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Journal of Molecular Catalysis B: Enzymatic 7 (1999) 191–205

JOURNAL OF MOLECULAR CATALYSIS B: ENZYMATIC

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Irreversible thermoinactivation of α-chymotrypsin in buffer and water miscible organic solvent. Comparison with a reverse micellar system

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Abstract

The mechanism of irreversible thermoinactivation of α-chymotrypsin in buffer medium, dimethylformamide, dimethylsulfoxide/buffer and reverse micelles was studied. Experiments for the enzyme thermoinactivation in the different systems were accompanied by studies on protein structure alterations. The formation of free SH groups was followed during the stability tests. This formation is due to the cleavage of the enzyme primary structure which leads to small peptides. These were identified by SDS-PAGE, in the case of the buffer medium, and also by HPLC in the three other systems. An increase of SH groups with the residence time of the enzyme in the buffer and organic solvent/buffer system was observed. This was corroborated by the disappearance of the electrophoresis bands of the protein and also by HPLC analysis. In the latter technique, the peaks corresponding to α-chymotrypsin disappeared with the concomitant appearance of small peaks in the chromatogram. For the enzyme encapsulated in reverse micelles, the formation of free SH groups was not detected and the HPLC analysis revealed that the protein peak stayed intact during the residence time in this system. The thermoinactivation of chemically modified α-chymotrypsin by the introduction of dianhydride pyromellitic on the Lys residues of the protein was also studied in some of the systems. The results showed that the chemical modification stabilized the enzyme when the system used was buffer or organic solvent mixed with buffer being this stabilization partially due to a slower process of cysteine bond cleavage.

Keywords: Degradation process; Reverse micelles; α-Chymotrypsin; Chemical modification; Organic solvent systems

1. Introduction

α-Chymotrypsin is one of most used enzymes in the catalysis of peptidic bond synthesis. Among the reaction media used to carry out the referred synthesis, dimethylformamide (DMF) and dimethylsulfoxide (DMSO) mixed with buffer solutions [1–4] and also reverse micellar systems [5–8] can be mentioned.

The use of this enzyme in a continuous process requires it to be in a stable form under the operational conditions. There are external factors to the protein that influence its stability in a given system, like temperature, buffer pH, ionic strength and also denaturating agents [9]. These external factors can alter the delicate forces balance that are responsible for the stable proteic structure [10].

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1381-1177/99/$ - see front matter
PII: S1381-1177(99)00029-6

To increase the protein stability in a given system, its mechanism of denaturation should be known in order to apply medium or protein engineering technologies to prevent its inactivation.

The thermal inactivation process of a protein is known to occur in two steps: first, the enzyme molecule partially unfolds in a reversible process and after prolonged heating, the enzyme gradually loses activity in an irreversible process, as it cannot be reactivated after cooling [11,12].

Among the mechanisms causing the irreversible thermoinactivation of proteins, the reduction of S–S bonds of the cystine residues, the deamidation of Asn and Gln residues and the hydrolysis of the peptidic bonds can be referred [13–15].

Considering that water is the main reagent in the degradative reactions of the protein structure [16], its replacement by an organic solvent has been referred as one method to achieve a higher protein stability.

The effect of an organic solvent on the enzyme stability has been correlated with several solvent parameters among which the dielectric constant [17] and the hydrophobicity through the log P parameter [18] can be referred. Exceptions to the several rules have also been found [19].

In the case of the reverse micellar systems, besides the effect of the organic solvent, the surfactant influence on the enzyme stability has also to be taken into consideration. By choosing adequately the system composition, the enzyme in a reverse micellar system can display a higher stability than in the buffer medium [20,21] or even higher to an organic solvent miscible with water [22]. In these systems, the water content is also crucial for the enzyme stability [23,24].

The replacement of water by an organic solvent and the composition of the system formed can be quoted among the medium engineering processes [16] to increase enzyme stability. The other strategy is protein engineering, either achieved by genetic or chemical processes [15].

Among the chemical modification of enzymes the hydrophilization of its surface has been referred as one process of greatly increasing the enzyme thermal stability [25]. This chemical modification is accomplished by introducing dianhydride residues on the Lys residues of the protein. α-Chymotrypsin was modified with this process and an increase of a factor of 1000 [25] in its stability in a buffer medium at 60°C was obtained.

The chemically modified α-chymotrypsin in carbonate buffer and in DMF or DMSO/carbonate buffer, previously used for peptide synthesis [22], was used to study the effect of hydrophilization in these systems. The same type of study was also done with the native enzyme. The stability of both enzyme forms in the referred systems was compared with the micellar system used in peptide synthesis [8] and known to confer a higher stability to the native form. The cause of this higher stability was studied by measuring the S–S bond cleavage in the α-chymotrypsin structure as well as its hydrolysis during the permanence of the enzyme in the different systems referred. The results of this study are described in the present work.

2. Materials and methods

2.1. Chemicals and enzyme

Tetradecyltrimethylammonium bromide (TTAB), α-chymotrypsin, acetylphenylalanine ethyl ester (AcPheOEt), 5,5′-dithiobis-(2-nitrobenzoic) acid (DTNB) and 2,4,6-trinitrobenzenesulfonic acid (TNBS) were obtained from Sigma. Dianhydride pyromellitic (DAP) was obtained from Aldrich. All organic solvents were of analytical grade.

2.2. Chemical modification of α-chymotrypsin

The enzyme modification was performed at room temperature by adding dropwise 100 μl of 0.567% DAP solution in dimethylsulfoxide

(DMSO) to an enzyme solution in 50 mM borate buffer at pH 10. The concentration of the enzyme solution was 0.1 mM for the stability studies in the buffer and in the reverse micelles and 0.5 mM when the stability in the organic buffer system was studied. After 15 min, the modification yield was determined by comparison spectrophotometrically the absorbance resulting from the NH_2 groups titration with TNBS [26]. When needed the borate buffer was exchanged for the carbonate buffer used in the reaction medium by an ultrafiltration membrane system. This procedure gave 98% of modified Lys residues.

