised in Table 1. The dialysed portion of the NH_4SO_4 precipitate and the bound portion on DEAE Sephacel gave specific activities of 4.05 and 4.55 with yields of 35% and 32% respectively. However, a 45-fold purification was achieved with a recovery of 15% and specific activity of 10.3 U/mg after the enzyme was eluted on Sephacryl S-200. The elution profiles on DEAE Sephacel and Sephacryl S-200 are shown in Figs. 2 and 3, respectively.

3.3. Effect of pH on phytase activity

The effect of pH on the phytase activity of *Aspergillus fumigatus* is presented in Fig. 4. The purified phytase was found to be active in all the pH investigated. The relative activity increased from 63% to 80% between pH 2.0–5.0 while the pH optimum was observed to be pH 6.0. A very high relative activity of 97.0% was observed at neutral pH; the phytase also exhibited a higher relative activity at alkaline pH compared to the activity at acidic pH.

3.4. Effect of pH on the stability of phytase from A fumigatus

The influence of pH on the stability of phytase is presented in Fig. 5. The residual activity of 70–75% was obtained at pH 4.0–6.0. There was a slight decrease in these values at neutral pH to yield a residual activity of 65%. However, residual activities of 19% and 11% of its initial activity were obtained at the alkaline pH of 8.0 and 9.0 respectively.

3.5. Effect of temperature on the Activity of phytase from A. fumigatus

The optimum temperature of activity was obtained at 40 °C while 69% relative activity was measured at 30 °C as shown on Fig. 6. A gradual decline in relative activity was observed from 50 °C to 80 °C with a relative activity of 24% at 80 °C.

3.6. Effect of Temperature on the stability of phytase from A. fumigatus

The thermal stability of phytase is presented in Fig. 7. The percentage residual activity of the enzyme was found to be about 48% after 1 h incubation at 50 °C while 27% and 20% remaining activities were recorded at 60 °C and 70 °C respectively. A minimal residual activity of 14% was measured at 80 °C after 1 h incubation period.

Table 1
Purification Table of phytase from *Aspergillus fumigatus*.

	Volume Ml	Protein Concentration (ml)	Total Protein (mg)	Activity U/ml	Total activity (U)	Specific activity U/mg	Yield %	Purification fold
Crude extract	600	3.72	2232	0.95	570	0.23	100	1
$(NH_4)_2SO_4$	20	2.48	49.6	10.05	201	4.05	35	18
DEAE Sephacel	25	1.58	39.5	7.2	180	4.55	32	20
Sephacryl S-200	15	0.34	5.1	5.64	85.6	10.3	15	45

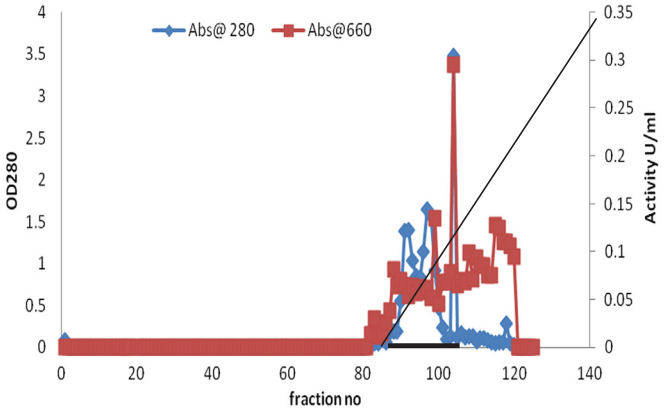

Fig. 2. Chromatogram of phytase on ion exchange column (1.25 × 12.5 cm) of DEAE Sephacel with single activity peak. Pooled fractions were indicated with black bold line.

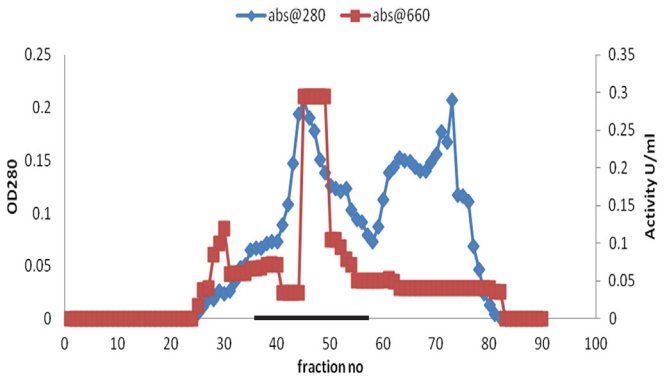

Fig. 3. elution profile of phytase with gel filtration (2.5 × 70 cm) on Sephacryl S-200 with single activity peak. Pooled fractions were represented with black bold line.

