Comparative Biochemistry and Physiology, Part B 158 (2011) 99–105

Contents lists available at ScienceDirect

Comparative Biochemistry and Physiology, Part B

journal homepage: www.elsevier.com/locate/cbpb

Purification and characterization of an intracellular lipase from pleopods of whiteleg shrimp (*Litopenaeus vannamei*)

Crisalejandra Rivera-Pérez, M. de los Ángeles Navarrete del Toro, Fernando García-Carreño *

Centro de Investigaciones Biologicas del Noroeste (CIBNOR), Apdo. Postal 128, La Paz, B.C.S. 23000, Mexico

ARTICLE INFO

Article history:
Received 12 July 2010
Received in revised form 8 October 2010
Accepted 10 October 2010
Available online 18 October 2010

Keywords:
Crustaceans
Intracellular lipase
Litopenaeus vannamei
Purification

ABSTRACT

An intracellular lipase present in the whiteleg shrimp *Litopenaeus vannamei* was detected in pleopods. The lipase from pleopods was purified and characterized by biochemical and kinetic parameters. Purified intracellular lipase has a molecular mass of 196 kDa, the polypeptide is assembled by two monomers, 95.26 and 63.36 kDa. The enzyme lacks glycosylation, and it has an isoelectric point of 5.0. The enzyme showed the highest activity at a temperature range of 30–40 °C at pH 8.0–10.0. Activity was completely inhibited by tetrahydrolipstatin and diethyl *p*-nitrophenyl phosphate, suggesting that the intracellular lipase is a serine lipase. The lipase hydrolyzes short and long-chain triacylglycerides, as well as naphthol derivatives at comparable rates in contrast to other sources of lipases. Specific activity of 930 U mg^{-1} and 416.56 U mg^{-1} was measured using triolein and tristearin at pH 8.0 at 30 °C as substrates, respectively. The lipase showed a $K_{M,app}$ of 41.03 mM and $k_{cat}/K_{M,app}$ ratio of 4.88 using MUF–butyrate as the substrate. The intracellular lipase described for shrimp has a potential role in hydrolysis of triacylglycerides stored as fat body, as has been shown in humans.

1. Introduction

Triacylglycerides (TAGs) are the predominant form of storage of fatty acids and comprise the main energy reserve in all animals (Wolins et al., 2006). The ability to store and use this energy involves a regulated balance between TAG synthesis and hydrolysis. Invertebrates, such as insects accumulate TAG as lipid droplets within the cytoplasm of fat body cells, from which they are released when required and transported to target tissues to support anabolism. Storage of TAG during the larval stages of crustaceans is primarily used to support metamorphosis that includes non-feeding periods during molting and in insects to meet energy demands during migratory flight (Beenakkers et al., 1985; Ziegler, 1991; Pistillo et al., 1998). The content of TAG in the fat body is influenced by several factors, including development stage, nutritional state, and sex (Beenakkers et al., 1985).

The whiteleg shrimp *Litopenaeus vannamei* is widely used as a crustacean model. The main storage site of lipids is the digestive gland (Dall et al., 1990); the lipid depots are stored in R-cells (Heffington Bunt, 1968). Enzymes responsible for the hydrolysis of lipid droplets are intracellular lipases. These enzymes have also been reported in insects (Arrese and Wells, 1994; Rajesh Patel et al., 2005), but not in crustaceans. Total, digestive and intracellular lipase activity has been reported in the same species with focus on the effect of oligotrophic and eutrophic pond water on digestive enzymes (Moss et al., 2001) and measuring the effect of probiotics on these enzymes (Yan-Bo, 2007). Currently, there are no studies on intracellular lipases in crustaceans. Indirect participation of these enzymes have been proposed in shrimp by measuring the changes of lipids of the digestive gland and hemolymph during fasting (Sánchez-Paz et al., 2007) and during ontogeny e.g. *Farfantepenaeus paulensis* (Lemos and Phan, 2001), and *Metapenaeus ensis* (Chu and Osvianico-Koulikowsky, 1994), showing that lipids are used as energy reserve during stressing conditions and during ontogeny.

Intracellular lipase is a member of the hormone-sensitive lipase family (PF06350) (Derewenda, 1994), which has an α/β-hydrolase fold (Ollis et al., 1992), a catalytic triad composed by Ser, Asp, and His that generates a charge relay system, a highly nucleophilic serine, a consensus sequence, GXSXG, and a *lid* that covers the active site and is displaced at the oil–water interface to allow the access of hydrophobic substrates (Grochulski et al., 1993).

TAGs are catabolized by sequential cleavage of the acyl chain from the glycerol backbone to release free fatty acids and generate diacylglycerides and monoacylglycerides intermediates which is allowed by intracellular lipases (Jaeger et al., 1994). TAG turnover in fat body cells is regulated by the endocrine system and hormones. In invertebrates, the adipokinetic hormone (AKH) plays this function (Gâde and Auerswald, 2003). Stimulus by AKH is associated with a rapid activation of fat body cAMP-dependent protein kinase A (PKA) and a sustained increase in calcium flux (Rajesh Patel et al., 2005), which activate the protein kinase A and phosphorylate the intracellular lipase,

Abbreviations: E-600, diethyl *p*-nitrophenyl phosphate; MUF, methylumbelliferone; PV_IL, *Penaeus vannamei* intracellular lipase; TAGs, triacylglycerides; THL, tetrahydrolipstatin; SDS, sodium dodecyl sulphate.

* Corresponding author. Tel.: +52 612 123 8484; fax: +52 612 125 3625.
E-mail address: fgarcia@cibnor.mx (F. García-Carreño).

1096-4959/$ – see front matter © 2010 Elsevier Inc. All rights reserved.
doi:10.1016/j.cbpb.2010.10.004

yielding an active enzyme. In crustaceans, activation of the intracellular lipase by hormonal stimulus remains unknown. Intracellular lipases of the fat body have been purified from insects, such as tobacco hornworm *Manduca sexta* (Arrese and Wells, 1994) and migratory locust *Locusta migratoria* (van der Horst et al., 2001).

Since digestive and intracellular lipases have the same catalytic function, the identification of lipase activity in different tissues has been useful to understand the role of lipases. In the fruit fly *Drosophila melanogaster*, lipase activity was present in male accessory glands, head, thorax, and gonad. Lipase activity in these tissues is related to lipids used to satisfy energy demands (Smith et al., 1994), while the lipase activity found in the gut is related to lipid digestion from feed.

