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Rearing of *Helicoverpa armigera* Hubner and Characterization of its Gut Proteinases

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ABSTRACT

Background: The genus *Helicoverpa* includes the Old-World species *Helicoverpa armigera*, Hubner (Lepidoptera: Noctuidae) generally considered to be the most important species within group. It has been recorded on more than 200 host species in India and is most damaging to cotton, pigeon pea, cow pea, chick pea, ground nut, sorghum, pearl millet, tomato and many other crops of economic importance. *H. armigera* is being polyphagous pest with high reproductive and damaging potential, its suppression became difficult over past decade due to development of resistance to several chemicals. The current study was aimed to isolate and characterize the gut proteinases of *Helicoverpa armigera*.

Methods: During the period 2016-2018, *H. armigera* larvae were collected from the field and reared on an artificial diet and maintained under controlled conditions in the laboratory at Dr. Panjabrao Deshmukh Krishi Vidyapith, Akola, India. The laboratory conditions were maintained at temperature 28±2°C, humidity 75±5% and 14:10 hours light: dark period. The gut proteinases were isolated and were estimated by Bradford method. The total *Helicoverpa* gut proteinase activity, trypsin like activity, chymotrypsin like activity and electrophoretic visualisation of proteinases were done.

Result: Total proteinases activity was found to be 2.10 U/gut. Trypsin like activity was found to be 1.89 U/gut and chymotryptic activity was observed as 1.81 U/gut. The trypsin like activity was slightly more than chymotryptic activity.

Key words: Characterization, *Helicoverpa*, Proteinases.

INTRODUCTION

An increase in crop yield, its management and preservation are among the main challenges standing before the human population that exceed 10 billion by the mid of 21st century. *H. armigera*, Hubner (Lepidoptera: Noctuidae), is a major polyphagous pest present widespread in Asia, Africa and Oceania (EPPO, 2006) responsible for significant levels of yield losses in many economically important crops. The major host crops of this pest include tomato, cotton, pigeon pea, chick pea, sorghum and cowpea. The other hosts are groundnut, okra, peas, soybeans, maize, *Phaseolus sps*. and wide range of vegetable crops (CAB, 2006; Multani and Sohi, 2002; Chandra and Rai, 1974; Gahukar, 2002; Kakimoto *et al*., 2003). *Helicoverpa* is the major pest in most parts of the country (Yadav *et al.,* 2021) and it has covered major part of the world from tropical climates to regions with cooler temperate climate and spread easily to natural migration. Ojha, *et al*., (2018) shown the use of neem and microbial pesticides combination in the control of *H. armigera*.

About 50% of the insecticides used in India and China are used to control *Helicoverpa* pest. Bajya, *et al*. (2015) shown 53.33% mortality rate of *H. armigera* larvae by the use of chemical pesticide as quinalphos. To tackle this serious problem, farmers spend over approximately 40% of their annual income to buy such costly and unaffordable chemical pesticides. Application of huge amount of the pesticides resulted in to high levels of resistance in *H. armigera*. Some of the examples of pesticides to which *Helicoverpa* has acquired resistance are pyrethroids, endosulfan, carbamates and organophosphates (Armes, 1993 and 1995; Armes *et al*. 1992, 1994 and 1996; Forrester *et al*. 1993; Kranthi *et al*. 2001; Martin *et al*. 2000 and 2003; Torres Villa *et al*. 2002a). There are many field failures have been reported from Australia, Thailand, Turkey, India, Indonesia and Pakistan resulting from pyrethroid resistance acquired by *H. armigera* (CAB International, 2006). In order to develop sustainable solutions for insect control more complex resistance management strategies that involve several proteins with different modes of action are needed. Proteinase Inhibitors adversely affect protein digestion by inhibiting gut proteinases as their target enzymes are most common in insect gut. Udamle, *et al*. (2013) shown the tryptic and chymotryptic proteinase activity in the gut of *H. armigera*. To compensate the inhibited protease activity, insect overproduce digestive proteases which leads to deficiency of essential amino acids (Broadway and Duffey, 1986). This

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creates physiological stress resulting in inhibition of growth of insects and thus become weak and easy targets for their natural enemies (Lewis *et al.,* 1997). Therefore, it is necessary to study different gut proteinases of the insect, *Helicoverpa armigera*.

