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Properties of soluble α-chymotrypsin in neat glycerol and water

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Abstract

UV scanning of α-chymotrypsin dissolved in neat glycerol and water showed no significant differences in its spectra at pH 7.8. Fluorescence scanning revealed a strong dependence on pH values (between 5.9 to 10.5) of the maximum wavelength emission in water and no pH-dependence in 99% glycerol supplemented with 1% of appropriate buffers. The profile of α-chymotrypsin activity dissolved in water-glycerol mixtures with phenyl acetate as substrate displayed two maximum: highest peak was found at 100% water, and the second one was observed in 99% glycerol concentration with about 40% of the relative activity. Optimum pH of the soluble α-chymotrypsin in glycerol showed a displacement of 1 pH/U towards the alkaline side compared to water at pH 8.0. Kinetic and thermodynamic analysis using kinetic measurements of the thermal stability of α-chymotrypsin showed a higher inactivation rate in neat glycerol as compared to water in 30 to 45°C range, however, when temperature increases enzyme stability in glycerol is better than water. Thermostability of trypsin and α-chymotrypsin dissolved in glycerol at 100°C showed a half reaction time of approximately 7 and 20 h, respectively, and less than 1 minute in aqueous buffer for both enzymes.

Keywords: Homogeneous biocatalysis; Enzymes in organic media; α-chymotrypsin; Trypsin; Glycerol; Enzyme thermostability

1. Introduction

Biocatalysis in organic solvents is an important area of current research in biotechnology. However, most of the research in nonconventional media was developed in enzymatic heterogeneous systems (i.e., suspended powders and micelles) [1]. Strong efforts have been developed to solubilize enzymes in nearly anhydrous media by using chemical modifications techniques such as coupling proteins with water-soluble molecules such as some polyols, or surfactants [2,3]. However, these methods have some drawbacks, such as system characterization, optimization and scaling-up, very important issues in industrial process.

On the other hand, aqueous solutions of polyols, as additives, are currently used to study thermostabilization of enzymes at concentrations lower than 40% [4–6]. In recent work, 73% polyol concentration was used to study enzyme specificity [7]. However, in most of the cases at high co-solvent concentration, aggregates and/or precipitates of proteins in buffer were observed [5,8]. Biocatalysis using dissolved proteases in neat organic solvents were recently reported for subtilisin, and the study was extended to α-chymotrypsin, thermolysin, and trypsin [9,10]. Glycerol was considered the most suitable solvent for these enzyme reactions because of high reaction rates and enzyme stability [9,10]. However, high viscosity of glycerol is the main problem in the mass transfer process associated with reaction kinetic at room temperature. Glycerol high viscosity can be overcome by increasing the working temperature. Enzymatic catalysis at high temperatures has some advantages, such as thermodynamic shifting to products, increasing of reaction rate, high solubility of substrates and products, reduction of microbial contamination, but one of the major problems is enzyme thermostability. To stabilize enzymes at high temperatures chemical modification of enzymes such as α-chymotrypsin was previously described [11], and the presence of additives in aqueous solutions, such as polyols on α-chymotrypsin thermostability were also reported [12].

The K_m and V_m of soluble α-chymotrypsin in neat glycerol were approximately 10× higher and 2× lower respectively compared to water, using phenyl acetate as substrate [10].

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In this study, the catalytic properties of soluble α-chymotrypsin and trypsin in neat glycerol and in aqueous solutions related to pH, solvent mixture composition and thermostability were examined and compared.

2. Materials and methods

2.1. Materials

Alpha-chymotrypsin (E.C. 3.4.21.1), and trypsin (E.C. 3.4.21.4) from bovine pancreas were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Commercial enzymes were dissolved at 5.0 mg/ml in 50 mM buffers containing 10 mM $CaCl_2$ at the following pH range: 5.9 and 6.6 (MES), 7.0 and 7.8 (MOPS), 8.2 and 9.0 (TAPS), and 9.7–10.5 range (CAPS). Enzyme solutions were lyophilized for 48 h (−50°C, 10 μm of Hg), and the dry powders stored at −20°C under Argon.

Analytical grade glycerol was stored with 3Å molecular sieves under Argon at room temperature before usage. The water content was measured by the titrimetric Fisher method [13]. Water did not exceed 0.05% (w/w) in pure glycerol.

2.2. UV—visible spectra

Wavelength scanning was performed by using 1.0 mg/ml enzyme concentration in buffer and 99% glycerol at pH 7.8. The sample readings were performed by using solvent controls in a double bean spectrophotometer Hitachi U-3110.

2.3. Fluorescence experiments

Alpha chymotrypsin solutions (100 μg/ml) were prepared dissolving 1.0 mg enzyme (containing 12 to 17 enzymatic units) in appropriate volume of solvent (glycerol or buffer systems) at room temperature, and fluorescence emission was measured immediately. Experiments were performed in a Flouromax-2 (Jobin Yvon-spec, Edison, NJ, USA) with an excitation wavelength of 280 nm. Emission wavelengths were scanned between 300 to 350 nm (1 nm increment, 1 s integration time), and slits of 5 nm. Light scattering produced by solvent was subtracted from the samples.

