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Higher order structure of *Mucor miehei* lipase and micelle size in cetyltrimethylammonium bromide reverse micellar system☆

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Abstract

The higher order structure of *Mucor miehei* lipase and micelle size in a cationic cetyltrimethylammonium bromide (CTAB) reverse micellar system was investigated. Circular dichroic (CD) measurement revealed that the lipase far-UV CD spectra changed markedly, going from buffer solution to the reverse micellar solution, and were very similar for any organic solvent used. The ellipticity of the solubilized lipase in the far-UV region markedly decreased with increasing water content (W_0: molar ratio of water to CTAB), indicating that the secondary structure of lipase changed with the water content. The linear correlation between the W_0 and the micelle size was obtained by measuring dynamic light scattering. From the linear correlation between the micelle size and W_0, the higher order structure of the solubilized lipase appears to be affected directly by the micellar interface. The species and concentration of alcohol as a cosurfactant had an inferior effect on lipase structure. Especially, at ratios of 1-pentanol to CTAB of less than 8, the secondary and tertiary structures of lipase were preserved in the reverse micelles. The CTAB concentration had little effect on the lipase structure in the micelles. The catalytic activity of the lipase solubilized in the CTAB reverse micelles increased with increasing the W_0.

Keywords: Reverse micelle; Lipase; Higher order structure; CTAB; Micelle size

1. Introduction

A reverse micelle consists of an aqueous micro-domain facing the polar heads of a surfactant whose hydrophobic chain interacts with a bulk organic solvent. Polar cores of micelles are nanometer-sized and are able to solubilize considerable amounts of water and hydrophilic proteins. From these features, reverse micelles are very attractive systems for some enzymes when substrates and/or products are hydrophobic and low water content is desired. This is often the case with lipase reactions, since the solubility of the substrate, e.g., a triglyceride, is markedly improved in an organic solvent [1,2]. Furthermore, the minute size (on a nanometer scale) of water pools in reverse micelles means that a reverse micellar organic solvent offers an enormous W/O interface area. In view of these advantageous features, the use of reverse micellar organic solvents and gels derived from them as reaction media for industrial processes involving a lipase-catalyzed reaction using a hydrophobic substrate is anticipated [3,4]. Similarly, the reverse micellar system is also very attractive for bioseparation. The extraction of protein using reverse micellar organic solvents is suitable for practical application on a large-scale and continuous processing because it corresponds to a liquid–liquid extraction [5,6].

It has been reported that several significant operating parameters govern the enzyme reaction in reverse micelles, such as water content [7–11], organic solvent [1,12,13], surfactant concentration [7,10,11,14] and cosurfactant concentration [14–16].

Protein has an intrinsic molecular architecture, which is crucial for protein function [17]. In particular, in the enzymatic

Abbreviations: AOT, aerosol-OT [bis(2-ethylhexyl) sulfosuccinate sodium]; CD, circular dichroism; CTAB, cetyltrimethylammonium bromide; DLS, dynamic light scattering; RM, reverse micelle; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis

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Nomenclature

A_0	molar ratio of alcohol to surfactant (mol-alcohol/mol-surfactant)
D_{rm}	diameter of reverse micelle (nm)
V_i	initial reaction rate (mM/min)
W_0	molar ratio of water to surfactant (mol-H_2O/mol-surfactant)

Greek letters

$[\theta]$	mean residue ellipticity (deg cm^2/dmol)
$[\theta]_{222,feed}$	mean residue ellipticity at 222 nm in feed solution (deg cm^2/dmol)
$[\theta]_{222,RM}$	mean residue ellipticity at 222 nm in reverse micellar phase (deg cm^2/dmol)

reactions using a reverse micellar system, the micellar environment may affect the higher order structure of an enzyme, leading to the alteration of its reaction behavior. Similarly, in protein extraction the experience of protein through the micellar environment is important to its structure recovered from the micelles. Therefore, an investigation of the effect of operating parameters on the enzyme structure is important for the application of reverse micelles to bioprocess media.

Lipase reactions using reverse micelles have been studied mainly in the systems formed by anionic surfactant bis(2-ethylhexyl) sulfosuccinate sodium (AOT) [18–20]. In the ionic reverse micellar systems, protein solubilization is governed mainly by electrostatic interaction between the protein and the polar head of the surfactants [21]. Many commercially available fungal lipases have a negative charge in a neutral pH range since they have an acidic isoelectric point (pI) [22]. Hence, from the relationship of the electrostatic interaction between the surfactant and the lipase, it is desirable to use a cationic surfactant for solubilizing the lipase in a micellar organic solvent.

