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Activation and Stabilization of Enzymes Entrapped into Reversed Micelles

Studies on Hydrolyzing Enzymes—Protease and α-Amylase

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ABSTRACT

Observations of the activity of two hydrolyzing enzymes—protease and α-amylase—entrapped inside the reversed micelles formed by surfactants in hexane, benzene, and cyclohexane are reported. The surfactants chosen for this study are: Tween 80, a nonionic surfactant, Cetyl pyridinium chloride, a cationic surfactant, and two anionic surfactants, sodium lauryl sulfate and Aerosol OT.

Tween 80 enhances the activity of both protease and α-amylase. Sodium lauryl sulfate and Aerosol OT, which are ionic surfactants, enhance the activity of protease, but inhibit the activity of α-amylase. Cetyl pyridinium chloride, however, enhances the activity of α-amylase, but inhibits the activity of protease. Enhanced activity is generally severalfold greater in comparison to the activity observed in the usual aqueous system in the absence of reversed micelles. It has also been observed that the enhanced activity of the enzymes entrapped inside the reversed micelles remains preserved for a much longer period of time in comparison to the activity in the usual aqueous systems. These observations, which support the view that with proper choice of surfactant and the organic solvent, reversed micelles act like a microreactor that provides a favorable aqueous microenvironment for enzyme activity, have biotechnological overtones.

Index Entries: Reversed micelles; enzyme stabilization; protease; α-amylase.

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INTRODUCTION

Replacement of water by organic solvents and hydrophobic media is normally accompanied by either a total denaturation of the enzymes, or dramatic decrease of their catalytic activity and also the loss of their substrate specificity (*1*). This fact impedes the application of enzymes in several biotechnological processes where hydrophobic media are required. In order that enzymes function in a nonaqueous hydrophobic medim one has to protect the enzyme molecule from the unfavorable action of organic solvents. This protection can be achieved by entrapping the water-soluble enzymes into the aqueous pockets of the reversed micelles formed by the surfactants in the hydrophobic medium. This idea was advanced by Martinek and associates (*1–4*)) and further substantiated by several workers (*5–10*). It has been shown (*1–10*) that the enzymes solubilized inside the reversed micelles retain their catalytic activity and substrate specificity.

In the present article, we report studies on two hydrolyzing enzymes, viz. protease and α-amylase, entrapped into the reversed micelles formed by the surfactants in hydrophobic medium. Anionic, cationic, and nonionic surfactants in three different hydrophobic solvents—hexane, benzene, and cyclohexane—have been experimented with. The data revealed that the activity of both protease and α-amylase is enhanced in the case of nonionic surfactant, Tween 80. With the ionic surfactants, however, trends were mixed. The activity of protease showed enhancement with anionic surfactants and a decrease with the cationic surfactant, whereas the activity of α-amylase increased in the case of the cationic surfactant and decreased in the case of anionic surfactants.

It was also observed that the enzymes entrapped in the reversed micelles retained their activity for much longer periods than that in the purely aqueous medium. Since catalytic properties of enzymes can be utilized in the fine organic synthesis, in the production of drugs, in the synthesis of important biochemicals, and so forth, these observations seem to have biotechnological overtones.

MATERIALS AND METHODS

Materials

Enzyme Extraction and Assay

PROTEASE

The enzyme protease (E.C. 3.4.21.14) used in the present study was extracted from germinating rice (*Oryza sativa* L.) seeds following the method described in literature (*11*). Seeds were surface-sterilized with 1% sodium hypochlorite solution for 10 min and soaked in water for 24 h. The

