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ARTICLE

Alkaline serine proteases from *Helicoverpa armigera*: potential candidates for industrial applications

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Abstract

We characterized trypsin- and chymotrypsin-like serine alkaline proteases from cotton bollworm, *Helicoverpa armigera*, for their probable potential application as additives in various bio-formulations. Purification was achieved by using hydroxylapatite, DEAE sephadex and CM sephadex columns, which resulted in increased enzyme activity by 13.76- and 14.05-fold for trypsin and chymotrypsin, respectively. Michaelis–Menten constants (K_m) for substrates of trypsin and chymotrypsin, BApNA and SAAPFpNA, were found to be 1.25 and 0.085 mM, correspondingly. Fluorescent zymogram analysis indicated the presence of five trypsin bands with molecular masses of ~21, 25, 38, 40, and 66 kDa and two chymotrypsin bands with molecular masses of ~29 and 34 kDa in SDS-PAGE. The optimum pH was 10.0 and optimum temperature was 50°C for proteolytic activity for the purified proteases. The proteases were inhibited by synthetic inhibitors such as PMSF, aprotonin, leupeptin, pefabloc, and antipain. TLCK and TPCK inhibited about 94 and 90% of trypsin and chymotrypsin activity, respectively, while EDTA, EGTA, E64, pepstatin, idoacetamide, and bestatin did not affect the enzymes. The purified enzymes exhibited high stability and compatibility with metal ions; oxidizing, reducing, and bleaching agents; organic solvents; and commercial detergents. Short life cycles, voracious feeding behavior, and production of multiple forms of proteases in the midgut with rapid catalytic activity and chemostability can serve *H. armigera* as an excellent alternative source of industrially important proteases for use as additives in stain removers, detergents, and other bio-formulations. Identification of enzymes with essential industrial properties from insect species could be a bioresource.

KEYWORDS

alkalophilic, chymotrypsin, halophilic, *Helicoverpa armigera*, industrial application, serine protease, trypsin, thermostable

1 | INTRODUCTION

With the recent advances in biotechnology, there has been a growing interest and demand for enzymes with novel properties. Proteases play an important role in metabolism of all forms of life, including prokaryotes, fungi, plants, and animals. Proteases constitute nearly 60% of the total industrial market for enzymes (Nunes & Martins, 2001). Proteases are used in various industries such as food, detergent, leather and waste processing, therapeutic, diagnostics, and silver salvaging (Abidi et al., 2008; Kumar & Takagi, 1999; Zambare et al., 2007). Other applications of alkaline protease include the utilization in peptide synthesis, resolution of the racemic mixture of amino acids, and hydrolysis of gelatin layers of X-ray films (Gupta et al., 2002; Singh et al., 1999).

Investigation on various properties of proteases is a continuous process in enzymology due to both their physiological importance and wide application in research and industries. Due to great industrial demand, there is always a search for alternative sources of proteases with suitable specificity and broad range of stability to various chemicals. Alkaline proteases from bacteria and fungi are the major source for industrial applications (Kumar & Takagi, 1999; Singh et al., 1999). However, insect proteases have received relatively lesser attention than the microbial proteases. Proteases from insects could be excellent candidates for industrial applications. Proteolysis is an essential part of food digestion in insects mediated by concerted action of several proteases (Anwar & Saleemuddin, 2002; Sanatan et al., 2013; Visweshwar et al., 2015). Serine protease from insect midguts have been characterized from *Osphranteria coerulescens* Redtenbacher, *Periplaneta americana* L., *Glyphodes pyloalis* Walker, and so on possess important features such as stability under high temperatures, and high activity under alkaline pH (Mahdavi et al., 2013; Sanatan et al., 2013; Sharifi et al., 2012).

Helicoverpa armigera (Hubner; Noctuidae: Lepidoptera), commonly called cotton bollworm or legume pod borer, is a polyphagous pest, and has been reported to attack more than 180 plant species including pigeonpea, chickpea, cotton, sorghum, groundnut, maize, fruits, vegetables, and forest trees (Sharma, 2005). Lepidopteran larvae consume more food and due to the presence of multiple isozymes of proteases in their midgut they digest and assimilate the ingested food more rapidly and thus grow much faster than mammals and birds, with high metabolic rate. Trypsins, chymotrypsins, cathepsin-B-like proteases, elastases, aminopeptidases, carboxypeptidases, and many serine proteases are dominant proteolytic enzymes in the larval midgut (Chougule et al., 2008; Patankar et al., 2001; Srinivasan et al., 2006; Tabatabaei et al., 2011). The primary digestive proteinases of many insect species are serine proteinases, predominantly trypsin (EC 3.4.21.4) and chymotrypsin (EC 3.4.21.1) (Reeck, et al., 1999; Terra & Ferreira, 1994). The larval digestive proteinases (trypsin and exopeptidases) were first studied in yellow mealworm, *Tenebrio molitor* L. (Applebaum et al., 1964). X-ray contact print technique has indicated the presence of 10 isozymes of proteases in the midgut of *H. armigera* (Parde et al., 2012). *H. armigera* when reared on a high-protein diet, cDNA library from the midgut showed two genes encoding elastase and 18 trypsin-like and 14 chymotrypsin-like proteinases (Gatehouse et al., 1997).

The digestive enzymes of insects are also of interest for insect control because of their unusual alkaline microenvironment of the midgut (pH 10.0–12.0) (Christeller et al., 1992). Since insect midgut enzymes exhibit highly alkaline pH optima (Anwar & Saleemuddin, 2002; Parde et al., 2012; Sanatan et al., 2013; Visweshwar et al., 2015), insects can be used as a promising source for isolating proteases that have industrial application. Therefore, in the present study we characterized the serine proteases, trypsin and chymotrypsin, from the midgut of *H. armigera* for their chemostability under extreme alkalinity and temperature conditions that are typically encountered during industrial processing.

2 | MATERIALS AND METHODS

2.1 | Insect culture

The *H. armigera* larvae were obtained from the *Helicoverpa* rearing laboratory, Entomology, International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Patancheru, Telangana, India. The larvae reared under

laboratory conditions (27 ± 1°C, 65 ± 5% RH, and 12-h photoperiod) on a chickpea-based diet (Chitti Babu et al., 2014).

2.2 | Isolation of midgut proteases

Fourth-instar larvae of *H. armigera* starved for 3 h were dissected midventrally. About 25 midguts were homogenized in a dounce homogenizer in ice-cold 0.1 M glycine–NaOH buffer, pH 10.0. The homogenate was centrifuged at 10,000 rpm for 20 min at 4°C, the supernatant stored at −20°C until use. The protein concentration of the midgut extract was estimated by Lowry's method using BSA as a standard (Lowry et al., 1951).

2.3 | Trypsin and chymotrypsin assay

Trypsin and chymotrypsin activity in the midgut extract was measured by incubating 10 μl of gut extract with 1 mM Nα-benzoyl-DL-arginine p-nitroanilide (BApNA) and 0.1 mM Suc-Ala-Ala-Pro-Phe-p-nitroanilide (SAAPFpNA) (Sigma-Aldrich, India) in 0.1 M glycine–NaOH buffer, pH 10.0, for 20 and 10 min, respectively, in 1.5 ml Eppendorf tubes at 37°C. The reaction was terminated by adding 300 μl of 30% acetic acid. The samples were centrifuged and the released pNA was read at 410 nm by means of spectrophotometer (Hitachi U-2900, Japan) (Purushottam & Vandana, 2013). One unit of enzyme activity was expressed as the amount of enzyme hydrolyzing 1 μmole of substrate/min at 37°C.