In this study, we investigate the operating parameter effects on the higher order structure of *Mucor miehei* lipase solubilized in a reverse micellar system formed with cationic surfactant cetyltrimethylammonium bromide (CTAB). The micelle size of the CTAB reverse micellar system is also examined. *M. miehei* lipase is a favorable model lipase because of its broad range of applications in bioindustrial processes.

2. Materials and methods

M. miehei lipase (MW = 30,000) was purchased from Sigma (L-9031, St. Louis, MO), and its purity was guaranteed as minimum 4000 units per mg-solid using olive oil. As the lipase exhibited a single, strong band by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), the purity seemed to be sufficient to discuss CD spectra. The reproductivity of the experimental data was checked by duplicate examinations. Pavlenko et al. also reported its sufficient purity based on SDS-PAGE [23]. The requisite purity of enzyme is depended on subjects, such as more detail investigations or practical bioprocess applications. In further detail presentation and discussions on optical analyses, a further purification level of enzyme is necessary in the preparation of experimental systems. Cetyltrimethylammonium bromide (CTAB) obtained from Tokyo Chemical Industry (Tokyo, Japan) was used as an amphiphilic molecule. The purity was guaranteed more than 98%. 1-Pentanol and 1-hexanol were purchased from Kishida Reagents Chemicals (Osaka, Japan), and used as cosurfactants after distillation. Organic solvents, 2,2,4-trimethylpentane (isooctane), *n*-octane and *n*-hexane were purchased from Wako Pure Chemical Industries (Osaka, Japan). A phosphate buffer solution (disodiumhydrogen phosphate–sodium dihydrogenphosphate, 0.1 M, pH 7.0) was used as an aqueous phase.

CTAB reverse micellar solutions were prepared by injecting the buffer solution into the alcohol–organic solvent solution containing CTAB, and transparent, stable solutions were obtained. Alcohol as a cosurfactant was contained in the main solvent at 10 vol%, at which a CTAB-alcohol–organic solvent solution has high water solubility.

Lipase solubilization in reverse micellar organic phases was conducted by injecting a lipase-containing buffer solution into a CTAB reverse micellar organic solvent (10 mL) in 100 mL screw-cap test tubes at 298 K to set the lipase concentration at 9.38 mM. The volume of the lipase-containing buffer solution was adjusted to obtain the micellar organic solution at the desired water concentration. The injected solution was mixed for 10 min at 250 rpm using a magnetic stirrer. After centrifugation at 2500 rpm for 3 min, the organic solution was measured by a UV–vis spectrophotometer (Shimadzu UV-2400PC) at 280 nm to determine the protein concentration.

A circular dichroic (CD) measurement was performed to investigate the higher order structure of lipase in the system. CD spectra were recorded with a Jasco J-820, using 1 cm cells at wavelengths from 250 to 350 nm and 1 mm cells at wavelengths from 200 to 250 nm. All spectra were corrected by subtracting a "blank" spectrum (without enzyme). The ellipticity is expressed in mean residue ellipticity.

The reverse micelle size was measured with a Sysmex Malvern HPPS system (Model HPPS5001), which is based on dynamic light scattering (DLS). The water content of the reverse micellar organic phase was measured by Karl-Fisher titration with a Hiranuma AQV-5S aquacounter.

The enzymatic activity of lipase was examined in terms of its hydrolysis of olive oil. Hydrolysis reactions were performed in 100 mL screw-cap test tubes at 303 K. The enzymatic reaction was initiated by injecting a lipase-containing buffer solution (0.1 M acetate buffer, pH 5.6) into a pre-incubated CTAB/solvent solution (39 mL) containing the substrate and buffer solution at desired concentrations. The enzymatic activity was determined by measuring the increasing profile of fatty acid produced as described by Lowry and Tinsley [24]. Samples (0.2 mL) taken at desired running times were put into 4.8 mL of benzene in a glass tube. Cupric acetate aqueous solution (1 mL) containing pyridine (5%, w/v, pH 6.0) was then added into the tube and the solution was vigorously mixed for 1 min using a magnetic stirrer. After centrifugation at 2500 rpm for 5 min, the upper organic

phase was measured by a UV–vis spectrophotometer (Shimadzu UV-2400PC) at 715 nm.

