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Effect of microwave irradiation on lipase-catalyzed reactions in ionic liquids

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Abstract: Microwave-assisted organic synthesis has gained a remarkable interest over the past years because of its advantages - (i) rapid energy transfer and superheating, (ii) higher yield and rapid reaction, (iii) cleaner reactions. Ionic liquids are well known for their unique properties such as negligible vapor pressure and high thermal stability. With these properties, ionic liquids have gained increasing attention as green, multi-use reaction media. Recently, ionic liquids have been applied as reaction media for biocatalysis. Lipase-catalyzed reactions in ionic liquids provide high activity and yield compared to conventional organic solvents or solvent free system. Since polar molecules are generally good absorbent to microwave radiation, ionic liquids were investigated as reaction media to improve activity and productivity. In this study, therefore, the effect of microwave irradiation in ionic liquids was investigated on lipase catalyzed reactions such as benzyl acetate synthesis and caffeic acid phenethyl ester synthesis. Comparing to conventional heating, microwave heating showed almost the same final conversion but increased initial reaction rate (3.03 mM/min) compared to 2.11 mM/min in conventional heating at 50 °C.

Key words: microwave, ionic liquids, lipase, activity, initial reaction rate

1. Introduction

Microwaves are electromagnetic waves with frequencies ranging between 0.3-300 GHz, and are mostly used for communication or energy transfer. The frequency range of microwaves is allocated for their specific use. Household microwave ovens or microwave reactors used for chemical reactions are mostly operated at a frequency of 2.45 GHz in order to avoid overlapping with mobile communications or wireless networks.[1]

Heating by microwave irradiation is induced by the interaction between microwaves and polar molecules, which is explained by two mechanisms, “dipole rotation” and “ionic conduction.” Dipole rotation is the process by which polar molecules like water absorb microwaves with periodic repetitions of electrically positive and negative waves, leading to

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molecular movements with continuous rearrangements of the positions and orientations of molecules, which results in heat generation. In contrast, ionic conduction is a process whereby charged ions collide with surrounding molecules or atoms while vibrating back and forth as an electrical response to microwaves, thus, generating heat.[2] Microwave heating method has many distinct features in comparison with conventional heating method. The largest difference is the heating efficiency. In conventional heating methods, heat generated from the heat source transmits through conduction or convection from the surface of the heat source. Thus, it takes a long time for targets to be heated evenly, and the vessel also needs to be heated if targets are contained in a reaction vessel, which requires extra energy. On the contrary, microwave heating utilizes heat generated from molecular movements of polar molecules and ions within heating substances or materials in the heating targets, which enables rapid energy transfer and simultaneous volumetric heating, and hence, heating of the reaction vessel or reaction accessories in the vessel is not required.

After Gedye group and Giguere group used microwaves in organic synthetics reactions in 1986, respectively, there has been an increase in the number of studies on the use of microwaves in organic reactions.[3,4] Most of the studies focused on the utilization of microwaves in synthetic reactions using chemical catalysts in organic solvents for fast, efficient, and uniform heating to high temperature in order to raise yield or reduce reaction time compared to conventional heating methods.[5] In the beginning of those studies, household microwave ovens were used, whereas exclusive microwave equipments have been developed for precise power control of microwaves and fine control of temperature and stirring speed. Besides the organic synthetic processes, microwaves have been applied in fields of biology such as the synthesis of peptides and carbohydrates, fields of proteomics, construction of libraries for drug candidates, PCR (polymerase-chain reaction) techniques, synthesis of immobilized enzymes, and biomass pretreatment.[5-8]

