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# Effect of quaternary ammonium salts on the hydrolysis of *N*-glutaryl-L-phenylalanine catalysed by α-chymotrypsin

Paolo Viparelli*, Francesco Alfani, Alberto Gallifuoco, Maria Cantarella

*Dipartimento di Chimica, Ingegneria Chimica e Materiali, Università di L'Aquila, Monteluco di Roio, 67040 L'Aquila, Italy*

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Dedicated to the memory of Prof. Francesco Alfani

**Abstract**

The hydrolysis of *N*-glutaryl-L-phenylalanine *p*-nitroanilide catalysed by α-chymotrypsin (α-CT) was studied in the presence of the following quaternary ammonium salts: tetrapentyl ammonium bromide (TPeABr), tetrabutyl ammonium bromide (TBABr), tetrapropyl ammonium bromide (TPABr), tetraethyl ammonium bromide (TEABr) and tetramethyl ammonium bromide (TMABr). The activity of the enzyme is strongly affected by the salts that act as activators. Superactivity has been detected in the presence of TPeABr, TBABr, TPABr and TEABr. The enzyme activity seems to depend on the molecular structure of the salts; the higher the molecular weight of the alkyl residues in the cationic ammonium group, the higher the superactivity. In the whole investigated range, the enzyme hydrolysis rate resulted to be a monotonic increasing function of the salt concentration. The model of a non-essential activator was adopted to describe the effect of the salts on the hydrolysis activity and good agreement was found between the experimental results and the model predictions. The dependence of the enzyme activity on the substrate concentration was also studied to further verify the applicability of the model. Finally, a preliminary study about the effect of these additives on α-chymotrypsin thermal stability was performed.


*Keywords:* Enzymes; Kinetics; Activator; Cationic additives; Ammonium salts

## 1. Introduction

The serine protease α-chymotrypsin (α-CT) (EC 3.4.21.1) has been widely studied as a catalyst for the hydrolysis of peptide bonds. Its structure [1] and mechanism of action are well known [2,3]. As this enzyme is commercially available in a pure form and also because of the large amount of work already done with this protease, it has also been chosen as a model enzyme in many studies [4–13].

α-Chymotrypsin retains its activity in numerous systems from buffered media to organic solvent media [14,15], reverse micelles [6,8–13,16,17] and nearly anhydrous organic systems [19]. The enzymatic activity can be either reduced [16,17] or remain equal or higher than in aqueous media [6,17].

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 [6,14–22]. Enzyme superactivity has been detected also in purely water/surfactant media owing to positive interactions between the enzyme and the surfactant [6–8,23,24].

In our previous paper we focused the investigation on the study of α-CT catalytic activity in aqueous buffered solutions enriched with self-organising amphiphilic whose concentration molecules are above the critical micellar one (CMC). We reported that some cationic surfactants (cetyltrialkylammonium bromides) induced a superactivity of this enzyme in the hydrolysis of *N*-glutaryl-L-phenylalanine *p*-nitroanilide (GpNA). The surfactant head group size mainly modulated the extent of α-CT superactivity: the higher the molecular weight of the head group, the higher the superactivity.

The effect of these molecules on the enzymatic activity were attributed to both hydrophobic and electrostatic interactions between the surfactant aggregates and the enzyme molecule.

The superactivity was strongly affected by the surfactant concentration: typically, bell-shaped curves were obtained when plotting the enzymatic reaction rate versus the total

* Corresponding author. Tel.: +39-0862-434215/434232; fax: +39-0862-434203.
*E-mail address:* viparell@ing.univaq.it (P. Viparelli).

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**Nomenclature**

| | |
|---|---|
| $A$ | enzyme activity (time $t$) (mol $mg_E^{-1}$ $s^{-1}$) |
| $A_0$ | initial enzyme activity (time 0) (mol $mg_E^{-1}$ $s^{-1}$) |
| $[E]_0$ | total enzyme concentration (M) |
| $E_a$ | active enzyme |
| $E_i$ | inactive enzyme |
| $E'$ | denaturated enzyme |
| $k_1$ | rate constant for the denaturation kinetics ($s^{-1}$) |
| $k'_1$ | parameter of the global deactivation mechanism ($s^{-1}$) |
| $k_2$ | rate constant of the self-digestion reaction (l $mol^{-1}$ $s^{-1}$) |
| $k'_2$ | parameter of the global deactivation mechanism (l $mol^{-1}$ $s^{-1}$) |
| $k_P$ | turnover number for the unactivated reaction ($s^{-1}$) |
| $k_{P,app}$ | apparent turnover number for the reaction in the presence of the activator ($s^{-1}$) |
| $K$ | equilibrium constant for the denaturation kinetics |
| $K_m$ | Michaelis–Menten constant of the self-digestion reaction (M) |
| $K_S$ | Michaelis–Menten constant for the unactivated reaction (M) |
| $K_{S,app}$ | apparent Michaelis–Menten constant for the reaction in the presence of the activator (M) |
| $K_X$ | dissociation constant of the enzyme-activator complex (M) |
| $r$ | reaction rate (μmol $l^{-1}$ $s^{-1}$) |
| $r_{buffer}$ | reaction rate in pure buffer (μmol $l^{-1}$ $s^{-1}$) |
| $[S]$ | substrate concentration (M) |
| $V_{max}$ | maximal velocity for the unactivated reaction (μmol $l^{-1}$ $s^{-1}$) |
| $V_{max,app}$ | apparent maximal velocity for the reaction in the presence of the activator (μmol $l^{-1}$ $s^{-1}$) |
| $[X]$ | activator concentration (M) |

*Greek letters*

| | |
|---|---|
| $\alpha$ | parameter that describes the effect of the activator on the enzyme–substrate affinity |
| $\beta$ | parameter that describes the effect of the activator on the enzyme activity |
| $\varepsilon_{410}$ | pNA extinction coefficient at 410 nm ($M^{-1}$ $cm^{-1}$) |

surfactant concentration in the system. This behaviour was explained on the basis of a substrate partition between the aqueous buffered phase and the organic core of the surfactant aggregates [25,26].

