Articles

Horseradish Peroxidase Activity in a Reverse Catanionic Microemulsion

Sekh Mahiuddin,[†] Audrey Renoncourt,[‡] Pierre Bauduin,[‡] Didier Touraud,[‡] and Werner Kunz*,[‡]

Material Science Division, Regional Research Laboratory, Jorhat-785 006, Assam, India, and Institut für Physikalische und Theoretische Chemie, Universität Regensburg, D-93040 Regensburg, Germany

Received November 18, 2004. In Final Form: February 17, 2005

In this paper we present the first results of enzymatic activities in a reverse microemulsion medium based on a mixture of an anionic and a cationic surfactant, called catanionic microemulsion. The studied system is composed of sodium dodecyl sulfate (SDS)/dodecyltrimethylammonium bromide (DTAB)/*n*-hexanol/citrate buffer/*n*-dodecane, with high SDS/(SDS + DTAB) weight fractions. It turns out that the results are similar to those obtained in classical reverse microemulsions, except that the presence of DTAB exerts an inhibiting effect on the enzyme. Nevertheless, enzymatic superactivities are found even at a DTAB to total surfactant ratio of 15%, corresponding to 3% weight fraction of cationic surfactant in the microemulsion. The influence of pH and hexanol content on the enzymatic activities is also studied.

Introduction

A mixture of oil, water, and an amphiphile at low concentrations forms a turbid and unstable emulsion. But the addition of a cosurfactant, generally a medium-chain alcohol, to this emulsion can convert it into an optically transparent and thermodynamically stable microemulsion.[1] A substantial amount of research work has been carried out to understand the physicochemical properties of microemulsions for their technical applications with either single or mixed (nonionic−nonionic, nonionic−anionic, anionic−anionic, anionic−cationic, or cationic−cationic) surfactants.[2−7] Studies of microemulsions containing anionic and cationic surfactants together, that is, catanionic microemulsions, are not frequent in comparison to other pairs of surfactants, and the studies are generally restricted to the solubilization and phase behavior.[6,7] As for other mixtures, the microstructure of the catanionic microemulsion can be tailor-made by varying the tail and head of the surfactants, the oil-to-water ratio, the surfactant-to-oil ratio, and the ratio between the amount of both types of surfactants.

Enzymes have extensive applications as catalysts for organic synthesis and for oxidation of environmentally harmful chemicals to value-added products at ambient temperature with large selectivity and specificity with minimum side products.[8−10] For example, horseradish peroxidase (HRP) can catalyze the oxidation of a variety of organic compounds such as phenols, biphenols, anilines, benzidines, and related heteroaromatic compounds. Due to low cost, easy availability, and activity over a broad pH and temperature range, HRP has been used as a catalyst in various media such as ionic liquids,[11] sol−gel hosts,[12,13] biphasic systems,[14,15] organic solvents,[14−16] microemulsions and reverse micelles,[11,15] supercritical carbon dioxide,[17] and emulsions.[18,19] Enzymes in reverse micelles are often used as a biological model for living cells. However, living cells contain not only anionic or uncharged components but also positively charged molecules. It is tempting to try enzymatic reactions in catanionic reverse microemulsions to see how far the interaction between oppositely charged surfactant molecules modifies the influence of both types of surfactants on the enzymatic activity.

The use of a catanionic microemulsion as a reaction medium for organic and particle synthesis is rare. The number of studies concerning the influence of a cosurfactant, which transforms the emulsion to a stable

[†] Regional Research Laboratory.
[‡] Universität Regensburg.

