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Stabilization of various α-chymotrypsin forms in aqueous-organic media by additives

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Abstract

The effect of different polyols (glucose, sorbitol or polyethylene glycol) on the stability of soluble and different covalently immobilized α-chymotrypsin forms in aqueous-organic solvent (ethanol, dioxane or acetonitrile) systems were investigated. Generally, immobilization resulted in remarkable improvement of the stability of the enzyme in aqueous-organic media: while immobilized forms retained 80–85, 70–90 or 30–60% of their initial activity in 50% (v/v) ethanol, dioxane or acetonitrile, respectively, the soluble enzyme showed only about 13, 35 or 2% remaining activity in the above aqueous-organic mixtures, respectively (after 60 min preincubation). Further improvement was found in the stability by the addition of polyhydroxy compounds. This effect depended on the feature of the support and the organic solvent. The most explicit increase in the stability was observed when the enzyme was immobilized on a silica-based support (Silochrome activated with *p*-benzoquinone) in aqueous-acetonitrile mixtures, where the activity of α-chymotrypsin was enhanced about 1.5–2 times by the addition of sorbitol or glucose as compared with its control.

Keywords: α-Chymotrypsin; Immobilization; Stability; Organic solvents; Additives

1. Introduction

There is growing interest in the use of biocatalysts in organic media, where hydrolytic enzymes can catalyze various synthetic reactions such as esterification, transesterification, peptide synthesis etc. [1–3]. However, enzymatic catalysis in organic media has a disadvantage that enzymes are often inactivated by the organic solvent. One of the main purpose of biotechnology is obtaining stable biocatalyst in organic solvent systems. According to Khmelnitsky et al. [4] organic solvent molecules tend to displace water from the hydration shell of the proteins and this destruction is one of the main reason of denaturation of proteins by organic solvents. Modifying of the immediate surroundings of the enzyme affects the water shell around the enzyme and its conformation. Possibilities of enzyme stabilization might be the modification [5,6] and immobilization of enzymes [7–9]. Notwithstanding, addition of polyhydroxy compounds to enzymatic reaction media may also display positive effect on the activity of several enzymes [10,11]. Numerous works have been performed with soluble enzymes in water [12,13] in order to estimate the influence of polyhydroxy compounds on the catalytic and thermal stability of the enzymes. The possible stabilizing effects of these substances on different enzymes in organic media have also been analyzed when the immobilization process was performed in the presence

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of the additive [14,15]. Additives to the enzymes are considered to affect stability primarily by affecting the distribution of water around the protein [16,17].

In the present study soluble and differently immobilized forms of α-chymotrypsin were preincubated with or without polyhydroxy compounds (sorbitol, glucose or polyethylene glycol) in aqueous-organic solvents (ethanol, dioxane or acetonitrile) and the effects of these materials on the stability of various α-chymotrypsin forms were investigated.

2. Experimental

2.1. Materials

α-Chymotrypsin (EC 3.4.21.1; type II from bovine pancreas; specific activity 50 U mg^{-1}), *N*-acetyl-L-tyrosine ethyl ester (ATEE), PEG 8000 and 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluene sulfonate (CMC) were from Sigma (Budapest, Hungary). Silochrome activated with *p*-benzoquinone, a silica-based support (quinone content 37 μmol g^{-1} solid; average pore size 50 nm; particle size 0.1–0.25 mm), was from NPO Biolar (Riga-Olaine, Latvia). Akrilex P-100, a polyacrylamide bead (100–320 μm) polymer, its derivative Akrilex C-100, a polyacrylamide bead (100–320 μm) polymer containing carboxylic functional groups (4 meq. g^{-1} xerogel), and all other chemicals were commercial reagent-grade preparations from Reanal (Budapest, Hungary).