To our knowledge, no intracellular lipase has been purified and characterized in penaeids; hence, the details of hydrolysis of TAG stored in fat body cells remain unknown in crustaceans. The study of metabolism and transport of lipid in crustaceans is of interest, but, to make a thorough study, the enzymes and genes involved must be characterized. Before attempting to isolate intracellular lipase genes from shrimp, as a step towards disentanglement of the physiology of lipid catabolism, it is necessary to know more about the tissue distribution and substrate specificity of intracellular lipases present in shrimp.

In this report, we present data on intracellular lipase activity in a selected shrimp tissue and purification as well as some properties of the intracellular lipase from pleopods of *L. vannamei*.

2. Materials and methods

2.1. Samples

Shrimp (*L. vannamei*) were maintained under controlled conditions (28 °C, 34‰ salinity, 7.4 mg L^{-1} dissolved oxygen) and fed twice daily with commercial feed containing 35% protein. From each specimen, the digestive gland, pleopods, uropods, and muscle were dissected and immediately homogenized individually in cold, distilled water (1:4 w/v). Individual homogenates were centrifuged at 10,000 *g* at 4 °C for 30 min. The supernatant of each crude extract was recovered and stored at 4 °C for further analysis. Protein concentration was determined using the Bradford method (1976). Bovine serum albumin (B-4287, Sigma) was used as the standard.

2.2. Determination of lipase activity

Lipase activity (EC 3.1.1.3) was measured by titrating free fatty acids liberated from TAG in a slightly alkaline solution using the pH-stat standard assay conditions described by Gargouri et al. (1984), using 0.1 N NaOH for titration. The reaction mixture containing 0.25 mL triolein (17 mM) (T-7140, Sigma) in 30 mL 2.5 mM Tris–HCl at pH 8.0, 150 mM NaCl, and 3% (w/v) gum arabic (G9752, Sigma) as an emulsifying agent which stabilizes the emulsion (Hoppe and Theimer, 1996), was prepared in a glass reaction vessel and constantly stirred using a magnetic bar. Then, 15 μg protein of PV_IL was incubated at 30 °C for 1 h with magnetic stirring. Lipase activity was calculated as:

$$U/mg = (A/t_A - B/t_B) \times 0.01 \times 10^3)/E,$$

where U is free fatty acids in micromoles per min, *A* and *B* are 0.01 N NaOH volumes (in μL); t_A and t_B are reaction time in min for the assay and blank, respectively. E is the protein concentration of the sample in mg mL^{-1}.

Lipase activity was also determined with a fluorescent substrate MUF–butyrate (19362, Sigma) according to Prim et al. (2003). The substrate concentration in the assay was 100 μM in a total volume of 100 μL 50 mM Tris–HCl buffer at pH 8.0. Fluorescence was measured at 355 nm (excitation) and 460 nm (emission) for 10 min with a fluorometer (Kontron SFM 25). Blanks were run in parallel. A standard curve was prepared with 4-methylumbelliferone (M1381, Sigma). Enzyme activity was calculated in relation to the soluble protein and the standard curve. Lipase activity was expressed in relation to the maximum value (= 100%) of the respective assay.

2.3. Purification of intracellular lipase

The crude extract from pleopod samples (17.18 mg mL^{-1}) was treated with 0.5 M ammonium sulphate. Then proteins were separated by hydrophobic chromatography with a FPLC system (GradiFrac System, Pharmacia Biotek) at 27 °C. The sample was loaded into a 1 mL Phenyl-Sepharose CL-4B column previously equilibrated with 50 mM Tris–HCl containing 0.5 M ammonium sulphate at pH 8.0. The column was washed with the same buffer and unbounded proteins were removed during this step. A first elution was performed by reduction of ammonium sulphate using 50 mM Tris–HCl at pH 8.0. The most hydrophobic proteins, including lipase, were eluted with a second elution using a gradient from 0 to 100% propanol. Simultaneously, absorbance at 280 nm and conductivity were monitored and recorded for the two elution processes. The flow rate was 1 mL min^{-1} and the final volume was 20 mL, fractions of 500 μL with lipase activity were collected. The propanol was removed using a filtration membrane (AMICON® Ultra-15, Millipore) by centrifugation at 4000 *g* for 15 min at 25 °C, followed by washing with Milli-Q water. The concentrated lipase solution named PV_IL, was stored at −80 °C.

2.4. SDS-PAGE and zymograms

Protein profiles were resolved by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) according to Laemmli (1970), under reducing and non-reducing conditions and by staining with silver nitrate (Merrill and Washart, 1998) in gels containing 8% acrylamide. For reducing conditions, samples were diluted (1:2) with a sample buffer (0.125 M Tris–HCl, 4% SDS, 20% v/v glycerol, 0.02% bromophenol blue, 0.2 M dithiothreitol at pH 6.8) and heated for 2 min at 100 °C. For non-reducing conditions, samples were mixed in the same buffer without both dithiothreitol and SDS and were not boiled. Electrophoretic separation was performed at a 15 mA constant current and at 2 °C. To visualize the protein, the gels were stained with 0.5% Coomassie brilliant blue R-250 in 7.5% (v/v) acetic acid and 40% (v/v) methanol at room temperature and distained in 10% (v/v) acetic acid and 40% (v/v) methanol.

The molecular weight of lipase was determined using SDS-PAGE under reducing conditions (Laemmli, 1970). The molecular weight was estimated by comparison with molecular markers (17-0615-01, Amersham Pharmacia Biotech).

Zymograms were developed using MUF–butyrate as the substrate (Prim et al., 2003). After electrophoresis separation of proteins, the SDS was soaked out from the gel by rinsing the gel with distilled water followed by an incubation at room temperature in Triton X-100 (2.5% v/v), allowing the renaturation of the enzyme. After 30 min, gels were rinsed 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 fluorescence signal was detected and captured using a UV transilluminator.

2.5. Determination of biochemical characteristics

Analytical isoelectric focusing (IEF) was performed with the PhastSystem™ (GE Healthcare Life Sciences, Uppsala, Sweden) using IEF gels at pH from 3 to 9. A protein mixture (Broad *pI* 3.5–9.3) was used as the *pI* marker (17-0471-01, GE Healthcare Life Sciences). The gel was treated with silver nitrate (Merrill and Washart, 1998).

The presence of carbohydrates associated with the isolated protein was analyzed by electrophoresis under non-reducing conditions

(Thornton et al., 1994). After electrophoresis, the gel was incubated for 30 min at room temperature in solution C (50% v/v ethanol) and thoroughly washed with distilled water for 10 min. The gel was incubated in 25 mL of solution A (1% v/v periodic acid in 3% v/v acetic acid) for 30 min, washed with distilled water for 30 min, and incubated for 20 min in 25 mL solution B (0.1% w/v sodium metabisulfite in 10 mM HCl). Then the gel was incubated in 25 mL Schiff's reagent (S-5133, Sigma) for 1 h in the dark and immersed in 25 mL solution B for 1 h in the dark. Finally, the gel was washed several times in 25 mL solution D (0.5% w/v sodium metabisulfite in 10 mM HCl) for at least 2 h in the dark. The gel was stored in solution E (7.5% w/v acetic acid, 5% v/v methanol).