In order to ascertain whether α-CT superactivity was mainly due to the positive electrostatic interactions of the cationic head with the enzyme molecule or to hydrophobic effects and also to eliminate the phenomenon of the substrate partition, in this work we focused our attention on a series of tetra-alkylammonium bromides $(NR_4)^+Br^-$. These salts have a molecular structure similar to that of the cationic surfactants previously employed, the main difference being the absence of the long hydrophobic tail that prevents the formation of any organised structure (micelle) and thereby the partition of the substrate.

These salts are promising additives as different alkyl residues in the $(R_4N)^+$ ammonium cation lead to different charge density and hydrophobicity and, on the basis of our previous studies, different effects on α-CT activity can be expected.

The main purpose of this paper was to gain knowledge on the influence of five tetra-alkyl ammonium salts (C1–C5 R-groups) on the kinetics of α-CT catalysed GpNA hydrolysis. To meet this end, a wide range of salt concentration was first investigated and then the effect of the substrate concentration at different fixed concentration of the additives was studied.

In order to explain the obtained experimental data, a suitable kinetic model was adopted and its ability to predict the effect of the ammonium salts on α-CT activity was tested.

Finally, the α-CT thermal stability in the presence of the ammonium salts in the storage medium was also investigated.

## 2. Experimental

α-Chymotrypsin (type II, 3× crystallised and lyophilised powder, EC 3.4.21.1) was 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) (p$K_a$ 8.3) and hydrochloric acid were from Aldrich (Germany) and Carlo Erba (Italy), respectively. Tetramethyl ammonium bromide, tetraethyl ammonium bromide, tetrapropyl ammonium bromide, tetrabutyl ammonium bromide and tetrapentyl ammonium bromide were from Sigma (USA) and were used without further purification.

### 2.1. Assay of enzyme activity

The GpNA hydrolysis catalysed by α-chymotrypsin was monitored by following the change in absorbance at 410 nm due to the formation 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 salt and substrate solutions (both prepared with buffer) to a final volume of 2.94 ml. The reaction was initiated adding 0.06 ml of the enzyme buffered stock solution (10 mg ml$^{-1}$) to reach a final concentration of 8 μM (0.2 mg ml$^{-1}$). The product extinction coefficient was found to be 10,015 M$^{-1}$ cm$^{-1}$ both in pure buffer and in the presence of salts. No auto-hydrolysis of the substrate was observed in the absence of enzyme 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 and the substrate conversion was always less than 5%. 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, $k_P$ (s$^{-1}$), moles of GpNA transformed per second and per mole of enzyme. The maximal velocity, $V_{max}$ and the Michaelis–Menten constant, $K_S$, in the presence of buffer and/or salts were determined from linear regression analysis of the data reported in 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. Enzyme storage stability

The storage stability of α-CT was studied incubating enzyme solutions (0.267 mg ml$^{-1}$) at different temperatures (25, 35 and 45 °C) in pure buffer or in buffer solution enriched with the cationic additive (0.133 M). These enzymatic solutions were incubated in a thermostated bath and periodically two cuvettes were withdrawn and rapidly inserted in the spectrophotomer cell, thermostated at the same temperature. The reaction was then started by adding the substrate, kept at the same temperature, so that the desired reaction conditions (pH 7.75, [Tris–HCl] = 0.1 M, $[E]_0$ = 0.2 mg ml$^{-1}$ and $[S]_0$ = 2.5 mM) were realised. The reaction was followed for a short time (3 min) and, as the reaction progress was linear over this period, the enzyme deactivation during the assay was considered as negligible. All the experiments were performed at least in duplicate and the mean value was reported.

## 3. Results and discussion

### 3.1. Effect of the ammonium salts on the catalytic activity

At first, a set of experiments was performed in a buffered (Tris–HCl = 0.1 M, pH 7.75) aqueous solution with a fixed concentration (0.1 M) of the ammonium salts. The temperature was set at 25 °C and the substrate concentration at 2.5 × 10$^{-3}$ M, a value higher than the saturation concentration for the reaction in pure buffer (absence of the ammonium salt). The hydrolysis rate in pure buffered, $r_{buffer}$, was also measured and used as reference.

Table 1
Effect of quaternary ammonium salts on GpNA hydrolysis catalysed by α-CT.

| System[a] | $r$ (μmol s$^{-1}$ l$^{-1}$) | $r/r_{buffer}$ |
|---|---|---|
| α-CT | 0.103 | 1 |
| α-CT + TMABr | 0.106 | 1.03 |
| α-CT + TEABr | 0.128 | 1.24 |
| α-CT + TPABr | 0.229 | 2.22 |
| α-CT + TBABr | 0.477 | 4.63 |
| α-CT + TPeABr | 0.775 | 7.52 |

pH: 7.75; $T$: 25 °C; [$S$]: 2.5 mM; [α-CT]: 8 μM and [$X$]: 0.1 M.
[a] α-CT in pure buffer.

Values of the hydrolysis rate, $r$, and of the ratio, $r/r_{buffer}$ are reported in Table 1. These data show that the presence of the ammonium salts enhanced α-CT activity being the activity ratio, $r/r_{buffer}$, always higher than unity.

Under the adopted experimental conditions, the superactivity increases in the series TMABr < TEABr < TPABr < TBABr < TPeABr, suggesting that the phenomenon should be correlated to the size of the alkyl residue in the cationic group of the ammonium salt (R-groups in Fig. 1). The more effective salt was found to be TPeABr that increased α-CT activity by a factor of 7.52.

During these experiments the pH of the buffered solution was not significantly affected by the presence of the salts in the whole explored range of concentrations, thereby α-CT activity changes cannot be attributed to pH effects.

