Bioprocess Engineering 21 (1999) 13–19 © Springer-Verlag 1999

Stabilization of acid phosphatase in DDDACl/n-butyl acetate reverse micelles

F. Alfani, M. Cantarella, D. Cifoni, N. Spreti, R. Germani, G. Savelli

Abstract Storage stability of acid phosphatase entrapped in reverse micelles was studied. Supramolecular systems were prepared with a cationic twin chain surfactant, didodecyldimethylammonium chloride (DDDAC1), n-butyl acetate as an organic solvent and different water percentages. The rate of enzyme deactivation was monitored in the temperature interval from 20 to 45 °C, at bulk pH from 4.8 to 6.4, either unstirred conditions or under convective mixing from 250 to 750 rev min^{-1}, water-to-surfactant molar ratio (w_0) equal to 11.4, 12.7, 14.2 and with the following buffers, Na-citrate, Li-citrate, K-citrate, Na-propionate. Acid phosphatase entrapped in buffer pools of reverse micelles exhibited enhanced stability in comparison with the enzyme in the pure aqueous phase. Half-life was up to 4 times larger. Both the chemicals used for buffer preparation and buffer pH change, within one unit, were found to influence the rate of acid phosphatase deactivation. The activation energy of enzyme deactivation process in micellar systems was slightly increasing with w_0 but the values were not very different from the one in aqueous phase (145.3 kJ mol^{-1}). The rate of deactivation of enzyme confined in the micelles when shear stress was applied was reduced in comparison with that of the free protein, even though the percentage loss was greater.

1 Introduction

Reverse micelles have been studied in a variety of applications such as biomolecule extraction from fermentation broths [1] and in vivo drug delivery [2] but particularly in the last decade a new research area under the name micellar enzymology has emerged. Many different enzymes have been studied in these systems and comprehensive reviews have also appeared in the literature [3, 4]. The research interest is related to potential applications since reverse micelles offer the possibility of solving problems peculiar of multi-phase systems. In the case of surface-active enzymes, such as lipases, micelles improve biocatalyst activity by increasing the interfacial area of an oil in water emulsion [5] and enzyme activity could be much higher than in organic solvent systems [6]. The phenomenon of superactivity of various enzymes has been reported some years ago [3, 7] while little or no activity was monitored for other enzymes in supramolecular systems [8]. Studies were undertaken to explain these conflicting reports [8] but the exact molecular level interpretation is not yet totally clear [9].

Enzyme stability was found to be dramatically dependent on the nature of the used surfactant [7], and the view emerges from the literature that some surfactants can stabilize active conformation of enzymes by entrapping them and protecting the proteins against denaturation by the surrounding organic solvent [7, 10]. On the other hand, it is also reported that activity decreases more rapidly in reverse micelles than in aqueous solutions [9] or in other organic solvent systems [6]. It was postulated that ionic interactions between the enzyme and the surfactant head group at the interface might play an important role in deactivation [11].

In view of these evidently conflicting reports in the literature regarding enzyme stability in reverse micelles and of the conclusion that, despite progress, we are still far from a fundamental and complete understanding of how stability of biocatalysts is affected in non-conventional media [12], this research was undertaken to contribute to the knowledge of the relationships between enzyme stability in reverse micelles and physico-chemical properties of the water pool.

Several surfactants, mainly cetyltrialkylammonium bromide, and various solvents were chosen with the aim of investigating new micellar systems. In fact, experiments till now reported in the literature were almost exclusively performed with n-hexanol and a few surfactants, specifically the anionic surfactant bis (2-ethylhexyl) sodium sulfosuccinate (AOT), the cationic surfactants cetyltrimethylammonium bromide (CTAB) and triethylmethylammonium chloride (TOMAC). Comparison of enzyme performances in a variety of micellar systems can provide additional useful informations on the extent of enzyme deactivation in these environments.

Received: 12 August 1998

F. Alfani, M. Cantarella, D. Cifoni, N. Spreti
Department of Chemistry,
Chemical Engineering and Materials,
University of L'Aquila,
67040 Monteluco di Roio AQ, Italy

R. Germani, G. Savelli
Department of Chemistry, University of Perugia,
60100 Perugia, Italy

Correspondence to: F. Alfani

This work was partially funded by Ministry of University and Scientific and Technological Research (MURST) and National Research Council (CNR, 95-00955-CT03).

The present study concerns with an experimental investigation in didodecyldimethylammonium chloride (DDDACl)/n-butyl acetate micelles and aims to measure the rate of enzyme deactivation as function of temperature, buffer pH, agitation speed and water-to-surfactant ratio (w_0), i.e. different sizes of micelles, in order to observe its dependence on some physical and chemical parameters, which are usually considered key factors for the engineering optimization of supramolecular systems.

