

Imidaclopride, a Potent Inhibitor of Array of Digestive Enzymes of *Helicoverpa armigera* (Hubner) (Lepidoptera: Noctuidae)



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Abstract

Imidacloprid, the first commercialized neonicotinoid, acts by systemic and contact insecticidal activity apart from its more specific and high agonistic affinity to insect nicotinic acetylcholine receptors (nAChR) for most of the lepidoptera including *Helicoverpa armigera*. Effect of Imidaclopride on digestive enzymes was evaluated. Imidacloprid inhibited the growth and development of the *H. armigera*. Moderate changes in total protease and aminopeptidase activities were observed whereas chymotrypsin, trypsin, elastase and alkaline phosphatase activities were significantly inhibited by imidacloprid in a dose dependent manner. However, increasing pattern for all the protease were recorded at sub lethal doses of imidacloprid.

Keywords: *H. armigera*; Imidacloprid; Proteases; Alkaline phosphatase

Abbreviations: LD: Lethal Dose; ALP: Alkaline Phosphatase; BBMs: Brush Border Membrane Vesicles

Introduction

The *Helicoverpa armigera* (Hubner) (Lepidoptera: Noctuidae) a phytophagous insect which feeds on more than hundred plant species, brought crop loss more than half the yield and annual loss of more than US \$500 million [1]. The devastating pest have developed resistance to almost all class of insecticides [2,3], have become serious constraint to crop production in Asia, Africa, Australia and Mediterranean Europe for the last four to five decades [4]. Enhanced metabolism rate in insects also plays an important role on fitness of the insect [5]. Digestive proteolytic activity in the insect gut might be the key in the adaptation of resistance against plant defensive compounds. Defensive role of luciferase aminopeptidases (LAPs) have extensively studied in lepidopteran gut. The decreased utilization of food, delayed growth, reduction in survival, weight or size and reproduction of new generation adults could be due to the interference of the toxicants with amino acid metabolism, inhibited by most of the plant metabolites [6]. The *H. armigera* has a large multigene family for serine proteinases, which

includes two main types, trypsin and chymotrypsin [7]. It has been shown [7] that the differential regulation and expression of protease enzymes in response to chronic exposure to protease inhibitors [8]. Studies were carried out with respect to the effect of imidacloprid on reproduction, fecundity, and survivorship of female in *H. armigera* [9]. The literature survey shown a minimal/lack of information regarding metabolic changes in *H. armigera* under imidacloprid stress conditions which can be correlated with the other neonicotinoids and lepidopteran insect pest. Here we report the effect of imidacloprid on serine proteases, aminopeptidase and detoxification enzymes on the fitness of the *H. armigera* larvae.

Materials and Methods

Chemicals

Rallis India Limited, Bangalore, India, kindly provided imidacloprid. Adenosine triphosphate (ATP), adenosine diphosphate (ADP), azocasein, bovine serum albumin (BSA),

1,4- leucine p-nitroanilide (LpNA), N- α -benzoyl-DL-arginine p-nitroanilide, N-glutaryl-L-phenylalanine p-nitroanilide, p-Nitroanisole (p-NA), phenazine methosulfate (PMS), were purchased from Sigma Aldrich (Mumbai, India). All other chemicals used were commercial products and of analytical grade of highest purity available.

Insects

The present study was carried out at International Crops Research Institute for Semi-Arid Tropics (ICRISAT), Patancheru, Telangana State, India. *H. armigera* larvae were reared on a chickpea based semi-synthetic diet under laboratory conditions at 27 \pm 1 $^{\circ}$ C, 65 \pm 5% RH, and 12 h photoperiod, obtained from the insect rearing laboratory.

Bioassay

Initial weights of third instar larvae were recorded before releasing on the diet containing different concentrations of imidacloprid (0-600 μ M). Three replicates for each treatment containing 10 larvae in each replication. The larvae fed with insecticide for 7 days and the final weights and mortality were recorded [10].

Enzyme preparations

Imidacloprid treated and untreated larval midguts were homogenised with two volumes of 0.1 M glycine-NaOH buffer (pH 10.0), centrifuged at 8000 \times g for 20 min at 4 $^{\circ}$ C. The supernatants were used as enzyme source for protease assay [11].

