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Snail Hepatopancreatic Lipase: A New Member of Invertebrates Lipases' Group

Sawsan Amara · Ahmed Fendri · Nadia Ben Salem · Youssef Gargouri · Nabil Miled

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Abstract Higher animal's lipases are well characterized; however, much less is known about lipases from mollusks. A lipolytic activity was located in the land snail (*Eobania vermiculata*) digestive glands (hepatopancreas), from which a snail digestive lipase (SnDL) was purified. Pure SnDL has a molecular mass of 60 kDa; it does not present the interfacial activation phenomenon. It was found to be more active on short-chain triacylglycerols than on long-chain triacylglycerols. The NH_2-terminal sequence of the SnDL shows 66% of identity with the 17 NH_2-terminal amino acids of a putative lipase from sea urchin (*Strongylocentrotus purpuratus*). No sequence identity was found with known lipases. Interestingly, neither colipase nor bile salts were detected in the snail hepatopancreas. This suggests that colipase evolved in vertebrates simultaneously with the appearance of an exocrine pancreas and a true liver which produces bile salts. Altogether, these results suggest that SnDL is a member of a new group of digestive lipases belonging to invertebrates.

Keywords Hepatopancreas · Snail · Invertebrate · Digestive lipase · Purification · Characterization

Abbreviations

AG	Arabic gum
BSA	Bovine serum albumin
DEAE	Diethylaminoethyl
FPLC	Fast protein liquid chromatography
NaDC	Sodium deoxycholate
NaTDC	Sodium taurodeoxycholate
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SnDL	Snail digestive lipase

S. Amara · A. Fendri · N. Ben Salem · Y. Gargouri · N. Miled (✉)
Laboratoire de Biochimie et de Génie Enzymatique des Lipases, ENIS route de Soukra,
BPW 3038 Sfax, Tunisia
e-mail: nmiled@yahoo.com

PVDF	Polyvinylidene difluoride
TC_3	Tripropionin
TC_4	Tributyrin

Introduction

Lipases are hydrolytic enzymes (EC 3.1.1.3) catalyzing the hydrolysis of the water-insoluble triglycerides into free fatty acids and glycerol and operate at the surface of emulsified lipid substrates.

Higher animal's lipases are well characterized [1–3]; in contrast, much less is known about lipases from lower ones. Since the detection of the presence of lipase activity in the gastric juice of some crustaceans (*Homarus americanus*) and mollusks (*Aplysia californica*) [4, 5], a number of investigations on invertebrate lipases have been reported. Recently, lipases were isolated from digestive glands of lower animals, such as the scorpion [6] and the crab [7]. The purified enzymes showed no similarity with any known lipases. Within the phylum of mollusks, gastropods are by far the largest class. Many studies have described the morphology as well as the digestive system of snails [8, 9]. The hepatopancreas, named also digestive gland, as well as the salivary gland of snails are the main source of digestive enzymes [9]. The digestive gland was shown to be composed of two main cell types, the digestive cells and secretory cells. The digestive cells appeared to be concerned with the absorption and digestion of nutrients, while secretory cells produced digestive enzymes and calcareous concretions. Undifferentiated cells were scattered between these two main cell types [9].

Many studies have attempted to purify peptidases and cellulases from snail hepatopancreas [10, 11] but, to our knowledge, no lipolytic enzymes from the snail digestive glands have been purified so far. In this study, the snail hepatopancreas represents the starting material to detect the existence of a lipolytic activity.

This paper reports the purification to homogeneity of an active lipase from the snail hepatopancreas. This lipase, tentatively named snail digestive lipase (SnDL), was characterized with respect to its biochemical properties.

Materials and Methods

Animals

Snails (*Eobania vermiculata*) were collected alive from the area of Sfax (Tunisia). After dissection of the snails, the hepatopancreas are immediately delipidated.

Enzymes

Pure dromedary pancreatic lipase was a generous gift from Dr. Ben Bacha (Ecole Nationale d'Ingénieurs de Sfax (ENIS), Tunisie).

Delipidation of Snails' Hepatopancreases

The snail hepatopancreases were cut into small pieces and delipidated according to the method described previously [12]. After delipidation, 20 g of delipidated powder were obtained from 100 g of fresh tissue. The delipidated powder was stored at −20 °C until use.