DDDACl is one of the non-commercial surfactant, synthesized and purified in the laboratory [13]. These precursors of aggregates vary in the number of chains (single chain and twin chain), the counter ion (both chloride and bromide) and the dimension of head groups. DDDACl, a cationic twin chain surfactant, was selected for this study since it allows solubilization of relatively large water volumes in the organic phase, up to 4% v/v (at 0.1 M DDDACl in solvent). Previous studies on acid phosphatase stability showed that the rate of enzyme inactivation in (DDDACl)/n-butyl acetate reverse micelles is smaller than in pure buffer and stabilization is specially improved at values of w_0 (water to surfactant molar ratio) below 14.2 [14, 15].

Acid phosphatase from potato (EC 3.1.3.2) is non-specific phosphomonoesterase [16], interesting for its capability to hydrolyze orthophosphoric monoester into alcohol and orthophosphoric acid and for its great clinical significance. It was also selected as model enzyme because several authors investigated its mechanism of action since many years [17, 18]. Stability of both the free and immobilized enzymes in pure buffer is also well known and recently it was also determined in non-conventional media such as water-acetyl esters and water-hydrocarbon solvent two-liquid phase systems [19, 20].

2
Materials and methods

2.1
Chemicals

Baker Chemicals (Holland) supplied substrate (p-nitrophenyl phosphate, sodium salt, m.w. 371.15), n-butyl acetate, citric acid and sodium citrate; lithium citrate, potassium citrate and sodium propionate were from Sigma chemicals Co. (USA). All other chemicals were of analytical grade and were used without further purification.

2.2
Enzyme

Acid phosphatase (EC 3.1.3.2) from potato was purchased from Boehringer Biochemia (Italy). The enzyme is a nonspecific phosphomonoesterase, molecular weight 114 KDa, a value in fair agreement with the results in [21], which suggested the presence of two nearly identical subunits with a molecular weight of about 46 KDa. Ion exchange chromatography excluded the presence of isoenzymes [22]. The commercial enzyme powder was dissolved either in distilled water or aqueous buffers at concentration of 5 mg ml^{-1}. The concentrated enzyme stock solutions were frozen and stored. The activity of stored samples was always measured before experiments in aqueous and supramolecular environments.

2.3
Synthesis of surfactant and preparation of reverse micelle systems

The synthesis and the purification of the twin chain surfactant, didodecyldimethylammonium chloride [m.w. 418.19], were performed according to the methods previously described [13, 23–25]. Reverse micelles were prepared following to what is a commonly used method, which consists of microinjection of enzyme, dissolved in aqueous medium, into stirred solvent-surfactant solutions. In such a way, the long time required for the solubilization of the protein and the uncertainties in determining the actual amount of water in the pool, typical of the other procedures, were avoided [26]. This was especially important to measure deactivation rate of acid phosphatase, an enzyme with fast activity decay. The injection method in comparison with the dry-addition and the phase transfer techniques presents two other benefits: more protein is solubilized and protein solubilization is not strongly dependent on the micelle size [27].

Water or buffer (50 μl) and the concentrated enzyme solution (50 μl, 5 mg ml^{-1}) were directly added at room temperature and while gently mixing to the solvent- surfactant solution (3.9 ml), which had been prepared in advance. The temperature of the system was successively varied very rapidly up to the desired experimental value. Clear and apparently homogeneous solutions were obtained in a very short time (roughly 1 min).

Two other approaches of injection method were also tested. In case i) organic solvent, surfactant and concentrated enzyme in buffer solution (100 μl) were gently mixed and stored 24 hours at 4 °C. The temperature of system was successively increased very rapidly up to the desired experimental value. In case ii) solvent, surfactant and a portion of water (80 μl of buffer solution) were vigorously mixed and stored 24 hours directly at the test temperature, then 20 μl of concentrated enzyme in buffer solution were added. These two methods were not adopted since solutions were cloudy and 60–90 minutes were required by the system to become clear. The presence of water droplets, the separation of surfactant and the formation of thin films on the vessel walls were observed.

In the investigated temperature range, 20–45 °C, clear micellar solutions of enzyme were obtained for w_0 between 11 and 15 and no turbidity was observed during 6 h of storage. The occurrence of reverse micelle formation was also monitored by conductivity measurements [15, 25] in an Orion Research meter with a 1 cm dipping cell. The microemulsion system remains stable also at the higher explored temperature (45 °C). Dynamic light scattering measurements proved that the reverse micellar core diameter ranged from 2.3 to 3.3 nm and depended on surfactant concentration following the equation: r_w (nm) = 0.23 w_0 [28]. This finding is in fairly good agreement with those reported in the literature [7] and particularly in [6] using lipase from wheat germ (m.w. 67,000) and similar overall enzyme concentration 0.1 mg ml^{-1}. Analysis of standard deviation (15–20%) suggested

that the system was monodispersed and the micellar aggregates had spherical shape [28].

