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Stability of hydrolytic enzymes in water–organic solvent systems

L.M. Simon [a,*], K. László [a], A. Vértesi [a], K. Bagi [a], B. Szajáni [b]

[a] *Department of Biochemistry, József Attila University, P.O. Box 533, H-6701 Szeged, Hungary*
[b] *COVENT Industrial Venture Capital Investment Co., Ltd., P.O. Box 335, H-1537 Budapest, Hungary*

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Abstract

The effects of organic solvents on the stabilities of bovine pancreas trypsin, chymotrypsin, carboxypeptidase A and porcine pancreas lipase were studied. Water-miscible solvents (ethanol, acetonitrile, 1,4-dioxane and dimethyl sulfoxide) and water-immiscible solvents (ethyl acetate and toluene) were used in 100 mM phosphate buffer (pH 7.0) or 100 mM Tris/HCl buffer (pH 7.0) in concentrations of 20–80% (v/v). All hydrolytic enzymes studied were inactivated by mixtures containing dimethyl sulfoxide at higher concentrations. Trypsin and carboxypeptidase A resisted solvent mixtures containing acetonitrile, 1,4-dioxane and ethanol. They preserved more than 80% of their starting activities during 20-min incubations. The activities of lipase and chymotrypsin decreased with increasing concentration of water-miscible polar organic solvents, but at higher concentrations (80%) 70–90% of the activity remained. In mixtures with water-immiscible solvents, the decrease in activity of carboxypeptidase A was pronounced. Trypsin and chymotrypsin underwent practically no loss in activity in the presence of toluene or ethyl acetate. In respect of stability, the polar solvent proved to be more favorable for lipase. These results suggest that the conformational stabilities of hydrolytic enzymes are highly dependent on the solvent–protein interactions and the enzyme structure. © 1998 Elsevier Science B.V.

Keywords: Trypsin; Chymotrypsin; Lipase; Carboxypeptidase A; Conformational stability; Organic solvent effects

1. Introduction

The application of organic media allows the use of hydrolytic enzymes for synthetic purposes to catalyze esterification, transesterification, transpeptidation, etc. Further, the solubilities of substrate and/or product may be improved. However, the solvent influences the catalytic properties and stabilities of enzymes to a large extent [1–3].

At present there is considerable interest in the utilization of enzymes as industrial catalysts in the presence of an organic liquid phase [4,5]. The choice of an organic solvent for a given reaction should be determined by the effects of the solvent on the reaction and on the biocatalyst stability. Some enzymatic biotransformations require that polar solvents be used to dissolve the substrates (sugars and their derivatives) because of their high polarities.

The aim of the present work was to study the effects of different water-miscible and water-immiscible organic solvents on the conformational stabilities of some hydrolytic enzymes in buffered aqueous solution.

* Corresponding author.

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2. Materials and methods

2.1. Materials

Bovine pancreas trypsin (EC.3.4.21.4), chymotrypsin (EC.3.4.21.1), carboxypeptidase A (peptidyl-L-amino-acid hydrolase, EC.3.4.17.1; CPA), *N*-benzoyl-L-arginine ethyl ester (BAEE), *N*-acetyl-L-tyrosine ethyl ester (ATEE) and hippuryl-L-phenylalanine were purchased from Sigma-Aldrich Company (Budapest). Lipase (triacylglycerol ester hydrolase, EC.3.1.1.3) from porcine pancreas was obtained from Fluka AG (Buchs). The specific activities were; chymotrypsin: 10.000, trypsin: 15.900, CPA: 55 and lipase: 2.55 units/mg. All other chemicals were reagent grade products (Reanal, Budapest).