surface-sterilized seeds were then spread in Petri plates lined with thin beds of sterilized moist cotton using uniform quantities of deionized distilled water. Seeds were germinated for 5 d at 28 ± 1°C with 80% relative humidity in a humidity cum B.O.D. incubator, maintaining a regular cycle of light followed by a dark period of 12 h each. For extraction of protease, 50 germinated dehusked seeds were homogenized in 50 mL of 0.1*M* sodium acetate buffer (pH 6.5) at 4°C and centrifuged at 22,000*g* for 15 min. Supernatant was used as enzyme extract. Total volume of the assay mixture for protease was 2 mL, which consisted of 1 mL enzyme extract and 1 mL casein solution (40 mg/mL). The contents were incubated at 37°C for 2 h after which the reaction was stopped by the addition of 3 mL of 5% trichloroacetic acid (TCA) (CAS 76-03-9) and centrifuged at 10,000*g* for 10 min. TCA soluble peptide fragments were estimated in the supernatant, using the method of Lowry et al. (*12*). The specific activity of protease enzyme is defined as μg of protein hydrolyzed min^{-1} mg^{-1} protein.

α-AMYLASE

The enzyme α-amylase (E.C. 3.2.1.1) was extracted from germinating rice (*Oryza sativa* L.) seeds and assayed following the method documented in the literature (*13,14*). Fifty germinated rice seeds were dehusked and homogenized in 50 mL of 0.1*M* sodium acetate buffer (pH 4.8) containing 25 μ*M* cysteine using prechilled glass pestle and mortar. The contents were centrifuged at 22,000*g* for 10 min at 4°C. Supernatant was used as enzyme extract. Assay mixture for α-amylase in a total volume of 2 mL contained 1.0 mL starch solution and 1.0 mL enzyme extract. After incubation at 30°C for 30 min, the reaction was stopped by adding 1 mL of 6*N* HCl. Amount of unhydrolyzed starch in this reaction mixture was estimated using the method described in literature (*13,14*). To a 0.1-mL aliquot in a 25-mL volumetric flask, 15 mL water and 0.5 mL IKI solution (0.2% I_2 in 2% KI) were added. Volume was made up to 25 mL with water, and absorbance was recorded at 660 nm.

A blank experiment containing 1 mL of starch solution and 1 mL of enzyme, where the reaction was stopped at 0 time, by adding 1 mL of 6*N* HCl was also run simultaneously. Amount of starch hydrolyzed was estimated from the difference between the values from the blank and the actual experimental run. The specific activity of α-amylase enzyme is defined as mg starch hydrolyzed min-1 mg-1 of protein.

Protein Estimation

In all enzyme preparations, protein was estimated by the method of Lowry et al. (*12*) using bovine serum albumin (BSA) (CAS 9049-46-8) as standard. Other prominent chemicals used in the present study were Aerosol OT (CAS 38916-42-6), BSA (CAS 9048-46-8), Casein (CAS 9000-71-9), Cetyl pyridinium chloride (CAS 6004-24-6), potato starch (CAS 9005-84-9), sodium lauryl sulfate (CAS 151-21-3), all from Sigma, and Tween 80

(CAS 9000-65-6). The organic solvents hexane (CAS 110-54-3), cyclohexane (CAS 110-82-7), and benzene used were of Analar grade. Water distilled twice in an all-Pyrex™ glass still was used for preparing solutions.

Methods

To 20 mL of organic solvent, hexane, benzene, or cyclohexane, contained in a glass beaker, 0.2 mL of Tween 80, or 0.1431 g of sodium lauryl sulfate (NaLS), or 0.0193 g of Cetyl pyridinium chloride (CPCl), or 0.2 mL of Aerosol OT (AOT) was added. To the turbid solution thus obtained, 0.2 mL of aqueous enzyme extract containing protease and 0.2 mL of aqueous casein solution of desired strength were added slowly with vigorous stirring until the system became transparent, indicating solubilization of the enzyme extract and its substrate inside the reversed micelles. Enzymes and the substrate entrapped inside the reversed micelles were incubated at 37°C using a thermostated water bath. After incubating for the desired period of time, the nonaqueous phase containing the reactants solubilized inside the reversed micelles was vigorously shaken with 10 mL of aqueous phase containing 7 mL of distilled water and 3 mL of 5% aqueous solution of trichloroacetic acid (TCA). The purpose of TCA was to denature the enzyme. It is expected that the products formed along with the unused reactants will come out in the aqueous phase, because on extraction with aqueous phase, the reversed micelles will either acquire the configuration of regular micelles or will be dissociated into monomers. In either case, the materials solubilized inside the reversed micelles will become available in the aqueous phase for estimation. In fact, a control experiment was performed to demonstrate that the amount of casein solubilized inside the reversed micelles present in the hydrophobic medium can be estimated by the method of aqueous extraction. Hydrolyzed substrate as peptide fragments was estimated by the method of Lowry et al. (*12*).