MATERIAL AND METHODS

Rearing of *H. armigera*

Healthy, actively feeding *H. armigera* larvae were collected from the field and reared on an artificial diet (Nagarkatti and Prakash, 1974 with modification) and maintained under controlled conditions in the laboratory at Dr. Panjabrao Deshmukh Krishi Vidyapith, Akola, India (Table 1 and 2). The laboratory conditions were maintained at 28±2°C temperature, humidity 75±5% and 14:10 h light, dark period. The pupae were collected and disinfected with 0.02% sodium hypochloride solution and transferred to the individual screw capped vials, containing soil bed. The adults emerged from the pupae after 7-10 days were then transferred to the bell jar covered with muslin cloth at its mouth. The sexes were differentiated on the basis of coloration pattern of the wings as female shows chocolate brown wings whereas males are pale brown. The adults were provided sterile cotton swabs imbibed with liquid diet of honey, sucrose and vitamin-E.

Extractions of helicoverpa gut proteinases

Helicoverpa armigera larvae of the late 3rd in starved were selected and dissected out midventrally. The midguts were isolated and stored at -20°C in deep freezer until required. For extraction of *Helicoverpa* gut proteinases, gut tissues were homogenized separately in 3 volumes of 0.2M Gly-NaOH buffer (pH 10.0) and kept for 2 hours. The gut luminal contents were removed by centrifugation at 12000 rpm at 4°C for 20 min and the resulting supernatant was analyzed for *H. armigera* gut proteinases (HaGPs) (Srinvasan *et al*. 2005). The proteins in gut extract were estimated by Bradford method keeping BSA as a standard (Bradford, 1976).

Estimation of proteins

The protein was estimated by the Bradford method (1976). It involves the addition of an acidic dye to protein solution and subsequent measurement at 595 nm with ELISA reader.

Standard curve

Bovine Serum Albumin (BSA) was used as a standard protein.

1. 1 mg/ml solution of BSA was prepared and taken in wells of microplate in different volumes ranging from 1 µl to 10 µl, to make the concentrations of BSA from 10 µg to 100 µg; each concentration was replicated three times.
2. Volume of the protein solution (BSA) was adjusted to 20 µl by adding 0.15 N NaCl.
3. 200 µl ready to use Bradford reagent (diluted 1:4 with distilled water) was added in each well.
4. Microplate was incubated for 15 min at room temperature.
5. Absorbance was recorded at 595 nm on ELISA Reader
6. Calibration curve was plotted between mean value of concentration on X - axis and mean value of absorbance on Y - axis.

Quantification of protein from unknown sample

The dye reagent was prepared by diluting one-part Bradford dye reagent (5X) with 4 parts of distilled water.

1. 10 µl of enzyme extract was added to each well.
2. 10 µl of 0.15 N NaCl was added in to that.
3. After, 200 µl 1X Bradford dye agent was added to each well.
4. The mixture was incubated at room temperature for 15 minutes.
5. The absorbance was recorded at 595 nm on (Parkin Elmer) Microplate reader.

Three experimental replications were maintained in this experiment.

Helicoverpa gut total proteinase activity

Helicoverpa gut proteinase activity was studied using casein as a substrate (Belew and J. Porath, 1970; used by Vinod Parde *et al.,* 2012). 50 µl of *Helicoverpa* Gut Proteinases was taken in 0.2 M Gly-NaOH buffer, pH 10.0. This reaction mixture was incubated for 10 min at 37°C in a water bath. After incubation, the residual caseinolytic activity was

Table 1: Composition of Artificial diet (700 ml) for rearing *H. armigera* larvae under laboratory condition (Nagarkatti and Prakash, 1974 with modification).

Ingredients	Quantity
Chickpea flour	110 gms
Ascorbic acid	2.6 gms
Methyl-p-hydroxy-benzoate	2.0 gms
Sorbic acid	0.5 gm
Casein powder	10 gms
Multi vitamin mixture	1 gm
Streptomycin sulphate	1 gm
Cholesterol	0.1 gm
Formaldehyde	1 ml
Vitamin E	0.6 gm
Yeast (dried granules)	20 gms
Agar	10 gm (in 300 ml of DW)
Distilled water	400 ml

Table 2: Composition of *Helicoverpa armigera* (Adult) Diet.

