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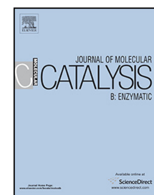

Lipase *Candida* sp. 99-125Coupled with β-cyclodextrin as additive synthesized the human milk fat substitutes

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ABSTRACT

Lipids with specific distribution of fatty acids could benefit people in various aspects. Structured lipids rich in palmitic acid at the sn-2 position and oleic acid at the sn-1,3 positions are a mimic of human milk fat, which has been used as human milk fat substitute (HMFS). The aim of this work was to optimize the production of HMFS from blends of lard and oleic acid by applying an improved enzymatic synthesis. A cheap lipase *Candida* sp. 99-125 was introduced, meanwhile β-cyclodextrin was chosen to improve the product yield. A significant increase of 1,3-dioleoyl-2-palmitoylglycerol (OPO) was obtained by coupling of β-cyclodextrin through optimization of the key parameters of the process. The influence of additives on the process was also discussed. The results indicated that β-cyclodextrin was an effective additive for OPO synthesis. Due to the function of β-cyclodextrin, the yield of OPO significantly increased from 40.2% to 55.3% under the optimal condition, and the thermal stability of the lipase was improved as well. The cost of biocatalyst was decreased by using lipase *Candida* sp. 99-125 coupled with additive, which would make the process attractive for HMFS production.

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1. Introduction

Functional lipids have now become a high research priority around the world because of their excellent physiological benefits, and potentiality to reduce the risk of chronic disease [1,2]. Among the functional lipids, one of the most popular lipids is human milk fat substitute (HMFS), which is a mimic of human milk fat (HMF). The structure of triacylglycerol (TAG) in HMFS is completely different from that in natural oils. In HMFS, palmitic acid primarily dominates at sn-2 position of TAG. However, palmitic acid oppositely predominates at sn-1 and sn-3 positions of TAG in plant oils [3–5]. The different locations of palmitic acid in TAG will lead to complete opposite digestion and absorption patterns in infants. These differences come from the catalytic process of pancreatic lipase in digestive system. Pancreatic lipase is a 1,3-specific lipase, which will selectively hydrolyze the fatty acids at sn-1 and sn-3 positions in TAG and produce 2-monoacylglycerol (2-MAG) [6]. TAGs with palmitic acids locating at sn-1,3 positions would be hydrolyzed to free palmitic acids and then accumulated in infants. However, these saturated fatty acids are easily bonded with calcium to form calcium soap, which will lead to energy and calcium loss and finally aggravate infant pediatric constipation [7]. As a result, TAG in a USU (U stands for unsaturated fatty acids, S stands for saturated fatty acids) structure, in particularly the 1,3-dioleoyl-2-palmitoylglycerol (OPO), will benefit infants better [8].

The productive and commercial technology HMFS synthesis has raised considerable attention in recent years. Generally, the modification of oils could be divided into three types: acidolysis, interesterification, and multiple-step enzymatic catalysis [9–16]. The yield of OPO in the multiple-step enzymatic catalysis was around 80% and was higher than that produced by other methods. However, interesterification and acidolysis were more available to carry out through simple procedures. In the modification of oils, raw materials are typically TAGs from natural resources or chemical modifications. In most cases, the biocatalysts are generally sn-1,3 specific lipases of commercial sources with high prices, for instance, Novozyme 435, Lipozyme RM, Lipozyme TL

Abbreviations: M(C14:0), myristic acid; P(C16:0), palmitic acid; Po(C16:1), palmitoleic acid; S(C18:0), stearic acid; O(C18:1), oleic acid; L(C18:2), linoleic acid; FA, fatty acid; FAME, fatty acid methyl ester; TAG, triacylglycerol; MAG, monoacylglycerol; PPP, tripalmitin; OPO, 1,3-dioleoyl-2-palmitoylglycerol; OPP, 1,2-dipalmitoyl-3-oleoylglycerol; POP, 1,3-dipalmitoyl-2-oleoylglycerol; OOP, 1,2-dioleoyl-3-palmitoylglycerol; OOO, triolein.

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Table 1
Analysis of fatty acid composition (mol%) and distribution of lard.

	C14:0	C16:0	C16:1	C18:0	C18:1	C18:2
Total	1.76 ± 0.14	26.87 ± 0.20	2.89 ± 0.26	15.71 ± 0.57	41.17 ± 0.85	11.6 ± 0.09
%sn-2[a]	78.01 ± 6.39	81.92 ± 1.08	49.68 ± 4.49	8.65 ± 0.90	12.97 ± 0.23	15.64 ± 0.91
sn-1,3[a]	0.58 ± 0.18	7.29 ± 0.47	2.18 ± 0.32	21.53 ± 0.92	53.74 ± 1.06	14.68 ± 0.24

[a] The results were calculated by Eqs. (1) and (2).

[17]. "InFat^{TM}" patented the process where *Rhizopus oryzae* lipase modified double-fractioned palm stearin and free fatty acids from fractioned palm kernel oil [18]. Furthermore, preparation of HMFS through acidolysis of lard and soybean fatty acids by Lipozyme RM was also recorded [10]. In another case, Srivastava et al. optimized the incorporation of tripalmitin with oleic acid and methyl oleate by using *Candida rugosa* lipase LIP1 and Lipozyme RM [19]. Lipase with high sn-1,3 selectivity is the key issue for the site-specific HMFS, and the cost of lipase dominated the whole cost of the production.

