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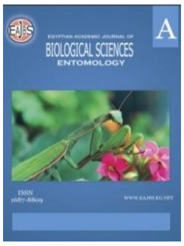

Efficacy of Partially Purified Soybean Protease Inhibitor on The Development of Cotton Leafworm, *Spodoptera littoralis* (Bosid.)

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ABSTRACT

Plant protease inhibitors (PIs) are one of the plant strategies to maintain favorable growth and survival. Soybean trypsin inhibitor is the most well-known of the plant serine proteinase. The present study aims to detect the inhibitory activity of two concentrations of partially purified trypsin inhibitors from seeds of soybean (Glycine max). Comparing the results of feeding larvae on partially purified soybean inhibitors with control, revealed that the larval weight reduction caused by soybean PI was noticed after seven days of treatment. The highest larval weight reduction was obtained due to treatment with SPI at levels SPI 30% and SPI 60%, and there was no significant difference between the two levels. Feeding larvae on SPI 60% caused higher larval mortality about 20% than the control followed by SPI 30% which caused 16% larval mortality. The pupation in treated larvae was delayed two days by comparing to control. The highest weight reduction in pupal weight was 3.5± 0.3 gm at SPI 60% compared with control. Besides, the pupation rate decreased from 83% at SPI 60% to 80% at SPI 30%. Also, the reduction in adult emergency % was recorded at SPI level 30% by 41% and at SPI level 60% by 16% compared with the control. Results reveal that soybean protease inhibitors inhibit the growth and development of *S. littoralis*, and may be used in integrated pest control strategies.

INTRODUCTION

The major biotic stresses to crops are insect pests, responsible for severing crop reduction despite the extensive use of chemical pesticides. Insects often append losses of 15 to 50% of the yield of some crops in various parts of the world and provide infection for various pathogens that append even greater damage (Ferry *et al*. 2004)

The Egyptian cotton leafworm, *Spodoptera littoralis* (Boisd.) (Lepidoptera: Noctuidae) is one of the most destructive pests of several crops such as cotton, corn, peanut, clover, vegetables, and various fruits in Africa, Asia, and Europe (Smagghe and Degheele, 1997; El-Aswad *et al*. 2003 and Ragaei and Sabry, 2011). The cotton leafworm is the destructive pest to about 112 host plants from different families in Egypt as well as in Mediterranean and Middle East countries (Kandil *et al*. 2003; El-Sinary *et al*. 2008 and El-Zoghby *et al*. 2011). In addition to its direct damage, reducing photosynthetic area and reduce the marketability of vegetables and ornamentals (Pluschkell *et al*. 1998)

During the evolution, plants have developed strategies to maintain favorable growth and also guarantee their survival. Enhancing the protective mechanisms, for example, one of these strategies is plant inhibitors) PIs) that allow them to successfully tolerate insects, phytopathogenic microorganisms, and other unfavorable conditions (Stotz *et al*. 1999). The natural products of plant origin are receiving considerable attention to avoid the different disadvantages of insecticide use, as they would be non-hazardous, easy to use, and specific in their action. Previous researchers demonstrated that plants were considered one of the richest sources which could be used as pest control agents. They attended to use plant extracts as toxicants, repellents, synergists, growth regulators, and antifeedant for cotton leafworm (El-Kholy *et al*. 2014).

Plant inhibitors are a common occurrence in the plant kingdom. They are generally small proteins that have mainly been described as occurring in storage tissues, such as tubers and seeds, but they have also been found in the aerial parts of plants (De Leo *et al.* 2002). Among the proteinaceous inhibitors, serine protease inhibitors are abundant in the Leguminosae. The most well-known of the plant serine proteinase inhibitors is the soybean Kunitz trypsin inhibitor (SKTI). It is a seed-specific protein that is expressed in high amounts during its development. The soybean Kunitz trypsin inhibitor has a molecular weight of 21 kDa (Abd El-Latif., 2015). The use of PIs in insect control strategies primarily depends on the inhibition of digestive gut proteinases. Most of the lepidopteran insects, which feed on the economically important crop plants, have alkaline midgut fluid with pH between 9 and 11. Serine proteinases like trypsin and chymotrypsin are the major digestive enzymes responsible for proteolytic activity in their midgut fluid (Prasad *et al*. 2010).

Protease inhibitors inhibit insect gut proteases by binding tightly to the active site, the complex formation being essentially irreversible. The inability to utilize ingested protein and to recycle digestive enzymes results in critical amino acid deficiency, which affects the growth, development, and survival of the herbivore (Abd El-Latif., 2014).

