Identification and Characterisation of Gut Proteases in the Fig Tree Skeletoniser Moth, *Choreutis nemorana* Hübner (Lepidoptera: Choreutidae)

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Abstract

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The biochemical properties of proteases from the digestive system of the fig tree skeletonizer moth, *Choreutis nemorana*, were determined. Gut extracts of *C. nemorana* larvae were analysed using different specific peptide substrates and proteinase inhibitors. The optimal pH and temperature for proteolytic activities using azocasein as substrate were obtained as pH 11 and 45°C, respectively. In the case of *N*-benzoyl-ʟ-arg-*p*-nitroanilide as substrate, the enzyme showed the maximum tryptic activity at pH 11. The kinetic parameters of trypsin-like proteases indicated that the K_m and V_{max} values of trypsin in the gut of *C. nemorana* were 0.157 ± 0.006mM and 0.188 ± 0.005 µmol/min/mg protein. Using specific proteolytic inhibitors, the inhibitors including phenyl methane sulfonyl fluoride, N-*p*-tosyl-ʟ-lys chloromethyl ketone and ethylene diamine tetraacetic acid showed the greatest inhibitory effect on total proteolytic activity. These results indicated that serine proteinases accounted for the major proteases in the gut of *C. nemorana*. Inhibition assays and zymogram analysis showed that only small amounts of cysteine proteases are present in the digestive system of *C. nemorana*.

Keywords: protease inhibitors; fig leaf roller moth; gut; trypsin; chymotrypsin

Insects need an adequate source of protein in order to grow and maintain themselves. Proteins comprised in amino acids and these organic compounds are fundamental for growth, reproduction, and as a structural element of the cell in the insect body. Proteases are very important enzymes in the alimentary canal of insects and they cleave the peptide bonds in the proteinous insect foods to release the amino acids that are then absorbed by epithelial cells of the insect midgut. Proteolysis plays an important role in insect physiology and food digestion, facilitated by serine, cysteine, aspartic proteinases or endopeptidases and metalloproteinases. It is very important to study insect proteases due to disorders of protein digestion in the digestive tract of insects by protease inhibitors (Josephrajkumar *et al.* 2006). Protease inhibitors are proteins or small polypeptides that may interfere with food digestion in the insect gut sufficiently to control them. Transgenic plants expressing protease inhibitors can kill the pest. These inhibitors can bind with dominant digestive proteases of insects feeding on the host plants, impairing their digestion and retarding growth and larval development in some insect species including lepidoptera (Gatehouse *et al.* 2000). Serine proteases and metallo-exopeptidase are dominant active proteases in the digestive system of lepidopteran larvae (Patankar *et al.* 2001; Josephrajkumar *et al.* 2006; Chougule *et al.* 2008). Also, serine proteases have been reported from the digestive tracts of many insects belong to different orders and families and these enzymes are inhibited by serine protease inhibitors available in legume and cereal plants. Also, serine protease inhibitors have been found in some plants and

showed insecticidal effects on several lepidopteran larvae (Ussuf *et al.* 2001).