Brush border membrane vesicles (BBMVs) were prepared by the method adopted from [12], where guts were removed from imidacloprid treated and untreated larvae were homogenised with 20mM Tris-HCl buffer (pH 7.5) containing 5mM EGTA, 1mM PMSF and 300mM mannitol, at cold condition. The homogenates were added with equal volumes of 20mM MgCl₂ and allowed to stand on ice for 20min followed by centrifugation at 2500 \times g for 15min at 4 $^{\circ}$ C. The supernatants were stand on ice where as the pelletes were resuspended with the extraction buffer and the process repeated for twice. The resulting supernatants were pooled and centrifuged at 30000 \times g for 30min at 4 $^{\circ}$ C. The pelletes containing BBMVs obtained were suspended in minimum volumes of extraction buffer for aminopeptidase assay.

Effect of imidacloprid on gut proteases

Aminopeptidase activity was measured by incubating brush border membrane vesicles (BBMVs) from imidacloprid fed and unfed larvae using leucine p-nitroanilide (LpNA) as a substrate. Reaction mixture consisted of 50 μ L of enzyme, 20 μ L of 2mM substrate and 630 μ L of Glycine-NaOH buffer (pH 10.0) incubated for 20 min at 37 $^{\circ}$ C. The reaction mixtures were centrifuged at 5000 \times g for 15min after adding 300 μ L of 30% acetic acid. The absorbance was measured at 410 nm [11].

Elastase activity was measured similarly as aminopeptidase, except using N-succinyl-alanine-alanine-alanine p-nitroanilide (SAAApNA), as a substrate. One unit of enzyme activity was defined as the micromoles of p-nitroaniline released per minute per milligram of gut protein [11].

Total protease activity was measured by using 1% azocasein as a substrate in 0.1M glycine-NaOH buffer (pH 10.0). 100 μ L of gut extract was incubated with 500 μ L of substrate and incubated for 30min at 37 $^{\circ}$ C. Then add 200 μ L of 5% TCA followed by centrifugation at 5000 \times g for 15min. Equal volumes of 1N NaOH were added to the supernatants to read the absorbance at 450 nm [11].

Chymotrypsin and trypsin activities were determined by using 30 μ L of 1mM N-glutaryl-L-phenylalanine p-nitroanilide and 10 μ L of 1.2mM N- α -benzoyl-DL-arginine-p-nitroanilide as substrates, respectively. Enzyme (50 μ L) mixed with respective substrates and 0.1M glycine-NaOH buffer (pH 10.0) (to make 700 μ L of final volume), was incubated for 20 min at 37 $^{\circ}$ C and then adds 300 μ L of 30% acetic acid and stand for 10min. The absorbance was measured at 410nm after centrifugation of the samples at 8000 \times g for 10min. Amount of enzyme catalyzing the hydrolysis of 1 μ M substrate per minute at 37 $^{\circ}$ C, was defined as one unit of enzyme activity [13].

Alkaline phosphatase assay

Alkaline phosphatase activity was measured using p-nitrophenyl phosphate (p-NP) as substrate [13]. In brief, assay mixture consisted 50 μ L of 0.1mM p-NP, 900 μ L of 0.1 M Tris-HCl buffer (pH 8.6) and 50 μ L of enzyme, change in the absorbance was measured at 405 nm for 3min. Protein concentrations for all the enzymes assays were measured using bovine serum albumin (BSA) as a standard [14].

Statistical analysis

Data were subjected to One-way Analysis of Variance (ANOVA) using Genstat (14th edition, Version 14.1.0.5943, VSN International Ltd, United Kingdom) software to judge the significance of differences between the treatments by F-test, while the treatment means were compared by least significant difference (LSD) at p < 0.05. Duncan's Multiple Range Test (DMRT) was used to know the differences between treatments. LD₅₀ values were determined by using EPA PROBIT analysis program (Version 1.5).

Results

Bioassay

Imidacloprid inhibited the growth of third instar *H. armigera* larvae in a dose-dependent manner. There was ~67.5% larval growth inhibition and 73.33% reduction in survival rate observed. LD₅₀ value for the insecticide was found to be 531.24 μ M (95% confidence limit) (data not shown).

Effect of imidacloprid on gut proteases

Dose dependent response was observed in gut protease profile of larvae fed with insecticide compared with the control larvae for 24h. A significant decrease in of chymotrypsin (57.14%), trypsin (57.07%) and total proteases (14.81%) were observed at 600µM of imidacloprid. The activities in the control larvae were found to be 0.133±0.008U/mg, 2.05±0.190U/mg and 0.027±0.001 UA, respectively, and the activities of imidaclopride

(600µM) treated larvae were found to be 0.057±0.005U/mg, 0.88±0.008U/mg and 0.023±0.005 UA, respectively (Table 1). Elastase (0.37±0.008 to 0.18±0.049U/mg) and aminopeptidase (0.13±0.002-0.11±0.003U/mg) activities were also inhibited significantly by 51.35%, and 15.38%, respectively, compared with the control larvae, in a dose dependent manner, of the larvae fed with increasing concentrations of imidacloprid (0-600µM) (Table 1).