2.6. The effect of pH and temperature on lipase activity

The effect of pH on PV_IL activity was measured using 2 μg lipase diluted in 15 μL universal buffer at 30 °C (Stauffer, 1989). To obtain the required pH, 20 mL stock solution (57 mM boric acid, 36 mM citric acid, 38 mM monobasic sodium phosphate, 310 mL 1 N NaOH) was adjusted with 1 N HCl and then filled with distilled water to 100 mL. Buffers were prepared for the pH range from 2 to 12. After incubation for 1 h at each pH value, lipase activity was assayed at 30 °C by fluorescence using MUF–butyrate as the substrate as describe in Section 2.2.

To measure the effect of the temperature on PV_IL activity, 2 μg lipase diluted in 15 μL buffer (50 mM Tris–HCl at pH 8.0) was incubated for 1 h at temperatures ranging from 20 to 70 °C. Immediately after incubation, lipase activity was measured by fluorescence using MUF–butyrate as the substrate, as described earlier.

2.7. Specific activity of lipase using different substrates

PV_IL substrate specificity was measured (Versaw et al., 1989) by assaying its hydrolytic activity on naphthyl derivatives and triacylglycerols with different chain lengths: tributyrin (C4:0; T-8626, Sigma); tripalmitin (C16:0; T-5888, Sigma); tristearin (C18:0; T-5016, Sigma), and triolein (C18:1; T-7140, Sigma). Specific activity was determined spectrophotometrically for naphthyl derivatives and pH-stat titration as described for TAG hydrolysis (Gargouri et al., 1984).

2.8. The effect of calcium concentration

The effect of Ca^{2+} on PV_IL was determined by measuring hydrolysis of MUF–butyrate in the presence of different calcium concentrations (0–100 mM $CaCl_2$) by fluorescence.

2.9. The effect of inhibitors on PV_IL

To determine if PV_IL is a serine lipase, tetrahydrolipstatin (THL, O-4139, Sigma), a hydrogenated lipstatin analogue isolated from *Streptomyces toxytricini* (Borgstrom, 1988) and diethyl *p*-nitrophenyl phosphate (E-600, PS610, Sigma) a phosphorylating agent (Müller and Petry, 2004), were used as specific inhibitors. To compare the efficacy of both inhibitors against lipase activity, different concentrations of inhibitors were used; the final concentrations of the mixtures were 0, 2.5, 5, 7.5, and 10 mM for THL and 0, 1, 2.5, 5, 7.5, and 10 mM for E-600. Equal volumes of inhibitor and enzyme (5 μg of protein in each enzyme solution) were incubated for 1 h at room temperature in a final volume of 20 μL. Residual lipase activity was measured by fluorescence using MUF–butyrate, as described earlier.

2.10. Determination of kinetic parameters

The kinetic parameters $K_{M,\ app}$ and $V_{max,\ app}$ of PV_IL were obtained at 30 °C and pH 8.0, using MUF–butyrate as the substrate. MUF–butyrate hydrolysis was assayed fluorometrically at concentrations of 0 to 100 μM for 10 min. $K_{M,\ app}$ and $V_{max,\ app}$ were determined with the Lineweaver–Burk transformation.

3. Results and discussion

3.1. Identification of intracellular lipases

The hormone-sensitive lipase (HSL) is an intracellular lipase in mammals found in several organs and tissues, including testis, heart, mammary gland, skeletal muscle, macrophages, intestinal mucosa, pancreas, and adipose tissue (Holm et al., 2000). In the fruit fly *D. melanogaster*, lipase activity was reported in a number of adult tissues located in various body parts (Smith et al., 1994). In crustaceans, the search for lipase activity has focused on digestive enzymes.

In our analyses, several protein bands with lipase activity were found in the digestive gland of the shrimp when we used substrate-SDS-PAGE analysis, while in muscle, pleopods and uropods, only one band with lipase activity was found (Fig. 1). Active bands inhibited by THL were considered serine lipases (Hadváry et al., 1991), since THL reacts with the nucleophilic serine residue from the catalytic triad by covalently blocking the active site, this serine residue is recognized as essential for the catalysis. Nine protein bands with lipase activity were observed from crude extract of the digestive gland with molecular mass >220 kDa (four bands) and ~80–45 kDa (five bands). The protein band of ~200 kDa found in all tissues including digestive gland and inhibited by THL, suggested a possible role of intracellular lipase. We focused on lipase band 4 found in all tissues (Fig. 1), since the band of 45 kDa was previously described as a digestive lipase (Rivera-Pérez et al., 2010) and the other bands were only found in the digestive gland with a potential role in digestion, while band 4 has a potential role as an intracellular lipase, which is the focus of this study.

Chymotrypsins and lipases share the same catalytic triad (Ser, His, and Asp) and a similar catalysis mechanism, which explains why chymotrypsins hydrolyze ester bonds (Stauffer, 1971). To distinguish chymotrypsin activity from lipase activity, we compared protein composition, zymograms of proteases (García-Carreño, 1993) and zymogram for lipases (Fig. 2). The zymogram for lipases showed four bands with high molecular weight and three with low molecular weight. Bands 1–5 did not have protease activity and band 5 was previously recognized as a digestive lipase. Bands 6 and 7 are chymotrypsins, since they hydrolyze casein; these proteases are well characterized in whiteleg shrimp (Hernández-Cortés et al., 1997); band 3 showed lipase activity but not protease activity, confirming that this protein is a lipase.

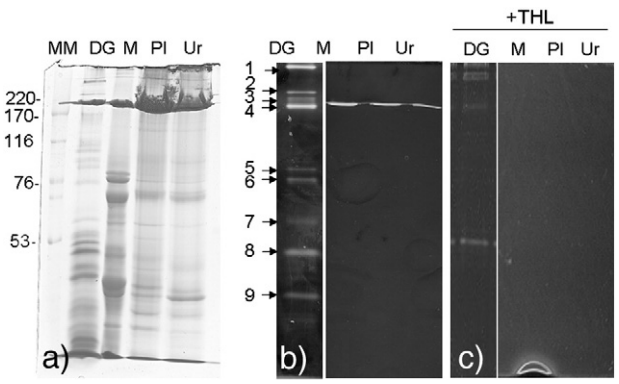

Fig. 1. Identification of lipases in whiteleg shrimp tissues. a) Protein profile, b) zymogram of lipase without inhibitor, and c) zymogram of lipase with THL as the inhibitor. SDS-PAGE 8% acrylamide, 15 μg protein per sample was loaded, MM: molecular marker, DG: digestive gland, M: muscle, PI: pleopods and Ur: uropods. THL: tetrahydrolipstatin 1 mM, MUF–butyrate was used as the substrate for lipase activity.