2.3. Stability studies in buffer medium

A 0.1 mM solution of α-chymotrypsin, in 50 mM borate buffer, pH 10, was thermostated at 65°C for 10 min for the native enzyme and 5 h for the chemically modified form. Aliquots were withdrawn at several time intervals and the enzyme activity was determined according to the procedure described under Section 2.6.

To follow the formation of free SH groups during an enzyme stability study, a 0.2 mM solution of α-chymotrypsin, native and modified was thermostated also at 65°C, in the same buffer. A blank without enzyme was simultaneously included in the bath. Aliquots were withdrawn from both tubes and the formation of free SH groups was measured according to the method described under Section 2.6.

To follow the formation of small peptides, a 0.1 mM enzyme solution, either native or chemically modified was thermostated at the same temperature and aliquots of 1 ml were withdrawn and analysed by HPLC and by electrophoresis according to Section 2.6.

2.4. Stability studies in DMF and DMSO / buffer

A 0.5 mM solution of α-chymotrypsin, either native or chemically modified, in DMF or DMSO in 50 mM carbonate buffer, pH 10 (30/70, v/v) was thermostated at 65°C for 2 h. Aliquots of 200 μl were withdrawn at several time intervals and the residual enzyme activity was determined by the methods described under Section 2.6.

To follow the formation of SH groups during the enzyme stability study, aliquots of 1 ml of the enzyme solution, either native or modified, were withdrawn at several time intervals. These aliquots were diluted to 2 ml and analysed for SH groups formation as described in Section 2.6.

The formation of small peptides was followed by HPLC, after having withdrawn aliquots of 1 ml at several time intervals.

2.5. Preparation of the reversed micellar system and stability studies

The reversed micellar system was prepared by injecting appropriate volumes of sodium carbonate buffer containing the enzyme into a solution of TTAB in heptane/octanol (80/20% v/v). The final volume was 8 ml. Ten microliters of 0.5 mM enzyme solution in the buffer reverse micelles system was added dropwise. The water content in the system, usually defined through the parameter $w_0 = [H_2O]/[\text{surfactant}]$, was controlled by adjusting the buffer volumes added and kept to a final value of 5.5. The temperature was kept at 25°C. The test tube was capped and left for 1 h. The enzyme activity was determined by cooling down the test tube to 15°C and adding 2 ml of the substrate AcPheOEt in heptane/octanol (80/20, v/v). The final concentration of the ester substrate was 6 mM and the enzyme concentration was 0.5 μM. In this stability test, the buffer molarity was 300 mM and the pH was 10.

To follow the formation of free SH groups inside the reverse micelles, a solution of 5 μM of native and modified α-chymotrypsin was subject to 25°C. Aliquots of 1 ml were withdrawn at several time intervals and analysed for SH as described under Section 2.6.

To follow the formation of small peptides, a 50 μM enzyme in a reverse micellar solution

was prepared. The system was thermostated at 25°C and aliquots of 1 ml were withdrawn. After adding 100 μl of methanol, the samples were subject to a vortex system and centrifuged at $2397 \times g$ for 2 min. The bottom phase was formed by a white solid that was dissolved in water, pH 3 by the addition of TFA. Both phases were analysed by HPLC as described in Section 2.6.

2.6. Analytical methods

2.6.1. Activity tests in organic/buffer solvent and in buffer medium

To a volume of 1 ml of enzyme solution, either in organic/buffer solvent or in buffer medium, were added 100 μl of a 16.1 mM BzArgpNA solution in DMSO, The reaction was followed spectrophotometrically at 410 nm [27] against the corresponding blank.

2.6.2. Activity tests in reverse micelles

The enzymatic hydrolysis of AcPheOEt was used to follow the α-chymotrypsin activity at the beginning of the stability test and during 1 h of residence in the reverse micellar system of TTAB/heptane/octanol. The hydrolysis reaction was followed by HPLC in a C_{18} reverse phase column with isocratic elution and detection at 220 nm, using as eluent, 35% acetonitrile in 20 mM sodium phosphate at a final pH of 3.5.

2.6.3. Measurement of free SH groups

The free SH groups were measured according to the method of Ellman [28]. To 2 ml aliquot of a 0.2 mM enzyme, either native or modified, in buffer solution, 40 μl of 3.8 mM DTNB solution in 0.5% $NaHCO_3$ were added and the free SH groups were measured spectrophotometrically at 412 nm against the corresponding blank. The same procedure was followed for the enzyme in organic/buffer mixture, the only difference being the enzyme concentration, 0.5 mM, in this latter case. For the enzyme, either native or modified, inside the reverse micellar system, 1 ml aliquot of a 5 μM enzyme solution in reverse micelles was withdrawn and 20 μl of DTNB were added. The product of this reaction was measured spectrophotometrically.

Table 1
Composition of the gradient used in the HPLC system to elute the small peptides

Time (min)	Composition (% B)	Flow rate (ml/min)
0	25	1.0
15	98	1.5
20	98	1.5

Eluent A: 1000 ml of water + 600 μl of TFA; Eluent B: 800 ml of AcCN + 520 μl of TFA + 200 ml of water.

2.6.4. Small peptides by SDS-PAGE

The presence of small peptides was analysed by electrophoresis in the mini-gel of Pharmacia Phastsystem using the SDS-PAGE technique in a 8–25% gradient gel.

2.6.5. Small peptides by HPLC

An HPLC system was also used to analyse the presence of small peptides. A RP-18 column was used and the detection was done at 210 nm. A sample volume of 100 μl was injected for all the analysed systems. The small peptides were eluted using a gradient of composition and flow rate according to Table 1.

3. Results and discussion

This work aims to identify the cause or causes that can lead to the different stabilities found for α-chymotrypsin, when catalysing the dipeptide synthesis in dimethylformamide/carbonate buffer [2,3] and reverse micelles of TTAB/heptane/octanol [8].

Previous work [20] showed that the native α-chymotrypsin was more stable in the reverse micellar system than in the DMF/buffer. The measured half-life value for the reverse micellar system was higher than 30 days, while for the organic buffer system a value of 6.6 days [22]

was found. The replacement of DMF by DMSO in the same proportion could increase considerably the enzyme stability [22].