2.4 | Purification

Fifty milliliters of crude midgut extract (~750 mg protein) of *H. armigera* larvae was concentrated using Amicon ultracentrifugal filters. The gut concentrate was applied on hydroxylapatite (HTP) column (2 × 10 cm), equilibrated with 0.1 M Tris–HCl buffer (pH 8.0). The column was washed with 10 ml of the 0.1 M of Tris–HCl buffer (pH 8.0). Soon after washing with buffer, proteins were eluted with a stepwise gradient of 0–1.0 M potassium phosphate buffer (pH 8.0). The fractions collected in aliquots of 2 ml. The trypsin activity eluted at 0.2 to 0.4 M phosphate buffer fractions. Active fractions containing trypsin activity were pooled together, concentrated using Amicon filters, and applied to a column of DEAE sephadex (2 × 10 cm) previously equilibrated with phosphate buffer (0.1 M), pH 8.0. After washing the DEAE sephadex column with 10 ml of the same buffer, proteins were eluted with a stepwise gradient of 0–1 M NaCl, and 2 ml fractions collected. Trypsin activity eluted at 0.5 and 0.6 M NaCl. Fractions encompassing trypsin activity were combined, and then concentrated using Amicon filters to a final volume of 2 ml.

Chymotrypsin was similarly purified as trypsin with certain modifications. After concentrating the midgut crude extract using Amicon ultracentrifugal filters, the concentrate was subjected to HTP column. Fractions of 2 ml each were collected and those having chymotrypsin activity were combined and applied on to the CM sephadex column (2 × 10 cm), previously equilibrated with 0.1 M phosphate buffer (pH 8.0). The column was washed with 10 ml of the same buffer, the bound proteins were eluted with a stepwise gradient of 0–1 M NaCl, and fractions of 2 ml each were collected. Chymotrypsin activity eluted at 0.6 to 1.0 M NaCl. Fractions containing chymotrypsin activity were pooled, and then concentrated using Amicon filters to a final volume of 4 ml. The purified fractions of trypsin and chymotrypsin were dialyzed against 0.1 M glycine–NaOH buffer (pH 10.0), and tested for homogeneity by SDS-PAGE (Laemmli, 1970).

2.5 | Kinetic studies

The kinetic parameters, K_m and V_{max}, for the enzyme substrates were determined by measuring the activities of trypsin and chymotrypsin in 0.1 M glycine–NaOH buffer, pH 10.0, using L-BApNA and SAAPFpNA, concentration ranging from

0.25 to 2 mM and 0.02 to 0.2 mM, respectively, using Lineweaver–Burk plot (LB plot). LB plot was obtained by linear regression analysis using MS Excel 2010 version.

2.6 | Fluorescent zymogram and molecular mass determination

Midgut proteases of *H. armigera* were run on 10% SDS-PAGE. After electrophoresis was performed, the gel was washed in 2.5% Triton X-100 and in cold distilled water for 10 min each. The gel was incubated in assay buffer (50 mM Tris–HCl, pH 8.0, containing 10 mM $CaCl_2$, 0.005% Triton X-100 and 50 μM Boc-Gln-Ala-Arg-MCA or 50 μM Suc-Ala-Ala-Pro-Phe-MCA for detection of trypsin and chymotrypsin, respectively) for 30 min at 37°C. The gel was then washed in distilled water and the fluorescent bands of trypsin and chymotrypsin were observed in Gel Documentation System under UV-B transillumination (Bio-Rad Laboratories, Hercules, CA; excitation wavelength for AMC group is 385 nm) (Yasothornsrikul & Hook, 2000). Standard molecular weight markers were loaded in one lane to determine the molecular mass of the purified enzymes. After electrophoresis, the lane was sliced and molecular weight markers were stained using Coomassie Brilliant Blue (R-250). Another lane was loaded with partially purified midgut extract, after electrophoresis the proteases were visualized by incubating the gel in 1.5% casein in 0.1 M glycine–NaOH buffer, pH 10, for 60 min; washed; stained with Coomassie Brilliant Blue; and then destained (Visweshwar et al., 2015).

2.7 | Optimum pH and pH stability

Trypsin and chymotrypsin activity in the midgut extract of *H. armigera* was evaluated by preincubating 10 μl of the enzyme in various buffers of pH ranging from pH 4.0 to 12.0 (acetate buffer pH 4.0–5.0; phosphate buffer pH 6.0–7.0; glycine–NaOH pH 8.0–12.0) for 30 min. at 37°C to determine the pH optima. pH stability was determined by incubating the purified enzymes in buffers with different pH range of 7.0–12.0 for 1 h at 37°C. The enzyme activities were assayed as stated above.

2.8 | Effect of temperature and thermal stability

The activity of purified trypsin and chymotrypsin was evaluated at different temperatures (20–100°C) by incubating 10 μl of the purified enzyme in 1 ml of 0.1 M glycine–NaOH buffer, pH 10.0, in the presence of respective substrates for 30 min. Stability of the enzymes to temperature was examined by incubating the purified trypsin and chymotrypsin at different temperatures ranging from 50 to 80°C for 1 h, and the enzyme activities were determined as described above.

2.9 | Effects of synthetic protease inhibitors and metal ions

The effect of protease inhibitors on the activity of purified proteases was studied using EDTA (5 mM), EGTA (5 mM), 1 mM each of PMSF, TPCK, TLCK, aprotonin, leupeptin, E64, pepstatin, iodoacetamide, pefabloc, bestatin, antipain, and chymostatin. The inhibitors were preincubated with the purified trypsin and chymotrypsin for 30 min at 37°C. Effect of metal ions Na^+, K^+, Mg^{2+}, Ca^{2+}, Fe^{2+}, Fe^{3+}, Mn^{2+}, Zn^{2+}, Cu^{2+}, Pb^{2+} Mo^{2+}, As^{3+}, and Hg^{2+} on the activities of trypsin and chymotrypsin at 1 mM was also investigated by preincubating the proteases with metal ions for 30 min at 37°C, and the proteolytic activities were assessed as stated above. Protease activity without the inhibitor or metal ions was considered as 100%.

2.10 | Effect of surfactants, oxidizing and reducing agents

The stability of the purified proteases in the presence of surfactants (SDS, CHAPS, Triton X-100, Tween 20, and Tween-80) and oxidizing (H_2O_2 and bleach) and reducing (β-mercaptoethanol, DTE, DTT, and urea) agents was determined by

preincubating them with the purified enzymes for 30 min at 37°C. Proteolytic activities were determined as described above and the relative activities determined in relation to the untreated control.

2.11 | Effect of organic solvents

Effect of various organic solvents such as methanol, ethanol, propanol, butanol, acetone, hexane, DMSO, DMF, benzene, toluene, diethylether, chloroform, benzaldehyde, and formaldehyde on the activity of trypsin and chymotrypsin was examined in a reaction mixture containing 0.1 M glycine–NaOH buffer (pH 10.0) and a final concentration of each solvent as 5%. The enzyme activity was determined by incubating the purified trypsin and chymotrypsin with the solvent for 30 min. Enzyme activity was calculated as a percentage in relation to the untreated control.

