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Protease Isoforms of Cotton Bollworm *Pectinophora gossypiella*: A Biochemical Insight

Heena L. Nadaf, Ashwini K. Sirsat and Vandana K. Hivrale*
Department of Biochemistry, Dr. Babasaheb Ambedkar Marathwada University, Aurangabad (Maharashtra), India.

(Corresponding author: Vandana K. Hivrale)*
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ABSTRACT: The cotton bollworm larva, *Pectinophora gossypiella* is a threat to cotton crops globally. The pest attack result in qualitative and quantitative loss. Gut proteases of pest are studied to target its digestive system for inhibition. The biochemical and electrophoretic properties of proteases from the digestive system of *Pectinophora gossypiella* were determined. By using a well-diffusion assay, it was confirmed that *P. gossypiella* gut extract contained proteases. The proteolytic activity obtained in the solution assay was 0.116 Umg. The impact of varying pH and temperatures on proteolytic activity was assessed. At pH 10 and a temperature of 50°C, it showed that proteolytic activity was at its peak. To see the isoform of proteases in gel, native PAGE gelatine zymography was used. There are a total of 8 protease isoforms in all instars. The total protein profile of *P. gossypiella* was also determined. There is currently no information available about the digestive proteases of *P. gossypiella*. Detailed insights into the gut proteases of *P. gossypiella* can be used to create and enhance successful pest management techniques based on protease inhibitors.

Keywords: *Pectinophora gossypiella,* Pink bollworm, Proteases, protease isoforms, Gelatine-zymography.

INTRODUCTION

The most significant crop raised for commercial purpose is cotton (*Gossypium hirsutum* L.), which is grown for fiber, fuel, and edible oil (Shrinivas *et al.* 2019). Due to its significance to the global economy, it is grown in more than 100 nations and is known as "silver fiber" (Rajput *et al.,* 2017; Shrinivas *et al.,* 2019). The main pest attacking this cash crop is the bollworms (Umer *et al.,* 2019). *Pectinophora gossypiella* (Saunders), an oligophagous pest commonly known as pink bollworm, belongs to the family of Lepidoptera called Gelechiidae (Umer *et al.,* 2019). *P. gossypiella* is one of the most damaging pests of cotton, resulting in significant economic losses to the cotton crop by causing the greatest quantity and quality loss globally. It is the main pest responsible for the tremendous harm to cotton bolls (Sarwar 2017; Khakwani *et al.* 2021). It is reportedly resulting in enormous losses with an average range of 3 to 50% in seed cotton output (Khakwani *et al.,* 2021).

After hatching from eggs, the pink bollworm larvae dig into the squares and bolls and generate rosette blooms (Khakwani *et al.,* 2021). The developing *P. gossypiella* larvae feed on and eat the seeds inside the cotton bolls (Umer*et al.* 2019). It also harms the lint by piercing it through its mouthparts (Khakwani *et al.,* 2021). Damage from *P. gossypiella* causes a subsequent bacterial infection that turns the boll black. Due to an increase in trash content and lint staining, the damage causes subsequent growth arrest, boll-rotting, early partial boll opening, and an overall drop in quality. Due to the *P. gossypiella* onslaught, the cotton crop is generally of inferior quality (Sarwar, 2017). In addition to its primary host i. e. cotton, *P. gossypiella* is also known to feed on hosts such as okra (*Abelmoschus esculentus*), *Abutilon* spp., and *Hibiscus* spp., and is reported as a pest on these hosts (Shrinivas *et al.,* 2019).

P. gossypiella has been found to be resistant to both pesticides used and the cry toxin present in commercial Bt cotton (Rajput *et al.,* 2017; Sarwar, 2017). Since the larva is present inside the cotton bolls and insecticide cannot get there, controlling it can be difficult (Umer *et al.,* 2019). Therefore, it is highly recommended that safer alternatives to chemical control be developed, such as the usage of transgenic plants that express proteinase inhibitors.