The chemical modification of α-chymotrypsin with dianhydride pyromellitic, following the procedure described by Mozahev et al. [25] was used and the modified enzyme encapsulated in the reverse micellar system described, however no increase in the thermal stability was found [20]. The interactions that exist between the negatively charged enzyme surface and the surfactant charged head groups may be one of the causes of the lesser stability found for modified α-chymotrypsin inside reverse micelles.

The stability of the enzyme modified by the same technique in the organic solvent miscible with water was also studied, together with the enzyme behaviour in the buffer medium.

The cleavage of the S–S bonds existing in the α-chymotrypsin structure, 5 cystine residues [29] was looked for by the formation of free SH groups, according to the procedure described by Zale and Klibanov [14]. This analysis was done for the native and chemically modified enzyme in all the systems under study. This cleavage should be accompanied by the formation of small peptides. These peptides are looked for in the HPLC analysis and also by gel-electrophoresis, in the case of the buffer medium. This study was done for both the native and the chemically modified enzyme in all the systems with the exception of the reverse micelles where the modification did not show to be effective. This work was accompanied by the corresponding stability experiments for the three systems.

3.1. Stability of native and chemically modified α-chymotrypsin stability in buffer medium

Although α-chymotrypsin is one of the most studied enzymes, its behaviour in a buffer system was not found in the literature. It is known [25] that the chemical modification strongly stabilises the enzyme but nothing is said about the enzyme decay in that system.

Table 2
Half-life time for the native and the modified α-chymotrypsin in different systems

System	Native enzyme	Modified enzyme
Buffer	2.1 min	> 12 h
DMF/buffer	0.8 min	2.8 min
DMSO/buffer	1.6 min	> 60 min

To study the stabilisation of the chemical modification, stability studies were carried out at 65°C. In Table 2, the half-life time for the native and the modified form are presented. By comparing the results of the half-lives of the enzyme, it can be seen that the chemical modification causes a strong stabilisation in the buffer medium.

The results obtained with the several studies of the α-chymotrypsin stability in the buffer system are in accordance with the results of the Mozahev group and corroborate their suggestions about the stabilisation by hydrophilization [25].

3.2. Stability of the native and chemically modified chymotrypsin in organic solvents miscible with water

One of the most studied systems for the peptide synthesis is formed by an organic solvent miscible with water, like dimethylformamide (DMF) and dimethylsulfoxide (DMSO). These solvents were mainly used in the proportion of 30% for several peptidic bonds formation in reactions catalysed by several enzymes [2,3] among which α-chymotrypsin can be referred.

The stability of the modified enzyme form was studied in this system at high temperature values (65°C) to accelerate the degradation process and to compare against the native form. In Table 2, the half life time for the native and modified enzyme in DMF and DMSO/buffer can be seen.

An increase in α-chymotrypsin stability in DMSO relatively to the DMF system can be

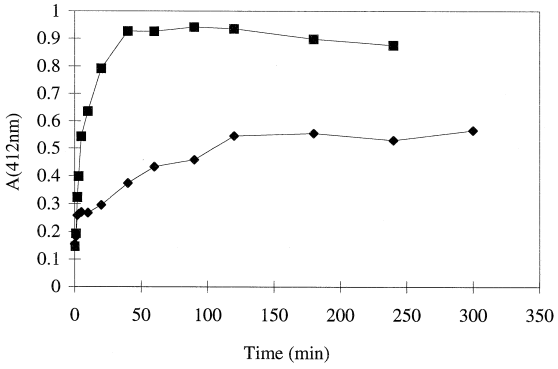

Fig. 1. SH-free group formation for the (■) native and (♦) modified α-chymotrypsin during the stability experiments in buffer medium.

noticed. The half-lives values were 0.8 and 1.6 min in DMF and DMSO (Table 2), respectively.

In these systems a stabilisation of the enzyme caused by the surface modification can be verified. The half-life of the modified enzyme in DMF/buffer was 2.8 min, while in the DMSO system an activation of the modified enzyme during all the experiment time was noticed and the half-life for the enzyme was higher than 60 min.

The higher stability found in DMSO/buffer system, comparatively to the DMF is probably related with the fact that DMSO is a solvent with a lower capacity to attract the water molecules from the enzyme surface than DMF [30].

The chemical modification of α-chymotrypsin led to an increase in the enzyme stabilisation, as it was the main objective with the introduction of the hydrophilic groups on the Lys residues. The stabilisation effect is more relevant in the DMSO system.

The increase in the enzyme stability by the chemical modification is explained [25] by a protective effect of the hydrophobic residues of the protein surface given by the new hydrophilic groups introduced on the enzyme surface. These hydrophilic groups strongly attract the water molecules, giving a more rigid structure to the protein. On account of this water molecules fixation, they will have less mobility to participate in auto-hydrolysis reactions and also in the other reactions where the water molecules can take part.

The chemical modification not only increases the enzyme stability but also produces an enzyme activation during the residence time under the tested conditions. This enzyme activation has also been verified with several enzymes in