A strain of *Candida* sp. 99-125 was obtained by our lab. It has been successfully applied on the synthesis of biodiesel, sugar fatty acid esters, wax esters, monoesters and diesters [20–24]. Recently, it was found that in some specific conditions, *Candida* sp. 99-125 lipase demonstrated sn-1, 3 specific selectivity that could firstly be applied for the production of HMFS. In this work, β-cyclodextrin was chose [25] to improve the production of HMFS from lard and oleic acid, and a series of experiments were carried out to optimize the synthesis process, and also to decrease the cost of biocatalyst.

2. Materials and methods

2.1. Materials

Lard was purchased from a local manufacturer, Tianjin TianYuan Oils & Fats Co., Ltd. Tripalmitin (PPP), 1,2-dipalmitoyl-3-oleoylglycerol (OPP), 1,3-dipalmitoyl-2-oleoylglycerol (POP), 1,2-dioleoyl-3-palmitoylglycerol (OOP), 1,3-dioleoyl-2-palmitoylglycerol (OPO), triolein (OOO), 37-Component FAME Mix, and *Pancreatic* lipase were purchased from Sigma–Aldrich, St. Louis, MO, USA. Oleic acid (O), β-cyclodextrin and all solvents were of analytical or chromatographic grade, and they were purchased from Beijing Chemical Works. Thin-layer chromatography (TLC, 10cm × 20 cm) was purchased from Qingdao Haiyang Chemical Co., Ltd. Lipase (*Candida* sp. 99-125) was obtained by our lab according to previous work [26].

2.2. Methods

2.2.1. Determination of fatty acid compositions in lard

Lard was converted into FAMEs following the method described by Nie and then analyzed on GC [20]. The result was illustrated in Table 1. All the results were the mean of triplicated experiments.

2.2.2. Lipase activity assay

The lipase activity assay was carried out according to a modified olive oil emulsion method [25].One activity unit of lipase was defined as the amount of enzyme that released 1 mmol of fatty acid per minute under the assay condition.

The activity of lipase *Candida* sp. 99-125 was 39680 U/g lard.

2.2.3. Enzymatic acidolysis

Reactions of lard and oleic acid were conducted by lipase *Candida* sp. 99-125 (added at 397, 1984, 3968, 5952, and 7936 U/g lard, respectively) at 35–55 °C for 16 h in a reaction mixture of lard/oleic acid(1/0.5–1/4, *w*/*w*) at 80 rpm. β-cyclodextrin was added as additive at 1–10% (to the weight of lard). Water was also added at 1–5% (to the substrates) to figure out its influence. Samples were taken every 2 h and then stored at −20 °C for further analysis.

2.2.4. Isolation of TAG

TLC was employed to isolate TAG from the samples. Developing solvent was petroleum ether/diethyl ether/acetic acid 90/10/0.5(v/v/v). The TAG band was script off and extracted with dichloromethane. The separated TAG was used for further analysis.

2.2.5. HPLC-ELSD analysis

The HPLC was equipped with an ELSD detector (LT II, Shimdzu, Japan), two high-pressure pumps (LC-20AT, Shimdzu, Japan), a column oven (CTO-10AS, Shimdzu, Japan), and an air generator (GAX-10L, Beijing HuiJia YingYi CO., LTD). The column connected to the detector was a ChromSpher Lipids column (250mm × 4.6 mm ID, 5 μm particle size, silver ion impregnated, Agilent J&W Technologies, USA).

The characterization of different fatty acids substitution in TAGs was performed with a gradient elution. Solvent A and B were acetone and dichloromethane, respectively. The HPLC program followed the work of Chen [27]. The injection volume was 20 μl at a flow rate of 0.8 ml/min. The yields of the products were qualified by external standards. The yield of OPO was its relative yield to the whole TAG yield. All the results were the mean of triplicated experiments of each point.

2.2.6. Determination of FA composition and location in TAGs

FA compositions at sn-2 and sn-1,3 positions were determined by *Pancreatic* lipase through hydrolysis according to the IUPAC method [28].

$$\%sn-2 = \frac{sn-2FA}{3 \times TotalFA} \times 100\% \quad (1)$$

$$sn-1,3 = \frac{3 \times TotalFA - sn-2FA}{2} \quad (2)$$

2.2.7. Lipase recovery

After the reaction, the lipids, lipase, and additive were separated by centrifugation at 4100 × *g* for 10 min. The liquid phase was structured lipids, and the solid phase was lipase and β-cyclodextrin. Lipase and β-cyclodextrin were put into repeated reaction without further purification. The activity of the lipase was expressed as the yield of OPO in the recycling process.

3. Results and discussion

3.1. Influence of raw material ratio

The contents of fatty acids of lard is shown in Table 1. C16:0 and C18:1 were the dominated fatty acids in lard. The composition of FA in lard was much similar to that of human milk fat [29]. Thus, it was a substrate both available and renewable to produce HMFS.

The influence of the substrate ratios (lard/oleic acid, w/w) on the enrichment of OPO is demonstrated in Fig 1. The increase of the molar ratio will promote the equilibrium. When the ratio of substrates was higher than 1/2, the yield of OPO kept steady and stayed around 40%. When the ratio of lard to oleic acid was 1/4, the best incorporation of OPO obtained at 43%. However, a higher

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