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RESEARCH ARTICLE

PURIFICATION AND CHARACTERIZATION OF TRYPSIN LIKE PROTEASE FROM THE MID GUTS OF *HELICOVERPA ARMIEGRA*

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ABSTRACT

A trypsin- like protease was purified and characterized from the mid guts *of Helicoverpa armigera* using ion exchange chromatography and gel filtration on QAE- sephadex column. The protease activity was found to reside in two protein peaks. High BAPNAase activity was associated with a second peak eluted with 0.3M NaCl. The enzyme was found to be homogeneous by the criteria of native PAGE. SDS-PAGE analysis in the presence of 2-mercaptoethaonol gave a single band corresponding to a molecular weight of about 30.4 kDa. Trypsin like protease from the mid gut was alkali-stable and its pH optimum for activity was about pH 11. The specific activity of the purified enzyme was 1426.92 TU per mg protein. The final yield was 52.81% with a fold purification of 3.81. The enzyme followed Michaelis-Menten kinetics when the protease activity was measured at different concentrations (0.9-4.0 mM) of BAPNA. Km and Vmax values for the protease were found to be 3.84 x 10^{-3} M and 45 nmol/10min/mg protein with BAPNA as the substrate respectively. Strong inhibition of protease activity by TLCK not by TPCK suggests that the isolated mid gut protease is trypsin like but not chymotrypsin like specificity of serine protease.

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INTRODUCTION

Proteolysis is an essential metabolic process required for protein processing and turnover and is crucial for cellular activities. Proteases are the important enzymes in insects which hydrolyze the peptide bonds in dietary proteins to liberate the amino acids needed for growth, survival and reproduction and detoxify protein toxins ingested as a consequence of plant and pathogen feeding (Terra *et al*., 1996). Depending upon the amino acids and metal ions present in their active site, these are basically divided into four classes- serine, cysteine, aspartic and metalloproteases. Serine proteases in particular are the major class of digestive proteases, accounting for 95 % of digestive activity in Lepidoptera (Srinivasan *et al*., 2006). The lepidopteran larvae need a proteolytic enzyme complex including trypsin, chymotrypsin, elastase, cathepsin-B like proteases, aminopeptidases and carboxypeptidases for protein digestion and many serine proteases are dominant in the larval gut (Chougule *et al.,* 2008; Tabatabaei *et al.,* 2011). *Helicoverpa armigera*, a lepidopteran, is a serious polyphagous pest of diverse economically important crops and is most widely distributed with broad host spectrum including commercially important crops like cotton, chickpea, pigeonpea, maize, sunflower, corn, tomato, okra and groundnut (Bhavani *et al*., 2007; Wondafrash *et al*., 2012). *Helicoverpa armigera*, also known as cotton bollworm or legume pod borer, is one of the most devastating crop pest of *Cicer arietinum* since it inflicts annual losses of over US$ 328 million. Losses caused only by this pest reported up to US$ 17 million in crops like cotton, pigeon pea, chickpea, groundnut, sorghum, pearl millet, tomato and others of economic importance (Chaturvedi, 2007). Because of its high mobility, survival rate under adverse conditions, capacity to complete several generations in a year and ability to develop resistance against insecticides, its management is very difficult. There is a need to develop certain strategies to manage this insect in an eco-friendly manner. Alkaline proteinases are responsible for gaining nutrients and energy in Lepidopteran insects (Broadway, 1989; Valaitis *et al.,* 1999). Among insect gut proteinases, serine proteinases function optimally at alkaline pH, whereas cysteine proteinases require acidic pH and aspartic proteinases usually occur together with cysteine proteinases (Terra and Ferreira, 1994). Since there is significant variation among the biochemical properties of insect digestive proteases, their isolation and characterization is necessary for designing a safe control strategy that utilizes plant-proteinaceous inhibitors (Wilhite *et al*., 2000). Protease inhibitors mediate plant defense against herbivores by inhibiting their midgut proteases, thus causing a reduction in the availability of amino acids necessary for their growth, survival and reproduction (Volpicella *et al.*,

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2003). The gut physiology of *Helicocerpa armigera* is an important subject for study and the design of new approach for its control, such as developing transgenic plants that express proteinase inhibitors. Knowledge of enzyme properties together with their sensitivity to various inhibitors can provide a base for the control methods. In the present paper, purification and characterization of trypsin like protease from the midgut of *Helicoverpa armigera* larvae along with its kinetic properties has been reported.