The fig tree skeletonizer moth, *Choreutis nemorana* Hübner (Lepidoptera: Choreutidae), is a generally common pest of fig trees in Guilan province, Iran. The larvae of this pest feed on leaves of the fig tree causing massive defoliation but, at times of severe infestation, it also feeds on developing leaves resulting in a huge loss of the crop. They are protected by a web of silken threads and feed on upper epidermal and parenchyma cells (Alford 2007) and penetration of pesticides to this protected area is considered to be hindered. Diazinon and chlorpyriphos were recommended as foliar insecticides against this pest by the Iranian Plant Protection Organization (IPPO). Also, the extensive and exclusive use of chemical pesticides may result in pollution and developing of resistance to such compounds and impair the balance between pests and natural enemies (Metcalf 1986). Heavy reliance on chemical methods is highly unsafe. Therefore, development of alternative or safe methods instead of the chemical control such as the use of transgenic plants expressing proteinase and carbohydrase inhibitors is highly advisable. Protease inhibitors are among the plant defence mechanisms against herbivorous pests. These proteins can inhibit proteases present in insect midguts or produced by symbionts, lead to a reduction in absorbable amino acids which are necessary for insect growth, reproduction and survival (Volpicella *et al.* 2003). Additionally, protease inhibitors from various plants have been used against different mechanistic classes of proteases in order to control pests for a long time. Their use in the insect control is notable based on several studies indicating their effectiveness in disruption of protease digestion. The lepidopteran larvae use a complex proteolytic enzymes including trypsins, chymotrypsins, elastases, cathepsin-B like proteases, aminopeptidases, and carboxypeptidases for protein digestion and many serine proteases are dominant in the larval gut (Patankar *et al.* 2001; Srinivasan *et al.* 2006; Chougule *et al.* 2008; Tabatabaei *et al.* 2011). Insect digestive proteases have different biochemical properties. Therefore, our knowledge of the properties of midgut digestive protease is necessary for developing rational control strategy based on transgenic plants expressing inhibitors (Wilhite *et al.* 2000). Protease inhibitors can be regarded as the best choice method against this insect and these inhibi-

tors can reduce the nutritional quality of food for *C. nemorana*. The first step for discovering the protease inhibitors is identification and biochemical characterisation of digestive proteases in the insect gut. Characterisation and identification of the type of digestive proteases is fundamental to studying nutritional physiology and degradation or activation of toxin protein such as Bt delta endotoxin (Knop Wrigth *et al.* 2006). Therefore, this paper reports biochemical properties of digestive proteases of *C. nemorana*. Also, we studied the effects of various inhibitors on enzyme activities with the aim of identification and application of new pest management technologies.

MATERIAL AND METHODS

Chemicals. Azocasein, BApNA (*N*-benzoyl-ʟ-arginine-*p*-nitroanilide), BTEE (benzoyl-ʟ-tyrosine ethyl ester), TLCK (*N-p*-tosyl-ʟ-lysine chloromethyl ketone), TPCK (*N*-tosyl-ʟ-phenylamine chloromethyl ketone), PMSF (phenyl methane sulfonyl fluoride), iodoacetate were purchased from Sigma (St. Louis, USA). Trichloroacetic acid and EDTA (ethylene diamine tetraacetic acid) were obtained from Merck Company (Darmstadt, Germany).

Insects. *C. nemorana* larvae were collected from fig trees in Rasht, Iran. This population has been maintained on fig leaves in optimum rearing conditions of $25 \pm 2°C$, $60 \pm 10\%$ RH with a photoperiod of 16 h light and 8 h dark.

Enzyme preparation. Last-larval instars were selected for gut extraction. The individuals were chilled and dissected on ice. The whole gut with lumen contents was used. The digestive system of ten individuals was used as one sample. Each sample was replicated at least 3 times and 3 samples were used for each measurement. The collected guts were then homogenized in a known volume of distilled water using plastic pestles, centrifuged at $10\,000 \times g$ and 4°C for 10 minutes. The supernatant was used as an enzyme solution.

Determination of general protease activity. Total proteolytic activity of gut enzyme extracts was evaluated using the proteinase substrate azocasein 2.5%. The reaction mixture consisted of 15 μl of enzyme and 43 μl of 50mM sodium acetate-phosphate-glycine buffer with the desired pH (pH = 11). After 5 min, 17 μl of the substrate azocasein was added and the reaction mixture was incubated at 35°C for 90 minutes. Then, the reaction was

stopped by adding 50 µl of 30% trichloroacetic acid (TCA) and then rested at 4°C for 30 minutes. Then all samples were centrifuged at 13 000 rpm and 4°C for 10 minutes. 100 µl of the supernatant was then transferred to the microplate well and added an equal volume of 1N NaOH before the absorbance was read. The absorbance was measured at 440 nm (with a microplate reader Stat Fax® 3200 (Awareness Technology Inc., Palm City, USA) and then converted to units of protease activity by the following equation:

$$(\text{absorbance/extinction coefficient}) \times 10^3 = \text{micromoles of dye}$$

The activity of protease was expressed as µmol dye/min/mg protein.