2.2. Preparation of immobilized enzymes

The covalent immobilization of α-chymotrypsin to Akrilex C-100 (the water-soluble CMC being used for the activation of the carboxylic functional groups) and the covalent coupling to Silochrome activated with *p*-benzoquinone was effected as described earlier [18]. The covalent binding of α-chymotrypsin to Akrilex P-100 was performed according to Kálmán et al. [19]. For the immobilization procedures 400–600 U of enzyme was used for 100 mg supports. The immobilized enzyme activities were found to be 204.1, 50.1 and 26.7 U g^{-1} dry gel on Akrilex C-chymotrypsin (AC-Chy), Akrilex P-chymotrypsin (AP-Chy) and *p*-Silochrome-bound α-chymotrypsin (*p*Sil-Chy), respectively. The 57.5, 81 or 15% activity left in the supernatants after the immobilization procedures in case of AC-Chy, AP-Chy or *p*Sil-Chy, respectively. Further loss of activity (39.1, 18 or 84.3%, respectively) was due to enzyme inactivation during the immobilization. The immobilized enzymes were stored in 0.05 M potassium phosphate buffer (pH 7.0) until use.

2.3. Assay of α-chymotrypsin activity

For the measurement of α-chymotrypsin hydrolytic activity [20], ATEE was used as substrate and the changes in absorbance at 237 nm were followed in a continuously stirred reaction mixture (3 ml) containing 50 mM potassium phosphate (pH 7.0) and 1 mM ATEE. The reaction was initiated with 1–5 U of soluble or immobilized enzyme. One unit of enzyme activity was defined as the amount of enzyme that catalyzes the hydrolysis of 1 μmol of ATEE per min at pH 7.0 and 25 °C.

2.4. Stability tests

The stability of the enzymes were determined in 50 mM potassium phosphate buffer (pH 7.0) containing different concentrations of organic solvents, 5–10 U of immobilized or soluble enzyme and the polyhydroxy compounds in indicated concentrations. The samples were preincubated for 60 min at 25 °C in closed vessels and aliquots were withdrawn at appropriate times and analyzed spectrophotometrically for hydrolytic activity. All the experimentally derived values reported in the text are averages of triplicate individual measurements.

3. Results and discussion

3.1. Dependence of enzyme stability on the concentration of organic solvents

An earlier study [21] demonstrated that soluble α-chymotrypsin stability was strongly influenced by the concentration of the organic solvent. The effect of the solvents on the enzyme stability is characterized with a minimum curve, where the lowest activities were measured in 50% (v/v) ethanol, dioxane or acetonitrile. The above results prompted us to study the

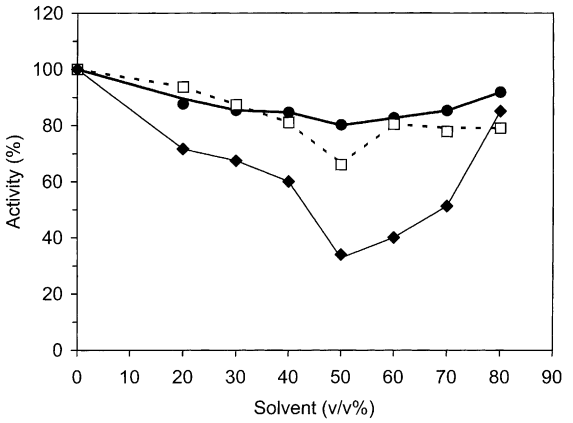

Fig. 1. Effect of different concentrations of ethanol (●), dioxane (□) or acetonitrile (◆) on stability of Akrilex C-bound α-chymotrypsin. Incubations were performed for 60 min at 25 °C.

stability of differently immobilized α-chymotrypsin forms in systems containing various concentrations of ethanol, dioxane or acetonitrile. As an example, the stability of AC-Chy are illustrated in Fig. 1. In agreement with the results of the previous study on soluble α-chymotrypsin [21] we found similar effects on immobilized enzymes, although, the changes in the stability of the immobilized enzyme forms were moderate compared to the soluble one. On the basis of these results, different enzyme forms were preincubated with various polyols in aqueous-organic media in further experiments in order to estimate whether these compounds might improve the stabilities of soluble and immobilized α-chymotrypsins in 50% (v/v) aqueous-organic solvents.

