

Enhanced activity of *Mucor javanicus* lipase in polyoxyethylene sorbitan trioleate containing microemulsion-based organogels

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Abstract

The efficacy of polyoxyethylene sorbitan trioleate (Tween 85) addition on the activity of *Mucor javanicus* lipase was investigated in sodium bis(2-ethylhexyl) sulfosuccinate (AOT) microemulsion-based organogels (MBGs). Gelatin was used as the gelling component of the MBGs. The maximal reaction rate was obtained at an AOT:Tween 85 molar ratio of 10:1. Under a fixed molar ratio system of AOT:Tween 85 = 10:1, the reaction rate also attained a maximum at a W_G ($=[\text{H}_2\text{O}]/[\text{AOT} + \text{Tween 85}]$ in MBG phase) value of 100 and an AOT concentration of 150 mM. The reaction proceeded under a reaction-controlled regime, and the reaction rate for the AOT/Tween 85 mixed system was about 2-fold higher than that for the AOT single system. The lipase activity was well maintained for 10 days and recovered by contacting the MBGs with concentrated amphiphile solutions.

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1. Introduction

Microemulsions, which consist of the dispersion of the nanometer-sized water droplets stabilized by amphiphilic molecules, have been widely reviewed as useful tools for enzyme reactions in hydrophobic solvent [1–3]. Especially, such media are effective for lipid transformations catalyzed by lipases [4,5].

Microemulsions gelled by adding natural polymers, i.e. microemulsion-based organogels (MBGs), have become of interest as novel hydrophobic gel matrices [6,7], and they are studied as enzyme immobilization and drug delivery carriers [8,9]. Sodium bis(2-ethylhexyl) sulfosuccinate (AOT) as amphiphile and gelatin as natural polymer are frequently employed for the stable MBG formation.

As enzyme immobilization carriers, MBGs containing lipases have been noted as effective catalysts for lipid transformations, and their activities are affected by the gel composition and maintained over a long period [8,10–12].

In developing MBG catalytic processes, the improvement of the enzyme activity is one of the main factors achieving high productivity. One approach to simplifying improvement of enzyme activity is to modify the microemulsion interface by adding nonionic surfactants. Addition of nonionic surfactants such as Span and Tween has been noted to improve the enzyme activity in an AOT microemulsion system [13,14,17]. Such co-surfactant additive methods are also expected to be effective for the improvement of enzyme activity in the microemulsion-based organogel systems.

In this paper, we report the effect of the addition of nonionic surfactant on the lipase activity in AOT microemulsion-based organogels. Lipase activity is also examined as a function of the water and amphiphile concen-

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Nomenclature

A_g	surface area of MBG particle (mm^2)
C_{AOT}	concentration of AOT in MBG phase (mM)
C_{gelatin}	concentration of gelatin in MBG phase (% (w/v))
C_s	concentration of substrate in organic phase (mmol dm^{-3} -organic phase)
$C_{\text{Tween 85}}$	concentration of Tween 85 in MBG phase (mM)
D_e	effective diffusion coefficient of substrate in MBG phase ($\text{m}^2 \text{s}^{-1}$)
$r_{(\text{MBG})}$	radius of MBG particle ($=3 V_g A_g^{-1}$) (mm)
t	time (day)
$V_{(\text{MBG})}$	reaction rate in MBG phase ($\mu\text{M s}^{-1}$)
V_g	volume of MBG particle (mm^3)
W_G	molar ratio of water to total amphiphiles in MBG phase (mol- H_2O /mol-AOT or mol- H_2O /mol-AOT + Tween 85 in MBG phase)

Greek letters

η	effectiveness factor (–)
ϕ	Thiele modulus (–)
Φ	observable modulus ($=\eta\phi^2 = V_{(\text{MBG})} r_{(\text{MBG})}^2/9D_e C_s$) (–)

Superscripts

BTA	butanol
DA	dodecanoic acid

trations in the gel. In addition, the lipase activity during the repeated batch reactions is observed.

2. Experimental

2.1. Chemicals

Sodium bis(2-ethylhexyl) sulfosuccinate (AOT, purity 99%) was purchased from Nacalai Tesque (Kyoto). Nonionic surfactant polyoxyethylene sorbitan trioleate (Tween 85) was obtained from Wako Pure Chemical Industries (Osaka). It has a hydrophile–lipophile balance (HLB) of 11 [22]. Lipase from *Mucor javanicus* (536 units/mg) and gelatin from porcine skin (Type A, Bloom 300) were supplied by Sigma (St. Louis, MO). 2,2,4-Trimethylpentane (isooctane), dodecanoic acid and butanol were also from Wako. All other chemicals were of analytical reagent grade.

