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ESI-MS in the study of the activity of α-chymotrypsin in aqueous surfactant media †

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The catalytic activity of α-chymotrypsin on a model and a peptide substrate, in the supramolecular system "enzyme–surfactant" in water solution, has been studied by electrospray ionization mass spectrometry. Hydrolysis of *N*-succinyl-L-phenylalanine *p*-nitroanilide as the model compound, catalysed by α-chymotrypsin in the presence of monomeric cetyltributylammonium bromide, has been followed by UV and ESI-MS detection. Kinetic data, which are essentially identical independent of their determination techniques, show a twelve fold improvement of the enzyme catalytic efficiency when compared with the reaction carried out in the absence of the additive. Once validated, the ESI-MS technique was used to study the hydrolytic activity of the enzyme on a peptide substrate like substance P; it is worth emphasising that the spectrophotometric detection cannot be employed on peptides, where the chromophores are untouched by the hydrolytic process. Substance P hydrolyses in aqueous surfactant following dichotomic kinetics, which are initially rapid but then slow down as the reaction progress. The results presented in this paper are expected to extend studies on biocatalysis in aqueous surfactant media to a wide range of substrates, independent of their spectroscopic properties.

Introduction

Enzymes have been widely used as catalysts in organic synthesis and several methods have been developed to carry out bioconversions in organic media.[1] However, many organic solvents lead to inactivation of the enzyme, its stabilization being thus an important goal in biocatalysis. In general, interactions between an enzyme molecule and the surrounding water are of crucial significance for enzymatic catalysis but the use of the water as solvent in biocatalysis has always represented a problem, because of the general low solubility of the reagents in this reaction medium as well as of the reaction products.

On the other hand, aqueous surfactant media can provide an aqueous phase for hydrolytic enzymes and an organic phase for hydrophobic substrates and products. Moreover, examples of the stabilization and/or superactivation of enzymes in water solutions, in the presence of functionalized synthetic surfactants, have recently been reported.[2] Among these, the superactivity of α-chymotrypsin (from now on referred to as α-CT) in water, in the presence of cetyltributylammonium bromide (CTBABr) is well documented.[3,4] The reaction progress, in all the cases reported, is followed by UV-Vis spectroscopy by using model substrates which have been selected according to their spectroscopic properties.

In this paper, we wish to report on the study of the catalytic activity of α-chymotrypsin in the supramolecular system "enzyme–surfactant", in water solution, on substrates such as amides and peptides, following the reaction progress by electrospray ionization mass spectrometry (ESI-MS). In the case of compounds, which after enzymatic digestion preserve the chromophore moieties unchanged, UV-Vis detection cannot be used to register directly the reaction progress. In these cases, spectroscopic methods require substrates modified by chromogenic agents. Such substrate modifications, however, often necessitate multistep chemical syntheses and could lead to altered enzyme kinetics.[5]

ESI-MS plays an important role in biological applications: it allows the primary structure determination of proteins and other biomolecules,[6,7] the elucidation of protein folding[8,9] and the detection of protein–protein interactions.[10] In recent years, ESI-MS has also been used for monitoring the kinetics of enzymatic reactions;[11–15] it is an accurate, rapid and sensitive tool to follow, in real time, the change of the concentrations of the substrate as well as of the products during the progress of the hydrolytic reaction.

We will show that ESI-MS can be employed with success as a tool to detect α-CT activity in water in the presence of a surfactant medium. Kinetic data are totally in agreement with those obtained by UV-Vis spectroscopy, at least in cases where this technique is applicable. Moreover, the presumed superactivation of the enzyme in water, promoted by the synthetic surfactant, is for the first time partially verified in its reaction against a natural substrate like the neuropeptide substance P.[16]

Results and discussion

The need for a model to validate, in an independent way, the ESI-MS data became mandatory during early experiments. As a preliminary study the activity of α-chymotrypsin, both without and with the presence of CTBABr, was checked on a reference substrate, the hydrolysis of which was followed by ESI-MS as well as by UV detection.

In previous papers,[3,4,17] *N*-glutaryl-L-phenylalanine *p*-nitroanilide (GPNA) was used as a model substrate and the effect of different ionic surfactants and additives, along with their concentration, was monitored on α-CT activity and stability. GPNA is poorly soluble in aqueous ammonium acetate, a volatile salt commonly used in the ESI-MS technique; *N*-succinyl-L-phenylalanine *p*-nitroanilide (SPNA) was thus selected for the MS experiments, as a more soluble model substrate. Nevertheless, 1% CH_3CN had to be added in order to improve substrate solubility.

