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Role of Environment on the Activity and Stability of α-Amylase Incorporated in Reverse Micelles

CLAUDIA SHAH, SUBRAMANI SELLAPPAN, AND DATTA MADAMWAR*

Post Graduate Department of Biosciences, Vallabh Vidyanagar—388 120, Gujarat, India

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ABSTRACT

α-amylase (3.2.1.1) was solubilized in reverse micelles formed by Triton X-100 in xylene. Although the enzyme shows decrease in specific activity in reverse micellar medium, it possesses significantly high stability in comparison to bulk aqueous medium. Water/Surfactant ratio (Wo) was found to play a crucial role in both activity and stability of the enzyme. The optimum water/surfactant ratio for the catalytic function of an enzyme in reverse micelles is 36, while the enzyme is stable at Wo 12 for a considerably long period, and at Wo above 20 the enzyme gets inactivated within a day. Glycerol and $CaCl_2$ improve the stability in both aqueous and reverse micellar medium. Thus the interior of the reverse micelles acts as a microreactor and provides favorable environment for the enzyme activity and stability.

Index Entries: α-Amylase; reverse micelles; Triton X-100; xylene; water/surfactant ratio; glycerol; $CaCl_2$.

INTRODUCTION

The use of enzymes to carry out reaction in organic solvents has gained considerable interest due to the many potential advantages *(1–3)*. However, replacement of conventional medium with organic solvents destroys the catalytic power of enzymes because of the exposure of the nonpolar groups *(1,3)*. A number of different alternatives are available for enzymatic catalysis in organic solvents with retention of catalytic activity

*Author to whom all correspondence and reprint requests should be addressed.

(1,2,4–7). Among these, entrapment of enzymes in reverse micelles has attracted attention in the recent past *(7)*.

Reverse micelles are associated colloids of surfactant molecules in water-immiscible organic solvents *(8)* and it has been suggested that the entrapped water resembles the water adjacent to biological membranes *(7)*. It has been selected as a tool to study enzymatic reactions. This system facilitates solubilization of materials of widely differing polarities in a single phase that is isotropic, optically clear, and thermodynamically stable *(9)*. Several fields of applications for protein containing reverse micelles have also been suggested by many workers *(10–12)*. Microenvironment around the enzyme in reverse micelles that includes the water–water interactions, water–surfactant interactions, and enzyme–water interactions plays an important role in the catalytic efficiency of an enzyme.

Stability of an enzyme at a given temperature is essential and is desirable in isolation, storage, and processing. Hence the concept of stabilizing the enzyme has gained considerable importance *(13)*.

In this article we have investigated the activity and stability of α-amylase in reverse micelles formed by Triton X-100/hexanol/xylene as a part of a continuing attempt to understand the cellular microenvironment.

MATERIALS AND METHODS

Chemicals

α-Amylase (E.C. 3.2.1.1) was procured from Sigma Chemical (St. Louis, MO). Hexanol was procured from Riedel de-Haen, Seelze, Germany. p-Xylene and all other chemicals were of analytical grade. Pyrex double glass distilled water was used throughout the experiment.

Assay of α-Amylase Activity in Reverse Micelles

To a tube containing 1 mL of 1*M* Triton X-100 prepared in xylene was supplemented with a predetermined amount of 0.02 *M* phosphate buffer (pH 6.9) and 200 μL hexanol. In addition, 150 μL of 1% starch and 50 μL of α-amylase (0.05 mg/mL) prepared in buffer were added and shaken well. This gave an optically transparent homogenous system after a few seconds indicating the complete solubilization of enzyme in reverse micelles.

Activity over a range of water/surfactant ratio (Wo) from 4 to 40 was determined. Effect of pH from 3.0 to 12.0 was also studied. Citric acid—NaOH buffer for the range 3.0 to 5.0, phosphate buffer for the range 6.0 to 9.0, glycine—NaOH for the pH 9.0 and 10.0 and disodium hydrogen phosphate—NaOH buffer for the pH 11.0 and 12.0 of 0.02*M* were used.

