

Short Communication

Substrate selectivity and optimization of immobilized SMG1-F278N lipase in synthesis of propylene glycol monooleateXingxing Li¹, Pengzhan Liu¹, Faez Iqbal Khan², Daoming Li¹, Bo Yang³ and Yonghua Wang¹¹ School of Food Science and Engineering, South China University of Technology, Guangzhou, P. R. China² School of Chemistry and Chemical Engineering, Henan University of Technology, Zhengzhou, P. R. China³ School of Bioscience and Bioengineering, South China University of Technology, Guangzhou, P. R. China

In the present study, the immobilized SMG1-F278N lipase was employed for the esterification of 1,3-propylene glycol with oleic acid. This was the first report of using mono- and diacylglycerol lipase for the production of propylene glycol monoesters (PGME). It was found that immobilized SMG1-F278N preferred 1,3-propylene glycol than 1,2-propylene glycol. Molecular docking of 1,2-propylene glycol and 1,3-propylene glycol into SMG1-F278N suggested that the 1,3-propylene glycol preferentially binds to the active pocket of SMG1-F278N as compared to 1,2-propylene glycol. The maximum 1,3-propylene glycol monooleate content of 70.67% was obtained under the reaction conditions of 1,3-propylene glycol/oleic acid ratio of 5:1 (mol/mol), enzyme loading of 7.5% (w/w, with respect to total substrates), and water addition of 7% (w/w, with respect to total substrates) at 30°C. The present work offers insights into the selectivity of immobilized SMG1-F278N towards 1,2-propylene glycol and 1,3-propylene glycol, and suggests the extended applications of immobilized SMG1-F278N for industrial purpose.

Practical applications: To our knowledge, the production of PGME using mono- and diacylglycerol lipases was reported for the first time. This study could contribute to develop potential applications of immobilized SMG1-F278N in industries.

Keywords: Enzymatic catalysis / Immobilized lipase / Molecular docking / Propylene glycol monooleate / Substrate selectivity

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1 Introduction

Propylene glycol monoesters (PGME) are good water-in-oil emulsifiers, possessing low hydrophile-lipophile balance (HLB) values [1]. In United States, PGME have been ratified by the Food and Drug Administration (FDA) for the application in the pharmaceutical and food industries [2]. Commonly, PGME mixed with mono- and diglycerides were utilized in the production of cake, margarine, and bread [3].

Unsaturated PGME have superior emulsibility than saturated ones and oleic acid has prominent effect in preventing cardiovascular disorders. Hence, PGME of oleic acid are more feasible than saturated PGME in food industry [4].

The production of PGME has been achieved by chemical methods. For example, esterification of fatty acid with propylene glycol could be catalyzed using acid or alkaline at a temperature over 220°C, however, those methods lead to the formation of complex byproducts such as lactones and isomers of PGME, and even toxic compounds [5, 6]. Enzymatic catalysis has been considered as a trend for the synthesis of PGME due to its mild reaction conditions, low energy consumption, high substrate selectivity, and regioselectivity [7–9]. In the past, in-depth and comprehensive researches on the enzymatic synthesis of unsaturated monoacylglycerols have been carried out, but very few studies on enzymatic synthesis of unsaturated PGME. Till date, there was no report on the synthesis of PGME using

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Abbreviations: FDA, Food and Drug Administration; HLB, hydrophile-lipophile balance; PGME, propylene glycol monoesters

mono- and diacylglycerol lipases. Therefore, it is essential to investigate the esterification of propylene glycol with oleic acid catalyzed by immobilized mono- and diacylglycerol lipase SMG1-F278N.

