Article

Biochemical characterization of pectinase activity from the digestive midgut fluid of larvae and adult of the Colorado potato beetle, *Leptinotarsa decemlineata* (Col: Chrysomelidae)

Razieh Karimi, **Majid Kazzazi**, **Mohammad Vatanparast**
Department of Plant Protection, College of Agriculture, Bu Ali-Sina University, Hamedan, Iran
E-mail: mkazzazi@basu.ac.ir

Received 17 November 2014; *Accepted 25 December 2014*; *Published online 1 March 2015*

Abstract
The study of pectinase enzyme in potato leaf beetle that is the most important pest of potatoes consider as an effective way to develop control methods because this enzyme is important in degrading plant cell wall. Pectinase enzyme was studied in midgut of Colorado potato beetle (CPB). This enzyme was extracted from the midgut of larvae and adult of CPB and then their important features were examined by specific substrate, pectin 1%. The optimum enzyme activity in 4th instar larvae and adult occurred at pH 5-6 range. Effect of temperature on enzyme activity were examined, the results suggest that the pectinase in midgut of 4th instar larvae and adult shows maximum activity at temperature 40°C and 35°C, respectively. Zymogram analysis showed presence of two activity bands for pectinase enzyme. The effect of various chemical compounds on the activity of enzyme showed that SDS, Urea and Tris reduced the enzyme activity. NaCl and $CaCl_2$ increased this enzyme activity in 4th instar larvae and adult of CPB. This is first report of pectinase activity in *L. decemlineata.*

Keywords activity; Colorado potato beetle; midgut; pectinase; Zymogram.

Arthropods
ISSN 2224-4255
URL: http://www.iaees.org/publications/journals/arthropods/online-version.asp
RSS: http://www.iaees.org/publications/journals/arthropods/rss.xml
E-mail: arthropods@iaees.org
Editor-in-Chief: WenJun Zhang
Publisher: International Academy of Ecology and Environmental Sciences

1 Introduction

Colorado potato beetle (CPB), *Leptinotarsa decemlineata* (Coleoptera: Chrysomelidae)] is one of the most important pests of potato in the world, that cause significant damage of quality and quantity value to this product (Hare, 1990). If this pest is not controlled causes all shrubs until middle of the season being defoliate, completely (Ferro and Boiteau, 1993). The complete removal of leaves, potato function decrease, more than 50% (IGRC-BARI et al, 1999). CPB management by Intensive applications of pesticides is the cause of resistant populations (Forgash, 1985; Ioannidis et al., 1991; Stewart et al., 1997), furthermore, this method has negative effects on food derived from sprayed plants, environment and non-target species (Sinden et al., 1991). So there is a need for alternative methods for pest control (Wolfson and Murdock, 1987). The study of

digestive enzymes in insects is important because enzymes are included important part of the digestive. Digestive enzymes are present in insects based on the type of food diet by them (Wigglesworth, 1997). Pectin is a main compound of the lamella of plant cell wall (Jarvis, 1984) that by pectinase enzymes break down into its constituent units (Galacturonic acid) (Collmer et al., 1988). Pectinases are compound group of enzymes that most of these enzymes are pectin methylesterase, polygalacturonases and pectin lyases (Djordjevic et al., 1986; Pifferi et al., 1989). Pectinolytic enzymes are produced in bacteria, fungi, yeasts, insects, nematodes, protozoa and plants. This enzyme exists in Ortopthera, Coleoptera, Lepidoptera, Heteropthera, Diptera and thrips (Vonk and Western, 1984). Pectinolytic enzymes has been reported in *Sitophilus oryzae* (Coleoptera: Curculionidae) (Shen et al., 1996), *Schizaphis graminum* (Homoptera: Aphididae) (Runlin et al., 1990), *Hypera postica* (Coleoptera: Curculionidae) (Vatanparast & hosseininaveh, 2010), *Diaprepes abbreviatus* (Doostdar et al., 1997), *Eurygaster integriceps* (Hem: Scutelleridae) (Vatanparast et al., 2010) and in Chrysomelidae family (Girard and Jouanin, 1999; Reek et al., 1999).

