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Purification and characterization of fat body lipase from the greater wax moth, *Galleria mellonella* (Lepidoptera: Pyralidae)

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Abstract

Background: Insect lipid mobilization and transport are currently under research, especially lipases and lipophorin because of their roles in the production of energy and lipid transport at a flying activity. The present study has been conducted to purify intracellular fat body lipase for the first time, from the last larval instar of *Galleria mellonella*.

Results: Purification methods by combination of ammonium sulfate [$(NH_4)_2SO_4$] precipitation and gel filtration using Sephadex G-100 demonstrated that the amount of protein and the specific activity of fat body lipase were 0.008633 ± 0.000551 mg/ml and 1.5754 ± 0.1042 μmol/min/mg protein, respectively, with a 98.9 fold purity and recovery of 50.81%. Hence, the sephadex G-100 step was more effective in the purification process. SDS-PAGE and zymogram revealed that fat body lipase showed two monomers with molecular weights of 178.8 and 62.6 kDa. Furthermore, biochemical characterization of fat body lipase was carried out through testing its activities against several factors, such as different temperatures, pH ranges, metal ions, and inhibitors ending by determination of their kinetic parameters with the use of p-nitrophenyl butyrate (PNPB) as a substrate. The highest activities of enzyme were determined at the temperature ranges of 35–37 °C and 37–40 °C and pH ranges of 7–9 and 7–10. The partially purified enzyme showed significant stimulation by Ca^{2+}, K^{+}, and Na^{+} metal ions indicating that fat body lipase is metalloproteinase. Lipase activity was strongly inhibited by some inhibitors; phenylmethylsulfonyl fluoride (PMSF), ethylene-diaminetetractic acid (EDTA), and ethylene glycoltetraacetic acid (EGTA) providing evidence of the presence of serine residue and activation of enzymes by metal ions. Kinetic parameters were 0.316 Umg^{-1} V_{max} and 301.95 mM K_m.

Conclusion: Considering the purification of fat body lipase from larvae and the usage of some inhibitors especially ion chelating agents, it is suggested to develop a successful control of *Galleria mellonella* in near future by using lipase inhibitors.

Keywords: *Galleria mellonella*, Purification, Characterization, Intracellular lipase, Fat body lipase

Background

Lipases are the enzymes responsible for the hydrolysis of lipid (triacylglycerol acylhydrolase, EC 3.1.1.3) which catalyze the fatty acids ester bonds hydrolysis. These enzymes have significant functions in the usage, storage, and transmission of lipids in insects. They are also essential in fundamental physiological processes such as reproduction, development, protection against pathogens and oxidative stress, and pheromone signaling (Horne et al. 2009). Insect lipases are classified into triacylglycerol lipases (TAG-lipases), alkaline and acid phosphatases in addition to phospholipases (Terra and Ferreira, 2012).

Lipases have a dynamic physiological role; the catabolism of triacylglycerols (TAGs) that stored as depots of fat and those from nutritional lipids. Henceforth, two basic groups of lipase are documented, lysosomal (intracellular) and digestive lipases (Miled et al., 2000). Intracellular lipases, stored as lipid droplets are responsible for TAGs hydrolysis, the chief endogenous energy source (Wolins et al. 2006), while digestive lipases hydrolyze TAGs in food.

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The greater wax moth, *Galleria mellonella* (*G. mellonella*), is one of the most destructive pests of honey bee colonies worldwide (Oh et al. 1995). It causes considerable economic losses to beekeepers by damaging wax combs. The destruction of the comb is responsible for leaking or contaminating stored honey, killing bee larvae or causing the spreading of honey bee diseases (Caron, 1992). However, an actual method of controlling this pest has not been developed. Physical and chemical methods are unsatisfactory (Burges, 1978). Therefore, many studies have been directed to find solutions to controlling it. It is mandatory to study the enzymes of the insect pests for developing biotechnological methods to provide perfect and effective control measures (Oh et al., 1995).

Recently, insect lipid mobilization and transport are currently under search, especially lipases and lipophorin because of their roles in the production of energy and lipid transport at a flying activity. Most of the researches carried out on insect lipase focused on midgut lipase, while little researches have been carried out on fat bodies (Orscelk et al. 2007; Patel et al. 2005). Lipases have been purified from some insects, such as *Manduca sexta* (Arrese and Wells, 1994); *Gryllus campestris* (Orscelk et al., 2007); *Locusta migratoria* (Van der Horst et al. 2001); and *Naranga aenescens* (Zibaee, 2012).

Detailed knowledge of the enzymatic environment of *Galleria* will provide new chances for a maintainable pest management to control this pest. The wax moth is a highly specialized insect; its larva feeds on bees wax and probably has a unique system for lipid transport and usage (Wlodawer and Lągwińska 1967).

According to the available literature, in wax moth, no intracellular lipase has been purified and characterized yet. Therefore, the current study aims to purify and characterize the fat body lipase of *G. mellonella* larvae.

Materials and methods

Rearing and maintenance of studied insect

The greater wax moth, *G. mellonella* (L.) was obtained from Plant Protection Research Institute, Agricultural Research Center, Egypt. A stock colony of *G. mellonella* was maintained for several generations in the insectary of Entomology Department, Faculty of Science, Ain Shams University and reared on artificial diet according to (Kulkarni et al. 2012). This colony was kept in constant darkness at 30 ± 2 °C in a 5-l screen gallon with the diet mixture containing pollen and bee wax. The larvae during the last stage were collected for further experiments.

Processing and preparation of larval tissue homogenates

Collection of fat body tissues of *G. mellonella* larvae was prepared according to Fuchs et al. (2010). Larvae were cut from the upper part of the body distally to the lower part. The larval body was squeezed to discard the hemolymph, and the fat body was dissected out under ice cold distilled water.

