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A PRELIMINARY CHARACTERIZATION OF DIGESTIVE PROTEASE IN THE MID-GUT OF THE THIRD LARVAL INSTAR OF GASTEROPHILUS INTESTINALIS

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SUMMARY

Protinases 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 chym trypsin 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- phenyl-lactic 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 mid-gut also showed activity of aspartic proteinase and identified it as cathepsin D. A drug (Banminth 12.5% pyrantel tartarate) used for the routine control of helminthes parasites of horses and donkies in Egypt was used in vitro to investigate its effect on the optimal activity of studied enzymes. It was found that the drug has no effect on trypsin and carboxypeptidase A while it decreases the activity of chymotrypsin, aminopeptidase and acidic proteinase and was also found to increase the activity of carboxypeptidase B greatly.

INTRODUCTION

The larvae of the botlfly *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, 1980, 1981, 1982 and 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* that are important in understanding protein digestion, as part of the overall nutritional process and to show the effect of pyrantel tartrate (a drug used for the routine control of helminthes parasites of horses and donkies in Egypt) on the optimum activity of these proteases in vitro.

MATERIALS AND METHODS

Collection of larvae:

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).

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 8000g for 10 minutes at 4°C. Then supernatant was used for carryingg the following experiments.

Buffers used:

0.2M phosphate-citrate buffer at pH 4, 5, 6 and 7, 0.05M and 0.01M Tris-HCl at pH8 and pH9, 1M sodium acetate-hydrochloric acid at pH 2, M glycine-HCl buffer at pH3.

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). The steps used for determination of enzyme activities were repeated by incubating the enzyme solution with 30 ul of 1 % of Banminth drug (12.5 % Pyrantel tartrate) for 15 minutes before addition of substrate and adjusting pH at 8 (optimal pH activity of trypsin).

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).

Aminopeptidase: 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 haemoglobin was prepared by dissolving 2 gm of haemoglobin

in 100 ml distilled water and mixing it with 100 ml of 0.06M HCl.

Bovine serum albumine (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).

The above steps were repeated by incubating equivalent amounts of 1 % of 12.5 % pyrantel tartrate at optimum pH levels for each enzyme.
Control contained all components and was under same conditions but without enzyme solution.

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.

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 Duncanís multiple range test (Duncan, 1955). Correlations between variables were calculated by Pearsonís method. The significance level was set at $p < 0.05$ or less.

RESULTS

I- 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, 2, 3, 4 & 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).

The drug (pyrantel tartrate) used showed that its effect on the highest activity of trypsin and carboxypeptidase A and carboxypeptidase B was not significant (Fig. 10, 11) while it was significant ($P< 0.05$) for aminopeptidase, highly significant ($P<0.01$) for acidic protease and extremely significant ($P < 0.001$) for chymotrypsin (Fig. 10, . 12).

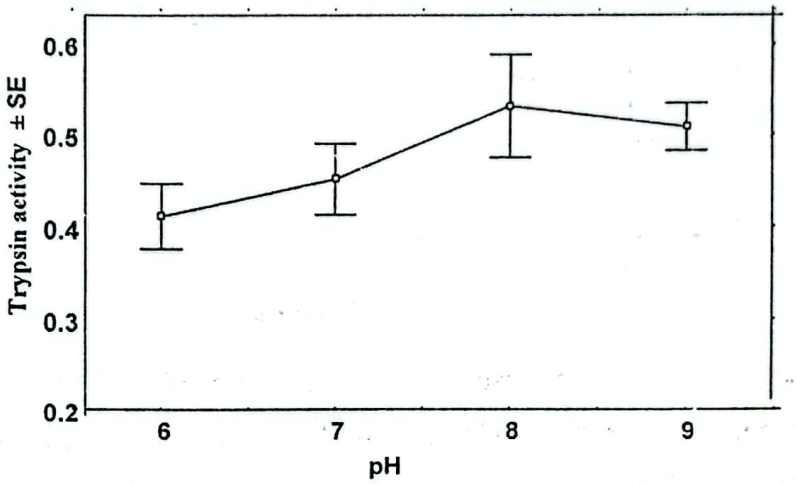

Fig. (1): Trypsin-like activity at different pH values using BApNA in homogenates of mid-gut of early third larval instars of *G. intestinalis*.
Activity of enzyme expressed as O.D. / 10 min / 0.001 mg protein.