These preliminary results suggested to study the influence of the salt concentration on α-CT activity and the wide investigated range was 0.001–0.2 M. The only exception was

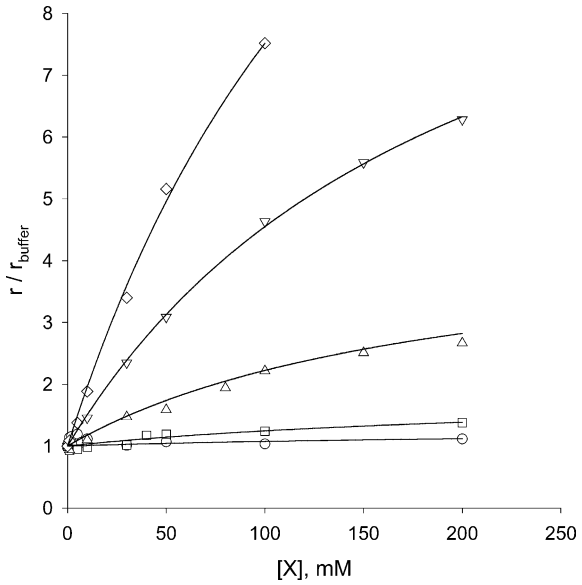


Fig. 1. Effect of quaternary ammonium salts on GpNA hydrolysis catalysed by α-CT. TPeABr (◇); TBABr (▽); TPABr (△); TEABr (□) and TMABr (○). Reaction conditions: $T$ = 25 °C, pH 7.75, [Tris–HCl] = 0.1 M, [GpNA] = 2.5 mM and [α-CT] = 8 μM.

TPeABr, because of its lower solubility in the presence of the substrate that did not allow to perform experiments at concentration higher than 0.1 M.

Results are shown in Fig. 1, where the ratio $r/r_{buffer}$ was plotted as a function of the salt concentration.

As illustrated, the enzyme activity is a monotonic increasing function of the salt content in the reaction medium, and the superactivity confirms the trend suggested by the preliminary results: TMABr < TEABr < TPABr < TBABr < TPeABr.

Interestingly, these results are in accordance with those obtained in the presence of the homologues series of cationic surfactants (cetyltrialkylammonium bromide) and are reported elsewhere [26]. In the presence of either ammonium salts or ammonium cationic surfactants, α-CT superactivity depends on the structure of the cationic group: the higher the molecular weight of the alkyl residue, the higher the superactivity.

This result suggests that the different charge density should play a primary role in the interaction between the cations and the enzyme molecule, even though it cannot be excluded that hydrophobic phenomena should also be important.

When salts were used as additives, in the absence of the surfactant tail, no substrate partition takes place and, as a consequence, the bell-shaped curve obtained in the presence of the cationic surfactants [25,26] was no more observed as the dependence of the enzyme activity on the activator concentration was studied.

### 3.2. Effect of the substrate concentration

In order to better understand the effect of the tetra-alkylammonium bromides, the subsequent part of this study was devoted to investigate the dependence of the enzyme activity on the substrate concentration in the presence of the salts.

The kinetic runs were performed at different substrate concentration and at fixed salt concentration, chosen in the range from 0.005 to 0.2 M. Results are showed in Fig. 2. Lineweaver–Burk plots were also build in Fig. 3 in order to evaluate the two overall parameters $V_{max,\,app}$ and $K_{S,\,app}$ and the secondary-order kinetic parameter, $k_{P,\,app}/K_{S,\,app}$, reported in Table 2 (for simplicity data regarding TMABr are not showed in the figures, but only quoted in the table). It can be observed that for TMABr and TEABr, $k_{P,\,app}/K_{S,\,app}$ is lower for higher salt concentration, [*X*], in the system; it always remains lower than for α-CT in the absence of additives. As Fig. 2 shows that the hydrolysis rate increases with increasing [*X*], it

