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A grey mullet enzyme displaying both lipase and phospholipase activities: Purification and characterization

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ABSTRACT

A lipase from the golden grey mullet viscera was purified to homogeneity by ammonium sulphate precipitation, gel filtration, anionic and cation exchange chromatographies. The pure enzyme tentatively named grey mullet digestive lipase (GmDL) is a monomer having a molecular mass of about 35 kDa, as determined by SDS-PAGE analysis. No similarity was found between the NH_2-terminal amino acid residues of GmDL and those of other known digestive lipases. GmDL is a serine enzyme, like all known lipases from different origins. Interestingly, GmDL has not only lipase activity but also a phospholipase activity which requires the presence of Ca^{2+} and bile salts. Specific activities of 64 U/mg, 55 U/mg and 63 U/mg were measured using tributyrin, olive oil emulsion or phosphatidylcholine as substrate, respectively at pH 8 and at 50 °C. GmDL is therefore a thermo-active enzyme as compared to other fish lipases studied so far. It is worth to notice that grey mullet lipase was active in the presence of salt concentrations as high as 0.8 M.

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1. Introduction

Lipases (triacylglycerol acylhydrolases; EC 3.1.1.3) constitute a group of enzymes defined as carboxylesterases that catalyse the hydrolysis of long-chain acylglycerols at the lipid–water interface [1].

Lipases are important because of the role they play in the post-mortem quality deterioration of seafood (and other foodstuffs) during handling, chilled, and frozen storages. Lipases are having increasing uses as food and other industrial processing aids [2]. Compared with other hydrolytic enzymes (e.g., proteases and carbohydrolases), lipases from fish are relatively poorly studied. Lipases from aquatic animals are less known than those from mammal, plant and microbial sources [2]. The search for new enzymes with novel properties, higher efficiency or new substrate selectivity is a growing trend and holds a pivotal position in the future of enzyme technology. Because of their habitate, marine animals might have enzymes that display distinguishable properties that make them better suited for specific applications.

The grey mullet (*Mugil auratus*) is an extremely active fish that travels in large areas searching for food. This marine specie mostly inhabits estuaries, fresh water, and shallow offshore waters, and is adaptable to a wide range of salinity. The grey mullet feeds on detritus, planktons, worms and insect larvae [3]. Its digestive system contains an oesophagus then a stomach, a dilated section of the digestive tract, and finishes by the intestine which digests food and absorbs nutrients [4]. A thermoactive lipase was partially purified from the viscera of grey mullet (*Mugil cephalus*) [2]. Two lipases having molecular masses of about 46 and 41 kDa were purified from the dorsal part of grey mullet (*Liza parsia* Hamilton, 1822) and some of their biochemical properties were determined [5].

Lipases from *Sardinella aurita* [6], Chinook salmon and New Zealand hoki [7] have been purified. Studies of both purified and partially purified lipases [8] indicate that carboxyl ester lipase (CEL, also known as bile salt activated lipase or bile salt-dependent lipase) is the dominant digestive lipase in fish.

We describe in this work the purification and some biochemical characteristics of a lipase named grey mullet digestive lipase (GmDL) purified from the golden grey mullet viscera. This work is a part of ongoing research to discover new enzyme sources for potential food and biotechnological applications.

2. Materials and methods

2.1. Chemicals

Tributyrin (99%, puriss), benzamidine were from Fluka (Buchs, Switzerland); pure triolein were from Acros Organics (Noisy-Le-Grand, France), phosphatidylcholine (PC), β-mercaptoethanol

Abbreviations: SaDL, sardine digestive lipase; GmDL, grey mullet digestive lipase; NaDC, sodium deoxycholate; AG, arabic gum; OO, olive oil; BSA, bovine serum albumin; PC, phosphatidylcholine; PVDF, polyvinylidene difluoride; SDS, sodium dodecyl sulphate.

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(βME), bovine serum albumin (BSA) (99%, puriss) and nitrocellulose membrane were from Sigma Chemical (St. Louis, USA); arabic gum was from Mayaud Baker LTD (Dagenham, UK); sodium deoxycholate (NaDC) was from Sigma Chemical (St. Louis, USA); Orlistat is a generous gift from F. Hoffmann-La Roche AG (Switzerland); acrylamide and bis-acrylamide electrophoresis grade were from Bio-Rad (Paris, France), marker proteins and supports of chromatography used for GmDL purification; Sephacryl S-200, Mono-Q Sepharose, Mono-S Sepharose were from Pharmacia (Uppsala, Sweden), PVDF membrane and protein sequencer Procise 492 equipped with 140 C HPLC system purchased from Applied Biosystems (Roissy, France); pH-stat was from Metrohm (Herisau, Switzerland).

2.2. Biological materials

Golden grey mullet (*M. auratus*) (1.5–2 kg) were bought from local fish market (Sfax, Tunisia). A stock of viscera (pyloric caeca, intestines) removed immediately after dissection of the grey mullet was stored at −20 °C.

2.3. Preparation of viscera extract

Extraction buffer A (25 mM Tris–HCl, 150 mM NaCl, 2 mM benzamidine, pH 8) was added to the viscera sample in the proportion of 10 ml per 1 g of fresh tissue. The fresh tissues were mixed using a blender (2 × 30 s) in an extraction buffer A then stirred with a magnetic bar during 45 min at 4 °C. The extract obtained was centrifuged for 30 min at 12,000 rpm and the clear supernatant was collected to measure the lipase and the phospholipase activities.

