Studying the Activity of Alkaline Phosphatase, Digestive Proteases and Some Carbohydrate Enzymes in the Mid-Gut of the Third Instar Larvae of *Gasterophilus intestinalis* and Comparing Some of Them with Pupae

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Abstract: Proteinases contained in the mid-gut of the early third instar of ***Gasterophilus intestinalis*** have been tentatively identified by midgut hydrolysis of synthetic substrates**.** Trypsin was identified by maximal hydrolysis of benzoyl-DL-arginine-p-nitroanilide (BApNA) at pH 8 and chymotrypsin by maximal hydrolysis of benzoyl-L-tyrosine ethyl ester (BTEE) at pH 9.Carboxypeptidase A and B were identified by their maximal hydrolysis of hippuryl-DL- phenyllactic acid and hippuryl-L-arginine at pH 9 and 8 respectively. Aminopeptidase was identified by maximal hydrolysis of leucine-p-nitroanilide at pH 9. The activity of alkaline phosphatase and some carbohydrate enzymes (invertase, amylase and trehalase) were determined in the midgut of 3rd instar larvae and pupae of *Gasterophilus intestinalis.* The activity of alkaline phosphatase as well as the trend of amylase and trehalase activity were higher in the larval stage than that of pupa. There were no significant changes in invertase activity between the larvae and pupae. The results are discussed in view of the utilization of metabolites during metamorphosis.

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Key Words*:* *Gasterophilus intestinalis*, alkaline phosphatase, digestive proteases, carbohydrate enzymes, larvae, pupae

1. Introduction:

The larvae of the botfly ***Gasterophilus intestinalis*** (De Geer) infect the alimentary tract of horses and donkies in Egypt and they are completely endoparasitic. The veterinary importance of ***G. intestinalis*** has largely dealt with the damage, which the larvae produce, in gastric tissue of the horse. Gastric disturbances include ulceration (Shefstad, 1978, Pandey *et al*., 1980) subserosal abscess formation (Waddell, 1972; Shefstad, 1978), and nodule or papilla formation (Ashizawa *et al.*, 1972; Pandey *et al.*, 1980).

Roy (1937) showed that the larvae of ***G. intestinalis*** contain amylase, proteinase and lipase in their mid-gut. Tatchell (1958) suggested that larvae of this insect contain maltase, invertase, dipeptidaes and polypeptidase in the mid-gut and amylase, maltase and invertase in the salivary glands, while the haemolymph contain lipase, amylase and an anticoagulant.

Proteinases are divided into subclasses on the basis of catalytic mechanism and they are serine proteinases with a serine and a histidine in the active site, cysteine proteinases possess a cysteine in the active site, aspartic proteinase with an acidic amino acid residue and metalloproteinases with an essential metal involved in the catalytic mechanism. Exopeptidases include enzymes, which hydrolyse single amino acids from the N-terminus (aminopeptidases) or from the C-terminus (carboxypeptidases) of the peptide chain, (Terra *et al*., 1996).

Digestive serine proteinases, including chymotrypsin and trypsin, and the aspartate proteinase pepsine were first studied in insects since these were the principle digestive enzyme in vertebrates and reviews (Houseman and Downe, 1983), pepsin-like enzymes in some Diptera (Greenberg and Paretsky, 1955; Sinha, 1975; Pendola and Greenberg, 1975) and trypsin-like enzymes with maximal activity at pH values greater than 9 are frequently reported in Lepidoptera (Applebaum, 1985).

The aim of the present work was to characterize the major protease activities present in the mid-gut of the early third larval instar of ***G. intestinalis.*** Also to study the activity of alkaline phoshatase and some carbohydrases in both larvae and pupae that are important in understanding protein digestion, as part of the overall nutritional process and utilization of metabolities in this insect.

2. Material and Methods

Collection of insect larvae and pupae:

Third larval instar of ***Gasterophilus*** was collected from the stomach of freshly slaughtered donkies and horses in the Zoo, Giza, Egypt. They

were identified according to Zumpt (1965).Pupae were obtained by incubating late third instars larvae at 30 °C for 10 days.

Preparation of mid-gut homogenate:

The selected larvae were immobilized by placing on ice and dissected in insect saline solution.