2.2. Assay of enzyme activities

The activity of trypsin was measured by following the increase in absorbance at 253 nm [6] in a reaction mixture (3 ml) containing 46.7 mM Tris/HCl buffer (pH 8.0), 19 mM $CaCl_2$ and 0.9 mM BAEE, the reaction being initiated by 100 μg of enzyme dissolved in 1 mM HCl. One unit of enzyme activity was defined as the amount of enzyme that hydrolyses 1 μmol of BAEE per min at pH 8.0 and at 25°C. For the measurement of chymotrypsin activity, ATEE was used and the changes in absorbance at 237 nm were followed [6] in a reaction mixture (3 ml) containing 40 mM Tris/HCl, 50 mM $CaCl_2$ and 0.5 mM ATEE. The reaction was initiated by 20 μg of enzyme. One unit of enzyme activity was defined as the amount of enzyme that catalyses the hydrolysis of 1 μmol of ATEE per min at pH 7.0 and 25°C. The CPA activity was determined by following the change in absorbance at 254 nm [7] in a reaction mixture (3 ml) containing 55 mM Tris/HCl buffer (pH 7.5), 500 mM NaCl and 0.5 mM hippuryl-L-phenylalanine, the reaction being initiated by the addition of 100 μg of enzyme (5 U). One unit of enzyme activity was defined as the amount of enzyme that hydrolyses 1 μmol of hippuryl-L-phenylalanine per min under the assay conditions. The activity of lipase was measured by pH-stat titration, using an automatic titrator (Radiometer, Copenhagen) as described earlier and with olive oil as substrate [8]. The titrant used was 10 mM NaOH and olive oil was emulsified by using arabic gum as emulsifier. One unit of enzyme activity was defined as the amount of enzyme that catalyses the formation of 1 μmol of fatty acid from olive oil as substrate per minute at pH 8.9 and 37°C.

2.3. Stability tests

The stabilities of the enzymes were determined in 100 mM phosphate buffer (pH 7.0) containing different concentrations of organic solvents and 20–50 units of enzymes. In the case of CPA, 100 mM Tris/HCl buffer (pH 7.0) was used instead of phosphate buffer. The samples were incubated for appropriate periods of time (up to 40 min) at 24°C and aliquots were then withdrawn. The residual activities of the enzymes were determined by using standard methods as described above.

3. Results

3.1. Effects of water-miscible solvents

In the presence of different concentrations of dimethyl sulfoxide, the hydrolytic enzymes were incubated for 40 min in 100 mM phosphate buffer at pH 7.0 and 24°C. The changes in activity were monitored at 5, 20 and 40 min. As may be seen from Fig. 1, up to a solvent concentration limit (chymotrypsin 30%, CPA 60%, trypsin 50% and lipase 40%) no changes were observed in the catalytic activity. Above these concentrations, the catalytic activities of all the enzymes studied decreased dramatically, with the exception of trypsin, which preserved about 60% of its starting activity even in the presence of 80% dimethyl sulfoxide, and a small activation of the enzyme was observed at lower

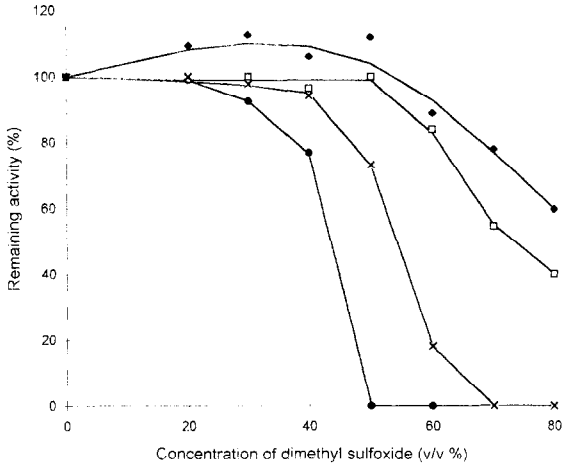

Fig. 1. Effects of dimethyl sulfoxide on activities of (♦) trypsin, (●) chymotrypsin, (□) CPA and (x) lipase. Incubations were performed for 5 min at 24°C. For details, see text.

