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Unsaturation at the surfactant head: Influence on the activity of lipase and horseradish peroxidase in reverse micelles

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Abstract

Influence of unsaturation present at the surfactant head on the activity of interfacially located enzyme, lipase, and horseradish peroxidase (HRP) is investigated in cationic reverse micelles of a series of surfactants having unsaturated (allyl and pyridinium moieties) as well as analogous saturated (*n*-propyl and piperidinium moieties) polar head. Lipase activity increases with *n*-propyl (saturated) substitution as the increase in the headgroup area (A_{min}) presumably provides greater space for the enzyme to attain flexible conformation and increases the local concentrations of enzyme and substrate at the interface. In contrast, activity of lipase decreases with increasing number of allyl (unsaturated) substitution though A_{min} gradually increased. Similar trend in deactivation was observed when unsaturation is present in cyclic ring (pyridine) at the surfactant head in comparison to the saturated analogue, piperidine. Circular dichroism (CD) spectra of lipase in reverse micelles indicate that ellipticity in the far-UV region increases with increasing unsaturation. Thus, lipase probably loses its α-helix content and thereby its activity. Inhibition of biocatalyst with increasing unsaturation at the polar head of surfactant is also observed in case of HRP, an oxidoreductase enzyme.

Keywords: Amphiphiles; Enzymes; Inhibition; Reverse micelles; Surfactant head; Unsaturation

Reverse micelles are optically transparent thermodynamically stable nanometer scale aggregates of water and surfactants dispersed in bulk apolar solvent. The surfactant molecules posed themselves with their polar head towards the aqueous core, popularly known as water pool and the hydrophobic tail in contact with the bulk apolar solvent [1–4]. These organized aggregates possess the potential for technological applications due to their enhanced interfacial area and increased ability to solubilize otherwise immiscible substrates [5–9]. Enzymology in reverse micelles has been an area of increasing interest during the past two decades owing to their wide-ranging biotechnological applications in different branches of science [10–18]. Lipases and HRP are the class of surface-active enzymes, widely employed for various transformations in water-in-oil (w/o) microemulsions [10–24].

Previous investigations by our group were intended to understand the effect of headgroup hydrophilicity, size, geometric constraints, and the nature of counterions on the activity of interfacially solubilized *Chromobacterium viscosum* (CV) lipase [25–29]. As a whole it was found that 'space' at the interface is the most crucial parameter, which largely regulates the lipase activity. Enzyme presumably attains flexible conformation at the augmented interface and with concomitant increase in the local concentrations of enzyme and substrate leading to higher activity of lipase [25–29]. Lipase activity was also found to alter with the variation in the substrate's chain length in reverse micelles [27,28]. While, the presence of unsaturation at the chain length of substrate (ester) is known to inhibit the hydrolytic activity of lipase [30–32]. Although, it was never discussed whether unsaturation present only in the substrate can inhibit lipase or

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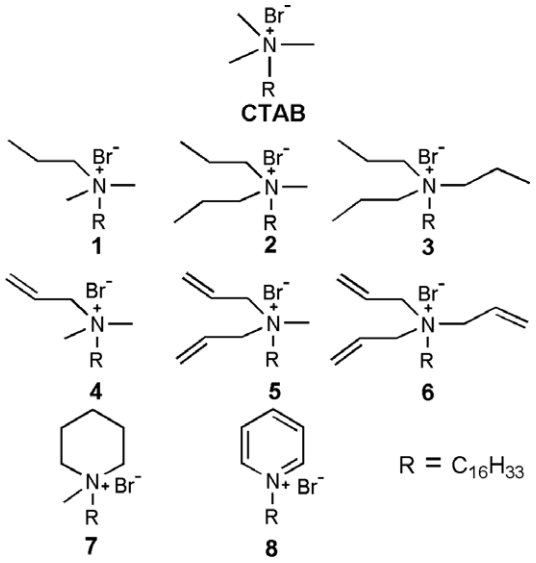

Chart 1. Structure of CTAB and cationic surfactants **1**–**8** with and without unsaturation at the head group.

anywhere in the vicinity of the enzyme also can do the similar resistance.

