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Production, purification and characterization of an extracellular lipase from *Aureobasidium pullulans* HN2.3 with potential application for the hydrolysis of edible oils

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Abstract

Lipase production (8.02 ± 0.24 U/ml) by the yeast *Aureobasidium pullulans* HN2.3 isolated from sea saltern was carried by using time-dependent induction strategy. The lipase in the supernatant of the yeast cell culture was purified to homogeneity with a 3.4-fold increase in specific lipase activity as compared to that in the supernatant by ammonium sulfate fractionation, gel filtration chromatography and anion-exchange chromatography. According to the data on SDS polyacrylamide gel electrophoresis, the molecular mass of the purified enzyme was estimated to be 63.5 kDa. The optimal pH and temperature of the purified enzyme were 8.5 and 35 °C, respectively. The enzyme was greatly inhibited by Hg^{2+}, Fe^{2+} and Zn^{2+}. The enzyme was strongly inhibited by phenylmethanesulphonyl fluoride, not inhibited by ethylene diamine tetraacetic acid (EDTA), but weakly inhibited by iodoacetic acid. It was found that the purified lipase had the highest hydrolytic activity towards peanut oil.

Keywords: Lipase production; Sea saltern; Lipase; *Aureobasidium pullulans*; Lipid hydrolysis

1. Introduction

Lipases catalyze a wide range of reactions, including hydrolysis, inter-esterification, alcoholysis, acidolysis, esterification and aminolysis. Therefore, lipases, especially microbial lipases have many industrial applications [1]. Following proteases and carbohydrases, lipases are considered to be the third largest group based on total sales volume. Numerous species of bacteria, yeasts and molds were found to produce lipases. Among the terrestrial yeasts, *Candida rugosa*, *C. tropicalis*, *C. antarctica*, *C. cylindracea*, *C. parapsilosis*, *C. deformans*, *C. curvata*, *C. valida*, *Yarrowia lipolytica*, *Rhodotorula glutinis*, *R. pilimornae*, *Pichia bispora, P. maxicana*, *P. sivicola*, *P. xylosa*, *P. burtonii*, *S. crataegenesis*, *Torulaspora globosa* and *Trichosporon asteroides* have been found to be able to produce lipase [2]. Extracellular lipases from several yeasts have been purified and characterized and the genes encoding lipase in *Candida*, *Geotrichum*, *Trichosporon* and *Y. lipolytica* have been cloned and overexpressed [2]. It has been found that most of lipases are serine hydrolyases according to their biochemical properties. Although lipases from *C. rugosa* and *C. antarctica* have been extensively used in different fields, very few studies exist on the lipase produced by yeasts isolated from sea salterns [3]. This study aims at production, purification and characterization of lipase produced by the yeast *Aureobasidium pullulans* HN2.3 isolated from sea saltern and uses of the purified lipase for hydrolysis of edible oils from different sources. This is the first report that lipase from the yeasts obtained from saltern could be used to hydrolyze edible oils.

2. Materials and methods

2.1. Yeast strain

Yeast strain HN2.3, which was identified to be a strain of *A. pullulans* according to the results of routine yeast identification and molecular methods, was isolated from the sea saltern in Qingdao [4]. This yeast strain was maintained in solid YPD medium (g/l) containing glucose 20.0, yeast extract 10.0, polypeptone 20.0, agar 20.0 at 4 °C.

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2.2. Lipase production

One loop of the cells of the yeast strain was transferred to 50 ml of YPD medium prepared with distilled water in a 250-ml flask and aerobically cultivated by shaking at 170 rpm and 25 °C for 24 h. The cell culture (0.2 ml, $OD_{600\,nm} = 20.0$) was transferred to 50 ml of the production medium (g/l) which contained olive oil 30.0, glucose 4.0, ammonium sulfate 6.0, K_2HPO_4 1.0, $MgSO_4.7H_2O$ 0.5, pH 7.0 and grown by shaking at 170 rpm and 25 °C for 4 days. In order to further enhance lipase production by the yeast, after the cells were grown in the production medium for 0, 3, 6 and 9 h, sterile olive oil (the final concentration was 30.0 (g/l) was added to the culture. Then, the culture was continued to be cultivated under the conditions described above. Five milliliters of the culture were taken at the intervals of 8 h, the culture was centrifuged at 14,000 × *g* and 4 °C for 20 min and the supernatant obtained was used as the crude lipase for determination of lipase activity.

