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PARTIAL CHARACTERIZATION OF MIDGUT ENZYMES IN BUTTERFLY *PAPILIO POLYTES POLYTES* L. (LEPIDOPTERA: PAPILIONIDAE)

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ABSTRACT
Characterization of digestive enzymes from the larval and adult midgut of *Papilio polytes polytes* L. was studied by using different assays. High enzymatic activity were found at pH 7.2 for amylase and invertase, pH 7.8 for trehalase and lipase and pH 9.8 for protease in fifth instar larvae where as it was at pH 6.4 for adult invertase and 40^{0}C for all studied enzymes. The linear period of enzyme activities were found at 60, 15, 90, 15 and 20 min for larval amylase, invertase, trehalase, lipase and protease respectively where as it was 30 min for adult invertase. The 50% inhibition at high temperature was found to be 13.5 min (amylase), 9.5 min (invertase), 6 min (trehalase), 8 min (lipase) and 6.5 min (protease) in larva and 6.5 min for adult invertase. The specific activities were 2.844 μg maltose/μg protein/hr, 10.56, 22.25 and 0.4657 μg glucose/μg protein/hr for larval amylase, invertase, trehalase and adult invertase respectively and 9.3474 μg palmitic acid/μg protein/hr (larval lipase) and 1.0285 μg tyrosine/μg protein/hr (larval protease). The measurement of maximal catalytic activities of studied enzymes determines the physiological capacities of the different metabolic pathways. Hence, results of such would be utilized in the formulating control strategies against various pests including the species under study.

INTRODUCTION

Insects are adapted to a wide range of diets and digestion of food is dependent on pH of alimentary canal. The pH of alimentary canal is strongly correlated with type of food consumed (Swingle, 1931; Grayson, 1951, 1958). Earlier work on digestive physiology of insects was concerned with the quantitative determination of enzyme activities in different parts of alimentary canal. Most of the vertebrate digestive enzymes are present in insects too and are classified as carbohydrases, proteases and lipases. Study on digestive enzymes of insects is one of the new and winning area to reach a safe and effective way to decrease the damage of the pest on agricultural products (Mahdavi *et al.,* 2013) and it offers an opportunity for developing appropriate and effective pest management strategies against the pests of agricultural products. The swallowtail butterfly, common mormon *Papilio polytes polytes* L. is a serious pest of *Citrus spp., Murayya koenigi* and other plants of rutaceae. If, enzymatic activities of such pest species are known then it will be helpful for formulating control strategies against this species. Hence, it was decided to work on characterization of enzymes in the species under study.

Many studies have been carried out on the digestive enzymes of adults and larvae of various insects (House, 1974).Amylase is one of the key enzymes involved in digestion and carbohydrate metabolism in insects (Daone *et al.,* 1975; Horie and Watanabe, 1980). Different workers carried out studies on amylase in lepidopterans like *Spodoptera littolaris* (Ishaaya *et al.,* 1971); silkworm *Bombyx mori* (Mori, 1930; Ito *et al.,* 1962; Nishida and Hayashiya, 1969; Kanekatsu, 1972) and *Antheraea proylei* (Kumbhar *et al.,* 2010). Bhuvaneshwari and Sivaprasad (2012b) studied the impact of photoperiod on circadian carbohydrate and amylase rhythms in the digestive system of *Bombyx mori* under 12h light-dark cycle, continuous light and continuous dark conditions. Invertase is one of the carbohydrase enzymes that cleave sucrose into glucose and fructose. Invertase activity has been demonstrated in lepidopterans like silkworm larvae (Mori, 1930; Horie, 1959; Muniv, 2012) and *Pieris rapae* (Nishide and Kusano, 1976).

Trehalase degrades trehalose to glucose which is major blood carbohydrate for internal energy supply (Wyatt, 1967). This enzyme has been extensively studied and substantially purified from several insects and non-insect sources (Gilby *et al.,* 1967; Fisher and McAlister, 1969; Friedmann, 1975; Rosinski *et al.,* 1979; Kumbhar *et al.,* 2009; Sarwade *et al.,* 2009; Dhalman, 1971).

Tryptic and chemotryptic activities have been described within the context of the complete digestive protease component and their identification is based on hydrolysis of specific substrate. A unique feature of the protease action is encountered in the digestive fluid of lepidopteran larval midgut (Ishaaya *et al.,* 1971; Ahmad *et al.,* 1976, 1980; Pritchett *et al.,* 1981; Sasaki and Suzuki, 1982). Bhuvaneshwari and Sivaprasad (2012a) studied the photoperiod-induced clock-shifting in circadian protein and protease rhythms in the larval

digestive system of *Bombyx mori* under 12h light-dark cycle, continuous light and continuous dark conditions. A trypsin substrate specificity and kinetics have been studied in *Manduca sexta* (Miller *et al.,* 1974) and *B. mori* (Eguchi and Iwamoto, 1976). Lipase activity has been reported in *Periplaneta americana* (Bollade *et al.,* 1970; Hoffman and Downers, 1979; Male and Storey, 1981).

Characterization of the digestive enzymes of insects offers an opportunity for developing appropriate and effective pest management strategies. Review on literature indicates, most of the work on digestive enzymes of lepidopteron insects is pertaining to moths. There is scant information is available on characterization of digestive enzymes of butterflies. Only information on digestive system of *P. polytes polytes* L. is available on anatomy and histology of adult alimentary canal which was studied by of Gaikwad *et al.* (2011). Therefore, efforts have been made to study on characterization of midgut digestive enzymes in *P. polytes polytes* L. which is a serious pest of *Citrus spp., Murayya koenigi* and other plants of rutaceae.