3.2. Effect of additive concentration

Enzyme activities were measured in 50% (v/v) ethanol, dioxane or acetonitrile with addition of 30–365 g l^{-1} glucose or sorbitol or 30–240 g l^{-1} PEG 8000 (depending on the solubility) at 25 °C in order to determine the optimal enzyme stabilizing concentration of the above polyols. We found that 180 g l^{-1} glucose, 182 g l^{-1} sorbitol or 90 g l^{-1} PEG was needed to achieve the highest stabilizing effects (data not shown), thus these polyol concentrations were used for the further assays.

3.3. Effects of polyols on stability of α-chymotrypsin in different organic solvents

We found considerable activity loss due to the immobilization procedure, as detailed under Section 2. It is possible that certain groups or residues of the enzyme, which are important for the activity, participate in coupling. Furthermore, the presence of the activating agent (e.g. CMC) and/or the character of the support (e.g. polyanionic microenvironment in case of AC) may also influence the change of the enzyme structure caused by the immobilization. Therefore, polyhydroxy compounds may play particularly important role in maintaining the catalytic structure in organic media. A time-course comparative study on the stability of soluble and different immobilized α-chymotrypsin forms were carried out in the presence and absence of glucose, sorbitol or PEG 8000 in media containing 50% (v/v) ethanol, dioxane or acetonitrile. Enzymes were preincubated with additives for 60 min in the above aqueous-organic solvent systems and activities were measured at stated intervals. The stability of AP-Chy in the presence and absence of polyols in aqueous-acetonitrile are presented in Fig. 2 as example, which shows that the stabilizing effect of the additives was significant, but the extent of stabilization was

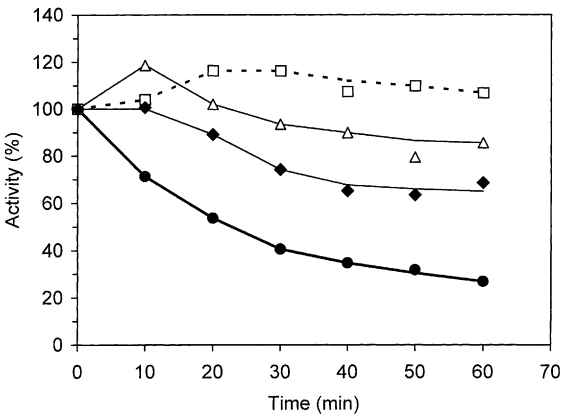

Fig. 2. Akrilex P-α-chymotrypsin activity in 50% (v/v) acetonitrile without additives (●) and in the presence of glucose (□), sorbitol (△) or PEG 8000 (◆). Experimental data were expressed as percentage of the activity of its control (immobilized enzyme was incubated in 50 mM potassium phosphate buffer (pH 7.0) for 60 min at 25 °C), which activity (6.70 μmol min^{-1} mg^{-1}) is regarded to be 100%. The glucose concentration was 180 g l^{-1}; sorbitol 182 g l^{-1}; PEG 90 g l^{-1}.

different. It is known that polyhydroxy compounds interact with water molecules for their hydration, decrease water activity and modify the physico–chemical properties of the medium, hereby they can alter the stability of the enzymes. However, the effects depend on the nature and the concentration of the additive [22,23], which may explain why these polyols display different effects on the stability of the enzymes.

We observed rather complex results for the stability of the soluble and the three types of immobilized α-chymotrypsins with additives in the examined organic solvent systems. The 60 min data of the above time-course measurements were expressed as percentage of the activity of their own control (soluble or immobilized enzymes were incubated in 50 mM potassium phosphate buffer (pH 7.0) for 60 min at 25 °C). The activity values of the above controls were 47.1, 5.61, 6.70 or 1.02 μmol min^{-1} mg^{-1} for soluble enzyme, AC-Chy, AP-Chy or *p*Sil-Chy, respectively, which activities are regarded to be 100%. Effects of solvents and additives together on the stability of different α-chymotrypsin forms are presented in Fig. 3.