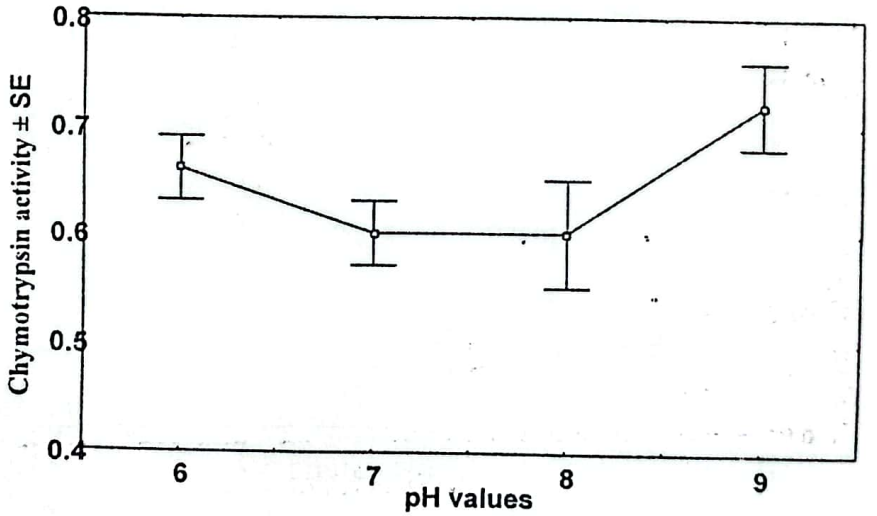

Fig. (2): Chymotrypsin-like activity at different pH values using BTEE in homogenates of midgut of early third larval instars of *G. intestinalis*
enzyme expressed as O.D. / 10 min / 0.003 mg protein.

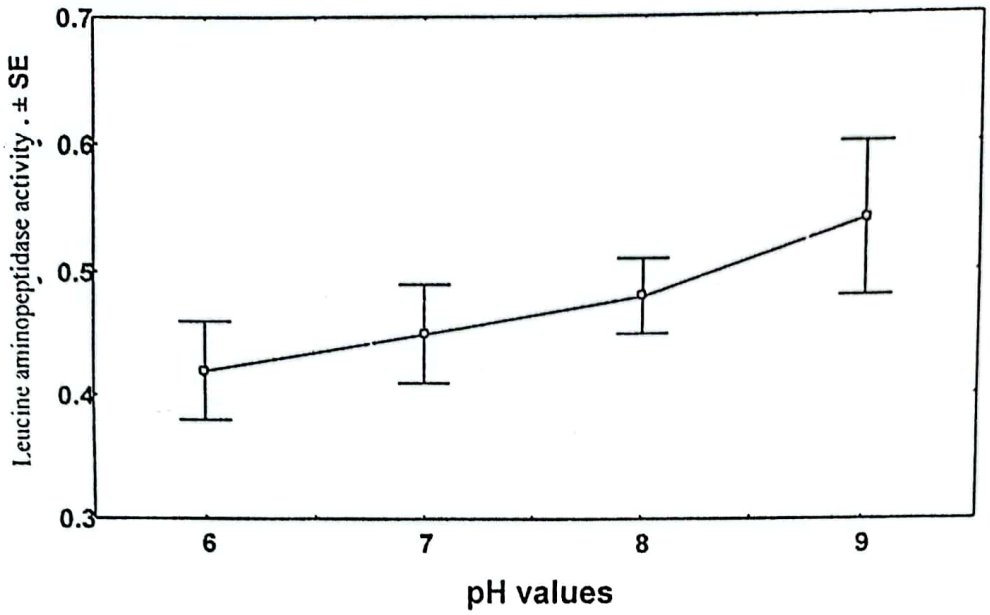

Fig. (3): Leucine-aminopeptidase activity at different pH values using LpNA in homogenates of mid-gut of early third larval instar of *G. intestinalis*
Activity of enzyme expressed as O.D. / 10 min / 0.0002 mg protein .

