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Purification and characterization of lipase from *Burkholderia* sp. EQ3 isolated from wastewater from a canned fish factory and its application for the synthesis of wax esters

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ABSTRACT

The aim of this study was to purify and characterize the lipase produced by *Burkholderia* sp. EQ3, a bacterium isolated from the wastewater pond of a canned fish factory. The lipase was purified to homogeneity with a 70-fold increase in its activity by chilled acetone precipitation, Q-Sepharose Fast Flow and Sephadex G-50 column chromatography, respectively. The molecular mass of the purified lipase was 30.7 kDa. The purified lipase showed optimal activity at pH 7.0–7.5 and 30 °C. The enzyme was stable over the pH range of 5.0–8.0 and temperatures between 30 and 55 °C for 1 h. Its half-life was 2 h at 55 °C. The lipase activity was enhanced in the presence of Ca^{2+}, Mg^{2+} and K^{+} while it was inactivated by Cu^{2+}, Fe^{2+} and Zn^{2+}. It hydrolyzed the natural triglycerides with its highest activity on coconut oil (120%), olive oil (101%) and palm olein (100%). Gum arabic (1%) significantly increased the lipase activity whereas 1.0 mM phenyl methyl sulphonyl fluoride strongly reduced the activity. The purified lipase EQ3 retained 80% activity in iso-propanol, but was inactivated by ethanol and iso-octane as well as other hydrophobic solvents. The lipase EQ3 was immobilized by adsorption with Accurel MP-100 and used for wax esters synthesis and compared with five commercial immobilized lipases (Lipozyme RM IM, Lipozyme TL IM, Novozyme 435, Lipase PS and Lipase AK). The transesterification reaction was carried out between coconut oil, palm olein and jatropha oil with oleyl alcohol by using 1 U of enzyme, a substrate molar ratio of oil and oleyl alcohol of 1:3 in hexane at 37 °C and 150 rpm for 72 h. The immobilized lipase EQ3 was most efficient in the synthesis of wax esters with 60.3, 49.6 and 50.1% wax esters from coconut oil, palm olein and jatropha oil, respectively. For the five commercial immobilized lipases, Lipozyme RM IM exhibited the highest wax esters synthesis with 49.2, 41.4 and 48.1% wax esters, respectively.

1. Introduction

Lipases (triacylglycerol acyl hydrolase, E.C. 3.1.1.3) are found widely in animals, plants, and microorganisms. Microbial lipases have become increasingly important in the last two decades for industrial applications [1]. Some novel biotechnological applications have been successfully established using lipases for the synthesis of biopolymers and biodiesel, the production of enantiopure pharmaceuticals, agrochemicals, flavor compounds and the conversion of natural fats and oils into high value products such as cocoa butter equivalent and oils enriched with omega-3 fatty acids [2]. Lipases from different sources display variations in properties in terms of their regioselectivity, fatty acid specificity, thermostability, optimum pH and kinetics in solvent systems [3]. Their characteristics have recently accounted for a marked increase in the industrial usage of lipases. The lipases from *Burkholderia* species are among the most widely used enzymes in biotechnology [4] especially from *Burkholderia cepacia* that shows very high transesterification activity in organic solvents [5,6].

Wax esters can be extracted from a variety of natural sources, including honeycomb, jojoba seeds, carnauba, sperm whale, skin lipids, sheep wool and seafowl feathers. They are used in lubricants, polishes, plasticizers and coating materials [7]. With the steadily growing demand for wax esters in the food, pharmaceutical and cosmetic industries, methods for the chemical or enzymatic synthesis of wax esters have been developed. The crude lipase from *Burkholderia* sp. EQ3 could synthesize 95% wax esters from crude fish fat and cetyl alcohol in 6 h [8]. In addition, the wax esters from palm kernel oil and oleyl alcohol were synthesized by Lipozyme RM IM and produced 84% wax esters after 10 h [9] and 90% oleyl oleate was synthesized by Lipozyme IM-20 in 2 h [10].

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In previous work, we isolated a mesophilic lipolytic bacterium from the wastewater pond of a tuna canning factory in southern Thailand. It was identified and named *Burkholderia* sp. EQ3 using 16S rDNA analysis. It synthesized high amounts of wax esters from crude fish fat and cetyl alcohol [8]. In this study the purification and characterization of the lipase of *Burkholderia* sp. EQ3 has been described. The application of the lipase EQ3 for the synthesis of wax esters from different oils in comparison with commercial lipases was also investigated.

2. Materials and methods

2.1. Bacterial strain and immobilized lipases

Burkholderia sp. EQ3 producing an extracellular lipase was isolated from a wastewater pond of the tuna canning factory in southern Thailand and was cultivated in the basal medium in g/L of distilled water (tryptone 1.0, yeast extract 1.0, K_2HPO_4·2.0, KH_2PO_4·1.0, $(NH_4)_2SO_4$·1.0, $MgSO_4$·0.2 and $CaCl_2$·0.15) containing 1% fish oil [8]. This strain was maintained at −20 °C in a medium containing 25% glycerol. The nucleotide sequence data for *Burkholderia* sp. EQ3 appeared in the DDBJ/EMBL/GenBank nucleotide sequence database with the accession number AB563172.1. Immobilized lipases, Novozyme 435 (from *Candida antarctica*), Lipozyme RM IM (from *Rhizopus miehei*) and Lipozyme TL IM (from *Thermomyces lanuginosus*) were obtained from Novozymes (Bagsvaerd, Denmark). Lipase AK (from *Pseudomonas fluorescens*) and lipase PS (from *Burkholderia cepacia*) were provided by Amano (Nagoya, Japan) and were immobilized in the laboratory according to Pawongrat et al. [11] by adsorption onto Accurel MP-100 (size 200–400 μm). The immobilized lipases were stored at 4 °C until used.

