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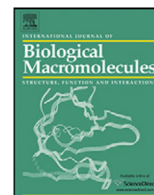

Purification and biochemical characterization of a halotolerant *Staphylococcus* sp. extracellular lipase

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ABSTRACT

We have isolated a lipolytic halotolerant bacterium, designated as CJ3, that was identified as a *Staphylococcus* sp. Culture conditions were optimized and the highest extracellular lipase production amounting to 5 U/ml was achieved after 24 h of cultivation. The extracellular lipase was purified 24-fold by ammonium sulfate precipitation and a Sephacryl S-200 chromatography, and its molecular mass was found to be around 38 kDa, as revealed by SDS-PAGE and gel filtration. The lipase substrate specificity was investigated using short (tributyrin) and long (olive oil) chain triglyceride substrates. The lipase was inhibited by submicellar concentrations of Triton X-100, and maximum specific activities were found to be 802 U/mg on tributyrin and 260 U/mg on olive oil at pH 8.0 and 45 °C. The lipase was fairly stable in the pH range from 6.0 to 9.0, and about 69% of its activity was retained after incubation at 45 °C for 60 min. The enzyme showed a high tolerance to a wide range of salt concentration and a good stability in organic solvents, especially in long chain-fatty alcohols.

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1. Introduction

Extremophilic organisms have drawn much interest in the scientific community because of the molecular adaptation they underwent during evolution and for their biotechnological potential [1–3]. The environmental challenges that extremophiles have to face necessitate, besides other physiological modifications, biosynthesis of macromolecules stable and active at environmental physicochemical extreme conditions. These macromolecules display clearly distinguished structural features when compared to their homologs from mesophilic microorganisms [4–6].

Lipases (EC 3.1.1.3) are soluble enzymes that act on insoluble triglycerides substrates and thus perform interfacial catalysis [7]. Lipases constitute the most important group of biocatalysts for biotechnological applications [8,9]. Since industrial processes are commonly carried out under harsh conditions, it would be of great importance to obtain lipases which retain their activity at adverse environments (i.e. extreme temperature, pH, salts concentrations, and in the presence of organic solvents) [10]. In this sense, lipases isolated from extremophiles constitute an excellent alternative for industrial processes [11].

Halophilic microorganisms are salt-loving extremophilic organisms that can often adapt to a broad salt concentration range by maintaining a proper osmotic pressure in their cytoplasm [12,13]. Hypersaline conditions favor protein aggregation and collapse, interfere with the electrostatic interactions between protein residues, and are responsible for a general decrease in the availability of water molecules [14]. Halophilic proteins, rather than being unfolded by these conditions, are distinguished by maintaining soluble and active conformations in an environment generally detrimental to other proteins [5]. For this reason, several lipolytic enzymes active and stable in extreme conditions of salinity have been recently characterized [15–17].

In this work, we isolated a halotolerant lipolytic bacterium, *Staphylococcus* sp. strain CJ3, from Tunisian biotope, and described the production and purification of its extracellular lipase. The enzyme was characterized with respect to substrate specificity and biochemical properties.

2. Materials and methods

2.1. Isolation of the lipolytic strain CJ3

Samples were collected from various saline and hypersaline environments such as salted vegetables, salted animal skin, soil

Abbreviations: TC4, tributyrin; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; OD, optical density; GA, gum arabic; rDNA, ribosomal DNA.

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samples from Chott Eldjerid (a salt lake in Tunisia), marine sediments, sedimentary rocks, and effluents from a fish processing industry. Successive decimal dilutions were made in 9 ml of distilled water containing 10% NaCl. After shaking, 0.1 ml of each suspension was spread on solid medium containing (% w/v): casein peptone, 0.5; meat extract, 0.5; NaCl, 10; glucose added aseptically, 0.5; agar, 2, pH 7.5. The plates were incubated at 30 °C for 48 h. The isolates were re-isolated several times to confirm their purity and then conserved in glycerol at 20% (v/v) at −20 °C. To detect lipase production, bacteria were cultured on agar plates containing the same solid medium, used for the isolation, supplemented with 1% olive oil and 1‰ Rhodamine B (Sigma Aldrich, Germany). The plates were incubated at 30 °C for 48 h. Strains that have a halo fluorescent under UV were regarded as putative lipase producers. A bacterium designated as strain CJ3, isolated from the soil sample of Chott Eldjerid, showed the most intense fluorescence and then was selected for further work on lipase production.

Salt tolerance assays were performed by growing the strain CJ3 in 250 ml Erlenmeyer flasks containing 50 ml of medium A containing (% w/v): $MgSO_4$–$7H_2O$, 2; Na_3 citrate, 0.3; KCl, 0.2; yeast extract, 0.5; casein peptone, 0.5; olive oil, 1, pH 7.5, plus various concentrations of NaCl from 0 to 5 M. The cultures were inoculated with 1% of a fresh pre-culture having an optical density (OD) equal to 1 at 600 nm and were incubated at 30 °C in a rotary shaker (160 rpm). Samples were taken aseptically at different times, and bacterial growth kinetics were determined by measuring the optical density at 600 nm along with the lipase activity using tributyrin as substrate.

