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The weakened 1,3-specificity in the consecutive microwave assisted enzymatic synthesis of glycerides

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Abstract

The nonthermal effect of microwave irradiation (MI) on the 1,3-specificity in the enzymatic esterification of glycerol and caprylic acid in the presence or absence of the solvent was studied, respectively. Comparison on the 1,3-specificity was made between reactions driven by low power consecutive microwave irradiation and conventional heating (CH). For the four assayed lipases, the microwave irradiation did not change but weaken the lipase's 1,3-specificity regardless the form of the lipase employed in the solvent-free medium. Novozyme 435 was then selected for the further investigation to reveal the changing of the 1,3-specificity resulted by varying reaction parameters under consecutive microwave irradiation and conventional heating, respectively. The influence of the molar ratio of the substrates, the initial water dosage, the solvents with different log *P*, and the power of the microwave irradiation on the 1,3-specificity was investigated. In any circumstance assayed, the consecutive microwave irradiation did not change but weaken the 1,3-specificity of Novozyme 435 in the presence or absence of the solvent.

Keywords: 1,3-Specificity; Microwave; Lipase; Nonthermal effect; Glyceride

1. Introduction

Glycerides are of functional additives in drug, food and cosmetics. The lipase 1,3-specificity and its regulation are very important in the enzymatic synthesis of glycerides, which results different product composition (Scheme 1) and consequential functions, as demonstrated in the well documented enzymatic synthesis of glycerides using conventional heating (CH) [1–6]. Meanwhile, microwave irradiation (MI) has been employed in chemical synthesis of glycerides but not reported for the enzymatic approach. However, as a fast growing technology, microwave has been approved as an efficient tool to accelerate reactions with or without enzyme. The knowledge or the utilizing of the nonthermal effect of microwave irradiation in chemical reaction has been a very curious and challenge objective for organic chemists [7–15]. For example, it is found that microwave can change the affinity of Lipozyme RM IM (LRI) (a lipase from *Mucor miehei* immobilized on an anionic resin) to the short chain alcohols [9]; and also the irradiation can influence the enzyme selectivity on esterification [12,13]. Therefore it would be necessary to study that how the nonthermal effect of microwave would affect the lipase 1,3-specificity. Unfortunately, most researchers barely take account of a constant microwave power when they kept the reaction temperature constantly; or did not consider temperature control during microwave irradiations [17]. In addition, there is still lack of detailed report regarding the effect of microwave irradiation on the 1,3-specificity. In this paper, to study the nonthermal effect of microwave on the 1,3-specificity, we first investigated the performance of some 1,3-specific lipases in the consecutive microwave assisted and conventional heating synthesis of glycerides without solvent, respectively. Subsequently, we studied the variety of the 1,3-specificity of Novozyme 435 in the esterification of glycerol and caprylic acid by means of consecutive microwave irradiation and conventional heating, in the presence or absence of the solvent, respectively.

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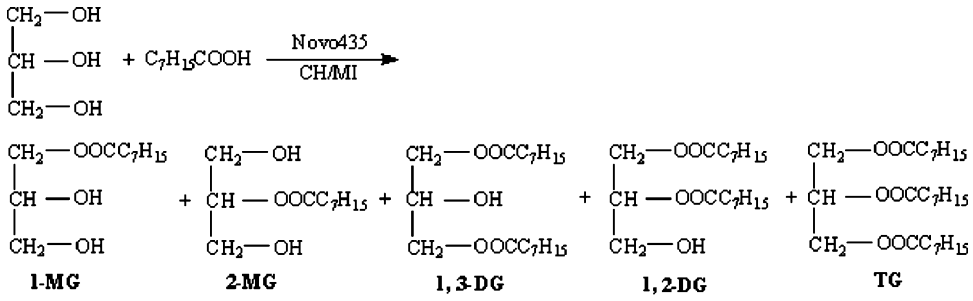


Scheme 1. The synthesis of glycerides.

