ORIGINAL ARTICLE

Purification and Biochemical Characterization of Digestive Lipase in Whiteleg Shrimp

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Abstract *Penaeus vannamei* lipase was purified from midgut gland of whiteleg shrimp. Pure lipase (E.C. 3.1.1.3) was obtained after Superdex 200 gel filtration and Resource Q anionic exchange. The pure lipase, which is a glycosylated molecule, is a monomer having a molecular mass of about 44.8 kDa, as determined by SDS-PAGE analysis. The lipase hydrolyses short and long-chain triacylglycerols and naphthol derivates at comparable rates. A specific activity of 1787 $U mg^{-1}$ and 475 $U mg^{-1}$ was measured with triolein and tributyrin as substrates, respectively, at pH 8.0 and 30°C in the absence of colipase. The lipase showed a $K_{m, app}$ of 3.22 mM and $k_{cat, app}/K_{m, app}$ of $0.303 \times 10^3 mM^{-1} s^{-1}$ using triolein as substrate. Natural detergents, such as sodium deoxycholate, act as potent inhibitors of the lipase. This inhibition can be reversed by adding fresh oil emulsion. Result with tetrahydrolipstatin, an irreversible inhibitor, suggests that the lipase is a serine enzyme. Peptide sequences of the lipase were determined and compared with the full-length sequence of lipase which was obtained by the rapid amplification of cDNA ends method. The full cDNA of the *pvl* was 1,186 bp, with a deduced protein of 362 amino acids that includes a consensus sequence (GXSXG) of the lipase superfamily of α/β-hydrolase. The gene exhibits features of conserved catalytic residues and high homology with various mammalian and insect lipase genes. A potential *lid* sequence is suggested for *pvl*.

Keywords Digestion · Lipase · Purification · *Penaeus vannamei* · Crustaceans

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Introduction

Lipases play an important role in lipid metabolism and energy homeostasis because fatty acids, mostly stored as triacylglycerides (TAG), are the major endogenous source of energy. In mammals, lysosomal and digestive lipases have been recognized by several authors (Roussel et al. 1999; Miled et al. 2000). Among digestive lipases, two major groups, gastric and pancreatic lipases, are of fundamental physiological interest because they are responsible for TAG hydrolysis (Miled et al. 2000). Studies of invertebrate lipid metabolism demonstrate lysosomal and digestive lipases (Arrese and Wells 1994; Slim et al. 2007).

Penaeid shrimp require food lipids to satisfy a variety of metabolic functions (González-Félix and Pérez-Velazquez 2002). Food lipid sources have been studied in penaeid species (González-Félix and Pérez-Velazquez 2002; Gonzalez-Baró and Pollero 1998). They demonstrate that shrimp have a limited ability to synthesize de novo the *n*-6 and *n*-3 highly unsaturated fatty acids. Lipids found in fish oils and plant oils rich in linoleic and linolenic acids are better utilized by crustaceans and have better nutritional value (Lim et al. 1997; González-Félix and Pérez-Velazquez 2002). The enzymes responsible for TAG hydrolysis from dietary lipids to free fatty acids are digestive lipases.

Lipases (EC 3.1.1.3) are characterized by the ability to hydrolyze hydrophobic long and short-chain glycerides (Jaeger et al. 1994). Lipases investigated so far vary

considerably in size and in their primary structure; however, all of them belong to the α/β-hydrolase superfamily, in which a nucleophilic serine amino acid residue is part of the catalytic triad (His, Ser, and Asp) and the consensus sequence (Gly-X-Ser-X-Gly). The active site of lipases is covered by a surface loop, named *lid*, which is displaced at the oil–water interface to permit the entry of hydrophobic substrates (Ollis et al. 1992).

To date, only a few studies are available about digestive lipases in crustaceans, their detailed function in lipid metabolism, and methods of purification and characterization. Lipases from crab *Carcinus mediterraneus* (Slim et al. 2007), scorpion *Scorpio maurus* (Zouari et al. 2005), and squid *Todarodes pacificus* (Park et al. 2008) have been purified. Some biochemical characteristics are similar to those found in mammalian digestive lipases: molecular masses of ∼40–50 kDa with an alkaline p*I* between 6.8 and 7.8 (Moreau et al. 1992; Miled et al. 2000).

In crustaceans, lipases are liberated by B cells of the midgut gland (Loizzi and Peterson 1971) into the tubule lumen where they move into the gastric chamber and initiate lipid digestion of ingested food. Because TAGs are hydrophobic, some emulsifiers may be required. In Penaeidae, fat emulsifiers have not been described, but other decapods secrete acyltaurines for this purpose (Dall and Moriarty 1983). Moreover, mammalian pancreatic lipases require colipase, a pancreatic protein, as a cofactor for enzymatic activity (Lowe 1997). In contrast, in invertebrates, which lack a true liver, colipase has not been found, e.g., the crayfish *Pacifastacus leniusculus* (Marcus and Talalay 1956) and crab *C. mediterraneus* (Slim et al. 2007). Accordingly, there are marked differences between invertebrate and mammalian digestive lipases.

Lipases from different sources exhibit broad substrate specificities. Mammalian lipases exhibit a preference for short fatty acids and depend on bile salts and colipase (Holm et al. 2000). Crustaceans, such as the penaeids, *Penaeus schmitti*, *Penaeus vannamei*, *Farfantepenaeus californiensis*, and *Farfantepenaeus notialis* have a tendency to hydrolyze longer chain TAG, such as triolein, tripalmitin, and triestearin (Forrellat Barrios et al. 2004; Del Monte et al. 2002), show different enzymatic specificities, as well as different catalytic rates. Inhibitors, such as bile salts (Borgstrom and Donner 1976), are related to the change in the conformation of lipase during catalysis. However, only a few specific inhibitors have been identified, such as tetrahydrolipstatin and di-isopropyl fluorophosphate. These inhibitors are used to characterize digestive enzymes from the scorpion *S. maurus* (Zouari et al. 2005) and crab *C. mediterraneus* (Slim et al. 2007) as serine lipases.

To our knowledge, no digestive lipases were purified and sequenced from penaeids; hence, the details of the lipolytic process in crustaceans are still unknown. This is important in terms of better understanding of the nutrition and trophic interactions in the field. Moreover, lipolytic enzymes are valuable catalysts in many biotechnological applications. Therefore, novel enzymes from shrimps might exhibit catalytic properties that may be beneficial in biotechnological process. Here, we isolated and characterized a lipase from the midgut gland of *P. vannamei* to obtain the gene that codifies this protein.