SMG1 is a mono- and diacylglycerol lipase, from *Malassezia globose*, and show specific activity on mono- and diacylglycerol but not on triacylglycerol. SMG1 has an atypical activation mechanism because only two positions residues 102 and 278 are responsible for a gating mechanism that regulates the active and inactive states of the lipase [10]. It was found that a single mutation (F278N) could increase the activity of SMG1 significantly [10]. In our previous study, the mutant SMG1-F278N was designed by site directed mutagenesis which employed PCR method and the pGAPZaA-SMG1 plasmid was as template [11]. Compared with wild type SMG1, the hydrolytic activity SMG1-F278N was increased sixfold [10]. And the esterification activity of SMG1-F278N was improved threefold relative to wild type SMG1 [12]. Compared with free lipase, the thermostability of SMG1-F278N was increased significantly after immobilization on the resin ECR8285. Moreover, immobilized enzyme could be reused for several times and the activity retention of esterification of glycerol and oleic acids by immobilized SMG1-F278N was 98% after seven consecutive cycles [13]. Therefore, the immobilized SMG1-F278N may have great potential applications in industries.

In the present work, the selectivity of immobilized SMG1-F278N towards 1,2-propylene glycol and 1,3-propylene glycol has been studied. The effects of molar ratio of substrates, temperature, enzyme loading, and water addition on esterification of 1,3-propylene glycol with oleic acid were also investigated.

2 Materials and methods

2.1 Materials

Oleic acid (85%) was purchased from Aladin Reagent (Shanghai, P. R. China), and 1,2-propylene glycol (99%), and 1,3-propylene glycol (98%) were from Macklin Biochemical Co., Ltd. (Shanghai, P. R. China). Acylglycerol standards of 1,2-propylene glycol monooleate and 1,2-propylene glycol dioleate were obtained from Alfa-Chemistry (NY, USA). 1,3-Propylene glycol monooleate and 1,3-propylene glycol dioleate were purchased from Sigma-Aldrich Co., LLC. (St. Louis, MO, USA). 2-Propanol, *n*-hexane and formic acid were of HPLC grade from Kermel Chemical Reagent Co., Ltd. (Tianjin, P. R. China). All other chemicals were of analytical grade.

2.2 Preparation of immobilized lipase SMG1-F278N

Immobilized SMG1-F278N was prepared according to the method suggested by Li et al. [13]. Briefly, SMG1-F278N was immobilized onto the ECR8285 resin. The supernatant

of crude lipase SMG1-F278N was mixed with equal volume of 1.5 M $\text{NaH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$ buffer (pH 6.0) before it was added to conical flask, and a certain amount of ECR8285 resin was also added to conical flask which lipase/support ratio is 20 mg/g, and then they were stirred by thermostatic air bath shaker at 200 r/min at 30°C for 7 h.

2.3 Selectivity of immobilized SMG1-F278N

Immobilized SMG1-F278N was applied to catalyze the esterification of 1,2-propylene glycol and 1,3-propylene glycol with oleic acid for producing propylene glycol monooleate. The reaction mixture consisted of 2 g substrates which was 2:1 (mol/mol) propylene glycol/ oleic acid ratio, 5% (w/w, with respect to total substrates) water addition, and 5% (w/w, with respect to total substrates) enzyme loading, and they were stirred at 30°C by a thermostatic air bath shaker at 200 r/min. Samples (20 μL each) were withdrawn periodically and diluted with 980 μL *n*-hexane, 2-propanol, and formic acid (21:1:0.003, v/v/v) for HPLC analysis.

2.4 Molecular docking study

Molecular docking is a method which predicts the preferred orientation of ligand into the protein to understand the mechanism of substrate selectivity [14]. The open conformation of SMG1-F278N was obtained by homology modeling methods using crystal structure of *Rhizomucor miehei* lipase (PDB ID: 4TGL) as template as predicted by Guo et al. [10, 15]. Open conformation of SMG1-F278N mutant was generated using mutate module of Swiss-PdbViewer [16]. After mutation, the structure was subjected to optimization and energy minimization. Molecular docking of 1,2-propylene glycol and 1,3-propylene glycol into SMG1-F278N were carried out. 1,2-propylene glycol and 1,3-propylene glycol were docked by defining the grid box with spacing of 1 Å and size of 60 × 60 × 60 pointing in *x*, *y*, and *z* directions around the protein following the standard docking protocol [17, 18] using AutoDockTools and AutoDock-Vina [19] with default docking parameters. The Lamarckian Genetic Algorithm [20] was used as the search algorithm with default parameter values. The most suitable docked conformation was taken for further analysis.

