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Purification and Characterization of Midgut Lipase from the Greater Wax Moth, *Galleria mellonella* (Lepidoptera: Pyralidae)

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ABSTRACT

In this study, purification and molecular weight of the digestive lipase was described for the first time in *Galleria mellonella*. It was extracted from the midgut of the last larval instar and partially purified by ammonium sulphate and sephadex G-100. The results indicated that the purified enzyme has specific activity of 2.68 U mg^{-1}, recovery of 18.3% and purification fold of 49.15 and has a 104.23kDa molecular weight. The purified enzyme was further characterized for its activity at different temperatures and pH values. The highest activity was at 37˚C and pH 7. Also, the enzyme activity was tested against some inhibitors (EDTA, PMSF and EGTA) and activators ($CaCl_2$, NaCl and KCl). It was observed that lipase was inhibited and activated with all used chelating agents and activators, respectively. The kinetic parameters of this enzyme were determined as they give us important information about the enzyme behavior and efficiency. The results revealed that the enzyme needs a high concentration of P-NPB (used as substrate) to achieve its maximal velocity. The enzyme showed K_m of 381.46 mM using P-NPB as a substrate. These results will help in developing new pest control strategies targeting pest physiology.

Key words: *Galleria mellonella*, midgut lipase, purification, biochemical characterization.

INTRODUCTION

Living organisms depend on carbohydrates, lipids and proteins for their physiological activities, but the proportion of requirements varies from species to species and based on their environmental adaptation. Although, lipids can be produced from carbohydrates even in insects, dietary lipids are the most important parts of ingested food (Zibaee and Fazeli-Dinan, 2012). Enzymes responsible for the hydrolysis of lipid are lipases (triacylglycerol acylhydrolase, EC 3.1.1.3) which catalyze the hydrolysis of fatty acid ester bonds. They are widely distributed among animals, plants and micro-organisms (Grillo *et al*., 2007; Zibaee *et al*., 2008).

Lipases of insects are divided into triacylglycerol lipases (TAG-lipases), alkaline and acid phosphatases as well as phospholipases (Terra and Ferreira, 2012). The majority of ingested lipids by insects are the storage lipids (TAGs) that must be digested to di- and monoglycerids in midgut (Zibaee *et al*., 2008). Lipases have a vital physiological role, catabolism of triacylglycerols (TAGs) stored as fat bodies and those from dietary lipids; hence, two main groups of lipase are recognized (Miled *et al*., 2000), lysosomal (intracellular) and digestive lipases. Intracellular lipases are responsible of TAGs hydrolysis stored as lipid droplet, the major endogenous source of energy (Wolins *et al*., 2006), while digestive lipases hydrolyze TAGs in food.

The greater wax moth, *Galleria mellonella*, is one of the most destructive pests of honey bee colonies in the world (Oh *et al*., 1995).There are different control methods to decrease pest damage, including

releasing biocontrol agents (Barakat, 2006), but no effective control has yet been found. The use of inhibitors against digestive enzymes is a promising method to disrupt growth and development of insect pests. Insect digestive enzymes are a potential target for reducing feeding ability, therefore complete characterization of digestive enzymes is necessary to study.

Galleria larva feeds on beeswax and probably has a unique system for lipid transport and utilization. Lipases have been purified from some insects, such as *Manduca sexta* (Arrese and Wells, 1994); *Drosophila melanogaster* (Smith *et al*., 1994), *Locusta migratoria* (Van der Horst *et al*., 2001); *Gryllus campestris* (Orscelk *et al*., 2007) and *Naranga aenescens* (Zibaee and Fazeli-Dinan, 2012). As yet, there is an almost complete lack of knowledge on the lipase purification in *G. mellonella.* Detailed knowledge on the enzymatic environment will provide new opportunities for a sustainable pest management. The current study aimed to purify and characterize the midgut lipase as a preliminary study on *G. mellonella* to gain a better understanding of its digestive physiology. The obtained findings could, subsequently, offer basic information for integrated pest management of this important pest.

MATERIALS AND METHODS

Insect rearing

The greater wax moth, *G. mellonella* (L.) was obtained from Plant Protection Research Institute, Agricultural Research Center, Dokki, Egypt and reared for several generations on an artificial diet, as reported by Kulkarni *et al.* (2012). The last larval stage was removed and used for sample preparation.

Sample preparation

The last larval instars were dissected and their midguts were removed and washed in ice-cold saline solution (NaCl, 15 mM). The midgut tissue rinsed in ice-cold buffer, placed in a pre-cooled glass homogenizer and ground in 1 ml of buffer solution. The homogenate was separately transferred to 1.5 ml centrifuge tubes and centrifuged at 13,000 rpm for 20 min at 4 °C The supernatant was pooled and stored at -20 °C for subsequent analyses.