Materials and Methods

Animals

Midgut gland tissue from whiteleg shrimp *P. vannamei* (= *Litopenaeus vannamei*) was obtained from aquaculture facilities. Midgut glands were dissected and immediately homogenized in cold, distilled water (1:4 *w/v*). The homogenates were centrifuged for 30 min at 10,000 g and at 4°C. The supernatant was lyophilized and stored at 4°C for further analysis.

Determination of Lipase Activity and Protein Concentration

Lipase activity was measured by titrating free fatty acids liberated from TAG at slightly alkaline conditions using the pH-stat standard assay conditions described by Gargouri et al. (1984). The reaction mixture consisted of 0.25 ml triolein (Sigma, T-7140) in 30 ml of 2.5 mM Tris–HCl, 150 mM NaCl, and 3% gum arabic at pH 8.0. The appropriate amount of enzyme was incubated at 30°C for 1 h with magnetic stirring. One activity unit corresponds to 1 μmol fatty acids released per minute. Activity profiles of pH, thermal stability, and inhibition were expressed in relation to the maximum value (= 100%) of the respective profile.

Lipase activity was also determined with a fluorescent substrate MUF-butyrate (Sigma, 19362). The substrate concentration in the assay was 100 μM in a total volume of 300 μl phosphate buffer (50 mM, 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 (Sigma, M1381). Activity profiles were expressed in relation to the maximum value (= 100%) of the respective profile. The protein concentration was determined using the Bradford method (1976). Bovine serum albumin (Sigma, B-4287) was used as a standard.

Chromatography

Gel Filtration

Lyophilized sample (200 mg) was dissolved in 1 ml PBS (50 mM phosphate, 150 mM NaCl, pH 7.5). Proteins from

the midgut gland extract were separated by gel filtration chromatography using a FPLC system (Pharmacia Biotech, Uppsala, Sweden). Small proteins were removed by loading the sample onto a NAP-10™ Sephadex G25 column (Amersham Biosciences, Uppsala, Sweden) and eluted with 1.5 ml PBS buffer. The processed extracts were loaded into a 124-ml gel filtration column (HiLoad Superdex 200 16/60, Amersham Biosciences) equilibrated in PBS buffer. To separate the proteins by size, the column was washed with 175 ml PBS buffer. Simultaneously, the absorbance at 280 nm and the conductivity were monitored and recorded. The flow rate was 1 ml min^{-1}. We collected 25 fractions of 5 ml each. Each fraction was concentrated through centrifugal filters (AMICON® Ultra-15, Millipore, Bellerica, MA, USA) at 4,000 g for 15 min as 25°C to a final volume of 500 μl.

Anionic Exchange Chromatography

Fractions with lipase activity from gel filtration were first loaded onto a NAP-5™ column containing Sephadex G25 (Amersham Biosciences). The samples were eluted with the standard phosphate buffer (50 mM, pH 8.0). Thereafter, the samples were loaded into a 1 ml Resource Q column (Pharmacia Biotech) that was first equilibrated with the standard phosphate buffer. Under these conditions, the enzyme adsorbed onto the ionic support and was eluted with an increasing concentration of NaCl (0–1 M) in standard phosphate buffer. Simultaneously, absorbance at 280 nm and conductivity were monitored and recorded. The flow rate was 1 ml min^{-1} and the final elution volume was 35 ml. Fractions of 500 μl each were collected.

The fractions were assayed for lipase activity by MUF-butyrate hydrolysis: 10 μl of each fraction were incubated at 30°C with 250 μl MUF-butyrate at a final concentration of 100 μM. The assay was carried out as described above. The purified lipase (hereafter named PVL) was stored at −80°C until further analysis.

Mass Spectrometry Analysis

To identify the pure enzyme, 3 μg PVL were electrophoretically separated on a 12% acrylamide gel. After separation, the gel was stained with Coomassie Blue to identify protein bands that were cut from the gel. The sequence analysis was performed at the Wistar Institute Proteomics Facility using a trypsin digestion and nanocapillary HPLC interfaced directly with a hybrid ion trap mass spectrometer (Gel/LC-MS/MS).

Electrophoresis

Proteins bands were analyzed by electrophoresis under nonreducing as well as reducing conditions using SDS-PAGE, as described by Laemmli (1970) and also by staining with silver nitrate (Merril and Washart 1998) in gels containing 12% acrylamide. For reducing conditions, samples were diluted (1:2) with sample buffer (0.125 M Tris–HCl, 2% SDS, 20% v/v glycerol, 0.04% bromophenol blue, 5% β-mercaptoethanol at pH 6.8) and heated for 2 min at 100°C. For nonreducing conditions, samples were mixed in the same buffer without mercaptoethanol and sodium dodecyl sulfate and were not heated. Electrophoretic separation was performed at 15 mA constant current and at 2°C. To detect protein bands, the gels were stained with 0.1% Coomassie brilliant blue R-250 in 7.5% acetic acid and 5% methanol at room temperature and destained in 10% acetic acid and 40% methanol.

Zymograms were developed using MUF-butyrate as substrate (Prim et al. 2003). After electrophoretic separation of proteins, the gels were rinsed in water and then incubated at room temperature in Triton X-100 (2.5% w/v) for 30 min. The gels were rinsed again with water and incubated in 100 ml MUF-butyrate solution (100 μM dissolved in phosphate buffer, 50 mM at pH 8.0). After 10 min, the fluorescence signal was detected and captured using a ChemiDoc XRS (Bio-Rad Laboratories, Hercules, CA, USA).

Biochemical and Kinetic Characteristics

Isoelectric Focusing

Analytical isoelectric focusing (IEF) was performed with the Phast System (Pharmacia Biotech, 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. The gel was treated with Coomassie brilliant blue R-250 using the method of Laemmli (1970).

Glycosylation Analysis

PVL was analyzed for glycosylation by electrophoresis under nonreducing conditions (Thornton et al. 1994). The gel was first incubated for 30 min at room temperature in solution C (50% v/v ethanol), rinsed with water, and thoroughly washed with distilled water for 10 min. Then, the gel was incubated in solution A (1% v/v periodic acid in 3% v/v acetic acid) for 30 min, washed with distilled water for 30 min, and washed for 20 min in solution B (0.1% w/v sodium metabisulfite in 10 mM HCl). Then the gel was incubated in Schiff's reagent for 1 h in the dark and immersed in solution B for 1 h in the dark. Finally, the gel was washed several times in 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% v/v acetic acid, 5% v/v methanol).

