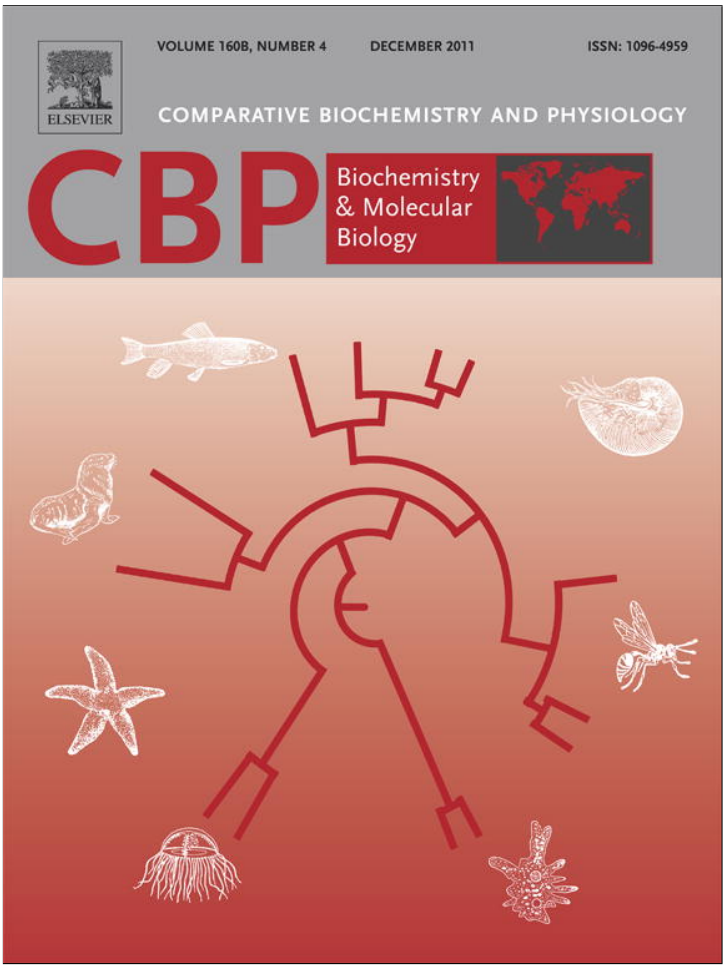

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Triacylglycerol catabolism in the prawn *Macrobrachium borellii* (Crustacea: Palaemoniade)

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ABSTRACT

While invertebrates store neutral lipids as their major energy source, little is known about triacylglycerol (TAG) class composition and their differential catabolism in aquatic arthropods. This study focuses on the composition of the main energy source and its catabolism by lipase from the midgut gland (hepatopancreas) of the crustacean *Macrobrachium borellii*. Silver-ion thin-layer chromatography of prawn large TAG deposit (80% of total lipids) and its subsequent fatty acid analysis by gas chromatography allowed the identification of 4 major fractions. These are composed of fatty acids of decreasing unsaturation and carbon chain length, the predominant being 18:1n-9. Fraction I, the most unsaturated one, contained mainly 20:5n-3; fraction II 18:2n-6; fraction III 18:1n-9 while the most saturated fraction contained mostly 16:0. Hepatopancreas main lipase (Mr 72 kDa) cross-reacted with polyclonal antibodies against insect lipase, was not dependent on the presence of Ca^{2+} and had an optimum activity at 40 °C and pH 8.0. Kinetic analysis showed a Michaelis–Menten behavior. A substrate competition assay evidenced lipase specificity following the order: 18:1n-9-TAG>PUFA-enriched-TAG>16:0-TAG different from that in vertebrates. These data indicate there is a reasonable correspondence between the fatty acid composition of TAG and the substrate specificity of lipase, which may be an important factor in determining which fatty acids are mobilized during lipolysis for oxidation in crustaceans.

1. Introduction

Lipids are major components in animals and play a vital role in cell structure and in the supply of energy for physiological processes. The high energy content of triacylglycerols (TAG) has made these compounds to be selected by most organisms as one of their main energy storage forms. In arthropods TAG support the energy needs for key physiological functions such as flight, metamorphosis and reproduction (Arrese and Soulages, 2010). Animals have therefore evolved complex enzymatic systems to utilize these lipids for the generation of energy. The first and key enzyme involved in TAG catabolism is lipase (triacylglycerol ester hydrolase, EC 3.1.1.3) that breaks down triacylglycerols into free fatty acids and glycerol. Due to its central role in TAG catabolism, it is not surprising that lipase activity has been found in species of all phyla studied (Berner and Hammond, 1970). However, among arthropods true lipases have only been purified and characterized in scorpion (Chelicerata) (Zouari et al., 2005), insects (Arrese and Wells, 1994; Arreguin-Espinosa et al., 2000; Ponnuvel et al., 2003) and in the midgut gland (hepatopancreas) of the crustaceans *Carcinus mediterraneus* (Cherif et al., 2007) and *Penaeus vannamei* (Rivera-Perez et al., 2011). Other studies in arthropods report only lipase activities (Vonk, 1960; Deering et al., 1996; González Baró et al., 2000; Lopez-Lopez et al., 2003; Auerswald et al., 2005; Lavarías et al., 2006), indicating an important lack of knowledge on this key enzyme of lipid metabolism in the largest animal Phylum.

Early studies on the assimilation of nutrients in crustaceans were limited to morphological and histological descriptions of their complex digestive systems. More recently, biochemistry and molecular biology studies have enlarged our knowledge about the processes of digestion and food utilization in these arthropods. As an outcome of these studies, crustacean hepatopancreas was recognized as possessing a very active lipid metabolism. This organ performs intestinal, hepatic, pancreatic and adipose tissue functions (Vogt, 1996) being somehow analogous to insect fat body (Gilbert and Chino, 1974; Arrese et al., 2001) or spider midgut diverticula (Laino et al., 2009). Crustacean lipid metabolism has probably been best studied in the decapod *Macrobrachium borellii* (Crustacea: Palaemonidae), thus this model has been used in this study. This endemic South American prawn has been characterized regarding its lipid and fatty acid composition. *M. borellii* hepatopancreas has been recognized as

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playing a central role in lipid metabolism, and a TAG lipase activity that changes with exposure to crude oil contaminants was identified (González Baró and Pollero, 1988, 1993, 1998; Heras et al., 2000; Lavarías et al., 2005, 2006, 2007, 2009).

