Enzyme and Microbial Technology 117 (2018) 56–63

Contents lists available at ScienceDirect

Enzyme and Microbial Technology

journal homepage: www.elsevier.com/locate/enzmictec

Synthesis of conjugated linoleic acid-rich triacylglycerols by immobilized mutant lipase with excellent capability and recyclability

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ARTICLE INFO

Keywords:
Conjugated linoleic acid
Triacylglycerols
Immobilization
Esterification
Recyclability

ABSTRACT

Conjugated linoleic acid (CLA)-rich triacylglycerols (TAG) have received significant attention owing to their health promoting properties. In this study, CLA-rich TAG were successfully synthesized by an immobilized mutant lipase (MAS1-H108A)-catalyzed esterification of CLA-rich fatty acids and glycerol under vacuum. MAS1-H108A was first immobilized onto ECR1030 resin. Results showed that the lipase/support ratio of 41 mg/g was suitable for the immobilization and the thermostability of immobilized MAS1-H108A was greatly enhanced. Subsequently, the immobilized MAS1-H108A was employed for the synthesis of CLA-rich TAG and 95.21% TAG with 69.19% CLA was obtained under the optimized conditions. The TAG content (95.21%) obtained by immobilized MAS1-H108A is the reported highest value thus far, which was significantly higher than that (9.26%) obtained by Novozym 435 under the same conditions. Although the TAG content comparable to the results obtained in this study could also be obtained by Novozym 435, the used enzyme amount is approximately 5-fold of the immobilized MAS1-H108A. Additionally, the immobilized MAS1-H108A exhibited excellent recyclability during esterification retaining 95.11% of its initial activity after 10 batches. Overall, such immobilized mutant lipase with superior esterification activity and recyclability has the potential to be used in oils and fats industry.

1. Introduction

Conjugated linoleic acid (CLA) is a family of isomers of linoleic acid with a conjugated double bond, which occurs in beef, daily foods, and dietary supplements [1,2]. CLA has 28 different isomers, of which 9c, 11t-CLA and 10 t, 12c-CLA are the most biologically active isomers [3,4]. It has been demonstrated that 9c, 11t-CLA and 10 t, 12c-CLA exert various potent physiological functions, such as anticarcinogenic, antiatherogenic, antidiabetic, antiobese, and antihypertensive properties [5,6]. With the America approval of CLA as general recognized as safe in 2008 [7], the consumption of CLA is increasing in recent years. CLA in supplements is mainly present in the forms of free fatty acids (FAs) and triacylglycerols (TAG). Both forms of CLA exhibit comparable absorption and biological activity [8,9]. However, compared with free FAs, CLA existing in the form of TAG is more acceptable, not only because of its good stability but also because of its better taste [9,10]. Therefore, there is a growing interest for the synthesis of CLA-rich TAG.

CLA-rich TAG can be synthesized by chemical or enzymatic methods. Compared with chemical methods, enzymatic synthesis of CLA-rich TAG has gained widespread attention owing to its mild reaction conditions, high specificity, and environmentally benign nature [11,12]. Typically, non-regiospecific or weak regiospecific lipases are employed for the synthesis of CLA-rich TAG by esterification of CLA and glycerol to obtain high TAG content product [4,12,13]. Among the commonly used lipases, Novozym 435 was found to be the most effective enzyme due to its high esterification activity and weak specificity [4,12,13]. Although Novozym 435 is the reported most effective lipase for the synthesis of CLA-rich TAG, a large quantity of Novozym 435 (10%, based on the total substrates) should be added to achieve high CLA-rich TAG content product [4,13]. Therefore, further exploration of novel enzyme resources with superior capability for the synthesis of CLA-rich TAG is of great significance.

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https://doi.org/10.1016/j.enzmictec.2018.06.007
Received 2 January 2018; Received in revised form 29 May 2018; Accepted 17 June 2018
Available online 18 June 2018
0141-0229/

A new lipase of MAS1 from marine *Streptomyces* sp. strain W007 is a thermostable and non-regiospecific lipase, which exhibited great potential in the synthesis of n-3 PUFA-rich TAG during esterification of glycerol and n-3 PUFA-rich FAs [14,15]. To our knowledge, lipases have specificities towards FAs based on the chain length, the number of double bonds, and the position or geometry of the double bonds [16,17]. Although MAS1 showed high specificities towards EPA, DPA, and DHA, the specificities of MAS1 towards CLA are still unknown. Our recent study found that a mutant lipase of MAS1 (MAS1-H108A) with 1.6-fold enzymatic activity of wild type was a thermostable and non-regiospecific lipase [18]. However, to date, less information is available about its catalytic performances in enzymatic reactions.

Generally, physical adsorption is the most commonly used immobilization method by virtue of its simple operation, low cost, and high stability of the immobilized enzymes [19–21]. Some commercial immobilized lipases are prepared by physical adsorption. For example, Novozym 435 is one of the most commonly used immobilized lipases in industries, which is immobilized onto a macroporous acrylic resin Lewatit VP OC 1600 by physical adsorption. Lipozyme RM IM, which has been widely used for the synthesis of structured lipids, is also immobilized onto a macroporous anion exchange resin Duolite 568 by physical adsorption [22–24].

ECR1030 resin, with a surface area of 103 m^2/g and a pore size of 28.6 nm, is a hydrophobic divinylbenzene-crosslinked polymer with methacrylic esters, which has been demonstrated as a new highly robust and cheap carrier for the immobilization of *Candida antarctica* lipase B [25,26]. Compared with other commercial carriers, ECR1030 resin exhibited unprecedented mechanical stability. Although ECR1030 resin was a robust carrier, there is no literature available on the immobilization of other lipases using ECR1030 resin as carrier.