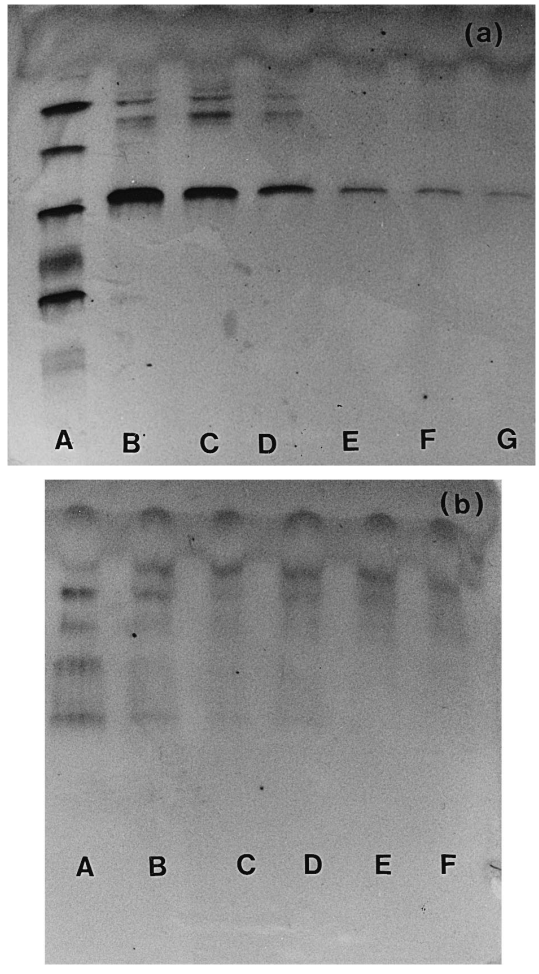

Fig. 2. SDS-polyacrylamide gel electrophoresis α-chymotrypsin during the stability studies in buffer medium. (a) Native α-chymotrypsin: **A**—molecular weight standards: 1—lactalbumine (14 000), 2—soy trypsin inhibitor (20 100), 3—carbonic anhydrase (30 000), 4—ovalbumine (43 000), 5—bovine serum albumine (67 000), 6—phosphorilase B (94 000); **B**—native enzyme at time zero, $t = 0$; **C**—$t = 1$ min; **D**—$t = 2$ min; **E**—$t = 3$ min; **F**—$t = 4$ min; **G**—$t = 5$ min. (b) Modified α-chymotrypsin: **A**—$t = 0$; **B**—$t = 10$ min; **C** = 30 min; **D**—$t = 50$ min; **E**—$t = 1$ h; **F**—$t = 3$ h.

different systems [31]. It is only noticed during the first period of residence in these systems and it is still a phenomenon not well explained.

3.3. Stability of α-chymotrypsin in reverse micellar medium

The α-chymotrypsin stability inside reverse micellar systems was studied elsewhere [20]. Its higher stability relatively to the other systems was noticed. In order to study the denaturation of the protein in reverse micellar systems to compare with the organic and buffer media, an experimental test to force the enzyme activity decay was setup. Due to the organic solvent volatility the enzyme stability in the reverse micelles system was evaluated at 40°C. The results of the stability of α-chymotrypsin in reverse micelles of TTAB/heptane/octanol indicated that after 4.4 min at that temperature the enzyme showed half of its initial activity. The chemical modified α-chymotrypsin had a lower half-life time in the reverse micelles than the native form. The chemical modification of the

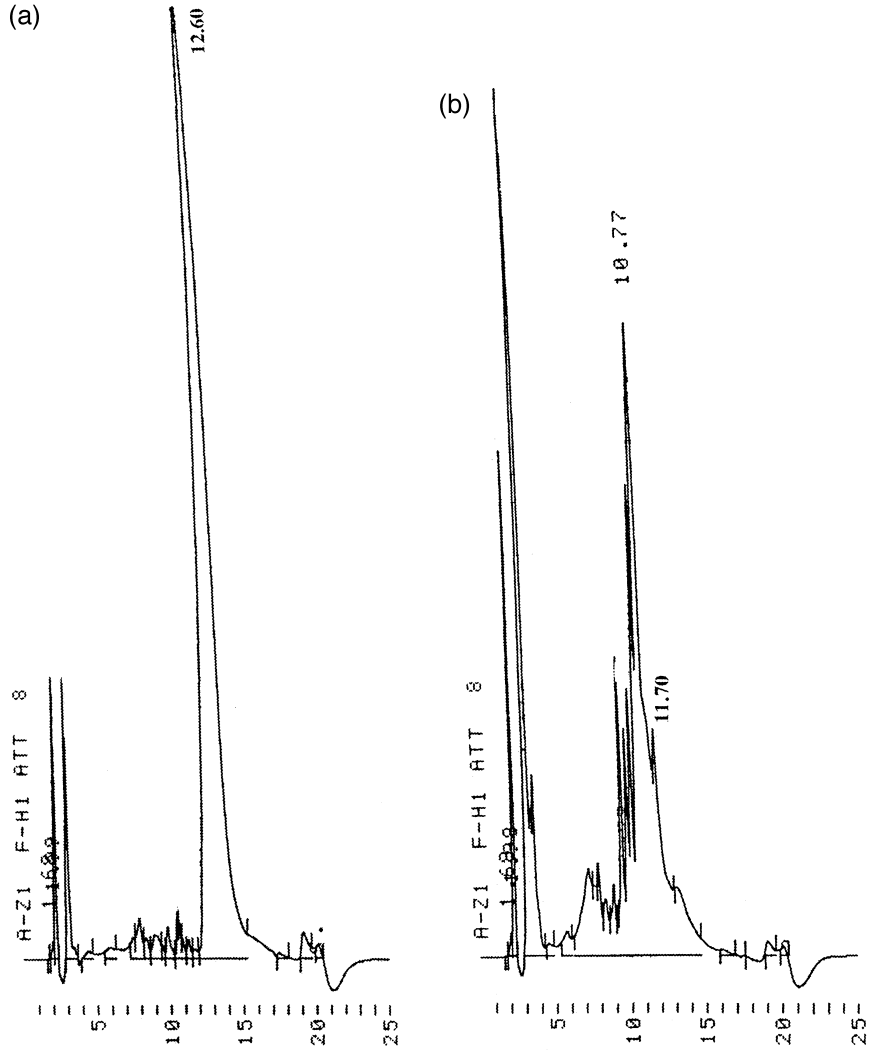

Fig. 3. HPLC chromatogram of samples withdrawn from the native α-chymotrypsin stability studies in buffer medium: (a) $t = 0$; (b) $t = 1$ min.

enzyme did not increase protein stability in this system.

From all the systems studied, the reverse micellar medium, allowed a higher stability.

A comparative study [21] between the α-chymotrypsin stability in buffer reaction medium and the micellar system indicated that the enzyme displayed higher stability in the former system. The enzyme micellar stability is dependent on the system parameters employed. In the reverse micelles described, a cationic surfactant (CTAB) similar to the one used in this work but dissolved in heptane/chloroform (50/50) was used. A previous work in which this enzyme encapsulated in a similar reverse micellar system also registered a lower half-life [7] compared to the value that can be obtained by replacing the chloroform by a long chain alcohol like octanol, for instance.

