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Isolation of Phytate Degrading Activity from Africa Giant Land Snail (*Archachatina marginata*)

David Morakinyo Sanni[1]*, Monsurat Bukola Jimoh[1], Toluwase Hezekiah Fatoki[1], Fatuyi Olanipekun Ekundayo[2] and Oluwasegun V. Omotoyinbo[1]

[1]Enzyme Biotechnology and Bioinformatics Unit, Department of Biochemistry, Federal University of Technology, PMB 704 Akure, Nigeria.
[2]Department of Microbiology, Federal University of Technology, PMB 704 Akure, Nigeria.

ABSTRACT

Phytase, chemically know as myoinositol (1,2,3,4,5,6)-hexakisphosphate phosphohydrolase, catalyses the hydrolysis of myo-inositol hexakisphosphate (phytate) to inorganic monophosphate and lower myo-inositol phosphates, and in some cases to free myo-inositol, rendering phosphorus available for absorption. Phytate degrading enzymes was isolated from different parts of African giant land snail (*Archachatina marginata*). Some physicochemical properties of the enzyme such as the effect of pH, effect of temperature, pH stability and thermostability of the phytase were investigated. The highest phytase degrading activity was found in the mouth. The optimal pH activity of pH 4.0 and 8.0, pH 5.0 and 8.0 were observed for phytases from the mouth and intestine respectively while both phytases has optimum temperature of activity at 60ºC. Phytases isolated from the mouth shows a lower thermal stability compared to intestinal phytases. This work demonstrate the presence of phytase degrading activity in land snail which might be responsible for the bioavailability of some essential divalent metal that are very important in the nutrition of the snail.

KEYWORDS: African giant land snail, *Archachatina marginata*, phytate, phytase, Characterization.

INTRODUCTION

Phytate is a free form of inositol hexakisphosphate (IP6), a polyanionic molecule with six phosphate groups that can strongly chelate with cations such as calcium, magnesium, zinc, copper, iron and potassium to form insoluble salt which affect the bioavailability of these minerals in fish (Papatryphon *et al.,* 1999), as well as phosphorus to monogastric animals (human, dogs, pigs, birds), and also to agastric animals because they lack the intestinal digestive enzyme phytase (Jackson *et al.,* 1996). Moreover, phytate can also integrate with cation groups on protein, amino acids, starch and lipids in feedstuff reducing the digestibility of these nutrients in fish, poultry and pig (Kumar *et al.,* 2012).

Phytase, chemically know as myoinositol (1,2,3,4,5,6)-hexaphosphate phosphohydrolase, catalyses the hydrolysis of myo-inositol haxakisphosphate (phytate) to inorganic monophosphate and lower myo-inositol phosphates, and in some cases to free myo-inositol, rendering phosphorus available for absorption (Kumar *et al.,* 2012; Mullaney and Ullah, 2003; Greiner *et al.,* 1997). There are two types of phytase based on the first phosphate group attacked by the enzyme; 3-phytase (EC 3.1.3.8) and 6-phytase (EC 3.1.3.26). 3-phytase is typical for microorganism while 6-phytase is found in plants. Phytase can also be divided into two types based upon their optimal pH these are acid phytase with optimum activity at pH about 5.0 and alkaline phytase with optimum activity at pH about 8.0 (Kumar *et al.,* 2012).

Phytase is widespread in nature, its activities in microorganisms, plants and animal tissues have been reported (Shieh and Ware, 1968; Yamada *et al.,* 1968) which include rye (Grenier *et al*., 1998), maize, rice and wheat (Hubel and Beck, 1996), *A. oryzae* (Fujita *et al.,* 2000), *Escherichia coli* and *Saccharomyces cerevisiae* (Moore *et al.,* 1995). The major problem in production of plant phytases is that a cost-effective and efficient production of the enzyme is yet to be developed. The higher pH and thermal stability of microbial phytase compared to plant phytases have made the microbial phytase more investigated for industrial purposes.