MATERIALS AND METHODS

Fifth instar larvae and adults of *P. polytes polytes* were dissected in chilled insect Ringer solution (Ephrussi and Beadle, 1936). Homogenates of the midgut were prepared in chilled 0.9% NaCl, unless otherwise indicated, which were cold centrifuged at 3000 rpm for 20 min (Tonapi, 1994). Aliquots of supernatants were used as enzyme source for the characterization (effect of pH, temperature, time, thermolability and substrate concentration) of amylase, invertase, trehalase, protease and lipase enzymes. The activity of amylase, invertase and trehalase was determined by using 3-5 dinitrosalicylic acid (DNSA) reagent (Bernfeld, 1955) and measured spectrophotometrically at 540nm (Ishaaya and Swirski, 1970). The assay for amylase, invertase and trehalase consisted of 1 ml substrate (1% starch for amylase, 1% sucrose for invertase and 1% trehalose for trehalase), 1 ml 0.2 M buffer with appropriate pH, 0.5 ml supernatant, 2.5 ml DNSA reagent and 2.5 ml distilled water. In blank 0.5 ml supernatant was replaced by distilled water. The standard curve obtained by direct reaction with glucose for invertase and trehalase and for amylase maltose using DNSA reagent under similar assay conditions. The activity of lipase was measured according to Hayase and Tappel (1970). The standard curve was obtained by using palmitic acid under similar assay condition. The procedure of Birk *et al.* (1962) as used by Ishaaya *et al.* (1971) was used to determine the protease activity and absorbance of the reaction mixture was read on UV-spectrophotometer at 280 nm. The standard curve was obtained by using different tyrosine concentrations. To study thermolability, supernatant was subjected to high temperature treatments *i.e.* 55ºC for amylase, 50ºC for invertase, 60ºC for trehalase and protease and 50ºC for lipase for different period of time. The activities of residual enzymes left after heat treatments were determined by respective method. The soluble protein content of the enzyme extract was determined by Lowry *et al.* (1951) using Bovine serum albumin as standard.

RESULTS

Characterization of midgut enzymes viz. amylase, invertase, trehalase, lipase and protease were studied in the fifth instar larvae and adults of *P. polytes polytes*. All the enzymes under study showed positive results in fifth instar larvae. However, in adult only invertase shows positive results.

Effect of pH

Measurement of the enzymatic activities in the different pH range showed the highest activity of enzyme in different pH for different enzyme. In midgut of fifth instar larvae, the activity of amylase and invertase was maximum at pH 7.2 (Fig.1, 2) whereas trehalase and lipase were most active at pH 7.8 (Fig. 3, 4) and protease at pH 9.8 (Fig. 5). The optimum pH of adult invertase was 6.4 (Fig. 6).

Effect of Temperature

The enzymes showed a steady increase in their activity by elevating of the incubation temperature from 10ºC - 40ºC and then decreased till 60ºC. The results showed that the temperature optima for activities of all the enzymes under study were at 40ºC (Fig. 7-12).

Effect of Time

The results showed different linear digestion period for different enzyme. The fifth instar larval midgut amylase,

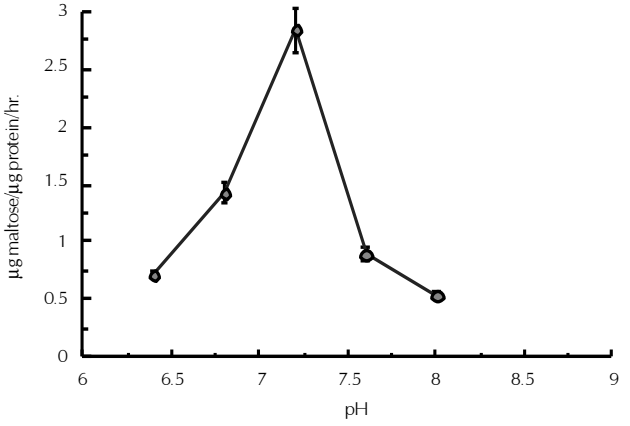

Figure 1: Optimum pH of amylase

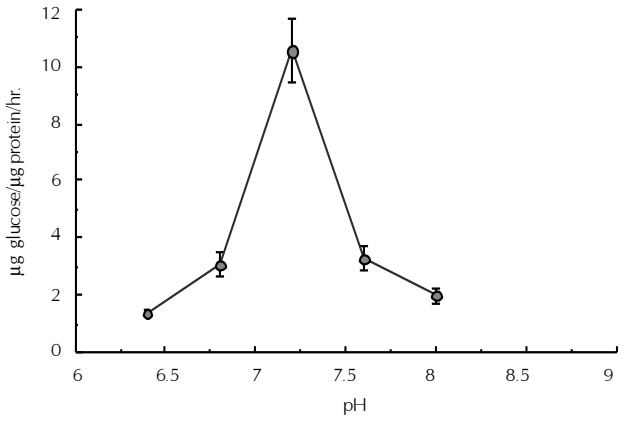

Figure 2: Optimum pH of invertase

invertase, trehalase, lipase and protease showed a digestion period of 60, 15, 90, 15 and 20 min respectively whereas midgut invertase of adult requires 30 min. for maximum activity (Fig. 13-18).

Thermolability

The theoretical duration of high temperature treatment for 50% loss of enzyme activity in fifth instar was found to be 13.5 min for amylase at 50ºC (Fig.19), 9.5 min for invertase at 50ºC (Fig. 20), 6 min for trehalase at 60ºC (Fig. 21), 8 min for lipase at 50ºC (Fig. 22) and 6.5 min at 60ºC for protease (Fig. 23) where as adult invertase showed 6.5 min for 50% loss of enzymatic activity at 50ºC (Fig.24).

Effect of Substrate concentration

The relationship between the substrate concentration (1/S) and rate of hydrolysis (1/V) were studied for all enzymes under study. The substrates maltose (for amylase), glucose (for invertase and trehalase), palmitic acid (for lipase) and tyrosine (for protease) and their rate of hydrolysis are shown in fig 25-30. Michaelis-Menten constant (Lineweaver-Burk) plots, enabling estimation of values for *Km* was obtained by plotting reciprocal of substrate concentration (1/S) and velocity (1/v). Lineweaver-Burk plot was employed by using regression equation $y = ax + b$ and the regression line obtained were $y = 0.0153x + 0.0082$ for amylase (Fig. 31), $y = 0.0768x + 0.0007$ for invertase (Fig. 32), y = 0.0189x 1.9253 for trehalase (Fig. 33), y = 0.4651x + 0.0942 for lipase (Fig. 34), $y = 0.0109x + 0.0332$ for protease (Fig. 35) and $y = 0.0384x + 0.0007$ for adult invertase (Fig. 36). The Km values obtained were 0.533% (amylase), $2.33X10^{-3}$M (invertase), $0.302X\ 10^{-3}$M (trehalase), $5.13X10^{-4}$M (lipase) and 0.4% (protease). In adult, midgut trehalase, lipase and protease activity was not observed, the midgut showed only invertase activity i.e. $2.59\ X\ 10^{-3}$M.