Pooled tissues from 50 individuals (These insects convey ~ 350 μg fat body tissue) were immediately transported to buffered saline (pH: 6.9) and weighed before analysis. Experimental fat body tissue was placed in a pre-cooled glass homogenizer and crushed in 1 ml of buffer solution (pH 7). The homogenate was transferred to 2 ml centrifuge tubes and centrifuged at 13,000 rpm (Human Centrifuge, TGL-16XYJ-2, 16,000 rpm, Korea) for approx. 20 min at 4 °C. The supernatant was stored at − 20 °C for subsequent analyses.

Estimation of protein concentration

The method of Bradford (1976) was applied to measure the protein concentration by using bovine serum albumin (BSA) as a standard protein.

Determination of lipase activity

Lipase activity was determined by using the continuous spectrophotometric rate determination method as documented by Tsujita et al. (1989) with some modifications. The enzyme activity for samples at the beginning of the purification procedure was assayed using the following substrate solution: unlabeled trioleoylglycerol (final concentration 2 mM) and Triton X-100 (final concentration 10 mM) were dissolved in toluene-ethanol 1:l (v/v), and purified; radiolabeled trioleoylglycerol was added to give a specific activity of about 7 × lo** dpm/mol. Paranitrophenyl butyrate (PNPB) (final concentration 27 and 50 mM). Thirty microliters of crude extracts (from fat body) and 100 μl of p-nitrophenyl butyrate (PNPB, 50 mM), as substrate were thoroughly mixed and incubated at 37 °C. For samples of negative control, the tubes were put in a boiling water bath for 20 min to destroy the activity of enzyme then cooled. Saline buffer (100 μl) were supplemented to each tube (control and treatment), and the absorbance was read at 405 nm with a spectrophotometer (UNICO, SP2100 UV, China) for approximately 5 min, then the absorbency/min (total activity) was obtained by means of the maximum linear rate for both the test and blank.

Specific enzyme activity as unit/min/ml protein was calculated according to the following equation:

$$\text{Units/mg protein} = \text{units/ml enzyme/mg protein/ml enzyme.}$$

Purification of fat body lipase

Purification of the tissue lipase extracted from the fat body was achieved in the two steps established on a

process described by Orscelk et al. (2007), with some adjustments.

Ammonium sulfate [$(NH_4)_2SO_4$] precipitation

Samples were first exposed to [$(NH_4)_2SO_4$] precipitation by making use of 40 and 80% of [$(NH_4)_2SO_4$] solution and the [$(NH_4)_2SO_4$] fraction was then gathered and centrifuged at 10,000 rpm for 20 min. All the precipitation steps were performed at 4 °C, and in each step, the activity of enzyme and content of protein were determined (Additional file 1).

Sephadex G-100 gel filtration chromatography

The final fraction of [$(NH_4)_2SO_4$] was exposed to gel filtration on a Dried Sephadex G-100 column. The dried gel was incubated in distilled water for 5 h at 90 °C. It was loaded onto the column (12 × 2 cm) at 27 °C, after its cooling and removal of air. Afterward, 20 mM universal buffer (pH 10) was used for equilibration of the column, containing 50 mM [$(NH_4)_2SO_4$]. Three milliliters enzyme fractions were collected at a flow rate of 20 ml/h with the same buffer. Content of protein and activity of lipase were measured and fractions that showed the highest activities were pooled for the last step. Sephadex G-100 was obtained from Pharmacia Fine Chemicals, and experiment applied at Regional Center for Mycology and Biotechnology, Al-Azhar University, Cairo, Egypt.

Purification fold and yield% was calculated according to the following equations:

$$\text{Purification fold} = \text{specific activity of purified enzyme} / \text{specific activity of unpurified enzyme.}$$

$$\text{Yield}\% = \left(\text{Total activity of purified enzyme} / \text{total activity of unpurified enzyme}\right) * 100.$$

Determination of molecular weight and purity of the purified lipase (electrophoretic analysis)

The purity and molecular weight of the purified enzyme were determined by using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), as explained by Laemmli (1970). A 4% stacking gel and 15% resolving gel were used.

The enzyme molecular mass was estimated using BL Uelf Prestained Protein Ladder (from Genedirex) as molecular mass standards (marker proteins).

A gel documentation system using a scanner was used to scan the gel, and then, analysis of bands was carried out using software: Gel-Pro Analyzer ver. 6.0.

Zymogram analysis

Lipase zymogram analysis was carried out according to methods of Prim et al. (2003), using MUF–butyrate (from Sigma) as the substrate. SDS-PAGE was performed using 15% resolving and 4% stacking gel. By ending of the run, the gel separated gently from glasses and rinsed immediately with distilled water then incubated in Triton X-100 (2.5% v/v) at room temperature, allowing the enzyme renaturation. Gels were rinsed again after 30 min, with distilled water, and incubated in 100 ml MUF-butyrate solution (100 μM in 50 mM phosphate buffer at pH 8.0). After 10 min, the gel was transferred to UV trans-illuminator to detect fluorescent bands in dark background.

Determination of biochemical characteristics of the purified lipases

Effect of pH on lipase activity

The pH effect on the activity of purified lipase was measured using lipase diluted in 15 μl universal buffer (Stauffer, 1989). Buffers were made for the pH range from 2 to 13. Lipase activity was assayed as described above, after incubation for 1 h at each pH value.

Effect of temperature on lipase activity

Lipase was diluted in 15 μl buffer (50 mM Tris–HCl at pH 7–7.5) then incubated at temperatures ranging from 20 to 70 °C for 1 h. Instantly post-incubation, the activity of lipase was determined using PNPB as the substrate, as described previously.

