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Fluorometric analysis on physicochemical properties of phosphatidylcholine-based W/O microemulsion involved with enzymatic reactivity in phospholipid hydrolysis

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Abstract−Interfacial tension and fluorometric analysis were employed for the investigation of the interfacial local fluidity and the hydrophobicity of the micro water pool in the PC-based water-in-oil (W/O) microemulsion. These micro-environment properties strongly influenced the phospholipase A_2 reactivity for phospholipid hydrolysis in the W/O microemulsion. The organic phase was prepared by mixing of isooctane as a main solvent and 1-butanol as a co-solvent. The critical micelle concentration (CMC) was dramatically decayed from 9mM to 0.025mM by the increasing of the 1-butanol content. The local interfacial fluidity of the micro water pool was measured by using fluorescence polarity indicated by 1,6-Diphenyl-1,3,5-hexatriene-4′-trimethylammonium tosylate (TMA-DPH) and Coumarin 343 (C343). It was apparently increased with increasing the molar ratio of additive 1-butanol. In contrast, the hydrophobicity of the water pool measured by C343 was almost constant throughout the molar ratio of additive 1-butanol. Additive alcohol influenced the micro fluidity and enhanced reactivity of phospholipase A_2 in lipid hydrolysis.

Key words: W/O Microemulsion, Phospholipid, Hydrophobicity, Interfacial Fluidity, Fluorometric Analysis

INTRODUCTION

W/O microemulsion was attractively utilized as a reaction medium for hydrophobic substrate enzyme lipase and phospholipase A_2 (PLA_2) [1-3]. Its reactivity was affected by the component of the organic phase, the water content and the amphiphilic molecule [2,4-6]. These parameters regulated the physicochemical properties of the W/O microemulsion.

In AOT-based W/O microemulsion, the water molecule in the micro water pool was strongly hydrated with negatively charged polar head of AOT molecules and its physicochemical property was different from that of bulk water [7].

The physicochemical properties of the W/O microemulsion referenced as "microenvironment" were examined by fluorescence analysis [8,9], dynamic laser scattering [10], and small angle X ray scattering [11]. Especially, fluorescence analysis was very fruitful for elucidating the microenvironment, because the fluorescent probe accurately indicates the local physicochemical properties at which the probe molecules are located. The hydrophobicity of the micro water pool [9], the viscosity of the micro water pool [12-14] and local fluidity of micro interface [8] were examined effectively by using an adequate fluorescent probe.

Phospholipid-based W/O microemulsion was employed as a reaction medium for the hydrolysis of phospholipid by PLA_2. The hydrolysis reactivity of phospholipase A_2 was significantly enhanced

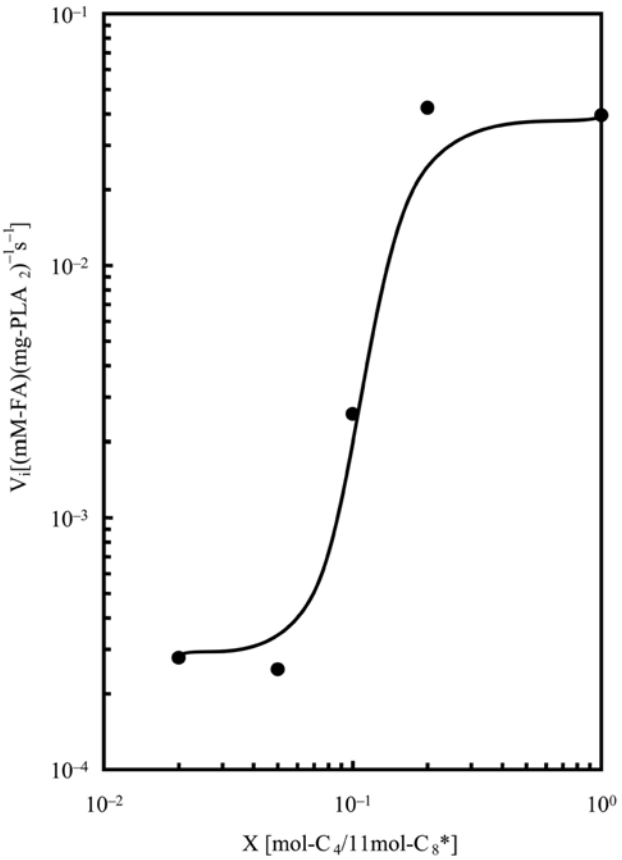

Fig. 1. Effect of the molar ratio of X [mol-C_4/11 mol-C_8^*] on the initial reaction rate. Molar ratio of C_8^* : C_4=11 : X, PC=27.6 mM, W=10, temperature=313 K, PLA_2=1.0 mg/50 ml in the W/O microemulsion phase. Data were quoted from our previous paper [2].

