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Cellulase activity in the larval digestive tract of the Colorado potato beetle, *Leptinotarsa decemlineata* (Coleoptera: Chrysomelidae) and the cigarette beetle, *Lasioderma serricorne* (Coleoptera: Anobiidae)

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Abstract: *Leptinotarsa decemlineata* (Say) and *Lasioderma serricorne* F. are destructive pre-harvest and post-harvest pests of many plants in the family Solanaceae, and stored foodstuffs and non-food items, respectively. In this study, some biochemical characteristics of cellulase in the larval digestive tract of these pests were studied. Endo-β-1, 4-glucanase activity was measured against the substrate carboxyl methyl cellulose. Maximum activity of the enzyme in *L. decemlineata* and *L. serricorne* occurred at pH 7.0 and pH 6.0, respectively. The enzymes from *L. decemlineata* and *L. serricorne* were maximally stable at pH 7.0 and pH 5-6, respectively. However, the enzyme extracted from *L. serricorne* is more stable than that of *L. decemlineata*. Cellulase activity was in the highest level at 50 °C in both species. EDTA and SDS reduced cellulase activity, while the Ca^{2+}, Mg^{2+} and Na^{+} ions had a significant increasing effect on cellulase activity. K^{+} did not have any significant effect on the enzyme activity. The values of K_m and V_{max} were 0.608 % and 0.0187 µmol min^{-1} mg^{-1} protein in *L. decemlineata*, and 0.99 %, and 0.0035 µmol min^{-1} mg^{-1} protein in *L. serricorne*, respectively. Zymogram studies revealed two bands of cellulase activity in the digestive tract of both species.

Keywords: *Leptinotarsa decemlineata, Lasioderma serricorne,* cellulase, digestion, zymogram

Introduction

The Potato leaf beetle (Colorado beetle), *Leptinotarsa decemlineata* (Col.: Chrysomelidae) is one of the most important pests of potatoes worldwide. This pest was a quarantine pest at first in Iran and Kazemi (Nouri-Ganbalani, 1989) found it in Ardabil province, in spring 1984. This pest prefers potato, however in the absence of the preferred host, it also feeds on eggplant, tomato, pepper, tobacco, pigweed, nettle and datura (Jacques, 2005). This pest is one of the 15 major pests of plants in the world whose different larval and adult stages feed on hosts leaves. Larvae and adults of the pest are able to eat 40 and 9.65 cm^2 of the leaves in their lifetime, respectively (Ferro *et al.*, 1991). The cigarette beetle, *Lasioderma serricorne* Fabricius (Coleoptera: Anobiidae), is a cosmopolitan pest of many raw and stored products especially spices, seeds, grains, dried potatoes, raisins and tobacco (Oppert *et al.*, 2002).

Despite the destructive effects of pesticides on humans, animals, environment, pest resistance to pesticides and the still residual pesticides in foods, these chemicals are broadly used in pest control programs (Ascher, 1993;

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Rodriguez *et al.*, 2003; Regnault-Roger *et al.*, 2004). Using insect-specific toxic proteins in genetically modified crops is a safe and new method of pest control. In this method, genes that are inhibitors of digestive enzymes are inserted into the plants (Oppert, 2000). The first step in the production of transgenic plants containing inhibitor proteins of digestive enzymes is the biochemical study of these digestive enzymes (Oppert *et al*., 2000). Insects need polysaccharides in their diet during their different developmental stages (Mendiola-Olaya *et al.*, 2000). Cellulose is a carbohydrate that is recognized as the most abundant compound of the cell walls (Maness and Mort, 1989). This compound is also the most abundant carbohydrate produced in the biosphere (Suto and Tomita, 2001; Guedon *et al*., 2002). Cellulose is a liner polysaccharide that is made up of hundreds and sometimes thousands of β-D-glucopyranosyl units that are joined by 1, 4 glycosidic bonds (Watanabe and Tokuda., 2010). Cellulase which is a set of three enzymes: endo-β-1, 4-glucanases, exo-β-1, 4-cellobiohydrolases, and β-1, 4-glucosidases, affects the cellulose and finally after hydrolysis by these enzymes, cellulose is broken down to glucose units. Endo-β-1, 4-glucanases attack the cellulose chain from the middle and hydrolyse β-1, 4 bonds. Exo-β-1, 4-cellobiohydrolases hydrolyse cellulose from the reducing end and produce the cellobiose units. Finally, β-1, 4-glucosidases convert cellobiose units to glucose monomers (Tokuda *et al*., 1997; Sukumaran *et al.*, 2005). The existence of these three enzymes together is necessary for insects to digest cellulose. These enzymes are also present in the insect's digestive system and have been studied in many species. For example, existence of endo-β-1, 4-glucanases in the grasshopper *Oxya chinensis* (Amtul *et al.*, 2010) and adult insects of the long-horn beetle *Anoplophora glabripennis* (Li *et al.*, 2010) has been demonstrated.

