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Lipase-catalyzed acylation of naringin with palmitic acid in highly concentrated homogeneous solutions

Sung Hun Youn, Hyun Jung Kim, Tae Han Kim, Chul Soo Shin *

Department of Biotechnology, College of Engineering, Yonsei University, 134 Shinchon-dong, Seodaemun-gu, Seoul 120-749, South Korea

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Abstract

Acylation reactions of naringin with palmitic acid were performed by a lipase after formation of highly concentrated homogeneous solutions. Their initial naringin concentration was 840–950 mM, which is 20–60 times greater than that in organic solvent media. The overall productivity of highly concentrated solutions was more than 15 times greater than those of organic phase media. The addition of DMSO (20–40%, w/w) to substrate mixtures lowered the melting temperature of a naringin–palmitic acid mixture (1:1 molar ratio) to about 40 °C. Reactions at 80 °C apparently followed Michaelis–Menten kinetics despite extremely high substrate concentrations. As the temperature increased from 60 °C to 80 °C, the apparent viscosity of the highly concentrated solution decreased remarkably from 4.31 Pa s to 0.063 Pa s. An activation energy of 7.65 kcal/mol obtained in a range of 60–75 °C suggests a diffusion-control. On the other hand, an activation energy of 17.09 kcal/mol in a range of 75–90 °C indicates a reaction-control. The highest product conversion yield of 33% (mol/mol) was obtained in a 10 h reaction at 80 °C. Addition of activated molecular sieves to the highly concentrated solution increased the product conversion yield by 7% (mol/mol), suggesting that the original equilibrium was disrupted by removing water and then a new equilibrium was reached.
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Keywords: Acylation; Naringin; Palmitic acid; Lipase; Activation energy

1. Introduction

Flavonoids are natural compounds originating from various plants. A variety of physiological activities of the flavonoids have been reported [1–4]. Among flavonoids, naringin (naringenine-7-rhamnosidoglucoside) extracted from citrus fruits has been used as an oriental herbal medicine. There are numerous reports of the physiological activity of naringin, e.g., antioxidant [5,6], anti-viral [7,8], anti-cancer [9], hepatoprotective [10,11], lipid-lowering [12–16], and blood pressure lowering [17].

In spite of various physiological activities, the flavonoids have had limited industrial applications due to their narrow solubility in hydrophobic organic solvents [18]. Thus, certain flavonoids have been modified with fatty acids [19]. Chemical reactions have been generally used and various methods including combinatorial chemistry to prepare chemical libraries have been developed. However, chemical methods have some disadvantages in that the reactions are carried out under extreme conditions, i.e., high pressure, high temperature, or toxic solvent. These harsh conditions can cause environmental pollution. In contrast, biocatalytic reactions, which usually proceed under mild conditions, are environment-friendly. Biocatalytic reactions also have the advantage of high regio- and/or stereoselectivity in reactions with biologically active compounds [20]. However, biocatalytic methods also have disadvantages, such as lower productivity than chemical methods.

Recently, enzymatic modifications of flavonoids have been reported. Reactions were performed in an organic phase, resulting in low productivity. Various key factors for enhancing reactions, e.g., water content [21], acyl donor type [22–24], temperature [25], or solvent type [26] were investigated. Despite these efforts to increase the conversion yield, problems such as low productivity due to low substrate concentration levels still remain to be solved.

The formation of eutectic mixtures is a well-known melting technique. This method has been used to prepare high concentrations of substrate mixtures in enzymatic reactions

* Corresponding author. Tel.: +82 2 2123 2886; fax: +82 2 362 7265.
E-mail address: csshin@yonsei.ac.kr (C.S. Shin).

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[27–31]. Eutectic mixtures are defined as homogeneous solutions, in which the melting temperature of the mixture is lower than that of each component. Adding small amounts of solvent adjuvants (generally 5–30%, w/w) can lower the melting temperature of the eutectic mixture. Thus, high concentrations can be maintained during enzymatic reaction, even at room temperature. The term 'eutectic media' had been previously used by our group for highly concentrated homogeneous solutions prepared from two solid substances using eutectic melting techniques [32]. However, since the palmitic acid used as one of substrates in this study exists in a liquid state at reaction temperatures of 60–80 °C, the term 'eutectic media' was not used here. Instead, the term 'highly concentrated homogeneous solution' was used.