SPNA is a substrate for α-CT; moreover, the anilide itself, as well as the product of the hydrolytic reaction, gives abundant

† Dedicated to Professor D. Spinelli on the occasion of his 70th birthday.

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Table 1 Kinetic constants for α-chymotrypsin at 25.0 °C

Reaction medium	$10^4 K_M$/M	k_{cat}/s^{-1}	(k_{cat}/K_M)/M^{-1} s^{-1}
0.05 M Tris-HCl pH 8.0[a]	9.10	16.0×10^{-3}	17.60
0.015 M Ammonium acetate[b]	7.30	5.4×10^{-3}	7.40
3×10^{-5} M CTBABr in 0.015 M ammonium acetate[b]	0.51	8.3×10^{-3}	163.33

[a] Ref. 18. [b] 1% CH_3CN.

[M + 1] ions under +ve ESI conditions. As to its spectroscopic characteristics there is, also, an evident bathochromic shift of the absorbance of the aromatic moiety after hydrolysis. *p*-Nitroaniline (PNA), one of the two reaction products, shows in fact a maximum UV absorbance at 410 nm, while SPNA has its maximum at 328 nm.

As reported in ref. 4, α-CT superactivity is observed both in monomeric and micellised CTBABr; positive enzyme–surfactant interactions taking place independent of the supramolecular organization of the medium. The large activity improvement induced by CTBABr was thus explained by the favourable interaction of the enzyme with the surfactant monomers. α-CT activity was then investigated at 3×10^{-5} M CTBABr, a concentration slightly lower than the cmc (cmc = 3.2×10^{-5} M), SPNA concentration being 1×10^{-4} M, as calculated by the K_S definition (see experimental: K_S = 3000 M^{-1}). The critical micelle concentration (cmc) and SPNA binding constant (K_S) were determined in 1% CH_3CN ammonium acetate.

Kinetic parameters were also determined both in the presence and absence of CTBABr, and compared with the literature data[18] (Table 1). All our data were obtained by following the reaction by UV detection (see experimental).

The data from the tris buffer and ammonium acetate experiments show that the K_M values are quite similar, while k_{cat} in the case of ammonium acetate decreases to a noticeable extent; consequently, the enzyme efficiency (k_{cat}/K_M) was quite lowered. This result could be explained taking into account that α-CT is very sensitive to the buffer nature and concentration;[3,19,20] the presence of the aprotic water-miscible organic solvent can also perturb the catalytically active conformation of the protein. Moreover, the presence of the monomeric surfactant caused a large increase in the α-CT–SPNA affinity (lower K_M) together with a higher k_{cat} value: a large superactivity (22-fold) in 3×10^{-5} M CTBABr was thus revealed.

In order to clarify the role of the salt and CH_3CN on α-CT activity, the effect of their concentrations on SPNA hydrolysis rate in the presence and absence of CTBABr was investigated. Fig. 1 reports the dependence of the SPNA hydrolysis rate on ammonium acetate concentration, ranging from 0.015 M to 0.2 M. In pure ammonium acetate, an increase in its concentration brought about a slight increase in the reaction rate, according to the literature.[3,19,20] On the contrary, in CTBABr solutions, the SPNA hydrolysis rate dropped with a non-linear trend as salt concentration increased: the superactivity, which occurred at 0.015 M ammonium acetate, was annihilated when a 0.2 M value was reached. The decrease of the reaction rate with increasing salt concentration can be attributed to interactions between the salt itself and the surfactant.[3] Ammonium acetate could shield the positively charged surfactant head group while the methyl group of the acetate anion could interact with the hydrophobic region of the surfactant molecule. Therefore, the favourable interactions between enzyme and surfactant, that induce superactivity, were altered by the presence of a high salt concentration.

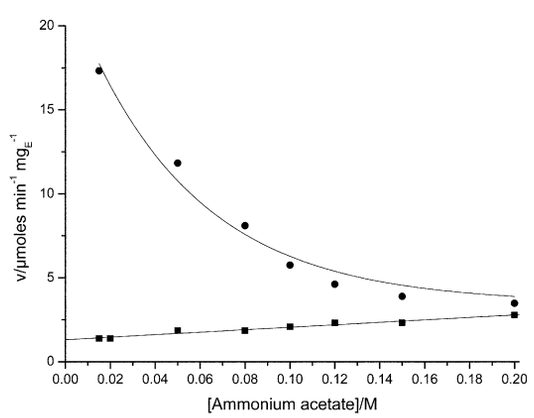

Fig. 1 Effect of ammonium acetate concentration on SPNA hydrolysis rate in 0.015 M ammonium acetate, pH 8 at 25.0 °C. [α-CT] = 0.2 mg mL^{-1}, [SPNA] = 1×10^{-4} M. (■) Pure ammonium acetate and (●) in 3×10^{-5} M CTBABr.

