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Effects of polyhydroxy compounds on the structure and activity of α-chymotrypsin

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Abstract

The effects of glycerol, polyethylene glycol, fructose, glucose, sorbitol, and saccharose on the conformation and catalytic activity of α-chymotrypsin were studied in 0.1 M sodium phosphate buffer and buffered aqueous 60% ethanol (pH 8.0). The enzyme activity was practically completely lost within 10 min in 60% ethanol, but in the presence of stabilizers the activity was retained. With the exception of polyethylene glycol, the stabilizing effect decreased with increase of the incubation time. The preservation of the catalytic activity was accompanied by changes in the secondary and tertiary structures of α-chymotrypsin.

Keywords: α-Chymotrypsin; Enzyme structure; Ethanol; Polyols; Stability; Sugars

The use of enzymes in organic solvents has extended their practical applications and allowed the syntheses of polymers, biologically active enantiomers that are difficult to obtain with conventional chemical catalysts [1,2]. The solvent influences the catalytic properties and stability of the enzyme to a large extent. The stability of enzymes is one of the most difficult problems in protein chemistry, in consequence of the great number of factors involved and the lack of experimental methods allowing an evaluation of their individual contributions. Protein molecules in aqueous solution are surrounded by a hydration shell, which is composed of water molecules attached to the protein surface. If an organic solvent is present, the solvent molecules tend to displace the water molecules both in the hydration shell and in the interior of the protein, thereby distorting the interactions responsible for maintaining the native conformation of the enzymes [3,4]. Different strategies have been developed to enhance the stability of enzymes: protein engineering, immobilization, chemical modifications, and the use of salts or polyols [5–8]. Sorbitol, polyethylene glycol (PEG), and different mono- or disaccharides are commonly used as protein stabilizers. Obtaining stable biocatalysts in organic media is one of the main targets of biotechnology.

Organic solvents can cause significant alterations in the secondary structures and activities of enzymes [9–12]. We earlier studied the effects of various organic solvents (ethanol, 1,4-dioxane, and acetonitrile) at different concentrations on the kinetic parameters and stability of hydrolytic enzymes [10,12]. With increasing concentrations of water-miscible polar solvents, the activities of α-chymotrypsin, trypsin, and lipase first decreased and then increased. We found that the rates of catalytic activities of trypsin and α-chymotrypsin at an organic solvent content of 95% were similar (or higher) to those of the native enzymes in water. α-Chymotrypsin proved to be least stable in ethanol, its stability reaching a minimum value in the interval 40–85% [10]. In the present work we have studied the effects of polyhydroxy stabilizers (glycerol, glucose, fructose, sorbitol, saccharose, and PEG 20000) on the catalytic activity of α-chymotrypsin in phosphate buffer (pH 8.0) and in a buffered water–ethanol (60%) mixture (pH 8.0). The changes in the secondary and tertiary structures were followed by means of circular dichroism (CD) measurements.

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Materials and methods

Materials. *N*-Acetyl-L-tyrosine ethyl ester (ATEE), α-chymotrypsin (from the bovine pancreas), D-fructose, D-glucose, D-sorbitol, saccharose, and PEG 20000 were obtained from Sigma–Aldrich. All other chemicals were reagent grade products of Reanal.

Assay of enzyme activity. 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 [13]. The reactions were initiated by 50 μl of 0.15 mg/ml enzyme.

Stability tests. The stability tests were performed at 25 °C in 60% ethanol. The incubation mixture (0.4 ml) contained 30 μl of 0.1 M sodium phosphate buffer (pH 8.0) and 10 μl of 6 mg/ml enzyme. The samples were incubated with one or other of the different stabilizers for appropriate periods of time, aliquots were then withdrawn and the residual activities of the enzymes were determined by using the standard methods, as described above.

CD measurements. CD spectra were recorded in the far-UV range from 190 to 260 nm in a 0.02 cm 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 concentration of each of the enzyme solutions was adjusted to 0.15 mg/ml. 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-analyzing algorithm [14].