A similar procedure was adopted for α-amylase; 0.2 mL of the enzyme extract and 0.2 mL of starch solution of desired strength were solubilized inside the reversed micelles. The solubilized reaction mixtures were incubated at 37°C for desired periods of time, and were then extracted with 5 mL of aqueous phase containing 4 mL of distilled water and 1 mL of 6*N* HCl. The purpose of HCl was to denature the enzyme. The amount of starch remaining unhydrolyzed was estimated from the contents of aqueous extract using the method described in literature (*13,14*). A control experiment was performed to make sure that the starch solubilized inside the reversed micelles can be estimated with reasonable accuracy by the method of aqueous extraction. For protease, pH = 6.5 and for α-amylase, pH = 4.8 were maintained using 0.1*M* sodium acetate buffer.

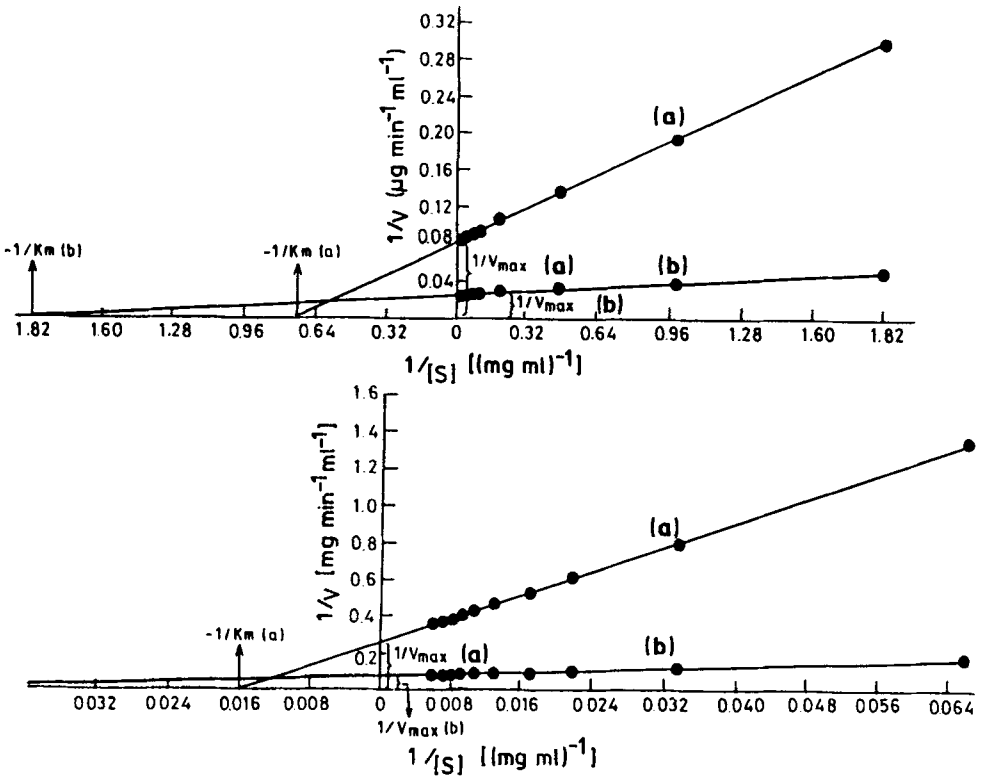

Fig. 1. Lineweaver and Burk (LB) plots. V is the initial velocity, and S is the substrate concentration. (**A**) LB plot for protease (a) in aqueous system without surfactants and (b) after entrapment in the reversed micelles of Aerosol OT in *n*-hexane. For aqueous system, 1 mL enzyme of 1 mL casein solution of strengths varying from 1 to 40 mg/mL were incubated for 2 h at 37°C, whereas in micellar system, 0.2 mL enzyme and 0.2 mL casein solution of strengths varying from 1 to 40 (mg/mL) were incubated for 2 h at 37°C. (**B**) LB plot for α-amylase (a) in aqueous system without surfactants and (b) after entrapment in the reversed micelles of Cetyl pyridinium chloride in *n*-hexane. For aqueous system, 1 mL enzyme and 1 mL starch solution of strengths varying from 15 to 150 (mg/mL) were incubated for 30 min at 30°C, whereas in micellar system, 0.2 mL enzyme and 0.2 mL starch solution of strengths varying from 15 to 150 (mg/mL) were incubated for 30 min at 30°C.

Determination of Maximum Velocity (V_{max}) and Michaelis Constant (K_m)

V_{max} and K_m were determined from the Lineweaver and Burk (LB) plots. Typical LB plots for both enzymes, viz. protease and α-amylase when the reaction was carried out inside the reversed micelles and when it was carried out in the usual aqueous system in the absence of reversed micelles, are shown in Fig. 1 as an illustration.