α-CT activity as a function of the percentage of CH_3CN added to the reaction medium is reported in Fig. 2. Both in the absence and in the presence of CTBABr, a decrease in reaction rate was observed; when the percentage of CH_3CN was equal to 20% no reaction occurred.

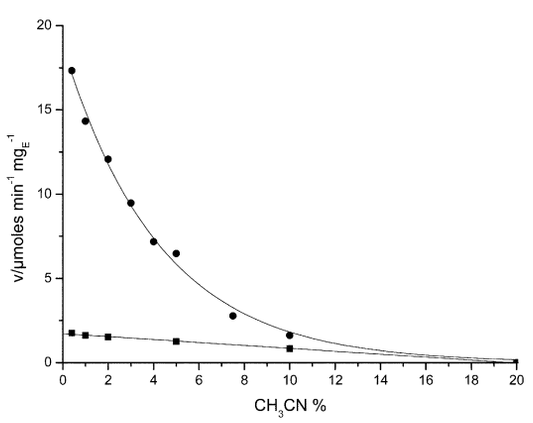

Fig. 2 Effect of CH_3CN percentage on SPNA hydrolysis rate in 0.015 M ammonium acetate, pH 8 at 25.0 °C. [α-CT] = 0.2 mg mL^{-1}, [SPNA] = 1×10^{-4} M. (■) Pure ammonium acetate and (●) in 3×10^{-5} M CTBABr.

Kinetic parameters were then determined in 5% CH_3CN (Table 2). The comparison with the data in 1% CH_3CN (Table 1) show that K_M values are not greatly affected by the increase of the added co-solvent and hence the decrease in α-CT activity is almost entirely due to a reduction of the k_{cat} values. The presence of CH_3CN does not modify the enzyme–substrate interaction, since their affinity is almost unchanged; it follows that the reaction rate decrease is probably due to a progressive protein denaturation induced by the higher solvent concentration. In fact, the effects of dipolar organic solvents on the enzyme activity are generally attributed to alterations of various non-covalent interactions in the protein, including solvations of ionic groups and dipoles, hydrogen bonding and hydrophobic interactions.[21–23] These alterations can end up in the modification of the tertiary structure of the protein to produce a variable degree of denaturation.

Having then set the reaction conditions for ESI-MS experiments and with a full knowledge of the kinetic parameters, we switched to mass spectrometry to follow the kinetics of the reaction, performed in ammonium acetate alone and in the presence of 3×10^{-5} M CTBABr both doped with 1% CH_3CN.

Table 2 Kinetic constants for α-chymotrypsin in the presence of 5% CH_3CN at 25.0 °C

Reaction medium	$10^4 K_M$/M	k_{cat}/s^{-1}	(k_{cat}/K_M)/M^{-1} s^{-1}
0.015 M Ammonium acetate	8.70	3.7×10^{-3}	4.3
3×10^{-5} M CTBABr in 0.015 M ammonium acetate	0.72	5.0×10^{-3}	69.0

FIA (flow injection analysis) was used for sample introduction, and SIM (selected ion monitoring) to acquire data as mass peak intensities. In particular, the ion corresponding to protonated PNA was selected to quantitatively monitor the α-CT activity since it shows an abundant peak at *m*/*z* 139.

SIM was used to generate a calibration curve for PNA (Figs. 3A, and 4A): an excellent correlation between concentration and ion peak intensity was obtained (r^2 = 0.999 in pure ammonium acetate, and r^2 = 0.996 in CTBABr solutions). Ion intensities of the analytes, throughout the experiments, are obviously affected by the presence of the surfactant; this phenomenon appears to be completely controlled by building up the calibration curves themselves, thus ensuring the complete reliability of the different experiments performed. The hydrolysis of SPNA was then performed at the same experimental conditions used for the spectrophotometric analysis: the ESI-MS results are shown in Figs. 3B and 4B. The change in intensity of the peak relevant to the product ion was determined as a function of time by repeated injections of the reaction mixture in the MS instrument, at increasing hydrolysis times. Data were then treated to give a measure of the reaction progress under both the reaction conditions. The initial rates were then measured and the results compared with those obtained in parallel by UV-Vis spectroscopy, both in the absence and in the presence of CTBABr. Data are reported in Table 3.