The Effect of pH and Temperature on Enzyme Activity

The effect of pH on PVL activity was measured using 2 μg lipase diluted in 15 μl universal buffer at 37°C (Stauffer 1989). To obtain the required pH, 20 ml stock solution (57 mM H_3BO_3, 36 mM citric acid, 28 mM NaH_2PO_4, 310 ml 1 N NaOH) was adjusted with 1 N HCl and then filled up with distilled water to 100 ml. Buffers were prepared for the pH range from 2 to 12. After incubation for 1 h at room temperature, lipase activity was assayed by titration using triolein as the substrate.

To measure the effect of temperature on PVL activity, 2 μg lipase diluted in 15 μl buffer (50 mM Tris–HCl 50, pH 8.0) were incubated for 1 h at temperatures ranging from 10°C to 80°C. Immediately after incubation, lipase activity was measured by titration using triolein as the substrate.

Substrate Specificity

PVL substrate specificity was measured (Versaw et al. (1989) by assaying its hydrolytic activity on naphthyl derivates and triacylglycerols with different chain length: tributyrin (Sigma, T-8626), tripalmitin (Sigma, T-5888), tristearin (Sigma, T-5016), and triolein (Sigma, T-7140). Specific activity was determined spectrophotometrically for naphthyl derivates and pH-stat titration as described for TAGs hydrolysis (Gargouri et al. 1984).

Effect of Ca^{2+} Concentrations on Enzyme Activity

The effect of Ca^{2+} on PVL was determined by measuring the hydrolytic activity of triolein in the presence of different calcium concentration (0–100 mM $CaCl_2$) by the pH-stat method.

Effect of Inhibitors and Bile Salts on Enzyme Activity

To determine if PVL is a serine lipase, tetrahydrolipstatin (THL, Sigma, O-4139) was used as a specific inhibitor. We used 0, 2.5, 5, 7.5, and 10 mM THL to measure its effect on lipase activity. Equal volumes of inhibitor and enzyme solution (0.2 mg ml^{-1}) were incubated for 1 h at room temperature, and the residual activity was measured by titration, as described above.

The effect of bile salt on PVL activity was measured with 0–4 mM sodium deoxycholate (NaDC, Sigma D-6750). Enzyme solution (0.2 mg ml^{-1}) was added to a triolein substrate solution which contained NaDC. To restore PVL activity, fresh oil emulsion was added after 30 min of reaction.

Kinetics Parameters

The kinetic parameters $K_{m, app}$ and $V_{max, app}$ of PVL were measured using triolein and MUF-butyrate as substrates. Triolein hydrolysis was assayed at concentrations of 0 to 20 mM during 1 h using the pH-stat method. MUF-butyrate hydrolysis was assayed fluorometrically at concentration of 0 to 100 μM for 10 min. $K_{m, app}$ (mM) and $V_{max, app}$ (U mg^{-1}) with the Lineweaver–Burk transformation graph.

Lipase cDNA Sequence

To amplify the full-length cDNA of *P. vannamei* lipase gene *pvl*, a 5′/3′ rapid amplification of cDNA ends was performed (GeneRacer^{TM} Kit, L1500, Life Technologies, Carlsbad, CA, USA). cDNA was prepared from 1 μg total RNA isolated from midgut gland tissue using Trizol reagent (Life Technologies). The primers based on a partial sequence of lipase (GenBank accession no. DQ858927) were designed as follows: PvLip_F1 (5′-GAAAGCG CTGGCTTACAAAC-3′) and PvLip_R1 (5′-TAGGAC CTTCGGCATTGTTC-3′).

The synthesized primers were used as internal gene-specific primers and 5′/3′ nested primers included in the GeneRacer^{TM} Kit were used as anchors at the cDNA ends. PCR amplification was performed using GoTaq$^{®}$ Green Master Mix (Promega, M7122, Madison, WI, USA), primers (10 μM each), cDNA template 1 μl (diluted 1:50), and nuclease-free water. All reactions were performed in a final volume of 25 μl. The thermal cycling program to amplify the lipase gene was performed on a thermocycler (Bio-Rad), configured as follows: 35 cycles of 1 min at 95°C, 30 s at 59°C, and 1 min at 68°C followed by a single cycle at 72°C for 10 min. PCR products were cloned (TOPO-TA cloning kit, Life Technologies K4500-01) and transformed into *Escherichia coli* DH 5α. Database nucleotide homology searches were carried out using BLAST in at the National Center of Biotechnology Information (Altschul et al. 1997). Gene translation of the deduced protein was performed with ExPASy (Gasteiger et al. 2003), and alignments were performed with the ClustalW software (Thompson et al. 1994). The nucleotide sequence of lipase gene (*pvl*) was deposited in GenBank (accession no. FJ619564).

Results

Purification of Lipase

PVL was purified by the two-step procedure described in the methods section. After the first step, the gel filtration