3. Results and discussion

3.1. Solubilization of lipase in CTAB reverse micelles

We attempted to solubilize *M. miehei* lipase into the CTAB/1-hexanol/isooctane micellar solution to examine the capability of enzyme solubilization by the CTAB reverse micelles. The lipase was solubilized by injecting the buffer solution containing lipase. Fig. 1 displays UV spectra of the micellar organic phase after solubilization and in the buffer aqueous solution. For comparison, a UV spectrum of CTAB/1-hexanol/isooctane solution before solubilization is also illustrated. The CTAB reverse micellar organic solution after solubilization was clear, transparent and thermodynamically stable over time. Peaks at an absorbance of 280 nm in both spectra, indicative of aromatic amino acid residues of lipase, reveal that lipase was solubilized in the CTAB/1-hexanol/isooctane reverse micellar system. In the 1-hexanol/isooctane solution without CTAB, no lipase solubilization was observed.

3.2. Effect of solubilization and organic solvents on lipase structure in reverse micellar organic phase

Fig. 2 illustrates the CD spectra of lipase in the far-UV region solubilized in CTAB reverse micellar organic phases formulated with various organic solvents, compared to that in the buffer aqueous solution. The organic solvents used to prepare the micellar phase were isooctane, *n*-hexane and *n*-octane, and 1-hexanol was used as a cosurfactant. There are marked CD changes in all the solvent systems in the far-UV region, going from the buffer solution to the reverse micellar organic phases. This indicates that the secondary structure change of lipase occurs on going from the buffer solution to the CTAB/1-hexanol/isooctane reverse micelles, because in general, CD spectra in the far-UV (200–250 nm) region indicate the secondary structure of a protein.

Several reports regarding the higher order structure of lipase in reverse micellar systems determined by the CD technique

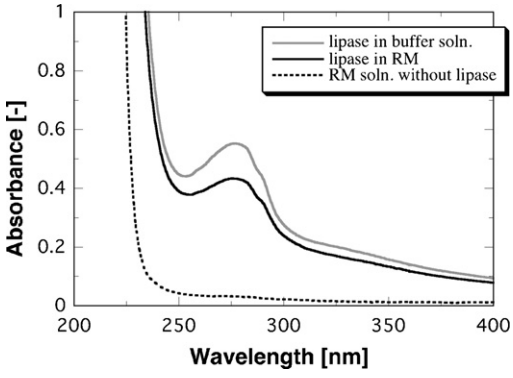

Fig. 1. UV spectra of *M. miehei* lipase in CTAB/1-hexanol/isooctane reverse micellar organic phase after solubilization and in the buffer aqueous solution. CTAB concentration = 0.1 M, 1-hexanol concentration = 10 vol%.

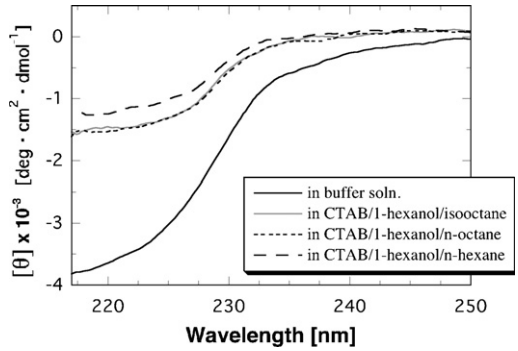

Fig. 2. CD spectra of *M. miehei* lipase in CTAB reverse micellar organic phases formulated with various organic solvents in the far-UV region. 1-Hexanol was used as a cosurfactant (at 10 vol%). $W_0 = 10$, CTAB concentration = 0.1 M. CD spectrum of native *M. miehei* lipase in the aqueous solution was shown for comparison.

have been published. Upon the solubilization of *Candida rugosa* lipase in anionic AOT reverse micelles, the blue shift of a negative maximum of the spectrum was observed, which indicates a decrease in the α-helix content [25]. Similarly, upon incorporation of *Rhizopus arrhizus* lipase into an AOT reverse micelle, dramatic changes in both secondary structural elements and accountable secondary structures were reported [26]. It appears that the higher order structure of lipase in the reverse micellar system depends on the system.

The lipase in the micellar system demonstrated very similar spectra whatever the organic solvent used, although a slight increase was observed in the ellipticity in the *n*-hexane system. This indicates that the secondary structure of the lipase was almost independent of the organic solvent forming the system. The effect of organic solvent on the higher order structure of proteins in the AOT system has been examined [27]. In the case of lysozyme, the organic solvent was not observed to affect the secondary structure of the protein. In the cytochrome *c* and ribonuclease A systems, the ellipticity in the *n*-hexane system was slightly different from that in other solvent systems. The effect of organic solvents on the proteins solubilized appears to depend on the protein species.

