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Properties of Amyloglucosidase in the Digestive Tract of *Periplaneta americana* L. (*Blattodea*: *Blattidae*)

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Authors' contributions

This work was carried out in collaboration between all authors. Author OJS designed the study, wrote the protocol together with author REO and supervised the work. Authors RSA and ATB carried out all laboratories assays and performed the statistical analysis. Authors OJS and REO managed the analyses of the study. Author OJS wrote the first draft of the manuscript. Authors OJS and REO managed the literature searches and edited the manuscript. All authors read and approved the final manuscript.

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Original Research Article

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ABSTRACT

Aim: Partial purification and characterization of amyloglucosidase from an insect were carried out to determine the physicochemical properties of the enzyme.
Study Design: It was designed to dissect digestive tracts from the American cockroach, *Periplaneta americana*, and to investigate the properties of the gut amyloglucosidase with a view to predicting possible industrial and pest control applications.
Place and Duration of Study: The study was carried out in the Insect Physiology Laboratory of the Department of Crop Production and Protection and Department of Biochemistry, Obafemi Awolowo University, Ile-Ife, Nigeria between June and October, 2013.

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Methodology: Newly emerged cockroaches were dissected in ice-cold phosphate buffer and digestive tracts were collected to prepare the crude enzyme extract. Standard bioassays were constituted to purify and characterize amyloglucosidase.
Results: The purification had a 71.6% yield and a specific activity of 2.53 U/mg protein. On soluble starch, the enzyme exhibited optimum activity at pH 4.0 with a Michaelis constant (K_m) of 1.67 mg/ml and a maximum velocity (V_{max}) of 10mg/ml/min. Amyloglucosidase activity was enhanced by Mn^{2+} but it was slightly inhibited by Sn^{2+}, Mg^{2+} and Ni^{2+}, while Zn^{2+} caused a 50% inhibition. Optimum temperature for the partially purified enzyme was 40°C and it lost about 90% of its original activity when incubated beyond 20 min at 60°C.
Conclusion: Obtained results suggested that starch degradation using amyloglucosidase from *P. americana* could be done around pH 4 and at temperature around 40°C. This work appears to give the first report on physicochemical properties of amyloglucosidase in insects. Further studies would be needed to determine the possibility of using molecular techniques in inducing amyloglucosidase-Zn^{2+} complex in *P. americana* and to find a probable source of thermophilic amyloglucosidase which would be of importance in an industrial context.

Keywords: Amyloglucosidase; characterization; optimum pH; optimum temperature; Periplaneta americana; purification.

1. INTRODUCTION

The American cockroach, *Periplaneta americana* L. (*Blattodea: Blattidae*), is the largest of the common peridomestic species measuring on average 4 cm in length. The insect can become a public health problem due to its association with human waste and diseases, and its ability to move from sewers into homes and commercial establishments. It can also spoil food and cause allergies [1]. At least 22 species of pathogenic human bacteria, virus, fungi and protozoans, as well as five species of helminthic worms, have been isolated from field-collected *P. americana* [2].

Periplaneta americana has been studied extensively due to its economic and medical importance [3,4] but some aspects of its nutrition biology, especially, characterization of its digestive enzymes are yet to be fully understood. The American cockroach is omnivorous and scavenges, eating almost anything. It prefers carbohydrate sources and has been observed eating paper, boots, hair, bread, fruit, old rice, cloth and dead insects.

Digestive tract of *P. americana* harbours xylanase, laminaribiase, cellobiase, maltase, sucrase, α- and β-amylase, α- and β-glucosidase, α- and β-glycosidase, β-fucosidase, chitinase and N-acetyl-β-glucosaminidase that break down ingested carbohydrates [5-7]. These enzymes, generally known as glycohydrolases, degrade different polysaccharides either directly or in step-wise manners to produce glucose. Glucose is an important carbohydrate in insect biology, serving as the primary source of energy in cells.

