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Effects of Ca^{2+} on catalytic activity and conformation of trypsin and α-chymotrypsin in aqueous ethanol

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Abstract

The effects of calcium ions on the conformation and catalytic activity of trypsin and α-chymotrypsin were studied in aqueous ethanol. The activity of α-chymotrypsin was practically lost within 10 min in the presence of 60% ethanol while trypsin preserved about 40% of its original activity even in 85% ethanol at pH 3. The catalytic activity of α-chymotrypsin did not decrease in the presence of 1.2 M $CaCl_2$ and 0.6 M $CaCl_2$ with trypsin in ethanolic solvent. In the latter case an activation of enzyme was observed. The stabilizing effects of calcium ions were accompanied by an increase in the helical content in both enzymes, as followed by circular dichroism measurements.

Keywords: α-Chymotrypsin; Trypsin; Stability; Structure; Ethanol; Calcium ion

The calcium ion regulates many biological systems by interacting with proteins with different affinities in different biological environments. The binding of Ca^{2+} to proteins leads to an increase in stability and to changes in conformation of the calcium-binding proteins. The Ca^{2+}-binding sites in proteins are highly irregular, with a great variation in ligand type [1,2]. In contrast, no Ca^{2+}-binding sites have been found in non-calcium-binding proteins with all natural residues. The stabilizing effect of calcium ions on some proteases is well known but the mechanism is not understood [3]. Recently, a weak Ca^{2+}-binding site was detected in subtilisin BPN [4]. The effects of metal ions on the conformational flexibility and thermal stability of calreticulin have also been studied [5].

Biocatalysis in organic media has been the object of intensive basic and application-oriented research. Although the ability of enzymes to act as selective catalysts for a broad spectrum of organic reactions has been known for many years, their application is rare because of the inappropriate stabilities of the biocatalysts. The attainment of high levels of catalytic activity of enzymes in organic solvents is one of the major challenges in non-aqueous enzymology. Protein engineering, immobilization, chemical modifications of enzymes, and the use of salts or polyols provide possibilities for the stabilization of enzymes [6,7]. We have previously shown that organic solvents (ethanol, acetonitrile, and 1,4-dioxane) exert a strong influence on the secondary structures of some hydrolytic enzymes [8]. It has also been established that the preservation of the catalytic activity of α-chymotrypsin by polyols in buffered aqueous ethanol is accompanied by changes in the secondary and tertiary structures of the enzyme [9]. The aim of the present work was to study the effects of calcium ions on the catalytic activities of trypsin and α-chymotrypsin in a water–ethanol mixture at pH 3.0. The changes in the secondary/tertiary structures of these enzymes were monitored by means of far and near-UV circular dichroism (CD) measurements.

Materials and methods

Materials. The synthetic substrates *N*-acetyl-L-tyrosine ethyl ester (ATEE), *N*-benzoyl-L-arginine ethyl ester (BAEE), α-chymotrypsin,

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and trypsin (from the bovine pancreas) were from Sigma–Aldrich. All other chemicals were reagent grade products of Reanal.

Assay of enzyme activities. The measurement of trypsin activity was based on differences between the spectra of BAEE and the carboxylate form of *N*-benzoyl-L-arginine [10]. The increase in absorbance at 253 nm was followed in a reaction mixture (3 ml) containing 46.7 mM Tris/HCl buffer (pH 8.0) and 0.9 mM BAEE. For the measurement of α-chymotrypsin activity, ATEE was used: the change in absorbance at 237 nm was followed in a reaction mixture (3 ml) containing 40 mM Tris/HCl (pH 7.0) and 0.5 mM ATEE [10]. In both cases, the reactions were initiated by 50 μl of 0.15 mg/ml enzyme.

Stability tests. The stability tests in organic solvents were performed at pH 3.0 in solutions with enzyme concentrations of 0.15 mg/ml at 25 °C. The samples were incubated for appropriate periods of time, aliquots were then withdrawn, and the residual activities of the enzymes were determined by using the standard methods described above.