Ingredients	Quantity
Sucrose	50 gm
Honey	50 ml
Aureomycin	2.5 gm
Streptomycin sulphate	1 gm
Vitamin E (Capsule)	1 gm
Methyl-p-hydroxy-benzoate	1 gm
Formaldehyde (10% V/V)	1 ml
Multivitamin	1 gm
Distilled water	500 ml

measured by adding 0.5 ml of 0.5% casein (in 0.2 M glycine-NaOH, pH 10.0) and incubated at 37°C for 20 minutes. The reaction was terminated by adding 750 µL of 5% tri-chloro acetic acid. After centrifugation at 10,000 rpm for 10 minutes, the absorbance of supernatant was measured at 280 nm.

Helicoverpa gut trypsin like proteinase activity

To study the trypsin like activity, 50 µl of *Helicoverpa* Gut Proteinase was taken in 200 µl of 0.01M Tris-HCl buffer of pH 8.0 containing 0.02 M $CaCl_2$. This reaction mixture was incubated for 10 min at 37°C in a water bath. After incubation, the residual trypsin activity was measured by adding 1 mL of 1 mM chromogenic substrate BApNA in pre-warmed (37°C) buffer 0.01 M Tris-HCl (pH 8.0) containing 0.02 M $CaCl_2$ and incubated at 37°C for 10 minutes (Erlanger *et al*., 1961). The reactions were stopped by adding 200 µL of 30% Glacial acetic acid. After centrifugation, the liberated p-nitroaniline in supernatant was measured at 410 nm. All the assays were performed in triplicate. 100% enzyme activity was seen in the reaction mixture containing enzyme and substrate lacking proteinase inhibitors. The value produces 100% enzyme activity and the difference between absorbance in reaction tubes and respective control was used for the calculation of the residual activity of Trypsin based upon µL of proteinase extract used (Celia *et al*., 2002). One proteinase unit was defined as the amount of enzyme that increases absorbance by 1 OD/ min under the given assay conditions.

Helicoverpa gut chymotrypsin like proteinase activity

To study the chymotrypsin like activity, 50 µl of *Helicoverpa* Gut Proteinase was taken in 200 µl of 0.01M Tris-HCl buffer of pH 8.0 containing 0.02 M $CaCl_2$. This reaction mixture was incubated for 10 min at 37°C in a water bath. After incubation, the residual chymotrypsin activity was measured by adding 1 mL of 1 mM chromogenic substrate N-Benzoyl-L-tyrosine ethyl ester (BTEE) in pre-warmed (37°C) buffer 0.01 M Tris-HCl (pH 8.0) containing 0.02 M $CaCl_2$ and incubated at 37°C for 10 minutes (Erlanger *et al.* 1961). The reactions were stopped by adding 200µL of 30% Glacial acetic acid. After centrifugation, the absorbance was measured at 410 nm. All the assays were performed in triplicate.

Electrophoretic visualization of HGPs

HGPs were detected by using by Non-reductive SDS-polyacrylamide gel electrophoresis. Enzyme extracted from the mid gut of *H. armigera* larvae in 0.2M Gly-NaOH buffer (pH 10.0) was diluted by 100 µl of 1X sample buffer and 25 µl of this sample mixture was electrophoresed on 10% SDS-polyacrylamide (Gujar *et al.,* 2004). After electrophoresis, SDS-polyacrylamide gel was washed in 2.5% (v/v) Triton X-100 for 10 min to remove SDS, then incubated in 2% casein in 200 mM Glycine-NaOH, 10 pH, gel was then stained with Coomassie brilliant blue R-250 (CBB R-250). HGP bands were revealed as white bands with dark blue background.

Sample buffer (1X)

Sample buffer for non-reductive SDS-PAGE (2X): 0.1ml
50% Sucrose: 0.4 ml
DW: 0.6 ml

RESULTS AND DISCUSSION

Rearing of *Helicoverpa armigera* is done and is shown in Fig 1 to Fig 8

Quantification of *H. armigera* gut proteins in different larval stages

The protein estimation of midgut was done by Bradford method. It was found that the protein present in 5th instar larvae of *H. armigera* was more *i.e.* 0.57µg/10 µl, as compared to the 3rd instar and 4th instar larvae which was 0.45 and 0.51µg/10 µl respectively.

***Helicoverpa armigera* gut proteinases activity**

As *Helicoverpa* is a polyphagous pest and produces diversity of gut proteinases, it is now essential to characterize the gut proteinases of *Helicoverpa*. It is seen that the total gut proteinase (Caseinase), trypsin like proteinases (BApNAase) and chymotrypsin like proteinases (BTEEase) activities were present in gut of *H. armigera* and were assayed given in (Table 3). Total proteinases activity was found to be 2.10 U/gut. Trypsin like activity was found to be 1.89 U/gut and chymotryptic activity was observed as 1.81 U/gut. The trypsin like activity was slightly more than chymotryptic activity.