Amyloglucosidase [glucoamylase; exo-1,4-α-glucosidase; EC 3.2.1.3] is an exo-enzyme which degrades starch from the non-reducing end and releases glucose as the sole end-product [8]. It does this by hydrolyzing α-1, 4 and α-1, 6 linkages in starch and related polymers. The production of glucose from starch by amyloglucosidase alone is a slow and inefficient process but its efficacy could be enhanced by adding α-amylase. This amylase is an endo-enzyme which increases the number of non-reducing ends by its random action on starch molecules, thereby, enhancing the rate of amyloglucosidase reaction [9]. On its own, α-amylase only degrades starch to maltose and maltodextrin, but not glucose [7]. Lima et al. [10] localized the amylase activity of *P. americana*in two regions of the midgut; the caecal epithelium and the anterior ventricular epithelium. In these regions, the enzyme was observed in the columnar cells, especially in areas above the nucleus and in the gut lumen near the brush border. Kouamé et al. [11] also reported synergism between α-amylase and α-glucosidase in *P. americana* starch hydrolysis. Dué et al. [7] obtained pure α-amylase from *P. americana* and did a comprehensive characterization of same. These and many other reports on α-amylase abound in literature whereas there is a dearth of information on amyloglucosidase. This work was, therefore, carried out to understand the properties of insect amyloglucosidase, using *P. americana* and to

provide additional information on starch degradation in the cockroach.

2. MATERIALS AND METHODS

All chemicals used were of analytical grade and they were purchased from Sigma Chemical Company, St. Louis, MO USA. Materials used are soluble starch, acetate and phosphate salts, sodium hydroxide, sodium and potassium tartarate, dinitrosalicylic acid and sodium chloride.

2.1 Insect Rearing

Cockroach colonies were established using specimens (oothecae, nymphs and adults) collected from the cafeteria and residential quarters of the Obafemi Awolowo University, Ile-Ife. Collections were made mostly at night from places such as stores, living rooms, ovens, kitchens, lockers, abandoned cartons, broken water pipes and septic tanks. Collected specimens were reared on biscuit and bread inside laboratory cages that were maintained at 26±1ºC and 73±3% RH. Cages were monitored on daily basis for newly emerged adults that were used for sample preparation.

2.2 Sample Preparation

Periplaneta americana adults (0-3 days old) were selected randomly and dissected under a stereomicroscope in ice-cold phosphate buffer (10mM, pH 3.0). The digestive tracts were removed carefully and 6.5g of this was homogenized in the buffer before centrifugation at 4000 rpm for 20 min at 4ºC. The supernatant was stored at 4ºC for subsequent analyses.

2.3 Determination of Amyloglucosidase Activity in Sample

The enzyme extract was incubated with 1% starch in 10mM phosphate buffer (pH 3.0) at 60°C and the amount of reducing sugar produced was determined according to the method described by Bernfeld [12]. The reaction mixture consisted of 0.2ml 1% (w/v) soluble starch and 0.1ml homogenate incubated for 5 min in a water bath at 60ºC. The mixture was then boiled at 100ºC for 5min and 8ml of distilled water was added. The reaction was halted by addition of dinitrosalicylic acid before estimating the reducing sugars released by hydrolysis. The optical density was read at 470nm. One unit of the enzyme activity is defined as the amount of amyloglucosidase which produced 1 µg of reducing sugar. Protein concentration was estimated according to the method of Bradford [13] using bovine serum albumin as standard.

2.4 Purification Procedure

Reactive Blue 2-Agarose resin was packed into 1.5×20.0cm column and equilibrated with 10mM phosphate buffer pH 3.0. The enzyme solution was layered on the packed column and eluted with 200ml linear gradient of 0-1M NaCl in 10mM phosphate buffer pH 3.0. Fractions of 3ml were collected from the column that was maintained at a flow rate of 30ml per hour. The activity and protein concentration of the enzyme in the collected fractions were then assayed.