3.7. Effect of metal ions on phytase activity

The effects of various metal ions at 5 mM, 10 mM, and 20 mM concentrations on the activity of phytase are shown in Fig. 8. Ca^{2+}, Cu^{2+}, and Fe^{2+} were observed to stimulate phytase activity at all concentrations except Cu^{2+} which inhibited the activity of phytase at higher concentration, 20 mM. Also, cations such as Hg^{2+}, Al^{3+}, Mg^{2+}, Zn^{2+} and Mg^{2+} inhibited the enzymatic activity at all the concentrations investigated.

3.8. Effect of inhibitors/activators on phytase

Inhibitory and stimulatory effects of different reagents on phytase from *A. fumigatus* are shown in Fig. 9. The results clearly indicate that Urea and SDS inhibited the activity of phytase from *A. Fumigatus* while EDTA and Ascorbic acid were shown to activate the enzyme at 5 mM and 20 mM concentrations.

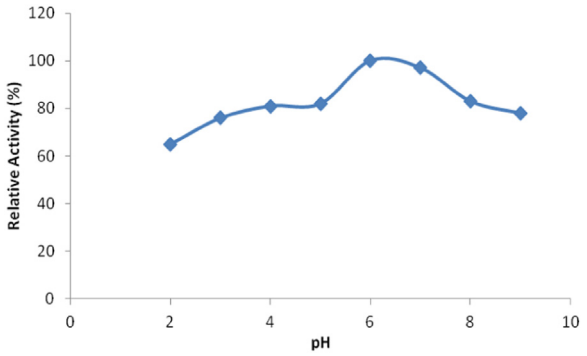

Fig. 4. Effect of pH on phytase enzyme. Optimum pH was taken as 100% and the rest of the pH was calculated relative to the optimum pH with the pH range from 2 to 11: in glycine-HCl buffer (pH 2.0–3.0), sodium acetate (4, 5 and 6), Tris-HCl (7, 8, 9). The results are taking in triplicate and expressed as mean ± standard deviation.

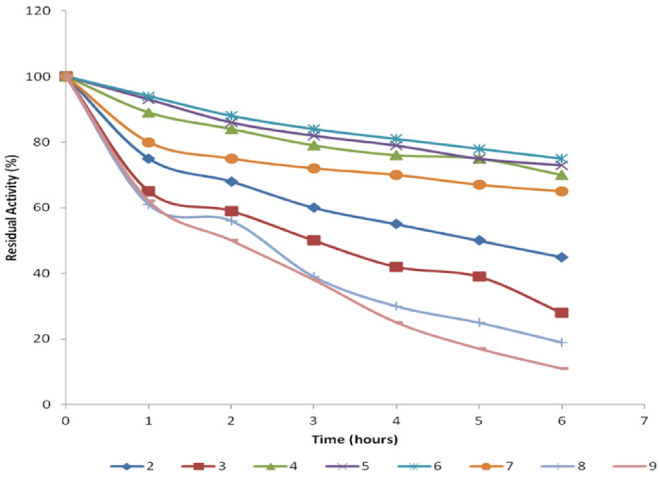

Fig. 5. Effect of on the stability of purified phytase. Initial activity was measured at 0 min while the residual activity of the enzyme was measured and calculated as percentage of the initial activity taken as 100% with the pH range from 2 to 9: in glycine-HCl buffer (pH 2.0–3.0), sodium acetate buffer (4,5 and 6), Tris-HC buffer (7, 8, 9). The results are taking in triplicate and expressed as mean ± standard deviation.

3.9. Kinetic analysis

The Lineweaver-Burk plot of $[V]^{-1}$ against $[S]^{-1}$ is presented in Fig. 10. The K_m and V_{max} values were found to be 7.2 mM and 35.7 μmol/min respectively using phytic acid as substrate.