The alimentary canals were separated and 0.23 gm of mid-gut was homogenized in 10 ml cold distilled water and was centrifuged at 8000 g for 10 minutes at 4 °C. Then supernatant was used for carrying the following experiments.

Determination of total protein:

The total protein was estimated according to the method specified by Lowry *et al.* (1951). The intensity of the colour was measured using Jenway 6100 spectrophotometer.

Buffers used:

0.2 M phosphate-citrate buffer at pH 4, 5, 6 and 7, 0.05M and 0.01M Tris-HCl at pH 8 and pH 9, 1M sodium acetate-hydrochloric acid at pH 2, 0.2M glycine-HCl buffer at pH 3

Determination of optimal activity of the following enzymes:

All substrate and chemicals were purchased from Sigma chemical company.

- **Trypsin:** The substrate N – benzoyl – DL – arginine – P- nitroanilide Hcl (BApNA) was prepared by dissolving 4.34 mg in 1 ml dimethyle sulphoxide Dimethyl sulphoxide (DMSO). The activity of trypsin was measured according to a modified method of Erlanger *et al*. (1961).The change in activity was measured at 410 nm (using Jenway 6100 spectrophotometer).

- **Chymotrypsin:** The substrate N – benzoyl – L – Tyrosine ethylester (BTEE) was prepared by dissolving 0.1567 gm in 50 ml of 50% methanol. The activity of chymotrypsin was determined according to the modified method of Hummel (1959).

- **Amino peptidase**: The substrate Leucine – p – nitroanilide (LpNA) was prepared by dissolving 4 mg in 0.1 ml DMSO. Activity of Leucine – aminopeptidase (LAP) was determined according to modified method of Houseman *et al*. (1985).

  The Change in activity of trypsin, chymotrypsin and aminopeptidase was measured at 410 nm (using Jenway 6100 spectrophotometer).

- **Carboxypeptidase:** A and B both substrate Hippuryl – DL – phenyl Lactic acid (HpLA) and Hippuryl – L – phenyl alanine (HA) were prepared by dissolving 0.01 gm of each substrate in 20 ml of 0.15 M NaCl. The activity of carboxyeptidase A and B was measured according to modified methods of Folk *et al*. (1960), and Gooding and Rolseth (1976).The change in activity was measured at 254 nm (using Shimadzu spectrophotometer).

- **Acidic protease:** Acid –denatured hemoglobin was prepared by dissolving 2 gm of hemoglobin in 100 ml distilled water and mixing it with 100 ml of 0.06M Hcl.
  Bovine serum albumin (BSA) was prepared by dissolving 2 gm in 100 ml distilled water.

  The activity of acidic proteases was measured according to modified method of Francisco *et al*. (1991).
  The change in activity was measured at 280 nm (using Shimadzu spectrophotometer).

- **Alkalinephosphatase**: Colorimeteric determination of alkalinephosphatase activity using phosphate alkaline-kit (ref, 61511) measured spectrophotometrically at an absorbance of 550nm.

- **Invertase and amylase**: Invertase and amylase activities were determined according to the method of Ishaaya and Swiriski (1970), Ishaaya *et al.* (1971) using 3-5 dintrosalicylic acid reagent for determining the free aldehylic groups of glucose formed after carbohydrate digestion, this reaction based on the reduction of dinitrosalysilic acid by the aldehydic groups of glucose units in a basic medium. A reduce dinitrosalysilic acid is measured spectrophotometrically at an absorbance of 550 nm.

  The reaction medium for invertase contained 0.2 ml 4% sucrose, 0.1ml 0.2M acetate buffer (pH 5.5) and 0.1ml 0.2% enzyme solution. The reaction medium for amylase contained 0.1ml 2% starch, 0.1ml 0.2M phosphate buffer (pH 6) and 0.2 ml 0.2% enzyme solution.

