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Effects of Ionic and Zwitterionic Surfactants on the Stabilization of Bovine Catalase

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The activity and stability of beef liver catalase have been investigated in the presence of different ionic and zwitterionic surfactants. All cationic and zwitterionic surfactants used in this work have no effect on the initial activity of catalase, but several of them allow the enzyme to retain a high residual activity for longer periods of time than those observed in the absence of any additives. However, the interactions between surfactants and catalase appear to be very peculiar, and certain zwitterionic surfactants have been found to remarkably slow down enzyme degradation, with the enzyme completely preserving its activity after several weeks at temperatures of up to 30 °C. This effect is probably due to an interaction between the surfactant and the intersubunit region of the protein; this interaction could stabilize the quaternary structure of the enzyme.

Introduction

A great deal of experimental work has been devoted to studying the interactions between detergents and proteins in recent years, essentially in regard to the effects of sodium *n*-dodecyl sulfate (SDS) on enzymes and, frequently, its denaturing effect (*1*, *2*). Different models have been proposed for the structures of these complexes (*3*), and it seems clear that anionic surfactants initially bind cationic sites on the enzyme surface by ionic interactions; moreover, surfactant alkyl chains bind hydrophobic regions of the protein close to the cationic sites. Denaturation occurs because many hydrophobic binding sites, initially buried inside the tertiary structure, are exposed outward (*1*).

Catalase has previously been studied in the presence of SDS. Jones *et al.* (*4*) found that while beef liver catalase is completely inactivated in the presence of 5 mM SDS, bacterial catalase (*Micrococcus lysodeikticus*) retains 80% of its activity after 24 h of incubation with 20 mM SDS. Measurements of the enthalpy of interaction show that no heat is evolved upon mixing bacterial catalase with SDS, while for bovine catalase the energy of interaction is comparable with that found for many globular proteins. The amino acid analysis of the two catalases shows that bacterial catalase contains more glutamyl and fewer lysyl residues than beef liver catalase. Since the initial interaction of anionic surfactants with globular proteins involves binding between the cationic sites of the lysyl, arginyl, and histidyl residues of the protein and the surfactant head groups, the smaller content of cationic sites in the bacterial catalase reduces the extent of interaction, thereby reducing the ability of the surfactant to split the enzyme into its subunits.

The activity of *Aspergillus niger* catalase (*5*) at pH 6.4 is reported to be increased up to 180% when about 150 molecules of SDS are bound per molecule of enzyme. As a possible activation mechanism, it has been proposed that the interaction between SDS and the protein could give rise to a small conformational change in the region between the surface of the enzyme and the heme site, with a subsequent increase either in the movement of substrate to the active site or the release of product. The content of cationic binding sites on the surface of *A. niger* catalase is lower than that in bovine and bacterial ones, but this resistance to SDS deactivation is probably due to the structural environment of the heme active site and, further, to the strength of subunit association.

Enzymes can be regarded as highly selective catalysts that are very useful in organic synthesis, expecially the synthesis of chiral compounds. Most of them, however, lose their activity in a short time, so that their use is restricted (*1*, *2*).

The following study was carried out to evaluate the effect of molecular changes of some ionic and zwitterionic surfactants on the activity and stability of bovine catalase (EC 1.11.1.6). Our interest is devoted to studying the activity and stability of this enzyme in the presence of synthetic surfactants under different experimental conditions.

Materials and Methods

The beef liver catalase used in this study is from Sigma; H_2O_2 is from Merck.

Activity of Catalase. Enzyme activity is assayed in 6.7 mM phosphate buffer (pH 7) and 29.4 mM H_2O_2. The disappearance of H_2O_2 under the experimental conditions is monitored spectrophotometrically at 240 nm.

Stability of Catalase. Catalase is stored at 30 °C; 10 μL of a 10.5 μg/mL enzyme solution is added to 790 μL of 6.7 mM phosphate buffer in water or in the presence of different surfactant concentrations. The measurement temperature is 30 °C unless otherwise specified. H_2O_2 is added to the solution at regular time intervals, and the residual activity is measured.

