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REGULATION AND CHARACTERIZATION OF AMYLASE ENZYME SECRETION IN THE DIGESTIVE TRACT OF *ANTHERAEA ASSAMENSIS* HELFER

Kishor Haloi[1], Moni Kankana Kalita[1]* and Dipali Devi[2]

[1]Department of Zoology, Digboi College, Digboi 786171, Assam, India
[2]Institute of Advanced Study in Science and Technology, Seribiotech Unit, Biological and Chemical Sciences Section, Life Sciences Division, Paschim Boragaon 781035, Assam, India
*Email: monikankana14@gmail.com (corresponding author): ORCID ID 0000-0002-2822-3842

ABSTRACT

***Antheraea assamensis* Helfer is an endemic non-mulberry lepidopteran species of North East India with high commercial demand for its golden hued silk. *Antheraea assamensis* suffers from a protozoan disease pebrine which lead to death of the silkworm. In this study, the effect of various factors (starvation, feeding, temperature, pH, Ca ion and larval stage) in the regulation of amylase secretion is compared in healthy and diseased muga silkworm. Moreover, enzyme zymography and purification of amylase from the midgut is also performed. Result shows that, the secretion of amylase in *Antheraea assamensis* in starved condition, significantly decreases. Refeeding experiments (after 2 days of starvation) suggest that amylase secretion sharply increases due to feeding stimulus. The purified enzyme molecular weight is confirmed as 56 kDa by SDS PAGE. The purification fold of purified enzyme is 34 times higher than the crude enzyme extract.**

Key words: *Antheraea assamensis*, pebrine disease, starvation, feeding, temperature, pH, Ca ion, larval stage, amylase activity, zymography, SDS PAGE, enzyme purification

Antheraea assamensis Helfer (*A. assamensis*) is one of the important non-mulberry Lepidopteran species which is a geographical indicator of Assam, India. It is reknown for producing magnificent golden huedmuga silk with high commercial demand. It is a semi domesticated, polyphagous insect which is primarily reared on *Persea bombycina* (Som) and *Litsea monopetala* (soalu) plant. (Tikader et al., 2013; Nath et al., 2013).The digestibility is mostly depends on enzyme amylase and (Tikader and Rajan, 2012). The ability of digestion lead to the growth, development and survival of the insect. In insects, Alpha-amylases (EC 3.2.1.1) catalyzes the hydrolysis of alpha-1,4 glycosidic bonds of starch and glycogen which further produced final products of maltose, maltotriose, and branched maltodextrins (Franco et al., 2000; Da Lage, 2018) followed by hydrolized to glucose by α-glucosidase(s) (Rahimi and Bandani, 2014). Activity of amylase has been investigated in different orders of insects (Hemmati et al., 2022; El-Didamony et al., 2022; Hori, 1970; Terra et al., 1988; Ferreira and Terra, 1989; Schumaker et al., 1993; Nagaraju and Abraham, 1995). In Lepidoptera, there are several reports showing the amylase enzyme activity in mulberry group (Yan et al., 2021; Yan et al., 2022; Abraham et al., 1992) but reports are less in non-mulberry silkworm.

Antheraea assamensis is often tainted with an intracellular parasite *Nosema assamensis* which causes the fatal disease pebrine. Pebrine infection in *A. assamensis* larva shows black spots in the whole body due to the melanin formation in the infected hypodermal cells. The larvae also become lazy, eat less compared to the healthy ones and growth decreases (Ganga, 2003). Similar symptoms were also reported in *Antheraea mylitta,* infected with protozoa *Nosema mylitta*. The larva showed extended development period, significant weight loss of infected larva and decrease in digestive enzyme secretion (Rath et al., 2003). Moreover, reduced digestive enzyme activity was also observed in young bees infected with *Nosema apis* (Malone et al., 1998). Therefore, this disease might be considered as an important factor which modulate the enzyme secretion in insects. Apart from disease, the other factors like feeding stimulus, temperature, pH, Ca ion etc. also modulate the secretion of enzymes from the endothelial cells of gut of insects. The activity and structural integrity of insect amylases are depend on Ca ion (Kaur et al., 2014). Starvation showed reduced synthesis of amylase leading to the lower secretion of amylase in *Acheta domesticus* (Teo and Woodring, 1985). Insect gut varies from acidic to alkaline in nature and therefore the secretion of digestive enzymes in the gut might depends on the pH. However, the role of these factors are less understood due to unavailability of investigation works. Nagaraju and Abraham (1995)

purified and characterized amylase enzyme from *Antheraea mylitta* but there is no report of amylase purification and characterization in other species of genus *Antheraea*, especially muga silkworm. Keeping these things in mind, the primary objective of this study is to determine the role of different factors like starvation and feeding, temperature, pH, Ca^{2+}, and developmental stages in crude amylase activity in the digestive tract of *A. assamensis* and to compare with pebrine infected silkworm. The study also aims for the purification and characterization of digestive amylase of *A. assamensis.*