2.4 Acid phosphatase storage stability

Rate of enzyme deactivation was determined under either stirred or unstirred conditions in thermostated and stoppered glass tubes. Stirring was used to mix the solutions and to test enzyme resistance to shear stress. Temperature was varied between 20 and 45 °C. Surfactant concentration in the organic solvent (3.9 ml) ranged from 0.100 to 0.125 M. Consequently, the addition of 100 μl of water phase led to w_0 values from 14.2 to 11.4. Experiments in pure aqueous media were performed with 0.0625 mg ml^{-1} of acid phosphatase equal to the overall enzyme concentration in the supramolecular system. All the experiments were conducted at least in duplicate, and the arithmetic mean of all determinations was reported. The largest data deviation was 10.8%. In order to improve internal consistency in each series of runs, all the pertinent experiments were carried out with a freshly prepared enzyme solution. The rate constant of the first order deactivation kinetics (k_d) in Eq. (1) was evaluated by the slope of the semilogarithmic plot of residual activity versus storage time. The two rate constants (k_{d1} and k_{d2}) of the series mechanism of enzyme deactivation and the ratio of specific activity of the intermediate form (a_2) and the native enzyme (a_1) in Eq. (2) were determined by regression of the residual activity data according to the procedure reported in [22, 29]. In the case of fast deactivation kinetics the half-life ($\tau_{1/2}$) was directly determined by residual enzyme activity versus storage time curves. In the cases of slow deactivation it was calculated using the following equations:

$$\tau_{1/2} = \frac{0.693}{k_d}, \text{ (First order deactivation)} \quad (1)$$

$$e^{-k_{d1}\cdot\tau_{1/2}} - \frac{a_2}{a_1}\frac{k_{d1}}{k_{d1}-k_{d2}}\left(e^{-k_{d1}\cdot\tau_{1/2}} - e^{-k_{d2}\cdot\tau_{1/2}}\right) = 0.5, \text{ (Series deactivation)} \quad (2)$$

according to the assumption of series deactivation model that the intermediate form concentration is nil at zero storage time.

Any important change of enzyme deactivation rate between aqueous system and reverse micelles it might be observed cannot be ascribed to the different concentrations and acid phosphatase in the water pool (2.5 mg ml^{-1}) and the pure aqueous phase (0.0625 mg ml^{-1}). In fact, results of a previous investigation [30] on the kinetic of thermal inactivation of acid phosphatase, free in buffer solution, proved that the highest observed variation was within 19.4% at 40 °C when enzyme concentration was reduced from 1 mg ml^{-1} to 0.01 mg ml^{-1}.

2.5 Enzymatic assays

The following standard procedure was adopted for determining the residual activity of enzyme after storage in micellar systems. At regular time intervals, samples (80 μl) were withdrawn from the storage medium and assayed for residual activity in stoppered test tubes (total volume 4 ml) filled with 50 mM Na-citrate/citric acid buffer at pH 5.6 and with 2 mM substrate. Incubation was carried out at 30 °C for 10 min. The small aliquot of organic solvent did not affect the enzyme activity measurement. The amount of p-nitrophenol liberated was determined spectrophotometrically at 405 nm after alkalization with 2 ml of NaOH 1 M. The surfactant tended to aggregate at liquid surface and to retain portion of the reaction product, consequently dilution with an equal volume of solvent was necessary to allow quantitative partition of the product in the organic phase [14, 15]. Calibration tests confirmed that the extinction coefficient in water (18.5 cm^2 μmol^{-1}) could be used also for the calculation of product concentration in n-butyl acetate.

3 Rate of enzyme deactivation

3.1 Effect of salt concentration

Rate of enzyme deactivation was monitored in different buffers at constant temperature (30 °C) and stirrer rotation speed (250 rev. min^{-1}) since several experimental evidences are available on the effects of salt on enzyme-micelle interactions. Protein extraction in reverse micelles depends on both pH and salt content of the water phase [31, 32]. Conductivity measurements also revealed that the formation of micelles in the absence of proteins was inhibited by addition of buffer to solvent-surfactant solutions (at all w_0 values) but the occurrence of micellar systems was detected when proteins were contained in the buffer [15]. Acid phosphatase was either free in aqueous solution or confined in reverse micelles prepared according to the above defined standard procedure. The following media were tested: a) 50 mM Na-citrate buffer pH 5.6; b) 2.5 mM Na-citrate buffer pH 5.6; c) distilled water.