MATERIALS AND METHODS

Chemicals and Reagents

α-N-benzoyl-DL-arginine-p-nitoanilide HCl (BAPNA), Phosphorylase b, Bovine serum albumin (BSA), Ovalbumin, Carbonic anhydrase SBTI (soybean trypsin inhibitor), Blue dextran, Acrylamide, N,N'-methylene bis acrylamide, Sodium dodecyl sulfate (SDS), N,N,N',N'-Tetramethylethylenediamine (TEMED), Coomassie brilliant blue R250, TLCK (Tosyl-L-lysyl-chloromethane hydrochloride) and TPCK (Tosyl phenylalanyl chloromethyl ketone) were purchased from Sigma Chemical company, St. Louis, Missouri, U.S.A. Sephadex G-200 and QAE-Sephadex were purchased from Pharmacia Fine Chemicals, Uppsala, Sweden. All other chemicals used were of analytical grade.

Larval mid gut enzymes

Helicovera armigera larvae grown on chick pea fields were collected from Central Tobacco Research Institute (CTRI), Rajahmundry. In order to avoid any fluctuations in activities due to physiological and environmental conditions, larvae were synchronized before assaying for the gut proteases. This was accomplished by selectively separating the fifth instar larvae and allowing them to feed on chick pea pods *ad libitum* for 24 h. The specimens killed by drowning in distilled water were immediately dissected over ice. The mid guts were transferred on to a watch glass containing ice- cold 0.1% NaCl and gently cleaned with a brush to remove any undigested food particles or adhering fat bodies. Mid gut tissue was then stored at -20^{o} C until further use.

Purification of serine proteinase from *Helicoverpa armigera*

The purification procedure has been carried out with several batches of larvae. Nearly identical protein profiles were obtained each time but with some variation (10-15% in enzyme activity). Serine proteinase from mid gut extracts of 5th instar larvae was isolated and purified following the procedure described below.

Extraction of trypsin like protease

Fifth instar larvae were immobilized on ice and dissected under a stereo-microscope in ice-cold distilled water. The midguts were separately removed, placed in distilled water and cleaned of adhering unwanted tissues and gut contents. Mid gut tissue obtained from fifth instar larvae was homogenized with one ml of ice cold extraction buffer, 10mM Phosphate buffer, pH 7.6 and centrifuged at 10,000 rpm at 4^{o} C for 10 min. The supernatant obtained was transferred to new tubes and frozen at -20° C for the purification of the enzyme.

Chloroform extraction: Four volumes of ice-cold chloroform was added to the supernatant from the previous step and mixed thoroughly before subjecting it to centrifugation at 3,000 rpm at 4^{o} C for 10 min. The aqueous fraction was then carefully pipetted out and collected. The pigments that occurred as a part of larval food and contaminating pigments from the larval cuticle were removed in this step.

Ammonium sulphate fractionation

To one ml of the aqueous fraction obtained in the above step, ammonium sulphate was added to obtain 60% saturation at 4^{o}C with constant stirring. The precipitated proteins were centrifuged at 10,000 rpm at 4^{o} C for 30 min. All the protease activity was found to reside in the pellet, which was then dissolved in a minimum volume of 20mM Tris-HCl buffer, pH 7.2. It was desalted on Sephadex G-25 (2 × 10 cm) column equilibrated and developed with 20mM Tris-HCl buffer, pH 7.2. The protein fractions obtained on Sephadex G-25 were pooled, lyophilized and stored at -20^{o}C until use.

Ion-exchange chromatography on QAE-Sephadex

The ammonium sulfate precipitated protein was then dissolved in one ml of 20mM Tris-HCl buffer, pH 7.2, loaded on to a QAE-Sephadex column (0.5 x 30cm) equilibrated with the same buffer. The column held at < 8^{o} C was washed with 20mM Tris-HCl buffer, pH 7.2 and then eluted with 0.1 - 0.3M NaCl prepared in the same buffer. Fractions, each 2ml, were collected at a flow rate of 20ml/h and the protein content was monitored at 280 nm. The enzyme activity in each fraction was determined using BAPNA as the substrate.