2.4. Analytical assays

Enzymatic cleavage of 30 mM phenyl acetate was assayed by HPLC by using Waters C_{18} reverse phase column (3.9 × 150 mm). Substrate and product were eluted isocratically with acetonitrile (38%) − water (62% containing 0.4% of CF_3CO_2H) solvent mixtures at 0.35 ml/min flow rate at room temperature. Concentrations of phenol and phenyl acetate were calculated from peak areas after calibration with standard solutions. Initial rates were determined by the increase of phenol concentration up to a 5.0% conversion at 30°C.

Enzymatic assays in glycerol were performed with 1% of appropriate buffers. For kinetic determinations in glycerol, samples of 50 μl were withdrawn and vortexed with 200 μl of acetonitrile for 2 min. In water kinetic measurements, 200 μl of samples were withdrawn, mixed with equal volume of isopropyl ether, and vortexed for 2 min. After centrifugation at 10 000 × *g* for 1 min, 20 μl of the top phase (acetonitrile or isopropyl ether) was analyzed by HPLC.

In water-glycerol mixtures, α-chymotrypsin activity in 0 to 50% glycerol concentration were measured using isopropyl ether system, and in glycerol concentration higher than 50% were assayed with acetonitrile extraction solvent as mentioned before.

2.5. Inactivation experiments

Time course of α-chymotrypsin deactivation was followed by measuring the concentration of *p*-nitroaniline liberated from succinyl-L-alanyl-L-alanyl-L-prolyl-L-phenylalanyl *p*-nitroanilide (SPN) at 410 nm by using Hitachi U-3110 UV-VIS spectrophotometer [14]. In the experiments, 1.0 mg of α-chymotrypsin (12–17 enzymatic units) or trypsin (was dissolved in 1.0 ml of solvent, glycerol or MOPS-Calcium buffer (pH = 7.0), and incubated at constant temperatures (30−65°C). At zero time, specific activities were in the range of 0.80 to 0.100 mM/h mg in all experiments. Periodically, aliquots were withdrawn, cooled, and residual activities were assayed in aqueous buffer system containing 1.0 mM SPN. Enzyme deactivation was followed by two steps first order model [11]:

$$\mathrm{E} \xrightarrow[\alpha_1]{k_1} \mathrm{E}_1 \xrightarrow[\alpha_2]{\mathrm{k}_2} \mathrm{E}_2$$

Where E, E_1, and E_2 are enzymes states, k_1 and k_2 are first-order deactivation rate constants, α_1 and α_2 are the ratio of specific activities in each state. The residual activity, a, is given by the equation:

$$a(t) = \alpha_2 + \left(1 + \frac{\alpha_1 k_1 - \alpha_2 k_2}{k_2 - k_1}\right) e^{-(k_1 t)} - k_1 \left(\frac{\alpha_1 - \alpha_2}{k_2 - k_1}\right) e_1^{-(k_2 t)}$$

Experimental deactivation parameters were calculated using iterative convergence method of non-linear regression (Marquardt–Levemberg method). Experimental and theoretical data was considered in good agreement when the correlation coefficient were higher than 0.90 for a single experiment.

Arrhenius plots were used to estimate the activation energy for inactivation in glycerol and water using the following equation [15]:

$$\ln k_1 = -\frac{E_i}{R} \cdot \frac{1}{T} + \ln C$$

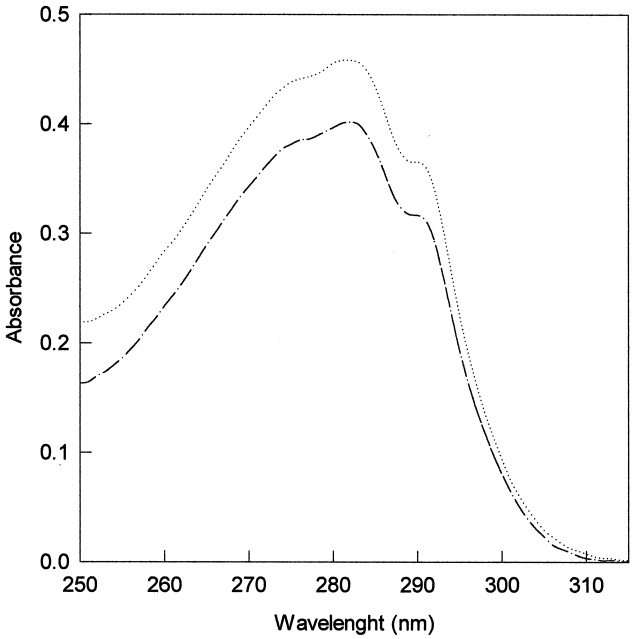

Fig. 1. UV spectra of α-chymotrypsin in glycerol (· · ·) and water (—).

