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RESEARCH REPORT

Determination of lipase activity in the larval midgut of *Bacterocera oleae* Gmelin (Diptera: Tephritidae)

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Abstract

In the current study, digestive lipase activity was determined and characterized in the third larval instars of olive fly, *Bactericera oleae* as the first time in dipteran order. By using two sample fractions, it was found that the enzyme had higher activity in membrane-bound fraction than that of soluble fraction. Optimal pH of soluble lipase was found to be 4 and 6 but membrane-bound lipase showed pH 4 as optimal value. Optimal temperatures for soluble and membrane-bound lipase were obtained to be 35 and 50 °C, respectively. Activities of digestive soluble and membrane-bound lipases decreased by using various mono- and di-valent ions. Since, fruits of olive are full of various oils, digestive lipases of *B. oleae* larvae have critical role in their digestion. So, these enzymes might be a good target for developing inhibitors and resistant varieties.

Key Words: *Bacterocera oleae*; digestive lipase; characterization

Introduction

Bacterocera oleae (Diptera: Tephritidae) is the most destructive pest of olive around the world (Richard *et al*., 2003). The insect have been introduced to north of Iran as a serious pest since 2004 (Jafari and Rezaee, 2005; Mirrahimi *et al*., 2008). Females lay their eggs in fruits especially large green ones, larvae feed on the fruit pulp causing severe loss and feasibility of pathogen entrances to fruits. Pest control tactics depend on chemical treatments against adults, biological control and physical control such as sticky and pheromone traps (Laskowski and Kato, 1980).

In insects, lipids are involved in several physiological functions like moulting during larval and adult development (Kawooya and Law, 1988), reproduction (Majumder and Sengupta, 1979), energy supplement during starvation (Cheeseman, 1976; Ziegler, 1991) and immunity. Lipases [EC 3.1.1.3] are the enzymes that hydrolyze the esteric bonds at the interface between insoluble substrate and water. The enzymes are synthesized by animals, plants, fungi and bacteria (Jaeger *et al.,* 1999; Gupta *et al*., 2004; Grillo *et al*., 2007). Digestive lipases of insects are divided into triacylglycerol lipases (TAG-Lipases), alkaline and acid phosphatases as well as phospholipases (Terra and Ferreira, 2012). Majority of ingested lipids by insects are the storage lipids (Triglycerids) that must be digested to di- and monoglycerids in midgut (Zibaee *et al*., 2008). During digestion process, triacylglyceride lipases hydrolyze certain positions of triacylglycerols while different phospholipases act on different ester bonds within phospholipids (Zibae e *et al*., 2008). Digestive lipases has been Studied in a few insects such as *Rhodnius prolixus* L. (Lepidoptera: Reduviidae) (Grillo *et al.,* 2007), *Chilo suppressalis* (Lepidoptera: Crambidae) (Zibaee *et al*., 2008), *Naranga aenescens* (Lepidoptera: Noctuidae) (Zibaee and Fazeli-dinan, 2012), *Pieris brassicae* (Lepidoptera: Pieridae) (Zibaee, 2012) and *Andrallus spinidens* (Hemiptera: Pentatomidae) (Zibaee *et al*., 2012). Although lipases have not been well studied like other digestive enzymes but the enzymes have critical role in digestion process of insects. Moreover, characterization of lipases is mandatory to find inhibitors against lipases. On the other hand, inhibitors do cause severe reduction in growth and development and even mortality due to the importance of long chain unsaturated fatty acids in essential dietary components. This could be discovered from host plants that insect feeding causes lower activity of digestive lipases. Moreover, biochemical behavior of the lipases must be elucidated before any exposure