Considering the importance of carbohydrases in the digestive of carbohydrates in insects such *L. decemlineata,* Any disorders in the enzymes function can be prevented digestion of carbohydrate sources; Pectinases are one of the most important carbohydrases, That interfere at the action of these enzymes can be considered as a way to control of this pest; Therefore, knowledge of the biochemical properties of digestive enzyme has highly the valuable. This paper reports some biochemical characteristics of one main polysaccharide degrading enzymes, pectinase, in the alimentary canal of the pest of elm, *L. decemlineata*. The resulted information confidently will guide to new strategies for the management of this pest. To the best of our knowledge, this is the first report on digestive pectinase in the Colorado potato beetle. In this study, pectinase enzyme was studied in midgut of 4th instar larvae and adult of Colorado potato beetle.

2 Materials and Methods

2.1 Insects

L. decemlineata were collected in two consecutive years at May from potato fields in Hamedan, Larvae were placed in ventilated containers and transported to the laboratory where they were reared on potato leaves. Fourth instar larvae and adults of CPB were used in experiments.

2.2 Sample preparation

Midgut of 4th instar larvae and adults separated according to Cohen (1993) method with some modifications, insects were immobilized on the ice and then their body dissected and their midgut separated by the described tools. Ten isolated midguts were transferred into a microtube containing 1ml citrate-phosphate buffer. The midguts were then homogenized using a hand-held glass grinder at 4˚C and then were centrifuged with 15,000 ×g for 15 minutes in 4˚c. The resulting supernatants were passed through a filter paper and then were transferred to new tubes and maintained at -20 °C for further uses.

2.3 Protein determination and pectinase activity assay

Protein concentration was determinate according to the Bradford method (1976). Bovine serum albumin was used as standard. In the first before any test, extracted protein of adult and larvae of CPB's midgut have been changed to similar concentrations. Pectin 1% was used as substrate of pectinase enzyme. Measuring of the enzyme activity performed at 40˚C temperature in specific buffer; 15 microliter of enzyme solution, 20 µl of specific substrate and 80 µl of buffer were incubated for 60 min in 40˚C. Then reaction stopped by adding 50 µl of 3, 5-Dinitrosalicylic acid (DNS) then was placed in boiling water for 10 min. The absorbance was measured at 540 nm.

2.4 pH profile of pectinase activity

The effects of pH on the activity of midgut pectinase were assayed. The optimum pH for pectinase activity was determined using citrate-phosphate-borate buffer at a pH range of 2 to 10. The assays were performed according to the section “Pectinase activity assay”.

2.5 Effect of temperature and stability on pectinase activity

The effect of temperature on activity of midgut pectinase was assayed. Incubation of the reaction mixture was done at a temperature set of 5, 27 and 40°C for 1, 2, 3, 4, 5, 6, 7 and 8 day. The assay was performed according to the section “Pectinase activity assay”.

2.6 Effect of activators and inhibitors on pectinase activity

To experiment the effect of several ions on enzyme activity, assays were performed in the presence of different concentrations of chloride salts of Na^+, K^+, Ca^{2+}, Mg^{2+} (5, 10, 20 and 40mM), sodium dodecyl sulfate (SDS) and Tris (10 and 20 mM) and 0.5, 4 and 8 M of Urea to the reaction mixture and activity was measured after 60 min.

2.7 Visualization of pectinase activity

In-gel pectinase assay was performed using SDS-PAGE for visualizing pectinase activity. Enzyme extract was diluted in electrophoresis sample buffer contained 25% stacking buffer (0.5 M Tris–HCl; pH6.8), 20% glycerol, 2% SDS, 0.005% (w/v) bromophenol blue) and loaded in gel of 5% stacking and 10% separating polyacrylamide gels. The substrate pectin was incorporated into the separating gel at final concentration of 1%.After electrophoresis, the gel was washed in citrate buffer containing 2.5% (v/v) triton X-100 for 45min and then incubated in citrate buffer for 60min with gentle shaking. Finally, it was stained with Ruthenium red (0.03%) and the bands of pectinase activity were appeared as clear areas in the field of red background of the gel.

