STABILITY AND OPERATION OF α-CHYMOTRYPSIN IN AQUEOUS-ORGANIC MEDIA

PHD THESES

KINGA LÁSZLÓ

SUPERVISOR: MÁRIA L. SIMON

UNIVERSITY OF SZEGED FACULTY OF SCIENCE
DEPARTMENT OF BIOCHEMISTRY

SZEGED 2002

INTRODUCTION

Enzymes catalyze chemical transformations with large specificity under mild conditions (pH, temperature, pressure). Nowadays, there are more than 3000 known enzymes, however the possibilities in biocatalysis are still not througly utilized. These days enzymes are used in clinical- and food analytical chemistry, production of drugs and foods and they play important role also in the modern biotechnology.

Using non-conventional media at low water content become increasingly important in biocatalysis. Non-conventional media can contain mainly organic substances (solvents, substrates, products, etc.), supercritical fluids or gaseous phase. Amongst them biocatalysis in organic solvents is the most dinamically developing area. There are some advantages of using organic media compared with aqueous solutions:

- possibility to apply higher concentrations of poorly water-soluble substrates and/or products
- facilitated recovery of products and biocatalyst, even when the latter is not immobilized
- shift the equilibria (as a result of the altered partitioning of substrates and products between the phases of interest or as a result of a substantially reduced water activity)
- less risk of microbial contamination
- lack of side reactions.

Shifting the equilibria of the reactions results that a hydrolase may act as synthetase in organic solvents. So hydrolases have shown to be useful for the preparation of condensation products such as esters, peptides in organic media.

Organic solvents effect the ***structure of the proteins*** and thus influence enzyme activity and stability. If solvent binds directly or nearby to the active center of the enzyme molecule, it can considerably influence the reaction catalyzed by the enzyme. Organic solvent molecules tend to draw off water from the hydration shell of the protein, so they may have unfavourable effect on the interactions, which are responsible for preserving the native conformation of the proteins (Arakawa and Godette, 1985). The amount of hydrogen bonds or the strenght of electrostatic interactions may change and consequently the power of the hydrophobic bonds may weakens (Battistel and Bianchi, 1993). Numerous literature data show that proportion of the secondary structure elements of the proteins in organic solvents, significantly differs from that is observed in water. This difference depends on the nature and the concentration of the organic solvent (Simon et al., 2001).

There is a great importance of the presence of ***water*** in organic solvent systems. Generally, some water have to be presence under biocatalytic processes (Adlercreutz, 1991). Water has a double role: it preserves the catalytically active conformation of the enzyme and it is also essential for the inactivation processes. The presence of water and its concentration in the systems considerably influence the activity/stability of the enzyme. Is is very important to optimize the water content for the optimal operation of the enzymes in organic media. If there is too much water in the aqueous-organic media, it is almost as unfavourable as the complete absence of the water. Too much water in the system is very disadvantageous, because it helps the inativation procedures and the reaction equilibrium shifts towards hydrolysis.

The inactivation of the enzyme caused by the organic solvents is a serious problem when using enzymes in organic media. It is essential to ***choose the*** appropriate ***solvent*** for the biocatalysis and optimalize the reaction conditions. Since the inactivation of the protein in organic solvents caused by damage of the hydration shell of the protein, so methods for stabilization of the enzymes have to decrease this effect and protect the enzyme from the inactivation. Stability of the enzyme may

improve by chemical modification, immobilization, protein engineering technology or adding stabilizers.

Protein engineering technology means to produce modified biocatalyst with site-specific or random mutagenesis.

Chemical modification of the enzyme means the modification of the microenvironment of the enzyme and it may favourable influence its properties. One of the most frequently used chemical modification is immobilization of the enzyme on a solid support. Immobilization of the biocatalyst can occur by adsorption, ionic interaction, intermolecular cross-binding, closing enzyme to the semipermeable membrane etc. The most often used method for enzyme immobilization is ***covalent binding*** to a solid support. The main advantage of this method is a formation of strong chemical bonds between the support and the enzyme. It has certain disadvantages such as the enzymes may be inactivated by the modifying reagent, because residues in the active center of the enzyme may also participate in the formation of the newly formed chemical bonds. Enzyme structure may change during the binding process, so availability of the substrate to the enzyme also may change.