Results showed trends at those reported in Fig. 1. The lines show correlations of experimental data using the single step and the two step model of deactivation pattern. The rate of enzyme thermal deactivation remained almost unchanged between aqueous medium and supramolecular systems when use was made of buffer at high ionic strength (a). A much lower stability was exhibited by acid phosphatase in both systems at lower or nil ionic strength, (b) and (c) and the protective effect of the micellar system against enzyme deactivation was quite evident only for distilled water pools. The results suggested that enzyme deactivation can depend on differences in protein-ion-surfactant interactions. This gives a possible explanation of the different stability reported in the literature for enzymes confined in reverse micelles.

3.2 Effect of shear stress

The acceleration of protein deactivation rate with convective mixing has been well known from many years [33]. The exact mechanism of enzyme inactivation by shear is not yet well understood. Recently, it was proved that enzyme monomer in solution could not be directly dena-

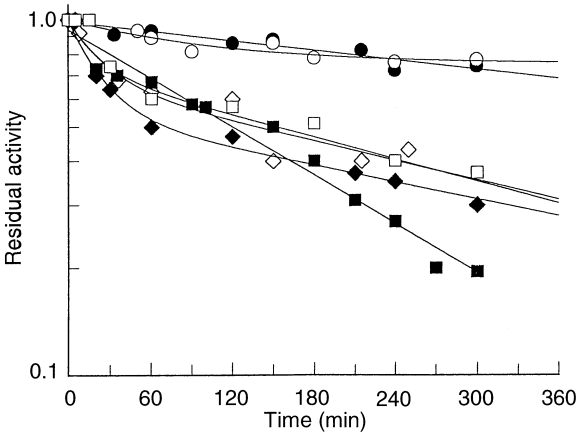

Fig. 1. Dependence of acid phosphatase deactivation on enzyme environment at 30°C, 250 rev.min^{-1}: open symbols, reverse micelles at w_0 = 14.2; closed symbols, aqueous solution. ●/○ = 50 mM Na-citrate pH 5.6, ◆/◇ = 2.5 mM Na-citrate pH 5.6, ■/□ = distilled water

tured by shear forces but, shear could indirectly influence their denaturation by accelerating the turnover of gas-liquid interfaces [34].

Reverse micelles might require some agitation in order to improve solution homogeneity. However, the enzyme being confined in the aqueous pool the protein could be protected from macroenviornment stirring. On the other hand, a dynamic equilibrium of micelle formation holds [26] and the exposure of protein to shear forces cannot be totally excluded. In order to verify the correctness of these hypotheses acid phosphatase deactivation rate was monitored at 30 °C and w_0 = 14.2 in micelles prepared either with Na-citrate 50 mM pH 5.6 buffer or distilled water. The experiments were carried out either in unstirred conditions or at two different stirrer rotations, 250 and 750 rev. min^{-1} at constant geometric characteristics of the apparatus. Semilogarithmic plots of residual activity *versus* process time have the shape of those reported in Fig. 2 and

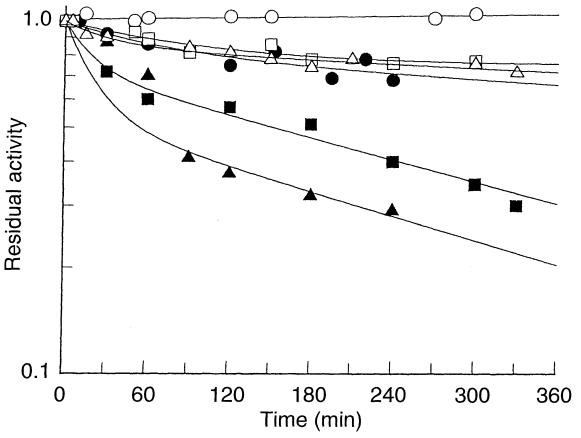

Fig. 2. Effect of convective mixing on acid phosphatase deactivation in reverse micelles at 30 °C and w_0 = 14.2. Open symbols, buffer pools (50 mM Na-citrate, pH 5.6); closed symbols, distilled water pools. ○/● = no stirring, □/■ = 250 rev.min^{-1}, △/▲ = 750 rev.min^{-1}

indicate a decrease activity due to both shear stress intensity and stress application time.

Acid phosphatase stability was also measured in totally pure buffer and in distilled water. The body of experimental results obtained at four storage conditions and at two mixing speeds are compared in Table 1. Both the values of enzyme half-life and of deactivation rate constants are listed. The visual inspection of enzyme residual activity *versus* storage time for several storage conditions suggested that the assumption of a first-order deactivation kinetics could be uncorrect. Therefore, the data fit was also performed on the hypothesis of the two-step mechanism. The choice between the two deactivation patterns was made on the basis of the best correlation coefficient and standard deviation. The biphasic kinetics of enzyme deactivation in the investigated storage time interval was quite evident for acid phosphatase confined in reverse micelles with the only exception of the buffer pool at unstirred conditions.