Surfactants. The anionic surfactant used in this study, sodium *n*-dodecyl sulfate (SDS) from Carlo Erba, was purified by crystallization (*6*). The cationic surfactant, cetyltrimethylammonium sulfate ($(CTA)_2SO_4$), was synthesized and purified following published procedures (*7*, *8*). The zwitterionic surfactants dodecyldimethylammonium propanesulfonate (C12MeBS) and tetradecyldimethylammonium propanesulfonate (C14MeBS) were from Fluka and were purified by crystallization (*9*); tetradecyldiethylammonium propanesulfonate (C14EtBS), trimethylammonium propanesulfonate (C1MeBS), butyldim-

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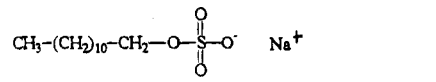
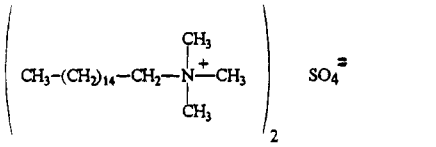
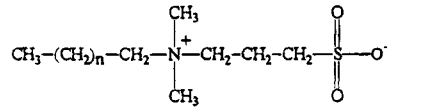
Scheme 1. Structure of the Surfactants Used in This Work



Sodium n-dodecylsulfate (SDS)

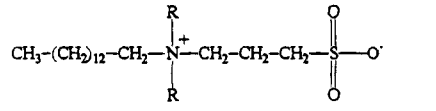


Cetyltrimethylammonium sulfate ($(CTA)_2SO_4$)



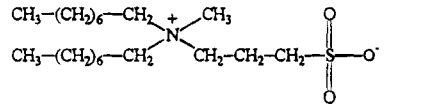
n=10, Dodecyldimethylammonium propanesulfonate (C12MeBS)

n=12, Tetradecyldimethylammonium propanesulfonate (C14MeBS)

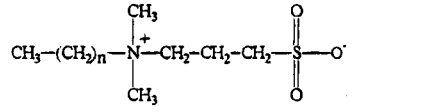


R=Et, Tetradecyldiethylammonium propanesulfonate (C14EtBS)

R=n-Pr, Tetradecyldipropylammonium propanesulfonate (C14PrBS)



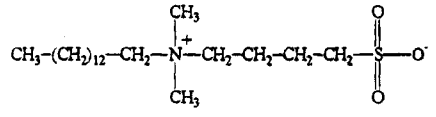
Dioctylmethylammonium propanesulfonate ($(C8)_2$MeBS)



n=0, Trimethylammonium propanesulfonate (C1MeBS)

n=3, Butyldimethylammonium propanesulfonate (C4MeBS)

n=5, Hexyldimethylammonium propanesulfonate (C6MeBS)



Tetradecyldimethylammonium butanesulfonate (C14MeBbS)

ethylammonium propanesulfonate (C4MeBS), hexyldimethylammonium propanesulfonate (C6MeBS), tetradecyldipropylammonium propanesulfonate (C14PrBS), dioctadecylmethylammonium propanesulfonate ($(C8)_2$MeBS), and tetradecyldimethylammonium butanesulfonate (C14MeBbS) were synthesized and purified following the general procedure reported here. All surfactants, shown in Scheme 1, were ≥99% pure.

Melting points were measured on a Büchi 510 melting point apparatus.

^{1}H NMR spectra were recorded in either $CDCl_3$ or D_2O at room temperature on a Bruker AC 200 (200 MHz) spectrometer; chemical shifts, given in ppm, were calculated with respect to the signal of TMS as an internal standard in $CDCl_3$ and to the signal of the solvent fixed at 4.60 for D_2O.

Critical Micelle Concentration. Values of the critical micelle concentration (cmc) were determined from plots of surface tension vs −log [surfactant]. No minima could be observed in these plots. Surface tensions were measured on a Fischer, du Noüy type tensiometer at room temperature; solutions were prepared using deionized, didistilled water.

General Procedure for the Preparation of the Propane- and Butanesulfonate Betaines (*10*). A solution containing 0.55 mol of propane or butane sultone and 0.6 mol of the appropriate tertiary amine in 100 cm^3 of dry toluene (dried over sodium) was refluxed for approximately 8 h. After cooling to room temperature, the solvent was evaporated under vacuum. The semisolid product was recrystallized from acetone or ethyl acetate 1−3 times until all impurities has been removed, followed by drying under vacuum (10^{-2} Torr) at 60 °C.