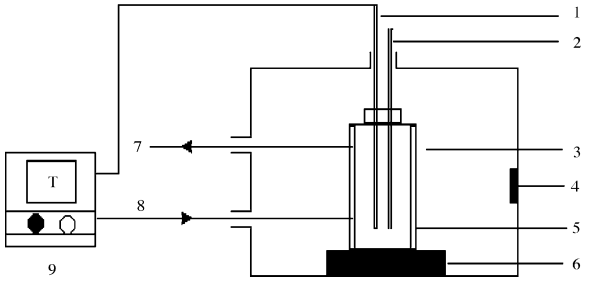
2. Experimental

2.1. Enzymes and chemicals

Novozyme 435 (a *Candida antarctica* lipase immobilized on a macroporous polyacrylic resin), Lipozyme RM IM (a *M. miehei* lipase immobilized on an anionic resin), and Lipolase 100 L (a liquid lipase) were generous gifts from Novozymes China. Lipase AY30 (a purified non-specific lyophilized *Candida rugosa* lipase) was presented kindly by Amano. All chemicals were of analytical grade. All liquid reagents were dehydrated with 4 Å molecular sieves before use. Deionized water was used, if any, throughout the all experiment.

2.2. Consecutive microwave reactor

Most commercial microwave reactors keep the temperature constant by providing intermittent or continuous but inconstant irradiation. Otherwise, they simply allow the temperature of reaction mixture to rise by offering consecutive irradiation with constant power. Therefore, a homemade consecutive microwave reactor [9] was employed in this study to investigate the non-thermal effect. We built a microwave reactor (Scheme 2) by modifying a variable frequency microwave oven with a cooling system and a temperature feedback loop, which can ensure the microwave irradiation providing constant power and keeping the reaction temperature constant as well. Our patented reaction system [16] contains a modified microwave oven (NS552, National Co., 2450 MHz) equipped with a magnetic stirring apparatus, configured with a cooling jacket glass cylinder reactor (20 mm in diameter, 60 mm in height) in the oven, a microwave-shielding sampling hole at the top of the oven, and a temperature control loop. The temperature control loop includes the cooling jacket, the pump, the microwave-shielding thermo sensor, and a temperature monitor. The thermo sensor is connected to the temperature monitor with an electromagnetic feedback loop, feeding back signals to the electromagnetic valve to control the flow of cooling media. Thus the temperature controlling system is able to keep the reaction at a desired temperature constantly (±1 °C) under consecutive microwave. The cooling media does not absorb microwave irradiation. The microwave irradiation was kept at 200 W constantly during the reactions unless otherwise stated.



Scheme 2. Sketch of the home-made microwave reactor. 1, microwave-shielding thermo sensor; 2, sampling hole; 3, jacket glass cylinder reactor; 4, microwave 14 generator; 5, cooling jacket; 6, magnetic stirring apparatus; 7 and 8, cooling media; 9, temperature monitor.

2.3. Microwave irradiation assisted enzymatic synthesis of glycerides

All reactions were conducted with both two heating modes, e.g. microwave irradiation and conventional heating, respectively.

In a typical experiment, the reaction mixture containing a mixture of caprylic acid (25 mmol), glycerol (25 mmol), solvent if any (in this case, 5 mmol of caprylic acid, 5 mmol of glycerol and 90 mmol of solvent) and water (12% of glycerol, w/w) was homogenized in a 20 mL glass reactor at 65 ± 1 °C, unless otherwise stated. The reactor was then placed into the microwave reactor chamber immediately. The reaction was initiated once the enzyme was added and well mixed with the reaction mixture under magnetic stirring (400 rpm), along with the continuous microwave irradiation (200 W, unless otherwise stated) simultaneously. The reaction mixture was sampled periodically, filtrated to remove the enzyme particles, if any, and analyzed by HPLC.

As for the reactions conducted using conventional heating, all reaction conditions were same as the aforementioned, but without microwave irradiation.

2.4. Analysis

The reaction was stopped by adding 15 mL of ethanol to the sampling vessel. After removing the unreacted glycerol from the samples, the compositions of the caprates were determined with HPLC. The conditions for the HPLC determination were as follows: Kromasil CN column, 4.6 mm × 250 mm, column temperature was 35 °C, mobile phase was hexane:isopropyl alcohol (9:1, v/v) with flow rate of 1.0 mL/min, and RI detector.