The presence of buffer improved enzyme stability in each system (aqueous media and micelles) and, independently of mixing conditions, half-life was much longer in buffer than in distilled water. The stability increase for enzyme confined in micellar pools was very evident at unstirred conditions. Acid phosphatase stability was affected also in micelles by shear since smaller half-lives were determined increasing convective mixing. Main difference in the half-life at the two investigated stirring speed is not determinable. The data also show that the percentage loss of acid phosphatase stability upon shear was in micellar system higher than when the enzyme is in aqueous solution. The hypothesis was made that agitation disrupts the supramolecular organisation and the enzyme undergoes to a more rapid deactivation.

3.3
Effect of temperature and of water to surfactant ratio

The molar ratio of water to surfactant (w_0) is a well known key parameter for both physical properties of reverse micelles and enzyme kinetics in these media [3, 10]. Acid phosphatase storage stability was studied in systems prepared with addition of a constant volume of buffer (2.5% v/v) to solutions of n-butyl acetate at different surfactant concentrations. DDDAC1 molarity in the solvent was 0.100, 0.110 and 0.125 M and consequently w_0 was 14.2, 12.7 and 11.4. Convective mixing was kept constant at 250 rev. min^{-1} and use was made of equal glass tubes and magnetic bars. Temperature from 20 to 45 °C were explored. Residual activity, at 45 °C, *versus* storage time was reported in Fig. 3. The higher the surfactant percentage the lower the rate of enzyme deactivation. Similar enzyme behaviour was observed at the lower temperatures and was characteristic of a series mechanism of enzyme deactivation usually related to differently active intermediate forms of the enzyme [29].

The enzyme deactivation at various temperature and three w_0 has been determined. The Arrhenius plot of Fig. 4 was prepared using only the k_{d1}-values, kinetic constant of first-step deactivation mechanism. In fact, the second deactivation step was observed only at high temperature and large w_0. The pertaining rate constant was almost inde-

Table 1. Deactivation rate constant and half-life of acid phosphatase under storage conditions at 30 °C

systems	k_d*10^3, min^{-1}			half-life, *h*		
	unstirred	250 rev. min^{-1}	750 rev. min^{-1}	unstirred	250 rev. min^{-1}	750 rev. min^{-1}
distilled water	3.43	5.20	13.24	3.40	2.30	0.87
50 mM, buffer	0.69	0.99	k_{d1} = 4.74 k_{d2} = 1.59	16.80	11.70	5.30
supramolecular (w_0 = 14.2, water pool)	k_{d1} = 2.52 k_{d2} = 0.85	k_{d1} = 10.00 k_{d2} = 2.65	k_{d1} = 10.80 k_{d2} = 2.70	13.60	2.15	1.15
supramolecular (w_0 = 14.2, 50 mM buffer pool)	0.15	k_{d1} = 1.96 k_{d2} = 0.67	k_{d1} = 2.79 k_{d2} = 0.67	75.00	15.50	13.60

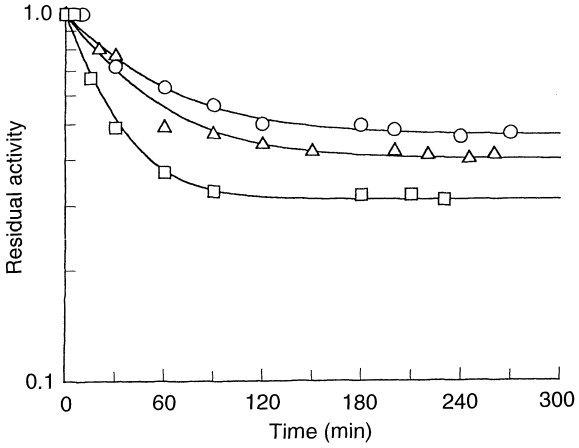

Fig. 3. Effect of water to surfactant molar ratio (w_0) on acid phosphatase deactivation in reverse micelles at 45 °C and 50 mM Na-citrate pH 5.6 pools. Stirring rate: 250 rev.min^{-1}
○, w_0 = 11.4; △, w_0 = 12.7; □, w_0 = 14.2