To obtain nutrition and a necessary source of protein, *P. gossypiella* larvae consume cotton bolls voraciously. In order to release the amino acids in the dietary proteins found in insect diets, proteases must first cleave and hydrolyze the peptide bonds (Srinivasan *et al.,* 2006). Therefore, proteolysis is crucial for insect physiology and food digestion. Serine proteases, metalloproteinases, cysteine proteases, and aspartate proteases are the four classes into which insect proteases are categorized (Gholamzadeh Chitgar *et al.,* 2013). The complex proteolytic enzymes used by lepidopteran larvae for protein digestion include

trypsins, chymotrypsins, elastases, cathepsin-B-like proteases, aminopeptidases, and carboxypeptidases (Chougule *et al.,* 2008; Patankar *et al.,* 2001). The gut of the larvae is dominated by several serine proteases (Tabatabaei *et al.,* 2011). Serine proteases are the predominant approach to food digestion in lepidopteran insects and account for roughly 95% of all digestive action (Srinivasan *et al.,* 2006; Chougule *et al.,* 2008).

Foregut, midgut, and hindgut are the three distinct parts of an insect gut. Due to the interactions between insects and their environment occurring in the gut region, the insect gut denotes one of the most significant areas in insect physiology (Senthil-Nathan, 2013). The production and secretion of midgut proteases can be hampered by protease inhibitors (Chougule *et al.,* 2005). Insect midgut proteases can be inhibited by these proteins (Gatehouse *et al.,* 2000). Insects' prominent digestive proteases can bind with protease inhibitors, hindering the digestion of the insects. Insect growth and development are slowed down by proteinase inhibitors' impairment of protein digestion (Volpicella *et al.* 2003). Numerous studies showing their efficiency in interfering with protease digestion make their usage in insect control remarkable (Gatehouse *et al.,* 2000).

Different biochemical characteristics can be observed in insect proteases. It is vital to characterize insect digestive proteases because of the variation in their biochemical characteristics (Gholamzadeh Chitgar *et al.,* 2013). Identification and biochemical characterization of the digestive proteases in the insect gut is the initial stage in the process of finding protease inhibitors (Wilhite *et al.* 2000). Consequently, understanding the characteristics of gut protease is essential for creating an effective pest control approach that relies on transgenic plants that express protease inhibitors (Josephrajkumar *et al.* 2006).

This study aimed to identify and characterize the *P. gossypiella* proteolytic enzymes. As a result, this research reveals the biochemical characteristics of *P. gossypiella*'s digestive proteases. To our knowledge, proteolytic diversity in the gut of *P. gossypiella* has not been discovered.

MATERIALS AND METHODS

Chemicals. Gelatine, Trichloroacetic acid, and other chemicals were purchased from Sisco Research Laboratory (SRL), Bombay, India. Azocasein and Bovine serum albumin was obtained from Sigma-Aldrich, USA.

Insects. *Pectinophora gossypiella* were collected from local cotton farms in the Marathwada regions of Maharashtra, India. Insects were reared on an artificial diet at 25±2°.

Enzyme extract and Protein Determination. The guts of the *P. gossypiella* larva were homogenized in a pre-chilled mortar and pestle with chilled 0.1 M Tris HCl buffer of pH 8 and centrifuged at 10,000 g for 12 min at 4°C. The obtained supernatant was collected, and filtered through a 0.2 µm syringe filter (Millipore) to remove cell debris. The filtrate was divided into aliquots and stored as a source of the enzyme at -20°C. The total protein concentration of the extracts was determined according to the Lowry method (1951) using bovine serum albumin as a standard in triplicate.

Confirmatory test. Confirmatory tests for protease were performed by gelatine well-diffusion assay. Plates were prepared with 2% gelatine and 3% agar; wells were prepared with the help of a sterile borer. 50 µl diluted insect extract was loaded into the wells; the buffer is used as a negative control. The plates were then incubated at room temperature overnight. The plate was flooded with 20% acidified mercuric chloride after incubation. The transparent zone of gelatine hydrolysis surrounding the well was observed and measured.

Total protease activity. The total protease activity was measured according to the method of Harsulkar *et al.* (1999). The total proteolytic activity of gut enzyme extracts was evaluated using the substrate 1% Azocasein prepared in Tris HCl buffer of pH 10. The reaction mixture consisted of 80 µl of enzyme extract and 120 µl of Tris HCl buffer of pH 10. The reaction mixture of 60µl was added into the 200µl of the substrate Azocasein. The reaction mixture was incubated at 37°C for 30 minutes. Then, the reaction was stopped by adding 300µl of ice-cold 5% Trichloroacetic acid (TCA) and then kept undisturbed at 4°C for 30 minutes. The reaction mixture was then centrifuged at 12000 rpm and 4°C for 10 minutes. An equal volume of 0.5 N NaOH was added to the obtained supernatant before taking measuring absorbance at 440 nm. (Nanodrop One C Spectrophotometer-Thermo Scientific) The total proteolytic activity was calculated according to the equation, Protease activity=Absorbance/Protein in mg ×Time in min.