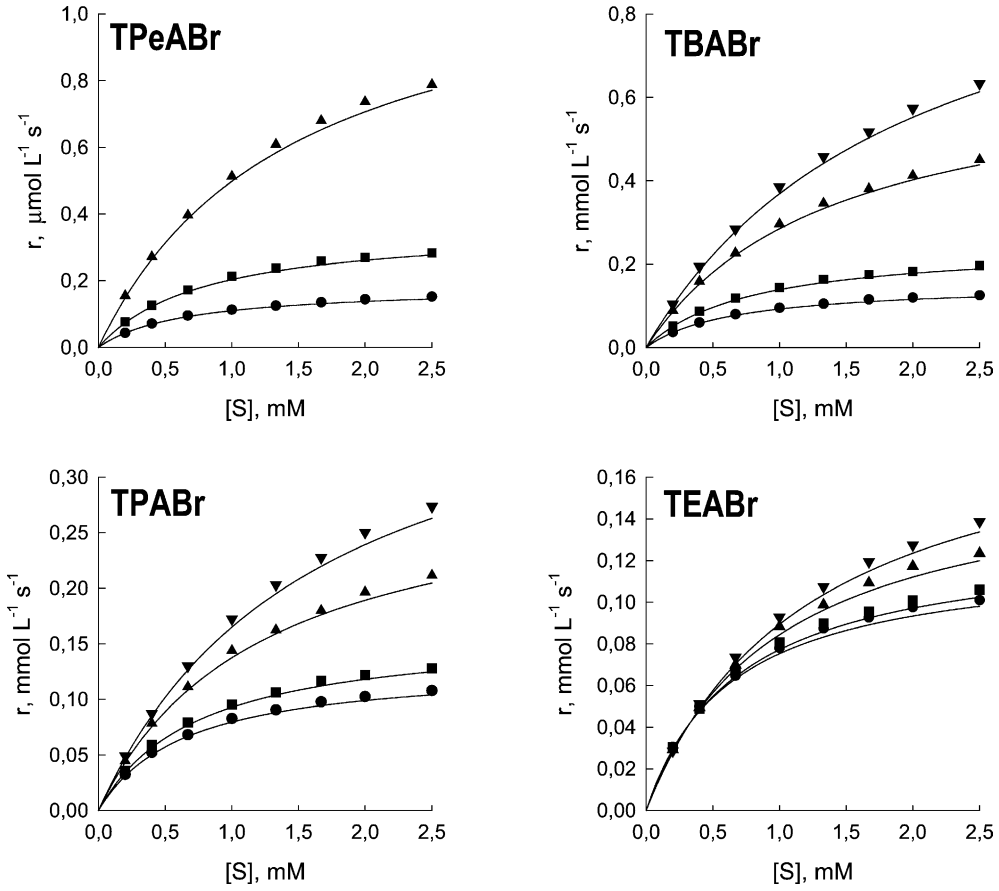


Fig. 2. Rate of hydrolysis as a function of GpNA concentration in the presence of quaternary ammonium salts: (●) 0.005 M; (■) 0.02 M; (▲) 0.1 M, and (▼) 0.2 M. Reaction conditions: $T = 25\,^{\circ}C$, pH 7.75, [Tris–HCl] = 0.1 M, [α-CT] = 8 μM.

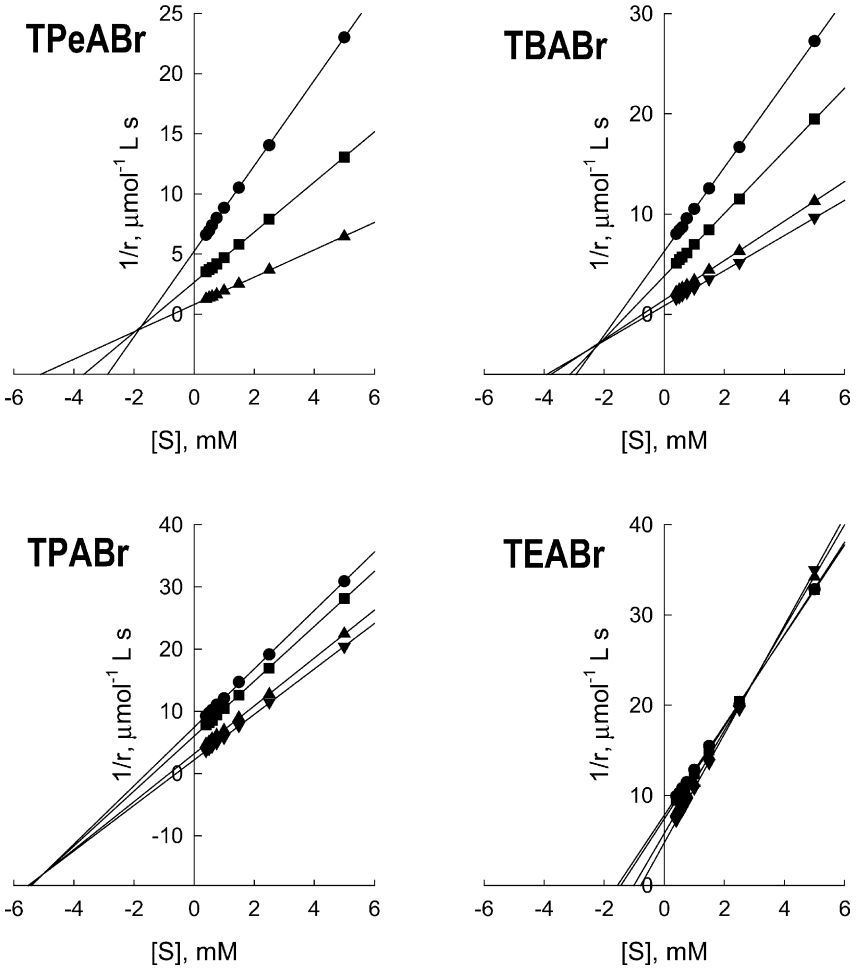


Fig. 3. The $1/r$ versus $1/[S]$ plot for GpNA hydrolysis catalysed by α-CT in the presence of quaternary ammonium salts: (●) 0.005 M; (■) 0.02 M; (▲) 0.1 M, and (▼) 0.2 M. Reaction conditions: $T = 25\,^{\circ}C$, pH 7.75, [Tris–HCl] = 0.1 M [α-CT] = 8 μM.

can be suggested that, in these two cases, the prevailing effect of the salt is to reduce the enzyme affinity for the substrate.

As TPABr, TBABr and TBABr are concerned, $k_{P,\,app}/K_{S,\,app}$ increases greatly with $[X]$: the superactivity phenomenon prevails on other effects induced by the presence of the ammonium salts.

Interestingly, for all salts, as the concentration of the activator, $[X]$, increases, the straight lines obtained by data regression in the reciprocal plots of Fig. 3 pivot counter clockwise about a common point of intersection.

### 3.3. Modelling the catalytic results

The experimental results shown in Figs. 1 and 3 suggest that a kinetic model based on a non-essential activation should be used to depict the effect of the tetraalkylammonium bromides on α-CT catalytic activity. This