Table 1
V_{max} and K_m Values for Protease Entrapped in the Reversed Micelles[a]

	Solvents					
	Hexane		Benzene		Cyclohexane	
Surfactants	V_{max}	K_m	V_{max}	K_m	V_{max}	K_m
Tween 80	80.00	1.00	74.08	1.00	69.24	1.17
NaLS	135.36	0.62	123.48	0.66	149.52	0.47
CPCl	40.00	3.57	33.32	0.50	37.60	1.59
AOT	157.80	0.51	150.40	0.54	83.52	1.12

[a] V_{max} expressed in μg protein hyrolyzed/min/mg protein and K_m expressed in mg/mL. For aqueous system without reversed micells, V_{max} = 50 μg/min/mg protein and K_m = 1.39 mg/mL.

Table 2
V_{max} and K_m Values for α-Amylase Entrapped in the Reversed Micelles[a]

	Solvents					
	Hexane		Benzene		Cyclohexane	
Surfactants	V_{max}	K_m	V_{max}	K_m	V_{max}	K_m
Tween 80	39.81	26.85	29.12	31.25	41.60	23.81
NaLS	16.00	62.50	13.88	69.45	14.88	66.67
CPCl	50.64	18.74	53.32	19.53	52.00	20.83
AOT	11.20	83.33	8.92	104.16	10.96	90.91

[a] V_{max} expressed in mg starch hyrolyzed/min/mg protein and K_m expressed in mg/mL. For aqueous system without reversed micells, V_{max} = 15 mg starch hydrolyzed/min/mg protein and K_m = 62.5 mg/mL.

RESULTS AND DISCUSSION

Three ionic surfactants, NaLS, CPCl, and AOT, and one nonionic surfactant, Tween 80, in combination with hydrophobic solvents, hexane, benzene, and cyclohexane, have been experimented with in the present studies. The data on V_{max} and K_m for protease and α-amylase, in various combinations of surfactants and the hydrophobic solvents, obtained from the LB plots are recorded in Tables 1 and 2. The LB plot in one typical case is shown in Fig. 1. The data (Table 1) reveal that the activity of protease is increased severalfold in case of all surfactants, except in the case of CPCl, a cationic surfactant, where a decrease in activity was observed. Similarly, the activity of α-amylase increased in the case of Tween 80 and CPCl, whereas in case of the anionic surfactants, NaLS and AOT, a decrease in activity was observed. The extent of increase or decrease in the activity of the two enzymes is summarized in Tables 3 and 4.