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150-fold by the additive co-solvent 1-butanol as shown in Fig. 1 [2]. The hydrophobicity of 1-butanol was evaluated as 0.8 of logP [15]. Based on the log P, 1-butanol has more hydrophilic character than that of main solvent isooctane. Partial added 1-butanol adsorbed on the microinterface [16] and other fraction of 1-butanol partitioned in the water pool. As a hypothetical speculation, the added 1-butanol changed the microenvironment of the W/O microemulsion.

In the present work, the interfacial local fluidity (interfacial fluidity) and the hydrophobicity of the inner water pool as microenvironments were examined by the interfacial tension and the fluorescence analysis. The influence of the microenvironment on the reactivity of phospholipase A_2 was discussed.

MATERIALS AND METHODS

1. Materials

Phospholipid (phosphatidylcholine; PC) (Epikuron 200) from soybean containing above 95% PC was provided by Nihon Sieber Hegner K.K. (Tokyo, Japan) and Lucas Meyer Co. (Hamburg, Germany). The organic phase of the W/O microemulsion employed 2,2,4-trimethylpentane (isooctane; C_8*) as a main solvent and *n*-alcohol (1-butanol; C_4) was employed as a co-solvent. They were analytical grade, and purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan).

TMA-DPH (1,6-Diphenyl-1,3,5-hexatriene-4′trimethylammonium tosylate, M.W.461.62) as the interfacial fluorescent probe was purchased from Fluka. C343 (Coumarin 343, M.W.285.3) as the hydrophilic fluorescent probe for the micro water pool was purchased from MP Biochemicals. Molecular formulas of fluorescent probes are shown in Fig. 2. The hydrophobicity of the water pool in the W/O microemulsion can be examined by fluorescence wavelength of C343. The shift of the wavelength to longer range referenced as "red-shift" presented hydrophilic character around C343 molecule [9].

2. Measurement of the Fluorescence Polarity of TMA-DPH and the Fluorescence Wavelength of C343

The composition of the organic phase was expressed by the molar ratio of isooctane (C_8^*) and 1-butanol (C_4), recorded as 11 [mol-C_8^*]: X [mol-C_4]. For the measurement of the fluorescence polarity, TMA-DPH (2 mg) was dissolved in the co-solvent (1 ml) in advance.

The W/O microemulsion was prepared in a 10 ml capped glass test tube at room temperature (296 K). Aqueous phase employed 50 mM Tris-HCl buffer (pH 8.0 at 313 K) containing 0.1 M $CaCl_2$.

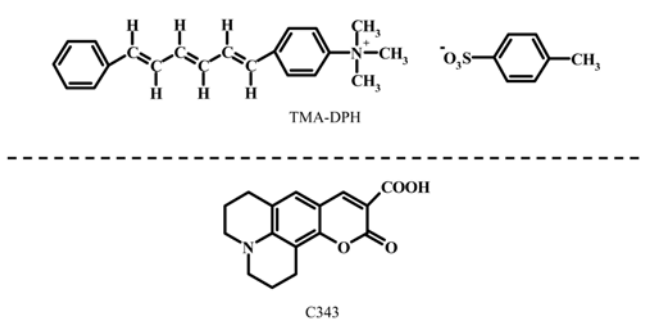

Fig. 2. Molecular structure of TMA-DPH and C343 as a fluorescence probe.

This temperature 313 K is adapted with the reaction condition of PLA_2 hydrolysis [2]. It was injected into the organic phase (5 ml) containing the desired amount of PC. For the measurement of the fluorescence wavelength, C343 (1.23 mg) was dissolved in the aqueous phase in advance. To dissolve the injected aqueous C343 phase, the mixture of aqueous and organic phase was stirred by a magnetic stirrer for 30 min at room temperature (296 K) in a darkroom. The transparent upper phase was employed for each measurement, and then the samples were centrifuged at 3,000 rpm for 10 min before measurement.