Endo-β-1, 4-glucanases in larval stage of *Diaprepes abbreviates* L. (Col.: Curculionidae) have also been identified (Doostdar *et al.*, 1997). Cellulase activity (endo-β-1, 4-glucanases) in some insect species feeding on different food resources has been compared (Shi *et al.*, 2010). In a review, cellulase activities in digestive fluids of 68 xylophagous and phytophagous species belonging to 8 insect orders have been assessed (Oppert *et al.*, 2010). Wharton *et al*., (1965) studied cellulase activities (endo-β-1,4-glucanases) in different species of cockroaches. Chararas *et al*. (1983) reported the purification of three enzymes forming cellulase complex in larvae of *Ergates faber* L. (Col.: Cerambycidae). Effects of β-glucosidases, endo-β-1, 4-glucanases and xylanases on digestion of plant fibers in several species of arthropods such as grasshoppers, crickets, cockroaches and some coleopterans were studied by Cazemier *et al*. (1997).

In the present study, we have shown some detailed biochemical characteristics of midgut digestive cellulolytic activity in larvae of two coleopteran pests, *L. decemlineata* and *L. serricorne*.

Materials and Methods

Insects

Last instars of the Colorado potato beetle, *L. decemlineata*, were collected from infected potato fields in the Hamedan province . *Lasioderma serricorne* was reared on pea (*Cicer arietinum*) at 30 °C. The infested peas were gently cut by a knife into two parts and last instars removed.

Enzyme sample preparation

Last instars of both species were cold-immobilized (ca. 4 ºC), dissected under a stereomicroscope, and their midguts were removed. The midguts were then cleaned of adhering unwanted tissues. The midguts, including contents, were collected into 100 µl distilled water and homogenized with a hand-held glass grinder on ice. The homogenates were centrifuged at 16000 × g at 4 °C for 18 min. The resulting supernatant were passed through a filter paper and then were transferred to new microtubes and maintained at -20 °C for further analysis.

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Cellulase activity assay

A slightly customized colorimetric assay was conducted for detecting cellulase activity using 3, 5-dinitrosalicylic acid (DNS) as the reagent and 1 % soluble carboxymethyl cellulose (CMC) as the substrate. Fifteen µl of the enzyme extract was incubated with 85 µl sodium citrate-phosphate-borate buffer (40 mM, pH 3 to 11) and 20 µl soluble CMC for 60 min. at 50 °C. The reaction was stopped by addition of 50 µl DNS and heating in boiling water for 10 min. The absorbance was finally measured at 540 nm. Relative cellulase activity was considered as the criterion in the tests. The assay was performed triplicate.

Effect of temperature on cellulase activity

The effect of temperature on the activity of midgut cellulases was assayed. Samples were incubated at different temperatures (20, 30, 35, 40, 45, 50, 60, 70, 80 °C) in water bath for 60 min. The assays were performed according to Tokuda *et al.,* (1997). All tests were conducted in three replicates of treatments and control.

pH stability of cellulases

Stability of the cellulases was determined at a pH set (3 to 11) and two incubation time periods. Enzyme extract was mixed with the buffer (sodium citrate-phosphate-borate) and incubated for 1 and 10 hours at 37 °C. The substrate was then added to the buffered enzyme extract and cellulase activity was determined as before.

