ACTIVITY OF CELLULASE IN THE MIDGUT HOMOGENATE OF THE PALM WEEVIL, *RHYNCHOPHORUS PHOENICIS* F. (COLEOPTERA: CURCULIONIDAE)

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ABSTRACT

Palm weevil, Rhynchophorus phoenicis F is a key pest of palm trees and sugar cane in the tropics and subtropical regions of the world where it causes great economic losses. The midgut of the larva and adult stages of this insect are rich in digestive enzymes. Cellulase, being one of the most important carbohydrases was investigated in the midguts of the larva and adult stages of this insect using dinitrosalicylic acid reagent method. The optimum condition for cellulase bioassay in the midgut of the larva of R. phoenicis involved 2 ml of 1% starch, 2 ml of phosphate buffer (pH 4.0) and 1 ml of enzyme extract incubated at optimum temperature of 45^{o} C were optimal for cellulase activity in both the larval and adult weevils. Michaelis-Menten constants (Km) of 2.5 mg/ml and 3.12 mg/ml were obtained for the larva and adult midgut cellulases respectively.

Keywords: *Rhynchophorus phoenicis, Cellulase, enzyme extract, optimum, homogenate, dinitrosalicylic acid, buffer*

INTRODUCTION

All living organisms are able to obtain and use energy very rapidly due to the presence of biological catalysts called enzymes. Biological reactions proceed much more quickly in the presence of enzymes. Just like inorganic catalysts, enzymes change the rate of chemical reactions but do not alter the final equilibrium of the reaction. In addition, only small quantities of enzymes are needed to bring about the transformation of a large number of molecules of food. Enzymes play very prominent roles in the digestion of food in insects (Delalibera, *et al.* 2005, Omotoso and Adedire, 2013). It has been reported that the use of neem oil and endosulfan have greatly reduced the activities of midgut enzymes in *Helicoverpa armigera* (Rashid War *et al.* (2014).

Plant materials contain significant amounts of structural polysaccharides, particularly cellulose and a range of hemicelluloses. These polymers contained sugar units, usually glucose which are joined together by β-1,4 and β-1,3 glycosidic bonds (Linton and Greenaway, 2004). Vertebrates generally lack the enzymes necessary to break these bonds but can only access these sugars through the

activity of micro-organisms that are present in their alimentary systems. Cellulose digestion is common among invertebrates, and an increasing number have been known to possess their own cellulases (Watanabe and Tokuda, 2001).

Enzymatic hydrolysis of cellulose to glucose occurs by the action of a mixture of three major classes of cellulase enzymes. They include Endo-1,4-β-glucanases (or endocellulases) which digest polymer chains, exo-1,4-β-glucanases (or exocellulases) which hydrolyze cellobiose polymer chain and β-glucosidase which hydrolyze cellobiose to glucose (Zhu, *et al.,* 2005, Oppert, *et al.* 2010). The conversion of cellulose to sugars and ethanol through fermentation is a process which has great economic potentials and it is environmentally friendly (Sheehan, 1994; Sree, *et al*., 2000). The ability to digest cellulose is a preserve attributes of a number of orders and families of insects. Several insect cellulases have been purified and characterized (Su, *et al.* 2013). Cellulase is a widespread enzyme in micro-organisms such as bacteria and fungi (Tomme, *et al*., 1995; Beguin and Aubert, 1994). Although the occurrence of cellulose digestion has been reported in cockroaches and higher termites from the subfamily Nasutitermitinae, most cellulose digestion in insects are usually mediated by microorganisms (Martin, 1991). The activity of cellulases have been reported in *Enterobacter cloacae*, (which produced maximum levels of cellulases after 96 h of fermentation, Sami, *et al.,* 2008), termites and cockroaches (Cruden and Markovetz, 1979). Also, it has been discovered that animals such as crustaceans, insects and molluscs are able to endogenously produce cellulase enzymes to digest cellulose (Watanabe and Tokuda, 2001; Davison and Blaxter, 2005, Linton *et al.,* 2006). The presence of cellulolytic microorganisms in the gut of the wood borer, *Saperda vestita*, and the Bark beetles, *Ips pini* and *Dendroctonus frontalis*, have been established by Delalibera, *et al.,* (2005). The activities of endo-β-1,4-glucanase and β-1,4-glucosidase have been responsible for the total hydrolysis of cellulose to glucose. The digestive juice of both species also contained laminarinase, lichinase and xylanase, which hydrolyzed laminarin, lichenin and xylan, respectively, to component sugars (Linton and Greenaway, 2004).