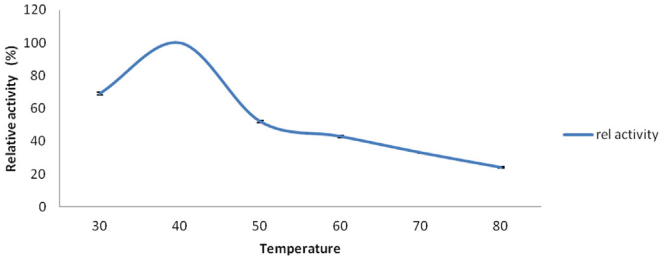

Fig. 6. Effect of temperatures on phytase. The optimum temperature was taken as 100% and the rest of the pH was calculated relative to the optimum activity. Assay mixture was incubated at temperature 30–80 °C and the activity was determined using standard assay procedure. The results are taking in triplicate and expressed as mean ± standard deviation.

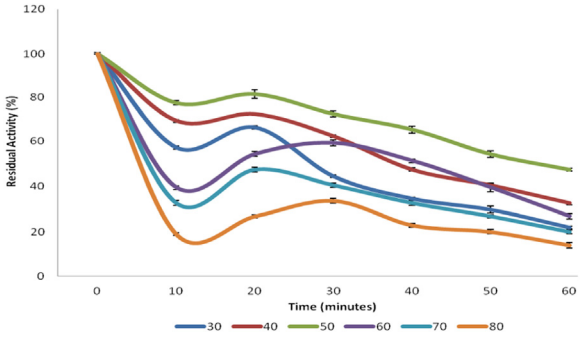

Fig. 7. Effect of temperature on the stability of purified phytase. Initial activity was measured at 0 min while the residual activity of the enzyme was calculated as percentage of the initial activity taken as 100%. The results are taking in triplicate and expressed as mean ± standard deviation.

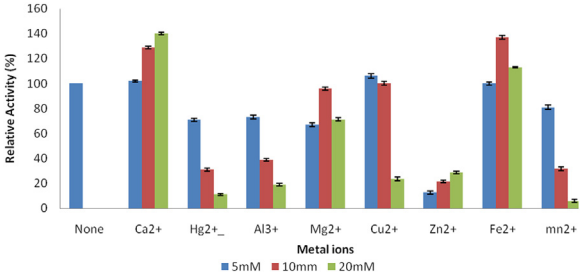

Fig. 8. Effect of metals on phytase activity. None was expressed as 100%. The results are taking in triplicate and expressed as mean ± standard deviation.

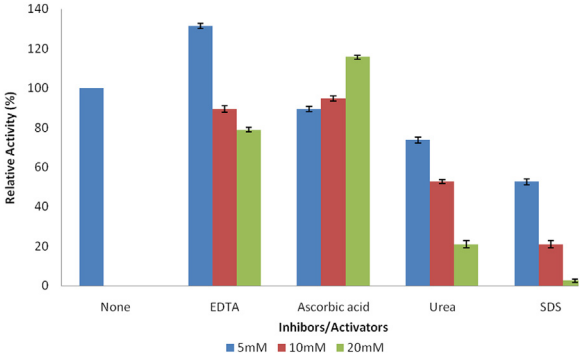

Fig. 9. Effect of inhibitors/activators on the activity of phytase. None was taken as 100%. The results are taking in triplicate and expressed as mean ± standard deviation.

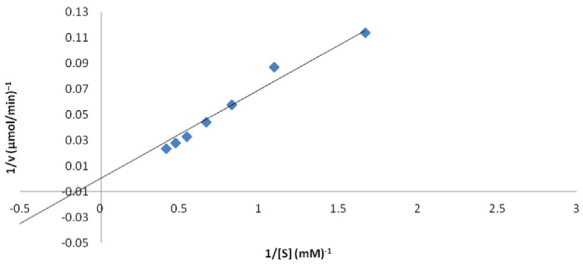

Fig. 10. Lineweaver-Burk Plot of purified phytase from *Aspergillus fumigatus* isolated from African giant snail.

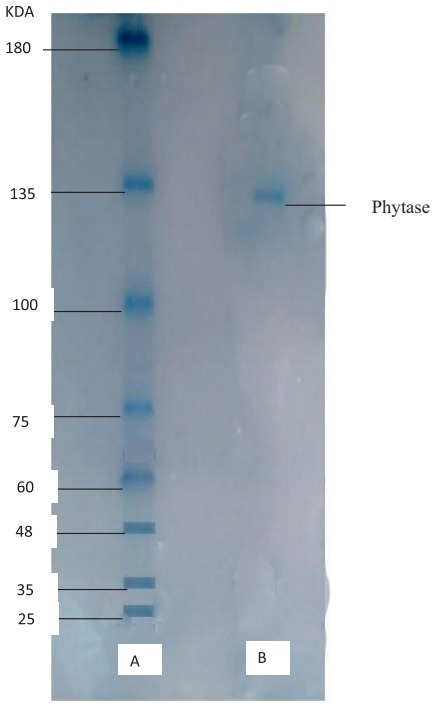

Fig. 11. SDS-PAGE of the purified fraction after gel filtration. Lane A is the molecular weight markers and Lane B is the purified phytase from *Aspergillus fumigatus*.