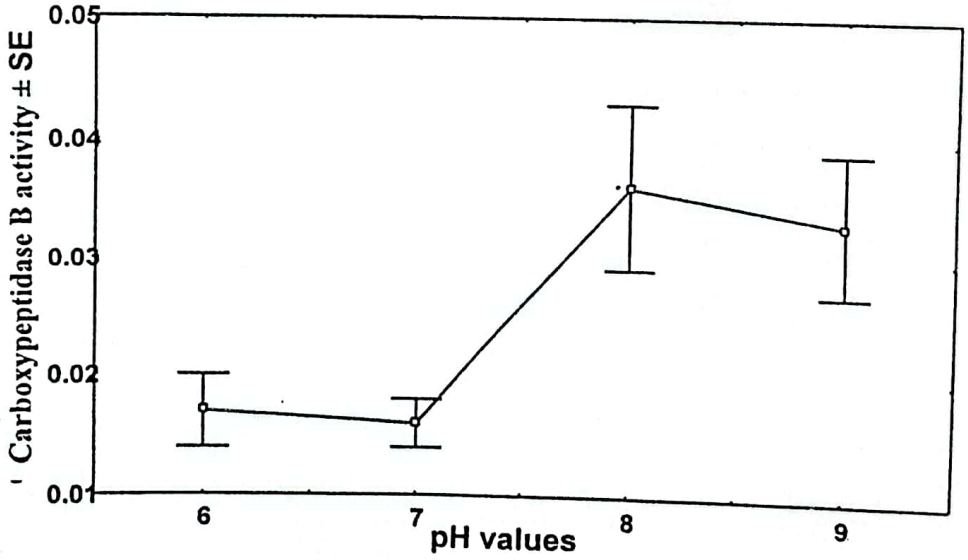

Fig. (4): Carboxypeptidase B at different pH values using HA in homogenates of mid-gut of early third larval instars of *G. intestinalis.*
Activity of enzyme expressed as O.D. / 5 min / 0.0013 mg protein.

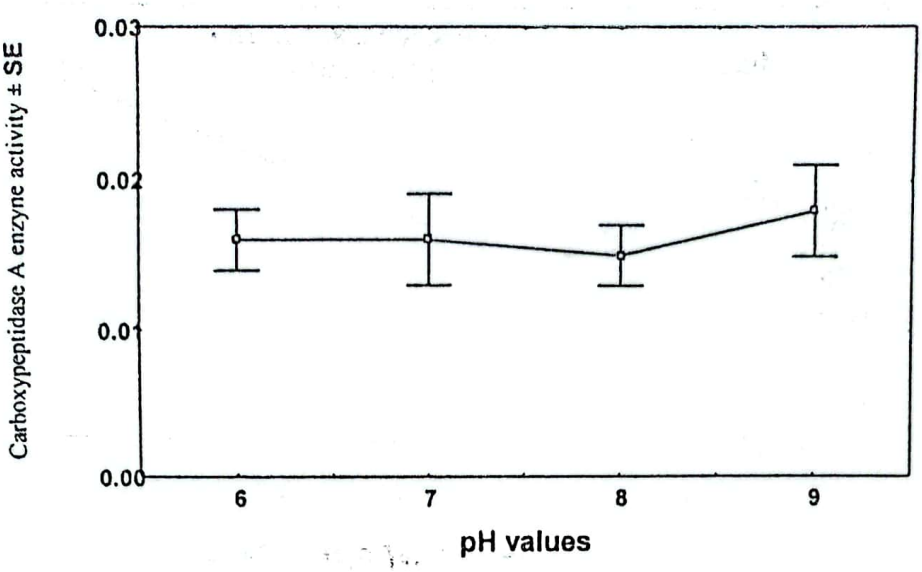

Fig. (5): Carboxypeptidase A activity at different pH values using HpLA in homogenates of mid-gut of early third larval instar of *G. intestinalis.*