2.5 Determination of Kinetic Parameters

The effect of substrate concentration on purified amyloglucosidase was determined using starch concentration ranging from 0.1-1.0%. Kinetic parameters (K_m and V_{max}) were determined from a double-reciprocal plot [14].

2.6 Effect of Divalent Metals on Amyloglucosidase Activity

The effect of metal on amyloglucosidase activity was studied using five cations: Zn^{2+}, Ni^{2+}, Sn^{2+}, Mn^{2+} and Mg^{2+}. A reaction mixture of 1ml contained 0.1mM metal concentration in typical enzyme assays.

2.7 Effect of pH and Temperature on Enzyme Activity

The effect of pH on amyloglucosidase activity was studied by assaying the enzyme at different pH values between 2 and 6. Effect of temperature on activity of the enzyme was also investigated between 25 and 100ºC. The assay mixture was first incubated at the indicated temperature for 5 min before the reaction was initiated by the addition of 50µl of the enzyme that had been equilibrated at the same temperature.

3. RESULTS AND DISCUSSION

Animals depend on digestive carbohydrases to break down and utilize starch in their food. This class of enzymes is very important to *P. americana* which feed mostly on carbohydrate sources and thus, an excellent understanding of

the enzymes' physicochemical properties could assist in formulating effective control strategies. A number of previous workers have characterized amyloglucosidase from microorganisms such as *Aspergillus niger* van Tieghem (*Eurotiales: Trichocomaceae*) [15,16], *A. candidus* Link [17], *Fusariumsolani* (Mart.) Sacc. (*Hypocreales: Nectriaceae*) [18] and *Halobacterium sodomense* (Oren) McGenity and Grant (*Halobacteriales: Halobacteriaceae*) [19] but the present study appears to give the first of such reports in insects.

3.1 Amyloglucosidase Activity and Purification

The summary of amyloglucosidase purification is presented in Table 1 and the elution profile is shown in Fig. 1. The affinity chromatography procedure resulted in a partially-purified amyloglucosidase with a specific activity of 2.53 U/mg and 71.6% yield but studies have shown that values could vary, among organisms and within species, depending on the efficacy of purification method used. For instance, Bhatti et al. [18] obtained a 31.8% yield of amyloglucosidase from *F. solani*using gel filteration on Fast Protein Liquid Chromatographic system (FPLC) with 26.2-fold increase in specific activity. A specific activity of 329 U/mg with a yield of 70-75% was obtained when amyloglucosidase was purified from *A. candidus* var. *aureus* using hydrophobic interaction chromatography and DEAE-cellulose treatment [17]. The amyloglucosidase from *H. sodomense* was purified using a combination of hydrophobic interaction chromatography and immobilized metal ion affinity chromatography with a 75% recovery [19]. In addition, Ramadas et al. [20] employed an aqueous two-phase extraction after solid state fermentation on fungal bran to recover 95% of amyloglucosidase with a purification factor of 11. Manera et al. [16] also obtained a purification fold of 9.9 from *A. niger* with a recovery of 47.6% using DEAE–cellulose ion exchange chromatography while Fogarty and Benson [21] reported a 61-fold purification and specific activity of 524U/mg from another strain of *A. niger* using ammonium sulphate fractionation, acetone precipitation and CM-BIO-GEL A chromatography.

3.2 Kinetic Parameters of Purified Amyloglucosidase

The values of Michaelis constant (K_m) and maximum velocity (V_{max}) derived from the double-reciprocal plot (Fig. 2) were 1.67mg/ml and 10mg/ml/min, respectively. This K_m value is comparable to 1.90mg/ml obtained for amyloglucosidase in *F. solani* [18]. Fogarty and Benson [21] noted the suitability of starch as a substrate for amyloglucosidase and it was reported that the enzyme possesses a greater ability to digest large molecular weight compounds as evidenced in the relative hydrolytic values for starch, maltotriose and maltose in descending order.