Cellulose and hemicellulose digestion requires two steps. The first step involves mechanical digestion whereby the cellulose fibres are broken down mechanically. Some insects possess structures such as biting and chewing mouthparts called mandibles and gastric mills in their stomachs and gizzards to do this. How this process aids in cellulose digestion is not understood. It is believed that it reduces the size of the cellulose fibres, digests individual cellulose molecules and provides more sites for the second digestive step which is the enzymatic attack. The primary reason why cellulose and hemicellulose are difficult to digest is that they require specialized enzymes called cellulases and hemicellulases to break the beta-glucosidic bonds and split the molecules up into their component sugars.

Most of the earlier studies on enzymes have been concentrated on the qualitative detection in the alimentary systems of insects. However, this investigation focused its attention on the properties of cellulase enzymes.

MATERIALS AND METHODS

Insects collection and laboratory maintenance of *R. phoenicis* used for this study.

Both live larva and adult stages of palm weevil, *R. phoenicis* were obtained from palm wine tappers at Igbokoda, (Latitude 6° 21° N and Longitude 4° 48° E) in Ondo State, Nigeria. The larvae were kept in a plastic container filled to two-third with raphia palm chips and the container was covered with nylon mesh. The adults were kept in another plastic container which was filled to two-third with raphia palm chips and the container was covered with nylon mesh. The weevils were transported to the laboratory immediately after collection. They were kept in the laboratory for 48 h at ambient temperature before being used in any experimental procedure. The weevils fed on the raphia palm chips while they acclimatized to the laboratory conditions. Measurements of the head capsule, body weight, body width and circumference of the larvae and adults were taken (Table 1.). The larvae and adults were killed, by asphyxiating them in a deep freezer for 2 h. The wings of the adults were removed with a pair of forceps.

Table 1. *Dimensions of the larval (n = 150) and adult (n = 150) stages of R. phoenicis used*

Stage	Weights (g)	Body length (cm)	Body width (cm)	Head capsule (cm)	Circumference (cm)
LS	8.14±0.140 (5.36-12.16)	4.61±0.063 (3.5-7.0)	2.05±0.036 (1.6-3.7)	1.10±0.013 (1.0-1.6)	5.47±0.076 (4.3-8.6)
ADS	6.98±0.132 (5.5-8.5)	5.70±0.041 (5.3-6.3)	1.59±0.021 (1.4-1.9)	2.88±0.049 (2.5-3.2)	4.49±0.037 (4.0-5.0)

Each value is a mean of 150 samples ± Standard error.

Cellulase activity in the gut homogenate of the palm weevil, *R. phoenicis*

The insects were carefully dissected in the laboratory. The alimentary systems of the insects were removed and their midguts were carefully separated from the rest of the alimentary system and homogenized with distilled water in an all glass homogenizer chilled with ice blocks. One midgut was homogenized in 1 ml of distilled water and the homogenate was centrifuged using Beckman Optima Model LE -80K refrigerated centrifuge at 10,000 rev/min. for 20 minutes in order to remove extraneous matters. The resulting supernatant was used as enzyme solution while the thick cloudy base was discarded.

Effect of pH on cellulase activity in the larval and adult stages of palm weevil

To determine the cellulase activity in the gut homogenate of palm weevil, the modified method of Miller (1959) described by Guliano and Khan (1984) was adopted. The buffer solutions used were acetate pH 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0 and phosphate pH 5.5, 6.0, 6.5, 7.0, 7.5 and 8.0 solutions. The reaction mixture

contained 2.0 ml of 1% cellulose solution, 2.0 ml of buffer solution and 1.0 ml of enzyme extract. The mixture was incubated for 2 h at 37^{o} C in a water bath. The control contained 1.0 ml of denatured enzyme. Aliquots of 0.5 ml of 2N NaOH and 0.5 ml of dinitrosalicylic acid reagent were added to both the control and the experimental tubes and the products of hydrolysis were read at 540 nm on Cecil CE 343 single sample spectrophotometer. Each assay was conducted in triplicates.

Effect of time of incubation on cellulase activity in the larval and adult stages of palm weevil

The reaction mixture contained 2.0 ml of 1% cellulose solution, 2.0 ml of buffer (pH 4.0 and 4.0 for larva and adult stages, respectively) and 1.0 ml of enzyme extract. The effect of incubation period on the cellulase activity was assayed at 15 minutes intervals over a period of 3 h at 37^{o} C in a water bath. In the control, 1.0 ml of denatured enzyme was used. To both the experiment and the control was 0.5 ml of 2N NaOH and 0.5 ml of dinitrosalicylic acid reagent added and the products of hydrolysis were read at 540 nm on Cecil CE 343 single sample spectrophotometer. Each assay was carried out in triplicates. The optimum time obtained for both larva and adult were used for subsequent experiments.