In order to observe the effect of unsaturation present in proximity of enzyme, activity of CV-lipase was investigated in the reverse micelles of a series of surfactants having acyclic saturated (*n*-propyl substitution, **1**–**3**) as well as analogous unsaturated (allyl substitution, **4**–**6**) and cyclic saturated (piperidinium, **7**), unsaturated (pyridinium, **8**) polar head (Chart 1). Sequential allyl substitution at the surfactant head (**4**–**6**) simultaneously increases the degree of unsaturation and area per headgroup (A_{min}). It will be interesting to find whether the increase in space at the interface improves lipase activity or presence of unsaturation deactivates lipase. Lipase activity increases with A_{min} for **1**–**3**, while it decreases from **4** to **6** with increase in unsaturation at the interface and is also notably lower compared to their saturated analogues (**1**–**3**). Although in previous instances, 'space' at the interface played the predominant role in regulating lipase activity, in the present study between space and unsaturation, later plays the crucial role. Similar trend in deactivation was observed when the enzyme activity is compared between cyclic saturated (**7**) and unsaturated (**8**) headgroup. Circular dichroism (CD) experiments were carried out to determine any possible correlation between the secondary structure of enzyme and the observed activity in w/o microemulsions. The ellipticity of lipase in the far-UV region increased with increasing unsaturation indicating its influence towards controlling the activity of lipase. Influence of unsaturation was also verified using a different category (oxidoreductase) of enzyme, HRP, whose activity was found to decrease with increase in degree of unsaturation at the interface.

Materials and methods

Materials. CV-lipase (EC 3.1.1.3, type XII) and Horseradish Peroxidase (HRP) (EC 1.11.1.7, Type II, RZ: 2.0) were purchased from Sigma and was used as received. Analytical grade CTAB from Spectrochem (India) was recrystallized three times from methanol/diethyl ether and the recrystallized CTAB was without minima in its surface tension plot. Allyl bromide and *n*-hexadecylamine were purchased from Spectrochem and SRL (India) and were of highest analytical grade. HPLC grade isooctane, *n*-hexanol, solvents, pyrogallol and all other reagents used in the syntheses were obtained from SRL (India). The UV–visible absorption spectra were recorded on Shimadzu UV-1700 spectrophotometer. ^{1}H NMR spectra were recorded on a Bruker Avance DPX-300 spectrophotometer. The synthetic procedures of different surfactants, ^{1}H NMR, elemental analysis and mass spectrometric information are available in Supplementary material).

Activity of interfacially solubilized CV-lipase. The second-order rate constant (k_2) in lipase-catalyzed hydrolysis of *p*-nitrophenyl-*n*-octanoate in cationic reverse micelles was determined at the isosbestic points (see Supplementary material) [20,33–35]. In a typical experiment, 4.5 μL of the aqueous enzyme stock solution (0.34 mg/mL) and substrate (10 μL, from 0.45 M stock solution in isooctane) were added to 1.5 mL of reverse micelles previously prepared with desired surfactant (50 mM) and pH (pH refers to the pH of the aqueous buffer solutions used for preparing the w/o microemulsions; pH within the water-pool of reverse micelles does not vary significantly, <1 U) [20,36], in a cuvette to attain the particular W_0 ([water]/[surfactant]). The reverse micellar solutions get clarified within 1 min. on gentle shaking. The initial linear rate of increase in absorbance of liberated *p*-nitrophenol in the reverse micelles of surfactants **1**–**8** was recorded in the UV–vis spectrophotometer. The overall concentration of lipase and *p*-nitrophenyl-*n*-octanoate are 1.02×10^{-6} g cm^{-3} and 3 mM, respectively. The concentrations of the reactants were referred to overall concentration to avoid complexity of the volume fraction of water droplets in reverse micelle and the partitioning of the substrate [20,23,34,35]. We have measured the second order rate constant (k_2) instead of first order Michaelis–Menten catalytic constant (k_{cat}), since the initial rate of lipase catalyzed hydrolysis of *p*-nitrophenyl-*n*-octanoate were observed to be first order with respect to substrate concentration [20,25,33–35]. The error in measured k_2 values were within the range of ±2–6%.