2.2. Preparation of MBGs

The microemulsion phase was prepared by the addition of lipase solution (70 mg powder lipase) to isooctane contain-

ing the desired concentrations of AOT and Tween 85. Gelatin swelled after the addition of buffer solution. The buffer consisted of 50 mM KH_2PO_4 –50 mM NaOH solution (pH 7.0). The microemulsion phase and gelatin in buffer were individually heated at 328 K, then vigorously mixed until homogeneous dispersion was achieved. The mixture was then cooled to room temperature. The MBG thus obtained was stored at 253 K. MBGs without Tween 85 and/or lipase were also prepared in a similar manner. The water content in the microemulsion phase was determined using a Karl Fisher titrator (MKS-1s; Kyoto Electronics, Kyoto). The water content in the gel phase was calculated from the above preparation procedure.

2.3. Reaction procedure

The prepared MBGs (4 cm^3) were cut into cubes of approximately 2 mm unit-length using a razor blade, and then immersed into 10 cm^3 isooctane. The reaction was initiated with the addition of 10 cm^3 isooctane solution containing the desired concentrations of dodecanoic acid and butanol. All the reactions were performed with magnetic stirring (4 s^{-1}) at 298 K.

Lipase activity was determined by measuring the production rate of butyl dodecanoate. Butyl dodecanoate in the sample solution was analysed by gas chromatography (GC-8A; Shimadzu, Kyoto) using a glass column packed with FFAP/Uniport S (60/80 mesh, GL Sciences, Tokyo) and a TCD detector.

2.4. Measurement of conductivity

The conductivity in the MBGs was measured with a conductivity meter using a 3552-10D cell (DS-12; Horiba, Osaka). The cell was placed in the sample gel being studied. All the conductivity measurements were performed at 298 K.

2.5. Measurement of effective diffusion coefficient

The effective diffusion coefficients of dodecanoic acid and butanol in the MBG phase were measured as described in the literatures [11,12].

A gel particle containing substrate was held by a stainless steel wire, and immersed into 5 cm^3 of fresh isooctane phase. Mixing of the isooctane phase in the test tube was done using a rotating magnetic bar at the bottom. Each mass transfer was accomplished in 3–5 s. The gel particle was allowed to remain in the final wash-out tube for at least 5 h for all remaining substrate to be recovered. All the experiments were performed at 298 K.

The concentration of dodecanoic acid eluted in the isooctane phase was determined by HPLC (TIM system; Shimadzu, Kyoto) with an ODS column ($4.6 \text{ mm} \times 250 \text{ mm}$; Shimadzu) and UV detection at 215 nm. Methanol containing 10% (v/v) water was used as the mobile phase. The concen-

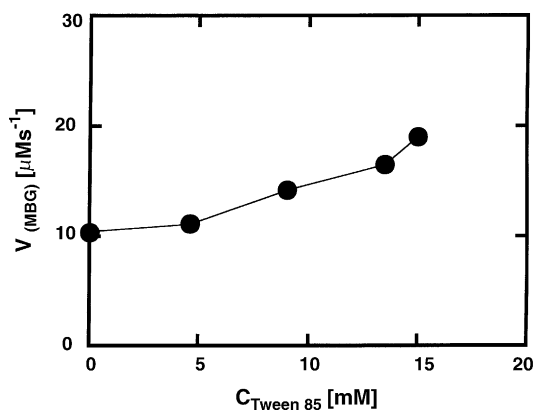


Fig. 1. Effect of Tween 85 concentration in MBG phase on reaction rate. $W_G = 100$; $C_{AOT} = 150$ mM; $C_{gelatin} = 18\%$ (w/v); dodecanoic acid = 100 mM; butanol = 100 mM.

tration of butanol was determined by gas chromatography as described above.

3. Results and discussion

3.1. Effect of Tween 85 concentration on lipase activity

Fig. 1 shows the effect of the Tween 85 concentration on the reaction rate. The reaction rates of this study were determined from the region where ester concentration exhibits a linear increase, not from the pre-steady state. The AOT concentration was set at 150 mM. The MBGs were formed at a Tween 85 concentration of 15 mM or below. When the Tween 85 concentration was more than 15 mM, the system separated into the gelatin phase and the organic phase.