Protein determination

Protein concentration was determined by the method of Bradford (1976) by using bovine serum albumin (BSA) as a standard protein.

Enzyme assay

Lipase activity was measured spectrophotometrically as described by Tsujita *et al.* (1985) with some modifications. P-nitrophenyl butyrate (pNPB) was used as substrate, reaction mixture contained 30 µl of gut extract and 100 µl of substrate mixed and incubated at 37 °C. 100 µl of homogenization buffer was added then absorbance was read at 405 nm. For control tube, samples were placed in boiling water path for 20 min to destroy enzyme activity.

Purification of crude extract

Purification process was performed as described by Orscelk *et al*. (2007), with some modifications. It was occurred in the two following steps:

Ammonium sulphate precipitation:

Samples were subjected to ammonium sulphate precipitation using 40 and 80% and fractions were collected by

centrifugation at 10000 rpm. Precipitation steps were carried out at 4°C.

Sephadex G-100 column:

The last ammonium sulphate fraction was collected and subjected to gel filtration on sephadex G-100 column (12 x 2 cm) equilibrated by 20 mM of universal buffer. Enzyme fractions of 3 ml were collected at a flow rate of 20 ml/h with the same buffer. Protein content and lipase activity were measured and the fractions showing the highest activities were pooled for further processing.

Gel electrophoresis and zymogram

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out according to the method of Laemmli (1970) by using 4% stacking gel and 15% resolving gel. Zymogram analysis of lipase was carried out according to method of (Prim *et al*., 2003) by using MUF–butyrate as a substrate. By the end of the run, gel was gently separated from glasses and rinsed in distilled water then incubated at room temperature in Triton X-100 (2.5% v/ v), 30 min later, gel was rinsed again in distilled water then incubated in 100ml of 100mM MUF-butyrate solution as fluorescent substrate. After 10 min, gel was put on a UV trans-illuminator to observe fluorescent bands in dark background.

Effect of temperature on enzyme activity

The effect of temperature on the activity of lipase enzyme was tested by incubating the reaction mixtures at different temperatures ranging from 20 to 70°C for 1 h and after incubation the activity was measured as described before.

Effect of pH on enzyme activity

The reaction mixtures were incubated at different pH values ranging from 3 to 13 for 1 h the enzyme activity was measured as mentioned.

Effect of inhibitors on enzyme activity

30 µl of enzyme was incubated with equal volumes of different concentrations (0, 0.5, 1, 1.5, 2.0 mM) of phenylmethylsulfonyl fluoride (PMSF), ethylene glycol bis N,N,N′,N′-tetraacetic acid (EGTA) and ethylenediaminetetraacetic acid (EDTA), incubated for 10 min and then activity was determined.

Effect of metal ions on enzyme activity

The effect of $CaCl_2$, NaCl and KCl was measured at the following concentrations 0, 10, 20, 30 and 40 mM. A 50 µl of buffer containing one concentration of these ions was incubated with 30 µl of enzyme for 1 h then 100 µl of universal buffer (pH 10) was added and activity was measured.

Determination of kinetic parameters

According to Walsh *et al.* (2010), the kinetic parameters were obtained at 37 °C and pH 7.5, using PNPB as substrate at concentrations of 10, 20, 30, 40, 50 and 60 mM.

Statistical analysis

All data were compared by one-way analysis of variance (ANOVA) followed by Turkey's test when significant differences were found at $p \leq 0.05$. Kinetic parameters of enzyme were estimated by Microsoft Excel®.

RESULTS

Purification of larval midgut crude extract

After second ammonium sulphate precipitation, midgut lipase showed specific activity of 0.675±0.0341 U/mg protein,

0.148±0.00721 mg/ml of protein, 85.22 % of recovery and 12.4 of purification fold (Table 1). When sample was loaded on sephadex G-100 column, fractions from 6 to 32 showed a lipase activity with the highest specific activity at fraction number 19 (Fig.1). The previous fraction had 0.008±0.001 mg/ml protein, specific activity 2.68±0.315 U/mg protein with 18.3 % recovery and 49.15 fold of purification (Table 1).

Table 1: Purification process of midgut lipase from *G. mellonella* larvae.