CD measurements. Circular dichroism spectra were recorded in the far-UV range from 190 to 260 nm in a 0.02 cm pathlength optical cell and in the near-UV range from 250 to 300 nm in a 1 cm cell on a Jobin-Yvon Mark VI dichrograph at 25 °C. Four spectra were accumulated and averaged for each sample. The concentrations of the protein solutions were adjusted to 0.15 mg/ml (pH 3.0) in the far-UV measurements and to 0.3 mg/ml in the near-UV measurements. Mean residue ellipticity, $[\Theta]_{MR}$, was expressed in deg cm^2 dmol^{-1}, using a mean residue weight of 110. Percentages of secondary structures were calculated by applying the Provencher and Glöckner curve-analysing algorithm [11].

Results and discussion

Enzyme stability

The effects of different concentrations of ethanol on the activities of α-chymotrypsin and trypsin at pH 3.0 are presented in Fig 1. In water, at pH 3.0 the enzyme activities did not decrease during the incubation period, but the activities of both enzymes decreased with the increasing ethanol concentration. Trypsin exhibited a higher stability than α-chymotrypsin. At an ethanol content of 60%, the α-chymotrypsin activity was practically completely lost, while the activity of trypsin was still about 40% of the initial level even in 85% ethanol (see also Fig. 3). The further experiments were aimed at establishing the effects of Ca^{2+} on the stability and conformation of α-chymotrypsin and trypsin at 60% and 85% ethanol, respectively.

Fig. 2 depicts the effects of different concentrations of Ca^{2+} on the α-chymotrypsin activity. With increasing Ca^{2+} concentration, the activity loss decreased. At higher $CaCl_2$ concentrations (1.1–1.2 M), no changes in the activity of α-chymotrypsin were measured in 60% ethanol in comparison with the control. The influence of Ca^{2+} on the trypsin activity was studied between concentrations of 0.3 and 0.6 M. In the presence of Ca^{2+}, the activity of trypsin increased and at 0.6 M calcium ion concentration not only was the catalytic activity of trypsin preserved, but even an activation was detected. Fig. 3 demonstrates the stabilizing effect of Ca^{2+}: the activities of these enzymes did not decrease an incubation period of 2 h.

CD measurements

The effects of the increasing Ca^{2+} concentration on the secondary and tertiary structures of α-chymotrypsin

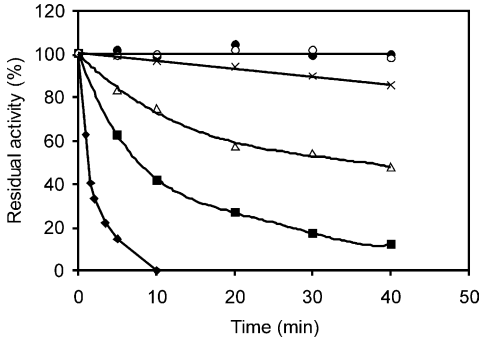

Fig. 2. Stabilities of α-chymotrypsin in 60% ethanol at pH 3 and 25 °C. The Ca^{2+} concentration was varied in the range 0–1.2 M (◆) 0 M, (■) 0.8 M, (△) 0.9 M, (×) 1.0 M, (●) 1.1 M, and (○) 1.2 M). Enzyme concentration: 0.15 mg/ml.

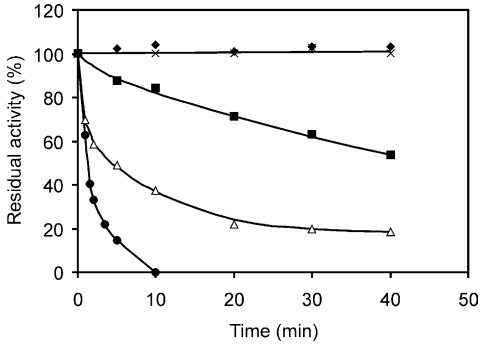

Fig. 1. Effects of different concentrations of ethanol on the activity of α-chymotrypsin at pH 3.0 and 25 °C. Enzyme concentration: 0.15 mg/ml. (×) Water, pH 3.0, (◆) 20%, (■) 30%, (△) 40%, and (●) 60% ethanol.

