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Substrate effect on α-chymotrypsin activity in aqueous solutions of "big-head" ammonium salts

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Abstract

The effect of two ammonium salts with a bulky head group, the tetrabutylammonium bromide (TBABr) and the surfactant cetyltributylammonium bromide (CTBABr) on α-chymotrypsin hydrolysis rate toward three peptidyl substrates was investigated. *N*-Succinyl-L-phenylalanine-*p*-nitroanilide (SP*p*NA), *N*-succinyl-L-alanyl-L-alanyl-L-phenylalanine-*p*-nitroanilide (SAAP*p*NA) and *N*-succinyl-L-alanyl-L-alanyl-L-prolyl-L-phenylalanine-*p*-nitroanilide (SAAPP*p*NA), which contain the same chromogenic and N-protecting groups, but a different number of amino acidic residues, were selected.

The relative activity showed a bell-shaped dependence on additive concentration, with a maximum which ranged from 1×10^{-3} M to 5×10^{-3} M for CTBABr and at 0.3 M for TBABr. Both the additives induced α-chymotrypsin superactivity, but their effect decreased as the peptide chain length of the substrate increased.

Analysis of the kinetic parameters indicated that the activation was mainly due to an increase in k_{cat} values, probably caused by enzyme conformational changes induced by the additives, while K_M remained almost unchanged. The very notable effect of both CTBABr and TBABr on SP*p*NA hydrolysis rate and the limited activation with SAAP*p*NA and SAAPP*p*NA could be probably due to non-specific interactions between the additives and the subsites next to the catalytic point within α-chymotrypsin active site, not allowed with the longer amino acidic chain substrates.
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Keywords: α-Chymotrypsin; Peptide substrates; Superactivity; Ammonium salts

1. Introduction

α-Chymotrypsin (α-CT) is one of the most studied enzymes and its structure and mechanism of action are well known [1–3]. Many papers in the literature deal with the effect of surfactants, or generally additives, on α-CT activity and stability [4–11]. In our previous papers the hydrolytic activity of α-CT toward *N*-glutaryl-L-phenylalanine-*p*-nitroanilide (GPNA) has been studied in the presence of ionic surfactants [12–14]. Enzyme catalytic properties, i.e. activity and stability, seem to be deeply dependent on the head group size of cationic cetyltrialkylammonium surfactants. In particular, GPNA hydrolysis rate was depressed in the presence of cetyltrimethylammonium bromide (CTABr), while a large superactivity was found with cetyltributylammonium bromide (CTBABr) [12]. Moreover, similar behaviour was obtained if the hexadecyl chain of the surfactants was substituted with *p*-octyloxybenzyl moieties, *p*-octyloxybenzyltrimethyl- and *p*-octyloxybenzyltributylammonium bromide (pOOTABr and pOOTBABr, respectively) [14].

Superactivity, observed both in micellized and monomeric CTBABr [13], was due to an increase in the turnover number, k_{cat}, whereas the Michaelis constant, K_M, was almost unaffected.

Abuin et al. [11] reported the effect of surfactant chain length on α-CT activity in the hydrolysis of GPNA. Superactivity was found with *n*-alkyltrimethylammonium bromide surfactants at concentration near to the critical micelle concentration (c.m.c.) and the longer the alkyl chain, the lesser the maximum catalytic efficiency.

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The structural modifications in the protein conformation induced by the surfactants, revealed by CD and fluorescence measurements, made the enzyme more active but, at the same time, more sensitive to inactivation. In both CTBABr and pOOTBABr, α-CT activity decreased more sharply than in pure buffer and after 24 h the reaction rate in the presence and without additives reached approximately the same values.

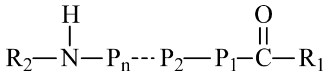
Superactivity was also observed in the presence of tetrabutylammonium bromide (TBABr): it possesses the same head group of CTBABr, but it is not able to form aggregates because of the lack of the hydrophobic chain [14]. Enzyme activity showed a bell-shaped curve and the maximum occurred at a concentration 80-fold higher than CTBABr. However, in TBABr solutions α-CT was able to preserve its activity for long period of time.

A bulky head group is therefore essential in determining the superactivity of α-CT and the hydrophobic chain promotes additive–enzyme interactions but, at the same time, causes the observed rapid inactivation. So, the influence of additives on enzyme catalytic properties strongly depends on the additive structure, i.e. the charge and the size of the head group.