While there are several studies in crustaceans reporting total lipid fatty acid composition (Ackefors et al., 1997; Ackman, 1989; Ackman and Eaton, 1967; Arts et al., 2001; Celik et al., 2004; Chen et al., 2007; Cherif et al., 2008; Napolitano and Ackman, 1989), to our knowledge there are no studies describing the fractionation and composition of arthropod TAG, the natural substrate for lipases.

This paper therefore focuses on the triacylglycerol catabolism in *M. borellii.* First, the fractionation and fatty acid composition of TAG, the major energy stores of hepatopancreas is reported, followed by the partial isolation, identification, kinetic and substrate specificity of the main triacylglycerol-lipase of hepatopancreas.

2. Materials and methods

2.1. Sample collection

Adults of *M. borellii* were collected in autumn from a watercourse of La Plata river estuary, Argentina (34°57′40″ S, 57°46′40″ O). They were taken to the laboratory and kept in dechlorinated running water at 22 ± 2 °C, 14:10 h L:D photoperiod for at least a week for acclimation. During acclimation, an artificial diet was fed daily "*ad libitum*" as pellets, formulated according to Collins and Petriella (1999). The animals were starved for 48 h before experiments, a standard practice in our laboratory.

2.2. Analysis of prawn triacylglycerols by silver-ion-thin-layer chromatography

2.2.1. Lipid extraction and TAG fraction preparation

Groups of adult prawns (n = 15) weighing 0.64–3.04 g (40–67 mm) were anesthetized on ice and hepatopancreas dissected. Tissues were homogenized and total lipids were extracted with a chloroform/methanol mixture following the method of Bligh and Dyer (1959) and quantified by gravimetry.

Lipids were then separated into their classes by thin-layer chromatography (TLC) on Silicagel G using hexane/diethyl ether/acetic acid (80:20:1.5, by vol). The plate was sprayed with 0.01% rhodamine and the TAG spot visualized under UV light, was scraped and extracted with chloroform:hexane (1:1, by vol). TAG were identified by comparing their R_f with those from standards run on the same plate.

2.2.2. Silver-ion HPTLC

Silver-ion HPTLC has been widely used in the fractionation of triacylglycerols according to their degree of unsaturation (Bottino, 1971; Hamilton and Hamilton, 1992; Christie and Han, 2010). The property of silver ions to form complexes of varying stability with the double bonds of fatty acids was employed in silver nitrate (argentation) chromatography to separate TAG into their different fractions. High performance thin-layer chromatography (HPTLC) plates impregnated with silver nitrate ($AgNO_3$) 15% in acetonitrile were developed with chloroform/methanol (97:3, by vol) as a mobile phase. After development, the plates were sprayed with 2′,7′-dichlorofluorscein and the TAG fractions were visualized under UV light, marked and scraped-off the plates. TAG elution and clean-up was performed following the procedure of Hamilton and Hamilton (1992).

2.2.3. Fatty acid composition of TAG classes

Fatty acids methyl esters (FAME) from TAG were prepared using a base-catalyzed transesterification microscale procedure with sodium methoxide in methanol (Christie, 1982). FAME were analyzed by gas–liquid chromatography (GC) in a HP-6890 capillary GC (Hewlett Packard, Palo Alto, CA, USA), fitted with an Omegawax 250 fused silica column, 30 m × 0.25 mm, with 0.25 μm phase (Supelco, Bellefonte, CA, USA) equipped with a flame ionization detector (FID). The column temperature was programmed for a linear increase of 3 °C/min from 175 to 230 °C. Fatty acids were identified by comparing their characteristic retention times with those from a mixture of standard methyl esters run under the same conditions.

2.3. Partial isolation of TAG lipase

Hepatopancreas from groups of 12 adults (45–67 mm, approx. 0.80–2.0 g) were obtained. Briefly, ice-cold-anesthetized adults were dissected, weighed, immediately cooled on ice and homogenized in a buffer (0.25 M sucrose containing 0.15 M KCl, 62 mM K-phosphate buffer, pH 7.4, 2.5 mM $MgCl_2$ and 1.4 mM N-acetyl-L-cysteine, containing a protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA)) as previously described (Lavarías et al., 2007). The ratio of buffer:sample was 6:1 v/w. The crude homogenate was first centrifuged at 1400 g for 10 min in a Sorvall RC-2 (Newtown, CT, USA) to remove cellular debris and nuclear fraction. The supernatant was filtered and centrifuged at 10,000 g for 20 min to remove mitochondrial fraction. The supernatant was filtered and centrifuged at 100,000 g for 50 min in a Beckman L8M ultracentrifuge (Beckman, Palo Alto, CA, USA) to obtain the cytosolic fraction. All centrifugations were done at 4 °C. The 100,000 g supernatant was then subjected to ammonium sulfate precipitation by adding a saturated ammonium sulfate solution to a final concentration of 45% v/v. After 90 min on ice, sample was centrifuged for 30 min at 13,000 g at 4 °C. The pellet was resuspended in 0.1 M phosphate buffer, pH 8.0. Total protein from all purification steps was quantified and stored at −20 °C or immediately assayed. The purification steps were followed by both zymographic and radiometric assays, as described below.

Protein concentration was determined according to Bradford (1976) with bovine albumin serum as standard.

2.4. Cross-reactivity between insect and prawn anti-lipase antibodies

To immunodetect lipase in *M. borellii* hepatopancreas cytosolic fraction, a chicken polyclonal anti-serum kindly provided by Dr. Arrese's laboratory was used. Anti-serum was raised against *Manduca sexta* purified fat body lipase (Arrese et al., 2006).