In this study, highly concentrated homogeneous solutions were made from the substrates naringin and palmitic acid then acylation reactions were performed by a lipase. The reaction yields of highly concentrated solutions were compared with those in organic phase. Their reaction patterns were also investigated.

2. Experimental

2.1. Lipase and chemicals

Naringin and dimethylsulfoxide (DMSO) were purchased from Sigma Chemical Co. Palmitic acid was purchased from Duksan and 2-methyl 2-butanol from Fluka. A commercially available lipase (Novozym 435) from *Candida antarctica* immobilized on acrylic resin was obtained from Sigma Chemical Co. (originally a product of Novozyme Co.). Chloroform and methanol (HPLC grade) were obtained from J.T. Baker. Molecular sieves (potassium, sodium alumino-silicate; 3 Å) were a product of Sigma Chemical Co. The sieves were used after incubation in a dry oven at 70 °C for 72 h to remove water.

2.2. Preparation of highly concentrated solutions

Equal amounts of substrates (each 0.2 mmol), naringin and palmitic acid, were put into a 10 mL screw-top glass vial. DMSO was added into the same vial as an adjuvant at 20–40% (w/w). Highly concentrated and homogeneous solutions were made by heating the vials in water or an oil bath (EYELA OSB-2000; Tokyo, Japan). The melting temperatures of the mixtures were roughly measured by observing disappearance of the last traces of solid phase. In other cases, the mole fractions of substrates in a mixture varied. Melting temperature–mole fraction (*TX*) phase diagrams were then constructed.

2.3. Acylation of naringin with palmitic acid

Acylation reactions using highly concentrated solutions or organic phase media were performed by adding *C. antarctica* lipase. Preparation of the highly concentrated solution was described in the previous section. Organic phase media were prepared by adding 0.25 mmol naringin and 1.25 mmol palmitic acid to 10 mL screw-top glass vials containing 5 mL of 2-methyl 2-butanol. Reactions proceeded in a shaking incubator at 200 rpm by adding lipase at 30% (of naringin; w/w) to the vials.

2.4. Qualitative and quantitative analyses of reaction mixture components

Thin layer chromatography (TLC) was used to monitor reaction progress qualitatively. Reaction samples (10 μL) were loaded on TLC plates (Kieselgel 60 F_{254}), and the plates were eluted with a mixture of chloroform/methanol/distilled water (4:1:0.15, v/v/v). The spots were detected using UV light (254 nm).

Substrates and products were analyzed by HPLC (Waters 2690: separation module, Waters 2487: Dual λ absorbance detector) with a 5 μm GOLD column (C_{18}, 250 mm × 4.6 mm, Hypersil, Kleinostheim, Germany). Samples were prepared for HPLC analysis as follows: the reacted solutions were dissolved in 5 mL of methanol. The solutions were then diluted 10-fold, followed by treatment with PTFE syringe filters (Whatman, 13 mm, 0.45 μm) to remove the enzyme. After injecting the samples into the HPLC, a mixture of acetonitrile, methanol and deionized water (65:25:10, v/v/v) was eluted at a flow rate of 0.7 mL/min. The components naringin and naringin–palmitic acid ester were detected at 285 nm.

The molecular mass of the reaction product was measured with a Quattro-LC triple quadrupole mass spectrophotometer (Micromass, Manchester, UK) equipped with an ESI probe and Z-spray interface.

The water content of the reaction mixtures was measured with a 37858-HYDRANAL® Moisture Testkit (Riedel-de Haën®) based on the Karl Fisher titration method. The product conversion yield was defined as the molar concentration ratio of naringin–palmitic acid ester to initial naringin.

2.5. Rheology analysis of reaction mixtures

After preparing a highly concentrated solution consisting of naringin–palmitic acid (1:1 molar ratio), and DMSO (30%, w/w), the shear rates and shear stresses of the media incubated at 60 °C, 70 °C, and 80 °C were measured with a rheometer (Brookfield, DV-III, equipped with a No. 31 spindle; MA, USA). Apparent viscosities were calculated from the slopes of shear stress versus shear rate.