2.5 Production of 1,3-propylene glycol monooleate

Enzymatic synthesis of 1,3-propylene glycol monooleate was carried out with mixture of 2 g substrates (propylene glycol to oleic acid molar ratio of 1:1, 2:1, 3:1, 4:1, 5:1, and 6:1), immobilized SMG1-F278N (1, 2.5, 5, 7.5, and 10% w/w, with respect to total substrates), and water (0, 1, 3, 5, 7, and 9% w/w, with respect to total substrates). The mixture was stirred at different temperatures (25, 30, 35, 40, 45, and 50°C) by a thermostatic air bath shaker at 200 r/min. Samples (20 μL each) were withdrawn periodically and diluted with

980 μ L solvent consisting of *n*-hexane, 2-propanol, and formic acid (21:1:0.003, v/v/v) for HPLC analysis.

2.6 HPLC analysis

HPLC analysis of the products was performed on a normal-phase HPLC with a refractive index detector according to the protocol described by Li et al. [13]. Isocratic elution was performed at 30°C with a flow rate of 1 mL/min, and mobile phase of *n*-hexane/2-propanol/formic acid (21:1:0.003, v/v/v) was employed. The retention times for 1,2-propylene glycol dioleate, 1,3-propylene glycol dioleate, oleic acid, 1,2-propylene glycol monooleate, and 1,3-propylene glycol monooleate were 3.10, 3.21, 3.73, 7.49, and 9.73 min, respectively. Waters 2695 integration software was applied to calculate the peak-areas percentages [13].

Propylene glycol monooleate content was defined as Eq. (1) in this study:

Propylene glycol monooleate content (%)

$$= \frac{A_1(\%)}{A_2(\%)} \times 100 \quad (1)$$

Where A_1 (%) was the peak-area percentage of propylene glycol monooleate and A_2 (%) was the summery of the peak-area percentages of propylene glycol monooleate, propylene glycol dioleate, and oleic acid in the chromatograms, respectively.

2.7 Statistical analysis

All experiments were conducted in triplicate. Significant differences in the means were evaluated by using significant differences test and variance analysis ($p < 0.05$). The results were showed as mean $\pm$ SD.

3 Results and discussion

3.1 Selectivity of immobilized SMG1-F278N

Changes of content of propylene glycol monooleate during the reaction are shown in Fig. 1a. The results revealed the

selectivity of immobilized SMG1-F278N towards 1,2-/1,3-propylene glycol. The content of 1,3-propylene glycol monooleate was significantly higher than that of 1,2-propylene glycol monooleate. This suggested the immobilized SMG1-F278N preferred 1,3-propylene glycol over 1,2-propylene glycol. The contents of conversion, monooleate, and dioleate used 1,3-propylene glycol and oleic acid as substrates are shown in Fig 1b. The results suggested that the content of 1,3-propylene glycol monooleate reached maximum after 12 h, while the content of 1,3-propylene glycol dioleate increased gradually. It was due to the fact that there were two processes of the reaction. The product of first process was 1,3-propylene glycol monooleate and the product of second process was 1,3-propylene glycol dioleate used monooleate as substrate. There was transformation between 1,3-propylene glycol monooleate and 1,3-propylene glycol dioleate. A portion of 1,3-propylene glycol monooleate was transferred into 1,3-propylene glycol dioleate with the extension of the reaction time.

Molecular docking is a tool which can suggest a preferential substrate for the enzyme. In this study, a molecular docking trial was carried out to help comprehend the selectivity of the substrate. The catalytic triad (Ser171–Asp228–His281) of SMG1 in open conformation are accessible for the substrate. The lid domain of SMG1 in closed conformation showed as a loop, while the generated model has lid in the form of α -helix [15]. To predict substrate selectivity, open conformation of SMG1-F278N was generated, and the substrate 1,2-propylene glycol and 1,3-propylene glycol were docked into the catalytic pocket of SMG1-F278N. The suitable docked pose was selected by mimicking the crystal structure of SMG1 F278N mutant complex with glycerol (PDB:4ZRD). The docked complex where 1,3-propylene glycol was present at the same position of glycerol in crystal structure was selected for further analysis. It was found that 1,3-propylene glycol preferentially binds to the active pocket of SMG1-F278N than 1,2-propylene glycol (Fig. 2). 1,3-propylene glycol shows interaction with the nucleophilic O_Y of Ser171 at a distance of 2.5 Å, while 1,2-propylene glycol shows no interaction with active site residue. The 1,3-propylene glycol has additional hydrogen bond with Asn278 at a distance of

