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Effect of cationic and non-ionic surfactants on the hydrolysis of *N*-glutaryl-L-phenylalanine catalysed by chymotrypsin iso-enzymes

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This paper is dedicated to the memory of Prof. Francesco Alfani, who suddenly died in November 2001.

Abstract

The hydrolysis of *N*-glutaryl-L-phenylalanine *p*-nitroanilide catalysed by various chymotrypsin (CT) iso-enzymes (α-CT, β-CT, δ-CT, and γ-CT) has been studied in the presence of cationic and non-ionic surfactants at concentration higher than the critical micellar concentration. The enzyme activity was tested in the presence of the following surfactants: cetyltrimethylammonium bromide (CTABr), cetyldimethylethylammonium bromide (CDMEABr), cetyltripropylammonium bromide (CTPABr), Triton X100 (TX100) and polyoxyethylene 9 lauryl ether (PO9). The activity of the iso-enzymes depends on the surfactant concentration and it varies with the surfactant head group dimensions (CTPABr > CDMEABr > CTABr). For all the iso-enzymes, superactivity has been detected only in the presence of CTPABr and CDMEABr. The extent of superactivity depends on the enzyme used (δ-CT > β-CT > γ-CT > α-CT). The observed reaction rate has been compared with the prediction of a theoretical model for enzymatic activity in the presence of surfactant aggregates in aqueous media developed in a previous paper. The results can be explained by introducing an equilibrium relation between the enzyme confined in the free bulk water and in the bound water pseudo-phase, and by allowing for different catalytic behaviours of the two forms of enzyme.

The theoretical model enables the initial reaction rate to be related to the substrate concentration with an overall Michaelis–Menten equation. Good agreement has been found between experimental and model predicted values of the kinetic parameters.
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Keywords: Enzymes; Micelles; Kinetics; Models

1. Introduction

The serine protease chymotrypsin (EC 3.4.21.1) is one of the most studied enzymes. Its structure [1] and mechanism of action are well known: it catalyses the hydrolysis of peptide bonds with an aromatic amino acid as the acyl donor component [2,3]. This enzyme also catalyses the reverse reaction with the same specificity [4]. These catalytic properties are often used at an industrial level, mainly in the pharmaceutical sector.

On the other hand, as the enzyme is commercially available in a pure form and also because of the large amount of work already done with this protease, it is also been chosen as a model enzyme in many studies [5–9].

α-CT retains its activity in numerous systems from buffered media to organic solvent media [10,11],

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Nomenclature

CMC	critical micellar concentration (M)
$[D_N]$	concentration of the surfactant present as aggregates (M)
$[E_0]$	total enzyme concentration (M)
$[E_b]$	enzyme concentration in the bound water pseudo-phase (M)
$[E_w]$	enzyme concentration in the free bulk water pseudo-phase (M)
$(H_2O)_x$	hydration number (M)
K_E	equilibrium constant of the association between the enzyme and the surfactant (M^{-1})
K_S	equilibrium constant of the association between the substrate and the surfactant (M^{-1})
$k_{CAT}^{b,w}$	turnover number for the hydrolysis of S_b catalyzed by E_w (s^{-1})
$K_M^{b,w}$	Michaelis–Menten constant for the hydrolysis of S_w catalyzed by E_b (M)
$k_{CAT}^{w,w}$	turnover number for the hydrolysis of S_w catalyzed by E_w (s^{-1})
$K_M^{w,w}$	Michaelis–Menten constant for the hydrolysis of S_w catalyzed by E_w (M)
k_{CAT}^{0}	turnover number for the hydrolysis in pure buffer (s^{-1})
K_M^{0}	Michaelis–Menten constant for the hydrolysis in pure buffer (M)
k_{CAT}^{OV}	overall turnover number for the hydrolysis in the presence of surfactant (s^{-1})
K_M^{OV}	overall Michaelis–Menten constant for the hydrolysis in the presence of surfactant (M)
$P_{b,s}$	partition coefficient of the equilibrium between the substrate in the surfactant core and in the bound water pseudo-phases (dimensionless)
r	hydrolysis reaction rate ($\mu mol\,l^{-1}\,s^{-1}$)
r_0	hydrolysis reaction rate in pure buffer ($\mu mol\,l^{-1}\,s^{-1}$)
r_{CMC}	hydrolysis reaction rate at the critical micellar concentration ($\mu mol\,l^{-1}\,s^{-1}$)
$[S_0]$	total substrate concentration (M)
$[S_b]$	substrate concentration in the bound water pseudo-phase (M)
$[S_s]$	substrate concentration in the surfactant core pseudo-phase (M)
$[S_w]$	substrate concentration in the free bulk water pseudo-phase (M)
[TS]	total surfactant concentration (M)
V_{max}	maximal reaction rate ($\mu mol\,l^{-1}\,s^{-1}$)

Greek letter

ε_{410}	pNA extinction coefficient at 410 nm ($M^{-1}\,cm^{-1}$)

reverse micelles [5–7,9,12–14] and nearly anhydrous organic systems [15]. The enzymatic activity can be either reduced [13,14] or remains equal or higher than in aqueous media [9,14]. In these systems, a Michaelis–Menten-type kinetic relation can be applied to describe substrate dependence of the reaction rate and the kinetic parameters are generally comparable with those in aqueous solutions [9]. Several models have been developed for the mechanism of enzyme-catalysed reactions in reverse micelles [16–18].

Enzyme superactivity has been detected also in purely water/surfactant media owing to positive interactions between the enzyme and the surfactant [8,9,19,20].

This considerable result could find interesting applications in the development of processes in continuous reactors. Moreover, new supports suitable for enzyme immobilization could be projected with properties similar to those of the surfactant aggregates giving rise to superactivity.

However, in spite of this theoretical interest, the modelling of enzyme kinetics in aqueous solutions enriched with surfactants received little efforts. Therefore, it was the purpose of an our previous study [21]. The pseudo-phase approach was adopted to describe the kinetic behaviour of the enzyme at surfactant concentrations higher than the critical micellar concentration (CMC). The model showed how the presence of direct micelles can lead to superactivity in enzyme reactions. From a comparison of the numerical results with α-CT activity in aqueous solutions of buffer and two surfactants, cetyltributylammonium bromide (CTBABr) or cetyltrimethylammonium bromide

(CTABr), it appears that (1) the pseudo-phase approach can be applied to the problem, (2) the surfactant concentration in direct micelle systems can play a similar role to the molar ratio of water to surfactant in reverse micelle systems, and (3) the enzyme must interact with both the micelles and the surfactant in the monomeric form in order to explain the evidence of an optimum surfactant concentration in certain cases.

