Gut Proteolytic Profile of Larval *Callosobruchus maculatus* (Coleoptera: Chrysomelidae) in Response to Feeding on Different Fabaceous Host Plants

S. Sarboland¹, F. Mehrkhou¹*, and M. Imani²

**ABSTRACT**

The impact of three different fabaceous host plants including cowpea (*Vigna unguiculata*), chickpea (*Cicer arietinum*), and mung bean (*Vigna radiata*) seeds was investigated using biochemical approaches on possible changes of gut proteolytic activity of the cowpea weevil, *Callosobruchus maculatus* at 30±1°C and 70±5% RH and a photoperiod of 8:16 (L:D). Results revealed that pH of 4.5-5 and 9 was optimal for the activity of larval gut proteases using azocasein and hemoglobin as general substrates. Different serine (*BApNA, SAAPFpNA, PMSF, TLCK, and TPCK*) and cysteine (*Z-Arg-Arg-pNA, Z-Phe-Arg-pNA, and DTT*) specific substrates inhibitors and activator were used as a further proof of the proteolytic profile in the gut of *C. maculates*. Although combinations of serine and cysteine proteases were observed, the cysteine proteases had the highest rate on the studied hosts. The protease activity, especially cystein protease, was the highest on cowpea, which was supported by hemoglobin (0.156±0.045 U mg⁻¹), *Z-Phe-Arg-pNA (2.85 U mg⁻¹)* substrates and DTT (90.00±0.10%) as an activator. Due to the importance and frequency of cysteine proteinases and their effects on biological and physiological process, it would be better to design pest management programs based on cysteine plant proteinase inhibitors as transgenic plants.

**Keywords:** Chickpea, Cowpea, Cysteine proteinases, Digestive enzymes, Mung bean.

**INTRODUCTION**

Legume grains such as common bean (*Phaseolus vulgaris* Linneaus), cowpea (*Vigna unguiculata* Linneaus), chick pea (*Cicer arietinum* Linneaus), lentil (*Lens culinaris* Linneaus) and mung bean (*Vigna radiate* Linneaus) have become important sources of dietary proteins and carbohydrates, but yields are often diminished by infestation of seed weevils. Among the most important pests of stored grains of legumes, the bruchid cowpea weevil, i.e. *Callosobruchus maculatus* (Fabricius) (Coleoptera: Chrysomelidae) has been widespread in most tropical and subtropic countries, namely, Africa, South America, USA, Europe, and Asia (Ouedraogo et al., 1996; Silva et al., 1999; Pereira et al., 2006; Kazemi et al., 2009). It causes serious damage to grains so that over 30 percent of peas are destroyed by this weevil annually in Iran. As legumes provide the cheapest and richest source of plant protein, the control of this pest is essential (Taheri, 1995).

Control of *C. maculatus* currently relies upon application of chemical insecticides (Murad et al., 2008), which has negative effects on environment and human health. One approach to control herbivorous insects is to enhance the resistance of important crops by plant proteinase inhibitors which

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are the major plant defense mechanisms against herbivorous insects (Lawrence and Koundal, 2002). Several proteinaceous inhibitors are present in plant seeds and vegetative organs and act on the digestive enzymes (e.g. amylases and proteinases) (Biggs and McGregor, 1996; Lawrence and Koundal, 2002). Making any interference and inactivation in enzymatic protein digestion by inhibitors could lead to blocking of gut enzymes, resulting in poor nutrient utilization, retarded growth and development, depriving the reproduction efficiency and death because of starvation (Jongsma and Bolter, 1997; Gatehouse and Gatehouse, 1999). These enzymes and correlated genes can be used in the development of efficient and harmless biotechnological tools such as transgenic resistant plants for agricultural insect pest control without negative environmental and human health effects (Pereira et al., 2006). Therefore, knowledge of insect digestive enzymes could be crucial in designing new control methods based on inhibition of digestive enzymes (Azzouz et al., 2005).

