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Enzymatic esterification between *n*-alcohol homologs and *n*-caprylic acid in non-aqueous medium under microwave irradiation

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Abstract

The enzymatic esterification between *n*-alcohol homologs and *n*-caprylic acid catalyzed by lipozyme RM IM (LRI) in microwave field was investigated. Some interesting findings were obtained. The optimum reaction temperature slightly shifted from that in enzymatic esterification by conventional heating. *n*-Alcohol homologs used in this experiment showed substrate specificity in terms of the odd and even carbon numbers. THF expressed abnormal solvent effect. Whereas in the contrastive enzymatic esterification by conventional heating, the above mentioned substrate specificity and solvent effect were not observed. All the above phenomena could be explained by both thermal and non-thermal effect of microwave on enzyme and substrates. Further investigation revealed that microwave irradiation reduced the apparent activation energy of the enzymatic reaction according to *Arrhenius* equation, which is considered as one of the causes increasing initial reaction rate.

Keywords: Microwave irradiation; Enzymatic esterification; Solvent effect; Apparent activation energy

1. Introduction

Since last decade, researchers have attempted to use microwave to improve enzymatic reactions in non-aqueous medium [1–3], which has been well reviewed recently [4]. As indicated by Parker et al. [2], the enzymatic transesterification rate between butanol and ethyl butyrate was enhanced 2–3 folds by microwave irradiation over conventional heating. Ipsita and Gupta [3] also observed that microwave irradiation increased the initial reaction rates by 2.1–4.7 times at all levels of (trans-) esterification and discussed the potential non-thermal effect of microwave on enzymatic reactions. It is generally believed that microwave irradiation (MI) has obvious advantages over conventional heating (CH) in some enzymatic reactions, in which reaction rate, yield and selectivity such as stereo-selectivity or region-selectivity are obviously influenced [1–6]. It is worth noting that most previous work focused on the microwave heating technique and the reaction parameter optimization under MI, while less attention has been paid on the non-thermal microwave effect on enzymatic reactions and its origin. Therefore, to dig out the special effects or phenomena in microwave-assisted enzymatic reaction, which do not exist in enzymatic reaction by conventional heating, will be beneficial to illustrating the mechanism of microwave-assisted enzymatic reaction.

We recently reported the lipase-catalyzed esterification between pentanol isomers and *n*-caprylic acid under discontinuous microwave irradiation [7]. The results showed that microwave irradiation increased the reaction rates by 2.5–4.5 times. In the present work, we further investigated microwave effect on enzymatic esterification of C_2–C_{10} *n*-alcohol homologs and *n*-caprylic acid in non-aqueous medium. The reactions were performed in a homemade microwave reactor characterized by continuous and steady irradiation with temperature control ($\pm 1\,^{\circ}C$).

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2. Experimental

2.1. Chemicals

Lipozyme RM IM (LRI), a lipase from *Mucor miehei* immobilized on an anionic resin was a gift sample from Novo Nordisk Co. Isolated *n*-alcohol homologs (C_4 to C_{10}) were of reagent grade, other reagents including ethanol and *n*-propanol were of analytical grade. All reagents were dried with 4 Å molecular sieves before use. The deionized water was used, if any, throughout the all experiment.

2.2. Microwave enzyme reactor

The microwave enzyme reactor was configured with a glass reactor, a microwave-generator and a temperature control system. The reaction mixture was placed into a glass cylinder reactor with cooling jacket. The enzymatic esterification was performed in a modified microwave oven (NN-S552, National Co., 2450 MHz) equipped with a magnetic stirring apparatus and a sampling hole. The reaction temperature was controlled with a microwave-shielding thermo sensor, which is connected with an electromagnetic feedback loop to monitor the flow of cooling medium.

2.3. Analysis

Conversion of caprylic acid was defined as the percentage of consumed caprylic acid and measured by titration. The initial reaction rate of the esterification was defined as the mmoles of ester produced from one gram of acid per minute in the initial phase of reaction, and was calculated based on the amount of consumed *n*-caprylic acid in the first 5–10 min, during which the conversion of caprylic acid was exactly in direct proportion to the reaction time.