A recent study [22] showed that cationic and non-ionic surfactants can stimulate or depress α-CT activity. The addition of CTABr, polyoxyethylene 9 lauryl ether (PO9) and TX100 resulted in a monotonically decrease of the rate of hydrolysis of two different substrates, *N*-glutaryl-L-phenylalanine *p*-nitroanilide (GpNA) and *N*-succinyl-L-phenylalanine *p*-nitroanilide (SpNA). In contrast, CTPABr stimulated the rate of hydrolysis of both the substrates up to 2–2.5-fold. The experimental data confirmed that the observed behaviours can be depicted with good accuracy by the aforementioned model [21] and that the model can give indications of enzyme partition between the bulk of the solution and the surfactant aggregates as well as information on the efficiency of the two-enzyme forms.

The purpose of the present work is to extend our previous investigation to other enzymes characterised by similar structure and mode of action. The kinetic behaviour of α-CT was then compared with that of three iso-enzymes, β-CT, δ-CT, and γ-CT. Chymotrypsin iso-enzymes were studied in the presence of aggregates of non-ionic and cationic surfactants in water on the basis that the iso-enzymes can differently interact with the surfactant aggregates. To meet this end different cationic and non-ionic surfactants were employed. The rate of GpNA hydrolysis was measured at constant temperature and pH. The effect of the surfactant and substrate concentration was investigated in a wide range.

The ability of the theoretical model previously developed in [21] to predict the experimental data obtained with the chymotrypsin iso-enzymes was also tested. This model was already found to simulate with good accuracy the hydrolysis of GpNA catalysed by α-CT. In this paper, the consistency of physical–chemical and kinetic parameters estimated by fitting procedures with the model assumptions was tested and the modelling results were used to compare the catalytic behaviour of the different iso-enzymes.

2. Materials and methods

Chymotrypsin iso-enzymes (EC 3.4.21.1): α-chymotrypsin (type II, 3× crystallised and lyophilised powder), β-chymotrypsin (crystallised and lyophilised powder), γ-chymotrypsin (type II, 2× crystallised and lyophilised powder) and δ-chymotrypsin from bovine pancreas, essentially salt free were supplied from Sigma (USA) and used without further purification. The substrate, *N*-glutaryl-L-phenylalanine *p*-nitroanilide was supplied by Sigma. Enzyme and substrate solutions were always freshly prepared in the appropriate buffer immediately before their use in experiments. The buffer chemicals, Tris(hydroxymethyl) aminomethane (Tris) (pKa 8.3) and hydrochloric acid were from Aldrich (Germany) and Carlo Erba (Italy), respectively. The commercial grade non-ionic surfactants *t*-octyl phenoxy polyethoxy ethanol (TX100) and polyoxyethylene 9 lauryl ether (PO9) were from Sigma.

Cetyltrimethylammonium bromide (CTABr) and cetyldimethylethylammonium bromide (CDMEABr) were from Fluka (Germany). All the commercial surfactants were used without further purification. Cetyltripropylammonium bromide (CTPABr) was part of a stock kindly supplied by Prof. Gianfranco Savelli (University of Perugia). The preparation and purification at laboratory scale of this synthesised surfactant are fully described in [23]. The critical micellar concentration in water of all the surfactants is reported in Table 1.

2.1. Assay of enzyme activity

The GpNA hydrolysis catalysed by the chymotrypsin iso-enzymes was monitored by following the change in absorbance at 410 nm due to the forma-

Table 1
Critical micellar concentration

Surfactant	CMC[a] (M)
CTABr	8.7×10^{-4}
CDMEABr	8.3×10^{-4}
CTPABr	5.5×10^{-4}
PO9	3.0×10^{-4}
TX100	2.5×10^{-4}

[a] In water.

tion of *p*-nitroaniline (pNA). Kinetic determinations were performed at 25 °C in 0.1 M Tris–HCl buffer, pH 7.75, using a Perkin-Elmer Lambda 2 UV-Vis spectrophotometer equipped with thermostated cell holders controlled within ± 0.1 °C. GpNA hydrolysis was carried out in 3 ml cuvettes, 1 cm pathlength, filled with surfactant and substrate solutions (both prepared with buffer) to a final volume of 2.94 ml. The reaction was initiated by addition of 0.06 ml of the enzyme buffered stock solution (10 mg ml^{-1}) to result in a concentration of 8 μM (0.2 mg ml^{-1}). The product extinction coefficient, ε_{410}, was found to be 10,015 M^{-1} cm^{-1} both in pure buffer and in the presence of surfactants, in the whole investigated range of surfactant concentration. No auto hydrolysis of the substrate was observed in the absence of enzyme in the cuvette at all the explored experimental conditions during the period of the experiments (initial 10 min). The pNA formation during the adopted period of hydrolysis was linearly time dependent as predicted by differential reactor theory. The initial reaction rate, *r*, moles of pNA formed per litre (l) and second, was calculated by the slope of changes in absorbance versus time records. The enzymatic activity was also evaluated as turnover number, k_{CAT} (s^{-1}), moles of GpNA transformed per second and per mole of enzyme. The maximal velocity, V_{max}, and the Michaelis–Menten constant, K_M, in the presence of buffer and/or surfactant were determined from linear regression analysis of the double reciprocal Lineweaver–Burk plot. All the experiments were performed at least in duplicate and each data point represents the average value of a set of results. The discrepancy in repeated experiments was always less than 5%.

2.2. Model equations

Under the experimental conditions of this study, carried out at concentrations higher than the CMC, the model, fully detailed in [21], assumes that the surfactant aggregates are in the realm of small aggregates with an aggregation number of roughly 100/200 molecules [24]. The number of aggregates is an increasing function of the surfactant concentration, while the aggregate size remains almost unchanged [21,22,24,25]. Surfactant aggregates are depicted as micro-structures with the hydrophilic heads of the surfactant oriented towards the water molecules and the hydrophobic tails oriented towards the inner part of the structure. The aggregates appear as the dominant form above the CMC, but free surfactant is also present in the system as monomers or small not organised assemblies.