2.2. Substrates and chemicals

Coconut oil, soybean oil, olive oil, palm stearin, sunflower oil and palm olein (saponification values; 226, 165, 170, 176, 168 and 173, molecular weights; 735, 1011, 982, 947, 987 and 952 g/mole, respectively) were from a local market (Hat Yai, Thailand). Fish oil and jatropha oil (saponification value; 127 and 558, molecular weight; 302 and 1200 g/mole, respectively) were purified in the laboratory.

Q-Sepharose Fast Flow and Sephadex G-50 were from Amersham Pharmacia Biotech (Uppsala, Sweden). Chromatein Prestained Protein Ladder® (10.5–175 kDa) was from Vivantis (Selangor Darul Ehsan, Malaysia). The Silver Stain Plus kit was from Bio-Rad Laboratories, Inc. (Hercules, California, USA). Celite 545 (200 μm) was from Fluka (Buchs, Switzerland) and Accurel MP-100 (size 200–400 μm) was from Akzo Nobel Membrana (Obernburg, Germany). Oleyl alcohol was supplied by Sigma–Aldrich (St. Louis, Missouri, USA). Other analytical reagent grade chemicals were from local commercial sources.

2.3. Analyses

2.3.1. Lipase activity

The lipase activity was assayed in a two-phase system according to Lee and Rhee [12] using palm olein as a substrate in isooctane. The reaction was incubated at 37 °C by shaking at 300 rpm for 30 min. The lipase activity was determined by measuring the amount of fatty acids liberated as palmitic acid. One unit of enzyme activity was defined as the enzyme necessary to release 1 μmol of palmitic acid per minute at the specified conditions [8].

2.3.2. Determination of protein

The protein in the supernatants and samples from the purification steps as well as the immobilized enzyme was determined using Folin–Ciocalteu reagent according to Lowry et al. [13]. Bovine serum albumin was used as a standard. During chromatographic purification steps, the protein concentration was monitored by measuring the absorbance of fractions at 280 nm (A_{280}) [14].

2.3.3. Determination of wax esters

The percentage of wax esters was determined by thin-layer chromatography and a flame ionization detector (TLC-FID) (IATROSCAN MK5, Iatron Laboratories Inc. (Tokyo, Japan)). The sample diluted in chloroform (1:2, v/v) was spotted onto the chromarod S III (coated with silica gel powder) from Mitsubishi Chemical Medicine Corporation (Tokyo, Japan) and developed for 30 min in a mixture of hexane/diethyl ether/formic acid (64:6.4:0.4, v/v/v) [15]. After development and drying, the rods were subjected to scanning with FID. The percentage of the peak area was assumed to be the percentage content of the corresponding compound [8].

3. Methods

3.1. Lipase production

Burkholderia sp. EQ3 was cultivated in the basal medium containing 1% fish oil [8] as described above. The pH of the medium was adjusted to 6.0 with 0.1 M HCl. Fifty milliliter of the medium in a 250 mL Erlenmeyer flask was seeded with a 10% inoculum and incubated on a rotary shaker at 200 rpm and 37 °C. After 12 h of incubation, the culture supernatant containing extracellular lipase was harvested by centrifugation at 6000 × *g* at 4 °C for 15 min. The cell-free culture supernatant was used for lipase purification.

3.2. Lipase purification

3.2.1. Acetone precipitation

The cell free supernatant was precipitated with 70% chilled acetone overnight [8,16]. The precipitate was collected by centrifugation at 6000 × *g* at 4 °C for 15 min and dissolved in a small volume of 20 mM Tris–HCl buffer pH 7.5. The crude enzyme suspension was dialyzed against the same buffer at 4 °C overnight and concentrated by absorption on carboxymethyl cellulose.

3.2.2. Ion exchange chromatography

The enzyme solution (2.0 mL) after dialysis was applied to the Q-Sepharose Fast Flow (10 mL, 2.2 cm × 3.2 cm) anion exchange column equilibrated with 20 mM Tris–HCl buffer pH 7.5. The column was washed with the same buffer, and the unbound and bound proteins were eluted with a linear gradient from 0 to 2.0 M NaCl. The flow rate was 1.0 mL/min. The collected fractions were assayed for lipase and protein (A_{280}). The lipase active fractions were pooled, dialyzed against 20 mM Tris–HCl buffer pH 7.5 and concentrated by absorption on carboxymethyl cellulose and used for Sephadex G-50 gel filtration chromatography.

3.2.3. Gel column chromatography

The enzyme obtained from the Q-Sepharose Fast Flow step was applied to the Sephadex G-50 column (0.8 cm × 50 cm) previously equilibrated with 20 mM Tris–HCl buffer (pH 7.5). The elution was done using the same buffer at a flow rate of 0.2 mL/min. Fractions of 1.0 mL were collected and assayed for both lipase activity and protein (A_{280}). The lipase active fractions were pooled and concentrated. The specific activity of the purified enzyme of each purification step was compared with that of the crude enzyme and the purification fold was calculated. The samples from the purification process were used for sodium dodecyl sulfate-polyacrylamide