3. Results and discussion

3.1. Formation of highly concentrated solutions with naringin and palmitic acid

High substrate concentrations are essential to achieve high productivities. Thus, we attempted to form highly concentrated solutions at low temperatures. First, various mole fractions of naringin–palmitic acid mixtures were prepared. Their melting temperatures were then measured. As shown in Fig. 1, the

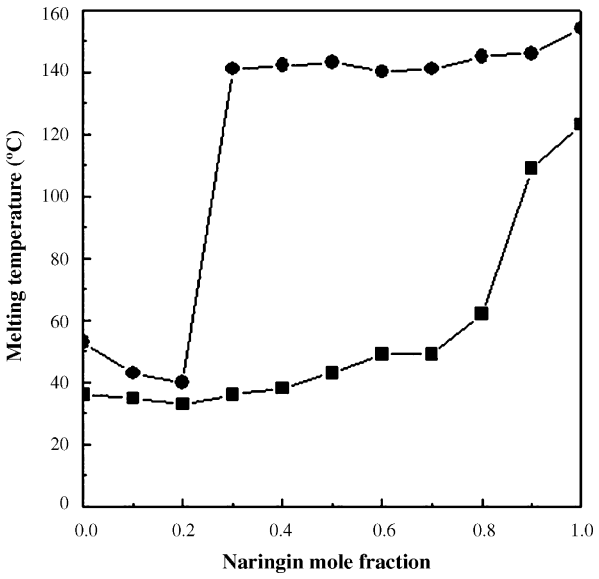

Fig. 1. Temperature–mole fraction (*TX*) phase diagrams of naringin–palmitic acid mixtures. (●) Without adjuvant and (■) with DMSO (20%, w/w).

melting temperatures of pure naringin and palmitic acid were 171 °C and 63 °C, respectively. As the mole fraction of naringin increased from 0 to 0.2, the melting temperature was reduced to 40 °C. When the mole fraction of naringin increased above 0.2, the melting point increased to 140 °C or higher. However, considering that the substrates usually react at 1:1 molar ratios, the high melting temperature (above 140 °C) obtained at a naringin mole fraction of 0.5 was not suitable for enzymatic reactions.

On the other hand, adjuvant addition can lower the melting temperature of the highly concentrated solutions. Through our preliminary experiments, DMSO was found to be the most suitable adjuvant for preparation of highly concentrated and homogeneous solutions with naringin and palmitic acid (data not shown). The addition of DMSO (20%, w/w) lowered the melting temperature of the media below 60 °C in naringin mole fractions of 0–0.8. When the DMSO content was increased to 30% or 40% (w/w), the melting temperature of the media at a naringin mole fraction of 0.5 was 39 °C or 37 °C, respectively. It indicates that enzymatic reactions can be accomplished even below 40 °C.

After highly concentrated solutions including different contents of DMSO were made, reactions were performed for 24 h at 60 °C. As shown in Table 1, at 20% (w/w) DMSO, a conversion yield of 18% (mol/mol) was obtained. At 30% and 40% (w/w), the yield increased to 25% and 28% (mol/mol), respectively. Based on these results, a concentration of 30% (w/w) DMSO was preferred and used in subsequent experiments. The naringin concentration of highly concentrated solutions with 30% DMSO was 20–60 times higher than those of organic solvent media (Table 1).

Enzymatic acylations of naringin with fatty acids have been usually performed in organic solvent media [21–24,33]. In those cases, the naringin concentration was in the range of 15–45 mM. These levels are extremely low compared to those of our highly concentrated solutions (840–950 mM).

3.2. Lipase-catalyzed acylation in highly concentrated solutions and organic media

The acylation reactions of naringin–palmitic acid mixtures were carried out in highly concentrated solutions and organic solvent media. As shown in Fig. 2, the product conversion yield of the highly concentrated solution reached 19% (mol/mol) at 24 h, corresponding to 171 mM naringin–palmitic acid ester. In organic media, a similar reaction pattern was obtained with a conversion yield of 19% (mol/mol) at 24 h corresponding to 9 mM naringin–palmitic acid ester. The concentration of reaction products in the highly concentrated solution was approximately 19 times greater than that obtained in the organic media.