In the work of Barbaric and Luisi [32], the reverse micellar system also led to a higher enzyme stability than the water medium.

Another comparative study of the enzyme stability in different systems [33] indicated that for the enzyme tyrosinase, a higher stability in the buffer medium relatively to the reverse micelles of AOT/isooctane or CTAB/hexane/chloroform was obtained. This enzyme stability in both reverse micellar systems was identical, indicating that the enzyme stability seemed to be independent of the type of surfactant, either anionic or cationic. When the same group compared the enzyme stability in the reverse micelles against its stability in a system containing an anhydrous organic solvent (an alkane) and the enzyme used in powder, as a suspension, the reverse micellar system allowed a higher enzyme stability.

These results show that the reverse micellar system is adequate to the enzyme stability, being even better than the organic solvent miscible with water and also the buffer medium. Several reasons can be proposed for this higher stability, for instance the lack of auto-hydrolysis, a slower rate of S–S bond cleavage can be referred among others [11,14].

3.4. Analysis of the enzymatic behaviour in the different systems used for the stability studies

Under this section, the results of the search for alterations in the α-chymotrypsin structure are described. For all the systems studied, the evolution in the SH group formation during the stability tests was analysed. The cleavage of the enzyme primary structure with the formation of smaller peptides was followed by HPLC for all the systems and also by electrophoresis in the buffer medium.

3.4.1. Buffer medium

Several aliquots were withdrawn during the stability studies performed at 65°C to evaluate the S–S bond cleavage in the α-chymotrypsin structure occurring in the buffer medium. The results can be seen in Fig. 1 for both enzyme forms. The formation of free SH groups occurs not only in the native form but also in the modified enzyme. In this last case, the rate of free SH appearance is much slower, than in the native form, 0.04 UA/min and 0.09 UA/min, respectively. It seems that the cystine residues in the α-chymotrypsin structure are cleaved during the enzyme incubation in the buffer. In the case of the native form, this could explain, at least in part, the loss of the enzyme activity after a few minutes at 65°C. The higher stability found in the modified form can, in part be

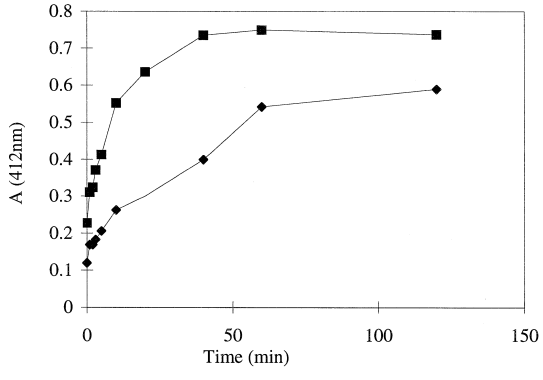

Fig. 4. SH-free group formation for the (■) native and (♦) modified α-chymotrypsin during the stability experiments in DMF buffer medium.

attributed to a cleavage in a lesser degree of these bonds.

The free SH groups formation will mean an increase in small peptides chains. The smaller peptides formation was detected by SDS-PAGE electrophoresis and also by HPLC.

During the stability studies described in the previous section, samples were also withdrawn to follow the changes in the α-chymotrypsin structure. The SDS-PAGE electrophoresis of the native and the chemically modified form, Fig. 2a and b, respectively, revealed the disappearance of the characteristic bands corresponding to the protein for both enzyme forms. In the modified form an increase in the intensity on the region of the small weight bands can be observed, while in the native form, all the bands disappear after a certain period in the buffer medium. This indicates the formation of very small peptides that cannot be retained by the gel porosity. The existence of four bands for the modified α-chymotrypsin may be due to the