Table 3
Percent Increase (+) or Decrease (−) in the Values of V_{max} and K_m for Protease After Entrapment in Reversed Micelles[a]

	Solvents					
	Hexane		Benzene		Cyclohexane	
Surfactants	V_{max}	K_m	V_{max}	K_m	V_{max}	K_m
Tween 80	160.00 (+)	72.00 (−)	148.16 (+)	72.00 (−)	138.48 (+)	84.16 (−)
NaLS	270.72 (+)	44.61 (−)	246.96 (+)	47.48 (−)	299.04 (+)	33.82 (−)
CPCl	80.00 (−)	256.84 (+)	66.64 (−)	179.86 (+)	75.20 (−)	114.38 (+)
AOT	316.00 (+)	36.70 (−)	300.80 (+)	38.85 (−)	167.04 (+)	80.58 (−)

[a]One hundred percent V_{max} and K_m values for protease correspond to 50 μg min/mg protein and 1.39 mg/mL, respectively, in the absence of reversed micelles.

Table 4
Percent Increase or Decrease in the Values of V_{max} and K_m for α-Amylase After Entrapment in Reversed Micelles[a]

	Solvents					
	Hexane		Benzene		Cyclohexane	
Surfactants	V_{max}	K_m	V_{max}	K_m	V_{max}	K_m
Tween 80	249.00 (+)	42.96 (−)	182.00 (+)	50.00 (−)	260.00 (+)	38.10 (−)
NaLS	100.00 (no increase or decrease)	100.00 (no increase or decrease)	86.75 (−)	111.12 (+)	93.00 (−)	106.67 (+)
CPCl	316.50 (+)	29.99 (−)	333.25 (+)	31.25 (−)	325.00 (+)	33.33 (−)
AOT	70.00 (−)	133.33 (+)	55.75 (−)	166.66 (+)	68.50 (−)	145.46 (+)

[a]One hundred percent V_{max} and K_m values for α-amylase correspond to 16 mg min/mg protein and 62.5 mg/mL, respectively, in aqueous system in the absence of reversed micelles.

In all cases where an increase in activity was observed, the interior of the reversed micelles acts like a microreactor that provides a favorable aqueous microenvironment for enzyme activity. In these cases, obviously, the reversed micellar aqueous cavities protect the enzyme against denaturation by the surrounding organic solvents, and also stabilize active conformation of the enzymes by entrapping them. The gross picture is depicted in Fig. 2.

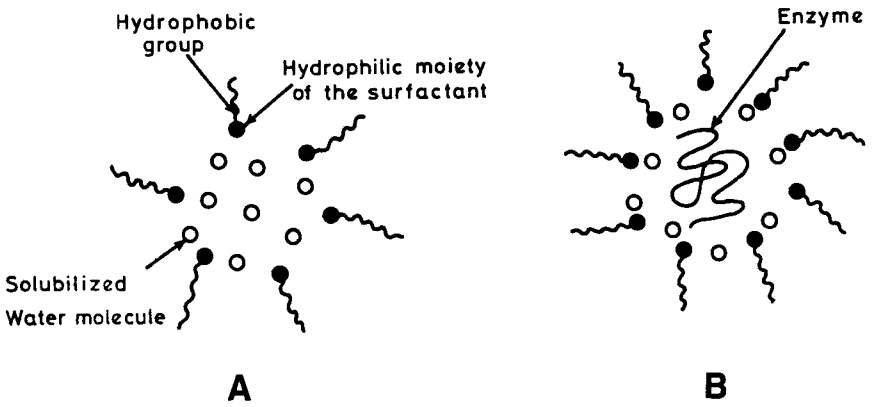

Fig. 2. Gross picture of (**A**) a reversed micelle and (**B**) enzyme entrapped into it.