Determination of specific protease (trypsin and chymotrypsin) activity. Tryptic activity was measured using the chromogenic substrate BApNA. The reaction was started with the addition of 10 µl enzyme, 85 µl of 25mM acetate-phosphate-glycine buffer (pH = 11) and 5 µl substrate in a microplate well. The absorbance was read at 405 nm continuously monitoring the change in absorbance *p*-nitroaniline release at 25°C for 10 min with a microplate reader (Stat Fax® 3200).

Chymotryptic activity was assayed according to Hummel (1959) as modified by Sharifi *et al.* (2012a) using 1mM *N*-benzoyl-L-tyrosine ethyl ester as substrate dissolved in 50% methanol (v/v), and in 0.08M Tris-HCl (pH 7.8) containing 0.1M CaCl$_2$ at room temperature. The increase in absorbance at 256 nm due to the hydrolysis of the substrate was recorded by monitoring the absorption at the wavelength. The aforementioned assays were carried out in triplicate and appropriate blanks were run for all assays.

Effect of pH and temperature on general proteolytic activity and tryptic activity. The pH profiles of general protease and trypsin were determined at room temperature in a mixed buffer containing phosphate, glycine and acetate (25mM of each) adjusted to various pHs (pH 3 to 12) by adding HCl or NaOH for acidic and basic pH values, respectively. Before determining the activity, the reaction mixtures were incubated at different pHs at room temperature for 5 minutes. The activities of general protease were determined by incubating the reaction mixture at different temperatures ranging from 15 to 75°C in 25mM sodium acetate-phosphate-glycine buffer with the desired pH. Enzyme activity was measured by the standard assay method mentioned above.

Kinetic parameters of trypsin. The Michaelis-Menten constant (K_m) and the maximal reaction velocities (V_{max}) of trypsin were determined by Lineweaver-Burk plots. The measurements were carried out at pH 11.0, measuring the initial rates of reaction with increasing substrate concentrations. BApNA was used as substrate at a final concentration range of 0.0156 to 1mM. The experiments were performed in triplicate (Sharifi *et al.* 2012b)

Effect of protease inhibitors. To analyse the effect of protease inhibitors, 10 µl of each freshly prepared protease inhibitor including 5mM PMSF, 1mM TLCK, 1mM TPCK, 2mM EDTA, and 5mM iodoacetate were added to 15 µl enzyme. After 15 min incubation at 35°C, 33 µl of sodium acetate-phosphate-glycine buffer with the desired pH was added. Residual protease activity was measured by the standard assay method. Three replicates for each compound were carried out and appropriate blanks were run for all assays. Controls have only the same amount of enzyme and aforementioned buffer.

Electrophoretic zymogram. Non-reducing SDS-PAGE (Laemmli 1970) of proteolytic enzyme was performed using resolving and stacking polyacrylamide gels of 12.5% (w/v) and 5% (w/v), respectively. A total of 10 µl of the enzyme extract was mixed with 10 µl of inhibitor stock solution. After incubation at room temperature for 20 min, 8 µl of sample buffer without mercaptoethanol were added and the samples were loaded in each well. Non-reducing SDS-PAGE was carried out at 4°C at a constant voltage of 100 V using electrophoresis was carried out at 4°C and 100 V constant voltages. After the run, the gel was removed and placed in phosphate buffer pH 8 containing 2.5% Triton X-100 for 20 minutes. After this step, the gel was immersed in 0.5–1% casein and shaken for 3 hours. Then, the gel was washed in distilled water and stained with 0.1% Coomassie Brilliant Blue R-250 in methanol-acetic acid-water (50:10:40). After 2 h, the gel was washed in water and destaining was done in methanol-acetic acid-water (50:10:40) for 1–2 h until clear bands could be visualised against the dark blue background.