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

The various ions ($CaCl_2$, NaCl, and KCl) effects on lipase activity were determined. Fifty microliters of buffer solution containing one concentration of ions (0, 10, 20, 30, and 40 mM) along with 30 μl of enzyme were pre-incubated for 1 h at pH 7.5 and 37 °C. The mixture that pre-incubated was supplemented to a solution including 100 μl of universal buffer (pH 7.5). Other steps were conducted out as mentioned before.

Effect of specific inhibitors on lipase activity

The enzyme inhibitors effects on activity of lipase were reported using different concentrations (0, 0.5, 1, 1.5, and 2 mM) of ethylene glycol-bis (β-aminoethylether) N, N, N′, N′-tetraacetic acid (EGTA), phenylmethylsulfonyl fluoride (PMSF), and Ethylenediamine tetraacetic acid (EDTA). The purified enzyme (30 μl) was incubated with equal volumes of each inhibitor for 10 min at pH 7.5 and 37 °C. The mixture was supplemented to a solution including 30 μl of substrate (PNPB, 50 mM), and then the activity was measured as mentioned above.

Kinetic parameters measurements

The kinetic parameters (The Michaelis constant, Km and the maximum velocity V_{max}) were determined according to (Walsh et al. 2010). Final concentrations for *p*-nitrophenol butyrate were 10, 20, 30, 40, and 60 mM,

using 30 ul of diluted enzyme preparations at 37 °C and pH 7.5 in each assay.

Statistical analysis

Data were expressed as mean ± standard error (SE) of 3 replicates for each determination and analyzed by using the Minitab 18 statistical software. The difference between means was analyzed by student *t* test after normality test and compared by the one-way analysis of variance (ANOVA) followed by Tukey's Test when $P \leq 0.05$. The K_m and V_{max} of the enzyme were estimated by Microsoft Excel®.

Results

Purification of intracellular fat body lipase from *G. mellonella* larvae

The amount of protein content in crude extract from fat body tissue of *G. mellonella* larvae was 1.6702 ± 0.023 mg/ml, and specific lipase activity was 0.016102 ± 0.000278 (μ/mg) (Additional file 1).

After the second phase of $[(NH_4)_2SO_4]$ precipitation, fat body lipase exhibited a specific activity of 0.0844 ± 0.00216 U/mg protein, 1.68 ± 0.0724 mg/ml of protein, 52.43% of recovery, and 5.3 fold purification (Table 1). The $[(NH_4)_2SO_4]$ fractions were then loaded to the Sephadex G-100 column. The sample was fractionated into 50 fractions from which 21 fractions showed lipase activity with three peaks of specific activities at fractions numbers 6, 8, and 17, and fraction number 6 showed the highest specific activity (Fig. 1). After the last purification step; protein and the specific activity amounts of fat body lipase were 0.008633 ± 0.000551 mg/ml and 1.5754 ± 0.1042 μmol/min/mg protein, respectively with 50.81% recovery and 98.9 fold purification. Statistically, there was no significant difference in specific lipase activity between crude sample and sample after ammonium sulfate $[(NH_4)_2SO_4]$ precipitation steps but a large significant difference was observed between the latter samples and sample that undergo final step of purification (Fig. 2). That means the sephadex G-100 step was more effective in the purification process.

Determination of molecular weight and purity of the purified lipases from *G. mellonella* larvae: SDS-PAGE and zymogram

SDS-PAGE showed that several protein bands were eliminated after each purification step, and the lowest number of bands that appeared on the gel was after the final step (Fig. 3). Zymogram analysis yielded two bands with molecular weights of 178.8 kDa and 62.6 kDa for fat body (Fig. 4).

Biochemical characterization of purified intracellular fat body lipase

The effect of pH on lipase activity

The pH effect on lipase activity is shown in (Fig. 5) with significant differences ($p < 0.05$). *F* values were 379.23. The activity of the enzyme gradually increased from 4 to 8 and then reduced until pH 13. Activity was high when assayed at pH 7–10, with the maximal activity at pH 8. Lipase activity was decreased at pH 3.

The effect of temperature on lipase activity

The temperature effect on the activity of purified lipase was assessed throughout a range from 20 to 70 °C (Fig. 6). The purified lipase represented a gradual increase in its activity by incubation temperature elevation from 20 to 40 °C and decreased until 70 °C. The Highest activity under these conditions was at both 37 and 40 °C. The statistical analysis demonstrated that the highest activity of the enzyme at 37 and 40 °C were similar to each other ($p \leq 0.05$) ($F = 60.81$).

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

Mono- and di-valent cations (in several concentrations) effects the purified lipase activity are shown in (Fig. 7). All different concentrations of Ca^{2+} significantly ($p \leq 0.05$) increased the activity of fat body lipase as it was 2.918 ± 0.0746 μmol/min/mg protein in the highest concentration that is 2.144-fold more than of the activity of enzyme without using Ca^{2+}. Effect of Na^+ and K^+ showed a significant increase ($p < 0.05$) from 0 concentration of Na^+ and K^+ to 40 mM concentration. *F* values

Table 1 Purification process of fat body lipase from *G. mellonella* larvae

Purification step	Protein[a] (mg/ml)	Total activity[b] (U)	Specific activity[b] (U/mg)	Purification fold	(%)
Crude extract	1.68 ± 0.0724 (A)	0.026767 ± 0.000681 (A)	0.015933 ± 0.000288 (B)	1	100
$(NH_4)_2SO_4$ (0–40%)	0.903 ± 0.00608 (B)	0.024 ± 0.0 (B)	0.0266 ± 0.000177 (B)	1.67	89.7
$(NH_4)_2SO_4$ (40–80%)	0.16633 ± 0.00551 (C)	0.014033 ± 0.000153 (C)	0.0844 ± 0.00216 (B)	5.3	52.43
Sephadex G-100	0.008633 ± 0.000551 (D)	0.0136 ± 0.0001 (C)	1.5754 ± 0.1042 (A)	98.9	50.81