- **Trehalase:** Trehalase activity was determined by a procedure similar to that of invertase and amylase using the dintrosalicylic acid reagent for determining the glucose formed after trehalase digestion, the reaction medium contained 0.2ml 3% trehalase, 0.1ml 0.2M acetate buffer (pH 5.5) and 0.1ml 0.2% enzyme extract. Dintrosalicylic acid reagent was prepared by the procedure similar to that of Ishaaya and Swiriski (1976). One gm of 3, 5 dintrosalicylic acid was dissolved in 20ml of 2N NaOH and 50 ml of distilled water with the aid of magnetic stirrer. Thirty grams of K-Na tartarate was added, and magnetic stirring was continued until a clear solution was obtained. Distilled water was then added to bring the final

volume to 100 ml of the reagent, when stored in the dark, is stable at least for three months.

Pupae:

In case of pupae, whole body (4 gm) was homogenized in cold distilled water and centrifuged at 5000 rpm, and the detection processes were performed as in larvae.

Statistical analysis:

All analyses were performed using the Statistical Package for the Social Sciences (SPSS, Chicago, IL USA). Data were expressed as mean ± standard error of 6 replicates in each experiment. Mean values of continuous variables were compared using t-test or analysis of variance (ANOVA) followed by Duncan's multiple range test (Duncan, 1955). Correlations between variables were calculated by Pearson's method. The significance level was set at $P < 0.05$ or less.

3. Results

Enzyme activity assays:

The results of enzyme activity assays showed clearly that the mid-gut of the early third larval instar of ***G. intestinalis*** is capable of digesting proteins.

Figs (1- 5) show that the effect of different pH value on the activity of trypsin, chymotrypsin, leucine- aminopeptidase, carboxypeptidase A and carboxypeptidase B was not significant. The highest enzyme activity of trypsin and chymotrypsin was at pH 8 and pH 9, respectively (Figs 1, 2) and that of leucine-aminopeptidase was at pH 9 (Fig 3). Carboxypeptidase B activity was low at pH 6 and pH 7, but it increased at pH 8 and pH 9 with highest activity was at pH 8 (Fig. 4). Figure (5) shows that the highest pH activity of carboxypeptidase A was at pH 9. The results also revealed a positive significant correlation ($r = 0.87$, $P < 0.05$) between leucine-aminopeptidase and chymotrypsin (Fig 6) and also a positive significant correlation ($r = 0.83$, $P < 0.05$) between trypsin and carboxypeptidase B (Fig. 7). Maximum hydrolysis of haemoglobin by acidic protease was at pH 4 (Fig. 8), while maximum hydrolysis of BSA was at pH 3 (Fig. 9).

Activity of alkaline phosphatase for *Gasterophilus* was high during third instar larvae and significantly decreased ($P < 0.01$) during pupal stages (Table 1).The results for carbohydrate enzymes (invertase, amylase and trehalse) varied among the larvae and pupae of *Gasterophilus.* Invertase activity has not significant change between larvae and pupae of *Gasterophilus* ($P < 0.2$) (Table 2 Fig. 11).The trend of amylase activity shows decreasing in pupal stages than third larval stages. This decrease was not significant in *Gasterophilus* ($P < 0.066$, Table 1).Trehalase activity in *Gasterophilus* was high in third instar larvae and significantly decreased in pupal stages ($P < 0.004$, Table 1)

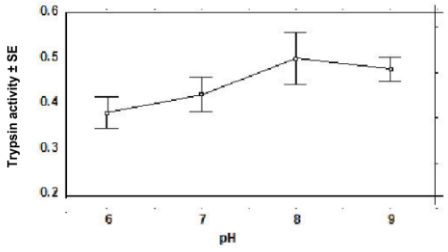

Fig. (1): Trypsin-like activity at different pH values using BApNA in homogenates of mid-gut of early third larval instars of ***G. intestinalis.***
Activity of enzyme expressed as O.D. / 10 min / 0.001 mg protein

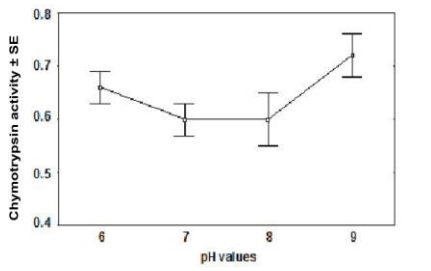

Fig. (2): Chymotrypsin-like activity at different pH values using BTEE in homogenates of midgut of early third larval instars of ***G. intestinalis.***
Enzyme expressed as O.D. / 10 min / 0.003 mg protein