DISCUSSION

The enzyme activity in alimentary canal is mainly depends on the gut pH. Enzymes have highest activity in their optimal pH and a small change in pH alters the catalytic mechanisms of the biochemical reactions (Terra and Ferriera, 2005). In insects, gut content pH ranges between 6 and 7, but there is considerable exception in lepidopteran larvae where it is between 7 and 12 due to disabling plant toxins ingested with nutrient parts of food (Zibaee, 2012). In the present investigation, the enzymes had the optimal pH between 5.8 and 9.8 coinciding with earlier reports on other lepidoptera. The optimal pH for α-amylase activity is 9.2 in *B. mori* L. (Abraham *et al.,* 1992)., Dow (1984, 1986) studied pH of midgut lumen and reported pH 12 for *Acherontia atropos*, 10.8 for *Lasiocampa quercus*, 11.3 for *M. sexta* and 10.8 for *Lichnoptera felina*. The pH optima for midgut amylase in larvae of *S. litturalis* was at pH 9.5 (Ishaaya *et al.,* 1971), in *Agrotis epsilon* it was at pH 8.2 (Lim and Teo, 1971) and in larvae of *A. proylei*, it was at pH 8.4 (Kumbhar *et al.,* 2010). Present study showed the optimum pH 7.2 for midgut amylase. The pH optima for midgut invertase in larvae and adult are 7.2 and 6.4 respectively. According to Wigglesworth (1953) invertase occurs in the digestive tract of several insects for digestion and utilization of sucrose. Maximum invertase activity has been reported at pH 6 to 6.5 in *P. rapae crucivora* (Nishide and Kusano, 1976). In *Heliothis zea* optimal pH for enzyme activity is 6.5 (Burton, 1975) and in multivoltine races of *B. mori*, it was 6.8 (Muniv et al., 2011). The enzyme trehalase works in acidic pH supporting observation of present study *i.e.* pH 5.8. Some notable reviews on pH optima are, pH 5.5 for *Calliphora erythrocephala* (Burton, 1975), pH 6 for fifth instar larvae of *A. proylei* (Kumbhar *et al.,* 2009) and pH 5.2 for *Platynotus belli* (Sarwade *et al.,* 2009a). The optimal pH for lipase activity is 7.8 in *P. polytes polytes*. Scant information exists on the insect lipase. The pH 10 as optimal pH for lipase was reported in the mdigut of *Chilo suppressalis* (Zibaee *et al.,* 2008b) and in *Naranga aenescens* (Zibaee and Dinan, 2012). According to Grilo *et al.* (2007) midgut lipase in *Rhodnius prolixus* has the maximal activity at pH 7-7.5. The larval mid gut protease showed 9.8 pH for maximum activity in *P. polytes polytes* i.e enzyme is active at highly alkaline pH. It has been previously reported that a gut proteases in insect have alkaline pH optima. These include those from *S. littoralis,* pH 11.0 (Ishaaya et al., 1971), *S.litura,* pH 9, 10.5 and 11.0 (Ahmad et al., 1976, 1980), *H. zea* pH 11 (Klocke and Chan, 1982), *P. rapae,* pH 8.0 (Broadway, 1989), *Helicoverpa armigera,* pH 9.5 and 10.0 (Johnston *et al.,* 1991), *M. sexta,* pH 8.5 (Samuels *et al.,* 1993), *H. virescens,* pH 10.0-11.0 (Johnston *et al.,* 1995), *Mamestra*