Results and discussion

Enzyme stability

The effects of different concentrations of ethanol on the activity of α-chymotrypsin in 0.1 M phosphate buffer (pH 8) are shown in Fig. 1. In the buffer, the enzyme activity did not change even in the 2-h incubation period (see also Fig. 4), but with increasing ethanol concentration the activity of α-chymotrypsin decreased. At 60% ethanol content, the enzyme activity was virtually lost after a 10-min incubation. Hence, in the further experiments an ethanol concentration of 60% was used to test the stabilizing effects of polyhydroxy compounds.

The effects of 0.4–1.4 M D-fructose and 5–100 mg/ml PEG on the α-chymotrypsin activity in buffered aqueous 60% ethanol at pH 8 are presented in Figs. 2 and 3. Both compounds exhibit a good stabilizing effect and are capable of preventing the inactivation of α-chymotrypsin against 60% ethanol in the 40-min incubation period. In the presence of 100 mg/ml PEG, practically no inactivation occurred. Fructose at concentrations >1.4 M probably exerts the same stabilizing effect, but the small amount of water present in the incubation mixture prevented the dissolution of a higher amount of fructose. Stabilization by sugars such as D-glucose, saccharose, D-sorbitol, and glycerol was also studied as a function of their increasing concentration. In most cases, the extent of stabilization at the maximum concentrations of polyols (D-sorbitol, 1.24 M; saccharose,

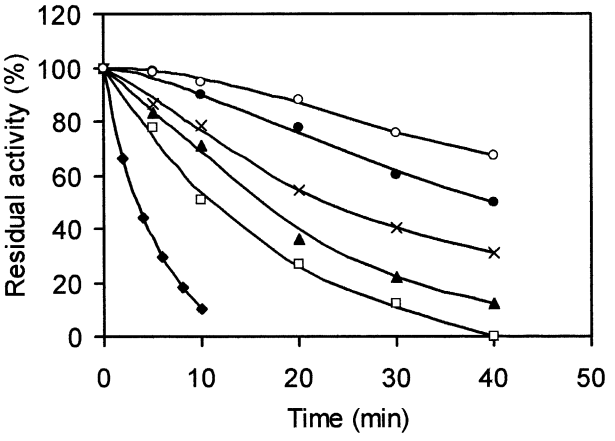

Fig. 2. Effects of different concentrations of D-fructose on the activity of α-chymotrypsin in buffered aqueous 60% ethanol at pH 8.0 and 25 °C. (◆) 0, (□) 0.4, (▲) 0.6, (x) 0.8, (●) 1, (○) 1.4 M D-fructose.

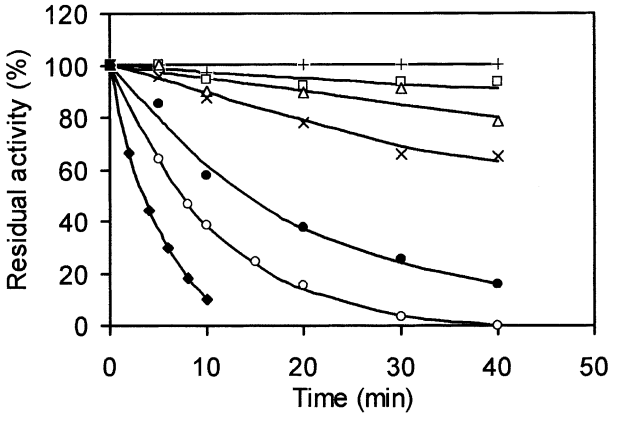

Fig. 1. Changes in activity of α-chymotrypsin in different concentrations of ethanol in 0.1 M phosphate buffer at pH 8 and 25 °C. (+) Buffer only, (□) 10%, (△) 20%, (x) 30%, (●) 40%, (○) 50%, and (◆) 60% ethanol. Enzyme concentration: 0.15 mg/ml.