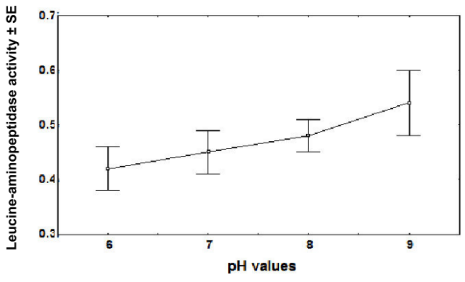

Fig. (3): Leucine-aminopeptidase activity at different pH values using LpNA in homogenates of mid-gut of early third larval instar of ***G. intestinalis***
Activity of enzyme expressed as O.D. / 10 min / 0.0002 mg protein

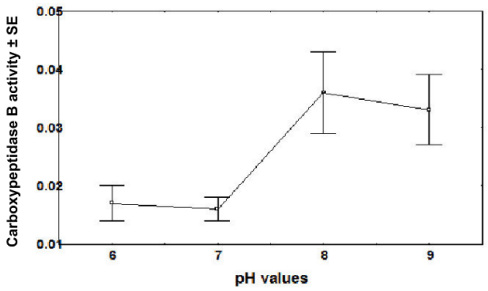

Fig. (4): Carboxypeptidase B activity at different pH values using HA in homogenates of mid-gut of early third larval instars of ***G. intestinalis***.
Activity of enzyme expressed as O.D. / 5 min / 0.0013 mg protein

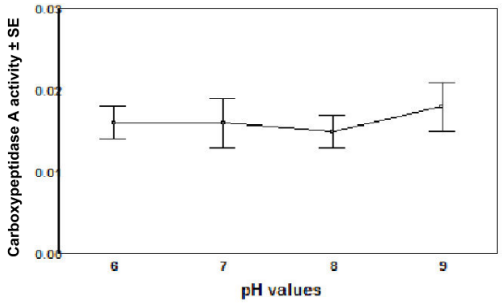

Fig. (5): Carboxypeptidase A activity at different pH values using HpLA in homogenates of mid-gut of early third larval instar of ***G. intestinalis***.

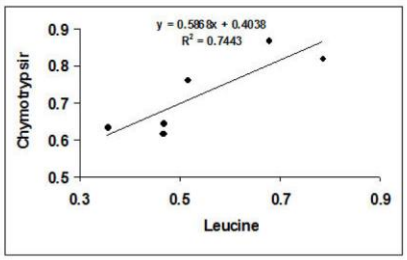

Fig. (6): Correlation in homogenates of mid-gut of early third larval instars of ***G. intestinalis*** between the activity of chymotrypsin and leucine–aminopeptidase at pH 9. Positive significant correlation can be observed ($r = 0.87$, $P < 0.05$).

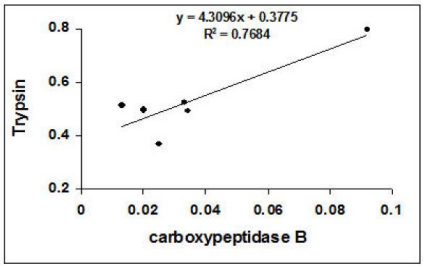

Fig. (7): Correlation between the activity of trypsin and carboxypeptidase B enzymes at pH 8 in homogenates of mid-gut of early third larval instars of ***G. intestinalis***. Positive significant correlation can be observed (r =

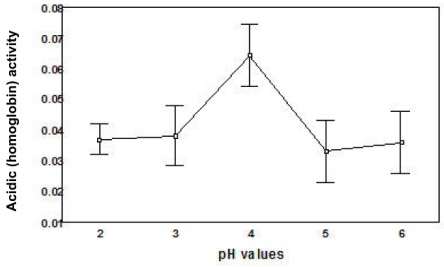

Fig. (8): Acidic protease activity at different pH values using acid– denatured haemoglobin in homogenates of mid-gut of early third larval instars of ***G. intestinalis***. Optimum pH at 4.
Activity of enzyme expressed as O.D. / 30 min / 0.003 mg protein

