Proteinase inhibitor in sunflower seed and its influence on growth and development of capitulum borer, Helicoverpa. armigera(Hubner)

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Abstract:

The presence of high proteinase activity was detected in the gut extracts of Helicoverpa armigera(Hubner) at pH 7.6. The seeds of sunflower lines viz., BRS-3(Helianthus annuus), a wild type of H. annuus, H. nuttalli, and H. maximiliani exhibited larval proteinase inhibitory activity in their seed meal extracts which varied from 0.8×10^4 to 28×10^4 inhibitory units per gram of soluble protein. The noctuid larva failed to gain significantl. the biomass, pupal weight and relative growth ratio compared to r. rmal ones when reared on diet added with different levels of inhibitors extracted from seeds of BRS-3. The presence of inhibitor in the larval food caused prolongation of larval period from 23 to 33 days and pupal period from 14 to 23 days. the growth index was reduced from 4.28 in normal ones to 1.15-3.68 under different inhibitory levels. The effect of inhibitors on larval period index pupal period index, pupation index, survival index and success index were also evident.

Introduction:

Helicoverpa armigera is a serious pest of several crop plants including sunflower and it has about 181 host plants (Manjunath et al. 1989). The intrinsic fecundity rate with a maximum of 4394 eggs per female (Hardwick, 1965), an incredible biotic potential, strategy to adopt and withstand various adversities, a challenging inherent survival capacity by acquiring resistance against many pesticides and suspected long range facultative migratory behaviour upto 2000 km have all gone in favour of this pest (Bowden and Johnson, 1976, Farrow and Dally, 1987). The ubiquitous presence of secondary plant chemicals implicated in plant defense against its enemies include either costitutive chemicals of tissues or synthesized ones in response to the pest attack. Among them, in recent past digestive enzyme inhibitors, especially, proteinase inhibitors(PIs) assumed great importance since they are wide spread in nature and most abundant class of proteins in the world (Ryan, 1983). The proteinase inhibitors of cowpea, potato and tomato are well known (Gatehouse and Hilder, 1990) and genes for these could be transferred to other plants using genetic engineering techniques. Further, an inhibitor in commercial formulation, E-64, against Colorado potato beetle and serine proteinase inhibitor against tobacco horn worm have been successfully used. Also, germplasm lines having enhanced resistance levels through PIs can be utilised in breeding

programmes to evolve resistant cultivars (Christeller and Shaw, 1989). Sunflower, Helianthus annuus L. is an important oil seed crop of the globe and in India it is next to groundnut and mustard in importance. H. armigera extensively damages sunflower capitulum and resistant lines are not in sight against this pest. So, an attempt to screen a few selected germplasm lines of these for the presence of PIs and its subtle influence on developmental biology of H. armigera has been envisaged in the present work.

Materials and methods

Insect culture: The larvae of H. armigera collected from field were transferred into vials containing semi-synthetic diet with following composition: whole Kabuli gram 80 g, Brewers yeast 40 g, ascorbic acid 5 g, multimineral and multivitamin capsules 1 each, methyl parabene 2 g, sorbic acid 1 g, gum arabica 2 g,

vitamin E 200 mg, cysteine 0.1 g, streptomycin 0.25 g, formaldehyde (10 percent in water) 2 ml, agar-agar 15 g and water 800 ml. On pupation these were removed from vials and placed in a oviposition chamber made of glass ($50x50x60^3 cm^3$) with a circular hole on the top of the cage. Emerging moths were provided with 10% honey solution in cotton swab placed on the floor of the glass chamber. A strip of wet muslin cloth hung in the chamber facilitated oviposition. Egg collected on these were sterilised with sodium hypochlorite, 0.3% and placed in perforated plastic jar. On hatching, larvae were released on bhendi fruits and allowed to feed for three days and later transferred to vials containing diet.