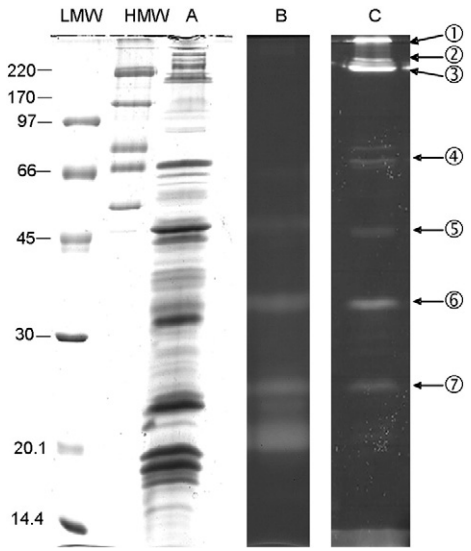

Fig. 2. Protein profile (A), zymogram of proteases using casein as the substrate (B), and zymogram of lipases using MUF–butyrate as the substrate (C) of the digestive gland of *L. vannamei*. SDS-PAGE 12%, 15 μg protein per sample was loaded, MM: molecular marker.

3.2. Purification of intracellular lipase

The isolation of the intracellular lipase was performed using pleopods as a starting material, since the protein mixture of muscle contained more muscular proteins than pleopods and uropods. After hydrophobic chromatography, three peaks of lipase activity were observed after elution with propanol (Fig. 3a), the analyzed fraction showed a single protein band by SDS-PAGE and zymography with MUF–butyrate as the substrate, yielded only one band with lipase activity with the same molecular mass (Fig. 3b). The propanol contained in the eluted fractions containing the intracellular lipase, named PV_IL was removed by centrifugation. The apparent molecular mass of the protein with lipase activity was 196 kDa (Fig. 3b), under reducing conditions, the isolated protein showed two monomers of 95.26 kDa and 63.36 kDa (Fig. 3b), the monomers showed that are not bound by cysteine residues given that when the enzyme was boiled without reducing agents, two single bands were observed; thus, the protein is a single polypeptide, as seen in other invertebrate (Arrese and Wells, 1994) and vertebrate intracellular lipases (Langin Dominique et al., 1993). This indicates that the intracellular lipase of whiteleg shrimp might be subjected to post-translational modifications by cleaving the polypeptide chain into two distinct chains that could be assembled by hydrogen bonds and some ionic interactions to form the mature intracellular lipase. The purification flow sheet is shown in Table 1. After purification, the enzyme was enriched ~58.25-fold and the specific activity of pure PV_IL reached 922.16 U mg^{-1}, using triolein as the substrate. Using 17.18 mg of protein from pleopods, 0.277 mg of PV_IL was recovered.

The *pI* of PV_IL is 5.0; similar results have been found in the intracellular lipase of tobacco hornworm *M. sexta* with a *pI* 5.6 (Arrese et al., 2006), while other intracellular lipases, including HSL from vertebrates have a *pI* between 6.7 and 6.9 (Holm et al., 1988; Arreguín-Espinosa et al., 2000), while the digestive lipase of shrimp has a *pI* 3.5 (Rivera-Pérez et al., 2010). The characteristic *pI* of shrimp intracellular lipase charges the protein negatively at pH 8.0 and it binds to the matrix CL-4B during isolation. Differences in *pI* between lipases suggest differences in the primary structure of the protein.

Glycosylation in lipases, from different sources of organisms, is considered an usual feature in lipases (Buscá et al., 1995). Glycosylated lipases have been described in organisms such as the digestive lipase of *L. vannamei* (Rivera-Pérez et al., 2010), human gastric lipase (Roussel et al., 1999) and human hepatic lipase (Stahnke et al., 1991).

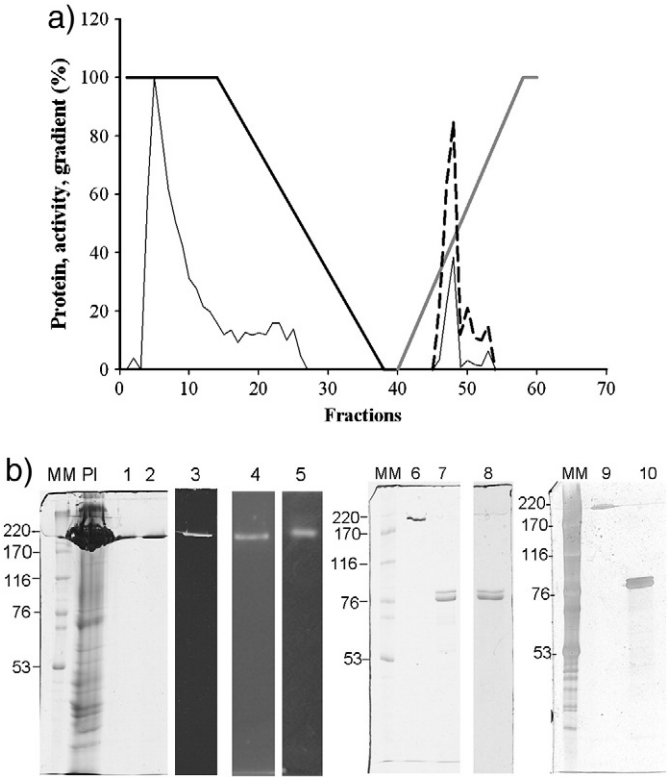

Fig. 3. Purification process of intracellular lipase (PV_IL). a) Chromatogram of hydrophobic chromatography; and b) protein composition and zymogram of eluted fractions. MM: molecular marker, PI: pleopods; 1, 2: concentrated fractions; 3: zymogram of concentrated fractions; 4: lipase with ammonium sulphate; 5: lipase with propanol; 6: pure PV_IL; 7: PV_IL treated with DTT; 8: protein boiled for 10 min; and 9, 10: silver nitrate stain of PV_IL isolated and treated with SDS and treated with DTT and boiled, respectively.

