ESTERIFICATION ACTIVITY OF LIPASE ENTRAPPED IN REVERSE MICELLES FORMED IN LIQUEFIED GAS

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For AOT-water-liquefied gas systems, we studied formation of reverse micelles, entrapping of lipase into water-pools of the reverse micelles, and esterification activity of the entrapped lipase. The formation of reverse micelles was confirmed with visual observation using a dye soluble in water for liquefied ethane and propane, but was not done for liquefied CO2. Water was solubilized as water-pools in large quantity for the system using liquefied propane and in small quantity for that using liquefied ethane. The entrapping of lipase was confirmed for the system of liquefied propane using lipase labelled with a fluorescence probe. The entrapped lipase exhibited high catalytic activity for esterification of lipophilic substrates, though the activity was a little lower than that in AOT reverse micellar solution formed using hexane as an organic medium. The activity varied with mole ratio of water to AOT, [H2O]/[AOT], and was maximized at a certain value of [H2O]/[AOT]. The value of [H2O]/[AOT] almost agreed with that in a AOT-hexane reverse micellar solution.

Introduction

Reverse micelles formed in a non-polar organic solvent solubilize water to form water-pools in their polar cores. Since Martinek et al. (1997), Luise et al. (1997) and Menger and Yamada (1979) reported that enzymes were solubilized into the water pools, much attention has been focussed on the catalytic behavior of such enzymes entrapped in reverse micelles. Many kinds of enzymes have been found to be entrapped into reverse micelles while they retained catalytic activities (Shield et al., 1986; Martinek et al., 1987a): they were active not only for hydrophilic substrates being soluble in the water-pools, but also for lipophilic substrates in an organic phase (Luther and Luise, 1984; Martinek et al., 1987b). The formation of reverse micelles was also found in non-polar supercritical fluids such as supercritical ethane (Lemert et al., 1990) and liquefied gases like liquefied propane (Yazdi et al., 1990). Further, these reverse micelles have been shown to be able to entrap proteins in their water-pools (Smith et al., 1990). To date, however, few reports have been found for enzymatic reactions in such reverse micelles.

In the present work, CO2, ethane and propane were mentioned as typical gases available and the followings were discussed: can they make reverse micellar solution under their liquefied conditions?, can the reverse micelles, if made, entrap lipase?, how active is the lipase, if entrapped, for esterification in comparison with lipase entrapped in reverse micelles made using organic solvents?

1. Experimental

1.1 Chemicals

The lipase was the same as that used in our previous work (Murakata et al., 1993; Sato et al., 1994). Bis (2-ethylhexyl) sodium sulfosuccinate (AOT) was purchased from Tokyo Kasei Kogyo Co., Ltd. and purified according to the usual technique (Menger and Yamada, 1979). Biebrich scarlet, dye, and fluorescamine, and a fluorescence probe were obtained from Schmid GmbH + Co. and Tokyo Kasei Kogyo Co., Ltd., respectively. Phosphate buffer (pH = 7.2, 0.067M) was used to form the water-pool in reverse micelles. All the organic solvents were distilled before use.

1.2 Equipments

Figure 1 (A) shows an autoclave used for the visual observation of the formation of water-pool in liquefied gas and for esterification experiments. The autoclave was made of stainless steel and had 100 ml of inner volume. It was equipped with a magnetically driven stirrer and optical windows which were set at upper and lower parts of the body. The lower window allowed the liquid phase to be seen, while the upper window allowed the gas-liquid interface to be viewed.

Figure 1 (B) shows a high-pressure fluorescence cell used for confirmation of entrapment of lipase in reverse micelles. It was made of stainless steel, and had 2 ml of inner volume and three optical windows. The windows were located so that an excitation beam passed from window 1 to window 2 and the emission beam emanated from window 3, which opened perpendicularly to the excitation beam path. The cell was 9 cm in diameter and the cylindrical part was 8 cm long. It was too large to mount on fluorescence spectrometers available and thus fluorescence measurements were carried out with handmade
apparatus as follows. An excitation beam (300-400nm) was obtained with a 150W Xe-lamp and a color filter (Toshiba UV-D35); the resulting emission beam was introduced into a monochromator (Jasco CT 10S) and light intensity was converted to electric signal with a photomultiplier tube (Hamamatsu R446).

1.3 Procedures

1) Confirmation of water-pool formation

Whether or not water-pools in liquefied gases were observed was observed at room temperature by the following procedure using the dye shown in scheme 1 (a). AOT (5.34 g) and 1.51 ml of phosphate buffer solution containing 0.20 g of the dye were placed in the autoclave. Then, liquefied gas was introduced until the liquid mixture reached 60 ml in volume, as measured from the height of the gas-liquid interface observed through the upper window. Under these conditions, the concentration of AOT was 0.2M, and the mole ratio of water to AOT ([H2O]/[AOT]) became 7.0. The liquid mixture was stirred with a magnetically driven stirrer for a while and then allowed to stand. Since the dye dissolves only in water and has strong absorption in the visible region to be red, the liquefied gas phase observed through the window must turn red if water-pools form. Thus, the color of the liquefied gas phase was observed through the windows.