2.12 | Stability of proteases to commercial detergents

The stability of purified trypsin and chymotrypsin was assessed in the presence of commonly available commercial detergents such as Ariel, Surf, Tide, Wheel, Vanish, and Rin. Enzyme activities for purified serine proteases was determined by incubating the enzyme with 5% detergent solution prepared in 0.1 M glycine–NaOH buffer (pH 10.0) for 30 min at 37°C. Commercial detergent solutions were heated prior to assay, at 100°C for 30 min to deactivate the already present proteases in them. Enzyme activity was estimated as a percentage in relation to control.

2.13 | Statistical analysis

Significant differences between the treatments and treatment means was analyzed by one-way analysis of variance (ANOVA) using *F*-test and least significant difference (LSD), respectively, in MS Excel 2010 version.

3 | RESULTS

3.1 | Purification of trypsin and chymotrypsin

Trypsin and chymotrypsin was purified up to 13.76- and 14.05-fold, with 11.93 and 13.62% recovery, respectively (Tables 1 and 2). The specific activity of the serine proteases was 39.37 and 172.56 U/mg protein, for trypsin and chymotrypsin, respectively. Purity of the proteases was also confirmed by SDS-PAGE.

3.2 | Kinetic studies

Trypsin and chymotrypsin showed a hyperbolic dependence on the utilization of their synthetic substrates. K_m and V_{max} were estimated to be 1.25 mM, 8.2 U and 0.085 mM, 13.5 U, with substrates of trypsin and chymotrypsin, respectively (Fig. 1).

TABLE 1 Purification of trypsin from midgut of *H. armigera*

	Volume (ml)	Total Protein (mg)[a]	Total Activity (U)[a]	Specific Activity (U/mg)[a]	Purification Fold	Yield (%)
Crude extract	50	750 ± 4.57	2,145.00 ± 21.58	2.86 ± 0.05	1.00	100.00
Amicon filters	20	680 ± 5.65	3,225.67 ± 15.24	4.74 ± 0.08	1.66	150.38
Hydroxyl apatite	12	25 ± 1.25	488.56 ± 8.24	19.54 ± 0.12	6.83	22.78
DEAE sephadex	2	6.5 ± 0.05	255.88 ± 9.25	39.37 ± 1.25	13.76	11.93

[a]Values in the table represent mean of three replications (mean ± SD).

TABLE 2 Purification of chymotrypsin from midgut of *H. armigera*

	Volume (ml)	Total Protein (mg)[a]	Total Activity (U)[a]	Specific Activity (U/mg)[a]	Purification Fold	Yield (%)
Crude extract	50	825 ± 10.23	10,135.82 ± 25.24	12.29 ± 0.08	1.00	100.00
Amicon filters	18	755 ± 15.23	15,252.64 ± 15.32	20.20 ± 1.25	1.64	150.48
Hydroxyl apatite	8	56 ± 8.21	5,752.45 ± 12.23	102.72 ± 7.23	8.36	56.75
CM sephadex	4	8 ± 0.05	1,380.5 ± 10.25	172.56 ± 8.32	14.05	13.62

[a]Values in the table represents mean of three replications (mean ± SD).

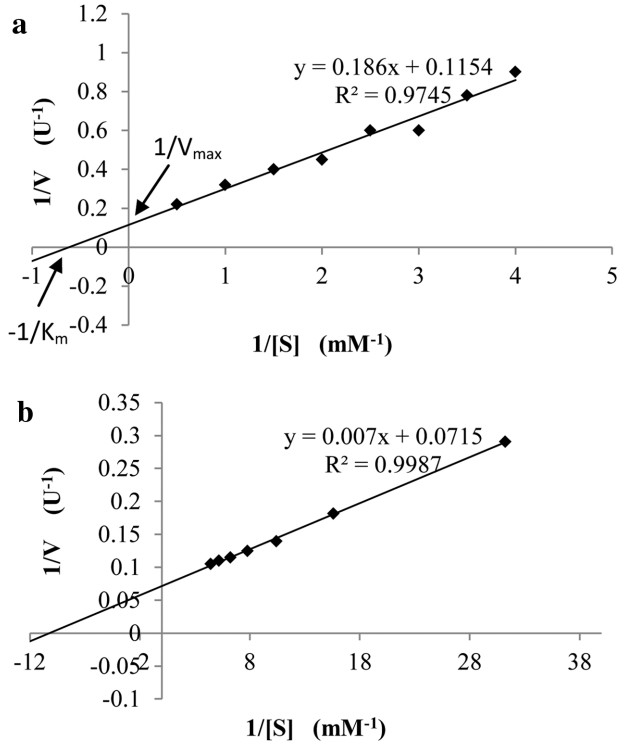

FIGURE 1 Double reciprocal plots for the determination of kinetic parameters of trypsin (a) and chymotrypsin (b) isolated from the midgut *H. armigera* larvae using BApNA and SAAPFpNA as their corresponding substrates

3.3 | Molecular masses of the trypsin and chymotrypsin

The fractions exhibiting trypsin and chymotrypsin activity were loaded on SDS-PAGE for separation. Trypsin and chymotrypsin were detected by fluorescent zymogram, where the liberated AMC (7-amino-4-methyl-coumarin) of the substrates, peptide-MCA, was envisaged as fluorescent bands on dark background under UV transillumination. Partially purified midgut extract showed the presence of ten proteases. Fluorescent zymogram analysis indicated that there were five trypsin bands with molecular masses of ~23, 27, 38, 40, and 66 kDa and two chymotrypsin bands with molecular masses of ~29 and 34 kDa in the midgut extract of *H. armigera* (Fig. 2).

3.4 | Influence of pH, temperature, and metal Ions on the activity of serine proteases

The proteases were highly active in the pH range of 8 to 12, the optimum being 10.0 for both the enzymes. The optimum activity at pH 10.0 for trypsin and chymotrypsin was found to be 4.01 and 4.95 U, respectively (Fig. 3A). The pH stability

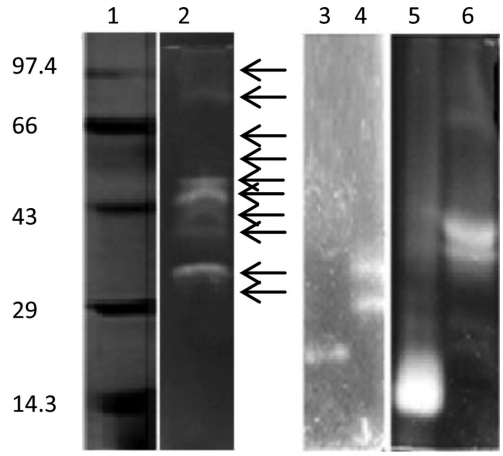

FIGURE 2 SDS-PAGE for detection of midgut serine proteases from *H. armigera*. Lane 1—molecular weight markers; lane 2—partially purified midgut extract, gel was stained with Coomassie Brilliant Blue after casein treatment; lane 3—bovine chymotrypsin (30 μg); lane 4—midgut extract (50 μg); lane 5—bovine trypsin (30 μg); lane 6—midgut extract (50 μg). Lanes 3 to 6 were stained for the detection of trypsin and chymotrypsin using specific fluorescent substrate as mentioned in the text

profile of the proteases indicated that these enzymes were stable in the pH range of 9.0 to 12.0 after 72 h of incubation, indicating the alkaline nature of the enzymes (Fig. 3B).