2.3. Determination of lipase activity

The substrate emulsions were prepared by dropwise addition of 0.2 ml solution A (40 mg of ρ-nitrophenyl-laurate was dissolved in 12.0 ml of isopropanol) into 3.0 ml solution B (0.4 g Triton X-100 and 0.1 g gum arabic were dissolved in 90 ml of 100 mM potassium phosphate buffer, pH 7.0) under intense vortexing. These emulsions were stable for 1 h at room temperature. 0.1 ml of the crude lipase or the purified lipase was added to 3.2 ml of the substrate emulsion and the mixture was incubated for 20 min in a shaking water bath at 35 °C. The mixture with 0.1 ml of the inactivated lipase (heated at 100 °C for 5 min) was used as control. Then, the mixtures were put into ice and OD value at 410 nm in the mixture was read by using spectrophotometer [5]. One unit (U) of enzyme activity was defined as the amount of enzyme required for the liberation of 1.0 μmol ρ-nitrophenol from ρ-nitrophenyl-laurate per minute under the assay conditions. The specific lipase activity was units per mg of protein. Protein concentration was measured by the method of Bradford, and bovine serum albumin served as standard [6]. All the chemicals were purchased from Sigma.

2.4. Enzyme purification

Enzyme purification was carried out at 4 °C. Eight hundred milliliters of 4-day-old culture mentioned in Section 2.2 was used as the starting material for lipase purification. After removal of the cells by centrifugation at 14,000 × *g* for 20 min, proteins in the resulting supernatant were precipitated by ammonium sulfate (the saturation concentration of ammonium sulfate was 80%) and dialyzed by using MD 25 (MWCO: 8000–14,000 Da, Solarbio) against 20 mM potassium phosphate buffer (pH 7.0). The dialyzed protein solution was then applied to Sephadex™ G-75 column (medium grade; Pharmacia 2.5 × 100 cm) and the column was eluted with 20 mM potassium phosphate buffer (pH 7.0) by using ÄKTA™ prime with Hitrap™ (Amersham Biosciences, Sweden). At a flow rate of 0.5 ml/min, 3.0 ml fractions were collected. The lipase-positive fractions were combined and dialyzed in 20.0 mM Tris–HCI buffer (pH 8.0) overnight. The dialyzed lipase-positive elute was applied to DEAE-Sepharose Fast Flow anion-exchange column (2.5 × 30 cm) which had been equilibrated with 20.0 mM Tris–HCl buffer (pH 8.0) and the column was washed with the same buffer for 1 h at a flow rate of 1.0 ml/min. The bound proteins were then eluted with a linear gradient of NaCl solution in the range of 0–1.0 M in the equilibrating buffer. The lipase-positive fractions were concentrated by filtration through an AmiconYM3 (MW cut-off 10,000) membrane.

2.5. Gel electrophoresis

The purity and molecular mass of lipase in the concentrated fractions showing the activity were analyzed in non-continuous denaturing SDS-PAGE [7] with a Two Dimensional Electrophoresis System (Amersham Biosciences, Sweden) and stained by Coomassie Brilliant Blue R-250 [8]. The molecular mass standards for SDS-PAGE comprised myosin (200 kDa), β-galactosidase (116 kDa), phosphorylase B (97.2 kDa), serum albumin (66.4 kDa), ovalbumin (44.3 kDa) and carbonic anhydrase (29 kDa).

2.6. Effects of pH and temperature on the lipase activity and stability

The effects of pH on the purified lipase activity were determined by incubating the purified enzyme (0.015 U/ml) between pH 4.0–10.0 using the standard assay conditions. The buffers used were 0.1 M citric acid–sodium citrate (pH 4.0–5.5), 0.1 M potassium phosphate (pH 6.0–8.0), 0.1 M glycine/NaOH (pH 8.5–10). The pH stability was tested by 6 h pre-incubation of the purified enzyme (0.015 U/ml) in appropriate buffers that had the same ionic concentrations at different pH values ranging from 4.0 to 10.0 at 0 °C. The remaining activities of lipase were measured immediately after this treatment with the standard method as mentioned above.

The optimal temperature for activity of the enzyme was determined at 25, 30, 35, 40, 45 and 50 °C in the same buffer [0.1 M glycine/NaOH (pH 8.5)] as described above. Temperature stability of the purified enzyme (0.015 U/ml) was tested by pre-incubating the enzyme at different temperatures (20, 30, 35, 40 and 50 °C) for 1 h and the residual activity was measured as described in Section 2.3 immediately. Here, an enzyme reaction incubated at 4 °C was included to act as the reference control to which all measured activities were compared.

2.7. Effects of different metal ions and enzyme inhibitors on the lipase activity

To examine effects of different metal ions on the lipase activity, the purified lipase (0.015 U/ml) was pre-incubated at 0 °C for 1 h with various metal ions at a final concentration of 1.0 and 5.0 mM, respectively, using the standard enzyme assay as described in Section 2.3. The activity assayed in the absence of metal ions was defined as control. The chemicals tested included $ZnSO_4$, $CuSO_4$, $MgSO_4$, $FeCl_3$,