Generally, phytate degrading enzymes of microbial origin are more pH and thermo stable than their plant counter parts. The stability of most of the plant enzymes decreased dramatically at pH value below 4 and above 7.5, whereas the majority of the corresponding microbial enzymes are rather stable even at pH values above 8 and below 3 (Grenier *et al.,* 1993). In purified form, most of the phytate degrading enzymes from plant have been irreversibly inactivate at temperature above 70ºC within minutes, whereas most of the corresponding microbial enzymes retained

Corresponding author: David Morakinyo Sanni, Enzyme Biotechnology and Bioinformatics Unit, Department of Biochemistry, Federal University of Technology, PMB 704 Akure, Nigeria. Email: moraksanni@yahoo.co.uk

significant activity even after prolonged incubation time. The phytate degrading enzymes must have resistance to high temperature reported so far have been isolated from *A. funmigatus* (Pasamontes *et al.,* 1977) and *S. castellii* (Seguilha *et al*., 1992).

Phytase activity of animals has been reported as insignificant in contrast to plant and microbial counterparts (Weremko *et al.,* 1997). Phytase activity was reported in young rats and pigs. It was found out that stomach is the major site of action of phytase in pigs (Lantzsch *et al.,* 1992) which possibly not improved phosphorus utilization but increased soluble phosphorus excretion. In laying hen, the phytase activity was high in caecum, intermediate in the small intestine and low in the crop and stomach (Marounek *et al.,* 2010). Also, it has been reported that addition of phytase to rice bran improved its nutritional quality as potential poultry feed (Lamid *et al.,* 2014). However, phytase activity has not been reported from the snail body itself.

Snails belong to the phylum mollusca, which are the second largest invertebrate after arthropods (South, 1992). *Achatina fulica* is of small size and fleshly part could be whitish or dark brown. It has low economic value compared to the giant land snail *Archachatina marginata* and *Achatina achatina* (Babalola and Akinsoyinu, 2009). African giant snails are fast growing, voracious wide herbivorous animal. It's generally consumed in West Africa and a great source of animal protein to the large population as well as an alternative to red meat. Snail is high in protein, iron and low in fat (Adeyeye, 1996). Divalent metal such as calcium are very important in the shell formation. However the dynamics of divalent metal utilization is not understood. Therefore, this study seek to investigate the presence of phytate degrading enzyme (Phytase) and some of its physicochemical properties which may probably provide a clue on the possible role it play in the bioavailability and utilization of nutritional significant divalent metal in African giant snail.

2.0 MATERIALS AND METHODS

2.1 Sources of Snail

The land snail obtained from Biological Garden of the Federal University of Technology Akure, Ondo state and fed for two weeks with waterleaves.

2.2 Preparation of Enzyme Extract

Snail was dissected and mouth, stomach, pancrease, and intestine were isolated. Each of those parts was homogenized with homogenizer at 8ºC in 1:3 weight/volume with sodium acetate buffer of pH of 5.5. The homogenate was firstly filtered with muslin cloth to remove the coarse particles and latter centrifuged at 6000 rpm for 30mins at 8ºC. The residue was discarded while the supernatant was stored as crude enzyme at 8ºC.

2.3 Assay for Phytase Activity

Phytase activity was determined based on the method of ammonium molybdate (Heinnen and Laht, 1981) by measuring the rate of phosphorus, as indicated by an increase in absorbance at 700 nm. The sample test tubes contained 1ml of enzyme solution, 2 ml of substrate solution incubated for 37ºCfor 30 minutes using a regulated Gallenhamp water bath. The reaction was stopped with 1ml of TCA (15% w/v) and color development by addition of 1 ml color reagent (3.66g of $FeSO_4.7H_2O$, 0.5g of $(NH_4)_6MO_7O_{24}.4H_2O$ and 1.6ml of concentrated H_2SO_4 in 50ml of distilled water).