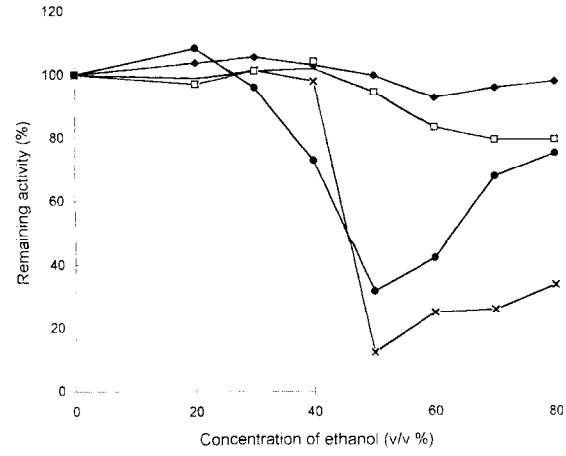

Fig. 3. Effects of ethanol on activities of (♦) trypsin, (●) chymotrypsin, (□) CPA and (x) lipase. Incubations were performed 20 min at 24°C. For details, see text.

dimethyl sulfoxide concentrations. It was also observed that the effect of the organic solvent on the enzyme conformation was relatively fast. On the other hand, the effects of this solvent on the enzyme activities were general and did not reveal high enzyme-dependent differences.

The effects of acetonitrile, ethanol and 1,4-dioxane on the enzyme activities are presented in Figs. 2–4. These organic solvents caused only slight decreases in the activities of trypsin and CPA. In contrast, the activities of chymotrypsin and lipase exhibited a sharp minimum with increasing concentrations of organic solvent. The lowest activities were measured in 40–50% acetonitrile and about 50% ethanol or 1,4-dioxane.

3.2. Effects of water-immiscible solvents

The effects of the water-immiscible organic solvents toluene and ethyl acetate are summarized in Fig. 5. Trypsin and chymotrypsin pre-

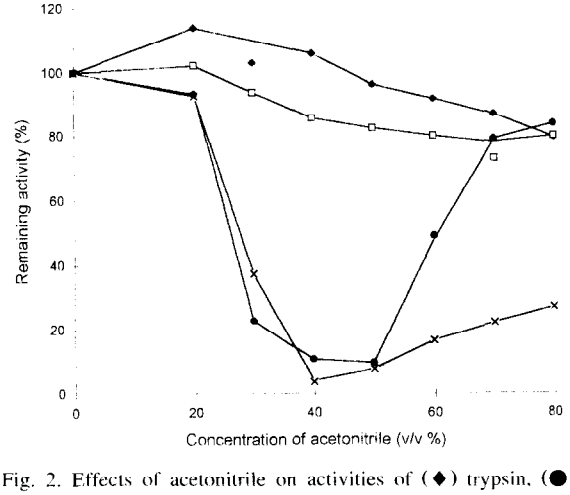

Fig. 2. Effects of acetonitrile on activities of (♦) trypsin, (●) chymotrypsin, (□) CPA and (x) lipase. Incubations were performed for 20 min at 24°C. For details, see text.

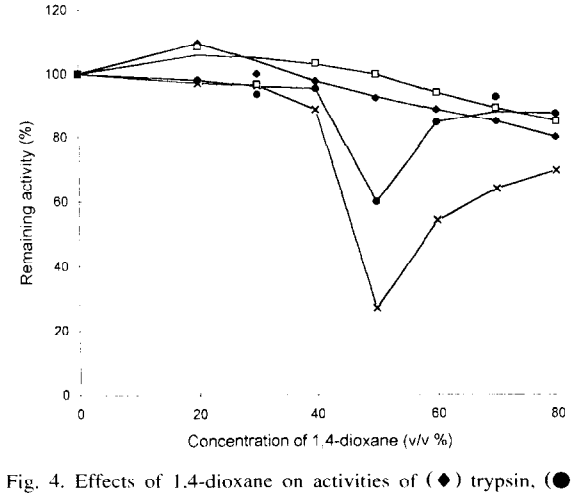

Fig. 4. Effects of 1,4-dioxane on activities of (♦) trypsin, (●) chymotrypsin, (□) CPA and (x) lipase. Incubations were performed for 20 min at 24°C. For details, see text.