Effect of pH. The effect of pH on total proteolytic activity was determined with 0.1 M buffers of different pH ranging from 3 to 12 for 10 min (pH 3 to 12 buffers; (i) glycine–HCl buffer pH 3; (ii) acetate buffer pH 4, 5; (iii) phosphate buffer pH 6, 7; (iv) Tris–HCl buffer pH 8, 9; and, (v) glycine–NaOH buffer pH 10, 11, and 12). The reaction mixture was incubated with different pH buffers for 5 mins. The protease activity at different pH was determined by the above method. All determinations were done in triplicate.

Effect of Temperature. The effect of temperature on total proteolytic activity was determined. The reaction mixture was incubated at different temperatures ranging from 20°C to 90°C for 30 mins. The protease activity at different temperatures was determined by the above method in triplicate.

Electrophoretic Visualization of protease isoforms. The native PAGE was used for zymographic detection of the number of protease isoforms present in *P. gossypiella* according to Lomate *et al.* (2013) with some modifications. The acetone-precipitated extract was resolved on native polyacrylamide gel (PAGE, 4% stacking, and 8% resolving). The electrophoresis was carried out at a constant voltage of 100 mV. After completion of electrophoresis, the gel was removed from the electrophoresis unit, rinsed with distilled water, and equilibrated in 0.1 M Tris HCl buffer of pH

8 for 10 min. The gel was then immersed in 1% gelatine solution prepared in 0.1 M Tris HCl buffer of pH 8 for 6 hrs with gentle shaking at 30 rpm. After incubation, the gel was washed with distilled water to remove excess gelatine. The gel was stained with Comassie brilliant blue R250 dye solution overnight. Protease activity-isoforms in the gel were detected in terms of gelatine hydrolysis, which served as the substrate for proteases. The gel was then observed for the appearance of bright colorless bands of proteases activity against the blue background. The gel was scanned and saved the image.

Protease isoforms in different instar larva. The detection of the number of protease isoforms present in different instars of *P. gossypiella* was done by the above method. The insect extract of 2nd, 3rd, 4th, and late 4th instar larva of *P. gossypiella* were resolved on native PAGE and detected zymographically.

Relative mobility. The mobility of amylase isoforms relative to that of bromophenol blue was measured according to the formula, migration distance of band/migration distance of dye front.

Protein profile of *P. gossypiella*. The enzyme extract was loaded on 8% polyacrylamide gel and separated the protein bands electrophoretically. The electrophoresis was carried out at a constant voltage of 100 mV. After completion of electrophoresis, the gel was removed from the electrophoresis unit, and rinsed with distilled water. The protein bands were visualized by staining with Comassie brilliant blue R250 (CBBR-250) overnight followed by destaining for 2 hrs. (Methanol: acetic acid: water- 50:10:40). The gel was scanned for the image.

Statistical analysis. All the experimental data were presented as the mean±SE (n=3) analysed in Microsoft Excel 2013.

RESULTS AND DISCUSSION

Insect development and growth are significantly aided by protease enzymes (Terra and Ferreira 1994). The targeted inhibition of insect pests' digestive enzymes is one of the key components of pest management. The target for an inhibition-based approach is the digestive protease enzyme (Volpicella *et al.* 2003). Development of control methods based on the selective inhibition of digestive enzymes requires in-depth knowledge of the properties of these enzymes. Accordingly, the gut proteases of *Pectinophora gossypiella* are biochemically characterized in this investigation.

Confirmatory test. The well containing extract of*P. Gossypiella* showed a gelatine hydrolysis zone of 1.9 cm, while no transparent zone was observed in the negative control well (Fig. 1). Using the gelatine agar plate test, we identified protease activity in *P. gossypiella* extracts. It is the main confirmatory test used to find protease activity initially. The results of this test showed that *P. gossypiella* larvae contain proteases.