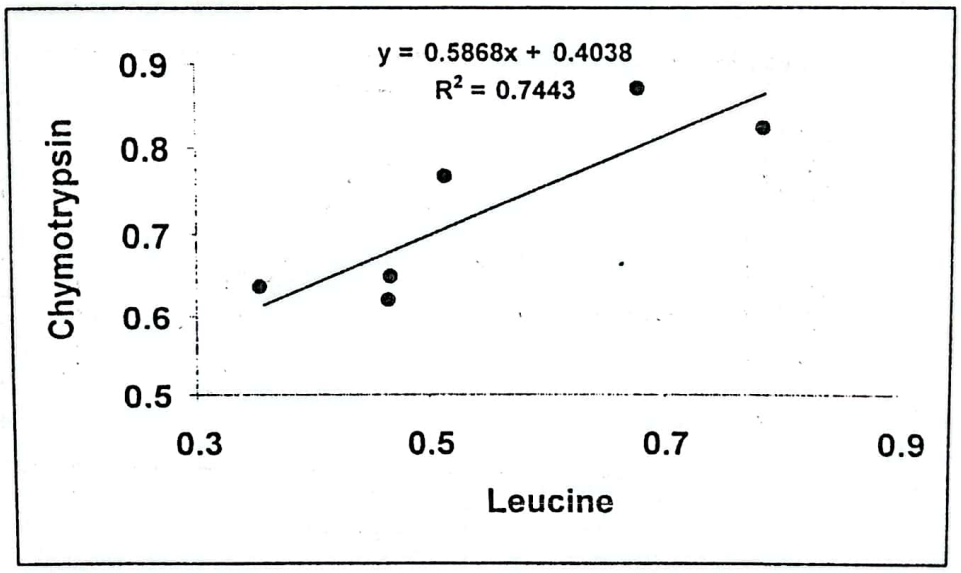

Fig. (6): Correlation in homogenates of mid-gut of early third larval instars of *G. intestinalis* between the activity of chymotrypsin and leucine -aminopeptidase at pH 9. Positive significant correlation can be observed ($r = 0.87$, $P < 0.05$).

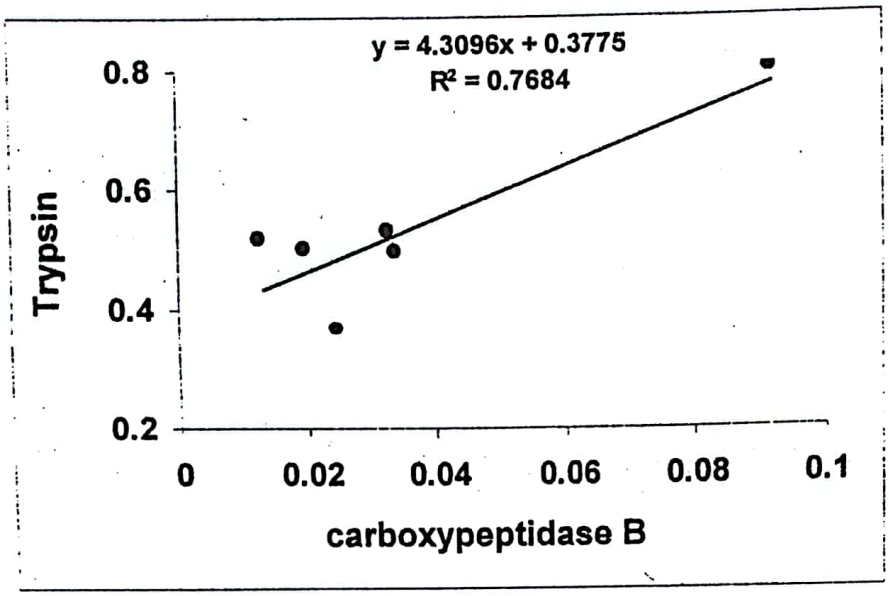

Fig. (7): Correlation between the activity of trypsin and carboxypeptidase B enzymes at pH 8 in homogenates of mid-gut of early third larval instars of *G. intestinalis*. Positive significant correlation can be observed ($r = 0.83$, $P < 0.05$).

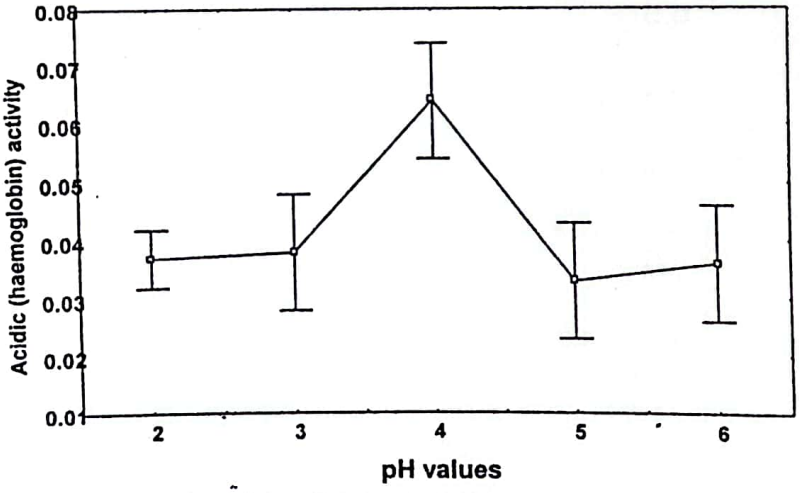

Fig. (8): Acidic protease activity at different pH values using acid- denatured haemoglobin in homogenates of mid-gut of early third larval instars of *G. intestinalis*. Optimum pH at 4.