2.4. Microwave irradiation-enzyme coupling catalysis (MIECC)

2.4.1. Solvent-free esterification

In a 20 ml glass reactor, caprylic acid (25 mmol), *n*-alcohol (25 mmol) and water (1%, w/w) were homogenized under the desired temperature. Then the reactor was placed into the modified microwave oven. The reaction started once the enzyme LRI (50 mg) was added and well mixed with the reaction mixture under magnetic stirring (400 rpm) accompanied by continuous microwave irradiation (200 W).

2.4.2. Esterification in solvent phase

In a 20 ml glass reactor, caprylic acid (5 mmol), *n*-alcohol (5 mmol), organic solvent (10 ml) and water (1%, w/w) were well mixed. All other operating conditions were the same as that in solvent-free esterification except the dosage of enzyme LRI was 10 mg.

2.5. Enzymatic esterification by conventional heating

In a 20 ml glass reactor, caprylic acid (25 mmol), *n*-alcohol (25 mmol), organic solvent (10 ml) if any, and water (1%, w/w) were homogenized under the desired temperature. The reaction started when the enzyme LRI (50 mg) was added and well mixed with the reaction mixture under magnetic stirring (400 rpm), the temperature of the reaction was maintained constant to the desired using a water bath.

3. Results and discussion

3.1. The optimum temperature for enzymatic esterification assisted by microwave

The initial reaction rates at different temperatures are shown in Fig. 1. The initial reaction rate under CH increased with increasing reaction temperature from 40 to 50 °C. The similar trend was observed under MIECC, where the highest initial reaction rate appeared at 55 °C. The reason that at each tested temperature the initial reaction rate under MIECC is higher than that under CH may be explained due to both thermal and non-thermal effect of microwave irradiation as follows. First of all, under MIECC, the polar molecules (the alcohol and the acid) collide with each other because of thermal effect and microwave effect. Therefore, the molecule collision under MIECC has extra driving force compared to that under CH, which results higher rate under MIECC as long as the enzyme is not deactivated by microwave. Since all MIECC reactions were applied under 200 w with the same amount and the same kind of reaction mixture, the contribution of microwave to each reaction are supposed to be the same, which is implied by the parallel curves in Fig. 1. One can assume that the bias in *Y*-axis of Fig. 1 is the contribution of microwave to reaction rate. Secondly, one of the non-thermal effects is that microwave energy can also modulate the configuration of enzyme molecules by accelerating the molecular rotation and electron spin oscillation of the polar parts of enzyme, which can provide more chance to make the substrates fit to the enzyme in unit of time.

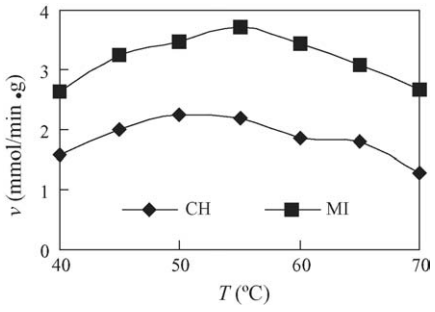

Fig. 1. Initial reaction rate of solvent-free enzymatic esterification (♦: CH, ■: MI).

Table 1
The effect of initial water content on the solvent-free enzymatic esterification

Initial water content (%)	v (mmol/min g)		v_{MI}/v_{CH}
	v_{CH}	v_{MI}	
0.5	1.51	1.59	1.05
1	2.3	3.46	1.51
2	1.98	3.09	1.56
4	1.87	2.88	1.54
8	1.14	1.43	1.26

3.2. The role of water under MIECC and CH

As mentioned by Klibanov [8], the minimum 'essential water' with the enzyme molecule was necessary to maintain the flexibility of enzyme conformation for catalyzing. Water content in enzymatic esterification influences the balance of esterification and adjusts the rigidity and flexibility of protein structure on enzyme surface that would be responsible for the catalysis characteristic of enzyme. Since the desired balance between rigidity and flexibility of enzyme is correlated with the optimum water content, we can adjust the water content to fulfill that. Further more, in microwave-filed, the polar water molecule will behave differently, and this may influence the reaction in a different way. So comparing the role of water in MIECC with that in CH will help us understand more about MIECC.