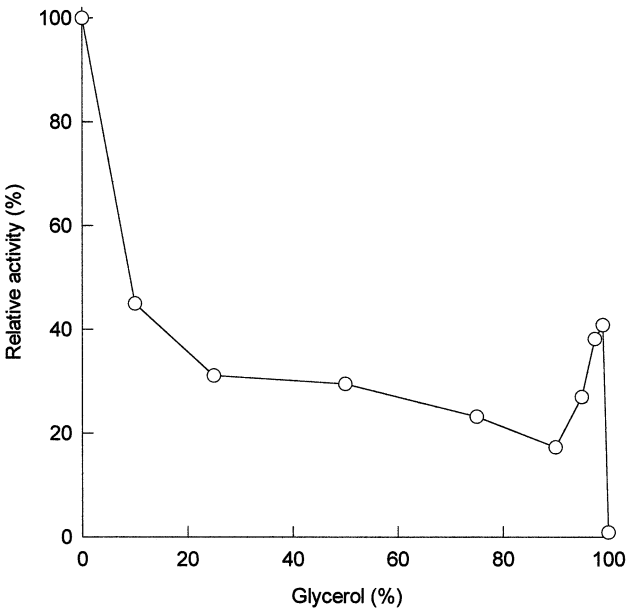

Fig. 2. Dependence of α-chymotrypsin activity with the solvent mixture composition at 30°C.

Where k_1 is the inactivation reaction initial rate constant (1/s), E_i is the activation energy for inactivation (kJ/mol), R is the gas constant (8.314 J/mol K), T is a temperature (K°), and C is a constant.

Experiments at 100°C were performed by using 2.0 mg/ml of α-chymotrypsin and trypsin lyophilized at pH 7.0 dissolved in glycerol or in buffer (50 mM MOPS, 10 mM $CaCl_2$, pH = 7.0). Initial specific rates were in the range of 0.120 to 0.165 mM/h. mg for trypsin and α-chymotrypsin experiments. Samples were incubated at 100°C for different times, and them cooled at 0°C for 5 min before measuring the residual activity at 30°C by using HPLC under standard conditions as described above.

2.6. Gel electrophoresis

SDS-PAGE was performed by using Fast System with 20% Phast gel homogeneous media according to instruction manual of the manufacturer (Amershan Pharmacia Biotech, Uppsala, Sweden). The molecular weights were estimated with low molecular weight standard markers (14,4–94,0 kDa.) (Amershan Pharmacia Biotech, Uppsala, Sweden). SDS-gels were stained using silver-Coomassie double staining procedure previously described [16].

3. Results and discussion

Comparative UV absorbance spectra of α-chymotrypsin dissolved in neat glycerol (99%) and in water at pH 7.0 did not show any significant changes in their profiles between 250 to 320 nm (Fig. 1). Considering that UV spectral properties of aromatic residues reflect their environments, it is possible to assume that conformations of α-chymotrypsin aromatic residues in neat glycerol and in water are practically the same at pH 7.8. Similar comparative profiles were found using trypsin dissolved in neat glycerol and in water (data not shown).

Soluble α-chymotrypsin in glycerol-water mixtures displayed a maximum emission UV spectra red-shifted (to longer wavelength and lowers energies) when the concentration of glycerol was increased between 5 to 90% (data not shown). Concomitant with the red shift, the enzyme activity decreased between 4 to 5 × in glycerol mixtures compared with activity in pure (Fig. 2).

Previous report describes α-chymotrypsin inactivation and aggregation at 10 M (about 73% v/v) and higher glycerol concentrations [8]. These results were not observed in our experimental conditions. On the contrary, comparative high activity, near to 40% relative to water, was found at 99% glycerol concentration in the mixture (Fig. 2). These discrepancies could be attributed to the pH of commercial enzymes, that it was not adjusted to optimum enzyme values before dissolve in organic media in previous work [8]. The "pH memory" of proteins plays an important role to solubilize enzymes in organic solvents as previously described [1].

In 100% glycerol concentration, α-chymotrypsin activity decreased more than 100-folds compared to water. Presence of small amount of water, 10% is essential for α-chymotrypsin activity (Fig. 2). Changes of enzyme activity and spectra related to the solvent composition of soluble polyols such as glycerol as a water co-solvents were reported previously, and attributed to water activity and protein hydration, but the mechanisms remains unclear [4,10,17,18].