Caprylic acid conversion was defined as the percentage of consumed caprylic acid and measured by alkaline titration.

The initial reaction rate of the enzymatic esterification was defined as the millimoles of the consumed caprylic acid per minute in 1 g reaction mixture using 1 g lipase (mmol g^{-1} min^{-1} g^{-1}) in the initial reaction phase, determined by alkaline titration and calculated from the linear portion of the time course of caprylic acid concentration. All measurements were made in triplicate.

The 1,3-specificity was defined as the molar ratio of 1-monoglyceride to 2-monoglyceride (1-MG/2-MG) and the molar ratio of 1,3-diglyceride to 1,2-diglyceride (1,3-DG/1,2-DG) in the product, and determined with the HPLC analysis based on the ratio of the peak areas.

3. Results and discussion

3.1. 1,3-Specificity of different lipases

The solvent-free esterification of glycerol with caprylic acid catalyzed by several commercial lipases was carried out under conventional heating or microwave irradiation, respectively. Each reaction was run at the optimal reaction temperature for the corresponding lipase [4].

As indicated in Table 1, all assayed lipases are of 1,3-sepecific in the synthesis of glycerides, and all of them presented weaker 1,3-specificity under microwave irradiation compared to their performance under conventional heating. This may be caused by the entropy increase of polar glycerol molecules under microwave irradiation, which also increased the collision between the 2-hydroxyl groups and the lipase.

Then we chose Novozyme 435 as the catalyst to further investigate that how the reaction parameters influence the 1,3-specificity under consecutive microwave irradiation and conventional heating, respectively.

3.2. Composition of the glycerides at different reaction phase

To observe a noticeable microwave effect from the experiment, first, we have to choose a suitable reaction temperature which is close to the optimum conventional reaction temperature and at which the microwave irradiation obviously accelerates the reaction rate. In addition, because of the reaction thermodynamics, the 1,3-specificity, which is presented by the ratio of glyceride isomers in the reaction mixture, is related to the sampling time for the measurement. Therefore, it is necessary to choose a suitable sampling phase at which the ratio of 1-monoglyceride to 2-monoglycerides is high enough for a reliable comparison, and the conversion of caprylic acid is high enough to provide enough 2-monoglyceride and diglycerides for accurate HPLC measurement. Hence, we tested the initial reaction rates at different temperature (Fig. 1) and the 1,3-specificity at different reaction phases (Fig. 2) under both heating modes. The optimum conventional heating reaction parameters [4] except the temperature were employed in the experiment.

Table 1
Effect of microwave irradiation on the 1,3-specificity of lipases

Lipases	1-MG/2-MG			1,3-DG/1,2-DG		
	CH	MI	MI/CH	CH	MI	MI/CH
Lipozyme RM IM	55.6	28.4	0.51	34.3	11.8	0.34
Novozyme 435	56.4	35.0	0.62	25.6	13.5	0.52
Lipolase 100 L	68.3	45.4	0.66	30.3	17	0.56
Lipase AY30	6.6	4.4	0.67	3.2	1.7	0.53

Glycerol:caprylic acid = 1:1 (mol:mol); initial water dosage: 12% of glycerol (w/w); lipase loading: 3.2 g/mol caprylic acid; caprylic acid conversion: 20%; reaction temperature: LRI (50 °C), Novozyme 435 (65 °C), Lipolase 100 L (40 °C) and lipase AY30 (40 °C).