The propanesulfonate betaines C1MeBS, C4MeBS, and C6MeBS were prepared in dry acetonitrile (dried over P_2O_5) at room temperature for 1−2 h. The zwitterions precipitated from the reaction medium in a sufficiently pure form. They were recrystallized further from anhydrous ethyl alcohol and dried under vacuum at 100 °C.

C1MeBS: yield, 94%; mp >300 °C (dec); δ_H (D_2O) 3.24−3.38 (m 2H, N^+CH_2), 2.98 (s, 9H, 3 CH_3), 2.81 (t, 2H, $CH_2SO_3^-$), 1.94−2.16 (m, 2H, N^+CCH_2).

C4MeBS: yield, 85% mp 275−278 °C; δ_H (D_2O) 3.24−3.38 (m, 2H, N^+CH_2), 3.09−3.22 (m, 2H, RCH_2N^+), 2.95 (s, 6H, $(CH_3)_2N^+$, 2.82 (t, 2H, $CH_2SO_3^-$), 1.95−2.16 (m, 2H, CCH_2C), 1.50−1.70 (m, 2H, CH_2CN^+), 1.12−1.33 (m, 2H, CH_3CH_2), 0.82 (t, 3H, CH_3).

C6MeBS: yield, 85%; mp 185−187 °C; δ_H (D_2O) 3.23−3.37 (m, 2H, NCH_2), 3.10−3.22 (m, 2H, RCH_2N^+), 2.95 (s, 6H, $(CH_3)_2N^+$), 2.81 (t, 2H, $CH_2SO_3^-$), 1.95−2.16 (m, 2H, CCH_2C), 1.50−1.70 (m, 2H, CH_2CN^+), 1.05−1.30 (m, 4H, $(CH_2)_2$), 0.75 (t, 3H, CH_3).

C14EtBS: yield, 80%; 186−188 °C; δ_H ($CDCl_3$) 3.59−3.72 (m, 2H, N^+CH_2), 3.28−3.46 (m, 4H, $N^+(CH_2CH_3)_2$), 3.05−3.20 (m, 2H, RCH_2N^+), 2.92 (t, 2H, $CH_2SO_3^-$), 2.09−2.28 (m, 2H, N^+CCH_2C), 1.58−1.78 (m, 2H, RCH_2CN^+), 1.38 (t, 6H, 2 CH_3), 1.20−1.39 (m, 22H, 11 CH_2), 0.89 (t, 3H, CH_3); cmc, 2.55 × 10^{-4} M.

C14PrBS: yield, 75%; mp 160−162 °C; δ_H ($CDCl_3$) 3.55−3.66 (m, 2H, N^+CH_2), 3.10−3.30 (m, 6H, $(CH_2)_3N^+$), 2.92 (t, 2H, $CH_2SO_3^-$), 2.10−2.30 (m, 2H, N^+CCH_2C), 1.58−1.89 (m, 6H, $(CH_2C)_3N^+$), 1.20−1.40 (m, 22H, 11 CH_2), 1.04 (t, 6H, 2 CH_3), 0.89 (t, 3H, CH_3); cmc, 1.99 × 10^{-4} M.

$(C8)_2$MeBS: yield, 68%; mp 158−160 °C; δ_H ($CDCl_3$) 3.51−3.66 (m, 2H, N^+CH_2), 31.5−3.30 (m, 4H, $(CH_2)_2N^+$),

Table 1. Residual Activity (%) of Catalase as a Function of Stocking Time and Temperature in 6.7 mM Phosphate Buffer

	temperature (°C)		
time (h)	30	40	50
1	56.0	61.1	48.6
4	55.2	33.2	15.9
15	7.9	7.0	1.5
72	3.9	0.5	1.0

3.13 (s, 3H, CH_3N^+), 2.92 (t, 2H, $CH_2SO_3^-$), 2.12–2.33 (m, 2H, CCH_2C), 1.55–1.80 (m, 4H, $(RCH_2C)_2N^+$), 1.15–1.50 (m, 20H, 10 CH_2), 0.88 (t, 6H, 2 CH_3).