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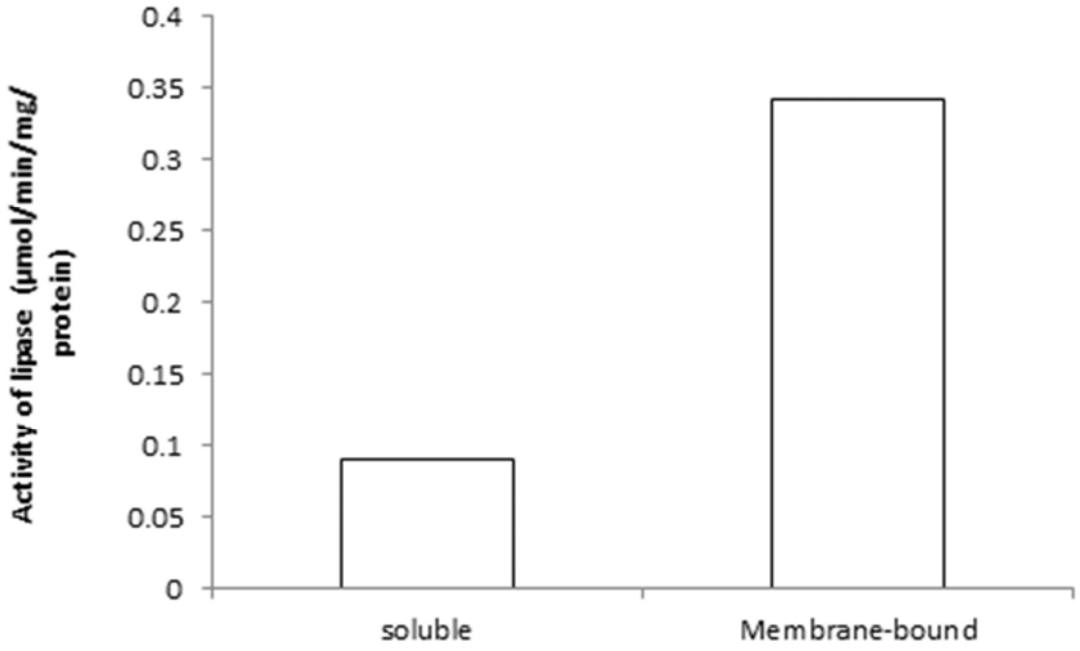

Fig. 1 Activity level of soluble and membrane-bound lipases in 3th instar larvae of olive fruit fly.

to inhibitors. Hence, a biochemical characterization of the digestive lipase from *B. oleae* has been made via evaluating pH and temperature, effects of different ions and specific inhibitors. This is the first report of digestive lipases in a dipteran.

Materials and Methods

Insect rearing

Larvae of *Bacterocera oleae* were reared on the olive fruits of Arbequina variety in containers of 20×12 cm under laboratory conditions of 25 ± 1 °C, 70 % of relative humidity and 16L:8D of photoperiod. Rearing containers were daily checked and cleaned to remove any contaminations. When the larvae reached to 3rd larval instars, they were randomly selected and used in biochemical experiments.

Sample preparation

Third larval instars were separated from olive fruits and dissected under a stereomicroscope in ice cold saline solution (NaCl, 10 mM). Whole guts were homogenized in distilled water by a glass homogenizer, centrifuged at 25000x*g* for 20 min at 4 °C and supernatants were used in lipase assay (Zibaee, 2012). Membrane preparations were exposed from pellets of centrifuged digestive tract (see above) to Triton X-100 for 20 h at 4 °C, in a ratio of 10 mg Triton X-100 per mg of protein. Samples were centrifuged at 25000x*g* for 20 min. Activity of the enzyme remains unchanged at -20 °C for at least a month (Zibaee, 2012).

Lipase assay

The enzyme assay was carried out as described by Tsujita *et al*. (1989). Five microliter of larval gut extract and 15 µl of p-nitrophenyl-butyrate (PNPB, 27 mM) as substrate were added into 40 µl of universal buffer (20 mM, pH 7), mixed thoroughly and incubated at 30 °C. For negative control, samples were placed in a boiling water bath for 15 min to destroy the enzymatic activity.Finally, absorbance was read after 10 min at 492 nm. One unit of enzyme released 1.0 nmol of p-nitrophenol per min at pH 7.2 and 37 °C when p-nitrophenyl butyrate was used as substrate.

Effect of pH and temperature on the activity and the stability of the enzyme

Effects of temperature and pH on lipase activity were examined by using p-nitrophenol-butyrate as substrate in various pH and temperature values. Optimal pH was determined using universal buffer (20 mM) with pH set at 3 - 12. The effect of temperature on the enzyme activity was determined by incubating the reaction mixture at 20, 25, 30, 35, 40, 45, 50 and 60 °C. The procedures for both pH and temperature assays were quite similar to mentioned lipase assay (See above).