The search for possible targets in bruchid physiology that could be used in control strategies made an important advance in the late 1970’s, when Gatehouse and his co-workers published results of their study suggesting that trypsin inhibitors were involved in resistance of a cultivar of V. unguiculata to the cowpea weevil (Gatehouse et al., 1979). It became evident from these studies that bruchid larvae utilize cysteine and aspartic proteinases to digest dietary proteins, which are not affected by serine proteinase inhibitors from legume seeds (Gatehouse et al., 1985; Kitch and Murdock, 1986; Wieman and Nielsen, 1988; Campos et al., 1989; Lemos et al., 1990; Silva and Xavier-Filho, 1991). Due to the importance of cystein protease activity on digestive system of C. maculatus, there is little information regarding the cystein protease activity. Characterization of cystein proteinases in response to the different host plants and inactivation of enzymatic protein digestion by inhibitors could lead to blocking of gut enzymes. The major purpose of this study was to determine the effect of different host plants on digestive proteinase activity of C. maculatus.

MATERIALS AND METHODS

Rearing of Insects

Stock culture of C. maculatus was prepared from stored pest lab in the Department of Agricultural Entomology (established in 2014) at Urmia University. The cowpea weevil was reared on the seeds of three different fabaceous plants such as cowpea (Vigna unguiculata), chickpea (Cicer arietinum), and mung bean (Vigna radiata) seeds. Stock cultures (9-cm diameter and 12-cm height), were reared in plastic jars containing seeds of the mentioned host plants for two generations before being used in the experiments. The insects were kept at 30±1˚C and 70±5% RH and photoperiod of 16:8 (D:L).

Preparation of Samples from Insects and Seeds

The fourth instar larvae were cold immobilized and dissected to remove the whole gut in 250 mM of NaCl (Silva et al., 1999). Only actively feeding larvae with their gut tracts filled with food were chosen for dissection. After the removal of the whole gut, the adhering unwanted tissues were removed, then, the gut tissue was homogenized and transferred to 1.5 mL microfuges and was centrifuged at 13,000 (g) for 20 minutes at 4˚C. The clear supernatant was transferred to a pre-chilled
microtube. The samples were stored at -20°C until further use. The seeds of fabaceous were ground by electrical grinder, and the resultant flour was kept at 4°C until further use.

**Protein Quantification**

Protein concentration of gut larvae was determined by the method of Bradford (1976) using Bovine Serum Albumin (BSA; Bio-Rad, biorad.com) as the standard (0.1, 0.3, 0.5, 0.7, 0.9, 1.0 and 1.2 mg mL⁻¹). Protein content of fabaceous flours were quantified using BSA as standard according to Bradford (1976) with minor modifications. In brief, a quantity (200 mg) of each host plant flour was homogenized in distilled water (10 mL), centrifuged at 13,000×g for 10 minutes, and 100 μL of the homogenate was added to 3 mL of Bradford reagent. The samples were incubated in darkness at 37°C, and absorbance was read at 595 nm (Bouayad et al., 2008).

**General Proteolytic Activity**

General proteinase activity of larval guts was determined using azocasein (Sigma-Aldrich, A2765) (pH 2–12) and hemoglobin (pH 2–12) as substrates at broad pH range. The universal buffer system (50 mM sodium acetate–phosphate–borate) was used to determine the optimal pH of proteolytic activity (Elpidina et al., 2001). For azocaseinolytic activity, the reaction mixture consisted of 30 μL of 2% azocasein solution in 50 mM universal buffer of specified pH and 15 μL enzymes. The reaction mixture was incubated at 37°C for 60 minutes. Proteolysis was stopped by addition of 30 μL of 30% TriChloroAcetic acid (TCA). Appropriate blanks in which TCA was added first to the substrate were prepared for each assay. Precipitation was achieved by cooling at 4°C for 60 minutes and the reaction mixture was centrifuged at 16,000×g for 10 minutes. An equal volume of NaOH 1N was added to the supernatant and the absorbance was recorded at 440 nm using a microplate reader (BioTek ELX808).

The assay with a hemoglobin substrate was performed according to Cohen (1993) with slight modifications. Hemoglobin solution (2%), 30 μL, was added to 90 μL of the desired pH universal buffer. Reactions were started with the addition of 15 μL enzyme extract and incubation at 37°C for 120 minutes. For reaction termination, 30 μL of 30% TCA was added to the reaction mixture. Precipitation was achieved by cooling at 4°C for 60 minutes and the reaction mixture was centrifuged at 16,000×g for 10 min. Appropriate blanks were used as described before. The peptides liberated from hemoglobin were estimated using Folin-Ciocalteu reagent at 650 nm (Folin and Ciocalteu, 1927).