The reaction rate gradually increased until the Tween 85 concentration was raised up to 15 mM, at which it attained about 2-fold that of the AOT single system. The addition of Tween 85 realized the improvement of the lipase activity in the MBG system as well as in the microemulsion system. In this system, the optimal molar ratio of AOT:Tween 85 for the reaction rate was found to be 10:1.

In conventional immobilized enzyme system, the influence of mass transfer on the overall reaction process can be viewed in relation to the effectiveness factor (η), which is a function of the observable modulus (Φ) as defined by

Eq. (1):

$$\Phi = \eta\phi = \frac{V_{(MBG)}r_{(MBG)}^2}{9D_eC_s} \quad (1)$$

The observable moduli within the experimental Tween 85 concentrations were in the range 0.05–0.13 for both substrates (Table 1), giving an effectiveness factor of 1 [21]. Thus, under the experimental Tween 85 concentrations, the reaction proceeded under a reaction-controlled regime. Therefore, the change in the reaction rate was dominantly caused by alterations in the kinetic factors, and not by diffusion phenomena in gel particles.

Tween 85 molecules are located at the interface of microemulsion and form mixed microemulsions with AOT molecules [13,17]. The lipase activity is improved by the decrease of the interface charge density of microemulsion, because of suppression of the hydrophobic and electrostatic interactions between the lipase and AOT polar heads and exclusion of AOT molecule from the microemulsion interface by bulky polyoxyethylene groups [13,14,17]. The AOT–MBG is composed of a three-dimensional network in which gelatin/water rod channels surrounded by a monolayer of amphiphile construct a basic framework [15,16]. Under such gel structure, Tween 85 molecules probably surround the gelatin/water rod channels with AOT molecules, and the lipase molecules are located at the interface of the rod channels. The improvement of the lipase activity in the AOT/Tween 85 mixed system was caused due to the decrease of the interface charge density around the gelatin/water channels by the addition of Tween 85.

Fig. 2 shows the conductivity of the MBG as a function of the Tween 85 concentration. The conductivity in the MBG decreased with increasing Tween 85 concentration. The electric charge carriers are expected to travel through the water-continuous channels in the MBGs [20]. The decrease of the conductivity reflected the decrease of the diameter and length of water-continuous channels. As the Tween 85 concentration was higher, there was apparently an increase of the interfacial area inside the MBG. In addition, a visual examination showed that the MBGs become from opaque to transparent with the increase of Tween 85 concentration. Although the relation between the MBG organization and the lipase activity has yet to be elucidated, the improvement of the lipase activity for the AOT/Tween 85 mixed system was also caused by a complex change of MBG networks.

Table 1

Observable modulus, reaction rate and effective diffusion coefficient for dodecanoic acid and butanol as a function of the Tween 85 concentration

C _{Tween 85} (mM)	ϕ^{DA} (–)	ϕ^{BTA} (–)	$V_{(MBG)}$ ($\mu\text{M s}^{-1}$)	D_e^{DA} ($10^{-10} \text{ m}^2 \text{ s}^{-1}$)	D_e^{BTA} ($10^{-10} \text{ m}^2 \text{ s}^{-1}$)
0.0	0.05	0.07	10.8	2.6	1.8
4.5	0.07	0.07	11.1	1.8	1.7
9.0	0.08	0.10	14.2	1.9	1.6
13.5	0.10	0.11	15.9	1.7	1.6
15.0	0.11	0.13	19.0	1.9	1.6

$\Phi = V_{(MBG)} r_{(MBG)}^2 / 9D_e C_s$, $r_{(MBG)} = 1$ mm, $C_s = 100$ mM for both substrates.

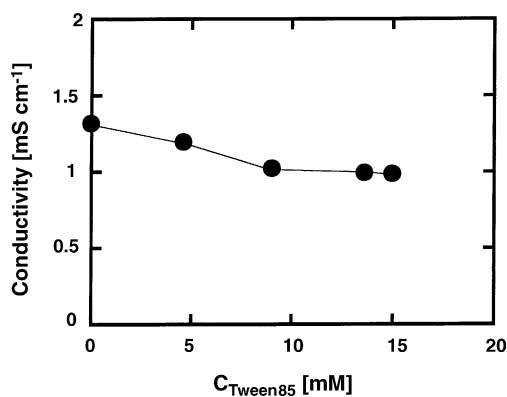


Fig. 2. Effect of Tween 85 concentration on conductivity in MBG phase. $W_G = 100$; $C_{\text{AOT}} = 150 \text{ mM}$; $C_{\text{gelatin}} = 18\% \text{ (w/v)}$.