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

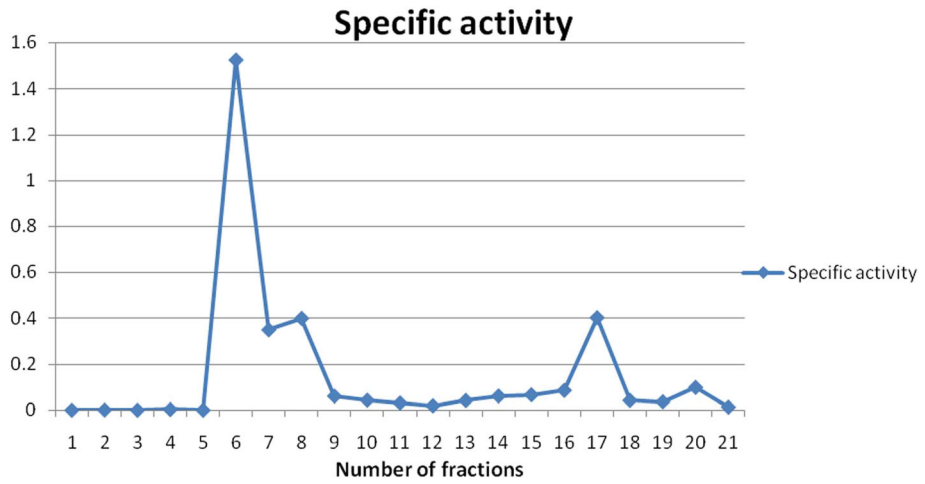

Fig. 1 Purification process of fat body lipase from *G. mellonella* larvae. Sephadex G-100 gel-filtration of lipase after ammonium sulfate **[$(NH_4)_2SO_4$]** (40 and 80%) treatment. Fractions number 6 to 21 contained the high enzymatic activity on PNPB (50 mM) and fraction number 6 with the highest specific activity (U/mg) was collected for next steps, (no. of replicates = 3)

of fat body lipase tests were 104.95, 47.90, and 83.25 for Ca^2, Na^+, and, K^+, respectively.

Effect of specific inhibitors on lipase activity of G. mellonella

We used PMSF (serine protease inhibitor), EGTA (calcium specific chelating agents), and EDTA (general chelating agent) to find the role of metal ions and serine residue in the active site of fat body lipase (Fig. 8). At different concentrations of PMSF, we recorded a significant inhibitory effect on fat body lipase with a sharp decrease in activity at 0.5 mM concentration of inhibitor in comparison with control. For EGTA, the enzyme activity decreased at all the tested concentrations. Also EDTA had a significant gradual inhibitory effect on fat body lipase with the lowest activity at a concentration of 2 mM. The purified lipase activity gradually reduced by increasing concentrations of PMSF, EGTA, and EDTA pointing to the presence of metal ions especially Ca^{2+} and a serine residue in the active sites of the enzymes. Statistically, all tests were significantly different with p value < 0.05.

Kinetic parameters of purified lipase from larval fat body of *G. mellonella*

The maximum velocity (V_{max}) and Michaelis constant (K_m) were calculated using several concentrations of p-nitrophenol butyrate to measure kinetic parameters of

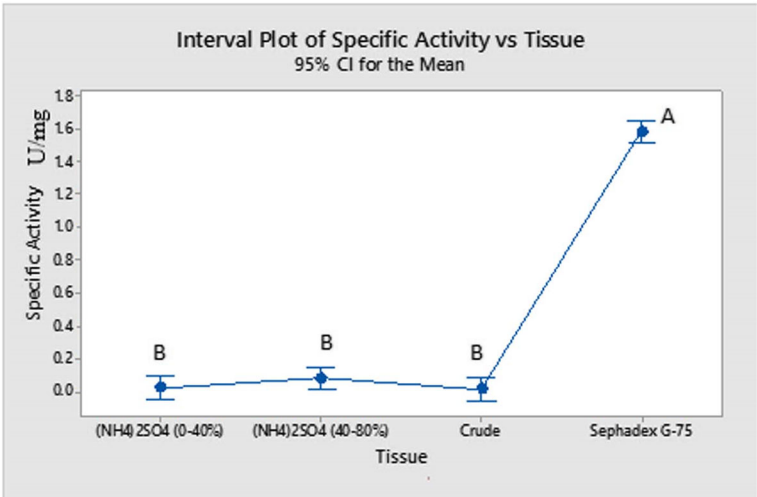

Fig. 2 Specific activity of fat body lipase before purification and during steps of purification. Different letters indicate significant differences ($p < 0.05$). (no. of replicates = 3)