3 Results and Discussion

In order to observe pectinase activity in this insect was used from the substrate-specific (pectin 1%). Pectinase activity was obviously determined in 4th instar larvae and adults. Specific activity was calculated, its values in 4th instar larvae and adults were determined 0.05 U/mg and 0.06U/mg, respectively. Zymogram analysis also shows that this enzyme is present in the midgut of CPB. Several different groups of enzymes are referred to as pectinase which are common at pectin degradation (Collmer et al., 1988). Pectinase seems are present in the Orthoptera, Coleoptera, Hemiptera, Diptera and Tricoptera (Vonk and Western, 1984).

The effect of pH on pectinase activity from pH 3 to 9 was performed using citrate-phosphate-borate buffer. The maximum enzyme activity in 4th instar larvae and adults occurred at pH 5-7 range, enzyme activity decreased after pH 7. Level of Enzyme activity reached less than 20% showed at Fig. 1 and 2.

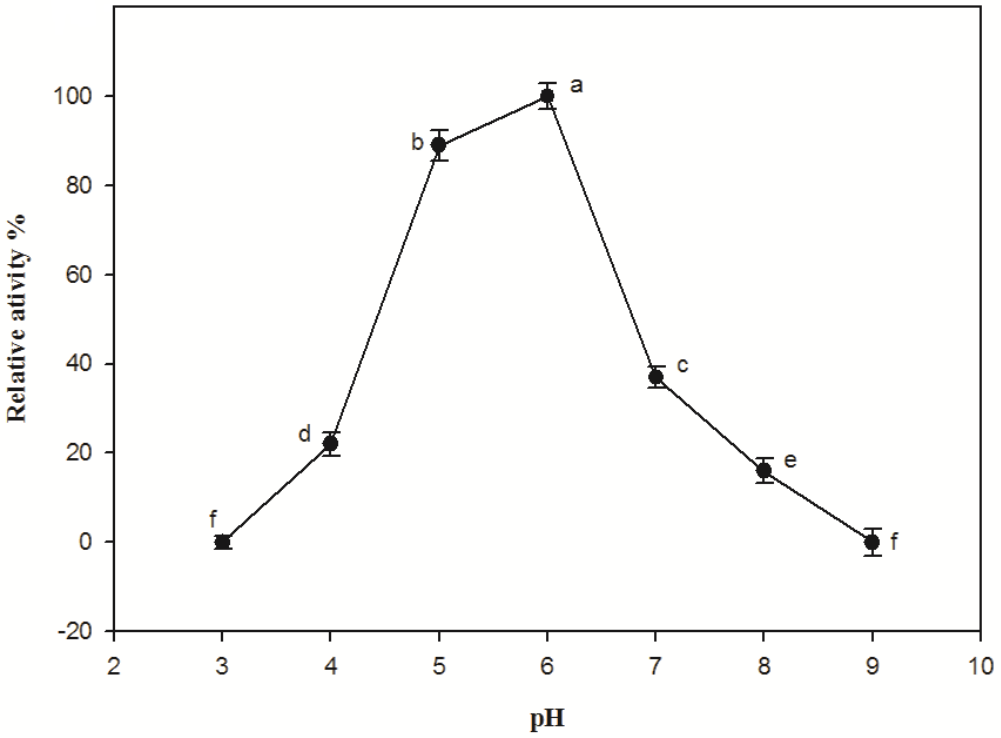

Fig. 1 Effect of pH on pectinase from the midgut of *Leptinotarsa decemlineata* larvae.