3.3 Action of Divalent Metals

In the present study Sn^{2+}, Mg^{2+}, Ni^{2+} and Zn^{2+} inhibited amyloglucosidase activity by 16%, 20%, 30% and 50%, respectively, while Mn^{2+} enhanced the performance of the enzyme (Table 2). This result corroborates that of Bhatti et al. [18] which stated that Ni^{2+} inhibited amyloglucosidase in *F. solani* but it differed with that of Fogarty and Benson [21] where Sn^{2+} and Zn^{2+} stimulated amyloglucosidase in *A. niger*. This is an indication that influence of metal ions on activity of a particular enzyme may vary from one species to the other.

3.4 Effect of Temperature

The purified amyloglucosidase exhibited maximum activity at 40°C (Fig. 3) and this compares very well with the range (40-45°C) obtained for *Aureobasidium pullulans* (de Bary) G. Arnaud (*Dothideales: Dothioraceae*) [22], *A. niger* [23] and *F. solani* [18]. James and Lee [24] also reported that the temperature optima of amyloglucosidases generally fall in the range of 30-60°C. However, Fogarty and Benson [21], Uhlich et al. [25] and Rani et al. [9] reported a surprisingly high optimum temperature of 70°C. A high operating temperature is of considerable significance in an industrial context since it offers the advantages of increased reaction rates, decreased viscosity, reduced contamination and better storage stability [21].

3.5 Thermal Stability

The effect of heat on the stability of purified amyloglucosidase, expressed as percent residual activity, is presented in Fig. 4. At 30°C and 60°C, the enzyme lost about 40% and 76% of its original activity, respectively, within 10min of incubation. When the period of incubation was doubled, loss in enzyme activity increased to 50% and 85%, respectively. Enzyme stability decreased with increasing incubation period, with a loss of about 90% of original activity when

incubation at 60°C exceeded 20min. This indicated that amyloglucosidase from *P. americana* may not be able to withstand high temperatures that usually characterize industrial activities.

3.6 Effect of pH

The partially purified amyloglucosidase had an optimum pH of 4.0 on soluble starch (Fig. 5). This acidic level is the same with that obtained for amyloglucosidase from a strain of *A. niger* [15] and it is comparable to pH 4.5 obtained for *F. solani* [18]. Amyloglucosidase is regarded as unstable above pH 7.0 [26,27] except those from *Corticium rolfsii* Curzi (*Atheliales: Atheliaceae*) [28] and *Humicola lanuginosa* (Tsiklinsky) Bunce (*Eurotiales*: *Incertaefamiliae*) [29] that possess considerable stability above the neutral pH level. The pH affects ionization of essential active site amino acid residues which are involved in substrate binding and catalysis. The ionization of these residues may cause distortion of active site cleft and hence indirectly affect the enzyme activity [18].

Table 1. Purification of amyloglucosidase from the digestive tract of *Periplaneta americana*

Purification step	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Purification factor	Yield (%)
Crude Extract	95.00	38.80	2.45	1.00	100.00
Affinity chromatography	70.20	27.80	2.53	1.02	71.60

Table 2. Effect of metal ions on activity of amyloglucosidase in the digestive tract of *Periplaneta americana*

Metal ion	Residual activity (%)
Mn^{2+}	107.0
Sn^{2+}	84.8
Mg^{2+}	80.0
Ni^{2+}	70.3
Zn^{2+}	50.6