Determination of protein concentration. Protein concentration was estimated by the method of Bradford (1976) using bovine serum albumin (BSA) as the standard.

Statistical analysis. Data was analysed using one-way analysis of variance (ANOVA) (SAS 1999). Differences between sample ($n = 3$) means were

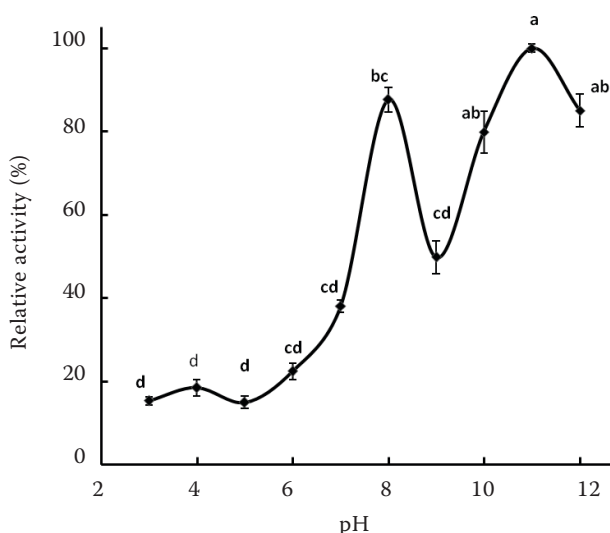evaluated using Tukey's test and were considered significant when the probability $P \leq 0.05$.

RESULTS

Protease activity

Proteolytic activity of azocasein as a protein substrate in the gut of *C. nemorana* was 7.267 ± 0.37 μmol/min/mg protein. In addition, the trypsin and chymotrypsin activity was 1.53 ± 0.03 and 1.42 ± 0.1 μmol/min/mg protein, respectively.

Effect of pH and temperature on general proteolytic activity and tryptic activity

The pH dependence of proteolytic activity with azocasein in the gut extract from *C. nemorana* larvae is presented in Figure 1. Enzyme activity increased from pH 3 to 8, then fell to pH 9 and increased again and reached the maximum at pH 11. The proteases in the gut of this insect are active more than 70% at pH 10–12. Trypsin showed higher activity in alkaline pH and optimal pH for its activity in the gut of *C. nemorana* larvae was 11 (Figure 2). As shown in Figure 3, the optimal temperature for proteolytic activity in the gut of *C. nemorana* using the proteinase substrate azocasein was 45°C.

Kinetic parameters of trypsin

Kinetic analysis of trypsin-like activity at pH 11.0 gave linear reciprocal Michaelis-Menten (Lineweaver-Burk) plots, enabling the estimation of values for K_m and V_{max}. Kinetic parameters of trypsin in the gut of larvae were measured by BApNA as substrate. The K_m and V_{max} values of trypsin in the gut were 0.1572 ± 0.006mM and 0.188 ± 0.005 μmol/min/mg protein, respectively.