Effect of activators and inhibitors on cellulase activities

To investigate the effects of several ions on the enzyme activity, assays were performed in the presence of 5 µl of different concentrations of chloride salts of Na^+, K^+, Ca^{2+}, and Mg^{2+}, sodium dodecyl sulfate (SDS) and Ethylenediaminetetraacetic acid (EDTA) in the reaction mixture. Activity was then measured after 60 min. We allowed the ions to react with enzyme for 15 min. and then the substrate was added. In controls, water was replaced with the ions.

Kinetic parameters of cellulase

The Michaelis–Menten constant (K_m) and the maximal reaction velocities (V_{max}) of cellulase was determined. The homogenate was incubated in an appropriate buffer (in the case of cigarette beetle; citrate buffer, 50 mM, pH 6 and in the case of the Colorado potato beetle; phosphate buffer, 50 mM, pH 7) at 50 °C toward the substrate cellulase in appropriate concentrations. The experiments were performed in triplicate. The K_m and V_{max} were evaluated by non-linear regression analysis using the software Sigmaplot 10.0.

Visualization of cellulase activity

Cellulase in-gel assays were performed using non-denaturing SDS-PAGE for visualizing the enzyme activity. Enzyme extract was diluted in electrophoresis sample buffer containing 25 % stacking buffer (0.5 M Tris–HCl; pH 6.8), 20 % glycerol, 2 % SDS, and 0.005 % (w/v) Bromophenol blue. The enzyme sample was loaded in 5 % stacking and 9 % separating polyacrylamide gels. The substrate CMC was incorporated into the separating gels at a final concentration of 0.1 % for detection of cellulase. After electrophoresis, the gels were washed in distilled water containing 2.5 % (v/v) Triton X-100 for 45 min. and then incubated in citrate buffer (pH 6) in the case of the cigarette beetle and phosphate buffer (pH 7) in the case of the Colorado potato beetle for 2 hr with gentle agitation. The gels were stained by Congo red (0.1 %) for 10–15 min. at room temperature (24 °C). The gels were destained by washing in 50 ml of 1 M NaCl until cellulase bands became obvious as clear zones where CMC had been ruined due to enzymatic activity. After 20 min. destaining, 100 µl glacial acetic acid was added to the gel for better visualization.

Protein determination

Protein content of the sample was measured according to the Lowry *et al.,* (1951). Bovine serum albumin was used as the standard.

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Results and Discussion

Optimum pHs for midgut cellulolytic activities in *L. decemlineata* and *L. serricorne* were determined at pHs 7 and 6 (Figure 1), respectively. Optimum temperature for cellulase activity in both insects was 50 °C (Figures 2). The test diagrams of pH in two insects show that most cellulase activity occurs in neutral and slightly acidic condition. The enzyme activities in highly acidic and highly alkaline condition were low so that the enzyme activity at pH 3 and 11 was minimized. Amtul *et al.* (2008) reported that the activity of cellulase in the Red pumpkin beetle, *Aulacophora foveicollis* Lucas (Col.: Chrysomelidae), was optimum at pH 7.8 which is higher than that we determined for *L. serricorne* and *L. decemlineata.* Studies of endo-β-1, 4-glucanase activity in adult of the Asian long-horn beetle, *Anoplophora glabripennis* Motschulsky (Col.: Cerambycidae), revealed that optimum activity was at pH 5.2 and assay was conducted at 40 °C as optimum temperature (Li *et al.*, 2010). The studies on the isolation of cellulase from beetles showed that optimum pH for maximum activity of cellulase from the larval gut of the Yellow-spotted longhorn beetle, *Psacothea hilaris* Pascoe (Col.: Cerambycidae) against CMC was 5.5 (Sugimura *et al.*, 2003). Also, optimum pH and temperature for endo-β-1, 4-glucanase activity in the *Tribolium castaneum* Herbst (Col.: Tenebrionidae) have been reported at 4.8 and 40 °C, respectively (Fayyaz-Ur-Rehman *et al*., 2009). All animal cellulases reported so far have optimal activities under the weak acidic conditions (Watanabe *et al*., 1997). In *Oxya chinensis* (Orthoptera: Acrididae), optimum pH and temperature for cellulase (endo-beta-1, 4-glucanase) activity were at pHs 2.0 and 7.0 and 50 °C. Due to these highly acidic pH optima, endoglucanase is able to hydrolyze cellulose into simple sugars completely by itself. That was the first report of cellulase activity in highly acidic conditions (Amtul *et al.*, 2010). Exo-β-1, 4-cellobiohydrolases from *Neotermes koshunensis* Shiraki, a termite of Kalotermitidae, reacted optimally at pH 5.0 and 45 °C (Ni *et al*. 2007). Exo-β-1, 4-cellobiohydrolases and endo-β-1, 4-glucanase optimal activities in *Planococcus citri* Risso (Hom.: Pseudococcidae) were reported at pH 5.5 for both enzymes (Fayyaz-Ur-Rehman *et al*., 2009). Many microbial and fungal cellulolytic enzymes have optimal activity at pH 4 to pH 6 and temperatures about 50 °C (Clarke, 1997). As in bacterial and fungal cellulases, EG (endo-β-1, 4-glucanase) enzymes from the Mulberry longhorn beetle, *Apriona germari* Hope (Col.: Cerambycidae), had optimum activity at 50 to 55 °C with an optimum pH = 6.0 (Lee *et al*., 2004).