Lipase Activity Determination

The lipase activity was measured titrimetrically at pH8 and 37°C with pH-Stat (Metrohm, Switzerland) using tributyrin (TC_4) as substrate (0.25 ml TC_4 in 30 ml of 2 mM Tris–HCl, 1 mM $CaCl_2$, and 2 mM sodium deoxycholate (NaDC)) or olive oil emulsified with Arabic gum as previously reported [13, 14]. Lipase assays were performed in the presence or in the absence of colipase. Lipase activity was also measured according to the method previously described [15] using tripropionin (TC_3). One unit (U) of lipase activity corresponds to 1 μmol of fatty acid released per minute. Specific activities are expressed as units per milligram of protein.

Phospholipase Activity Determination

The phospholipase activity was measured titrimetrically at pH8 and 37°C, with a pH-Stat under the standard assay conditions described previously [16], using phosphatidylcholine emulsion as substrate in the presence of 3 mM NaDC and 7 mM $CaCl_2$. One unit of phospholipase activity was defined as 1 μmol of fatty acid released per minute.

Determination of Protein Concentration

Protein concentration was determined using the Bradford method as described previously [17]. Bovine serum albumin (BSA) solutions of known concentrations were used as reference.

Bile Salts Titration

Bile salts titration was checked using 3α-hydroxysteroid dehydrogenase: hepatopancreas homogenates (400 μl) containing 4–100 mg of fresh tissue were mixed with 600 μl buffer containing 0.03 U dehydrogenase and 0.3 mg NAD^+ in 15 mM Tris–HCl (pH9.4), 0.5 mM ethylenediaminetetraacetic acid. Standard assays were carried out under the same conditions using various concentrations of sodium taurodeoxycholate (NaTDC) ranging from 0.005 to 0.5 mM. After incubation for 1 h at 37°C, the absorbance at 340 nm was measured [18].

Analytical Methods

Analytical polyacrylamide gel electrophoresis (PAGE) of proteins in the presence of sodium dodecyl sulfate (SDS)-PAGE was performed by the method of Laemmli [19]. Samples for sequencing were electroblotted on the polyvinylidene difluoride, according to Bergman and Jörnvall [20]. Protein transfer was performed during 1 h at 1 mA/cm^2 at room temperature.

NH_2-terminal Sequence Analysis

To determine the NH_2-terminal sequence, protein bands from SDS gels were transferred to a problott membrane (Applied Biosystems). Automated Edman protein degradation was performed with a protein sequencer (Applied Biosystems Protein sequencer Procise 492/610A [21, 22]. The NH_2-terminal sequence was established by Pr. Hafedh Mejdoub (Faculté des Sciences, Sfax, Tunisia).

Results

Investigation of Colipase and Bile Salts Levels in the Snail Hepatopancreas

In order to check the presence of colipase and bile salts in the snail's digestive gland, the hepatopancreas homogenate was treated during 30 min at pH 4 and/or at 60 °C to inactivate lipase and maintain only colipase activity. This homogenate failed to reactivate the dromedary pancreatic lipase inhibited by bile salts. Furthermore, the snail pancreatic lipase was also not activated by its own pH- and/or temperature-treated homogenate. These results suggest that the snail hepatopancreas contains no colipase (data not shown). We also used a colorimetric assay to check the presence of bile salts in the snail hepatopancreas following a previously described procedure [18]. Our data (not shown) indicated that unlike vertebrates, no detectable yields of bile salts were found in the snail digestive glands. This result is in line with that found in the case of other invertebrates, such as scorpion [6] and crab [7].

Lipolytic Activities of the Snail Hepatopancreas

The lipase activity level measured in the snail hepatopancreas extract was 100 U/g of fresh digestive tissue using TC_4 as substrate. Furthermore, phospholipase activity detected in the snail hepatopancreas was about 20 U/g of fresh hepatopancreas, measured with phosphatidylcholine emulsion (data not shown). One can say that the purified SnDL does not present any phospholipase activity. This enzyme is different from mammalian pancreatic lipase related protein 2 which has both lipase and phospholipase activities [23]. Besides, snail hepatopancreas seems to be equipped with separated lipase and phospholipase enzymes assuming the hydrolysis of dietary lipids.

