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°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 determined 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 littoralis (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 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 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 thermostability, 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,
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^-4M (invertase), 0.302X 10^-4M (trehalase), 5.13X10^-4M (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^-4 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. littoralis 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 midgut 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 pHoptima. 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](image1)

![Figure 4: Optimum pH of Lipase](image2)
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. aenesens (35-40ºC) for lipase. However, optimal temperatures
for soluble and membrane lipases were observed at 50°C and 35°C respectively by Roudsari (2014) in Bacterocera oleae. Kumbhar et al. (2009, 2010) reported optimum temperature of 40°C in A. proylei for trehalase and amylase. Muniv et al., (2011) reported optimum temperature 40°C for invertase in fifth instar larvae of B. mori. However, digestive enzymes are stable even at higher temperature i.e. 60°C in midgut of B. mori (Mori, 1930) and S. littoralis larva (Ishaaya et al., 1971) and 50°C in Glyphodes pyloalis (Mahdavi et al., 2013).

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

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
On the basis of results obtained on thermolability, 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°C which shows that the amylase of this insect is rather heat stable (Ishaaya et al., 1971).
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% denaturation at 60ºC (Teo, 1973a, where as protease in *Acheta domesticus* requires 62 min for 50% denaturation 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
Km values for mid gut amylase are 0.13% of starch in *S. zeamise* (Baker, 1983), 2.82 x 10^-2 M in *P. rapae* (Nishide and Kusano, 1971), 0.27 mg/mL in *Callosobruchus chinensis* (Podolar and Applebaum, 1971).

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 x 10^-3 M Km for gut invertase in *P. rapae* larval and Burton (1975) reported 1.12 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 trehalase respectively in *A. proylei*, Muniv et al. (2011) reported 0.011 M and 0.058 M 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

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**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 trehalase activity

**Figure 34:** Line weaver burk plot for lipase activity

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\[ y = 0.0153x + 0.0082 \]

\[ y = 0.0768x + 0.0007 \]

\[ y = 0.0189x + 1.9253 \]

\[ y = 0.4651x + 0.0492 \]
Km is $6.47 \times 10^{-4}$ M (Dhalman, 1971). The Km values for lipase in midgut of present insect is $5.13 \times 10^{-4}$ M of triolein indicating major source of lipase. The Km for lipase in V. nigrocornis is $2.68 \times 10^{-4}$ M of triolein (Teo, 1973b), in Chrysoma rufilaceus it is $4 \times 10^{-4}$ 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 $19.538 \text{mg/ml}$ of casein (Teo, 1973b). Mahdavi et al. (2013) reported Km 50.5 ± 2.0 M in the alimentary canal of Clyphodes 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 flyer 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.

REFERENCES


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