Any value>100 indicate enhancement of enzyme activity

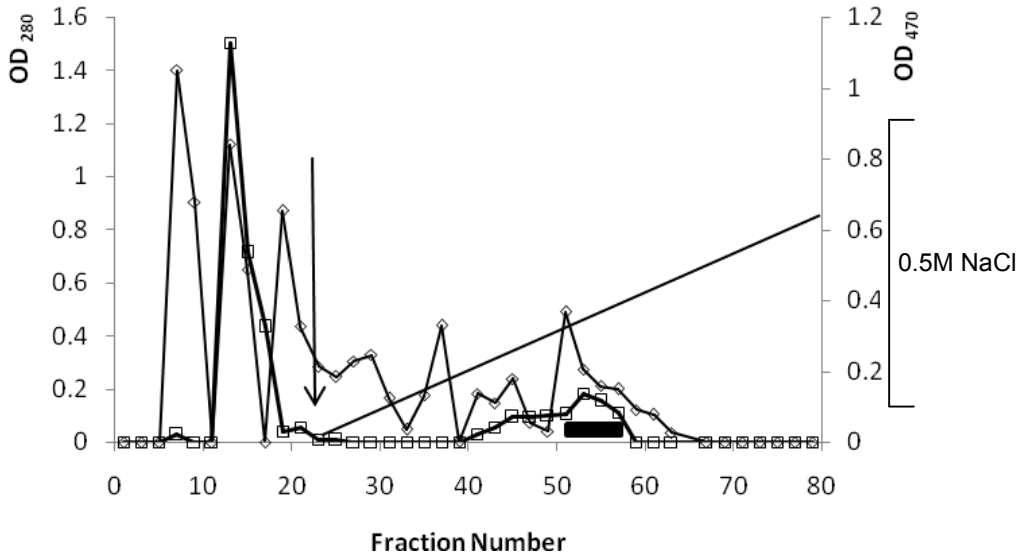

Fig. 1. Reactive Blue 2-Agarose resin chromatography of amyloglucosidase in the digestive tract of *Periplaneta americana*. The packed resin was equilibrated with 10mM Phosphate buffer, pH 3. The enzyme solution was layered on the packed column and eluted with a 200ml linear gradient of 0-1M NaCl in 10mM Phosphate buffer, pH 3. Fractions of 3ml were collected from the column

-□-□-□-□- *Activity profile (OD 470 nm)*; -◊-◊-◊-◊- *Protein profile (OD 280 nm)*
↓ *Salt concentration gradient;* ■ *Pooled enzyme fraction*

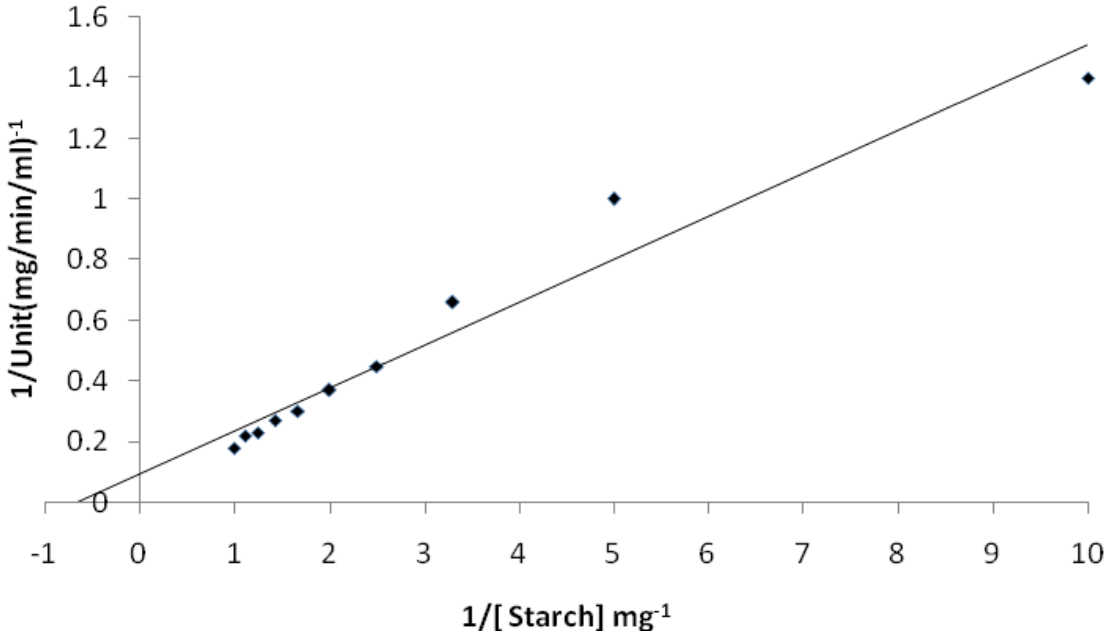

Fig. 2. Lineweaver-Burk plot showing the effect of varying concentrations of starch on the reaction velocity of amyloglucosidase in the gut of *Periplaneta americana*