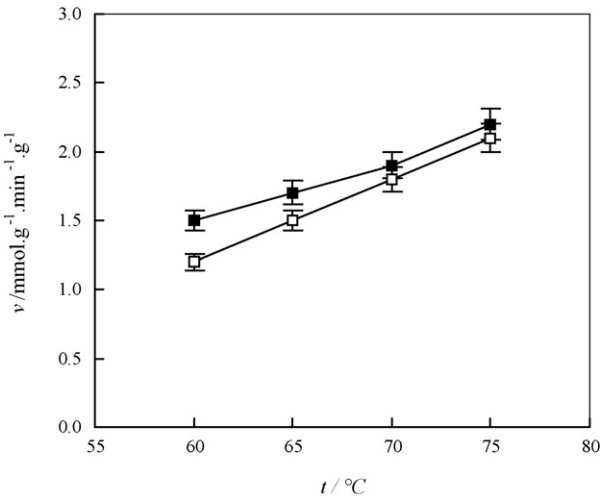


Fig. 1. The initial reaction rate vs. the reaction temperature; *n*(glycerol):*n*(caprylic acid) = 1:1; water dosage: 12% of glycerol (w/w); Novozyme 435 loading: 1.6 g/mol caprylic acid. (□) CH; (■) MI.

As shown in Fig. 1, the microwave irradiation notably accelerated the reaction rate at the optimum conventional heating reaction temperature (65 °C, at which the lipase performed a nice stability and activity [4]), so the subsequent experiments will be conducted at 65 °C.

Fig. 2 indicates that the microwave irradiation affected the reaction course (Fig. 2a and b) obviously at the conversion of caprylic acid of 20–40%. The influence tends to decrease with the proceeding of the reaction; Besides, the yields of MG and DG are almost in parallel (Fig. 2c) at the conversion of caprylic acid of 20–40%, therefore, which could be chose as the sampling period for the subsequent studies to provide reliable analysis.

3.3. Effect of initial water dosage

Initial water dosage can strongly influence the enzymatic esterification rate and equilibrium conversion, which is one of the key factors for the reaction kinetics and thermodynamics. Besides, water is a very strong microwave absorber, so that the initial water dosage may also influence the reaction not only on the reaction rate but also on the 1,3-specificity.

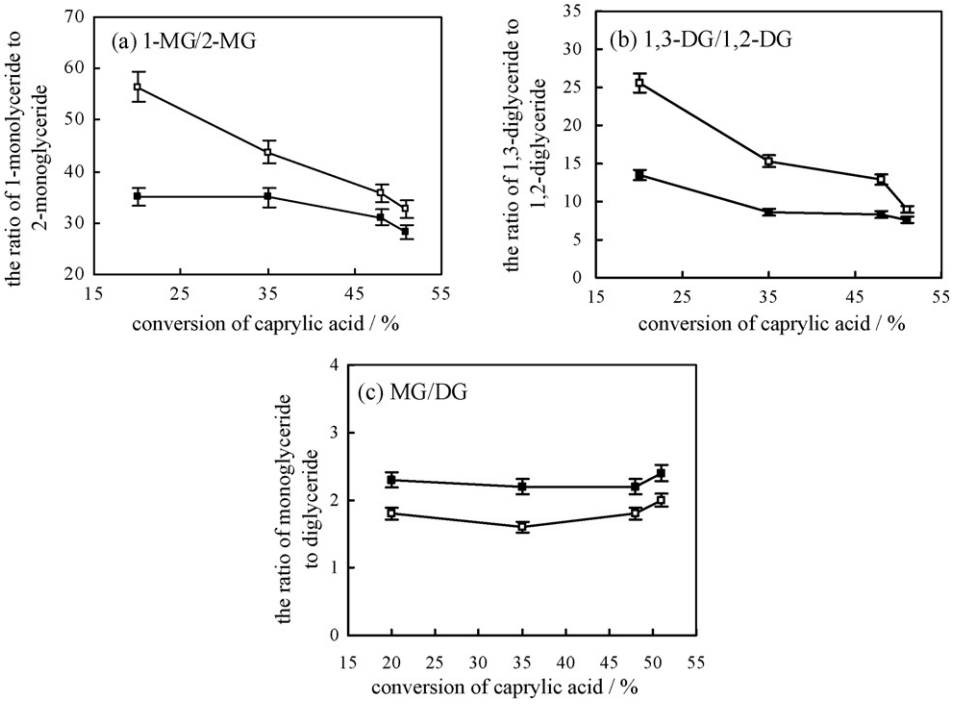


Fig. 2. Composition of the glycerides at different reaction phase. Solvent-free; glycerol:caprylic acid = 1:1 (mol:mol); 65 °C; Novozyme 435 loading: 3.2 g/mol caprylic acid. (□) CH; (■) MI.