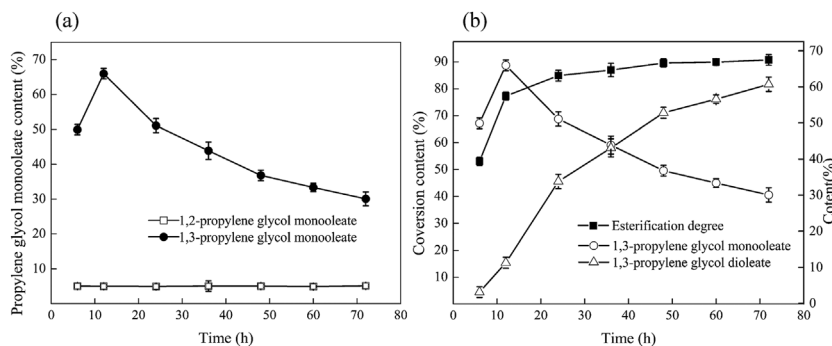


Figure 1. Selectivity of immobilized SMG1-F278N towards 1,2-propylene glycol and 1,3-propylene glycol. (a) Changes of content of 1,2-/1,3-propylene glycol monooleate during the reaction; (b) Change of esterification degree and contents of 1,3-propylene glycol monooleate and dioleate during reaction. Reaction conditions: Molar ratio of propylene glycol to oleic acid (mol/mol), 2:1; water addition, 5% (w/w); enzyme loading, 5% (w/w); temperature, 30°C; shaking speed, 200 rpm.

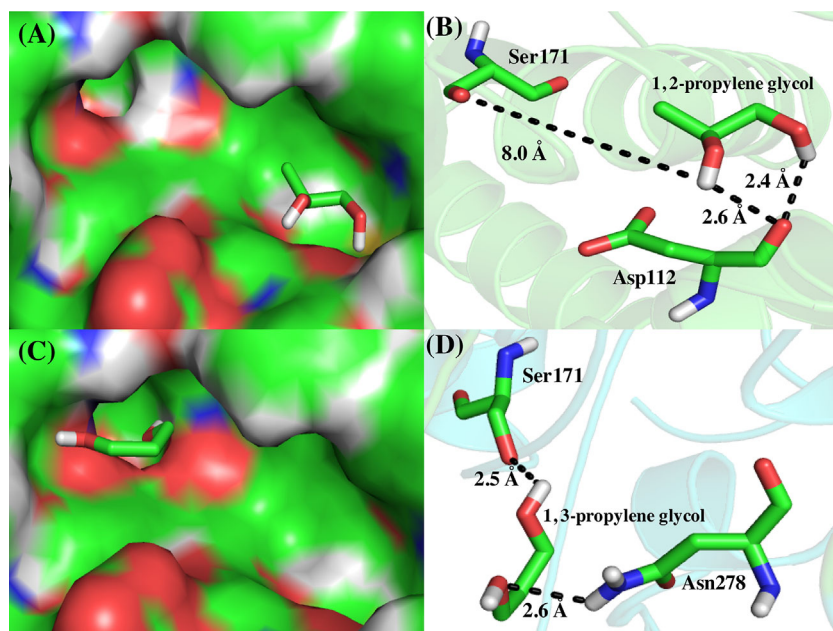


Figure 2. Molecular docking analysis. Surface view of most suitable binding position and orientation of (A) & (B) 1,2-propylene glycol and (C) & (D) 1,3-propylene glycol docked into the catalytic pocket of SMG1-F278N.