MATERIALS AND METHODS

The eggs of prevalent semi domesticated stock (healthy) silkworms were collected from several parts of *A. assamensis* rearing areas of Assam ($25^0$52´N $91^0$14´E, $26^0$02´N$90^0$51´E, $26^0$27´N $92^0$02´E, $26^0$45´N $92^0$06´E) of Assam-Hahim, Kushdhoa, Mangaldoi odalguri, and Sapkhaity and Tura and Adakgiri of Meghalaya ($25^0$30´N$90^0$16´E, $25^0$15´N $91^0$23´E). Pebrine infected (diseased) silkworms were collected from Mangaldoi Seri culture farm, Assam. Otherwise all others were of green morphs. The study was conducted in the *Persea bombycina* garden of IASST, Paschim Boragaon, Guwahati, Assam. The eggs were incubated at room temperature and after hatching they were transferred to the Som trees. Experiments were set up in the wild condition and both the healthy stock and diseased larva were reared separately.

The gut of *A. assamensis* was dissected out and removed gut was rinsed three times in 1 X PBS, pH 7.4 and divided into foregut, midgut and hindgut. The gut sections were stored in 0.1 M sodium acetate buffer (pH 5.6) at -20°C (Blakemore et al., 1995). For the preparation of crude enzyme, the stored gut sections were homogenized in 500 µl of 0.1 M sodium acetate buffer (pH 5.6) on ice (Zeng et al., 2012). The opened gut tissues were centrifuged at 2000g for 2 minutes at 4^0 C. To test enzyme activity in tissue of different The tissue homogenate (TH) of individual healthy and diseased larvae were centrifuged at high speed (16,000 × *g*) for 10 min at 4^0 C separately and supernatant was subsequently used for enzyme assay. The control was prepared by same volume of 0.1 M sodium acetate buffer for both healthy and diseased larvae. Amylase activity was determined by the method adopted by Bernfeld, (1955) with minor modifications. The regulation of secretion of amylase of *A. assamensis* was determined by starvation and feeding assay by the method of Weidlich et al., (2013). For control groups, 5thinstar larvae for healthy and diseased silkworms were starved for 2 days and the guts were dissected. For refed groups, 5th instar larvae after 2 days of starvation feed them for the next 24 hours with *Persea bombycina* leaves and gut were removed. The optimum temperature and pH for amylase activity was performed by DNS method (Ma et al., 2014). The effect of Ca ion on amylase secretion was determined in both healthy and diseased groups (Weidlich et al., 2013; Ma et al., 2014).

Zymography for amylase activity was carried out according to the method of García-Carreño et al., (1993). The SDS Page gel was prepared by the method of Laemmli, (1970) and Kotkar et al., (2009) with minor alterations. For purification, initially from the larval midgut of *A. assamensis* crude extract was prepared (Zeng et al., 2012). Then the extract was precipitated with ammonium sulfate (30% and 70%) at 4 °C. Followed by centrifugation at 6000 g for 15 min, then 5mL of universal buffer was applied for dilution and dialyzed overnight at 4 °C. A Sepharyl G-100 column was run at a flow rate of 0.5mL/ min by applying the dialyzed sample equilibrated with universal buffer (Zibaee et al., 2012). Protein concentration of all the purified samples were measured by the method of Lowry et al. (1951). All the experiments were performed in 3 replications and results are considered as mean ± standard deviation (n=30). Standard deviations and all the graphical presentations were done using Origin version 6.1. Means were compared by using ANOVA (significance at $p<0.05$ and $p<0.01$) with individual Post Hoc Tukey analysis by SPSS version 18 software package (www.winwrap.com).

RESULTS AND DISCUSSION

Current study reported the activity and regulation of amylase in the digestive tract of *A. assamensis*. The present study is the first report on regulation of amylase enzyme in different parts of the gut of *A. assamensis* and compared with diseased silkworm. Results showed more amylase activity the all three parts of the gut of healthy *A. assamensis* as compared with the diseased larvae ($p<0.01$) and in both cases the mid gut showed highest activity ($p<0.01$, Fig. 1). Similar result was also observed in *Antheraea mylitta* where amylase secretion was decreased due to infection that alter their digestion process leading to impaired growth. (Rath et al., 2003; Madhusudhan et al., 2018).