Table 2
Michaelis–Menten parameters for GpNA hydrolysis catalysed by α-CT in the presence of different fixed concentrations of quaternary ammonium salts

| α-CT | TMABr | | TEABr | | TPABr | | TBABr | | TPeABr | |
|---|---|---|---|---|---|---|---|---|---|---|
| $k_{P,\,app}/K_{S,\,app}$ ($l\,mol^{-1}\,s^{-1}$) | $[X]$ (M) | $k_{P,\,app}/K_{S,\,app}$ ($l\,mol^{-1}\,s^{-1}$) | $[X]$ (M) | $k_{P,\,app}/K_{S,\,app}$ ($l\,mol^{-1}\,s^{-1}$) | $[X]$ (M) | $k_{P,\,app}/K_{S,\,app}$ ($l\,mol^{-1}\,s^{-1}$) | $[X]$ (M) | $k_{P,\,app}/K_{S,\,app}$ ($l\,mol^{-1}\,s^{-1}$) | $[X]$ (M) | $k_{P,\,app}/K_{S,\,app}$ ($l\,mol^{-1}\,s^{-1}$) |
| 26.2 | 0.005 | 24.2 | 0.005 | 25.8 | 0.005 | 25.0 | 0.005 | 30.3 | 0.005 | 36.0 |
| | 0.02 | 25.0 | 0.02 | 23.6 | 0.02 | 28.3 | 0.02 | 40.6 | 0.02 | 59.4 |
| | 0.1 | 23.3 | 0.1 | 21.9 | 0.1 | 32.0 | 0.1 | 62.5 | 0.1 | 107.6 |
| | 0.2 | 22.6 | 0.2 | 21.2 | 0.2 | 46.5 | 0.2 | 72.6 | 0.2 | – |

$T$: 25 °C; pH: 7.75; [Tris–HCl]: 0.1 M and [α-CT]: 8 μM.

model, reported by Segel in [27], assumes the following reaction sequence:

$$\begin{array}{lll} E + S \overset{K_S}{\rightleftharpoons} & ES \overset{K_P}{\rightleftharpoons} E + P \\ + & + \\ \times & \times \\ \Updownarrow K_X & \Updownarrow \alpha K_X \\ EX + S \overset{\alpha K_S}{\rightleftharpoons} & EXS \overset{\beta k_P}{\rightarrow} EX + P \end{array} \quad (1)$$

The above reaction schema generates the rate expression hereafter reported:

$$r = \frac{V_{max}(1 + \beta([X]/\alpha K_X))[S]}{K_S(1 + ([X]/K_X)) + [S](1 + ([X]/\alpha K_X))} \quad (2)$$

The apparent Michaelis constant and the apparent maximum reaction velocity at fixed $[X]$ are both affected as follows

$$V_{max,\,app} = \frac{V_{max}(1 + (\beta[X]/\alpha K_X))}{1 + ([X]/\alpha K_X)} \quad (3)$$

$$K_{S,\,app} = \frac{K_S(1 + ([X]/K_X))}{1 + ([X]/\alpha K_X)} \quad (4)$$

On these basis, data reported in Figs. 1–3 were used to estimate the parameters of the proposed kinetic model. The following iterative fitting procedure was adopted: a first set of values for the three parameters was obtained using data of Fig. 1; then data of Fig. 3 were used to evaluate $V_{max,\,app}$ and $K_{S,\,app}$.These parameters were plotted as functions of $[X]$, and new values of $\alpha$ and $\beta$ were estimated using Eqs. (3) and (4) where $K_X$ was forced to the previously obtained value. These three values of $\alpha$, $\beta$ and $K_X$ were used to start a fitting session of the data plotted in Fig. 2 making use of Eq. (2). Finally, $\alpha$ and $\beta$ were forced in Eq. (2) to obtain from data of Fig. 1 a new $K_X$ estimate. The procedure was iterated to obtain the definitive values for the three parameters.

All the regression of the above described fitting procedure were performed making use of a data analysis software, Systat Sigmaplot©, which automatically generates the confidence intervals for the estimated parameters. Highest values of the confidence intervals obtained for each parameter in the various regressions of the fitting procedure were used to calculate the lower and upper limits of the parameters quoted in Table 3.

Values for the pure buffer kinetics, $V_{max} = 0.12\,\mu mol\,l^{-1}\,s^{-1}$ and $K_S = 0.61\,mM$, were evaluated in kinetic runs performed in pure buffer.

The estimated parameters were used to build the solid lines in Figs. 1 and 2 that show how the model describes the experimental behaviour with good accuracy. The higher discrepancy was found for TMABr and TEABr data, because of the very little effect of these additives.

Values of the kinetic parameters are quoted in Table 3. The parameter $\alpha$ depicts the effect of the activator on the enzyme–substrate affinity while $\beta$ describes the effect on the turnover number. Finally, $K_X$ is an association constant that describes the affinity between the enzyme and the activator.

Table 3
Parameters for the kinetic model

| Salt | $\alpha$ | $\beta$ | $K_X$ (M) |
|---|---|---|---|
| TMABr | 2.05 | 1.59 | 0.198 |
| Upper limit | 2.15 | 1.68 | 0.211 |
| Lower limit | 1.95 | 1.50 | 0.186 |
| TEABr | 4.76 | 3.56 | 0.121 |
| Upper limit | 4.99 | 3.82 | 0.134 |
| Lower limit | 4.53 | 3.29 | 0.108 |
| TPABr | 7.08 | 10.70 | 0.076 |
| Upper limit | 7.19 | 11.08 | 0.079 |
| Lower limit | 6.98 | 10.32 | 0.073 |
| TBABr | 9.87 | 33.75 | 0.061 |
| Upper limit | 10.17 | 35.33 | 0.063 |
| Lower limit | 9.57 | 32.17 | 0.059 |
| TPeABr | 10.47 | 66.23 | 0.062 |
| Upper limit | 11.46 | 70.90 | 0.066 |
| Lower limit | 9.48 | 61.56 | 0.058 |

Inspection of data in Table 3 allows the following conclusions. The enzyme–substrate affinity decreases in the presence of the ammonium quaternary salts, being $\alpha > 1$. The higher the molecular weight of the alkyl residues in the salt, the lower the affinity.

In the presence of the activators, the turnover number is always higher than in pure buffer ($\beta > 1$), and it increases in the series TMABr < TEABr < TPABr < TBABr < TPeABr.

The superactivity exhibited by the enzyme in the presence of the tetra-alkylammonium salts is explained because $\beta$ is always higher than unity.

The affinity of the enzyme for the activator also depends on the alkyl group present in the $(NR_4)^+$ cations: the higher the chain length of the R-group, the lower the affinity (lower $K_X$). This last result suggests that hydrophobic effects improve the catalytic efficiency, but, on the other hand, reduce the ability of the activator to interact with the enzyme molecule.