Effect of mono- and di-valent cations on lipase activity

Different concentrations of cations (1, 3 and 5 mM) were assayed to find their effects on lipase activity in *B. oleae* larvae. Used cations were Ca^{2+}, Cu^{2+}, Fe^{2+}, K^{+}, Mg^{2+}, Na^{+} and Zn^{2+}. Briefly, 5 µl of a solution containing each concentration of ions and 5

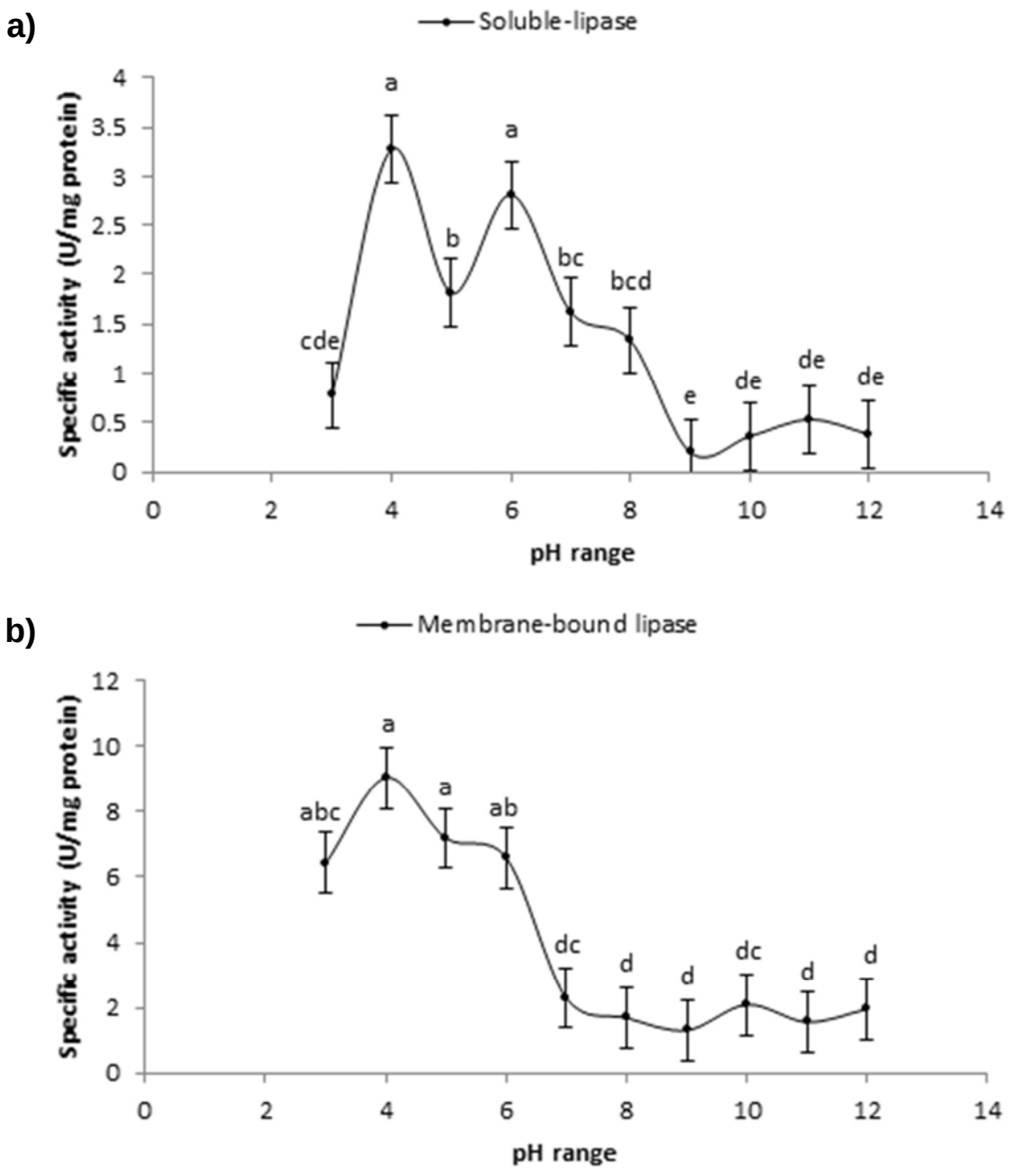

Fig. 2 Effect of pH on the activities of soluble and membrane-bound lipases extracted from the digestive system of *B.oleae* larvae. Statistical deifferences have been shown by various letters (Tukey's test, $p \leq 0.05$).