C14MeBbS: yield, 78% mp 286–289 °C; δ_H ($CDCl_3$) 3.50–3.60 (m, 2H, N^+CH_2), 3.15–3.35 (m, 8H, $CH_2N^+(CH_3)_2$), 2.86 (t, 2H, $CH_2SO_3^-$), 1.78–2.05 (m, 4H, CH_2CH_2), 1.55–1.77 (m, 2H, CH_2CN^+), 1.15–1.41 (m, 22H, 11 CH_2), 0.88 (t, 3H, CH_3); cmc, 3.72 10^{-4} M.

Results and Discussion

Experiments in Buffer. A set of experiments was carried out at 30, 40, and 50 °C in 6.7 mM phosphate buffer; after 72 h the enzyme lost its activity at all temperatures considered (Table 1).

Experiments in the Presence of Additives. The effect exerted by a surfactant or, more generally, by an additive on enzymes can be one of either maintaining their residual activity for longer periods of time than these observed without any additives, provided that enzyme specific activity remains unchanged, or lowering or (rarely) raising the latter. In the first instance, it is possible to carry out experiments, at constant temperature, by varying the additive concentration, thereby determining the percent activity relative to that in the absence of additive. This procedure has been used often in the literature (*5*), and the results have been referred to as activation effects by the additive.

In Figure 1, the percent activity of catalase (relative to activity in buffer) vs [C12MeBS] after 72 h is shown; it seems clear that this quantity is strongly dependent upon [C12MeBS] and that there is a 2500-fold *activation* for [C12MeBS] = 10^{-2} M. Once optimum [surfactant] values have been determined, the analysis may also be carried out by plotting percent residual activity vs time at a fixed [surfactant], i.e., percent activity relative to that shown by the enzyme at zero time; in this case, activity profiles can be as shown in Figure 2. With reference to C12MeBS (9 mM, after 72 h stocking), catalase residual activity is about 90%, whereas it is about 5% without surfactant; in other words, this means that a surfactant preserves enzyme activity for much longer periods than buffer alone, making the protein less prone to denaturation with time, and thus more stable, as set forth earlier. This does not mean, in our opinion, that the enzyme becomes *activated*; in fact, it is *stabilized*. True activation occurs when enzyme specific activity is enhanced by the additive.

In all of our experiments, surfactants did not show any effect upon enzyme specific activity, which remained unchanged at all [surfactant] used (except for SDS, see Table 2). For this reason, we prefer to deal with percent residual activity as a function of time at fixed [surfactant], because the effect exerted by the additive is more clearly visualized.

Effect of Anionic Surfactant. The presence of low concentrations of SDS does not affect the specific activity of catalase, but at 5 mM SDS the denaturation of the enzyme is very fast. However, if catalase is stored in the presence of 2 mM SDS in phosphate buffer at 30 °C, the enzyme loses its activity after 1 h (Figure 3).

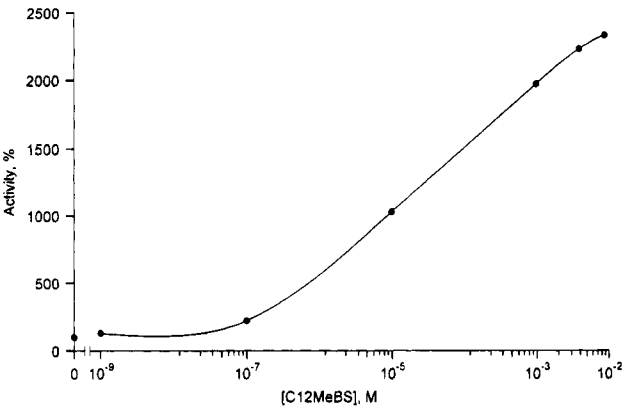


Figure 1. Percent activity of catalase (relative to the activity in buffer) vs [C12MeBS] (M) after 72 h of stocking at 30 °C.