In Fig. 4, the estimated kinetic parameters were plotted as a function of the number of methylene units of the tetra-alkylammonium bromides. The non-linear dependence of $\beta$ and $K_X$ suggests that the methyl/methylene groups adjacent to the nitrogen atom are not as effective as the subsequent methylene groups in higher homologues.

This seems to indicate that the role played by hydrophobic and electric interaction of $(NR_4)^+$ with the enzyme molecule and its active site strongly depends on how the electric charge is shielded, due to the higher chain length of the alkyl groups: the higher the electric charge is shielded, the lower the affinity protein for the salt (lower $K_X$).

On these basis, it was hypothesised that the ionic conductivity in water of the tetra-alkylammonium salts could result to be a good parameter to correlate our experimental results; indeed it usually depends on both the electric charge and the chemical structure of the cations. In our case, the higher

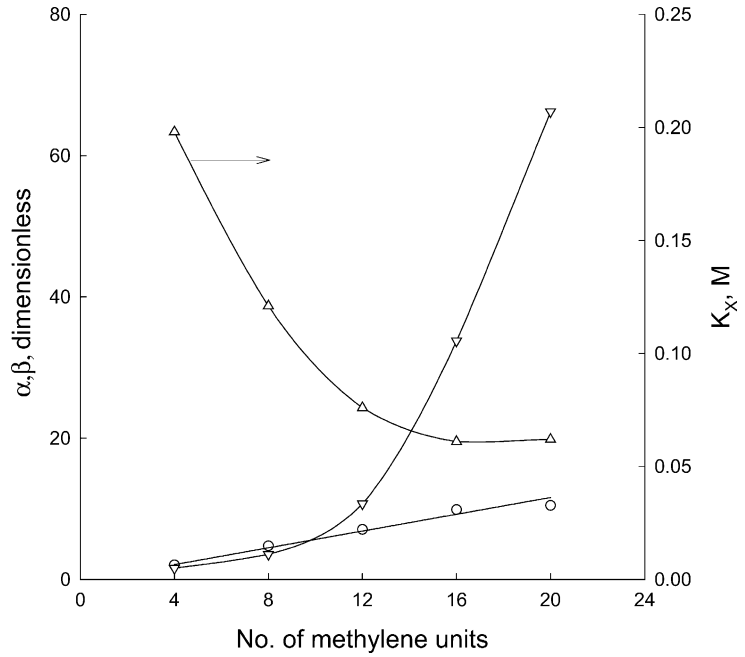


Fig. 4. Model parameters as a function of the number of methylene units of tetra-alkylammonium salts. (○) = $\alpha$, (▽) = $\beta$ and (△) = $K_X$.

the molecular weight of the R-group, the more the electric charge is shielded, and the lower the ionic conductivity.

For this reason, the secondary-order kinetic parameter $k_{P,app}/K_{S,app}$ was reported in Fig. 5 as a function of the ionic conductivity of the cationic additives [28]. As it can be seen,

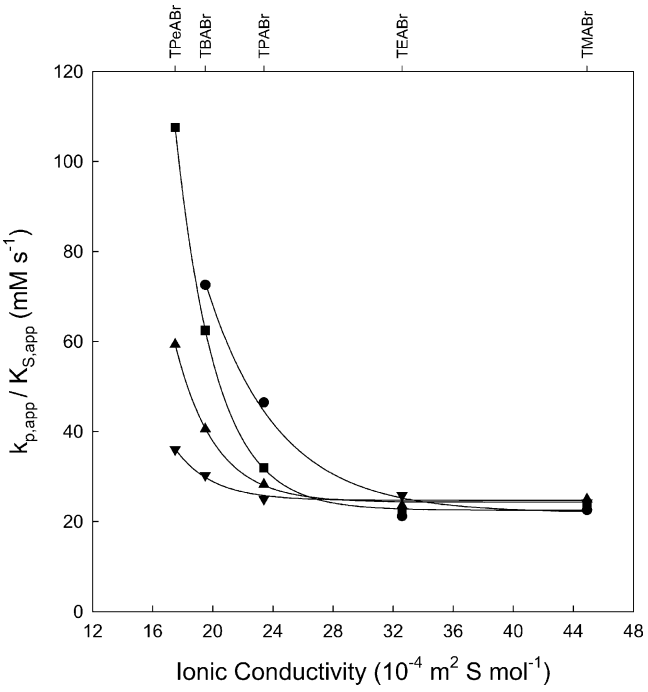


Fig. 5. Secondary-order kinetic parameter as a function of the ionic conductivity of the quaternary ammonium salts. [$X$] = 0.2 M (●); 0.1 M (■); 0.05 M (▲) and 0.02 M. (▼) Reaction conditions: $T$ = 25 °C, pH 7.75, [Tris–HCl] = 0.1 M, [GpNA] = 2.5 mM, [α-CT] = 8 μM.

an exponential decay occurs as the conductivity increases. Such a behaviour confirms that in the compromise between a lower enzyme-salt affinity, and a higher induced activity, the latter effect prevails when the hydrophobic interactions are greatly favoured (high alkyl chain length, low ionic conductivity).

### 3.4. Effect of the salts on α-CT storage stability

The stability of α-CT was assessed in experiments performed at different temperatures (25, 35 and 45 °C) in pure buffer aqueous medium. At time intervals, the α-CT exposed to the denaturing conditions, in the absence of GpNA, was assayed to measure the initial reaction rate. The time course of the residual activity ($A/A_0$ activity ratio) is plotted in Fig. 6. As expected, the higher the temperature, the higher the activity loss with time.

As more rapid deactivation occurs at 45 °C, we studied the effect of the ammonium salts at this temperature. The enzyme was stored at the selected temperature in the presence of the appropriate buffer and at 0.1 M salt concentration. The initial reaction rate of GpNA hydrolysis was assayed at defined time intervals.