Table 1 shows that the optimum initial water content in the solvent-free esterification are the same in both MIECC and CH, which is about 1% of total mass. But the v_{MI}/v_{CH} ratio that indicates the increase of reaction rate due to microwave irradiation is nearly the same when the initial water contents changed from 1 to 4%, although it is different at 0.5 and 8% of water content.

It is worthy of noting that those two typical parameters—the optimum temperature and the optimum water content are just the same or approximate under MIECC and CH.

3.3. The microwave effect on substrate specificity of alcohol

Fig. 2 shows the effect of the *n*-alcohol chain length on the initial reaction rate. In solvent-free enzymatic esterification, the homologous *n*-alcohol expresses different initial reaction rates.

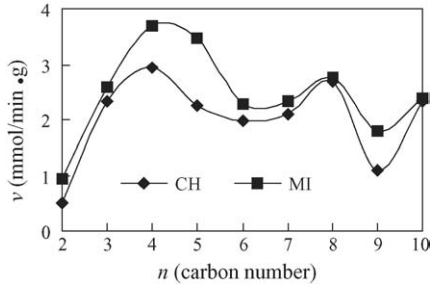

Fig. 2. The microwave effect on substrate specificity in solvent-free enzymatic esterification.

In case of CH, the initial reaction rate changed with alcohol chain lengths with a declined zigzag shape, which represented the ordinary substrate specificity. The same trend was observed under MIECC. The reaction rate altering with the alcohol carbon number can be explained in respect of two important parameters—polarity and steric hindrance effects. First, as indicated by Gorman and Dordick [9], solvents or substrates with high polarity are harmful to enzyme and result in irreversible loss of enzyme activity, which is a negative influence on the reaction rate. Second, the higher the molecular polarity, the stronger the activation resulted from microwave irradiation, in a certain range, which makes a positive influence on the enzymatic reaction. The third, the ever increasing of alcohol carbon numbers makes the polarity effect shrunk and the steric hindrance effect dominant. Thus, ethanol and *n*-propanol substrates have stronger negative effect on enzymatic esterification under both models due to their toxicity to enzyme. When the carbon number of alcohol increased to four, the positive effect of molecular polarity of the substrate gradually declares itself and becomes dominant, which makes the initial reaction rate the highest. With the carbon number more than four, the steric hindrance effect plays a major role impacting the rates, and the more the carbon number, the stronger the role. This may explain that the initial reaction rate becomes similar when the carbon number is 8 or 10 under both MIECC and CH. As for the fact that microwave effect is comparatively more obvious when the carbon number of substrate is odd except heptanol, it can also be explained by polarity effect that the odd one is more polar than the even one.

3.4. The solvent effect under microwave irradiation

Lanne et al. [10] summarized the influence of organic solvents on the enzymatic reactions. He concluded that the enzyme activity is higher in the environment surrounding by non-polar ($\log P > 4$) and mid-polar solvents ($2 < \log P < 4$), whereas the lowest activity is expressed in polar solvents ($\log P < 2$). It is then interesting to investigate how the solvent performed under MIECC. Fig. 3 shows that solvent effect on the irradiated samples and the non-irradiated ones is similar in alkanes. The initial reaction rate in solvent of *n*-hexane and *n*-octane is much higher than that in THF, which coincides with the conclusion by Lanne et al. As shown in Table 2, the highest microwave effect is expressed in the THF solvent instead of in the non-polar solvents, although polar solvent