3.3. Effect of water content on lipase structure and micelle size

The water content in the reverse micellar phase is an important operating parameter in lipase reactions using reverse micelles [8,10]. In addition, the water content strongly influences the stability of an encapsulated enzyme [28].

Fig. 3 exhibits far-UV CD spectra of *M. miehei* lipase in 0.1 M CTAB/1-hexanol/isooctane reverse micellar systems at various water contents. The water content in the micellar organic phase is expressed as the molar ratio of water to surfactant CTAB, W_0. The spectrum of lipase depended on the water content W_0, and the mean residue ellipticity at wavelengths of 220–250 nm decreased with increasing W_0. In general, CD spectra in the far-UV (200–250 nm) region indicate the secondary structure of a protein. In particular, the mean residue ellipticity at 222 nm is used as an indicator of a protein's α-helix content [29]. Fig. 4a

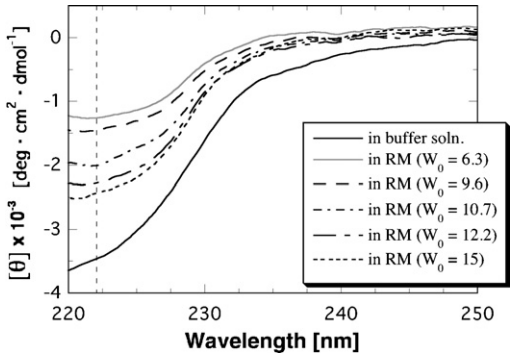

Fig. 3. CD spectra of *M. miehei* lipase in CTAB/1-hexanol/isooctane reverse micellar organic phases at various water contents in the far-UV region. CTAB concentration = 0.1 M. CD spectrum of native *M. miehei* lipase in the aqueous solution was shown for comparison.

and b reveal the effect of water content on the mean residue ellipticity at 222 nm of lipase solubilized in the CTAB reverse micelles, and the micellar size. These data were obtained in the 0.1 M CTAB/1-hexanol/isooctane reverse micellar system. The ellipticity at 222 nm increased with decreasing W_0 (Fig. 4a). The increase in the ellipticity at 222 nm with the W_0 indicates that the lipase loses its α-helix content with decreasing water content.

On the other hand, the reverse micellar size linearly increased with increasing W_0 as presented in Fig. 4b. The following empirical equation was obtained:

$$D_{rm} = 3.2 + 0.17\ W_0 \quad (5 \le W_0 \le 15) \tag{1}$$

The proportional constant between the mean diameter D_{rm} and the water content W_0 is 0.17. In the 0.1 M CTAB/1-pentanol/isooctane system, an empirical equation was not established in this study. Giustini et al. [30] measured the size of reverse micelles in the 0.1 M CTAB/1-pentanol/*n*-hexane system by pulsed field gradient spin-echo NMR and reported a proportionality constant of 0.15 with W_0. Vos et al. [31] reported a constant proportionality of 0.22 from the time-resolved fluorescence quenching technique in 0.2 M CTAB/1-hexanol/isooctane system. Despite the difference in analytical techniques, a similar dependency was obtained.

From the linear correlation, the interfacial curvature of reverse micelles may be larger at low W_0 values. Fugal lipase, *Penicillium simplicissimum* lipase is solubilized at the micellar interface [32]. Thus, these data imply that the higher order structure of lipase in reverse micelles is affected directly by the micellar interface. In Fig. 4a, the ellipticity increased more steeply below a W_0 of 14, indicating that the structure of lipase is more affected by the micellar size. From the linear correlation between the micelle diameter and the W_0 value, the diameter at $W_0 = 14$ is calculated as 5.6 nm. In order to compare the diameter of the micelle with that of native lipase, the size of native lipase was measured by DLS. The size of native lipase in the buffer solution (pH 7) is ca. 6.9 nm from our DLS measurement. This indicates that the lipase is solubilized in the reverse micelle smaller than the size of the native one. It seems that the lipase molecule is solubilized in a cramped state in the system.