Although the naringin concentration of highly concentrated solutions was much greater than that in the organic media, similar product conversion yields were obtained in both cases (Fig. 2), and the reaction rate of the highly concentrated solutions was much faster than that of the organic media (Figs. 2 and 6). Additionally, the overall productivity in the highly concentrated solution (9.4 mM/h) was about 15 times greater than that in organic media at 60 °C. Preparation techniques of highly concentrated solutions are considered to be useful for industrial biocatalysis because high substrate concentrations can be used.

Table 1
Enzymatic acylation of naringin with fatty acids in organic solvents and highly concentrated solutions

Media	Solvent or adjuvant	Naringin concentration (mM)	Temperature (°C)	Reaction time (h)	Enzyme content (g)	Conversion yield (%, mol/mol)	Reference
Organic solvent media	*tert*-Butanol, 5 mL	15.3	45	240	0.05	60	[22]
Organic solvent media	2-Methyl 2-butanol, 25 mL	45.2	60	48	0.25	40	[21]
Organic solvent media	2-Methyl 2-butanol, 25 mL	43.9	60	60	1.00	85	[24]
Organic solvent media	*tert*-Butanol or acetone, 10 mL	18.4	50	96	0.10	60	[33]
Highly concentrated solution	DMSO, 20% (w/w)	953	60	24	0.04	18	This study
Highly concentrated solution	DMSO, 30% (w/w)	901	60	24	0.04	25	This study
Highly concentrated solution	DMSO, 40% (w/w)	843	60	24	0.04	28	This study

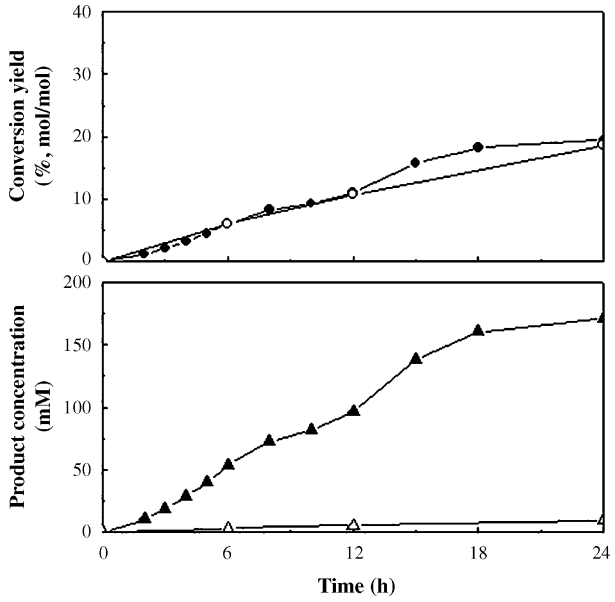

Fig. 2. Conversion yields and product concentrations of both highly concentrated solutions and organic solvent media at 60 °C. (●) Conversion yield of highly concentrated solution; (◯) conversion yield of organic solvent medium; (▲) product concentration of highly concentrated solution; (□) product concentration of organic solvent medium. The highly concentrated solution was prepared by mixing naringin and palmitic acid (each 0.2 mmol) as substrates at 1:1 then by adding DMSO as an adjuvant to the substrate mixture at 30% (w/w). In the case of organic solvent medium, 2-methyl 2-butanol was used as a solvent and the molar ratio of naringin/acyl donor was 1:5. Lipase was added at 30% (w/w) in both cases.

3.3. Reaction patterns of highly concentrated solutions

Reactions using highly concentrated solutions were performed at 60 °C and 80 °C, respectively. Initial reaction rates were plotted against naringin concentrations (Fig. 3). The initial reaction rate of the highly concentrated solution at 60 °C increased as the naringin concentration increased up to 840 mM. The media was highly viscous in the whole naringin concentration range at 60 °C. However, the initial reaction rate at 80 °C increased further until 960 mM. Above 960 mM, the reaction media changed to a highly viscous solution, similar to that at 60 °C. The effect of the initial reaction rate on substrate concentration in the media at 80 °C apparently followed the Michaelis–Menten equation, which can be usually applied to low substrate concentration reactions [34].