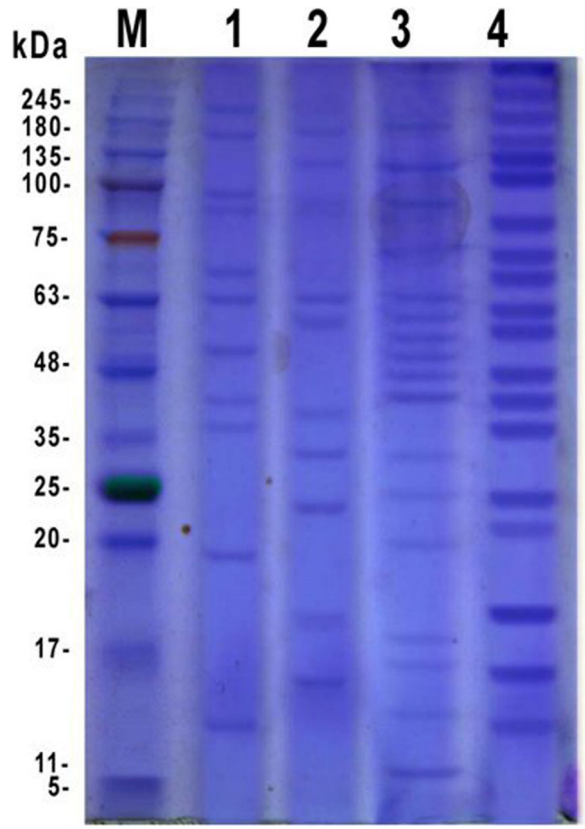

Fig. 3 SDS-PAGE of purified fat body lipase from *G. mellonella* larvae. M: molecular marker, 1: Fraction 17 after sephadex G-100 column, 2: Fraction 6 after loading column, 3: Sample after amm. Sulfate percipitation (40–80%), 4: Unpurified fat body extract

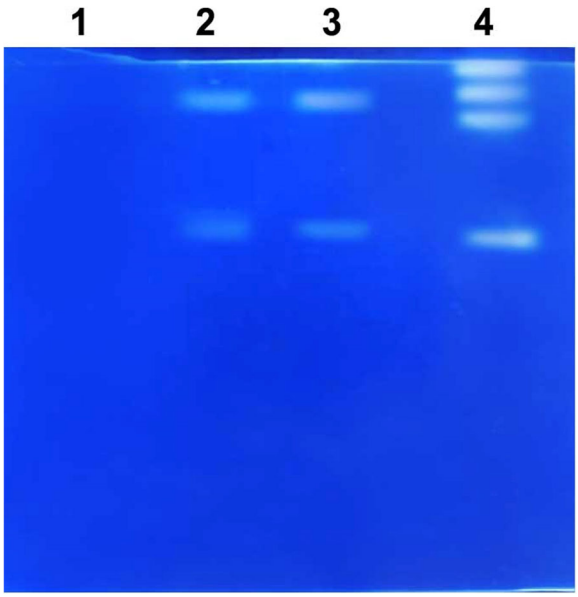

Fig. 4 Zymogram of purified fat body lipase from *G. mellonella* larvae. 1: Fraction 17 after sephadex G-100 column, 2: Fraction 6 after loading column, 3: Sample after amm. Sulfate percipitation (40–80%), 4: Unpurified fat body extract

the purified lipase. We found that; the V_{max} of fat body lipase was 0.316 ± 1.24 U/mg protein while K_m was 301.59 ± 24.973 mM (Fig. 9). The statistical analysis showed p value of 0.02 (Additional file 1).

Discussion

The presented study describes for the first time the possibility to use *G. mellonella* larvae for the identification of intracellular lipase extracted from the fat body. This study also provides direct tests for the characterization of enzyme properties by purification performance and biochemical approaches, which is an advantage for studies related to physiology and pest control.

The extracted lipase from larval and fat body was purified by using two steps. Non-protein components were separated from proteins using ammonium sulfate $[(NH_4)_2SO_4]$ in a salting-out process. Adding $[(NH_4)_2SO_4]$ decreased solvent molecules that interact with proteins by interacting with them (Robinson, 2015). Some proteins coagulate as precipitate because of decreasing the number of solvent molecules. Also, the separation of a specific enzyme from other proteins can be done through this step.

A gel filtration followed the previous step which separates large proteins according to molecular weights. Very large proteins were excluded while the smaller ones entered between gel particles. Larger protein molecules passed down the column and recovered with shorter elution time (Robinson, 2015). It was found that the selected procedures for purification of the larval fat body lipase are operative to abolish some of contaminating proteins and non-protein molecules so that the final preparation showed to be electrophoretically more partially pure than crude by presentation few number bands on SDS-PAGE.

Various purification methods depend on non-specific techniques, precipitation, filtration of gel, ion exchange, affinity, and hydrophobic interaction chromatography have been used to isolate and purify different lipases. Orscelk et al. (2007) have purified the total body lipase from *Gryllus campestris* by ammonium sulfate $[(NH_4)_2SO_4]$ precipitation followed by gel filtration. Ranjbar et al. (2015) has purified digestive lipases purified from several insects by three purification steps, such as *Ectomyelois ceratoniae* (Ranjbar et al., 2015); *Naranga aenescens* by Zibaee (2012) and from *Antheraea drury* by Marepally and Benarjee (2016).

In the current study, protein profiles were resolved by (SDS-PAGE). Electrophoretic separation was achieved at a 15 mA constant current and at 2 °C. Coomassie brilliant blue R-250 was used to visualize the separated proteins. In our study, the analyzed fractions show fewer numbers of bands than crude by SDS-PAGE that is due