The function of glycosylation is to tag the enzyme for biological recognition of structures other than the substrate (Nelson and Cox, 2000), usually to generate an allosteric function or for auto-catalytic protection on some secreted glycoproteins. In contrast to the digestive lipase previously described in whiteleg shrimp (Rivera-Pérez et al., 2010), the intracellular lipase reported in this study did not show apparent glycosylation. This suggests that PV_IL is not for secretion, which agrees with the function of the enzyme (Kraemer and Shen, 2002), which is to hydrolyze TAGs stored in the fat body cells. Other post-translational modification reported for these enzymes is phosphorylation (Anthonsen et al., 1998), which allows the enzyme to be active against TAG; however, this property was not assessed in this work.

3.3. Specific activity of PV_IL using different substrates

The activity of lipase was also measured using naphthol derivatives; PV_IL showed a higher hydrolytic activity on α-naphthyl acetate (85 U mg^{-1}) than β-naphtyl acetate and caprylate (Table 2). Lipase activities toward TAG were assayed under identical experimental conditions (pH 8.0 and 30 °C). The enzyme hydrolyzed all TAGs at different rates; the kinetic of triolein is shown in Fig. 4. Significant differences between activities for tristearin and triolein were observed, showing a higher activity (930 U mg^{-1}) with triolein, a triacylglyceride composed of *cis*-9-octadecanoic acid, than tristearin (416.56 U mg^{-1}). Similar results were observed for the digestive lipase of whiteleg shrimp (Rivera-Pérez et al., 2010). Similar preference for medium chain TAGs have been reported for bacterial lipases, such as *Bacillus subtilis* 168, *Aeromonas hydrophila* and *Acinetobacter* sp. (Anguita et al., 1993; Lesuisse et al., 1993; Snellman et al., 2002), invertebrate lipases, such as the beetle *Cephaloleia presignis* (Arreguín-Espinosa et al., 2000), fruit fly *D.*

Table 1
Purification process of intracellular lipase of *Litopenaeus vannamei*.

Step	Protein[a] (mg/mL)	Total protein (mg)	Total activity[b] (U)	Specific activity[b] (U/mg)	Purification fold	(%)
Crude extract	17.18 ± 0.12	17.18 ± 0.12	271.95 ± 0.11	15.83 ± 0.1	1	100
Phenyl sepharose CL-4B	0.231 ± 0.04	0.277 ± 0.05	255.44 ± 0.03	922.16 ± 0.5	58.25	93.29

[a] Protein content was measured by the Bradford method (1976).
[b] One unit of activity corresponds to a: 1 μmol of fatty acid released per min using TC_{18} as the substrate.

Table 2
Specific activity of shrimp intracellular lipase.

Substrate	Specific activity (U mg^{-1})
Naphthyl esters	
α-Naphthyl acetate	85.1 ± 7.2
β-Naphthyl acetate	71.5 ± 5.0
β-Naphthyl caprylate	27.2 ± 2.0
Triacylglycerides	
Tributyrin (C:4)	215.0 ± 1.9
Tripalmitin (C:16)	213.6 ± 3.2
Tristearin (C:18)	416.5 ± 3.3
Triolein (C18:1)	930.0 ± 1.6

melanogaster (Smith et al., 1994), and vertebrate lipases, including a fish *Trematomus newnesi* (Hazel and Sidell, 2004) and human HSL (Kraemer and Shen, 2002). Our data suggest that the intracellular lipase may better hydrolyze TAGs containing unsaturated fatty acids than TAGs containing saturated fatty acids.

3.4. The effect of pH and temperature on lipase activity

The effect of pH on PV_IL is presented in Fig. 5a. Significant differences ($p<0.05$) were observed. Activity was highest when assayed at pH 8–10; 90% of lipase activity was lost at pH 6. At pH 2, 4, and 12, no lipase activity was measured, while the digestive lipase of shrimp showed 10% of lipase activity (Rivera-Pérez et al., 2010); these result from electrostatic repulsion and the disruption of some hydrogen bonding, since extremes of pH alter the net charge on the protein. Lipases from organisms other than crustaceans also have optimum activity at slightly alkaline pH, such as the kissing bug *Rhodnius prolixus* at pH 7.0–7.5 (Grillo et al., 2007), the large-clawed scorpion *Scorpio maurus* at pH 9 (Zouari et al., 2005), and the tobacco hornworm *M. sexta* at pH 7.9 (Arrese and Wells, 1994), including enzymes from bacterial sources (Deb et al., 2006), which is related to the ionization properties of histidine ($pKa = 6.5$). The amino acid residues of the catalytic triad (Ser, His, and Asp) of lipases (Roussel et al., 1999) play an important role in the charge relay system involving the catalytic triad and the enhancement of the nucleophilic character of the serine residue.

The effect of the temperature on PV_IL activity was assayed over a range from 20 °C to 70 °C (Fig. 5b). Maximum activity under these conditions was at 30 °C. At 50 °C and 60 °C, 50% of the activity was lost and at 60 °C, activity fell abruptly by denaturation. Similar results have been shown in other invertebrate lipases, as in Japanese flying squid *Todarodes pacificus* (Park et al., 2008) and red flying squid *Ommastrephes bartramii* (Sukarno et al., 1996) and gypsy moth *Lymantria dispar* (Mrdakovic et al., 2008). Other invertebrate lipases have shown higher activity at 60 °C such as the Mediterranean green crab *Carcinus mediterraneus* (Cherif et al., 2007) and at 40 °C the Antarctic krill *Euphasia superba* (Barriga Gonzalez, 2006). This suggests that invertebrate lipase has a broad range of optimum lipase activity in contrast to mammalian lipases, which have a narrow optimum temperature, such as HSL from humans and rats, 37 °C and 30 °C, respectively (Laurell et al., 2000).

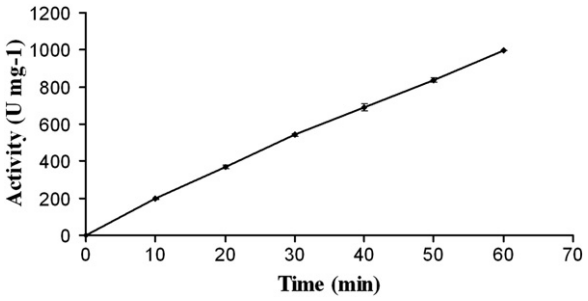

Fig. 4. Hydrolysis of triolein by PV_IL using the pH-stat method.

To date, it is not conclusive whether invertebrate lipases depend on a moderate concentration of calcium for maximum activity and stability, as observed in mammalian lipases e.g. lipase from the adipose tissue showed its maximum activity at 10 mM $CaCl_2$ (Matsumura et al., 1976). Lipase from crab *C. mediterraneus* (Slim and Gargouri, 2009), squid *T. pacificus* (Park et al., 2008), and gypsy moth *L. dispar* (Mrdakovic et al., 2008), digestive lipase from shrimp *L. vannamei* and intracellular lipase described in this study, are not dependent on Ca^{+2} for activation or stability. In fact, a calcium-binding site composed by two carboxylate groups of Asp242 and Asp288 and two carbonyl groups of Gln292 and Val296 has been described as necessary to stabilize the triad structure for *Pseudomona cepacia* lipase (Kim et al., 1997). However, it seems that this calcium-binding site does not occur in most of the invertebrates including shrimp lipases.