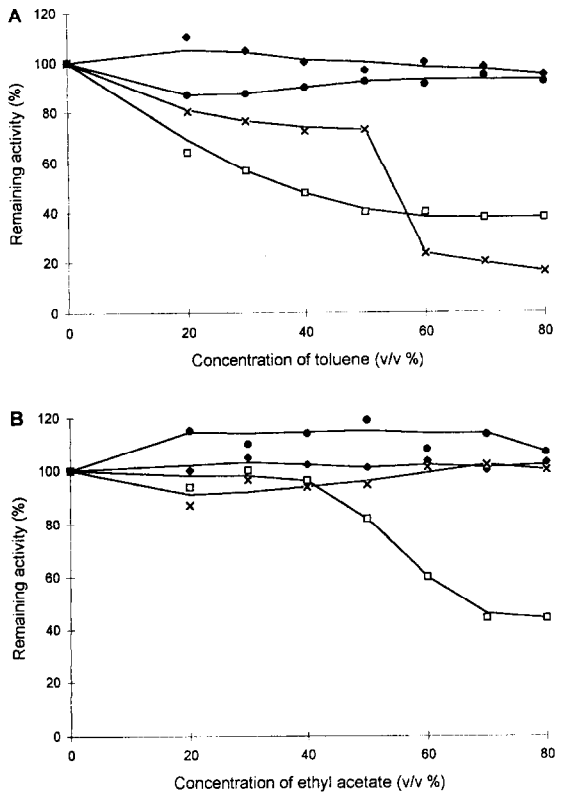

Fig. 5. Effects of (A) toluene and (B) ethyl acetate on activities of (♦) trypsin, (●) chymotrypsin, (□) CPA and (x) lipase. Incubations were performed for 20 min and at 24°C. For details, see text.

served practically all of their starting activities in both solvents. The CPA activity decreased in both solvents with increasing organic solvent concentration, but more quickly in toluene than in ethyl acetate. A decrease in activity of lipase was observed in toluene, but not in ethyl acetate.

4. Discussion

There is great interest in the use of enzymes as industrial catalysts in an organic liquid phase. Current research is directed partly towards the study of the effects of different solvents on the activities and stabilities of biocatalysts. It seems that there might be high yields of reverse reactions not only in low water–organic co-solvent systems (1–5%) [9–11]. We therefore studied the effects of organic solvents in the concentration range 20–80%. Organic solvents may affect the conformational stabilities of biocatalysts in several ways, including interactions with the hydration layer essential for catalysis and proper folding, as well as alterations of the protein structure by direct interactions with protein solvation sites, either hydrophobic or H-bonding. Selection of the appropriate solvent for a reaction, with the enzyme being active and stable, has been a critical point and many studies have attempted to relate enzyme activity in an organic solvent selected for a catalytic reaction with a molecular parameter of the solvent. The logarithm of the partition coefficient (log P) of an organic solvent in the standard water/octanol system has proved a useful factor for the prediction of enzyme activity and/or stability in nonpolar solvents [12,13]. The disadvantages of using solvents having a log P lower than 2 relating to their denaturing properties on proteins resulted in taking into account new parameters (water solubility of organic solvents, three-dimensional solubility parameter, etc.). In spite of the efforts that have been made, only some general rules can be applied to define the interactions between different solvents and enzyme stability. The solvent may also inhibit enzymes directly. With a bulk organic phase, the activity and stability are generally favorable in solvents with a higher log P value. Solvents may influence enzyme enantioselectivity not only through their bulk properties, but also by binding directly to the protein molecule. The X-ray crystal structures of subtilisin in acetonitrile [14] and of γ-chymotrypsin in n-hexane [15] have demonstrated directly that solvent molecules are bound to the enzyme molecules. In the former case, four of the twelve subtilisin-bound acetonitrile molecules were found in the active center, while in the latter case, two of the seven chymotrypsin-bound hexane molecules were localized near the active site.

Dimethyl sulfoxide, which is known as a good solvent for proteins, exerts very dramatic

effects on enzyme stabilities when it is present at high concentrations.