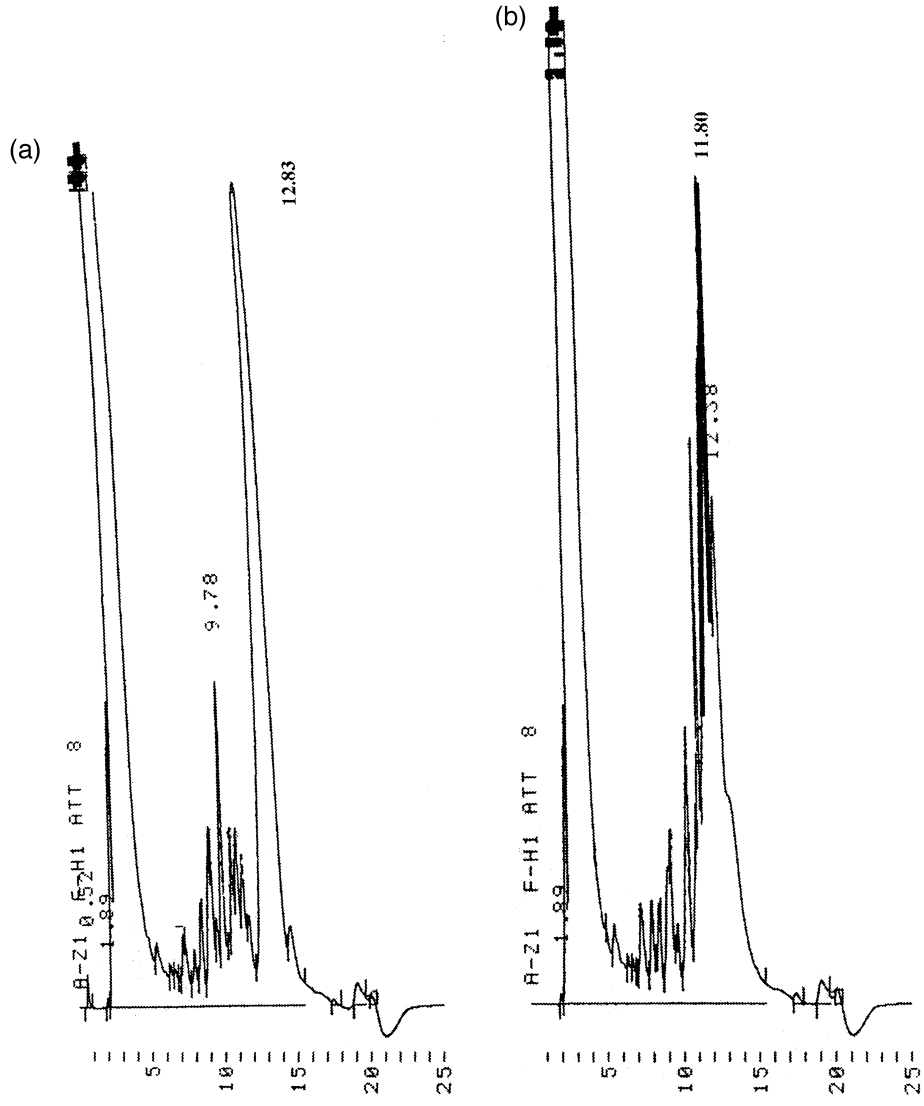

Fig. 5. HPLC chromatogram of samples withdrawn from the native α-chymotrypsin stability studies in DMF/buffer medium: (a) $t = 0$; (b) $t = 2$ min.

modification not only of α-chymotrypsin itself, but also to the B and C chains modification. These chains could also be seen in the electrophoresis from the native enzyme. Alfa-chymotrypsin consists of three polypeptide chains connected by two interchain disulfide bonds [29] that can be cleaved during the experimental conditions for the gel elctrophoresis.

The small peptides were also analysed by HPLC. If the α-chymotrypsin peak disappeared in the chromatogram this would indicate that the enzyme was being cleaved into smaller peptides.

A HPLC chromatogram of the native α-chymotrypsin can be seen in Fig. 3a. The protein has a retention time of approximately 12.60 min. The chromatogram corresponding to the sample withdrawn after 1 min at 65°C, time at which the enzyme has lost 40% of its initial activity can be seen in Fig. 3b. These results show that α-chymotrypsin completely disappeared, leading to a lot of small peaks spread over the chromatogram. These results are in accordance with those obtained by electrophoresis, meaning that α-chymotrypsin was split not only in the S–S bonds, but also in several very small peptides, that the electrophoresis gel was not able to retain them.

3.4.2. Water miscible organic solvent

The formation of SH groups due to the cleavage of the cystine bonds in α-chymotrypsin was studied in the protein incubation in DMF/buffer medium at 65°C. The results are shown in Fig. 4.

By comparing the ratio of increase in absorbance (SH formation) for native and modified enzyme, it can be verified once again that this ratio is higher in the native form, reaching a value of 36% higher than the initial value. The slower cleavage in the S–S bond in the modified enzyme may explain its higher stability.

The cleavage of the cystine bonds must increase the number of smaller peptides, at least those corresponding to the A, B and C chains [29] of α-chymotrypsin. These peptides were analysed by HPLC due to the difficulty in using organic solvents with electrophoresis gels [34]. The chromatogram of the native α-chymotrypsin in DMF/buffer can be seen in Fig. 5. The native protein has one main peak with a retention time of approximately 12.8 min. The peaks coming in front of the chromatogram belong to the solvent DMF/carbonate buffer.

The analysis of the aliquots withdrawn after 2 min at 65°C, time at which the enzyme lost 75% of its initial activity, can be seen in Fig. 5b. The peak with a retention time (12 min) corresponding to α-chymotrypsin shows a reduction of 43% in its area with an increase in some of the peaks in the protein vicinity.

The formation of SH free groups were also analysed in the DMSO/buffer medium for both enzyme forms (Fig. 6). In this system the rate of S–S bond cleavage is similar for the native and the modified enzyme, 0.05 and 0.04 UA/min, respectively, reaching the same value of SH free groups after 1 h. In this case it seems that the appearance of SH groups is not related with a loss in enzyme activity, because after incubation for 1 h at 65°C, the modified enzyme had a higher activity than at the beginning of the experiment. It seems that as long as the enzyme can maintain intact its catalytic triad it will display its activity, independently of some of the changes of its structure.

The HPLC chromatogram for the native α-chymotrypsin in DMSO/buffer is shown in Fig.

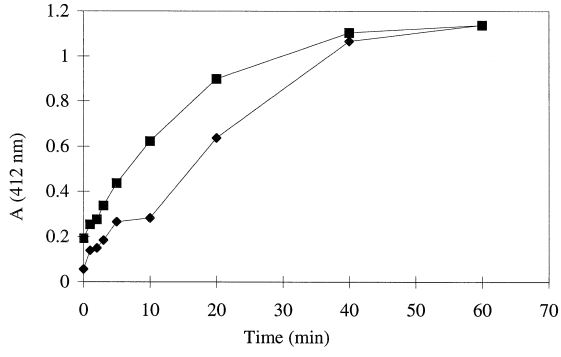

Fig. 6. SH-free group formation for the (■) native and (♦) modified α-chymotrypsin during the stability experiments in DMSO buffer medium.

7a. The protein has a retention time of approximately 12.2 min. The disappearance of the corresponding peak after 2 min, under the experimental conditions, can be seen in Fig. 7b.

3.4.3. Reverse micelles

The α-chymotrypsin stability inside reverse micelles of TTAB/heptane/octanol was higher, comparatively to the other two systems studied [20]. The cleavage of S–S bonds was also analysed in this system. Previous studies [20] showed that long chain alcohols, hexanol, octanol and decanol, used as co-surfactants for the TTAB reverse micelles formation had a strong influence on the α-chymotrypsin stability. Octanol led to a higher stability than the other two alcohols. The influence of the long chain alcohol in the cleavage of the cystine residues of the enzyme was studied.