Ionic liquids (ILs) which are composed of organic cations and appropriate anions exist liquid at a wide range of temperature and their physical and chemical properties can be tailored by the right choice of cations and anions.[9,10] They have recently emerged as green solvents that can replace organic solvents because they have negligible vapor pressure at room temperature, and are non-flammable. In addition, ILs have excellent thermostability and conductivity as well as high polarity. Numerous studies have been reported on the use of ILs as solvents and the enzyme pretreatment in various enzymatic reactions including lipase resulting in improved activity, thermostability, and reusability of enzymes compared to organic solvents or aqueous solutions.[9,11,12] ILs can be rapidly heated at low-power microwaves by ionic conduction, and their temperatures increase faster as power becomes larger within some ranges. Applications of ILs using microwave heating include reaction solvents or auxiliary solvents for increasing temperature in the field of organic synthesis, catalysts, auxiliary absorbents for solvents with low microwave absorption rates, material extraction, and the manufacture of nanomaterials and cellulose-based nanomaterials.[13-17] Nonetheless, microwaves have been seldom used for enzymatic reactions. The use of microwaves were reported in the conversion of small organic molecules, and there are few reports on the application of microwaves for enzymatic reactions in ILs.[18-21] Therefore, there is lack of understanding on the mechanism of enzymatic reactions using microwaves, and the interpretations on results still remain controversial as well.

Considering that ILs are ideal solvents for interacting with microwaves, it is necessary to study the thermal effect of microwave heating as well as non-thermal effect by the induction of electromagnetic waves through the use of microwaves as an energy source for enzymatic reactions in ILs. Thus, in this study, we investigated the effect of microwave irradiation using a microwave reactor on lipase-catalyzed reactions in ILs by comparing with lipase-catalyzed reactions in ILs based on conventional heating methods.

2. Experiments

2.1. Materials

Novozym 435 (*Candida antarctica* type B lipase immobilized on porous acrylic resin) was provided by Novo Nordisk (Bagsvaerd, Denmark). Amano PS-D (*Burkholderia cepacia* lipase immobilized on diatomite powder) was purchased from Sigma (St. Louis, USA).

Benzyl alcohol, vinyl acetate, and benzyl acetate were purchased from Sigma Aldrich (St. Louis, MO, USA). 1-Ethyl-3-methylimidazolium bis[(trifluoromethyl) sulfonyl]amide ([Emim][Tf_2N]), 1-butyl-3-methylimidazolium bis[(trifluoromethyl) sulfonyl]amide ([Bmim][Tf_2N]), 1-hexyl-3-methylimidazolium bis[(trifluoromethyl) sulfonyl]amide ([Hmim][Tf_2N]), and 1-octyl-3-methylimidazolium bis[(trifluoromethyl) sulfonyl] amide ([Omim][Tf_2N]) were obtained from C-TRI (Korea). All other chemicals used in this work were of analytical grade.

2.2. Experimental methods

2.2.1. Lipase-catalyzed synthesis of benzyl acetate

For the lipase-catalyzed reaction, 1 mL dried ILs, 10 mg Novozym 435, and 0.1 M benzyl alcohol were mixed in 10 mL Septa-glass vial, and preheated at 40~50 °C for 5 mins with stirring at 200 rpm. Enzyme reaction were initiated by adding 0.3 M vinyl acetate to the vial. Periodically, aliquots were taken from the reaction vial and the concentration of the products was quantified by HPLC.[22]

2.2.2. Lipase-catalyzed synthesis of caffeic acid phenethyl ester

For the lipase-catalyzed reaction, 1 mL dried [Emim][Tf_2N], 30 mg Novozym 435, and 2.16 mg caffeic acid were mixed in a 10 mL Septa-glass vial, and preheated at 70 °C for 5 mins with stirring at 200 rpm, and 45 μL 2-phenylethanol was added to the vial to initiate the reaction. Periodically, aliquots were taken from the reaction vial and the concentration of the products was quantified by HPLC.[23]

2.2.2. Reactor operating conditions

In the reaction block (Variomag, Germany) using the conventional heating method where heat is transmitted to the reaction vessel through a heated plate, constant temperature is maintained by an external heating bath circulator, and contents are stirred at 200 rpm using a 7 mm spin magnetic bar.

In the microwave reactor (CEM Discover microwave system (Matthews, USA)), microwave power, irradiation time, irradiation method, temperature and flow rate of air for cooling were adjusted to reach and maintain the desired reaction temperature and then the reaction was carried out.

2.2.3. HPLC analysis

The concentration of benzyl acetate produced in lipase-catalyzed trans-esterification was measured by HPLC, and used to determine enzyme activity.[12] The concentrations of benzyl acetate were measured by HPLC. Separation was accomplished with a Younglin HPLC system equipped with a Symmetry C-18 column (Waters, USA) and a UV detector (Younglin, Korea, 250 nm). The mobile phase consists of methanol : water = 7 : 3 with a flow rate of 0.5 mL/min.