2.4 Estimation of Protein Content

Protein concentration was measured according to the Bradford method (1976) using bovine serum albumin (BSA) as a standard.

2.5 Effect of pH on the Activity of Phytase

Effect of pH on the phytase activity of the snail extract was carried out using buffer solution pH 2.0 -9.0. 0.05M of the buffer solution were prepared using different buffer system; glycine (2.0-3.0), acetate (4.0, 5.0 and 6) and (7.0, 8.0 and 9.0). The substrate solutions used in the assay mixture were prepared by dissolving sodium phytate in each of the buffer solution. The activity of phytase was determined using the standard procedure earlier described.

2.6 Effect of pH on the Stability of Phytase

The enzyme solution was incubated for 24hours of buffer solution of pH (2.0-9.0). Different buffer solutions 0.05M were prepared using different buffer system; glycine (2.0-3.0), sodium acetate (4.0, 5.0, and 6.0) and Tris/HCL (7.0, 8.0 and 9.0). 2ml each of the aliquot of the enzyme were withdrawn at 2 hr, 4 hr, 6hr and 24hr for the determination of phytase activity.

2.7 Effect of Temperature on Activity of Phytase

The assay mixtures were incubated at different temperature from 40ºC-80ºC at 10ºC interval to determine the effect of temperature on enzyme activity. The assay mixture consist of 1ml enzyme solution, 2 ml of substrate solution was incubate at 37ºC. The phytase activity was measured at 700 nm after 30 minutes incubation followed by the addition of TCA and color reagent.

2.8 Effect of Temperature on the Stability of Phytase

The enzyme solution was incubated for 1hour at different temperatures; 50, 60 and 70ºC. Two milliliter (2ml) of aliquot of the enzyme were withdrawn at 0min and subsequently at 10mins time interval while the phytase activity was carried out according to the standard procedure.

3.0 RESULTS AND DISCUSSION

The phytase activity from the different parts of snail is illustrated in figure 1. The highest activity of 0.54µg/ml was found in the mouth while 0.41 and 0.28µg/ml were obtained in the intestine and pancreas. The stomach gave the lowest phytase activity. The results obtained indicate the presence of phytate degrading enzyme in intestine and mouth of snail respectively. Intestine seems to produce more of enzyme compared to mouth of snail as shown by the result of phytase activity. This may be due to occurrence of microorganism have been reported by several author; *Aspergillus niger* (Shield and Ware, 1968; Gargova *et al.,* 1997), *Escherichia coli* (Greiner *et al.,* 1993), *Saccharomyces cerevisia* (Moore *et al.,* 1995). *A. niger* has been report as potent phytase producer. The phytase gene originating from *Aspergillus* species almost exclusively encodes most commercial phytases and many other microorganisms are able to produce phosphatase with phytase activity.

Effect of pH on activity of phytase crude enzyme from mouth and intestine of snail is illustrated in figure 2 and 3. Two optimum activities was observed of phytase from mouth of snail at acidic at pH 4.0 and basic at pH 8.0 while the intestine of snail showed optimum activity acidic at pH5.0 and basic at pH8.0. Enzymatic activity was observed in both acidic and basic pH region. Cao et al., 2007; reported that most phytase showed optimum pH between 4.5 and 6.0. Alkaline phytate degrading enzymes having a pH optimum at 8.0 are described in legume seed (Scott, 1991) and cattail (Hara *et al.,* 1985) as well as the rat intestine (Yang *et al.,* 1991). The phytase of mouth of snail showed optimum temperature activity at 40ºC and 60ºC while intestine of snail showed optimum temperature activity at 60ºC.

Effect of pH stability on phytase activity from the mouth and intestine of Giant African snail is illustrated in 4 and 5. The enzyme from the intestine has highest activity at pH of 2 and pH of 9, which are above 50% of the residual activity while other pH values are at 40% and below the residual activity of the enzyme. The enzyme from the mouth shows stable activity at pH 7, 8 and 9 which is at 60% and above while other pH values are below 40% of the residual activity. Simon and Igbasan, (2002) reported a high pH stability in a broad pH range of pH (4.0-7.3) for *Aspergillus fumigatus*.