Table 2
E_a of enzymatic esterification under CH or MI with/without solvent

Solvent	E_a (kJ/mol)	
	CH	MIECC
Solvent-free	29.76	23.29
n-Octane	22.31	20.26

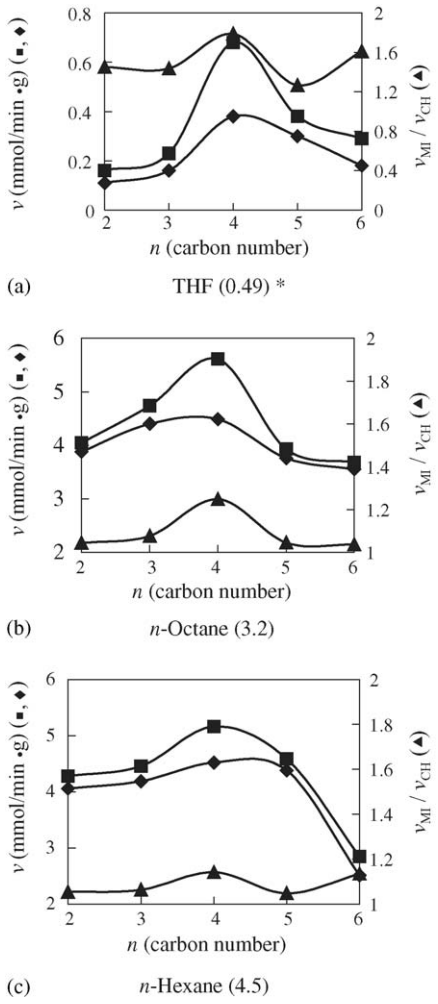

Fig. 3. The effect of solvent on esterification under microwave irradiation (◆: CH, ■: MI, ▲: v_{MI}/v_{CH}).'*' Values in brackets represent the log *P* values of the corresponding solvents.

like THF was anticipated as more 'unfavorable' to enzymatic reactions under CH [10]. We deduce that the abnormal solvent effect may be related to the microwave characteristics of selective heating. As we know that the microwave heating mechanism was accomplished by the dipole molecular rotation and ion conduction, microwave irradiation exerted a stronger effect on such polar solvent as THF than other non- or micro-polar solvents, which makes the reaction in THF solvent more active. So in this case the abnormal solvent effect of polar solvent under microwave irradiation is favorable to MIECC. This also coincides with the fact in Section 3.3 where a bigger v_{MI}/v_{CH} increment appears in odd carbon alcohol substrates which have higher polarity than neighbor even carbon alcohol.

3.5. The apparent activation energy (Ea)

The apparent activation energy (E_a) of enzymatic reaction can be calculated from the *Arrhenius* equation $\ln(k_2/k_1) = E_a/R\ (1/T_1 - 1/T_2)$, here k_2 and k_1 are reaction rate constants at temperature T_2 and T_1, respectively; R is the ideal gas constant. Table 2 shows E_a of enzymatic esterification between *n*-caprylic acid and *n*-pentanol. Apparently microwave irradiation decreased the E_a in both solvent (*n*-octane) and solvent-free system. The decrement of E_a affected by *n*-octane was smaller under MIECC than that under CH. This can be explained as the less microwave effect on the non-polar solvent (*n*-octane) as mentioned above in Section 3.4. Microwave effect in these reactions can be partly expressed as the decrease of E_a, which depends on solvent type.

4. Conclusion

Microwave irradiation increases the initial reaction rate overall comparing to conventional heating due to both thermal and non-thermal effect of microwave on enzyme and substrates. The MIECC effect was influenced by several factors such as temperature, water content, solvent and substrates. These phenomena can be explained with following microwave characteristics. One is the particular heat-transfer accomplished by dipole molecular rotation and ion conduction in regular alternating microwave field, which make the heat-transfer efficiency higher than that of conventional heating. The other is electron spin oscillation of polar molecules caused by the special microwave energy level, which may delicately modulate the local configuration of enzyme molecules to favor efficient binding of substrates according to the well-known lock–key theory. The apparent activation energy (E_a) of enzymatic reaction decreases in microwave field, which is considered as one of the reasons resulting in the increase of initial reaction rate.

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