µl of enzyme were pre-incubated for 10 min at pH 7 and room temperature. The pre-incubated mixture was added to a solution including 30 µl of universal buffer and 10 µl of substrate (PNPB, 27 mM). Other steps were carried out as mentioned earlier.

Effect of specific chelating agent on lipase activity

The effects of enzyme inhibitors on lipase activity were studied using different concentrations (2, 4, 6, 8 and 10 mM) of ethylene glycolbis (β-aminoethylether) N,N, N′, N′-tetraacetic acid (EGTA), triethylenetetramine hexa acetic acid (TTHA), Phenylmethylsulfonyl fluoride (PMSF), diethyldithiocarbamate (DTC), and Ethylenediaminetetraacetic acid (EDTA). The enzyme (5 µl) was pre-incubated with inhibitors for 10 min at pH 7 and room temperature. The pre-incubated mixture was added to a solution including 10 µl of substrate. Other steps were carried out as mentioned earlier.

Protein determination

Protein concentrations were assayed according to the method described by Lowry *et al*. (1951).

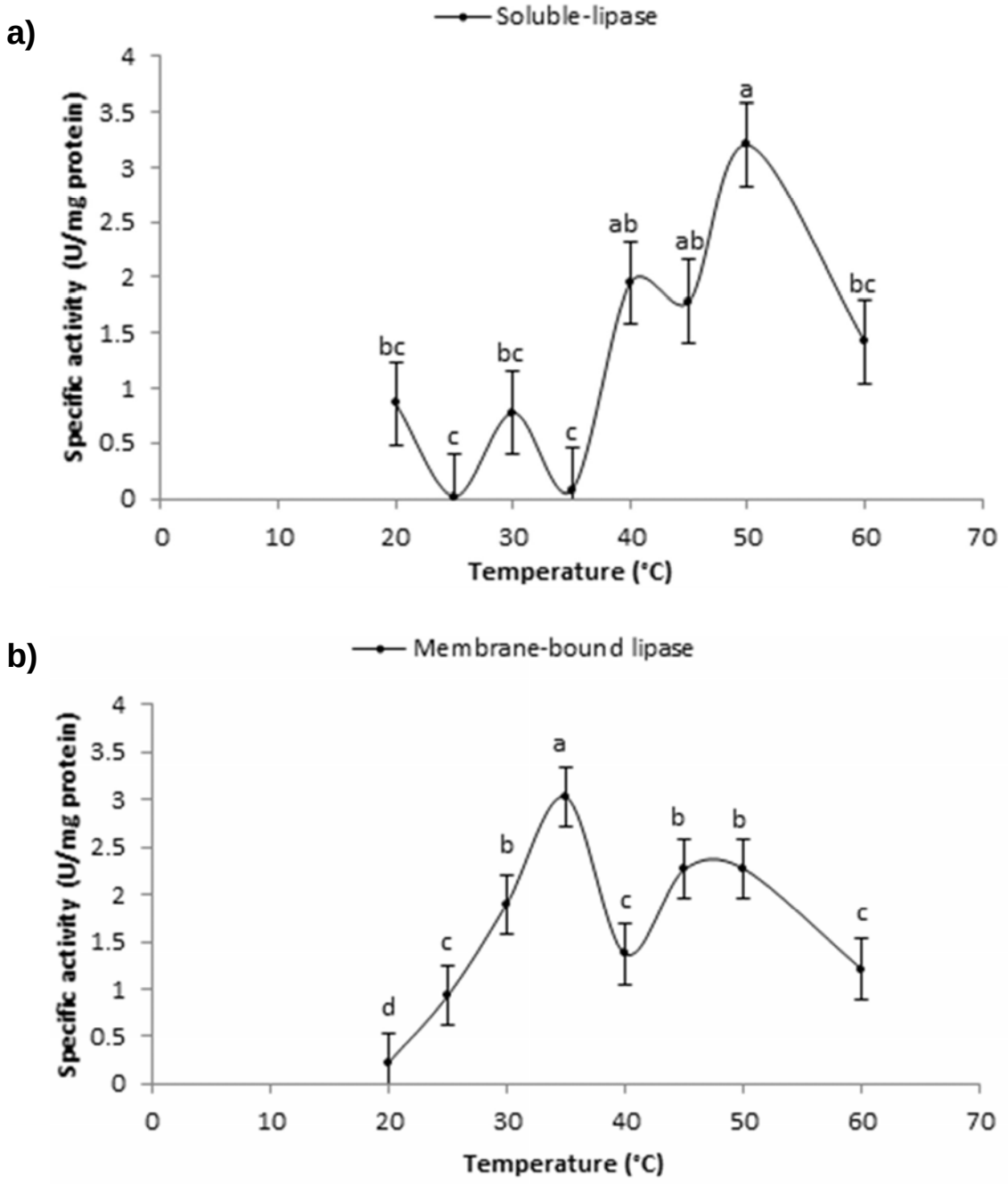

Fig. 3 Effect of temperature on the activities of soluble and membrane-bound lipases extracted from the digestive system of *B.oleae* larvae. Statistical deifferences have been shown by various letters (Tukey's test, $p \leq 0.05$).

Statistical analysis

The data were compared by one-way analysis of variance (ANOVA) followed by Tukey's test when significant differences were found at $p \leq 0.05$ using SAS program (SAS, 1997).

Results and Discussion