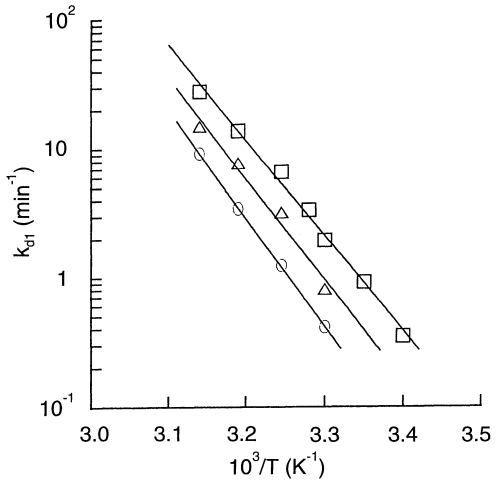

Fig. 4. Arrhenius plot of initial fast deactivation rate constant of acid phosphatase in reverse micelles in 50 mM Na-citrate pH 5.6 pools and different w_0. Stirring rate: 250 rev.min^{-1}.
○, w_0 = 11.4; △, w_0 = 12.7; □, w_0 = 14.2

pendent of surfactant molarity and increased very little with temperature. Activation energies (E_a) at the three surfactant concentrations are: 141.1 KJ mol^{-1} (DDDAC1 0.100 M), 152.2 KJ mol^{-1} (DDDAC1 0.110 M) and 162.2 KJ mol^{-1} (DDDAC1 0.125 M). These values allow the state that E_a is slightly dependent on w_0 and does not differ significantly from the activation energy (145.3 KJ mol^{-1}) measured from initial rates of acid phosphatase deactivation in totally aqueous buffered media [30].

The results of the present investigations are consistent with those of other studies on similar and different enzymes in reverse micelles. The enhancement of activity stabilisation in small size micelles was already observed for lipases from both *Candida cylindracea* and *Rhizopus delemar* in [35] and for lipase from *Candida rugosa* in [5].

3.4
Effect of pH and buffer composition

pH inside the water pools might be devoid of significance because of the very limited amount of free water and anyhow cannot be directly measured. Since, there are considerable evidences in the literature that enzyme properties in the micelles depend on pH of the solution used for preparation, an empirical acidity scale for water pools in reversed micelles was defined and the difference with pH of pure buffer was within 0.4 pH units [37]. Therefore, acid phosphatase stability in reverse micelles was studied at different buffer pH and at w_0 greater than 7, a value at which the water pool size can be considered intermediate to large. In this condition, with the pH buffer near the pK_a of the buffer salt, the difference in pH between free water and water in micellar pool should be limited to 0.1–0.2 units [35]. According to the previous references, the pH in the water pool was assumed as the analytical pH of the stock buffer solution used to prepare the microemulsions.

The rate of acid phosphatase deactivation in reverse micelles was measured under storage conditions at 30 °C. The water pool was prepared with sodium, lithium and potassium citrate buffer and sodium propionate buffer. DDDAC1 concentration in n-butyl acetate was 0.1 M and w_0 was fixed at 14.2. The pH of buffers in bulk water was changed from 4.8 to 6.4. Enzyme deactivation was also measured in pure buffer at the same temperature and pH. Several interesting observations arise from the experimental results plotted in Fig. 5. Large differences existed between the enzyme stability in totally aqueous buffer and in the micellar water pool. Storage stability in reverse

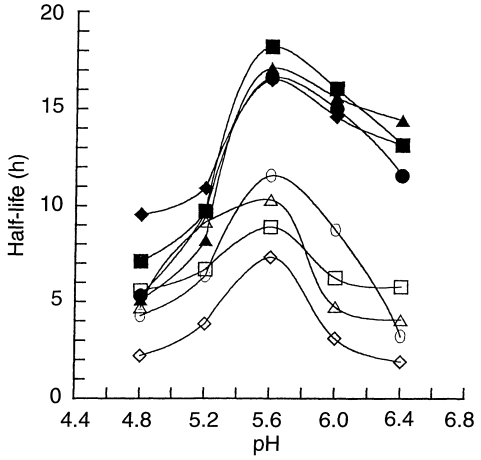

Fig. 5. Acid phosphatase half-life, at 30 °C, versus (50 mM) stock buffer at different pH. Stirring rate: 250 rev.min^{-1}. Closed symbols, reverse micelles (w_0 = 14.2); open symbols, totally aqueous solutions. ●/○ = Na-citrate, ■/□ = Li-citrate, ▲/△ = K-citrate, ◆/◇ = Na-propionate

micelles was always improved in comparison with that in purely aqueous media. In both systems the highest enzyme stability was monitored at pH 5.6 but the dependence of half-life on pH was larger in the micelles than in totally aqueous media. A shift of optimum pH was not observed among the different buffers.