Effect of temperature on cellulase activity in the larval and adult stages of palm weevil

Cellulase activity was determined over a temperature range of 25^{o} C to 60^{o} C at an increment of 5^{o} C. The reaction mixture contained 2.0 ml of 1% cellulose solution, 2.0 ml of buffer (pH 4.0 and 4.0 for the larva and adult stages, respectively) and 1.0 ml of enzyme extract. In the control, 1.0 ml of denatured enzyme was used. To both the experiment and the control was 0.5 ml of 2N NaOH and 0.5 ml of dinitrosalicylic acid reagent added and the products of hydrolysis were read at 540 nm on Cecil CE 343 single sample spectrophotometer. Each assay was carried out in triplicates. The optimum temperature obtained for both larva and adult were used for subsequent experiments.

Effect of starch concentrations on cellulase activity in the larval and adult stages of palm weevil

The reaction mixture contained 2.0 ml of graded cellulose solution containing 0-12 mg/ml (0, 2.0, 4.0, 5.0, 8.0, 10.0 and 12.0 mg/ml), 2.0 ml of buffer (4.0 and 4.0 for larva and adult, respectively) and 1.0 ml of enzyme extract. The mixture was incubated for 1 h at the optimum temperature (i.e. 45^{o} C and 45^{o} C for larva and adult, respectively) in a water bath. In the control, 1.0 ml of denatured enzyme was used. To both the experiment and the control was 0.5 ml of 2N NaOH and 0.5 ml of dinitrosalicylic acid reagent added and the products of hydrolysis were read at 540 nm on Cecil CE 343 single sample spectrophotometer. Double reciprocal plot of the starch concentration was plotted against the absorbance to determine the Lineweaver-Burk's graph. Each assay was carried out in triplicates.

RESULTS

Optimum pH for cellulase activity

Cellulase activity was detected at pH 2 – pH 8 with optimum activity at pH 4 for both larva and adult (Fig. 1). Activity was higher in acidic than in alkaline media in the midgut of both larva and adult weevils.

Optimum time for cellulase activity

The effect of duration of incubation on the hydrolysis of cellulose is given in Fig. 2. Low levels of cellulase activity were detected in both larva and adult weevil within 30 minutes of incubation. The optimum time for cellulase incubation was 120 minutes and 115 minutes for the larva and adult, respectively. At periods longer than the optimum time, the relationship between the quantities of cellulose hydrolyzed was no longer linear with the duration of incubation.

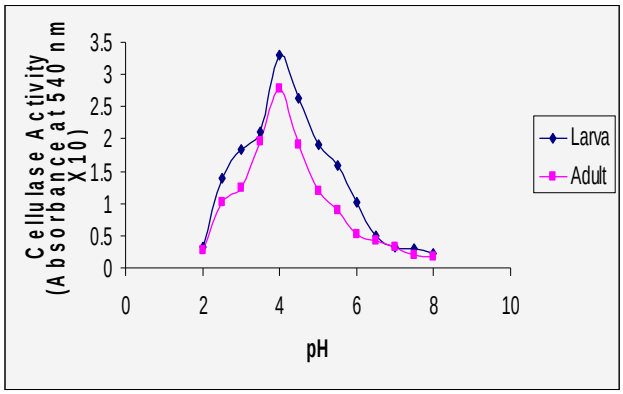

Figure 1. *Effect of pH on cellulase activity in the midgut of palm weevil, R. phoenicis.*

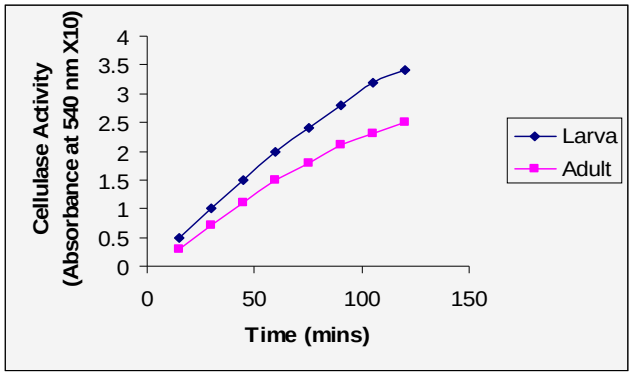

Figure 2. *Effect of time of incubation on cellulase activity in the midgut of palm weevil, R. phoenicis.*

Optimum temperature for cellulase activity

The effect of temperature on cellulase activity in the gut homogenate of *R. phoenicis* were determined over a range of 25^{o} C to 60^{o} C at an incremental

temperature range of 5° C (Fig. 3). The optimum temperature of 45° C was obtained for both larva and adult. Most of the cellulase activity occurred between 35° C and 50° C. Between 25° C and 30° C as well as between 55° C – 60° C cellulases activities were relatively low. A linear relationship was observed between 25° C and 45° C in both stages. A sharp drop in enzyme activity was observed between 45° C and 50° C. This was followed by a gradual drop in activity.

