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Production of lipase SMG1 and its application in synthesizing diacylglyecrol

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ABSTRACT

Exploration and evaluation of lipases from different sources will always be required by the swiftly developing industries for modification of oils and fats. Lipase SMG1 from *Malassezia globosa* was expressed in recombinant *Pichia pastoris*, and its catalytic activity in the hydrolysis and synthesis of partial glycerides was evaluated. Lipase SMG1 could not hydrolyze soybean oil; however, enhanced the hydrolysis of soybean oil by combining with Palatase 20000L, suggesting it showed strict specificity on mono- and diacylglycerol. Hydrolysates of soybean oil were esterified with glycerol to produce diacylglycerol, and the optimized results for the esterification reaction were with an fatty acids/glycerol molar ratio of 1:4, lipase SMG1 at a concentration of 120 U/g (U/w, with respect to total reactants), initial water content 1% (w/w, with respect to total reactants) and 30 °C, which yielded 62.03% of diacylglycerol in reaction mixture (80.5% in acylglyceride) after 12 h of reaction. The results showed that lipase SMG1 is a prospective enzyme which could be used in the oils and fats industry.

1. Introduction

Diacylglycerols (DAGs) are esters of glycerol in which two of the hydroxyl groups are esterified with fatty acids. Compared with traditional cooking oils (rich in triacylglycerols, TAGs), intake of DAGs can reduce body weight and visceral fat accumulation in rats and humans [1]. Consumption of DAG oil may also produce less postprandial elevation in plasma TAG levels in humans and lower fasting serum TAG concentrations in animals and humans [2]. Studies in animals and humans suggest that DAG, especially 1,3-DAG, have a number of beneficial effects on lipid metabolism [3]. The beneficial functions of DAG are probably attributable to differences in DAG and TAG absorption and metabolism. So the synthesis of DAG becomes more significant because of the minor content of DAG in natural form.

DAG can be produced chemically or enzymatically through esterification, glycerolysis and partial hydrolysis processes. The enzymatic approach provides an alternative process due to its mild performance conditions, the regioselectivity of the lipases and the low environmental impact [4]. Various methods have been reported for the production of DAG using the enzymatic approach such as the glycerolysis of triglycerides [5] and ethyl esters [6], the partial hydrolysis of oils [7], the re-esterification of monoacylglycerol (MAG) and fatty acids [8], and the esterification of fatty acids and glycerol in organic solvents [9] and in a solvent-free system [10]. However, much more attention has been focused on the development of reaction process, and less work has been carried out to evaluate the potential of lipases from different resources in the production of DAG. Among published enzymatic approaches for DAG preparation, lipase RM IM (*Rhizomucor miehei*), Novozym 435 (*Candida Antarctica*) and lipozyme TL IM (*Thermomyces lanuginous*) are mainly selected to catalyze the reaction. So, further exploration and evaluation of lipases from different sources will always be required by the swiftly developing industries for modification of oils and fats.

Lip1 from *Malassezia globosa*, has specific property which shows positive activity to diolein, but negative activity to triolein [11]. It may be a potential enzyme which could be used in oils and fats industry. In this work, lipase SMG1 (Lip1) was expressed in recombinant *pichia pastoris*, and its potential application in the production of DAG was evaluated in detail. Lipase SMG1 catalyzed esterification of glycerol and soybean oil hydrolysates were adopted to produce DAG, and conditions for the reaction process were investigated.

2. Materials and methods

2.1. Materials and reagents

Soybean oil was supplied by Kerry Oils & Grains Ltd. (Shenzhen, China). Glycerol (99.5%) contained 0.2% water was purchased from Guangzhou Chemical Reagent Factory (Guangzhou, China). The

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liquid lipase from *M. miehei* (Palatase 20000L, 8050 U/g) was kindly donated by Novozymes A/S, Bagsvaerd, Denmark. *n*-Hexane and 2-propanol were of HPLC grade from Kermel Chemical Reagent Co., Ltd. (Tianjin). Acylglycerol standards (triolein, diolein, and monoolein) and oleic acid were obtained from Sigma (Shanghai, China).

2.2. Crude *M. globosa* Lip1 production by recombinant pichia pastoris

Lipase SMG1 gene (GenBank accession number XM_001732152.1) was artificially synthesized by Sangon Biotech, Inc. (Shanghai, China) and cloned into the *Kpn*I and *Not* I restriction sites of the pUC57 plasmid. To subclone *smg1* gene into *P. pastoris* expression vector pGAPZαA (Invitrogen), the *smg1* gene fragment was released from pUC57 vector by digestion of *Kpn*I and *Not* I and inserted into the same site of pGAPZαA to generate pGAPZαA-*smg1* vector. The *P. pastoris* X-33 transformants harbouring the pGAPZαA-*smg1* constructive expression vector was used to produce lipase SMG1. Strains were stored in 50% glycerol solution (v/v) at −80 °C.

Seed medium consisting of yeast extract/peptone/dextrose (YPD) medium was inoculated and incubated at 30 °C in a 250 rpm rotary shaker for 24 h. After the cultivation, 50 μL of the seed culture was inoculated in 50 mL liquid medium of YPD in a 300 mL glass flask and this culture was grown under the same conditions above. The YPD medium contained (per liter): 1% (m/v) yeast extract, 2% bactopeptone (m/v) and 2% glucose (m/v). After 72 h of cell culture, the supernatant was clarified by centrifugation (1500 rpm, 4 min, 4 °C), and was filtered through a 0.45 mm cellulose acetate filter and then concentrated by ultrafiltration through a 5 kDa relative molecular mass membrane (Vivaflow 200, Sartorius, Germany). The concentrated supernatant was collected and lyophilized. The lyophilized lipase SMG1 powder was kept at 4 °C.