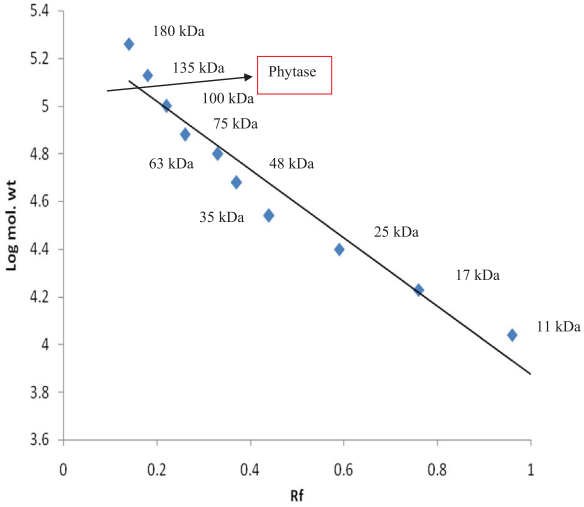

Fig. 12. Graph of log of molecular weight of protein markers against their R_f. The molecular weight of purified phytase was extrapolated from the straight line equation generated from standard graph.

3.10. Molecular weight of phytase from Aspergillus fumigatus

Figs. 11 and 12 showed the sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and graph of molecular weight against R_f respectively of the purified phytase. The protein bound occurred as a single band. The subunit molecular weight of the purified enzyme was estimated to be 118 kDa by SDS-PAGE.

4. Discussion

The isolation and production of phytase from *Aspergillus fumigatus* isolated from the gut of African giant snail in this study coroborated the report of other authors: Ullah (1998), Greiner et al. (2009), Casey and Walsh (2003), Vats and Banerjee (2005) that microorganisms obtained from different sources have the potential of producing phytase. Phytate degrading enzymes occur widely in plants, microorganisms and in some animal tissues. Konietzny and Greiner (2002) in their paper discussed the detection and isolation of phytate degrading enzymes in various bacteria, yeast and fungi. The isolation of *A. fumigatus* from the gut of African giant snail also confirmed the reported observation that the gut of the snail contains diversities of microbial flora (Ekperigin, 2006; Pawar et al., 2012; Oyeleke et al., 2012). Pawar et al. (2012) isolated about thirty-three bacteria genera in different regions of gasro-intestinal tract of African giant snail (*Achatina fulica*). These micobial populations have been reported to produce hydrolytic enzymes that aid in the degradation of complex macromolecules to release chemicals and molecules that are useful in biosynthesis of various macromolecules in African giant snail (Ekperigin, 2006; Pawar et al., 2012). Production of endocellulase from *Acinetobacter anitratus* and *Branhamella spp* isolated from the African giant snail was reported by Ekperigin (2006) while Oyeleke et al. (2012) reported the production of cellulase and protease from microorganisms isolated from the gut of the African giant snail. The work on microbial diversity in the different regions of the gastro intestinal tract of African giant snail (*Achatina fulica*) by Pawar et al. (2012), further inferred that the microbial communities are metabolically active. However, it has also been demonstrated by Charrier and Rouland (1992), Flari and Charrier (1992), and Alikwe et al. (2013), that plant materials such as native cellulose, laminaran and mannan, and leaves are highly degraded by these snails which harbour large sets of microorganisms that produce enzymes for the digestion of plant materials. Hence, gastro-intestinal tract microbiota plays a very important role in metabolism as it provides the African giant snail with a battery of digestive enzymes to hydrolyse the plant (Pawar et al., 2012). Therefore, dependence of digestive ability of African giant snail on the microbial activity within gastro-intestinal tract would explain their extraordinary efficiency in the utilization of plant nutrient.