Purification step	Protein [a] (mg/ml)	Total activity [b] (U)	Specific activity [b] (U/mg)	Purification fold	(%) of recovery
Crude extract	2.15±0.18 (A)	0.11723±0.00632 (A)	0.05453±0.0027 (A)	1	
$(NH_4)_2SO_4$ (0-40%)	0.4242±0.0266 (B)	0.11233±0.00681 (A)	0.2648±0.026 (B)	4.86	95.82
$(NH_4)_2SO_4$ (40-80%)	0.148±0.00721 (C)	0.0999±0.000100 (B)	0.675±0.0341 (B,C)	12.4	85.22
Sephadex G-100	0.008±0.001 (C)	0.021433±0.000208 (C)	2.68±0.315 (C)	49.15	18.3

a Protein content was measured by the Bradford method (1976).
b One unit of activity corresponds to 1 µmol of pNPB released per min using 50 mM pNPB as the substrate at 37°C.
Data were showed as (Mean±SE), n=3 replicates.
Different letters indicate significant differences ($p<0.05$)

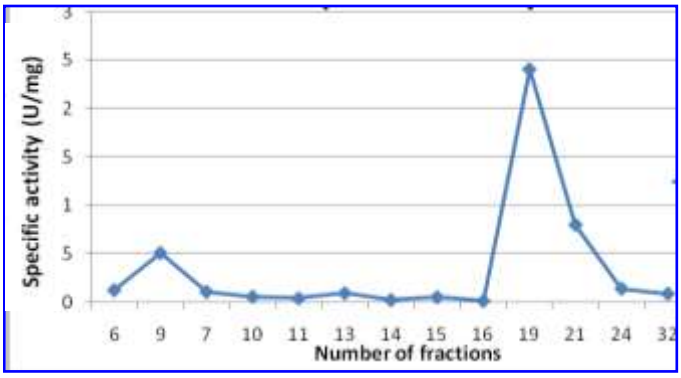

Fig.1. Specific activity of different fractions of lipase from purification process of midgut lipase of *G. mellonella* larvae.

Gel electrophoresis and zymogram:

Protein profile and zymogram Showed that midgut lipase had a molecular weight of 104.23 kDa (Fig. 2).

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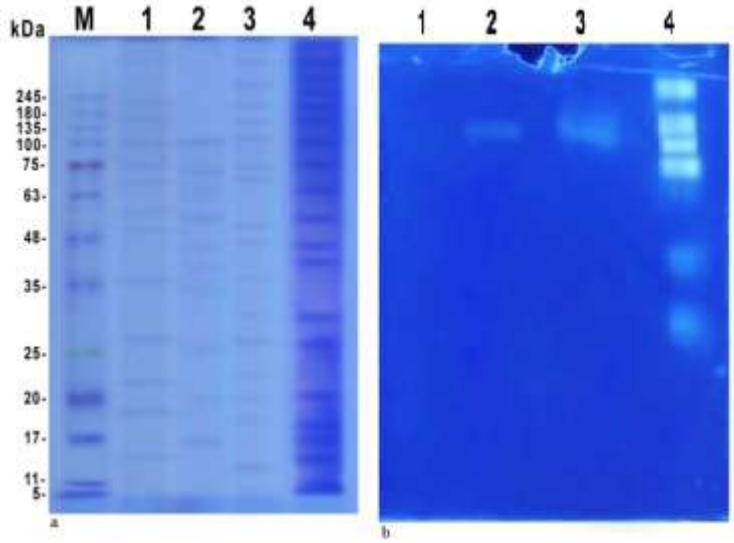

Fig. 2: SDS-PAGE of the purified midgut lipase from *G. mellonella* larvae. M: Molecular marker, 1: Fraction 9 after sephadex G-100 column, 2: Fraction 19 after loading column, 3: Sample after amm. sulphate precipitation (40-80%) 4: Crude extract.

Effect of temperature on enzyme activity

The purified lipase showed a steady increase in its activity by elevating the incubation temperature from 20-37°C then activity decreased till 70°C. The maximum activity was at 35 and 37°C (Fig. 3).

Effect of pH on enzyme activity

Lipase showed high activity at pH range from 7 to 9 with the maximal activity at pH 7 (Fig. 4).