Proteins were transferred from SDS PAGE gels onto nitrocellulose membranes (Amersham) in a Mini Trans-blot Cell (Bio Rad Laboratories, Inc.), using 25 mM Tris–HCl, 192 mM glycine, 20% (v/v) methanol, pH = 8.3 buffer. After blocking for 90 min with 3% (w/v) non fat dry milk in PBS-Tween, the membranes were incubated overnight at 4 °C with the anti-sera dilutions in 3% (w/v) non fat dry milk in PBS-Tween. Specific antigens were detected by chicken anti-rabbit IgG horseradish peroxidase conjugate (Bio Rad). Immunoreactivity was visualized by electro-chemi-luminiscence.

2.5. Lipase assay methods

The following assay methods were employed to identify and characterize lipase activity.

2.5.1. Zymographic assay

A zymographic method based on direct fluorescence was routinely used for lipase activity estimation throughout the purification steps (Diaz et al., 1999). First, proteins were analyzed by PAGE in a 4–20% gradient polyacrylamide gels under native conditions, according to the method of Laemmli (1970). Gels were then briefly washed (1 min.) in 50 mM phosphate buffer pH 7.0 and covered by a solution of 100 μM 4-methylumbelliferone-butyrate (MUF-butyrate) in the same buffer. Lipase activity was visualized as fluorescent bands under UV illumination. Immediately after zymographic analysis the gel was stained with Coomassie blue.

2.5.2. Colorimetric assay

The pH and temperature optimization of lipase activity was performed using a colorimetric assay. The procedure used was a modification of that applied by Versaw et al. (1989), using β-naphthyl caprate as substrate. The assay mixture contained: 30 μL of 100 mM sodium taurocholate, 570 μL of 50 mM Tris–HCl, 6 μL (15 μg) of lipase fraction and 6 μL of 200 mM β-naphthyl caprate in dimethyl sulfoxide (DMSO), at 2 mM final concentration. The mixture was incubated for 30 min, and 6 μL of 100 mM fast blue BB (in DMSO) was added. DMSO final concentration was less than 2% v/v. The reaction was stopped with 60 μL of 0.72 N trichloroacetic acid (TCA), the final volume was adjusted to 1.5 mL with ethanol/ethyl acetate 1:1 v/v and the colored product read at 540 nm in a plate reader. Blanks were prepared by substituting sample by Tris–HCl buffer. Temperatures were assayed between 10 and 60 °C and pH between 5.0 and 10.0 using 100 mM phosphate buffers.

The kinetic parameters of the enzyme were assayed using *p*-nitrophenylpalmitate (pNPP) (C16) as substrate following the hydrolysis of pNPP by lipase fraction. The released yellow p-nitrophenol was then measured at 410 nm in a spectrophotometer Agilent 8453 (PA, USA). A reaction mixture (150 μL) containing 0.25 to 4 mM of p-NPP (in isopropanol), 50 mM Tris–HCl, pH 8.0, 1% Triton X-100 and 17 μg of lipase fraction was incubated at 37 °C. Measurements were performed at 37 °C during 5 min and the absorbance in each assay was measured against a substrate–buffer mixture.

2.5.3. Radiometric assay

The radiometric assay of Arrese and Wells (1994) was used to measure lipase activity. Briefly, a mixture of [carboxyl-^{14}C] triolein (labeled at the carboxyl carbon of the oleic acid esterified to each of the three carbons of glycerol) (0.18 μCi, 3.3 nmol); unlabeled triolein (2.8 nmol) was used as substrate in a final volume of 500 μL. In order to form a micellar triolein substrate Triton X-100 (final concentration 0.05%) was added. After addition of 0.3 mL of reaction mixture (40 mM Tris–HCl buffer, pH 8.0, containing 0.5 M NaCl and 0.1% (w/v) delipidated bovine serum albumin) substrate was dispersed by vortexing for 1 min and then sonicated. The reaction was initiated by adding 40 μg protein/mL of reaction mixture and proceeded at 37 °C for 1 h. The reaction was stopped by addition of 2.5 mL of an extraction mixture consisting of chloroform–methanol–benzene 2:2.4:1 (v/v/v) containing 0.5 μmol of unlabeled oleic acid as a carrier. Then 100 μL of 1 N NaOH was added, the mixture was vortexed for 20 s, and then centrifuged at 2000 g for 5 min at room temperature. Aliquots (150 μL) of the upper aqueous phase were transferred to scintillation vials for counting. Blank reactions did not contain enzyme.

In the case of evaluating calcium dependence, $CaCl_2$ was added at a final concentration 1.5 mM, and activity determined using labeled triolein as described above.

A radiometric assay was also used to evaluate lipase substrate specificity against either different lipid classes or different TAG. The specificity of the enzyme towards different molecular species of TAG was inferred from the competition of non-radiolabeled substrates on the rate of hydrolysis of radiolabeled triolein (substrates with higher affinity than triolein are expected to reduce the amount of radioactivity recovered in the assay). Mixtures containing a fixed amount of 3.3 nmol (0.18 μCi) [^{14}C]triolein and 2.8 nmol of the competing unlabeled substrate, namely tripalmitin (807.2 MW), and prawn hepatopancreas fraction I TAG (901.4 avg MW) (a TAG polyunsaturated-enriched fraction purified from the prawn hepatopancreas, see below). Unlabeled triolein (885 MW) was employed as control. The assay was performed as described above.

In another set of experiments, a mixture of [carboxyl-^{14}C] cholesteryl-palmitate (0.18 μCi, 3 nmol) and unlabeled cholesteryl-palmitate (3.1 nmol) was assayed against triolein as control. Mixtures were assayed at a final concentration of 6.1 nmol and the assay was performed as described above in a final volume of 0.5 mL.

2.6. Statistical analyses

Data collected from all experiments were analyzed by either *t* test or ANOVA using Instat v.3.05 (Graphpad Software Inc.). Where significant differences between samples occurred, a post-hoc Tukey's HSD test was performed to identify the differing means. Results were considered significant at the 5% level.

3. Results

3.1. Composition of hepatopancreas triacylglycerols

The analysis of prawn hepatopancreas TAG with silver-ion HPTLC allowed the separation of 4 TAG fractions hereafter named fractions I–IV according to their increasing R*f* (Table 1).

