ISSN 0023-1584, Kinetics and Catalysis, 2011, Vol. 52, No. 1, pp. 6–10. © Pleiades Publishing, Ltd., 2011.

Effect of Cationic Surfactants on the Enzymatic Activity of α-Chymotrypsin[1]

S. K. Verma and K. K. Ghosh

School of Studies in Chemistry, Pt. Ravishankar Shukla University, Raipur (C.G.) 492010, India

e-mail: kallolkghosh@yahoo.com

Received July 14, 2009

Abstract—The hydrolysis of *p*-nitrophenyl benzoate catalyzed by α-chymotrypsin in the presence of cetyltriphenylphosphonium bromide, cetyltributylphosphonium bromide and cetyltrimethylammonium bromide (pre and post micellar regions) has been studied. The ester is hydrolyzed readily by α-chymotrypsin in all the surfactants with the highest activity shown in cetyltributylphosphonium bromide. The dependences of the Michaelis constant and the catalytic constant with surfactant concentration have also been discussed.

DOI: 10.1134/S0023158411010216

Recently there has been increased attention focused on micellar enzymology. Enzymes are highly specific and efficient biocatalysts with a wide range of biotechnological and industrial applications [1–3]. Among the different enzymes, proteolytic enzymes have been intensively studied because of their unique role for hydrolysing amide and peptide bonds under mild conditions. One of the most studied serine proteases enzyme is α-chymotrypsin (α-CT) and its structure and mechanism of action are well known [4, 5]. The active site contains three amino acids, which are essential for catalysis of the cleavage of peptide bonds. The hydrolysis is realized by the unique collaboration [6] between histidine-57 (His_{57}), serine-195 (Ser_{195}) and aspartate-102 (Asp_{102}) (Scheme 1).

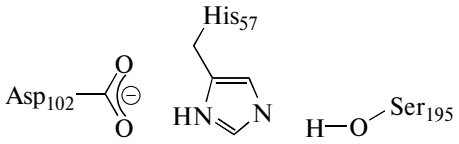
The search for activity enhancement has been an important aspect in vitro micellar enzymology. The kinetics of enzymatic reactions of esters, amides and anilides in selr-organizing assemblies like micelles and microemulsions are well documented in literature [7, 8]. Surfactants in aqueous solution could affect the kinetic behavior of enzymatic reactions below or above the critical micelle concentration (CMC) [9]. In previous works [10, 11] we have studied α-CT catalyzed hydrolysis of *p*-nitrophenyl acetate in the presence of different cationic surfactants and observed a greater catalytic efficiency in the presence of surfactants.

In order to collect more information about micellar enzymology, herein we present kinetic study for the enzymatic hydrolysis of *p*-nitrophenyl benzoate (PNPB) at pH 7.75 (Scheme 2) in the presence of different cationic surfactants, i.e., cetyltriphenylphosphonium bromide (CTPB), cetyltributylphosphonium bromide (CTBPB) and cetyltrimethylammonium bromide (CTAB) (Scheme 3).

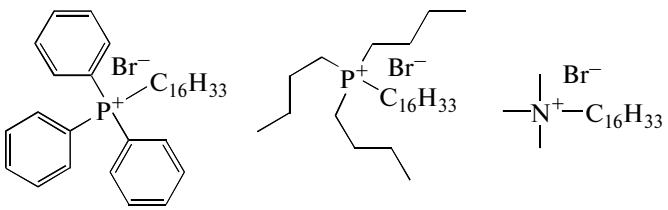
EXPERIMENTAL

Materials

Bovine pancreatic (Type-II) α-chymotrypsin enzyme (molecular mass 25 kDa, isoelectric point pI 8.8) was procured from Sigma and used without further purification. The substrate, *p*-nitrophenyl benzoate was obtained from Lancaster. Enzyme and substrate solutions were freshly prepared in the appropriate buffer immediately before their use in experiments. Tris-(hydroxymethyl)aminomethane (Tris) (pK_a 8.3) and HCl were obtained from Qualigens (Bombay). The surfactants, i.e., cetyltrimethylammonium bro-



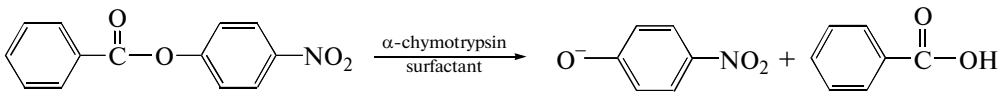
Scheme 1. Structure of α-chymotrypsin active site.