Caffeic acid phenethyl ester produced in the lipase-catalyzed synthesis reaction was also analyzed by HPLC.[13] Separation was accomplished with a Younglin HPLC system equipped with a Symmetry C-18 column (Waters, USA) at constant temperature of 30 °C and a UV detector (Younglin, Korea, 210 nm). The mobile phase consists of methanol : water = 7 : 3 with a flow rate of 0.5 mL/min.

3. Results and Discussion

3.1. Lipase-catalyzed reactions in the microwave reactor

The lipase-catalyzed synthesis of benzyl acetate in ionic liquids (ILs) was performed in a microwave reactor and a conventional heating reactor under the same conditions. The operating conditions controlling the reaction rate such as reaction temperature and

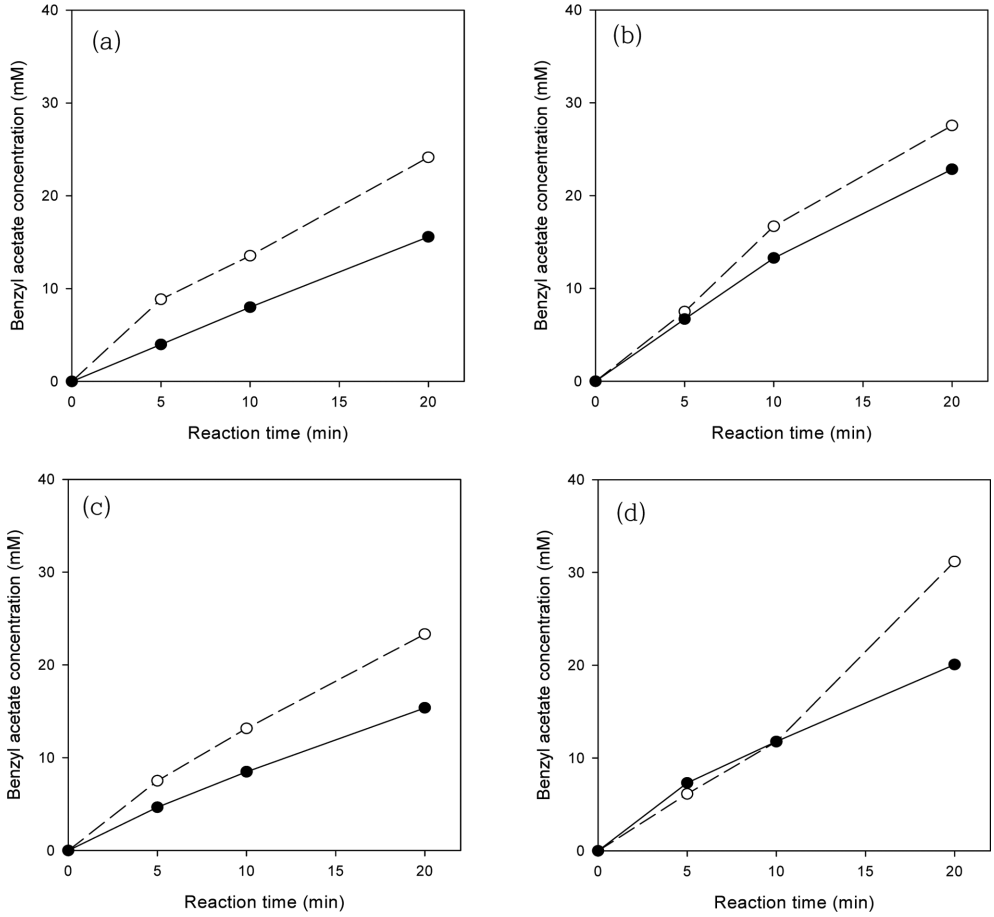

Fig. 1. Lipase-catalyzed benzyl acetate synthesis reactions at 50 °C in (a) [Emim][Tf_2N], (b) [Bmim][Tf_2N], (c) [Hmim][Tf_2N] and (d) [Omim][Tf_2N]. (● microwave reactor, ○ conventional heating reactor)

stirring speed were set to be the same, and the same reaction substrates were added to the same type of glass vial to conduct the reaction.

