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SUCCESSIVE USE OF PROTEINASE INHIBITOR OF *Zizyphus jujuba* LEAVES FOR EFFECTIVE INHIBITION OF *Helicoverpa armigera* GUT ENZYMES AND LARVAL GROWTH

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ABSTRACT

Helicoverpa armigera is a major pest of many tropical crop plants and a voracious feeder of leaves, developing seeds, fruits or bolls leading to drastic reduction in yield. The efficacy of proteinase inhibitor (PI) from *Zizyphus jujuba* (ZJ) leaves in retarding the growth of *H. armigera* larvae and a complete inhibition of digestive and mitochondrial enzymes has been reported. The PI responsible for mortality of *H. armigera* was found to be a 16.8 kD protein having PI, ^{2}-glucosidase and haemagglutinating activity.

Key words: *Zizyphus jujuba*, proteinase inhibitor, *Helicoverpa armigera.*

INTRODUCTION

Helicoverpa armigera of the Lepidoptera family is a serious pest of many important crops and claims a major share in crop losses every year. It is a polyphagous insect pest that feeds on important major crop plants like cotton, tomato, tobacco, chickpea and pigeon pea[1]. Larvae of *H. armigera* are voracious foliar feeders as early instars and later shift to the developing seeds, fruits, or bolls leading to drastic reductions in yield[2].

In recent years, it has assumed the status of national pest. So far this national pest is controlled with the massive application of synthetic pesticides, which not only leaves harmful residues in the food, but also causes adverse effects on non-target organisms and the environment[3]. To circumvent such problems, there is a continuous search for a safe and environmentally friendly alternative to combat the infestation and losses due to the pests. Exogenous chemical means to counteract *H. armigera* attack have become less feasible, mainly due to the development of pesticide resistance in insects and inherent possible environment hazards[4]. The use of genetic engineering technology for the transformation of crop plants for insect resistance has created a new scope in plant breeding. Possibility of utilizing PI gene(s) of plant origin as bio-insecticide for developing insect resistant transgenic crop plant has been fairly accepted line of approach in crop biotechnological program. PIs are polypeptides present in many plants, which in addition to their physiological function provide a natural defense against insect attack[5-7]. PIs block digestive proteinases in insect gut and starve them of essential amino acids[8,9]. They also affect a number of vital processes including proteolytic activation of enzymes and molting[10].

Our initial studies have shown that the PI from seeds of ZJ has a potential to prevent the growth of *H. armigera* larvae (results submitted for publication). In the present study, we report the larvicidal action of PI (trypsin/chymotrypsin) of ZJ leaves against *H. armigera.* On further screening, it was found that the PI is a small molecular weight

protein (16.8 kD) possessing its own b-glucosidase and haemagglutinating activity. Haemagglutinins (lectins) are reported to be widely distributed in the botanical kingdom present in root, stem, bark, seed, fruit coat etc in plants[11]. A few types of heaemagglutinins (chimero lectins) are reported to posses two kinds of domains[12].

The present paper reports the action of ZJPI on digestive and mitochondrial enzymes, identifying itself to be a potent insecticidal protein against *H.armigera.*

MATERIALS AND METHODS

Chemicals

The substrates of all enzymes were obtained from Sigma Chem. Co. U.S.A. All other chemicals were of analytical grade. Double distilled water was used throughout the experiments.

Extraction PIs

Ten percent extract of mature and undamaged ZJ leaves was prepared in 0.2 M sodium phosphate buffer (PH 7.6) containing 0.9% NaCl (PBS) following the method of Varghese and Patil (2005)[13]. The supernatant was subjected to 90% ammonium sulphate precipitation (w/v)[14]. The precipitate obtained after centrifugation was further dissolved in minimum amount of PBS and passed through trypsin ligated Sepharose-4B column prepared according to Hou and Lin (1998)[15]. Molecular weight of the affinity purified fractions was determined by the method of Laemmli (1970)[16].

Rearing of *H. armigera*

Adults of *H. armigera* were collected and reared for two generations on the chick pea based semi-synthetic diet as per the method of Arms *et al* (1972), under the laboratory conditions in the BOD incubator at 27± 2°C and photoperiod of approximately L : D = 13 : 11 [4]. Five to six days old larvae of the subsequent generation were used for the bioassay.

The bioassay studies for larvicidal action were conducted by using diet incorporation method by Czapla and Lang (1990) (17). PI solubilized in PBS was included into the artificial diet in five different doses at 100, 200, 300, 400 and 500 mg/ 5g of the diet at the interval of 24 h to the experimental groups. The control group received the artificial diet without PI. Larval weight and mortalities were meticulously recorded up to seven days. Assay of digestive, mitochondrial and respiratory enzymes were conducted in both experimental and control groups. All the enzymes were assayed in both live and dead larvae to understand the mechanism of action of PI. The experiments were repeated thrice for reproducibility and analysed by single linear regression equation.