The results of the possible formation of SH groups in the enzyme encapsulated in the re-

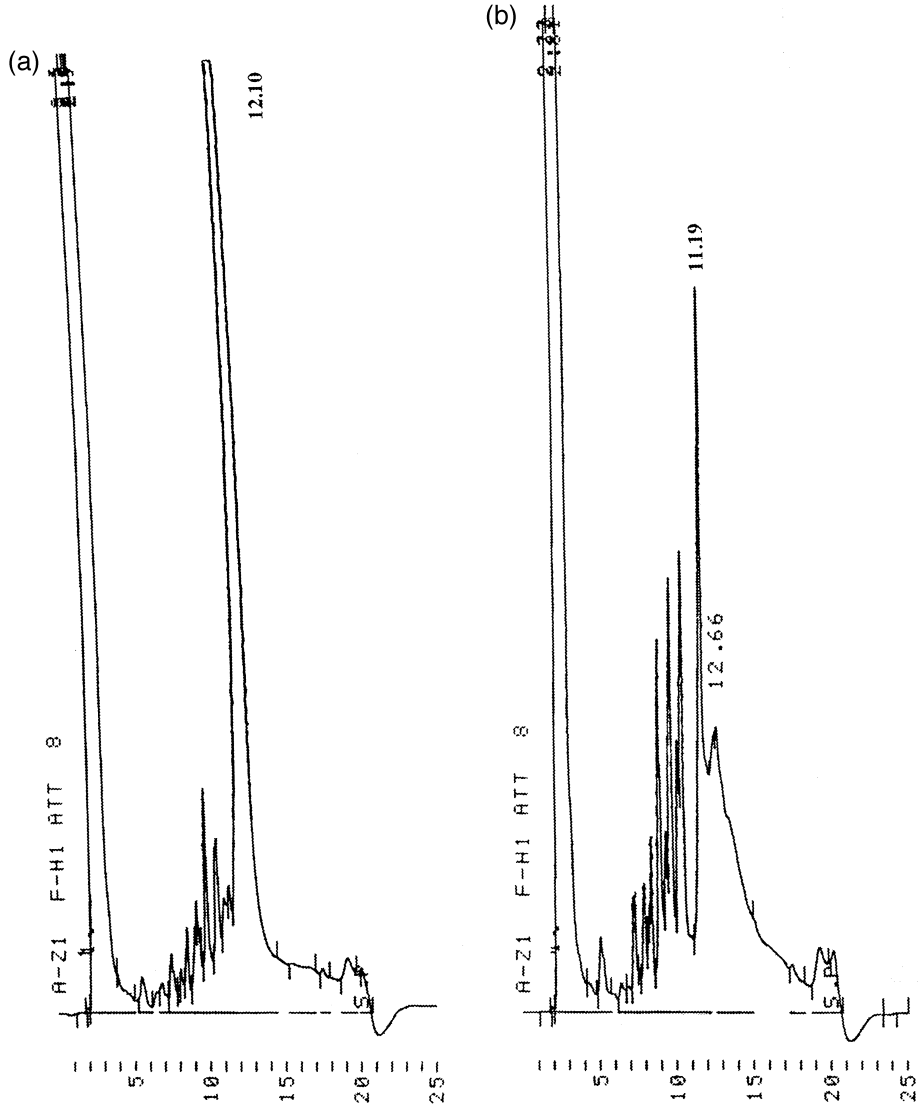

Fig. 7. HPLC chromatogram of samples withdrawn from the native α-chymotrypsin stability studies in DMSO/buffer medium: (a) $t = 0$; (b) $t = 2$ min.

verse micelles of TTAB/heptane/ hexanol, octanol and decanol can be seen in Fig. 8. These measurements were done against a blank of the corresponding reverse micellar system. In the octanol containing system, no increase was detected in the SH formation. In the system with decanol as co-surfactant, a slight increase was noticed during the first 10 min and as compared to a decrease in the SH groups formation. In the hexanol containing system, there is an increase in the absorbance till approximately 20 min and after that a slight increase in this value.

Although the enzyme is in equal concentration for all the reverse micellar systems, different values were found for the absorbance at 412 nm at the beginning of the experiment. This led to set up an experiment with the chemical stability of DTNB reagent inside the reverse micelles. These results are shown in Fig. 9. The initial values of absorbance at 412 nm increase with the decrease in the alcohol chain length, 0.12, 0.15 and 0.20 for the decanol, octanol and hexanol, respectively. During the first 20 min, a high rate of decrease in absorbance can be noticed. Taking into account that the compound (DTNB) has a disulphide bond and that its colour (yellow) increases with the extent of hydrolysis and this in turn, increases with the pH value, it seems that there is an increase in the actual pH inside the reverse micelles with

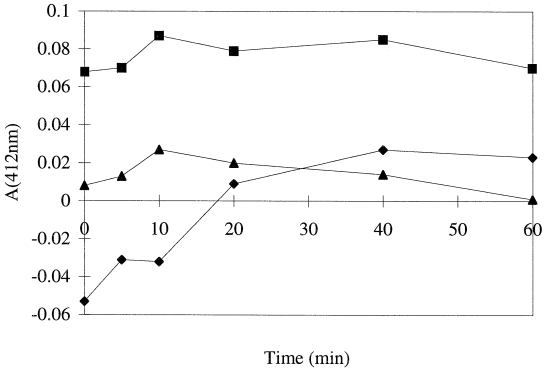

Fig. 8. SH-free group formation for α-chymotrypsin in reverse micelles of TTAB/heptane/long chain alcohol used as co-surfactant during the stability tests: (▲) decanol, (■) octanol and (♦) hexanol.

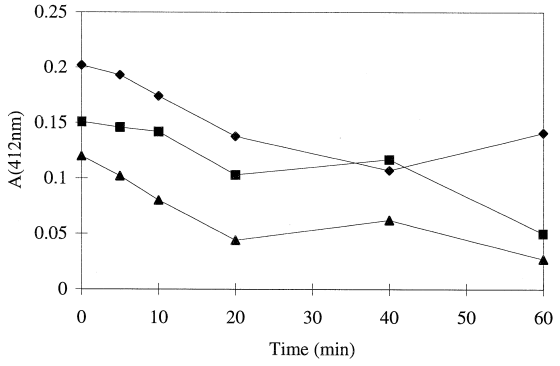

Fig. 9. SH-free group formation for the chemical reagent DTNB in reverse micelles of TTAB/heptane/long chain alcohol used as co-surfactant during the stability tests: (▲) decanol, (■) octanol and (♦) hexanol.

the decrease in the alcohol chain length, although all the systems have the same carbonate buffer, pH 10. The decrease in absorbance found for all the systems seems to indicate that after the initial moment, a synthesis of the disulphide bond takes place, leading to the formation of the S–S bond again. The pattern obtained for the equivalent study with the enzyme inside the reverse micelles is different, but it seems that after 40 min, a slight tendency for the decrease in absorbance is also noticed. During the first 20 min, the fluctuations found in the A_{412} seem to be due to the reagent itself and no SH free group formation is detectable in the α-chymotrypsin inside reverse micelles. These findings should be corroborated by HPLC analysis.