Fig. 1 Pure fraction from ion exchange chromatography. **a** The fractions showing lipase activity (37–40) were collected. **b** The zymogram of fractions (37–40) using MUF-butyrate as the substrate. **c** Fractions containing pure *Penaeus vannamei* lipase, separated by SDS-PAGE 12% and stained with Coomassie brilliant blue R-250. **d** Zymogram using MUF-butyrate as the substrate. **e** Silver-stained gel showing a single isolated protein band
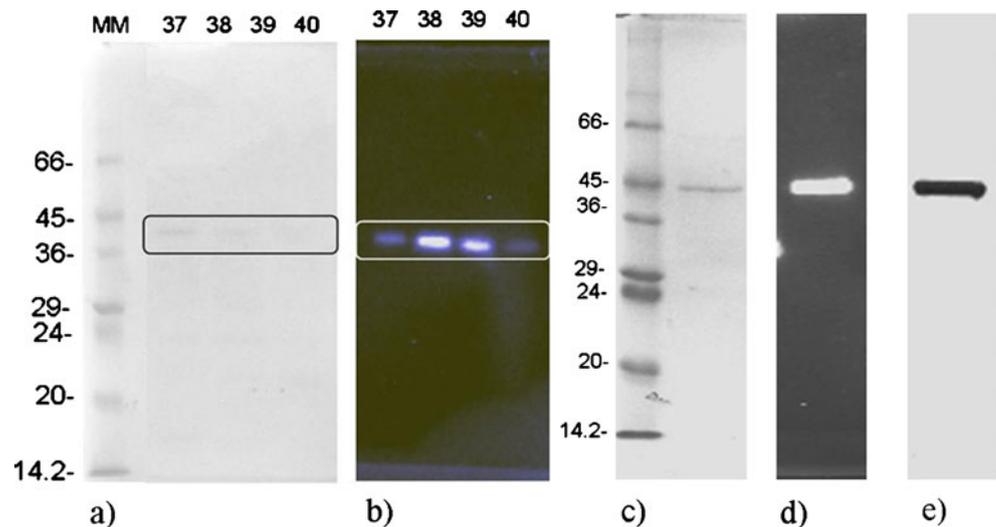
over a Superdex 200 column, the collected fractions contained different molecular weight proteins were revealed by SDS-PAGE and only those fractions containing PVL were subjected to anionic exchange chromatography on a Resource Q column. The fractions containing the eluted PVL (Fig. 1a, b) were pooled. A single band in SDS-PAGE (Fig. 1c), in zymogram analysis (Fig. 1d), and silver stain (Fig. 1e) was observed. A flow sheet of the purification process for PVL is presented in Table 1. The enzyme was enriched 48-fold and the yield was 0.12%. The apparent molecular mass of the band with lipase activity was 44.8 kDa (Fig. 1c). Electrophoresis analysis of PVL under reducing conditions showed a single band, demonstrating that PVL is a monomeric protein, which is also glycosylated and has a pI of 3.5. The sequence analysis (Gel/LC-MS/MS) results of PVL matched the deduced amino acid sequence of PVL.

General Characteristics

In whiteleg shrimp, PVL from the digestive system is capable of hydrolyzing naphthol esters, such as naphthyl acetate, and triacylglycerides, such as triolein. Four triacylglycerides were assayed to measure the specific activity of PVL. The pure enzyme shows 73% higher hydrolytic activity on long-chain triacylglycerols (TC_{18}) than on shorter triacylglycerols. The specific activity of PVL was 1787 Umg^{-1} for TC_{18} and 475 Umg^{-1} for TC_4 at pH8.0 and 30°C (Table 2).

Preliminary experiments with PVL indicated that the enzyme had low phospholipase activity. To confirm that this activity was inherent to PVL rather than to a contaminant protein, a zymogram of PVL was performed using PED6 (Life Technologies, D-23739) as substrate and compared to MUF-butyrate zymogram (data not shown). The zymogram showed significantly higher hydrolyzing activity for MUF-butyrate than for PED6. Both activity bands had the same molecular weight of 44.8 kDa, confirming the enzyme responsible for the activity is PVL.

The effect of temperature on PVL activity was assayed over a range from 10°C to 80°C (Fig. 2a). Maximum activity under these conditions took place at 30°C. Between 10°C and 20°Cm, activity was less than 10%. Above 40°C, there was a continuous drop in PVL activity caused by denaturation.

Table 1 Summary of *Penaeus vannamei* lipase purification

Step	Total protein (mg)	Total activity (U)	Specific activity (U mg^{-1})	Purification (fold)	Yield (%)
Freeze-drying	197.6±2.5	14,162±70.7	71.6±0.65	1	100
S-200 chromatography	1.82±0.06	2,285±4.08	1,252±45.2	17.46±0.47	0.16±0.0
Source 15 Q chromatography	0.51±0.02	1,783±5.11	3,447±174	48.12±2.8	0.12±0.0

Protein concentration was estimated by the Bradford method. The experiments were conducted three times; standard errors are shown

1 unit 1 μmol of fatty acid released per minute using TC_{18}

Table 2 Lipase activity of *Penaeus vannamei*

Substrates	Specific activity (Umg^{-1})
Naphthyl ester	
α-Naphthyl acetate	429.5±2.3
β-Naphthyl acetate	441.0±1.3
β-Naphthyl caprylate	15.1±0.1
Triacylglycerides	
Tributyrin (C:4)	475.8±4.9
Tripalmitin (C:16)	661.0±9.8
Tristearin (C:18)	780.6±9.8
Triolein (C18:1)	1,787.3±7.9

The effect of pH on PVL is presented in Fig. 2b. PVL has a maximum activity on TC$_{18}$ at pH 8.0 to 9.0. PVL lost activity below pH 4.0 and above pH 10.0. After 2 h incubation, 60% of activity of PVL was lost at pH 10.0. PVL does not rely on calcium for activity (data not shown).

Kinetic Parameters

There are only a few reports describing kinetic parameters of lipase in invertebrates. Triolein and MUF-butyrate were used as substrates under optimal conditions (pH 8.0 and 30°C). The kinetic values of PVL were obtained using the Michaelis–Menten model. Catalytic properties of PVL were $K_{m, app}$=3.22 mM and $V_{max, app}$=3404 Umg^{-1} for triolein and 0.24 mM and 3042 Umg^{-1} for MUF-butyrate (Table 3). The low $K_{m, app}$ value indicates high affinity between the enzyme and the substrate. PVL showed >150-fold higher catalytic efficiency $\left(k_{cat, app}/K_{m, app} = 52.8 \times 10^3 \text{mM}^{-1}\text{s}^{-1}\right)$ for MUF-butyrate than for triolein $(0.303 \times 10^3 \text{mM}^{-1}\text{s}^{-1})$. The higher catalytic efficiency of PVL on MUF-butyrate is related to higher affinity for this substrate.

Colipase and Effect of Bile Salts on PVL Activity

To corroborate that shrimp cosynthesize a colipase as a cofactor for PVL, a crude extract of midgut gland was treated for 30 min at pH 4.0 and 65°C to denature lipase, but preserving colipase activity (Slim et al. 2007). At the same time, porcine lipase (Lipase type II, Sigma, L-3126), used as a control, was inhibited with 3 mM NaDC and the reactivation was tried by mixing the putative colipase from whiteleg shrimp with the inhibited porcine lipase in the same reaction mixture containing triolein as substrate; the hydrolysis of triolein was measured by pH-stat. There was no reactivation of porcine lipase (data not shown), so, under the conditions used here, we concluded that shrimp do not possess colipase as a cofactor of PVL.