(1) Schwuger, M.-J.; Stickdorn, K.; Schomäcker, R. *Chem. Rev.* **1995**, *95*, 849.
(2) Kahlweit, M.; Strey, R.; Haase, D.; Firman, P. *Langmuir* **1988**, *4*, 785.
(3) Olsson, U.; Wennerström, H. *Adv. Colloid Interface Sci.* **1994**, *49*, 113.
(4) Neto, A. A. D.; Dantas, T. N. C.; Moura, M. C. P. A.; Neto, E. L. B.; Duarte, L. J. N. *Ind. Eng. Chem. Res.* **2003**, *42*, 1994.
(5) Jönsson, B.; Jokela, A.; Khan, B.; Lindman, B.; Sadaghiani, A. *Langmuir* **1991**, *7*, 889.
(6) Li, X.; Ueda, K.; Kunieda, H. *Langmuir* **1999**, *15*, 7973.
(7) Li, X.; Kunieda, H. *Curr. Opin. Colloid Interface Sci.* **2003**, *8*, 327.
(8) Dordick, J. S., Ed. *Biocatalyst for Industry*; Plenum Press: New York, 1991.
(9) Schmid, A.; Dordick, J. S.; Hauer, B.; Kiener, A.; Wubbolts, M.; Witholt, B. *Nature* **2001**, *409*, 258.
(10) Alexander, M. *Biodegradation and Bioremediation*; Academic Press: San Diego, CA, 1994.
(11) Laszlo, J. A.; Compton, D. L. *J. Mol. Catal. B* **2002**, *18*, 109.
(12) Kadnikova, E. N.; Kostic, N. M. *J. Mol. Catal. B* **2002**, *18*, 39.
(13) Smith, K.; Silvermail, N. J.; Rodger, K. R.; Elgren, T. E.; Castro, M.; Parker, R. M. *J. Am. Chem. Soc.* **2002**, *124*, 4247.
(14) Akkara, J. A.; Kaplan, D. L.; Ayyagari, M. U.S. Patent 19,960,-116, 2000.
(15) Akkara, J. A.; Kaplan, D. L.; Ayyagari, M. U.S. Patent 19,990,-204, 2001.
(16) Klibanov, A. M. *Nature* **2001**, *409*, 241.
(17) Ryu, K.; Kim, S. *Korean J. Chem. Eng.* **1996**, *13*, 415.
(18) Kalra, B.; Gross, R. A., *Green Chem.* **2002**, *4*, 174.
(19) Kamiya, N.; Inoue, M.; Goto, M.; Nakamura, N.; Naruta, Y. *Biotechnol. Prog.* **2000**, *16*, 52.

10.1021/la047161r CCC: $30.25
Published on Web 04/29/2005

microemulsion,[1] on the enzymatic activity is also limited.[20−22] In this paper we report on the enzymatic activity of HRP in a catanionic microemulsion consisting of sodium dodecyl sulfate (SDS)/dodecyltrimethylammonium bromide (DTAB)/*n*-hexanol/buffer/*n*-dodecane as a function of SDS, amount of buffer, and cosurfactant.

Experimental Section

Materials. Sodium dodecyl sulfate (≥99%, Merck, Germany), dodecyltrimethylammonium bromide (99%, Aldrich, Germany), *n*-hexanol (99%, Merck, Germany), *n*-dodecane (>99%, Aldrich, Germany), and hydrogen peroxide (extra pure, 33% w/w, Merck, Germany) were used without further purification. The enzyme was peroxidase type VI from horseradish (Sigma, batch 10K7430, 263 purpurogallin units/mg). The substrate for enzymatic activity was 2,2-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) (>99%, Fluka Germany). A buffer of pH 5 consisting of 0.009 mol L^{-1} citric acid monohydrate (>99.5%, Acros, Germany) and 0.016 mol L^{-1} sodium citrate dihydrate (>99.5%, Acros, Germany) was used for preparation of the microemulsion and the enzyme solutions. The Millipore water used in this study had a conductivity $<10^{-6}$ S m^{-1}. pH values were determined with the help of a glassy electrode. The conductivity of the catanionic microemulsion was measured with a dip-type cell having a cell constant of 109.1 m^{-1} and a LCR bridge (model 6440A, Wayne Kerr, U.K.)

Preparation of Microemulsions. Our microemulsions consisted of five components: In a first series of experiments, the total amount of surfactant and cosurfactant was kept at 20.2% (w/w) each, and the buffer:*n*-dodecane ratio was varied from 0.155 to 0.702 (w/w) to examine the effect of water on the enzymatic activity. To study the influence of the cosurfactant on the enzymatic activity, a second series of microemulsions was prepared with a total amount of surfactants = 20.2% (SDS weight fraction of 0.922, correspondingly 7.79 wt % DTAB with respect to the total amount of surfactant), a buffer total weight fraction of 25.0%, and *n*-hexanol:*n*-dodecane weight ratios varying from 0.584 to 1.21. The microemulsions were prepared by simple mixing of all components. The surfactant counterions (Na^+ and Br^-) were not removed.

Preparation of Reaction Mixtures. The reaction mixtures were prepared in a rectangular UV−vis cell with a 3 mL capacity containing 2.9 mL of buffer solution or microemulsion. To this medium were added 10 μL of 0.012 mol L^{-1} ABTS and 10 μL of 0.0262 mol L^{-1} H_2O_2 solutions. The system was thoroughly mixed, and finally 10 μL of an aqueous HRP solution at a concentration of 31.25 mg L^{-1} enzyme was added to initiate the oxidation of ABTS. The oxidation of ABTS with hydrogen peroxide is a classical reaction to characterize peroxidase activities. Peroxidase type VI from horseradish and ABTS are both soluble in the water core of the reverse catanionic microemulsion.

