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Immobilized MAS1 lipase showed high esterification activity in the production of triacylglycerols with n-3 polyunsaturated fatty acids

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ABSTRACT

Immobilization of lipase MAS1 from marine *Streptomyces* sp. strain W007 and its application in catalyzing esterification of n-3 polyunsaturated fatty acids (PUFA) with glycerol were investigated. The resin XAD1180 was selected as a suitable support for the immobilization of lipase MAS1, and its absorption ability was 75 mg/g (lipase/resin ratio) with initial buffer pH value of 8.0. The thermal stability of immobilized MAS1 was improved significantly compared with that of the free lipase. Immobilized MAS1 had no regiospecificity in the hydrolysis of triolein. The highest esterification degree (99.31%) and TAG content (92.26%) by immobilized MAS1-catalyzed esterification were achieved under the optimized conditions, which were significantly better than those (82.16% and 47.26%, respectively) by Novozym 435. More than 92% n-3 PUFA was incorporated into TAG that had similar fatty acids composition to the substrate (n-3 PUFA). The immobilized MAS1 exhibited 50% of its initial activity after being used for five cycles.

1. Introduction

Long-chain n-3 polyunsaturated fatty acids (n-3 PUFA), especially eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), could reduce the risk of atherosclerosis (Abdukeyum, Owen, & Mclennan, 2008) and sudden death (Albert et al., 1998), contribute to reduce the incidence of cardiovascular disease (Cleland, Caughey, James, & Proudman, 2006), chronic inflammatory diseases (Giudetti & Cagnazzo, 2012) and cancer (Corsetto et al., 2011). In recent decades, EPA and DHA concentrates in triacylglycerols (TAG) form have attracted great interest due to their higher stability than those in free fatty acids (FFA) form and higher bioavailability than those in ethyl esters (EE) form (Valenzuela, Valenzuela, Sanhueza, & Nieto, 2005). Therefore, there is a growing demand for n-3 PUFA in the form of TAG with high purity in the pharmaceutical and food industry (Borg, Binet, Girardin, Rovel, & Barth, 2001).

n-3 PUFA-rich TAG can be synthesized chemically or enzymatically. Lipases are most commonly used in enzymatic process due to their high catalytic efficiency, mild reaction conditions, substrate specificity and positional selectivity (Akanbi, Adcock, & Barrow, 2013; Lyberg & Adlercreutz, 2008). Enzymes in immobilized form consume less enzyme, enable their reuse, increase stability, improve the ability to resist stress conditions and facilitate continuous operations (Zhang et al., 2014). Many researchers have reported the synthesis of n-3 PUFA-rich TAG in the solvent-free systems, but the resulting TAG content was low (Moreno-Perez, Luna, Señorans, Guisan, & Fernandez-Lorente, 2015; Sun et al., 2015). Other researchers used solvent systems to synthesize n-3 PUFA-rich TAG. Although high content of n-3 PUFA-rich TAG was obtained in solvent systems, these solvents could result in environmental pollution and food safety (Liu, Zhang, Hong, & Ji, 2007; Wang, Li, Ning, et al., 2012). Different lipases from various sources have been employed to synthesize n-3 PUFA-rich TAG. However, TAG content was less than 90% and maximum n-3 PUFA incorporated into TAG was reported to be 84.5% (Kosugi & Azuma, 1994). Therefore, it is necessary to develop new reaction systems as well as novel lipases with better catalytic properties.

In this paper, a thermostable lipase (named MAS1) from marine *Streptomyces* sp. strain W007 found in our laboratory (Yuan, Lan, Xin, Yang, & Wang, 2015) was immobilized on macroporous resins. The effects of lipase/support ratio and initial buffer pH on immobilization efficiency were studied. Then, the thermal stability, regiospecificity and catalytic ability of lipase MAS1 in free and immobilized form were investigated. Subsequently, immobilized MAS1 was employed in the esterification of glycerol with n-3 PUFA for the production of TAG and the effects of reaction conditions, including temperature, enzyme loading and glycerol/n-3 PUFA ratio were investigated. Furthermore, the reusability of

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immobilized MAS1 was evaluated. Finally, the catalytic properties of immobilized MAS1 were compared with those of Novozym 435 under the optimized conditions.

2. Materials and methods

2.1. Materials

The X33/MAS1 strain was stored in 50% glycerol solution (v/v) at −80 °C. Novozym 435 was obtained from Novo Nordisk (Denmark). DHA/EPA-rich EE were kindly provided by Sinomega Biotech Engineering Co., Ltd. (Zhejiang, China). Lauric acid was obtained from Aladdin Industrial Corporation (Shanghai, China). *n*-Hexane, 2-propanol and formic acid of chromatographic grade were purchased from Kermel Chemical Reagent Co., Ltd. (Tianjin, China). Three kinds of resins (Amberlite XAD1180, HP20 and HP2MGL) were obtained from Rohm and Haas Company (USA). The resins AB-8 and DA201 were purchased from Chemical Plant of Nankai University (Tianjin, China) and Zhengzhou Qinshi Technology Co., Ltd. (Henan, China), respectively. Bovine serum albumin (BSA) was purchased from Shanghai Bio Science & Technology Company (Shanghai, China). Bradford reagent was obtained from Sigma (Wuhan, China). All other chemicals were of analytical grade unless otherwise stated.