The effect of increasing concentrations of bile salt (NaDC) on the rate of hydrolysis of TC$_{18}$ by PVL is shown in Fig. 3a. When NaDC was placed at the water/lipid interface, below the critical micellar concentration (the concentration of surfactant above where micelles are formed), the apparent activity of lipase decreased to 40% of the initial activity with 1 mM of NaDC. This inactivation is related to the critical concentration of NaDC in the micelle (5 mM), which may shift downward in the presence of a water–lipid interface, which results from adsorption of detergent molecules (Hermoso et al. 1996). This effect was observed in PVL when a fresh emulsion of triolein was added after 30 min of reaction (Fig. 3a).

Effect of Inhibitors on Enzyme Activity

THL is a specific inhibitor of lipases (Gargouri et al. 1991), which react with the catalytic serine of active site. To determine if the active site of PVL contains a serine, we assayed the effect of THL on PVL activity (Fig. 3b). PVL lost 95% of its activity when incubated with 2.5 mM THL, demonstrating that PVL is a serine lipase, which agrees with the deduced amino acid sequence obtained, including the consensus sequence GXSXG.

Lipase cDNA Sequence

The full cDNA encoding triacylglycerol lipase *pvl* from the midgut gland of whiteleg shrimp was uploaded from the

Fig. 2 The effect of **a** temperature and **b** pH on PVL using TC18 by the titrimetric method. The activities were calculated in relation to the mean of all measurements which was set to be 100% (means±SD, *n*=3)
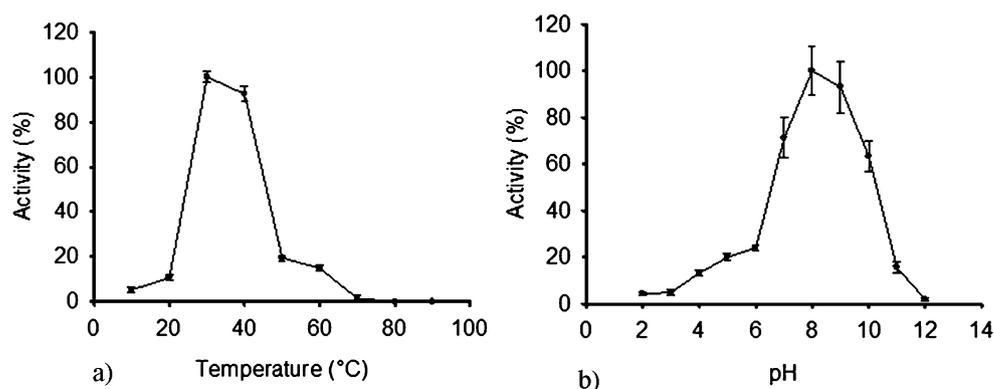
Table 3 Kinetic values of *Penaeus vannamei* lipase

Substrate	V_{max} (Umg^{-1})	K_m (mM)	k_{cat} ($\times10^3$ s^{-1})	k_{cat}/K_m ($\times10^3$ mM^{-1} s^{-1})
TC$_{18}$	3,404±29.51	3.22±0.12	1.01±0.04	0.303±0.02
MUF-butyrate	3,042±2.88	0.24±0.00	12.67±0.27	52.8±2.33

GenBank database (GenBank accession no. FJ619564). We obtained a 335-bp fragment of DNA with specific primers (PvLip_F1 and PvLip_R1), a 242 bp 5′ end fragment and a 609 bp 3′ end fragment with a polyadenylation (A)$^+$ tail. We obtained a 1,186-bp full-length cDNA using overlapping PCR that included a 59-bp 5′ untranslated region and a 1,089-bp open reading frame (ORF). The 3′ end contained a 20-bp polyadenylation (A)$^+$ tail that was 14 bp from ATAA, the eukaryotic consensus polyadenylation signal. The ORF encoding a 362-amino acid protein was 41.2 kDa and a putative isoelectric point of 4.7. The protein included a consensus sequence composed of GXSXG and a catalytic triad composed of Ser, Asp, and His (Fig. 4). The *lid* domain was recognized by comparing the aligned sequence of invertebrate and mammal lipases between residues 219–241 of PVL (Table 4). BLASTX analysis demonstrated that 45% of the sequence of *pvl* is identical to the amino acid sequence of *A. aegypti* lipase 1, 46% of *Culex quinquefasciatus* lipase 1, 42% of *Ixodes scapularis* triacylglycerol lipase, and 40% of *Drosophila melanogaster lip4*; these lipases showed amino acid residues of the active site at homologous positions (Fig. 5). The deduced amino acid sequence of *pvl* coincides with the four peptide sequences obtained by mass spectrometric procedures. The pattern [DY] [L] [G] XXX [VYY] X [GXSXGX] was identified as part of the amino acid sequences of PVL that corresponds to a α/β-hydrolase structure (Fig. 4) of this kind of enzyme.

Discussion

This is the first detailed report of the purification of a lipase from the midgut gland of whiteleg shrimp. The lipase is a glycoprotein with a molecular mass of 44.8 kDa, which is similar to that for other lipases from the redclaw crayfish *Cherax quadricarinatus* (López-López et al. 2003), squid *Ommastrephes bartramii* (Sukarno et al. 1996), and scorpion *S. maurus* (Zouari et al. 2005). Within the conserved domain identified in the deduced amino acid sequence of *pvl*, a catalytic triad and a *lid* were identified, which shared similarity in residues to those seen in other lipases, and the level of similarity seen here and the catalytic properties of the pure lipase supports *pvl* encoding a lipase protein with novel biotechnological applications.

Glycosylation in PVL is a usual feature of lipases (Buscá et al. 1995). Other glycosylated lipases have been described in invertebrates such as the scorpion *S. maurus* (Zouari et al. 2005). However, lipases shown a different degree of glycosylation which differs among species (Rathelot et al. 1981). The carbohydrate portion of PVL could play a functional role in controlling its secretion by B cells, as in mammals, in which the main role of glycosylation have been attributed to the control of secretion of the protein and stability (Buscá et al. 1995). The nature of the carbohydrate and its functional role in PVL remains to be investigated.