3.2. Effect of water content on lipase activity

In this study, the water content in MBG phase was indicated by the molar ratio of water to amphiphile, $W_G (= [\text{H}_2\text{O}]/[\text{AOT}]$ for AOT single system and $[\text{H}_2\text{O}]/[\text{AOT} + \text{Tween 85}]$ for AOT/Tween 85 mixed system). Fig. 3 shows the effect of the W_G value on the reaction rate in the AOT/Tween 85 mixed system. The AOT concentration and molar ratio of AOT:Tween 85 were set at 150 mM and 10:1. As a comparison, the reaction rates in the AOT single system are also shown. In both MBG systems, the MBGs were formed at W_G values of 80 or above.

The reaction rate in the AOT/Tween 85 mixed system was dependent on the W_G value and took a maximum at a W_G value of 100. This profile corresponded to that of the AOT single system. It was also observed that over the experimental W_G conditions the reaction rates for the AOT/Tween 85 mixed system were about 2-fold higher than those for the AOT single system.

Table 2 shows the observable modulus for both substrates as a function of the W_G value in the AOT/Tween 85 mixed

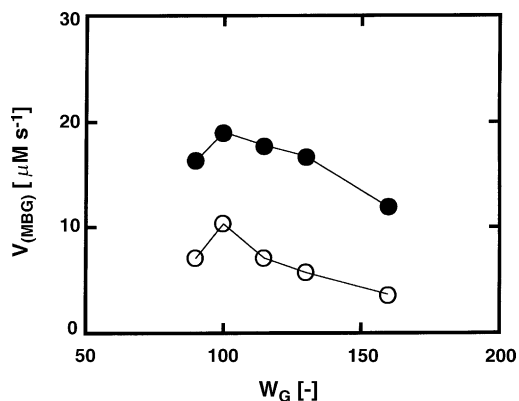


Fig. 3. Effect of W_G value on reaction rate in AOT/Tween 85 mixed and AOT single systems. $C_{\text{AOT}} = 150 \text{ mM}$; $C_{\text{gelatin}} = 18\% \text{ (w/v)}$; dodecanoic acid = 100 mM; butanol = 100 mM. For the AOT/Tween 85 mixed system, $C_{\text{Tween 85}}$ was 15 mM. The W_G value was defined as $[\text{H}_2\text{O}]/[\text{AOT} + \text{Tween 85}]$ for the AOT/Tween mixed system and as $[\text{H}_2\text{O}]/[\text{AOT}]$ for the AOT single system. Symbols: closed circles, AOT/Tween 85 mixed system; open circles, AOT single system.

Table 2

Observable modulus, reaction rate and effective diffusion coefficient for dodecanoic acid and butanol as a function of the W_G value in AOT/Tween 85 mixed and AOT single systems

W_G (-)	AOT/Tween 85 mixed system				AOT single system				
	ϕ^{DA} (-)	ϕ^{BTA} (-)	$V_{(\text{MBG})} \text{ (}\mu\text{M s}^{-1}\text{)}$	$D_e^{\text{BTA}} \text{ (}10^{-10} \text{ m}^2 \text{ s}^{-1}\text{)}$	ϕ^{DA} (-)	ϕ^{BTA} (-)	$V_{(\text{MBG})} \text{ (}\mu\text{M s}^{-1}\text{)}$	$D_e^{\text{DA}} \text{ (}10^{-10} \text{ m}^2 \text{ s}^{-1}\text{)}$	$D_e^{\text{BTA}} \text{ (}10^{-10} \text{ m}^2 \text{ s}^{-1}\text{)}$
85	0.12	0.11	16.3	1.5	0.03	0.05	7.1	2.3	1.7
100	0.11	0.13	19.0	1.6	0.05	0.07	10.8	2.6	1.8
115	0.12	0.12	17.8	1.6	0.04	0.04	7.1	2.0	1.8
130	0.11	0.11	16.7	1.7	0.04	0.04	5.8	1.5	1.8
160	0.10	0.08	11.9	1.7	0.04	0.02	3.6	1.0	1.7

$\phi = V_{(\text{MBG})} \tau_{(\text{MBG})}^2 / 9D_e C_s$, $\tau_{(\text{MBG})} = 1 \text{ mm}$, $C_s = 100 \text{ mM}$ for both substrates.

system. As a comparison, the data in the AOT single system is also shown. From the observable modulus for both substrates, the reaction proceeded under the reaction-controlled regime over the experimental W_G range.