Since highly concentrated solutions include tremendously high substrate concentrations and are highly viscous, diffusion difficulty may occur. This phenomenon appeared to occur seriously at 60 °C rather than at 80 °C (Fig. 3), resulting in limited progress of the reaction. This suggests that reaction rates at high naringin concentrations would be significantly retarded by the high viscosity of the highly concentrated solutions.

3.4. Analysis of diffusion limitation in highly concentrated solutions

The apparent viscosities of the highly concentrated solutions at different temperatures (60 °C, 70 °C, and 80 °C) were estimated (Fig. 4). The media included a DMSO adjuvant at 30% (w/w). Apparent viscosities were estimated as the slope of shear stress plotted against shear rate. The apparent viscosity of highly concentrated solutions sharply decreased from 4.31 Pa s at 60 °C to 0.15 Pa s at 70 °C then 0.063 Pa s at 80 °C (Fig. 4). The mixture solution at 80 °C showed a Newtonian fluid property similar to water. Under these conditions, diffusion limitation may not be serious.

The possibility of diffusion limitation which would occur during reaction in the highly concentrated solutions can be determined by activation energy. An Arrhenius plot of initial

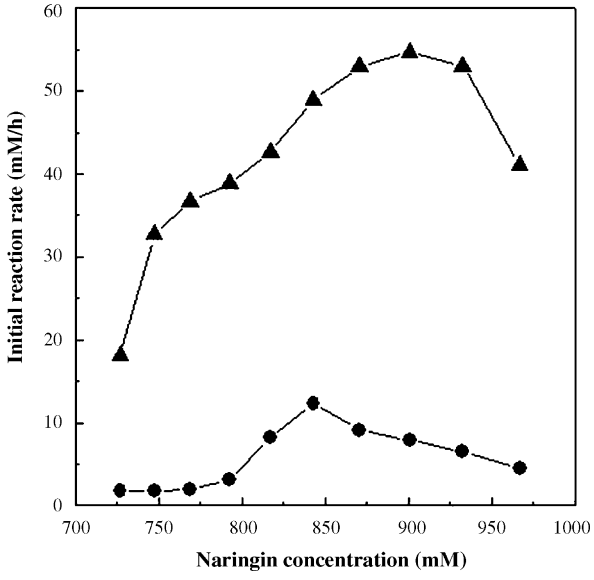

Fig. 3. Initial reaction rates at various naringin concentrations. Reactions were performed at 60 °C (●) and 80 °C (▲). Naringin and palmitic acid (0.2 mmol each) were mixed as substrates at 1:1. Naringin concentrations were adjusted by controlling DMSO content. Enzyme was added at 30% (w/w) in all cases.

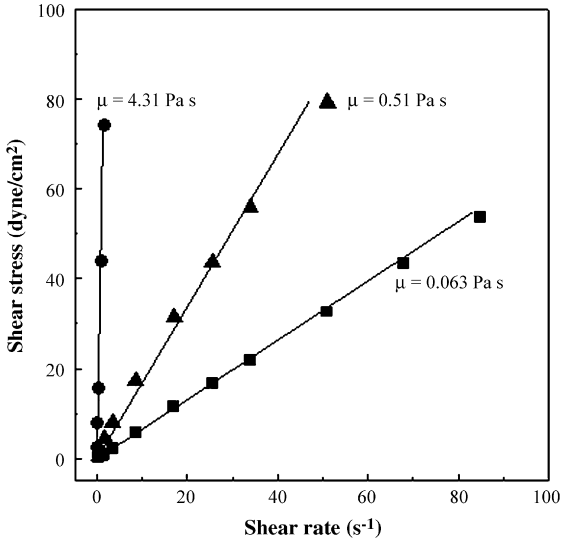

Fig. 4. Changes in apparent viscosity of highly concentrated solutions at various temperatures: (●) 60 °C; (▲) 70 °C; (■) 80 °C. Naringin and palmitic acid (0.2 mmol each) were mixed as substrates at 1:1. DMSO (30%, w/w) was used as an adjuvant.