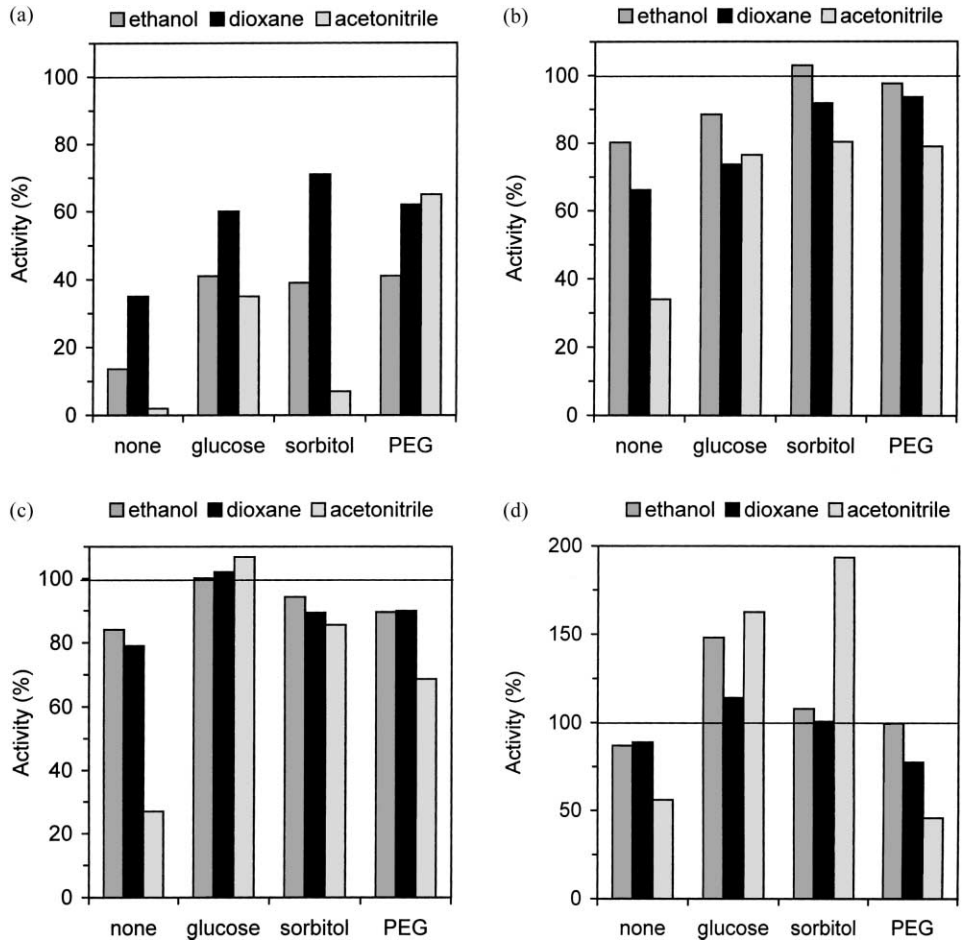

Fig. 3. Comparison of stability of different α-chymotrypsin forms in the presence and absence of glucose, sorbitol, or PEG in 50% (v/v) ethanol, dioxane or acetonitrile at 25 °C after a 60 min incubation. Experimental data were expressed as percentage of the activity of their own control (enzyme forms were incubated in 50 mM potassium phosphate buffer pH 7.0) for 60 min at 25 °C and the activity values were 47.1, 5.61, 6.70 or 1.02 μmol min^{-1} mg^{-1} for soluble enzyme, AC-Chy, AP-Chy or *p*Sil-Chy, respectively), which activity values are regarded to be 100%. (a) Soluble chymotrypsin; (b) Akrilex-C-chymotrypsin (AC-Chy), (c) Akrilex-P-chymotrypsin (AP-Chy), (d) *p*-Silochrome-chymotrypsin (*p*Sil-Chy).