Each surfactant aggregate in the buffered medium is shaped by three pseudo-phases: free bulk water, bound water and surfactant core. Thermodynamic equilibrium is assumed for both substrate and enzyme distribution between the free water (subscript w) and the whole aggregates (m). The substrate can partition into three pseudo-phases, free water, bound water (b) and surfactant core (s). In contrast, the enzyme partition occurs only between the free and the bound water pseudo-phases, the enzyme molecular dimensions being of the same order of magnitude as the surfactant aggregates:

$$[S_w] + [D_N] \overset{K_S}{\rightleftharpoons} [S_m] \quad (1)$$

$$[E_w] + [D_N] \overset{K_E}{\rightleftharpoons} [E_b] \quad (2)$$

where $[D_N] = [TS] - CMC$ is the concentration of the surfactant present as aggregates, $[S_m] = [S_b] + [S_s]$ is the concentration of the substrate associated to the surfactant aggregates, K_S and K_E are association constants. Values for K_S can be experimentally determined as shown in [20]. A further equilibrium between the substrate in the bound water and that associated with surfactant is then introduced.

$$[S_b] \overset{P_{b,s}}{\rightleftharpoons} [S_s] \quad (3)$$

$P_{b,s}$, an unknown parameter of the model, is the coefficient of substrate partition between these two pseudo-phases. Finally, the overall mass balance for substrate and enzyme can be written:

$$[S_0] = [S_w] + [S_b] + [S_s] \quad (4)$$

$$[E_0] = [E_w] + [E_b] \quad (5)$$

where $[S_0]$ and $[E_0]$ are the total substrate and enzyme concentration.

The model makes the hypothesis that the enzyme kinetic parameters depend on enzyme location in the system. The uncertainty about the geometry of the aggregate is not a limitation since the model only needs the assumption of interactions of both substrate and enzyme with the surfactant molecules, present either

as aggregates or as free molecules at the equilibrium concentration (CMC), that modulate the kinetic and catalytic behaviour of the system.

The number of adjustable parameters in the model may be reduced on the basis of the following considerations. In the case of the cationic surfactants, their strong affinity for the substrate allows one to assume that $P_{b,s} \gg 1$ and $[S_b] \approx 0$. Moreover, it would appear reasonable to further assume that the substrate associated with the surfactant core, S_s, cannot participate in the reaction. If the active site of the enzyme is hypothesised to be accessible to the substrate molecules in each pseudo-phase, the reaction rate is the sum of two contributions:

$$r = \frac{k_{CAT}^{w,w} \frac{[E_0]}{1+K_E[D_N]} \frac{[S_0]}{1+K_S[D_N]}}{K_M^{w,w} + \frac{[S_0]}{1+K_S[D_N]}} + \frac{k_{CAT}^{b,w} \frac{K_E[D_N][E_0]}{1+K_E[D_N]} \frac{[S_0]}{1+K_S[D_N]}}{K_M^{b,w} + \frac{[S_0]}{1+K_S[D_N]}} \quad (6)$$

where the first and the second superscripts of the kinetic parameters refer to the pseudo-phase for the enzyme and the substrate, respectively. In the case of the non-ionic surfactants, the reaction rate can be still expressed as in Eq. (6) with $K_S \approx 0$. In fact, previous experiments reported in [22] showed that no association occurs between the substrate and the non-ionic surfactants, so that $[S_w] \approx [S_0]$.

3. Results and discussion

At first, a characterization of the four iso-enzymes in pure buffer system was performed, investigating GpNA concentration from 0.2 to 2.5 mM. The reaction was carried out at 25 °C in 0.1 M Tris–HCl buffer, pH 7.75, using an enzyme concentration of 0.2 mg ml^{-1}. The formation of the product, pNA, was spectrophotometrically followed for 5 min. From these data, the hydrolysis rate, r_0 (μmol l^{-1} s^{-1}), was evaluated. The kinetic parameters, k_{CAT}^0 (s^{-1}) and K_M^0 (M), were evaluated from the double reciprocal linear plot, known as Lineweaver–Burk plot. Values for r_0, k_{CAT}^0, and K_M^0 are shown in Table 2.

Under the experimental conditions of this study, r_0, k_{CAT}^0 and K_M^0 vary in the decreasing series α-CT > β-CT > γ-CT > δ-CT. The enzyme second-order kinetic parameter, k_{CAT}^0/K_M^0, is also reported in Table 2.

3.1. Effect of surfactant concentration on the reaction rate

In a second set of experiments, the initial reaction rate of GpNA hydrolysis catalysed by the four iso-enzymes was measured in buffer and in the presence of a fixed surfactant concentration. The hydrolysis conditions were similar to those currently adopted in the literature: substrate concentration (2.5×10^{-3} M) was higher than the saturation concentration in pure buffer, and surfactant concentration (1×10^{-3}/0.1 M) was well above the CMC.

Results of a comparison of the hydrolysis rates in the presence of surfactant aggregates, r, and in pure buffer, r_0, are shown in Table 3. Because in all the systems prepared with the enzymes and the surfactants the rate of GpNA hydrolysis in pure buffer was equal to the reaction rate at a surfactant concentration approximately equal to the CMC, r_{CMC}, the values of Table 3 provide also a comparison of the hydrolysis rate at saturating surfactant concentration (CMC) and in the presence of a surfactant excess.

The activity of all the iso-enzymes was lower with the non-ionic surfactants than in pure buffer. These

Table 2
Initial reaction rate and Michaelis–Menten parameters for the hydrolysis of GpNA catalysed by chymotrypsin iso-enzymes in pure buffer system

Enzyme	r_0[a] (μmol l^{-1} s^{-1})	k_{CAT}^0 (s^{-1})	K_M^0 ($\times 10^{-3}$ M)	k_{CAT}^0/K_M^0 (mol^{-1} l s^{-1})
α-Chymotrypsin	0.103	0.0160	0.61	26.2
β-Chymotrypsin	0.097	0.0147	0.52	28.3
δ-Chymotrypsin	0.083	0.0118	0.34	34.7
γ-Chymotrypsin	0.093	0.0133	0.36	36.9

$[E_0] = 8$ μM, [buffer] = 0.1 M, $T = 25$ °C, pH = 7.75, $[S_0] = 0.2$–2.5 mM.
[a] r_0 was measured at $[S_0] = 2.5$ mM.