The influence of additives on α-chymotrypsin activity seems to depend also on the nature of the substrate [8,15]: the effect of CTBABr on α-CT hydrolysis of the neuroundecapeptide substance P is much less pronounced as regards the model substrate [15]. Moreover, α-CT–CTABr interactions led to an increase in enzyme catalytic activity and affinity for the substrate *p*-nitrophenyl acetate [8].

It is well known that α-CT, such as other proteases, protein kinases and phosphatases, possesses a broad selectivity toward its substrates; moreover, it contains an extended binding site for peptide substrates, partitioned into a series of subsites, each corresponding to a residue of substrate [16–19], and hydrolysis rate is markedly enhanced by an increase in the peptide chain length from the catalytic point to the N-terminus in the substrate [17]. So, α-CT substrates can be divided in "poor" and "good" substrates [20], depending on their kinetic parameters, especially k_{cat}/K_M; this "specificity constant" has been properly used to obtain information about enzyme–substrate specificity [21].

The structure of a model substrate can be simply depicted as in the following scheme.



where R_1 and R_2 represent the chromophore moiety for UV–vis detection and the protecting group of N-terminal amino acid, respectively, while P_1–P_2···P_n, according to the nomenclature proposed by Schechter and Berger [22], are the amino acidic residues of the substrate, which can interact with corresponding enzymatic subsites within the active site ($S_1, S_2, \ldots, S_n$).

The present kinetic study was undertaken to investigate the effect of CTBABr and TBABr, which significantly enhance the hydrolysis rate of GPNA, on α-CT activity toward a series of substrates with the same chromogenic and protecting groups but that differ in the number of amino acid residues: *N*-succinyl-L-phenylalanine-*p*-nitroanilide (SP*p*NA), *N*-succinyl-L-alanyl-L-alanyl-L-phenylalanine-*p*-nitroanilide (SAAP*p*NA) and *N*-succinyl-L-alanyl-L-alanyl-L-prolyl-L-phenylalanine-*p*-nitroanilide (SAAPP*p*NA). The use of *p*-nitroaniline as leaving group allows the hydrolytic reaction to be simply followed and succinyl residue as N-protecting group to enhance substrates solubility due to the free carboxylic group. Moreover, the presence of the proline residue in SAAPP*p*NA instead of a further alanine notably increases enzyme–substrate specificity; indeed, as already reported by Case [19], in pure buffer the catalytic efficiency of α-CT is sevenfold and only twofold higher with respect to SAAP*p*NA with the model substrate containing the proline and the alanine residue in P_2 position, respectively. Model substrates with marked differences in the corresponding k_{cat}/K_M values were then chosen to better evaluate the influence of the different additives on enzyme activity.

Kinetic parameters with and without additives were also compared to relate the substrates structure and the effect of the additives on α-CT catalytic properties.

2. Materials and methods

2.1. Materials

Crystalline bovine pancreatic α-chymotrypsin (EC 3.4.21.1) was purchased from Sigma and used without further purification. The commercial Tris and *p*-nitroaniline (*p*-NA) used for buffer preparation and for molar absorption coefficient (ε) determination, respectively were from Aldrich. The substrates *N*-succinyl-L-phenylalanine-*p*-nitroanilide (SP*p*NA) and *N*-succinyl-L-alanyl-L-alanyl-L-prolyl-L-phenylalanine-*p*-nitroanilide (SAAPPpNA) were from Sigma, while *N*-succinyl-L-alanyl-L-alanyl-L-phenylalanine-*p*-nitroanilide (SAAP*p*NA) from Bachem. Enzyme and substrates buffered solutions were always freshly prepared immediately before their use and kept in ice during the experiments.

The commercial grade salt tetrabutylammonium bromide (TBABr) was supplied by Aldrich and it was dried at 50 °C under reduced pressure (50 mTorr) in a BÜCHI TO-51 drying oven before use. The preparation and purification at laboratory scale of the surfactant cetyltributylammonium bromide (CTBABr) have already been described [23].

2.2. α-Chymotrypsin activity assay

The α-chymotrypsin activity measurements were carried out spectrophotometrically at 25.0 ± 0.1 °C, following the increase in absorbance at 410 nm due to the formation of *p*-nitroaniline. The molar absorption coefficient (ε_{410}) is 8800 M^{-1} cm^{-1} in pure buffer and in CTBABr solutions (below and above c.m.c.), while in the presence of TBABr it varies from 10,600 M^{-1} cm^{-1} to 13,700 M^{-1} cm^{-1} in the concentration range of 0.01–1 M.