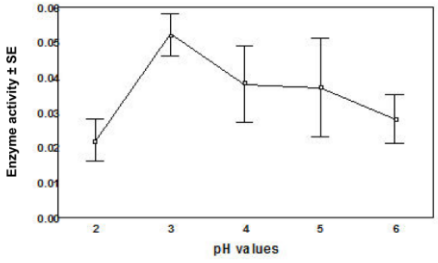

Fig. (9): Acidic protease activity at different pH values using BSA in homogenates of mid-gut of early third larval instars of ***G. intestinalis*** optimum pH at 3.
Activity of enzyme expressed as O.D. / 30 min / 0.003 mg protein

Table (1): Activity of alkaline phosphatase, invertase, amylase and Trehalase of 3rd instar larvae and pupae of ***Gasterophilus intestinalis***

Stage	Alk. Phos. activity* (mean±SD)	Invertase activity** (mean±SD)	Amylase activity** (mean±SD)	Trehalase activity** (mean±SD)
Larvae	**16.7 ± 1.15**	**0.13 ± 0.006**	**0.21 ± 0.008**	**0.15 ± 0.007**
Pupae	**6.5 ± 0.25**	**0.13 ± 0.006**	**0.17 ± 0.006**	**0.13 ± 0.002**

*Enzyme activity expressed as O.D./min/gm protein
**mg glucose/min/gm protein

4. Discussion

The mid-gut of the early third larval instars of ***G. intestinalis*** showed activity of some proteolytic enzymes at different pH values. It was clear from the results of the present work that trypsin, chymotrypsin, leucine- aminopeptidase, carboxypeptidase A and carboxypeptidase B showed great activity in the alkaline pH range mainly around pH 8 and 9.

The highest activity of trypsin in the mid-gut of the early third larval instars of ***G. intestinalis*** was at pH 8. This pH value matches those values (pH 7.8–10) of other insects recorded by different authors *e.g.* ***Pterostichus melanarius*** (Gooding and Rolseth, 1976), ***Tenebrio molitor*** (Levinsky *et al.*, 1977), ***Vespa crabo*** (Jany *et al.*, 1978), ***Attagenus megatoma*** (Baker, 1981b) ***Hypoderma lineatum*** (Tong *et al.*, 1981), ***Bombyx mori*** (Sasaki and Suzuki, 1982), ***Aedes***

aegypti (Graf and Briegel, 1985), ***Costelytra zealandica*** (Christeller *et al.,* 1989), ***Locusta migratoria*** (Sakal *et al.,* 1989), ***Muscat domestica*** (Lemos and Terra , 1992), ***Thrombi domestica*** (Zinkler and Polzer, 1992), ***Choristoneura fumiferana*** (Milne and Kaplan, 1993), ***Nauphoeta cinerea*** (Elpidina *et al*., 2001) and ***Mamestra configurata*** (Hegedus *et al* ., 2003) .

Trypsin is a serine proteinase. Lehninger (1970) stated that trypsin catalyzes the hydrolysis of peptide bonds in which the carbonyl function is donated by a basic amino acid residue like lysine or arginine. Trypsin – specific substrate (N- benzoyl –DL – arginine – p – nitroanilide) has a bond that is susceptible to trypsin hydrolysis because the carbonyl function is contributed by the basic residue arginine. This may indicate that the trypsin- like nature of the enzyme in the early third larval instar of ***G. intestinalis*** is responsible for the basic proteolytic activity in the mid- gut.

Digestive trypsin-like activity has been reported in most insect species. Important exceptions are Hemiptera species and species belonging to the series Cucujiformia of Coleoptera. The optimum pH of trypsin in most insects are always alkaline (mostly between 8 and 9), irrespective of the pH prevailing in mid-guts from which the trypsins were isolated. Nevertheless, trypsin isolated from Lepidopteran insects have higher optimum PH' corresponding to the higher pH values found in their mid-guts (Terra *et al.,* 1996). Also cleavage specificity against polypeptides was studied in trypsins from several insects (Terra and Ferreira, 1994). Results showed that specificities of these enzymes are similar (but not identical) to that of vertebrate trypsins. Nevertheless, some properties of insect trypsins contrast with those of vertebrate trypsins. Insect trypsins are not activated or stabilized by calcium ions (Levinsky *et al*., 1977; Jany *et al.,* 1978; Lemos and Terra, 1992), in most cases they are unstable in acid pH (Sakal *et al.,* 1989) and have different sensitivities to natural trypsin inhibitors (Purcell *et al*., 1992).