Adding ***polyhydroxy compounds*** (polyols, saccharides), other polimers and salts to the enzyme reaction may increase the stability of the enzyme by modification of the microenvironment of the biocatalyst (Gray, 1988). Effects of additives are not known yet at the molecular level, but presumably they influence the enzyme activity by affecting the distribution of the water in the system and the microenvironment of the enzyme molecules. There are numerous literature data available about the effects of polyhydroxy compounds on the ***soluble enzyme*** activity and thermostability ***in water***, as well as about the stabilizing effect of these compounds on different enzymes ***in organic media***, when these additives are ***co-immobilized*** or co-lyophilized with enzyme.

AIMS

The aim of our work was to study the stability and operation of bovine pancreas α-chymotrypsin in various organic solvents. We tried to answer the following questions:

- how the stability of soluble enzyme is influenced by organic solvents differing from each other in chemical characters (water-miscible and water- immiscible organic solvents were used)
- are there any correlation between the polarity of the solvent and the stability of the enzyme in it
- what is the role of the immobilization (support material, immobilization methods) on the stability of the enzyme in organic media
- may additives (polyols) protect enzyme against inactivation caused by organic solvents
- which kind of direct- and transesterification reactions may be catalyzed by α-chymotrypsin in organic media
- a comparative study of direct esterification and transesterification concerning the optimal reaction conditions (tempertaure, pH, substrate concentration, enzyme concentration, water content etc.) was performed
- which effect immobilization has on the synthetic activity of the enzyme in organic solvents.

METHODS

Immobilization of α-chymotrypsin on different supports

Immobilization of α-chymotrypsin to Akrilex C-100 support was performed according to Szajáni et al. (1980). 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluene sulfonate was used for the activation of the carboxyl functional groups. Activation was performed in one-step method, because α-chymotrypsin is not sensitive for carbodiimide.

Immobilization of the enzyme to Akrilex P-100 support was carried out by Kálmán et al. (1983). Before the immobilization the support had to be activated by p-benzoquinone.

Immobilization to the Silochrome activated with p-benzoquinone was performed by Vértesi et al. (1999).

Determination of the protein content

The protein content of the solutions was determined by method of Lowry et al. (1951).

Assay of α-chymotrypsin activity

For the measurement of α-chymotrypsin hydrolytic activity, N-acetyl-L-tyrosine ethyl ester (ATEE) was used as substrate and the changes in absorbance at 237 nm were followed in a continuously stirred reaction mixture (3 mL) containing 0.05 M potassium phosphate buffer (pH 7.0) and 1 mM ATEE (Schwert és Takenaka, 1955). The reaction was initiated with 1-5 units 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.

Stability tests

The stability of the enzymes was determined in 0.05 M potassium phosphate buffer (pH 7.0) containing 10-90% (v/v) concentrations of organic solvents. In case of soluble enzyme the concentration of the enzyme in the incubation mixtures was 1 mg mL^{-1}. In case of immobilized enzymes 5-15 units of the enzyme was present in the incubation mixtures. In the reaction mixtures polyhydroxy compounds were present at the following concentrations: glucose 180 g L^{-1}, sorbitol 182 g L^{-1}, lactose 137 g L^{-1} and polyethylene glycol 90 g L^{-1}. The samples were incubated for 60 or 120 min at 25°C and aliquots were withdrawn at appropriate times and analyzed spectrophotometrically for hydrolytic activity.

Ester syntheses and their analyzes

The standard reaction mixtures (5.125 mL) contained 0.05 mmol amino acid, 0.5 mL alcohol, 4.5 mL solvent and 0.5 mg mL^{-1} α-chymotrypsin dissolved in 0.05 M potassium phosphate buffer (pH 7.0). The reaction mixtures were incubated and magnetically stirred (450 rpm) at 30°C for 24 h. The amounts of N-acetyl-L-tyrosine esters were determined by quantitative TLC on activated silica gel plates (F_{254}), using a 1-butanol:acetic acid:water (4:1:1) developing system. The Gelbase Pro&Gelbase/Gelblot (UVP/Ultra Violet Products) computer program was used for analysis of the plates.

Determination of the water content of the reaction mixtures

Water content of the reaction mixtures was determined by the iodometric method of Fischer (1935).

SUMMARY OF RESULTS

We investigated the activity and stability of α-chymotrypsin in various organic solvent systems. Different methods (immobilization, additives) were applied to increase the stability of the enzyme. Both the esterification and transesterification reactions of the α-chymotrypsin were studied in different organic media.