Table 1
Major fatty acids of triacylglycerol fractions of prawn hepatopancreas.

Fatty acid	Total (100)	Fraction I (19.1 ± 0.4)	Fraction II (30.3 ± 3.1)	Fraction III (14.3 ± 0.8)	Fraction IV (36.3 ± 2.8)
14:0	4.5	1.9 ^{a}	2.3^{b}	2.9 ^{b}	7.7 ^{c}
16:0	16.8	9.1 ^{a}	9.4 ^{a}	12.2 ^{b}	28.0 ^{c}
18:0	5.5	4.0 ^{a}	4.1 ^{a}	4.6 ^{a}	7.7 ^{b}
16:1 n-7	10.7	8.1 ^{a}	10.2 ^{a,b}	13.4 ^{c}	12.1 ^{b,c}
18:1 n-7	9.4	7.1 ^{a}	10.7 ^{b}	17.2 ^{c}	14.9 ^{c}
18:1 n-9	23.2	14.4 ^{a}	18.4 ^{b}	27.4 ^{c}	22.3 ^{c}
18:2 n-6	11.0	12.7 ^{a}	19.8 ^{b}	9.4 ^{c}	ND
20:2 n-6	1.5	1.3 ^{a}	2.6 ^{b}	1.3 ^{a}	ND
18:3 n-3	2.9	6.6 ^{a}	10.0 ^{b}	1.3 ^{c}	ND
20:4 n-6	2.3	7.4 ^{a}	6.7 ^{a}	ND	ND
20:5 n-3	3.5	16.2 ^{a}	1.9 ^{b}	0.6 ^{b}	0.5 ^{b}
22:6 n-3	0.4	1.8	ND	ND	ND
Others*	6.7	5.4 ^{a}	4.2 ^{b}	5.0 ^{a}	4.0 ^{b}
Unidentified	1.6.	4.0 ^{a}	0.7 ^{b}	4.7 ^{c}	2.8 ^{a}
Σ Saturated	28.7	16.0 ^{a}	16.7 ^{a}	20.7 ^{b}	46.1 ^{c}
Σ Monounsaturated	44.6	30.5 ^{a}	40.1 ^{b}	58.8 ^{c}	50.5 ^{c}
Σ Polyunsaturated	25.3	47.8 ^{a}	43.3 ^{a}	14.8 ^{b}	0.5 ^{c}
Σ n-6	14.8	21.4 ^{a}	30.0 ^{b}	10.8 ^{c}	ND
Σ n-3	8.2	25.5 ^{a}	11.9 ^{b}	1.8 ^{c}	0.5 ^{d}

Values between brackets represent the relative mass % of each TAG fraction as mean ± SD; ND: Not detected. FA values are the mean of triplicate independent analyses expressed as % w/w; Differences of FA among fractions ($p<0.05$) are indicated by different letters. SD has been omitted for clarity but never exceeded 10% of the mean.

* Amount of all other minor fatty acids.

Fatty acid composition of each TAG fraction was analyzed by capillary GC. The relative proportions of fatty acids found in total TAG as well as in each TAG fraction are given in Table 1. The fatty acid profiles were significantly different among TAG fractions.

On the whole, the fatty acid profile of prawn hepatopancreas TAG was dominated by monounsaturated fatty acids (45.2%) oleic acid (18:1 n-9) being the dominant one (23.2%). Total TAG saturated fatty acids accounted for a 28.9%, and were dominated by palmitic acid (16:0) and stearic acids (18:0). Regarding TAG total polyunsaturated fatty acid (PUFA) it amounts to 25.3% linolenic acid (18:2 n-6) being the major one. The n-3 fatty acid family accounted for a 29.2% of PUFA, dominated by eicosapentaenoic acid (EPA) (20:5 n-3) (Table 1).

The general tendency of TAG fractions in TLC plates was to decrease unsaturation and carbon chain length along with an increase of R_f or fraction. Thus, 20:5 n-3 PUFA prevailed in fraction I followed by 18-carbon PUFA in fraction II. In fraction III predominated monounsaturated 18-carbon fatty acid (18:1n-7 and 18:1n-9), and fraction IV (the PUFA-poorest fraction) had high amounts of both monounsaturated and saturated fatty acid (50.5% and 46.1%, respectively) and was dominated by 16:0 and 18:1n-9.

The n-6 family was well represented in TAG, particularly in fractions I and II, while it represents only 12% in fraction III and was barely present in fraction IV. The n-3 family displayed a decreasing pattern along with the TAG fraction from its highest value in fraction I (25.5%) to less than 2% in fraction IV (Table 1).

3.2. Identification of the main hepatopancreas TAG lipase

Lipase activity was determined along the enrichment steps, namely the 10,000 g supernatant, the 110,000 g supernatant (cytosolic fraction) and the "lipase fraction" (the resuspended pellet from the 45% ammonium sulfate precipitation) (Fig. 1). Zymographic analysis showed several positive bands in the 10,000 g supernatant and the cytosol, while a single band was obtained in the resuspended pellet after 45% ammonium sulfate precipitation (hereafter named lipase) (Fig. 1A). This band has a relative mass of 72 kDa (Fig. 1C) and cross-reacted with a polyclonal antibody directed against insect fat body TAG lipase (Fig. 1D). To confirm that this esterase activity was due to a true TAG lipase, activity was probed radiometrically measuring ^{14}C-triolein hydrolysis. It is worth recalling that TAG containing fatty acids of >16C are not hydrolyzed by esterases other than TAG lipases (Bornscheuer et al., 2002). Results showed that lipase specific activity towards triolein in the purified fraction was more than twice the cytosolic one, confirming the presence of a true lipase (Fig. 1B).