Electrophoretic visualization of *H. armigera* gut proteinases

Electrophoretic visualization of *H. armigera* gut proteinases (HaGPs) fed on artificial diet were extracted in 0.2 M glycine-NaOH buffer, pH 10.0 and separated out by 12% SDS-polyacrylamide. As revealed from the Fig 9 total *H. armigera* gut proteinase activity was recorded in ten different isoforms, ranging from molecular weight 118 kDa to 16 kDa. The appearing density of P_1, P_2, P_3, P_7, P_8, P_9 and P_{10} found to be high while that of P_4, P_5 and P_6 were low.

Proteinases are a group of hydrolytic enzymes present in insects and are involved in different activities like digestive processes, activation of proenzymes, liberation of active peptides, complement activation and inflammation processes in others (Neurath, 1984). These enzymes belong to the class endopeptidases (EC 3.4.21-24 and EC 3.4.99) and involved in cleavage of internal peptide bonds of polypeptides (Fan and Wu, 2005; Rawlings *et al.,* 2004).

Table 3: *H. armigera* gut proteinases activity.

Proteinases	Enzyme activity (U/gut)
Total proteinase activity	2.10±0.001
Trypsin like activity	1.89±0.002
Chymotrypsin like activity	1.81±0.002

All the figures are mean of triplicate values±SE.

Fig 1: Rearing of *Helicoverpa armigera* larvae collected from the fields of Dr. Panjabrao Deshmukh Krishi Vidyapeeth, Campus Akola. The collected larvae were reared on the synthetic media (Table 1) and maintained under laboratory conditions.

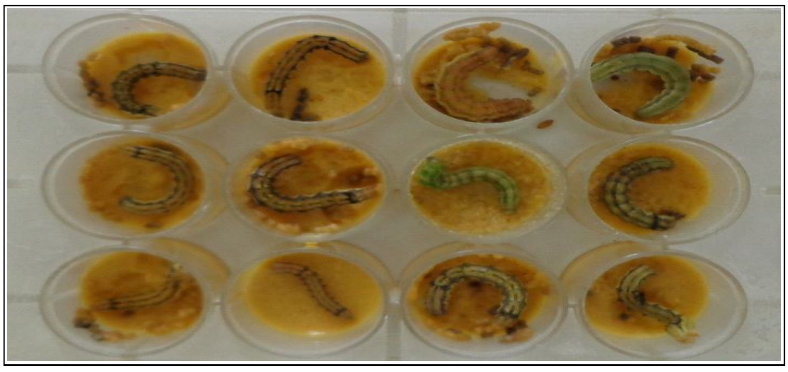

Fig 2: *Helicoverpa armigera* larvae in 3rd, 4th and 5th instar stage feeding on artificial diet (Composition of artificial diet is given in Table 2).

Fig 3: Pupae of *Helicoverpa armigera* disinfected with 0.02 per cent sodium hypochloride solution shown brown in colour.

Fig 4: Release of *Helicoverpa armigera* adult moths from pupae after 9 days on sterile soil bed.

Fig 5: Mating chamber containing male and female adult moths of *H. armigera*.

In the previous study, Johnston *et al*. (1991) showed the presence of proteolytic activity due to diverse properties of the proteinases which are found in the lepidopteran insect mid gut in various isoforms and also showed trypsin like proteolytic activity in the gut of *H. armigera*. In 1998, Harsulkar *et al*. showed the presence of proteolytic activity in six different isoforms in the gut of *H. armigera*. Haq *et al*. (2004) studied Serine and Cysteine proteinases as the major classes of proteolytic enzymes found in the digestive system of phytophagous insects. While working on the pest *H. armigera*, Potdar in 2008 found the presence of proteolytic activity distributed in ten different proteinase isoforms.

The presence of the proteolytic activity of the different proteinases found in the gut of insects showed its polyphagous nature also has a great impact on the adaptation and the survival of the insects. The adaptation of the different insects to the host plant PIs may results due to the selection pressure acting on the insect pests on encountering the PIs of their host plants (Harsulkar *et al*. 1999). Patankar *et al*. in 2001 showed the polyphagous nature of the *H. armigera* by studying the expression of *Helicoverpa* gut proteinases. Thus, like the earlier and present study, the presence of different isoforms of the proteinase in *H. armigera* shows its polyphagous nature.