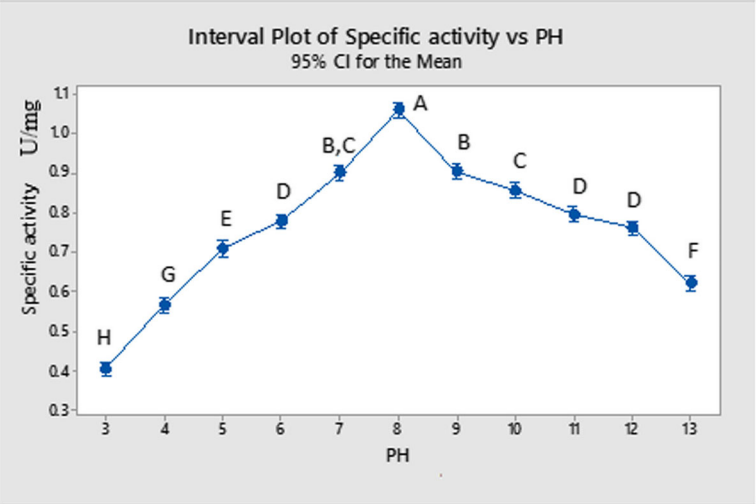

Fig. 5 Effect of pH on activity of purified fat body lipase from larvae of *G. mellonella* by using different pH range. Different letters indicate significant differences ($p < 0.05$), (no. of replicates = 3)

to the elimination of unwanted proteins from fractions through purification processing. Invertebrate lipases vary widely in their molecular mass. Zymographic analysis showed that intracellular fat body lipase consisted of two monomers with molecular mass of 178.8 kDa and 62.2 kDa. Indeed, nature of enzyme varies with food and organism from which they extracted (Agusti and Cohen, 2000; Hubert et al. 1999; Torres and Bayd, 2009; Zeng and Cohen 2000a, 2000b). Other molecular masses were observed for many insects such as 76 kDa for fat body lipase of *M. sexta* (Arrese and Wells, 1994), 30 kDa for lipase of *Cephaloleia presignis* (Auerswald and Gäde, 2006), 28 kDa for lipase from digestive juices of *Bombyx mori* (Ponnuvel et al., 2003) and 196 kDa for intracellular lipase from *Litopenaeus vannamei* pleopods (Rivera-Pérez and García-Carreño, 2011).

The pH is one of the most vital factors which biochemical reactions depend on. Fat body lipase showed the greatest activity at pH 8 followed by 9 while most activity was lost at pH 3. It seems that lipases from *G. mellonella* have a slightly alkaline optimum pH, these results are in conscience with that of kissing bug, *R. prolixus* which have high lipase activity at pH 7.0–7.5 (Grillo et al. 2007), and tobacco hornworm *M. sexta* at pH 7.9 (Arrese and Wells, 1994).

The high activity of the enzyme at certain pH indicates where the organism feeds in an environment (Grillo et al., 2007). The pH affects the enzyme activity by

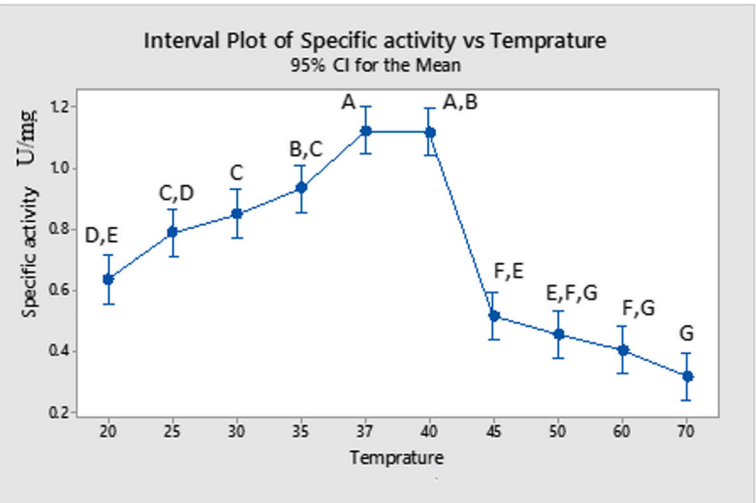

Fig. 6 Effect of temperature (°C) on activity of purified fat body lipase from larvae of *G. mellonella* by using different temperature range. Different letters indicate significant differences ($p < 0.05$), (no. of replicates = 3)

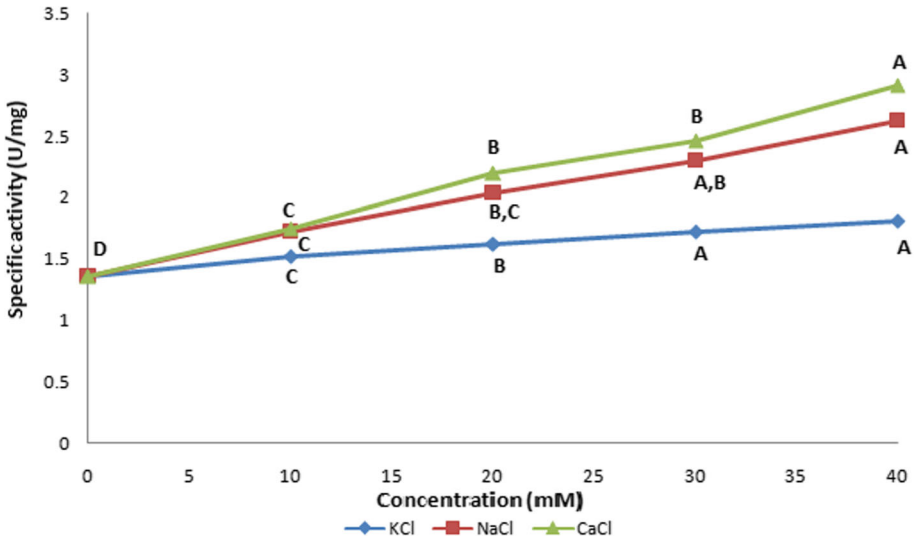

Fig. 7 Effect of KCl, NaCl and $CaCl_2$ on purified lipase from larval fat body tissue of *G. mellonella*. All experiments were carried out at 37 °C. PNPB (50 mM) was used as substrate. The enzyme reaction was incubated with ions separately for 1 h. Different letters indicate significant differences ($p < 0.05$), (no. of replicates = 3)

charge altering of substrate or enzyme active site. Very high and low pH can cause electrostatic repulsion (Roussel et al. 1999). The pH extremes can also interrupt the hydrogen bonds that keep the enzyme in its three-dimensional structure (Zeng and Cohen, 2000a). However, these changes may be canceled if the enzyme returned to its optimal conditions (Robinson 2015).