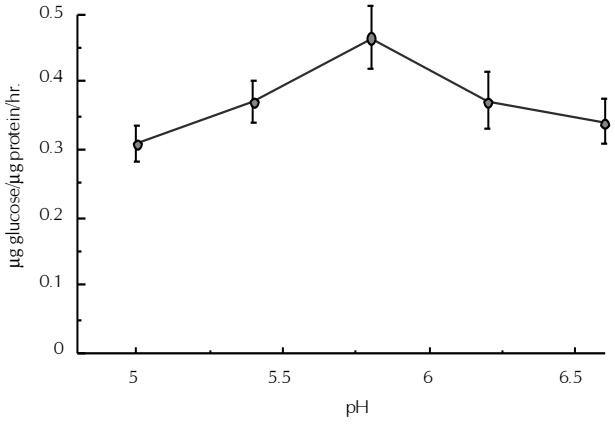

Figure 3: Optimum pH of Trehalase

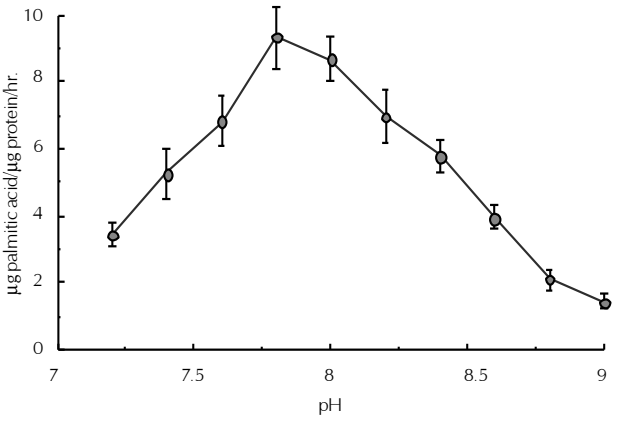

Figure 4: Optimum pH of Lipase

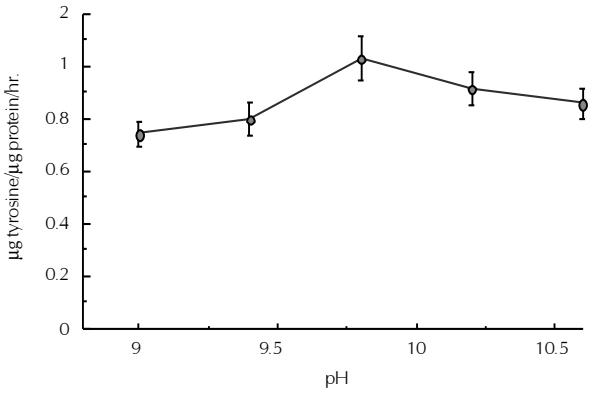

Figure 5: Optimum pH of Protease

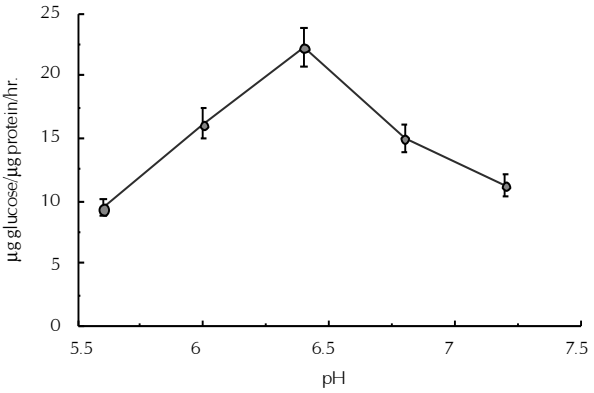

Figure 6: Optimum pH of adult invertase

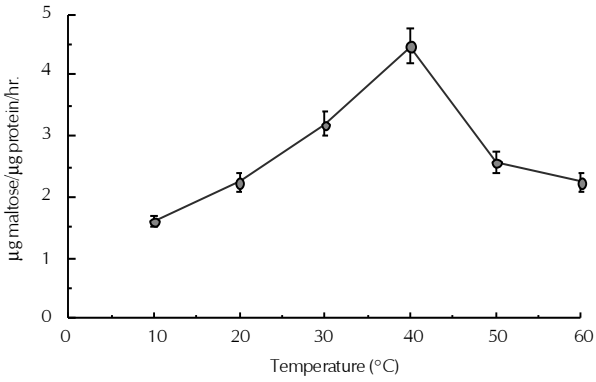

Figure 7: Optimum temperature of amylase

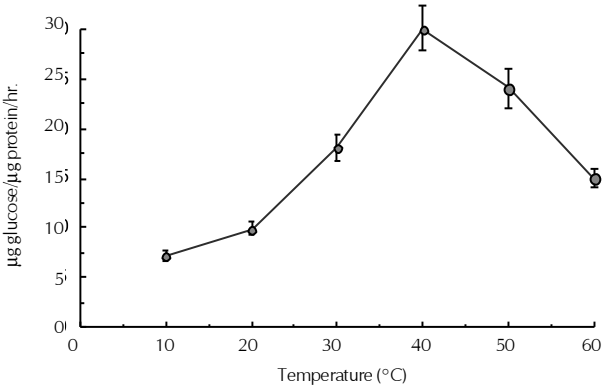

Figure 8: Optimum temperature of invertase

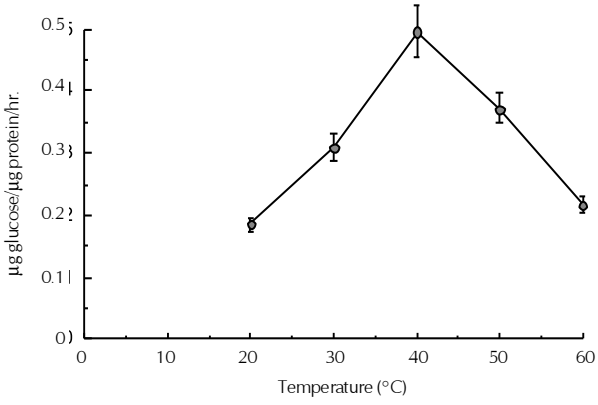

Figure 9: Optimum temperature of trehalase

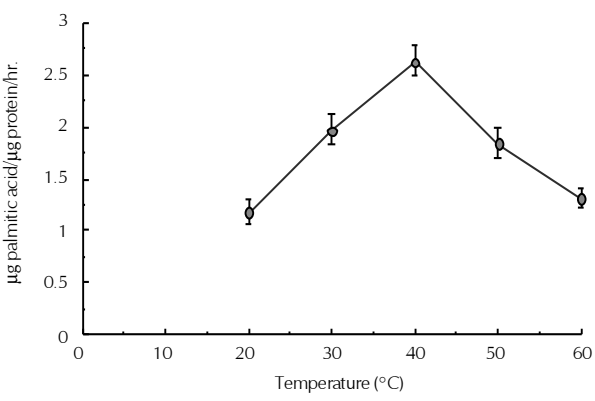

Figure 10: Optimum temperature of lipase

brassicae, pH 11.0 (Chougule *et al.,* 2008) and *Glyphodes pyloalis* pH 10.0 (Mahdavi *et al.,* 2013). The results of optimal pH in the current study confirmed Terra and Ferreira (1994) in relation to high pH of lepidopteran gut to an adaptation of leaf eating lepidopteran ancestors for extraction of hemicellulose of plant cell walls.

The enzyme activity of the enzymes under study, increase as temperature was raised from 20ºC to 40ºC. There was significant drop in enzymatic activity when temperature was further raised to 60ºC. Temperature optima for all five enzymes studied is 40ºC. These results are consistent with the findings of Burton (1975) in *H. zea* (37ºC) for invertase , Bhawane and Mandlik (1992) in *Holtrichia serrata* (40ºC) and Kumbhar *et al.* (2009) in *A. proylei* (40ºC)for trehalase, Zibaee *et al.* (2008) in *C. suppressalis* (37-40ºC), Zibaee and Dinan (2012) in *N. aenescens* (35-40ºC) for lipase. However, optimal temperatures