CTPB CTBPB CTAB

Scheme 2. Structure of surfactants.

[1] The article is published in the original.



p-nitrophenyl benzoate (PNPB) *p*-nitrophenolate ion benzoic acid

Scheme 3. Reaction scheme of PNPB hydrolysis.

mide, cetyltributylphosphonium bromide were procured from Sigma and cetyltriphenylphosphonium bromide was obtained from Prof. R.M. Palepu (St. Francis Xavier University, Antigonish, Canada) as gift. The CMC of these surfactants were determined in aqueous medium by conductometric method. Absorbances were recorded in a Varian Cary 50 UV-visible spectrophotometer equipped with Pelteir Temperature controller unit. The α-chymotrypsin solution was freshly prepared in acetate buffer (pH 4.6). To improve solubility of substrate, the PNPB was prepared in 100% (*v*/*v*) acetonitrile. The presence of acetonitrile in reaction mixture (4%, *v*/*v*) does not modify the enzyme-substrate interaction.

The rate of PNPB hydrolysis, catalyzed by α-CT, 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 ($\varepsilon = 12000\ M^{-1}\ cm^{-1}$). The initial reaction rate V_0 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

Enzymatic Hydrolysis

An interaction between the enzyme and the surfactant and the partitioning of the substrate between the micelle and the external medium plays a significant role to decide enzyme activity [13]. The influence of additives on the catalytic properties of enzymes strongly depends upon the additive structure, i.e. the charge and the size of the head group and also on the nature of the substrate [14, 15]. As known the superactivity of α-CT correlates with surfactant hydrophobicity [16]. In pure buffer hydrolysis rate enhances with the increase of substrate amino acidic residue, owing to the marked raise of the rate constant for the formation of the acyl enzyme. The dependence of superactivity on the substrate could be attributed to hydrophobic non-specific interaction between additives and the enzymatic subsides next to the catalytic point, which produces a more hydrophobic microenvironment [17].

Substrate Binding to the Micelles

Surfactant aggregates in aqueous solutions are usually depicted as microstructures with the hydrophilic heads and hydrophobic tails oriented towards outer and inner part of the surfactant respectively. Each surfactant aggregate was shaped by three different microenvironments or pseudo-phases: the free water, the bound water and the system core formed by the surfactant core [14, 15]. The distribution of substrate between the bulk water and the micellar aggregate can be determined by thermodynamic equilibrium [12]. The association constant K_s is defined as:

$$S_F + D_N \underset{K_S}{\rightleftharpoons} S_M,$$

where S_F and S_M represent the free and micelle-bound substrate (PNPB), respectively, and D_N is the concentration of micellar surfactant, determined from the difference of the total concentration of surfactant from CMC. The CMC of CTPB, CTBPB, and CTAB were determined at 27°C by conductometric method (Table 1). K_s was calculated using the equation

$$A_{270} = (\varepsilon_w + \varepsilon_M K_s[D_N])/(1+ K_s[D_N]),$$

where A_{274} is the absorbance at 274 nm, and ε_w and ε_M are the absorption coefficients of PNPB in buffer (ε_w 0.78) and in the presence of CTAB, CTPB, and CTBPB ε_M values are 0.88, 1.31, and 0.79, respectively. The association constants of PNPB in different surfactants are summarized in Table 1.

The substrate distribution between the aqueous phase and the micelle interface affects the observed rate of the enzyme-catalyzed reaction. The substrate in the bound water pseudo-phase shows strong affinity in the case of CTPB. Taking into account that the activation behavior indicates that the bound α-CT reacts with the free substrate, higher K_s values lead to lower concentrations of the free substrate in the water pseudo-phase.