Table 3
Effect of various surfactant on the activity of chymotrypsin iso-enzymes

Enzyme	Surfactant	r/r_0		
		[TS] = 0.001 M	[TS] = 0.005 M	[TS] = 0.1 M
α-CT	CTABr	0.96	0.48	0.03
	CDMEABr	1.13	0.87	0.11
	CTPABr	1.96	2.37	0.15
	PO9	0.96	0.88	0.29
	TX100	0.94	0.85	0.25
β-CT	CTABr	0.86	0.62	0.06
	CDMEABr	0.98	0.82	0.08
	CTPABr	3.3	2.8	0.22
	PO9	0.84	0.70	0.14
	TX100	0.96	0.83	0.22
δ-CT	CTABr	0.93	0.75	0.04
	CDMEABr	0.91	0.68	0.09
	CTPABr	3.10	4.45	0.46
	PO9	0.98	0.68	0.12
	TX100	0.98	0.83	0.23
γ-CT	CTABr	0.95	0.69	0.09
	CDMEABr	0.98	0.80	0.09
	CTPABr	2.70	2.80	0.31
	PO9	0.86	0.70	0.13
	TX100	0.94	0.83	0.17

$[E_0] = 8\,\mu M$, $[S_0] = 2.5\,mM$, [buffer] = 0.1 M, $T = 25\,^{\circ}C$, pH = 7.75.

surfactants lead to a considerable reduction in enzyme activity that ranges from 4 to 75% for α-CT and from 2 to 88% for δ-CT.

More interesting was the effect of the cationic surfactant aggregates on the reaction rate, as compared with the values in pure buffer. CTABr was found to give rise to a loss of activity with all of the iso-enzymes (4–97%) compared with that observed in pure buffer. CTPABr always leads to a significant increase of enzyme activity, from 2.37 to 4.45 times the value in pure buffer. In the presence of CDMEABr aggregates, increase and reduction in activity were found to depend on the iso-enzyme. Inspection of Table 3 shows conclusively that, in the presence of aggregates of all the tested surfactants, the activity of both α-CT and the three iso-enzymes depends markedly on the surfactant concentration.

As both CTPABr and CDMEABr were found to induce a certain level of superactivity of the four iso-enzymes, a more in depth study was performed investigating the effect of the surfactant concentration and the results are shown in Figs. 1 and 2.

The ratio of substrate hydrolysis rate in aqueous solutions of CTPABr and CDMEABr to those in buffer, (r/r_0), is plotted versus the total surfactant concentration, [TS]. Bell-shaped curves of (r/r_0) versus [TS] were obtained, clearly indicating the dependence of superactivity on the surfactant concentration.

The optimum CTPABr concentration to be used in the system ranges between 2 and 3 mM. The highest (r/r_0) ratio reached with δ-CT was 4.8 ($r = 0.45\,\mu mol\,l^{-1}\,s^{-1}$), 3.2 with β-CT ($r = 0.31\,\mu mol\,l^{-1}\,s^{-1}$), 3.1 with γ-CT ($r = 0.26\,\mu mol\,l^{-1}\,s^{-1}$) and 2.6 with α-CT ($r = 0.27\,\mu mol\,l^{-1}\,s^{-1}$). For all CTPABr concentrations, the superactivity exhibited by δ-CT is higher than that reached by the other enzymes.

The addition of CDMEABr causes a slight superactivity only with of α-CT and δ-CT, while determines a monotonically decrease in β-CT and γ-CT activity. Enzyme superactivity still reached the highest value with δ-CT.

For all the enzymes, the addition of CTABr and of the non-ionic surfactants leads to a monotonically decrease of the activity as compared to the value in pure buffer (data not shown).

3.2. Results of model predictions

The theoretical model introduced in [21] and described in Section 2.2 is able to predict with reasonable accuracy the experimental results of Figs. 1 and 2. The solid lines in these figures were calculated using Eq. (6) and the adjustable parameters values obtained by regression of the experimental data quoted in Table 4.

An important result is that, under our experimental conditions, the model successfully predicts the enzyme activity in aqueous solutions of surfactant aggregates through the estimation of only three adjustable parameters: $k_{CAT}^{b,w}$, $K_M^{b,w}$ (kinetic parameters of the reaction between the enzyme confined in the bound water and the substrate confined in the free water) and K_E. In fact, $k_{CAT}^{w,w}$ and $K_M^{w,w}$ can be determined from kinetic tests at surfactant concentration equal to the CMC. Since, under the experimental conditions of this study, reaction rates at the CMC were the same as in pure buffer, in the model it was assumed for the enzymatic hydrolysis in the free water