The enzymes were active at temperatures ranging from 40 to 80°C, with optimum activity at 50 and 60°C for trypsin (6.72 U) and chymotrypsin (5.68 U), respectively. The enzymatic activity was significantly reduced beyond 80°C (Fig. 3C). These enzymes remained active at 50 to 70°C even after 5 h of incubation. Incubation of trypsin and chymotrypsin at 80°C for 1 h reduced the enzymatic activity to 95 and 82% relative to the control, respectively (Fig. 3D).

Activity of trypsin was unaffected even at 3 M concentration of NaCl, but lost 60% of its activity at 5 M concentration, whereas chymotrypsin was unaffected even at 5 M of NaCl (Table 3). Both the proteases retained their activities in the presence of K^+, Ca^{2+}, Zn^{2+}, Cu^{2+}, Mn^{2+}, As^{3+}, Mo^{2+}, Hg^{2+}, and Fe^{2+}. There was a difference in trypsin activity in presence of Mg^{2+} and Fe^{3+} but the increase in activity was nonsignificant. Trypsin activity was significantly affected in presence of Pb^{2+} and retained only 58% of residual activity, but the effect was nonsignificant on chymotrypsin activity.

3.5 | Influence of synthetic inhibitors on serine proteases

Activities of trypsin and chymotrypsin were completely inhibited by the PMSF, aprotonin, leupeptin, pefabloc, and antipain. TLCK inhibited about 94% of trypsin activity, while TPCK and chymostatin inhibited about 90% of chymotrypsin activity. EDTA, EGTA, E64, pepstatin, idoacetamide, and bestatin did not affect the activities of trypsin and chymotrypsin (Table 4).

3.6 | Effect of surfactants and oxidizing and reducing agents on serine proteases

Effect of anionic surfactant (SDS), nonionic surfactants (Tween-20, Tween-80, and Triton X-100), and oxidizing (H_2O_2, and bleach) and reducing (β-mercaptoethanol, DTE, DTT, urea) agents on the activities of trypsin and chymotrypsin is summarized in Table 5. In the presence of 1 mM of SDS, trypsin and chymotrypsin retained 66.5 and 94.5% of activities as compared to the control, whereas other surfactants did not affect the enzyme activities. In presence of 5% H_2O_2 and bleach, about 15–25% of the enzyme activities were inhibited, which was statistically nonsignificant. In presence of 1 mM of β-mercaptoethanol, about 20 and 30% of trypsin and chymotrypsin activities were inhibited, respectively. Nearly 10–25% of the enzyme activities were inhibited in presence of DTE and DTT at 1 mM, but urea (1 mM) did not affect the enzyme activities.

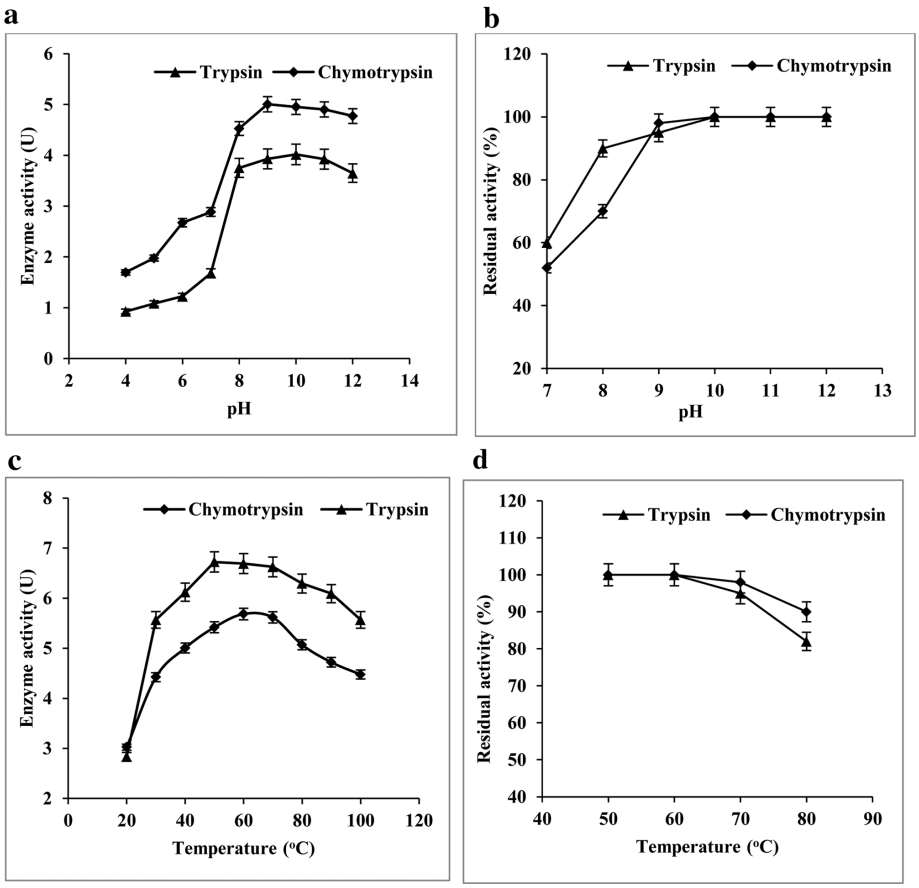

FIGURE 3 Influence of pH and temperature on the activity and stability of trypsin and chymotrypsin isolated from *H. armigera*. Effect of pH on the activities of trypsin and chymotrypsin (a) and their stability to alkaline pH (b) was evaluated spectrophotometrically by incubating the purified enzymes in various buffers as stated in the text. Effect of temperature on the activities of trypsin and chymotrypsin (c) and their stability at extreme temperatures (d) was evaluated by incubating the enzymes in 0.1 M glycine–NaOH buffer, pH 10.0, at different temperatures as described in methodology. The data represent the mean of three replications (mean ± SD)

3.7 | Stability of the serine proteases in the presence of organic solvents

Activities of trypsin and chymotrypsin increased from 5 to 15% in presence of 5% organic solvents, but the effects were nonsignificant (Table 6).

3.8 | Effect of commercial detergents

Trypsin and chymotrypsin exhibited 80% of the activities in presence of Ariel, Tide, and Surf detergents as compared to the control, but the activities were unaffected in the presence of Rin, Vanish, and Wheel (Table 7).