The present work aims to study the biological activity of partially purified soybean inhibitor on larval, pupal, and adult cotton leafworm, *Spodoptera littoralis*.

MATERIALS AND METHODS

A. Insect Culture:

Culture of *Spodoptera littoralis* was originally obtained from the Plant Protection Research Center, Giza, Egypt, that had no history of pesticides. The larvae were reared on clean fresh castor bean leaves, *Ricinus communis* L., in a controlled environmental chamber at 25±2ºC and 65±5% R.H. and a photoperiod of 12:12 hrs (L: D) according to (El-Defrawi *et al*. 1964).

B. Soybean Protease Inhibitor Extraction:

Seeds of soybean (Glycine max) were obtained from the Ministry of Agriculture, Giza, Egypt. The crude extract of seeds was obtained according to Abd El-Latif (2015). Finely ground seeds were defatted by using ice-cold acetone (–20°C). After one hr in acetone, the flour was separated using a Buchner funnel this process was repeated twice. The defatted flour was air-dried overnight and then was extracted by homogenization in a 0.01 M sodium phosphate buffer (1: 10 w/v) which had a pH 7.0, and which contained 0.15 M NaCl. After 10-15 min extract stirred for two hrs at room temperature. The homogenate was centrifuged at 10,000 rpm for 30 min at 4°C. The supernatant (crude extract) passed through two-three layers of cheesecloth. Solid ammonium sulfate was added to the crude extract to obtain a precipitate formed at 0–30%, 30–60%, and 60–90% saturation for this salt. The pellet collected in all fractions (F0-30, F30-60, and F60-90) and was dissolved in a minimal volume of extraction buffer and dialyzed overnight with the same extraction buffer at 4°C.

C. Insect Treatment:

The leaves of castor bean (*R. communis*) were cut into discs dipped in protease inhibitors of soybean partially purified by ammonium sulfate saturation at (SPI 30%) and (SPI 60%) without dilution for 15 seconds then placed in dryness on tissue papers at room temperature. Twenty starved 4^{th} instar larvae of *S. littoralis* were placed in each jar, fed on fresh control or treated leaves for 24 hrs then fresh leaves (untreated) added when the larvae are required. Three replicates for each concentration were used.

Larval weights and mortality were recorded daily through12 days. Pupal weight, pupation %, and adult emergency % were also daily recorded. The obtained data were statistically analyzed.

D. Statistical Analysis:

The results were analyzed using SPSS version 17.0 for windows. Data represented as mean ± SD. Comparison among groups was done using a one-way analysis of variance (ANOVA) followed by LSD test. Statistical significance was set at P≤0.05.

RESULTS

Table (1) showed the effect of partially purified soybean inhibitors SPI 30**%** and SPI 60**%** on the 4^{th} larval instar *S. littoralis*. There are no significant differences in the larval weight between untreated larvae (control), and that treated with SPI 30% through the first seven days post-treatment except at day six which increased significantly. The larval weight reduction caused by soybean PI was noticed on days eight, nine, and ten. On day eight, control larvae gained 8.3± 0.1gm which was significantly higher than larvae fed on SPI F30 %. On day nine control larvae gained 12.5±0.6 gm which was significantly higher than larvae fed on SPI 30 % and SPI 60%, but there was no significant difference between the larval weights of both treatments. After day 10 the control larvae begin to pupate while the pupation of treated larvae was delayed two days on both levels of treatment (SPI 30% and SPI 60%). Feeding larvae on SPI 60% caused higher larval mortality about 20% than the control followed by SPI 30% which caused 16% larval mortality.

Table 1: effects of soybean protease inhibitors on growth and development of 4^{th} larval instar of *Spodoptera littoralis*