In both healthy and diseased silkworm, refed larvae of 24 h showed highly significant result as compared to the 2 days starved 5th instar larvae ($p<0.01$) except

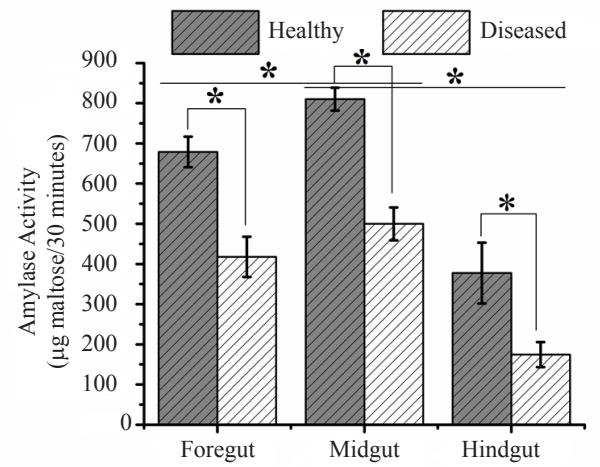

Fig. 1. Amylase activity in the different parts of the gut of healthy silkworms compared with diseased silkworms. Data showed significant difference among the groups of foregut, midgut and hindgut, ANOVA with post hoc tuckey**p<0.01 level of significance.

the hindgut of diseased ones. Prsent study findings indicated feeding as an important parameter for amylase activity. Here, the sharp increase in amylase activity after refeeding suggests that the presence of food content might act as a stimulus which increase amylase action (Fig. 2). It was also earlier reported that the presence of food in the gut acts as a prandial response which regulate the control release of digestive enzymes (Chapman, 1985; Lehane et al., 1995). Similar kind of result was also observed in *Gryllus bimaculatus* where the secretion of amylase is increased shortly

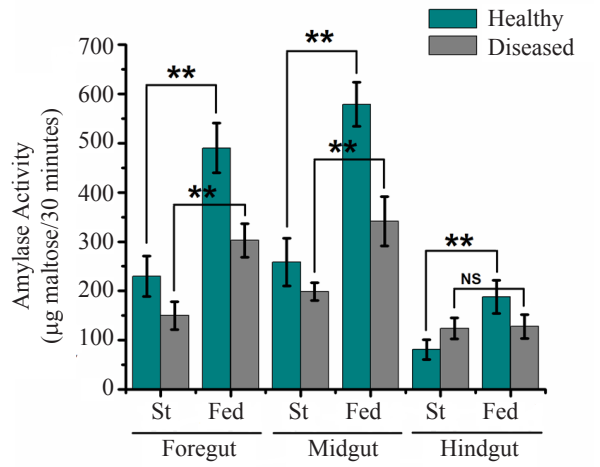

Fig. 2. Amylase activity in the different parts of the gut of healthy and diseased silkworm. In Refeeding experiment silkworms were starved for 2days and again reefed for 24 hours to observe amylase enzyme activity Data are significantly different among different groups of feed and starved, ANOVA with post hoc tuckey**p<0.01 level of significance.In the hind gut of amylase activity of infected larve showed no significant difference. Here St- Starved, Fed- feeding.

after molting as the larvae become voracious eater and consume increase amount of food (Weidlich et al., 2013) and in *Tribolium castaneum* (dos Santos et al., 2022). Likewise in lepidoptera also, with the advancement of developmental stages of different instars amylase activity is also increased (Nakonieczny et al., 2006; Willis et al., 2010). As the substrate is more, the enzyme activity is also more (Zadeh et al., 2020) and unavailability of substrate decreased or shut down the enzyme activity. This statement can be further supported with starvation experiment where the starved *A. assamensis* larvae showed less enzyme activity in comparison to the feeded ones. The food stimulus induce the secretion of neuropeptides which are released into the haemolymph and triggers the secretion of digestive enzymes (Sakai et al., 2006; Woodring et al., 2009; Mikani et al., 2011).