Several deactivation models are available in the literature to explain enzyme activity decay with time. The activity loss of α-CT solutions is more complex, because α-CT also catalyses its own hydrolysis.

With the only purpose of describing the obtained experimental results, we have tentatively adopted a model proposed by Kawamura *et al.* [29]. The authors proposed a mechanism consisting of two reactions, hereafter described. The first reaction that is the only one evident at low temperature, is a monomolecular deactivation:

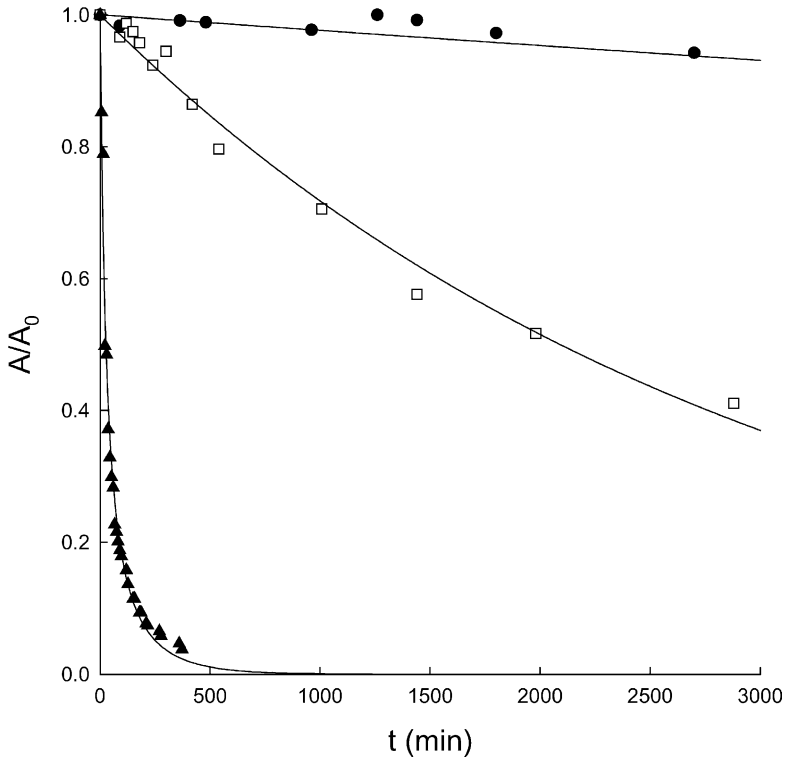


Fig. 6. Time course of α-CT deactivation at (●) 25 °C, (□) 35 °C and (▲) 45 °C. Conditions of the activity assay: $T = 25$, 35 and 45 °C, respectively, pH 7.75, [Tris–HCl] = 0.1 M, [GpNA] = 2.5 mM, [α-CT] = 8 μM.

$$E_a \overset{K}{\rightleftharpoons} E_i \overset{k_1}{\rightarrow} E' \tag{5}$$

where $E_a$ and $E_i$ are active and inactive forms, $E'$ is the denaturated form (random coil), $K = [E_i]/[E_a]$ is an equilibrium constant and $k_1$ is a denaturation constant.

The second reaction (an auto-digestion step) is more evident at higher temperature, due to the higher enzyme activity. Kawamura *et al.* assumed that the reversibly inactivated form $E_i$ is the one that is susceptible to be attacked by the protease form $E_a$, with a Michaelis–Menten type mechanism:

$$E_a + E_i \overset{k_1}{\rightleftharpoons} E_aE_i \overset{k_2}{\rightarrow} E_a + \text{products} \tag{6}$$

where $K_m = [E_a][E_i]/[E_aE_i]$, $k_2$ is the rate constant of the digestion reaction, which leads to the decomposed products. Integration of the model equations gives:

$$\frac{A}{A_0} = \frac{k_1'}{k_1' + k_2'[E]_0 e^{k_1' t} - k_2'[E]_0} \tag{7}$$

where $A$ and $A_0$ are the activity evaluated at time $t$ and 0, respectively, $k_1' = k_1 K/(1 + K)$ and $k_2' = k_2 K/[K_m(1 + K)^2]$.

Fig. 6 shows how the model applies to our experiments: solid lines obtained by Eq. (7) well fit in the experimental data. Values for $k_1'$ and $k_2'$ are quoted in Table 4. The second reaction is evidenced only at higher temperature (45 °C).

Table 4
Parameters for the deactivation process

| System | $T$ (°C) | $10^3 \times k_1'$ ($s^{-1}$) | $k_2'$ ($l\,g_{cat}^{-1}\,s^{-1}$) |
|---|---|---|---|
| α-CT | 25 | 0.03 | – |
| α-CT | 35 | 0.33 | – |
| α-CT | 45 | 2.04 | 0.21 |
| α-CT + TMABr | 45 | 1.50 | 0.07 |
| α-CT + TEABr | 45 | 2.77 | 0.12 |
| α-CT + TPABr | 45 | 2.58 | 0.21 |
| α-CT + TBABr | 45 | 21.30 | 0.27 |

Similarly, the effect of the cationic additives on the enzyme deactivation was investigated. A series of experiments was performed at 45 °C and at fixed salt concentration of 0.1 M. Fig. 7 reports the results obtained in these experiments.

An interesting feature of the presence of the salts in the storage medium is the possibility to modulate α-CT stability. Indeed, the ability of the additive of either stabilise or destabilise the protein appears to depend on the alkyl chain length of the R-group in the tetra-alkylammonium salt: the longer the chain, the lower the stability.