Kinetic determinations were performed using a Shimadzu UV-160A UV–vis spectrophotometer equipped with a thermostated cell.

Enzyme activity was always assayed in 0.1 M Tris–HCl buffer at pH 7.75 and, for the experiments with SAAP*p*NA and SAAPP*p*NA, 0.01 M $CaCl_2$ was also added. The concentration of both α-CT and substrate was properly varied depending on the substrate itself: for SP*p*NA: $[S] = 2.5 \times 10^{-3}$ M, [α-

CT] = 8 μM; for SAAP*p*NA: [S] = 5×10^{-4} M, [α-CT] = 60 nM; for SAAPP*p*NA: [S] = 2.5×10^{-4} M, [α-CT] = 8 nM.

Kinetic parameters k_{cat} and K_M in pure buffer and in the presence of additives were obtained by both non-linear and linear regression analysis of the double reciprocal Lineweaver–Burk plot and results were within the experimental error; the range of substrate concentration was 5×10^{-4} M to 2×10^{-3} M for SP*p*NA, 5×10^{-5} M to 4×10^{-4} M for SAAP*p*NA and 1×10^{-5} M to 2×10^{-4} M for SAAPP*p*NA.

All sets of experiments were reproduced several times and the differences between duplicates were always below 5%.

2.3. Determination of the binding constant (K_S)

The distribution of the substrate between the bulk water and the micellar aggregates of CTBABr was determined. The binding constant (K_S) is related to surfactant and substrate concentrations by the following equation:

$$K_S = \frac{[S_M]}{[S_W][D_n]} \quad (1)$$

where $[S_M]$ and $[S_W]$ are the micelle-bound and the free substrate molar concentrations, respectively and $[D_n]$ ($[D_T]$-c.m.c.) is the concentration of micellized surfactant. K_S values were calculated from absorbance measurements following a procedure already reported in the literature [24].

3. Results and discussion

The activity of α-CT toward SP*p*NA, SAAP*p*NA and SAAPP*p*NA in pure buffered solutions was first investigated to find the suitable enzyme and substrate concentrations to obtain a linearly time dependence during the first minutes of reaction. As expected, the optimized experimental conditions were quite different depending on the substrate used because of the differences in their water solubility and specificity toward the enzyme.

Then, the effect of the additives on the hydrolysis rate of the three substrates was evaluated.

3.1. Effect of TBABr on α-chymotrypsin activity

Fig. 1 shows the relative activity, expressed as the ratio of specific hydrolysis rate in the presence of additive (r_{TBABr}) to that in pure buffer (r_b), as a function of TBABr concentration.

These plots were very similar to those previously observed with *N*-glutaryl-L-phenylalanine-*p*-nitroanilide (GPNA) [14], but the figure highlighted the considerable decrease of the additive effect as the peptide chain length increased. In fact, at 0.3 M TBABr, i.e. the optimal salt concentration, the specific reaction rate of SP*p*NA hydrolysis was sixfold higher with respect to buffer, while a slight increase in activity occurred with SAAPP*p*NA, the observed activity enhancement being only 60%.

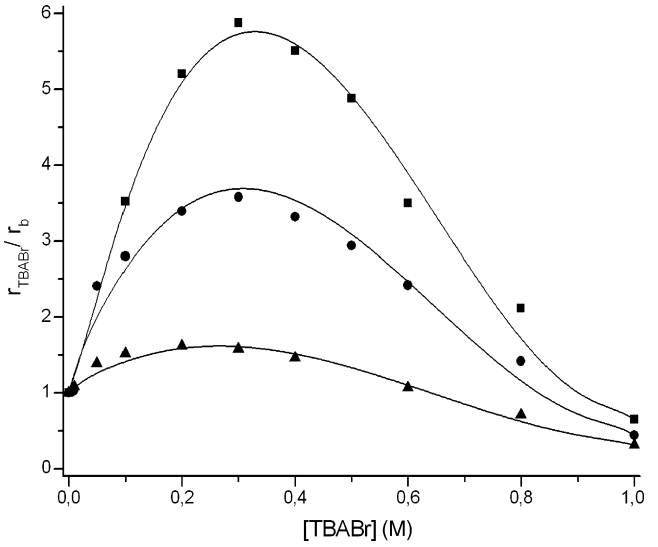


Fig. 1. Effect of TBABr concentration on α-chymotrypsin activity at 25.0 °C: (■) SP*p*NA, (●) SAAP*p*NA and (▲) SAAPP*p*NA. The experimental conditions are reported in Section 2.