**Specific Protease Activity**

Trypsin- and chymotrypsin-like activities (as two sub-classes of serine proteases) were assayed using final concentrations of 1 mM BApNA (Nα-Benzoyl-L-Arginine4-NitroAnilide hydrochloride; Sigma-Aldrich, B3279) and 1 mM SAAPFpNA (N-Succinyl-Alanine-Alanine-Proline-phenylalanine-p-NitroAnilide; Sigma-Aldrich, S7388) as substrates, respectively. A reaction mixture consisted of 10 μL enzyme, 85 μL of universal buffer at broad pH range (8-12), and 5 μL of the above mentioned substrates. The reaction mixture was incubated at 37°C for 10 minutes before adding 30 μL 30% acetic acid to terminate the reaction. The absorbance of the resulting mixture was measured spectrophotometrically at 405 nm by p-nitroaniline release.

Two specific substrates benzyloxycarbonyl-Arg-Arg-pNA (Sigma-Aldrich, C8536) and benzyloxycarbonyl-Phe-Arg-pNA (Sigma-Aldrich, B2133) were used for cysteine proteinase assays. Hydrolysis of the substrates was determined by measuring the absorbance by p-
RESULTS

Protein Quantification of Larval Gut Extracts and Fabaceous Seed Flours

Statistical tests indicated significant differences in the content of protein among flour of various host plants tested (F=1.219; df=2.6; P<0.05). The highest content of protein was obtained in cowpea (0.871±0.021 mg mL$^{-1}$) followed by chickpea (0.866±0.014 mg mL$^{-1}$) and mung bean (0.777±0.052 mg mL$^{-1}$). The protein concentration of gut extracts of the larvae reared on different fabaceous host plants was significantly different (F=1.568; df=2.6; P<0.05). The protein content in gut of the larvae fed on cowpea, chickpea and mung bean were 1.43, 1.10, and 1.50 mg mL$^{-1}$, respectively (Table 1).

Protease Activity

The effect of pH on proteolytic activity of the gut extract from C. maculatus larvae is presented in Figure 1. Proteolytic activity with azocasein as a protein substrate showed that the substrate was hydrolyzed over a broad range of acidic and alkaline pH values with a peak at 4-5 and 9 (Figure 1-a). Similarly, the same pattern was observed using hemoglobin substrate at acidic (pH 4) and alkaline (pH 8) (Figure 1-b) on the three mentioned host plants. Serine and cysteine proteolytic activity data (P<0.05) by general substrates from C. maculatus fourth instar larvae reared on

Table 1. Mean (±SE) protein contents (mg mL$^{-1}$) of flours and gut extract from fourth instar gut of Callosobruchus maculatus, feeding on different fabaceous host plants.$^a$

<table>
<thead>
<tr>
<th>Hosts</th>
<th>Protein content of flours (mg mL$^{-1}$)</th>
<th>Protein content of gut larvae (mg mL$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cowpea</td>
<td>0.871±0.021$^a$</td>
<td>1.43 ± 0.011$^a$</td>
</tr>
<tr>
<td>Chickpea</td>
<td>0.866±0.014$^b$</td>
<td>1.10 ± 0.023$^b$</td>
</tr>
<tr>
<td>Mung bean</td>
<td>0.777±0.052$^c$</td>
<td>1.50 ± 0.047$^a$</td>
</tr>
</tbody>
</table>

$^a$The means followed by different letters in the same columns are significantly different (Tukey, P<0.05).
The effect of pH on the proteolytic activity of larval gut extracts from *Callosobruchus maculatus* on the substrates azocasein (a) and hemoglobin (b). Various fabaceous are shown in Table 2. The lowest serine proteolytic activity was in larvae reared on chickpea using azocasein (0.002±0.0001 U mg⁻¹) and hemoglobin (0.032±0.001 U mg⁻¹), whereas the highest activity was on cowpea and mung bean using both substrates (Table 2). In the case of cysteine proteinase activity, although the cysteine activity was not affected using hemoglobin substrate, it was higher than serine proteinase activity by both substrates (Table 2).

**Specific Proteolytic Activity**

The results obtained when assaying larval digestive extracts (susceptible hosts: cowpea) with specific substrates showed the presence of both trypsin- (100.00±10.20%) and chymotrypsin-like (4.00±0.001%) activities (Figure 2-a). The substrates BApNA and SAAPFpNA were hydrolyzed at alkaline pH with maximum activity at pH 8-10, respectively. The gut extract showed hydrolytic activity against the substrates (Z-Arg-Arg-pNA) and (Z-Phe-Arg-pNA), indicating the presence of cysteine protease which hydrolyzed at acidic pH, with maximum activity at pH 3-4, respectively (Figure 2-b) on cowpea.