The tubes containing the system were incubated at 37°C for 75 min. To terminate the reaction 1 mL of DNSA reagent was incorporated followed by 3 mL chloroform and centrifuged at 3000*g* for 5 min to separate the aqueous layer. A known amount of aliquot was taken from the aqueous

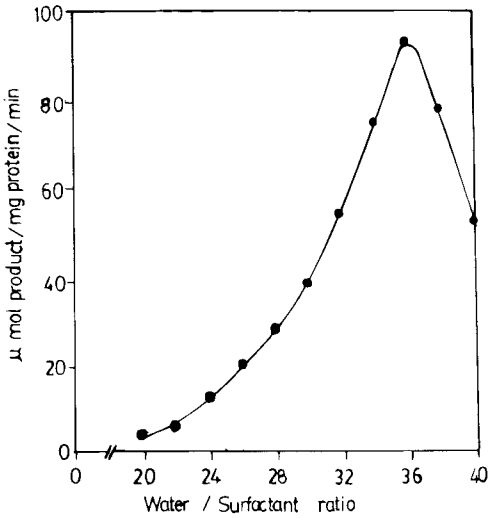

Fig. 1. Effect of Water/Surfactant ratio (Wo) at 37°C in reverse micelles.

phase and estimated for reducing sugar *(14)*. Protein was estimated by the Folin-Lowry method *(15)*.

Study of α-Amylase Stability in Reverse Micelles

To 1*M* Triton X-100 in xylene, 50 μL of α-amylase (2 mg/mL) per mL of organic solvent was introduced. Depending upon the Wo required, varying amounts of phosphate buffer were added to the system. The system was incubated at 37°C. Known amount of sample from the bulk solution was withdrawn at regular intervals of 24 h. To this tube, buffer was added to make 500 μL of aqueous content. Further, 3 mL of chloroform was added to the system and centrifuged at 3000*g* for 5 min to separate the aqueous phase.

Twenty μL sample from the aqueous phase containing α-amylase was withdrawn and incubated with 400 μL of 1% starch at 37°C for 75 min. Reducing sugar was estimated by DNSA method (*14*).

Effect of 7m*M* $CaCl_2$ and 16% glycerol on the stability of the enzyme was also studied.

RESULTS AND DISCUSSION

Overall activity of α-amylase was found to be lower in reverse micelles of Triton X-100 prepared in xylene (specific activity 93.13 μ mol/mg protein/min) (Table 1) than in that of aqueous system (specific activity 122.96 μ mol/mg protein/min) however, possessed higher stability.

As seen in Fig. 1, the amount of α-amylase that can be solubilized in reverse micelles depends on the water content. Activity was influenced by water/surfactant ratio (Wo), giving optimum activity at Wo of 36. At low water content, hardly any water is available in the micelles for hydrolysis,

Table 1
α-Amylase Activity at Different Water/Surfactant Ratio (W_0) at 37°C in Reverse Micelles

Wo	μ mol/mg protein/min	μ mol/mL solvent/min
20	3.64	0.002
22	4.49	0.002
24	12.30	0.007
26	20.06	0.011
28	27.64	0.015
30	38.09	0.021
32	54.00	0.031
34	74.79	0.043
36	93.13	0.053
38	78.00	0.044
40	52.50	0.029

as initially major portion of the solubilized water firmly binds to the surfactant molecules and forms a hydration shell, that is to say insufficient water solvatization shell *(7)*. Alternatively, it could be that at low Wo values the micelle becomes too small to properly accommodate the protein with the consequent distortion of the protein conformation and loss of activity *(16)*. Free water becomes available with an increase in Wo to carry out hydrolytic reaction by amylase. However, at Wo higher than 36 activity of amylase goes down where the micelles will tend to become larger where the properties of micellar water approach those of bulk water *(17)*. Presence of enzyme molecule in the aqueous pool of reverse micelles can considerably modify the physico-chemical characteristics of water which in turn may alter the activity of enzyme.

At optimum Wo the effect of pH from 3.0 to 12.0 was studied. Optimum pH remained unaltered from that of aqueous system (Fig. 2). However, α-amylase is slightly active in the alkaline region in reverse micelles whereas activity is lost in the aqueous system, thus reverse micelles provide little better stability.