Effect of protease inhibitors

The effect of various inhibitors on protease activity is given in Table 1. Results showed that PMSF (5mM) and TLCK (1mM) inhibited 62.06% and 57.89% of proteolytic activity in the gut of *C. nemorana*, respectively. Considerable inhibition of proteolytic activity by PMSF (serine protease inhibitor) and TLCK (trypsin-like protease inhibitor) compared to other inhibitors suggested that the serine proteinases were the major proteases in the gut of *C. nemorana*. Also, EDTA (metalloprotease inhibitor) caused a significant inhibitory effect (53.83%) on hydrolysing azocasein compared to TPCK and iodoacetate. This data suggest that metalloproteases and serine proteases play the main roles in protein digestion in the gut of *C. nemorana*.
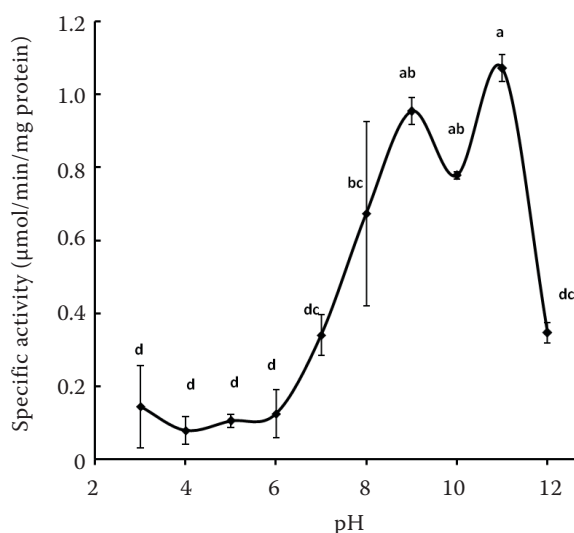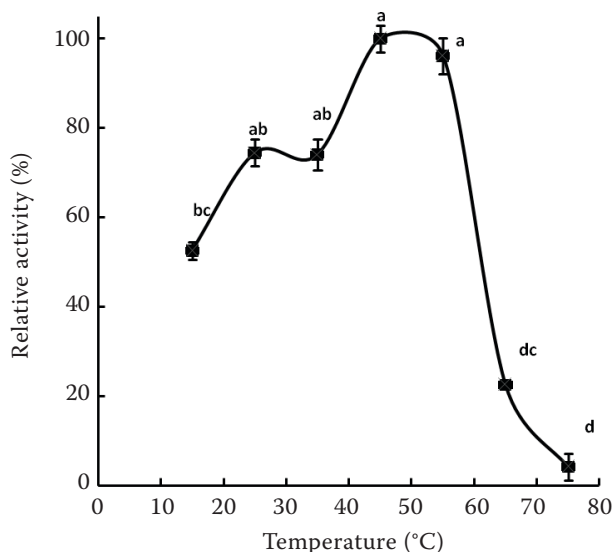

Different letters indicate that the activity of enzymes in different pH is significantly different from each other by Tukey's test ($P < 0.05$)

Figure 1. Effect of pH on the proteolytic activity of larval gut extract from *C. nemorana* on the substrate azocasein

Figure 2. Effect of pH on trypsin specific activity (mean ± SE) extracted from the gut of *C. nemorana* larvae

Different letters indicate that the activity of enzymes at different temperatures is significantly different from each other by Tukey's test ($P < 0.05$)

Figure 3. Effect of temperature on the activity of gut proteases in *C. nemorana*

Electrophoretic zymogram

The effect of proteinase inhibitors on proteolytic activity in gel is shown in Figure 4. The crude *C. nemorana* larval extracts were analysed by SDS-PAGE. After protease activity staining, at least 5 major bands were detected based on their electrophoretic mobility. The results of SDS-PAGE confirm the effects of inhibitors in their absence and presence in inhibition assay. However, as depicted in Figure 4, PMSF revealed complete disappearance of the bands p2, p3, p4, and p5 and reduced the intensity of p1 band compared to the control. According to this result, the zymogram made using azocasein as substrate showed the presence of trypsin as the main protease in the gut of *C. nemorana*.
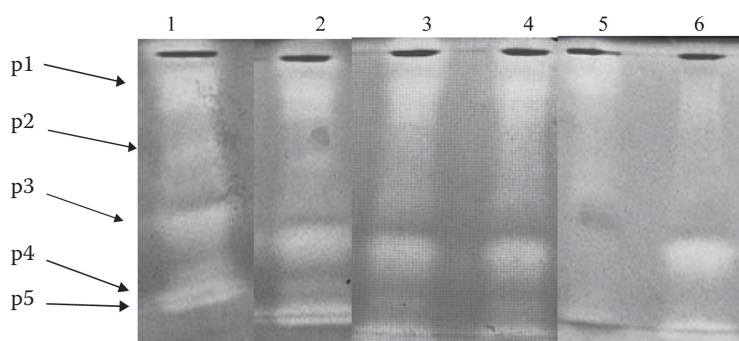
Table 1. Effect of some proteinase inhibitors on the gut protease activity in *C. nemorana* larvae