2) Confirmation of lipase entrapment

Entrapment of lipase into water-pools was studied using the lipase labelled with fluorescence probe. The fluorescence-labelled lipase was prepared by a reaction of lipase with fluorescamine according to a procedure described in the literature (Bohlen et al., 1973). Scheme 1 (b) shows the reaction. The reagent bonds covalently with an amino group of the enzyme to become fluorescent. The excess reagent not binding the enzyme is hydrolyzed rapidly and thus only the labelled lipase emits fluorescence. For fluorescence measurements, AOT and the buffer solution of the labelled lipase were loaded into the cell and then the cell, was filled with liquefied gas. Concentrations of the lipase and AOT in the resultant liquefied gas mixture were kept constant at 0.32 g/l and 0.2M, respectively, while [H2O]/[AOT] was varied from 5 to 20.

When reverse micelles are formed and solubilize the labelled lipase, fluorescence must emanate from the liquefied gas mixture by UV illumination. However, if reverse micelles are not made, or even if made they can not solubilize fully the buffer solution added, the whole or a part of the buffer solution will not be solubilized, and it will remain at the cell bottom as a discrete phase. Since the labelled lipase must naturally be distributed into this discrete phase if it exists, the lipase entrapment should be confirmed with attention that the labelled lipase in the discrete phase is excited as little as possible. Therefore, the excitation beam was directed to the upper part of window 1 so as to pass through only a liquefied gas phase. For comparison, a similar fluorescence measurement was done on conventional micellar solutions using a quartz cell employing hexane in a range of 3.0 to 20 of [H2O]/[AOT], while the other conditions were kept the same as those for the liquefied gas. All the fluorescence measurements were done at room temperature.

3) Esterification

Esterification in the liquefied gas mixtures was carried out with the autoclave described above. Lauric acid and lauryl alcohol were used as substrates. AOT, the substrates and the buffer solution of lipase were placed in
upper window of the autoclave. The phase was red for propane, slightly red for ethane but colorless for CO₂. The coloration and difference in its degree informs which liquefied gas can make reverse micelles and what amount of water can be solubilized: liquefied propane and ethane made reverse micelles and enabled water to be solubilized in that order, and liquefied CO₂ could not make reverse micelles, and therefore no water was solubilized. Such information coincided with the observation through the lower window: the discrete water phase of the buffer solution was present for liquefied CO₂ and ethane after vigorous stirring. The above results suggest that of the gases used, liquefied propane is the most suitable for making reverse micelles and therefore entrapping lipase.

Incidentally, it is very interesting to ascertain the maximum water amount solubilized as the critical property of reverse micellar solutions made of liquefied gases. Such a property can be measured by a spectroscopic method using a dye. It, however, was not measured because the apparatus employed was not suitable for the purpose and because it was beyond the scope of the present work.

2.2 Entrapment of lipase into reverse micelles

On the basis of the above finding, propane was mentioned as a liquefied gas and the entrapment of lipase was studied. During the UV-illumination, the liquefied propane solution was emitting blue fluorescent light, which was observed directly with naked eyes through window 3. Since liquefied propane itself does not solubilize lipase, the fluorescence had to be emitted by the labelled lipase solubilized in water-pools. This implies that the reverse micelles formed in liquefied propane are able to entrap lipase.

Figure 2 (A) and (B) show the fluorescence spectra measured at various values of [H₂O]/[AOT] for AOT-liquefied propane and AOT-hexane systems, respectively. The intensity in both the systems was found to change against [H₂O]/[AOT] variation while λ_max seemed almost unchanged. For conventional reverse micellar solutions made using ordinary organic solvents like hexane and examined with a fluorescence probe such as 8-anilino-1-naphthalene sulfonic acid, a similar property change accompanying [H₂O]/[AOT] variation has been reported (Wong et al., 1976). It has been explained in terms of the variation in microscopic environment around the probe, i.e. property variations in water pools: for example, microscopic viscosity and polarity in water pools changed synchronously with [H₂O]/[AOT] variation, affecting an excited state of the probe molecules and resulting in the spectral change (Martinek et al., 1987a). If this explanation is applicable to the case of present liquefied propane reverse micellar solution as well, fluorescence spectra of the solution in question should change in a way similar to that for the hexane reverse micellar solution. Thus, their intensity was mentioned as a representative spectral property and compared between the two kinds of reverse micellar solution.