Barillas – Mury *et al.,* (1991) sequenced what seems to be the precursor of mid-gut trypsin in ***Aedes aegypti*** .The sequence shows significant differences from the vertebrate trypsin precursors in the region of the activation peptide. Similar results were found with a putative trypsinogen from ***Simulium vittatum*** (Ramos *et al.,* 1993). These differences suggest that the processing of precursors of insect trypsins may be different from that of vertebrates. In ***Erinnyis ello*** (Santos *et al.,* 1986) and in ***Musca domestica*** (Lemos and Terra, 1992; Jordao *et al.,* 1996b), trypsin is synthesized in the mid-gut cells in an active form, but is associated with the membranes of vesicles. These vesicles then migrate to the cell apex and trypsin precursors are processed to a soluble form before being secreted.

Secretory granules isolated from the opaque zone cells from ***Stomoxys calcitrans*** adults contain trypsin precursor, which is also different from that found in vertebrates. (Moffat and Lehane, 1990).

Determination of the chymotrypsin esterase- like activity in the early third larval instar of *G. intestinalis* indicates a maximum activity at pH 9. This pH value is similar to recorded values (pH 8 – 10) in other insects *e.g.* ***Pieris brassicae*** (Lecadet and Dedonder, 1966), ***Vespa orientalis*** (Jany and Pfleiderer, 1974), ***Glossina morsitans*** (Gooding and Rolseth, 1976), ***Locusta migratoria*** (Sakal *et al*., 1988), females of ***Anopheles*** (Horler and Briegel, 1995), ***Nauphoeta cinerea*** (Elpidina *et al.,* 2001) and ***Mamestra configurata*** (Hegedus *et al.,* 2003).

Chymotrypsin is a serine proteinase. Lehninger (1970), reported that chymotrypsin catalyzes the hydrolysis of peptide bonds in which the carbonyl function is contributed by an aromatic amino-acid residue like phenylalanine, tyrosine or tryptophane This also may indicate that a chymotryptic-like enzyme is also responsible for the basic proteolytic activity in ***G. intestinalis*** mid-gut.

It seems that the distribution of chymotrypsin-like enzymes among insect taxa is similar to that of trypsin (Applebaum, 1985). The optimum pH of chymotrypsin in most insects is in the range (8 – 9), irrespective of the pH prevailing in the mid-guts from which the chymotrypsins were isolated (Terra *et al.,* 1996). The sequences of the chymotrypsin-like proteinases were determined from ***Vespa orientalis*** and ***Lucilia cuprina*** and are similar to vertebrate chymotrypsins (Jany *et al.,* 1983; Casu *et al*., 1994). Also, insect chymotrypsins act on glucagon and B-chain of oxidized insulin in a manner similar to vertebrate chymotrypsins. However, some properties of insect chymotrypsins contrast to those of vertebrate chymotrypsins, such as their instability at acid pH and their strong inhibition by soyabean trypsin inhibitor. (Terra *et al*., 1996).

Maximum activity of leucine-aminopeptidase in the early third larval instar of ***G. intestinalis*** was at pH 9. This pH is more or less similar to pH values (7.2 – 8.5) in other insects *e.g.* ***Glossina morsitans*** (Gooding and Rolseth, 1976, Cheeseman and Gooding, 1985), ***Attagenus megatoma*** (Baker and Woo, 1981), ***Rhodinus prolixus*** (Houseman and Downe, 1981; Ferreira *et al.,* 1988), ***Trinervitermes trinervoides*** (Van der Westhuizen *et al.,* 1981), ***Rhynchosciara americana*** (Ferreira and Terra, 1984, 1985, 1986a, b; Klinkowstron *et al.,* 1994), ***Costelytra zealandica*** (Christeller *et al.,* 1989), ***Pheropsophus aequinoctiolis*** (Ferreira and Terra, 1989), *Teleogryllus commodus* (Christeller *et al*., 1990), ***Anopheles stephensi***

(Billingsley , 1990b) ***Spodoptera littoralis*** (Lee and Anstee, 1995). However, Leucine–aminopeptidase activity from ***Acanthoscelides obtectus*** (Osuala *et al.*, 1994) was maximum between pH ranges of 5.5 – 8.