Treatments	Mean fresh weight of larvae (gm ± SD)														Larval [%] mortality
	Before treatment	12 h	D1	D2	D3	D4	D5	D6	D7	D8	D9	D10	D11	D12	
Con.	1 ± 0.7^{a}	1.2 ± 0.1^{a}	1.2 ± 0.1^{a}	1.4 ± 0.05^{a}	2.1± 0.1^{a}	3.1 ± 0.3^{a}	3.9 ± 0.5^{a}	4 ± 0.3^{a}	5.2 ± 0.1^{a}	8.3 ± 0.1^{a}	12.5± 0.6^{a}	14.3± 0.9^{a}	pupated	pupated	11 %
SPI 30 %	1 ± 0.1^{a}	1.1 ± 0.1^{a}	1.1 ± 0.1^{a}	1.6 ± 0.2^{a}	2.2± 0.5^{a}	2.9± 0.6^{a}	3.6± $o.5^{a}$	4.5± 0.6^{b}	5.7 ± 1.1^{a}	7.5 ± 1.4^{b}	10.2± 1.6^{b}	12.1± 1^{b}	12.5± 1^{a}	12.5± 1^{a}	16 %
SPI 60 %	0.9 ± 0.05^{a}	1± 0.1^{a}	1 ± 0.05^{a}	1.7± 0.15^{a}	2.2± 0.3^{a}	3 ± 0.3^{a}	3.9± 0.2^{a}	5.2 ± $o.1^{c}$	6.3 ± 0.2^{b}	8.3 ± 0.6^{a}	10.9± 0.2^{b}	12.5± 0.8^{b}	13 ± 0.8^{a}	13. ± 0.8^{a}	20 %

SPI – soybean protease inhibitor

Values are presented as mean ± SD. Means within the same column carrying different letters are significantly different at P≤0.05.

In the table (2), the SPI treatment caused a reduction in pupation and pupal weight in the treated larvae. The highest weight reduction was 3.5± 0.3 gm at SPI 60% compared with 4.4 ± o.4 gm in control, but the reduction was 4 ± 0.5 gm at SPI 30% with no significant differences with control. The pupation rate decreased from 83% to 80% at SPI 60% and SPI 30%, respectively, while in the untreated larvae it recorded 92%. Besides, adult emergency begins after ten days of pupation in control as same as SPI 60%, but it delayed about 36 hrs in SPI 30%. Adult emergency rates recorded 75% and 50% in SPI 60%, and SPI 30% respectively compared with 91% recorded in control.

Table 2: Effects of soybean protease inhibitors on pupal weight, pupation %, and adult emergency of 4th larval instar of *Spodoptera littoralis*

Treatments	Mean pupal weight (gm ±SD)	Pupation (%])	Adult emergency (%)
control	4.4 ± 0.4^{a}	92%	91%
SPI 30%	4 ± 0.5 ^{ac}	80%	50%
SPI 60%	3.5 ± 0.3 ^{c}	83%	75%

SPI – soybean protease inhibitor
Values are presented as mean ± SD. Means within the same column carrying different letters are significantly different at P≤0.05.

DISCUSSION

Protease inhibitors have been determined as natural control agents against herbivorous insects and could reduce the digestive proteolytic enzyme activity and larval development in different species of Lepidoptera and Coleoptera (Chen *et al*. 2007; Ramesh Babu *et al*. 2012 and Aghaal *et al*. 2013).Protease inhibitors have an inhibitory or activating function on serine protease in the midgut of insects, destroying coordination among the proteases which disrupt the digestion process of the insect. This affects their growth, development, and reproduction (Lawrence and Koundal, 2002).

In the current study, the larval weight reduction caused by soybean protease inhibitors (SPI) was observed after seven days of treatment at SPI 30% (at days eight, nine & ten). There was a significant reduction in larval weight, about 15 % smaller than the control. Larval mortality was increased due to treatment with SPIs, the higher mortality was in SPI 60% followed by SPI 30%, respectively. The obtained data coincided with (Abd El-Latif, 2015; Arti and Satwinder(2016). They showed a significant reduction in growth and development among larvae of *Spodoptera litura* fed on an artificial diet with an amount of soybean protease trypsin inhibitors (SPTI) at all stages of the larval growth period. Moreover, Broadway and Duffey (1986) found a significant reduction in growth and development of *Helicoverpa zea* and *Spodoptera exigua* larvae when given a diet incorporated with SPTI. Also, *Chougule et al*. (2008), found that soybean trypsin inhibitor against *Mamestra brassicae* larvae did not cause any mortality during the three days feeding trial but there was a significant effect on the mean larval weight of about 25% smaller than the control.

Furthermore, Johnston *et al*. (1993) found that after 14 days the biomass of larvae fed on soybean kunit trypsin inhibitors (SKTI) was more than 50% lower than that of the control larvae. On the other hand, Gatehouse *et al*. (1999) detected a decrease in *Lacanobia oleracea* (tomato moth) larval growth, survival, and retarded development by incorporation of single concentration SKTI protein into a potato leaf-based artificial diet at two % of total protein. Faktor and Raviv (1997) reported that slower biomass production of *S. littoralis* larvae fed a diet containing two % soybean Bowman-Birk inhibitor (SBBI) compared to the controls.