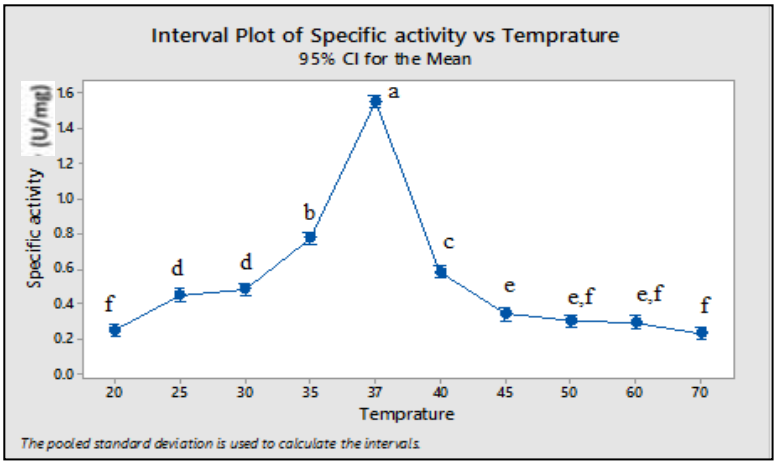

Fig. 3: Effect of temperature (°C) on specific activity of purified midgut lipase from *G. mellonella* larvae by using different temperature range. Different letters indicate significant differences ($p<0.05$).

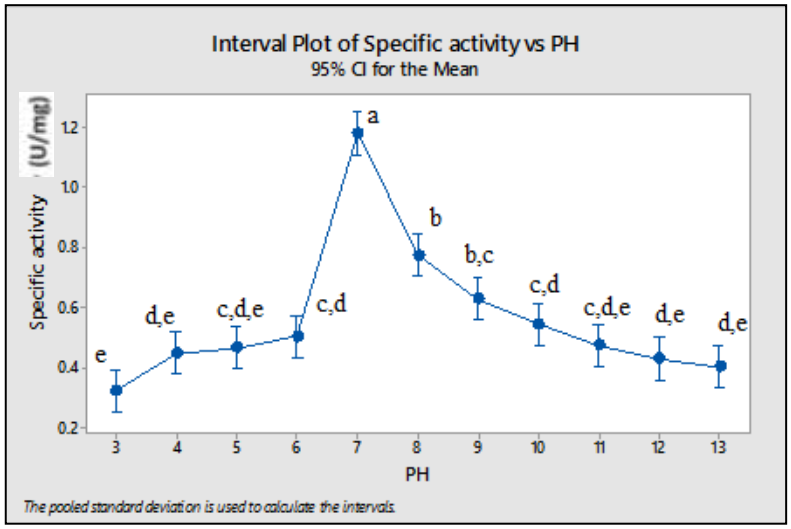

Fig.4: Effect of pH on specific activity of purified midgut lipase from *G. mellonella* larvae by using different pH range. Different letters indicate significant differences (p<0.05).

Effect of metal ions on enzyme activity

The highest concentration of Ca^{2} increased the enzyme activity 2.7 fold than 0 concentration with specific activity of 4.889 ± 0.192 for 40 mM of $Ca2^{+}$ and 1.795±0.08231 for absence of $Ca2^{+}$ (Fig. 5). Also K^{+} and Na^{+} had a significant effect on activity of midgut lipase (Fig. 6 and Fig. 7), respectively.

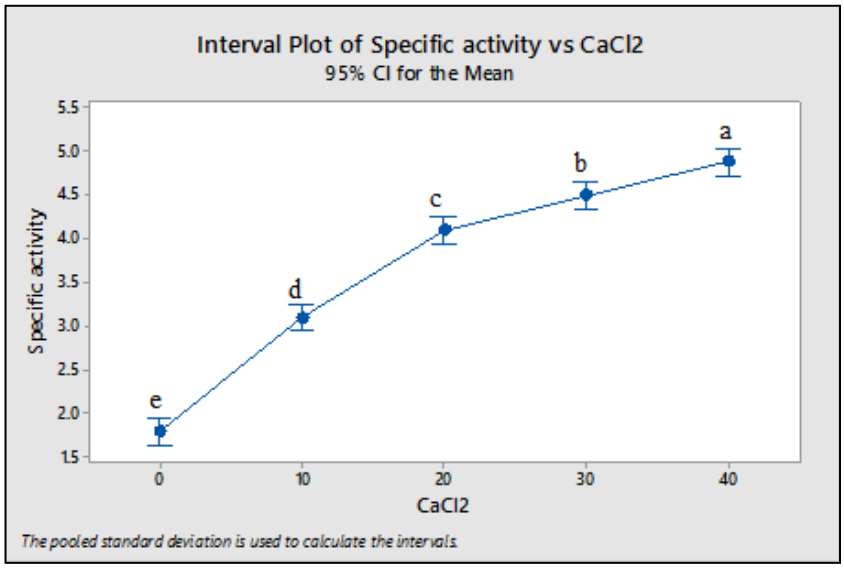

Fig. 5: Effect of $CaCl_2$ on specific activity (U/mg) of purified lipase from larval midgut tissue of *G. mellonella*. All experiments were carried out at 37°C. pNPB (50 mM) was used as substrate. The enzyme reaction was incubated with ions separately for 1 h. Different letters indicate significant differences (p<0.05).