Activity of enzyme expressed as O.D. / 30 min / 0.003 mg protein.

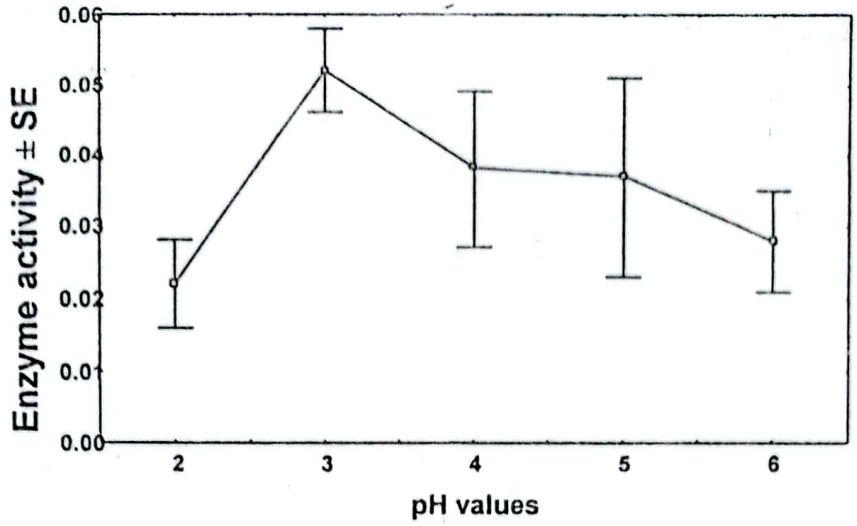

Fig. (9): Acidic protease activity at different pH values using BSA in homogenates of mid-gut of early third larval instars of *G. intestinalis* optimum pH at 3. Activity of enzyme expressed as O.D. / 30 min / 0.003 mg protein.

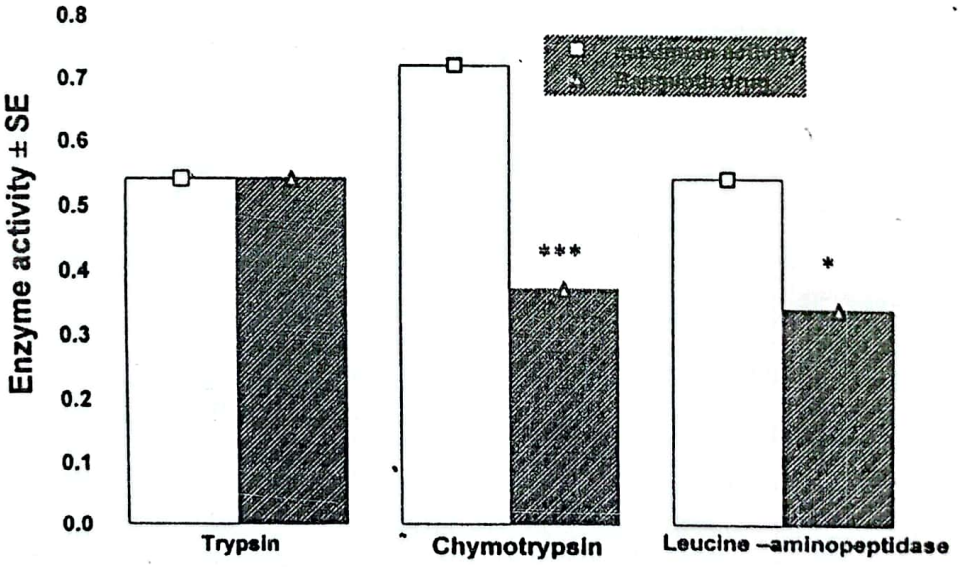

Fig. (10): Effect of Banminth drug on the maximum activity of trypsin (pH8) chymotrypsin (pH 9) and leucine - aminopeptidase (pH 9) in homogenates of mid-gut of early third larval instars of *G. intestinalis*