2.3. Lipase activity assay

Lipase assay was performed with DAG oil (purchased from the Kao Corporation of Japan) emulsion. One unit of lipase (U) is the amount of enzyme which releases 1 μmol of titratable fatty acids per minute under the described conditions. Substrate solution: DAG oil and 2% polyvinyl alcohol solution were emulsified at a volume ratio of 1:3 at 10,000 rpm for 10 min. Analysis conditions: 4 mL of DAG oil emulsion, 5 mL of 0.05 M KH_2PO_4/Na_2HPO_4 buffer (pH 6.0) buffer and 1 mL of enzyme solution were mixed and incubated at 25 °C for 15 min. The reaction was terminated with the addition of 95% ethanol (15 mL) after incubation, and the liberated fatty acids were titrated with 0.05 M NaOH. Blanks were measured with a heat-inactivated enzyme sample, for which an enzyme stock solution was kept at 100 °C for 15 min. After cooling to ambient temperature, the solution was used as described for the active enzyme sample. Lipase activity of Palatase 20000L was determined according to the olive emulsion method [12]. The substrate solution was the emulsion of olive oil and polyvinyl alcohol.

2.4. Effect of temperature on the lipase activity of lipase SMG1

The effect of temperature on the enzyme activity was investigated. Lipase SMG1 was kept under each temperature condition (20–60 °C) for 2 h before activity assay. The activity of the lipase was measured by the method mentioned above.

2.5. Preparation of soybean oil hydrolysates

50 g of soybean oil and 5 g of distilled water were added into a 250 mL Florence flask, and stirred magnetically (IKA RH Basic 2 IKAMAG Magnetic Stirrers) at 25 °C, 200 rpm. 100 U/g (lipase with respect to the soybean oil) of Palatase 20000L and lipase SMG1 were employed as biocatalysts. About 0.1 g of the reaction mixture was withdrawn periodically and centrifuged at 10,000 × *g* for 5 min. The upper layer was transferred into another centrifugation tube and was mixed with anhydrous sodium sulfate and 1 mL of *n*-hexane and 2-propanol (15:1, v/v) by swirling. The mixture was underwent centrifugation at 10,000 × *g* for 1 min, and the supernatants was removed for HPLC analysis.

Reaction mixture was separated after 36 h of hydrolysis of soybean oil, and it was extracted with 100 mL of *n*-hexane, then the *n*-hexane layer was dried over anhydrous sodium sulfate. Soybean oil hydrolysates were obtained by removing hexane with a rotary evaporator at 40 °C under vacuum.

2.6. Lipase SMG1-catalyzed esterification of soybean oil hydrolysates and glycerol to produce DAG

2.8 g of extracted hydrolysates of soybean oil together with different amount of glycerol and lipase were added into 25 mL stoppered conical flask for esterification on magnetic stirrer at a stirring rate of 200 rpm. The reaction conditions of enzyme load (30, 60, 90, 120 and 150 U/g), temperature (25, 30, 35 and 40 °C), initial water content (0, 1%, 2%, 3% and 4%, w/w with respect to total reactants) and substrate molar ratio of glycerol to fatty acids (1:1, 2:1, 3:1, 4:1 and 5:1) were varied for optimization. The reactions were allowed to proceed for 24 h. 20 μL of the reaction mixture was withdrawn at periodic intervals and mixed with 1 mL of *n*-hexane and 2-propanol (15:1, v/v). The mixture was centrifuged at 10,000 × *g* for 5 min to remove the glycerol then the upper layer was filtered through a 0.45 μm nylon membrane to remove the enzyme. 10 μL of supernatants was drawn for HPLC analysis.

2.7. Composition analysis by HPLC

Reaction products were analyzed by Normal Phase HPLC (NP-HPLC, refractive index detector) to separate and quantify the acylglycerols and fatty acids. A phenomenex Luna column (Phenomenex Corporation, 4.6 mm i.d. × 250 mm, 5 μm particle size) was used. The mobile phase was *n*-hexane and 2-propanol (15:1, v/v). The flow rate was 1 mL/min. Peaks in HPLC were evaluated by comparison of their retention times with those of known standards. Peak percentages and areas were calculated using Waters 2695 integration software. Analysis was carried out in triplicate, and the values were of the average of triplicate measurements.

3. Results and discussion

3.1. Preparation of crude lipase SMG1

A recent genome sequence analysis of *M. globosa* showed that the *M. globosa* genome encodes abundant hydrolase genes (e.g. lipases, phospholipases, proteases and sphingomyelinases) [13]. Until now, biochemical properties of Lip1 and Lip2 from *M. globosa* were reported, and Lip1 showed lipase activity [11], Lip2 was verified as an esterase [14]. In this work, Lip1 (named as lipase SMG1 in this study) were overexpressed in recombinant *P. pastoris*. The recombinant lipase SMG1 was secreted into culture media, and then concentrated according to the method described in the materials and methods section. The SDS-PAGE electrophoresis (Fig. 1) showed the band of lipase SMG1 in the crude lipase SMG1 solutions after 72 h cell culture. In contrast to the transformant harbouring plasmid without lipase gene, the recombinant *P. pastoris* cells have a good expression of lipase SMG1. 99.5% of lipase SMG1 yield was obtained when the culture medium was concentrated by ultrafiltration through 5-kDa membrane. The molecular weight was