There was a significant difference among different host plants, regarding specific activity value of trypsin (F= 2.439; df= 2.6; P< 0.05), Cathepsin L (F= 1.003; df= 2.6, P< 0.05) and Cathepsin B (F= 0.465; df=...
Table 2. Mean (±SE) serine (U mg⁻¹) and cysteine proteolytic (U mg⁻¹) activities of the gut extracts from the fourth instar larvae of *Callosobruchus maculatus* on different fabaceous at optimal pH using general substrates.

<table>
<thead>
<tr>
<th>Hosts</th>
<th>Serine activity (U mg⁻¹)</th>
<th>Cysteine activity (U mg⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Azocasein</td>
<td>Hemoglobin</td>
</tr>
<tr>
<td>Cow pea</td>
<td>0.003±0.0002</td>
<td>0.037±0.002</td>
</tr>
<tr>
<td>Chick pea</td>
<td>0.002±0.0001</td>
<td>0.032±0.001</td>
</tr>
<tr>
<td>Mung bean</td>
<td>0.0035±0.001</td>
<td>0.077±0.014</td>
</tr>
</tbody>
</table>

*The means followed by different letters in the same columns are significantly different (Tukey, P<0.05).*

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Figure 2. Tryptic (a); chymotryptic (b); cathepsin L (c), and cathepsin B (d) activity of larval gut extract of *Callosobruchus maculatus* fed on susceptible host (cowpea).

The specific activity values of cathepsin L determined for Z-Phe-Arg-pNA were 2.855, 1.721, and 1.517 (U mg⁻¹) substrate hydrolyzed on cowpea, chickpea, and mung bean, respectively. The specific activity values of cathepsin B determined for Z-Arg-Arg-pNA were 1.60, 2.252, and 1.936 (U mg⁻¹) substrate hydrolyzed on cowpea, chickpea and mung bean, respectively, which was the highest rate by the larvae that fed on chickpea (Figure 3).
**Gut Proteolytic Profile and Fabaceous Host Plants**

**Effects of Inhibitors and Activator on Protease Activity**

The total proteolytic activity of larval gut extract was further characterized using general and specific serine proteinase inhibitors and cysteine activator (Table 3). It was found that DTT compound affecting Cysteine proteases significantly changed proteolytic activity, confirms the presence of cysteine proteases in the gut extract of *C. maculatus* (Table 3). Therefore, the highest cysteine activation was observed by DTT on cowpea. PMSF, as a serine protease inhibitor, significantly decreased proteolytic activity along with trypsin-like (TLCK) and chymotrypsin-like (TPCK) (Table 3). However, considerable inhibition of larval azocaseinolytic activity by PMSF, TLCK, and TPCK was observed for trypsin-like proteinases compared with chymotryosin-like proteinases. The highest inhibition by PMSF and TLCK inhibitors were observed in gut of larvae fed on cowpea as (13.63±0.01%) and (11.93±1.15%), respectively (Table 3).

**DISCUSSION**

Host plants have a critical role in the fitness of herbivorous insects because their

**Table 3.** The effects of some protease inhibitors and activator, on the general proteolytic activity from gut extract of *Callosobruchus maculatus* on different fabaceous at optimal pH using general substrates.\(^a\)

<table>
<thead>
<tr>
<th>Hosts</th>
<th>PMSF</th>
<th>Inhibition (%)</th>
<th>TPCK</th>
<th>Activator</th>
<th>DTT (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cow pea</td>
<td>13.63±0.01(^a)</td>
<td>11.93±1.15(^a)</td>
<td>3.70±0.005(^b)</td>
<td>90.00±0.10(^a)</td>
<td></td>
</tr>
<tr>
<td>Chick pea</td>
<td>13.50±0.06(^a)</td>
<td>11.65±0.03(^a)</td>
<td>4.20±0.023(^b)</td>
<td>12.10±3.20(^b)</td>
<td></td>
</tr>
<tr>
<td>Mung bean</td>
<td>5.28±0.001 (^b)</td>
<td>4.70±0.01(^b)</td>
<td>6.10±0.32(^a)</td>
<td>12.00±2.20(^b)</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) The means followed by different letters in the same columns are significantly different (Tukey, \(P<0.05\))
components could directly affect the digestive processes of insect pests (Chown and Nicolson, 2004). Changes of proteolytic activity of different host plants and their transgenic varieties have been reported in different insects (Chen, 2008). Results of the current study demonstrated the presence of major proteases in the gut of *C. maculatus*, and their changes after feeding on different host plants.