Total protease Activity. By quantitative means we have detected significant proteolytic activity in extracts of *P. gossypiella*. The protease assay by using Azocasein as substrate revealed that the total protease activity of *P. gossypiella* was 0.116 U mg of insect protein. The highest activity was observed at 80µl of insect extract (Fig. 2). Azocasein hydrolysis was shown to occur at a rate of 7.267± 0.37 mol/min/mg protein in *C. nemorana* gut extract (Gholamzadeh Chitgar *et al.* 2013). The midgut extract of *A. janata* had a proteolytic-specific activity of 1200±90 nmol/min/mg protein (Budatha *et al.* 2008). In the whole gut preparations of 4th instar lesser mulberry pyralid, the proteolytic activity 2.48±0.04 µmol min^{-1} mg^{-1} protein was recorded by Mahdavi *et al.* 2013. The proteolytic activity is quantitatively reported in different lepidopteron, viz. *Spodoptera littoralis*(Egyptian cotton worm), *Plutella xylostella*, *Spodoptera cosmioides* (El-latif 2014; Zhao *et al.,* 2019; Duarte Rocha *et al.,* 2020). The quantitative estimations from the above reports are corroborating our findings.

Effect of pH and temperature. The relative activity at different pH values is shown in Fig. 3. The protease activity was higher at pH 10. A significant amount of enzyme activity is observed in the alkaline range of pH 8 to 11. Beyond pH 11, there is a slight decrease in activity. Considerable protease activity was also observed in pH 3 to 7 but it was relatively lower. The surrounding pH of enzymes affects their activity. The term optimum pH refers to the pH level at which an enzyme is most active. The stability of enzymes is influenced by pH. The overall loss of enzyme activity is typically caused by pH values that are excessively high or low (Murray *et al.,* 2003). Our findings indicated that the alkaline range had a higher level of proteolytic activity. The higher activity at alkaline pH is suggestive of the presence of serine-type proteases in the gut of *P. gossypiella.* Other lepidopteran larvae have reportedly produced similar findings (Chougule *et al.,* 2008; Mahdavi *et al.,* 2013). According to Zibaee *et al.* (2012), the lepidopteran gut digestive enzymes are functional in alkaline conditions present in their guts at pH 10–12. Our findings coincide with these report accounts. According to Terra and Ferreira (1994), the high pH of tissue-chewing Lepidopteran insects may be an adaptation for removing hemicelluloses from plant cell walls. However, several lepidopteran insects have reported having alkaline pH values for activity since the digestive system of insects naturally has an alkaline pH (Ferry *et al.,* 2005). The proteolytic activity was found to be high at 50 °C. A decrease in activity was observed at both sides of 50 °C as shown in Fig. 4. Temperature values have a significant impact on how quickly enzymatic reactions occur (Gholamzadeh Chitgar *et al.,* 2013). The kinetic energy required for a reaction up to the minimum optimum value increases with increasing temperature, enhancing the rate of an enzyme-catalyzed reaction (Nadaf *et al.,* 2022). The rate of azo dye release from Azocasein by *P. gossypiella* proteases increases when the temperature is raised, just like other enzymatic reactions. The maximum activity was noted at 50°C, however, the proteases of *P. gossypiella* were found to be active over a wide temperature range. Similar results were obtained for the amylase enzymes of *P. gossypiella* (Nadaf *et al.,* 2022). Temperatures between 30 and 40 °C were found

to be ideal for other Lepidoptera protease activities (Josephrajkumar *et al.,* 2006; Budatha *et al.,* 2008). At a temperature of 30°C, *Achaea janata* (Lepidoptera: Noctuidae) exhibited the highest midgut protease activity (Budatha *et al.,* 2008). The maximum proteolytic activity in lesser mulberry pyralid was reported to be 50°C (Mahdavi *et al.,* 2013). Due to the denaturation of enzymes at high temperatures, enzyme activity gets negatively impacted (Nadaf *et al.,* 2022). The temperature ranges that produced a substantial quantity of protease activity in *P. gossypiella* correlate to the Marathwada region's sunny-field temperatures. In vitro experiments that show high enzyme activity at a given temperature typically reflect the temperature of the environment where the insects feed on their host plants (Zibaee *et al.,* 2012).