Measurement of Enzymatic Activities. The kinetics of ABTS oxidation was monitored spectrophotometrically with a Cary 3E spectrometer (Varian). The progress of the reaction was estimated at 414 nm (λ_{max} of the oxidized product of ABTS) for 4 min just after addition of HRP. The initial velocity, V, of the enzymatic reaction was inferred from the slope of the absorption intensity versus time, which was linear at least during the first few minutes. To reduce the uncertainty of the measured velocity of the reaction due to the variable reproducibility of the mother enzyme solution, relative activity, $A = V/V_0$, is reported, where V_0 is the initial velocity of the enzymatic reaction in the standard buffer solution. For each microemulsion and buffer solution, at least three reactions were measured at 25.0 ± 0.1 °C. The reproducibility of the results was within ±5%.

Results and Discussion

Phase Diagram. As discussed in the Experimental Section, our system contained five major components: SDS/DTAB/*n*-hexanol/buffer/*n*-dodecane. Li et al.[6,7] have studied a part of the phase diagram of this system except that they used an aqueous NaBr solution instead of a buffer. We started from their results as a reference and found stable microemulsions for a total amount of 20.2% surfactant and 20.2% *n*-hexanol in a range of 8−25% citric buffer (the remaining 51.6−34.6% being *n*-dodecane) and for SDS/(SDS + DTAB) ratios ranging from 0.78 to 0.92 for each buffer-to-oil ratio. This homogeneous, transparent low-viscosity microemulsion phase was detected by visual observation.

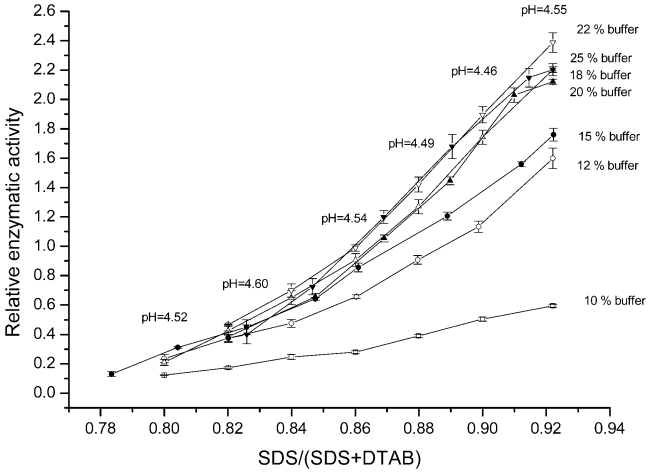

Figure 1. Variation of the relative enzymatic activity of HRP, *A*, in the catanionic microemulsion containing different amounts of buffer as a function of SDS weight fraction in the surfactant composition. Total surfactant concentration was 20.2% (w/w); *n*-hexanol concentration was 20.2% (w/w). The *n*-dodecane amount varies depending on the buffer content. The indicated pH values refer to the curve of 25% buffer. The pH values corresponding to the 22% buffer systems are very similar.

Enzymatic Activity as a Function of DTAB Concentration, pH, and Buffer Content. Figure 1 shows the relative enzymatic activities *A* of HRP in the microemulsion for different percentages of citrate buffer (initial pH before mixing with the other components equal to 5) as a function of SDS weight fraction. *A* sharply decreases as the percentage of DTAB increases from 0.08 to 0.22 weight fraction and the percentage of buffer decreases. In an independent experiment it was found that the relative activity *A* of HRP is nearly 0, when DTAB is added to a citrate buffer solution ($c = 0.025$ M, pH 5) at concentrations close to or higher than its critical micellar concentration.[23]

The enzyme exhibits superactivity (i.e., $A > 1$) at low DTAB weight fraction [i.e., SDS/(SDS + DTAB) > 0.86] and high buffer content (>10%). This superactivity can reach values that are even higher than in the SDS/water/*n*-hexanol/*n*-dodecane microemulsion systems where the maximum value is around 1.6.[24]

In Figure 2 we report the relative enzymatic activities *A* for a constant ratio SDS/(SDS + DTAB) and a subsequent replacement of dodecane by buffer. The indicated *A* values are the same as those shown in Figure 1 at the SDS/(SDS + DTAB) ratio of 0.922. We have also plotted the specific conductivities measured in the respective microemulsion systems and the corresponding pH values (Figure 2).

Our interpretation of these results is as follows:

The cationic surfactant DTAB is an inhibitor of the enzyme.[23] With increasing DTAB concentration, the inhibition of the enzyme increases also. But even at 2.8%

(20) Lopez, F.; Cinelli, G.; Ambrosone, L.; Colafemmina, G.; Ceglie, A.; Palazzo, G. *Colloids Surf. A: Physicochem. Eng. Aspects* **2004**, *237*, 49.