3.2. Kinetic analysis with TBABr

The effect of TBABr on α-CT kinetic parameters was also investigated at the additive concentrations which induced the maximum of superactivity. All data points obey to a Michaelis–Menten kinetics and K_M and k_{cat} values were reported in Table 1.

In the absence of the additive, both the rate constant and the enzyme–substrate affinity raised with the increase of amino acidic residues and therefore the specific constant (k_{cat}/K_M) for α-CT catalysis was more than four order of magnitude as the substrate was lengthened from SP*p*NA to SAAPP*p*NA. Our k_{cat} and K_M values are very close to those of the literature [19,20,25,26] and the enhancement in α-CT selectivity with the increase of the peptide chain length was dependent on the noticeable raise of the rate constant for the formation of the acyl-enzyme, as well reported by Case [19].

The kinetic parameters obtained with the organic salt TBABr suggested that, except in the case of SP*p*NA, enzyme–substrate affinity was very similar to that determined in pure buffer and then the increase in the relative activity showed in Fig. 1 can

Table 1
Effect of TBABr on kinetic parameters of α-chymotrypsin[a]

	K_M (10^{-3} M)	k_{cat} (s^{-1})	k_{cat}/K_M (M^{-1} s^{-1})
SP*p*NA			
Buffer	1.09 ± 0.04	0.014 ± 0.001	13 ± 1.4
TBABr	10.70 ± 0.5	0.27 ± 0.03	25 ± 4
SAAP*p*NA			
Buffer	0.14 ± 0.012	7.8 ± 0.4	$(5.6 \pm 0.77) \times 10^4$
TBABr	0.13 ± 0.01	27.5 ± 0.9	$(2.11 \pm 0.23) \times 10^5$
SAAPP*p*NA			
Buffer	0.044 ± 0.005	39 ± 4	$(8.9 \pm 1.9) \times 10^5$
TBABr	0.057 ± 0.005	60 ± 6	$(1.05 \pm 0.2) \times 10^6$

[a] At 25.0 °C, [TBABr] = 0.3 M.

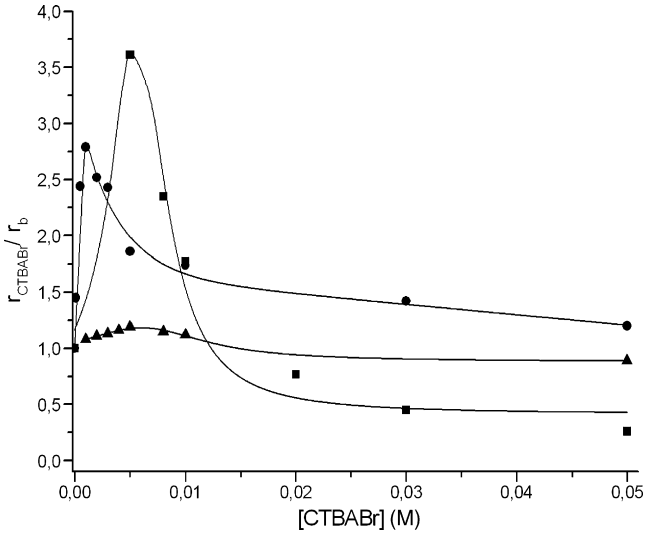


Fig. 2. Effect of CTBABr concentration on α-chymotrypsin activity at 25.0 °C: (■) SP*p*NA, (●) SAAP*p*NA and (▲) SAAPP*p*NA. The experimental conditions are reported in Section 2.

be related to a k_{cat} enhancement which decreased from SP*p*NA to SAAPP*p*NA. The high K_M value observed with SP*p*NA was probably due, as previously hypothesized [14], to the presence of a large amount of TBA^+ ions near the enzyme active site which could interact with protein residues and bind to the negatively charged substrate hampering the reaction.

3.3. Effect of CTBABr on α-chymotrypsin activity

The effect of CTBABr concentration on α-CT relative activity is shown in Fig. 2.