Effect of Surfactants

The rate of PNPB hydrolysis, catalyzed by α-CT, was measured in the absence of surfactant and in the presence of CTPB, CTBPB, CTAB at pH 7.75. The data show a high initial rate that readily decays to a steady situation. A similar behavior was observed under all the experimental conditions employed. The effect of CTAB, CTPB, and CTBPB micelles on the catalytic constant k_{cat} and Michaelis constant K_M for PNPB hydrolysis was also studied. In buffer only, we

Table 1. Surfactants CMC and association constants K_s of PNPB between the micelles and the aqueous phase

Surfactant	CMC, 10^{-3} M	K_s, M^{-1}
CTAB	1.0	60.8
CTPB	0.16	154
CTBPB	0.20	22.7

found that α-CT catalyzed hydrolysis of PNPB showed k_{cat} of 0.75×10^{-2} s^{-1} and K_M of 0.53×10^{-4} M (Table 2). With the increasing concentration of CTAB from 0.5 to 2.0 mM, k_{cat} increases from 0.86×10^{-4} to 1.28×10^{-4} s^{-1}, respectively, and upon further increasing CTAB up to 10 mM k_{cat} decreases to 0.49×10^{-4} s^{-1}. On the other hand, the K_M values for CTAB falls from 0.5 to 2.0 mM and further rises up to 10 mM as presented in Table 2.

The same trend was followed in the presence of CTPB and CTBPB, i.e., increment was observed in k_{cat} values from 0.1 to 2.0 mM and further decreases, whereas K_M falls down to 2.0 mM, further increment was indicating that micelle-bound α-CT presents 1.70, 6.22, and 2.85 times higher catalytic efficiency for CTAB, CTPB, and CTBPB, respectively, than the free enzyme. Larger values of k_{cat} denote a higher maximum activity of enzyme. The K_M value represents the affinity of the α-CT for the PNPB. Smaller values of K_M indicate that the enzyme and substrate are tightly bound and form the enzyme–substrate complex more quickly. Larger values of the K_M constant indicate that the components are loosely bound and form the enzyme–substrate complex more slowly.

The data of Fig. 1 indicates that the values of V_0 are strongly dependent on the CTPB concentration. A notable increase in reaction rate with the CTPB concentration up to 2 mM has been observed. When the CTPB concentration increases further (5 mM) V_0 decreases. All the data points obey to Michaelis–Menten kinetics. Kinetic parameters k_{cat} and K_M in absence and presence of surfactants were obtained by the linear regression analysis of Linweaver–Burk plot (Fig. 2a) and Eadie–Hofstee plot (Fig. 2b). The calculated results are presented in Table 2.

Table 2. Effect of head group of cationic surfactants on α-CT catalyzed reaction of PNPB

Surfactant	[Surfactant], mM	k_{cat}, 10^{-2} s^{-1}	K_M, 10^{-4} M	k_{cat}/K_M, M^{-1} s^{-1}	k_{cat}, 10^{-2} s^{-1}*	K_M, 10^{-4} M*	k_{cat}/K_M, M^{-1} s^{-1}*
Aqueous	–	0.75 ± 0.06	0.53 ± 0.13	141	0.79	0.55	143
CTAB	0.5	0.86 ± 0.15	0.60 ± 0.25	143	0.92	0.64	144
	1.0	1.04 ± 0.01	0.41 ± 0.10	253	1.20	0.47	255
	2.0	1.28 ± 0.08	0.35 ± 0.01	365	1.41	0.38	371
	5.0	0.71 ± 0.03	0.43 ± 0.21	165	0.78	0.44	177
	10.0	0.49 ± 0.03	0.51 ± 0.21	96.0	0.56	0.55	101
CTPB	0.1	1.72 ± 0.06	1.16 ± 0.04	148	1.81	1.20	151
	0.5	2.43 ± 0.17	0.98 ± 0.13	247	2.51	1.00	251
	1.0	2.75 ± 0.15	0.84 ± 0.01	327	2.90	0.87	333
	2.0	4.67 ± 0.24	0.80 ± 0.13	583	4.89	0.84	582
	5.0	2.11 ± 0.07	0.91 ± 0.01	231	2.33	1.00	233
CTBPB	0.1	0.93 ± 0.05	0.57 ± 0.12	163	1.09	0.65	167
	0.5	1.66 ± 0.20	0.48 ± 0.18	345	1.72	0.50	344
	1.0	1.99 ± 0.10	0.38 ± 0.07	523	2.19	0.41	534
	2.0	2.14 ± 0.10	0.32 ± 0.06	668	2.39	0.35	682
	5.0	1.98 ± 0.03	0.66 ± 0.06	300	2.24	0.71	315
	10.0	1.75 ± 0.04	0.84 ± 0.08	208	1.85	0.88	210