Ions affected in a different way the acid phosphatase stability in the two systems. When Na-propionate was used, acid phosphatase loosed in pure buffer almost completely its initial activity during 2–7 hour storage depending on pH, and the enzyme showed in the whole pH range poorer stability than in the other buffers. On the contrary, when micelles were prepared with Na-propionate the enzyme half-life does not differ from that measured with the other buffers. The hypothesis was made that hydrophobic interactions between the enzyme and the propionate molecule can determine the faster rate of deactivation in aqueous media. The presence of both hydrophobic and electrostatic interactions between the propionate molecule and the cationic surfactant in the micellar water pool could reduce the availability of buffer moieties for interaction with acid phosphatase. The hypothesis well agrees with experimental observations in aqueous micellar systems [37].

Similar values of acid phosphatase half-life in micelles were measured using sodium, lithium and potassium citrate buffers near the optimum pH. On the contrary, in pure buffer the half-life largely depended on the salt ion. The lowest rate of deactivation was observed in Na-citrate at pH 5.6 which is the best pH for enzyme activity and stability in purely aqueous media [37]. The best improvement in stabilization of acid phosphatase activity by solubilization in reverse micelles was achieved in the upper pH range (5.6–6.4).

These experimental evidences supported the view that DDDAC1 can stabilize the active conformation of the enzyme by entrapping it in the micelles but the interaction with the buffer salt also plays an important role. Besides, this finding can be partially related to results discussed in [32] which showed that NaCl, KCl and LiCl exert a significant specificity in transfer of protein from fermentation broth into micellar systems.

4 Conclusions

The results of the experiments on acid phosphatase stabilization upon entrapment in DDDACl-butyl acetate reverse micelles pointed out the importance of water pool composition on the rate of enzyme deactivation and proved that the higher the water to surfactant molar ratio the higher the rate of acid phosphatase activity decay. The loss of enzyme activity in buffered pools occurred at a lower rate than in distilled water pools and varied with the type of buffer and the pH.

These results support the hypothesis that enzyme stability in supramolecular systems is affected by micellar size which depends on both surfactant to water ratio and the presence of ions in the medium [39]. Furthermore, the different stabilization obtained with the used buffers might be attributed not only to specific interactions between the cationic surfactant and propionate or acetate molecule but also to the content of water solubilized in the organic phase, in a similar way to the observations reported in [40].

The comparison of acid phosphatase half-life when stored in reverse micelles and in pure buffer confirms the possibility to improve enzyme stability. The ratio of the enzyme half-life when stored in the system at unstirred conditions is roughly 4.5 and therefore, neither protein-surfactant interaction nor the presence of n-butyl acetate causes enzyme denaturation. This result is in tune with the finding on enzyme stability in water-organic solvent media [19].

The large reduction of enzyme stability observed in stirred systems can be attributed to enzyme distribution in four phases in equilibrium (free water, bound water, surfactant tails, and organic solvent) as suggested in [26]. According to this model only the portion of the protein present in the inner part of the micellar pool might be protected by the shear stress generated by mixing. This hypothesis could explain why only a partial enzyme stability improvement is observed and the rate of deactivation remains lower than in pure buffer under the same stress conditions.

Finally, the body of the results obtained making use of a new synthesized precursor of aggregates (DDDAC1) confirmed the need of investigating surfactant different from those already widely reported in the literature for a more in depth understanding of enzyme behavior in micellar systems.

References

1. **Hatton, T.A.:** In: J.F. Scamehorn and J.H. Harwell (eds.) Surfactant-based separation processes. Marcel Dekker, N.Y., (1989) 55
2. **Speiser, P.:** In: P.L. Luisi and B.E. Straub (eds.) Reverse micelles: Biological and techonological relevance of amphiphilic structure in apolar media. Plenum Press, N.Y., (1984) 339
3. **Martinek, K.; Klyachko, N.L.; Kabanov, A.V.; Khmelnitsky, Y.L.; Levashov, A.V.:** Biochim. Biophys. Acta. 981 (1989) 161
4. **Bru, R.; Sanchez-Ferrer, A.; Garcia-Carmona, F.:** Biochem. J. 310 (1995) 721