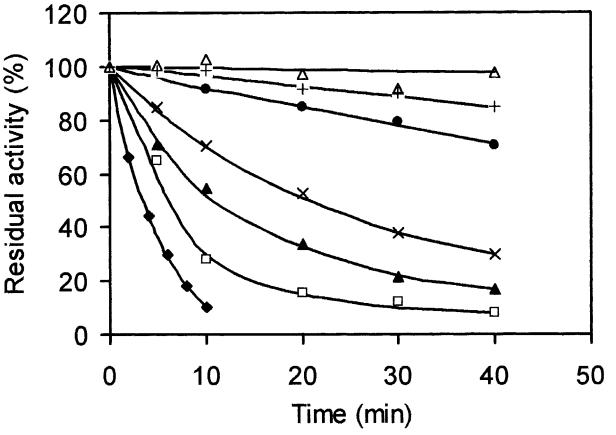

Fig. 3. Effects of different concentrations of PEG 20000 on the activity of α-chymotrypsin in 60% ethanol. (◆) 0, (□) 5, (▲) 10, (x) 20, (●) 40, (+) 80, (△) 100 mg/ml PEG.

0.7 M; glycerol, 12.5% w/v) was similar to that obtained with D-fructose, and the enzyme retained around 50% of its activity in the 2-h incubation period (Fig. 4). However, in the presence of 0.77 M D-glucose, α-chymotrypsin lost almost 90% of its activity. Glycerol could be used as stabilizer at 10–15%: higher or lower concentrations had no stabilizing effect, or decreased the enzyme activity (Fig. 5). Thus, the stabilizing effects of the different saccharides and glycerol decreased with increasing incubation time, while that of PEG underwent only a negligible change. This difference in stabilization might be due to the fact that PEG is essentially a nonpolar polyol and can bind to the hydrophobic sites of proteins [15]. A study of the intra- and intermolecular bonds within the protein conformation by Raman methods revealed that the polyols (glycerol, sorbitol, and PEG) have little effect on the structural organization of water, suggesting that their protective effects arise from direct interactions (specific or non-specific) with the enzyme polypeptide [16]. The interaction between lysozyme and sorbitol was studied by NMR spectroscopy, and anomalous relaxation properties of Ala and Thr methyl groups were observed, indicating the modifications of local motions. Water displacement was also found in the protein structure, which reveals a complex interplay of different interactions [17]. In contrast with polyols, sugars appear to enter the lattice structure of water surrounding the protein molecules and in this way strengthen it, thereby stabilizing the protein structure [18].

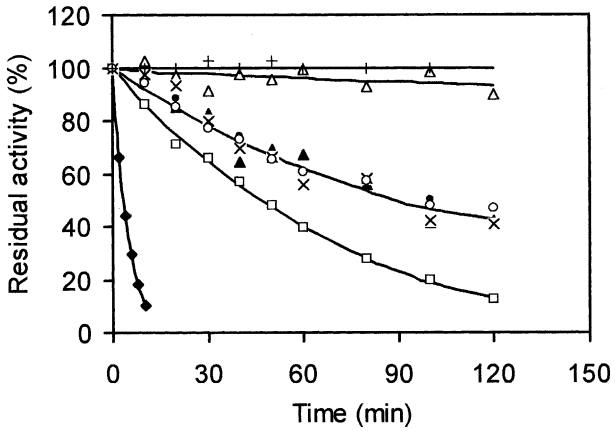

Fig. 4. Comparison of the stabilizing effects of the different polyols at their maximum concentration applied in the assays of the activity of α-chymotrypsin in buffered 60% ethanol at pH 8 and 25 °C. (◆) No stabilizer in ethanol, (□) 0.77 M D-glucose, (▲) 1.4 M D-fructose, (x) 0.7 M saccharose, (○) 12.5% glycerol, (●) 1.24 M D-sorbitol, (△) 100 mg/ml PEG 20000, (+) enzyme activity in 0.1 M sodium phosphate buffer (pH 8) without stabilizers.