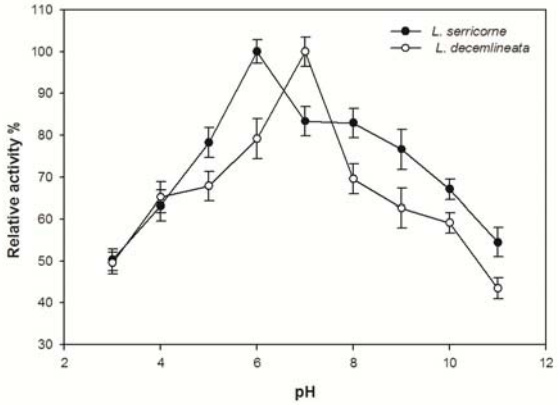

Figure 1 Midgut digestive cellulase activity of *Leptinotarsa decemlineata* and *Lasioderma serricorne* at different pHs.

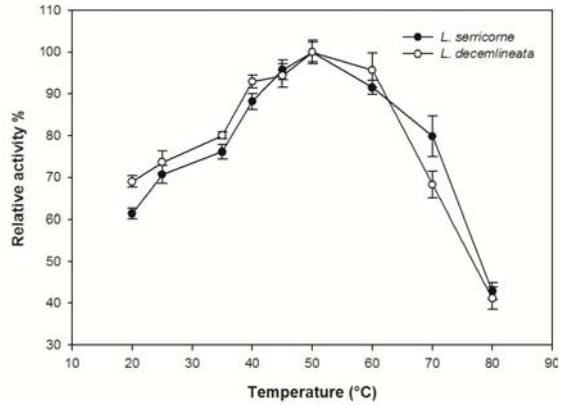

Figure 2 Midgut cellulolytic activity in *Leptinotarsa decemlineata* and *Lasioderma serricorne* at different temperatures.

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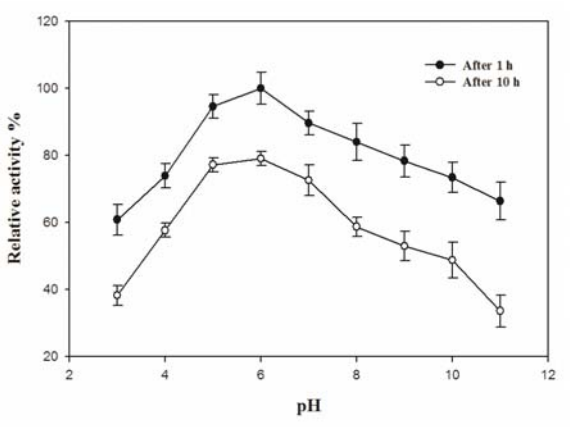

Figure 3 Stability of digestive cellulase from *Lasioderma serricorne* at different pHs after 1 and 10 hrs incubation period.