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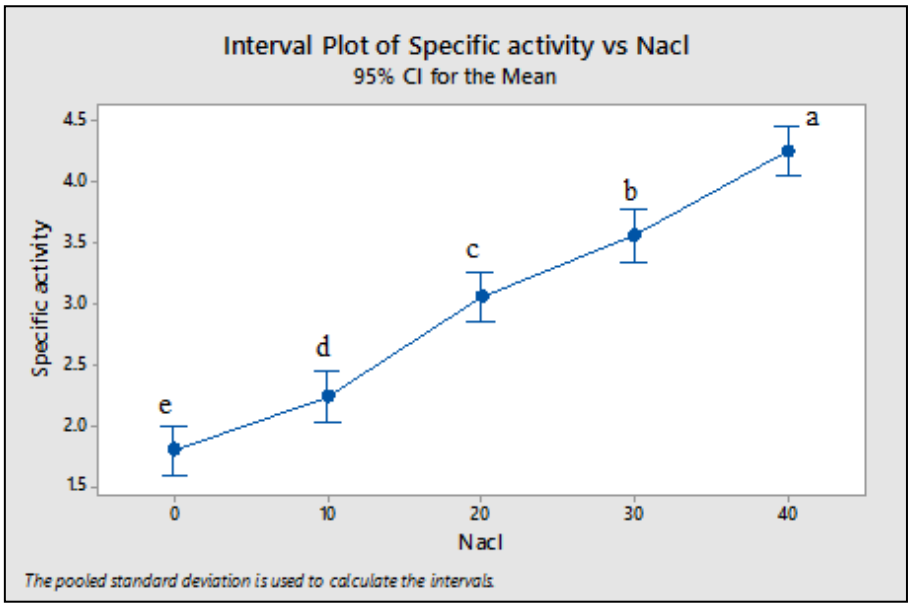

Fig. 6: Effect of NaCl on specific activity (U/mg) of purified lipase from larval midgut tissue of *G. mellonella*. All experiments were carried out at 37°C. pNPB (50 mM) was used as substrate. The enzyme reaction was incubated with ions separately for 1 h. Different letters indicate significant differences ($p<0.05$).

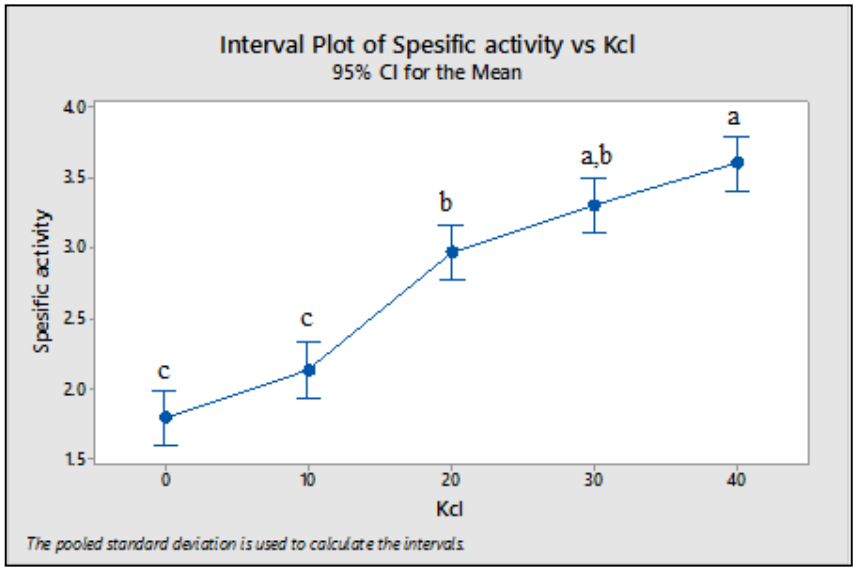

Fig. 7: Effect of KCl on specific activity (U/mg) of purified lipase from larval midgut tissue of *G. mellonella*. All experiments were carried out at 37°C. pNPB (50 mM) was used as substrate. The enzyme reaction was incubated with ions separately for 1 h. Different letters indicate significant differences ($p<0.05$).

Effect of inhibitors on enzyme activity

Different concentrations of EDTA, EGTA and PMSF had significant inhibitory effect on purified enzyme (Figs. 8, 9 and 10), respectively, with a sharp decrease in activity at 0.5 mM concentration of EDTA and PMSF in comparison with control.