In this study, MAS1-H108A was immobilized onto ECR1030 resin and the potential of immobilized MAS1-H108A in the synthesis of CLA-rich TAG was evaluated. The immobilization process of MAS1-H108A and the properties of immobilized MAS1-H108A were studied. Subsequently, the immobilized MAS1-H108A was used for the synthesis of CLA-rich TAG and effects of reaction variables such as enzyme loading, reaction temperature, and substrate molar ratio on the esterification were investigated. Additionally, the recyclability of immobilized MAS1-H108A was assessed and the capability of Novozym 435 and immobilized MAS1-H108A in the synthesis of CLA-rich TAG was compared. Finally, the positional distribution of FAs in the synthesized TAG was analyzed.

2. Materials and methods

2.1. Materials

CLA was purchased from Qingdao Auhai Biotech Co., Ltd. (Shandong, China). ECR1030 resin was purchased from Purolite Co., Ltd. (Zhejiang, China). MAS1-H108A was produced in our lab. Glycerol was purchased from Richjoint Co., Ltd. (Shanghai, China). Novozym 435, the immobilized *Candida antarctica* lipase B with an esterification activity of 9760 U/g, was purchased from Novozymes A/S (Bagsvaerd, Denmark). Porcine pancreatic lipase was purchased from Sigma-Aldrich (Shanghai, China). Thin layer chromatographic (TLC) plate was purchased from Qingdao Haiyang Chemical Co., Ltd. (Shandong, China). *n*-Hexane, 2-propanol, and formic acid were of chromatographic grade from Kermel Chemical Reagent Co., Ltd. (Tianjin, China). Standard compounds of trioleoyl glycerol, dioleoyl glycerol, monoolein, and the 37-component FAME mixture (C4:0-C24:0) were purchased from Sigma-Aldrich (Shanghai, China). Heptadecanoic acid was purchased from Sigma-Aldrich (Shanghai, China). All other solvents and chemicals were of analytic grade.

2.2. Production and immobilization of MAS1-H108A

2.2.1. Production of MAS1-H108A

MAS1-H108A was produced according to the method described previously [18]. Briefly, the expression vector pGAPZαA containing the mutant gene of MAS1-H108A was electroporated into *P. pastoris* strain X-33, and then the transformants were cultured on an YPD medium at 30 °C on a rotatory shaker (200 rpm) for 72 h. The broth supernatant was collected by centrifugation at 4 °C (10,000 × g, 10 min) and concentrated by across flow cassette with a 10 kDa cut-off membrane (Sartorius, Germany). The protein concentration of the fermentation broth was 1.05 mg/mL. The hydrolytic activity of the fermentation broth was determined as described by Li et al. [27]. The hydrolytic and specific activity of the fermentation broth were 1000 U/mL and 952.38 U/mg, respectively.

2.2.2. Immobilization of MAS1-H108A

The crude fermentation broth of MAS1-H108A was directly immobilized onto ECR1030 resin. Briefly, 5 g ECR1030 resin, a certain volume of enzyme solution (1.05 mg protein/mL) with an equal volume of phosphate buffer (20 mM, pH 7.5) were added into a 2-L conical flask. Subsequently, the flask was placed into an air bath shaker with an agitation speed of 200 rpm and temperature of 30 °C for the immobilization of MAS1-H108A. In this study, to study the effects of different lipase/support ratios on the immobilization of MAS1-H108A, five different lipase/support ratios (17, 25, 33, 41, and 49 mg/g, means 1 g of support adding 17, 25, 33, 41, or 49 mg protein for immobilization) were chosen. Before and after immobilization, the supernatant was withdrawn for the determination of protein concentration according to the Bradford method [28]. After immobilization, the immobilized MAS1-H108A was collected through a Buchner funnel and dried in a vacuum oven at 30 °C for 8 h. The moisture content of the immobilized MAS1-H108A was detected by IR-35 moisture analyzer at 110 °C for 10 min. The protein loading of immobilized MAS1-H108A was calculated according to the method of Zhao et al. [29]. The esterification activity of immobilized MAS1-H108A was determined according to the Novozymes propyl laurate unit (PLU) method EB-SM-1069.02 [25]. The specific activity of immobilized MAS1-H108A was expressed as units per mg of protein (U/mg). The immobilized MAS1-H108A was stored in the refrigerator at 4 °C until use.

2.3. Characterization of the free and immobilized MAS1-H108A

2.3.1. Effects of pH on the activity of free and immobilized MAS1-H108A

Effects of pH on the activity of free and immobilized MAS1-H108A were studied. The selected pH values of buffer solution ranging from 5.0 to 9.0. The used buffer solutions were as follows: citrate buffer (25 mM, pH 5.0, 5.5), phosphate buffer (25 mM, pH 6.0, 6.5, 7.0, 7.5), Tris-HCl buffer (50 mM, pH 8.0, 8.5, 9.0). The hydrolytic activity of free and immobilized MAS1-H108A was determined according to the method of Li et al. [30]. Briefly, the substrates were a mixture of olive oil and 4% (w/w, based on the total weight of polyvinyl alcohol and distilled water) polyvinyl alcohol solution with the mass ratio of olive oil to 4% polyvinyl alcohol of 1:3. The mixture was emulsified at 10,000 rpm for 10 min before being used for the following activity assay. After emulsification, 4 g emulsion and 5 mL of buffer solution were added into a 100-mL conical flask and incubated at 60 °C for 5 min. Reactions were initiated when free or immobilized MAS1-H108A was added. For the control groups, heat-inactivated enzyme samples were added for the hydrolytic reactions. One unit of hydrolytic activity (U) was defined as the amount of enzyme which releases 1 μmol FAs per min under the described conditions.

2.3.2. Effect of temperature on the activity of free and immobilized MAS1-H108A

Effects of temperature on the activity of free and immobilized