** $P < 0.05$

*** $P < 0.001$

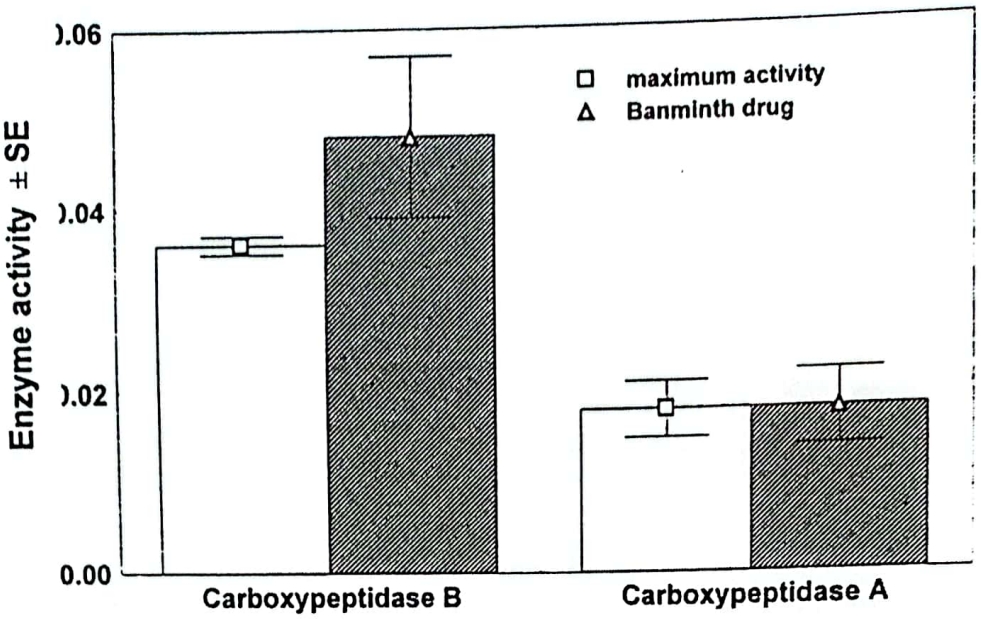

Fig. (11): Effect of Banminth drug on the maximum activity of carboxypeptidase B (pH 8) and carboxypeptidase A (pH 9) in homogenates of mid-gut of early third larval instars of *G. intestinalis*.

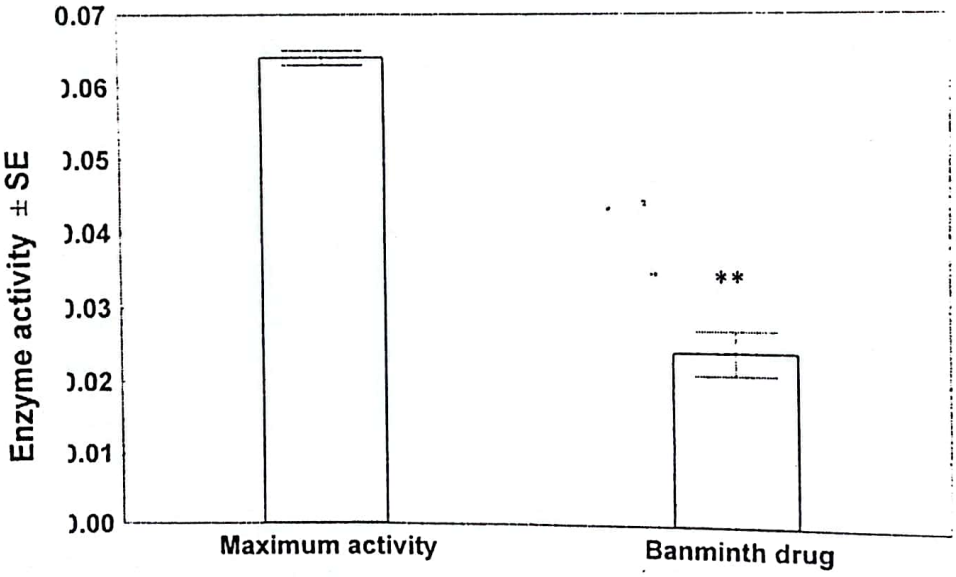

Fig. (12): Effect of Banminth drug on the optimum activity of acidic protease (pH 4 in-case of acid-denatured haemoglobin) in homogenates of mid-gut of early third larval instars of *G. intestinalis* .

** $P < 0.01$.

DISCUSSION

Digestive enzymes in the early third larval instars of *G. intestinalis* showed, that the larvae can digest proteins.

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 PHí 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., 1996), 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 ñ 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 ñ 9), irrespective of the pH prevailing in the midguts 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, 1990 b) *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). A drug (Banminth 12.5% pyrantel tartarate) used for the routine control of helminthes parasites of horses and donkies in Egypt. It has no effect on the highest activity of trypsin and carboxypeptidase A while it decreases the activity of chymotrypsin, aminopeptidase and

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