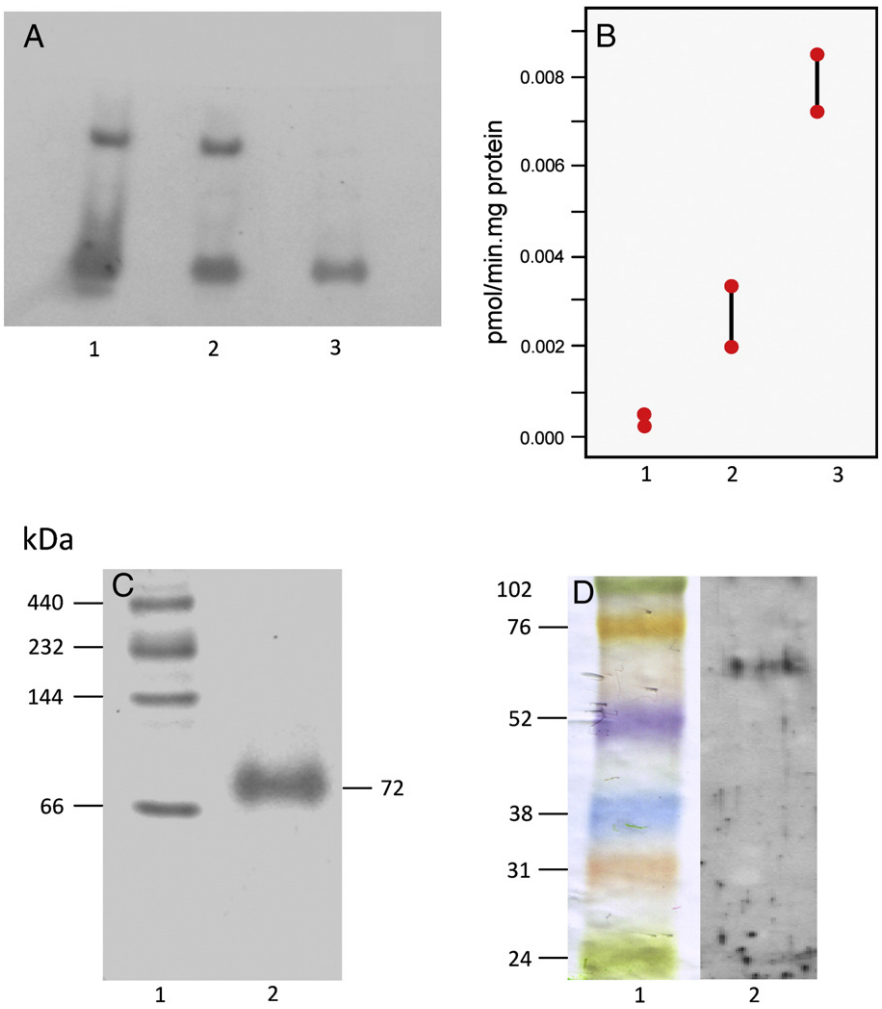

Fig. 1. Identification of lipase activity in hepatopancreas of *M. borellii*. Panel A: Native PAGE zymogram using MUF-butyrate as substrate, lane 1: 10,000 g supernatant; lane 2: 110,000 g supernatant (cytosolic fraction); lane 3: lipase fraction. Panel B: Purification of lipase followed by the release of oleic acid using ^{14}C triolein as substrate, 1: 10,000 g supernatant, 2: cytosolic fraction, 3: lipase fraction. Error bars represent the range of determinations. Panel C: Native PAGE electrophoresis zymogram of purified lipase. Lane 1: Molecular weight marker (Pharmacia), lane 2: lipase fraction. Panel D: Western blot analysis of purified lipase. Lane 1: Rainbow molecular weight marker (GE Healthcare), lane 2: lipase fraction. Nitrocellulose membrane was probed with an anti-insect lipase polyclonal antibody.

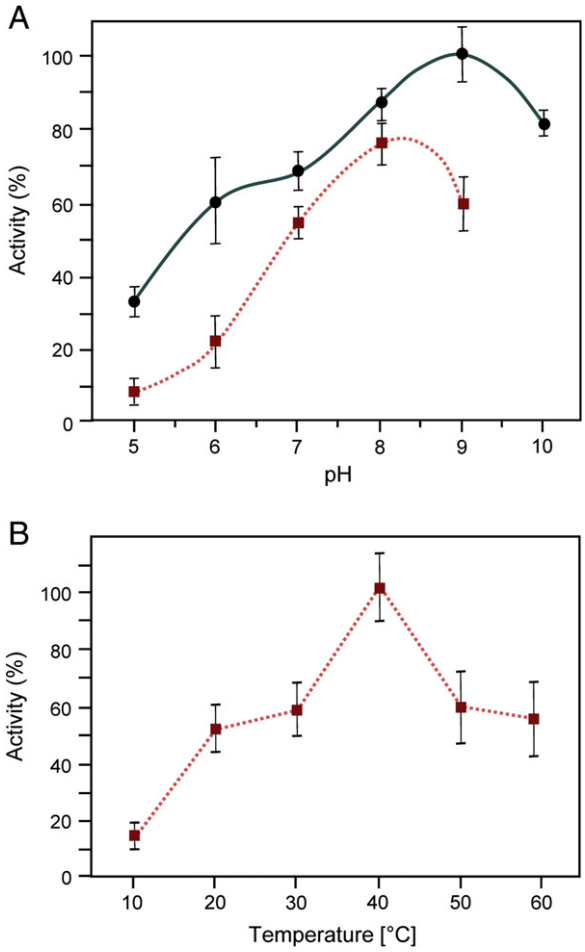

Fig. 2. Effect of pH (panel A) and temperature (panel B) on lipase activity using β-naphthyl caprate as substrate. Activity was normalized to 100% for maximum activity recorded. (—) 110,000 g supernatant (▪▪▪) lipase fraction. Values represent the average of three experiments ± SD.

3.3. Lipase optimum pH, temperature and kinetic characterization

Lipase optimal pH was 8.0 for lipase fraction and biphasic (pH 6 and 9) for cytosolic fraction (Fig. 2A) indicating the presence of more than one β-naphthyl caprate hydrolytic activity in the 110,000 g supernatant. Optimal temperature was between 35 and 40 °C (Fig. 2B). Based upon these results, the assay conditions selected for subsequent assays were 38 °C and pH 8.0. Lipase kinetics using pNPP, a substrate containing a long-chain fatty acid (16:0), showed a Michaelis–Menten behavior with a Vmax of 0.384 ± 0.060 mmol.min^{-1} and a *Kmapp* of 1.40 ± 0.46 mM (Fig. 3). *M. borellii* lipase does not require calcium for activity (0.330 ± 0.025 and 0.276 ± 0.026 mmol.min^{-1}) with and without Ca, respectively (n = 3, *p* 0.176).