Besides other factors, activity and stability of an enzyme are functions of temperature and pH. In whiteleg shrimp,
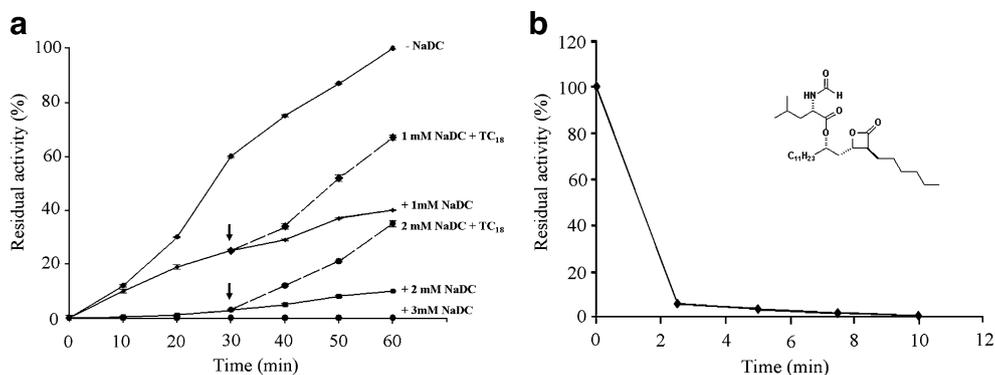
Fig. 3 The effect of bile salts and inhibitors on PVL. **a** The effect of increasing the concentration of bile salts (NaDC) on the rate of hydrolysis of TC18 by *Penaeus vannamei* lipase. **b** Effect of increasing concentration of THL on the rate of hydrolysis of TC18 by *Penaeus vannamei* lipase. Lipase activity was measured at pH 8.0 and 30°C using TC18 as the substrate. The *arrows* indicate the addition of TC18 fresh emulsion. The activities were calculated in relation to the mean of all measurements which was set to be 100% (means±SD, *n*=3)

Fig. 4 Nucleotide and deduced amino acid sequence of *Penaeus vannamei* lipase cDNA (GenBank accession no. FJ619564). Consensus sequence (GXSXG) is shaded in *gray*. Residues of catalytic (*shaded triangle*) and pattern of α/β-hydrolase structure (*asterisk*) are indicated. The peptide sequences obtained by mass spectrometry are *boxed*. *Underlining* in the nucleotide sequence indicates polyadenylation

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 -59  gaaggagtagaaaaaaattctcataatctcctttcttgtattgtatctgatagttgaatg
   1                                                             M

  63  gataaatggcaaaaatatggaaatctaataaatcgatatagaagaaacgttaatgaatgc
   2   D  K  W  Q  K  Y  G  N  L  I  N  R  Y  R  R  N  V  N  E  C

 123  acgatggaatacggaaggatattctcattctcctttacgaaaacaaaatagttaaagca
  22   T  M  E  Y  G  R  I  F  S  F  S  F  Y  E  N  K  I  V  K  A

 183  atggagaggagagacagaccgggagacagagaaagacaacagacacaaagaaaggcaacg
  42   M  E  R  R  D  R  P  G  D  R  E  R  Q  Q  T  Q  R  K  A  T

 243  aaagcgctggcttacaaactcgccgatgcgggctatgatgtgtggctttcgaacatgcgc
  62   K  A  L  A  Y  K  L  A  D  A  G  Y  D  V  W  L  S  N  M  R

 303  ggaaacacctattccagaaaccatgtggagttggatcctgaagacatcagcttctggcag
  82   G  N  T  Y  S  R  N  H  V  E  L  D  P  E  D  I  S  F  W  Q

 363  ttcagctgggacgaattagcttactacgatgttcctgcgagcattgattatgttctcggc
 102   F  S  W  D  E  L  A  Y  Y  D  V  P  A  S  I  D  Y  V  L  G
                                           *     *        *

 423  atgactgggggctgaagcagtatactatgctggctggagcatggggaccaccgtcttctgg
 122   M  T  G  A  E  A  V  Y  Y  A  G  W  S  M  G  T  T  V  F  W
          *                    *  *     *     ▲        *

 483  gcgatgatgagtgaaaaacctgagtataatgaaaaggttcgagccatggccgctatggct
 142   A  M  M  S  E  K  P  E  Y  N  E  K  V  R  A  M  A  A  M  A

 543  cctgtcgccttcatgaacaatgccgaaggtcctatcatggcgctggctccttactctgat
 162   P  V  A  F  M  N  N  A  E  G  P  I  M  A  L  A  P  Y  S  D

 603  gatcttgatttcatggccaccccttctcggcgtcggggagtttctgccaagttcggacctg
 182   D  L  D  F  M  A  T  L  L  G  V  G  E  F  L  P  S  S  D  L

 663  cttgatcacttcgtcgagacgtactgcgactcggaggccgtgactgccgaagtctgctac
 202   L  D  H  F  V  E  T  Y  C  D  S  E  A  V  T  A  E  V  C  Y

 723  aacttcctcctcctcctcgccggcccggaccccgacgaaattccgaaggactttctccct
 222   N  F  L  L  L  L  A  G  P  D  P  D  E  I  P  K  D  F  L  P

 783  ataatcctggctcacacacctgccggagcgtctgttcacaccgtcaatcactacgcacag
 242   I  I  L  A  H  T  P  A  G  A  S  V  H  T  V  N  H  Y  A  Q

 843  ctggtcatgtcaggtgtttttcgacaaatacgattacggcctcattggcaacctgaaccac
 262   L  V  M  S  G  V  F  D  K  Y  D  Y  G  L  I  G  N  L  N  H

 903  tacggacagaacacgccgcccttgttcaacctcagcagagtcgccgcgcccgttggcttg
 282   Y  G  Q  N  T  P  P  L  F  N  L  S  R  V  A  A  P  V  G  L

 963  ttctggggctccacggactggctggctgatcctacggacgtggcaaggctcgctgaagga
 302   F  W  G  S  T  D  W  L  A  D  P  T  D  V  A  R  L  A  E  G
                      ▲

1023  cttccaaatctcgcccttaatcacaaggtggataaggaagagttcaaccatctagacttt
 322   L  P  N  L  A  L  N  H  K  V  D  K  E  E  F  N  H  L  D  F
                                                    ▲

1083  ggctgggggatccacgccgacgaactggtgtaccgccacattctcgatttcttcgccaac
 342   G  W  G  I  H  A  D  E  L  V  Y  R  H  I  L  D  F  F  A  N