4 | DISCUSSION

Serine proteases in insects are comparable to those from mammals. Serine proteases are involved in the protein digestion and constitute nearly 95% of the total proteinase activities in a wide variety of insects (Chougule et al., 2008). Proteolytic enzymes play vital roles in development, growth, and reproduction processes in insects' life cycle (Terra

TABLE 3 Effect of metal ions on the activities of trypsin and chymotrypsinisolated from the midgut of *H. armigera*

Metal Ion (1 mM)	Trypsin Activity (%)[a]	Chymotrypsin Activity (%)[a]
Control	100	100
NaCl (1 M)	105.12 ± 2.34	100.05 ± 2.45
NaCl (3M)	85.23 ± 2.54	101.12 ± 2.67
NaCl (5 M)	42.00 ± 1.44[b]	100.04 ± 2.34
$MgCl_2$	127.45 ± 2.45	109.20 ± 3.45
KCl	102.12 ± 2.66	99.45 ± 2.56
$CuSO_4$	102.05 ± 2.33	82.45 ± 2.67
$ZnCl_2$	104.24 ± 4.23	96.78 ± 2.66
$CaCl_2$	87.35 ± 5.55	106.34 ± 2.56
$FeSo_4$	84.45 ± 6.43	98.23 ± 3.22
$FeCl_3$	120.00 ± 4.34	109.45 ± 3.33
$MnCl_2$	83.00 ± 5.44	93.54 ± 2.33
Sodium arsenite	89.00 ± 4.34	100.04 ± 0.44
Lead acetate	58.00 ± 3.44[b]	82.49 ± 2.33
Molybdenum	81.00 ± 4.44	82.56 ± 2.45
Mercuric chloride	83.00 ± 3.43	90.56 ± 3.54

[a]Values in the table represents mean of three replications (mean ± SE).
[b]Significantly different from control at $P < 0.05$.

& Ferreira, 1994). Serine proteases are characterized by a catalytic serine residue which in fact is a catalytic triad (serine, histidine, and aspartic acid) (Hedstrom, 2002). Trypsin (EC 3.4.21.4) and chymotrypsin (EC 3.4.21.1) are the major proteolytic enzymes belonging to this family (Reeck et al., 1999; Terra & Ferreira, 1994). The present study describes the characterization of serine proteases from the midgut of *H. armigera*. The proteases were purified by employing column chromatographic techniques such as hydroxyl apatite, CM sephadex, and DEAE sephadex. K_m for the substrates BApNA and SAAPFpNA was found to be 1.25 and 0.085 mM, with a low K_m for the latter with chymotrypsin. Zymogram analysis performed for the detection of proteases in the partially purified midgut extract indicated the presence of 10 proteases and the results were in agreement with that of earlier studies (Parde et al., 2012). Fluorescence zymogram analysis performed using specific fluorescent substrates for trypsin and chymotrypsin indicated that among the ten proteases, there were five trypsin bands with molecular masses of ~23, 27, 38, 40, and 66 kDa and two chymotrypsin bands with the molecular masses of ~29 and 34 kDa in the midgut extract of *H. armigera*. Trypsin and chymotrypsin are the most common enzymes characterized in insect digestive system (Lopes & Terra, 2003; Mazumdar-Leighton & Broadway, 2001). About four to seven active proteolytic enzymes have been detected with casein zymogram in insects: *T. molitor* (Zwilling, 1968), *Astacus fluviatilis* (Pfleiderer et al., 1967), *Carcinus maenas* (Linke et al., 1969), *Apis mellifica* L. (Giebel et al., 1971) and *Rhyzopertha dominica* (F.) (Zhu & Baker, 1999).

The proteases from *H. armigera* exhibited a parabolic dependency on pH of the medium, and were highly active in the pH range of 8 to 12, the optimum being 10.0 for both the enzymes. Activities of both the enzymes were reduced under acidic pH (<6.0). Alkaline pH optima observed for the proteases was due to the intrinsic alkaline pH conditions of the insect midgut. Midgut proteases reported from *Spilosoma obliqua* Walker (pH 11.0) (Anwar & Saleemuddin, 2002), cockroach (pH 8.0) (Lopes & Terra, 2003; Sanatan et al., 2013), *H. armigera* (pH 10.0) (Parde et al., 2012), and *T. molitor* Linnaeus (pH 8.5) (Tsybina et al., 2005) were active in the pH range of 7.0 to 12.0. The alkaline pH of insects' midgut has been attributed to digestion and absorption of the nutrients from plants, an adaptation for extracting hemicelluloses from plant cell walls (Tsybina et al., 2005). Trypsin and chymotrypsin from *H. armigera* were resistant to thermal inactivation at high temperatures up to 80°C. These enzymes were active at temperatures ranging from 40 to 80°C, with an optimum activity at 50°C. The alkaline proteases from other insects have also been found to be thermostable. Optimum

TABLE 4 Effect of synthetic protease inhibitors on the activities of trypsin and chymotrypsin in *H. armigera*

Treatment	Relative Activity (%)[a]	
	Trypsin	Chymotrypsin
Control	100.00	100.00
PMSF	3.20 ± 2.55[b]	2.88 ± 2.45[b]
EDT	95.00 ± 3.45	96.40 ± 3.56
aEGT	100.23 ± 2.56	98.00 ± 4.23
aTPCK	96.12 ± 2.45	11.18 ± 2.56[b]
TLCK	6.80 ± 4.56[b]	102.13 ± 3.22
Aprotonin	19.49 ± 4.56[b]	15.00 ± 4.67[b]
Leupeptin	8.19 ± 3.34[b]	13.00 ± 3.21[b]
E64	94.08 ± 2.45	98.99 ± 3.56
Pepstatin	91.84 ± 2.67	100.00 ± 0.04
Iodoacetamide	87.20 ± 2.87	99.00 ± 1.11
Pefabloc	12.00 ± 2.98[b]	14.00 ± 1.45[b]
Bestatin	92.35 ± 3.56	101.26 ± 1.56
Antipain	11.08 ± 6.56[b]	28.05 ± 2.32[b]
Chymostatin	92.00 ± 3.32	12.75 ± 1.45[b]

[a]Values in the table represents mean of three replications (mean ± SE).
[b]Significantly different from control at $P < 0.01$.

TABLE 5 Effect of surfactants and oxidizing and reducing agents on the activities of trypsin and chymotrypsin isolated from the midgut of *H. armigera*

Treatment	Relative Activity (%)[a]	
	Trypsin	Chymotrypsin
Control	100.00	100.00
Surfactants		
SDS	66.50 ± 0.25[b]	94.50 ± 2.77
CHAPS	119.70 ± 1.23	101.22 ± 3.21
Triton X-100	115.11 ± 2.22	84.52 ± 3.65
Tween 20	102.35 ± 2.67	95.23 ± 2.88
Tween 80	110.14 ± 1.87	90.74 ± 1.45
Oxidizing agents		
H_2O_2	87.98 ± 2.65	86.44 ± 1.76
Bleach	75.12 ± 2.67	82.14 ± 0.87
Reducing agents		
β-mercaptoethanol	81.73 ± 3.76	68.26 ± 2.44[b]
DTE	85.69 ± 4.76	92.73 ± 1.67
DTT	89.77 ± 1.67	91.72 ± 2.45
Urea	102.38 ± 2.56	101.54 ± 1.45

[a]Values in the table represents mean of three replications (mean ± SE).
[b]Significantly different from control at $P < 0.05$.