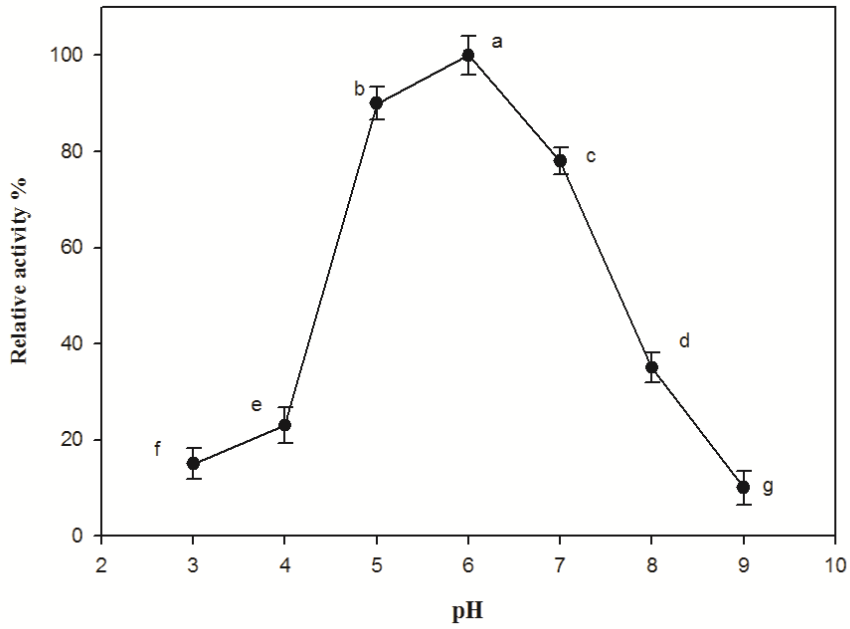

Fig. 2 Effect of pH on pectinase from the midgut of *Leptinotarsa decemlineata* adult.

The results of enzyme stability showed, pectinase was stable relatively in the pH 5-7 range, after 8hr incubation, the activity of enzyme was reduced to 40% in the 4th instar larvae and to 30% in adult (Fig. 3 and 4).

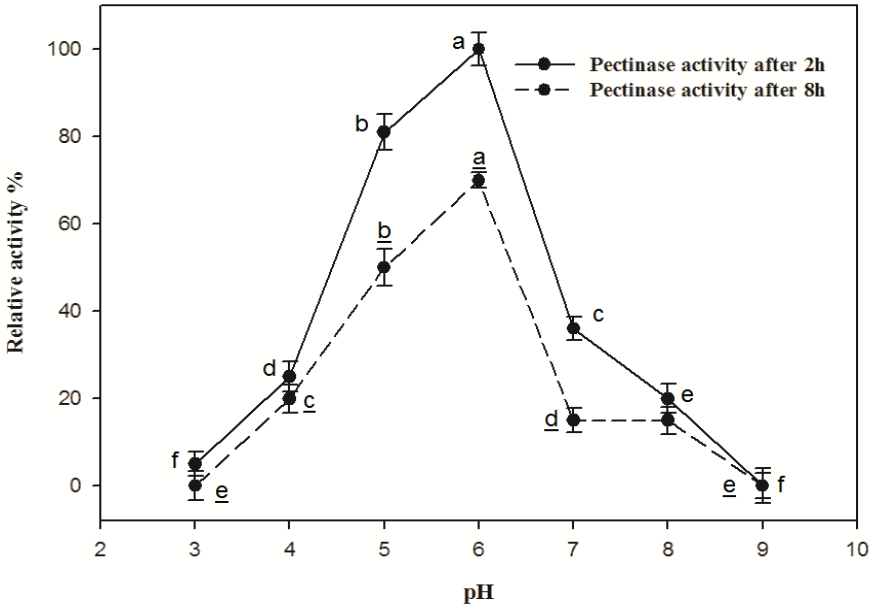

Fig. 3 The stability of the midgut pectinase at different pHs and incubations periods in *Leptinotarsa decemlineata* larvae.

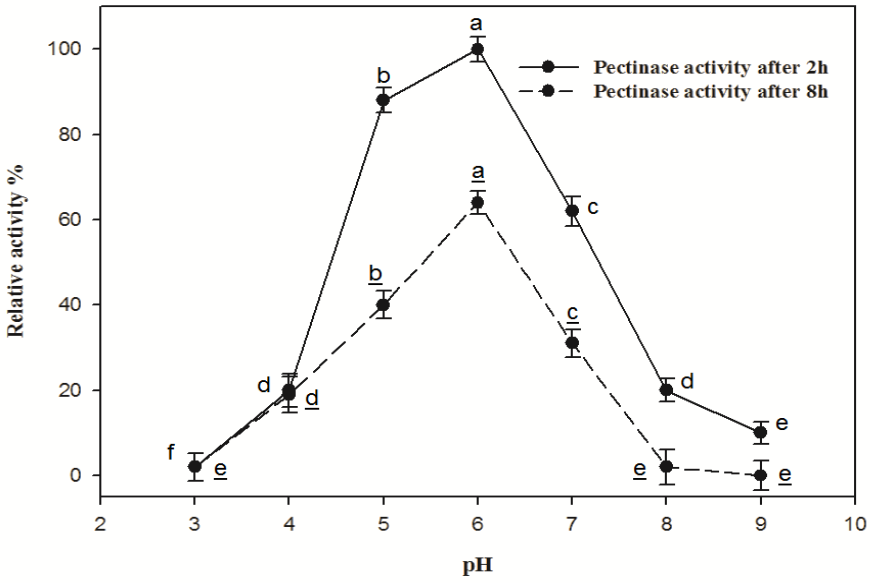

Fig. 4 The stability of the midgut pectinase at different pHs and incubations periods in *Leptinotarsa decemlineata* adult.