Estimation of trypsin activity

Amidolytic activity of trypsin was assayed by the method of Kakade *et al.,* (1969) using BAPNA as the substrate. Substrate solution was prepared by dissolving 30 mg of BAPNA in 2 ml of dimethyl sulfoxide and the solution was made up to 100 ml with 50 mM Tris-HCl buffer, pH 8.2 containing 20 mM $CaCl_2$. Aliquots of trypsin solution containing 10-50 µg of the enzyme in 2 ml distilled water was incubated with 7 ml of BAPNA solution at 37°C for 10 min. The reaction was stopped by adding 1 ml of 30% acetic acid. The absorbance of the samples were then measured at 410 nm by continuously monitoring the change in absorbance p-nitroaniline release for 10 min at 25°C against a blank incubation sample containing 2 ml of water instead of trypsin solution.

Protein estimation

Protein was estimated by the method of Lowry *et al*., (1951) using bovine serum albumin as the standard. The protein content in the column effluents collected during chromatographic separation was determined by measuring the absorbance at 280 nm.

Molecular Weight by Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE was carried out by the method of Laemmli (1970) in slab gels. The marker proteins, phosphorylase b (97 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), chymotrypsinsogen A (25 kDa), soybean trypsin inhibitor (20.1 kDa) and lysozyme (14 kDa) in 0.1 ml of phosphate buffer were kept at 100°C for 2 min with 0.1 ml of sample buffer (0.5 M Tris-HCl buffer, pH 6.8 containing 4% SDS and 10% 2-mercaptoethanol). The protease in sample buffer was

kept at 100°C for 2 min, then subjected to electrophoresis. After electrophoretic run, proteins were fixed in glacial acetic acid 10% (v/v), methanol 30% (v/v) and were visualized using coomassie brilliant blue.

Optimum pH and pH stability of protease

Protease activity was determined using different buffers in a pH range of 3-14 using BAPNA as the substrate. Buffers used were sodium acetate, 0.1M (pH 3-4), sodium citrate, 0.1 M (pH 5-6), 0.1 M Sodium phosphate buffer (pH 7-8), Tris- HCl, 0.1M (pH 9-10) and 0.1M glycine-NaOH (pH 11-14). Aliquots of the enzyme were diluted with phosphate buffer, pH 7.6 and assayed as described above for protease. In order to determine the pH stability of protease, enzyme in an appropriate buffer was kept at 5^0C for 24 h, aliquots were then taken and assayed for enzyme activity.

Optimum temperature and temperature stability of protease

Optimum temperature for protease was determined by measuring the activity in the temperature range 20^o-80^o C using the activity assay procedure. 3 ml samples of 100 µg/ml solution of the protease in 0.1 M sodium phosphate buffer, pH 7.6, were separately incubated in a water bath at different temperatures for 10 min. After the heat treatment, the solutions were quickly cooled in ice and appropriate aliquots were used for the assay of protease activity using BAPNA as the substrate. In order to determine the temperature stability of protease, enzyme (2ml in 0.1M sodium phosphate buffer, pH 7.6) was incubated for 30min at different temperatures (20-80°C). After cooling for 2h, enzyme activities were determined from the aliquots.

Effect of various metal ions and protease inhibitors

Metal ions (Mg^{2+}, Zn^{2+}, Ca^{2+}, Cu^{2+}, Cd^{2+}, Mn^{2+}, Hg^{2+}) were tested for their inhibitory efficiency. Reaction mixture containing 50 µl enzyme, 5 mM metal ion and 150 µl assay buffer was incubated for 20 min at 37°C. Reaction was started by adding 800 µl BAPNA and trypsin activity was measured.
The effects of various protease inhibitors such as TLCK, TPCK, Iodoacetate and EDTA each 5mM on proteolytic activity of isolated enzyme were investigated. After 30 min of pre-incubation of inhibitors with enzyme at room temperature, substrate was added and residual protease activity was measured by the standard assay method.

Kinetic parameters of trypsin like protease

The Michaelis–Menten constant (K_m) and the maximum reaction velocities (V_{max}) of trypsin were determined by Lineweaver–Burk plots. The protease activity was measured at different concentrations (0.9-4.0 mM) of BAPNA.

RESULTS

A trypsin like protease has been isolated and purified from the midguts of fifth instar larvae of *Helicoverpa armigera* following ammonium sulphate fractionation and Ion-exchange chromatography on QAE-Sephadex. When ammonium sulfate precipitated protein was subjected to Ion-exchange chromatography on QAE-Sephadex, a single symmetrical peak with a corresponding enzyme activity was eluted with 0.3M NaCl in 20mM Tris-HCl buffer, pH 7.2. The elution profile of trypsin like enzyme from crude gut protease revealed maximum trypsin-like activity. The protein profile on ion exchange chromatography is shown in Fig. 1. Recoveries and relative purification at each step for a typical purification from five larvae are shown in Table -1. The specific activity of the purified enzyme was 1426.92 TU per mg protein. The final yield was 52.81% of the original protease with a fold purification of 3.81.