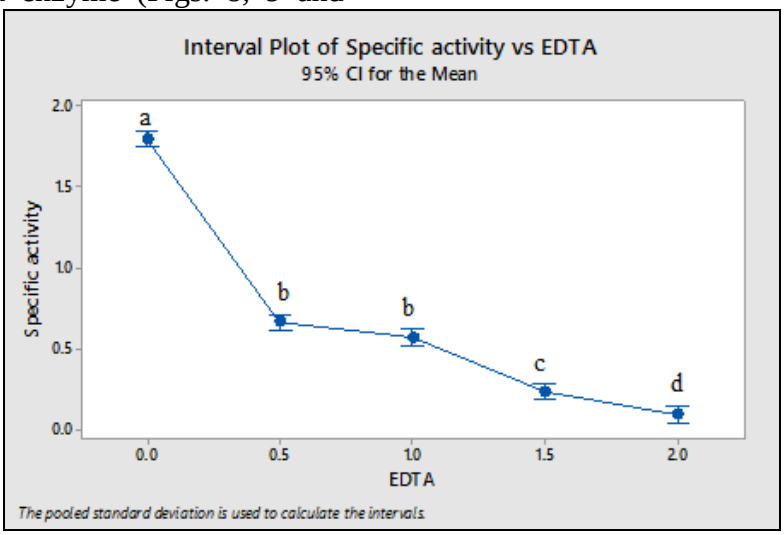

Fig. 8: Effect of EDTA on specific activity (U/mg) of purified lipase from larval midgut tissue of *G. mellonella*. All experiments were carried out at 37°C. pNPB (50 mM) was used as substrate. The enzyme reaction was incubated with ions separately for 1 h. Different letters indicate significant differences ($p<0.05$).

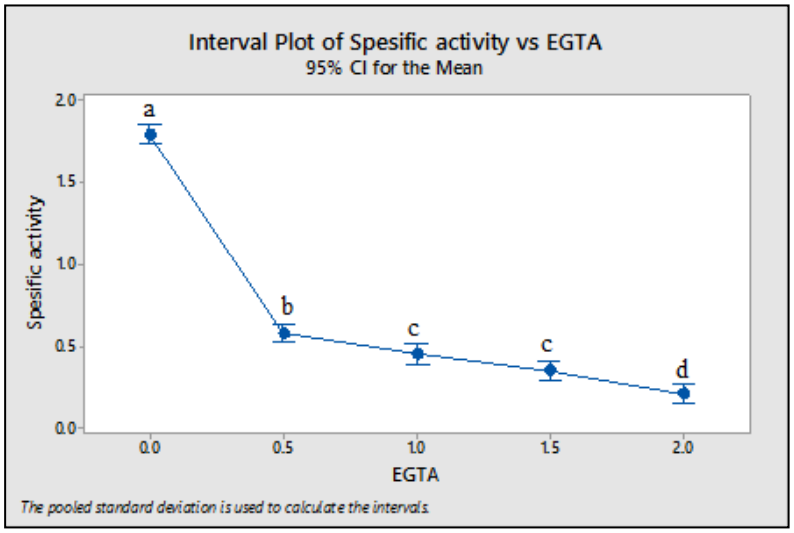

Fig. 9: Effect of EGTA on specific activity (U/mg) of purified lipase from larval midgut tissue of *G. mellonella*.All experiments were carried out at 37°C. pNPB (50 mM) was used as substrate. The enzyme reaction was incubated with ions separately for 1 h. Different letters indicate significant differences ($p<0.05$).

