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Rhizopus chinensis lipase: Gene cloning, expression in *Pichia pastoris* and properties

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ABSTRACT

Lipases are the most attractive enzymes for use in organic chemical processes. In our previous studies, a lipase from *Rhizopus chinensis* CCTCC M20102 was found to have very high ability of esterification of short-chain fatty acids with ethanol. In this study, we reported the cloning and expression of the lipase gene from *R. chinensis* in *Pichia pastoris* and characterization of the recombinant lipase. The lipase gene without its signal sequence were cloned downstream to the alpha-mating factor signal and expressed in *P. pastoris* GS115 under the control of *AOX1* promoter. In the induction phase, two bands of 37 kDa and 30 kDa proteins could be observed. The amino-terminal analysis showed that the 37-kDa protein was the mature lipase (30 kDa) attached with 27 amino acid of the carboxy-terminal part of the prosequence (r27RCL). The pH and temperature optimum of r27RCL and mRCL were pH 8.5 and 40 °C, and pH 8 and 35 °C, respectively. The stability, reaction kinetics and effects of metal ions and other reagents were also determined. The chain length specificity of r27RCL and mRCL showed highest activity toward *p*-nitrophenyl hexanoate or glyceryl tricaproate (C6) and *p*-nitrophenyl acetate or glyceryl triacetate (C2), respectively. This property is quite rare among lipases and gives this new lipase great potential for use in the field of biocatalysis.

1. Introduction

Lipases (triacylglycerol ester hydrolases EC 3.1.1.3) are well known hydrolases capable of hydrolyzing the ester bonds of water-insoluble substrates at the interface between substrate and water. Furthermore, contrary to many other enzymes, they show remarkable levels of activity and stability in non-aqueous environments, which facilitates the catalysis of several unnatural reactions such as esterification and transesterification. Because of these unique properties, lipases are the most attractive enzymes for use in organic chemical processes [1].

Lipase-mediated synthesis of aliphatic esters of longer chain substrates has shown their easy esterification abilities [2]. However, the synthesis of low molecular flavor ethyl esters from shorter chain substrate comparatively received less attention with no satisfaction as short fattyacids easily strip the essential water around enzymes to cause their deactivation or cause dead-end inhibition reacting with the serine residue at the active site of lipase [3]. In our previous studies, a fungal strain was isolated from leaven (mouldy grains) samples with very high ability of esterification of short-chain fatty acids with ethanol and was identified as *Rhizopus microsporus* var. *chinensis*, also named as *Rhizopus chinensis* [4]. In further studies two kinds of intracellular lipases were purified from this strain (RCL-lip1 and RCL-lip2), and RCL-lip2 showed a preference to hydrolyze short chain esters and its N-terminal sequence has a high homology with *Rhizopus oryzae* mature lipase [5].

Rhizopus species was mainly divided into three groups, including *R. microsporus*, *R. oryzae*, *Rhizopus stolonifer*, among which *R. oryzae* was investigated more widely. Many individually named isolates, such as *R. oryzae*, *Rhizopus arrhizus*, *Rhizopus delemar* and *Rhizopus javanicus* latterly recognized to be the same organism, in spite of some minor variations, were combined into *R. oryzae* [6]. *R. oryzae* lipase, *R. delemar* lipase and *R. javanicus* lipase have a substitution in the His134 and the Leu234 by an Asn and a Leu, respectively [7]. The lipase gene from *R. stolonifer* (Genebank No. DQ139862) was reported with 84% amino acid sequence identity to *R. oryzae* lipase. However, there is no report on the molecular characterization of lipase from *R. microsporus*.

The production of an active *Rhizopus* lipase has been performed in *Escherichia coli* [8], in *Saccharomyces cerevisiae* [9] and in *Pichia pastoris* [10,11]. Over the last few decades, the *P. pastoris* expression system has been used successfully for production of various recombinant heterologous proteins. This process offers several advantages, such as alcohol oxidase 1 (*AOX1*) gene promoter tightly regulated by methanol, easy growth to high cell densities, high levels of protein expression at the intra- or extra-cellular level, and the ability to perform eukaryotic protein modifications [12]. Several

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other microbial lipases have also been successfully expressed in *P. pastoris*, such as *Candida rugosa* lipase 1[13], *Candida parapsilosis* lipase/acyltransferase [14] and *Candida antarctica* lipase B [15].