According to the obtained results, the flour of mentioned fabaceous hosts considerably influenced the digestive enzymatic activity of fourth instars of *C. maculatus*. It is generally accepted that the insect larvae regulate the release of digestive enzymes in response to the level of metabolites ingestion (Lwalaba et al., 2010). Since variations in protein content of the hosts may lead to differences in the proteolytic activity of *C. maculatus*, the highest protein content of gut extract and proteolytic activity were detected in the larvae reared on cowpea, which is attributed to the high protein content of this host.

The azocasein and hemoglobin are routinely used as conventional substrates for detecting general proteolytic activity in many cases (Cohen, 1993; Elpidina et al., 2001). Our data show that the proteinases in gut extracts from *C. maculatus* larvae are capable of hydrolyzing both substrates. Gut pH is a major factor affecting the regulation of enzymatic reactions in digestion (Terra and Ferreira, 1994). Experiments to determine the optimal pH for general proteolytic activity in the gut of *C. maculatus* demonstrated two peaks for the gut extract at acidic and alkaline pH using azocasein and hemoglobin (2%), suggesting that there were cysteine and serine proteinase activities in the gut extract of larvae. According to the obtained results, the most conventional proteinase activity happens at acidic pH (2-5) compared with alkaline pH (8-9), and it seems that the cysteine proteinases were the major hydrolyzing enzyme in the gut extract of *C. maculatus* compared with serine proteinase. This was due to the fact that in many Coleoptera, the main endopeptidases have cysteine or aspartic acid at their active centers (Murdock et al., 1987), which are primarily responsible for protein digestion in *C. maculatus* (Lawrence and Koundal, 2002). Investigations of other coleopteran species also show that the cysteine proteases are the prevailing proteolytic enzymes in their gut according to optimal pH of their activity, specific substrates and inhibition assays (Kitch and Murdock, 1986; Lemos et al., 1990; Murdock et al., 1987; Campos et al., 1989).

The ability to hydrolyze specific synthetic substrates, the elucidation of the pH at which maximal hydrolysis occurs, and their sensitivity to protease inhibitors confirmed the presence of trypsin-like and chymotrypsin-like activities in gut extracts of insects (Hosseininaveh et al., 2007). The specific substrates including trypsin, chymotrypsin, cathepsin L and cathepsin B were used to prove the presence of protease activity which is involved in digestion. In the case of serine proteases, trypsin-like, and chymotrypsin-like proteases were active in gut extract, but trypsin-like proteinase had significant activity in the gut compared with the chymotrypsin-like proteases, which is supported by specific serine proteinase inhibitors on cowpea (Table 3). Larval proteinases with alkaline pH optima (8 and 10) hydrolyzed BApNA and SAAPFpNA, respectively. Alkaline pH values are optimal for trypsin and chymotrypsin-like proteases activities. The presence of serine proteases has been previously reported in different species of beetles, such as *Lasioderma serricorne* Fabricius (Oppert et al., 2002), *Tribolium castaneum* Herbst (Oppert et al., 2003), *Tenebrio molitor* L. (Vinokurov et al., 2006), *Trogoderma granarium* Everts (Dermestidae: Coleoptera) (Hosseininavesh et al., 2007) and *Plagiodera versicolora* Laicharting (Coleoptera: Chrysomelidae) (Zibaee and Hajizadeh, 2013), *Oryctes elegans* Prell (Coleoptera: Scarabaeidae) (Saberi Riseh et al., 2014).

Further studies with specific cysteine proteinase substrates (Z-Phe-Arg-pNA and Z-Arg-Arg-pNA) with optimal pH (4 and 3, respectively), found in the gut. Although the cathepsin B had higher activities in the gut extract of larvae fed on chickpea, the larvae fed on cowpea had the highest cathepsin L
activity in their gut. Combinations of serine and cysteine proteases have been reported in many coleopteran beetles (Oppert et al., 2002, 2003; Vinokurov et al., 2006; Hosseininaveh et al., 2007; Zibaee and Hajizadeh, 2013).