As one can see from Fig. 3, the microwave irradiation did not change but weaken the 1,3-specificity of Novozyme 435 independent on the initial water dosage. The increase of initial water dosage did not obviously enhance the MG/DG and 1,3-DG/1,2-DG. However, it did decrease the 1-MG/2-MG, which is reasonable according to the fundamental reaction thermodynamics. In addition, the parallel curves indicate that the microwave irradiation weakened the 1,3-specificity with a very similar strength regardless the water dosage even at a caprylic acid conversion of 40% (meaning the yield of water was 40% also). This can only tell us that in this polar reaction mixture, the role of water under

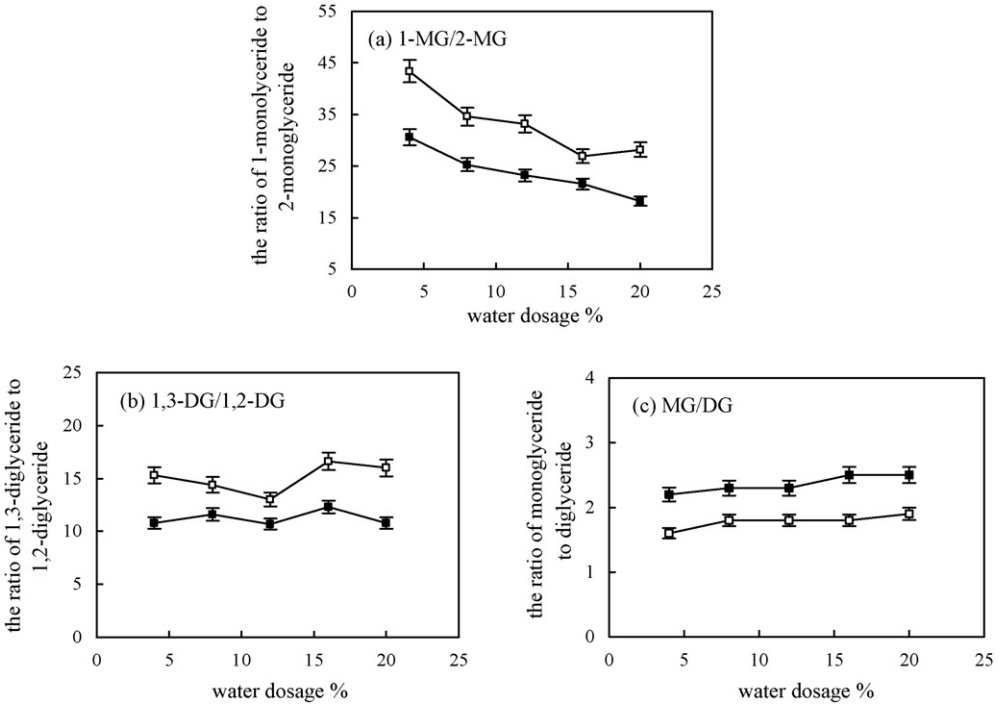


Fig. 3. Effect of microwave irradiation on the 1,3-specificity of Novozyme 435 with different water dosage. Solvent-free; glycerol:caprylic acid = 1:1 (mol:mol); 65 °C; lipase loading: 3.2 g/mol caprylic acid; caprylic acid conversion: 40%. (□) CH; (■) MI.