Effect of cellulose concentration on cellulose activity

The effect of cellulose concentration on cellulase activity in the gut of palm weevil is given in Fig. 4. There were hydrolytic activities in all the substrate concentrations tested. Cellulolytic activity was greater in the larval than in the adult. However, higher concentrations of cellulose did not correspond directly to higher hydrolysis of the substrate. The reaction velocity followed Michaelis-Menten type kinetics. The lineweaver-Burk plot was used to determine the value of k_m for the larva and the adult (Fig. 5) and the k_m obtained for larva and adult were 2.5 mg/ml and 3.12 mg/ml, respectively.

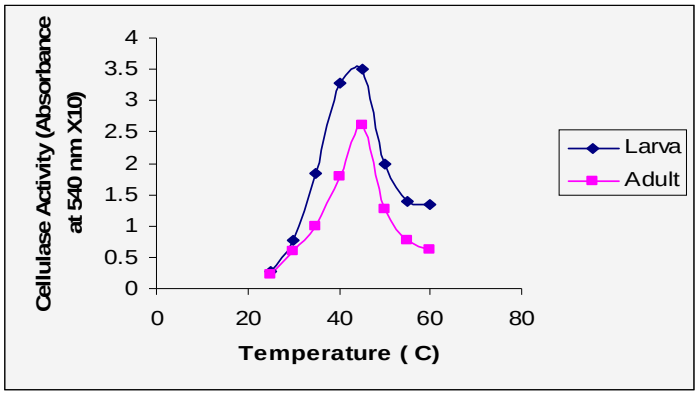

Figure 3. *Effect of temperature on cellulase activity in the midgut of palm weevil, R. phoenicis.*

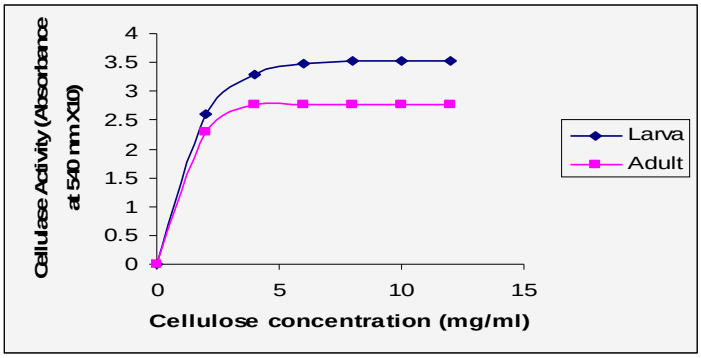

Figure 4. *Effect of cellulose concentrations on cellulase activity in the midgut of palm weevil, R. phoenicis*

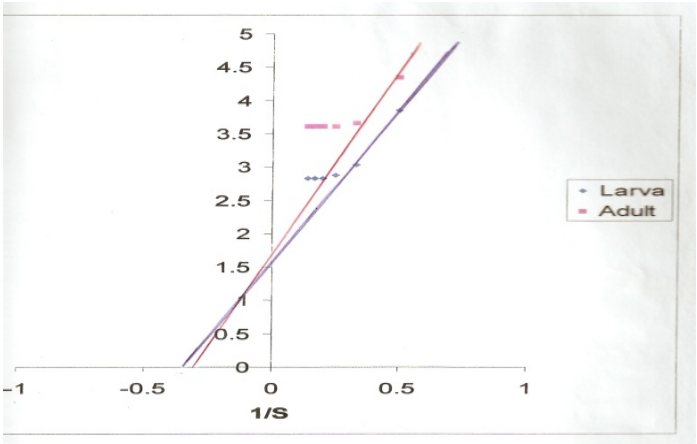

Figure 5. *Lineweaver-Burk plots for cellulose concentration against cellulase activity in the gut of palm weevil, R. phoenicis.*