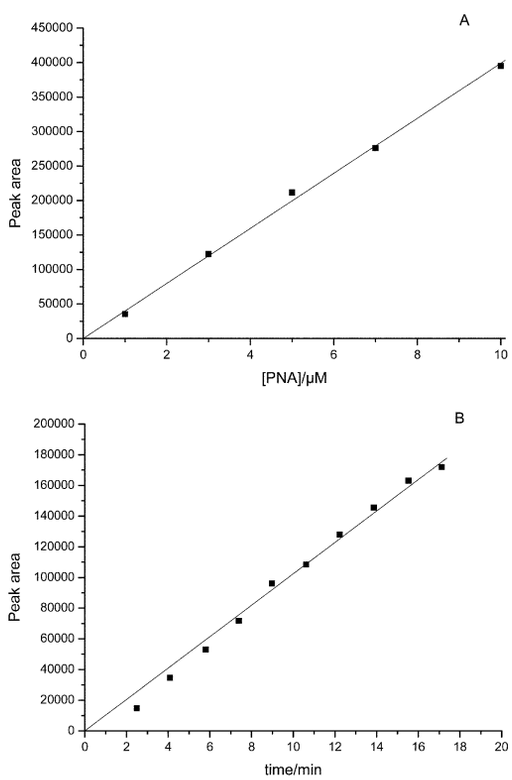

Fig. 3 ESI-MS Plots; SIM of *m*/*z* 139 (protonated PNA): calibration curve for PNA (A) and kinetic plot for the hydrolysis of SPNA (B) in 0.015 M ammonium acetate, pH 8. [α-CT] = 0.2 mg mL^{-1}, [SPNA] = 1×10^{-4} M, 1% CH_3CN.

Despite the change of the experimental conditions, *i.e.* the buffer/salt (tris or ammonium acetate) and the substrate used, and the addition of the organic solvent, we have confirmed the

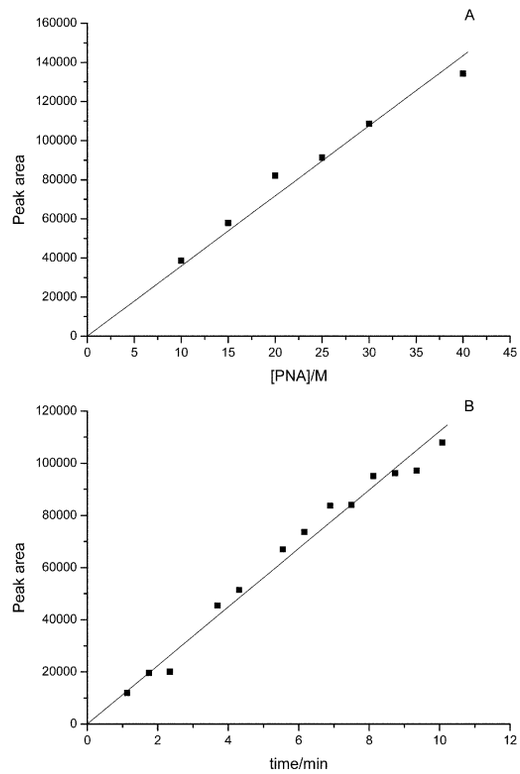

Fig. 4 ESI-MS Plots; SIM of *m*/*z* 139 (protonated PNA): calibration curve for PNA (A) and kinetic plot for the hydrolysis of SPNA (B) in 0.015 M ammonium acetate, pH 8 in the presence of 3×10^{-5} M CTBABr. [α-CT] = 0.2 mg mL^{-1}, [SPNA] = 1×10^{-4} M, 1% CH_3CN.

Table 3 Comparison of the initial reaction rates obtained by UV-Vis and ESI-MS monitoring in 0.015 M ammonium acetate alone and in the presence of 3×10^{-5} M CTBABr

	Reaction rate/μM min^{-1}	
	UV-VIS	ESI-MS
Kinetics in ammonium acetate	0.258	0.259
Kinetics in surfactant solution	3.330	3.130

positive interactions between α-CT and CTBABr that induce superactivity.[4] Moreover, the results obtained by UV-Vis spectroscopy and ESI-MS are practically identical; it can be thus concluded that the ESI-MS technique can be efficiently used to follow an enzymatic hydrolytic reaction.