The water content in the W/O microemulsion phase was expressed by W_{add}. It was defined as a molar ratio of the added water to the added PC (W_{add}=[mol-H_2O]/[mol-PC]) [17].

The fluorescence polarity of TMA-DPH was measured with a Hitachi spectrofluorophotometer (F-7000). The excitation wavelength (λ_{ex}) was 360 nm, and the fluorescence wavelength (λ_{em}) was 435 nm. The fluorescence polarity of C343 measured using the excitation wavelength (λ_{ex}) was 430 nm, and the fluorescence wavelength (λ_{em}) was 470 nm.

The fluorescence wavelength of C343 was measured with a Shimadzu spectrofluorophotometer (RF-1500). The excitation wavelength (λ_{ex}) was 425 nm, and the maximum wavelength of fluorescence spectrum was measured in the range from 425 nm to 550 nm.

3. Measurement of the Interfacial Tension between the Organic Phase and the Aqueous Phase

The interfacial tension between the aqueous phase and the organic phase containing the desired concentration of PC was measured at room temperature by the drop-weight method [18,19]. In brief, the volume of one aqueous droplet separated from the tip of the needle immersed into the organic phase was measured. Then the interfacial tension was evaluated by the following equation:

$$\gamma = \frac{V \cdot (\rho_w - \rho_o) \cdot g}{r} \cdot F\left[\frac{N}{m}\right]$$

Where γ is the interfacial tension, V is the volume of the aqueous droplet, ρ_w is the density of the aqueous phase, ρ_o is the density of the organic phase, g is the gravitational constant (9.8 [m/s^2]), r is the radius of the needle and F is the correction factor [18,19].

RESULTS AND DISCUSSION

1. Physicochemical Properties of W/O Microemulsion

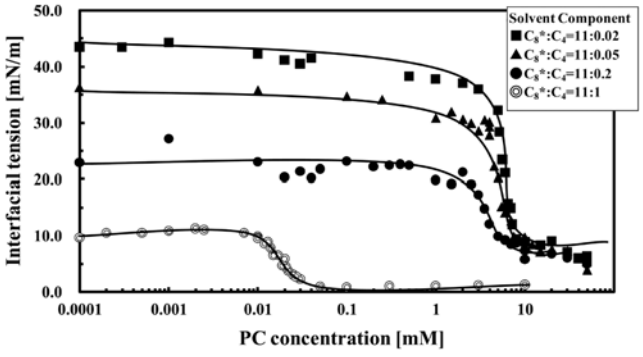

Fig. 3. Interfacial tension in variable PC concentration in each co-solvent content.

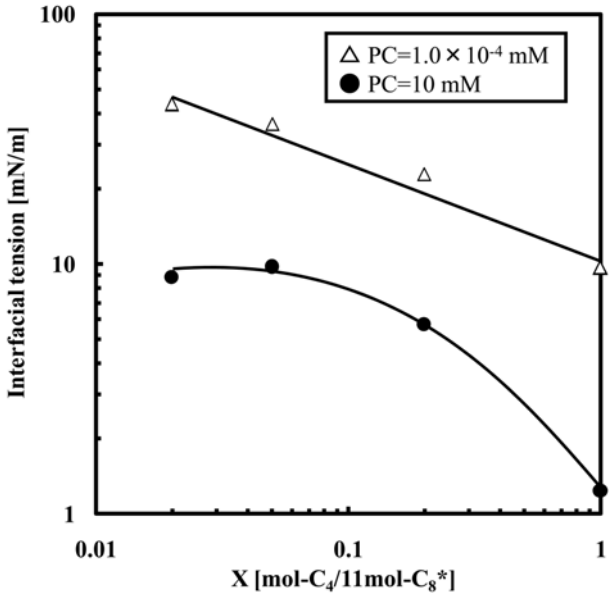

Fig. 4. Interfacial tension at PC=10 mM (●) and PC=1.0×10^{-4} mM (△) as a function of the 1-butanol content.