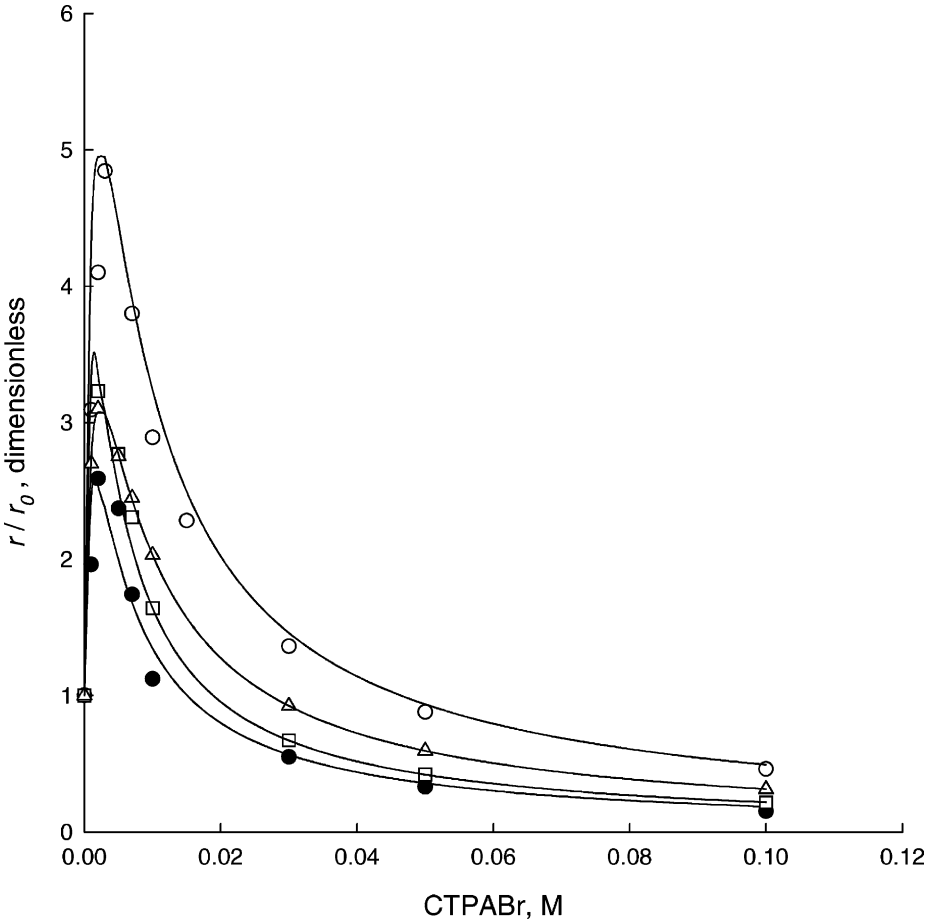

Fig. 1. Activity of α-CT (●), β-CT (□), γ-CT (△) and δ-CT (○) as a function of CTPABr concentration. $[E_0] = 8\,\mu M$, $[S_0] = 2.5\,mM$, [buffer] = 0.1 M, $T = 25\,^{\circ}C$, pH = 7.75 (points: experimental data; solid lines: model predictions).

pseudo-phase: $k_{CAT}^{w,w} = k_{CAT}^{0}$ and $K_M^{w,w} = K_M^0$, which are the kinetic parameters for GpNA hydrolysis in pure buffer (see Table 2). Moreover, the values for K_S quoted in Table 4 were experimentally measured following the method reported in [20].

Inspection of Table 4 leads to the following conclusions. The partition constant of all the iso-enzymes, K_E, is generally much lower with non-ionic surfactants than with cationic surfactants. This implies that electrostatic interactions between the enzyme and the surfactant aggregates play the most important role. However, the small partition constant with the non-ionic surfactants suggests that hydrophobic interactions of the enzyme with the aggregates should also have a role to play.

Being the average value of the association constant between enzyme and cationic surfactant aggregates approximately equal to $0.4\,mM^{-1}$, roughly 50% of the total enzyme is located in the bound water. From Figs. 1 and 2, it can be seen that the rate of GpNA hydrolysis reaches the highest value at a surfactant concentration of the order of magnitude of 2.5 mM. Under the hypothesis that the dominant form is small aggregates, with an aggregation number of roughly 150 molecules, it can be deduced that the chymotrypsin molecule must simultaneously interact with four surfactant aggregates to show the highest superactivity. Lower values of the ratio between the number of surfactant aggregates and the number of enzyme molecules lead to a lower superactivity,

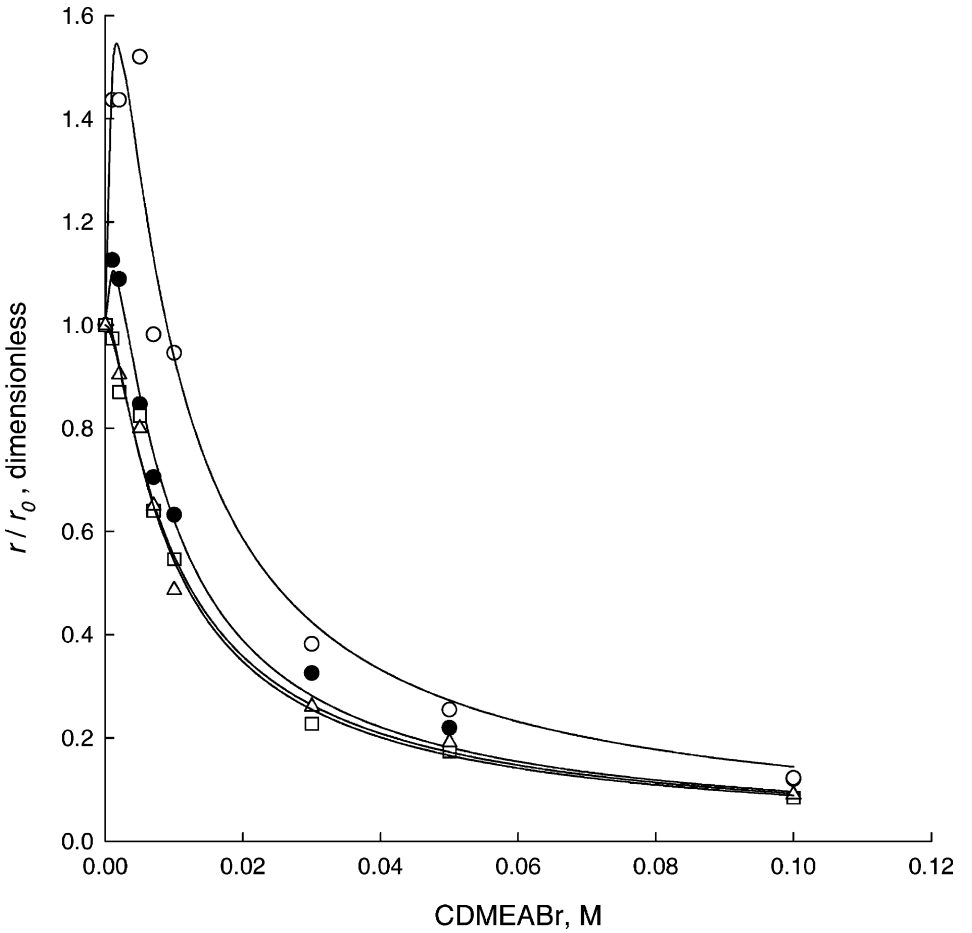

Fig. 2. Activity of α-CT (●), β-CT (□), γ-CT (△) and δ-CT (○) as a function of CDMEABr concentration. $[E_0] = 8\,\mu M$, $[S_0] = 2.5\,mM$, [buffer] = 0.1 M, $T = 25\,^{\circ}C$, pH = 7.75 (points: experimental data; solid lines: model predictions).

while higher ratio can even depress enzyme activity as compared to the value in pure buffer.