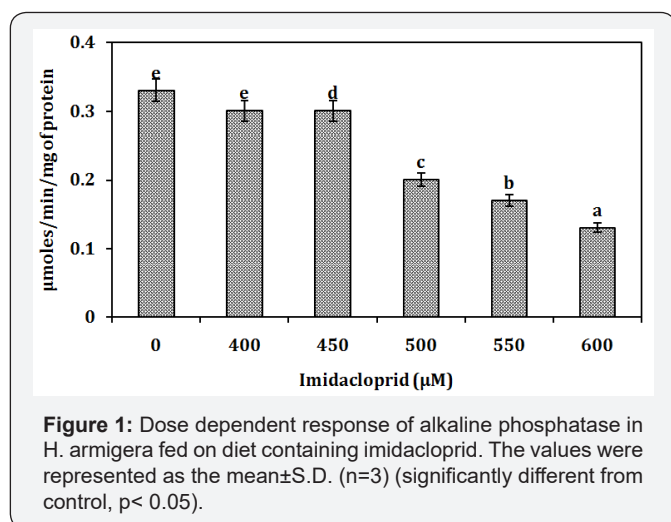
Table 1: Dose dependent response of protease enzymes in *H. armigera* fed on diet containing fimidacloprid. The values were represented as mean±SE (n = 3).

Dose (µM)	Chymotrypsin (U/mg)	Trypsin (U/mg)	Total protease (UA)	Elastase (U/mg)	Aminopeptidase (U/mg)
0	0.133±0.008c	2.05±0.190c	0.027±0.001bcd	0.37±0.008e	0.13±0.002ab
400	0.127±0.003c	1.52± 0.032b	0.031±0.001d	0.35±0.058de	0.16±0.005b
450	0.117±0.008c	1.45±0.127b	0.026±0.006bcd	0.32±0.045cd	0.14±0.005b
500	0.113±0.004bc	1.37±0.054b	0.025±0.007ab	0.30±0.142bc	0.14±0.002ab
550	0.097±0.005b	0.94±0.026a	0.024±0.001a	0.26±0.001b	0.13±0.013ab
600	0.057±0.005a	0.88±0.008a	0.023±0.005a	0.18±0.049a	0.11±0.003a

Column values followed by same letters are not significantly different from each other at p<0.05%.

Alkaline phosphatase activity (ALP)

Significant dose-dependent reduction of alkaline phosphatase activity in the larvae fed on diet containing increasing concentration of imidacloprid (0-600µM). The decline was measured to be 0.33±0.0004 to 0.13±0.001µM/min/mg of protein (Figure 1).



Discussion

Proteases play a vital role in the proper development of insects as they hydrolyze the peptide bonds to liberate free amino acids by the action of endopeptidases and exopeptidases, as they are needed for the growth, survival and reproduction [15]. The decreased activities of chymotrypsin, trypsin and total

protease confers to the antifeedant nature of imidacloprid where the larvae unable to metabolise the diet provided for its survival, growth, and reproduction.

Insects when exposed to protease inhibitors alter their midgut composition by monitoring the proteases secretion [16]. Studies have shown that the food, temperature, and acidity are key factors responsible for production of energy needed for larval development. The food containing imidacloprid directly affected the feeding behaviour of the insect thereby leading to the reduced body weight and delayed growth in a dose dependent pattern (data not shown). Aminopeptidases, catalyses the amino acids hydrolysis from N-terminus of the peptides and proteins was moderately inhibited at higher concentration of insecticide. The physiological condition of insects are related to levels of alkaline phosphatase which can also reflects digestion, absorption, and positive transport of nutrients in the midgut [17]. Tyrosine O-phosphate was shown to be the natural substrate for Alkaline phosphatase [18] in *Drosophila*. Decreased levels of ALP activities provides the lower levels of tyrosine, precursor of dopamine and octopamine, play a vital role in regulation of juvenile hormone (JH), and 20-hydroxyecdysone (20E) [18]. The decreased level of alkaline phosphatase activity in response to increased concentrations of imidacloprid indicates insecticide's harmful effect on insect's digestive system and development.

Conclusion

Our results demonstrate that imidacloprid acts as potent protease inhibitor by inhibiting elastase, aminopeptidase, chymotrypsine, trypsin, total proteases and ALP.

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