Catalytic activity of enzyme in water/organic cosolvent mixtures for the hydrolysis of \( p \)-nitrophenyl acetate and \( p \)-nitrophenyl benzoate

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The ability of enzymes to efficiently carry out highly specific reactions on a broad range of substrates has been exploited in many diverse applications, such as pharmaceutical development, protein engineering, and polymer templating and synthesis. The effect of common organic solvents on micellar enzymology is highly promising for basic research and medical biotechnology. It is of interest to investigate the effect of organic solvents on the kinetics of enzyme catalyzed reaction. The possibility of enzymatic reactions in organic media has greatly increased the industrial uses of enzymes. The enzymatic reactions in water can extend the capabilities of enzymes providing it tunable specificity and selectivity. Since native enzymes do not commonly posses a significant catalytic activity in organic media, different strategies have been proposed to prevent enzyme denaturation and thus their application in organic solvents. It is generally believed that the introduction of additional hydrophobic group on the protein surface may improve its stability in such systems owing to the enhanced ability of the hydrophilized surface to keep the hydration shell and form additional electrostatic interaction and hydrogen bonds. The activity of enzyme is higher in hydrophobic solvents than in hydrophilic ones. It is also pertinent to add that most often the medium effects are explained in terms of more hydrophobic solvents having fewer tendencies to strip water from enzymes. The addition of water miscible organic solvents to aqueous medium would provide an efficient and easy strategy to carry out enzymatic reactions with water insoluble substrates. Water plays an important role in enzyme structure and function in aqueous media. In addition to its role as a solvent, water molecules can mediate enzymatic catalysis either directly by taking part in the reaction or indirectly through providing a solvation medium for reactants, transition state, and products. The motivation for fundamental studies in the field of nonaqueous enzymology stems partly from a variety of potential applications. By manipulating the microenvironment of an enzyme through solvent, it has become possible to modulate enzyme activity, tailor catalyst selectivity, and alter enzyme stability. Several factors contribute to the behavior of catalytic activity including the loss of critical water residues from the enzyme’s surface, a drastic decrease in the polarity of the enzyme’s microenvironment, the decreased conformational mobility of the protein structure, ground-state stabilization of enzymic substrates in organic versus aqueous media, and, the loss of activity in the preparation of enzymes for use in non-aqueous media. Many solvent effects, in fact, arise from substrate solvation. If a substrate is more soluble in a solvent, the substrate molecules will be less available to the enzyme. Many studies have been focused on the role of the reaction media on enzymatic catalysis in organic solvents. However, information on the enzymatic reaction of carboxylic esters using \( \alpha \)-chymotrypsin and cationic surfactants in the presence of different organic solvents is rather limited. In previous studies, we have reported \( \alpha \)-CT catalyzed hydrolysis of \( p \)-nitrophenyl acetate in the presence of cationic surfactants.
In the present study we report the effects of different organic solvents, i.e., acetonitrile, dimethyl sulfoxide, dimethylformamide, ethylene glycol, methanol, ethanol, propan-2-ol, tert-butanol (2-14 %) (v/v) on the α-chymotrypsin (α-CT) catalyzed hydrolysis of p-nitrophenyl acetate (PNPA) and p-nitrophenyl benzoate (PNPB) (Scheme 1) at pH 7.75 in the presence of cetyltrimethylphosphonium bromide (CTPB) surfactant (cmc: 0.16 mM).

![Scheme 1](image)

**Experimental**

α-CT (EC 3.4.21.1) from bovine pancreas (Type-II, mol. wt. 25 kDa, isoelectric point pI 8.8) was procured from Sigma and used without further purification. The substrate, p-nitrophenyl acetate (PNPA), was obtained from Sigma-Aldrich (St. Louis Missouri, USA) and other substrates like p-nitrophenyl benzoate (PNPB) procured from Lancaster (Newgate, Lancashire, England). Enzyme and substrate solutions were always freshly prepared in the appropriate buffer immediately before their use Tris-(hydroxymethyl) aminomethane (Tris) (pKₐ 8.3), hydrochloric acid and some organic solvents, viz., dimethylformamide (DMF), ethylene glycol (EG), ethanol and propan-2-ol were obtained from Qualigens Fine Chemicals (Mumbai, India). Other solvents like acetonitrile (ACN), dimethyl sulfoxide (DMSO) and tert-butanol were from SD Fine Chemicals (Mumbai, India) while methanol was from Merck (Mumbai, India). The cationic surfactant, cetyltrimethylphosphonium bromide (CTPB) was obtained from Prof. R. M. Palepu, St. Francis Xavier University, Antigonish, Canada as a gift.