Purification of SnDL

Delipidated powder (12 g) of snail hepatopancreases was suspended in 120 ml of buffer A (25 mM Tris–HCl pH 8, 25 mM NaCl, 2 mM benzamidine). The mixture was stirred during 30 min at 4 °C. After centrifugation for 30 min at 10,000 rpm, the supernatant was used as a starting material for subsequent purification steps.

Anion Exchange Chromatography

The supernatant was loaded on a column (3×20 cm) of diethylaminoethyl (DEAE)-cellulose equilibrated with buffer A. Under these conditions, the enzyme was fixed on the cationic support. Non-fixed proteins were washed out with buffer B (25 mM Tris–HCl pH 8, 100 mM NaCl, 2 mM benzamidine). SnDL is then eluted by washing the column with the same buffer containing 300 mM NaCl. The recovery yield of SnDL from this step was about 70% of the starting enzymatic activity.

Ammonium Sulfate Precipitation

Active fractions eluted from DEAE-cellulose were pooled. Then, they were brought to 60% saturation with solid ammonium sulfate under stirring conditions and maintained for 30 min at 4 °C. After centrifugation (30 min at 8,500 rpm), the pellet was resuspended in 10 ml of buffer A.

Filtration on Sephacryl S-200

The enzymatic solution, issued from ammonium sulfate precipitation, was loaded on a gel filtration Sephacryl S-200 column (2.5×120 cm) equilibrated with buffer A. Elution of the lipase was performed with the same buffer at 30 ml/h. SnDL was eluted between 1.9 and 2.4 of void volumes. The fractions containing 47% of the starting activity of the lipase were pooled together (Fig. 1a).

Anion Exchange Chromatography

The pooled fractions containing SnDL activity, issued from the Sephacryl S-200 column were applied to a Mono-Q fast protein liquid chromatography (FPLC) column (2.6×20 cm)