5. **Han, D.; Rhee, J.S.:** Biotechnol. Bioeng. 28 (1986) 1250
6. **Yang, F.; Russell, A.J.:** Biotechnol. Bioeng. 47 (1995) 60
7. **Khmelnitsky, Y.L.; Levashov, A.V.; Klyachko, N.; Martinek, K.:** Enzyme Microb. Technol. 10 (1988) 710
8. **Creagh, A.L.; Prausnitz, J.M.; Blanch, H.W.:** Enzyme Microb. Technol. 15 (1993) 383
9. **Sarcar, S.; Jain, T.K.; Maitra, A.:** Biotechnol. Bioeng. 39 (1992) 474
10. **Martinek, K.; Levashov, A.V.; Klyachko, N.; Khmelnitsky, Y.L.; Berezin, I.V.:** Eur. J. Biochem. 155 (1986) 453
11. **Steinmann, B.; Jackle, H.; Luisi, P.L.:** Biopolymers 25 (1986) 1133
12. **von Stockar, U.:** In: J. Tramper et al. (eds) Biocatalysis in non-conventional media. Elsevier, NL, (1992) 201
13. **Cipiciani, A.; Germani, R.; Savelli, G; Bunton, C.A.; Mhala, M.; Moffatt, J.R.:** Chem. Soc. Perkin II (1987) 541
14. **Alfani, F.; Cantarella, M.; Cutarella, N.: Spreti, N.; Germani, R.; Savelli, G.:** J. Biotechnol. 24 (1992) 129
15. **Cantarella, M.; Cifoni, D.; Spreti, N.; Alfani, F.; Germani, R.; Savelli, G.:** Ann. N.Y. Acad. Sci. 750 (1995) 97
16. **Schmidt, G.; Laskowski, M. Sr.:** In: P.D. Boyer, H.A. Lardy and K. Myrback, (eds), The enzymes, 2nd edition, Vol. 5, Academic Press, N.Y., (1961) 3
17. **Hsu, R.Y.; Cleland, W.W.; Anderson, L.:** Biochemistry 5 (1966) 799
18. **Jørgensen, O.B.:** Acta Chem. Scand. 13 (1959) 900
19. **Cantarella, M.; Cantarella, L.; Alfani, F.:** Enzyme Microb. Technol. 13 (1991) 547
20. **Cantarella, L.; Alfani, F.; Cantarella, M.:** Enzyme Microb. Technol. 15 (1993) 861
21. **Kubicz, A.:** Acta Biochem. Pol. 20 (1973) 223
22. **Toscano, G.:** Ph.D. Thesis, University of Napoli, Italy (1992)
23. **Bonan, C.; Germani, R.; Ponti, P.P; Savelli, G.; Cerichelli, G; Bunton, C.A:** J. Phys. Chem. 94 (1990) 5331
24. **Cipiciani, A.; Germani, R.; Savelli, G.; Bunton, C.A.:** J. Chem. Soc. Perkin II (1987) 553
25. **Germani, R.; Ponti, P.P; Spreti, N.; Savelli, G; Cipiciani, A.; Cerichelli, G.; Bunton, C.A.; Si, V.:** J. Colloid Interface Sci. 38 (1990) 443
26. **Sanchez-Ferrer, A.; Pérez-Gilabert, M.; Garcia-Carmona, F.:** In: J. Tramper et al. (eds) Biocatalysis in non-conventional media. Elsevier, NL, (1992) 181
27. **Matzke, S.F.; Creagh, A.L.; Haynes, C.A.; Prausnitz, J.M.; Blanch, H.W.:** Biotechnol. Bioeng. 40 (1992) 91
28. **Di Profio, P.:** (1996) Private communication
29. **Gianfreda, L.; Marrucci, G.; Grizzuti, N.; Greco, G.:** Biotechnol. Bioeng. 27 (1985) 877
30. **Alfani, F.; Cantarella, L.; Cantarella, M.; Gallifuoco, A.; Scardi, V.:** Latin Am. Appl. Res. 20 (1990) 47
31. **Dekker, M.; van't Riet, K.; Weijers, S.R.; Baltussen, J.W.A.; Laane, C.; Bijsterbosch, B.H.:** Chem. Eng. J. 33 (1986) B27
32. **Marcozzi, G.; Correa, N.; Luisi, P.L.; Caselli, M.:** Biotechnol. Bioeng. 38 (1991) 1239
33. **Charm, S.E.; Wong, B.L.:** Enzyme Microb. Technol. 3 (1981) 111
34. **Lencki, R.W.; Tecante, A.; Choplin L.:** Biotechnol. Bioeng. 42 (1993) 1061
35. **Hayes D.G.; Gulari, E.:** Biotechnol. Bioeng. 35 (1990) 793
36. **Smith, R.E.; Luisi, P.L.:** Helv. Chim. Acta 63 (1980) 2302
37. **Fringuelli, F.; Germani, R.; Pizzo, F.; Santinelli, F.; Savelli, G.:** J. Org. Chem. 57 (1992) 1198
38. **Alvarez, E.F.:** Biochim. Biophys. Acta 59 (1962) 663
39. **Leodidis, E.B.; Hatton, T.A.:** In: M.P. Pileni (ed.) Structure and reactivity in reverse micelles, Elsevier, NL, (1989) 270
40. **Hilhorst, R.; Wolbert, R.; Fijneman, P.; Heering, D.; Rietveld, P.; Dekker, M.; van't Riet, K.; Bijsterbosch, B.H.:** In: J. Tramper et al. (eds.) Biocatalysis in non-conventional media. Elsevier, NL, (1992) 299