The hydrophobic interactions in immobilization carriers play a significant role in the conformation of enzymes. In AOT microemulsions, the α -helix content of *Chromobacterium viscosum* lipase approaches a maximum at the optimal water content for catalytic activity [18], while the α -helix content of *Candida rugosa* lipase decreases with increasing water content [19]. It has been reported that the amount of the bounded water yielded by the electrostatic interaction of AOT polar head varies with the size of water droplet depending on the water content, which correspond to the change in the hydrophobicity near the microemulsion interface [23,24]. These findings indicated that the conformation of lipase is affected by the change of microenvironment near the microemulsion interface.

In the AOT–MBG system, the geometric parameters, e.g. diameter and length, of the water/gelatin rod channels increases with increasing water content [15]. Such changes in the rod channels seem to cause a change of the amount of bounded water as well as in the microemulsion system. Under the optimal water content ($W_G = 100$) in the present study, there was a geometrical structure in which provide the adequate hydrophobic environment for catalytic activity of lipase.

3.3. Effect of AOT concentration on lipase activity

Fig. 4 shows the effect of the AOT concentration on the reaction rate in the AOT/Tween 85 mixed and AOT single MBG systems. The W_G value and molar ratio of AOT:Tween 85 were set at 100 and 10:1. The MBGs were formed at an AOT concentration of 100 mM or above for the AOT/Tween 85 mixed system, while at 80 mM or above for the AOT single system. In the AOT/Tween 85 mixed system, the maximal reaction rate was obtained at an AOT concentration of 150 mM,

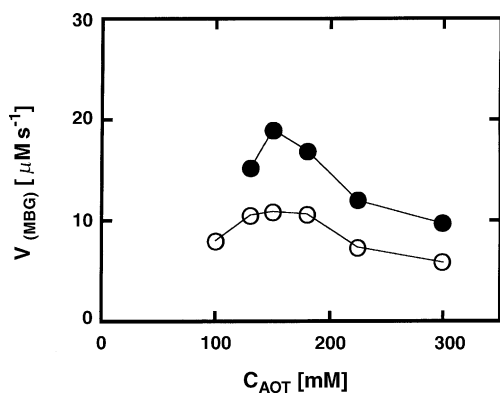


Fig. 4. Effect of AOT concentration on reaction rate in AOT/Tween 85 mixed and AOT single systems. $W_G = 100$; $C_{\text{gelatin}} = 18\%$ (w/v); dodecanoic acid = 100 mM; butanol = 100 mM. The molar ratio of $C_{AOT}:C_{\text{Tween 85}}$ was 10:1. The symbols are the same as those shown in Fig. 3.

as well as the AOT single system. Over the experimental AOT concentrations, the reaction rates for the AOT/Tween 85 mixed system were about 2-fold higher than those for the AOT single system.

Table 3 shows the observable modulus for both substrates as a function of the AOT concentration in the AOT/Tween 85 mixed system. The data in the AOT single system is also shown as a comparison. From the observable modulus for both substrates, under the experimental AOT concentrations, the reaction also remained under the reaction-controlled regime.

In the case of low AOT concentration, the MBG shrinks, and as a result the lipase activity decreased by the enhancement of the hydrophobic surroundings within the gel. On the other hand, in high AOT concentration the lipase activity seems to be affected by the enhancement of the electrostatic interaction because of the abundance of AOT molecules around the gelatin/water rod channels. However, this point is necessary to discuss based on exact pI of *M. Javanicus* lipase in future study.

3.4. Re-use reaction of immobilized lipase

Fig. 5 shows the reaction rate during repeated batch reactions in the AOT/Tween 85 mixed system. As a comparison, the reaction rates in the AOT single system are also shown. In each run, the reaction was continued for 24 h. After the reaction, MBGs were washed five times with fresh isooctane (20 cm^3) and then reused. The reaction rate in the AOT/Tween 85 mixed system remained ca. 80% of initial value after 10-day and about 2-fold that in the AOT single system.