* Eadie–Hofstee method. [α-CT] = 0.02 mM, [Tris-HCl buffer] = 10 mM, T = 27°C, pH 7.75, [PNPB] = (0.02–0.50) mM.

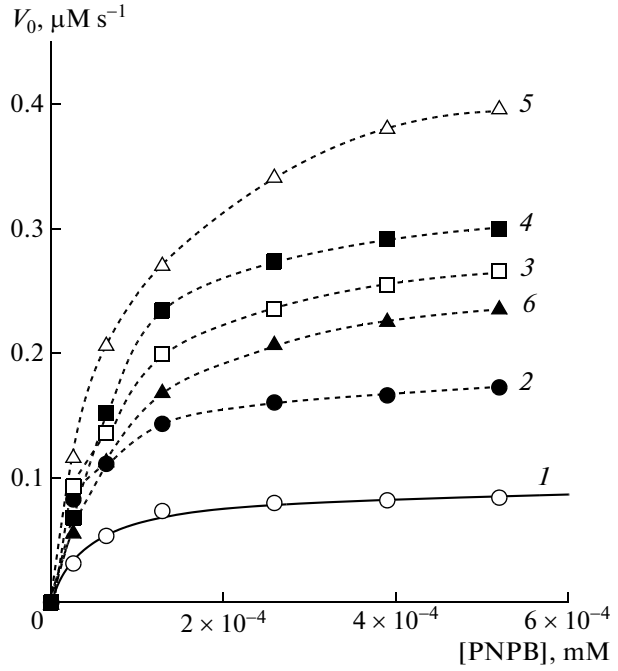


Fig. 1. Effect of CTPB concentration for hydrolysis of PNPB; [CTPB], mM: *1*—0, *2*—0.1, *3*—0.5, *4*—1.0, *5*—2.0, *6*—5.0. [α-CT] = 0.02 mM.