TABLE 6 Effect of solvents on the activities of trypsin and chymotrypsin isolated from the midgut of *H. armigera*

	Relative Activity (%)[a]	
Treatment	**Trypsin**	**Chymotrypsin**
Control	100.00	100.00
Methanol	114.50 ± 4.45	114.39 ± 3.36
Ethanol	118.80 ± 3.32	105.77 ± 3.36
Propanol	125.55 ± 2.25	97.33 ± 2.25
Butanol	127.00 ± 5.42	105.30 ± 1.25
Acetone	112.63 ± 2.65	112.55 ± 1.25
Hexane	115.17 ± 1.25	111.62 ± 1.25
DMSO	93.41 ± 0.06	118.98 ± 2.69
DMF	118.87 ± 2.23	110.17 ± 1.25
Benzene	125.56 ± 3.36	112.59 ± 0.36
Toluene	86.75 ± 1.25	108.69 ± 1.25
Diethylether	117.68 ± 2.69	90.00 ± 5.58
Chloroform	116.72 ± 1.15	106.06 ± 6.65

[a]Values in the table represents mean of three replications (mean ± SE).

TABLE 7 Effect of commercial detergents on the activities of trypsin and chymotrypsin isolated from the midgut of *H. armigera*

	Relative Activity (%)[a]	
Treatment	**Trypsin**	**Chymotrypsin**
Control	100.00	100.00
Ariel	78.00 ± 1.23	75.00 ± 2.25
Tide	80.00 ± 1.56	78.00 ± 1.15
Surf	82.00 ± 1.58	80.00 ± 2.36
Rin	96.06 ± 1.65	97.17 ± 2.22
Vanish	92.00 ± 2.63	92.56 ± 2.23
Wheel	95.00 ± 2.20	98.00 ± 1.45

[a]Values in the table represents mean of three replications (mean ± SE).

temperature for the proteolytic activity of alkaline serine proteases in *S. obliqua*, *O. coerulescens*, and *P. americana* was reported to be 50, 55, and 60°C, respectively (Anwar & Saleemuddin, 2002; Sanatan et al., 2013; Sharifi et al., 2012). Activities of trypsin and chymotrypsin were unaffected at higher concentration of NaCl (3 M). Serine alkaline protease in *P. americana* was unaffected in presence of NaCl (Sanatan et al., 2013), whereas in *G. pyloalis* larvae, the proteolytic activity was reduced by about 38% in presence of 5 mM NaCl (Mahdavi, et al., 2013). Protease with salt-tolerant nature is the exclusive characteristic of halotolerant microbes. Proteases with proteolytic activities in a wide range of pH, and stability to salt (approx. 3 M) and solvents have been reported from microbial sources (Kumar & Takagi, 1999; Santos et al., 2013). Proteases with stability at high temperature and salt concentrations, and alkaline pH optima are desirable for applications in detergents and tanning (Joo et al., 2003).

Activities of both trypsin and chymotrypsin were unaffected in presence of metal ions except Pb^{2+}. Protease activity was affected due to metals ions in *Conogethes punctiferalis* (Guenee) larvae (Josephrajkumar et al., 2006). Na^{+}, Ca^{2+}, Co^{2+}, and Mn^{2+} reduced the protease activity in lesser mulberry pyralid, *G. pyloalis* (Mahdavi, et al., 2013). Proteases having extreme stability toward various metal ions are suitable in tanning processing and sewage treatment (Li et al., 2010). TLCK, an irreversible trypsin inhibitor, decreased hydrolysis of L-BApNA but did not

affect chymotrypsin in *H. armigera*. TLCK inhibited trypsin-like enzyme activity in *R. dominica* and *Anticarsia gemmatalis* (Hübner) (Xavier et al., 2005; Zhu & Baker, 1999). TPCK and chymostatin, being potent inhibitors of chymotrypsin, inhibited the chymotrypsin-like enzyme activity in the present study. In contrast, TPCK and TLCK did not affect the chymotrypsin-like enzyme activity in *T. molitor* larvae and *Apis mellifera* (Elpidina et al., 2005; Matsuoka et al., 2015). Serine protease inhibitors, PMSF, aprotonin, leupeptin, pefabloc, and antipain completely inhibited the activities of both the enzymes. Also, the proteases were insensitive to EDTA and EGTA, suggesting that the enzymes did not require metal ions for their catalytic activity, in contrary to the mammalian proteases. PMSF, aprotinin, and leupeptin inhibited trypsin-like activity in lesser grain borer, *R. dominica* (Zhu & Baker, 1999). PMSF completely inhibited the chymotrypsin-like enzyme, but EDTA did not affect the enzyme activity in *T. molitor* larvae (Elpidina, et al., 2005). Trypsin-like activity was inhibited by PMSF, EDTA, benzamidine, and aprotonin in velvetbean caterpillar, *A. gemmatalis* (Xavier et al., 2005). A nonsignificant reduction in the enzyme activities of both the enzymes was observed in presence of oxidizing agents. Among the tested surfactants, SDS and β-mercaptoethanol slightly affected the trypsin and chymotrypsin activity, respectively. Chymotrypsin-like enzyme activity was substantially inhibited by 2-mercaptoethanol in *T. molitor* larvae (Elpidina, et al., 2005). Surfactants and oxidizing agents did not affect the serine alkaline protease in *P. americana* at 1% (Sanatan et al., 2013). Thus, there was a differential response of alkaline serine proteases to the metal ions, synthetic protease inhibitors, and surfactants from insects.

There is great industrial demand for organic solvent-stable proteases in making of pharmaceutical products and peptide synthesis in molecular biology (Gupta et al., 1999; Motyan et al., 2013). Usually in aqueous environments, enzymes possess the conformational mobility to perform the catalytic activity optimally, which is lost during organic solvents treatment in industrial processing. Purified trypsin and chymotrypsin from the midgut of *H. armigera* were resistant to tested solvents and commercial detergent. These properties of serine proteases from *H. armigera* can be exploited in industrial application. Industrially important proteases with tolerance to alkalinity and extreme temperatures have been known to be reported from microbial source. Thus, it can be expected that the serine alkaline proteases purified from the midgut of *H. armigera* could belong to the symbiotic bacteria residing in the midgut. However, Visweshwar et al. (2015) has successfully demonstrated that zymogram analysis of midgut proteases in *H. armigera* when administered on antibiotic cocktail for exclusion of midgut bacteria exhibited inhibition of only one protease among the nine proteases reported in control larvae suggests that most of the observed proteases in zymogram belong to insect larvae. Therefore, insects could be a better option over microbial source in producing multiple forms of proteases in their midgut for application in industrial purposes.

Serine alkaline proteases are a commercially important group of enzymes for biotechnological applications, including clinical, pharmaceutical, and analytical chemistry (Joo & Chang, 2005; Joo et al., 2004; Samal et al., 1990). Alkaline proteases are also an important constituent in detergent formulations to remove a variety of stains such as blood, tea, curd, and so on, from clothes at elevated pH and temperatures. Nonenzymatic removal of proteins may lead to a permanent stain on the clothes due to oxidation caused by bleaching and drying. Proteases breakdown the proteins into soluble polypeptides or free amino acids and effectively remove the stains along with the surfactant from fibers. Enzymes to be used in detergents must be effective at lower concentrations and compatible with various detergent components and remain active in presence of a wide range of chemicals and temperatures (Kumar & Takagi, 1999). The larvae of *Sarconesiopsis magellanica* (Le Guillou) and *Lucilia sericata* (Meigen) used in healing necrotic wounds release proteolytic enzymes in their excretion and secretion that help in wound healing (Pinilla et al., 2013).