Seed materials: seeds of BRS-3, Acc.no. 1037, 1471, 1214, 916, H. annuus wild type, H. nuttalli (wild type), H. maximiliani (wild type) were obtained from Project Coordinator (Sunflower), University of Agricultural Sciences, Bangalore, India.

Larval enzyme solution: the larval enzyme solution was prepared according to Applebaum et al, (1964) and Ishaaya et al(1971). A day old sixth instar larvae of H. armigera were dissected in ice cold conditions under binocular microscope and midgut of each larva was separated and transferred to micro-centrifuge tubes(1.5 ml) and stored at -20°C. Midgut tissues suspended in phosphate buffer (7.6 pH) were homogenised and centrifuged in the cold (3°C) at 10,000 rpm for 15 minutes. Supernatant was used as crude enzyme.

Proteolytic activity: The determination of enzyme activity was made following casein digestion method (Birk et al., 1962) and activity was measured at an absorbency of 280 nm (Kunitz,,1947). The reaction mixture consisting of 0.9ml of buffer, 0.1ml of enzyme solution (50 μg protein) was incubated at room temperature for 5 min. One ml of pre-incubated 1% casein solution at 37°C was used to start the reaction. After 30 min by addition of 2ml of 10% trichloro acetic acid (TCA) the reaction was terminated. The reaction mixture on filtering was measured for absorbance at 280

nm per min of digestion of casein under the assay conditions (Birk,1976).

Proteinase inhibitor activity: The dry powdered seeds of sunflower were thoroughly defatted using cold acetone at room temperature. Defatted mixture was suspended in phosphated buffer (0.1M) containing sodium chloride (0.15N) and was shaken for 2 hrs in cold and later centrifuged at 5000 rpm for 30 min at 30° C. Supernatant was taken for assay. Procedure adopted by Kakade et al. (1969) using known amount of casein as substrate for proteinase inhibitory activity was adopted here. A known amount of enzyme extract was taken in an assay system containing phosphate buffer (0.1 M) and sodium chloride (0.15N). To this was added known amount of inhibitor extract. Enzyme and inhibitor were incubated for 10 min at room temperature and the reaction was started by adding known amount of 1% casein. After 30 min at 37° C reaction was terminated by adding 10% TCA. To the control TCA was added before casein. The absorbance of filtrate was read at 280 nm. Decrease in caseinolytic activity in the test system compared with control was taken as measure of inhibitory activity. One proteinase inhibitory unit is that amount of inhibitor which inhibits one enzyme unit under assay conditions.

Developmental studies on diet containing inhibitors: Basic diet without cystein (Gatehouse and Boulter, 1983 and Broadway and Duffy, 1986) was added with three levels of crude inhibitory protein extracted from BRS-3 var. of sunflower @ 40.5, 81.01 and 121.51 mg per 106.5 g of diet through 5,10, and 15 ml of phosphate buffer. These diets in w/w ratio contained inhibitory proteins at 0.038, 0.076 and 0.1140 percent. Three day old larvae were released in each vial on the diet for the study. Each treatment was replicated six times. Larval weight, pupal weight, adult emergence, larval duration, pupal duration and larval mortality, were noted. Several growth indices were also computed (Waldbaur, 1968 and Veerareddy and Bhattacharya, 1990).

Results

The proteinase activity measured at pH 7.6 showed an activity of 1.8×10^5 units per ml of gut extract. The gut of *H. armigera* had 10,891 µg protein per ml extract. The quantitative assay of protein was made using bovine serum albumin as standard. The soluble protein in seed meal extract of sunflower varied from 9.7247 mg/ml in *H. annus* (wild type) to 43.8880 mg/ml of extract in Acc.No. 1037 (Table I). The total soluble protein in defatted seed meal was lowest at 46.03 mg/g in *H. maximiliani* (wild type) to 79.95 mg/g in Acc.No. 1471. The proteinase inhibitory activity was noticed in highest amount in seeds of BRS-3 (2.8×10^5 IU/g) followed by *H. annuus* (wild type) (1.36×10^5 IU/g), *H. nuttalli* (1.04×10^5 IU/g) and *H. maximiliani* (0.8×10^5 IU/g).