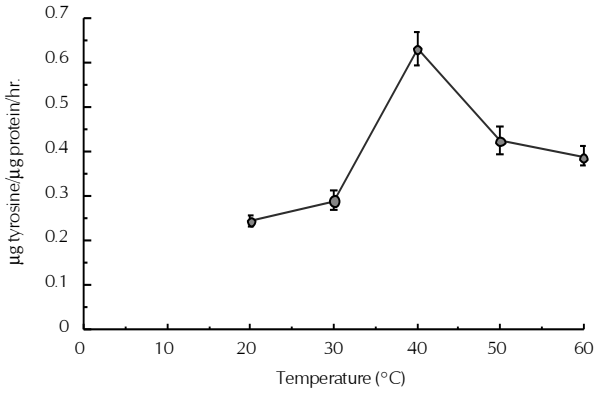

Figure 11: Optimum temperature of protease

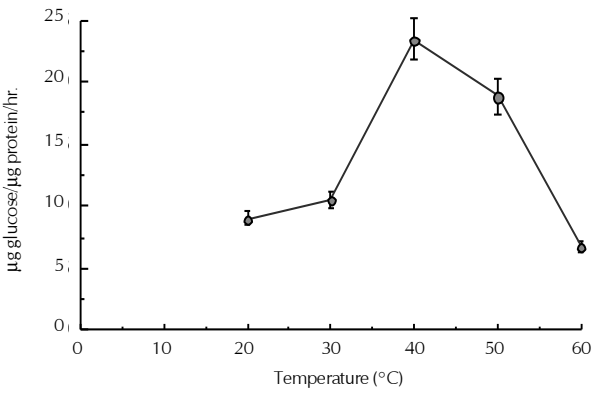

Figure 12: Optimum temperature of adult invertase

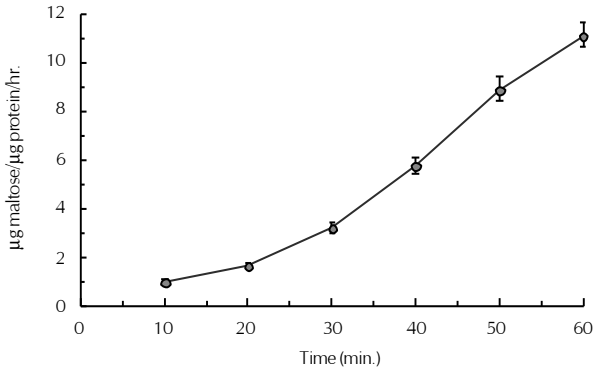

Figure 13: Effect of time on amylase activity

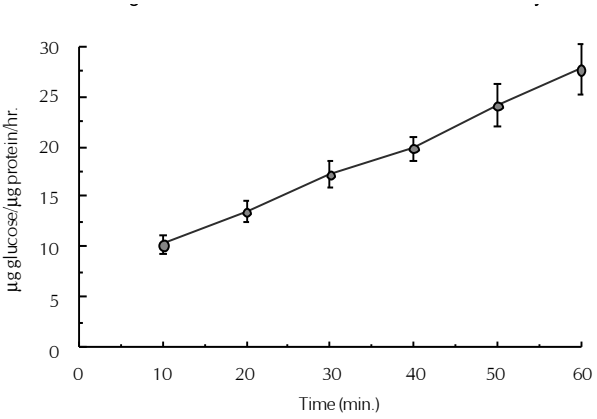

Figure 14: Effect of time on invertase activity

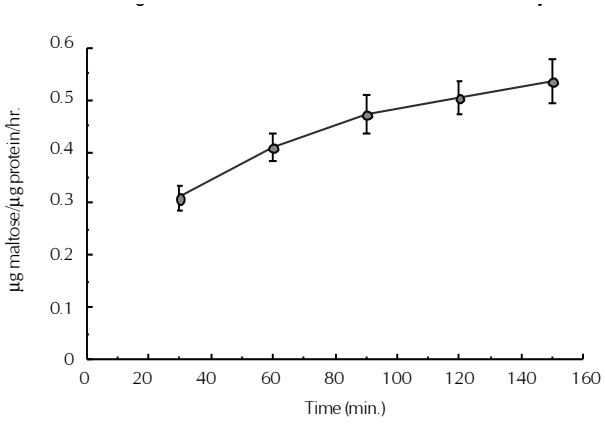

Figure 15: Effect of time on trehalase activity

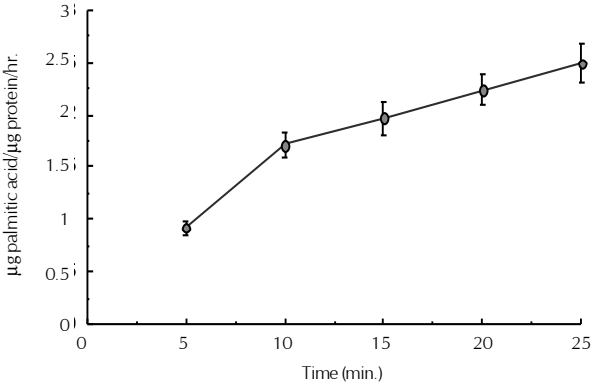

Figure 16: Effect of time on lipase activity

for soluble and membrane lipases were observed at 50ºC and 35ºC respectively by Roudsari (2014) in *Bacterocera oleae*. Kumbhar *et al.* (2009, 2010) reported optimum temperature of 40ºC in *A. proylei* for trehalase and amylase. Muniv *et al.,* (2011) reported optimum temperature 40ºC for invertase in fifth instar larvae of *B. mori*. However, digestive enzymes are stable even at higher temperature *i.e.* 60ºC in midgut of *B. mori* (Mori, 1930) and *S. littoralis* larva (Ishaaya *et al.,* 1971) and 50ºC in *Glyphodes pyloalis* (Mahdavi *et al.*, 2013).

Larval midgut amylase, invertase, trehalase, lipase, protease shows linear digestion period of 60 min, 15 min, 90 min, 15 min and 20 min respectively where as midgut invertase activity was linear with time upto 15 min in adult of *P. polytes polytes*. The digestion period in *A. proylei* is 30 min for amylase and trehalase (Kumbhar *et al.,* 2009, 2010), in *B. mori* 50 min for invertase (Muniv *et al.,* 2011) and in *P. belli* 20 min and 30