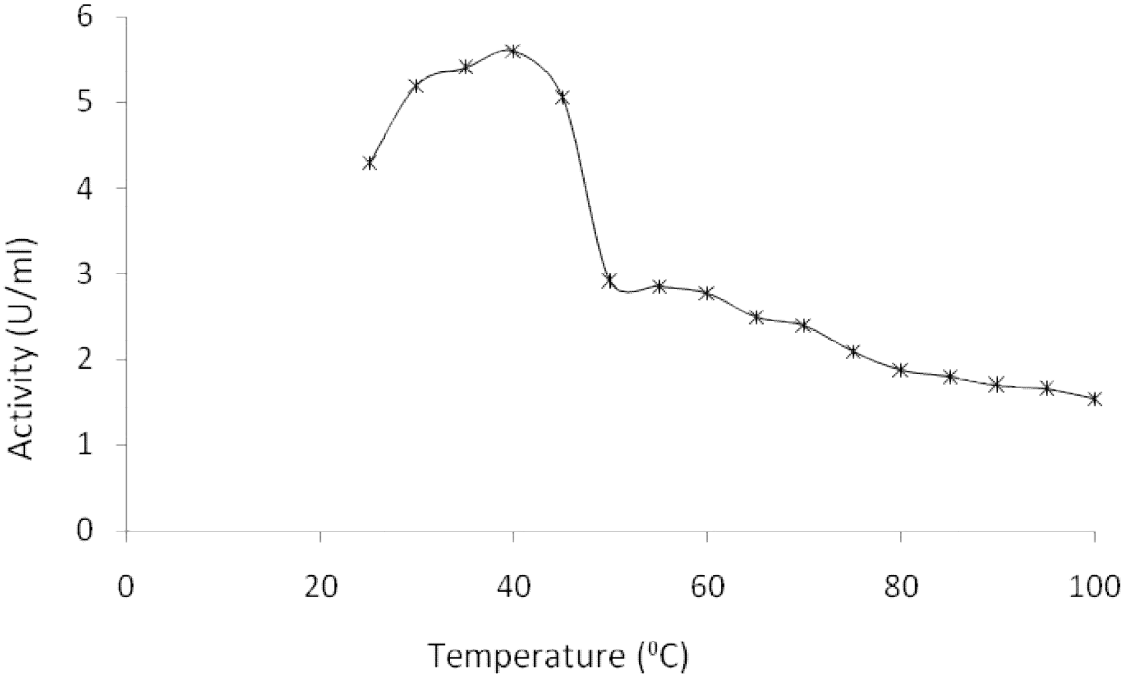

Fig. 3. Effect of temperature on the amyloglucosidase of *Periplaneta americana*. Reaction mixtures were incubated at temperatures between 25 and 100°C for 5min