(21) Schirmer, Chr.; Liu, Y.; Touraud, D.; Meziani, A.; Pulvin, S.; Kunz, W. *J. Phys. Chem. B* **2002**, *106*, 7414.

(22) Meziani, A.; Touraud, D.; Zradba, A.; Pulvin, S.; Pezron, I.; Clausse, M.; Kunz, W. *J. Phys. Chem. B* **1997**, *101*, 3620.

(23) Pinna, C.; Monduzzi, M.; Touraud, D.; Bauduin, P.; Kunz, W.; Ninham, B. W. Manuscript in preparation.

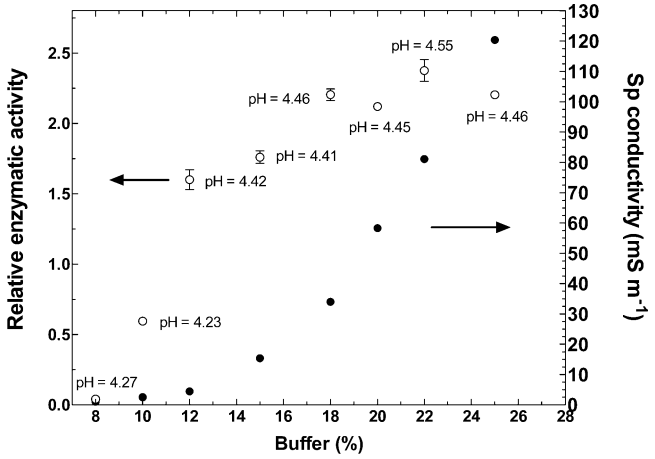

Figure 2. Influence of the amount of buffer on the relative enzymatic activity of HRP, *A*, in the catanionic microemulsion at 0.922 weight fraction of SDS with respect to the total amount of surfactant. Total surfactant concentration was 20.2% (w/w); *n*-hexanol concentration was 20.2% (w/w). The *n*-dodecane amount varies depending on the buffer content. (●) Specific conductivities of the microemulsions.

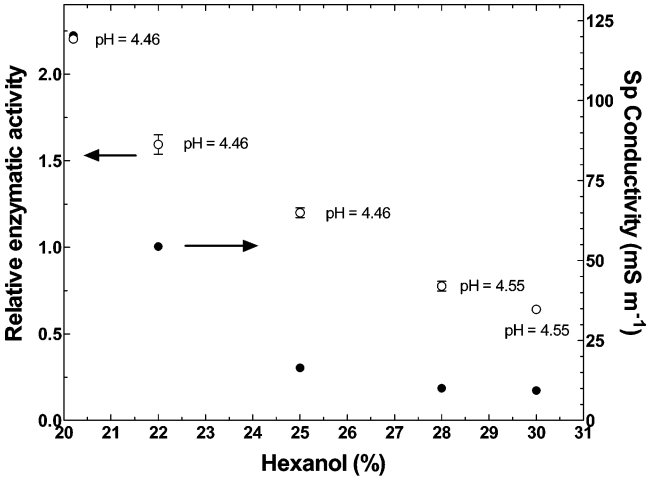

Figure 3. Effect of gradual replacement of *n*-dodecane by an equivalent weight of *n*-hexanol on the enzymatic activity of HRP, *A*, in the catanionic microemulsion at 0.922 weight fraction of SDS with respect to the total amount of surfactant (20.2% w/w) and at 25% buffer. (●) Specific conductivities of the systems.

(w/w) DTAB in the system (corresponding to 14% of the total amount of surfactant) a relative activity of 1 is found. A reasonable explanation for this surprisingly small inhibition effect is that the cationic surfactant is incorporated within the micellar surface layer and its influence on the enzyme is reduced by the strong catanionic interaction of the surfactants. Note that DTAB is only very slightly soluble in the organic pseudophase, so that it must be either in the aqueous or in the interfacial pseudophase. But in the aqueous pseudophase it cannot be present in large quantities, because it was shown[23] that DTAB concentrations as low as 10^{-2} M strongly inhibit the enzyme.