Benzyl acetate synthesis was performed at 50°C in 4 different ILs ([Emim][Tf_2N], [Bmim][Tf_2N], [Hmim][Tf_2N], and [Omim][Tf_2N]). It was found that the initial reaction rates in the microwave reactor were lower than those in the conventional heating reactor (*Fig*. 1). For caffeic acid phenethyl ester synthesis in [Emim][Tf_2N], the enzyme activity in the microwave reactor was much lower than that in the conventional heating reactor (*Fig*. 2). Increase of microwave power from 1 watt to 10 watt resulted in much lower enzyme activity.

3.2. Effects of microwaves on the enzyme-catalyzed reactions in the microwave reactor

So far, there have been little known about the effect of microwave irradiation on enzyme-catalyzed reactions in both ILs and organic solvents. Some researchers have reported that enzymatic reactions in organic solvents using microwave heating yielded improved initial reaction rates, product yields, and enantioselectivity compared to the conventional heating method.[5,24-26] In the study on the effects of microwaves in lipase-catalyzed transesterification of methyl acetoacetate in toluene, on the other hand, Leadbeater *et al*. reported that there was no enzyme activity difference between microwave heating and

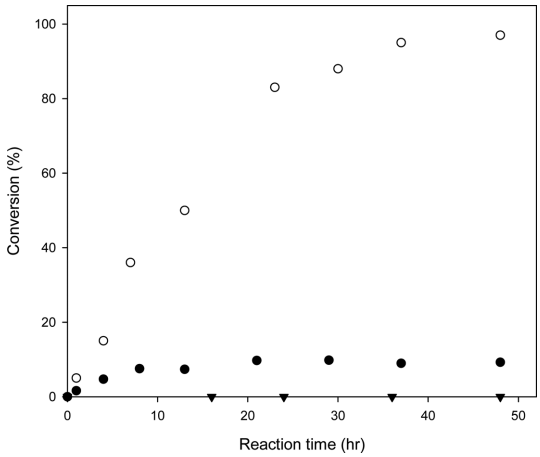

Fig. 2. Effect of microwave heating and microwave power on lipase-catalyzed caffeic acid phenethyl ester synthesis in [Emim][Tf_2N] at 70 °C. (○ conventional heating reactor, ● microwave reactor with 1 watt, ▼ microwave reactor with 10 watt).

conventional heating in lipase-catalyzed transesterification of methyl acetoacetate in toluene.[19] Kerep and Ritter reported that the enzyme activity in the lipase-catalyzed ring-opening polymerization reaction of ε-caprolactone in organic solvents was either enhanced or diminished by microwave radiation depending on the type of reaction solvent heated.[27]

Although the enzyme-catalyzed reactions in ILs were carried out under the same operating conditions, the enzyme activity and initial reaction rate in the microwave reactor were lower than in the conventional heating reactor, as described in Section 3.1. Based on the report of Kerep and Ritter, it was hypothesized that the temperature of the reaction solvents in the reactor might be related to the enzyme activity and initial reaction rate. Thus, temperature change in the reactor were monitored during heating.

The microwave reactor used in this study allows the monitoring of the real-time temperature of the reaction solution by using Synergy® which is a control program. As shown in *Fig*. 3, a sudden increase in temperature was observed during the synthesis of caffeic acid phenethyl ester in [Emim][Tf_2N] when temperature was monitored with Synergy®. An asymmetrical peak pattern was observed, which showed that the temperature increased instantaneously by 20~40 °C higher than the target temperature and slowly recovered. This instantaneous increase in temperature, frequency of occurrence, duration time and range of temperature increase were different at each time. Although its cause has not been determined yet, it occurred even when IL alone without any