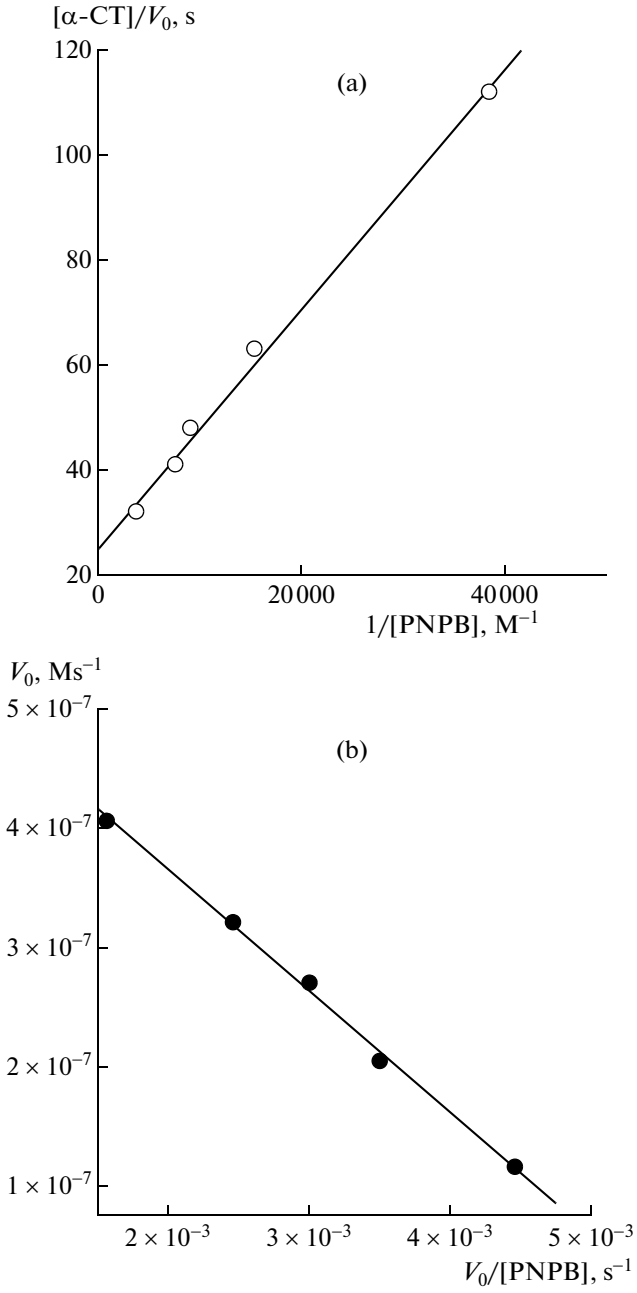


Fig. 2. The Linweaver–Burk plot (a) and Eadie–Hofstee plot (b) for the hydrolysis of PNPB catalyzed by α-CT. (•) 2 mM CTPB. [α-CT] = 0.02 mM, [PNPB] = (0.02–0.50) mM.

The results given in Table 2 support the observation that the catalytic rate of hydrolysis increases with increasing surfactant concentrations up to 2 mM for CTAB, CTPB, and CTBPB and then decreases. A bell-shaped profile is observed. This apparent loss of activity at high surfactant concentrations could be due to a decreased thermodynamic activity of the substrate resulting from its incorporation into the micellar pseudo-phase. The binding of the substrate to the enzyme and the enzyme turnover increases with surfactant concentrations.

For the assessment of superactivity, the ratio of the catalytic rate constant k_{cat} and the Michaelis constant K_M (obtained from Lineweaver–Burk plot) are plotted against surfactant concentration (Fig. 3). As seen in Fig. 3, the catalytic efficiency k_{cat}/K_M increases with surfactant concentration above the CMC in the presence of CTAB, CTPB, and CTBPB. The CTBPB shows higher catalytic reactivity than CTPB and CTAB, and the catalytic activity follows the order CTBPB > CTPB > CTAB. The present results show that the superactivity observed in this series of surfactants above the CMC is due to an increase in k_{cat}. The decrease in activity observed with higher surfactant concentration is due to an increase in the number of micelles in solution and a decrease in the concentration of free substrate [12]. Since the enzyme reacts with free substrate, the unfavorable partition leads to a decrease in activity when the concentration of surfactant is increased. The maximum superactivity increases in the order of changing the surfactant head group indicating that the surfactant structure exerts a larger influence on the CMC than upon its association with the enzyme.

Superactivity of α-CT is observed in the presence of the three surfactants, with maximum catalytic efficiencies occurring above the corresponding critical micelle concentrations. The catalytic efficiency k_{cat}/K_M showed a bell-shaped profile with surfactant concentration. The highest catalytic efficiencies are