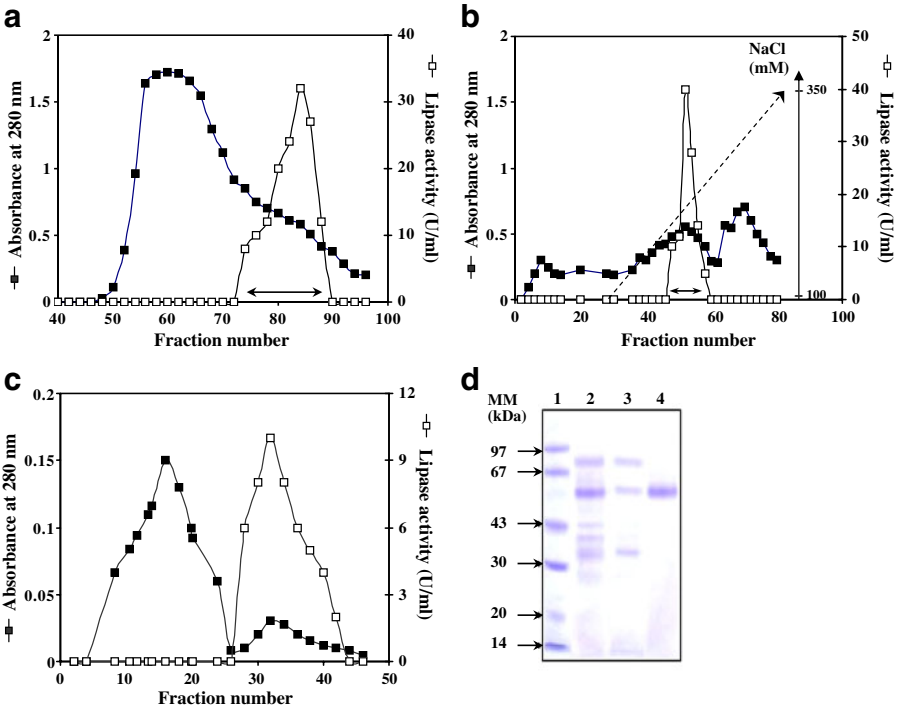

Fig. 1 **a** Chromatography of snail digestive lipase (SnDL) on Sephacryl S-200. The column (2.5×120 cm) was equilibrated with buffer A: 25 mM Tris–HCl pH8, 25 mM NaCl and 2 mM benzamidine. The elution of lipase was performed with the same buffer at a rate of 30 ml/h and 6 ml by fraction. SnDL activity was measured as described previously using TC_4 as substrate. Active fractions (72–88) were gathered. **b** Chromatography of SnDL on Mono-Q fast protein liquid chromatography. The column (2.6×20 cm) was equilibrated with buffer A. The elution of the adsorbed proteins was then performed with a linear gradient of NaCl (100–350 mM). The flow rate was 180 ml/h, and the fraction size was 3 ml. SnDL activity was measured using TC_4 as substrate. Active fractions (50–58) were gathered. **c** Chromatography of SnDL on Mono-S Sepharose. The column (2.6×20 cm) was equilibrated with buffer C (25 mM acetate pH6.5, and 2 mM benzamidine) containing 25 mM NaCl. The elution of lipase was performed with the buffer C containing 50 mM NaCl at a rate of 30 ml/h. SnDL activity was measured using TC_4 as substrate. **d** Analysis of purified SnDL (sodium dodecyl sulfate-polyacrylamide gel electrophoresis (15%)). *Lane 1*, molecular mass markers (Pharmacia); *lane 2*, 10 μg of SnDL solution obtained after Sephacryl S-200 chromatography; *lane 3*, 10 μg of SnDL solution obtained after Mono-Q Sepharose chromatography; *lane 4*, 10 μg of purified SnDL obtained after the last step. The gel was stained with Coomassie blue

equilibrated with buffer A. Non-fixed proteins were washed out with buffer B. The elution of the adsorbed proteins was then performed with a linear gradient of NaCl (100–350 mM). As shown in the elution diagram (Fig. 1b), SnDL activity emerged between 150 and 250 mM NaCl. The recovery yield of SnDL from Mono-Q column was 27% of the starting activity.

Cation Exchange Chromatography

The pooled fractions containing SnDL activity of Mono-Q FPLC column were concentrated and then diluted 25 times in the buffer C (25 mM acetate pH6.5, 2 mM benzamidine) in order to decrease its pH and ionic strength. The enzymatic extract was applied to a Mono-S column (2.6× 20 cm) equilibrated with buffer C containing 25 mM NaCl. SnDL was then eluted with buffer C containing 50 mM NaCl. The fractions containing the SnDL activity (Fig. 1c) were pooled and analyzed on SDS-PAGE. The purified enzyme was represented by a single band corresponding to a molecular mass of about 60 kDa (Fig. 1d). This result was in line with the molecular mass determined under native conditions, using gel filtration. These data suggested that SnDL was a monomeric protein. The purification flow sheet (Table 1) shows that the enzyme purification factor was 345, and the final recovery yield of enzymatic activity was 12%.

NH_2-terminal Sequence of SnDL

The NH_2-terminal sequencing allowed the identification of the NH_2-terminal 17 residues of the SnDL: DSVEYGDLGEFATDAPF. No similarity of the SnDL NH_2-terminal amino acid sequence was found with those of known digestive lipases. Whereas, an identity of 66% was found with the 17 NH_2-terminal amino acids of a putative lipase from sea urchin (*Strongylocentrotus purpuratus*; gene bank accession no.XP_793065; Table 2). Signal peptide (SignalP) predicts that residue 17 of the sea urchin lipase is also likely to be the NH_2-terminal of the mature lipase (after cleavage of a 16 residue signal sequence).

Therefore, SnDL appears to be a member of a new invertebrates' lipases group.

General Characteristics

The SnDL was found to be three times more active on short-chain triacylglycerols (TC_4) than on long-chain ones (olive oil emulsion). Indeed, the maximal specific activity of SnDL reaches 500 U/mg using TC_4 as substrate in the presence of 2 mM NaDC and 1 mM calcium at pH8 and 37°C. Whereas, 145 U/mg were obtained when olive oil was used as

Table 1 Flow sheet of snail digestive lipase purification.