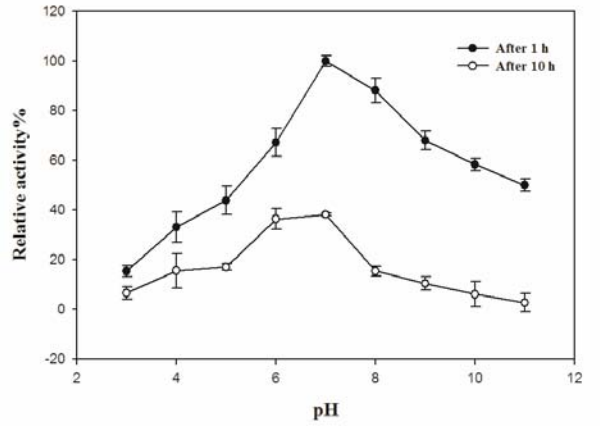

Figure 4 Stability of digestive cellulase from *Leptinotarsa decemlineata* at different pHs after 1 and 10 hrs incubation period.

The data obtained from the enzyme stability at different pHs show that cellulase in *L. serricorne* retained its maximum activity at pH 6 after 1 and 10 hours (Figure 3), while endo-β-1, 4-glucanase in *L. decemlineata* retained its maximum activity at pH 7 (Figure 4). As it is clear in figure 4, in *L. decemlineata* enzyme activity at pH 6 to pH 9 is high after 1 hour but after 10 hours it is reduced greatly, so that after 10 hours at pH 7, enzyme activity is less than 40 %. Cellulase from the cigarette beetle is more stable at pHs 5-7 than at highly acidic and alkaline pHs. Cellulase from *L. serricorne* retained 80 % of its maximal activity after 10 hours incubation at pH 6.

Kinetic parameters of cellulase were estimated at pH 6 and 7 for the cigarette beetle and the Colorado potato beetle, respectively, against CMC at 50 °C. The K_m and V_{max} values for *L. serricorne* and *L. decemlineata* were 0.608 % and 0.0187 µmol min^{-1} mg^{-1} protein and 0.99 % and 0.0035 µmol min^{-1} mg^{-1} protein, respectively. Analysis of three cellulase enzymes in *Ergates faber* (Col.: Cerambycidae) showed that the K_m values for endo-β-1, 4-glucanase and cellobiohydrolase were 2.55 and 1.6 mM, respectively (Chararas *et al.*, 1983). K_m of endo-β-1, 4-glucanase in long-horn beetle *Pyrophorus divergens* larvae has been reported as 1.3 mM (Colepicolo-Neto *et al*., 1987).

Some ions and compounds showed different impacts on endo-β-1, 4-glucanase activity in the cigarette beetle and the Colorado potato beetle (Figure 5). Increase in concentration of $MgCl_2$, NaCl, KCl and $CaCl_2$, enhanced the activity of cellulase. In contrast, increased concentrations of SDS and EDTA inhibited the enzyme activity. As far as the authors are aware, there aren't any studies on the effects of ions on exo-β-1, 4-cellobiohydrolases and endo-β-1, 4-glucanase. However, some studies have shown effects of some compounds on β-glucosidase and other carbohydrases. Studies on activators and inhibitors on digestive fluid from adults of *Rhynchophorus palmarum* revealed that $ZnCl_2$, $CuCl_2$ and $FeCl_3$ had an inhibitory effect on β-glucosidase activity, while $MnCl_2$, $SrCl_2$, $CaCl_2$, $BaCl_2$ and $MgCl_2$ didn't have a significant effect (Yapi Assoi Yapi *et al.*, 2009). Studies on carbohydrases from *Lygus hesperus* (Hem.: Miridae) showed that Cu^{2+} had an enhancing impact on α-glucosidase and β-glucosidase, while Mg^{2+} didn't have significant effect on either enzymes (Zeng and Cohen, 2001). Ca^{2+} showed positive effect on α-glucosidase activity and increased the enzyme activity in *Glyphodes pyloalis* Walker, effectively (Ghadamyari *et al.*, 2010). Vatanparast and Hosseininaveh (2010) reported that KCl and NaCl increased α-amylase activity in the larval midgut of *Hypera postica*.