Lipases have important roles in digestion of lipids by insects and intermediary metabolism including lipid storage and mobilization (Horne *et al*., 2009). Our results clearly demonstrated presence of a digestive lipase in the gut of *B. oleae*. On the other hands, it could be inferred that alimentary canal of larval olive fruit fly has ability to digest lipid by the action of soluble and membrane-bound lipases.In the current study, lipase activity was observed in both soluble and membrane-bound fractions by higher activity of membrane-bound fraction (Fig. 1) althopugh, majority of studies have been determined

in soluble content of the midgut. Since digestive enzyme secretion is a secretagogue process, digestive enzymes are presence as enzymatic vesicle inside of epithelial cells during starvation (Klowden, 2007). During ingestion, these vesicles are released into midgut lumen. Hence, higher activity of membrane-bound lipase could be attributed to the higher amounts of enzyme as vesicles inside the epithelial cells.

Temperature and pH are the two factors that affect biochemical reactions via various approaches

Table 1 Effect of mono- and divalent cations on soluble lipase activity of *B.oleae*

compound	concentration	Specific activity
	C	1.857±0481 a
	1	0.312±0.064 b
	3	0.122±0.093 b
Ca^{2+}	5	0.047±0.037 b
	C	1.857±0481 a
	1	0.442±0.034 b
	3	0.289±0.75 b
Cu^{2+}	5	0.537±0.074 b
	C	1.857±0481 a
	1	1.088±0.194 b
	3	1.598±0.119 ab
Fe^{2+}	5	1.714±0.167 ab
	C	1.857±0481 a
	1	0.741±0.134 ab
	3	0.224±0.081 b
K^{+}	5	0.047±0.013 b
	C	1.857±0481 a
	1	8.913±0.649 b
	3	2.415±0.350 b
Mg^{2+}	5	1.592±1.354 b
	C	1.857±0481 a
	1	0.340±0.018 b
	3	0.115±0.013 b
Na^{+}	5	1.769±0.426 ab
	C	1.857±0481 a
	1	1.585±0.204 a
	3	0.122±0.031 b
Zn^{2+}	5	0.394±0.013 b

*Statistical differences have been shown by various letters (Tukey's test, $p \leq 0.05$).