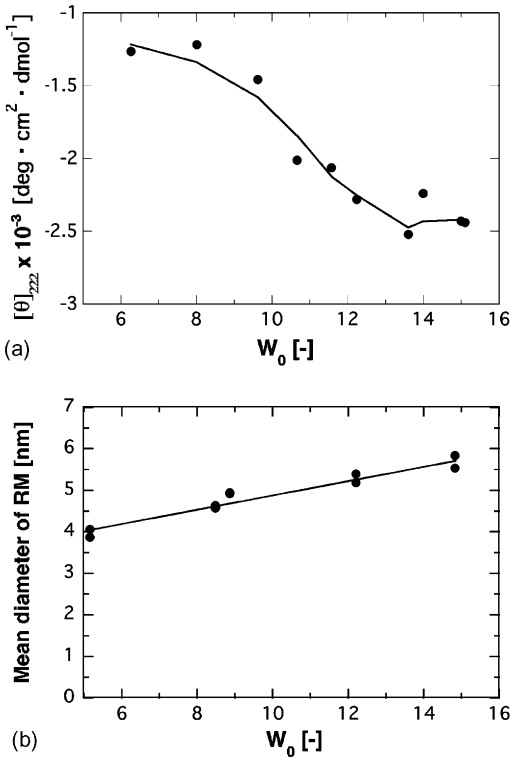

Fig. 4. Effect of water content on (a) mean residue ellipticity at 222 nm of the solubilized *M. miehei* lipase and (b) mean diameter of reverse micelles in the CTAB/1-hexanol/isooctane reverse micellar system. CTAB concentration = 0.1 M.

3.4. Effect of alcohol as cosurfactant on lipase structure and micelle size

CTAB requires a cosurfactant to facilitate the formation of reverse micelles. Hence, a cosurfactant is a requisite component for the application of CTAB reverse micellar media to biocatalysis. In spite of the importance of cosurfactants in the CTAB biocatalysis, no investigation on the effect of cosurfactant on the enzyme structure has been reported. Accordingly, the effect of the species and content of cosurfactants on the higher order structure of lipase solubilized in the CTAB reverse micelles was investigated. Fig. 5a and b illustrate far-UV CD spectra of *M. miehei* lipase solubilized in the CTAB reverse micellar system with various amounts of cosurfactants 1-hexanol and 1-pentanol. The CD spectrum of native lipase in the aqueous buffer solution is displayed for comparison. Alcohol content in the micellar phase is expressed as the molar ratio of alcohol to CTAB, A_0. Under the same A_0 conditions, the spectra in the 1-pentanol system were closer to that in the buffer solution than those of the 1-hexanol system, indicating that the secondary structure of lipase solubilized in the 1-pentanol system is closer to the native structure.

In both alcohol systems, the spectrum of lipase depended on the alcohol content A_0 and the mean residue ellipticity values increased with increasing A_0, indicating the decreasing of

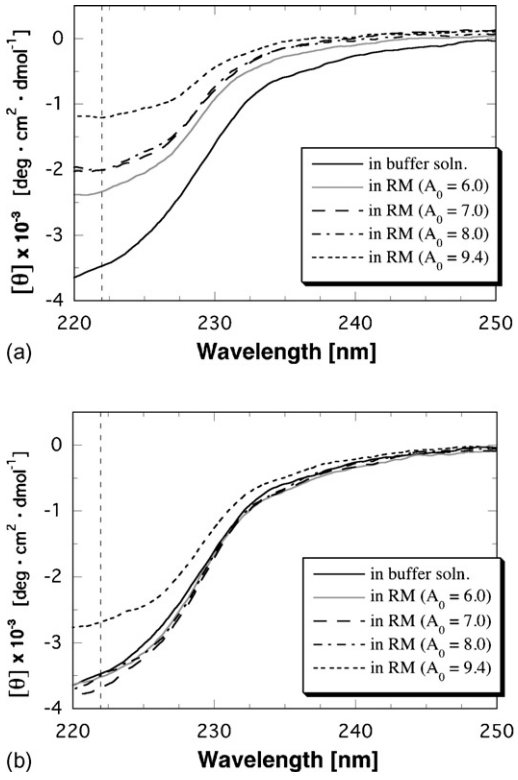

Fig. 5. Far-UV CD spectra of *M. miehei* lipase in CTAB/alcohol/isooctane reverse micellar organic phases with various contents of (a) 1-hexanol and (b) 1-pentanol. Alcohol content was expressed as A_0 = [alcohol]/[CTAB]. W_0 = 10, CTAB concentration = 0.1 M. CD spectrum of native *M. miehei* lipase in the aqueous solution was shown for comparison.

α-helix content of lipase with increasing alcohol content. In other words, at smaller A_0 values the spectrum appeared more like the native one. The 1-hexanol system indicated a stronger dependency on A_0 than the 1-pentanol system.