The obtained results coincided with Gatehouse *et al*. (1999) who feeding *P. brassicae* on cabbage leaf discs coated with different concentrations of SPI there were deleterious effects on the growth and development of the larvae. They attributed their result to the direct inhibition of the digestive enzyme and the depletion of essential amino acids.

In contrast, the present result disagrees with Amorim *et al.* (2008). They reported that there was no effect on larval mass and mortality on *Plodia interpunctella* (pyralid moth) toward SPI when added to the diet.

The present data showed a two-day deletion in the pupation by feeding on SPI and deletion in adult emergency feed on SPI 30% about 36 hrs compared with control, on the other side there was no effect on adult emergency due to feeding on SPI 60% compared with control. It was a great significant difference between the pupal weight of control and treatments; the higher reduction was in SPI 60%. The total life period did not show any reduction with partially purified SPI treatment but show a reduction in the percentage of pupation and adult emergence. The current result was in agreement with Arti and Satwinder (2016). On the other hand, Pompermayer *et al.* (2001) which in agreement with the current result, detected a delaying effect in the developmental time of pupation and adult emergence of *Diatraea saccharalis* (sugarcane borer) feeding on a diet incorporated with SPI. Furthermore, Kuwar *et al.* (2020) observed the significant inhibitory effects of SKTI on larval growth of *Helicoverpa armigera.* This inhibition was reversed and completely compensated for after 48 hrs. They also observed a significant reduction of pupal weight after the 4th day of pupation.

Zhao *et al.* (2019) pointed that the mode of action of SPI may be due to inhibition of the synthesis and secretion of protease enzymes, interfered with the protease activity, and negatively affects the absorption of nutrients, delaying the growth and development.

In conclusion, feeding assay results demonstrated that partially purified soybean inhibitors isolated from glycine max are effective in inhibiting the growth and development of *S. littoralis* larvae.

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ARABIC SUMMARY

فاعلية مثبط بروتييز فول الصويا المنقى جزئيًا على تطور دودة ورق القطن

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مركز ابحاث وقاية النباتات – الجيزة – القاهرة- مصر

تعتبر مثبطات البروتييز النباتية (PIs) من إحدى استراتيجيات النبات للحفاظ على النمو والبقاء المناسبين له. ومثبط التربسين بفول الصويا هو أشهر بروتينات السيرين النباتي. والدراسة الحالية تهدف الى الكشف عن النشاط التثبيطي لمثبطات التربسين المنقاة جزئيًا من بذورفول الصويا (glycine max). وبمقارنة نتائج تغذية اليرقات على مثبطات فول الصويا المنقى جزئياً فقد لوحظ انخفاض فى وزن اليرقات المعامله وقد تم رصد هذا الانخفاض بعد سبعة أيام من المعامله. وتم الحصول على أعلى انخفاض في وزن اليرقات بسبب المعاملة بـ SPI عند التركيزين30٪ SPI و60٪ SPI من البروتين، ولم يسجل فرق معنوي بين المستويين. تغذية اليرقات على مثبط فول الصويا بتركيز60٪SPI ادت الي اعلي وفيات بنسبه 20% مقارنه باليرقات الغير معامله يليها مثبط فول الصويا بتركيز 30٪ SPIوقد أدى إلى 16% نسبة اليرقات المتوفاه. وقد تأخر التعذر يومين مقارنة بالحشرات الغير معامله. كما لوحظ أعلي انخفاض فى وزن العذاري بنسبه 3.5 ±0.03 جرام عند المعامله بالتركيز60 ٪SPI . كما انه قد لوحظ انخفاض في معدل التعذر من 83% الي 80% عند المعامله بتركيز 60٪ SPI يليه 30٪SPI . كذلك تم تسجيل انخفاض في النسبة المئوية لخروج الحشرة الكاملة عند المعامله بمثبط فول الصويا بتركيز30٪SPI بنسبة 41٪ ، وعند تركيز60٪ SPIبنسبة 16٪ مقارنة مع الحشرات الغير معامله. وقد كشفت النتائج أن مثبطات التربسين المنقاة جزئيًا من بذور فول الصويا تمنع نمو وتطور دودة ورق القطن، كم أنه يمكن استخدامها في استراتيجيات مكافحة الآفات المتكاملة.