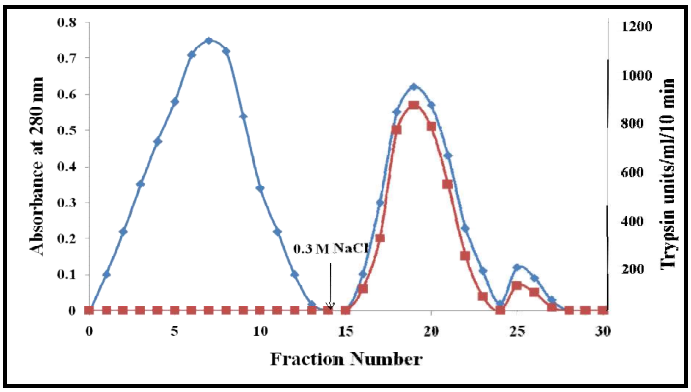

Figure 1. Ion exchange chromatography on QAE-Sephadex of the ammonium sulphate fraction

About 4.5 mg of the ammonium sulfate precipitated protein was loaded on to a column (0.5 x 30 cm) in 20mM Tris-HCl buffer, pH 7.2. Elution was done with 0.3 M NaCl in the same buffer. Two ml fractions were collected at a flow rate of 20 ml/h. Protein was monitored by measuring absorbance at 280nm (- ♦ -) and the trypsin- like activity (- ■ -) was assayed using BAPNA as substrate. One trypsin unit (TU) is arbitrarily defined as an increase in 0.01 absorbance unit at 410nm per 2.5 ml reaction mixture, under the assay conditions. Yield and fold purification were calculated from activity units and specific activity respectively.

Molecular weight determination of trypsin like protease

SDS-PAGE was carried out under denaturing conditions to determine the molecular weight of the protease. Fig 2 shows the protein band pattern of CGTI on 5-15% gradient gel. The enzyme when treated with SDS and 2-mercaptoethanol for 24 h at room temperature showed a single coomassie blue stainable, broad protein band corresponding to a molecular weight of 30.4 kDa.

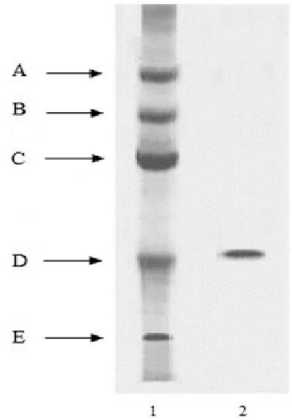

Figure 2. Molecular weight determination of *Helicoverpa armigera* protease by SDS-PAGE at pH 8.3 on 5-15 % slab gels under denaturing conditions

Table-1. Purification table of trypsin-like protease from mid guts of *Helicoverpa armigera*

Preparation	Total volume (ml)	Total protein (mg)	Total activity (TU)	Specific activity TU/mg protein	Yield %	Fold purification
Crude Extract	1.0	7.5	2810	374.67	100	1
Ammonium sulphate precipitate	0.25	4.5	1812	402.67	64.48	1.07
QAE Sephadex fraction	1.0	1.04	1484	1426.92	52.81	3.81