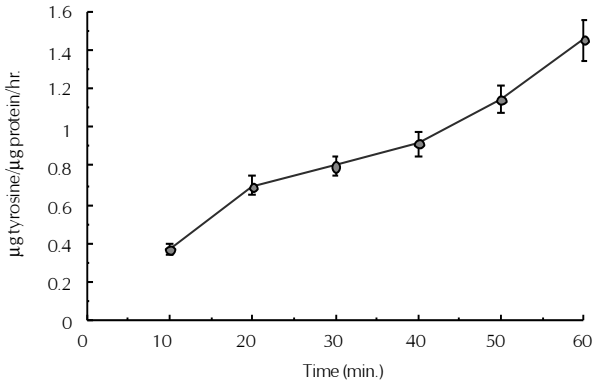

Figure 17: Effect of time on protease activity

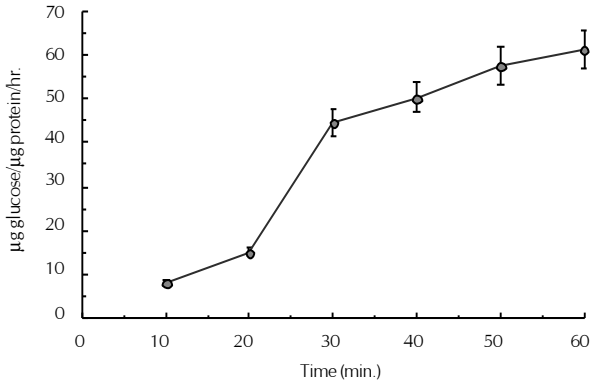

Figure 18: Effect of time on invertase activity

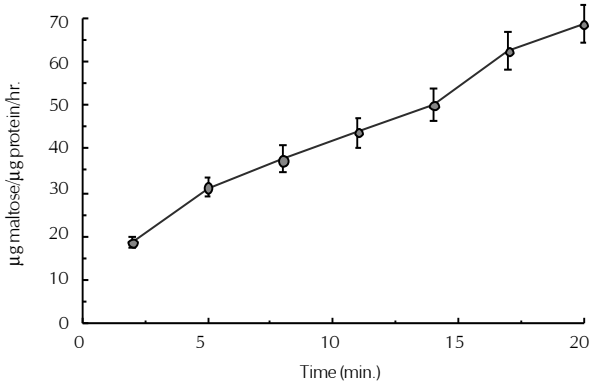

Figure 19: Thermolability of amylase

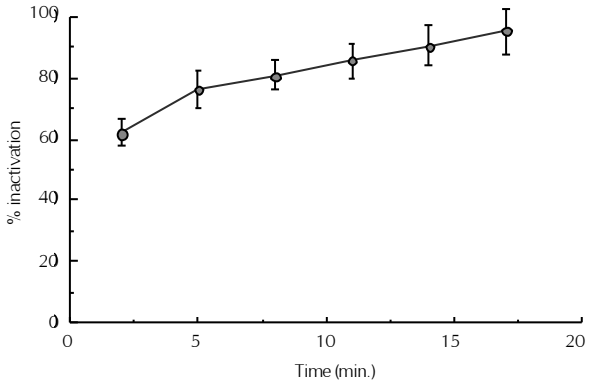

Figure 20: Thermolability of invertase

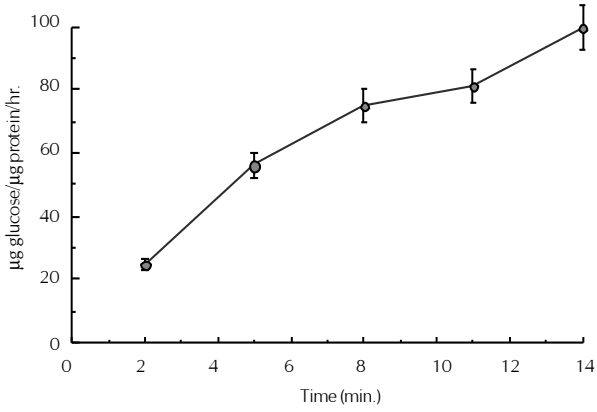

Figure 21: Thermolability of trehalase

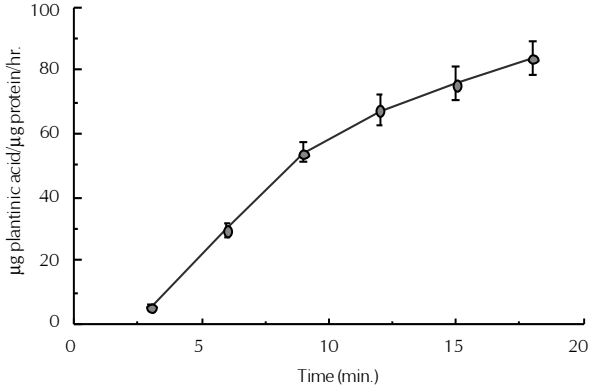

Figure 22: Thermolability of lipase

min for invertase and trehalase respectively (Sarwade *et al.,* 2009b).

On the basis of results obtained on thermoliablity, it was very much clear that amylase is more heat stable than the other studied enzymes requiring more time for its 50% theoretical degradation. Lipase shows that it is more heat stable than the adult invertase, larval trehalase and protease. The protease is more heat stable than the trehalase and adult invertase. The adult invertase and larval trehalase and protease show that these enzymes are requiring more or less similar time for their 50% theoretical degradation. This aspect is very little investigated in insects for few enzymes. Amylase of *S. littoralis* lost its activity only above 65^{0}C which shows that the amylase of this insect is rather heat stable (Ishaaya *et al.,* 1971). Midgut