Structural characterization

The effects of the stabilizers on the secondary structure of α-chymotrypsin were also studied by means of UV CD measurements. The far-UV CD spectra of α-chymotrypsin in buffer, in buffered 60% ethanol without and with the different polyols are shown in Fig. 6. The spectrum of α-chymotrypsin in buffer has a negative maximum at 204 nm; the Provencher–Glöckner curve-analyzing algorithm yielded a β-sheet content of 47%. α-Chymotrypsin belongs among the all-β-proteins, with distorted or short irregular strands, which may cause the negative CD band to shift from the ideal β-sheet position of 210–220 nm to lower wavelengths [19]. In buffered 60% ethanol, the spectrum displays a more typical β-sheet character (λ_{max} at 216 nm). In the presence of different stabilizers in 60% ethanol, the spectral changes can be divided into two groups. Glucose, fructose, and sorbitol have only slight effects on the secondary structure of α-chymotrypsin, while in the cases of saccharose, PEG, and glycerol the low-intensity distorted spectra reflect the presence of aggregates.

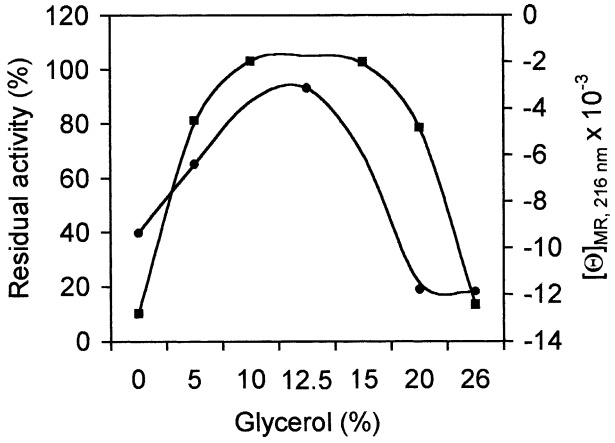

Fig. 5. Catalytic activity (after 10-min incubations) (■) and mean residue ellipticity $[\Theta]_{MR}$ at 216 nm (●) of α-chymotrypsin as a function of the glycerol concentration.

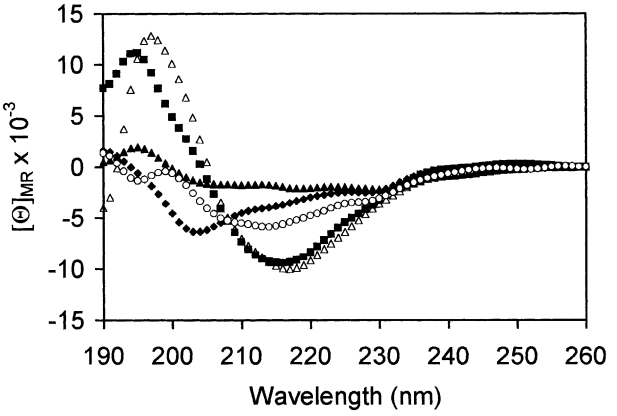

Fig. 6. Far-UV CD spectra of α-chymotrypsin in 0.1 M sodium phosphate buffer (pH 8.0) (◆), in buffered 60% ethanol without stabilizer (■) and with 1.4 M D-fructose (△), 100 mg/ml PEG (▲), or 0.7 M saccharose (○).

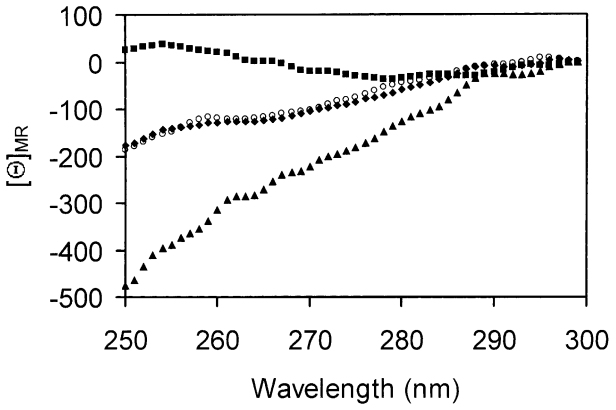

Fig. 7. Near-UV CD spectra of α-chymotrypsin in 0.1 M buffer (◆), in buffered 60% ethanol without stabilizer (■), or with 100 mg/ml PEG (▲) and 0.7 M saccharose (○).