Figure 3 shows the intensity changes against

### Figure 2

![Fluorescence spectra of fluorescence-labelled lipase solubilized in reverse micellar solutions made by AOT-liquefied propane (A) and AOT-hexane (B) under various values of [H₂O]/[AOT]: (a) 3.5, (b) 7.0, (c) 14, (d) 20, (e) 5.0, (f) 7.0, (g) 15, (h) 20.](image-url)
The values at 480 nm are used as a measure of intensity and, for convenience, were reduced to the relative ones which were the quotients of the intensities at individual values of [H₂O]/[AOT] divided by the largest intensity: the intensity at [H₂O]/[AOT] = 3 for the liquefied propane micellar solution and at [H₂O]/[AOT] = 5 for the other micellar solution. The figure indicates that, as [H₂O]/[AOT] increased, the intensity for the two micellar solutions decreased similarly. For higher values of [H₂O]/[AOT], however, the intensity decrease for the case of the former micellar solution was rather greater than that for the latter micellar solution. This may be caused from the unintended drop, as described below, in the amount of the labelled lipase being entrapped in water pools at higher values of [H₂O]/[AOT] for the former micellar solution. That is to say, at higher values of [H₂O]/[AOT], the former micellar solution did not enable to solubilization as much buffer solution as the latter micellar solution: thus the amount of labelled lipase entrapped in water pools might be less than that for the latter micellar solution. Such speculation should be supported by the observation of whether or not a part of the buffer solution remaining unsolubilized was present as the discrete phase. However, the observation was not able to be made, because the amount of unsolubilized buffer solution was not so significant as to be observed with the present cell: the unsolubilized buffer solution, if present, had to settle at the bottom of the cell, being able to be observed only when its amount was more than a certain value because of the cell structure.

The visual observation of the fluorescence stated above and the spectral property change as shown in Fig. 3 lead to the conclusion that lipase was entrapped within reverse micelles formed in liquefied propane and subjected to micro-environmental variation accompanying [H₂O]/[AOT] change.

2.3 Esterification by entrapped lipase

Figure 4 shows results for the esterification in micellar solutions formed with liquefied ethane and propane, and several organic solvents. Liquefied ethane, as shown earlier, and chloroform cannot solubilize the buffer solution so much as the others. Therefore, when chloroform was utilized, experimental values of [H₂O]/[AOT] were limited to values at which the discrete water phase was not formed. However, when liquefied ethane was utilized, they were not limited, especially in spite of the formation of the discrete phase because of the convenience of comparison. Thus, the reaction in liquefied ethane proceeded as a biphase system and not as a reverse micellar system.

As seen in Figure 4, the esterification activity of entrapped lipase depended on the kinds of organic phase and value of [H₂O]/[AOT]. For all the reverse micellar solutions except for the case of chloroform where low activity was exhibited, the activity was maximized at a respective value of [H₂O]/[AOT] depending on the kinds of organic phase as reported by many workers (Martinek et al., 1989). For the cases of chloroform and benzene, irrespective of their advantage of making reverse micellar solutions, the activity was lower than that for the case of liquefied ethane forming a biphase system. This implies that the micro-environment within the reverse micelles formed in the two former solvents was unfavorable for lipase to retain its natural conformation. The activity for the case of liquefied propane was comparable to that for the case of hexane, though slightly lower than that for the case of hexane. The value of [H₂O]/[AOT] at which the activity was maximum was about 15 for both cases. These results indicate that lipase entrapped in reverse micelles formed using liquefied propane has a high esterification activity similar to that entrapped in reverse micelles made in hexane. They also indicate that the consideration of the propanes suitability for making reverse micelles and entrapping the enzyme, based on the coloration experiments using the dye, was reasonable. It was worth noting that the experimental finding obtained using a low molecular weight compound of the dye was applicable to experiments using macromolecules of the enzyme. This observation, however, should be studied further for its application to
other systems.

Since propane, when liquefied, can dissolve many kinds of lipophilic substrates, be controlled in volume by pressure, and be separated easily from reaction systems, reverse micelles formed using it may provide a new biocatalytic conversion process.

Conclusions

Water-pools were formed in liquefied propane and ethane, but not formed in liquefied CO₂. The amount of water solubilized as water-pools was large in liquefied propane, and small in liquefied ethane. The water-pools formed in liquefied propane were able to entrap lipase. The entrapped lipase showed high catalytic activity for esterification of lipophilic substrates. The activity was comparable, though somewhat lower, to that in the hexane micellar solution. It was maximized at a certain value of \([\text{H}_2\text{O}]/[\text{AOT}]\), which almost agreed with a value of \([\text{H}_2\text{O}]/[\text{AOT}]\) maximizing activity in the hexane reverse micellar solution.

Literature Cited