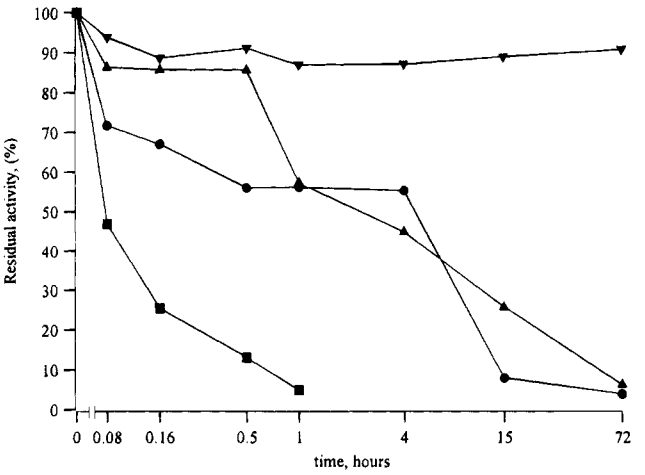


Figure 2. Percent residual activity vs time for catalase at 30 °C in the presence of (■) 2 mM SDS, (●) H_2O, (▲) 9 mM $(CTA)_2SO_4$, and (▼) 9 mM C12MeBS.

Table 2. Specific Activity (%) of Catalase as a Function of Anionic, Cationic, and Zwitterionic Surfactant Concentrations in 6.7 mM Phosphate Buffer at 30 °C

	concentration (mM)					
	0	1	2	4	5	9
SDS	100	100	92.8	28.6	0	0
$(CTA)_2SO_4$	100	100	100	100	100	100
C12MeBS	100	100	100	100	100	100

Effect of Cationic Surfactant. In the presence of a cationic surfactant ($(CTA)_2SO_4$), after 15 h catalase retains 50% of its activity, but after 72 h the stabilization is not significant (Figure 2).

Effect of Zwitterionic Surfactants. The results obtained with zwitterionic surfactants are very interesting. In the presence of 1 mM or higher C12MeBS, at 30 °C after 72 h catalase retains most of its activity (Table 3). The addition of two methylene groups to the hydrophobic chain does not seem to significantly modify the stabilizing effect. In 9 mM C14MeBS, catalase still preserves the full activity after several weeks (Table 3). Further modifications to the structure of the zwitterionic surfactant were made to establish the extent to which the stabilizing effect is due to the head group and/or to the alkyl chain length. We synthesized sulfobetaines with a short chain of from one to six methylene residues, and the results show that, after 15 h of incubation, the residual activity in the presence of C1MeBS, C4MeBS, and C6MeBS is still about 50% (7.9% in phosphate buffer), but after 72 h it decreases to about 3–4% with C1MeBS and C4MeBS and to 15.4% with C6MeBS.

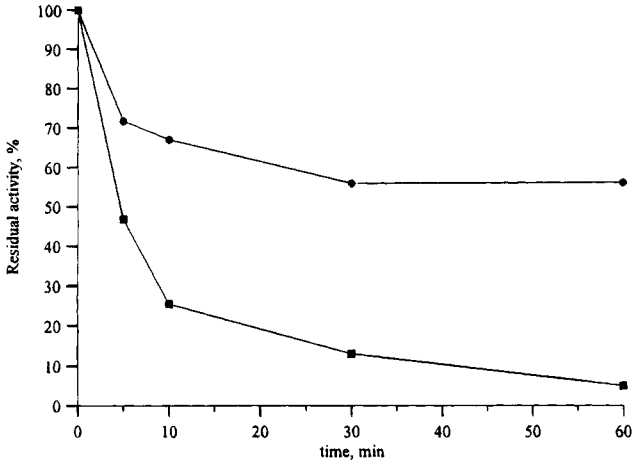


Figure 3. Percent residual activity vs time for catalase at 30 °C in (●) phosphate buffer and in (■) 2 mM SDS in phosphate buffer.

Table 3. Residual Activity (%) of Catalase as a Function of Sulfobetaine Chain Length at 30 °C ([surfactant] = 9 mM)

	time (h)		
	1	15	72
buffer[a]	56.0	7.9	3.9
C1MeBS	91.5	40.6	3.0
C4MeBS	84.7	58.0	4.3
C6MeBS	92.9	51.6	15.4
C12MeBS	91.0	88.8	90.6
C14MeBS	100	100	100[b]

[a] 6.7 mM phosphate buffer. [b] Residual activity (%) remains unchanged after several weeks.