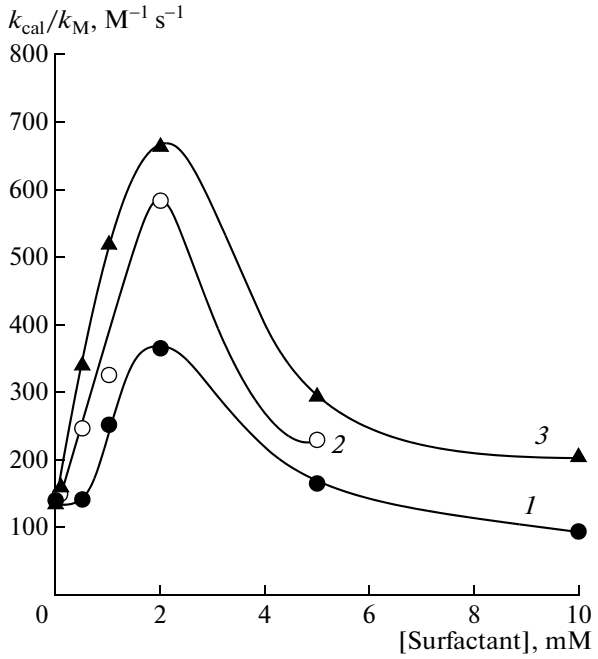


Fig. 3. Effect of surfactant concentration on the k_{cat}/K_M: *1*—CTAB, *2*—CTPB, *3*—CTBPB. [α-CT] = 0.02 mM, [PNPB] = (0.02–0.50) mM.

observed for CTBPB. The effect of the surfactant is interpreted in terms of an interaction between enzyme and the surfactant, which favor the catalytic hydrolysis of the *p*-nitrophenyl benzoate. The micellar binding of the substrate in different surfactants governs the hydrolytic reaction rate.

ACKNOWLEDGMENTS

Financial support of this work by Council of Scientific and Industrial Research, New Delhi (CSIR Ref. no. 01 (2143)/07/EMR-II) is gratefully acknowledged.

REFERENCES

1. Rezaei-Ghaleh, N., Ramshini, H., Ebrahim-Habibi, A., Moosavi-Movahedi, A.A., and Nemat-Gorgani, M., *Biophys. Chem.*, 2008, vol. 132, p. 23.
2. Katz, E. and Willner, I., *Angrew. Chem. Int. Ed. Engl.*, 2004, vol. 43, p. 6042.
3. Banerjee, D. and Pal, S.K., *Langmuir*, 2008, vol. 24, p. 8163.
4. Hedstrom, L., *Chem. Rev.*, 2002, vol. 102, p. 4501.
5. Blow, D.M., Birktoft, J.J., and Hartley, B.S., *Nature*, 1969, vol. 221, p. 337.
6. Banerjee, D. and Pal, S.K., *Langmuir*, 2008, vol. 24, p. 8163.
7. Roy, S. and Dasgupta, A., Das P.K, *Langmuir*, 2006, vol. 22, p. 4567.
8. Mitra, R.N., Dasgupta, A., Das, D., Roy, S., and Debnath, S., Das P.K, *Langmuir*, 2005, vol. 21, p. 12115.
9. Abuin, E., Lissi, E., Biasutti, M.A., and Duarte, R., *Protein J.*, 2007, vol. 26, p. 475.
10. Ghosh, K.K. and Verma, S.K., *Int. J. Chem. Kinet.*, 2009, vol. 41, p. 377.
11. Ghosh, K.K. and Verma, S.K., *Indian J. Biochem. Biophys*, 2008, vol. 45, p. 350.
12. Celej, M.S., D'andea, M.G., Campana, P.T., Fidelio, G.D., and Bianconi, M.L., *Biochem. J.*, 2004, vol. 378, p. 1059.
13. Sarkar, R., Ghosh, M., Shaw, A.K., and Pal, S.K., *J. Photochem. Photobiol., B*, 2005, vol. 79, p. 67.
14. Viparelli, P., Alfani, F., and Cantarella, M., *J. Mol. Catal. B: Enzym.*, 2003, vol. 21, p. 175.
15. Viparelli, P., Alfani, F., and Cantarella, M., *J. Mol. Catal. B: Enzym.*, 2001, vol. 15, p. 1.
16. Spreti, N., Profio, P.D., Marte, L., Bufani, S., Brinchi, L., and Savelli, G., *Eur. J Biochem.*, 2001, vol. 88, p. 6491.
17. Abuin, E., Lissi, E., and Calderon, C., *J. Colloid Interface Sci.*, 2007, vol. 308, p. 573.