It has been well understood that insect reared on different host plants or transgenic plants containing heterologous anti-feedants like plant protease inhibitors had different digestive enzymatic responses (Saikia et al., 2011). Plant species have many allelochemicals that may affect the digestive physiology of insects. Polyphagous insects, which are associated with a wide range of host plants, are more likely to encounter a wide range of biosynthetically unrelated allelochemicals (toxins) in their host (Zibaee and Hajizadeh, 2013). In fact, lower activities of specific proteases in the gut of insects may indicate that seed proteins are not efficiently utilized due to the presence of allelochemicals and plant inhibitors. Plant inhibitors of proteinaceous enzymes are part of the reserve storage proteins of seeds and have been also considered to be a part of the constitutive and inducible array of defense mechanisms against attack by insects (Blanco-Labra et al., 1996).

The effect of four leguminous species (cowpea, green gram, chickpea, and lentil) on biological and demographic parameters of C. maculatus has been studied by Kazemi et al. (2009). Based on their findings, the larvae reared on cowpea had the shortest developmental time and the highest population parameters (e.g. net reproductive rate and intrinsic rates of increase), suggesting the suitability of cowpea compared with the other tested leguminose species. The enzymatic current study can be interpreted in line with their findings, as compared with the cystein protease, it is due to their inhibition by trypsin inhibitors (Lemos et al., 1990; Silva and Xavier-Filho, 1991), resulting in hyperproduction of cystein protease. There is some evidence regarding the importance and frequency of these enzymes, which are responsible for life process (Murdock et al., 1987; Lawrence and Koundal, 2002).

**CONCLUSIONS**

The results of this study showed that C. maculatus had different types of specific proteases in the gut extracts of larvae, suggesting the adaptation of this insect to different host plants. Changes of proteolytic activity due to feeding on different host plants indicate a correlation between digestive proteolytic activity and nutrient composition of the various diets, and reflect the adaptive nature of the polyphagous pest (Zibaee and Hajizadeh, 2013).

This study indicates that the fabaceous hosts affect the type and frequency of digestive enzymes in C. maculatus larvae. Although, both serine and cysteine proteinases activity were observed in gut extract, the cysteine proteinases were the common proteinases in gut of C. maculatus larvae and the cathepsin L was the main endoproteinases in the larval digestive system which fed on cowpea. For pest management programs, it is recommended to use transgenic plants with cysteine protease through biotechnological approaches, because of their importance and frequency in digestive system process.

**ACKNOWLEDGEMENTS**

We would like to thank Dr. Arash Zibaee and Dr. Sorayya Naem at Faculty of Agriculture and Veterinary in Guilan and Urmia University, respectively for allowing us to use some laboratory equipment for enzyme assays work.

**REFERENCES**


پروفایل پروتئینی روده لارو سوسک وی‌کارنی‌های حیوانات *Callosobruchus maculatus* (Coleoptera: Chrysomelidae) در واکنش به تغذیه از میزان‌های مختلف حیوانات

س. سرنلند، ف. مهرخو، و. ایمانی

چکیده

تأثیر سه میزان مختلف حیوانات شامل لوبیای چشم بلبلی (*Cicer arietinum*), نخود، (*Vigna unguiculata*), و *Vigna radiata* (وری تغییرات احتمالی فعالیت پروتئزیکی سوسک *Callosobruchus maculatus* (Coleoptera: Chrysomelidae) با استفاده از روش‌های به‌شیمیایی در شرایط دما ۳۰±۱ درجه سانتی‌گراد، رطوبت نسبی ۵۰±۱۰ و دوره روش‌سنجی ۱۶۰ روز‌سنجی به تاریکی مورد مطالعه قرار گرفت. نتایج نشان داد که بهینه پایی فعالیت پروتئزی روده لاروها با استفاده از سوبرترهای PH به‌طور گسترده‌ای عملی آروکارکنی و هموگلوبین ۴ و ۹ بود. از سوبرترها، مهارکننده فعال یک‌نظامی تهیه شده‌است. فعالیت پروتئزی سیستمی و اثرات آنها در حفظ دمای و فعالیت آنزیم‌های آنزیم‌پذیر و زیستی، به‌طور مستقیم یا باعث بهبود بیماری‌های مدربریت بر اساس مهارکننده‌های پروتئزی سیستمی در قالب گیاهان تراریخته طراحی شد.