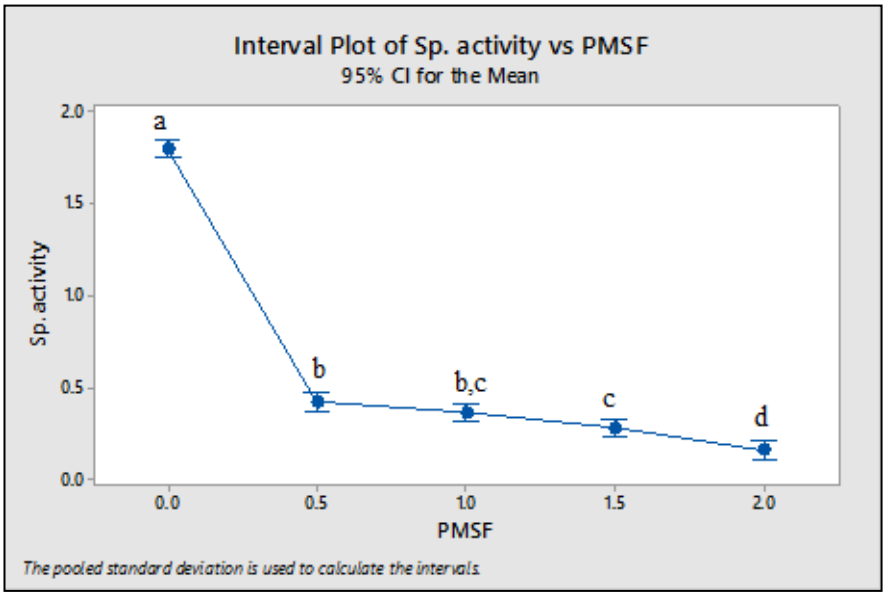

Fig. 10: Effect of PMSF on specific activity (U/mg) of purified lipase from larval midgut tissue of *G. mellonella*.All experiments were carried out at 37°C. pNPB (50 mM) was used as substrate. The enzyme reaction was incubated with ions separately for 1 h. Different letters indicate significant differences ($p<0.05$).

Kinetic parameters

Midgut lipase showed a K_m 381.46 ± 6.8199 mM of and V_{max} 0.7893 ± 0.34Umg^{-1} for pNPB (Fig. 11).

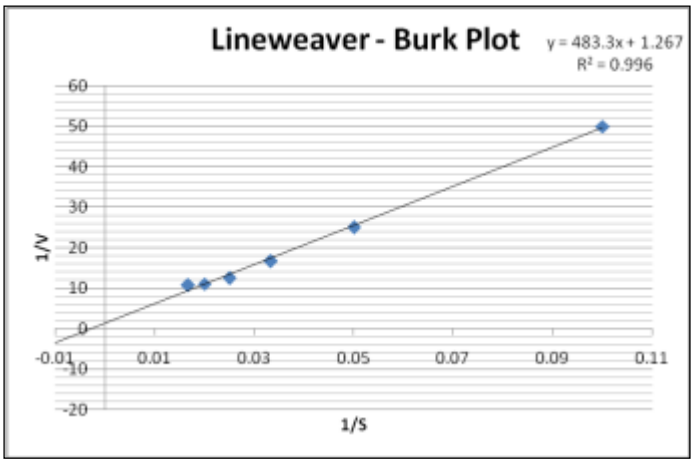

Fig. 11: Double reciprocal plot to show the kinetic parameters of purified lipase from the larval midgut tissue of *G. mellonella* by using pNPB (from 10 to 60 mM) ($1/V_{max}$= intercept on the $1/V_0$ ordinate, K_m/V_{max} = the slope of the regression line.

DISCUSSION

The current study describes the possibility to use *Galleria* larvae for identification of digestive lipase extracted from the midgut, and provides direct tests to clarify the enzyme properties by performing purification and biochemical approaches, which is an advantage for studies related to the digestive physiology. Various purification methods depend on nonspecific techniques, precipitation, gel filtration, ion exchange chromatography, affinity chromatography and hydrophobic interaction chromatography have been used to isolate and purify different lipases. Total body lipase was purified from *Gryllus campestris* by ammonium sulphate precipitation followed by gel filtration (Orscelk *et al*., 2007). Digestive lipase was purified from many insects such *Ectomyelois ceratoniae* (Ranjbar *et al*., 2015) by three purification steps. The increase in enzyme specific activity and decrease in total protein content after each purification step indicate the success of purification process of salting out and gel filtration. In the present study; after the final purification step, the enzyme was enriched 49.15 fold and the specific activity reached 2.68 U mg^{-1}. Also the partial purification of lipase was confirmed by screening the protein profile of enzyme before and after each purification step through elimination of some protein bands after each step. Then the molecular weight of enzyme was estimated by zymogram and it was supposed to be 104.23 kDa. Other molecular masses were observer for many insects such 76 kDa for fat body lipase of *Manduca sexta* (Arrese and wells 1994), 30 kDa for lipase of *Cephaloleia presignis* (Auerswald and Gäde 2006).