Comparison of the kinetic parameters for GpNA hydrolysis in the presence of surfactant aggregates and in pure buffer makes an important point: $K_M^{b,w}$ values for the enzyme in the bound water are approximately equal to the values in pure buffer. By contrast, in the presence of surfactant aggregates, the turn over number of the enzyme in the bound water, $k_{CAT}^{b,w}$, was found to depend markedly on the surfactant and the enzyme. Consequently, the second-order kinetic parameter of the enzymes in the bound water pseudo-phase is either much lower (for PO9 and TX100) or higher (for cationic surfactants) than in pure buffer. In the presence of cationic surfactant aggregates, the higher the number of carbon atoms in the surfactant polar head, the higher $k_{CAT}^{b,w}$.

In Fig. 3 the model predicted kinetic parameters of the enzyme confined in the bound water, $k_{CAT}^{b,w}$ and $K_M^{b,w}$, were plotted versus the hydration number of the surfactant aggregate, $(H_2O)_x$. This was defined by Soldi et al. as the molarity of the water that hydrates the anion, Br^-, in the interfacial region (bound water) of the surfactant aggregate [26]. In Fig. 3, the diagrams of $k_{CAT}^{b,w}$ and $K_M^{b,w}$ refer to α-CT and δ-CT. For both iso-enzymes the increase of the hydration number of the surfactant aggregates leads to a monotonically decrease of $k_{CAT}^{b,w}$ and $K_M^{b,w}$ values. This

Table 4
Results of modelling

Enzyme	Surfactant	K_S (M^{-1})	K_E (M^{-1})	$k_{CAT}^{b,w}$ (s^{-1})	$K_M^{b,w}$ ($\times 10^{-3}$ M)	$k_{CAT}^{b,w}/K_M^{b,w}$ ($mol^{-1}\,l\,s^{-1}$)
α-CT	CTABr	2000	383	0.018	0.43	41.9
	CDMEABr	1504	370	0.039	0.49	79.6
	CTPABr	2160	391	0.16	0.61	262.3
	PO9	–	40.3	0.0014	0.61	2.3
	TX100	–	37.8	0.00091	0.60	1.5
β-CT	CTABr	2000	292	0.024	0.39	61.2
	CDMEABr	1504	410	0.022	0.31	71.0
	CTPABr	2160	340	0.21	0.88	238.6
	PO9	–	78	0.00024	0.6	0.4
	TX100	–	40	0.00097	0.61	1.6
δ-CT	CTABr	2000	306	0.020	0.30	66.7
	CDMEABr	1504	450	0.044	0.46	95.0
	CTPABr	2160	409	0.21	0.44	477.3
	PO9	–	78	0.00019	0.33	0.6
	TX100	–	46.8	0.00076	0.33	2.3
γ-CT	CTABr	2000	351	0.021	0.28	75.0
	CDMEABr	1504	348	0.021	0.32	65.6
	CTPABr	2160	390	0.13	0.40	325.0
	PO9	–	72	0.00063	0.36	1.7
	TX100	–	56.4	0.0010	0.36	2.8

result confirms that hydrophobic interactions between the enzyme and the aggregate also play an important role: higher hydrophobicity—lower $(H_2O)_x$—rises the catalytic activity but reduces the affinity between the enzyme and the substrate.

3.3. Effect of the substrate concentration

Other part of this study aimed to investigate the dependence of the enzyme activity on the substrate concentration. The kinetic investigation was restricted to α-CT and δ-CT that showed a catalytic behaviour significantly different in the presence of CTPABr.

The specific rate of GpNA hydrolysis was measured at three surfactant concentrations (1, 5 and 20 mM) and in the interval of substrate concentration from 0.2 to 2.5 mM.

In all the tested experimental conditions, the reaction rate, r, followed an overall Michaelis–Menten-type behaviour:

$$r = \frac{k_{CAT}^{OV}[E_0][S_0]}{K_M^{OV} + [S_0]} \quad (7)$$

The term overall indicates that the value of the reaction rate depends on the activity of the enzyme in the whole system (both the free and the bound water pseudo-phases). The overall kinetic parameters, k_{CAT}^{OV} and K_M^{OV}, were determined from a Lineweaver–Burk regression of the experimental data. The overall second-order kinetic parameter of the enzyme, k_{CAT}^{OV}/K_M^{OV} was also calculated. Values for these parameters are quoted in Table 5.

In the presence of PO9 and TX100, an increase of the surfactant concentration leads to a decrease of the turnover number of both iso-enzymes. The Michaelis–Menten constant remains almost unchanged. Therefore, the overall enzyme second-order kinetic parameter, k_{CAT}^{OV}/K_M^{OV}, is lower than in pure buffer.

In reactions carried out in the presence of the cationic surfactants, K_M^{OV} is always higher than in pure buffer and increases with the surfactant concentration. The k_{CAT}^{OV} is higher than in pure buffer with the only exception of CTABr. The higher the surfactant concentration, the higher the turn-over number.

For a better comparison of the results among the different surfactants and the pure buffer, it should be