Data obtained on the effect of incubation time on the specific activity (Figs. 3 and 4) are consistent with the trends on the catalysis of the enzymic reactions inside the reversed micelles (Tables 1 and 2). In all systems (surfactant–organic solvent combinations) where an activation was observed, less time was required for the specific activity to reach a maximum in comparison with the time required in the purely aqueous system where no reversed micelles were present. For example, in the case of AOT-hexane system where the activity of protease was enhanced most, maximum product was formed in the least time (Fig. 3A). A similar trend was observed for α-amylase in the case of the CPCl-cyclohexane system (Fig. 4C) where maximum enhancement in the activity was observed. A decline in the activity after the attainment of the maximum (Figs. 3 and 4) is obviously the result of the depletion of the substrates inside the reversed micelles.

Experiments were performed to demonstrate that the activity of the enzymes entrapped into the reversed micelles remains preserved for a much longer period of time in comparison to that in the purely aqueous medium. The enhanced activity of protease entrapped into the reversed micelles of AOT in *n*-hexane remained intact up to 21 d, whereas in the purely aqueous system under the same conditions of the temperature, i.e., at 8°C, the activity declined after 5 d and on the 10th d, it was totally lost (Table 5). Similarly, α-amylase entrapped into the reversed micelles of CPCl in *n*-hexane remained at its enhanced activity for 7 d, whereas in the purely aqueous medium, the activity declined after 2 d, and after 4 d, it was totally lost (Table 6).

The observations that among the ionic surfactants sodium lauryl sulfate and Aerosol OT enhance the activity of protease, but inhibit the activity of α-amylase, whereas Cetyl pyridinium chloride enhances the activity of α-amylase, but inhibits the activity of protease call for an explanation.