3.4. Substrate specificity

A substrate competition assay evaluated different rates of oleic acid release from radiolabeled triolein in the presence of non-radiolabeled cosubstrates. The addition of tripalmitin (16:0-TAG) as cosubstrate significantly stimulated the rate of triolein hydrolysis by 1.8 ± 0.17 (n = 3) fold as compared with those observed with triolein as the sole substrate, indicating that 16:0-TAG is a poorer substrate. Likewise, rates of radiolabeled triolein hydrolysis were also significantly stimulated by the competition of triolein with PUFA-enriched TAG fraction I (see Table 1), though the rate of lipolysis was less than when competition was performed using the saturated TAG species (1.3 ± 0.1-fold), indicating a higher specificity towards this TAG mixture than to 16:0-TAG.

In another set of experiments, the capacity to hydrolyze cholesteryl esters was investigated. Though lipase was found to be capable of hydrolyzing such kind of compounds, its hydrolysis rate was several orders of magnitude lower than towards TAG ($0.286 \times 10^{-3} \pm 0.031 \times 10^{-3}$ pmol/min.mg protein; n = 3).

4. Discussion

Crustacean bioenergetics relies mainly on the use of TAG as energy source (Harrison, 1990). In this regard, the present results showed that 18C monounsaturated fatty acid containing TAG fractions particularly containing oleic acid are the most abundant. Considering the relative abundance of oleic acid in TAG hepatopancreas, lipase competition studies were performed to determine if there was specificity toward a particular fraction of TAG. The enzyme was able to hydrolyze a variety of TAG containing long-chain fatty acids regardless of their unsaturation, in agreement with reports on *P. vannamei* lipase (Rivera-Perez et al., 2011). Competition between a totally saturated molecular species of TAG and triolein clearly evidenced that at least tripalmitin TAG is a poor substrate for prawn lipase. On the other hand competition between triolein and a natural mixture of TAG species enriched in PUFA purified from hepatopancreas, significantly diminished the rate of labeled oleate release from triolein, but not as much as a competition using non-labeled triolein TAG as cosubstrate. Therefore, above data support the following rank of preference for the hydrolysis of molecular species of prawn TAG: 18:1n-9-TAG > PUFA-enriched-TAG > 16:0-TAG. *P. vannamei* lipase also showed more specificity towards 18:1n-9-TAG > than towards 16:0-TAG, but PUFA-TAG

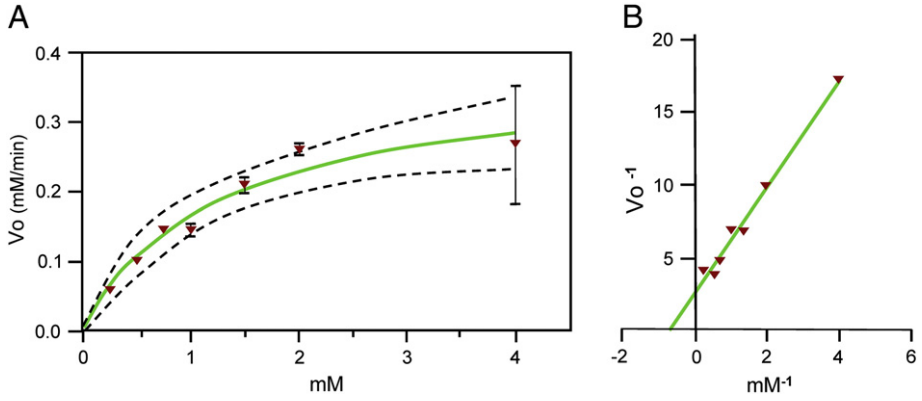

Fig. 3. Kinetic characterization of TAG lipase from hepatopancreas of *M. borellii* using pNPP (C16:0) as substrate. Panel A: Effect of substrate concentration. Values represent the mean of tripliclate analysis ± SD. Dotted lines (envelope) represents 95% confidence intervals. Vo: Initial velocity. Panel B: Lineweaver–Burk plot of the data depicted in A. $R^2 = 0.96$.

were not tested in that report (Rivera-Perez et al., 2011). There are no other data available on the specificity of other invertebrate lipases for comparison, but the specificity of *M. borellii* lipase contrasts with those reported for fish and mammals, where saturated fatty acids, along with oleic acid, were much less mobilized than the entire fraction of PUFA-containing TAG (Connor et al., 1996; Hazel and Sidell, 2004). Our data would indicate that prawn lipase release fatty acids from TAG in a fashion proportional to their content of oleic acid.

The TAG composition dominated by oleic acid-containing TAG may explain the fact that *M. borellii* lipase hydrolyzes preferentially monoenoic containing TAG, which in term would provide an important advantage for lipid mobilization in these arthropods generating adequate amounts of this vital fatty acid for β-oxidation. Prawn lipase was also capable of hydrolyzing cholesteryl esters. Though its hydrolysis rate was very low, still contrasts with *M. sexta* which lacks this capability. (Arrese and Wells, 1994). As *M. sextas* is the only other arthropod true lipase tested using this lipid as substrate no other comparisons are possible.

M. borellii hepatopancreas main TAG lipase activity was localized in the cytosol, like the principal fat body lipase of *M. sexta* moth (Arrese and Wells, 1994). Moreover, an antibody raised against this *M. sexta* lipase cross-reacted with prawn lipase-positive band, probably indicating that both proteins may share domain homology, sharing both lipases and also with a similar mass of around 72–76 kDa (Arrese and Wells, 1994). This MW is higher than those of other crustacean lipases (Brockerhoff et al., 1970; Cherif et al., 2007; Rivera-Perez et al., 2011).