2.6 Å, while 1,2-propylene glycol shows interaction with Asp112. The distance between 1,2-propylene glycol and nucleophilic O_Y of Ser171 was found to be 8 Å, that is not favorable distance for enzymatic catalysis. This might be the reason for higher selectivity of SMG1-F278N towards 1,3-propylene glycol than 1,2-propylene glycol.

3.2 Optimization of production of 1,3-propylene glycol monooleate

3.2.1 Effect of molar ratio of 1,3-propylene glycol to oleic acid on esterification

The effect of molar ratio of substrates on esterification was evaluated and the results are shown in Fig. 3. By the end of the reaction, the content of 1,3-propylene glycol monooleate raised according to the increasing of 1,3-propylene glycol/oleic acid ratio. However, there were no significant different in the content of 1,3-propylene glycol monooleate when the 1,3-propylene glycol/oleic acid ratio was raised from 5:1 to 6:1. It could be explained that the transformation equilibrium between 1,3-propylene glycol monooleate and 1,3-propylene glycol dioleate was achieved and the content of 1,3-propylene glycol monooleate was achieved saturation at these ratio. Therefore, the 1,3-propylene glycol/oleic acid ratio was determined at 5:1 (mol/mol) for the subsequent studies by overall consideration.

3.2.2 Effect of temperature on esterification

Effect of temperature on the 1,3-propylene glycol monooleate content was investigated (Fig. 4). During the whole process, the contents of 1,3-propylene glycol monooleate in the reaction at 25°C and 30°C were much higher than those

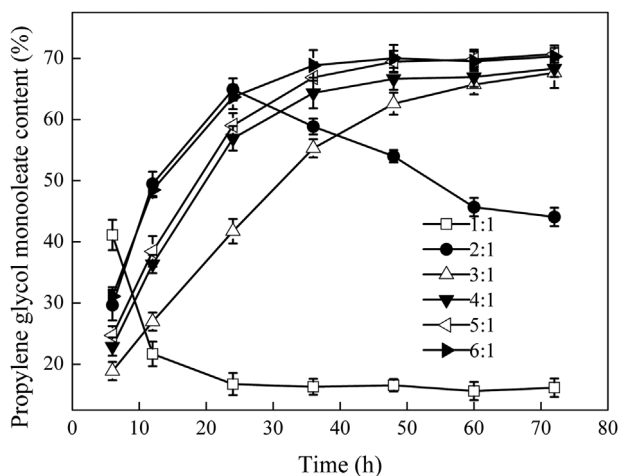


Figure 3. Effect of molar ratio of 1,3-propylene glycol to oleic acid on esterification. Reaction conditions: Different molar ratio of 1,3-propylene glycol to oleic acid (mol/mol); water addition, 5% (w/w); enzyme loading, 5% (w/w); temperature, 30°C; shaking speed, 200 rpm.

of other temperatures, and by the end, the reaction at 30°C produced more 1,3-propylene glycol monooleate than that at 25°C. This showed that the immobilized lipase SMG1-F278N had its highest catalytic activity at 30°C.

The content of propylene glycol monooleate was found to be decreased, when the temperature was increased from 30°C. Especially when the temperature raised from 40 to 50°C, it was declined sharply and the content of 1,3-propylene glycol monooleate was about 10% merely. This might be caused by the denature of lipase SMG1-F278N at

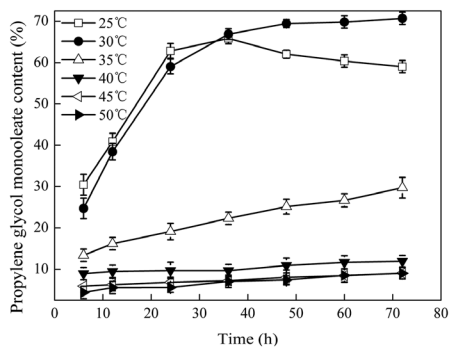


Figure 4. Effect of temperature on esterification. Reaction conditions: Molar ratio of 1,3-propylene glycol to oleic acid (mol/mol), 5:1; water addition, 5% (w/w); enzyme loading, 5% (w/w); different temperature; shaking speed, 200 rpm.