The larval development was also studied on the diet added with phosphate buffer at 5,10 and 15 ml as to verify whether the buffer has any influence on the larval development since it is through this amount of buffer that the three levels of inhibitor were added to the diet for the developmental studies. The larval weight in the normal diet increased from 0.96 mg on fourth day to 450.5 mg on the 19th day of development. The larval development on buffer added diet showed an increase from 0.09-0.92 mg on the 4th day to 448-452 mg on 19th day of the development.

The larval and pupal duration, maximum weight gained by the larva, pupal weight, growth index and relative growth ratio on these diets were not showing any significant differences(Table 2). Other parameters such as larval and pupal period index, pupation index, survival index and success index showed no perceptible differences among buffer added diets and normal diet. Following the above finding, we proceeded with the larval development studies on the diets added with different levels of inhibitors along with the buffer solutions. The results of this experiment revealed that larval weight continuously increased under all the treatments with a loss during prepupal period (Table 3). The normal diet supported faster growth than inhibitor added diets. For example, on seventh day larval weight lowered from 4.03 mg to 2.16 mg with the increase in the level of inhibitors from level 1 to 3 in the diet. On the tenth day, larval weight on the normal diet was almost double of its weight under inhibitor level 1 and 2 and four times of its weight under inhibitor level 3. On the 19th day, larval weight on normal diet gained a maximum weight of 458.2 mg which was significantly higher than 200.2 mg and 185.8 mg respectively, attained on diets with inhibitory levels 1 and 2 and it was only 83.2 mg when reared on diets with inhibitory level 3. The prepupal weights were 296.8, 296.1, 272.0 mg (2 larvae) under inhibitor level 1,2 and 3. The larval biomass of 150 mg was accumulated between 19th day and 22nd day of its growth under inhibitor level 1 whereas under inhibitor level 2 and 3 weight gain was maximum during 16th day and 19th day (95.5 mg) and 22nd and 25th day, respectively. The mean of larval weight at six intervals of growth from 4th to 19th day under normal diet was significantly higher than weights under inhibitor level 1 and 2. The maximum weight gain of the larvae of controlled diet and diet added with inhibitor level 1,2 and 3 are 458, 350, 370 and 354 mg, respectively.

The pupal weight corresponding to the above treatments were 289,286,278 and 262 mg, respectively (Table 4). There were clear cut differences in the larval period and pupal period among the treatments. The larval period which was 23 days in control diet prolonged to 26.5, 30.0 and 33.0 days on diets with inhibitor level 1,2 and 3. There was a gradual reduction in relative growth ratio from 0.087 on the control diet to 0.066 under inhibitor level 3. The indices such as larval period index, pupal period index, pupation index, survival index were computed by comparing

the values of these periods on standard diet with those on the test diet added with inhibitors. The average value of above indices was termed as success index. The results on growth indices (Table 5) revealed that with the increasing inhibitor level there was a gradual decrease in larval period index, pupal period index, pupation index, survival index and success index.

Discussion

It had been observed that the midgut of sixth instar larva of *H. armigera* had high level of alkaline proteinase ($1.8\times10^5 U$). The presence of serine proteinase and alkaline proteinase were reported to be present in this insect (Johnston *et al*, 1991). Both trypsin and chymotrypsin were reported by other workers (Rubinstein and Polson, 1983 and Baicheng and Cha-yunsha, 1990). The proteinase inhibitors were recorded on three wild lines viz. *H. annuus* wild type, *H. nuttalli*, *H. maximiliani* and BRS-3 variety of sunflower at 6.8, 5.2, 0.4 and 14.0 units per 50 µg protein and corresponding inhibition of gut proteinase by them was 38.4, 28.6, 2.2 and 79 percent. This constitutes the first report of proteinase inhibitor in sunflower seeds. However, proteinase inhibitors such as trypsin and chymotrypsin were reported in other oilseed crops like groundnut (Sinai *et al*,1972) and mustard (Menegatti *et al*, 1985). *H. armigera* which preferably feeds on reproductive parts of sunflower is likely to depend on proteins from seeds. The presence of proteinase inhibitor in the above lines is likely to act as secondary plant defensive chemical and cause antibiosis in larvae feeding upon them.