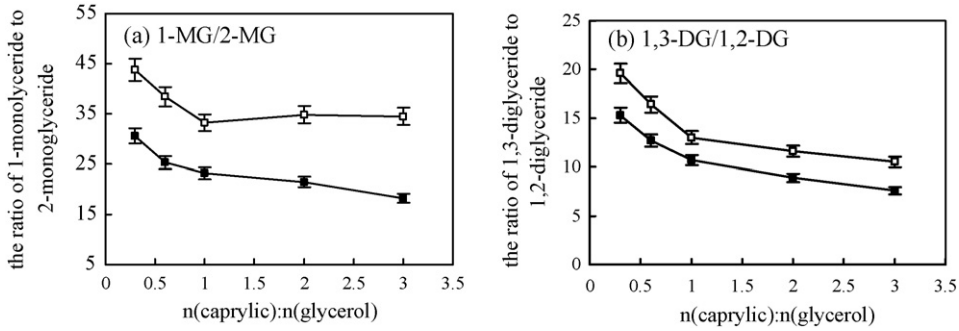


Fig. 4. Effect of microwave irradiation on the 1,3-specificity of Novozyme 435 with different ratio of substrate. Solvent-free; water dosage: 12% of glycerol (w/w); 65 °C; lipase loading: 3.2 g/mol caprylic acid; caprylic acid conversion: 40%. (□) CH; (■) MI.

microwave irradiation is not notable because of its relative small amount.

3.4. Effect of molar ratio of caprylic acid to glycerol

Although the reaction thermodynamics encourages a high 1,3-specificity with a lower molar ratio of caprylic acid to glycerol, but considering the different performance of the acid and glycerol under microwave irradiation, it may cause some difference; hence, we investigated the effect of the molar ratio on the 1,3-specificity (Fig. 4).

When the ratio of caprylic acid to glycerol was lower than 1, which is in the thermodynamic range of favoring the formation of MG, the curves resulted from CH and MI are just parallel. This means the changes of the molar ratio contributed equally to both heating modes, but still, microwave irradiation weakened the 1,3-specificity at any tested range of the molar ratio. However, in Fig. 4a, the difference on the 1-MG/2-MG between the two heating modes became lager and larger with the increasing molar ratio when the ratio is higher than 1, implying that the excessive acrylic acid performed stronger microwave effect, which is apparently a nonthermal effect. It is interesting that we observed a different phenomenon with the same change of the molar ratio of caprylic acid to glycerol in Fig. 4b: the two curves are almost parallel in the entire tested range, although the microwave irradiation also weakened the 1,3-specificity at any tested range. This could be explained with entropy increase and molecules collision probability, as diagrammatized in Scheme 3.

3.5. Effect of the solvent

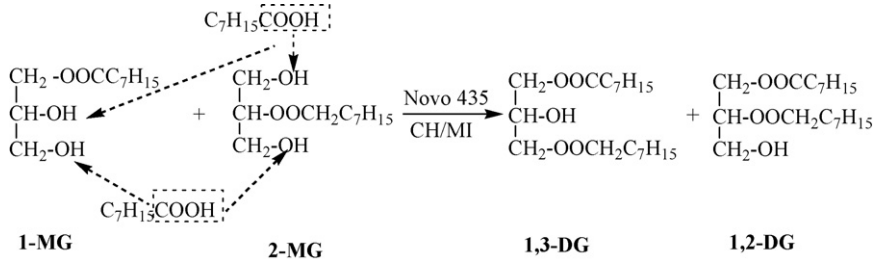
All the aforementioned experiments were performed without additional solvent. Nevertheless, how it will be if we conducted the esterification in organic solvents? As we all know, the enzymatic reaction is related with solvent polarity, so aiming to reveal the influence of the solvent polarity on the 1,3-specificity under both heating modes, experiments were carried on as shown in Table 2 with solvents of different log *P*.

Table 2
Effect of microwave irradiation on the 1,3-specificity of Novozyme 435 with different solvents

Solvents	log *P*	1-MG/2-MG			1,3-DG/1,2-DG		
		CH	MI	MI/CH	CH	MI	MI/CH
No solvents (control)	–	56.4	35	0.62	25.6	13.5	0.52
Dioxane	−0.27	45.3	43.5	0.96	26.3	17.8	0.68
Ethylene glycol dimethyl ether	−0.21	33.6	27.8	0.83	27.5	18.8	0.68
Tert-butanol	0.35	30.8	19.8	0.64	13.9	7.5	0.54
Ethyl acetate	0.73	44.6	36.2	0.81	25.6	19.0	0.74

Glycerol:caprylic acid:solvent = 5:5:90 (mol:mol:mol); 65 °C; lipase loading: 8 g/mol caprylic acid; caprylic acid conversion: 20%.