In the early third larval instar of ***G. intestinalis*** carboxypeptidase A showed maximum peak at pH 9 while carboxypeptidase B showed maximum activity at pH 8. The specificity for trypsin hydrolysis of peptide bonds on the carboxyl side of basic L-aminoacids such as arginine or lysine means that the products, a carboxyl terminal of arginine or lysine is generated, which is the preferred substrate for carboxypeptidase B. This may indicate that these two enzymes may act in a sequential manner. This may be true in case of the early third larval instar of ***G. intestinalis***, as results revealed a positive significant correlation between trypsin and carboxypeptidase B.

Also the same may be true for chymotrypsin, which hydrolyze peptide bonds that give amino acids (as phenylalanine, tyrosine or tryptophane), these amino acids are preferred as a substrate to carboxypeptidase A. This also may be true in case of the early third larval instar of ***G. intestinalis***, as the results showed that both enzymes work optimally at the same pH value.

The optimum activity of carboxypeptidase A is near to the optimum pH (8-8.5) found in other insects *e.g.* ***Teleogryllus commodus*** (Christeller *et al*., 1990), ***Attagenus megatoma*** (Baker, 1981 a), and ***Costelytra zealandica*** (Christeller *et al.*, 1989). Also the optimum activity of carboxypeptidase B is more or less similar to the optimum activity (pH 7.8) of ***Glossina morsitans*** (Gooding and Rolseth, 1976).

Briegel and Lea, (1975) suggested that trypsin is the major primary hydrolytic protease in the mosquito mid-gut and is responsible for the initial breakdown of proteins and peptides in the mosquito mid-gut.

Billingsley (1990 a) stated that three aminopeptidases are responsible for the post-tryptic digestion of peptides throughout the mid-gut in ***Anopheles stephensi***. Billingsley and Hecker (1991) suggested that although trypsin is responsible for primary proteolytic events in the mid-gut, secondary digestion of peptides is brought about by aminopeptidases and carboxypeptidases.

The mid-gut of the early third larval instar of ***G. intestinalis*** also possessed proteinases that have optimum pH below 5. Terra. *et al.*, (1996) reported that aspartic proteinases have an optimum pH below 5, due to the involvement of a carboxyl residue in catalysis. The first report of aspartic proteinase in insects was made by Greenberg and Paretsky (1955), where they found a strong proteolytic activity at pH (2.5 –3.0) in homogenates of whole bodies of ***Musca domestica***. They hypothesized that this activity may be due to a pepsin-like enzyme. Lemos and Terra (1991) were able to demonstrate that the enzyme is cathepsin–D- like. Sequence studies have shown that pepsin may have evolved from the same archetypical gene as cathepsin D in vertebrates. A similar evolutionary trend seems to have occurred in Cyclorrhaphous Diptera, which apparently use cathepsin D as a digestive enzyme in the acid zone of their mid-guts.

The aspartic proteinase in the early third larval instar of ***G. intestinalis*** hydrolyzes haemoglobin maximally at pH 4.

The optimum pH at which haemoglobin was hydrolyzed in the early third larval instar of ***G. intestinalis***, was near to those pH values (3 – 4.5) reported in ***Rhodinus prolixus*** (Terra *et al.*, 1988), ***Leptinotarsa decemlineata*** (Thie and Houseman, 1990), Musca ***domestica*** (Lemos and Terra, 1991) *Aedes aegypti* (Cho *et al.*, 1991), ***Parasarcophaga surcoufi*** (Dorrah *et al.*, 2000) ***Callosobruchus maculatus*** (Silva and Xavier – Fiho, 1991).

The present study identifies an aspartic proteinase but does not distinguish between cathepsin D and pepsin. Cathepsin D (as an aspartic proteinase) can be differentiated from pepsin by the fact that bovine serum albumin is hydrolysed at 10 % or less of the rate for haemoglobin, whereas pepsin hydrolyzes both substrates equally (Barrett, 1977).