Miscible polar solvents such as acetonitrile, ethanol and 1,4-dioxane exerted different effects on the activities of the enzymes. Trypsin and CPA behaved in a similar manner: increasing amounts of the organic solvent resulted in slight decreases in the activities, probably causing small changes in the conformations of the enzymes. It was surprising that chymotrypsin characterized by a certain homology, with the trypsin in the primary and three-dimensional structure displayed quite different behavior. Hydrophobic interactions generally play a dominant role in maintaining the catalytically active conformations of enzymes. At higher concentrations, the solvents can replace water in the hydration shell, and they possess the ability to maintain the solvophobic interactions to a sufficient degree. It may be concluded that the replacement of water in the bulk causes a reduction in activity at certain concentrations, but at higher solvent concentrations the properties and interactions of the solvents might contribute significantly to the preservation of the catalytic activities of the enzymes. It is known that the enzymes are stable at solvent concentrations up to 98–99% [16]. These results suggest the significance of the hydration shell and/or the deleterious effects of the solvent on the enzyme molecules.

In the water-immiscible polar solvent ethyl acetate, trypsin, chymotrypsin and lipase preserved practically all of their activities. The activity of CPA decreased with increasing amount of ethyl acetate.

The apolar toluene influenced the stabilities of lipase and CPA significantly. Slight decreases were observed in the activities of chymotrypsin and trypsin.

It may be stated that the conformational stabilities of enzymes in highly polar solvents are relatively poor. This may be related to the ability of the solvent to solubilize and strip away from the enzymes the water essential for protein folding. On the other hand, enzymes dispersed in hydrophobic, water-immiscible organic solvents display stabilities greater than those in aqueous solution. Although these liquids are nonsolvents for protein, they tend to interact less extensively with the protein molecules. The decrease in stability of CPA is the opposite. The behavior of lipase in toluene is also surprising, because the lipases are known to be active in hydrophobic organic solvents with a limited water content.

These results show that, under given conditions, the effects of organic solvents depend partly on their interactions with the water molecules in the micro- and/or macroenvironments of enzymes. The changes in stability of enzymes in organic solvents are also greatly affected by the individual structures of the enzymes.

References

[1] Yu.L. Khmelnitsky, A.V. Levashov, N.L. Klyachko, K. Martinek, Enzyme Microb. Technol. 10 (1988) 710.
[2] G. Carrea, G. Ottolina, S. Riva, Trends Biotechnol. 13 (1995) 63.
[3] G. Bell, P.J. Halling, B.D. Moore, J. Partridge, D.G. Rees, Trends Biotechnol. 13 (1995) 468.
[4] K. Farber, M.C. Franssen, Trends Biotechnol. 11 (1993) 461.
[5] F. Terradas, M. Teston-Henry, P.A. Fitzpatrick, A.M. Klibanov, J. Am. Chem. Soc. 115 (1993) 390.
[6] G.W. Swert, Y. Takenaka, Biochim. Biophys. Acta 16 (1955) 570.
[7] Biochemica Information, Boehringer Mannheim GmbH, 1973, I.55.
[8] K. Bagi, L.M. Simon, B. Szajani, Enzyme Microb. Technol. 20 (1997) 531.
[9] K. Nilsson, K. Mosbach, Biotechnol. Bioeng. 26 (1984) 1146.
[10] J.M. Guisan, R.M. Blanco, A. Bastida, R. Fernandez-Lafuente, C. Cuesta, P.J. Halling, Ann. NY Acad. Sci. 672 (1992) 158.
[11] T. Su, R. Furutani, H. Ooshima, J. Kato, Enzyme Microb. Technol. 17 (1995) 1067.
[12] C. Laane, S. Boeren, K. Vos, Trends Biotechnol. 3 (1985) 251.
[13] R.H. Valivety, G.A. Johnston, C.J. Suckling, P.J. Halling, Biotechnol. Bioeng. 38 (1991) 1137.
[14] P.A. Fitzpatrick, A.C.U. Steinmetz, D. Ringe, A.M. Klibanov, Proc. Natl. Acad. Sci. USA 90 (1993) 8653.
[15] N.H. Yennawar, H.P. Yennawar, G.K. Farber, Biochemistry 33 (1994) 7326.
[16] A. Zaks, A.M. Klibanov, J. Biol. Chem. 263 (1988) 3194.