In most of the conventional heating reactions, the addition of the solvent decreased the 1,3-specificity; it may be caused by the increased freedom of the glycerol molecules otherwise it is hard to explain with any current theory. Nevertheless, all the values of MI/CH obtained in Table 2 were higher than the control, meaning the solvents assayed in microwave field, more or less, their addition improved or less decreased the 1,3-specificity, which may be because that in the diluted environment, the enhanced molecule oscillation of the polar substrate caused by the irradiation turns to be dominating. But still, it is out of our expectation that the influence on the 1,3-specificity is independent of the



Scheme 3. Schematic formation of DG with increased entropy and molecules collision probability caused by microwave irradiation.

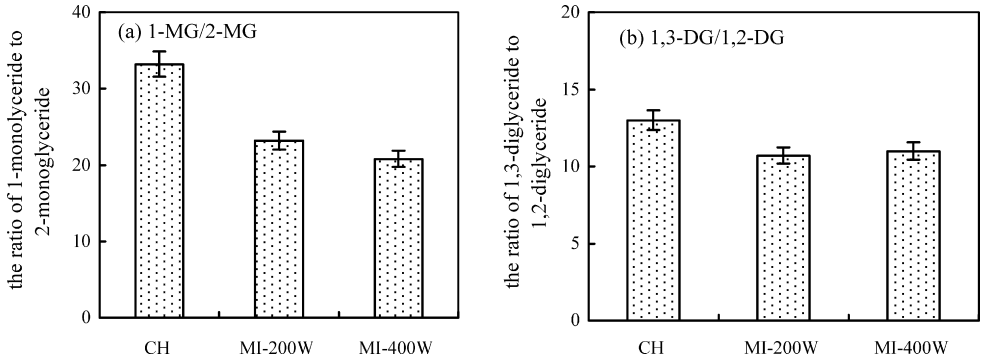


Fig. 5. Effect of microwave irradiation on the 1,3-specificity of Novozyme 435 with different power; *n*(glycerol):*n*(caprylic acid) = 1:1; water dosage: 12% of glycerol (mass); reaction temperature: 65 °C; lipase loading: 3.2 g/mol caprylic acid; conversion of caprylic acid: 40%.

log *P*, maybe in this case, log *P* is not a good polarity parameter to be used for the judgment.

3.6. Effect of the microwave power

Fig. 5 shows a similar result with above sections that increasing the power of the irradiation did not induce any obvious change; However, it also weakened the 1,3-specificity.

4. Conclusions

The microwave irradiation presented some nonthermal effects on the 1,3-specificity in enzymatic esterification of glycerol and caprylic acid, in the presence or absence of the solvents. First, the microwave irradiation can accelerate the enzymatic esterifacation of glycerol and capryic acid. Second, all tested lipases did not change but weaken their 1,3-specificity when the microwaved esterification was conducted under the corresponding optimum conventional heating reaction conditions, no matter the lipase was immobilized or formulated in aqueous solution. For a immobilized lipase Novozyme 435, varying the reaction parameters such as the molar ratio of the substrates, the initial water dosage, the reaction media with different polarity, and the microwave power did not change the 1,3-spcifity of Novozyme 435 in the microwaved esterification, but weaken it either, comparing to its performance in the conventional heating esterification.

Theoretically, parts of the above findings can be explained as following: due to the entropy increase of the polar substrate molecules under microwave irradiation, the accelerated molecules oscillation caused by microwave irradiation can raise the molecules collision probability, and subsequently increase the reaction rate. In the meantime, this enhanced oscillation also increases the chance for the substrates binding to the active sites on the enzyme and decreases the tightness of the binding. Since the results were obtained in the circumstance that the microwave accelerated the esterification rate, which means the overall result is the increase of the probability that the substrates hit the active sites on the enzyme. Therefore, the space hindered 2-hydroxyl got more chance to bind the active sites on the enzyme, that is why that the microwave irradiation weakened the 1,3-specificity in the esterification of glycerol.

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