The necessary extension of the ESI-MS methodology was to study the kinetics of the enzymatic digestion of a real substrate. We chose the neuroundecapeptide substance P, a widely studied substrate already investigated in our laboratories by following its partial acid hydrolysis by mass spectrometry.[24,25] It is cleaved by α-CT at the level of the aromatic amino acids. Five fragments are formed, as listed in Table 4; the calculated *m*/*z* values, relevant to single and doubly protonated molecular ions, along with the amino acid sequence, are also reported.

The ESI mass spectrum of substance P reveals the presence of the single and doubly protonated molecular ion at *m*/*z* 1348.7 and 674.9 respectively. Since the latter peak is more intense than the former one, we decided to monitor the intensity of this peak when following the kinetics of the α-CT digestion of the peptide.

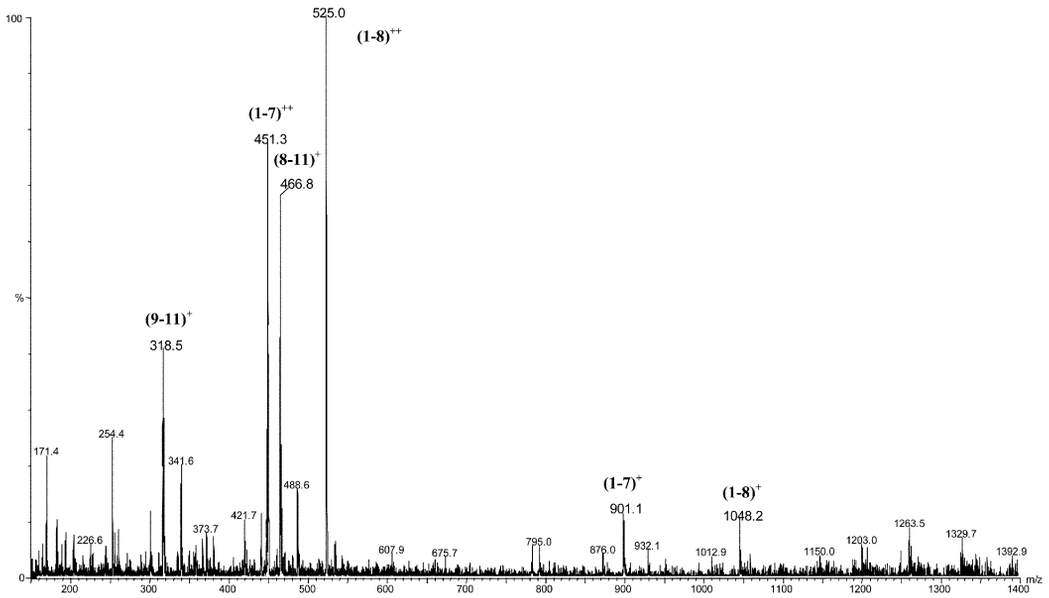

Fig. 5 ESI-Mass spectrum of the hydrolytic products of substance P.

Table 4 Hydrolytic fragments of substance P along with their single and doubly protonated molecular ions

		m/z	
Fragments	Sequence	$(M + H)^+$	$(M + 2H)^{++}$
8	H-Phe-OH	166.1	83.6
9–11	H-Gly-Leu-Met-OH	320.2	160.6
8–11	H-Phe-Gly-Leu-Met-OH	467.2	234.1
1–7	H-Arg-Pro-Lys-Pro-Gln-Gln-Phe-OH	900.5	450.8
1–8	H-Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-OH	1047.6	524.3
Substance P	H-Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-OH	1348.7	674.9

The hydrolysis of substance P was initially carried out both with and without CTBABr, following the same experimental conditions used for the model substrate. A few minutes after the addition of the enzyme, the peak relevant to the doubly protonated substrate had disappeared while the fragment peaks were easily recognizable. The mass spectrum of the products is reported in Fig. 5.

The high hydrolysis rate is probably due to a high enzyme–substrate affinity; thus, to adequately follow the substrate consumption, the enzyme concentration was 100-fold decreased. A calibration curve was performed both in ammonium acetate and in surfactant solutions (Figs. 6A and 7A); the correlation coefficients between the substrate concentration and its ion peak intensity were 0.996 and 0.997 respectively. Kinetics were then performed; experiments were carried out in triplicate, in order to ensure their reproducibility. Figs. 6B and 7B report the change in abundance of substance P as a function of time in pure ammonium acetate and in 3×10^{-5} M CTBABr, respectively. The reaction rate values are reported in Table 5. In the absence of surfactant, a linear decrease of the substrate concentration is observed; conversely, in the presence of CTBABr, a 64% enhancement of the enzyme activity is observed only at the very beginning of the reaction progress, while the reaction kinetic rapidly slows down with time, becoming even lower with respect to the reference reaction.