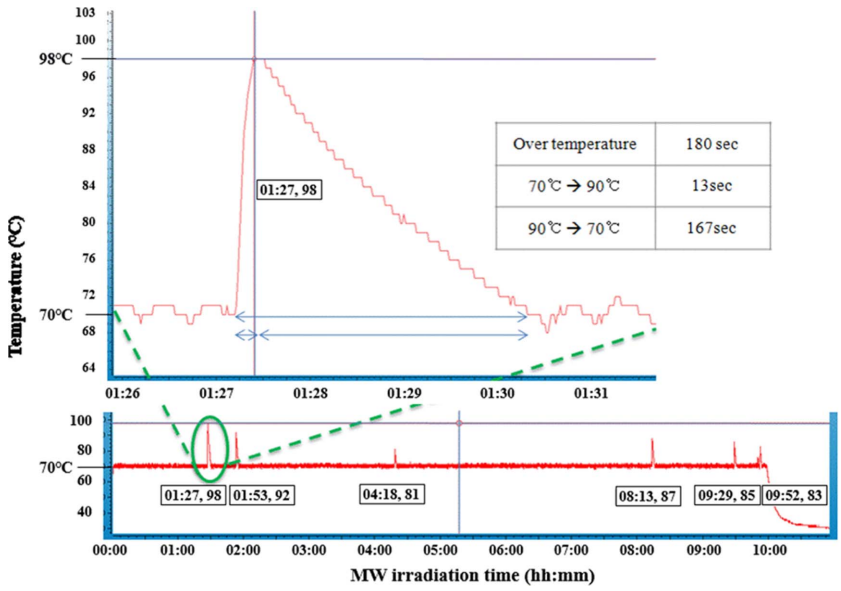

Fig. 3. Temperature profile during lipase-catalyzed caffeic acid phenethyl ester synthesis in [Emim][Tf_2N] at 70 °C in microwave reactor.

other reagents and substances in the reaction vials was heated in the microwave reactor. From this result, it can be concluded that instantaneous temperature increase was not caused by external substances such as magnetic bar or the resin of the immobilized enzymes. This phenomenon tended to occur frequently when the ILs were heated in the microwave reactor even either at low power or when acceptable deviation from the target temperature was minimized in order to maintain constant target temperature. This might be caused by the high interaction between the microwaves and ILs.

3.3. Temperature changes in the reactor and enzymatic reactions in different microwave irradiation types

Unlike chemical catalysts, enzymes are inactivated at temperatures higher than their optimal temperature. Therefore, enzymes are catalytically active within a limited range of temperature. While the combination between microwaves and ILs can raise the temperature of the reaction solution rapidly and efficiently, the optimal temperature of Novozym 435, the immobilized enzyme used in this study, is 40-60 °C. Hence, an instantaneous elevation of temperature over the target temperature during the reaction in the microwave reactor can thermally inactivate the enzyme. Thus, we tried an alternative microwave irradiation method in order to keep the temperature stable for a long time during microwave heating of the reaction solution containing ILs.

The microwave irradiation in the reaction solution generates energy through the interaction with polar materials in the solution. The generated heat is equilibrated to a specific temperature by the surrounding temperature of the reactor or the cooling system connected to the reactor. Since microwave irradiation without a cooling system leads to equilibration at a high temperature, the following two methods can be applied to maintain optimal temperature for the enzymatic reactions. One is the intermittent microwave irradiation once the target temperature has been exceeded. The other is the continuous removal of heat using a cooling system once the target temperature

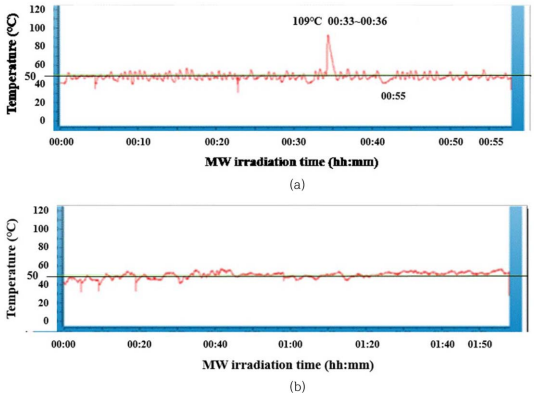

Fig. 4. Temperature profile during microwave heating of [Emim][Tf_2N] in a microwave reactor with (a) SPS mode at 1 watt without air-cooling and (b) fixed power mode at 1 watt with air-cooling, respectively.

has been exceeded under the continuous microwave irradiation. In Synergy®, a control software of the microwave reactor from CEM Co. used in this study, the former is called SPS (solid phase synthesis) mode, while the latter is called the fixed power mode. To investigate the effects of the intermittent irradiation and continuous irradiation on the activity of lipase enzyme, benzyl acetate synthesis were carried out under two different modes in the microwave reactor, and changes in reaction temperature were also analyzed.