M. borellii lipase optimum temperature is similar to that of the prawn *P. vannamei* though lower than that of the crab *C. mediterraneus* which holds the remarkable optimum temperature at 60 °C. *M. borellii* lipase alkaline pH optimum is similar to *P. vannamei* and *C. maditerraneus* lipases (Cherif et al., 2007; Rivera-Perez et al., 2011) and the gastric juice lipase of *Homarus americanus* (Brockerhoff et al., 1970). Moreover, considering that an optimum pH>7.0 was also found for lipases in other arthropod (Arrese and Wells, 1994; Zouari et al., 2007), data supports the idea that arthropod lipases can be considered of the alkaline type, regardless of the taxa considered.

Unlike vertebrate lipases, *P. vannamei* and *M. borellii* lipases do not need calcium as a cofactor for activity. This feature is also shared by the insect lipases of *Rhodnius prolixus* (Grillo et al., 2007) and *C. presignis* (Arreguin-Espinosa et al., 2000). This would suggest that arthropod and vertebrate lipases are dissimilar. However, more work on other lipases from this large Phylum is needed to validate this hypothesis.

The kinetics of *M. borellii* lipase displayed a Michaelis–Menten behavior, like the ones from the scorpion *Scorpio maurus* and *P. vannamei* (Zouari et al., 2005; Rivera-Perez et al., 2011). Its kinetic properties indicate a good capacity for TAG hydrolysis, which is important considering the large stores of TAG in hepatopancreas (more than 80% of total lipid mass). Although lipases have not been reported in other tissues of *M. borellii*, considering that oleic acid is also the most abundant fatty acid in female gonads, whose lipids are dominated by TAG (González Baró and Pollero, 1988), we could suggest that if gonad lipase selectivity is similar to that of hepatopancreas, this fatty acid would be the main energy supply during vitellogenesis.

5. Conclusions

In summary, this work provides evidence about the mechanism through which fatty acids are released from TAG in crustaceans, indicating that prawn lipase releases fatty acids from a TAG emulsion in a nonrandom fashion. 18:1n-9-TAG are preferentially released, followed by PUFA-enriched-TAG; saturated species of TAG are hydrolyzed more slowly. This substrate specificity displays differences with vertebrates. In addition, these results indicate that lipase is a potentially important determinant of the specific fatty acids released from the storage depots of crustaceans and that there is a reasonable correspondence between the fatty acid composition of TAG and those fatty acid preferentially released by lipolysis. More work on arthropod lipases is needed to better understand their properties, evolution, and make further generalizations and comparisons.

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References

Ackefors, H., Castell, J.D., Orde-Ostrom, L., 1997. Preliminary results on the fatty acid composition of freshwater crayfish, *Astacus astacus* and *Pacifastacus leniusculus*, held in captivity. J. World Aquacult. Soc. 28, 97–105.

Ackman, R.G., 1989. Nutritional composition of fats in seafoods. Progr. Food Nutr. Sci. 13, 161–241.

Ackman, R.G., Eaton, C.A., 1967. Fatty acid composition of the decapod Shrimp, *Pandalus borealis*, in relation to that of the euphasid. *Meganyctiphanes norvegica*. J. Fish. Res. Board Can. 24, 467–471.

Arreguin-Espinosa, R., Arreguin, B., Gonzalez, C., 2000. Purification and properties of a lipase from *Cephaloleia presignis* (Coleoptera, chrysomelidae). Biotechnol. Appl. Biochem. 31 (Pt 3), 239–244.

Arrese, E.L., Soulages, J.L., 2010. Insect fat body: energy, metabolism, and regulation. Annu. Rev. Entomol. 55, 207–225.

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.

Arrese, E.L., Canavoso, L.E., Jouni, Z.E., Pennington, J.E., Tsuchida, K., Wells, M.A., 2001. Lipid storage and mobilization in insects: current status and future directions. Insect Biochem. Mol. Biol. 31, 7–17.

Arrese, E.L., Patel, R.T., Soulages, J.L., 2006. The main triglyceride-lipase from the insect fat body is an active phospholipase A(1): identification and characterization. J. Lipid Res. 47, 2656–2667.

Arts, M.T., Ackman, R.G., Holub, B.J., 2001. "Essential fatty acids" in aquatic ecosystems: a crucial link between diet and human health and evolution. Can. J. Fish. Aquat. Sci. 58, 122–137.

Auerswald, L., Siegert, K.J., Gade, G., 2005. Activation of triacylglycerol lipase in the fat body of a beetle by adipokinetic hormone. Insect Biochem. Mol. Biol. 35, 461–470.

Berner, D.L., Hammond, E.G., 1970. Phylogeny of lipase specificity. Lipids 5, 558–562.

Bligh, E.G., Dyer, W.J., 1959. A rapid method of total lipid extraction and purification. Can. J. Biochem. Physiol. 37, 911–917.

Bornscheuer, U.T., Bessler, C., Srinivas, R., Krishna, S.H., 2002. Optimizing lipases and related enzymes for efficient application. Trends Biotechnol. 20, 433–437.

Bottino, N.R., 1971. The composition of marine-oil triglycerides as determined by silver ion-thin-layer chromatography. J. Lipid Res. 12, 24–30.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. Anal. Biochem. 72, 248–274.

Brockerhoff, H., Hoyle, R.J., Hwang, P.C., 1970. Digestive enzymes of the American lobster (*Homarus americanus*). J. Fish. Res. Board Can. 27, 1357–1370.

Celik, M., Tureli, C., Celik, M., Yanar, Y., Erdem, U., Kucukgulmez, A., 2004. Fatty acid composition of the blue crab (*Callinectes sapidus* Rathbun, 1896) in the north eastern Mediterranean. Food Chem. 88, 271–273.

Chen, D.W., Zhang, M., Shrestha, S., 2007. Compositional characteristics and nutritional quality of Chinese mitten crab (*Eriocheir sinensis*). Food Chem. 103, 1343–1349.

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.

Cherif, S., Frikha, F., Gargouri, Y., Miled, N., 2008. Fatty acid composition of green crab (*Carcinus mediterraneus*) from the Tunisian Mediterranean coasts. Food Chem. 111, 930–933.