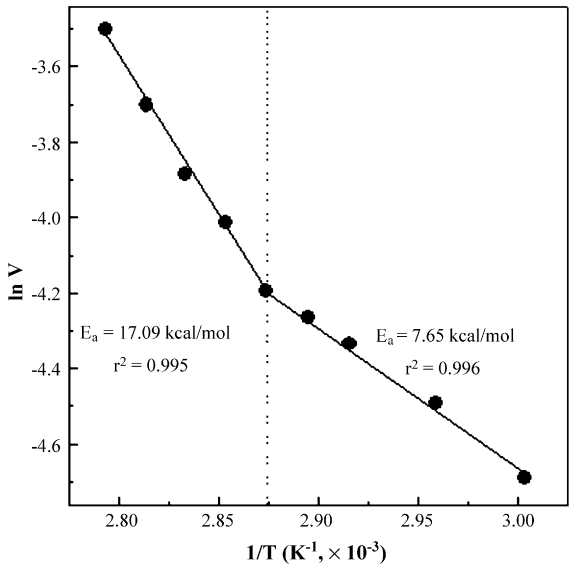

Fig. 5. An Arrhenius plot for reactions using highly concentrated solutions of naringin and palmitic acid. Dashed line: 75 °C. Naringin and palmitic acid (each 0.2 mmol) were mixed as substrates at 1:1. DMSO was added as an adjuvant to the substrate mixture at 30% (w/w).

reaction rates versus reverse temperatures was made (Fig. 5). From the slope, the activation energy was estimated to be 7.65 kcal/mol ($r^2 = 0.996$) below 75 °C, and 17.09 kcal/mol ($r^2 = 0.995$) over 75 °C. The activation energy value at 60–75 °C indicated the existence of a diffusion limitation whereas the value at 75–90 °C indicated no diffusion limitation [35]. Effective reactions seemed to require a temperature above 75 °C.

The conversion yields over time at different temperatures were obtained (Fig. 6). As the temperature increased from 50 to 80 °C, the conversion yield increased remarkably. The highest productivity, 29.5 mM/h, was obtained from 10 h of reaction at 80 °C, which is approximately 47 times greater than that in organic solvent media [24]. At 90 °C, a certain degree of enzyme inactivation probably occurred, causing the reaction to stop [25,36]. Consequently, the reaction temperature is considered to be a key determinant of the reaction by controlling viscosity of the highly concentrated solutions.

3.5. Additive effect of activated molecular sieves on conversion yield

As the reaction progressed, the highly concentrated solutions changed to highly viscous and cloudy solutions. This phenomenon should have inhibited further progress of reaction [28–31].

We next determined whether the final conversion yield depended on either phase change or reaction equilibrium. Reactions were performed at 80 °C. The conversion yield increased rapidly up to 6 h then the rate was slowed until 10 h when the water content reached about 0.25% (Fig. 7). During the early reaction stage at 80 °C, the highly concentrated solutions were kept in a homogeneous state thus the reactions were thought to be kinetically controlled. However, as the reaction proceeded considerably, the media got highly viscous and cloudy. And the reaction did not proceed further after 10 h. However, the addition of activated molecular sieves to the media decreased water content from 0.25 to 0.2%. Simultaneously, the conversion yield increased from 31 to 38% (mol/mol), suggesting that the equilibrium state at 10 h was shifted by the water removal allowing establishment of a new equilibrium at 12 h. Thus, the final conversion yield was probably determined by equilibrium state, not by the phase change of reaction mixtures.