Water molecules as a by-product during the reactions were accumulated in the MBGs. This accumulation of water within the MBGs is considered to be one of the main factors responsible for the decrease in lipase activity [8,10,11]. Residual water is removed effectively from the MBGs by contacting

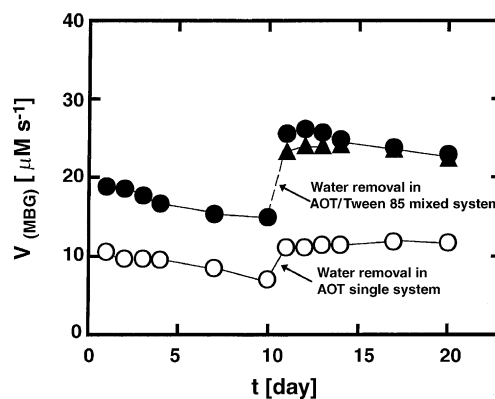


Fig. 5. Stability of lipase activity in AOT/Tween 85 mixed and AOT single systems. $W_G = 100$; $C_{AOT} = 150\text{ mM}$; $C_{\text{gelatin}} = 18\%$ (w/v); dodecanoic acid = 100 mM; butanol = 100 mM. For the AOT/Tween 85 mixed system, $C_{\text{Tween 85}}$ was 15 mM. The MBGs were contacted with (●) 1 M AOT/0.1 M Tween 85 solution or (▲) 1 M AOT solution for the AOT/Tween 85 mixed system and (○) 1 M AOT solution for the AOT single system.

Table 3
Observable modulus, reaction rate and effective diffusion coefficient for dodecanoic acid and butanol as a function of the AOT concentration in AOT/Tween 85 mixed and AOT single systems

C_{AOT} (mM)	AOT/Tween 85 mixed system					AOT single system				
	ϕ^{DA} (–)	ϕ^{BTA} (–)	$V_{(\text{MBG})}$ ($\mu\text{M s}^{-1}$)	D_e^{DA} ($10^{-10} \text{ m}^2 \text{ s}^{-1}$)	D_e^{BTA} ($10^{-10} \text{ m}^2 \text{ s}^{-1}$)	ϕ^{DA} (–)	ϕ^{BTA} (–)	$V_{(\text{MBG})}$ ($\mu\text{M s}^{-1}$)	D_e^{DA} ($10^{-10} \text{ m}^2 \text{ s}^{-1}$)	D_e^{BTA} ($10^{-10} \text{ m}^2 \text{ s}^{-1}$)
100	–	–	–	–	–	0.05	0.04	8.0	1.4	1.8
130	0.11	0.11	15.2	1.6	1.5	0.08	0.06	10.5	1.5	1.8
150	0.11	0.13	19.0	1.9	1.6	0.05	0.07	10.8	2.6	1.8
180	0.11	0.12	16.9	1.7	1.6	0.05	0.07	10.7	2.3	1.7
225	0.08	0.09	12.0	1.6	1.5	0.03	0.04	7.3	2.3	1.7

$\phi = V_{(\text{MBG})} r_{(\text{MBG})}^2 / 9D_e C_s$, $r_{(\text{MBG})} = 1 \text{ mm}$, $C_s = 100 \text{ mM}$ for both substrates.

them with the concentrated amphiphile solution [8,10,11]. In the AOT/Tween 85 mixed system, two different concentrated amphiphile solutions, i.e. 1 M AOT solution or 1 M AOT/0.1 M Tween 85 solution, were individually used to remove the residual water from the MBGs. The residual water was removed from the MBGs by using both concentrated amphiphile solutions. When these treated MBGs were reused, the reaction rates were about 1.5-fold higher than that in the beginning of the repeated reactions. The improvement of the reaction rate was due to overextraction of water from the MBG after treatment with the concentrated amphiphile solutions [8]. However, no significant differences in the reaction rate could be observed between the two concentrated amphiphile solutions. After the water removal procedure, the lipase activity in the AOT/Tween 85 mixed system remained again about 2-fold that in the AOT single system.

4. Conclusions

The effect of the addition of Tween 85 on the catalytic activity of *M. javanicus* lipase in AOT microemulsion-based organogels was investigated. The reaction rate was found to be maximal at the molar ratio of AOT:Tween 85 = 10:1. In the fixed molar ratio system at AOT:Tween 85 = 10:1, the maximal reaction rate was obtained at $W_G = 100$ and $C_{\text{AOT}} = 150 \text{ mM}$, which correspond that in the AOT single system. Based on an estimation of the effectiveness factor, the reaction was shown to be under a reaction-controlled regime for all MBG compositions. The reaction rate for the AOT/MBG mixed system was about 2-fold in comparison with that for the AOT single system. Also, the lipase activity was maintained over a long period by the removal of water accumulated in the MBGs.

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