Measurement of peroxidase activity. In a typical experiment, 2.25 μL of the pyrogallol stock solution (from 0.2 M stock in acetone) and 3.0 μL of the aqueous enzyme stock solution (0.5 mg/mL) were added to the 1.5 mL of reverse micelle previously prepared with 25 mM surfactant at pH 7, in a cuvette to attain the particular W_0. Gentle shaking produced clarification of the microemulsion within 1 min. The overall concentration of the substrate and the enzyme inside the cuvette was maintained at 0.3 mM and 1 μg/mL. Lastly, 1.0 μL H_2O_2 stock solution (0.15 M in aqueous buffer) was added to attain $[H_2O_2] = 0.1$ mM inside the cuvette. The progress of the reaction was monitored by the formation of purpurogallin, the oxidized product of pyrogallol at 420 nm (λ_{max} of purpurogallin) for initial 5 min. The initial velocity (V) of this enzymatic oxidation was determined from the slope of the absorption intensity versus time curve, using the molar absorption coefficient of purpurogallin at 420 nm (4400 M^{-1} cm^{-1}) [37]. In triplicate experiments, the activity values were found to be varied within ±2–5%. To reduce the possible uncertainties associated with the measured velocity of the reaction, enzyme activity was expressed in terms of relative activity as reported earlier [38]. Relative enzyme activity, $A = V/V_0$ is reported, where V_0 is the initial velocity of the enzymatic reaction in the standard buffer solution.

Circular dichroism spectra. The CD spectra of lipase in reverse micelles were recorded in Jasco J-815 using 2 mm path length cell at wave length 220–250 nm with a scan speed 50 nm/min. All the spectra were corrected subtracting a blank spectrum (without enzyme) and accumulated for 6 times. Results were expressed in terms of mean residue ellipticity (deg cm^2 dmol^{-1}). Lipase (21.6 μL) (from a stock of 5 mg/mL at pH 7, 20 mM phosphate buffer) was injected for preparation of 2 mL microemulsions at $W_0 = 12$, $z = 4.8$ (for CTAB $W_0 = 40$, as it does not form macroscopically homogeneous solution below this W_0). The final concentration of lipase in w/o microemulsion was 54 μg/mL. CD spectra of surfactant **6** and **8** could not be measured due off-scale signal.

Results and discussion

In deciphering the role of unsaturation present at the interface on the efficiency of the surface-active enzymes we have synthesized a series of cationic surfactants having acyclic saturated (*n*-propyl substitution, **1**–**3**) as well as analogous unsaturated (allyl substitution, **4**–**6**) and cyclic saturated (piperidinium, **7**), unsaturated (pyridinium, **8**) polar head (Chart 1). Unsaturation at the headgroup increases from **4** to **6** and from **7** to **8**.

The aqueous critical micelle concentration (cmc) of the surfactants (**4**–**8**) has been measured by tensiometry and conductometry (Table 1) from the breaks in the plots of surface tension (γ) vs. [surfactant] and specific conductivity (κ) vs. [surfactant], respectively, indicating the onset of aggregation (see Supplementary material). The minimum area per surfactant headgroup at the micellar interface, A_{min} was calculated following the procedure as reported earlier [39–41]. Cmc is expectedly found to decrease with increasing headgroup size of the surfactant (Table 1). Cmc and A_{min} for surfactant **1**–**3** were taken from our previous report [27]. In general, introduction of unsaturation at the interface lowers the cmc in **4**–**6** compared to their saturated analogues (**1**–**3**).

To determine how the structural variation at the head of surfactant **1**–**8** in presence and absence of π electron influences the efficiency of surface-active enzyme, the catalytic activity of CV-lipase was examined in the reverse micelles of 0.05 M surfactant (**1**–**8**)/isooctane/*n*-hexanol/water at pH 6.0 (20 mM phosphate), z ([alcohol]/[surfactant]) = 4.8 and 25 °C across a varying range of W_0 (at which the isotropic solutions are formed). The second order rate constant, k_2 (Figs. 1 and 2) for the lipase-catalyzed hydrolysis of *p*-nitrophenyl-*n*-octanoate was found to be independent of W_0 for all the surfactants.

In case of *n*-propylated surfactants (acyclic saturated headgroup) k_2 expectedly increases from **1** to **3** (595–831 $cm^3\ g^{-1}\ s^{-1}$, Fig. 1) with increase in A_{min} 1.23–2.2 nm^2 (Table 1) in concurrence with our previous observation. The k_2 values were multiple folds higher than in widely used cationic CTAB based reverse micelles. As described earlier, with increase in interfacial area the enzyme may attain flexible conformation and also the local concentrations of enzyme and substrate increases at the augmented interface leading to higher activity of lipase [26–29]. A_{min} similarly improves with allyl substitution for unsaturated analogues **4**–**6**, 1.09–1.62 nm^2 (Table 1). At the same time unsaturation also increases from **4** to **6**. Although earlier reports revealed that presence of unsaturation at the substrate chain decrease the activity of lipase possibly due to difficulty of formation of the activated complex [30–32]. In the present study, the substrate chain (*p*-nitrophenyl-*n*-octanoate) does not contain any unsaturation, however π-electrons of the allyl group (**4**–**6**) are localized in vicinity of surface-active lipase at the interface. This time it would be really interesting to find whether lipase activity still increases with A_{min} from **4** to **6**. k_2 was found to be comparable 547 and 563 $cm^3\ g^{-1}\ s^{-1}$, respectively (Fig. 1), in monoallylated (**5**)