3.5. The class of intracellular lipase as assessed by specific inhibitors

THL and E-600 have been recognized as a serine lipase inhibitor since both inhibitors react with the nucleophilic serine residue, however E-600 acts on the serine residue of serine proteases as well as

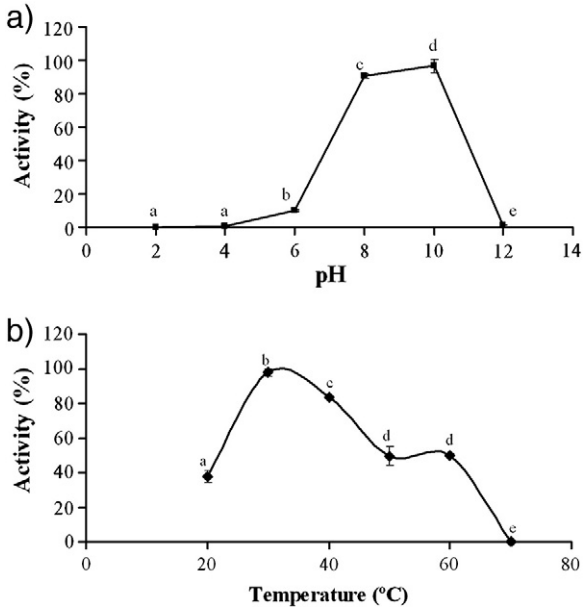

Fig. 5. a) Effect of pH and b) temperature on lipase activity (mean ± SD, n = 3). Different letters indicate significant differences ($p<0.05$).

Table 3
Kinetic parameters of different sources of lipases.

	$V_{max,app}$ (μM min^{-1})	$K_{M,app}$ (mM)	$k_{cat,app}$ (s^{-1})	$k_{cat}/K_{M,app}$ ($\times 10^3$ mM^{-1} s^{-1})	Reference
PVL	3042 ± 2.88	0.24 ± 0.00	12,670 ± 0.27	52.8 ± 2.33	Rivera-Pérez et al. (2010)
PV_IL	1226 ± 1.23	41.03 ± 0.02	200.24 ± 0.87	4.88 ± 1.1	This study
Paenibacillus sp. BP-23	ND	0.0155	ND	ND	Prim et al. (2003)
Bacillus sp. BP-7	ND	0.026	ND	ND	Prim et al. (2003)
Bacillus subtilis LipA	ND	0.031	ND	ND	Prim et al. (2003)

*MUF–butyrate was used as the substrate at pH 8.0 and 30 °C.

lipases (Müller and Petry, 2004), while THL does not inactivate serine proteases such as trypsin and chymotrypsin (Hadváry et al., 1988). Thus, in order to evaluate the effect of these two irreversible inhibitors different concentrations were tested. THL and E-600 inhibited the intracellular lipase, which indicates that a serine residue is essential for its catalytic function. E-600 inhibited half of the lipase activity at 1 mM, while THL inhibited all activity at the same concentration, however at 10 mM, E-600, maintained only 10% of the lipase activity. Because several lipases are susceptible to serine-directed reagents, a similarity between their respective active sites has been proposed in which a consensus sequence (GXSXG) has been identified in the primary structure of lipases of mammals and prokaryotes (Holm et al., 1988; Gupta et al., 2004) and invertebrate lipases such as holometabolous insects (Horne et al., 2009), and digestive lipase of *L. vannamei* (Rivera-Pérez et al., 2010).

3.6. Determination of kinetic parameters of intracellular lipase

There are only a few reports describing the kinetic parameters of lipase in invertebrates. MUF–butyrate was used as the substrate under optimal conditions (pH 8.0 and 30 °C). The kinetic values of PV_IL were obtained using the Michaelis–Menten model. Catalytic properties of PV_IL were $V_{max,\ app} = 1226$ μM min^{-1} and $K_{M,\ app} = 41.03$ mM (Table 3). The high $K_{M,\ app}$ value indicates lower affinity between the enzyme and the substrate than other lipases from different organisms shown in Table 2.

4. Conclusions and perspectives

The recent characterization of the intracellular lipase of whiteleg shrimp is a major advancement because relatively little was known regarding the enzymes that catalyze the hydrolysis of stored TAGs in crustaceans. The presence of the intracellular lipase in different body tissues in shrimp could be related to energy demands for motion. The availability of pure intracellular lipase of shrimp, presumably the first enzyme involved in lipid mobilization from the fat body, will permit additional studies on the mechanism of the hormonal regulation of this important metabolic pathway in crustaceans, including an investigation of the role of phosphorylation of the enzyme. This information will provide clues to understanding changes in the lipid catabolism during fasting periods that shrimp show during molting.

Acknowledgement

This work was supported by the Consejo Nacional de Ciencia y Tecnologia (CONACYT grant 80935). C.R.P. is a recipient of a CONACYT fellowship 173780.