Preparation of *H. armigera* gut extract

Midguts isolated by dissecting the larvae were extracted in PBS to get a 30% homogenate. The gut luminal contents were removed by centrifugation at 9670 g for 10 min. at 4 °C. The resulting supernatant was analysed for digestive and respiratory enzymes.

Enzyme assays

Activity of trypsin was assayed by the method of Kakade *et al* (1989), using casein as the substrate (18). Activity of chymotrypsin was assayed following the method of Prabhu and Pattabhiraman (1977), using *N*-acetyl L-tyrosine ethyl ester (ATEE) as the substrate[19]. Activtiy of proteinases was assayed by the method of Kunitz (1977) using casein as the substrate[20]. The activities of a and b-glucosidase were estimated by the method of Murray (1983), using p-nitro phenyl a-D glucopyranoside and p-nitro phenyl b-D glucopyranoside respectively, as substrates[21]. a-amylase activity was determined by the method of Bernfeld (1995), using starch as the substrate[22].

Mitochondrial enzymes succinate dehydrogenase was assayed by the method of Slater *et al* (1952), using sodium succinate as the substrate[23]. Glutamate dehydrogenase activity was assayed by the method of Fahein *et al* (1968), using sodium 2-oxoglutarate as the substrate[24].

To study the *in vitro* effects of PI of ZJ leaves on various enzyme activities, 10 mg of PI was incubated with 500 mg of crude enzyme protein in supernatant for one hour at 37 °C. The residual percentage enzyme activity of the experimental group was compared with enzyme activities of the control group receiving no PI.

RESULT AND DISCUSSION

ZJPI inhibiting both trypsin/chymotrypsin and exhibiting b-glucosidase and HA was purified by affinity chromatography on Sepharose-4B resin. Data presented in table 1 shows the effect of PI of ZJ leaves on mortality and weight loss of *H. armigera* larvae when fed on diet receiving various concentration of PI. Every dose of PI resulted in 100% mortality. The highest dose of PI (500 mg) kills the larvae at an average of 18 h, while the lowest dose (100 mg) killed the larvae at average of 38 h after exposure to the treated diet.

Table - 1: Effect of PI of ZJ leaves on mortality and weight loss of larvae of *H. armigera*

Dose (mg)	Mortality of larvae (h)	% wt. Loss of larvae
100	38	73
200	26	64
300	22	51
400	20	42
500	18	36

It is observed that as the concentration of PI in the diet increases, the time required for the mortality decreases, which is simultaneously followed by a decrease in the body weight of the individual larvae.

Table - 2: Effect of PI of ZJ leaves on enzyme activities of midgut of *H. armigera*

Enzyme assayed	Live larvae % inhibition	Dead larvae % activity
Proteinases	100	0
α-glucosidase	98	0
β-glucosidase	99	0
α-amylase	98	0
Lipase	96	0
Acid phosphatase	95	0
Alkaline phosphatase	100	0
Succinate dehydrogenase	100	0
Glutamate dehydrogenase	60	0

Table 2 presents the percent inhibition of digestive and mitochondrial enzymes of *H. armigera* in both *in-vitro* and *in-vivo* analysis. The gut proteinases, showed 100% inhibition suggesting that the primary site of action of proteinase inhibitors is the digestive system of insect larvae. Reports indicate that *H. armigera* gut proteinases has appreciable amount of trypsin like activity[25]. Trypsin is involved in developmental processes such as molting and synthesis of neuro-peptides, thus trypsin inhibitors can disrupt these processes leading to major damage to the growth and development of the larvae[26].

ZJPI having its own â-glucosidase activity was also found to inhibit other digestive enzymes like a-glucosidases, a-amylase, lipase, acid and alkaline phosphatase. Inhibition of enzymes of digestive tract may cause the indigestion of food leading to the poor intake causing starvation and death of larvae. These type of results were also reported by Deshpande and Patil for proteins of *Dregea volubilis*[27].

Mitochondrial enzyme succinate dehydrogenase (SDH) showed total inhibition when the larvae were fed on a diet incorporated with ZJPI. SDH is an electron carrier of electron transport chain. Complete inhibition of SDH leads to mitochondrial disfunction and energy metabolism of the cells possibly leading to the death of cells and larvae in general[28]. Glutamate dehydrogenase (GDH) on the other hand, showed 60% inhibition. Inhibition of GDH suggests the interference in amino acid metabolism and in synthesis of structural protein leading the loss in body weight of treated Larvae[29].

ZJPI apart from having PI activity also shows lectin like property. Lectins are known to specifically bind to carbohydrate residues of glycoprotein present on the cell surface.

Glycoproteins are the major cell wall constituents of epithelial cells of the luminal gut linings, where they act as ion channels. If these channels are blocked by binding with lectin like proteins, the whole metabolic function of the cell comes to stand still causing deleterious effect and ultimate death of the cell[11].

ZJPI thus appears to be a potent inhibitor of *H. armigera* gut proteinase and mitochondrial enzymes which completely inhibits the larval growth. The gene of such small molecular weight ZJPI once isolated and cloned can effectively be exploited for developing transgenic plants.

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