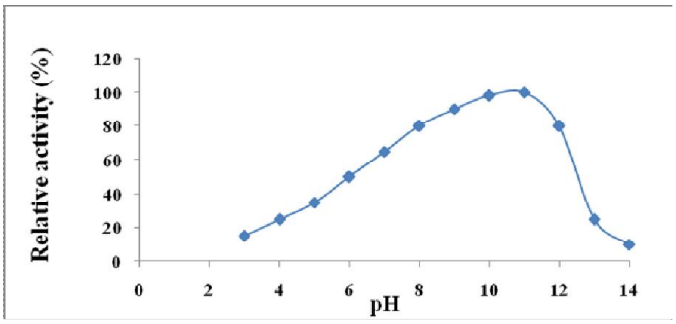

Figure 3a. pH optima of trypsin like protease of *Helicoverpa armigera*

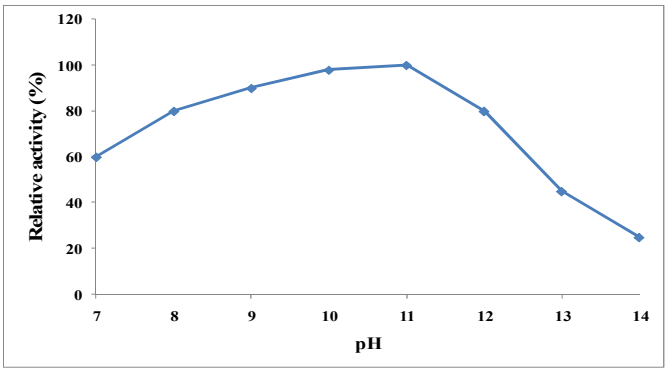

Figure 3b. pH stability of trypsin like protease of *Helicoverpa armigera*

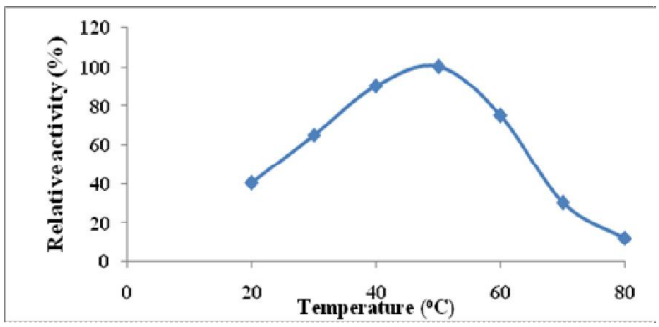

Figure 4a. Temperature optima of trypsin like protease of *Helicoverpa armigera*

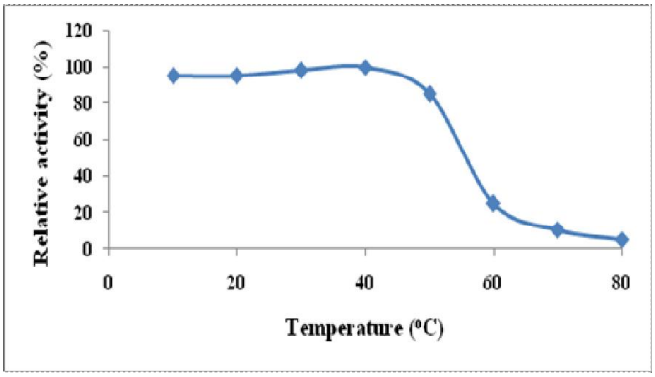

Figure 4b. Thermal stability of trypsin like protease of *Helicoverpa armigera*

The presence or absence of 2-mercaptoethanol in the sample buffer did not show any difference in the band pattern as well as in the relative band intensities on SDS-PAGE.

1. Standard proteins
   - Phospohorylase b (97kDa)
   - Bovine serum albumin (67 kDa)
   - Ovalbumin(43kDa)
   - Carbonic anhydrase (29kDa)
   - SBTI (20.1kDa)
2. *Helicoverpa armigera* protease

Effect of pH and temperature of trypsin like protease on protease activity

The optimum pH for trypsin activity was found to be 11.0 (Fig 3a). The enzyme was found to be unstable at acidic pH, whereas at alkaline pH it shows wide range of activity. The maximum enzyme activities were observed at a pH range of 9.0– 11.0 (Fig 3b). The optimum temperature of proteolytic activity of purified gut trypsin like enzyme was maximum at 50^{o}C (Fig 4a). A decline in enzyme activity was found at temperature above 50^{o}C. The thermal stability of trypsin-like protease in the midgut samples pre-incubated for 5 min remained unchanged at temperatures up to 40^{o}C. However, preincubation for 30 min at temperatures of 40^{o}C sharply reduced the proteolytic activity, which was almost abolished above 60^{o}C (Fig 4b).

Effect of various metal ions on protease activity

$ZnSO_4$, $CuSO_4$, $CdCl_2$ and $HgCl_2$ at 5 mM concentration inhibited the trypsin like activity of gut protease, whereas Ca^{2+}, Mn^{2+} and Mg^{2+} at that concentration elevated the enzyme activity (Table 2). Maximum inhibition was observed with Cd^{2+} (82 %) and Hg^{2+} (79%) and minimum inhibition with Cu^{2+} (45.2 %). Metal ions such as Cd^{2+} and Hg^{2+} act as strong inhibitors of enzymatic activity. Addition of $CaCl_2$, $MgCl_2$ and $MnCl_2$ at low concentrations increased the rate of trypsin activity whereas the reaction rate was progressively lowered with the increase in ionic strength (Results not shown).