Another factor that biochemical reactions rely on is temperature. In the current study, the temperature effect on lipase activity was assessed over a range from 20 to 70 °C. The maximum activity for fat body intracellular lipase was at 37–40 °C while the minimum activity was at 70 °C. It was concluded that the enzyme activity reached its peak at the most suitable range of temperature then this activity decreased by increasing the temperature above the suitable one till reached the point at which enzyme denatured then activity decreased sharply. High temperature may interfere with the hydrogen bond of enzyme causing denaturation of this enzyme (Zeng and Cohen, 2000a). Parallel results have been revealed in other insect lipases, as in gypsy moth *Lymantria dispar* (Mrdaković et al. 2008), *Rhynchophorus palmarum* (Santana et al. 2017) and *N.*

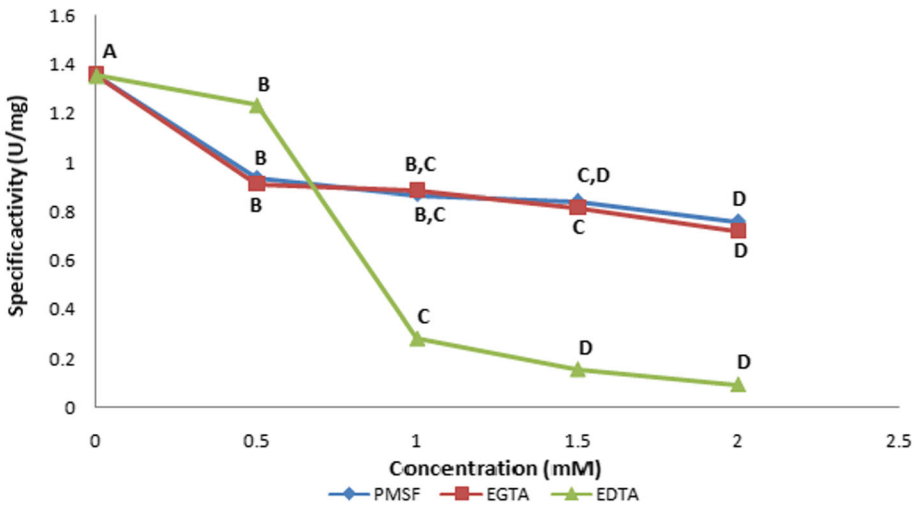

Fig. 8 Effect of PMSF, EGTA and EDTA on purified lipase from larval fat body tissue of *G. mellonella*. All experiments were carried out at 37 °C. PNPB (50 mM) was used as substrate. The enzyme reaction was incubated with various concentrations of inhibitor separately for 10 min. Different letters indicate significant differences ($p < 0.05$), (no. of replicates = 3)

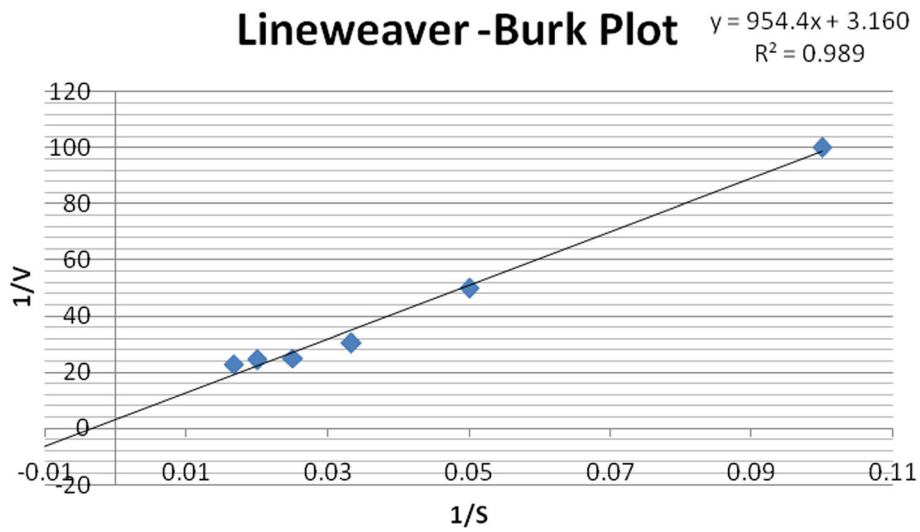

Fig. 9 Double reciprocal plot to show the kinetic parameters of purified lipase from the larval fat body tissue of *G. mellonella* by using PNPB (from 10 to 60 mM) ($1/V_{max}$ = intercept on the $1/V_0$ ordinate, *Km*/*V*max = the slope of the regression line

aenescens (Zibaee, 2012). In contrast, some invertebrates showed the highest activity of lipase at 60 °C such as the Mediterranean green crab *Carcinus mediterraneus* (Cherif et al., 2007) and the Antarctic krill *Euphasia superba* (Barriga González, 2006).

In our investigation, it was found that $CaCl_2$, NaCl, and KCl had an increasing effect on the partially purified lipase activity from the fat body of *G. mellonella* larvae. All different concentrations of Ca^{2+}, Na^{+}, and K^{+} significantly increased the activity of fat body intracellular lipase.