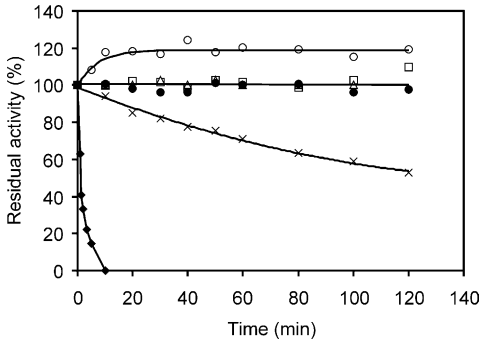

Fig. 3. Comparison of the stabilizing effects of Ca^{2+} on the activities of trypsin and α-chymotrypsin at pH 3.0 and 25 °C. α-Chymotrypsin: water, pH 3.0 (△), 60% ethanol (◆), and 1.2 M $CaCl_2$ (□). Trypsin: water, pH 3.0 (●), 85% ethanol (×), and 0.6 M $CaCl_2$ (○).

and trypsin were studied by means of UV CD measurements. The far-UV CD spectra of the two enzymes in ethanol (pH 3) without and with the highest concentrations of Ca^{2+} applied are shown in Fig. 4. The spectra of α-chymotrypsin in 60% ethanol and of trypsin in 85% ethanol are characteristic of β-sheet conformation. The Provencher–Glöckner curve-analysing algorithm yielded 52% and 61% β-sheet contents for α-chymotrypsin and trypsin, respectively. However, the appearance of a new peak at 208 nm in the presence of 0.6 M Ca^{2+} (in trypsin) and 1.2 M Ca^{2+} (in α-chymotrypsin) reflects an increase in α-helix content. The mean residue ellipticities at 208 nm (proportional to the α-helix content) and 215 nm (proportional to the β-sheet content) show different responses with increasing Ca^{2+} concentration in the ethanolic solutions of the enzymes: the α-helix and β-sheet contents of trypsin change almost in parallel, while those of α-chymotrypsin change in the opposite way with increasing Ca^{2+}concentration (Fig. 5). This resulted in a higher α-helix/β-sheet ratio at the maximum Ca^{2+} concentration for α-chymotrypsin than for trypsin. An increase in the helical content on the addition of Ca^{2+} has likewise been detected for Ca^{2+}-binding proteins such as parvalbumin or calmodulin [12]. Ca^{2+} has also been shown to play an important role in stabilizing the non-Ca^{2+}-binding protein subtilisin by binding to the backbone carbonyl oxygens [4]. We presume that the ligation of Ca^{2+} by backbone oxygens may increase the proportion of the helical conformation in α-chymotrypsin and trypsin.

The near-UV CD spectra (250–300 nm) were also monitored in order to obtain information on the tertiary structures of the enzymes. This spectral region is dominated by aromatic side-chain and disulfide contributions and the band intensity is affected by oligomerization and/or local conformational changes around these chromophores [13]. The intensity of this band is increased when the aromatic residues come into closer contact with each other [14]. Since both enzymes contain tyrosines, tryptophans, and cystines, the assignment of the spectra and interpretation of the spectral changes are rather difficult. The near-UV CD spectra of trypsin and α-chymotrypsin in water and in ethanol, without and with Ca^{2+}, are shown in Figs. 6 and 7. The significantly smaller CD signals in ethanol, as compared with those measured in water, reflect the less compact structures of both enzymes. The presence of 1.2 M Ca^{2+} in a 60% ethanolic solution of α-chymotrypsin results in partial

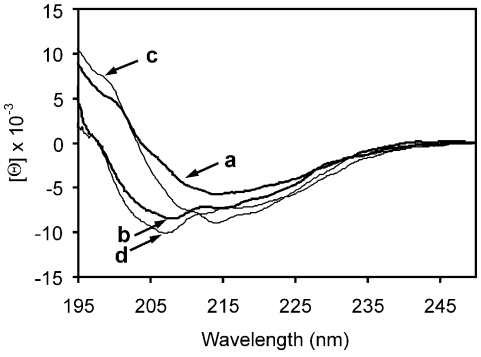

Fig. 4. Far-UV CD spectra of trypsin in 85% ethanol at pH 3 without (a) and with 0.6 M Ca^{2+} (b) and α-chymotrypsin in 60% ethanol at pH 3 without (c) and with 1.2 M Ca^{2+} (d).