The weight of the larvae reared on normal diet and on diets added with increasing levels of inhibitor, monitored at 3 day interval, indicated that larvae could put forth the biomass at a poorer pace on inhibitory diets than on normal diet. All larvae survived on controlled diet and the diet with inhibitor level 1. The percent survival of larvae reduced to 66.6% and 50% when reared on diet with inhibitor level 2 and 3. The growth retardation due to the presence of proteinase inhibitors was evident in case of *Bombyx mori* fed with crude extract of soybean seed meal (Ito *et al*. 1975). Reese (1978) reported inhibition of larval growth of black cut worm due to the inhibition of assimilation and efficiency of conversion of assimilated material or both. The larval growth inhibition due to proteinase inhibitor from soybean was also reported by Shade (1986) on bruchid and Broadway and Duffy (1988) on *Spodoptera exigua*. The two classes of digestive proteinases, serine and cystein endopeptidases are considered to be the most likely targets for inhibition. Possibly serine proteinases which is highly prevalent in lepidopterans is the likely targets of the inhibitors. The reasons for the reduction of the growth of the larvae fed with the diet containing proteinase inhibitors is probably that these inhibitors cause a pernicious hyper-production of trypsin which when coupled with insufficient dietary availability of specific amino acids such as methionine needed for enzyme synthesis results in theinhibition of

growth (Broadway and Duffy, 1986). Larcoque and Houseman (1990) subscribed to the same phenomenon taking place in case of corn borer, Austrania nubilalis fed with soybean inhibitor. Wherever the antimetabolic effects are induced toxicity was more pronounced. It might have caused the death of larvae. The detrimental effect of sunflower inhibitors in the diet on the development of H. armigea larvae was also reflected in the failure of those individual to attain a maximum weight eqivalent to the maximum weight attained by these on the normal diet. The other developmental disarray like prolonged larval period (by 5 days), prolonged pupal period by 9 days and reduction in the pupal weight are also as a consequence of antimetabolic effect of the added inhibitors in the diet.

As to gain further understanding on the nature of the proteinase inhibitor in sunflower seeds there is a need to purify the inhibitor and also test it against purified enzyme. The antimetabolite effect of BRS-3 seeds against H. armigera possibly would impart field resistance to this pest.

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Table 1. Larval protienase inhibitory activity in seed meal extract of sunflower.

Var./Acc.No.	Total soluble protein in seed meal extract (mg/ml)	Total soluble protein in defatted seed (mg/g)	Inhibitory Units per g of soluble protein
BRS-3	24.3033	67.563	2.80×10^5
1037	43.8880	46.813	-
1471	38.8312	79.946	-
1214	38.3576	78.971	-
916	16.7096	52.217	-
H.annuus	9.7247	79.062	1.36×10^5
H.nuttalli	15.9656	49.892	1.06×10^5
H. maximiliani	14.2404	46.029	0.80×10^4

Table 2. Mean duration, maximum weight gained by larval and pupal stages and growth indices of *H. armigera* on normal and buffer added diets.