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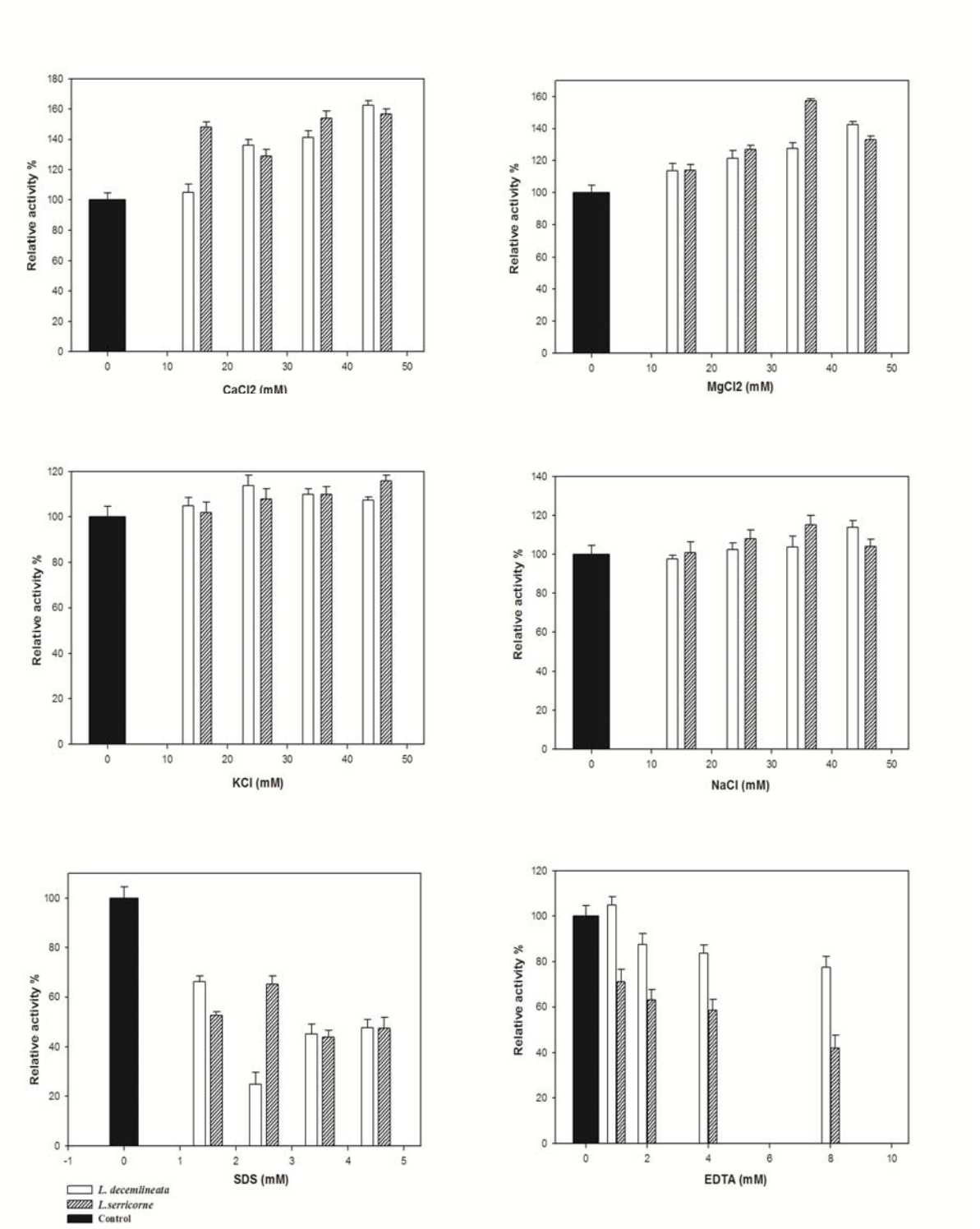

Figure 5 Effect of different ions and compounds on the midgut digestive cellulase activity in *Leptinotarsa decemlineata* and *Lasioderma serricorne*.

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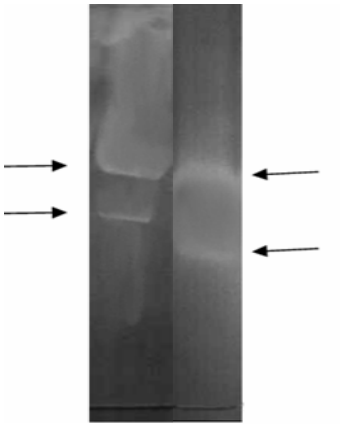

Figure 6 Zymogram analysis of cellulase activity from *Lasioderma serricorne* (Left) and *Leptinotarsa decemlineata* (Right).