Maximum activity after 8h incubation occurred at pH 5 and 6, that it show acidic condition is proper for pectinase activity in the larvae and adult of CPB. These results was consistent with optimum pH extracted pectinase from midgut of *H. postica* (Vatanparast and Hosseininaveh, 2010). Pectinase enzyme from *S. oryzae*

(Shen et al., 1996), *Eurygaster integriceps* (Vatanparast et al., 2010) and polygalacturonase of larvae gut of *Diaprepes abbreviates* (Doostdar et al., 1997) showed the maximum activity at pH 5.5, 6 and 5.5, respectively. The polygalacturonase of Salivary glands of *Lygus lineolaris* and *L. hesperus* (Agblor et al., 1993) also showed high activity at acidic pH. These results and studied suggested that optimum pectinase activity, in coleopteran and homopteran insects that their pectinase enzyme families detected, is occurred at middle acidic range pH that it can relate to food diet of these orders. Probably this conditions help and vital to digestive.

The effect of temperature on pectinase activity with day effect on *L. decemlineata* was examined. The maximum enzyme activity in midgut of 4th instar larvae and adults was observed at temperatures of 40°C in first day. Over time enzyme activity decreased gradually (Fig. 5 and 6).

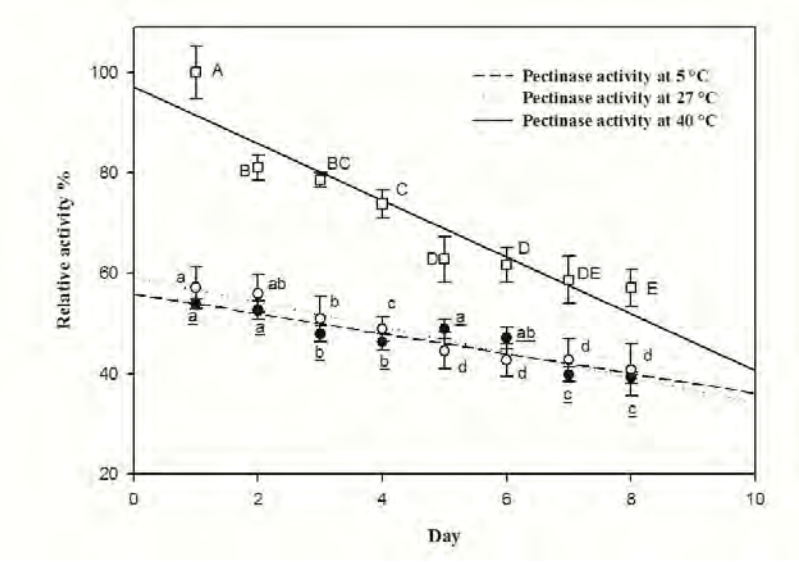

Fig. 5 Simultaneous effect of temperature and day on pectinase from the midgut of *Leptinotarsa decemlineata* larvae.