Electrophoretic Visualization of protease isoforms. Protease isoforms in the whole gut were studied in gel using native gelatine PAGE zymography. In zymography, bright colorless bands against a blue-colored background were observed and considered protease isoforms. In *P. gossypiella*, a total of 8 isoforms of protease activity was observed. Protease isoforms were named PgP1- PgP8 and referred to by arrows (Fig. 5). The isoforms PgP3, PgP6, and PgP8 were observed to be highly active in the gel relatively. It has been asserted that the midgut digestive enzyme profile varies depending on the order and species of the insects (Terra and Ferreira 1994). Also, the gut protease complement in insects varies according to feeding pattern and is developmentally regulated at larval growth (Srinivasan *et al.,* 2006). The varying number of protease isoforms in different pests was reported. In *Helicoverpa armigera* 10 isoforms, while in *Choreutis nemorana* 5 isoforms were reported (Gholamzadeh Chitgar *et al.,* 2013; Lomate *et al.,* 2013). The multiple protease isoforms are the result of multi-copy genes of protease due to gene duplication in the insect genome. Functional diversity and exposure to naturally existing host defense chemicals are connected to how effectively insects have adapted to their varied host plants (Srinivasan *et al.,* 2006).

Protease isoforms in different instar. The variation in protease isoforms in different instars of *P. gossypiella* was also observed by Gelatine zymography. Highly active protease bands were observed in the 2nd and 3rd and 4th instar larva of *P. gossypiella.* In the late 4th instar, reduced protease activity was found in the gel. Due to reduced feeding in the late fourth instar, the low expression of proteases in the gel can be seen as shown in Fig. 6.

Relative mobility. The relative mobility of each protease isoform on the native PAGE zymogram of *P. gossypiella* is measured and shown in Table 1. Isoform PgP8 was more anionic according to mobility, relative to isoform PgP1-PgP4.

Protein profile of *P. gossypiella*. The electrophoretic profile of *P. gossypiella* crude extracts showed distinct protein bands. The protein profile of *P. gossypiella* is shown in Fig. 7. The protein profile of insects from Tenebrionidae family shown by Flores *et al.* (2020). Apart from Lepidoptera, protease activity was reported previously in different insect orders including Coleoptera, Diptera, and Hemiptera. But proteases in cotton pink bollworm *P. gossypiella* were not explored to date.

Table 1: Relative mobility of protease isoforms of *P. gossypiella.*

Sr. No.	Isoform	Relative mobility
1.	PgP1	0.042
2.	PgP2	0.125
3.	PgP3	0.194
4.	PgP4	0.264
5.	PgP5	0.43
6.	PgP6	0.486
7.	PgP7	0.569
8.	PgP8	0.667

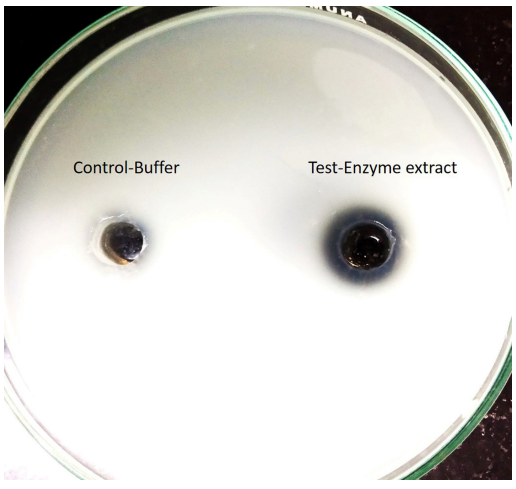

Fig. 1. Confirmatory well-diffusion assay on gelatine agar plates for the presence of proteases in *P. gossypiella*. The clear Zone of gelatine hydrolysis was 1.9 cm. The transparent zone around Test-well indicates the presence of proteases in insect extract. While in negative control no such zone appeared. (Negative control- Buffer, Test- Insect extract).

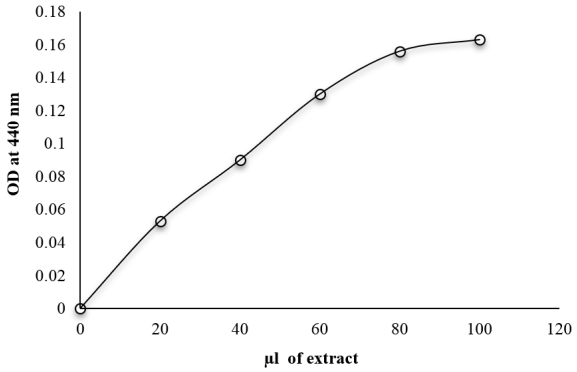

Fig. 2. *P. gossypiella* total protease assay by Harsulkar *et al.* (1999) method.