Inhibitor	% inhibition (mean ±SE)
PMSF	62.06 ± 0.22^c
TLCK	57.89 ± 0.59^c
EDTA	53.83 ± 0.14^{bc}
TPCK	28.09 ± 0.12^{ab}
Iodoacetate	25.92 ± 0.7^{ab}
Control	0^a

Different letters indicate that the activity of enzymes is significantly different from each other by Tukey's test ($P < 0.05$); PMSF – phenyl methane sulfonyl fluoride; TLCK – *N*-*p*-tosyl-ʟ-lysine chloromethyl ketone; EDTA – ethylene diamine tetraacetic acid; TPCK – *N*-tosyl-ʟ-phenylanaline chloromethyl ketone

DISCUSSION

The digestive enzymes such as proteolytic enzymes are of interest as a target for insect pest management (Christeller *et al.* 1992). These enzymes play important roles in insect growth, development and reproduction, enzyme activation, toxin activation/detoxification, and inflammation processes (Terra *et al.* 1996). In this research we characterised the digestive proteases in the gut of *C. nemorana*. Our data showed that the optimal pH for proteolytic and tryptic activity was 11. Similar results have been reported for other lepidopteran larvae such as *Spodoptera littoralis* Fabricius (Lepidoptera: Noctuidae) pH 11 (Ishaaya *et al.* 1971); *Conogethes punctiferalis* Guenée (Lepidoptera: Pyralidae) pH 10 (Josephrajkumar *et al.* 2006); *Parnassius apollo* Linnaeus (Lepidoptera: Papilionidae) pH 9–10 (Nakonieczny *et al.* 2007); *Mamestra brassicae* Linnaeus (Lepidoptera: Noctuidae) pH 11 (Chougule *et al.* 2008). Our results are in agreement with these reports on

Figure 4. Effect of some proteinase inhibitors on azocasein hydrolytic activity of the larval digestive system extract in 12.5% non-reducing SDS-PAGE: 1 – control, 2 – iodoacetate, 3 – control, 4 – TPCK (*N*-tosyl-ʟ-phenylalanine chloromethyl keton), 5 – PMSF (phenyl methane sulfonyl fluoride), 6 – TLCK (*N*-*p*-tosyl-ʟ-lysine chloromethyl ketone)

the presence of proteases with alkaline optima in gut extracts of lepidopteran larvae. Christeller *et al.* (1992) reported that the digestive enzymes of lepidopteran larvae are active in an alkaline environment in their guts at pH 10–12 in which serine proteases and metalloexopeptidases are most active. Terra and Ferreira (1994) attributed the high pH of the lepidopteran gut to an adaptation of herbivorous lepidopteran larvae for releasing hemicellulose from plant cell walls. The pH of gut contents is a major factor that affects digestive enzymes (Terra & Ferreira 1994). It seems that the pH value in the digestive system of this pest is alkaline like in the other lepidopteran insects (Hegedus *et al.* 2003); however, the gut pH in this pest was not studied.

Digestive system extracts of *C. nemorana* have azocaseinolytic activity within a broad range of temperatures (15–75°C) with an optimum temperature of 45°C, while optimum temperatures for protease activity from other lepidoptera were obtained at temperatures of 30–40°C (Josephrajkumar *et al.* 2006; Budatha *et al.* 2008). The optimum midgut protease activity in *Achaea janata* (Lepidoptera: Noctuidae) was obtained in a pH range of 8–10.5 and at a temperature of 30°C on the basis of the initial studies using buffers with pH ranging from 7 to 11 (Budatha *et al.* 2008).