DISCUSSION

In both the larva and adult *R. phoenicis,* the enzyme exhibited high activity in acidic conditions while slightly lower activities were observed in alkaline conditions. More than 50% of activity was observed in acidic media and the optimum pH was 4.0. The same trend was observed in the adult where more than 50% of activity occurred in acidic media and the pH was 4.0. The present study revealed that cellulase prefers acidic pH for its optimum activity in the guts of palm weevil. Similar acidic conditions have been reported for the cellulases of animals by other workers (Maglione *et al.,* 1997; Jianchu *et al.,* 2004). Sugimura, *et al.* (2003) reported that the optimal pH for the cellulase of the yellow spotted longicorn beetle, *Psacothea hilaris,* was 5.5. Cellulose digestion has been reported in the some insect orders (Martin, 1983). Insects possess most of the major enzymes found in other animals. Cellulolytic activities have been reported in the gut fluids of 54 insect species that belong to 7 different taxonomic orders by Su *et al.,* (2013). These authors reported that the highest CMC gut fluid activities were found in Coleoptera and Orthoptera. Aslo, cellulase activity has been reported in insects such as the Tiger beetle, *Cicindela scutellaris*, Red cotton bug, *Dysdercus koenigii*, Blue pumpkin beetle, *Aulacophora atripennis*, Red pumpkin beetles, *Aulacophora foveicollis* and *Aulacophora hilaris,* and Grasshopper, *Chrotogonus trachypterus trachypterus* (Sami and Shakoori, 2006).

Cellulase is an enzyme that is very important in wood eating animals and the enzyme is usually produced by symbiotic microorganisms living in the guts of insects (Weimer, 1992; Tokuda, *et al.* 2004, 2005, 2012, Omotoso, 2009). Oppert *et al.,* (2010) described the quantitative determinations of cellulolytic activity in the gut and head-derived fluids from 68 phytophagous or xylophagous insect species belonging to eight different taxonomic orders. These authors reported the highest carboxymethyl cellulose (CMC) gut fluid activities in Dictyoptera, Coleoptera, Isoptera, and Orthoptera, while the highest MCC gut fluid activities were found in Coleoptera, Hymenoptera, Lepidoptera, and Orthoptera. They reported further that in most cases, gut fluid activities were greater in CMC substrate than in MCC

substrate, except in Diptera, Hymenoptera, and Lepidoptera. Non-microbial cellulases are expected in insects with large foreguts and midgets and small hindguts as observed in the stick insect (Shelomi, *et al.* 2014), whereas insects dependent on microbial cellulases tend to have enlarged hindgut pouches where bacterial fermentation occurs (Shelomi, *et al.* 2010). The cellulases utilized for the hydrolysis of cellulose are mainly produced by endosymbiotic bacteria in the hepatopancreas of the woodlouse, *Porcellio scaber* (Zimmer and Topp, 1998). The cellulase activities in wood-eating termites are concentrated in the midgut (Li, *et al.,* 2013). The cellulases of some animals are endogenous in origin. Many insects are able to synthesize their own Cx-cellulases and cellobiases, but few (if any) can synthesize C1-cellulases. Thus, insects compensate for their inability to synthesize the C1-cellulases by exploiting the cellulolytic potential of protozoa, bacteria or fungi (Martin, 1983). Some cellulolytic anaerobes possess the unique ability to hydrolyze cellulose materials to cellobiose and glucose in higher quantities (Giuliano, *et al.,* 1983). These anaerobes are potentially useful in co-culture in the conversion of cellulose to sugars or to other useful end products (Khan and Murray, 1982). The quantitative difference in the cellulolytic activity in both the larva and adult palm weevil indicated that the enzymes were more active in the larval than in the adult. Low cellulose concentrations have been reported in the stick insect, by Shelomi *et al.* (2014).

The degradation of cellulose obeyed first kinetic order within the first 120 minutes and 115 minutes of incubation in the larva and the adult weevil respectively. The relationship between the time of incubation and the quantity of cellulose hydrolyzed was linear up to 120 and 115 minutes of incubation for the larval and the adult. The relationship was no longer linear when the incubation period was extended beyond these periods. This result showed that not much cellulose would have been hydrolyzed if the incubation time had been extended beyond 120 minutes.

The optimum temperature for cellulase activity was 45^{o} C for both larva and adult weevil. At higher temperatures of 50^{o} C-60^{o} C, some of the enzymes were denatured and this was responsible for the low levels of hydrolysis observed under this condition. The temperature range of 35^{o} C-45^{o} C resulted in the hydrolysis of about 50% of the total cellulose degraded.

The k_m values of 2.5 mg/ml and 3.12 mg/ml obtained for the larval and adult weevil are comparable to those reported for other animals. Linton and Greenaway (2004) reported the k_m value of 3.03 mg/ml carrboxymethyl cellulose for the land crab, *Gecarcoidea natalis*.

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