2.2. Identification of the isolate

Standard morphological and physiological tests were performed to characterize this new isolate, including Gram reaction, cell morphology, motility, growth under anaerobic conditions, catalase and oxidase production, hydrolysis of gelatin, starch and Tween 80, as well as other tests that are included in the species description. Procedures for all tests have been described previously [18]. Unless otherwise indicated, tests were performed in media that contained 10% (w/v) salt using an API® Staph (bioMérieux, France).

To identify the isolate, genomic DNA was extracted by Wizard® genomic DNA purification kit (Promega) according to the manufacturer's procedure, and the 16S rDNA was amplified using the universal primers FD1 (5′-AGAGTTTGATCCTGGCTCAG) and RD1 (5′-AAGGAGGTGATCCAGCC), as described previously [19]. Direct sequencing of the PCR product was performed with the ABI Prism Big Dye terminator cycle sequencing Ready Reaction Kit (ABI Prism/PE Biosystems), and products were resolved on ABI Prism 3100-Avant. 16S rDNA sequence analyses were performed with the ARB software package [20].

2.3. Production and purification of the lipase from the bacterium CJ3

One liter of culture medium, obtained after 24 h of cultivation, was centrifuged for 20 min at 8000 rpm to remove the microbial cells. The supernatant containing extracellular lipase was used as the crude enzyme preparation.

The crude enzyme solution (1 L) was brought to 60% saturation with solid ammonium sulfate (390 g) under stirring conditions at 4 °C. After centrifugation (30 min at 10,000 rpm), the precipitate was resuspended in 15 ml of buffer A (25 mM Tris–HCl, 25 mM NaCl, 2 mM benzamidine, pH 8). Insoluble material was removed by centrifugation at 10,000 rpm during 10 min. The supernatant (∼15 ml) was applied to a Sephacryl S-200 (1.8 × 100 cm) gel filtration column (Amersham Biosciences) equilibrated in buffer A. The elution of the lipase was performed with the same buffer at a flow rate of 18 ml/h.

The flow rate was adjusted to 18 ml/h. The protein elution profile was recorded spectrophotometrically at 280 nm. Fractions with lipase activity eluted from the column were pooled and concentrated using a Vivaspin centrifugal concentrator. The purified enzyme was used for further biochemical characterization.

2.4. Lipase activity measurements

Lipase activity was assayed potentiometrically by automatically titrating the free fatty acids released from a mechanically stirred emulsion of trioctanoin, using 0.1 N NaOH and a pH-stat device (Metrohm 718 Stat Titrino, Zofingen, Switzerland). Each assay was performed in a thermostatted vessel containing 250 μL tributyrin emulsified in 25 ml of 5 mM $CaCl_2$ solution under mechanical stirring. Unless otherwise mentioned, the pH and the temperature of the emulsion were kept at 8.0 and 45 °C, respectively. Various concentrations of NaCl or Triton X-100 were added when required. Specific activities are expressed here in international units (U) per milligram of lipase. 1 U corresponds to 1 μmol of fatty acid released per minute.

Long-chain triglycerides (e.g. olive oil) had first to be pre-emulsified with gum arabic (GA) by mixing 5 ml of olive oil with 45 ml of a 10% (w/v) GA solution prepared as previously described [21]. 10 ml of this olive oil–GA emulsion were then mixed in the pH-stat vessel with 20 ml of 7.5 mM $CaCl_2$ solution. The final amounts of long-chain triglyceride and GA in the pH-stat vessel were 0.5 ml and 3% (w/v), respectively. The lipase activity was measured under agitation at 45 °C and pH 8.0.

2.5. Protein analysis

The purified lipase was analyzed electrophoretically on 12% polyacrylamide gels in the presence of SDS as described by Laemmli [22]. Protein concentration was measured by the method of Bradford using BSA as standard [23].

2.6. Study of lipase stability as function of pH and temperature

To study the effects of the pH on the enzyme stability, buffered 1-ml samples containing 100 μg lipase were incubated at various pH values ranging from 4 to 11 at 25 °C. During the incubation period, the residual lipase activity was measured at different times in order to determine the changes with the time in the residual lipase activity. Each measurement was performed twice. The thermal stability was studied by incubating the lipase at pH 8 and at various temperatures and measuring the residual activity with time under standard titrimetric assay conditions.

2.7. Effect of organic solvents on the lipase stability

Buffered samples (pH 8.0) containing 100 μg lipase were incubated in the presence of 25% (v/v) of each organic solvent. After incubation for 60 min at 25 °C and 200 rpm, the residual enzyme activity was measured using tributyrin as substrate. Enzyme activity in the absence of organic solvents was marked as the control. All experiments were performed in triplicate.

3. Results and discussion

3.1. Characterization of the strain CJ3

The strain CJ3 was isolated from a salt lake in Chott Eldjerid, Tunisia. Strain CJ3 is a Gram-positive, non-motile, rod shaped and

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