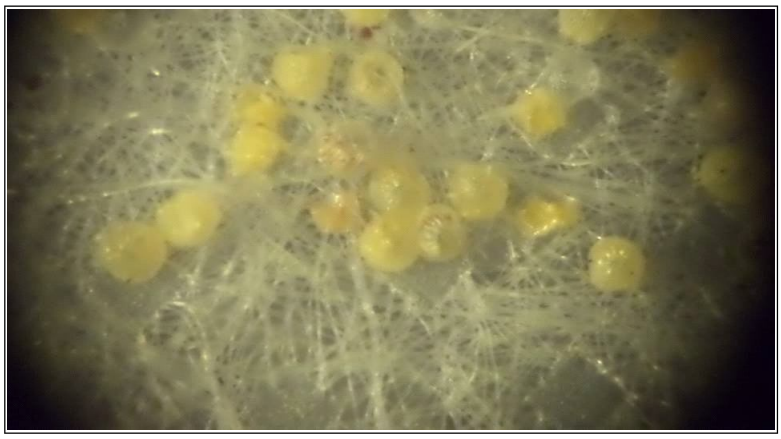

Fig 6: Mass of fertile roundish pale green eggs of *Helicoverpa armigera* on muslin cloth.

Fig 7: Fertile roundish pale green egg of *Helicoverpa armigera*.

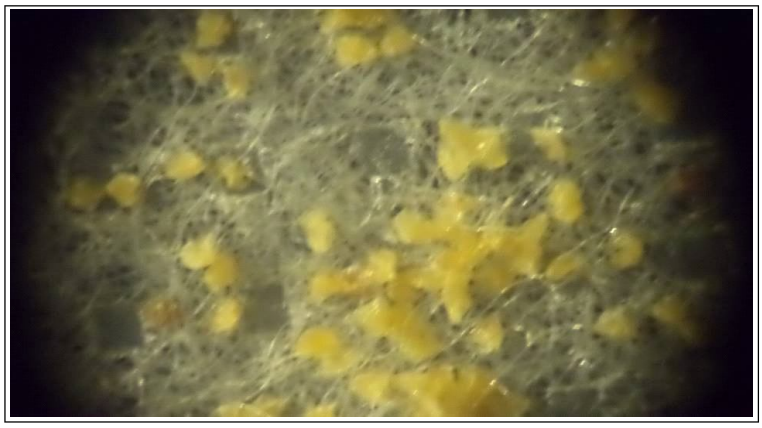

Fig 8: Non-fertile and irregular pale green eggs of *Helicoverpa armigera*.

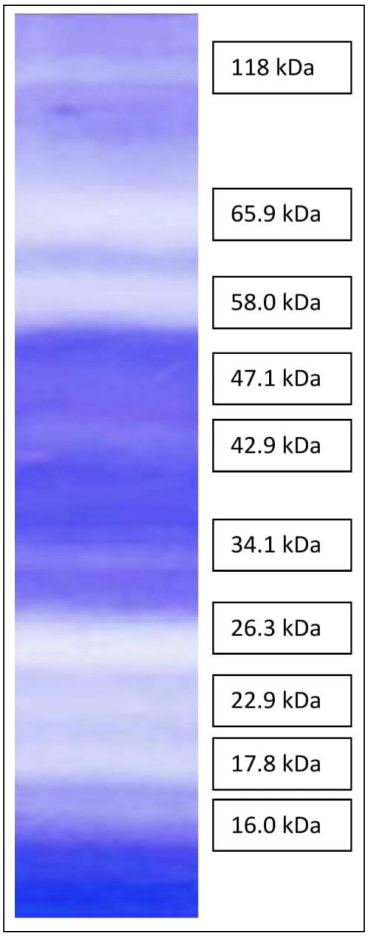

Fig 9: Gut proteinase profile of *H. armigera* fed on chickpea based artificial diet separated on 12% native-PAGE.

The gut shows trypsin and chymotrypsin like activities were assayed and given in Results.

CONCLUSION

In the larval stage of the *Helicoverpa armigera* ten different isoforms of the proteinases have been found which indicates its polyphagous nature. The gut of this insect found to have both trypsin and chymotrypsin proteinases like activity. These proteinases were involved in protein digestion and provided the source of essential amino acids and energy to the insect. Thus, such proteinases become a target for the proteinase inhibitors having tryptic and chymotryptic inhibitory potential.

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