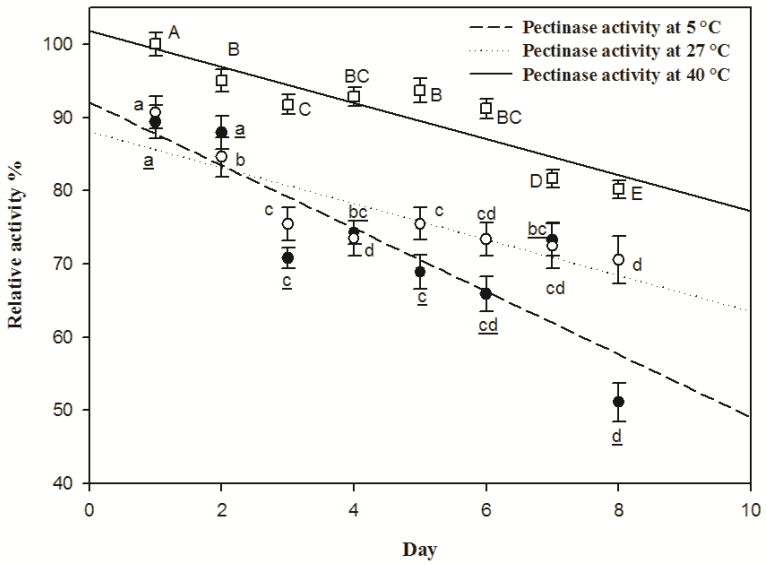

Fig. 6 Simultaneous effect of temperature and day on pectinase from the midgut of *Leptinotarsa decemlineata* adult.

The lowest activity happened after eighth day at 5°C that it was expected. The maximum enzyme activities were reported in larvae midgut of *H. postica* at temperature of 35-45°C (Vatanparast & Hosseininaveh, 2010) endo-polygalacturonase of *D. abbreviates* at 30°C (Doostdar et al., 1997) and polygalacturonase of salivary glands of *L. lineolaris* and *L. hesperus* at 40°C (Agblor et al., 1994). This result is consistent with experiments performed on *L. decemlineata.* Almost certainly increasing temperature more than 50°C, cause enzyme denatured and relative activity decreased. In general, according to us study that done on digestive carbohydrase activity of beetles on the temperature, the results indicate that the maximum activity in the range of 35 to 50°C has been accrued. High temperature can denature enzyme probably.

Results of the effect some of compounds on pectinase activity have been shown in Table 1.

Table 1 Relative activity of *Leptinotarsa decemlineata* pectinase toward different compounds[a]. Values are means±S.E (Standard error), n = 3

Compound	**Concentration**	**Relative activity%**	
Control	-	100	100
NaCl	5 mM	109.82±1.24	104.23±2.05
	10 mM	125±1.84	108.91±1.02
	20 mM	123.55±1.95	109.05±3.02
	40 mM	130.11±2.06	112.73±2.04
KCl	5 mM	97.64±1.96	89.94±1.82
	10 mM	81.79±1.74	90.62±1.63
	20 mM	100.17±2.08	93.48±1.11
	40 mM	98.03±2.04	94.54±1.61
$CaCl_2$	5 mM	144.84±1.83	104.48±1.21
	10 mM	126.02±0.73	107.35±1.33
	20 mM	148.58±1.08	128.2±1.34
	40 mM	178.98±1.99	133.1±1.56
$MgCl_2$	5 mM	83.81±1.56	95.65±1.62
	10 mM	80.58±1.64	77.67±1.96
	20 mM	59.57±2.03	70.55±1.85
	40 mM	54.59±2.01	55.28±2.01
Tris	10 mM	95.4±2.12	94.14±2.42
	20 mM	95.65±2.05	77.81±2.03
SDS	10 mM	14.05±1.83	83.91±3.02
	20 mM	6.48±1.43	74.17±3.12
Urea	0.5 M	95.27±1.94	68.85±2.33
	4 M	92.32±1.92	47.49±1.52
	8 M	62.84±1.05	14.35±1.83

The addition of Na^+ and Ca^{2+} ions, increased activity of this enzyme, The Ca^{2+} ion (concentration 40mM) was increased pectinase activity to 79% and 33% in the adult and 4th instar larvae, respectively. The K^+ ion had no effect on the activity of this enzyme. The Mg^{2+} reduced relatively high (approximately 45% at 40mM concentration) activity of pectinase in the 4th instar larvae and adults. Tris, urea and SDS decreased enzyme activity in *L. decemlineata*. SDS decreased enzyme activity to 93% (concentrations of 20mM) in the adults and was strongly inhibiting of this enzyme, also the urea decreased pectinase activity at concentration of 20mM to 85 % in 4th instar larvae. Terra et al (1996) suggested that the SDS with associate with amino acids of insect digestive enzymes, inhibit and decrease their activity significantly. Results obtained in this study confirms with the above mentioned studies.

Zymogram of pectinase activity using the SDS-PAGE showed that in midgut of the 4th instar larvae and adults two protein bands visualized (Fig. 7).