Table 2 Effect of mono- and divalent cations on membrane-bound lipase activity of *B. oleae*

compound	concentration	Specific activity
	C	6.800±0.322 a
	1	0.628±0.081 b
	3	0.052±0.028 b
Ca^{2+}	5	0.231±0.087 b
	C	6.800±0.322 a
	1	0.304±0.066 b
	3	1175±62.915 b
Cu^{2+}	5	0.555±0.277 b
	C	6.800±0.322 a
	1	0.463±0.176 b
	3	1.283±0.162 b
Fe^{2+}	5	0.972±0.251 b
	C	6.800±0.322 a
	1	0.807±0.184 b
	3	142.719±719 b
K^{+}	5	140.778±12.165 b
	C	6.800±0.322 a
	1	3.380±0.587 b
Mg^{2+}	3	1.574±0.767 b
	5	1.316±0.324 b
	C	6.800±0.322 a
	1	0.469±0.017 b
	3	0.205±0.102 b
Na^{+}	5	0.608±0.283 b
	C	6.800±0.322 a
	1	1.223±0.160 b
Zn^{2+}	3	0.205±0.102 b
	5	0.641±0.509 b

*Statistical differences have been shown by various letters (Tukey's test, $p \leq 0.05$).

like substrate and enzyme stability, their combination, tertiary structure of the enzyme and etc. Both of these factors provide optimal conditions to more adjustment of enzyme leading to better affinity and velocity during enzymatic reactions (Zibaee *et al*., 2012). It was found that optimal pH of the digestive soluble- and membrane-bound lipases in the gut of *B. oleae* larvae were obtained 4 and 6 for soluble lipase and 6 for membrane-bound one (Fig. 2). Meanwhile, optimal temperatures for soluble and membrane lipases were observed at 50

°C and 35 °C, respectively (Fig. 3). Grillo *et al.* (2007) reported that digestive lipase from the midgut of *Rhodnius prolixus* (Hemiptera: Reduviidae) had maximal activity in pH 7-7.5. Zibaee *et al*. (2008) found the optimal pH and temperature of 10 and 37 - 40 °C in larvae of *Chilo suppressalis*. Zibaee (2012) found optimal pH and temperature of digestive lipasein *P. brassicae* as pH 11 and temperature of 30 °C. The optimal pH of 10 and temperature of 35 - 40 ˚C observed for a lipase from larvae of *N. aenescens* (Zibaee and Fazeli-Dinan, 2012). The optimal pH for TAG-lipase activity was obtained at pH 9 and temperature 40 °C for *A. spinidens* (Zibaee *et al*., 2012). The optimal temperature for activity of an enzyme reflects temperature in which organism is living in it. Also, activity of enzymes increase along with elevation of temperature up to optimal value then it decrease because the hydrogen bonds in enzyme structure break in extreme temperature and disrupt three-dimensional structure leading to enzyme denaturation (Zeng and Cohen, 2000).

Ions are one of the significant components in active sites of enzymes. Ions can take and release electrons; affect electrophiles and nucleophiles, increase efficiency of enzyme-substrate complex and stability of enzymes (Zibaee, 2012). In the present study, different concentrations of ions and chelating agents caused various effects on lipase activity in *B oleae* larvae (Tables 1 - 4). Activities of the digestive soluble and membrane-bound lipases in the gut of *B. oleae* larvae were decreased by using all ions (Tables 1, 2). The effects of several chelating agent and PMSF were examined to find their possible effects on digestive soluble- and membrane-bound lipase activities (Tables 3, 4). All the chelating agents except for TTHA in soluble lipase had no inhibitory effects on the enzymatic activity but PMSF sharply decreased enzymatic activity in both fractions (Tables 3, 4). Applebaum 1985) reported that Ca^{2+} ion increased lipase activity

Table 3 Effect of specific inhibitors on soluble lipase activity of *B. oleae*

compound	concentration	Specific activity
DTC	C	0.217±0.013 a
	10	0.442±0.114 a
PMSF	C	1.857±0.481 a
	10	0.421±0.118 b
EGTA	C	925±0.156 b
	10	1.809±0.119 a
EDTA	C	0.217±0.013 a
	10	0.442±0.114 a
TTHA	C	1.687±0.203 a
	10	0.591±0.142 b

*Statistical differences have been shown by various letters (Tukey's test, $p \leq 0.05$).