Moreover, for glycerol a noteworthy spectral response of the enzyme was observed. Fig. 5 shows a plot of the mean residue ellipticity values at 216 nm as a function of the glycerol concentration in the range 0–26% (w/v). At low or high glycerol contents, the CD spectra indicate characteristic β-sheets with high negative $[\Theta]_{MR}$ values at 216 nm, while the small negative $[\Theta]_{MR}$ values at around 12.5% glycerol concentration (where the highest enzyme activities were observed) reflect the formation of β-aggregates from the β-sheets.

Near-UV CD spectra (250–300 nm) were also monitored in order to acquire further insight into the overall change in the enzyme structure. This spectral region includes major contributions of aromatic–aromatic interactions of tyrosine molecules [20]. The intensity of the peak here is sensitive to the distance between the aromatic groups and in turn to the compactness of the protein. Enhancement of the intensity of this band occurs when the aromatic residues come into closer contact with each other upon aggregation. The near-UV CD spectra of α-chymotrypsin in 60% ethanol with or without stabilizers and in buffer are shown in Fig. 7. The significantly smaller CD signal at around 275 nm in 60% ethanol, compared to that measured in buffer, reflects the loss in tertiary structure in ethanolic solution. The presence of 0.77 M saccharose and 100 mg/ml PEG results either in restoration of the tertiary structure (saccharose) or in the formation of an even more compact structure (PEG). The increases in the band intensities in the near-UV range are in accordance with the decreased intensities of the far-UV negative maxima in the presence of these stabilizers (Fig. 6).

Conclusions

Our comparative studies on the stabilities and steric (secondary/tertiary) structures of α-chymotrypsin permit the following conclusions:

In aqueous buffer at pH 8, α-chymotrypsin proved to be stable in the 2-h incubation period; the native secondary structure of the active enzyme is a distorted β-sheet conformation.

In buffered 60% ethanol at pH 8, the rapid loss of enzyme activity within 10 min is accompanied by a structural rearrangement (distorted β-sheet → classical β-sheet). The increased proportion of the classical β-sheet conformation might be partly responsible for the deactivation of the enzyme. The near-UV CD spectrum suggests that the classical β-sheet conformation is less compact than the distorted β-sheet. The aromatic–aromatic interactions are weakened in the presence of ethanol, presumably as a result of the solvent-induced perturbations of the enzyme molecules.

In the presence of different stabilizers in buffered 60% ethanol, either the secondary/tertiary structures of the enzyme are changed (glucose, fructose, and sorbitol) or a subtle change is observed in the tertiary structure (β-aggregation with saccharose, PEG, and glycerol).

Preservation or loss of enzyme stability is not accompanied by an unequivocal change in the steric structures in the presence of the highest concentrations of stabilizers applied:

(i) Although a significant β-aggregation of α-chymotrypsin occurs in the presence of PEG, saccharose, and glycerol, PEG proved to be a very efficient stabilizing agent, whereas the latter two did not.

(ii) A ~50% loss of stability was detected within 2 h either in the presence of sorbitol and fructose, without a significant change in the secondary structure of the enzyme, or with saccharose and glycerol, inducing an increased tertiary structure.

(iii) In the presence of glucose, a 90% loss of enzyme activity occurred, with no alteration in the secondary structure.

Our results suggest that the restoration of the activity and stability of α-chymotrypsin in the presence of the polar organic solvent ethanol requires the formation of a very compact structure, which exists in the presence of PEG.

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