α-Chymotrypsin is encapsulated inside reverse micelles and the surfactant may produce a protective sphere against the interactions between the enzyme and the reverse phase HPLC column, preventing the protein to be adequately eluted at its appropriate retention time. To avoid this situation the protein was taken out the water pool and after that analysed by chromatography.

To withdraw the protein from the inside of the reverse micelles, methanol was added to disrupt the reverse micellar structure [35]. With the addition of methanol, two phases are obtained. The bottom phase, a white precipitate, is

completely dissolved in water at pH 3. After the analysis by HPLC a peak with the characteristic retention time of α-chymotrypsin, approximately 11.5 min, can be seen (Fig. 10a). The top phase is a clear solution and the analysis by HPLC gives a peak in the beginning of the chromatogram in which the TTAB is the main component.

The sample withdrawn after 1 h of incubation under the experimental conditions was analysed by HPLC and the results can be seen in Fig. 10b. In these chromatograms, the peak corresponding to the protein has a retention time of approximately 11.6 min. This peak appears in all the chromatograms done for the aliquots withdrawn from the enzyme stability tests inside reverse micelles. A decrease in the area of α-chymotrypsin peak is not detected, which is corroborated by the fact that small peaks corresponding to small peptides were not formed. This means that the loss of α-chymotrypsin activity is not due to a cleavage of S–S bonds or to auto-hydrolysis. In this system, the loss of activity must be due to conformational changes,

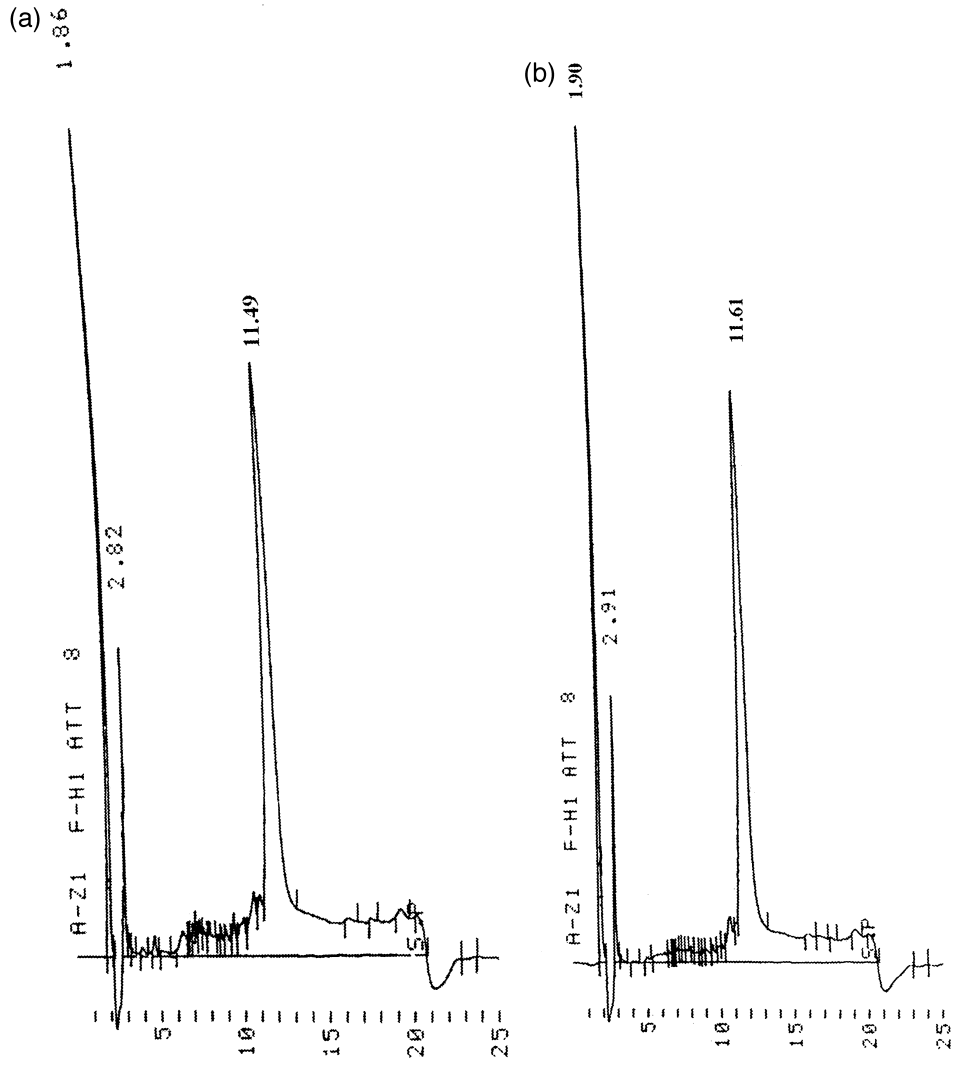

Fig. 10. HPLC chromatogram of samples withdrawn from the native α-chymotrypsin stability studies in reverse micelles of TTAB/heptane/octanol: (a) $t = 0$; (b) $t = 1$ h.

because the other cleavage reactions [14], such as loss of Asn residues, etc. would also lead to smaller peptides that were not detected in this system.

4. Conclusions

The higher stability found for α-chymotrypsin encapsulated in the reverse micellar system relatively to the buffer medium and the organic solvent miscible with water can be explained by the absence of S–S bond cleavage of its cystine residues and also by the absence of auto-hydrolyses of the protein structure. The formation of small peptides and also of SH free groups was detected for the enzyme incubated in the aqueous and organic solvent systems. The chemical modification of the enzyme stabilised its activity for the buffer and the organic buffer medium. Part of this stabilising effect may be due to a slower rate of cystine bonds cleavage found for the modified enzyme.

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