To study the effect of solvents related to the protein

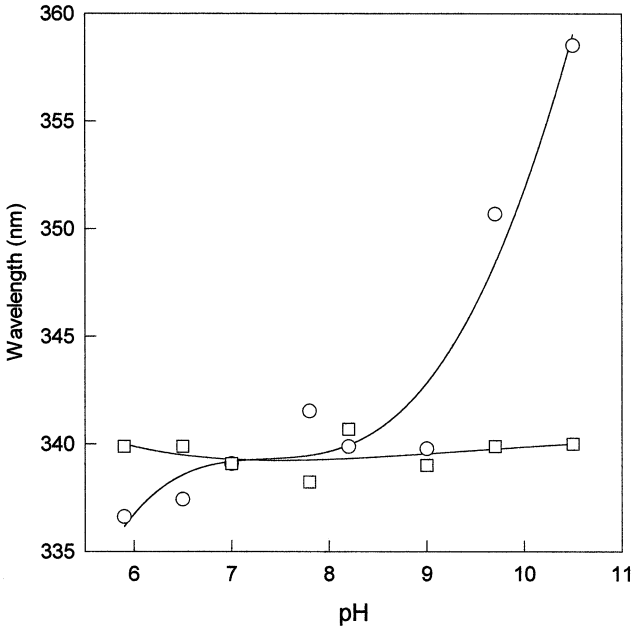

Fig. 3. Effect of pH on maximum wavelength emission of α-chymotrypsin in glycerol (□), and water (○) at 30°C.

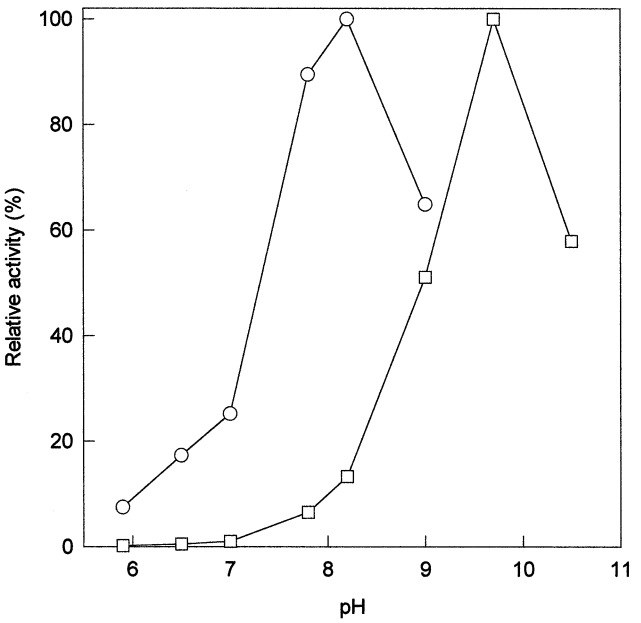

Fig. 4. Effect of pH on the activity of α-chymotrypsin in glycerol (□), and water (○) at 30°C.

conformation changes associated with pH, α-chymotrypsin lyophilized at different pHs were dissolved in 99% glycerol and water and analyzed by fluorescence spectroscopy.

Displacement of approximately 25 nm maximum emission wavelengths in water was observed between 5.9 to 10.5 pH range (Fig. 3). On the contrary, fluorescence emission profile of α-chymotrypsin was almost pH-independent in neat glycerol (Fig. 3). Fluorescence of proteins is a very sensitive indicator of the tryptophan residues microenvironment. Alpha chymotrypsin has eight tryptophan residues, six located at the surface, and other two buried in the interior of the aqueous native protein. In water above pH 9.0, the emission wavelength (λ_{max}) showed an abrupt red shift, to high wavelengths and lower energies values, indicating an important structural change (Fig. 3). This result confirmed previous finding of α-chymotrypsin conformation changes in water related to pH using Raman spectroscopy [19]. However, maximum emission wavelength against pH of α-chymotrypsin dissolved in glycerol was approximately constant in all pH range, indicating a more rigid enzyme conformation in this solvent (Fig. 3).

Using α-chymotrypsin lyophilized at different pH between 5.9 to 10.5, the dependence of the soluble enzyme activity associated to the pH showed sigmoidal and bell shaped curves in water and in neat glycerol respectively (Fig. 4). In water, the maximum activity of α-chymotrypsin was detected at pH 8.0, and fast decay of enzyme activity was observed above this pH, as previously reported [20]. Low enzymatic activity at alkaline pH in buffer is in agreement with the fluorescent shift, and usually associated to protein denaturation.

The α-chymotrypsin activity dependence with pH in glycerol was shifted in approximately one pH unit to the alkaline side compared to the aqueous systems. The effect of pH buffer solution before lyophilization plays a crucial role in enzyme activity in organic solvents [1]. Deprotonation of Ile-16 residue and destruction of salt bridge between Ile-16 and Asp-194 were considered the cause for α-chymotrypsin inactivation at alkaline pH in water [20]. In neat glycerol, proton exchange of Ile-16 residue with the surrounding solvent could not be favored until strong alkaline condition was reached, 10× higher compared to water. Alpha-chymotrypsin was more resistant to the inactivation at higher pH in glycerol probably because the constraint of enzyme structure in organic solvent is higher than in water as suggested by fluorescence experiments.