Optimum Wo for maximum enzyme stability varied from that of maximum enzyme activity. Stability of amylase at different Wo was determined and it was observed that enzyme was not stable at Wo above 20 while the enzyme retained 10% activity at Wo 12 even on d 12 in reverse micelles. Maximum enzyme stability was found to be at Wo 12. Enzyme in aqueous medium was stable only for 2 d at 37°C (Fig. 3) indicating that enzyme has better stability in reverse micellar medium. As shown in Fig. 3 the enzyme is more stable at Wo 12 as compared to Wo 4 and Wo 8. One of the possible reasons for the long-term stability in reverse micelles at Wo 12 can be that this small amount of water is neces-

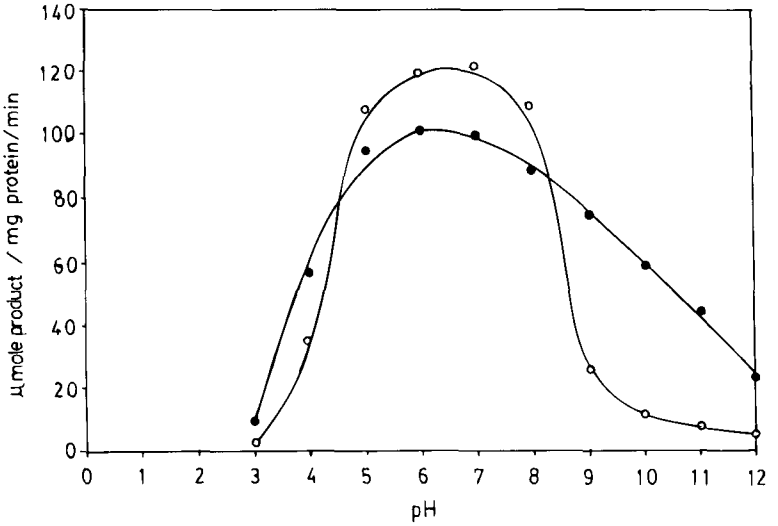

Fig. 2. Effect of pH at 37°C (●—●) in reverse micelles and (○—○) in aqueous medium.

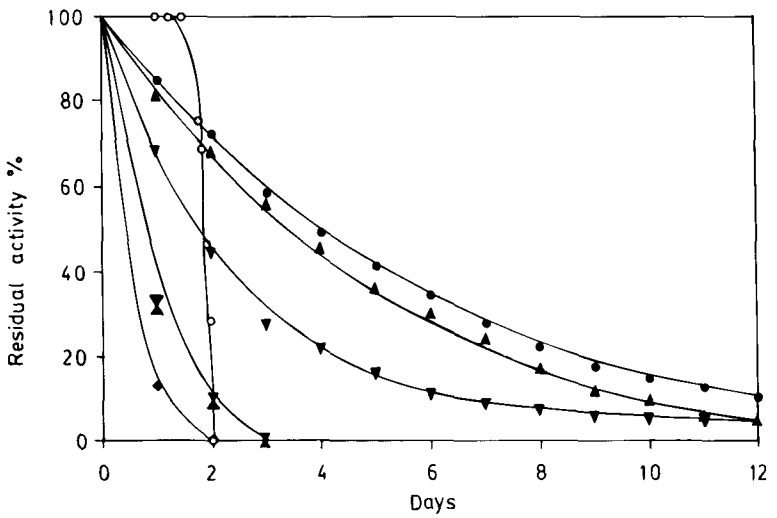

Fig. 3. Stability at 37°C (W_o 4 ▼—▼), (W_o 8 ▲—▲), (W_o 12 ●—●), (W_o 16 ⧗—⧗) and (W_o 20 ◆—◆) in reverse micelles and (○—○) in aqueous medium.

sary to maintain the intact configuration of the enzyme. In general it has been reported that enzymes encapsulated in reverse micellar system have a longer storage stability than in water solution *(18)*. The increase in the water level leads to a decrease in the enzyme stability. This water effect may be explained by the loss in the interactions between the enzyme and the surfactant polar heads that stabilize the enzyme at the optimum Wo *(19)*.