Purification step	Total activity[a] (units)	Proteins[b] (mg)	Specific activity (U/mg)	Activity recovery (%)	Purification factor
Extract of SnDL	3,000	2,069	1.45	100	1
DEAE-cellulose chromatography	2,100	600	3.5	70	2.4
S-200 chromatography	1,400	23	60	47	41
Mono-Q chromatography	800	8	100	27	69
Mono-S chromatography	350	0.7	500	12	345

[a] 1 unit: 1 μmol of fatty acid released per minute using TC_4 as substrate in the presence of 1 mM NaDC.

[b] Proteins were estimated by Bradford method [17]. The experiments were conducted three times.

Table 2 Alignment of the NH_2-terminal sequence of snail digestive lipase with that of the putative *Strongylocentrotus purpuratus* lipase.

	1	17
Snail digestive lipase (SnDL)	**D S V** E **Y G D L G - -E** F A T D **A P** F	
	17	34
Strongylocentrotus purpuratus lipase	**D S V** T **Y G D L G** T F **E** Y G N G **A P** S	

For comparison, residues in bold indicate identical amino acids.

substrate under the same conditions. As it was previously found for many digestive lipases [24], SnDL was inactivated after incubation for 30 min at a temperature higher than 45 °C.

Effect of pH on SnDL Activity

The effect of pH on enzyme activity was determined for pH values ranging from 6 to 10, using TC_4 as substrate. The purified enzyme was mostly active between pH7.5 and 8.5 with an optimum at pH8 (data not shown). Lipase activity decreased significantly above pH 9.0, and only 5% of the residual activity was measured at pH9.5. The optimum pH for SnDL was similar to that reported for the crab lipase [7] and was lower than that of the scorpion lipase (pH9) [6].

Interfacial Activation of SnDL

TC_3 was selected in this study to assess whether the SnDL displays the interfacial activation phenomenon, as was suggested in a previous work [15]. The hydrolysis rate by SnDL of TC_3, emulsified in 0.33% GA and 150 mM NaCl, as a function of substrate concentration shows a normal Michaelis–Menten dependence of the activity on the substrate concentration (Fig. 2). These results indicate that SnDL, unlike classical pancreatic lipases [25], does not present the interfacial activation phenomenon.

Effect of Amphiphiles on SnDL Activity

Effect of Bile Salts

When increasing the NaTDC concentration in the TC_4 lipolysis medium, the SnDL activity increased to reach a maximal value of 500 U/mg at 2 mM NaTDC (Fig. 3). The activity then decreased slightly, but the lipase remained active (350 U/mg) even at NaTDC concentration as high as 8 mM. The same results were obtained when using NaDC instead of NaTDC (data not shown). Interestingly, the SnDL preserved 80% of its maximal activity in presence of a high concentration of NaTDC (8 mM) when using the olive oil emulsion as substrate (Fig. 3).

Effect of Triton X-100 and Bovine Serum Albumin (BSA)

The effects of varying concentrations of synthetic detergents like triton X-100 or amphiphilic proteins like BSA (Fig. 4) on the rate of hydrolysis of TC_4 by SnDL were studied. The activity of the SnDL decreased slightly even at a triton X-100 concentration as high as 1 mM (Fig. 4a). Besides, SnDL was found to be insensitive to the BSA addition even at a concentration as high as 50 μM (Fig. 4b).

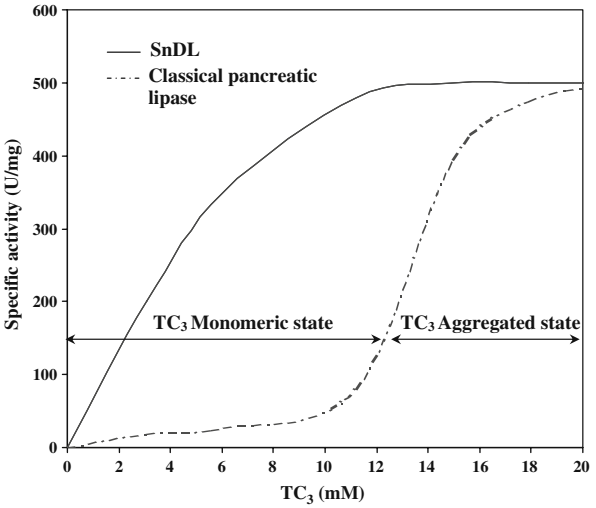

Fig. 2 Hydrolysis rate of TC_3 by snail digestive lipase as function of substrate concentration. The TC_3 solutions were systematically prepared by mixing (3×30 s in a warring blender) a given amount of TC_3 in 30 ml of 0.33% Arabic gum and 0.15 M NaCl. The release of propionic acid was recorded continuously at pH7 and 37°C using a pH-stat. The limit of TC_3 solubility (12 mM) is indicated. For the sake of comparison, the curve representing the hydrolysis rate of TC_3 by a classical pancreatic lipase is shown (*dashed line*)