It was found that soluble enzyme activities were decreased a lot more than those of immobilized ones in the absence of polyhydroxy compounds in all organic solvent systems. This way, our results also support the literature facts that immobilization can improve the enzyme stability in water, as well as in organic solvents [9,24]. One of the possible reasons for the activity loss of soluble proteases may be the autolysis, which is influenced by the temperature and the pH of the solution [25]. The immobilization of a protease may hinder its autodigestion by protecting the enzyme molecules from one another, hereby the stability of the enzyme may increase. On the other hand, the enzyme inactivation by organic solvents often starts with protein unfolding, therefore multipoint covalent attachment of the enzyme to a support affords high conformational rigidity and consequently high enzyme activity might be retained [26]. In the presence of additives, it was experienced that each one displayed stabilizing effect on the soluble chymotrypsin, AC-Chy and AP-Chy, as well. In case of *p*Sil-Chy polyols (except PEG) also exerted stabilizing effect in all solvents. The influence of the additives (except glucose) was similar on the stability of AC-Chy and AP-Chy in all organic solvent systems, however, their stabilizing effects were weaker than in systems containing soluble enzyme or *p*Sil-Chy. It can be established that the different effects of additives on the stability of immobilized enzymes may bring into connection with the character of the support [7,8]. The cause of similar behavior of Akrilex-bound enzymes is probably in connection with the fact that Akrilex C and P are structurally similar hydrophilic polyacrylamide-based supports with high water-retaining capacity. The small differences in AC-Chy and AP-Chy stability might be related to the presence of different functional groups on the supports participating in the immobilization procedure. Akrilex C contains carboxylic functional groups and it is more hydrophilic than Akrilex P, which contains amide groups to bind the enzyme. When the enzyme was immobilized to the silica-based silochrome support, which can be characterized by a low water-retaining property and has quinone groups for immobilizing α-chymotrypsin, it was observed that stabilizing effect of additives was more pronounced than in case of Akrilex-Chys. According to Adlercreutz [7] the enzyme activity depends on the aquaphilicity of the support in organic media and increased reaction rates were obtained when supports with low aquaphilicity in water-immiscible solvents were used. On the other hand we should take into account that the character of the solvent also influences the stabilizing effect of additives. It is interesting that acetonitrile was the least favorable solvent for all the enzymes without additives, but generally, the stabilizing effects of the additives were the highest in this solvent. This stabilizing effect in acetonitrile containing media was the most pronounced by PEG action for soluble enzyme, but by sorbitol for *p*Sil-Chy and by glucose for AP-Chy. Simon et al. [27] demonstrated that in 50% (v/v) acetonitrile the secondary structure of soluble α-chymotrypsin considerably altered as compared with that in aqueous-buffered system. It supports our finding, that enzymes were the least stable in 50% (v/v) acetonitrile as compared with the other solvents. Generally, the extent of stabilization by the additives was very similar in media containing ethanol or dioxane. PEG proved to be the most efficient stabilizer for soluble chymotrypsin in all solvents, but for immobilized enzymes glucose or sorbitol were more efficient. Adlercreutz [14] found similar results with Celite-bound α-chymotrypsin, where sorbitol proved to be the best additive for immobilized α-chymotrypsin-catalyzed alcoholysis and hydrolysis too. Obviously, we should consider the stability of the protein in a complex way, which include the protein itself and the other components of the system, such as support, additive, organic solvent and water. Stabilizing effect of polyols is considered to be the consequence of the modified distribution of the water in the system, because these compounds draw away water from their environment for own hydration. By this means they also influence the distribution of the water around the enzyme molecules, which in turns modify their stability [28].

4. Conclusions

In summary, we can state that immobilization improved the stability of the α-chymotrypsin in organic solvent containing media and additives can be used effectively to stabilize α-chymotrypsin in organic solvents. However, the degree of stabilization depends on the type of the solvent and the additive and it is related to the form of the enzyme (soluble or immobilized)

existing in the system. If the enzyme exists in immobilized form, its stability is depended also on the nature of the support. The physico–chemical property of the media containing organic solvent and/or additive is modified as compared with a buffered system in view of hydrophobicity, diffusion and distribution of water. We establish that organic solvents used influenced the secondary structure of the α-chymotrypsin hereby affect the stability of the enzyme. Polyols influence the structure of the enzyme environment and thus its biocatalytic behavior. These highly polar polyhydroxy compounds probably protect the native structure of the hydrolases in organic media by maintaining the essential interactions in the system providing the active conformation for the enzyme for catalysis.

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