The influence of temperature on the enzyme activity of crude enzyme extracts is illustrated in figure 6 and 7 for mouth and intestine of snail. The figures showed two optimal temperatures for mouth phytase activity to be 40ºC and 60ºC and one optimum activity for intestine phytase activity at 60ºC. The result obtained for the thermal stability shows that, both phytase from mouth and intestine of snail higher thermal stability in which 60% and 70% of relative activity retained at 50ºC and 60ºC respectively. The temperature optima of phytate degrading enzyme vary from 35ºC to 77ºC. Generally, phytate degrading enzymes from plants exhibit maximum activity at lower temperatures compared with their microbial counterparts (Yang *et al.,* 1991). Tsang *et al* (2000) reported an optimum temperature of 55ºC for *P. simplicissimium.*

The effect of thermo stability of phytase of crude enzyme of mouth and intestine of snail are illustrated in figure 8 and 9. It revealed over 60% residual activities at three different temperatures after one-hour incubation. However, less than 40% residual activity was observe as the residual activity for the three different temperatures after 60 minutes of incubation for both phytases. Simon and Igbasan (2002) reported a high thermostability up to 80% for phytase from *Aspergillus fumigatus* and *A. Niger ATTC.*

CONCLUSION

The occurrence of phytase in the various part of *Archachatina maginata* showed that it can digest phytic acid from plant leaves efficiently, which may impact on the bioavailability and utilization of plant minerals in the feed stock, and the contribution of microorganism to phytate degrading ability has not been investigated in snail.

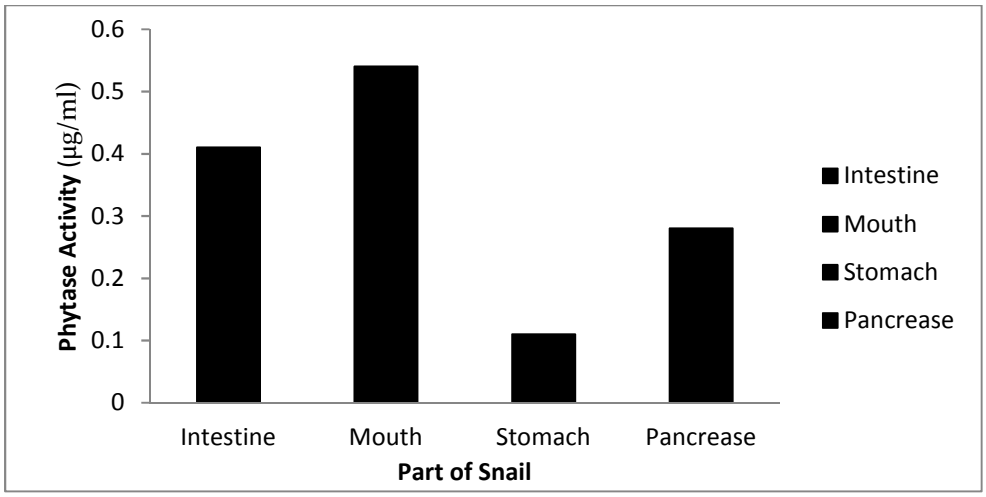

Figure 1: Distribution of phytase activity in different part of snail

The assay mixture contain 1ml of enzyme solution, 2ml of substrate solution prepared by dissolving 0.2% of sodium phytate substrate concentration in sodium acetate buffer of pH 5.5 and was incubated for 30 minutes according to standard assay condition.1ml of T.C.A to stop reaction and 1ml of color reagent was added. The activity was expressed that there is higher phytase activity in intestine and mouth of while lower phytase activity in stomach and pancrease of snail.