2.2. Production of crude lipase MAS1

The pPICZαA-MAS1-X33 expression strain was provided by the enzyme engineering group in our laboratory, and fermentation was carried out according to the method described previously (Wang et al., 2014). After fermentation, the broth was centrifuged (10,000*g* for 30 min, 4 °C) and filtered. Then, the resultant supernatant was concentrated and kept at 4 °C for subsequent immobilization. The hydrolytic activity of the concentrated crude extract containing lipase MAS1 was 940 ± 18 U/mL, according to the olive oil emulsion method described previously (Yang, Wang, & Yang, 2006). The specific activity of free lipase MAS1 was defined as units per mg of protein.

2.3. Immobilization of lipase MAS1 by physical adsorption

The pretreatment processes of macroporous resins were carried out as follows: each macroporous resin (10 g) was initially wetted with 30 mL of 95% (v/v) ethanol for 24 h. After decanting ethanol, the resins were washed with distilled water several times until no ethanol was detected in the supernatant. Then, the resins were mixed with 30 mL of 5% HCl (w/w) for 4 h. After that, the supernatant was filtered out and the resins were rinsed with distilled water repeatedly until the supernatant had a neutral pH value. Subsequently, the resins were immersed in 30 mL of 2% NaOH (w/w) for 4 h and recovered from the supernatant by filtration, washing with distilled water until the pH value of the filtrate was around 7.0. Subsequently, the resins were treated by 0.02 M sodium phosphate buffer (various pH values) for 4 h. Finally, the filtrate was removed and the resins were used for the immobilization of lipase MAS1. The purpose of using ethanol, acid and alkali was to remove bubbles, residual monomers and compounds in the resin pores, respectively.

The immobilization of lipase MAS1 on each macroporous resin was carried out as follows: lipase MAS1 solution (50 mg protein/g resin) was mixed with an equal volume of sodium phosphate buffer (0.02 M pH 8.0), and then 3 g of the pretreated each macroporous resin using sodium phosphate buffer (0.02 M pH 8.0) ahead of time was added to form a mixture. Then, the mixture was stirred by a shaking water bath at 30 °C with a speed of 200 rpm/min for 8 h. After that, the supernatant was filtered out and the obtained immobilized MAS1 was washed with 0.02 M pH 8.0 sodium phosphate buffer several times to remove the enzyme that loosely bound to the resins until no protein was detected in the filtrate. Finally, the immobilized MAS1 was vacuum-dried at 40 °C for 8 h and stored in closed vials at 4 °C before use.

The immobilization conditions such as initial pH (6.0, 7.0, 8.0, 9.0) of buffer, and lipase/support ratio (25, 50, 75, 100, 150 mg/g resin) were varied for optimization.

2.4. Determination of esterification activity and protein content of immobilized MAS1

The esterification activities of immobilized MAS1 and Novozym 435 were determined according to Novozymes Standard Method EB-SM-1069.02 (Basso, Froment, Hesseler, & Serban, 2013). The protein contents of free lipase MAS1 and immobilized MAS1 were determined according to the Bradford assay and a calibration curve using bovine serum albumin was used for protein quantification (Bradford, 1976). The specific activity of immobilized MAS1 was defined as units per mg of protein (U/mg). Protein amount in immobilized enzyme (mg/g) and protein recovery (%) was calculated according to Zhao, No, Kim, Garcia, and Kim (2015).

2.5. FT-IR spectroscopy of immobilized MAS1

Fourier transform infrared (FT-IR) analysis of immobilized MAS1 and XAD1180 resin was carried out using a Nicolet 8210E FT-IR spectrometer in the frequency range of 4000–400 cm^{-1}. The resolution was 2 cm^{-1} and 128 scans. The standard KBr pellet technique was applied for sample preparation. The free enzyme was lyophilized and then mixed with KBr in a mortar. Finally, the mixture was ground and pressed into a pellet for FT-IR analysis.

2.6. Thermal stability studies

The effect of temperature on the stability of free lipase and immobilized MAS1 was investigated by measuring the residual hydrolytic activities of lipases after incubation at 65 °C with continuous shaking for 3 h. Samples were withdrawn every 30 min and their hydrolytic activities were determined as described in Section 2.2. The hydrolytic activity at the beginning was set as 100% and the residual activity of the lipases after incubation was calculated accordingly.

2.7. Regiospecificity of immobilized MAS1

The determination of the regiospecificity of free lipase and immobilized MAS1 was carried out in the hydrolysis of triolein under the conditions of enzyme amount of 30 U/g triolein (U/w, with respect to oil mass), sodium phosphate buffer (0.1 M, pH 7.0) of 20% (w/w, with respect to oil mass) and reaction temperature of 65 °C according to the method described previously (Li et al., 2015). A commercial non-regiospecific enzyme (lipase AYS) was also used to catalyze the hydrolysis of triolein at a temperature of 40 °C. Samples were withdrawn at selected times for high-performance liquid chromatography (HPLC) analysis. The ratio of 1,2(2,3)-DAG to 1,3-DAG was used as an indicator to evaluate the regiospecificity of the lipases. The hydrolytic activities of the lipases were determined as described in Section 2.2.

2.8. Comparison of the catalytic ability of immobilized MAS1 and its free form in the esterification of glycerol and n-3 PUFA

The esterification activities of immobilized MAS1 and lyophilized free lipase MAS1 were measured, respectively, according