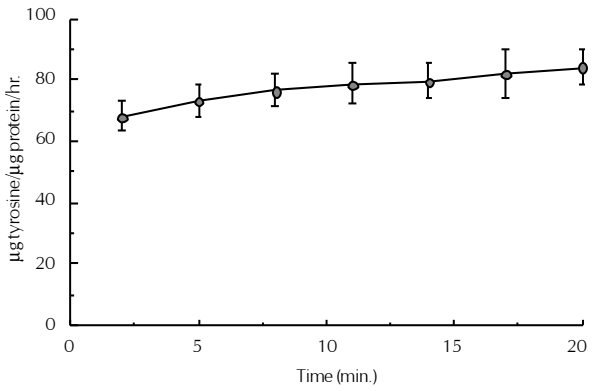

Figure 23: Thermolability of protease

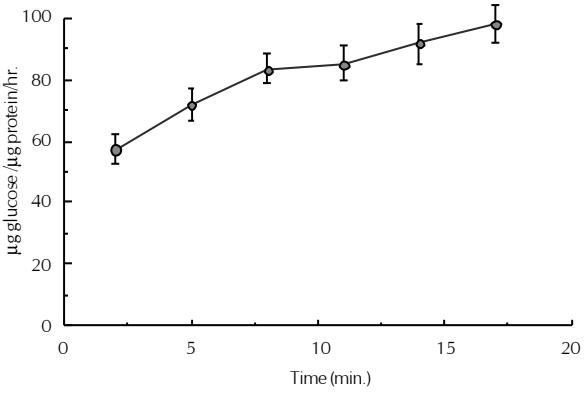

Figure 24: Thermolability of adult invertase

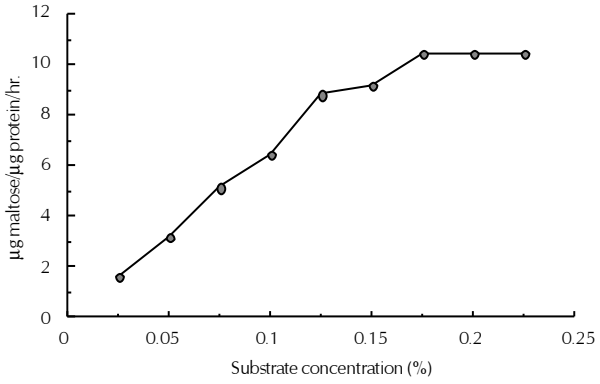

Figure 25: Effect of substrate concentration on amylase activity

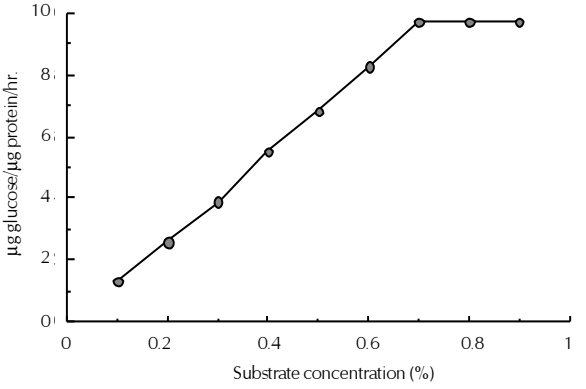

Figure 26: Effect of substrate concentration on invertase activity

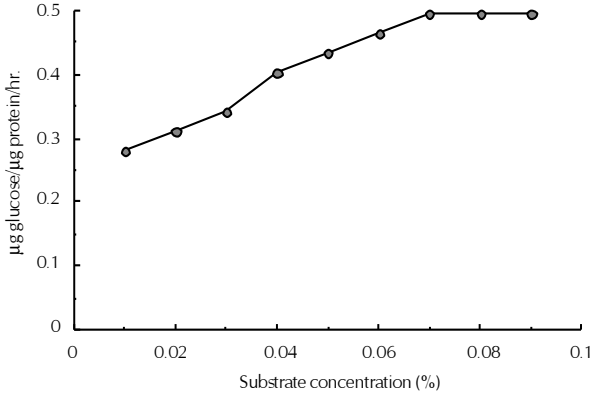

Figure 27: Effect of substrate concentration on trehalase activity

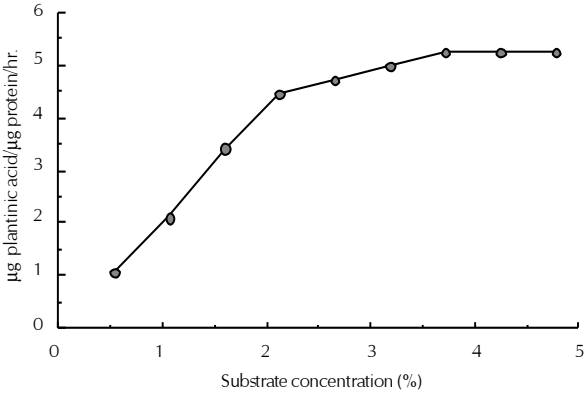

Figure 28: Effect of substrate concentration on lipase activity

amylase and trehalase of *A. proylei* requires 17 min and 11 min respectively for 50% denaturation (Kumbhar *et al.,* 2009, 2010) and in multivoltine race of *B. mori,* 50% loss of activity of amylase was 13 min (Muniv *et al.,* 2011). Trehalase is very unstable in tobacco horn worm *M. sexta* at temperature above 57ºC (Dhalman, 1971). Invertase of *Valanga nigrocornis* requires time of 29 min for 50% denataration at 60ºC (Teo, 1973a, where as protease in *Acheta domisticus* requires 62 min for 50% denataration at 50ºC (Teo and Woodring, 1988). The trehalase and protease enzymes of species under study shows heat labile results than above mentioned species. Generally insect amylases are capable of hydrolyzing starch, amylopectin and solubalised amylase at similar rate and with similar Km values (Applebaum and Konijn, 1965). Reported