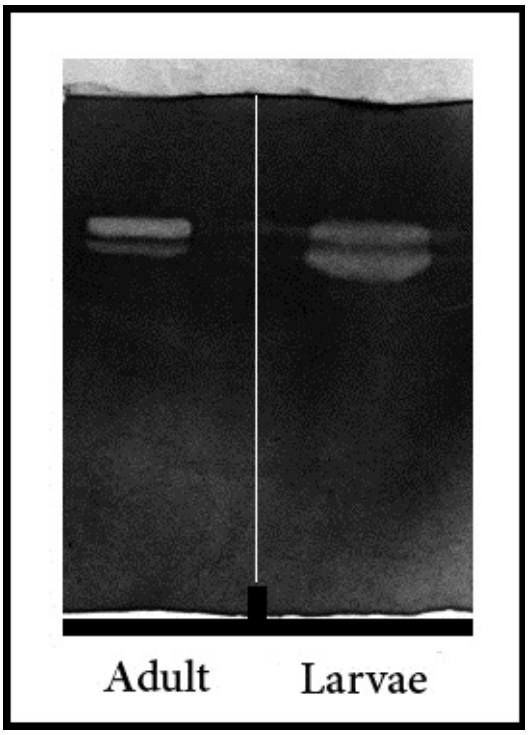

Fig. 7 Zymogram analysis of the midgut pectinase from adult and larvae of *Leptinotarsa decemlineata*.

Biological activity of pectinase enzyme has been studied in some of insects. Shen et al (1996) observed one protein band of the polygalacturonase in *S. oryzae* by staining the gel in a solution of ruthenium red. Also, Shen et al. (1999) using the SDS-PAGE method on a10% separating gel and the 4% stacking gel was observed only one protein band for pectin methylesterase of *S. oryzae*. Pectinase enzyme is necessary in CPB to breakdown pectin of plant cell wall.

Our study indicated that Pectinase activity exist in the gut extract of the CPB. Surely, these enzymes, have an important role in digestion of insect. Insect-resistant crops have been one of the key attainments of applying plant genetic engineering ability to agriculture (Sharifi at al., 2011). The secondary metabolites in plants can take action as defensive agents next to insects either by repellence or during directly toxicity. A lot of dissimilar types of secondary metabolites, as well as alkaloids, terpenes, steroids, iridoid glycosides, aliphatic

molecules, phenolics (Hsiao, 1985) and others, have been established to confer resistance to different plant species in contradiction of insects. Among them, carbohydrase inhibitors seem to play an important role in host plant resistance to insects (Sharifi at al., 2011). Study of the carbohydrates in insects is vital not only for considerate digestion biochemistry but also for rising insect pest management strategies.

References

Agblor A, Henerson HM, Madrid FJ. 1994. Characterization of alpha-amylase and poltgalacturonase from *Lygus spp*. (Heteroptera: Miridae). Food Research International, 27(2): 321-326

Bradford MM. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry, 72(3): 248-254

Cohen AC. 1993 Organization of digestion and preliminary characterization of salivary trypsin-like enzymes in a predaceousheteropteran, *Zelus renardii*. Journal of Insect Physiology, 39(2): 823-829

Collmer A, Ried JL, Mount MS. 1988. Assay methods for pectic enzymes. Methods in Enzymology, 161(3): 329-335

Djordjevic SP, Batley M, Redmond JW. 1986. Preparative gel chromatography of acidic oligosaccharides using a volatile buffer. Journal of Chromatography A, 354(1): 507-510

Dootsdar H, McCollum TG, Mayer RT. 1997. Purification and characterization of an endo-polygalacturonase from the gut of West Indies sugarcane rootstalk borer weevil (*Diaprepes abbreviatus* L.) larvae. Comparative Biochemistry and Physiology, Part B. 118(4): 861-867

Ferro DN, Boiteau G. 1993. Management of insect pests. Potato Health, 65(2): 112-128

Forgash AJ. 1985. Insecticide resistance of the Colorado potato beetle. In: Proceedings of the Symposium on the Colorado Potato Beetle, XVIIth International Congress of Entomology (Ferro DN, Voss RH). 33-52, Massachusetts Agricultural Experiment Station, Amherst, MA, USA