1143  tactgattaacaagaagaaactttaaaaaaaaaaaaaaaaaaaaa
 362   Y  *
```

90% of lipase activity occurs in a range of 30–40°C. Temperatures of maximum lipase activity in other organisms are also quite narrow. Digestive lipase of the neon fly squid *O. bartramii*, displays its highest activity between 30–40°C (Sukarno et al. 1996) and in the Indian fish, the rohu *Labeo rohita* at 45°C (Nayak et al. 2004). The crab *C. mediterraneus* exhibits maximum lipase activity at 60°C (Slim et al. 2007), while activity in whiteleg shrimp lipase decreases above 40°C.

The pH range over which an enzyme will be stable varies from one protein to another; lipase from whiteleg shrimp has maximum activity from pH 7 to 8. The pH for maximum activity of digestive lipases in the scorpion is between 8.5 and 9 (Zouari et al. 2005), which is related to the ionization properties of histidine (pKa=6.5) and the fact that the amino acid residues of the catalytic triad (Ser, His and Asp) (Roussel et al. 1999) play an important role in the charge relay system involving the catalytic triad and the

Table 4 Sequence alignment of endothelial, hepatic, lipoprotein, and *Penaeus vannamei* lipase *lid* region

Protein	*lid* sequence																						GenBank accession number
EL	C	G	L	N	D	V	L	G	S	I	A	Y	-	G	-	T	I	T	E	V	V	K	C	Q9Y5X9
LPL	C	N	I	G	E	A	I	R	V	I	A	E	R	G	L	D	V	D	Q	L	V	K	C	P06858
HL	C	H	F	L	E	L	Y	R	H	I	A	Q	H	G	F	N	A	I	T	Q	T	K	C	A2RUB4
PVL	C	Y	N	F	L	L	L	L	A	G	P	D	P	D	E	I	P	K	D	F	L	P		This study
	a	•		:	•	•	•	:	•	•					•			•		•	•			

a highly conserved amino acid, • and : semiconservative amino acid replacement

enhancement of the nucleophilic character of the serine residue. Digestive lipases from organisms other than crustaceans also have maximum activity in the alkaline range of the pH scale: bovine pancreatic lipase, pH 8 (Shahani et al. 1976), human hepatic lipase, pH 8 (De Caro et al. 1977), and the bacterium *Mycobacterium tuberculosis,* pH 8–9 (Deb et al. 2006). PVL lost activity below pH 4.0 and above pH 10. After 2 h of incubation, 60% of activity of PVL was lost at pH 10. Lipase of scorpion *S. maurus* lost only 35% of its activity after 4 h incubation at pH 11 and pH under 5 (Zouari et al. 2005), as well as lipase from the Asian gypsy moth *Lymantria dispar* (Mrdakovic et al. 2008) and squid *O. bartramii* (Sukarno et al. 1996). Lipase activity decreased in *Rodnius prolixus* and *Cephaloleia presignis* at pH>8.0 (Arreguín-Espinosa et al. 2000; Grillo et al. 2007); these evidences showed that the pH stability of lipases differs between species.

Cationic cofactors are required for lipase activity of vertebrates. Divalent cations, such as calcium, increase enzyme activity. For example, maximum lipase activity in adipose tissue of pig occurs at 10 mM $CaCl_2$ (Matsumura et al. 1976). PVL does not rely on calcium for activity, indicating that PVL catalytic properties are dissimilar to mammalian lipases. This is supported by reports showing that lipase in insects *R. prolixus* (Grillo et al. 2007) and *C. presignis* (Arreguín-Espinosa et al. 2000) have the same activity regardless of the calcium concentration. To date, it is not certain that invertebrate digestive lipases require a moderate concentration of calcium for activity or stability, as observed in mammalian digestive lipases.

The pure shrimp lipase shows properties of serine lipases. It is inhibited by THL, which is consistent with the presence of serine at the catalytic site. This result is consistent with the consensus sequence GXSXG obtained from shrimp lipase cDNA which characterizes the lipase gene family which is composed by pancreatic, lipoprotein, and hepatic lipases, based on amino acid sequence similarity (Hide et al. 1992), confirming its identity.

In spite of the absence of a true liver in invertebrates, emulsifiers such as NaDC act as an inhibitor of PVL; its inactivation may be shifted downward, as are mammalian lipases. This evidence suggests similar structural properties with the vertebrate lipases. However, the lack of colipase in invertebrates such as shrimp, we suggest that colipase and bile salts evolved after the divergence of invertebrate and vertebrate.

The feeding behavior of marine invertebrates has been described for some organisms. There are differences in feeding behavior among penaeids and among the stages of their development. The use of different lipids, such as TAG, as sources of energy may depend on its digestibility, energy content, and fatty acid composition.

PVL was able to hydrolyze long-chain TAG better than short ones, in contrast to crab digestive lipase with 500 U mg^{-1} for TC_4 and 130 U mg^{-1} for olive oil (Slim et al. 2007). The tendency of PVL to hydrolyze long-chain TAG is similar to other shrimp lipases (Del Monte et al. 2002; Forrellat Barrios et al. 2004) and could be related to hydrophobicity of the PVL *lid* (residues 219–241), which differ from those of mammalian lipases, such as human endothelial lipase, lipoprotein lipase, and hepatic lipase (Table 3). In previous work done on lipoprotein and hepatic lipase in humans, it (Dugi et al. 1995) found that the sequence of the *lids* and its hydrophobicity affect enzymatic activity. The capability of whiteleg shrimp to hydrolyze long-chain TAG from alimentary lipids is an important advantage for lipid utilization by these invertebrates because hydrolysis of long-chain TAG provides essential fatty acids, such as linoleic and linolenic acids, as long as these are intended for β-oxidation.