Temperature and pH are critical factors that must be taken in consideration as they affect the biological reaction. In current study; the optimum pH and temperature ranges were 7-9 and 35-37°C, respectively. These results are in consistence with the findings of Zibaee *et al*. (2008) on *C. suppressalis*, Zibaee and Fazeli-Dinan (2012) on *N. aenescens*, and Santana. (2017) on *Rhynchophorus palmarum*. The high activity of enzyme at certain pH range gave information about the environment where enzyme locates (Roy, 1937) which seems to be alkaline in *G. mellonella*. The pH affects the enzyme activity by altering the charge of substrate or the active site of the enzyme (Zheng and Cohen, 2000). Very high and very low pH disrupts the hydrogen bond which help enzyme to maintain its three-dimensional structure (Zheng and Cohen, 2000). The enzyme activity was decreased by increasing the temperature up to point of enzyme denaturation. These results are similar to that on gypsy moth, *Lymantria dispar* (Mrdakovic *et al*., 2008) and *N. aenescens* (Zibaee and Fazeli-Dinan, 2012). The increasing effect of metal ions on lipase activity especially Ca^{2+} was also showed by Applebaum (1985), Grillo *et al*. (2007) which indicates that lipase is metalloproteinase. Ions enhance enzyme activity by making enzyme and substrate close to each other or put the active groups of enzyme and substrate in the most perfect position leading to increase the ability of enzyme-substrate complex and enzyme stability. This was supported by using EDTA and EGTA chelating agents. Also PMSF had a negative effect on lipase activity by increasing its concentration. Rosseto *et al*. (2003) showed similar results. The effect of PMSF indicates the presence of a serine residue for these activities as concluded by Brady *et al*. (1999) on analyses of the three-dimensional structure of lipase.

Enzyme kinetics is the study of the chemical reactions that are catalyzed by

enzymes. Kinetic parameters of enzyme give us important information about enzyme behavior and efficiency. Therefore the reaction rate is measured and the effects of varying the conditions of the reaction are investigated (Walsh*et al*., 2010). Studying an enzyme's kinetics in this way can reveal the catalytic mechanism of this enzyme, its role in metabolism, how its activity is controlled, and how a drug or an agonist might inhibit the enzyme (Fromm and Hargrove, 2012).

Results of midgut lipase from larvae of *G. mellonella* revealed that it needs a high concentration of P-NPB (used as substrate) to achieve its maximal velocity. Increasing substrate concentration means an increasing rate at which the enzyme and substrate molecules encounter one another. However, at relatively high substrate concentrations, the reaction rate asymptotically approaches the theoretical maximum; the enzyme active sites are almost all occupied by substrates resulting in saturation, and the reaction rate is determined by the intrinsic turnover rate of the enzyme (Wrighton and Ebbing, 1993). The substrate concentration midway between these two limiting cases is denoted by K_M. Thus, K_m is the substrate concentration value in which the substrate concentration is reaching halfway of the maximum reaction velocity (Schnell and Maini, 2004). The purified midgut lipase from larvae of *G. mellonella* had 381.46 mM K_m. Kinetic parameters vary according to the used substrate and are achieved using various concentrations of the substrate.

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تنقية وتوصيف ليبيز المعى المتوسط من يرقات فراشة الشمع الكبرى، *جاليريا ميلونيلا* (حرشفية الأجنحة: بايراليدى)

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المستخلص

تم في هذه الدراسة وصف طريقة تنقية إنزيم الليبيز الهضمى وتعيين الوزن الجزيئى له لأول مرة في حشرة فراشة الشمع الكبرى. تم إستخلاص الإنزيم من المعى المتوسط لليرقات في العمر الأخير وتنقيته جزئياً بواسطة كبريتات الأمونيوم والسفادكس جـ - 100. وأظهرت النتائج أن الإنزيم المنقى له نشاط خاص يبلغ 2.68 وحدة/ملج، وقوة إستعادة 18.3٪ وقوة تنقية قدرها 49.15 ضعفا، وله وزن جزيئي 104.23 كيلو دالتون. تم كذلك وصف الإنزيم المنقى وتعيين نشاطه عند درجات حرارة ودرجات حموضة وقلوية مختلفة. وكان أعلى نشاط عند 37 درجة مئوية و **pH 7**. كما تم اختبار نشاط الإنزيم ضد بعض المثبطات مثل (**EGTA**،**PMSF** ،**EDTA**) وبعض المحفزات مثل (**$CaCl_2$** ،**NaCl** و **KCl**). وقد لوحظ أن الليبيز يتم تثبيطه وتنشيطه مع جميع العوامل المخلبية والمحفزات المستخدمة على التوالي. وأخيراً، لأنه من المهم تحديد العوامل الحركية للإنزيم لأنها تعطى معلومات مهمة عن سلوك وفعالية الإنزيم فقد وجد من النتائج أن الإنزيم يحتاج إلى تركيز عال من الركيزة المستخدمة **(pNPB)** لتحقيق أقصى سرعة تفاعل. هذه النتائج سوف تساعد في تطوير استراتيجيات مكافحة الآفات الجديدة التي تستهدف فسيولوجيا الآفات.