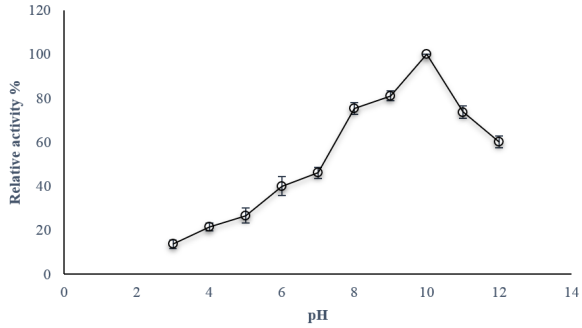

Fig. 3. Effect of various pH on the protease activity of *P. gossypiella*

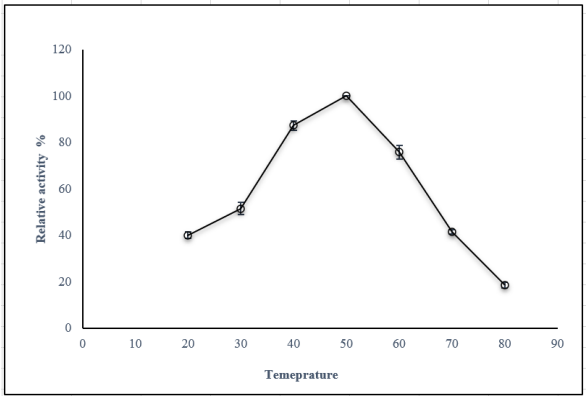

Fig. 4. Effect of different temperature on the protease activity of *P. gossypiella.*

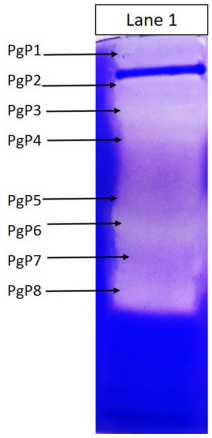

Fig. 5. Native PAGE gelatine zymography of proteases of *P. gossypiella.* Native PAGE was performed on 8% resolving and 4% stacking gel. Upon electrophoresis gel was treated with a gelatine solution prepared in buffer and stained with CBBR250 solution. The gel was observed for clear bands of protease activity. Arrows refer to protease isoforms numbered as PgP1-PgP8.

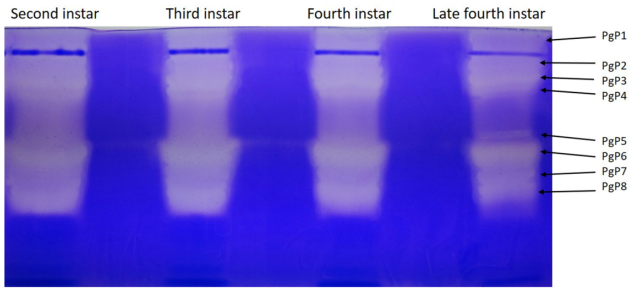

Fig. 6. Native PAGE gelatine zymography of proteases of different instars of *P. gossypiella*. Lane 1-2nd instar, Lane 2-3rd instar, Lane 3-4th instar, Lane 4-Late 4th instar.

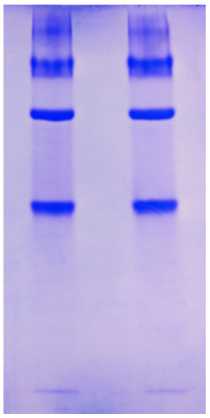

Fig. 7. Protein profile of *P. gossypiella* larva. The total proteins were resolved on 8% resolving and 4% stacking gel, stained with CBBR250 solution followed by destaining.

CONCLUSIONS

The presence of 8 protease isoforms suggests enormous dependency of *P. gossypiella* on proteases for food digestion. Any impairment in protein digestion can hamper the growth and survival of *insects.* The protease inhibitors are specific in action and it can inhibit to its complementary proteases only. In this view, to find a potent inhibitors against *P. gossypiella*, detailed knowledge of its gut proteases is required. This study provides biochemical characterization of *P. gossypiella* proteases along with detailed map of protease isoforms on gel.

FUTURE SCOPE

The characterization of *P. gossypiella*'s gut enzymes can be utilized to design appropriate and efficient pest management tactics. The insights in gut proteases of cotton bollworm will be helpful in developing a cotton crop protection strategy based on protease inhibitors.

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Conflict of Interest. None.

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