All reactions were followed spectrophotometrically by observing the appearance of p-nitrophenoxide ion at 400 nm at pH 7.75 (10 mM Tris/HCl buffer, C = 12000 M⁻¹ cm⁻¹). The α-chymotrypsin solution was freshly prepared in acetate buffer (pH = 4.6). To enhance the rate of hydrolysis, PNPA and PNPB were prepared in 14 % (v/v) organic solvents, i.e., acetonitrile (ACN), dimethyl sulfoxide (DMSO), dimethylformamide (DMF), ethylene glycol (EG), methanol, ethanol, propan-2-ol and tert-butanol. The rate of hydrolysis of PNPA and PNPB catalyzed by α-chymotrypsin was measured in aqueous solution and in surfactants solution at pH = 7.75 (10 mM Tris/HCl buffer) following the formation of p-nitrophenoxide ion (PNP) at 400 nm (C = 12000 M⁻¹ cm⁻¹). The initial reaction rate, V₀, was determined from the slope of the PNP concentration versus time profiles using enzyme kinetics software (Varian). The rates of all enzyme-catalyzed reactions were corrected for the rate of spontaneous non-enzymatic hydrolysis (buffer/surfactant) determined under identical conditions.

**Results and discussion**

The catalytic activity of α-CT was studied using PNPA and PNPB in different solvents at three temperatures. Representative results obtained for V₀ (per enzyme) versus substrate concentration in some of the organic solvents are shown in Figs 1 and 2. The value of the catalytic rate constant, k_cat and the Michaelis-Menten constant, K_M, were obtained from the linear Lineweaver–Burk plot. A summary of the results obtained in the kinetics experiments is presented in Tables 1 and 2. The data of these tables show that the k_cat and K_M values are strongly dependent on the solvent nature and polarity. It is also evident from Table 1 that the catalytic efficiency of the α-CT shows a maximum at 27 °C.

In this present investigation we have used some organic solvents, viz., ACN, DMSO, DMF, EG, methanol, ethanol, propan-2-ol, and tert-butanol. Results obtained at a fixed surfactant concentration of CTPB (1 mM) with various organic solvents are summarized in Tables 1 and 2. The rate of α-CT catalyzed hydrolysis of PNPA in aqueous medium was compared to that in organic solvents at 27 °C (Table 1). The catalytic constant, k_cat, for the aqueous medium (1.72 s⁻¹) is 3.5 times lower than in DMF (5.94 s⁻¹). On the other hand, the k_cat/K_M ratio for the EG (514 M⁻¹s⁻¹) and tert-butanol (454 M⁻¹s⁻¹) are 3 and 2.5 times higher than in aqueous medium (171 M⁻¹s⁻¹).
The catalytic efficiency of DMSO (170 M⁻¹s⁻¹) was equal to the aqueous medium. Other organic solvents, i.e., ACN (321 M⁻¹s⁻¹), DMF (297 M⁻¹s⁻¹), methanol (277 M⁻¹s⁻¹), ethanol (239 M⁻¹s⁻¹), and propan-2-ol (279 M⁻¹s⁻¹) show the nearly equal kcat/KM ratio. This can be explained on the basis that protein activity is sensitive to type of organic solvent used. As the polarity of the solvent is increased (e.g. octane → THF → acetonitrile) the enzymatic activity drops dramatically. Similarly dependence of activity on the polarity of solvent has been reported for the other enzymes that were either solubilized by the surfactants or simply suspended in organic media. It has been suggested that the key determinant of the enzymatic activity is the amount of hydration water that is available for solvation of enzyme. This solvation water can affect catalytic activity by changing enzyme flexibility as well as by effecting specific details of active site hydration. The observed decrease in enzymatic activity by increased solvent polarity shows the tendency of organic solvents to strip water molecules from the enzyme surface and the extent of stripping increases with the polarity of the organic solvent. The pH of the reaction medium was maintained at 7.75. The correct protonation state of side chains of amino acid residues of enzymes is important in organic solvents, hence, pH tuning (placing the enzyme in water at optimum pH of the enzyme, and lyophilizing) results in higher rates in organic solvents.