α-CT efficiency strongly depends on the substrate used. In general, substrates with an extended peptide chain show a low K_M value, since the enzyme contains an extended binding site for peptide substrates.[26,27] The hydrolysis of a more sensitive (low K_M and high k_{cat}) synthetic substrate, the tetrapeptide succinyl-L-Ala-L-Ala-L-Pro-L-Phe-*p*-nitroanilide, was studied in our laboratories to evaluate the role of the substrate on surfactant-induced enzyme activation:[28] only a 73% increase in k_{cat} was observed thus showing that CTBABr did not significantly affect the high enzyme–substrate affinity. Conversely, the α-CT efficiency observed for GPNA hydrolysis in the presence of monomeric CTBABr was enhanced by about 20-fold.[4] It can be concluded that superactivity induced by CTBABr depends on the substrate, the higher the enzyme–substrate affinity the lower being the activating effect of the surfactant.

Table 5 Comparison of the initial reaction rates obtained by ESI-MS monitoring in ammonium acetate alone and in the presence of 3×10^{-5} M CTBABr

	Reaction rate/μM min^{-1}
Kinetics in ammonium acetate	4.11
Kinetics in surfactant solution	6.75[a]
	1.25[b]

[a] 0–3 min. [b] 3–11 min.

Coming back to substance P hydrolysis in the presence of CTBABr, the increase in the enzyme activity observed at the early stage of the reaction is very similar to that obtained with the model tetrapeptide substrate. The subsequent abrupt decrease in substrate hydrolysis can be explained taking into consideration the high product concentration which could inhibit the reaction progress. This possibility was verified by following substance P hydrolysis by HPLC (under the same reaction conditions), by monitoring the substrate consumption and the lower peptide formation by UV detection, at the backbone absorption at 214 nm. Actually, the reaction rate slows down after three minutes, thus exhibiting a feedback inhibition by product formation. It is worth considering, however, that the HPLC detection appears extremely time consuming as compared to the ESI-MS method.

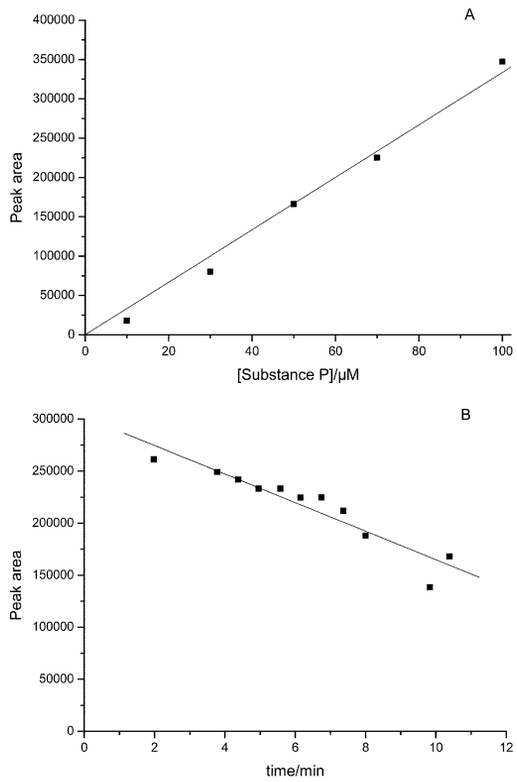

Fig. 6 ESI-MS Plots; SIM of *m*/*z* 674.9 (doubly protonated substance P): calibration curve for substance P (A) and kinetic plot for the hydrolysis of substance P (B) in 0.015 M ammonium acetate, pH 8. [α-CT] = 2 μg mL^{-1}, [Substance P] = 1×10^{-4} M.