Table 4 Effect of specific inhibitors on membrane-bound lipase activity of *B. oleae*

compound	concentration	Specific activity
DTC	C	0.555±0.052 b
	10	1.223±0.152 a
PMSF	C	6.800±0.322 a
	10	1.706±0.558 b
EGTA	C	1.574±0.220 a
	10	1.488±0.045 a
EDTA	C	0.840±0.262 a
	10	1.303±0.304 a
TTHA	C	1.580±0.077 a
	10	0.077±0.0.056 a

*Statistical differences have been shown by various letters (Tukey's test, $p \leq 0.05$).

of *Callosobruchus chinensis*. Zibaee (2012) found that Mg^{2+}, Na^{+}, EDTA and TTHA significantly affect digestive lipase activity in *P. brassicae*. Grillo *et al*. (2007) found that Ca^{2+} increase activity of lipases in *R. prolixus.* Similar results were found in case of *C. suppressalis* and *N. aenescens* (Grillo *et al.,* 2007; Zibaee *et al.*, 2008; Zibaee and Fazeli-Dinan, 2012). Ca^{2+}, Mg^{2+}, K^{+}, Na^{+}, Mn^{+}, PMSF and EGTA showed significant effects on lipase activity in *A. spinidens* (Zibaee *et al.,* 2012).

Although digestive lipases of insects have been less studied but they have a great potential to be inhibited like amylases and proteases. This property could be used to develop resistant varieties in sustainable agricultural system. There are many reports on inhibition of vertebrate pancreatic lipases by secondary metabolites of plants but relevant studies on insect digestive lipases are few (Markwick *et al*., 2011). Senthil-Nathan *et al*. (2006) and Zibaee and Bandani (2010) reported that feeding of *Eurygaster integriceps* (Hemiptera: Scutelleridae) and *Cnaphalocrocis medinalis* on the diets containing botanical extracts lead to significant inhibition of digestive lipases. Also, Markwick *et al*. (2011) demonstrated effects of tetrahydrolipstatin (THL), on neonate *Epiphyas postvittana* (Lepidoptera, Tortricidae) larvae by feeding on control artificial diets and diets containing one of three concentrations of THL (0.011 %, 0.037 % and 0.11 %). The authors reported significant decrease in growth, pupation and time to pupation in comparison with control.

Although reports on digestive lipases of insects revealed promising results but it is necessary to carried out more studies to confirm that digestive lipases of insects could be a new control for insect pest or not. Lipase inhibitors do cause severe reduction in growth and development and even mortality due to the importance of long chain unsaturated fatty acids being essential dietary components. Hence, a plant breeding program might

be adopted to increase levels of naturally occurring lipase inhibitors. The current study was the first one to characterize a digestive lipase of dipteran larvae. This is a basic study that will continue to develop inhibitors leading to an efficient control of *B. oelae*.

References

Applebaum SW. Biochemistry of digestion. In: Kerkut GA, Gilbert LL (eds), Comprehensive insect physiology, biochemistry and pharmacology, Vol. 4, Regulation, digestion, excretion, Pergamon Press, Oxford, pp 279-307, 1985.

Cheeseman P, Jutsum AR, Goldsworthy GJ. Quantitative studies on the release of locust adipokinetic hormone. Physiol. Entomol. 1: 115-121, 1976.

Grillo LA, Majerowicz D, Gondim KC. Lipid metabolism in Rhodnius prolixus (Hemiptera: Reduviidae): role of a midgut triacylglycerol-lipase. Insect Biochem. Mol. Biol. 37: 579-588, 2007.

Gupta R, Gupta N, Rathi P.Bacterial lipases: an overview of production, purification and biochemical properties. Appl. Microbiol. Biotechnol. 64: 763-781, 2004.

Horne I, Haritos VS, Oakeshott JG. Comparative and functional genomics of lipases in holometabolous insects. Insect Biochem. Mol. Biol. 39: 547-567, 2009.

Jaeger KE, Dijkstra BW, Reetz MT. Bacterial biocatalysts: molecular biology, three-dimensional structures, and biotechnological applications of lipases. Ann. Rev. Microbiol. 53: 315-351, 1999.

Jafari Y, Rezaee V. First report of import olive fly to country. New Entomol. Soc. Iran. 6: 1-22, 2004 [in Persian].