Pancreatic proteases from animal origin such as trypsin, chymotrypsin, renin, and pepsin, though are readily available, the availability of enzymes depends on the livestock for slaughter, which is also governed by government policies (Rao et al., 1998). Therefore, alkaline proteases from *H. armigera* with high stability under higher temperatures and salt concentrations can play an important role in industrial processes. Another advantage of *H. armigera* is to easily produce copious numbers of the insect larvae under laboratory conditions using an inexpensive artificial diet. Insects can be more advantageous over microbial proteases due to their short life cycles, voracious feeding behavior, and production of multiple forms of proteases in the midguts for digestion. Insect proteases with rapid catalytic ability, wide range of physical and chemical characteristics, and stability under extreme environmental conditions and chemicals

make insect proteases more suitable biocatalysts for various industrial applications. Protein engineering from insects will broaden the applications of proteases for various purposes. Characterization of proteolytic digestive enzymes of insect origin also offers opportunities for developing suitable and effective pest management strategies via serine protease inhibitors for deployment in transgenics for sustainable crop production.

5 | CONCLUSION

In the present study, alkaline serine proteases from *H. armigera* were characterized for their potential applications to industrial usage. Zymographic analyses revealed the presence of multiple isozymes for trypsin and chymotrypsin, and the purified proteases were thermophilic, alkalophilic, and salt tolerant, and exhibited high stability and compatibility with solvents and detergents and oxidizing and bleaching agents. The results suggested that proteases from *H. armigera* have unique properties, which can be exploited in various industrial applications.

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CONFLICT OF INTEREST

All the authors declare no conflict of interest, financial or otherwise, that might possibly bias this work.

LITERATURE CITED

Abidi, F., Limam, F., & Nejib, M. M. (2008). Production of alkaline proteases by *Botrytis cinerea* using economic raw materials: Assay as biodetergent. *Process Biochemistry*, *43*, 1202–1208.

Anwar, A., & Saleemuddin, M. (2002). Purification and characterization of a digestive alkaline protease from the larvae of *Spilosoma obliqua*. *Archives of Insect Biochemistry and Physiology*, *51*, 1–12.

Applebaum, S. W., Birk, Y., Harpaz, I., & Bondi, A. (1964). Comparative studies on proteolytic enzymes of *Tenebrio molitor* L. *Comparative Biochemistry and Physiology*, *11*, 85–103.

Chitti Babu, G., Sharma, H. C., Madhumati, T., Raghavaiah, G., Murthy, K. V. M. K., & Rao, V. S. (2014). A semi-synthetic chickpea flour based diet for long-term maintenance of laboratory culture of *Helicoverpa armigera*. *Indian Journal of Entomology*, *76*, 336–340.

Chougule, N. P., Doyle, E., Fitches, E., & Gatehouse, J. A. (2008). Biochemical characterization of midgut digestive proteases from *Mamestra brassicae* (cabbage moth; Lepidoptera: Noctuidae) and effect of soybean Kunitz inhibitor (SKTI) in feeding assays. *Journal of Insect Physiology*, *54*, 563–572.

Christeller, J. T., Laing, W. A., Markwick, N. P., & Burgess, E. P. J. (1992). Midgut protease activities in 12 phytophagous lepidopteran larvae: Dietary and protease inhibitor interactions. *Insect Biochemistry and Molecular Biology*, *22*, 735–746.

Elpidina, E. N., Tsybina, T. A., Dunaevsky, Y. E., Belozersky, M. A., Zhuzhikov, D. P., & Oppert, B. (2005). A chymotrypsin-like proteinase from the midgut of *Tenebrio molitor* larvae. *Biochimie*, *87*, 771–779.

Gatehouse, L. N., Shannon, A. L., Burgess, E. P. J., & Christeller, J. T. (1997). Characterization of major midgut proteinase cDNAs from *Helicoverpa armigera* larvae and changes in gene expression in response to four proteinase inhibitors in the diet. *Insect Biochemistry and Molecular Biology*, *27*, 929–944.

Giebel, W., Zwilling, R., & Pfleiderer, G. (1971). The evolution of endopeptidases – Xii. The proteolytic enzymes of the honeybee (*Apis mellifica* L.). *Comparative Biochemistry and Physiology*, *38B*, 197–210.

Gupta, R., Beg, Q., & Lorenz, P. (2002). Bacterial alkaline proteases: Molecular approaches and industrial applications. *Applied Microbiology and Biotechnology*, *59*, 15–32.

Gupta, R., Gupta, K., & Khan, S. (1999). Bleach-stable alkaline protease from *Bacillus sp*. *Biotechnology Letters*, *21*, 135–138.

Hedstrom, L. (2002). Serine protease mechanism and specificity. *Chemical Reviews*, *102*(12), 4501–4524.

Joo, H. S., & Chang, C. S. (2005). Oxidant and SDS stable alkaline protease from a halotolerant *Bacillus clausii* I-52: Enhanced production and simple purification. *Journal of Applied Microbiology*, *98*, 491–497.

Joo, H. S., Kumar, C. G., Park, G. C., Paik, S. R., & Chang, C. S. (2003). Oxidant and SDS-stable alkaline protease from *Bacillus clausii* I-52: Production and some properties. *Journal of Applied Microbiology*, *95*, 267–272.

Joo, H. S., Kumar, C. G., Park, S. R., & Chang, C. S. (2004). Bleach resistance alkaline protease produced by a *Bacillus* species isolated from the Korean Polychaete *Perriserrula leucophryna*. *Process Biochemistry*, *39*, 1441–1447.

Josephrajkumar, A., Chakrabarty, R., & Thomas, G. (2006). Midgut proteases of the cardamom shoot and capsule borer *Conogethes punctiferalis* (Lepidoptera: Pyralidae) and their interaction with aprotinin. *Bulletin of Entomological Research*, *96*, 91–98.

Kumar, C. G., & Takagi, H. (1999). Microbial alkaline proteases: From a bioindustrial viewpoint. *Biotechnological Advances*, *17*, 561–594.

Laemmli, U. K. (1970). Cleavage of structural proteins during assembly of the head of bacteriophage T4. *Nature*, *227*, 680–685.

Li, Z. Y., Youravong, W., & H-Kittikun, A. (2010). Protein hydrolysis by protease isolated from tuna spleen by membrane filtration: A comparative study with commercial proteases. *LWT – Food Science and Technology*, *43*(1), 166–172.

Linke, R., Zwilling, R., Herbold, D., & Pfleiderer, G. (1969). Immunologische untersuchungen des trypsins und der niedermolekularen protease aus drei decapoden crustaceen.*Zeitschrift fur Physiologische Chemie*, *350*, 877–885.

Lopes, A. R., & Terra, W. R. (2003). Purification, properties and substrate specificity of a digestive trypsin from *Periplaneta americana* (Dictyoptera). *Insect Biochemistry and Molecular Biology*, *33*, 407–415.

Lowry, O. H., Rosenbrough, N. J., Farr, A. L., & Randal, A. J. (1951). Protein measurements with the folin phenol reagent. *Journal of Biological Chemistry*, *193*, 265–275.

Mahdavi, A., Ghadamyari, M., Sajedi, R. H., Sharifi, M., & Kouchaki, B. (2013). Identification and partial characterization of midgut proteases in the lesser mulberry pyralid, *Glyphodes pyloalis*. *Journal of Insect Science*, *13*, 81.