Fig. 3 depicts the change of the interfacial tension between the isooctane-1-butanol mixed organic phase and the aqueous phase with the PC concentration. In the lower molar fraction of 1-butanol (0.02≤X[mol-C_4/11 mol-C_8^*]≤0.2), the CMC was almost constant 9 mM. At the 1-butanol content equal 1.0 [mol-C_4/11 mol-C_8^*], the interfacial tension was drastically decayed and the CMC was obtained as 0.025 mM.

The change of interfacial tension for the PC=1.0×10^{-4} mM and the PC=10 mM is compared in Fig. 4. In the condition of PC=1.0×10^{-4} mM, the interfacial tension was gradually decreased from 43 mN/m to 10 mN/m, which depended on the −0.3 power of molar ratio of 1-butanol. In contrast, around the CMC condition (PC=10 mM), it largely depended on the molar ratio of 1-butanol in especially higher range of 1-butanol ratio. The effect of 1-butanol on the interfacial tension strongly appeared in higher PC concentration range over CMC.

In our previous work [17], the occupied interfacial area of single PC molecule was enlarged by the molar ratio of 1-butanol (Fig. 5). The interfacial area of X=1.0 [mol-C_4/11 mol-C_8^*] system was 5.5-fold larger than the X=0.02 [mol-C_4/11 mol-CC_8^*] system.

At the micro-scaled interface of microemulsion droplets, some geometrical irregularities was induced by two different size molecule, i.e., PC has 16-18 carbons chain and 1-butaol has 4 carbons chain. And then, the change of fluidity at the interface resulted by the additive alcohol.

2. Interfacial Fluidity of the W/O Microemulsion Droplet Measured by TMA-DPH

Fluorescence probe TMA-DPH participated on the interface of the W/O microemulsion droplet as well as amphiphilic molecule [20,21]. The signal of fluorescence polarity of TMA-DPH effectively indicates the interfacial fluidity of the W/O microemulsion [22]. Large value of fluorescence polarity indicates the lower fluidity around TMA-DPH molecule, and degradation of fluorescence polarity indicates the becoming higher fluidity of around TMA-DPH molecule.

As presented in Fig. 6, the fluorescence polarity of TMA-DPH significantly decreased from 0.22[−] to 0.16[−] in the range of X from 0.02 to 0.2. And then it was gradually decreased from 0.16[−] to 0.12[−] in the range of X from 0.2 to 1.0. This result was well consistent with the increasing profile of the occupied interfacial area as indicated in Fig. 5. Occupied interfacial area enlarged by the added 1-butanol molecule induced the increasing of the interfacial fluidity.

As previously depicted in Fig. 1, PLA_2 reactivity was remarkably enhanced 150-fold in the range of X from 0.05 [mol-C_4/11 mol-C_8^*] to 0.2 [mol-C_4/11 mol-C_8^*] by the 1-butanol addition. The interfacial fluidity was also considerably increased in the above-mentioned ranges of X. For example, according to the Arrhenius equation, the 150-fold enhancement of reaction rate under the constant

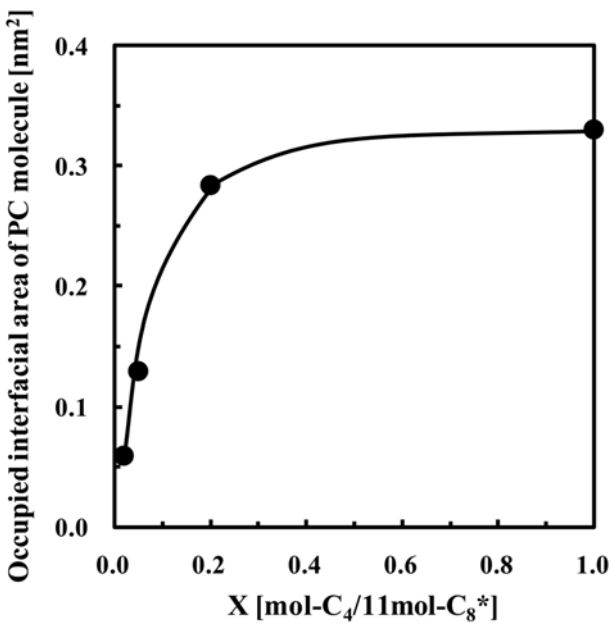

Fig. 5. Occupied interfacial area of the PC molecule at the micro-interface as a function of the molar ratio of X [mol-C_4/11 mol-C_8^*]. Data were quoted from our previous paper [17].