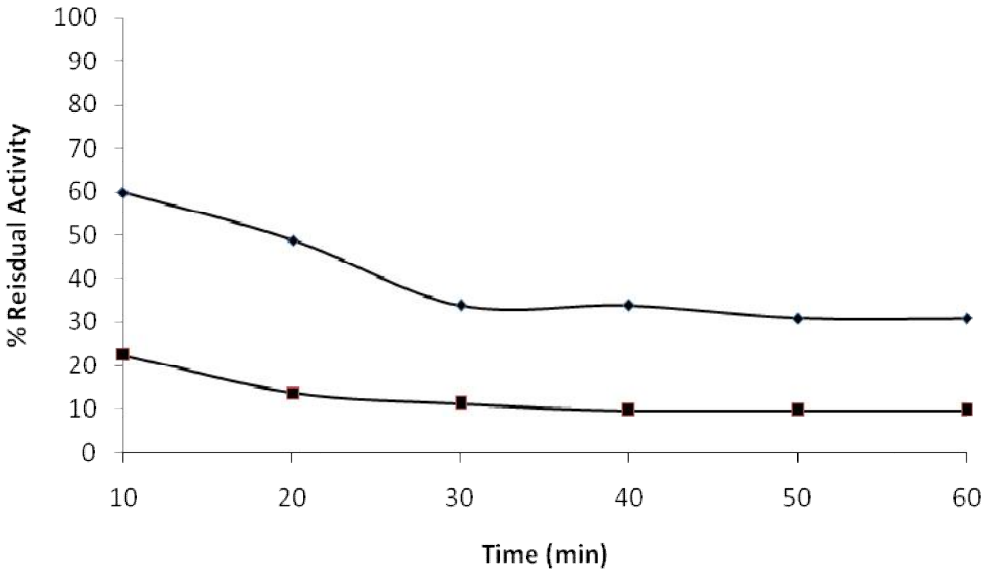

Fig. 4. Thermic inactivation of amyloglucosidase from *Periplaneta americana* at 30°C and 60°C. Thermal stability of the enzyme was followed for 1 h. Residual activity, determined under standard test conditions, is expressed as percentage activity of zero-time control of untreated enzyme

-♦-♦-♦- Activity at 30°C; -■-■-■- Activity at 60°C

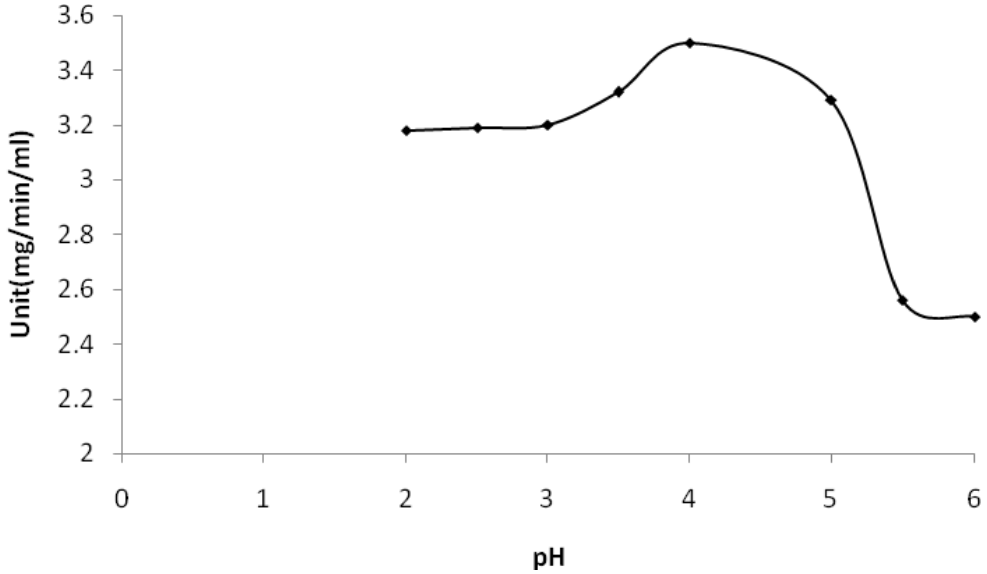

Fig. 5. Effect of pH on amyloglucosidase of *Periplaneta americana*. Reaction mixtures were assayed at pH 2-6

4. CONCLUSION

There is a dearth of information on amyloglucosidase of insect origin and this work has endeavoured to give a framework by determining different physicochemical properties of the enzyme in *P. americana*. Further studies would be necessary to examine amyloglucosidase from other insect species with

a view to determining a thermotolerant source. Induction of enzyme-inhibiting ion complex would also be an interesting line of research.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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