Under normal physiological conditions phytic acid chelates essential minerals such as calcium, magnesium, iron, zinc, (Hurrell et al., 2003). It is a major storage form of phosphorus in different plant tissues, thereby, causing essential minerals needed for metabolic activity in animals unavailable (Hurrell et al., 2003). The production of phytate degrading enzymes by *Aspergillus fumigatus* and other microorganism in gut of African giant snail may play a significant role in the hydrolysis of phytic acid by this snail. African giant snail is a voracious herbivorus animal (Charrier and Rouland, 1992) which develops well in the wild with a strong shell formation, which means lack of phytate degrading enzyme system may create a major issue in bioavailability of minerals needed for the metabolic activity. Thus, the inherent ability of *Aspergillus fumigatus* and other microbial floral in the GIT of the snail to produce phytase which hydrolyses the phytate salt into phosphorus and phosphorylated compounds, also releasing minerals essential for snail metabolic activity (Oyeleke et al., 2012) may be closely linked to its effective utilization of minerals for growth and shell formation (Flari and Charrier, 1992).

Optimum phytase production obtained at 24 h incubation time is consistent with reported optimum period of phytase production exhibited by *Candida parapsilosis* (Ranjan and Sahay, 2013). However, optimum phytase production was achieved at much higher hour of incubation by Coban and Demirci (2014), for *A. ficuum* at 120 h; Kalieva et al. (2017), for*A. niger*, at 216 h and Gaind and Singh (2015) for *A. flavus* at 144 h. The reason for short hour of incubation and high enzyme yield could be attributed to the ability of the fungus to immediately adapt and effectively utilize the new screening medium and thereby released the enzyme into the medium while in exponential phase. However, as phytic acid which served as inducer in the medium was depleting, the ability to release the enzyme also decreased while the exponential growth of the fungus was still maintained since other components in the medium supported the growth of the fungus until after 36th hour when the growth began to decrease.

A 45-fold purification with 15% recovery achieved after the enzyme was eluted on sephacryl S-200 is higher compared to that of Gaind and Singh (2015), with 34.72-fold purification and 2.17% recovery, Suresh and Dash (2014) obtained 0.76 purification fold and 24% recovery from *A. fumigatus* and Fasimoye et al. (2014) who reported 10% recovery and 39-fold purification. However, other authors reported much higher percentage recovery; El-Toukhy et al. (2013), 57.7% yield and 3.97-fold purification from *Bacillus subtilis* MJA and Roy et al. (2012) gave approximate 41% recovery with 133-fold purification and specific activity of 780 Umg^{-1} protein from *Shigella* spp. CD2. This suggests that, the success of purification depends on purification fold and percentage recovery and is proportional to the sources from which enzymes are produced, type of resins and factors such as purification conditions including elution rate, pH of the buffer and size of the resin.

The purified phytase has a wide range of pH activity between pH 3–9 with the optimum pH observed at pH 6.0 which suggests that the enzyme is an acidic phytase. Suresh and Dash (2014) also reported optimum phytase activity at pH 6 from *A. fumigatus* isolated from soil sample. Other authors have also reported optimum pH of 5.0 and 6.0 for phytase from *A. fumigatus* (Rodriguez et al., 2000; Wyss et al., 1999; Pasamontes et al., 1997). However, the optimum activity is slightly higher than pH 5.5 reported by Rodriguez (2000) for *A. fumigatus*. It has been reported by Rao et al. (2009) that most phytases of fungal origin exhibited optimal activity at pH 4.5–5.5. However, Tran (2010), Gaind and Singh (2015), reported that phytase from *Bacillus spp* and Aspergillus flavus ITCC 6720 have optimum pH between pH 6 and 7. The work of Fasimoye *et al.* (2010) also asserted the optimum activity of phytase from *B. lichiformis PFBL-03* to be pH 6.0. The purified phytase was very stable at the acidic and neutral pH of 4.0–7.0, but, demonstrated drastic instability in alkaline region with a minimal residual activity at pH 8.0 and 9.0 after 6 h of incubation. The influence of pH on phytase stability conformed with the results of Fasimoye et al. (2014) in which pH stability was obtained between pH 4.0–7.5. However, the phytase from *A. fumigatus* was still active at extreme acidic and alkaline medium with significant phytase activity recorded.