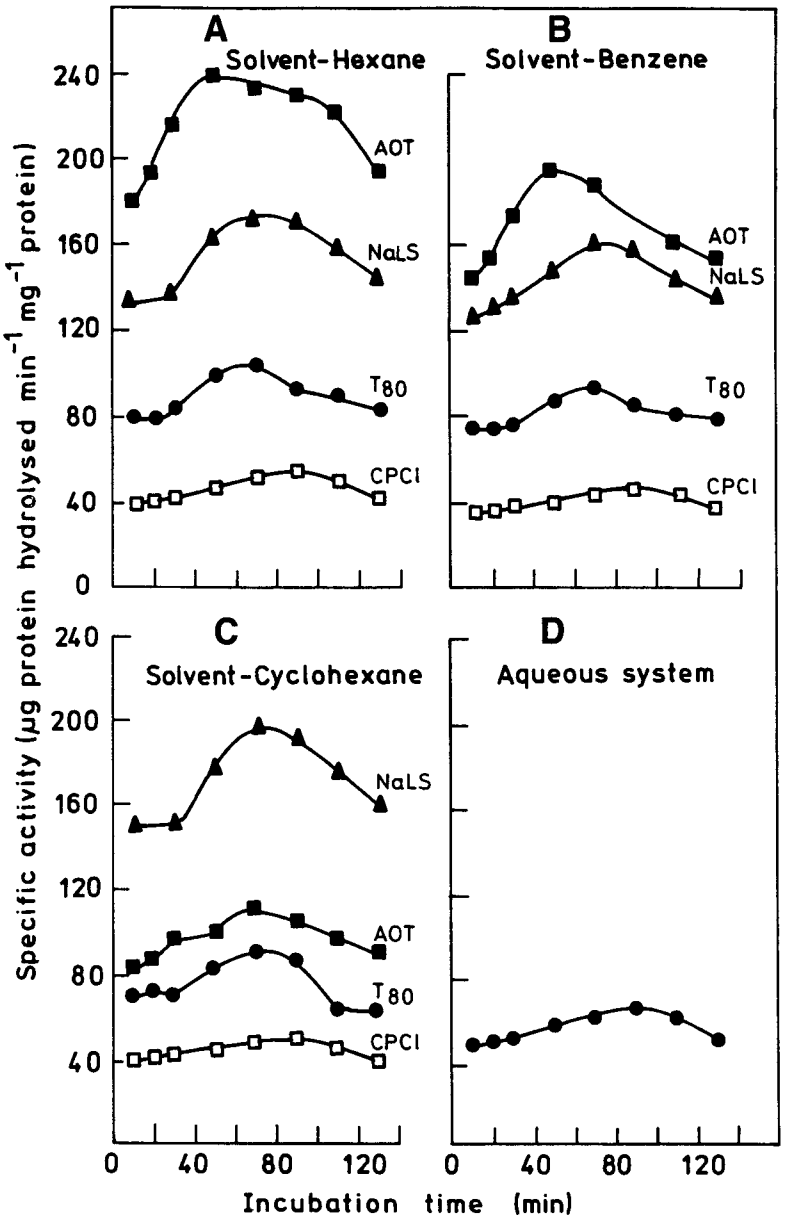

Fig. 3. Effect of incubation time on the specific activity of protease. Reversed micelles prepared in the solvents: (A) hexane, (B) benzene, (c) cyclohexane using the surfactants Aerosol OT (■), sodium lauryl sulfate (▲), Tween 80 (●), and Cetyl pyridinium chloride (□). In each case, 0.2 mL enzyme and 0.2 mL casein solution (40 mg/ml) were entrapped in the reverse micelles and incubated for different time periods. Data for aqueous system (D). In this case, 1 mL enzyme and 1 mL substrate solution (40 mg/mL) were incubated for varying time periods.