Table 2. Percent activity of purified trypsin like protease after treating with metalions

Sl. No.	Metal ion (5 mM)	Relative activity (%)
1	Control	100
2	Mg^{2+}	122.5
3	Zn^{2+}	65.3
4	Ca^{2+}	106
5	Cu^{2+}	55.2
6	Cd^{2+}	17.8
7	Mn^{2+}	115.4
	Hg^{2+}	21.3

Effect of inhibitors on protease activity

Larval midgut proteases were further characterized using protease specific inhibitors. Calculating of inhibition

percentage of BAPNA hydrolysis in the presence of various inhibitors offers information about the relative contribution of the inhibited class of protease to isolated gut protease activity. As shown in Figure 5, inhibition was effected by TLCK but not TPCK indicating trypsin like but not chymotrypsin like specificity of serine protease.

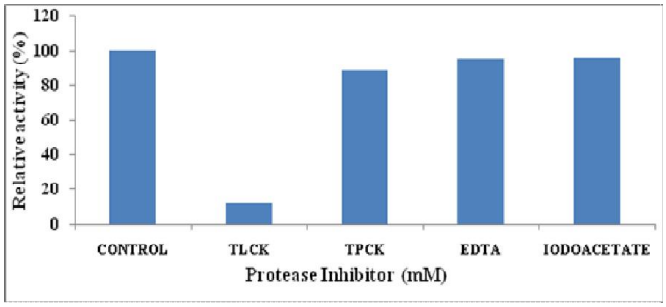

Figure 5. Effect of protease inhibitors on trypsin like protease

Kinetic studies

The protease activity was measured at different concentrations (0.9-4.0 mM) of BAPNA. The enzyme followed Michaelis-Menten kinetics. The double reciprocal plots of the kinetics data are shown in Fig. 6. K_m value for the protease was determined to be 3.84 x 10^{-3} M BAPNA. Vmax value for the protease was determined to be 45 nmol/10min/mg protein.

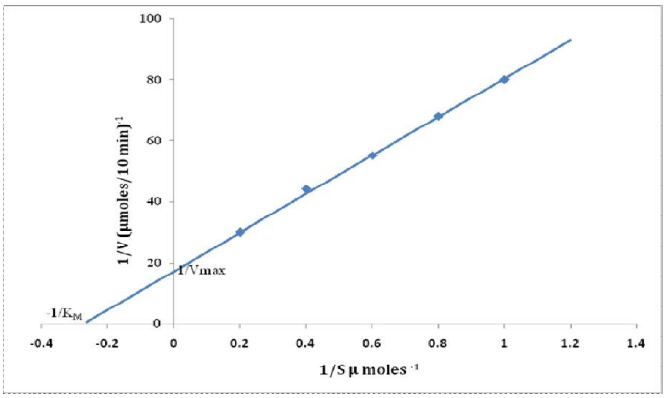

Figure 6. Lineweaver-Burk plot for Km and Vmax determination using BAPNA as substrate