References

Anguita, J., Rodríguez Aparicio, L.B., Naharro, G., 1993. Purification, gene cloning, amino acid sequence analysis, and expression of an extracellular lipase from an *Aeromonas hydrophila* human isolate. Appl. Environ. Microbiol. 59, 2411–2417.
Anthonsen, M.W., Ronnstrand, L., Wernstedt, C., Degerman, E., Holm, C., 1998. Identification of novel phosphorylation sites in hormone-sensitive lipase that are phosphorylated in response to isoproterenol and govern activation properties *in vitro*. J. Biol. Chem. 273, 215–221.
Arreguín-Espinosa, R., Arreguín, B., González, C., 2000. Purification and properties of a lipase from *Cephaloleia presignis* (Coleoptera, Chrysomelidae). Biotechnol. Appl. Biochem. 31, 239–244.
Arrese, E.L., Patel, R.T., Soulages, J.L., 2006. The main triglyceride-lipase from the insect fat body is an active phospholipase A1: Identification and characterization. J. Lipid Res. 47, 2656–2667.
Arrese, E.L., Wells, M.A., 1994. Purification and properties of a phosphorylatable triacylglycerol lipase from the fat body of an insect *Manduca sexta*. J. Lipid Res. 35, 1652–1660.
Barriga Gonzalez, A.A., 2006. Enzimas lipolíticas de krill antártico: purificación y caracterización, ¿enzimas adaptadas al frío? Facultad de Ciencias Químicas y Farmaceúticas. Universidad de Chile, Santiago, Chile, p. 181. vol. Doctor.
Beenakkers, A.M.T., van der Horst, D.J., van Marrewijk, W.J.A., 1985. Insect lipids and lipoproteins, and their role in physiological processes. Prog. Lipid Res. 24, 19–67.
Borgstrom, B., 1988. Mode of action of tetrahydrolipstatin: a derivative of the naturally occurring lipase inhibitor lipstatin. Biochim. Biophys. Acta 962, 308–316.
Bradford, M.M., 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein dye binding. Anal. Biochem. 72, 248–254.
Buscá, R., Pujana, M.A., Pognonec, P., Auwerx, J., Deeb, S.S., Reina, M., Vilaró, S., 1995. Absence of N-glycosylation at asparagine 43 in human lipoprotein lipase induces its accumulation in the rough endoplasmatic reticulum and alters this cellular compartment. J. Lipid Res. 36, 939–951.
Cherif, S., Fendri, A., Miled, N., Trabelsi, H., Mejdoub, H., Gargouri, Y., 2007. Crab digestive lipase acting at high temperature: purification and biochemical characterization. Biochimie 89, 1012–1018.
Chu, K.H., Osvianico-Koulikowsky, N.N., 1994. Ontogenetic changes in metabolic activity and biochemical composition in the shrimp *Metapenaeus ensis*. J. Exp. Mar. Biol. Ecol. 183, 11–26.
Dall, W., Hill, B.J., Rothlisberg, P.C., Staples, D.J., 1990. The biology of the Penaeidae. In: Blaxter, J.S.A. (Ed.), Advanced Marine Biology. Academic Press, London, pp. 1–489.
Deb, C., Daniel, J., Sirakova, T.D., Abomoelak, B., Dubey, V.S., Kolattukudy, P.E., 2006. A novel lipase belonging to the hormone-sensitive lipase family induced under starvation to utilize stored triacylglycerol in *Mycobacterium tuberculosis*. J. Biol. Chem. 281, 3866–3875.
Derewenda, Z.S., 1994. Structure and function of lipases. Adv. Protein Chem. 45, 1–52.
Gâde, G., Auerswald, L., 2003. Mode of action of neuropeptides from the adipokinetic hormone family. Gen. Comp. Endocrinol. 132, 10–20.
García-Carreño, F.L., 1993. Protease inhibition in theory and practice. Biochem. Educ. 146–150.
Gargouri, Y., Julien, R., Pieroni, G., Verger, R., Sarda, L., 1984. Studies on the inhibition of pancreatic and microbial lipases by soybean proteins. J. Lipid Res. 25, 1214–1221.
Grillo, L.A.M., Majerowickz, D., Gondim, K.C., 2007. Lipid metabolism in *Rhodnius prolixus* (*Hemiptera:Reduviidae*): role of a midgut triacylglycerol-lipase. Insect Biochem. Mol. Biol. 37, 579–588.
Grochulski, P., Li, Y., Schrag, J.D., Bouthillier, F., Smith, P., Harrison, D., Rubin, B., Cygler, M., 1993. Insight into interfacial activation from an open structure of *Candida rugosa* Lipase. J. Biol. Chem. 268, 12843–12847.
Gupta, R., Gupta, N., Rathi, P., 2004. Bacterial lipases: an overview of production, purification and biochemical properties. Appl. Microbiol. Biotechnol. 64, 763–781.
Hadváry, P., Lengsfeld, H., Wolfer, H., 1988. Inhibition of pancreatic lipase *in vitro* by the covalent inhibitor tetrahydrolipstatin. Biochem. J. Rev. 256, 357–361.
Hadváry, P., Sidler, W., Meister, W., Vetter, W., Wolfer, H., 1991. The lipase inhibitor tetrahydrolipstatin binds covalently to the putative active site serine of pancreatic lipase. J. Biol. Chem. 266, 2021–2027.
Hazel, J.R., Sidell, B.D., 2004. The substrate specificity of hormone-sensitive lipase from adipose tissue of the Antarctic fish *Trematomus newnesi*. J. Exp. Biol. 207, 897–903.
Heffington Bunt, A., 1968. An ultrastructural study of the hepatopancreas of *Procrambarus clarkii* (Girard) (Decapoda, Astacidea). Crustaceana 15, 282–288.
Hernández-Cortés, M.P., Whitaker, J., García-Carreño, F.L., 1997. Purification and characterization of chymotrypsin from *Penaeus vannamei* (Crustacea: Decapoda). J. Food Biochem. 2, 497–514.
Holm, C., Kirchgessner, T.G., Svenson, K., Fredrikson, G., Nilsson, S., Miller, C.G., Schotz, M.C., 1988. Hormone-sensitive lipase: sequence, expression and chromosal localization to 19 cent-q13.3. Science 241, 1503–1506.
Holm, C., Osterlund, T., Laurell, H., Contreras, J.A., 2000. Molecular mechanism regulating hormone-sensitive lipase and lipolysis. Annu. Rev. Nutr. 20, 365–393.
Hoppe, A., Theimer, R.R., 1996. Titrimetric test for lipase activity using stabilized triolein emulsions. Phytochem. Oxf. 42, 973–978.
Horne, I., Haritos, V.S., Oakeshott, J.G., 2009. Comparative and functional genomics of lipases in holometabolous insects. Insect Biochem. Mol. Biol. 39, 547–567.
Jaeger, K.-E., Dijkstra, B.W., Colson, C., Heuvel,, M.v., Misset, O., 1994. Bacterial lipases. FEMS Microbiol. Rev. 15, 29–63.

Kim, K.K., Song, H.K., Shin, D.H., Hwang, K.Y., Suh, S.W., 1997. The crystal structure of a triacylglycerol lipase from *Pseudomonas cepacia* reveals a highly open conformation in the absence of a bound inhibitors. Structure 5, 173–185.

Kraemer, F.B., Shen, W.-J., 2002. Hormone-sensitive lipase: control of intracellular tri-(di-) acylglycerol and cholesteryl ester hydrolysis. J. Lipid Res. 43, 1585–1594.

Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680–685.

Langin Dominique, L.H., Lena, Stenson Holst, Per, Belfrage, Cecilia, Holm, 1993. Gene organization and primary structure of human hormone-sensitive lipase: possible significance of a sequence homology with a lipase of *Moraxella* TA144, an Antarctic bacterium. Proc. Natl Acad. Sci. USA 90, 4897–4901.