Buffer (ml/106.5g diet)	Max.wt. gained by larva (mgs) 1	Pupal weight in mgs 2	Larval period in days 3	Pupal period in days 4	Relative gowth ratio 5	Growth index
Control	450.50	272.20	23.00	18.33	0.846	4.34
5ml	450.60	276.20	23.00	17.83	0.832	4.34
10ml	448.04	273.70	23.16	17.50	0.862	4.31
15ml	452.02	270.18	23.50	18.66	0.850	4.25
Mean of 5,10,15 ml	450.22	273.36	23.22	17.99	0.848	4.30

Test of significance and SEm values

	1	2	3	4	5
Buffer level	NS	15.6 NS	9.1 NS	0.4 NS	0.5 NS
Control vs Buffer level	NS	18.04 NS	10.5 NS	0.4 NS	0.6 NS

Table 3. Mean weight(mg) of H. armigera larva during successive days of development on control diet and diet with three levels of inhibitory protein from sunflower seed meal.

Inhibitor level in the diet	larval age(days) 4	7	10	13	16	19	22	25	28	31	Mean
Control(nil)	1.00 ±0.14	6.77 ±3.18	34.77 ±11.46	150.02 ±24.10	282.92 ±57.08	458.22 ±14.87	310.72 ±6.79				155.62
Level 1	1.05 ±0.12	4.02 ±1.97	14.15 ±6.67	40.45 ±3.04	94.87 ±35.58	200.02 ±83.22	350.40 ±21.51	296.80 ±30.14			59.09
Level 2 *	1.00 ±0.18	3.27 ±1.62	14.32 ±4.12	42.67 ±17.95	90.32 ±15.58	185.80 ±47.70	262.35 ±11.14	306.20 ±141.47	296.07 ±56.74	297.10 ±0.00	56.23
Level 3	1.03 ±0.15	2.16 ±0.40	8.33 ±2.48	19.65 ±9.09	42.50 ±9.80	83.20 ±12.92	135.96 ±6.65	302.70 ±10.91	343.20 ±11.35	311.50 ±69.79	26.14
Mean of L1&L2	1.02	3.65	14.23	41.56	92.60	192.91					57.66

	SEm±	CD at 5%
Inhibitor levels	9.05	25.10
Larval age	12.80	35.49
Level x age	9.05	25.10
Control vs Inhibitor level	11.09	30.74

Note: * not included in the statistical analysis
each analysed value is based on four observations

Table 4. Mean duration, maximum weight gain of larval and pupal stages and growth indices of H. armigera on control diet and diet added with different levels of inhibitors extracted from sunflower seed meal.

Inhibitor level	Max.larval wt.gain (mg) 1	Pupal wt. gain(mg) 2	Larval period (days) 3	Pupal period (days) 4	Relative growth 5	Growth index 6
Control diet	458.2±14.9	289.8±5.8	23.0±1.4	14.4±1.1	0.0870±0.005	4.28
Level 1	350.4±21.5	286.8±42.7	26.5±0.6	17.2±1.3	0.0755±0.001	3.68
Level 2	370.2±62.6	278.0±28.9	30.0±1.4	22.7±2.1	0.0665±0.003	2.22
Level 3	354.5±30.9	262.5±47.2	33.0±1.7	23.0±0.00	0.0600±0.002	1.15
Mean of L1 & L2	360.31	282.41	28.25	20.95	0.071	

	1 SEm±	1 CD	2 SEm±	2 CD	3 SEm±	3 CD	4 SEm±	4 CD	5 SEm±	5 CD
Treatment	23.98	70.5	18.28	55.45	1.007	2.96	0.58	1.78	0.0015	0.004
Control vs Inhibitors	25.91	76.2	19.66	NS	1.08	3.2	0.67	2.06	0.0017	0.005

Note: CD is given at 5% level
Level 3 is not included in the statistical analysis.
Each analysed value is based on four observations except pupal wt.(3 obsn.)

Table 5. Success index and its component indices of H. armigera on diets added with different levels of inhibitors from sunflower seed meal.

Inhibitor level	Larval period index	Pupal period index	Pupation index	Survival index	Success index
Level 1	0.858	0.80	1.00	1.00	0.91
Level 2	0.776	0.61	0.66	0.50	0.63
Level 3	0.706	0.60	0.13	0.33	0.49

Indices are ratios between parameters under control diet and test diet and success index is average of all the indices.