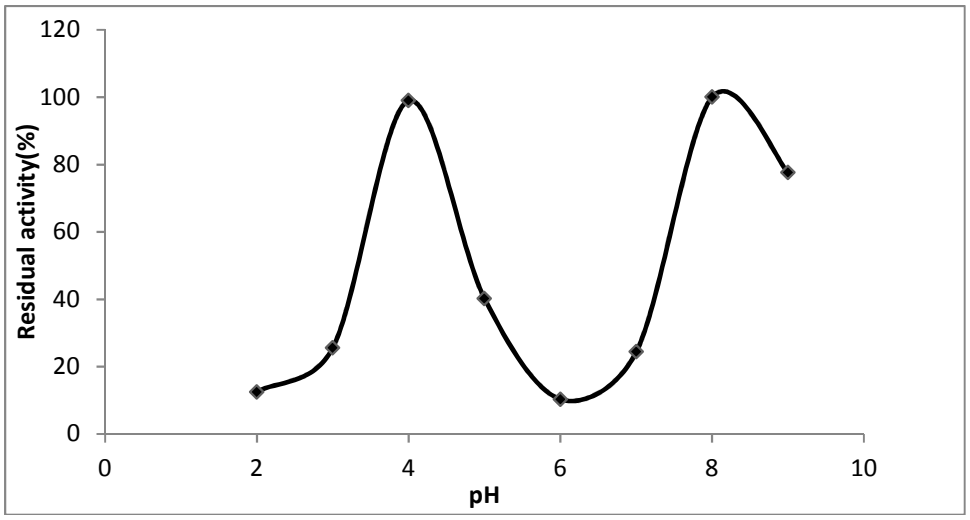

Figure 2: Effect of pH on activity of phytase on mouth of snail

The assay mixture contain 1ml of enzyme solution, 2ml of substrate prepared by dissolving the substrate in a buffer with the pH of interest and was incubated for 30 minutes according to n`standard assay condition, 1ml of TCA to stop reaction and 1ml of color reagent was added.

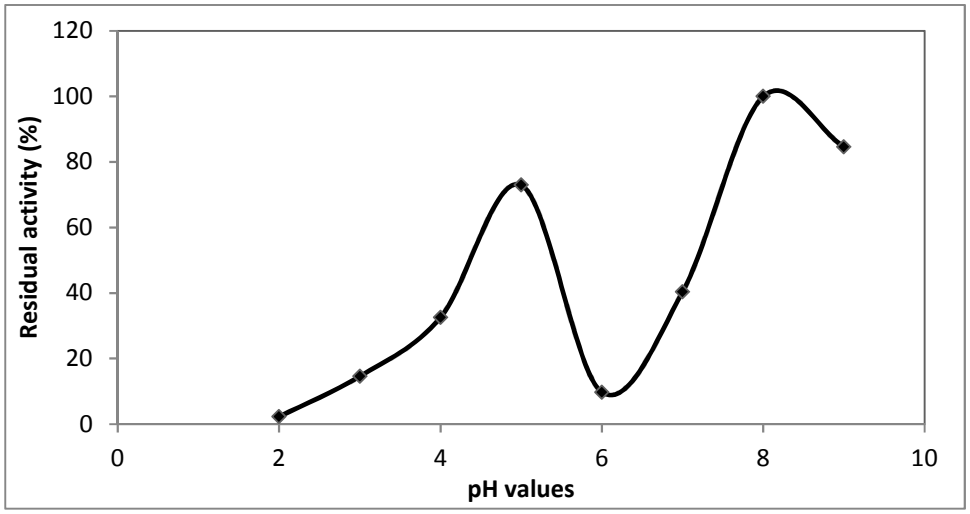

Figure 3: Effect of pH on activity of phytase on intestine of snail

The assay mixture contain 1ml of enzyme solution, 2ml of substrate solution prepared by dissolving the substrate bin a buffer with pH of interest and was incubated for 30 minutes according to the standard assay condition, 1ml of TCA to stop the reaction and 1ml of color reagent was added.