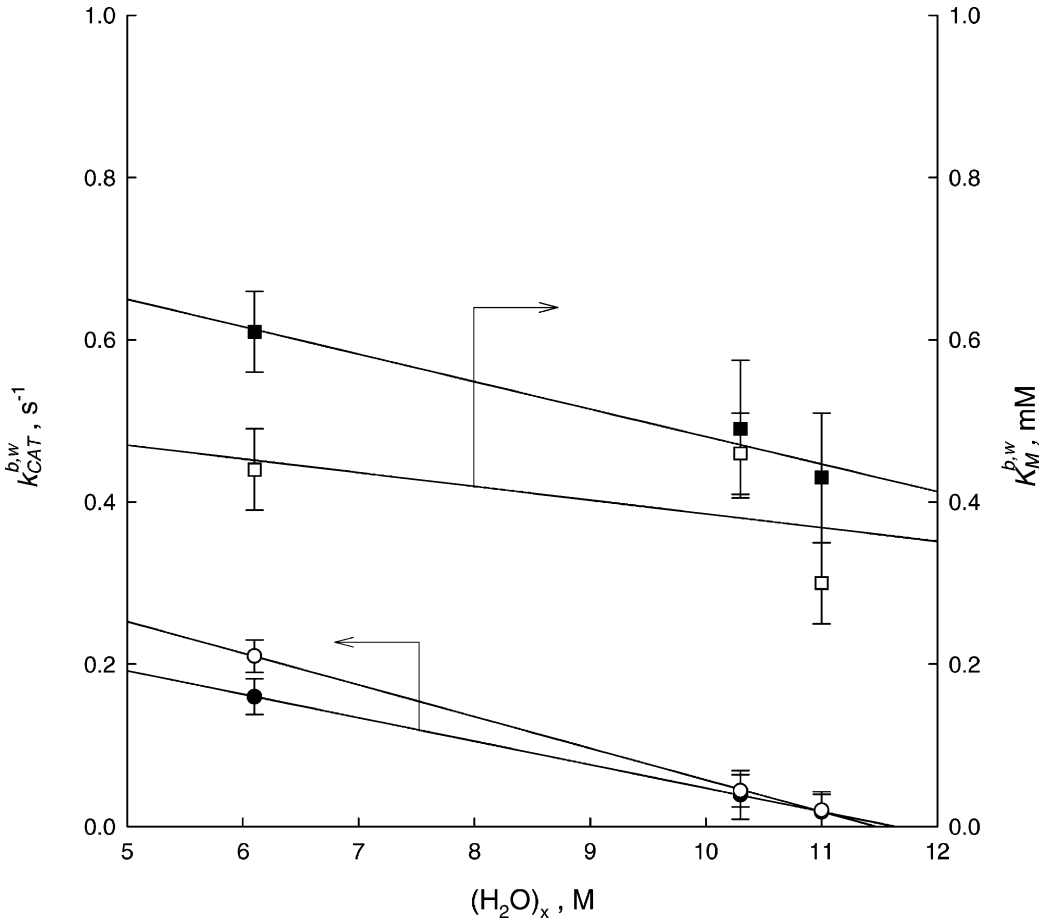

Fig. 3. Estimated values of (○, ●) $k^{b,w}_{CAT}$ and (□, ■) $K^{b,w}_{M}$ as a function of the degree of hydration of the polar head group of cetyltrialkylammonium bromide $(H_2O)_x = 11$, 10.3, and 6.1 M for CTABr, CDMEABr, and CTPABr, respectively. Open symbols: δ-CT; full symbols: α-CT.

clarified the importance of the substrate partition. Data of Table 4 show that $K_S \approx 0$ with the non-ionic surfactants. The substrate is totally available in the free water pseudo-phase, and the rate of hydrolysis is experimentally determined at the effective substrate concentration in the system (S_0). A direct comparison with the reaction in pure buffer is easy.

By contrast, most of the substrate is associated to the surfactant aggregates ($K_S \gg 1$, $P_{b,s} \gg 1$) when the hydrolysis is carried out in the presence of cationic surfactants. The concentration of the substrate available for the bioconversion, S_w, largely differs from the nominal substrate concentration, [S_0]. The values of the kinetic parameters are therefore apparent. The increase of K^{OV}_M with the surfactant concentration can be attributed to the corresponding decrease of [S_w].

The second-order kinetic parameter, k^{OV}_{CAT}/K^{OV}_M, decreases when the cationic surfactant concentration increases in the system.

The theoretical model previously introduced was also tested to predict values of k^{OV}_{CAT} and K^{OV}_M. In order to obtain these predictions, the values of K_E, $k^{b,w}_{CAT}$, and $K^{b,w}_M$, previously determined from simulation of experiments at constant substrate concentration (2.5 mM) with different surfactant concentrations (Table 4) and the kinetic parameters for GpNA hydrolysis in pure buffer (Table 2) were put in Eq. (6) to calculate the theoretical value of r at the various

Table 5
Results of Lineweaver–Burk regression

[TS] (M)	Surfactant	α-CT					δ-CT				
		k_{CAT}^{OV} (s^{-1})		K_M^{OV} ($\times 10^{-3}$ M)		k_{CAT}^{OV}/K_M^{OV} ($mol^{-1}\,l\,s^{-1}$)	k_{CAT}^{OV} (s^{-1})		K_M^{OV} ($\times 10^{-3}$ M)		k_{CAT}^{OV}/K_M^{OV} ($mol^{-1}\,l\,s^{-1}$)
		Measured	Estimated	Measured	Estimated		Measured	Estimated	Measured	Estimated	
–	None	0.016		0.61		26.2	0.0118		0.34		34.7
0.001	CTABr	0.016	0.016	1.64	1.61	9.8	0.014	0.015	0.98	1.03	13.9
	CDMEABr	0.023	0.022	1.38	1.36	16.7	0.021	0.022	0.99	1.01	21.3
	CTPABr	0.057	0.056	1.96	1.93	29.1	0.071	0.069	1.38	1.34	51.4
	PO9	0.016	0.015	0.66	0.61	24.2	0.011	0.011	0.34	0.34	32.4
	TX100	0.016	0.015	0.65	0.61	24.6	0.011	0.011	0.35	0.35	31.4
0.005	CTABr	0.018	0.017	5.17	5.13	3.5	0.016	0.018	3.41	3.49	4.8
	CDMEABr	0.031	0.031	4.40	4.31	7.0	0.039	0.045	3.40	3.80	8.7
	CTPABr	0.111	0.111	7.28	7.20	15.2	0.16	0.14	5.54	5.14	28.9
	PO9	0.014	0.014	0.62	0.61	22.6	0.0089	0.0085	0.35	0.34	25.4
	TX100	0.014	0.014	0.65	0.61	21.5	0.0097	0.0097	0.35	0.34	27.7
0.02	CTABr	0.018	0.018	18.11	18.04	1.0	0.018	0.021	12.4	13.00	1.5
	CDMEABr	0.036	0.036	15.40	15.34	2.3	0.055	0.041	19.0	14.30	2.9
	CTPABr	0.144	0.144	27.01	26.96	5.3	0.24	0.19	25.08	19.40	9.6
	PO9	0.010	0.0095	0.66	0.61	15.2	0.0047	0.0047	0.33	0.34	14.2
	TX100	0.010	0.0095	0.64	0.61	15.6	0.0064	0.0065	0.34	0.34	18.8

$[E_0] = 8\,\mu M$, [buffer] = 0.1 M, $T = 25\,^{\circ}C$, pH = 7.75.