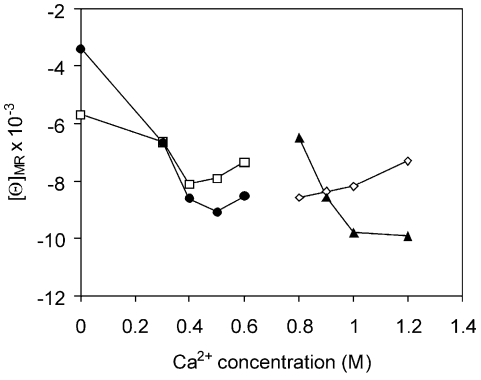

Fig. 5. Values of mean residue ellipticities as a function of the Ca^{2+} concentration. $[\Theta]_{MR}$ of trypsin in 85% ethanol at 208 nm (●) and 215 nm (□). $[\Theta]_{MR}$ of α-chymotrypsin in 60% ethanol at 208 nm (▲) and 215 nm (◇).

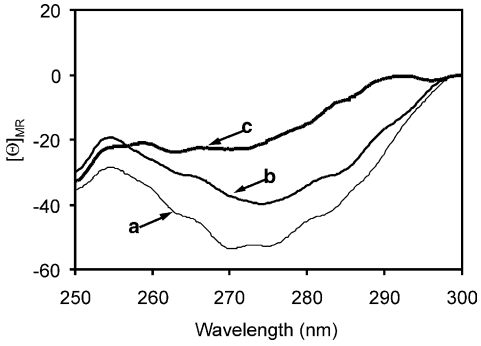

Fig. 6. Near-UV CD spectra of trypsin in water (a) and in 85% ethanol without (b) and with 0.6 M Ca^{2+} (c).

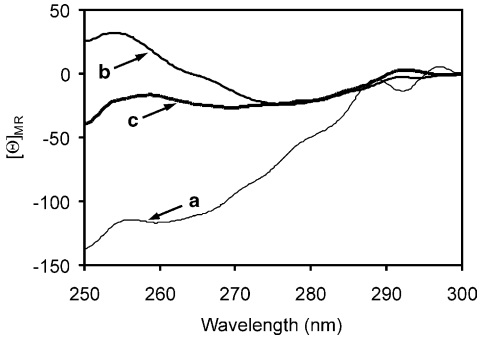

Fig. 7. Near-UV CD spectra of α-chymotrypsin in water (a) and in 60% ethanol without (b) and with 1.2 M Ca^{2+} (c).

restoration of the tertiary structure, but 0.6 M Ca^{2+} in an 85% ethanolic solution of trypsin apparently decreases of the compactness of the enzyme.

Conclusions

Our studies have shown that the activity of α-chymotrypsin was virtually lost within 10 min in the presence of 60% ethanol, whereas trypsin preserved about 40% of its original activity even in 85% ethanol at pH 3. In the presence of 1.2 M Ca^{2+} in 60% ethanol, the enzyme activity of α-chymotrypsin was restored and the same rate was measured as in water. In the case of trypsin, in the presence of 0.6 M Ca^{2+} in 85% ethanol, even activation was observed relative to the level detected in water. The restoration of the enzyme activity in the presence of calcium ions in the ethanolic solvent was accompanied by changes in the secondary and tertiary structures. In the ethanolic solvent, both enzymes are present mainly in the β-sheet conformation, but a high Ca^{2+} concentration increases the helical content to different extents, resulting in a higher α-helix/β-sheet ratio for α-chymotrypsin than for trypsin. It may be assumed that the stabilization of the α-helix in aqueous ethanol at pH 3 is due to the binding of calcium to the backbone carbonyl groups.

In an earlier study, the stability of α-chymotrypsin in ethanol was maintained by polyols and (particularly by polyethylene glycol) via the formation of a very compact structure following an increase in β-sheet content [9]. The results presented in this paper show that enzyme stabilization might be achieved in a different way. The increases in stability of α-chymotrypsin and trypsin in aqueous ethanol in the presence of Ca^{2+} appear to be due to an increased α-helical content and the formation of a less compact overall structure.

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