Zymogram analysis revealed the presence of endo-β-1,4-glucanase in the cigarette beetle and the Colorado potato beetle. Figure 6 clearly shows the existence of two isoforms of cellulase in both insects. A study on cellulolytic activity of digestive fluids in insects showed two bands in zymogram analysis (Oppert *et al.*, 2010). Also, in another study on cellulolytic enzymes in the midgut of *Tribolium castaneum*, zymogram analysis showed two bands of cellulase activity in both native -PAGE and non-denaturing SDS-PAGE systems (Fayyaz-Ur-Rehman *et al*., 2009).

Production of transgenic plants helps us to reduce toxic chemicals used against pests. Finally, we will have a healthier environment. To establish a control strategy based on the inhibitory power, the first step is to identify the chemical properties of digestive enzymes (Strobl *et al.*, 1998). On the other hand, it is very dangerous to use pesticides for controlling the pests of stored products. So, all attention is being focused on finding alternative solutions. Biochemical identification of enzymes involved in digestion raises our understanding of designing new control approaches using the enzyme inhibitor proteins of plant origin. Using resistant cultivars and biochemical studies on pest nutrition is very important. The present study is the first report on cellulolytic activity in the Colorado potato beetle and the cigarette beetle. It is hoped that this study will provide a new perspective to help us in controlling of the pests.

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فعالیت سلولازی در لوله گوارش سوسک برگخوار سیب‌زمینی، *Leptinotarsa decemlineata* (Coleoptera: Chrysomelidae)، و سوسک توتون، *Lasioderma serricorne* (Coleoptera: Anobiidae)

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چکیده: سوسک برگخوار سیب‌زمینی، *Leptinotarsa decemlineata* Say و سوسک توتون، *Lasioderma serricorne* F. به‌ترتیب از آفات مخرب پیش از برداشت گیاهان تیره‌ی سیب‌زمینی، Solanaceae، و پس از برداشت فراورده‌های غذایی و انباری می‌باشند. در این پژوهش، برخی ویژگی‌های بیوشیمیایی سلولاز در روده میانی لاروهای سن آخر این آفات مورد بررسی و مقایسه قرار گرفت. فعالیت اندو- بتا- ۱،۴- گلوکاناز با استفاده از بستره کربوکسیل متیل سلولز سنجیده شد. بیشینه‌های فعالیت آنزیم در سوسک کلرادوی سیب‌زمینی و سوسک توتون به‌ترتیب در pHهای ۷ و ۶ به‌دست آمدند. آنزیم در سوسک کلرادوی سیب‌زمینی و سوسک توتون بیشترین پایداری را به‌ترتیب در pHهای ۷ و ۵ تا ۶ نشان داد، اما نتایج نشان دادند که آنزیم سوسک توتون از آنزیم سوسک کلرادوی سیب‌زمینی پایدارتر بود. بیشترین فعالیت سلولاز در هر دو گونه، در دمای ۵۰ درجه سلسیوس مشاهده گردید. EDTA و SDS فعالیت سلولاز را کاهش دادند درحالی‌که یون‌های Ca^{2+}، Mg^{2+} و Na^{+} بر فعالیت سلولاز اثر افزایشی قابل‌توجهی داشتند. یون K^{+} روی فعالیت آنزیم اثر معنی‌داری نداشت. مقادیر K_m و V_{max} آنزیم در سوسک کلرادوی سیب‌زمینی به‌ترتیب ۰/۶۰۸ درصد و ۰/۰۱۸۷ میکرومول بر دقیقه بر میلی‌گرم پروتئین، و در سوسک توتون به‌ترتیب ۰/۹۹ درصد و ۰/۰۰۳۵ میکرومول بر دقیقه بر میلی‌گرم پروتئین به‌دست آمدند. بررسی زایموگرام‌ها وجود دو باند با فعالیت سلولازی را در روده‌ی میانی هر دو گونه مشخص نمود.

واژگان کلیدی: سوسک کلرادوی سیب‌زمینی، سوسک توتون، سلولاز، گوارش، زایموگرام.

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