Discussion

Unlike higher animals, there are few studies reporting the presence of lipolytic activity or the partial characterization of lipase from lower animals. Lipases from scorpion and crab were purified from these animal's hepatopancreas. Some of their catalytic properties were determined showing interesting features. However, much is less known about lipases from

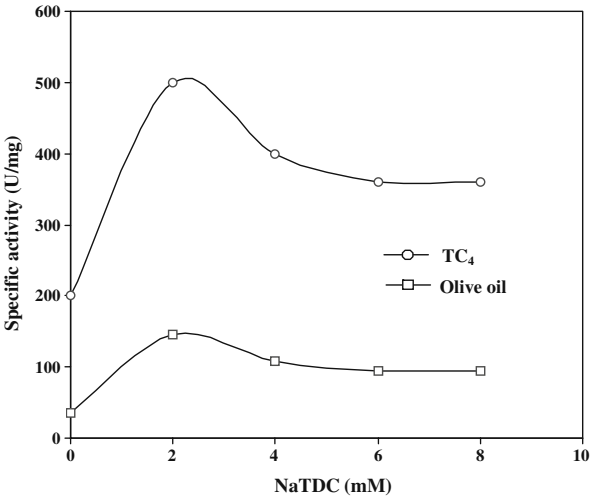

Fig. 3 Effect of increasing concentrations of bile salts (sodium taurodeoxycholate) on the rate of hydrolysis of TC_4 or olive oil emulsion by snail digestive lipase. Lipolytic activity was measured at pH8 and 37°C

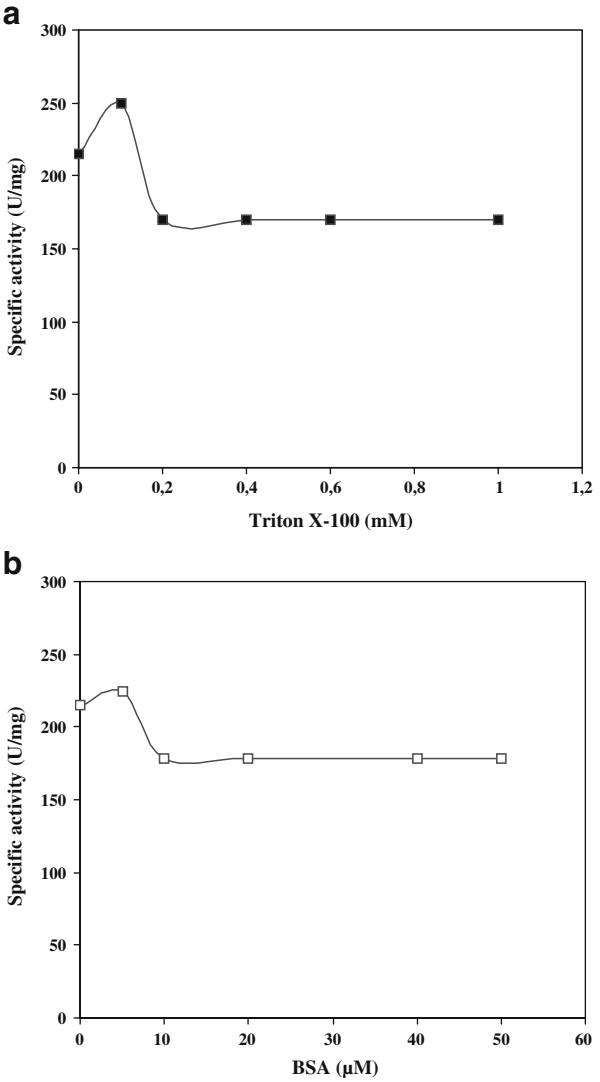

Fig. 4 Effect of amphiphiles on snail digestive lipase (SnDL) specific activity. Effect of triton X-100 (**a**) and bovine serum albumin (**b**) on the SnDL specific activity using TC_4 as substrate. SnDL activity was measured at 37°C, at pH8, and in the absence of bile salts