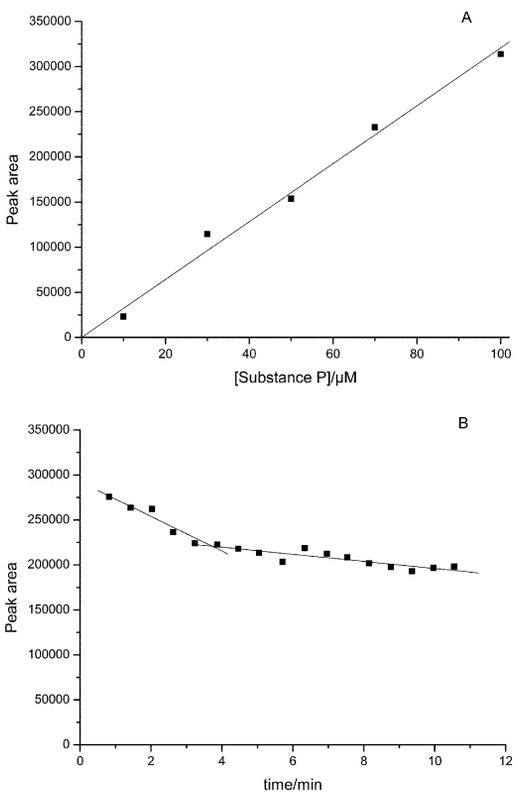

Fig. 7 ESI-MS Plots; SIM of *m*/*z* 674.9 doubly protonated substance P): calibration curve for substance P (A) and kinetic plot for the hydrolysis of substance P (B) in 0.015 M ammonium acetate, pH 8 in the presence of 3×10^{-5} M CTBABr. [α-CT] = 2 μg mL^{-1}, [Substance P] = 1×10^{-4} M.

Conclusions

As far as the activity of α-chymotrypsin in water is concerned at the presence of a surface active compound, it can be concluded that superactivity itself is not only related to the enzyme–surfactant interaction; it strongly depends, in fact, also on the nature of the substrate itself.

We have used SPNA as a model substrate, which can be considered typical "organical" in nature; in this case α-CT–surfactant cooperativeness is clearly observable. When using a more "biological" substrate, like the neuropeptide substance P, a much more complicate interaction amongst the three reaction actors (substrate, α-CT and surfactant) is to be conceived.

We also emphasize that our ESI-MS investigation on the enzyme activity can contribute to extend the field of application of ESI-MS to the study of biocatalysis in sustainable organic reactions, carried out in water.

Experimental

Materials

α-Chymotrypsin (α-CT) from bovine pancreas (EC 3.4.21.1, type II, MW 24.8 KDa, pI 8.8) and the substrates, *N*-succinyl-L-phenylalanine *p*-nitroanilide (SPNA) and substance P, were purchased from Sigma and used without further purifications. Enzyme and substrate solutions were freshly prepared immediately before their use. Ammonium acetate was from Aldrich. The preparation and purification at laboratory scale of the synthesized surfactant, cetyltributylammonium bromide (CTBABr), have already been described.[29] CTBABr was chemically pure as tested by spectroscopic and elemental analysis, and the absence of minima in surface tension *vs.* concentration plots excluded the presence of hydrophobic impurities.

α-Chymotrypsin activity assay

The hydrolytic activity of α-CT toward SPNA was monitored by following the increase in absorbance at 410 nm due to the formation of *p*-nitroaniline (PNA). Kinetic determinations were measured at 25.0 °C, using a Shimadzu UV-160A UV-Vis spectrophotometer equipped with a thermostated cell (3 mL volume and 1 cm pathlength). The product extinction coefficient was 10 800 M^{-1} cm^{-1}, either in ammonium acetate or in the presence of CTBABr.

α-CT activity was typically assayed in 0.015 M ammonium acetate, taken at pH 8 with a few drops of diluted ammonium hydroxide, with 1×10^{-4} M SPNA (solubilized in ammonium acetate plus CH_3CN) and 0.2 mg mL^{-1} (8 μM) enzyme. The reaction was started by adding 60 μL of α-chymotrypsin stock solution (10 mg mL^{-1}) to a thermostated solution of the substrate. PNA formation was recorded as a function of time (spontaneous hydrolysis of SPNA did not occur during the kinetic tests). The specific reaction rate, *v*, defined as moles of PNA formed per unit of time and mass of enzyme, was calculated from the slope of initial linear curve of PNA concentration *vs.* time. The Michaelis–Menten parameters (Michaelis constant, K_M and rate constant, V_{max}) were obtained by linear regression analysis of the double reciprocal Lineweaver–Burk plots.

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

Determination of the critical micelle concentration (cmc)

The cmc of the surfactant was determined at 25 °C by measuring the surface tension of the solution at different surfactant concentrations. A Krüss du Nouy type tensiometer was used.