Girard C, Jouanin L. 1999. Molecular cloning of cDNAs encoding a range of digestive enzymes from a phytophagous beetle *Phaedon cochleariae*. Insect Biochemistry and Molecular Biology, 29(4): 1129–1142

Hare JD. 1990. Ecology and management of the Colorado potato beetle. Annual Review of Entomology, 35(3): 81-100

Hsiao H. 1985. Feeding behaviour. In: Comprehensive Insect Physiology Biochemistry and Pharmacology (Kerkut GA, Gilbert LI, eds). Pergamon Press, Oxford, UK

Igrc-bari J, Dobrini R, Maceljski M. 1999. Effect of insecticides on the Colorado potato beetles resistant to OP, OC and P insecticides. Australia and New Zealand School, 72(2): 76-80

Ioannidis PM, Grafius E, Whalon ME. 1991. Patterns of insecticide resistance to azinphosmethyl, carbofuran, and permethrin in the Colorado potato beetle (Coleoptera: Chrysomelidae). Journal of Economic Entomology, 84(2): 1417-1423

Laemmli UK. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature, 277(4): 680-688

Pifferi PG, Buska G, Manenti I, Lo Presti A, Spagna G. 1989. Immobilization of pectinesterase on c-alumina for the treatment of juices. Belgian Journal of Food Chemistry and Biotechnology, 44(4): 173-182

Reek G, Oppert B, Denton M, Kanost M, et al. 1999. Insect Proteinases. In: Proteases (Turk V, ed). 125-148, Birkhauser, Basel, Switzerland

Sharifi M, Ghadamyari M, Mahdavi M, Saiidi F. 2011. Biochemical characterization of digestive carbohydrases from Xanthogaleruca luteola and inhibition of its α-amylase by inhibitors extracted from the common bean. Archives of Biological Sciences, 63(3): 705-716

Shen Z, Reese JC, Reeck G. 1996. Purification and characterization of polygalacturonase from the rice weevil, *Sitophilus oryzae* (Coleoptera: Curculionidae). Insect Biochemistry and Molecular Biology, 26(2): 427-433

Shen Z, Manning G, Reese JC, Reeck GR. 1999. Pectin methylesterase from the rice weevil, *Sitophilus oryzae* (L.) (Coleoptera: Curculionidae): Purification and characterization. Insect Biochemistry and Molecular Biology, 29(2): 209-214

Sinden SL, Cantelo WW, Sanford LL, Deahl KL. 1991. Allelochemically mediated host resistance to the Colorado potato beetle, *Leptinotarsa decemlineata* (Say) (Coleoptera: Chrysomelidae). Memoirs of the Entomological Society of Canada, 159(5): 19-28

Stewart JG, Kennedy GG, Sturz AV. 1997. Incidence of insecticide resistance in populations of Colorado potato beetle, *Leptinotarsa decemlineata* (Say) (Coleoptera: Chrysomelidae), on Prince Edward Island. Canadian Entomologist, 129(2): 21-26

Terra WR, Ferreira C, Jordao B.P, Dillon RJ. 1996. Digestive enzymes. In: Lehane, M.J., Billingsley, P.F. (Eds.), Biology of the Insect Midgut. 153-194, Chapman and Hall. London, UK

Vatanparast M, Hosseininaveh V. 2010. Digestive amylase and pectinase activity in the larvae of alfalfa weevil *Hypera postica* (Coleoptera: Curculionidae). Entomological Research, 40(2): 328-335

Vatanparast M, Hosseininaveh V, Hosseininaveh F, Pakarpour F. 2010. Digestive pectinolytic activity in the Sunn pest, *Eurygaster integriceps* Puton (Hemiptera: Scutelleridae). Archives of Phytopathology and Plant Protection, 44(14): 1390-1398

Vonk HJ, Western JRH. 1984. Comparative Biochemistry and Physiology of Enzymatic Digestion. Academic Press. New York, USA

Wigglesworth VB. 1974. The Principles of Insect Physiology (8th edn). Methuen, London, UK

Wolfson JL, Murdock LL. 1987. Method for applying chemicals to leaf surfaces for bioassay with herbivorous insects. Journal of Economic Entomology, 80(2): 1334-1336