mollusks. In the present study, we have located a lipolytic activity in the snail (*E. vermiculata*) digestive glands, so called hepatopancreas. The lipase, named SnDL, responsible for this activity has been purified. Purification of lipase was carried out by anion exchange chromatography (DEAE-cellulose), ammonium sulfate precipitation, Sephacryl S-200 gel filtration, anion exchange chromatography (Mono-Q Sepharose), and cation exchange chromatography (Mono-S sepharose). Pure SnDL has a molecular mass of 60 kDa. Its NH_2-terminal sequence was determinate, and no similarity was found with those of known digestive lipases; whereas, an identity of 66% was found with the 17 NH_2-terminal amino acids of a putative lipase from *S. purpuratus*. Altogether, these results

suggest that SnDL is a member of a new group of digestive lipases belonging to invertebrates.

The activities of SnDL on TC_4 and olive oil emulsion were measured under the optimal conditions (2 mM NaDC and 1 mM calcium at pH 8 and 37 °C). The data obtained clearly show that this enzyme has a preference for short-chain triacylglycerols than for long-chain triacylglycerols substrates. SnDL was found to be about three times more active on TC_4 than on olive oil emulsion (500 and 145 U/mg, respectively).

In order to maintain full lipolytic activity in the presence of the physiological concentrations of bile salts, pancreatic lipases need a cofactor, the colipase [26, 27]. Colipase has been found in the pancreatic juice of all mammals, avian, and in many lower vertebrates like dogfish (*Squalus acanthius*) [28, 29]. It was the purpose of this study to find out whether SnDL is functionally related to classical pancreatic lipases. Interestingly, neither colipase nor bile salts were detected in the snail hepatopancreas. Unlike higher animals, invertebrates do not possess a true liver and consequently do not secrete bile salts [30]. The occurrence of colipase in the pancreatic juice of higher animals may be seen as an evolutionary feature that allows lipase to function in the presence of bile salts in a weak acidic intestinal content [31] suggesting that colipase evolved in vertebrates simultaneously with the appearance of an exocrine pancreas and a true liver which produces bile salts.

It was earlier established [15, 32] that the interfacial activation cannot be used, as originally proposed by Sarda and Desnuelle [25], as the unique criterion to distinguish lipases from esterases. Lipases are defined as a subfamily of esterases enzymes able to hydrolyze long-chain triacylglycerols independently of the presence or the absence of the interfacial activation phenomenon [15]. In this study, SnDL, which was found to hydrolyze efficiently olive oil, can be said to be a true lipase.

The effect of some amphiphiles on SnDL activity was investigated using TC_4 or olive oil emulsion as substrates. SnDL was found to preserve 70% of its maximal specific activity at NaTDC concentrations beyond 8 mM when TC_4 is used as substrate. This behavior differs therefore from that of pancreatic lipases which are strongly inhibited by bile salts [33]. Analogous results have been obtained with the scorpion digestive lipase [6]. These findings might be explained by the invertebrates' digestive glands lacking the bile salt/colipase system. When olive oil emulsion is rather used in the reaction medium, 80% of SnDL residual activity was measured at high NaTDC concentrations. Similar behavior was previously described in the case of the human gastric lipase when TC_4 was used as substrate [34].

The effects of a synthetic detergent (triton X-100) and an amphiphilic protein (BSA) on the rate of hydrolysis of TC_4 by SnDL were also studied. Our results clearly show that SnDL is insensitive to the BSA and triton X-100 addition at high concentrations (50 μM and 1 mM, respectively). This behavior differs from that of pancreatic lipases which were fully inhibited by BSA using TC_4 as substrate [33]. The resistance of SnDL to high concentrations of surfactants might allow its use in detergence, in moderate temperature conditions.

This study and previous ones [6, 7] give additional data for a better understanding of the digestion pathway of mollusks. It will be interesting to clone and express SnDL in a prokaryotic system. Producing the recombinant snail lipase at a large scale will make it possible to deepen the characterization of this novel enzyme.

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