Inactivation of soluble α-chymotrypsin in neat glycerol and buffer was examined at pH 7.0 between 30 to 65°C. Deactivation of α-chymotrypsin between 30 and 37°C could be considered as an autolysis process. In water, autolysis of α-chymotrypsin at 37°C was relative slow: less than 10% was inactivated in 7 h (Fig. 5a). On the contrary, a second order inactivation curve of soluble α-chymotrypsin in neat glycerol was found, 41% of residual activity was detected after 7 h incubation (Fig. 5a). Degradation of α-chymotrypsin by autolysis mechanism in glycerol was confirmed by gel electrophoresis (Fig. 5b). The second order autolysis process in glycerol could be explained by presence of almost two enzyme fractions with different sensitivity to degradation [11].

At temperatures higher than 42°C, α-chymotrypsin inactivation proceeded at comparable rates in water and 99% glycerol, with similar profiles as previously showed in Fig. 5a (data not shown). Thermal deactivation mechanism was in agreement with two-steps kinetic model reported previously for α-chymotrypsin in water [12]. However, the ex-

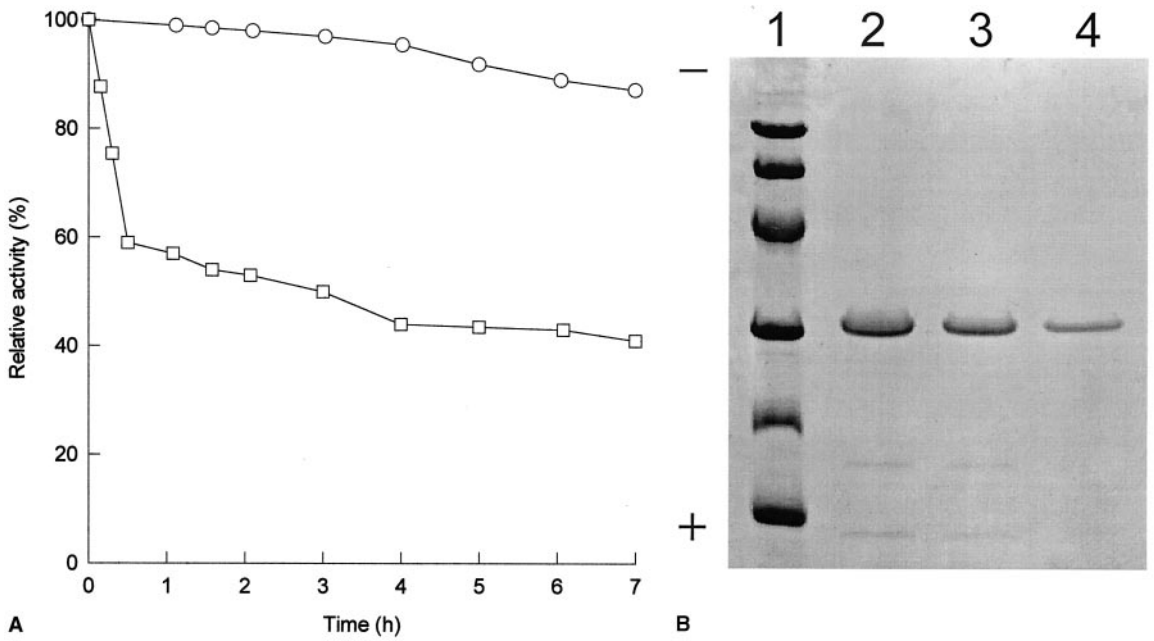

Fig. 5. Autolysis of α-chymotrypsin at 37°C. A enzyme inactivation in glycerol (□) and water (○). B, SDS-PAGE of α-chymotrypsin, lane 1, MW markers; lanes 2 and 3, 0 and 7 h incubation in water, respectively; lane 4, 7 h incubation in neat glycerol.

perimental results showed differences between first, and second inactivation constant rates higher than 10^4-folds (data not shown). In addition, specific activity ratios for the first stage (α_1) were near to the unit, and for the second stage (α_2) were more than 100× lower (data not shown).