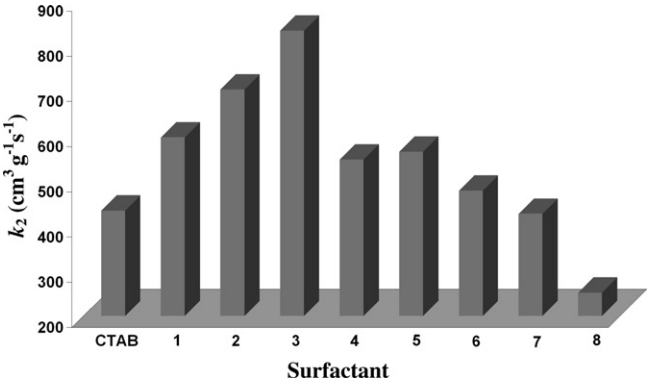

Fig. 1. Variation of the second order rate constant (k_2) for the lipase catalyzed hydrolysis of *p*-nitrophenyl-*n*-octanoate in different cationic reverse micelles formed at $z = 4.8$, 25 °C and pH 6.0 (20 mM phosphate). [Surfactant] = 50 mM, [enzyme] = 1.02×10^{-6} gm L^{-1}, [substrate] = 3 mM. The error in measured k_2 values were within the range of ±2–6%.

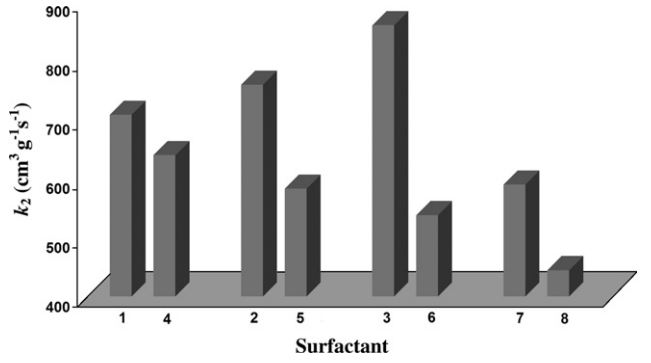

Fig. 2. Variation of the second order rate constant (k_2) for the lipase catalyzed hydrolysis of *p*-nitrophenyl-*n*-octanoate in different cationic reverse micelles of surfactants using least amount of *n*-hexanol required for each corresponding pair at 25 °C and pH 6.0 (20 mM phosphate). [Surfactant] = 50 mM. [enzyme] = 1.02×10^{-6} gm L^{-1}, [substrate] = 3 mM. For **1** and **4**, $z = 3.5$; **2** and **5**, $z = 2.8$; **3** and **6**, $z = 3.2$; **7** and **8**, $z = 3.2$. The error in measured k_2 values were within the range of ±2–6%.

Table 1
Critical micelle concentration (cmc), area minimum (A_{min}) of the surfactants **1**–**8**

Surfactant	$10^4 \times$ cmc (M)[a]		A_{min} (nm^2)
	Conductometric	Tensiometric	
1	9.5	6.12	1.23
2	7.26	2.04	1.4
3	6.33	1.38	2.2
4	8.49	5	1.09
5	5.99	4.27	1.25
6	5.5	3.38	1.62
7	6.46	2.87	1.09
8	8.78	6.19	1.05

[a] Experimental error within ±2% and ±3–5% for cmc measurements by tensiometric and conductometric method, respectively, in duplicate experiment.