Christie, W.W., 1982. A simple procedure for rapid transmethylation of glycerolipids and cholesteryl esters. J. Lipid Res. 23, 1072–1075.

Christie, W.W., Han, X., 2010. Chp. 10. Molecular species of triacylglycerols, diacylglycerols derived from complex lipids, and related lipids. in: Christie, W.W., Han, X. (Eds.), Lipid analysis. Isolation, separation, identification and lipidomic analysis. The oily press. Barnes & Associates, Cornwall, pp. 215–244.

Collins, P.A., Petriella, A., 1999. Growth pattern of isolated prawns of *Macrobrachium borellii* (Crustacea, Decapoda, Palaemonidae). Invertebr. Reprod. Dev. 36, 1–3.

Connor, W.E., Lin, D.S., Colvis, C., 1996. Differential mobilization of fatty acids from adipose tissue. J. Lipid Res. 37, 290–298.

Deering, M.J., Hewitt, D.R., Brock, I., 1996. Triacylglycerol digestion by the leader prawn *Penaeus monodon*. J. World Aquacult. Soc. 27, 359–361.

Diaz, P., Prim, N., Javier Pastor, F.I., 1999. Direct fluorescence-based lipase activity assay. Biotechniques 27 696–8, 700.
Gilbert, L.I., Chino, H., 1974. Transport of lipids in insects. J. Lipid Res. 15, 439–456.
González Baró, M.R., Pollero, R.J., 1988. Lipid characterization and distribution among tissues of the freshwater crustacean *Macrobrachium borellii* during an annual cycle. Comp. Biochem. Physiol. B 91, 711–715.
González Baró, M.R., Pollero, R.J., 1993. Palmitic acid metabolism in hepatopancreas of the freshwater crustacean *Macrobrachium borellii*. Comp. Biochem. Physiol. B 106, 71–75.
González Baró, M.R., Pollero, R.J., 1998. Fatty acid metabolism of *Macrobrachium borellii*: dietary origin of arachidonic and eicosapentaenoic acids. Comp. Biochem. Physiol. A 119, 747–752.
González Baró, M.R., Heras, H., Pollero, R.J., 2000. Enzyme activities involved in lipid metabolism during embryonic development of *Macrobrachium borellii*. J. Exp. Zool. 286, 231–237.
Grillo, L.A., Majerowicz, 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.
Hamilton, R.J., Hamilton, S., 1992. Thin layer chromatography. in: Hamilton, R.J., Hamilton, S. (Eds.), Lipid analysis. A practical approach. IRL Press, Oxford University Press, Oxford, UK, pp. 65–111.
Harrison, K.E., 1990. The role of nutrition in maturation, reproduction and embryonic development of decapod crustaceans: a review. J. Shellfish Res. 9, 1–28.
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.
Heras, H., González Baró, M.R., Pollero, R.J., 2000. Lipid and fatty acid composition and energy partitioning during embryo development in the shrimp *Macrobrachium borellii*. Lipids 35, 645–651.
Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680–685.
Laino, A., Cunningham, M.L., Garcia, F., Heras, H., 2009. First insight into the lipid uptake, storage and mobilization in arachnids: role of midgut diverticula and lipoproteins. J. Insect Physiol. 55, 1118–1124.
Lavarías, S., Dreon, M.S., Pollero, R.J., Heras, H., 2005. Changes in phosphatidylcholine molecular species in the shrimp *Macrobrachium borellii* in response to a water-soluble fraction of petroleum. Lipids 40, 487–494.
Lavarías, S., Pollero, R.J., Heras, H., 2006. Activation of lipid catabolism by the water-soluble fraction of petroleum in the crustacean *Macrobrachium borellii*. Aquat. Toxicol. 77, 190–196.
Lavarías, S., García, F., Pollero, R.J., Heras, H., 2007. Effect of the water-soluble fraction of petroleum on microsomal lipid metabolism of *Macrobrachium borellii* (Arthropoda: Crustacea). Aquat. Toxicol. 82, 265–271.
Lavarías, S., Pasquevich, M.Y., Dreon, M.S., Heras, H., 2009. Partial characterization of a malonyl-CoA-sensitive carnitine O-palmitoyltransferase I from *Macrobrachium borellii* (Crustacea: Palaemonidae). Comp. Biochem. Physiol. B 152, 369.
Lopez-Lopez, S., Nolasco, H., Vega-Villasante, F., 2003. Characterization of digestive gland esterase-lipase activity of juvenile redclaw crayfish *Cherax quadricarinatus*. Comp. Biochem. Physiol. B 135, 337–347.
Napolitano, G.E., Ackman, R.G., 1989. Lipids and hydrocarbons in *Corophium volutator* from Minas Basin, Nova Scotia. Mar. Biol. 100, 333–338.
Ponnuvel, K.M., Nakazawa, H., Furukawa, S., Asaoka, A., Ishibashi, J., Tanaka, H., Yamakawa, M., 2003. A lipase isolated from the silkworm *Bombyx mori* shows antiviral activity against nucleopolyhedrovirus. J. Virol. 77, 10725–10729.
Rivera-Perez, C., Garcia-Carreno, F.L., Saborowski, R., 2011. Purification and biochemical characterization of digestive lipase in Whiteleg shrimp. Mar. Biotechnol. (NY) 13, 284–295.
Versaw, W.K., Cuppeit, S.L., Winters, D.D., Williams, L.E., 1989. An improved colorimetric assay for bacterial lipase in nonfat dry milk. J. Food Sci. 54, 1557–1568.
Vogt, G., 1996. Morphology and physiology of digestive epithelia in Decapod crustaceans. Pflugers Arch. 431, 239–240.
Vonk, H.J., 1960. Digestion and metabolism. In: Smith, R.I., Waterman, T. (Eds.), The Physiology of Crustacea. Academic Press, New York, pp. 291–316.
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.
Zouari, N., Miled, N., Rouis, S., Gargouri, Y., 2007. Scorpion digestive lipase: a member of a new invertebrate's lipase group presenting novel characteristics. Biochimie 89, 403–409.