In this study, we report the cloning and expression of the lipase gene from *R. chinensis* in *P. pastoris* and characterization of the recombinant lipase.

2. Materials and methods

2.1. Strains, plasmids and growth conditions

R. chinensis CCTCC M201021 producing lipase (RCL) was from our lab. *E. coli* DH5α (*sup*E44Δ*lac*U169(φ80 *lacZ*ΔM15)*hsd*R17 *rec*A1 *end*A1 *gyr*A96 *thi*-1 *rel*A1) has been used for DNA propagation. *P. pastoris* GS115 (*His*$^-$ *Mut*$^+$) and the pPIC9K expression vector are from Invitrogen BV (*Pichia* Multi-Copy Expression Kit, version A, Invitrogen BV, The Netherlands).

R. chinensis CCTCC M201021 was cultivated in the medium contained 10 g/L maltose, 60 g/L peptone, 2 g/L K_2HPO4, 0.5 g/L $MgSO_4{\cdot}7H_2O$ and 20 g/L olive oil, pH of the medium was adjusted to 5.5. *E. coli* was cultivated in Luria–Bertani medium. *P. pastoris* GS115 was grown and maintained in YPD medium (10 g/L yeast extract, 2 g/L peptone, 2 g/L dextrose). The YPD-G418 plates containing different concentrations (0.25–1.0 g/L) of geneticin (G418 sulfate, Invitrogen BV) were used for selection of *Pichia* transformants.

2.2. Cloning of rcl gene and construction of the lipase expression vector

The open reading form of the *rcl* gene was amplified directly from *R. chinensis* genomic DNA using a pair of primers, RCL-F1 (CCG**ATG**GTTTCATTCATTTCCATTTCTC) and RCL-R1 (*GC***TTA**CAAACAGCTTCCTTCGTT) according to the consensus sequence of lipases from other *Rhizopus* sp. (GenBank accession nos. AB013496, DQ139862, AF229435). The PCR fragment was DNA sequenced. Based on the sequence of *rcl* gene, the *rcl* gene without its own signal peptide (*prorcl*) was amplified using a pair of primers, RCL-F2 (ATC*CCTAGG*GTTCCTGTTGCTGGTCATAAAGGTTC) and RCL-R2 (CAGT*GCGGCCGC***TTA**CAAACAGCTTCCTTCGTT). The restriction sites *Avr*II and *Not*Iwere incorporated into the forward and reverse primer sequence, respectively. The PCR fragment was ligated into the respective sites of pPIC9K resulting in pPIC9K-proRCL under the control of the methanol inducible alcohol oxidase 1 promoter (P_{AOX1}) and fused in-frame with the α-factor secretion signal peptide of *S. cerevisiae*.

2.3. Transformation of P. pastoris and selection of His$^+$ multicopy recombinants

P. pastoris GS115 was transformed with 7 μg of *Bgl*II-linearized pPIC9K-proRCL vector by electroporation, and selection of *His*$^+$ transformants was done on minimal dextrose medium (MD, Invitrogen BV) plate. The screening of geneticin resistant was performed on solid YPD-G418 medium. The insertion and methanol metabolization was checked by PCR. The PCR amplifications were carried out according to Invitrogen's recommendations with genomic DNA and primers complementary to the 5′ and 3′ region of the *AOX1* gene. Lipase secretion was assayed at 28 °C on agar plate composed of minimal methanol medium (MM, Invitrogen BV) with 10 g/L olive oil and 1 mg/L fluorescent dye rhodamine B (MM-rhodamine) by the appearance of a fluorescent halo around colonies under UV light.