DISCUSSION

Insects have all the mechanistic classes of proteolytic enzymes that exist in vertebrates (Reeck *et al.,* 1999) and play important role in insect growth, reproduction, development and health (Terra *et al.,* 1996). In insects, serine proteases have been detected in order Lepidopetra, Diptera, Orthoptera, Hymenoptera and Coleoptera. Serine alkaline proteases active at highly alkaline pH represent the largest subgroup of serine proteases and include trypsins, chymotrypsins, elastases, cathepsin-B like proteases, aminopeptidases and carboxypeptidases, which are all responsible for protein digestion. Trypsin is the main intestinal digestive enzyme. Based on the ability of various protease inhibitors to inhibit this enzyme from insect gut, trypsin has received major attention as a target for biocontrol of the insects. Insect trypsins share similar, but not identical specificities with vertebrate trypsins (Muhlia-Almazan *et al.,* 2008). Serine proteases are among the best studied proteases from the insect midgut (Hau and Benjakul 2006; Mohammadi *et al.,* 2010). Studies on the digestive proteases of lepidopteran insects have revealed that 95% of total digestive activity relies on serine proteases. Serine proteases, trypsin and chymotrypsin predominate digestive tract of *H. armigera*. Proteases are being targeted these days for various pest management strategies. Their inhibition can starve insects to death or may disrupt their normal metabolism (Padul *et al.,* 2012, Mittal *et al.,* 2014). The gut of *H. armigera* contains about 20 different types of active serine protease isoforms at any given moment (Bown *et al.,* 1997). A trypsin- like protease has been purified from the mid guts of *Helicoverpa armigera* using ion exchange chromatography and gel filtration on QAE- sephadex column. The protease activity was found to reside in two protein peaks. High BAPNAase activity was associated with a second peak eluted with 0.3M NaCl. The enzyme was found to be homogeneous by the criteria of native PAGE. The specific activity of the purified enzyme was 1426.92 TU per mg protein. The final yield was 52.81% with a fold purification of 3.81. SDS-PAGE analysis in the presence of 2-mercaptoethaonol gave a single band corresponding to a molecular weight of about 30.4 kDa. The protein peak obtained in the wash through fractions could thus be a polymerized form of the protease itself or association of the protease with other mid gut proteases. Proteases with molecular weights ranging from 41.6 - 172.9 kDa from mid gut extracts of the insect were reported by Johnston *et al.*(1991) and Harsulkar *et al*. (1998). Telang *et al*. (2005) reported two trypsin like proteases in the mid guts of *H. armigera* with molecular weights 24 and 29kDa. A trypsin like protease from *H.armigera* gut was purified 37 fold with 22 % yield and its molecular weight was found to be 18.8 kDa (Grover *et al.,* 2016). The optimum pH for trypsin like protease was found to be 11.0. The enzyme was found to be unstable at acidic pH, whereas at alkaline pH it shows wide range of activity. The optimum pH (11.0) is in agreement with previous reports of salivary gland extract of *Lygus lineolaris* and gut extracts of *A. gemmatalis* showing 10.0 and 10.5, respectively (Pereira *et al.,* 2005; Zeng *et al.,* 2002).

The alkaline pH range of the midgut is similar to that reported in other investigations including lepidopterans (black cutworm, corn earworm, and tobacco budworm, *A. gemmatalis*) and the boll weevil with maximal midgut proteolytic activity *in vitro* at pH 10–11 (Pereira *et al.,* 2005; Purcell *et al.,* 1992). The alkaline pH of the lepidopteran gut is attributed to an adaptation of leaf-eating lepidopteran ancestors for extracting hemicellulose from plant cell walls (Terra & Ferreira, 1994). Alkaline pH values for activity are due to intrinsic alkaline pH of the insect digestive system, and have been reported for many lepidopteran insects (Ferry *et al.,* 2005; Budatha *et al.,* 2008; Chougule *et al.,* 2008). Tabatabaei *et al*. (2011) showed that proteolytic activity of the carob moth, *Ectomyelois ceratoniae*, with hemoglobin as protein substrate occurred over a broad alkaline pH range (pH 8.0–11.0), with maximum activity at pH 10.The loss of enzymatic activity at pH values outside the range is probably caused by protein conformational changes due to change in state of ionization of amino acid residues at catalytic sites as a result of charge repulsion. This affects the catalytic activity. Serine proteases are found to be more active in alkaline conditions. Proteolytic activity of purified gut trypsin like enzyme was maximum at 50°C. A decline in enzyme activity was found at temperature above 50°C. The thermal stability of trypsin-like activity in the midgut samples of *Helicoverpa armigera* pre-incubated for 5 min remained unchanged at temperatures up to 40°C.