Proteolytic specific activity with azocasein as a protein substrate in the gut of *C. nemorana* was 7.267 ± 0.37 μmol/min/mg protein. The azocasein hydrolysis by the midgut extract of *A. janata* resulted in the specific activity of 1200 ± 90 nmol/min/mg protein (Budatha *et al.* 2008). Our results showed that the specific activity of *C. nemorana* was higher than that od *A. janata.*

The K_m and V_{max} values of trypsin in the gut of *C. nemorana* using BApNA as substrate were 0.157 ± 0.006mM and 0.188 ± 0.005 μmol/min/mg protein, respectively. K_m and V_{max} values for *Mamestra brassicae* trypsin were determined using Z-Arg-7-amido-4-methylcoumarin hydrochloride as 0.069 and 3.83mM/min/μg protein, respectively (Chougule *et al.* 2008).

This study has shown that PMSF as the serine-protease inhibitor and TLCK as the trypsin-like protease inhibitor caused a significant decrease in proteolytic activity in the gut of *C. nemorana* compared to the other inhibitors. This result showed that serine proteinases are the major hydrolyzing enzyme in the gut of *C. nemorana*. Similarly like in our study, Budatha *et al.* (2008) reported that PMSF had the greatest inhibition effect (almost

80%) on proteolytic activity in the gut of *A. janata*. According to this finding, he concluded that serine proteinases are the dominant enzyme in the gut of *A. janata*. In another study, George *et al.* (2008) found that PMSF reduced proteolytic activity to 90% in the gut of *Busseola fusca* (Lepidoptera: Noctuidae) compared to the other inhibitors. Also, Hegedus *et al.* (2003) reported that PMSF caused 90% inhibition of proteolytic activity in the gut of *Mamestra configurata* (Lepidoptera: Noctuidae). Similar study were reported by Amorim *et al.* (2008) where TLCK caused 96% inhibition of proteolytic activity in the gut of *Plodia interpunctella* (Lepidoptera: Pyralidae) larvae. Our results showed that the inhibitory effect of TLCK as the trypsin-like protease on total protease activity was higher than that of TPCK as the chymotrypsin inhibitor; it seems that trypsin is the predominant and most active protease enzyme in the gut of *C. nemorana* larvae and chymotrypsin activity is lower than that of trypsin. Similar results were reported by Broadway and Duffey (1986), when they worked on midgut proteolytic activity in larval *Pieris rapae* and *Trichoplusia ni*. In contrast, Budatha *et al.* (2008) using a combination of "synthetic substrates and specific inhibitor, photometric assays and activity blots detected three trypsin-like and one elastase-like serine proteases but no chymotrypsin-like activity in *A. janata*".

The results of SDS-PAGE are in close agreement with those obtained by inhibition assays. PMSF is the strongest inhibitor and a zymogram made using casein as substrate shows the presence of serine proteinases as the main proteases in the larval gut of *C. nemorana (*Figure 4). The data resulting from zymogram studies clearly revealed that PMSF and TLCK reduced the intensity of bands compared to the control in the gel electrophoresis zymogram. Also, in the case of lepidopteran species, trypsin, chymotrypsin, elastase-like proteinases, aminopeptidases and carboxypeptidases comprise the gut proteolytic profiles dominantly (Terra & Ferreira 1994) and lepidopteran larvae use serine protease for protein digestion (Patankar *et al.* 2001; Josephrajkumar *et al.* 2006; Chougule *et al.* 2008). In contrast, Nakonieczny *et al.* (2007) showed a minor role of trypsin and chymotrypsin in protein digestion in Apollo butterfly, *Parnassius apollo* ssp. *frankenbergeri* larvae. The low serine protease activity in this insect referred to the limited availability of plant proteins and very high carboxypeptidase in the gut tissue.

The biochemical analysis of *C. nemorana* larval digestive enzymes using specific peptide substrates and inhibitors shows that serine proteinases and metalloproteases were predominant in the digestive system of last instar larvae. Serine proteases have been identified from the digestive tracts of insects from many families and these enzymes are inhibited by protease inhibitors available in some plants. Therefore the evaluation of these natural protease inhibitors of tryptic activity in *C. nemorana* provides the knowledge needed for making transgenic plant resistant to this pest.

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