A bell-shaped behaviour was found again and the highest enzyme activity occurred at surfactant concentration equal to 5×10^{-3} M for the hydrolysis of SP*p*NA and SAAPP*p*NA and to 1×10^{-3} M for the reaction of SAAP*p*NA, even if, as previously reported with TBABr, the superactivity induced by CTBABr diminished with the increase of the peptide chain length of the substrate.

3.4. Role of CTBABr micelles on α-chymotrypsin activity

At the experimental conditions used, all the substrates are negatively charged due to the free carboxylic group of the succinyl moiety and they can strongly interact with the positively charged aggregates. Consequently, the substrate distribution between the aqueous phase and the micellar interface lowered the substrate concentration effectively available for the enzyme-catalyzed reaction. The binding constants (K_S) of the three substrates with CTBABr aggregates were then determined and the values are reported in Table 2.

K_S value of SP*p*NA was the same as GPNA [12] and it lowered with the increase of the peptide chain length; so the more is the size of the substrate and the less is its incorporation into CTBABr micelles. Therefore, at any surfactant concentration, the free substrate amount available for catalysis $[S_W]$ was dependent on the nature of the substrate and can be obtained from the following equation:

$$[S_W] = \frac{[S_T]}{1 + K_S[D_n]}$$

where $[S_T]$ is the analytical concentration of the substrate and $[D_n]$ is the concentration of the micellized surfactant.

The relative rate values (r_{CTBABr}/r_b) reported in Fig. 2 and determined in terms of the analytical concentration of the substrate must be corrected to take into account of the reduced free substrate concentration in the presence of CTBABr micelles. The reaction rates in pure buffer were then calculated with the already reported kinetic parameters (Table 1) at the effective substrate concentration (r_b^*); the results, along with the experimental data, are shown in Table 3.

The corrected data revealed that the positive effect of the surfactant on α-CT activity was found at all the selected CTBABr concentrations, hydrolysis rate being always higher than pure buffer.

The outcomes of the correction indicated that the effect of substrate removal by aggregates was more evident for SP*p*NA hydrolysis; in fact, at 5×10^{-2} M CTBABr, the highest concentration value investigated, r_{CTBABr}/r_b^* values changed, with respect to the experimental data, by factors of about 20, 10 and 2.5 for SP*p*NA, SAAP*p*NA and SAAPP*p*NA, respectively.

Moreover, the effect of CTBABr concentration on hydrolysis relative rates showed opposite trends by comparing SP*p*NA with the other two substrates. In fact, an increase of surfactant concentration produced, in the case of SP*p*NA, a decrease of α-CT superactivity, even if less evident with respect to the experimental data, while a rise in r_{CTBABr}/r_b^* values was found

Table 2
Binding constant values in CTBABr micelles at 25.0 °C

Substrate	K_S (M^{-1})
SP*p*NA	1500 ± 200
SAAP*p*NA	800 ± 90
SAAPP*p*NA	200 ± 30

Table 3
Effect of CTBABr concentrations on experimental (r_{CTBABr}/r_b) and calculated after correction for free substrate concentration (r_{CTBABr}/r_b^*) relative rate

[CTBABr] (M)	r_{CTBABr}/r_b	r_{CTBABr}/r_b^*
SP*p*NA		
5×10^{-3}	3.6	11.8
1×10^{-2}	1.8	9.8
5×10^{-2}	0.3	6.2
SAAP*p*NA		
1×10^{-3}	2.8	3.3
5×10^{-3}	1.9	3.5
1×10^{-2}	1.7	4.8
5×10^{-2}	1.2	11.7
SAAPP*p*NA		
1×10^{-3}	1.1	1.1
5×10^{-3}	1.2	1.4
1×10^{-2}	1.1	1.5
5×10^{-2}	0.9	2.2