To clarify the difference between the alcohol systems, we calculated the ratio of the ellipticity at 222 nm of solubilized lipase in the micelles to that of the native one in the aqueous solution in each alcohol system (Fig. 6a). The ratio means the extent to which the secondary structure of lipase alters due to solubilization into the reverse micelles. In both alcohol systems, the ratio was almost constant for $A_0 < 8$ and declined for $A_0 = 9.4$. This indicates that the alteration of lipase structure due to solubilization depends on the alcohol content. The A_0 tendency of the ratio is very similar for the two alcohol systems.

Comparing the alcohol systems revealed that the ratio in the 1-pentanol system was larger than that of the 1-hexanol system. This implies that the extent of the alteration in the lipase structure due to solubilization also depends on the alcohol species forming the micellar system. A difference of even one carbon of a straight-chain alcohol forming the system markedly altered the higher order structure of lipase solubilized in the system. Despite the investigation on the two straight-chain alcohol systems in this study, it appears that the structure alteration depends on the carbon length of the alcohol forming the system.

The effect of each alcohol on the size of reverse micelles was investigated by DLS. Fig. 6b indicates the effect of alcohol content on the mean diameter of reverse micelles in each alcohol system. In the 1-pentanol system, the diameter decreased with increasing alcohol content A_0 while in the 1-hexanol system the diameter was almost constant at ca. 5.6 nm (i.e., independent of A_0). Thus, the cosurfactant effect on the micellar size was observed only in the 1-pentanol system.

Comparing the cosurfactant effects on the lipase structure (Fig. 6a) and the micellar size (Fig. 6b) indicates that the content effect on them is different. In the 1-pentanol system, although both the lipase structure and the micelle size decreased with increasing cosurfactant content, the decreasing trend differs. In the 1-hexanol system, the lipase structure changed with the cosurfactant content at $A_0 > 8$ despite the fact that the micelle size was constant in the corresponding content range. This suggests that the alteration of the lipase structure with cosurfactant content is not strongly involved with the change in the micellar size.

In previous papers, it was reported that the cosurfactant influences the size of CTAB reverse micelles [14,33]. Lopez et al. [16] also reported that the micelle size in the

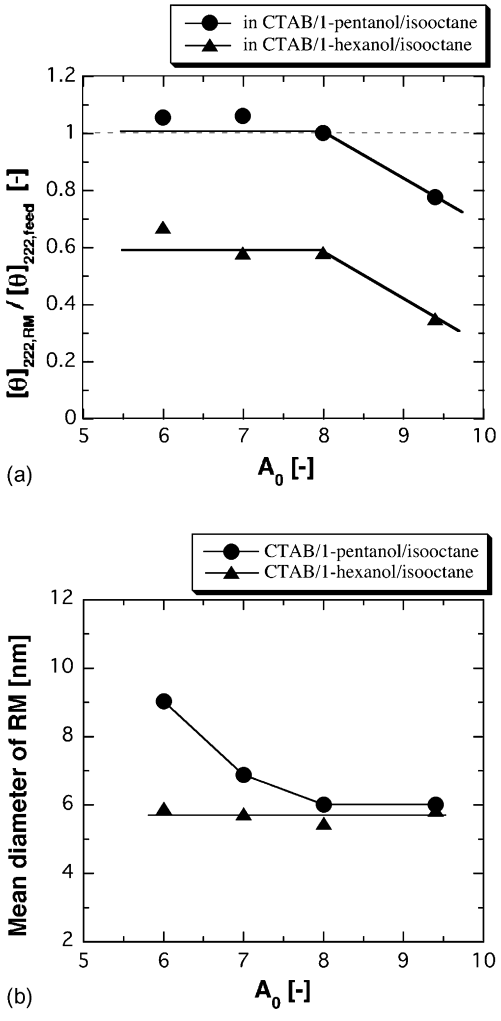

Fig. 6. Effect of alcohol content on (a) the ratio of the ellipticity at 222 nm of *M. miehei* lipase solubilized in CTAB micellar phases to that of the native one in the buffer solution and (b) mean diameter of reverse micelles in each alcohol system. CTAB concentration= 0.1 M, W_0 = 10.