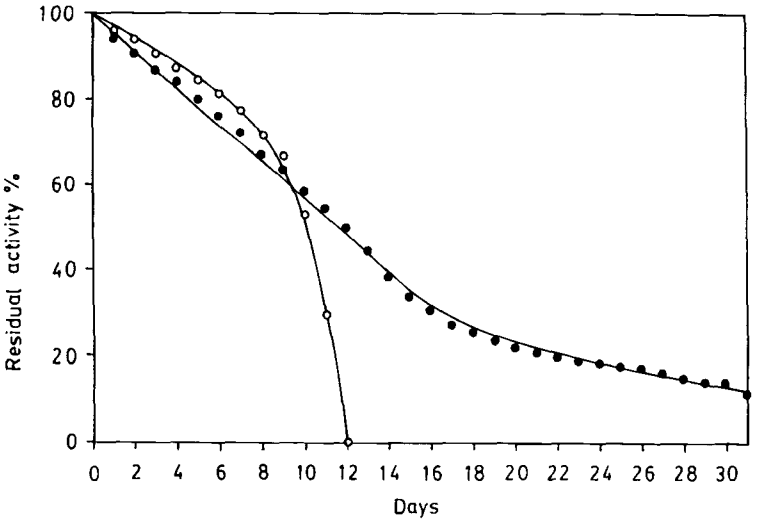

Fig. 4. Effect of Glycerol on stability at 37°C (●—●) in reverse micelles and (○—○) in aqueous medium.

In general, organic solvents, except polyhydric alcohols and some polar solvents are known to destabilise proteins *(20)*. The enzyme stability can be enhanced by several additives, glycerol is the most studied among the polyhydric alcohols used as stabilizers *(21)*.

We have studied the effect of glycerol on the activity of α-amylase and it was found that the stabilizing effect of glycerol was more pronounced in reverse micellar medium than in bulk aqueous medium (Fig. 4). Enzyme in aqueous medium was stable up to 11 d in the presence of glycerol which otherwise becomes inactivated in 2 d, while in reverse micellar medium it was stable for 31 d in the presence of glycerol as compared to 12 d without glycerol. Thus glycerol has a stabilizing effect in both bulk aqueous and reverse micellar medium but it has a stronger effect in reverse micellar medium. It may be expected that in low water systems the influence of polyols becomes substantial because of the rigidity of the protein molecule. Thus the specific interactions between the biomolecule and glycerol play a critical role in providing a stabilizing effect.

Many enzymes are stabilized by calcium ions like thermolysin and α-amylase *(13)*. Calcium chloride also has stabilizing effects in both reverse and aqueous medium but it is a more effective stabilizing agent in aqueous medium. In presence of $CaCl_2$ the enzyme retains 64% activity after 41 d in bulk aqueous medium while 8% activity is retained in reverse micellar medium (Fig. 5).

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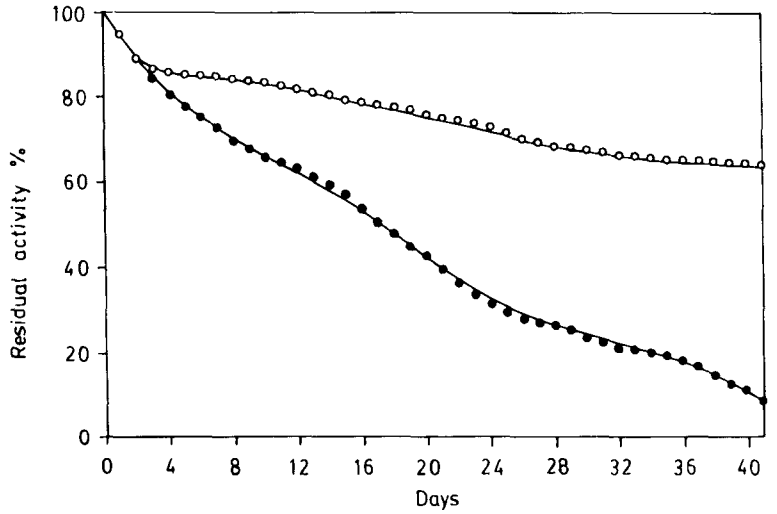

Fig. 5. Effect of $CaCl_2$ on stability at 37°C (●—●) in reverse micelles and (○—○) in aqueous medium.

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