The observed optimum temperature of 40 °C in this study was lower compared to other reported temperature optimum from *A. fumigatus,* 50 °C (Suresh and dash, 2014) and 60 °C (Roy et al., 2012), Rodriguez et al. (2000) and Wyss et al. (1999), *A. terreus,* 55 −58 °C (Suresh and das, 2014). The purified phytase retained 48% of its initial activity after 6 h at 50 °C while lower activity was observed at 60 °C. Mullaney and Ullah (2003) observed that phytase has a limited thermal stability and that activity begins to decrease at 60 °C. El-Toukhy et al. (2013) also reported that enzyme activity decreased with increasing incubation time within the same temperature whereas Fasimoye et al. (2014) reported a thermostable phytase from *B lichiformis PFBL-03* which retained 55% of its activity at 80 °C after 1 h incubation. However, Nunes and Guggenbuhl (1998) previously reported that phytase from *Aspergillus fumigatus* has the ability to refold into native-like fully active structure after heat denaturation (20 min at 90 °C).

The fact that phytase activity was enhanced in the presence of Ca^{2+}, $Cu^{2+}$and Fe^{2+} but was greatly inhibited by Zn^{2+}, $Hg^{2+}$and Al^{2+} revealed an observable difference in the report of Casey and Walsh (2003)

which showed that extracellular phytase isolated from *A. niger* ATCC 9142 was enhanced in the presence of Cd^{2+}, Cu^{2+}, Hg^{2+}, Mg^{2+}, Mn^{2+} and Zn^{2+} but was inhibited by Ca^{2+} and was not significantly affected by Fe^{2+} and Fe^{3+}. Li et al. (2008), reported phytase activity from *A. niger* van Teighem to be severely inhibited by Al^{3+} and Mn^{2+} at 10 mM and was enhanced by Ba^{2+} at 1–20 mM. Fasimoye et al. (2014) and Gaind and Singh (2015) as well observed the Ca^{2+} enhancement of activity of phytase by from *B. lichiformis PFBL-03* and *Aspergillus flavus ITCC 6720* respectively but the activity was greatly inactivated by Fe^{2+}, Mn^{2+} and Cu^{2+} by *B. lichiformis PFBL-03*. Phytase from *Aspergillus fumigatus* isolated from African giant snail showed promiscuity in modulating various metal ions even at high concentration establishing its uniqueness and exceptional ability.

Phytase was activated in the presence of EDTA (5 mM concentration) and ascorbic acid (20 mM concentration) while the activity was severely inhibited by SDS. Phytases from *K. ohmeri* and *Pantoea agglomerans* were reported to be inhibited by EDTA at concentration of 1 mM according to Li et al. (2008), whereas phytase from *A. niger van teighem* revealed about 50% enhancement in presence of EDTA with 0.1–2.0 mM (Vats and Banerjee, 2005).

K_m and V_{max} estimated in this work was similar to the values obtained by Wyss et al. (1999) from *Aspergillus niger*. The molecular weight of 118 kDa of purified phytase obtained from *A. fumigatus* was higher than molecular weights reported for phytase from *B subtilis* N-77 (36–38 kDa) (Shimizu, 1992), *Bacillus laevolacticus* (44 kDa) (Gulati et al. (2007)), *Escherichia coli* WC7 9142, (Choi et al., 2002)) and *Aspergillus flavus ITCC 6720,*(30 kDa) (Gaind and Singh, 2015). The predicted size of fungal phytases is around 50 kDa and experimental size is between 65 and 70 kDa, indicating heavy glycosylation. *A. niger* NRRL 3135 native phytase is 27% glycosylated. It contains a substantial proportion of N-linked mannose chains and galactose (Vohra and Satyanarayana, 2001). Molecular masses between 35 and 70 kDa of the phytases so far characterized suggests that they behave like monomeric proteins. However, some phytases appear to be made up of multiple subunits. Seguilha et al. (1992) gave an account of phytase from *Schwanniomyces castellii* which was identified as a tetrameric protein with large subunit (Mr 125 kDa) and three identical subunits (Mr 70 kDa).

5. Conclusion

The study revealed the exceptional ability of phytase from *Aspergillus fumigatus* isolated from African giant snail in maximising and utilizing production media within short incubation of 24 h thereby yielding high activity of 23 U/ml suggesting its time-effectiveness and source of high-yielding phytase. It also maintains pH stability between pH 4–7, thermal stability at 50 °C, neutral pH optimal and displayed various metal ions and inhibitors modulating effect at high concentration suggesting the enzyme tolerance to severe conditions. However, the enzyme has distinct high molecular weight compared to the reported works. Since, the interest of industrial enzyme is in high yield and the one which could withstand harsh conditions, therefore, phytase of *Aspergillus fumigatus* could be considered useful for commercial purposes such as feed supplementation and tackling environmental pollution.

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