and diallylated (**6**) surfactant though A_{min} increases from **5** to **6** (Table 1). The activity of lipase even decreases to 477 $cm^3\ g^{-1}\ s^{-1}$ for triallylated surfactant, **6** with $A_{min} = 1.62\ nm^2$. In comparison to the corresponding acyclic saturated analogues (**1**–**3**), lipase activity drops sharply with increasing unsaturation in **4**–**6**. Increase in π-electron at the interface makes the difference in k_2 higher between saturated and unsaturated headgroup (596–546 $cm^3\ g^{-1}\ s^{-1}$ from **1** to **4**; 701–563 $cm^3\ g^{-1}\ s^{-1}$ from **2** to **5**; 831–477 $cm^3\ g^{-1}\ s^{-1}$ from **3** to **6**). Thus, not only the presence of unsaturation at the substrate chain, also in the vicinity of enzyme/reacting site has notable influence on the lipase activity. This observation is consistent with the report that unsaturation present with in first five carbon atoms in substrate chain has prominent resistance on lipase activity.

To ascertain the observed inhibition is not limited to the acyclic unsaturated surfactant head, lipase activity was measured in the reverse micelles of cyclic saturated (**7**) and corresponding unsaturated (**8**) headgroup containing surfactants, keeping all the experimental conditions identical. k_2 plummets significantly from 426 $cm^3\ g^{-1}\ s^{-1}$ in **7** to 251 $cm^3\ g^{-1}\ s^{-1}$ in **8** (Fig. 1). Here also lipase looses ~40% of its activity moving from saturated to unsaturated polar head. Unlike substrate, unsaturation present at the interface is not likely to participate directly in the enzymatic hydrolysis, yet it inhibits the activity of lipase presumably resisting the formation of activated complex [30].

In this context, short chain alcohols are known to be competitive inhibitor of lipase [25,42]. Hence to discriminate the inhibitory effect of unsaturation from alcohols, it is essential to prepare w/o microemulsion with least amount of *n*-hexanol (co-surfactant), which will be at common z value for each saturated and analogous unsaturated surfactant. The activity of lipase in presence of least amount of hexanol for each pair of analogous surfactant (for **1** and **4**, $z = 3.5$; **2** and **5**, $z = 2.8$; **3** and **6**, $z = 3.2$; **7** and **8**, $z = 3.2$) was compared in Fig. 2. As expected, k_2 improved, though in a varying extent in reverse micelle of each surfactant due to lowering of *n*-hexanol. In case of saturated surfactant head, lipase activity increases with increasing number of *n*-propyl substitution (**1**–**3**). However, similar trend in deactivation was found in presence of olefin as observed at $z = 4.8$. In each pair of surfactants, lipase activity drops progressively with increasing number of unsaturation. The trend is consistent with cyclic analogues of saturated and unsaturated surfactant head (Fig. 2).

At this point we were curious to understand the influence of unsaturation on the higher order structure (secondary structure) of CV-lipase if any, and find a correlation with its activity. Fig. 3 illustrates the CD spectra of lipase in the far-UV region solubilized in the reverse micelles of different surfactants. The CD spectra in the far-UV region are indicative of the secondary structure of the protein and the mean residue ellipticity (MRE) at 222 nm denotes the protein's α-helix content [43]. The MRE was found to decrease with increasing *n*-propyl substitution (**1**–**3**) indicating increase in α-helix content and thereby improvement in lipase activity as described above (Figs. 1 and 2). In contrast, the increase in ellipticity at 222 nm with unsaturation implies that lipase loses it α-helix content as well as its activity. In concurrence with the observed trend in lipase activity, difference in ellipticity between saturated and unsaturated surfactant head becomes higher with increasing unsaturation. Among all the surfactants lipase had the lowest MRE and highest α-helical content in reverse micelle of surfactant **3** where the enzyme is most efficient. Thus, influence of unsaturation on lipase activity can be correlated to a certain extent with the secondary structure of enzyme. On the other hand, the MRE values in CTAB based microemulsion follow a pathway closer to that in **4** and **5** based system. Ellipticity value cannot be measured in **6** and **8** due to off-scale signal in CD spectra. Thus, no comparison can be made between cyclic analogues **7** and **8**. Relation between higher α-helical content and increased enzyme activity was further confirmed by taking CD spectrum of lipase in AOT based w/o microemulsions, which is always a proficient host for high lipase activity similar to surfactant **3** in present study. As to our expectation, ellipticity at 222 nm in AOT microemulsion is comparable to that observed in **3**.