However, preincubation for 30 min at temperatures of >40°C sharply reduced the proteolytic activity, which was almost declined at 60°C. The results were consistent with those reported by Grover *et al*., (2016) in *Helicoverpa armigera.* Metals are known to play an important role in various physiological processes. They serve as cofactors for metalloproteases, may mediate efficient substrate binding required for catalysis or possess role in protein folding maintaining the functional structure of proteins. $ZnSO_4$, $CuSO_4$, $CdCl_2$ and $HgCl_2$ at 5 mM concentration inhibited the trypsin like activity of gut protease, whereas Ca^{2+}, Mn^{2+} and Mg^{2+} at that concentration elevated the enzyme activity. Maximum inhibition was observed with Cd^{2+} (82 %) and Hg^{2+} (79%) and minimum inhibition with Cu^{2+} (45.2 %). Metal ions such as Cd^{2+} and Hg^{2+} act as strong inhibitors of enzymatic activity by structural destabilization of the protein due to their interference with sulfhydryl residues in proteins and are responsible for the breakdown of disulfide bonds. Addition of $CaCl_2$, $MgCl_2$ and $MnCl_2$ at low concentrations increased the rate of trypsin activity whereas the reaction rate was progressively lowered with the increase in ionic strength. Grover *et al*. (2016) reported that metal ions Cu^{2+}, Zn^{2+}, Cd^{2+} and Hg^{2+} were found to have the potential to inhibit the mid gut protease of *Helicoverpa armigera.* Maximum inhibition was observed with Cu^{2+} (73 %) and minimum inhibition with Cd^{2+} (47 %). Copper inhibited trypsin non-competitively and zinc inhibited uncompetitively. Costa *et al*. (2013) characterized trypsin from crevalle jack (*Caranx hippos*) and reported Cd^{2+} and Al^{3+} as strong inhibitors of trypsin (95 %), whereas Zn^{2+}, Cu^{2+}, Pb^{2+}, Hg^{2+} inhibited the trypsin from 50 to 85 % at 1 mM. Ca^{2+} has been found to positively modulate the activity of proteases. It is the cofactor of many trypsin-like proteases, from different sources. Calcium might play a role in stabilizing the structure of protease (Oliveira *et al.,* 2005). Mahdavi *et al*. (2013) reported that metal ions on the activity of proteases in *Glyphodes pyloalis* showed that NaCl, $CaCl_2$, $CoCl_2$ (5 and 10 mM), and $MnCl_2$ (5mM) reduced the protease activity. However, metals do have an effect on larvae of another lepidopteran, namely *Conogethes punctiferalis* (Joseph *et al.,* 2006).

The biochemical analysis of *Helicoveroa gut* protease using specific peptide substrates and inhibitors showed that serine proteinase especially trypsin like protease was predominant in midguts of fifth instar larvae. When the trypsin like protease was treated with various classes of inhibitors, strong inhibition was effected by TLCK but not TPCK indicating trypsin like but not chymotrypsin like specificity of serine protease. No inhibition of protease activity was observed with other inhibitors. TLCK has a maximum inhibitory effect on protease activity compared to TPCK (10% inhibition), suggesting that trypsin has a more important role than chymotrypsin in protein digestion in the larval midgut. The inhibitory effect of idoacetate and EDTA was very low. Azocaseinase gut activity in the Indian meal moth, *Plodia interpunctella*, at pH 9.5 was inhibited by serine proteinase inhibitors SBTI (soybean trypsin inhibitor) and TLCK, which are specific to trypsin-like enzymes, at 96% and 89%, respectively (Amorim *et al.,* 2008). Mahdavi *et al*. (2013) reported that EDTA, PMSF and TPCK showed considerable inhibition of larval azocaseinolytic activity of *Glyphodes pyloalis* suggesting that the midgut of larvae contains mainly metalloproteases and serine proteases, mainly chymotrypsin. Kinetic studies performed with different concentrations (0.9-4.0 mM) of BAPNA revealed that the enzyme followed Michaelis-Menten kinetics. K_m value for the protease was determined to be 3.84×10^{-3} M BAPNA. Vmax value for the protease was determined to be 45 nmol/10min/mg protein with BAPNA as substrate. Trypsin isolated from the midgut of *Helicoverpa armigera* had a K_m of 2300 µM and V_{max} of 430 nmol/ min/mg protein with BAPNA as substrate (Ozgur *et al.,* 2009). The Km value of trypsin in midgut of *Eurygaster integriceps* determined using BAPNA as substrate was found to be 600 µM (Hosseininaveh *et al.,* 2009). The Km and Vmax values of trypsin in the alimentary canal of *G. pyloalis* using BAPNA as substrate were 50.5 ± 2 µM and 116.06 ± 1.96 nmol/ min/ mg protein, respectively (Mahdavi *et al.,* 2013).

Conclusion

A trypsin- like protease was purified from the mid guts of *Helicoverpa armigera* using conventional methods of protein purification. The enzyme was found to be homogeneous by the criteria of native PAGE. SDS-PAGE analysis in the presence of 2-mercaptoethaonol gave a single band corresponding to a molecular weight of about 30.4 kDa. The specific activity of the purified enzyme was 1426.92 TU per mg protein. The final yield was 52.81% with a fold purification of 3.81. Proteinase activity from the mid gut was alkali-stable and its pH optimum for activity was about pH 12. K_m and V_{max} values for the protease were determined to be 3.84×10^{-3} M and 45 nmol/10min/mg protein with BAPNA as substrate respectively. Strong inhibition of protease activity by TLCK but not by TPCK suggests that the isolated mid gut protease is trypsin like but not chymotrypsin like specificity of serine protease.

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