Similar results were shown for other insects; Grillo et al. (2007) and Santana et al. (2017) show that the activity of lipase from *R. prolixus* and *Rhynchophorus palmarum* increases by increasing calcium ion concentrations respectively. Researches on *C. suppressalis* by Zibaee et al. (2008) and on *N. aenescens* by Zibaee (2012) reveal the same conclusion. All previous results indicate that the purified lipase is metalloproteinase, an enzyme whose catalytic mechanism involves metal ions, and this result is confirmed by using EDTA and EGTA in the current study.

On the other hand, Rivera-Pérez and García-Carreño (2011) conclude that invertebrate lipases are not influenced by calcium moderate concentration for maximum activity and stability, as noticed in mammalian lipases, for example, adipose tissue lipase show its maximum activity at 10 mM $CaCl_2$. The same conclusion is stated by Mrdaković et al. (2008), on the digestive lipase from gypsy moth, *L. dispar* that is not governed by Ca^{+2} for activation or stability. These authors depending on the observation of Kim et al. (1997) about lipase from *Pseudomonas cepacia* which state that the stabilization of lipase triad structure is due to the structure of calcium-binding site which consists of two carboxylate groups of Asp242 and Asp288 and two carbonyl groups of Gln292 and Val296, taking in consideration that not all invertebrates have this site.

There are several ways by which ions can affect enzyme activity; one of these ways is keeping the enzyme and substrate near to each other to enhance enzyme activity. Also, they preclude the unwanted reaction of nucleophiles and put the active groups of enzyme and substrate in the most perfect location leading to an increase in the ability of enzyme-substrate complex and enzyme stability (Santana et al., 2017).

Inhibitors are chemicals that diminish enzyme activity. They affect the catalytic characteristics of active site directly or indirectly (Robinson, 2015). In this study, three chelating agents were used; EDTA is a general chelating agent, EGTA is a calcium specific chelating agent, and PMSF is a serine protease inhibitor. All previous inhibitors were utilized to find the role of metal ions and serine residue in the enzyme active site, respectively.

In the current study, the tested inhibitors that are synthetic had a significant decline in the lipase activity purified from *G. mellonella* larvae fat body. The previous results indicated that fat body lipase requires metal ions especially calcium, and had a serine residue at their active site. The PMSF effect was also revealed in TAG-lipase activity from the sand fly *Phlebotomus papatasi* reproductive accessory glands (Rosetto et al., 2003) and *Rhynchophorus palmarum* (Santana et al., 2017). Also it was proven that the active-site serine residue is portion of a preserved sequence (GXSXG) that has been discovered in most lipase sequences of mammals and

prokaryotes (Gupta et al. 2004; Hide et al. 1992; Holm et al. 1988) and invertebrate lipases such as insects (Grillo et al. 2007; Horne et al. 2009) and crustacean (Rivera-Pérez and García-Carreño, 2010).

It is important to determine the kinetic parameters of the enzyme as it gives us important information about enzyme behavior and efficiency. The rate of reaction is measured and the influences of changing the reaction conditions are investigated (Walsh et al., 2010). Studying enzyme kinetics from this point of view can provide us with information about the mechanism by which enzyme catalyzes a reaction, metabolic role of enzyme and how enzyme activity can be controlled or inhibited using agonist or drugs (Fromm and Hargrove, 2012). K_m is the substrate concentration at which enzyme reaches to half of its maximal velocity (Schnell and Maini 2004). K_m values of enzymes that activate a specific reaction vary according to the organism from which these enzymes were derived.

In the present study, K_m values for both fat body lipase was high. High K_m values indicate that the enzyme has a low affinity to the substrate. In another word, an enzyme will not be saturated with low concentrations of substrate and needs high concentrations to reach its maximal velocity so enzyme activity depends on substrate concentration (Robinson, 2015). At high substrate concentrations, enzyme reaction rate may reach to its theoretical maximal rate, saturation of enzyme achieved by occupying all of their active sites with substrates and the determination of reaction rate achieved by the intrinsic turnover rate of the enzyme (Wrighton and Ebbing, 1993).

Integrated pest management programs paid attention to the using of enzyme inhibitors. This is because of the wide range use of synthetic pesticides and the appearance of environmental risks such as pest resurgence, pesticide resistance, and poisonous effects on non-target organisms such as human and biocontrol agents. Enzyme inhibitors naturally exist in many food plants and are especially plentiful in cereals like rice and legumes (Zibaee et al. 2008; Zibaee and Bandani, 2010). For example, when the action of digestive enzymes is inhibited, insect's nutrition is impaired, growth, and development are lagging and ultimately death takes place due to starvation. Therefore, a combination of the inhibitors targeting digestive enzymes probably will be more efficient. The enzymatic properties of lipases must be described earlier in considering inhibitors as a control approach.

Conclusion

A better understanding of *Galleria* digestive physiology was obtained through purification and characterization of intracellular fat body lipase. With 98.9 fold of purification, this enzyme was found to consist of two monomers of 178.8 and 62.6 kDa; Ca^{2+} dependent with a serine residue and its highest activity was shown at 37 and 40 °C and pH 8. The purified enzyme had a 0.316 ± 1.24 U/mg protein *V*max and 301.59 ± 24.973 mM Km, and these results will open new research fields such as investigation of hormonal regulation and the effect of various plant extracts on insect enzymes and physiology. That will help in obtaining new strategies for pest management that aim to disrupt pest physiology.

Supplementary information

Supplementary information accompanies this paper at https://doi.org/10.1186/s41936-019-0134-y.

Additional file 1. Screenshots of the abbreviation of the chemicals; supplementary materials for Figs. 7 and 8.

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Authors' contributions

RM had performed the practical section of this work, analyzed the data and revised the manuscript. ShM had written and revised the manuscript. MA had written and revised the manuscript. EB had designed the work, analyzed the data, written, and revised the manuscript. All authors had read the manuscript and approved it for submission.

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