Results showed that amylase activity was highest at 35^0 C for both healthy and diseased larvae (p<0.01, Fig. 3 A). The optimum pH for the amylase activity was peak at pH 9 for both healthy and diseased larvae (p < 0.01, Fig. 3 B).The optimal temperature for amylase activity in insects is reported in the range of 30–60^0 C (Nadaf et al., 2022, Syakuro et al., 2022; Sarkar and Sikdar 2020; Kaur et al., 2014; Sharifloo et al., 2016) and in this regard, the present finding in *A. assamensis* also showed the same range in both healthy and diseased silkworm. *Antheraea mylitta* also showed highest activity of digestive amylase in gut at pH 9-10 (Nagaraju and Abraham, 1995) and in non-lepidoptera, *Rhynchophorus ferrugineus* and in *Eristalis megacephala* showed highest activity at pH 7-8 (Abd El-latif, 2020; Mohamed et al., 2019). The present study is in agreement with the observation that the high pH optima for digestive enzymes is the characteristics of polyphagous lepidopteran larvae (Syakuro et al., 2022, Nadaf et al., 2022, Zibaee et al., 2012).

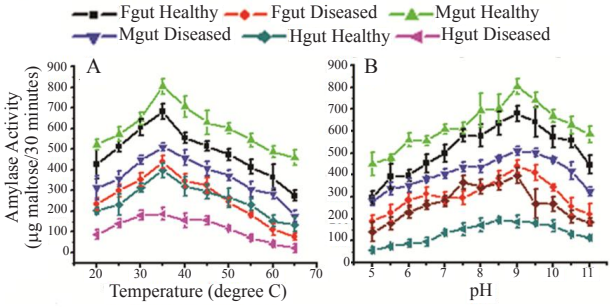

Fig. 3. Activity of amylase in respect to in a range of temperature (A) and pH (B) for both healthy and diseased silkworm. Data in terms of mean±sd, ANOVA with post hoc tuckeyp<0.01 level of significance.

The present investigation attempts to find out the mechanism of enzyme secretion (either apocrine or exocytosis mode) in both healthy and diseased muga silkworm. Current study showed an inhibitory effect of calcium ions on amylase activity which indicates that Ca ion might play a role in amylase secretion (Fig. 4 A, B). The possible mechanism of amylase secretion in the gut of *A. assamensis* is apocrine secretion instead of exocytosis as reported by several researchers in different insects (Cristofoletti et al., 2001; Weidlich et al., 2013).

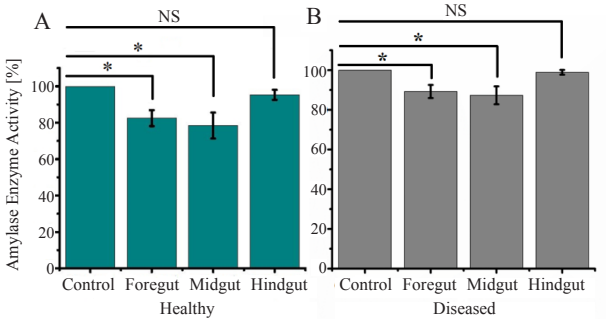

Fig. 4. Effect of calcium ion on amylase activity in the fore gut, mid gut and hind gut of healthy and diseased silkworm. Control hasn't incubated with calcium ions and converted to percentage enzyme activity, ANOVA with post hoc tuckey *p<0.05 level of significance and NS- not significant

The secretion of amylase had been significantly increased from 3rd instar to 5th instar stage in both healthy and diseased larvae (p<0.01, Fig. 5 A, B and C). The various developmental stages of insects act as one of the important factor in regulating enzyme activity (Weidlich et al., 2013). When *A. assamensis* silkworm emerges out from the eggs they start feeding with the tender leaves and after each molting they feed more. This implies that with the advancement of their age (1st, 2nd, 3rd, 4th, 5th instar) their food consumption rate is high and subsequently digestive enzymes activity also increase in both healthy and diseased silkworm. Study on amylase activity in *G. bimaculatus* also indicated high activity of enzyme in the last instar stage due to its high intake of food (Weidlich et al., 2013). Moreover, in current study the presence of amylase enzyme in gut of *A. assamensis* was confirmed by zymography. The mid gut of both diseased and healthy silkworm showed four similar peptide bands which indicated the presence amylase enzyme activity in both groups. However, the bands in diseased silkworm were faint in comparison to healthy one which indicated less amylase activity. Therefore, it might be stated that due to pebrine infection although, there was no change in the amylase enzyme but the overall amylase activity reduced.