Besides the physiological role of lipases in lipid catabolism, theses enzymes constitute one of the most important groups of biocatalyst for biotechnological applications, useful for improving food, detergents, pharmaceuticals, oleochemicals, textiles, cosmetics, and paper. The digestive gland of shrimp constitutes an alternative

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A.aegypti          ----MLTASLTTLLITAGAIQ----------------------------------------- 17
I.scapularis       ------------------------------------------------------------- 
D.melanogaster     MSVKLLIVALSLCLLQSGIVEGISLSLGDITLFNVNFKTPTFLGRSVAVDSNVRLENDVD 60
C.quinquefasciatus MAQLLPSIPILLALISWACGVPTARNISATRHFQVD----------------------- 36
P.vannamei         ------------------------------------------------------------- 

A.aegypti          -----RALAGTEQLVKRMGLPVEKHRAVTSDGYVLTMFRIPANNTNSP--------VAFL 64
I.scapularis       ------------SLIASKGYQVEEYEVTTSDGYILAVQRIPEGRSNALRIQDTPKKVVFL 48
D.melanogaster     PNIQEDSHLNTYSLIKKYGYPAENHTLETDDGYILTLHRIARPGATP---------VLL 110
C.quinquefasciatus ---AEDAHLSVPQLILKYGYGAEVHHATTEDGYILELHRIPKPGAPV---------VLL 83
P.vannamei         ----MDKWQKYGNLINRYRRNVNECTMEYGRIFSFSFYENKIVKAME-----------R 44

A.aegypti          QHGLIASSADWVILGPGKSLAHSLVTAGYDVWMGNFRGNTISRKHVSLDPAQPQFWDFSW 124
I.scapularis       LHGLLGSSADWVLNYPPQSLGFILADAGYDVWLGNVRGNTYSR-HVKYNRRSKEFWNFSV 107
D.melanogaster     VHGLLDSSATWVMMGPNKGIGYLLYDQGYDVWMANVRGNTYSRKHVKYSTHHAKFWDFTF 170
C.quinquefasciatus MHGLLCSSADWVSIGPGNGLAYLLADQGYDVWLGNARGNRYSRKHRTLTPKMFAFWQFSW 143
P.vannamei         RDRPGDRERQQTQRKATKALAYKLADAGYDVWLSNMRGNTYSRNHVELDPEDISFWQFSW 104

                                                GXSXG
A.aegypti          HEIGLYDLEAMIDYVLKKTGQKTLHYVGHSQGTTAFFVMASMKFEYNSKILSMQALAFIA 184
I.scapularis       DEMIERDLPETLDFVLKRTGRRKLFFVGHSQGTSIMFGLLSLRPEYSERIKLFCALGFVS 167
D.melanogaster     HEMGKHDISATMDYILNSTGVSQLHYIGHSQGTVVFWIMASEKPEYMDGIILMQGLAFVA 230
C.quinquefasciatus HEIGFYDLEASIDYVLEKTGRSKLHYIGHSQGTSFFFVMTSTRFEYNAKIALAQALAFVA 203
P.vannamei         DELAYYDVEASIDYVLGMTGAEAVYYAGWSMGTTVFWAMMSEKPFYNEKVRAMAAMAFVA 164
                                                   ▲

A.aegypti          FMGQMKSEFIRAIAPFSTQIEWTMRMLGVNELLFSHKMMIAGGQKAGEDTSTLQEVCVNV 244
I.scapularis       AITNTRSE-MRYMSPFGKYIGAFVNSLGRYEFLNNFVMKLLADAVGRHEG-PRDVCGNI 225
D.melanogaster     FLKHCRSEVVNFLAEWHLSVSLVLKLIGVHFFLFKNEFISMFNRIIGDETTITKEICSNV 290
C.quinquefasciatus FTENMQSELLRIMALFQDTLAALFETFGVAEFAFSNAILHDISKLLG-TTQISNNLCLNV 262
P.vannamei         FMNNAEGF-IMALAPYSDDLDFMATLLGVGEFLFSSDLLDHFVETYGDSEAVTAEVCYNF 223

A.aegypti          IFLIQGYDSAQLNRTLLETIVQHTPAGASVKQLAEYAKGINSGRERQFLHGVVGNVMNYG 304
I.scapularis       VFLIYGPETMELNVTRLFVFLCHVPAGTSVRTMVEYSKILISGREQKFLFGENRKQLVYG 285
D.melanogaster     IFLTTGFDKLQLNETMLFVIVGHSPACASTKQMQHFGKLNRSGGFRQYLHGWLRKHWIYG 350
C.quinquefasciatus LFQLAGANPDQVDLKLIFILMGHTPACASTKQIVFYAKGVRSGRERQYLHGTIKNRFVYG 322
P.vannamei         LLLLAGPDPDEIPKDFLFIILAHTPACASVHTVNEYAKLVMSGVFDKYLYGLIGNLNHYG 283

A.aegypti          SSTPPSYPLKRITAPVFLHYGDNLWLAAVSDVRLLYRQLGNGTRLLRVPEKQWNFLDFIY 364
I.scapularis       ASTPPEYDVSRVAVPVALFWSEGLWMADPRDVALLRRRLPNVVLDFKVSQPKFSHIDFAA 345
D.melanogaster     TIDPPSYHLENVRAKVALYYGQNLWLAPPELVEMCNRKLPNVVEKYLVDDKEFNELDFIW 410
C.quinquefasciatus TADPPVYNLTQVTAPVVFYYALNDYILAVPVDVERLSRGIGNLAGYRQVRMETFNELDFLF 382
P.vannamei         QNTPPLFNLSRVAAPVGLFWGSTDWLADPTDVARLAEGLPNLALNHKVDKEEFNELDFGW 343
                                           ▲                                 ▲

A.aegypti          ATGAKSLLYNRVMDLMNRYNNAMPEKYRMEE 395
I.scapularis       GIHAKALVYEPMMKLMASYKSAGE------- 369
D.melanogaster     GIDARELLWDRMLEIMRNHENSII------- 434
C.quinquefasciatus AKDVRTLLYEEILGNVRRYGG---------- 403
P.vannamei         GIHADELVYRHILDFFANY------------ 362
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Fig. 5 Multiple alignments of *pvl* with other lipase proteins. Lipases from *Culex quinquefasciatus* (GenBank accession no. XP_001844302), *Aedes aegypti* (GenBank accession no. XP_001662499), *Ixodes scapularis* (GenBank accession no. EEC17018), and *Drosophila melanogaster* (GenBank accession no. NP_609418). *Numbers* on the *right* indicate the amino acid position of different sequences. Identical amino acids are shaded in *black*. *Shaded triangle* indicates amino acid of catalytic triad: serine (*S*), aspartic acid (*D*), and histidine (*H*). The consensus sequence is represented by GXSXG

source of lipase that possesses a potential application in the oleochemical industry, since the kinetic values of PVL (Table 3) are in the range for most industrially relevant enzymes, $K_{m, \text{app}}$: between 10^{-1} and 10^{-5} M (Fullbrook 1996). Our data also showed that PVL may be used in the detergent industry, since it can hydrolyze TAG as efficiently as commercial lipases from bacteria *Mycobacterium tuberculosis* and fungi *Oryza sativa*, with $K_{m, \text{app}} =$ 7.57 and 6.71 mM, respectively (Deb et al. 2006; Bhardwaj et al. 2001).

Conclusions

The purification of a digestive lipase from midgut gland of the whiteleg shrimp *P. vannamei* and the full length of the

lipase cDNA are described for the first time. It contributes to understanding of lipase of invertebrates, since the biochemical properties differ from mammalian organisms. Also, the results show a new source of lipolytic enzymes with potential biotechnological applications.

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