Determination of the binding constant (K_S)

The following binding equilibrium between SPNA and the micellised surfactant was assumed:

$$[S_W] + [D_n] \overset{K_s}{\rightleftharpoons} [S_M]$$

where S_W corresponds to the free substrate, D_n to the micellised surfactant and S_M to the substrate coordinated by the surfactant aggregates. The determination of the binding constant was conveniently performed by the spectrophotometric method,[30] at two different substrate concentrations ($[S_T] = 6 \times 10^{-5}$ M and 8×10^{-5} M), according to the following equation:

$$A_\lambda = \frac{(\varepsilon_W + \varepsilon_M K_S[D_n])}{1 + K_S[D_n]} [S_T]$$

(ε_W and ε_M are the SPNA molar absorptivities in pure ammonium acetate and in concentrated surfactant solutions, respectively—the absorbance reaches a limit value with the surfactant concentration) and was refined with the least square fit. Determinations at 328 nm provided the following values: in pure ammonium acetate $\varepsilon_W = 10\,800$ M^{-1} cm^{-1}, in ammonium acetate plus surfactant $\varepsilon_M = 14\,500$ M^{-1} cm^{-1}; $K_S = 3000$ M^{-1} ($r^2 = 0.97$).

ESI-MS Experiments

Mass spectral data were obtained on a QUATTRO triple-quadrupole mass spectrometer (Micromass, Manchester, UK), operating in the positive ion mode and equipped with a Z-spray electrospray source. A HPLC system (Hewlett Packard Series 1100) with autosampler, without chromatographic column, was used for sample introduction. Aliquots of sample solution were directly injected into the ion source (flow injection analysis: FIA), kept at 140 °C, *via* a 20 μL loop, at a flow-rate of 30 μL min^{-1} (H_2O–CH_3CN–HCOOH 50 : 50 : 1 *v*/*v*/*v*), using nitrogen as drying gas at 250 °C. The mass spectrometer operated with a capillary voltage of 3.7 kV, and the sampling cone at 28 V for SPNA, and at 42 V for the neuropeptide substance P. Full-scan spectra were recorded by scanning from *m*/*z* 100 to 500 at 3 s per scan (SPNA), and from *m*/*z* 150 to 1400 at 4 s per scan (substance P).

The mass spectrometer was set and routinely employed in selected ion monitoring (SIM, dwell time 0.1 s) to quantitatively monitor the α-CT activity: at *m*/*z* 139 (protonated *p*-nitroanilide) and *m*/*z* 674.9 (doubly protonated substrate molecular ion) to follow hydrolysis of SPNA and substance P, respectively.

Data acquisition and analysis were performed using the software MassLynx (v. 3.5) running under Windows NT.

Hydrolytic activity of α-CT towards SPNA

Calibration curves. SIM was used to generate two calibration curves for PNA both in ammonium acetate alone and in surfactant solution. The curve of Fig. 3A was constructed by injecting 1 μL of PNA solution 1, 3, 5, 7 and 10 μM in 0.015 M ammonium acetate. The curve of Fig. 4A was generated by injecting 20 μL of PNA solution 10, 15, 20, 25, 30 and 40 μM in 0.015 M ammonium acetate and 3×10^{-5} M CTBABr.

Kinetic analyses. Enzymatic reactions were monitored as follows: i) in ammonium acetate alone, 1 μL of the reaction mixture was injected every 2 minutes (Fig. 3B) for an overall time of 10 minutes; ii) in 3×10^{-5} M CTBABr, 20 μL of the reaction mixture were injected every 1 minute (Fig. 4B) for an overall time of 15 minutes. In both cases, SIM profiles were smoothed and peak areas were used to give the kinetic plots of peak areas *versus* time.

Hydrolytic activity of α-CT towards substance P

Calibration curves. The two calibration curves were constructed by injecting 1μL of the substance P solution of 10, 30, 50 and 100 μM in (i) 0.015 M ammonium acetate (Fig. 6A) and in (ii) 0.0015 M ammonium acetate and 3×10^{-5} M CTBABr (Fig. 7A), respectively.

Kinetic analyses. α-CT activity was assayed with 100 μM solution of substance P, 2 μg mL^{-1} of enzyme in (i) 0.015 M ammonium acetate (Fig. 6B) and in (ii) 0.015 M ammonium acetate and 3×10^{-5} M CTBABr (Fig. 7B). Enzymatic reactions were monitored by injecting 1 μL of the reaction mixture every 1 minute for an overall time of 15 minutes.

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