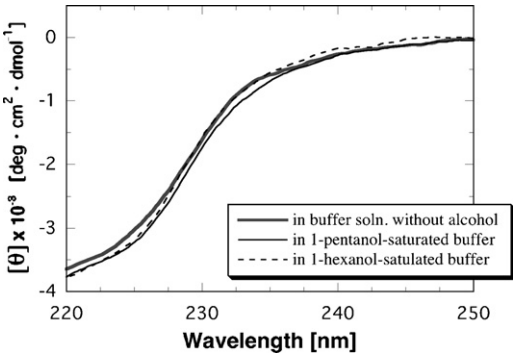

Fig. 7. Far-UV CD spectra of *M. miehei* lipase in alcohol-saturated buffer solutions. CD spectrum of native *M. miehei* lipase in the buffer solution was shown for comparison.

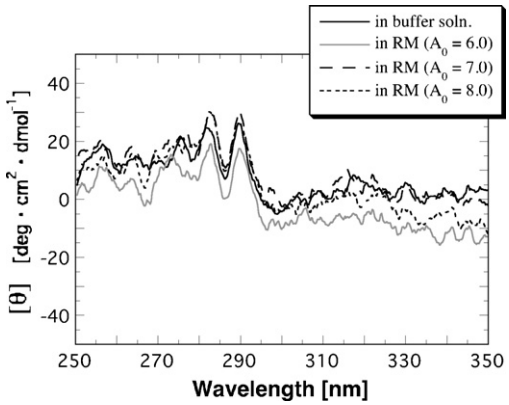

Fig. 8. Near-UV CD spectra of *M. miehei* lipase in CTAB/1-pentanol/isooctane reverse micellar organic phases with low1-pentanol contents. $W_0 = 10$, CTAB concentration = 0.1 M. CD spectrum of native *M. miehei* lipase in the aqueous solution was shown for comparison.

CTAB/pentanol/*n*-hexane system decreased with an increasing cosurfactant/surfactant ratio. They commented that this was due to the effective polar head area of CTAB increasing with the cosurfactant 1-pentanol content and, as a consequence, the micellar size decreases. Further discussion about geometric characteristics, such as aggregation number and alcohol distribution at micellar interface is necessary.

Fig. 7 illustrates the CD spectra of *M. miehei* lipase in a buffer solution saturated with each alcohol. The concentrations of each alcohol are 0.31 M for 1-pentanol, and 0.057 M for 1-hexanol. The CD spectra of lipase in the alcohol-saturated buffer solutions were similar with that of the native one without alcohol, indicating that each alcohol in the aqueous buffer solution does not affect the higher order structure of lipase. From this, it seems likely that the alcohol molecules distributed in the micro water pool of reverse micelles may not affect the lipase structure. A comparison in size between lipase and CTAB reverse micelles in the corresponding A_0 range (Fig. 6b) indicates that the lipase molecule can be solubilized in a micelle smaller than the native one (ca. 6.9 nm), except at $A_0 = 6$. This means that in this case the lipase interacts with the micellar interface, although the localization site of lipase in reverse micelles depends on the lipase species [32]. These experimental findings suggest that the alcohols at the micellar interface change the microenvironment of lipase, e.g., the hydrophobicity and the flexibility of the interface, resulting in the structural alteration.

It is of particular interest that for A_0 below 8 in the 1-pentanol system the ratio is almost unity. This indicates that the solubilized lipase has a secondary structure very close to that of the native one. Furthermore, the authors measured near-UV CD spectra of the lipase solubilized in the CTAB/1-hexanol/isooctane system with low alcohol contents $A_0 < 8$ (Fig. 8). As observed in Fig. 8, the spectra of the solubilized lipases are very similar to that of the native lipase in the buffer solution, indicating that the solubilized lipases have a tertiary structure very similar to that of native one. The CD data in both regions imply that at low 1-pentanol contents the lipase molecule is solubilized with an almost native structure. In the case of lysozyme in the CTAB reverse micellar system, a similar preservation of the enzyme structure after solubilization has been reported. Steinmann et al. [34] measured the near-UV CD spectra of lysozyme solubilized in CTAB/butanol/isooctane (1:4) and CTAB/chloroform/isooctane (1:1) reverse micellar systems. Lysozyme demonstrated positive CD spectra in both systems with only small differences from the aqueous solution. They concluded that slight conformational changes may occur when going from water to CTAB reverse micelles, but the tertiary structure of the enzyme is basically conserved.