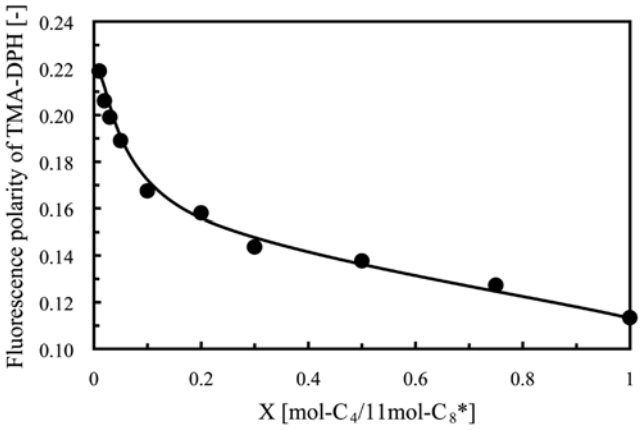

Fig. 6. Effect of the 1-butanol content on the fluorescence polarity of TMA-DPH in the W/O microemulsion. Measurement was carried out in PC=4.6 mM and W_{add}=10.

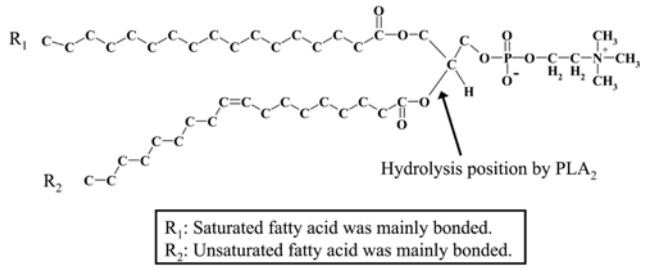

Fig. 7. Reaction scheme and hydrolyzed position in molecular chain by PLA_2.

reaction temperature resulted by decay of activation energy of 13 [kJ·mol^{-1}·K^{-1}]. This energy level was similar with the hydrodynamic fluid property of water, e.g., viscosity. The speculation strongly suggested that the change of fluidity of microinterface affected the reactivity of PLA_2.

PLA_2 hydrolyzes catalytically the ester bond between the fatty acid chain group and the glycerol group of the PC as illustrated in Fig. 7. This structural position is recognized as a boundary site of hydrophilic part of the PC and hydrophobic tail. In the higher fluidity condition at the micro-interface, PLA_2 can easily attack the targeting position, and then higher reactivity resulted.

3. Hydrophobicity and Fluidity of the Inner Water Pool

3-1. Influence of the Co-solvent on the Hydrophobicity of the Inner Water Pool

Fig. 8 depicts the influence of the molar ratio of X on the fluorescence wavelength (λ_{max}) of C343. In the aqueous buffer solution (50 mM Tris-HCl, pH 8.0, $CaCl_2$=100 mM), the fluorescence wavelength of C343 was obtained at λ_{max}=476 nm. This wavelength is recognized as a typical level of bulk water. In contrast, the wavelength appeared was almost constant level within narrow range 473 nm-470 nm for the W/O microemulsion (PC=46 mM, W_{add}=10 and C343=35.6 μM). The slightly change was induced by the partition