(With C12MeBS after 72 h, the enzyme retains 90.6% activity.) These results show that the alkyl chain as well is responsible for the stabilizing effect of sulfobetaines (Table 3). An increase in the head group size, by substituting methyl with ethyl and propyl residues (C14EtBS and C14PrBS), causes a decrease in stability (61.0 and 47.9% after 72 h, respectively, Table 4).

In Table 5, stabilizing effect of 9 mM C14MeBS and C14EtBS, when the enzyme is stored at 4 °C, is reported. The results show that, in the presence of surfactant, catalase still retains 100% activity after at least 2 weeks, compared with buffer where residual activity falls down to 15.9% after the same time. This result makes it possible to preserve the enzyme from denaturation for a very long time. By increasing the number of hydrophobic chains ($(C8)_2$MeBS), the residual activity of the enzyme is about 80% after 15 h, but it decreases to about 40% after 72 h (Table 6).

Another structural change was made to the intercharge spacer of betaines with the addition of a methylene residue between the ammonium and sulfonate groups (C14MeBbS). Even if in 1 and 9 mM C14MeBbS the activity is about 80% after 15 h, the enzyme retains only 33% activity in 9 mM C14MeBbS after 72 h (Table 6).

Table 7 gives some information on the thermal stability of catalase in the presence or absence of C12MeBS. It is readily seen that, after 72 h, residual enzyme activity at 30 °C relative to 9 mM C12MeBS is much higher than that observed without surfactant, whereas both residual activities are nearly zero at 50 °C after the same period of time; this thermal behavior, together with the preceding evidence, is in agreement with weak interactions taking place between detergent and enzyme that allow only particular molecular geometries to stabilize the enzyme to a high level. In particular, in order to achieve the best stabilizing effect, zwitterionic surfactants having a well-defined intercharge distance (i.e., three CH_2) and a low hydrophobicity of the substituents on the nitrogen of the head group (i.e., dimethyl-substituted ammonium) have to be used.

Table 4. Residual Activity (%) of Catalase as a Function of Sulfobetaine Head Group Size at 30 °C ([surfactant] = 9 mM)

	time (h)		
	1	15	72
C14MeBS	100	100	100
C14EtBS	100	81.9	61.0
C14PrBS	97.1	78.4	47.9

Table 5. Residual Activity (%) of Catalase in the Presence and Absence of 9 mM Sulfobetaines at 4 °C

	time (h)				
	1	15	72	168	336
buffer[a]	82.7	54.8	61.5	51.1	15.9
C14MeBS	100	100	100	100	100
C14EtBS	100	100	100	100	100

[a] 6.7 mM phosphate buffer.

Table 6. Effect of Modifications to the Structure of Zwitterionic Surfactants (9 mM) on Enzyme Stability: Percent Residual Activity Measured at 30 °C

	time (h)		
	1	15	72
C14MeBS	100	100	100
C14MeBbS	95.2	82.5	33.0
$(C8)_2$MeBS	93.2	81.3	41.8

Table 7. Residual Activity (%) of Catalase as a Function of Temperature in 6.7 mM Phosphate Buffer and in C12MeBS after 15 h

	temperature[a] (°C)		
	30	40	50
buffer	7.9 (3.9)	7.0 (0)	1.0 (0)
9×10^{-3}M C12MeBS	88.8 (90.6)	92.9 (66.4)	8.9 (4.4)

[a] Values in parentheses are after 72 h.

The effect of surfactant on the enzyme could be explained either with a conformational change in the environment of the active site or with an increase/decrease in the strength of subunit association. When the enzyme activity is increased, it is reasonable to think that the surfactant can modify the accessibility of the active site (*5*). On the other hand, the stabilizing effect on bovine catalase by zwitterionic surfactants could be explained by an interaction between the surfactant and the intersubunit region of the protein, with consequent stabilization of the quaternary structure.

Acknowledgment

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