Results are summarized as follows:

1. Stability of α-chymotrypsin was different in water-miscible and water-immiscible organic solvents. In water-immiscible organic solvents used (ethyl acetate or toluene) the stability of the enzyme was higher than in water-miscible ones (acetone, dioxane, ethanol, acetonitrile, dimethyl sulfoxide). α-Chymotrypsin showed very similar behaviour in acetone, dioxane, ethanol or acetonitrile. In all four solvents the activity of the enzyme changed according to a minimum curve by increasing concentration of the solvent (minimum point is at 50% organic solvent concentration). In case of dimethyl sulfoxide enzymatic activity decreased also by increasing the amount of the solvent, and at 50% dimethyl sulfoxide concentration α-chymotrypsin was totally inactivated after 5-min incubation.

   Among water-miscible organic solvents the stability of the enzyme decreased in the following order: dioxane > acetone > ethanol > acetonitrile > dimethyl sulfoxide. Dielectric constant of the solvent is inversely proportional to the stability of α-chymotrypsin.

2. Inactivation of the enzyme retained its two steps characteristics in the investigated organic solvents just as in water.

3. The enzyme was immobilized successfully on all the supports investigated. The immobilized enzyme activities were found to be 204.1, 50.1 and 26.7 U g^{-1} dry gel with Akrilex C-, Akrilex P- and Silochrome-chymotrypsin, respectively. We established that the highest immobilized activity and immobilized protein amount was achieved when the enzyme was attached to Akrilex C-100 support.

4. Several methods (immobilization, adding stabilizers) were used to increase the stability of the enzyme in organic solvents (ethanol, dioxane or acetonitrile). Each of the immobilized α-chymotrypsin forms were more stable in all three solvents than the soluble one, thus we concluded that immobilization increased the stability of the enzyme in these cases. The enzyme was the most stable on Silochrome support. The highest increase in stability was observed in ethanol. Using ethanol as a solvent we could not detect any difference in the stability of α-chymotrypsin on the three supports investigated. In dioxane and acetonitrile the stability of Akrilex P- and C-bound α-chymotrypsin was similar.

5. The effect of polyhydroxy compounds (glucose, sorbitol, lactose or polyethylene glycol 8000) was investigated on the stability of immobilized enzyme forms in media containing 50% acetonitrile, ethanol or dioxane. The highest stabilizing effect was shown in case of 180 g L^{-1} glucose, 182 g L^{-1} sorbitol, 137 g L^{-1} lactose and 90 g L^{-1} polyethylene glycol. Almost all the additives had stabilizing effect on the immobilized enzyme forms in all solvents. Additives stabilized the Silochrome-enzyme more than the Akrilex-enzymes. Effects of additives were similar on the two Akrilex-bound enzyme forms in each solvents, except for glucose. Effects of additives were similar on all support-bound enzyme forms in ethanol and dioxane. The enzyme was most inactivated by acetonitrile (after 60-min incubation activity of Akrilex C-enzyme was 34%, Akrilex P-enzyme 27%, Silochrome-enzyme 56%), however the stabilizing effect of the additives was strongest in acetonitrile (after 60-min incubation activity of the Akrilex C-enzyme was 75-80%, Akrilex P-enzyme 70-85%). Silochrome-chymotrypsin was

significantly activated by additives (except for polyethylene glycol) in acetonitrile. The large stabilizing effect in acetonitrile was achieved with sorbitol in case of Akrilex C-bound enzyme (80%), with glucose in case of Akrilex P-chymotrypsin (107%) and with lactose for Silochrome-enzyme.

The enzymatic activity did not decreased more than 30% during 60 minutes incubation in the presence of additives, thus inactivation caused by the organic solvents was succesfully prevented by polyhydroxy compounds.

6. The synthetic activity of α-chymotrypsin was also investigated in organic solvents. Two model reactions were studied: direct esterification between N-acetyl-L-tyrosine and alcohols and transesterification between N-acetyl-L-tyrosine ethyl ester and alcohols. We compared the dependence of the reactions on the alcohol- and solvent type, temperature, pH, water content, substrate concentration and enzyme concentrations. The optimal alcohol and amino acid concentration, pH or water content were similar in both types of reactions. Maximal conversion was achieved in both cases with 10% alcohol- and 9.75 mM amino acid concentration. It was found only very small difference between the optimal pH and water content of the reactions: maximal yield of ester was achieved by direct esterification at pH 7.0 and with 2.8% water content, and by transesterification at pH 7.5 and with 3.3% water content. However, there was a considerable difference in the optimal temperature and enzyme concentration between direct- and transesterification. Maximal conversion was achieved at 30°C and with 1.5 mg mL^{-1} enzyme concentration in case of direct esterification and at 40°C and with 0.5 mg mL^{-1} enzyme concentration in case of transesterification.