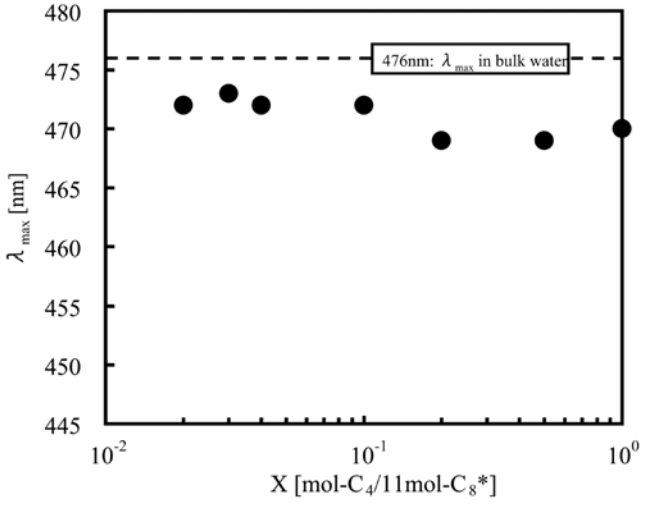

Fig. 8. Effect of the 1-butanol content (X [mol-C_4/11 mol-C_8^*]) on the λ_{max} of C343 in the W/O microemulsion. Measurement was carried out in PC=27.6 mM, W_{add}=10, C343=21.4 μM in the W/O microemulsion phase.

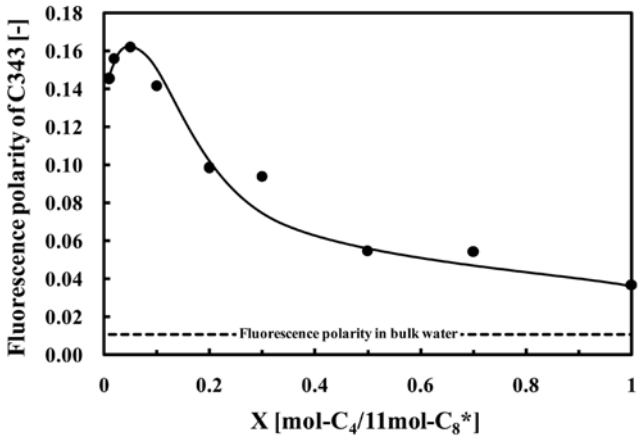

Fig. 9. Effect of the 1-butanol content on the fluorescence polarity of C343 in the W/O microemulsion. Measurement was carried out in PC=4.6 mM, W_{add}=10 and C343=3.56 μM.

of the 1-butanol into the water pool. This result suggested that the microenvironment of the water pool was similar to that of the bulk water throughout experimental range of 1-butanol.

3-2. Influence of the Co-solvent on the Fluidity of the Water Pool

Fig. 9 depicts the influence of the molar ratio of X on the fluorescence polarity of C343. The fluorescence polarity of C343 indicates fluidity of the water pool, because C343 is solubilized in the water pool [23]. The W/O microemulsion was prepared by PC= 46 mM, W_{add}=10 and C343=35.6 μM. Large value of fluorescence polarity indicates the lower fluidity around C343 as well as the case used TMA-DPH, and degradation of fluorescence polarity indicates the becoming higher fluidity.

In the range of X from 0.01 [mol-C_4/11 mol-C_8*] to 0.05 [mol-C_4/11 mol-C_8*], the fluorescence polarity showed a little bit higher. And then it decreased with increasing molar ratio of 1-butanol. As referenced in Fig. 1, the reactivity of PLA_2 was remarkably enhanced in the X from 0.05 to 0.2. In the same range, the fluidity of the micro water pool was apparently increased. This well agrees with the result indicated by TMA-DPH. The increasing of the fluidity at the micro water pool also induced the enhancement of reactivity of PLA_2 that cooperated with interfacial fluidity. The fluid environment would promote PLA_2 to attack the hydrolysis position more frequently.

CONCLUSION

The interfacial tension, the interfacial local fluidity and the hydrophobicity of the micro water pool in the PC-based water-in-oil (W/O) microemulsion was demonstrated and discussed the reactivity of phospholipase A_2. These microenvironment properties strongly influenced the phospholipase A_2 reactivity for phospholipid hydrolysis in the W/O microemulsion.

The critical micelle concentration (CMC) was clearly obtained throughout the 1-butanol mixed molar ratio (0.02≤X [mol-C_4/11 mol-C_8^*]≤1.0). In the range less than X of 0.2, the CMC was almost 9 mM. In the system of X=1.0, the CMC appeared as a very lower value of 0.025 mM. The occupied interfacial area of the PC molecule was enlarged by increasing of molar ratio of 1-butanol. The part of 1-butanol molecule co-operatively acted as an amphiphile with

the PC molecule to form the PC-based W/O microemulsion.