Therefore, deactivation process of α-chymotrypsin was governed by the first inactivation constant. Above 45°C in water, α-chymotrypsin activity showed a strong dependence of the first inactivation constant rate (k_1) with the temperature compared with neat glycerol (99%) (Fig. 6a). At temperatures below 45°C (318.15 K), inactivation process is more favorable in glycerol than water and the k_1 are ap-

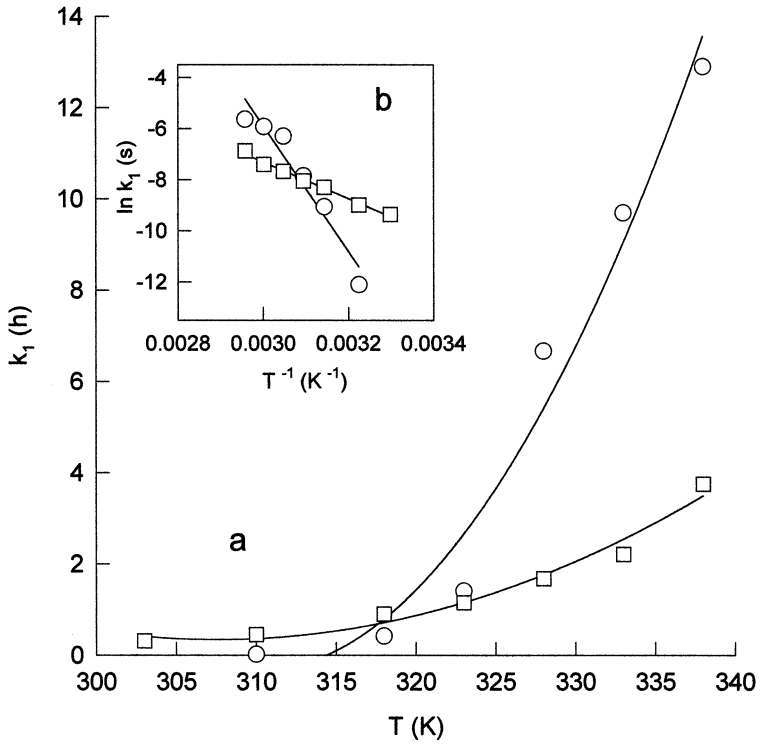

Fig. 6. Effect of temperature on the inactivation specific rate constant (k_1) of α-chymotrypsin in glycerol (□), and water (○).

Table 1
Effect of temperature on inactivation parameters of soluble α-chymotrypsin in glycerol and water

Solvent	Temperature (K)	$T_{1/2}$(h)	$\Delta G^{\#}$ (kJ/mol)
Glycerol	303.15	2.24	97.9
	310.15	1.54	99.2
	318.15	0.77	100.0
	323.15	0.60	101.0
	328.15	0.41	101.6
	333.15	0.31	102.4
	338.15	0.18	102.5
Water	310.15	34.66	107.3
	318.15	1.65	102.1
	323.15	0.49	101.0
	328.15	0.10	97.8
	333.15	0.07	98.3
	338.15	0.05	99.0

proximately constant for both solvents. However, there is a fast increase of k_1 over 50°C (323.15 K), but it is more marked in water compared with glycerol (Fig. 6a). Thermoinactivation at temperatures higher than 65°C (338.15 K) is very fast and k_1 can be not experimentally estimated. The relationship between specific reaction rate and the inverse of temperature can be expressed by a function formulated previously by Arrhenius. Alpha chymotrypsin activation energy for inactivation estimated by Arrhenius plot was 59.6 kJ/mol and 204.7 kJ/mol for glycerol and water respectively (Fig. 6b). Decrease of activation energy for inactivation in glycerol compared with water could be linked to the low availability of water molecules a surrounding the enzyme molecule.

Using inactivation kinetic data, half-life reaction time ($t_{1/2}$) and standard free energy ($\Delta G^{\#}$), of soluble α-chymotrypsin in neat glycerol and water were calculated (Table 1). The dependence of half-life reaction time with temperature was higher in water than in glycerol. The $t_{1/2}$ of α-chymotrypsin between 37 and 45°C decreased 21× in water, and only 2× in glycerol in the same temperature range (Table 1).

Standard free energy of soluble α-chymotrypsin in neat glycerol and water showed very similar absolute values, but a positive slow trend with the increase of temperature could be observed in the non-aqueous solvent (Table 1). On the contrary, the standard free energy for the inactivation process of α-chymotrypsin in buffer showed a negative trend with the temperature. These trends indicated that the energy barrier for soluble α-chymotrypsin inactivation in 99% glycerol became higher with the temperature, so the non-aqueous solvent stabilizes the enzyme against thermoinactivation. If α-chymotrypsin unfolding is considered as first step in thermoinactivation process, changes of free energies between maximum and minimum work temperatures in both solvents less than 10 kJ/mol are in good agreement with the small amount of free energy required for stabilization against protein unfolding as previously reported [4].