Similar results were found by Jain *et al.* when studying the effect of tetra-alkylammonium halides on lysozyme [30]. The authors attribute this effect to the hydrophobic interactions of the salt with the non-polar groups of the protein, which is, substantially, the same hypothesis we

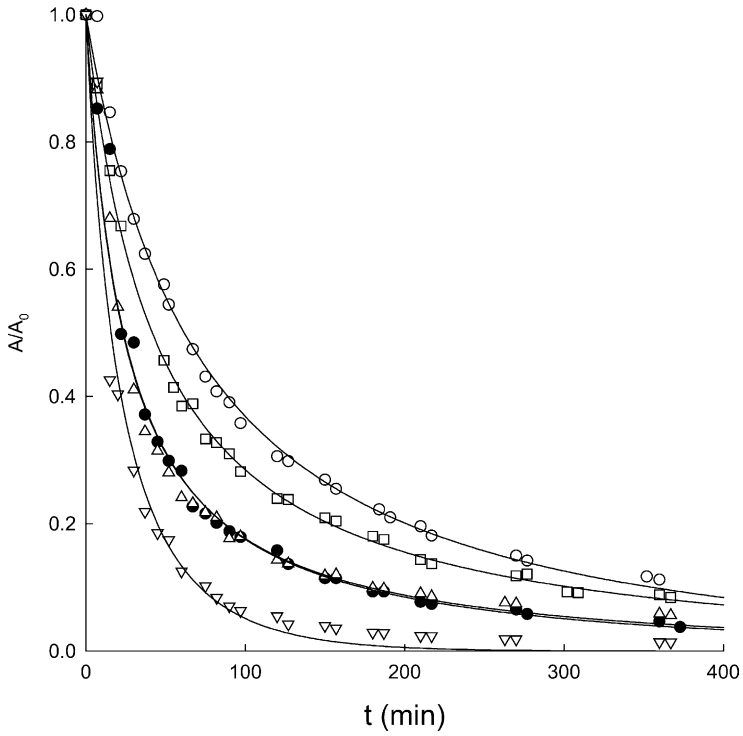


Fig. 7. Time course of α-CT deactivation in the presence of: no salt (●); TBABr (▽); TPABr (△); TEABr (□); TMABr (○) at 45 °C. Conditions of the assay activity: $T = 45\,^{\circ}C$, pH 7.75, [Tris–HCl] = 0.1 M, [GpNA] = 2.5 mM, [α-CT] = 8 μM, $[X] = 0.1$ mM.

have explored to explain the superactivity of α-CT in the presence of these additives.

Fig. 7 clearly shows that both TMABr and TEABr stabilise at different extent the enzyme. Unfortunately, both these salts are unable to induce α-CT superactivity. On the other hand, TBABr, which gives the highest superactivity, accelerates at the same time the deactivation process. Finally, TPABr seems to have a negligible effect on α-CT stability.

These results were also investigated with the model Eq. (7), as in the case of pure α-CT, the solid lines of Fig. 7 were computed and the fit resulted quite satisfactory. The corresponding parameters are also quoted in Table 4: $k'_1$ strongly increases in the series: TMABr < TEABr < TPABr < TBABr. On the other hand, $k'_2$ is scarcely affected by the presence of the additive, even if a similar trend as $k'_1$ might be suggested.

For TMABr and TEABr, $k'_1$ is lower than that measured in pure buffer, suggesting that these salts are able to slow down the irreversible thermal deactivation of the enzyme.

In the case of TPABr, the auto-digestion seems to be slightly favoured, probably because of the superactivity effect. The irreversible deactivation occurs at a lower velocity as compared with pure α-CT. The global effect is that the overall deactivation process is similar to that of the pure enzyme.

As far as TBABr is concerned, the whole deactivation phenomenon is strongly accelerated by the presence of the salt, and the deactivation constants are consequently increased.

It has to be observed that the ionic strength of the storage solution of the experiments carried out without additives (Fig. 6) was lower than in the presence of the ammonium salts (Fig. 7) and this can have a considerable effect on the enzyme deactivation.

Preliminary results of experiments performed with non-quaternary salts (data not shown) indicate that α-CT thermal stability depends on the ionic strength of the storage medium, while hydrophobic interactions contribute to deactivate the enzyme.

On these basis, we can hypothesise that the stabilising effect of the cationic salts results from a compromise between the positive effect of the higher ionic strength and the destabilising effect due to hydrophobic interactions with the cations. This hypothesis needs further investigations and will be discussed in a forthcoming paper.

As far as the quaternary ammonium salts are concerned, our experimental results indicate that, from an operational point of view, a good compromise between the superactivity and the denaturing effect might be represented by TPABr that, under the experimental conditions here adopted, induces superactivity, but, at the same time, do not significantly affect the velocity of the deactivation process.

## 4. Conclusions

The tetra-alkylammonium bromide salts were found to affect the activity of α-CT in the hydrolysis of GpNA. Under

the diluted conditions adopted, these salts are dissociated in the solution, so their effect has to be related to the presence of tetra-alkylammonium cations in the reaction medium. These cations seem to act as activator for the enzyme. The superactivity effect depends on the molecular weight of the alkyl residues present in the cationic group: the higher the molecular weight, the higher the activity.

This result is quite similar to that obtained with an homologue series of cetyltrialkylammonium bromide cationic surfactants.

The superactivity in the salted systems increases monotonically with the additive concentration. On the contrary, in the case of the cationic surfactants, typical bell-shaped curved were obtained, indicating that a segregation of the substrate occurred in the system. The main difference between the two similar series of additives being that the salt has no amphiphilic behaviour, so that no partition of the substrate occurs.

A non-essential activator model was able to describe the dependence of the enzyme activity on the salt concentration. The estimated model parameters can be correlated with the ionic conductivity of the cations, suggesting that both electric and hydrophobic interactions could be responsible of the enzyme superactivity in the presence of these additives.

These molecules also affect the storage stability of the enzyme: higher molecular weight of alkyl residues of the cations induce a lower stability, so that the more efficient additive, TBABr, also gives rise to a more rapid enzyme deactivation.

The best compromise is represented by TPABr that causes a lower superactivity but essentially does not affect the velocity of α-CT deactivation.

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