   Transesterification reached the saturation point more rapidly (4 hours) than direct esterification (24 hours). The hydrolytic activity of the enzyme was also studied in the reaction mixtures and it was found that enzyme retained 80% of its starting activity by the time it reached the maximal yield of ester in case of either direct esterification (83.9%) or transesterification (91.4%).

7. We compared the synthetic activity of different immobilized forms of α-chymotrypsin under optimalized conditions for soluble enzyme. It was found that the immobilized enzymes yielded considerable amount of ester. For direct esterification the best catalyst was the Silochrome-bound α-chymotrypsin (31.3% ester yield) and for transesterification the Akrilex-P-enzyme (50.5% ester yield).

Our results call attention to importance of choosing appropriate organic solvent for the enzymatic reactions in respect for both enzyme stability and reaction efficiency. Results of stabilization experiments may serve as a basis of studying biocatalysis in non-conventional media. Optimalization of the conditions of direct- and transesterification reactions contribute to wide-range use of enzymatic reactions in organic media.

References:

Adlercreutz, P. (1991) On the importance of the support material for enzymatic synthesis in organic media. Support effects at controlled water activity. *Eur. J. Biochem.* **199**, 609-614

Arakawa, T., Goddette, D. (1985) The mechanism of helical transition of proteins by organic solvents. *Arch. Biochem. Biophys.* **240**, 21-32

Battistel, E., Bianchi, D. (1993) Influence of the solvent properties on protein stability in organic media. In: *Stability and Stabilization of Enzymes* (Eds. Tweel, W.J.J., Harder, A., and Buitelaar, R.M.), Elsevier, Amsterdam, 13-20

Fischer, K. (1935) Neues Vefahren zur massanalytischen Bestimmung des Wassergehaltes von Flüssigkeiten und festen Körpern. *Angew. Chem.* **48**, 394-396

Gray, C. (1988) Additives and enzyme stability. *Biocatalysis* **1**, 187-196

Kálmán, M., Szajáni, B., Boross, L. (1983) A novel polyacrylamide-type support prepared by p-benzoquinone activation. *Appl. Biochem. Biotechnol*. **8**, 515-522

Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J.: Protein measurement with the folin phenol reagent. *J. Biol. Chem*. **193**, 265-275 (1951)

Schwert, G.W., Takenaka, Y. (1955) A spectrophotometric determination of trypsin and chymotrypsin. *Biochim. Biophys. Acta* **16**, 570-575

Simon, L.M., Kotormán, M., Garab G., Laczkó I. (2001) Structure and activity of α-chymotrypsin and trypsin in aqueous organic media. Biochem. Biophys. Res. Commun. **280**, 1367-1371

Szajáni, B., Ivony, K., Boross, L. (1980) Comparative studies on soluble and immobilized pig kidney aminoacylase. *J. Appl. Biochem*. **2**, 72-80

Vértesi, A., Simon, L.M., Kiss, I., Szajáni, B. (1999) Preparation, characterization and application of immobilized carboxypeptidase A. *Enzyme Microb. Technol*. **25**, 73-79

PUBLICATIONS

a) Publications in connection with the dissertation:

1. L.M. Simon, **K. László**, A. Vértesi, K. Bagi, B. Szajáni: Stability of hydrolytic enzymes in water-organic solvent systems. *J. Mol. Catal. B: Enzymatic* **4** (1998) 41-45
2. **K. László,** L.M. Simon: α-Chymotrypsin-catalysed synthesis of N-acetyl-L-tyrosine esters in organic media. *Prog. Biotechnol.* **15** (1998) 713-718
3. L.M. Simon, M. Kotormán, K. Maráczy, **K. László**: N-Acetyl-L-arginine ethyl ester synthesis catalysed by bovine trypsin in organic media. *J. Mol. Catal. B: Enzymatic* **10** (2000) 565-570
4. **K. László**, A. Szava, L.M. Simon: Stabilization of various α-chymotrypsin forms in aqueous-organic media by additives. *J. Mol. Catal. B: Enzymatic* **16** (2001) 141-146
5. **K. László**, L.M. Simon: Comparative study on α-chymotrypsin-catalyzed direct- and transesterification in organic solvents. *Hung. J. Ind. Chem.* (2002) (in preparation)

b) Posters and papers in connection with the dissertation:

1. K. Bagi, **K. László**, L.M. Simon, B. Szajáni: Immobilization and characterization of lipase from porcine pancreas (1995), 2nd International Conference of the Hungarian Biochemical Society, **P 15** (Szeged, Hungary)
2. A. Vértesi, **K. László**, L.M. Simon, B. Szajáni: Studies on an immobilized carboxypeptidase A (1995), 2nd International Conference of the Hungarian Biochemical Society, **P 16** (Szeged, Hungary)
3. **K. László**, A. Vértesi, K. Bagi, L.M. Simon, B. Szajáni: Effects of polar organic solvents on stabilities of some hydrolytic enzymes (1997), 8th European Congress on Biotechnology, **P 3121** (Budapest, Hungary)
4. **K. László,** L.M. Simon: Synthesis of N-acetyl-L-tyrosine esters catalysed by alpha-chymotrypsin in organic media (1998), Stability and Stabilization of Biocatalysts, **P 70** (Córdoba, Spain)

c) Other publications:

1. L. Simon M., K. Deér A., Bagi K., Vértesi A., **László K.**: Hidroláz enzimek szerepe xenobiotikumok detoxifikálásában balatoni halakban. *A Balaton kutatásának 1997-es eredményei*, MTA Veszprémi Ter. Biz. és MEH Balatoni Titkárság, (1998) 194-198
2. L.M. Simon, **K. László**, M. Kotormán, A. Vértesi, K. Bagi, J. Nemcsók: Effects of synthetic pyrethroids and methidation on activities of some digestive enzymes in carp (*Cyprinus carpio* L.). *J. Environ. Sci. Health B* **34** (1999) 819-828
3. L. Simon M., K. Deér A., Kotormán M., **László K.**: Xenobiotikumok hatása balatoni halak hidroláz enzimeinek aktivitására. *A Balaton kutatásának 1998-as eredményei*, MTA Veszprémi Ter. Biz. és MEH Balatoni Titkárság, (1999) 109-112
4. M. Kotormán, **K. László**, J. Nemcsók, L.M. Simon: Effects of Cd^{2+}, Cu^{2+}, Pb^{2+} and Zn^{2+} on activities of some digestive enzymes in carp (*Cyprinus carpio* L.). *J. Environ. Sci. Health A* **35** (2000) 1517-1526
5. L.M. Simon, **K. László**, M. Kotormán, B. Szajáni: A comparative study of the conformational stabilities of trypsin and α-chymotrypsin. *Acta Biol. Szeged*. **45** (2001) 43-49
6. Zs. Varanka, K. A. Deér, I. Rojik, I. Varanka, **K. László**, T. Bartók, J. Nemcsók, M. Ábrahám: Influence of the polyphenolic tannic acid on the toxicity of the insecticide deltamethrin to fish. A comparative study examining both biochemical and cytopathological parameters. *Acta Biol. Hung.* (2002) (közlésre elfogadva)
7. **K. László**, K. Hantosi, L. Nagy, M. Fekete: Anaemia in pregnancy: iron deficient or not? *Clin. Chem.* (2002) (in press)

d) Other posters and papers

1. **László K.**, Kovács Gy., Fekete M.: Beckman Synchron CX7 és Hitachi 717 kémiai analizátorokon végzett összehasonlító vizsgálatok (2000), 50th Congress of the Hungarian Society of Clinical Pathology, **P 2734** (Debrecen, Hungary)
2. Hantosi K., **László K.**, Kovács Gy., Fekete M.: Szolubilis transzferrin receptor mint a vashiányos anémia megbízható markere (2001), X. Centenáriumi Fejér Megyei Orvosnapok, **P 9** (Székesfehérvár, Hungary)
3. Sztrida J.I., Bors R. K., Horváth P., **László K.**, Fekete M.: A diabetes mellitus laboratóriumi diagnosztikája (2001), MOLSZE VII. Nagygyűlése, **P 69** (Bükfürdő, Hungary)