After adding 1 mL reaction solution into a reaction vial, 1 watt power of microwave was irradiated either intermittently or continuously to maintain the reaction temperature at 50 °C. Under the intermittent irradiation mode (SPS mode) with 1-2 on and off counts per min, temperature in reaction solution was maintained with only ± 1-2 °C deviation from the target temperature, but the temperature was instantaneously elevated to 109 °C at 34 min after the beginning of the reaction (*Fig.* 4(a)). Under the continuous irradiation mode (fixed power mode) using an auxiliary cooling system, the reaction temperature was maintained as a waveform (*Fig.* 4(b)). This waveform might be explained by the volumetric microwave heating mechanism with surficial cooling mechanism from the outer surface. As shown in *Fig.* 5, there was no difference in initial reaction rates of the lipase-

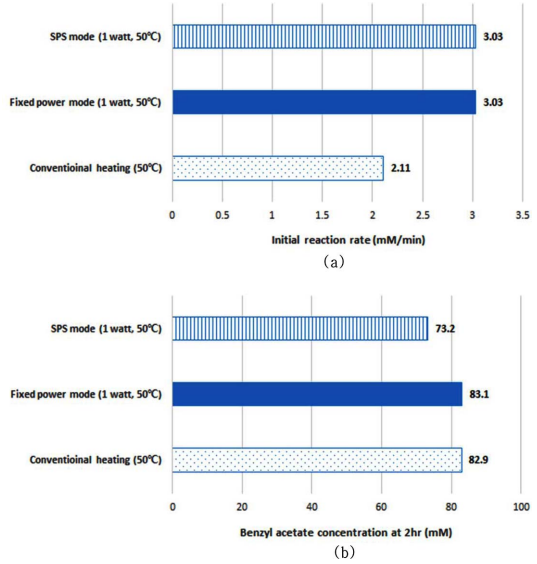

Fig. 5. Effect of microwave irradiation mode on (a) initial reaction rate and (b) benzyl acetate concentration at 2 hrs in lipase-catalyzed benzyl acetate synthesis during microwave heating of [Emim][Tf_2N] in a microwave reactor.

catalyzed reaction between fixed power mode and SPS mode. However, the yield of benzyl acetate, the final reaction product produced at 2 hrs in fixed power mode without instantaneous elevation of temperature was about 1.14 times higher yield than that produced at 2 hrs in SPS mode. However, microwave irradiation time under SPS mode was shorter than that under fixed power mode. Therefore, the non-thermal effect of microwaves on enzyme performance in the lipase-catalyzed synthesis of benzyl acetate appears to be weak, and the major determining factor on enzyme performance in the lipase-catalyzed synthesis of benzyl acetate was the reaction temperature itself.

4. Conclusions

The microwave heating is an energy-efficient heating method which allows homogeneous reactions. Since ILs composed of positive ions and negative ions are highly interact with microwaves, they can be used as ideal solvents in processes utilizing microwaves. Previous studies on the organic reactions using microwaves were conducted in organic solvents that are relatively less affected by the intensity and control of microwave power and chemical catalysts that are less sensitive to high temperature. While there were several reports on the use of microwaves for ILs or enzymes, studies utilizing microwaves for enzyme-catalyzed reactions in ILs are extremely rare.

In this study, temperature control was considered as the main factor to apply the advantages of the microwave heating method as the next generation heating method, and ILs as the next generation clean solvent to enzymatic reactions. The results of the enzymatic reactions in ILs in the microwave reactor were similar to or lower than those of the enzymatic reactions conducted in the conventional heating reactor, mainly owing to inadequate temperature control. Furthermore, the non-thermal effect of microwaves was negligible. The development of enzymes with high thermostability is expected to result in further applications of the highly interactive combination of microwaves and ILs.

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