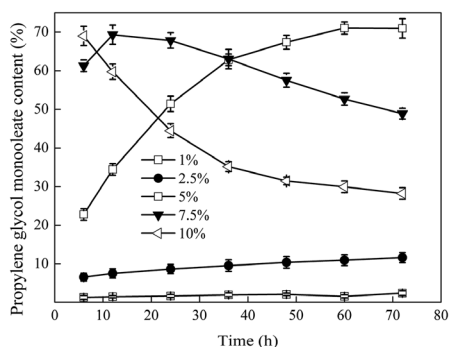


Figure 5. Effect of enzyme loading on esterification. Reaction conditions: Molar ratio of 1,3-propylene glycol to oleic acid (mol/mol), 5:1; water addition, 5% (w/w); different enzyme loading; temperature, 30°C; shaking speed, 200 rpm.

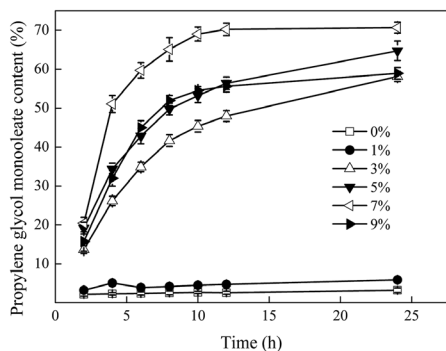


Figure 6. Effect of water addition on esterification. Reaction conditions: Molar ratio of 1,3-propylene glycol to oleic acid (mol/mol), 5:1; different water addition; enzyme loading, 7.5% (w/w); temperature, 30°C; shaking speed, 200 rpm.

this temperature. So, the optimum temperature of 30°C was selected for further study.

3.2.3 Effect of enzyme loading on esterification

The impact of enzyme loading on the esterification reaction was also studied. The results are presented in Fig. 5. When the enzyme loading was less than 5% (w/w), although the content of 1,3-propylene glycol monooleate increased with time, however, after 3 days reaction, the contents of the products were still less than 10%. The content of propylene glycol monooleate could be as high as 70% after 72 h, when 5% of lipase SMG1-F278N was used. The speed of 1,3-propylene glycol monooleate production was accelerated with increasing enzyme loading and a portion of 1,3-propylene glycol monooleate was transferred into 1,3-propylene glycol dioleate with the extension of the reaction time. When 7.5% and 10% (w/w) of enzyme loading were added, the content of monooleate reached about 70% in 12 and 6 h, respectively, but after that the content of 1,3-propylene glycol monooleate reduced gradually. This was due to the generation of 1,3-propylene glycol dioleate.

The optimal enzyme loading of 7.5% (w/w) was chosen because the maximum content of 1,3-propylene glycol monooleate (69.32%) was achieved at 12 h. Therefore, enzyme loading of 7.5% (w/w) was used in subsequent experiments and the reaction time was shortened to 24 h.

3.2.4 Effect of water addition on esterification

The yield of 1,3-propylene glycol monooleate reached the maximum value with water addition of 7% (w/w) (Fig. 6). When the water addition was over 7%, the yield of 1,3-propylene glycol monooleate decreased dramatically which might be explained by hydrolysis of products. Hence, the optimum water addition was found to be 7% (w/w).

4 Conclusions

During the process of esterification of 1,2-propylene glycol and 1,3-propylene glycol with oleic acid, the content of 1,3-propylene glycol monooleate was found to be significantly higher than that of 1,2-propylene glycol monooleate. The results of molecular docking revealed that 1,3-propylene glycol preferentially binds to the active pocket of SMG1-F278N than 1,2-propylene glycol. Therefore, immobilized SMG1-F278N may preferred 1,3-propylene glycol than 1,2-propylene glycol. The 1,3-propylene glycol monooleate content was up to 70.67% and the optimal reaction condition was 5:1 (mol/mol) 1,3-propylene glycol/oleic acid ratio, temperature of 30 °C, 7.5% (w/w) enzyme loading, and 7% (w/w) water addition. In summary, this study offers insights

into the selectivity of immobilized SMG1-F278N towards 1,2-propylene glycol and 1,3-propylene glycol, and provide a vital information about the industrial application of immobilized SMG1-F278N.

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