A series of purification steps lead to isolation of a molecule with recovery of 18.72%, specific activity of 19.72 U/mg and purification fold of 34. Electrophoresis by SDS page followed by amylolytic activity revealed a molecular weight of 56 kDa for amylase enzyme of *A. assamensis*. (Table 1, Fig. 6). Ferreira and Terra, 1989 reported that in many insect the molecular weight of amylase ranges from 28 to 87 kDa, however in *Orius insidiosus* and *L. longipalpis*the molecular weight of

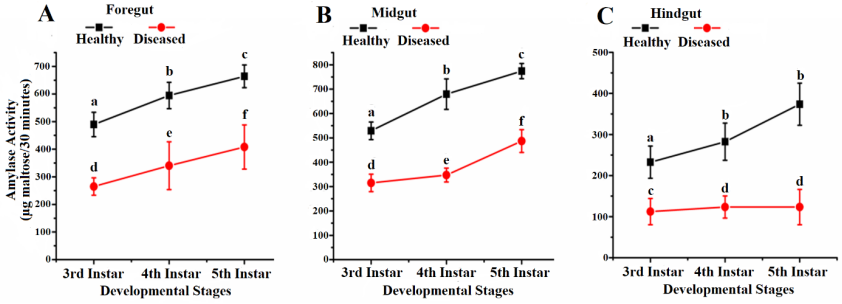

Fig. 5. Amylase in the gut of healthy/ diseased larvae -3rd instar to 5th instar

Table 1. Purification of amylase enzyme by different steps showing unit activity, specific activity, recovery percentage and purification folds

Purification steps	Unit activity (U± SE)	Amount of Protein (mg)	Specific Activity (U/mg Protein ± SE)	Recovery (%)	Purification fold
Crude extract	26.33± 0.57	45	0.58± 0.012	100	1
Ammonium sulfate (30%)	10.83± 0.28	20	0.54± 0.014	41.13	0.93
Ammonium sulfate (70%)	8.83± 0.15	6	1.47± 0.025	33.53	2.53
SepharylG-100	6.6± 0.173	0.45	14.66± 0.37	25.06	25.27
DEAE-Cellulose	4.93± 0.11	0.25	19.72± 0.44	18.72	34

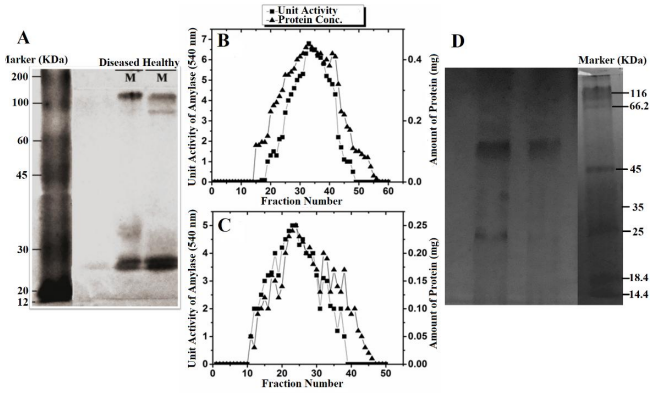

Fig. 6. Protein concentration of crude extract purified proteins in healthy and diseased larvae

amylase were 132 and 103 kDa respectively (Zeng and Cohen, 2000; Vale et al., 2012). A recent molecular study in Bark Beetle *Dendroctonus rhizophagus* (Curculionidae: Scolytinae) revealed the molecular weight of amylase was 53 kDa (Soto-Robles et al., 2021. In Lepidoptera, molecular weight of amylase was recorded as 51.2, 55 kDa for *Helicoverpa armigera* Hubner (Bhide et al., 2015), for *P. gossypiella* amylases was around 30 to 294 kDa (Nadaf et al., 2022) and 58 kDa for *Antheraea mylitta* (Nagaraju and Abraham, 1995). Furthermore, the variation of molecular weights of amylase enzyme due to compatibility properties of physiological role, interaction with dietary components and also depends on isoforms of the enzyme (Sharifloo et al., 2016).

Current study showed an alteration of amylase activity in diseased silkworms compared to healthy ones, but further in depth investigation is needed to understand the mechanism and characterization of amylase for their potential biotechnological applications is needed.

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AUTHOR CONTRIBUTION STATEMENT

Moni Kankana Kalita: Conceptualization, methodology, supervision, reviewing and editing; Kishor Haloi: Investigation, methodology and writing and original draft preparation; Dipali Devi: Editing and reviewing.

CONFLICT OF INTEREST

No conflict of interest.

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