2.4. Delipidation of grey mullet viscera

After unfreezing, viscera of grey mullet were delipidated according to the method described previously [9]. After delipidation, 14 g of powder were obtained from 140 g of fresh tissue. Delipidated powder was used for enzyme purification.

2.5. Determination of lipase activity

The lipase activity was measured titrimetrically at pH 8 and 50 °C with a pH-stat, under the standard assay conditions described previously, using tributyrin (0.25 ml) in 25 ml of 2 mM Tris–HCl pH 8, 150 mM NaCl or olive oil emulsion (10 ml olive oil emulsion in 20 ml of 2.5 mM Tris–HCl pH 8.5, 150 mM NaCl, 1 mM $CaCl_2$), as substrate [10,11]. The olive oil emulsion was obtained by mixing (3× 30 s in a Waring blender) 10 ml of olive oil in 90 ml of 10% arabic gum. Lipolytic activity was expressed as international units. Specific activities are expressed as U/mg of protein. One unit corresponds to 1 μmol of fatty acids released per minute.

2.6. Determination of phospholipase activity

The phospholipase activity was measured titrimetrically at pH 8 and 50 °C with a pH-Stat, under the standard assay conditions described previously [12], using phosphatidylcholine (PC)/bile salt mixed micelles as substrate in the presence of 10 mM NaDC, 8 mM $CaCl_2$ and 2 mM Tris–HCl. One unit of phospholipase activity was defined as 1 μmol of fatty acids released per minute.

2.7. Determination of protein concentration

Protein concentration was determined as described by Bradford [13], using bovine serum albumin ($E^{1\%}_{1cm} = 6.7$) as reference.

2.8. Purification of GmDL

2.8.1. Delipidated powder extraction

Twenty mullet were used as a starting material for each purification procedure. Delipidated powder (14 g) of mullet viscera was suspended in 140 ml of buffer A: 25 mM Tris–HCl pH 8, 25 mM NaCl, and 2 mM benzamidine. The mixture was stirred during 45 min at 4 °C, then centrifuged for 30 min at 12,000 rpm.

2.8.2. Ammonium sulphate precipitation

The supernatant (150 ml) was brought to 60% saturation with solid ammonium sulphate under stirring conditions and maintained for 45 min at 4 °C. After centrifugation (12,000 rpm during 30 min at 4 °C), the pellet was resuspended in 10 ml of buffer A. Insoluble material was removed by centrifugation at 12,000 rpm during 25 min. Approximately 93% of the starting total activity of GmDL was recovered in the supernatant.

2.8.3. Filtration on Sephacryl S-200

The supernatant containing the lipolytic activity was loaded on a column (2.5 cm × 140 cm) of gel filtration Sephacryl S-200 equilibrated with buffer A. Elution of enzyme was performed with the same buffer at a rate of 35 ml/h. The fractions containing the lipase activity (eluted between 1.9 and 2.1 void volume) were pooled.

2.8.4. Anion exchange chromatography

The pooled fractions containing GmDL activity issued from the Sephacryl S-200 column were injected onto a Mono-Q column (2.5 cm × 10 cm) equilibrated with buffer A: 25 mM Tris–HCl pH 8, 25 mM NaCl, and 2 mM benzamidine. GmDL activity was not adsorbed onto the cationic support and was eluted with the washing buffer at 80 ml/h. This is probably due to the presence of a high ionic strength in the active fractions of Sephacryl S-200. The ionic strength, applied by ammonium sulphate, decreased the adsorption capacity of GmDL onto the cationic support. The elution of the adsorbed proteins was then performed with a linear gradient of NaCl (50–400 mM of NaCl). The recovery yield of GmDL activity after the Mono-Q column purification step was 32% of the starting enzymatic activity.

2.8.5. Cation exchange chromatography

Active fractions of Mono-Q were applied to a Mono-S column (2.5 cm × 6 cm) equilibrated with buffer B (20 mM sodium acetate pH 5, 20 mM NaCl, 2 mM benzamidine). Under these conditions, the enzyme was not adsorbed onto the cationic support and was recovered with the washing buffer, using the same buffer at 80 ml/h. The fractions containing the lipase activity were pooled.

2.9. Analytical methods

Analytical polyacrylamide gel electrophoresis of proteins in the presence of sodium dodecyl sulphate (SDS/PAGE) was performed by the method of Laemmli [14]. Samples for sequencing were electroblotted according to Bergman and Jörnvall [15]. Protein transfer was performed during 1 h at 1 mA/cm^2 at room temperature.

Zymograms for lipase and phospholipase activities were carried out on polyacrylamide native gel (data not shown) according to the method described by Laemmli [14]. Protein bands were detected by staining with 0.25% of Coomassie brilliant blue R-250. For lipase activity, zymogram was accomplished with olive oil according to the method of Gilbert et al. [16]. In summary, gel overlays were prepared from a 10% olive oil emulsion in 150 mM Tris/HCl (pH 8.5) containing 0.3 mM red phenol as a pH indicator and 2% agarose. For phospholipase activity, zymogram was performed by layering the native gel in 4 mM NaTDC, 0.3 mM phenol red as a pH indicator, 100 mM NaCl, 8 mM $CaCl_2$, 0.1% PC, pH 8.5, and incubating for

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