2.4. Expression of lipase in P. pastoris in shake flasks

The *P. pastoris His*$^+$ transformants were cultured in 25 mL of buffered glycerol-complex medium (BMGY, 10 g/L yeast extract, 20 g/L peptone, 100 mM potassium phosphate, pH 6.0, 13.4 g/L YNB, 4×10^{-4} g/L biotin, 10 g/L glycerol) shaken at 28 °C and 250 rpm in 250 mL glass flasks. When cultures reached an OD_{600} of about 6, the cells were centrifuged and resuspended in 20 mL of buffered methanol-complex medium (BMMY, 10 g/L yeast extract, 20 g/L peptone, 100 mM potassium phosphate, pH 6.0, 13.4 g/L YNB, 4×10^{-4} g/L biotin, 5 g/L methanol) to an OD_{600} of 1.0, shaken at 28 °C and 250 rpm in 100 mL glass flasks for 120 h. The cultures were supplemented with methanol (5 g/L) to induce the expression of lipase every 12 h. The culture was centrifuged, and the supernatant was collected for SDS-PAGE and lipase activity assay.

2.5. Expression of lipase in P. pastoris in bioreactor

Fermentation experiments were performed at 28 °C with 2.8 L volume in a 7-L bioreactor (New Brunswick, BioFlo 110, USA). The inoculums were grown for 18 h at 28 °C in shake flasks at 220 rpm with BMGY medium. In glycerol batch phase, 200 mL of inoculums was directly added into 2.6 L of a Fermentation Basal Salts Medium (40 g/L[1] glycerol, 22.7 g/L H_3PO_4, 0.93 g/L $CaSO_4$, 18.2 g/L K_2SO_4, 14.9 g/L $MgSO_4{\cdot}7H_2O$, 4.13 g/L KOH, 7.0 g/L K_2HPO_4) and trace solution, 12 mL. Trace solution consisted of 6 g/L $CuSO_4{\cdot}5H_2O$, 0.08 g/L NaI, 3.0 g/L $MnSO_4{\cdot}H_2O$, 0.2 g/L $Na_2MoO_4{\cdot}2H_2O$, 0.02 g/L H_3BO_3, 0.5 g/L $CoCl_2$, 20 g/L $ZnCl_2$, 65 g/L $FeSO_4{\cdot}7H2O$, 0.2 g/L biotin, and concentrated sulfuric acid, 0.5% (v/v). The medium was sterilized by filtration. The pH of the medium was adjusted and controlled at 5.0 with the addition of 28% (v/v) ammonium hydroxide. Dissolved oxygen (DO) concentration was maintained always above 30% saturation and controlled in cascade mode: Aeration was kept constant at 1.0 vvm and pure oxygen was supplied as needed, agitation rate kept between 300 rpm and 700 rpm.

The fermentation was operated in glycerol batch phase at 28 °C until all of the glycerol was consumed, which was indicated by a sharp increase in DO. Then Proceed to glycerol fed-batch phase at 28 °C, feeding with 50% (v/v) glycerol containing 1.2% (v/v) trace solution at the average rate of 12.4 g/(L h^{-1}) was performed until the desired biomasses reached (OD_{600} = 90), and when needed, glycerol feeding rates were adjusted to control DO. After half an hour, start the methanol fed-batch phase at 20 °C, in which supplied with 100% (v/v) methanol containing 1.2% (v/v) trace solution and the methanol concentration was kept at about 2.5 g/L controlled by an on-line methanol analyzer (FC2002, Shanghai Super-xinxi, China). Biomass was determined by measuring the optical density (OD 600 nm). The correlation between OD_{600} and dry cell weight (g/L) was a factor of about 3.

2.6. Lipase activity determination

Lipase activity was measured on emulsified *p*-nitrophenyl palmitate (pNPP) according to Kordel et al. [16]. One volume of a 1.08-mM solution of pNPP in 2-propanol was mixed just before used with 9 volumes of 50 mM Tris–HCl buffer pH 8.0 containing 4 g/L Triton X-100 and 1 g/L arabic gum. The standard reaction was started by pre-equilibration of 2 mL of above mixture at 37 °C and addition of 0.1 mL of enzyme solution at an appropriate dilution in 50 mM pH 8.0 Tris–HCl buffer. The variation of the absorbance at 410 nm of the assay against a blank without enzyme was monitored for 2–5 min using a UV–vis spectrophotometer (UNICO UV-3102 PC, China). The reaction rate was calculated from the slope of the curve absorbance versus time, using a molar extinction coefficient of 44,656 cm^{-1} M^{-1} for *p*-nitrophenol. One enzyme unit was defined