A variety of strategies for improving conversion yield in organic solvent media can be applied to reactions using highly

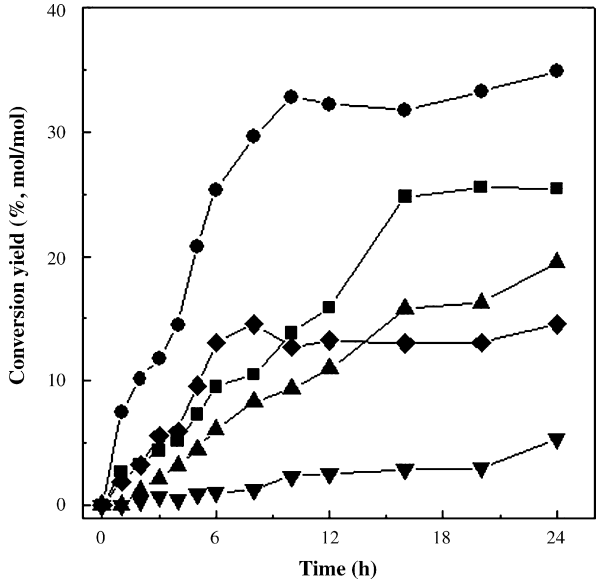

Fig. 6. Profiles for reactions using highly concentrated solutions of naringin and palmitic acid at various temperatures: (▼) 50 °C; (▲) 60 °C; (■) 70 °C; (●) 80 °C; (♦) 90 °C. Naringin and palmitic acid (each 0.2 mmol) were mixed 1:1 as substrates, DMSO (30%, w/w) was added as an adjuvant to the substrate mixture.

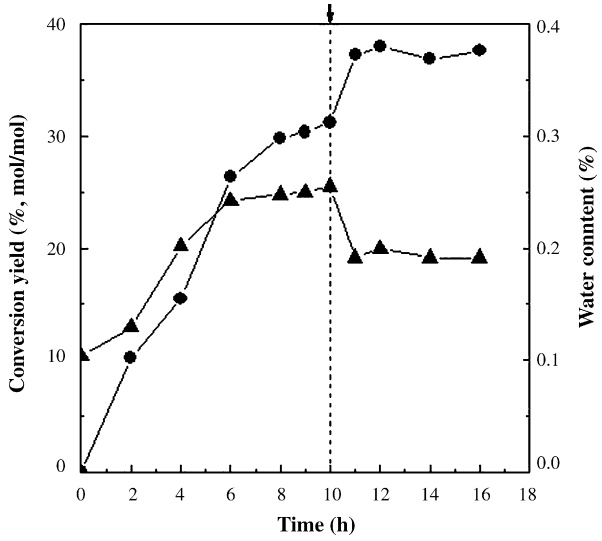

Fig. 7. Reactions profiles using highly concentrated solutions of naringin and palmitic acid at 80 °C: (●) conversion yield; (▲) water content. Naringin and palmitic acid (0.2 mmol each) were mixed 1:1 as substrates. DMSO (30%, w/w) was added as an adjuvant to the substrate mixture. An arrow indicates the input of activated molecular sieves.

concentrated solutions. For example, the enzyme content and molar ratio between naringin and palmitic acid can be varied. Alternatively, a by-product such as water can be removed, causing a shift of equilibrium state that allows further reaction progress [21].

When other fatty acids (C_4–C_{18}) instead of palmitic acid were used as acyl donors, highly concentrated solutions were formed (data not shown). We also found that other flavonoids, such as rutin could form highly concentrated solutions with various fatty acids (data not shown). Thus, various flavonoid derivatives can be synthesized via biocatalytic reactions at relatively high concentrations.

Reactions using highly concentrated solutions have important advantages against the organic media for industrial applications. High product yields or enhanced productivities can be accomplished. However, since it is not always possible to achieve highly concentrated solutions, many strategies for them should be developed for various substrate mixtures.

4. Conclusion

Highly concentrated and homogeneous solutions of naringin and palmitic acid were successfully formed below 40 °C by adding DMSO as an adjuvant. Substrate concentrations of the highly concentrated solutions were more than 20 times greater than those in the organic phase media, resulting in more than 15-fold increase in overall productivity. As the reaction proceeded, the media changed to highly viscous and disperse solutions. However, above 80 °C, the overall reaction was kinetically controlled and the final product yield was determined by equilibrium rather than phase change of the media.

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