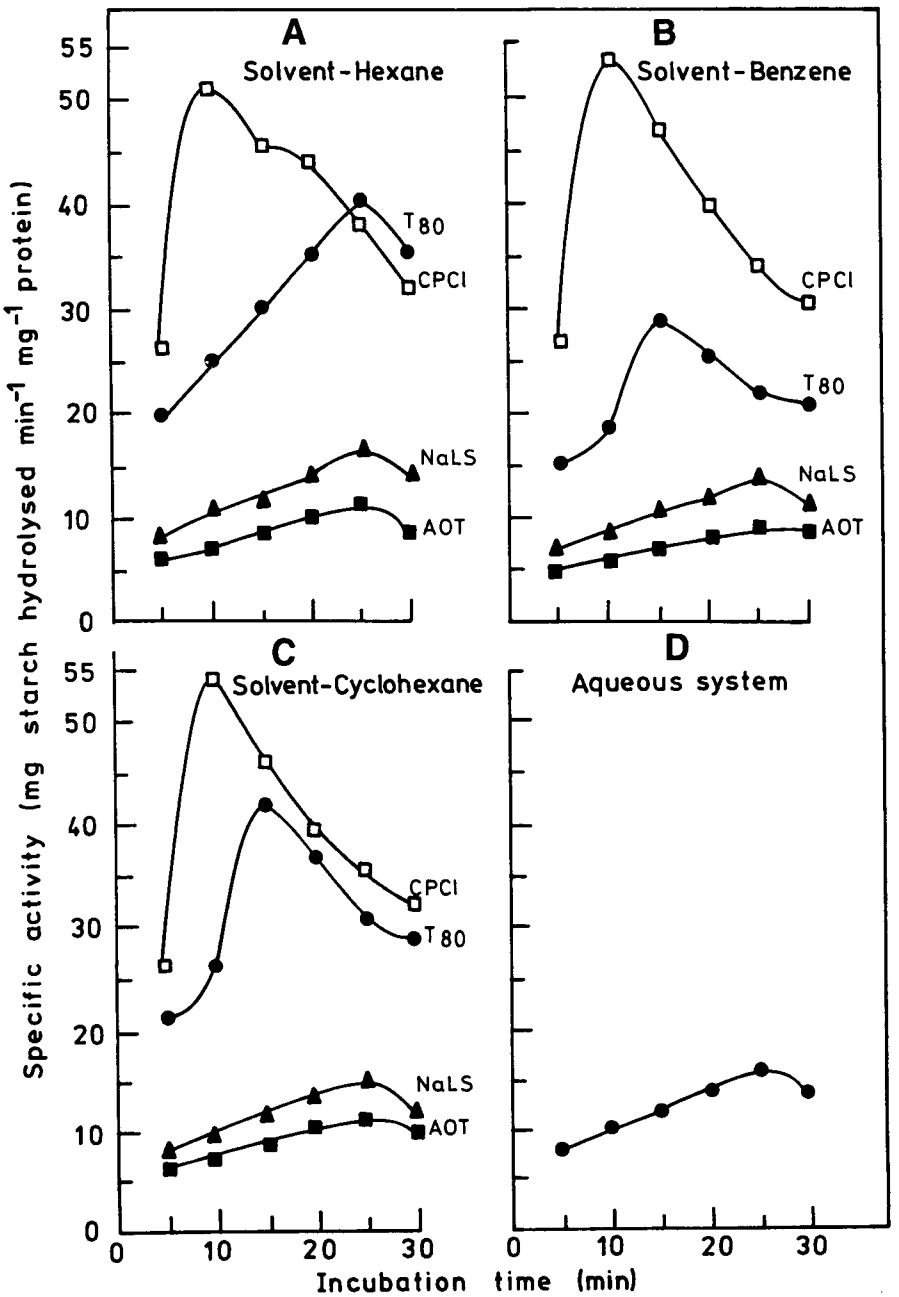

Fig. 4. Effect of incubation on the specific activity of α-amylase. Reversed micelles prepared in the solvents: (A) hexane, (B) benzene, (c) cyclohexane using the surfactants Aerosol OT (■), sodium lauryl sulfate (▲), Tween 80 (●), and Cetyl pyridinium chloride (□). In each case, 0.2 mL enzyme and 0.2 mL starch solution (150 mg/ml) were entrapped in the reverse micelles and incubated for different time periods. Data for aqueous system (D). In this case, 1 mL enzyme and 1 mL substrate solution (150 mg/mL) were incubated for varying time periods.

Table 5
Data Showing Stability of Protease in Reversed Micelles

Days	Specific activity of protease: In aqueous system without surfactants, μg/min/mg protein	Specific activity of protease: Entrapped in reversed micelles of AOT + *n*-hexane μg/min/mg protein
1	50	157.80
3	50	157.80
5	50	157.80
6	46.05	157.80
10	Activity completely lost	157.80
21	—	156.40
25	—	112.60

Table 6
Data Showing Stability of α-Amylase in Reversed Micelles

Days	Specific activity of α-amylase: In aqueous system without surfactants, mg/min/mg protein	Specific activity of α-amylase: Entrapped in reversed micelles of Cetyl pyridinium chloride + *n*-hexane, mg/min/mg protein
1	16	50.64
2	16	50.64
3	15.20	50.64
4	10.45	50.64
5	Activity completely lost	49.50
6	—	49.50
7	—	48.64
8	—	Activity completely lost

Unfortunately, we have none at present. Since the reversed micelles of CPCl, which is a cationic surfactant, will have a positively charged interior, whereas those formed by the anionic surfactants, NaLS and AOT, will have negatively charged interiors, we thought that probably charge on the enzyme fractions may provide an explanation. With this end in view, we conducted electrophoresis experiments at the same pH at which enzyme activities were assayed, i.e., pH = 6.5 for protease and pH = 4.8 for α-amylase. The electrophoretic movement revealed that the enzyme

fractions containing protease and α-amylase were both negatively charged. Thus, charge on the enzyme fraction cannot explain the present observation of the enhancement and inhibition of the activities of protease and α-amylase by ionic surfactants. This observation, which is a well-reproduced one, nonetheless calls for a deeper investigation.

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