Table 4
Effect of CTBABr on kinetic parameters of α-chymotrypsin

[CTBABr] (M)	K_M (10^{-3} M)	k_{cat} (s^{-1})	k_{cat}/K_M ($M^{-1}\,s^{-1}$)
SP*p*NA			
Buffer	1.09 ± 0.04	0.014 ± 0.001	13 ± 1.4
5×10^{-3}	10.1 ± 0.9	0.15 ± 0.011	15 ± 2.4
5×10^{-3}[a]	1.19 ± 0.11	0.15 ± 0.011	126 ± 20
SAAP*p*NA			
Buffer	0.14 ± 0.012	7.8 ± 0.4	$(5.6 \pm 0.8) \times 10^4$
1×10^{-3}	0.36 ± 0.02	26.4 ± 1.3	$(7.3 \pm 0.8) \times 10^4$
1×10^{-3}[a]	0.20 ± 0.011	26.4 ± 1.3	$(1.3 \pm 0.14) \times 10^5$
SAAPP*p*NA			
Buffer	0.044 ± 0.005	39 ± 4	$(8.9 \pm 0.19) \times 10^5$
5×10^{-3}	0.125 ± 0.013	57 ± 6	$(4.6 \pm 0.96) \times 10^5$
5×10^{-3}[a]	0.063 ± 0.007	57 ± 6	$(9.05 \pm 1.89) \times 10^5$

[a] Data after correction to free substrate concentration.

with SAAP*p*NA and SAAPP*p*NA. Different enzyme–substrate affinities along with different monomeric CTBABr/α-CT molar ratios could be probably involved to determine the observed behaviour.

3.5. Kinetic analysis with CTBABr

The effect of CTBABr on α-CT kinetic parameters was investigated and the results, along with those in the absence of additive, are reported in Table 4.

Analysis of the kinetic data obtained in the presence of CTBABr indicated that the activation of α-CT was mainly due to an increase in k_{cat}, whereas the enzyme affinity, after correction for substrate partitioning, remained almost unchanged, independently of the substrate. Moreover, as previously seen with TBABr, the ratio between the catalytic activity (k_{cat}) in CTBABr and in the absence of the surfactant noticeably lowered from SP*p*NA to SAAPP*p*NA.

In any case both the additives caused positive changes in α-CT conformation, as revealed by spectroscopic data (CD and fluorescence) [12,14] and consequently higher activity, even if the whole effect depended on the substrate.

In the absence of additives, α-CT kinetic parameters strongly depended on both the number and the nature of the substrate amino acid residues. Case [19] proposed that, after the formation of the Michaelis–Menten complex, substrates with a long peptide chain, as SAAPP*p*NA, could interact with remote subsites within the α-CT active site. This interaction, not available to SP*p*NA, induces a distortion of the stable, planar geometry of the scissile amide bond and it causes the carbonyl moiety to be more reactive toward the nucleophylic attack of the active site serine residue. The relatively slight decrease in K_M value could be explained with a negative enthalpy of association due to the hydrogen bonds and van der Waals interactions between substrate and α-CT active site residues, even if this effect is countered by a negative entropy of association due to the restricted conformational mobility of both enzyme and substrate, along with unfavourable solvation effects.

The very notable effect of both CTBABr and TBABr on SP*p*NA hydrolysis rate could be attributed to an interaction of the additives with the subsites next to the catalytic point. In particular, hydrophobic, non-specific interaction between amino acids with bulky side chains in P_2 position and the S_2 subsite of the enzyme seem to decrease the activation energy of the reaction [27]. Therefore, it is reasonable to suppose that "big-head" additives, such as CTBABr and TBABr, could positively interact with the subsites next to the catalytic point and consequently enhance the nucleophilicity of the catalytic serine residue simply by producing a more hydrophobic microenvironment. In the case of SAAP*p*NA and SAAPP*p*NA, their amino acidic chains could set positive interactions with the same enzymatic subsites and then the described additive effect should be probably less and less evident, being the additive incoming into the α-CT active site sterically prevented.

4. Conclusions

Activity of α-CT in the hydrolysis of three peptidyl substrates was promoted in the presence of two "big-head" ammonium additives, TBABr and CTBABr. In pure buffer, hydrolysis rate notably enhanced with the increase of substrate amino acidic residues, owing to the marked raise of the rate constant for the formation of the acyl-enzyme.

The addition of both TBABr and CTBABr did not affect enzyme–substrate affinities and α-CT activation was mainly due to the increase of k_{cat} values, probably related to a catalytically more favourable conformation of the enzyme induced by the additives.

Moreover, the extent of superactivity was strongly dependent on the chain length of the substrate: the more is the number of amino acidic residues, the less is the activation effect. The dependence of superactivity on the substrate could be attributed to hydrophobic non-specific interactions between the additives and the enzymatic subsites next to the catalytic point, which produced a more hydrophobic microenvironment and a consequent increase in the nucleophylicity of the catalytic serine residue. Therefore, as the substrate was lengthened, the additive effect was sterically hampered, because of the interactions between a long peptide chain and the above-mentioned enzymatic subsites.

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