Matsuoka, T., Takasaki, A., Mishima, T., Kawashima, T., Kanamaru, Y., Nakamura, T., & Yabe, T. (2015). Expression and characterization of honeybee, *Apis mellifera*, larva chymotrypsin-like protease. *Apidologie*, *46*, 167–176.

Mazumdar-Leighton, S., & Broadway, R. M. (2001). Identification of six chymotrypsin cDNAs from larval midguts of *Helicoverpa zea* and *Agrotis ipsilon* feeding on the soybean (Kunitz) trypsin inhibitor. *Insect Biochemistry and Molecular Biology*, *31*, 633–644.

Motyan, J. A., Tóth, F., & Tőzsér, J. (2013). Research applications of proteolytic enzymes in molecular biology. *Biomolecules*, *3*, 923–942.

Nunes, A. S., & Martins, M. L. (2001). Isolation, properties and kinetics of growth of a thermophilic *Bacillus*. *Brazilian Journal of Microbiology*, *32*, 271–275.

Parde, V. D., Sharma, H. C., & Kachole, M. S. (2012). Inhibition of *Helicoverpa armigera* gut pro-proteinase activation in response to synthetic protease inhibitors. *Entomologia Experimentalis et Applicata*, *142*, 104–113.

Patankar, A. G., Giri, A. P., Harsulkar, A. M., Sainani, M. N., Deshpande, V. V., Ranjekar, P. K., & Gupta, V. S. (2001). Complexity in specificities and expression of *Helicoverpa armigera* gut proteinases explains polyphagous nature of the insect pest. *Insect Biochemistry and Molecular Biology*, *31*, 453–464.

Pfleiderer, G., Zwilling, R., & Sonneborn, H.-H. (1967). Eine Protease vom Molekulargewicht 11,000 und eine trypsinaihnliche Fraktion aus *Astacus fluviatilis* Fabr. *Zeitschrift fur Physiologische Chemie*, *348*, 1319–1331.

Pinilla, Y. T., Moreno-Pérez, D. A., Patarroyo, M. A., & Bello, F. J. (2013). Proteolytic activity regarding *Sarconesiopsis magellanica* (Diptera: Calliphoridae) larval excretions and secretions. *Acta Tropica*, *128*, 686–691.

Purushottam, R. L., & Vandana, K. H. (2013). Effect *Bacillus thuringiensis* (Bt) Cry1Ac toxin and protease inhibitor on growth and development of *Helicoverpa armigera* (Hubner). *Pesticide Biochemistry and Physiology*, *105*, 77–83.

Rao, M. B., Tanksale, A. M., Ghatge, M. S., & Deshpande, V. V. (1998). Molecular and biotechnological aspects of microbial proteases. *Microbiology and Molecular Biology Reviews*, *62*(3), 597–635.

Reeck, G., Oppert, B., Denton, M., Kanost, M., Baker, J., & Kramer, K. (1999). Insect proteinases. In: V. Turk (Eds.), *Proteases: New perspectives* (pp. 125–148). Basel: Birkhauser Verlag.

Samal, B. B., Karan, B., & Stabinsk, Y. (1990). Stability of two novel serine proteinases in commercial laundry detergent formulations. *Biotechnology and Bioengineering*, *35*, 650–652.

Sanatan, P. T., Lomate, P. R., Giri, A. P., & Hivrale, V. K. (2013). Characterization of a chemostable serine alkaline protease from *Periplaneta americana*. *BMC Biochemistry*, *14*, 32.

Santos, A. F., Valle, R. S., Pacheco, C. A., Alvarez, V. M., Seldin, L., & Santos, A. L. S. (2013). Extracellular proteases of *Halobacillus blutaparonensis* strain M9, a new moderately halophilic bacterium. *Brazilian Journal of Microbiology*, *44*(4), 1299–1304.

Sharifi, M., Chitgar, M. G., Ghadamyari, M., & Ajamhasani, M. (2012). Identification and characterization of midgut digestive proteases from the rosaceous branch borer, *Osphranteria coerulescens* Redtenbacher (Coleoptera: Cerambycidae). *Romanian Journal of Biochemistry*, *49*(1), 33–47

Sharma, H. C. (2005). Heliothis/Helicoverpa *management: Emerging trends and strategies for future research*. New Delhi: Oxford and IBH Publishing.

Singh, J., Vohra, R. M., & Sahoo, D. K. (1999). Alkaline protease from a new obligate alkalophilic isolate of *Bacillus sphaericus*. *Biotechnology Letters*, *21*, 921–924.

Srinivasan, A., Giri, A. P., & Gupta, V. S. (2006). Structural and functional diversities in lepidopteran serine proteases. *Cellular and Molecular Biology Letters*, *11*, 132–154.

Tabatabaei, P. R., Hosseininaveh, V., Goldansaz, S. H., & Talebi, K. H. (2011). Biochemical characterization of digestive proteases and carbohydrases of the carob moth, *Ectomyelois ceratoniae* (Zeller) (Lepidoptera: Pyralidae). *Journal of Asia-Pacific Entomology*, *14*, 187–194.

Terra, W. R., & Ferreira, C. (1994). Insect digestive enzymes: Properties, compartmentalization and function. *Comparative Biochemistry and Physiology*, *109B*, 1–62.

Tsybina, T. A., Dunaevsky, Y. E., Belozersky, M. A., Zhuzhikov, D. P., Oppert, B., & Elpidina, E. N. (2005). Digestive proteinases of yellow mealworm (*Tenebrio molitor*) larvae: Purification and characterization of a trypsin-like proteinase. *Biochemistry (Moscow)*, *70*, 300–305.

Visweshwar, R., Sharma, H. C., Akbar, S. M. D., & Sreeramulu, K. (2015). Elimination of gut microbes with antibiotics confers resistance to *Bacillus thuringiensis* toxin proteins in *Helicoverpa armigera* (Hubner). *Applied Biochemistry and Biotechnology*, *177*, 1621–1637.

Xavier, L. P., Oliveira, M. G. A., Guedes, R. N. C., Santos, A. V., & De Simone, S. G. (2005). Trypsin-like activity of membrane-bound midgut proteases from *Anticarsia gemmatalis* (Lepidoptera: Noctuidae). *European Journal of Entomology*, *102*, 147–153.

Yasothornsrikul, S., & Hook, V. Y. H. (2000). Detection of proteolytic activity by fluorescent zymogram in-gel assays. *BioTechniques*, *28*, 1166–1173.

Zambare, V. P., Nilegaonkar, S. S., & Kanekar, P. P. (2007). Production of an alkaline protease and its application in dehairing of buffalo hide. *World Journal of Microbiology and Biotechnology*, *23*, 1569–1574.

Zhu, Y. C., & Baker, J. E. (1999). Characterization of midgut trypsin-like enzymes and three trypsinogen cDNAs from the lesser grain borer, *Rhyzopertha dominica* (Coleoptera: Bostrichidae). *Insect Biochemistry and Molecular Biology*, *29*, 1053–1063.

Zwilling, R. (1968). Zur Evolution der Endopeptidasen – IV. α- and ß-Protease aus *Tenebrio molitor*. *Zeitschrift fur Physiologische Chemie*, *349*, 326–332.

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