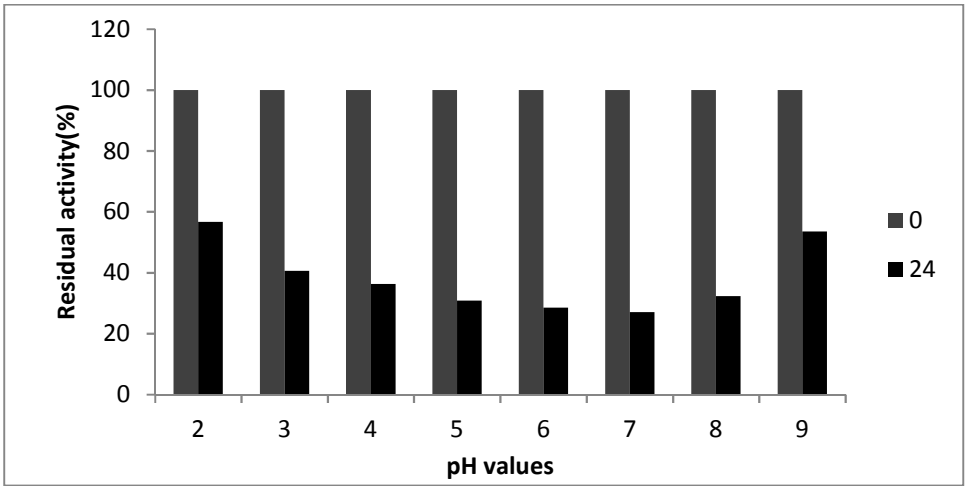

Figure 4: Effect of pH stability on intestine of snail

The enzymes solution incubated at different pH values, at pH 2 to 9 for 24hours. 2mls of the enzymes was withdrawn first at 0minutes and latter at 24 hours, the assay according to standard assay procedure.

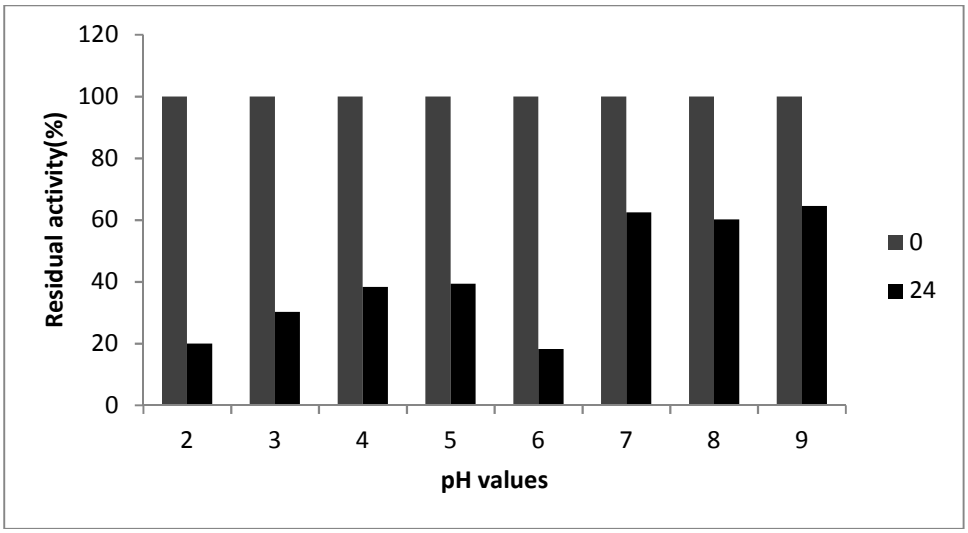

Figure 5: Effect of pH stability on mouth of the snail

The enzyme solution incubated at different pH, between pH 2 and pH 9 for 24 hours. 2 mls of the enzyme was withdrawn first at 0 minute and latter at 24 hours, then assay according to standard assay procedure.