Since that the aspartic proteinase in the mid-gut of the early third larval instar of ***G. intestinalis*** hydrolyses haemoglobin maximally at pH 4, and that the hydrolysis of bovine serum albumin is 59.4 % that of haemoglobin, (at optimum pH of hemoglobin hydrolysis), therefore the characters of the acidic proteinase in ***G. intestinalis*** may be that of cathepsin D (further investigation is needed to confirm this result).

Phospatases are involved in the process of digestion and cellular replacement (Srivastava and Saxena 1967, Dadd 1970).Alkaline phosphatase may act as hydrolase during the final stages of digestion (Chung and Low, 1975). Furthermore, it has been suggested that alkaline phosphatase isozymes are involved with nutrient absorption and membrane transport (Eguchi 1995). On the same time alkaline phosphatase is one of the enzymes with relatively broad specificity, capable of acting on a number of different structurally related substrates as well as hydrolyzes many different esters of phosphoric acid (Lehninger, 1993).

The present study revealed that the alkaline phosphatase activity in the mid-gut of larvae is higher than that of pupae. Sridhara and Bhat (1963) in their studies of the variation in the alkaline phosphatase activities of the silkworm ***Bombyx mori*** in all stages of life cycle reported a steady increase in the enzyme activity. Also, Srivastava and Saxena (1967) found alkaline phosphatase to be widely distributed in the gut, salivary glands and Malpigian tubules of both

nymph and adults of ***Periplaneta Americana***. They reported that the presence alkaline phosphatase activity at those sites indicates a role in active transportation. Moreover, Beadle (1971) found that alkaline phosphatase was associated with those mid-gut cells containing lipid droplets in ***Carausius morasus*** and he reported that alkaline phosphatase probably plays a role in lipid absorption in insects. Likewise, Nath and Butler (1973) and Barker and Alexander (1958) compared the alkaline phosphatase in larval stage of two insect species, the black carpet beetle and house fly. The larval longevity of black carpet beetle requires 9 months while that of house fly requires 6 days. The maximum alkaline phosphatase activity possessed towards the end of the larval stages and species with shorter larval duration show a peak at a much earlier age. Those findings could also explain the increase of alkaline phosphatase activity in the mature larvae of ***Gasterophilus*** than pupae, as ***Gasterophilus*** larvae remain attached for nine months in the stomach of donkies and then pupate in sand.

The increase of the alkaline phosphatase activity in the mid-gut of *Gasterophilus* third instar larvae could indicate its important in transport of materials like glucose, fatty acid etc… across the mid-gut walls because during the third instars, the larvae consume their food voraciously and need mechanisms for the speedy transport of the metabolites across the mid-gut walls, to be stored for the adult tissue development.

The activity of amylase, trehalse was higher in the mid-gut of third instar larvae than that of pupal stages. The value of invertase activity was almost the same for all third instar larvae and pupae. Most of the dipterous insects e.g. blow flies are known to rely mainly on carbohydrates as a metabolic fuel (Sacktor, 1965).

Trehalose sugar plays an important role in carbohydrate metabolism insects. These disaccharides are the main sugar reserve in insect haemolymph being a specific substrate for trehalase enzyme to hydrolyze to glucose for internal energy supply (Wyatt 1967 and Takesue *et al*. 1989).

The larvae feed on blood rich in conjugated disaccharides during their attachment of the hosts. The high activities of the investigated enzymes result from the adaptability of the larvae to the diet. This explanation is supported by the observations of other authors (Frouz *et al*. 2002 and Taha 2003).

The amylase and trehalase activities in the third instar larvae could therefore used as additional parameters for assessing the availability of di and polysaccharides as nutrients and adaptability of these larvae to their hosts.

The assumption, which is generally applicable, that a correlation can be drawn between the presence of a high concentration of a specific substrate in the natural milieu of an insect and the presence of the corresponding digestive enzyme is true in this case. The assumption has been confirmed by Applebum *et al*. 1961 who stated that the high starch content of stored grains indicates important role of amylase in the digestive enzyme of ***Tenbrio molitor***. In view of the biochemical tests for the presence of these enzymes in the mid-gut of the larvae, it was found that dissaharides and polyssacharides present in the diet are able to support larval life and it was established that ***Gasterophilus*** larvae could be dependent on carbohydrates as a major source of energy. The high activities of these enzymes in the larvae than pupae indicate that the larvae are metabolically more active than pupae.

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