Free standard energy of enzyme deactivation ($\Delta G^{\#}(T)$) for the first stage may be calculated using the following equation from experimental data:

$$\Delta G^{\#}(T) = RT \ln (k_b T/k_1 h)$$

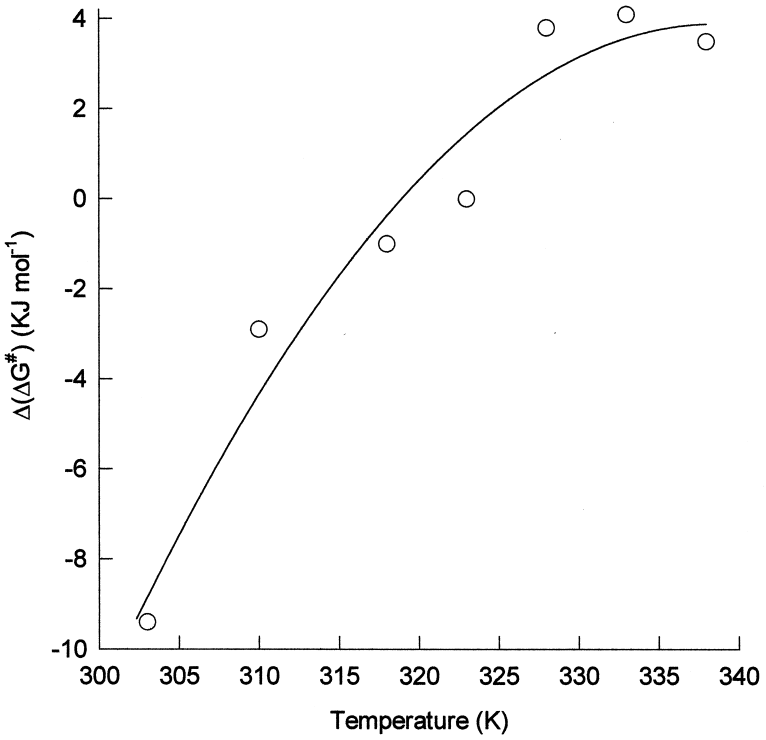

Fig. 7. Temperature dependence on $\Delta(\Delta G^{\#})$ of α-chymotrypsin deactivation in glycerol compared with water.

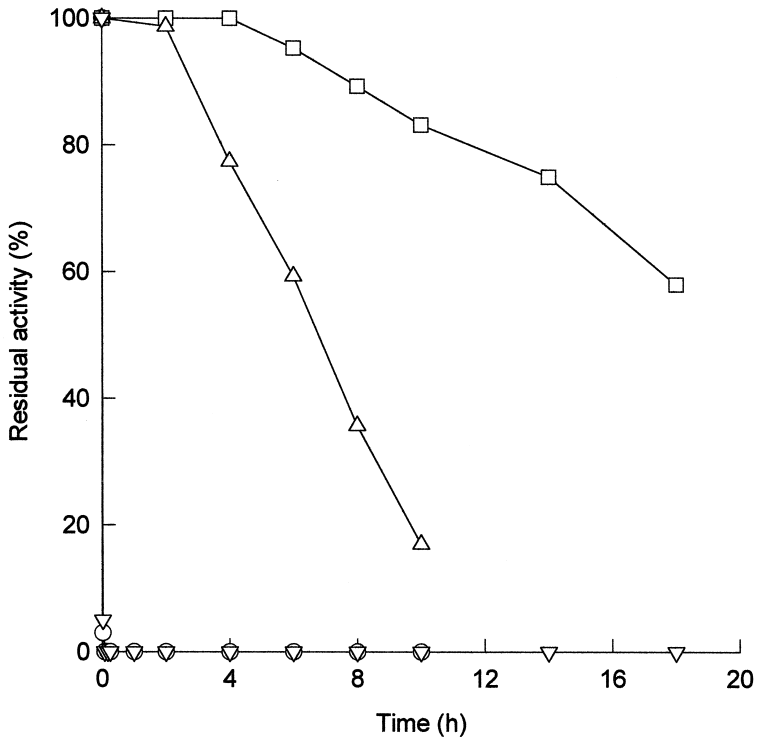

Fig. 8. Thermostability at 100°C of α-chymotrypsin in glycerol (□), and water (○), and trypsin in glycerol (Δ), and water (∇).

Where R, k_b and h are Universal gas, Boltzmann and Planck constants, respectively.

Comparative dependence of α-chymotrypsin stabilization in neat glycerol and water with the temperature can be described by changes on standard free energy ($\Delta G^{\#}(T)$) for the first stage inactivation at each temperature [4].

$$\Delta(\Delta G^{\#}(T)) = (\Delta G^{\#}_{Glycerol}(T)) - (\Delta G^{\#}_{water}(T))$$

A positive variation of $\Delta(\Delta G^{\#})$ with the temperature would be observed for α-chymotrypsin in glycerol compared with water when the temperature was increased (Fig. 7).

Thermoinactivation of soluble α-chymotrypsin at 100°C displayed very dramatic differences in water and in glycerol (Fig. 8). To confirm that the enzymatic activity in the samples was not related to small amount of trypsin that is usually present in commercial samples of α-chymotrypsin, trypsin thermoinactivation assays were performed. In water, α-chymotrypsin and trypsin were fully inactivated in less than 1 min. However, after 10 h of incubation in neat glycerol the residual activity of soluble α-chymotrypsin was approximately 80% (Fig. 8). Trypsin showed higher thermodeactivation sensitive profile than α-chymotrypsin. About 80% of trypsin activity was found after 4-h incubation time at the same experimental conditions (Fig. 8). These results of soluble α-chymotrypsin and trypsin in neat glycerol can be explained by reversible unfolding at high temperature and partial refolding after cooling the samples.