Kawooya JT, Law JH.Role of lipophorin in lipid transport to the insect egg. J. Biol. Chem. 263: 8748-8753, 1988.

Klowden MJ. Physiological systems in insects, 2nd ed., Elsevier, New York, NY, 2007.

Laskowski JR, Kato M. Protein inhibitors of proteinases. Ann. Rev. Biochem. 49: 593-626, 1980.

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193: 265-275, 1951.

Majumder UK, Sengupta A. Triglyceride composition of chrysalis oil, an insect lipid.J. American. Oil Chem. Soc. 56: 620-623, 1979.

Markwick NP, Poulton J, McGhie TK, Wohlers MW, Christeller JT. The effects of the broadspecificity lipase inhibitor, tetrahydrolipstatin, on the growth, development and survival of the larvae of *Epiphyas postvittana* (Walker) (Tortricidae, Lepidoptera). J. Insect Physiol. 57: 1643-1650, 2011.

Mirrahimi S, Khalaghani J, Nouri H. Effect of time, direction and site of sampling on olive infestation by olive fruit fly. Proceedings of 18th Iranian Plant protection, 2008.

Richard R, Phillips PA, Stewart-Leslie JS ibbett GS. Olive fruit fly population in central and southern California. 57. Doi: 10.3733/ca.v057n04p122, 2003.

SAS institute.*SAS/STAT* User's guide for personal computers. SAS Institute, Cary, Nc, 1997.

Senthil-Nathan S, Chung PG, Murugan K.Combined effect of biopesticides on the digestive enzymatic profiles of *Cnaphalocrocis medinalis* (Guenee) (the rice leaffolder) (Insecta: Lepidoptera: Pyralidae). Ecotox. Environ. Safe. 64: 382-389, 2006.

Tan-Kristanto A. Characterisation of lipase genes in *Helicoverpa armigera*. Department of Genetics. The University of Melbourne, 2006.

Terra WR, Ferriera C. Biochemistry of digestion. In: Gilbert LI (ed.), Insect molecular biology and biochemistry, Elsevier, pp 365-418, 2012.

Tsujita T, Ninomiya H, Okuda H. p-Nitrophenyl butyrate hydrolyzing activity of hormonesensitive lipase from bovine adipose tissue. J. Lipid Res. 30: 997-1004, 1989.

Zeng F, Cohen AC. Partial characterization of α-amylase in the salivary glands of *Lygus Hesperus and L. lineolaris*. Comp. Biochem. Physiol. 126B: 9-16, 2000.

Zeng F, Zhu Y, Cohen AC. Molecular cloning and partial characterization of a trypsin-like protein in salivary gland of *Lygus hesperus (Hemiptera: Miridae)*. Insect Biochem. Mol. Biol. 32: 455-464, 2002.

Zibaee A. A digestive lipase of *Pieris brassicae L*. (Lepidoptera: Pieridae): Purification, Characterization and Host plants effects. Arch. Insect Biochem. Physiol. 81: 1-19, 2012.

Zibaee A, Bandani AR.Effects of *Artemisia annua* L. (Asteracea) on digestive enzymes profiles and cellular immune reactions of sunn pest, *Eurygaster integriceps (Heteroptera: Scutellaridae),* against *Beauvaria bassiana.* Bull. Entomol. Res. 100: 185-196, 2010.

Zibaee A, Bandani AR, Ramzi S. Lipase and invertase activities in midgut and salivary glands of *Chilo suppressalis* (Walker) (Lepidoptera: Pyralidae), rice striped stem borer. Inv. Surv. J. 5: 180-189, 2008.

Zibaee A, Fazeli-dinan M. Purification and characterization of a digestive lipase in *Naranga aenescens* Moore (Lepidoptera: Noctuidae). SOAJ 1: 38-54, 2012.

Zibaee A, Hoda H, Fazeli-Dinan M. A TAG-Lipase activity in the salivary secretions of a predaceous bug, *Andrallus spinidens* Fabricius (Hemiptera: Pentatomidae).Trends Entomol. 9: 48-57, 2012.

Ziegler R. Changes in lipid and carbohydrate metabolism during starvation in adult *Manduca sexta.* J. Comp. Physiol. 161: 125-131, 1991.