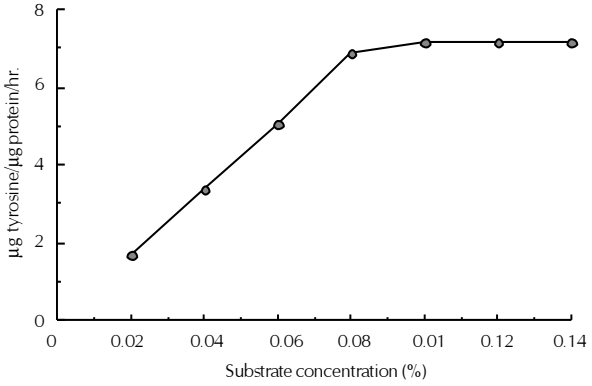

Figure 29: Effect of substrate concentration on protease activity

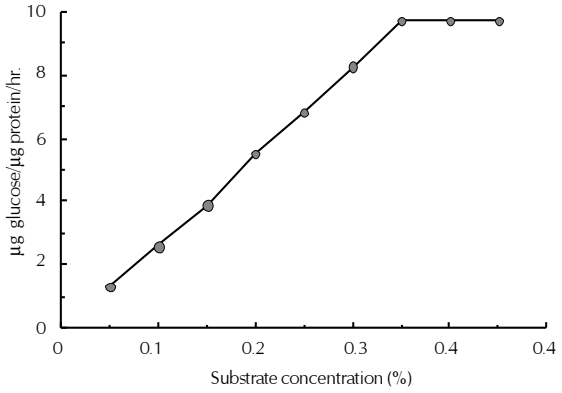

Figure 30: Effect of substrate concentration on adult invertase activity

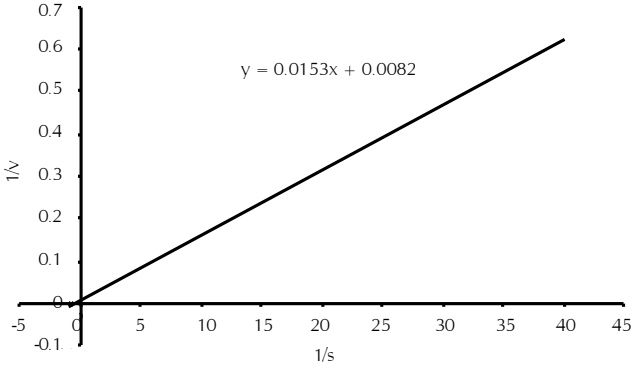

Figure 31: Line weaver burk plot for amylase

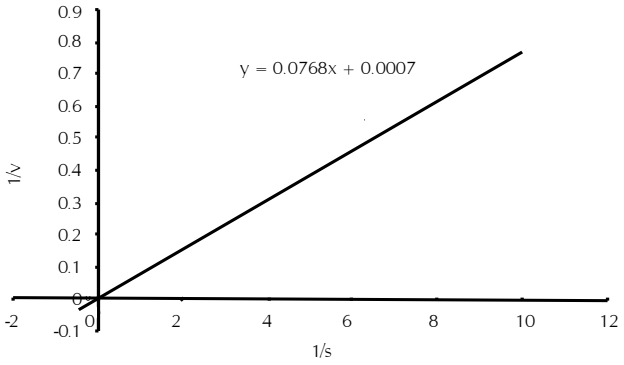

Figure 32: Line weaver burk plot for invertase

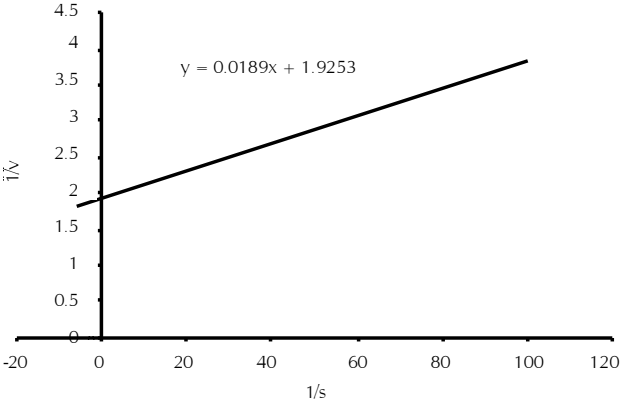

Figure 33: Line weaver burk plot for tre halase activity

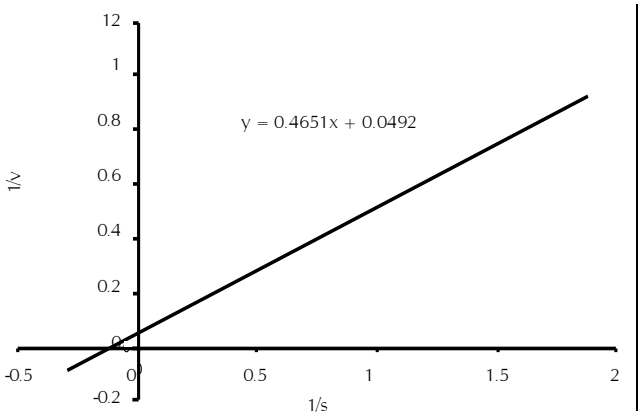

Figure 34: Line weavver burk plot for lipase activity

Km values for mid gut amylase are 0.13% of starch in *S. zeamise* (Baker, 1983), 2.82x10^{-2} M in *P. rapae* (Nishide and Kusano, 1971), 0.27mg/mL in *Callosobruchus chinensis* (Podolar and Applebaem, 1971).

The Km value of species under study is 0.533% of starch indicating amylase is more efficient. The Km value for invertase in fifth instar larvae and adults of *P. polytes polytes* were 2.333 x 10^{-3} M and 2.59 x 10^{-3} M of sucrose respectively. Earlier workers Nishide and Kusano (1971) reported 3.92 x10^{-3} M Km for gut invertase in *P. rapae* larval and Burton (1975) reported 11.2m M Km in salivary glands invertase of *H. zea*. Kumbhar *et al.* (2009,2010) reported 0.8% Km 2.11 x 10^{-3}M for midgut amylase and trehalse respectively in *A. proylei,* Muniv *et al.* (2011) reported 0.011 M and 0.058M Km values for midgut invertase in Pure Mysore and Kolar Gold races of *B. mori* respectively. The Km of midgut trehalase for species under study is 0.302 x 10^{-3} M indicates that the trehalase is more efficient than the other studied enzymes. In *M. sexta* the

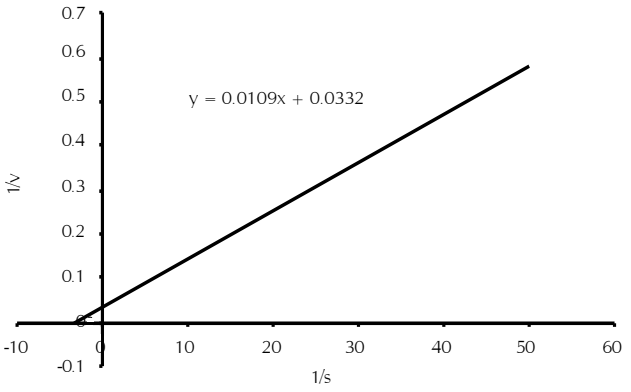

Figure 35: Line weaver burk plot for protase

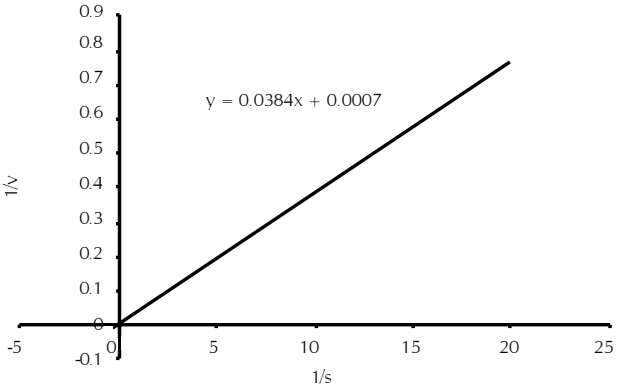

Figure 36: Line weaver burk plot for adult invertase

Km is 6.47 x 10^{-4} M (Dhalman, 1971). The Km values for lipase in midgut of present insect is 5.13 x10^{-4} M of triolene indicating major source of lipase. The Km for lipase in *V. nigrocornis* is 2.68x10^{-2}M of triolene (Teo, 1973b), in *Chrysomia rufifacies* it is 4x10^{-4}M (Pol, 1984). The protease has Km 0.4% of caesin in midgut of *P. polytes polytes*. Very scant information is available on Km of gut protease. In *V. nigrocornis,* Km value is 19.538mg/ml of casein (Teo, 1973b). Mahdavi *et al.* (2013) reported Km 50.5 ± 2.0 ì M in the alimentary canal of *Glyphodes pyloalis*. The results of the present study show that midgut is the major source for the most digestive enzymes. These results agree with the general view that the midgut is the chief site of digestion (Dadd, 1970, Law *et al.,* 1977., Engelmann and Geraets, 1980). Adult is active flier due to which sugary rich liquid nectar from the flowers is siphoned which fulfills the energy demand. The butterfly is semiautogenous insect because of this most of its nutritional requirement for maturation of gonads is fulfilled from the reserves accumulation during larval period. Hence, in the adult only invertase was detected in the midgut. Other enzymes amylase, trehalase, lipase and protease were not detected even at higher concentration of tissue.

The measurement of maximal catalytic activities of studied enzymes determines the physiological capacities of the different metabolic pathways. Such studies may be utilized in the formulating control strategies against the species under study and related species.

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