Thermoinactivation of enzymes takes place by unfolding and covalent alterations in the primary structure of the molecule.

In summary, glycerol can be used as alternative solvent to carry out enzymatic reactions in homogeneous phase with α-chymotrypsin. The results show that the use of glycerol has the advantage of providing enzyme activity at high temperatures and alkaline pH, physicochemical conditions that can be not used in aqueous solutions.

Acknowledgments

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References

[1] Dordick JS. Principles and applications of non-aqueous enzymology. In: Dordick JS, editor. Applied biocatalysis. New York: Marcel Dekker Inc., 1991, p. 1–53.
[2] Paradkar VM, Dordick JS. Aqueous-like activity of α-chymotrypsin dissolved in nearly anhydrous organic solvents. J Am Chem Soc 1994;116:5009–10.
[3] Yang Z, Domach M, Auger R, Yang FX, Russell AJ. Polyethylene glycol-induced stabilization of subtilisin. Enzyme Microb Technol 1996;18:82–9.
[4] Gekko K, Timasheff SN. Mechanism of protein stabilization by glycerol: preferential hydration in glycerol-water mixtures. Biochemistry 1981;20:4667–76.
[5] Baigorí MD, Castro GR, Siñeriz F. Purification and properties of extracellular esterase from *Bacillus subtilis* MIR-16. Biotechnol Appl Biochem 1996;24:7–11.
[6] Breccia JD, Moran AC, Castro GR, Siñeriz F. Thermal stabilization by polyols of β-xylanase from *Bacillus amyloliquefaciens*. J Chem Technol Biotechnol 1998;71:241–5.

[7] Ligné T, Pauthe E, Monti JP, Gacel G, Larreta–Garde V. Additional data about thermolysin specificity in buffer- and glycerol-containing media. Biochim Biophys Acta 1997;1337:143–8.
[8] Khmelnistky YL, Mozhaev VV, Belova AB, Sergeeva MV, Martinek K. Denaturation capacity: new quantitative criterion for selection of organic solvents as reaction media in biocatalysis. Eur J Biochem 1991;198:31–41.
[9] Xu K, Griebenow K, Klibanov AM. Correlation between catalytic activity and secondary structure of subtilisin dissolved in organic solvents. Biotechnol Bioeng 1997;56:485–90.
[10] Castro GR. Enzymatic catalysis in non-aqueous media. Proc Pew Ann Meet 1997, p. 62.
[11] Mozhaev VV, Melik–Nubarov N, Levitsky VYu, Siksnis VA, Martinek K. High stability to irreversible inactivation at elevated temperatures of enzymes covalently modified by hydrophilic reagents: α-chymotrypsin. Biotechnol Bioeng 1992;40:650–62.
[12] Lozano P, Combes D, Iborra JL. Effects of polyols on α-chymotrypsin thermostability: a mechanistic analysis of the enzyme stabilization. J Biotechnol 1994;35:9–18.
[13] Laitinen HA, Harris WE. Determination of water. In: Laitinen HA, Harris WE, editors. Chemical analysis (2nd Edition). New York: McGraw–Hill, 1975. p. 361–3.
[14] Erlanger BF, Kokowsky N, Cohen W. The preparation and properties of two new chromogenic substrates of trypsin. Arch Biochem Biophys 1961;95:271–8.
[15] Segel IH. Effects of pH and Temperature. In: Segel IH, editor. Enzyme kinetics. New York: J. Wiley and Sons Inc., 1993. p. 931–4.
[16] Dzandu JK, Deh ME, Barrat DL, Wise GE. Detection of erythrocyte membrane proteins, sialoglycoproteins, and lipids in the same polyacrylamide gel using a double staining technique. Proc Natl Acad Sci USA 1984;81:1733–7.
[17] Halling P. Thermodynamic predictions for biocatalysis in nonconventional media: theory, tests, and recommendations for experimental design and analysis. Enzyme Microb Technol 1994;16:178–206.
[18] Hertmanni P, Picque E, Thomas D, Larreta–Garde V. Modulation of protease specificity by a change in the enzyme microenvironment. FEBS Lett 1991;279:123–31.
[19] Heremans L, Heremans K. Raman spectroscopic study in secondary structure of chymotrypsin: effect of pH and pressure on the salt bridge. Biochim Biophys Acta 1989;999:192–7.
[20] Fersht A, Requena Y. Equilibrium and rate constant for the interconversion of two conformations of α-chymoptrypsin. J Mol Biol 1971; 60:279–90.