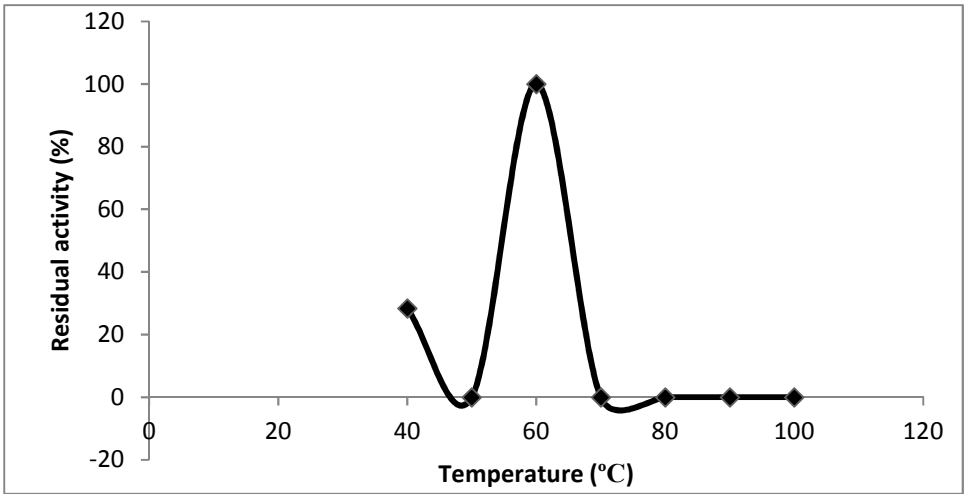

Figure 6: Effect of temperature on the activity of phytase on intestine of snail

The assay mixture, which consists of 1ml of enzyme solution and 2 ml of substrate solution, were incubated at different temperatures: 40°C to 100°C at 10 minutes interval. The activity was measured at 700 nm after 30 minutes of incubation addition of 1 ml of TCA to stop the reaction and 1ml color reagent.

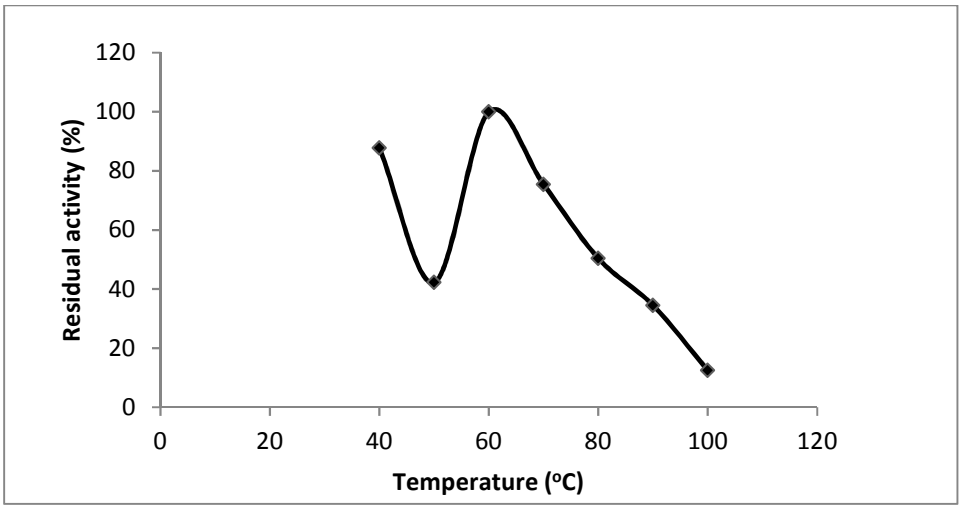

Figure 7: Effect of temperature of phytase activity on mouth of snail

The assay mixture, which consists of 1ml of enzyme solution and 2ml of substrate solution, incubated at different temperature between 40ºC to 100ºC interval. The was measured at 700nm after 30 minutes incubation, addition 1ml of TCA and 1ml color reagent.