A meaningful observation of the hydrolysis of PNPB shows similar behavior in nature as compared to PNPA in organic solvent medium as shown in Table 2. The kcat/KM ratio for the hydrolysis of PNPB in DMSO (278 M⁻¹s⁻¹) was higher than other in organic solvents used for experiment. This is because that the organic solvents, i.e., DMF, DMSO, ACN, EG and ethanol are well known solvents capable of solubilizing enzymes.

Surfactants are very often possess the ability to generate extraordinary properties in enzymes when used in small concentration, as little as 1 mM. In some cases enzymes can be extracted into hydrophobic organic phases in the presence of surfactant to give highly active and stable organic solvent-soluble enzyme preparations. Surfactants can also be added directly to suspended enzyme preparations in organic media. In the present investigation, surfactant CTPB has been used. The enhanced catalytic activity of α-CT may be attributed to increased conformational flexibility of the enzyme by CTPB addition.

It is well-known that many enzymes are able to retain their catalytic activity in non-aqueous, hydrophobic solvents at higher temperatures in comparison to water. One reason for this may be that the enzyme is kinetically trapped in its active conformation in the hydrophobic solvent due to the lack of water. As shown in Tables 1 and 2, the parameters obtained by PNPA and PNPB hydrolysis...
indicate that the catalytic activity of the enzyme decrease exponentially after 27 °C. It is demonstrated that changes in temperature between 27 and 37 °C also have little effect on the catalytic activity of α-CT. In enzyme catalysis, $k_{\text{cat}}$ gives an idea of the turnover rate and $K_M$ gives an indication of the formation of the enzyme-substrate complex in Figs 1 and 2. The inverse of the $K_M$ is associated with the possibility of dissociation of the enzyme-substrate complex at 37 °C suggesting that at higher temperature, even when enzyme-substrate complex is formed favorably, product formation is hindered. This indicates conformational perturbation of the enzyme-substrate complex at higher temperature. Structure and properties of enzymes in polar solvents are highly dependent on temperature. However, these measurements cannot be performed in polar solvents other than aqueous medium, because their structure and properties are highly dependent on the temperature.

Determination of the thermodynamic free energy of activation, $\Delta G^*$, parameters may support this proposal. It is interesting to observe the implication of the conformational dynamics of protein to the energetics of catalysis. The overall free energy of activation for the enzymatic catalysis can be estimated from the catalytic efficiency ($k_{\text{cat}}/K_M$) according to the relation, $\Delta G^* = -RT \ln (k_{\text{cat}}/K_M) (h/k_B T)$(ref.20). The decrease in the $\Delta G$ values with an increase in temperature suggests that the enzymatic activity is thermodynamically more favorable at higher temperatures.

From Figs 3 and 4, it can be seen that a unique intercept is obtained irrespectively of the temperature.

**Fig. 1** — Michaelis-Menten plots of initial-rate data for the α-CT hydrolysis of PNPA with CTPB (1 mM) in aqueous (A), DMSO (B), ACN (C). [(○) 15 °C, (■) 27 °C, (△) 37 °C].

**Fig. 2** — Michaelis-Menten plots of initial-rate data for the α-CT hydrolysis of PNPB with CTPB (1 mM) in DMSO (A), ACN (B). [(○) 15 °C, (■) 27 °C, (△) 37 °C].
The linearity of the plot indicates that, under the condition employed, the Michaelis-Menten mechanism applies in both aqueous and DMSO media. This is as expected for the case in which the enzyme is totally incorporated into the micellar media at certain temperature. This suggests that the differences in the kinetic profiles shown in Figs 1 and 2 are due to the partitioning of the substrate between the micellar pseudophase and the organic solvent.

In the present study, the effect of different organic solvents on the \( \alpha \)-chymotrypsin catalyzed hydrolysis of \( p \)-nitrophenyl acetate and \( p \)-nitrophenyl benzoate at \( pH \) 7.75 in the presence of cetyltriphenylphosphonium bromide surfactant has been studied. The results suggest that the hydration water is removed from the enzyme to different extents by organic solvents depending on the solvent polarity. Inverse dependence of enzymatic activity on polarity of organic solvents is observed. The active site of the proteolytic enzyme \( \alpha \)-CT has been correlated with its temperature-dependent catalytic efficiency. The catalytic efficiency, \( k_{cat}/K_M \), of the enzyme at different temperatures shows a maximum at 27 \( ^\circ\)C and the catalytic activity of the enzyme decrease exponentially after 27 \( ^\circ\)C. The enhanced catalytic activity of the enzyme is also attributed to the presence of surfactant, CTPB.

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**References**