The fluidity of the interface was obviously increased in the range of X from 0.02 [mol-C_4/11mol-C_8^*] to 0.2 [mol-C_4/11 mol-C_8^*]. And the fluidity of the water pool was also increased in the similar range of X.

The hydrophobicity of the water pool was almost constant throughout the experimental range of the co-solvent 1-butanol. 1-butanol is apparently an effective reagent to increase the fluidity of the interface. Especially, at the X from 0.05 to 0.2, the fluidity of the interface was obviously increased. The fluidity increasing of nano-scaled interface induced the enhancement of enzymatic reactivity of PLA_2 in the W/O microemulsion.

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REFERENCES

1. K. Nagayama, S. Matsuura, T. Doi and M. Imai, *J. Mol. Catal. B: Enzymatic*, **4**, 25 (1998).
2. K. Yamazaki, M. Imai and I. Suzuki, *Biochem. Eng. J.*, **19**, 171 (2004).
3. C. Yao, S. Tang, J. Zhang and Y. Yu, *J. Mol. Catal. B: Enzymatic*, **18**, 279 (2002).
4. M. M. R. Talukder, Y. Hayashi, T. Takeyama, M. M. Zamam, J. C. Wu, T. Kawanishi and N. Shimizu, *J. Mol. Catal. B: Enzymatic*, **22**, 203 (2003).
5. D. Han and J. S. Rhee, *Biotechnol. Bioeng.*, **28**, 1250 (1986).
6. K. Naoe, M. Tamai, M. Kawagoe, M. Imai and M. Shimizu, *Trans IChemE*, **75**, 143 (1997).
7. A. Chattopadhyay, *Chemistry Physics Lipids*, **122**, 3 (2003).
8. R. Chaudhuri, P. K. Sengupta and K. K. R. Mukherjee, *J. Photochem. Photobiol. A: Chemistry*, **108**, 261 (1997).
9. T. Yamasaki, O. Kajimoto and K. Hara, *J. Photochem. Photobiol. A: Chem.*, **156**, 145 (2003).
10. M. Hakoda, N. Shiragami, A. Enomoto and K. Nakamura, *Bioprocess Biosyst Eng.*, **25**, 243 (2003).
11. S. Sugiura, S. Ichikawa, Y. Sano, M. Nakajima, X. Q. Liu, M. Seki and S. Furusaki, *J. Colloid Interface Sci.*, **240**, 566 (2001).
12. R. Bermejo, E. M. Talavera, C. delValle and J. M. Alvarez-Pez, *Colloids Surfaces B: Biointerfaces*, **18**, 51 (2000).
13. M. Hasegawa, T. Sugimura, Y. Suzaki and Y. Shindo, *J. Phys. Chem.*, **98**, 2120 (1994).
14. M. Hasegawa, T. Sugimura, Y. Suzaki and Y. Shindo, *Colloids Surfaces A: Physicochem. Eng. Aspects*, **109**, 305 (1996).
15. C. Laane, S. Boeren, K. Vos and C. Veeger, *Biotechnol. Bioeng.*, **30**, 81 (1987).
16. N. Garti, D. Lichtenberg and T. Silberstein, *Colloids Surfaces A: Physicochem. Eng. Aspects*, **128**, 17 (1997).
17. K. Yamazaki, M. Imai and I. Suzuki, *Colloids Surfaces A: Physicochem. Eng. Aspects*, **293**, 241 (2007).
18. W. D. Harkins and F. E. Brown, *J. Am. Chem. Soc.*, **38**, 288 (1916).
19. *idem.*, *ibid.*, **41**, 499 (1919).
20. R. Abuin, E. Lissi, A. Ceròn and M. A. Rubio, *J. Colloid Interface Sci.*, **258**, 363 (2003).
21. M. P. Mora, C. Tourne-Peteilh, M. Charveron, B. Fabre, A. Milon and I. Muller, *Chem. Phys. Lipids*, **101**, 255 (1999).
22. L. Silva, A. Coutinho, A. Fedorov and M. Prieto, *Biochimica et Biophysica Acta - Biomembranes*, **1758**, 452 (2006).
23. K. Bhattacharyya, K. Hara, N. Kometani, Y. Uozu and O. Kajimoto, *Chem. Physics Letters*, **361**, 136 (2002).