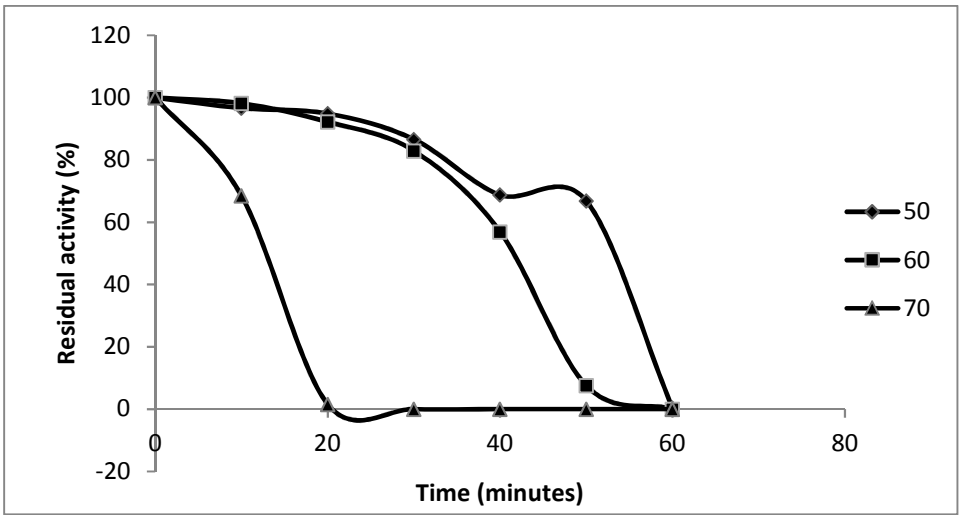

Figure 8: Effect of temperature on the stability of phytase from intestine of snail

Enzyme solution incubated at different temperatures of 50ºC, 60ºC and 70ºC for 60 minutes. 2ml of enzyme was withdrawn first at 0 minute and later at an interval of 10 minutes and assay according to standard assay procedure.

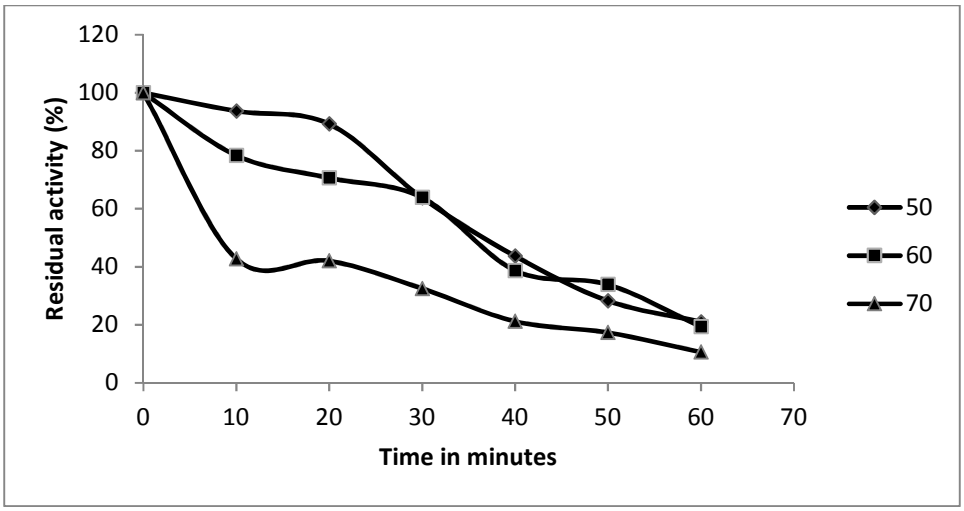

Figure 9: Effect of temperature on the stability of phytase from mouth of snail

Enzyme solution incubated at different temperatures of 50^{o}C, 60^{o}C and 70^{o}C for 60 minutes. 2ml of enzyme was withdrawn first at 0 minute and later at an interval of 10 minutes and assay according to standard assay procedure.

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