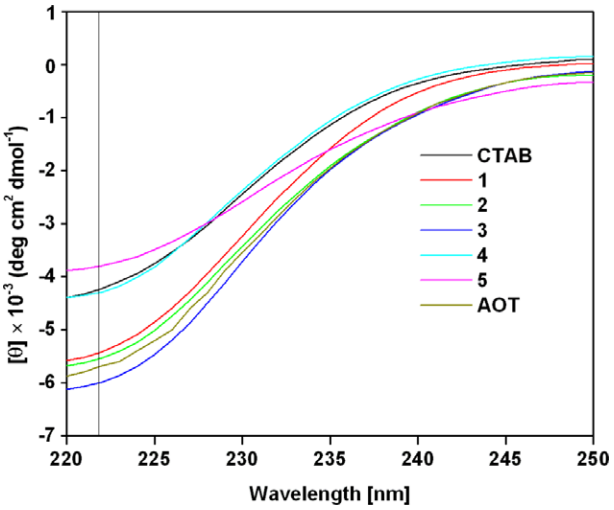

Fig. 3. CD spectra of CV-lipase in CTAB, AOT, surfactant **1**–**5**/1-hexanol/isooctane reverse micelles at $W_0 = 12$ (for CTAB $W_0 = 40$), $z = 4.8$, pH 6.0 (20 mM phosphate) and [surfactant] = 50 mM.

To find out whether the observed inhibiting role of unsaturation present at the interface is only specific to hydrolytic enzyme, lipase, we have estimated the catalytic activity of an oxidoreductase enzyme, HRP in the reverse micelles of **1**–**8** (25 mM, $z = 9.6$. pH 7.0) across wide range of W_0. Following the similar trend as it was observed in case of lipase here too HRP activity enhances (Fig. 4) with number of *n*-propyl substitution from **1** to **3** as a result of increased A_{min}. However, for unsaturated headgroups it decreases with increasing allyl substitution from **4** to **6**. Interestingly, for saturated polar head, HRP activity also increases with W_0 while that is almost unchanged across

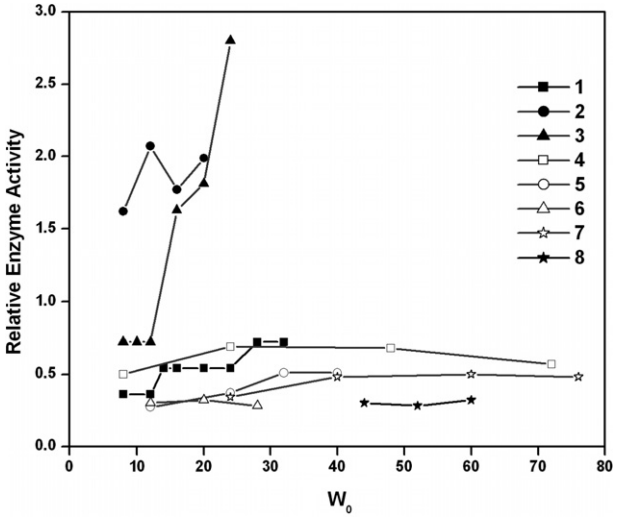

Fig. 4. Variation of the relative enzyme activity with W_0 for the HRP catalyzed oxidation of pyrogallol in cationic reverse micelles of **1**–**8** at pH 7.0 (25 mM phosphate buffer), [surfactant] = 25 mM, [pyrogallol] = 0.3 mM, [HRP] = 1 μg/mL, [H_2O_2] = 0.1 mM and $z = 9.6$.

wide range of W_0 for unsaturated headgroup. HRP showed almost comparable activity in case of **1** and **4** while difference in activity gets larger on moving to di and tri substituted analogous saturated (**2**,**3**) and unsaturated (**5**,**6**) surfactant head with W_0. Similar deactivation of HRP was also observed in analogous cyclic unsaturated headgroup (**8**) compared to the activity observed in saturated polar head (**7**).

In conclusion, for the first time the role of unsaturation present in proximity of enzyme on its activity has been questioned. Unsaturation introduced sequentially at the head group of surfactants constituting the reverse micelles was found to be inhibitory for the interfacially located enzymes viz lipase and HRP. The results also clarify that the inhibition by unsaturation overwhelms the activating effect of 'space' at the interface i.e. in presence of both the parameters, unsaturation plays the predominant role. CD spectroscopy also corroborated the results as lipase had lower α-helix content in the reverse micelles of unsaturated surfactants (**4**–**6**) while higher activity of lipase in **1**–**3** was supported by higher α-helix content.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2007.02.132.

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