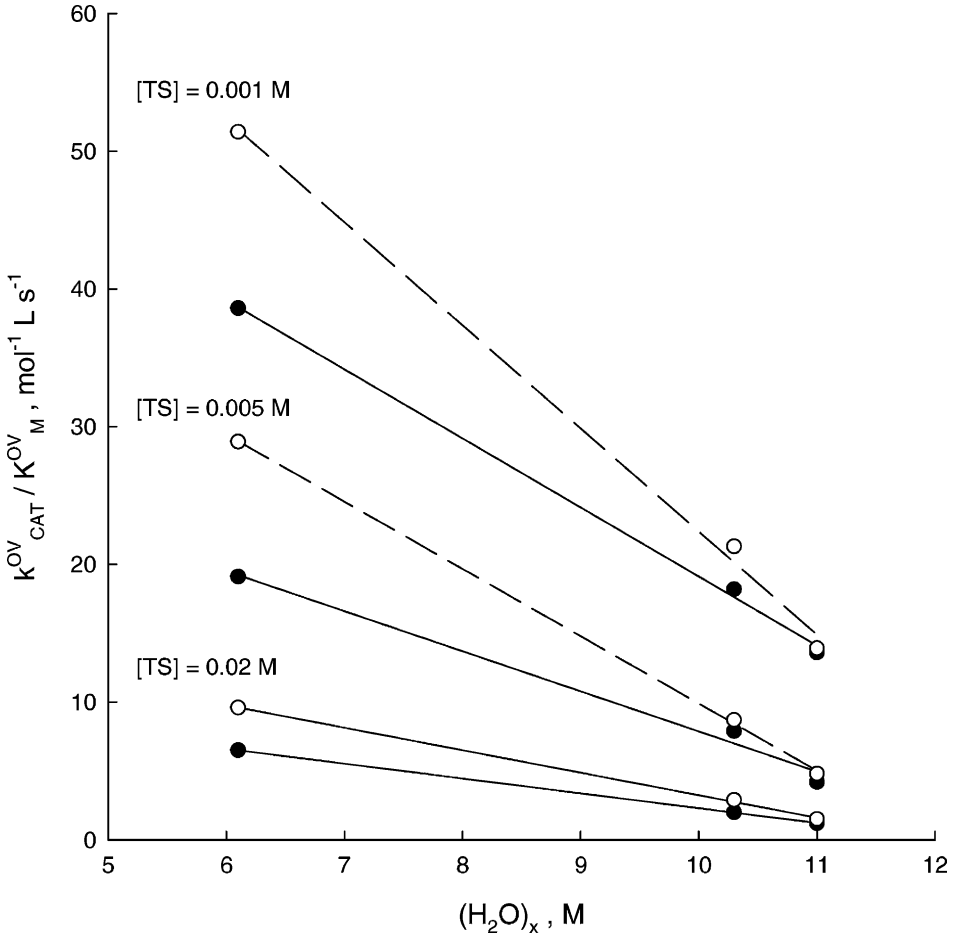

Fig. 4. Dependence of measured k^{OV}_{CAT}/K^{OV}_{M} at various surfactant concentrations on the degree of hydration of the polar head group of cetyltrialkylammonium bromide $(H_2O)_x$ = 11, 10.3, and 6.1 M for CTABr, CDMEABr, and CTPABr, respectively. Open symbols: δ-CT; full symbols: α-CT.

substrate concentrations. Simulated double reciprocal plots were then built and theoretical values for the overall parameters were estimated. Comparison of the results reported in Table 5 shows a fairly good agreement between measured and predicted values of the kinetic parameters.

Fig. 4 shows the dependence of the parameter k^{OV}_{CAT}/K^{OV}_{M} on the hydration number of the surfactant aggregates. The higher the hydration number, the lower the overall efficiency of both α-CT and δ-CT. The presence of cationic surfactants always leads to a higher efficiency of δ-CT than that of α-CT in the entire investigated range of surfactant concentration.

4. Conclusions

Results presented in this paper show that chymotrypsin iso-enzymes in aqueous solution of cationic surfactants aggregates can reach an activity higher than in pure buffer. The diagram of superactivity versus surfactant concentration shows a bell-shaped curve. By contrast, aggregates of non-ionic surfactants can only hinder the enzymatic activity and the curve of activity versus surfactant concentration shows a monotonically decreasing behaviour. The rate of GpNA hydrolysis is well predicted by a model developed on the basis of the pseudo-phase approach.

Overall bioconversion rate is the sum of two patterns catalysed by the enzyme present in the free water and in the bound water pseudo-phase. Comparison of enzyme catalytic behaviour in direct and in reverse micellar media suggests that the concentration of aggregated surfactant in direct micelle systems can play the same role of w_0 in reverse micelle systems.

The observed change in superactivity between α-CT and δ-CT with direct micelles of cationic surfactants presents similarity with the study reported in [27]. The only difference between α-CT and δ-CT is the N-terminal Ala149 that is not present in the δ-intermediate. This is sufficient to alter stability and activity of the enzymes in reverse micelles of anionic surfactants [27]. The different reduction of activity and thermal stability of α-CT and δ-CT in reverse micelles with respect to pure buffer was reported in [27] as an evidence of a specific interaction of a protein group with the anionic detergent and of a protein-interface interaction. Similarly, the results of the present study in direct micelles lead to the hypothesis that cationic surfactants (mainly the head group) can produce different positive effects on the activity of the two iso-enzymes, and thus that some specific interaction must occur between the surfactant aggregates and the enzyme molecule. In this interaction, both electric and hydrophobic properties of the surfactant seem to play an important role: at the pH of our experiments, higher than the enzyme iso-electric point, the surfactant aggregates ability of interacting with the protein molecule depends on the positive charge of the surfactant cationic head. On the other hand, hydrophobic interactions of the surfactant cationic head with the enzyme lipophilic residues seem to play a primary role in the observed variations of the catalytic activity of the chymotrypsin iso-enzymes: the higher the hydrophobicity, the higher the activity. As reported in [8,20], this could be probably explained on the basis of some structural modification of the enzyme molecule induced by the interactions with the surfactant aggregates.

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