A second factor is important: the actual pH value of the microemulsion is no longer 5.0 (the initial value of the buffer) but ≈4.5, which is very close to the optimum pH value of the enzyme.[25,26] Increasing enzymatic activities with decreasing pH (at least down to pH 4) of the medium have already been found.[25,26] To see how sensitive the enzyme is toward small pH changes, let us compare V_0, that is, the initial velocity of the enzymatic reaction in the standard buffer solution at pH 5 (0.0108 mol L^{-1} s^{-1}), with $V_{pH=4.5}$, the initial velocity of the enzymatic reaction in the standard buffer solution at pH 4.5 (0.0211 mol L^{-1} s^{-1}). The difference in the initial velocities of the enzymatic reaction in the citrate buffer solution at pH 5 and at pH 4.5 is nearly 100% and thus significant enough to prove that the lower pH (≈4.5) in the microemulsion, in comparison to the pH of the standard buffer solution (pH 5), can explain the HRP superactivity in the microemulsion.

The third factor is the minimum buffer content. Following Figure 2, the relative enzymatic activity sharply increases between 8% and 15% buffer and then nearly remains constant. If the pH were the decisive factor, the enzymatic activity should decrease instead. Obviously, a minimum amount of buffer solution is necessary to ensure enzymatic activity. Therefore, we believe that the same argument as for classical reverse microemulsion holds here: the enzyme must be sufficiently hydrated to be fully active.[27−29] According to the conductivity data, also shown in Figure 2, the minimum amount of water for sufficient enzyme hydration correlates with the onset of some percolation. Between 10% and 15% buffer, the conductivity goes up and our interpretation is that this is related to a structural change of the medium where water becomes sufficiently mobile to increase conductivity and to hydrate enzymes.

Effect of *n*-Hexanol. Figure 3 shows the effect of *n*-hexanol content on the enzymatic activity in the catanionic microemulsion at fixed SDS/(SDS + DTAB) weight fraction, fixed total surfactant concentration, and fixed 25% buffer content. For a better orientation, note that the first point of Figure 3 is the last point in Figure 2. As the amount of hexanol is increased, the relative enzymatic activity decreases, whereas the pH of the medium remains roughly constant. It can be expected that with increasing total hexanol concentration there is also an increasing concentration of hexanol in the interfacial film. Consequently, more and more hexanol comes into close contact with the enzyme. Since it was found[24] that alcohols are inhibitors of this enzyme, Figure 3 mainly reflects the increasing enzyme−alcohol interactions with increasing alcohol concentrations. A second reason might be a change in the structure of the microemulsion, which can be deduced from the conductivity data (Figure 3). However, in any case the systems remain stable and transparent.

Conclusions

Although this is a very complex system, some general conclusions can be drawn:

(1) At low DTAB concentration, the system behaves much as a typical enzymatic system in reversed anionic or nonionic microemulsions. Roughly the same arguments can be used to explain the enzymatic activities in both cases.

(24) Bauduin, P.; Touraud, D.; Kunz, W.; Savelli, M. P.; Ninham, B. W. The influence of structure and composition of a reverse SDS microemulsion on enzymatic activities and electric conductivities. *J. Colloid Interface Sci.*, submitted.

(25) Goodwin, D. C.; Yamazaki, I.; Aust, S. D.; Grover, T. A. *Anal. Biochem*. **1995**, *231*, 333.

(26) Bauduin, P.; Nohmie, F.; Touraud, D.; Neueder, R.; Kunz, W.; Ninham, B. Hofmeister Specific-Ion Effects on Enzyme Activity and Buffer pH: Horseradish Peroxidase in Citrate Buffer. *J. Mol. Liquids* (in press).

(27) Avramiotis, A.; Papadimitriou, V.; Cazianis, C. T.; Xenakis, A. *Colloids Surf. A: Physicochem. Eng. Aspects* **1998**, *144*, 295.

(28) Rees, G. D.; Robinson, B. H. *Biotechnol. Bioeng*. **1995**, *45*, 344.

(29) Ayala, G.; Mendoza-Hernandez, G. *Biochem. Int*. **1990**, *22*, 717.

(2) At higher DTAB concentration, an additional effect must be taken into account: the inhibiting influence of the cationic surfactant on the enzyme. However, the inhibiting effect of DTAB is attenuated by the strong interactions between DTAB and SDS. This attenuation is an example of how ionic interactions between oppositely charged surfactants can lead to partial preservation of biological activity, and this may occur in nature. Such an effect is of potential importance for disinfecting and bactericidal products based on cationic surfactants.

(3) The fact that DTAB is insoluble in the oil phase and that its inhibition of the water-soluble enzyme is attenuated in the microemulsion systems strongly suggests that DTAB is incorporated in the interfacial film.

Acknowledgment. We are grateful to the Deutscher Akademischer Austauschdienst, Bonn, for financial support. S.M. is thankful to the Director of the Regional Research Laboratory (Council of Scientific and Industrial Research), Jorhat, Assam, for granting leave.

LA047161R