3.5. Effect of CTAB concentration

A surfactant is also a requisite component for the formation of reverse micelles. The effect of the concentration of surfactant, of which CTAB was used in this study, on the higher order structure of lipase solubilized in reverse micelles was investigated. Fig. 9 exhibits far-UV CD spectra of lipase in CTAB/1-hexanol/isooctane reverse micellar phases in the CTAB concentration range of 0.1–0.3 M. The CD spectra did not strongly depend on the CTAB concentration. The spectrum of lipase at a CTAB concentration of 0.1 M was slightly similar in shape to the native one by comparison to those at higher CTAB concentrations. In the CTAB concentration range of 0.2–0.3 M, similar spectra were obtained. These indicate that lipase solubi-

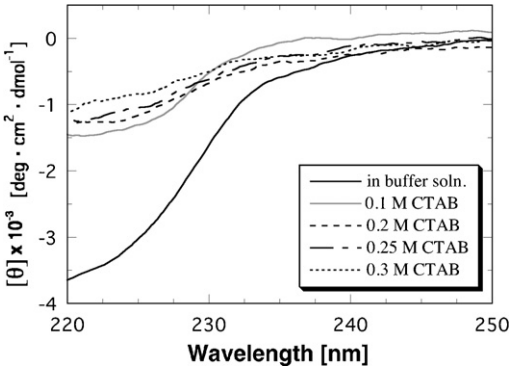

Fig. 9. Far-UV CD spectra of *M. miehei* lipase in CTAB/1-hexanol/isooctane reverse micellar organic phases at various CTAB concentrations. $W_0 = 10, A_0 = 8$. CD spectrum of native *M. miehei* lipase in the aqueous solution was shown for comparison.

Table 1
Initial reaction rates of olive oil hydrolysis by *Mucor miehei* lipase in CTAB/1-hexanol/isooctane reverse micellar system at various water contents

W_0	V_i (mM/min)
8	0.192
10	0.205
15	0.279

CTAB concentration = 0.2 M.

lized at 0.1 M CTAB is not comparatively affected by the CTAB concentration.

Reverse micelles constantly coalesce and redisperse, resulting in the exchange of material between the water pools [35]. From the collisions, the surfactant layers of reverse micelles interact one another and fuse together, and are finally reconstructed in discrete droplets. As the micellar size is linearly proportional to the water content W_0 as mentioned previously, increasing the surfactant concentration while maintaining the same W_0 causes increases the concentration of micelles. The increase of micelle concentration would strengthen the interaction between the micelles. Consequently, the fusion and reconstruction of the surfactant layers may be enhanced, thereby affecting the structure of lipase that strongly interacts with the micellar interface of the CTAB/1-hexanol/isooctane system as presented in the water content section. In fact, the higher order structure of lipase was affected at CTAB concentrations above 0.1 M. In the cases of cytochrome *c* and lysozyme in the AOT system that are solubilized at the micellar interface [36–39], the effect of AOT concentration on the protein structure was observed [27].

3.6. Lipase activity in CTAB reverse micellar system

The catalytic activity of *M. miehei* lipase solubilized in the CTAB reverse micellar system was examined in terms of its hydrolysis of olive oil. Table 1 shows the initial reaction rates of olive oil hydrolysis by *M. miehei* lipase in the CTAB/1-hexanol/isooctane system at several water contents. The solubilized lipase in the system exhibited a sufficient catalytic activity. The catalytic activity of lipase in CTAB reverse micellar systems has been reported by other research groups [15,16,40–42]. As well known, the ionic surfactants, such as AOT, CTAB, etc., inhibit strongly the enzymatic activity of lipase and cutinase in aqueous solution [43,44]. It is important to note that the lipase in the CTAB reverse micellar system exhibits a sufficient catalytic activity in spite of the strong inhibition effect of ionic surfactants in aqueous solution.

The lipase activity increased with increasing the water content W_0. The dependence of lipase activity on water content in reverse micellar systems has been widely reported in the literature, especially, the bell-shaped dependence of lipase activity is well known [8–10].

In the CD measurement of lipase at various water contents, the mean residue ellipticity of lipase decreased with increasing the water content, and approached the value of the native lipase in the aqueous solution (Fig. 3). These results imply that the solubilized lipase exhibits a higher catalytic activity when it has a closer structure to the native one.

In the *Chromobacterium viscosum* lipase reaction in anionic AOT reverse micellar systems, Yamada et al. [45] reported that the addition of non-ionic surfactant increased the lipase activity. They concluded that the improvement of the lipase activity was owing to the suppression of the hydrophobic and electrostatic interaction between micellar interface and lipase. In this study, it appears that the increase in micelle size with water content suppressed the interaction between micellar interface and lipase, which less influenced the lipase structure, thereby a higher lipase activity was obtained.

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