Laurell, H., Contreras, J.A., Castan, I., Langin, D., Holm, C., 2000. Analysis of the psychrotolerant property of hormone-sensitive lipase through site-directed mutagenesis. Protein Eng. 13, 711–712.

Lemos, D., Phan, V.N., 2001. Ontogenetic variation in metabolism, biochemical composition and energy content during the early life stages of *Farfantepenaeus paulensis* (Crustacea: Decapoda: Penaeidae). Mar. Biol. Berl. 138, 985–997.

Lesuisse, E., Schanck, K., Colson, C., 1993. Purification and preliminary characterization of the extracellular lipase of *Bacillus subtilis* 168, an extremely basic pH-tolerant enzyme. Eur. J. Biochem. 216, 155–160.

Matsumura, S., Matsuo, M., Nishizuka, Y., 1976. Partial purification and characterization of a triacylglyceride lipase from pig adipose tissue. J. Biol. Chem. 251, 6267–6273.

Merrill, C.R., Washart, K.M., 1998. Gel Electrophoresis of Proteins: A Practical Approach. Oxford University Press, New York.

Moss, S.M., Divakaran, S., Kim, B.G., 2001. Stimulating effects of pond water on digestive enzyme activity in the Pacific white shrimp, *Litopenaeus vannamei* (Boone). Aquacult. Res. 32, 125–131.

Mrdakovic, M., Lazarevic, J., Peric-Mataruga, V., Ilijin, L., Vlahovic, M., 2008. Partial characterization of a lipase from gypsy moth (*Lymantria dispar* L.) larval midgut. Folia Biol. Cracow 56, 103–110.

Müller, G., Petry, S., 2004. Lipases and Phospholipases in Drug Development. WILEY-VCH Verlag, Weinheim.

Nelson, L., Cox, M., 2000. Lehninger Principles of Biochemistry. New York.

Ollis, D.L., Cheah, E., Cygler, M., Dijkstra, B., Frolow, F., Franken, S.M., Harel, M., Remington, S.J., Silman, I., Schrag, J., Sussman, J., 1992. The a/b hydrolase fold. Protein Eng. 5, 197–211.

Park, J., Soon-Yeong, C., Suk-Jung, C., 2008. Purification and characterization of hepatic lipase from *Todarodes pacificus*. BMB Rep. 41, 254–258.

Pistillo, D., Manzi, A., Tino, A., Pilo Boyl, P., Graziani, F., Malva, C., 1998. The *Drosophila melanogaster* lipase homologs: a gene family with tissue and developmental specific expression. J. Mol. Biol. 276, 877–885.

Prim, N., Sánchez, M., Ruiz, C., Pastor, F.I.J., Diaz, P., 2003. Use of methylumbeliferyl-derivate substrates for lipase activity characterization. J. Mol. Catal. B Enzym. 22, 339–346.

Rajesh Patel, J.L.S., Hariharasundaram, Balaji, Arrese, Estela L., 2005. Activation of the lipid droplet controls the rate of lipolysis of triglycerides in the insect fat body. J. Biol. Chem. 22624–22631.

Rivera-Pérez, C., García-Carreño, F.L., Saborowski, R., 2010. Purification and biochemical characterization of digestive lipase in whiteleg shrimp. Mar. Biotechnol.10.1007/s10126-010-9298-7

Roussel, A., Canaan, S., Egloff, M.-P., Riviére, M., Dupuis, L., Verger, R., Cambillau, C., 1999. Crystal structure of human gastric lipase and model of lysosomal acid lipase, two lipolytic enzymes of medical interest. J. Biol. Chem. 274, 16995–17002.

Sánchez-Paz, A., García-Carreño, F., Hernández-López, J., Muhlia-Almazán, A., Yepiz-Plascencia, G., 2007. Effect of short-term starvation on hepatopancreas and plasma energy reserves of the Pacific white shrimp (*Litopenaeus vannamei*). J. Exp. Mar. Biol. Ecol. 340, 184–193.

Slim, C., Gargouri, Y., 2009. Thermoactivity and effects of organic solvents on digestive lipase from hepatopancreas of the green crab. Food Chem. 116, 82–86.

Smith, G.M., Rothwell, K., Wood, S.L., Yeaman, S.J., Bownes, M., 1994. Specificity and localization of lipolytic activity in adult *Drosophila melanogaster*. Biochem. J. Rev. 304, 775–779.

Snellman, E.A., Sullivan, E.R., Colwell, R.R., 2002. Purification and properties of the extracellular lipase, LipA, of *Acinetobacter sp.* RAG-1. Eur. J. Biochem. 269, 5771–5779.

Stahnke, G., Davis, R.C., Doolttle, M.H., Wong, H., Schotz, M.C., Will, H., 1991. Effect of N-linked glycosylation on hepatic lipase. J. Lipid Res. 32, 477–484.

Stauffer, C.E., 1989. Enzyme Assays for Food Scientists. New York.

Stauffer, C.E., 1971. Mechanism of chymotrypsin hydrolysis of an ester and an anilide. FEBS Lett. 16, 45–47.

Sukarno, Takahashi, K., Hatano, M., Sakurai, Y., 1996. Lipase from neon flying squid hepatopancreas: purification and properties. Food Chem. 57, 515–521.

Thornton, D.J., Carlstedt, I., Sheehan, J.K., 1994. Identification of glycoproteins on nitrocellulose membranes and gels. In: Walker, J.M. (Ed.), Methods Mol. Biol., vol. 32. Humana Press, Totowa, NJ, pp. 119–129.

van der Horst, D.J., van, M., Diederen, J.H., 2001. Adipokinetic hormones of insect: release, signal transduction, and responses. Int. Rev. Cytol. 211, 179–240.

Versaw, W.K., Cuppert, S.L., Winter, D.D., Williams, L.E., 1989. An improved colorimetric assay for bacterial lipase in non fat dry milk. J. Food Sci. 54, 1557–1558.

Wolins, N.E., Brasaemle, D.L., Bickel, P.E., 2006. A proposed model of fat packaging by exchangeable lipid droplet proteins. FEBS Lett. 580, 5484–5491.

Yan-Bo, W., 2007. Effect of probiotic on growth performance and digestive enzyme activity of the shrimp *Penaeus vannamei*. Aquacult. Res. 269, 259–264.

Ziegler, R., 1991. Changes in lipid and carbohydrate metabolism during starvation in adult *Manduca sexta*. J. Comp. Physiol. B Biochem. Syst. Environ. Physiol. 161, 125–131.

Zouari, N., Miled, N., Cherif, S., Mejdoub, H., Gargouri, Y., 2005. Purification and characterization of a novel lipase from the digestive glands of a primitive animal: the scorpion. Biochim. Biophys. Acta 1726, 67–74.