Collapse of stimuli-sensitive enzyme-containing polymeric matrix and immobilized enzyme activity

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Introduction

Kinetical behaviour of α-chymotrypsin in non-denaturating water/organic solvent mixtures strongly affects by the substrate used for residual enzyme activity determination. In the same mixture depending on substrate structure one can report about either monotonic decrease in enzyme activity (Tomiuchi, Y. et al, 1993) or its independence up to denaturation of enzyme (Mozhaev, V.V. et al., 1989), or even activation effects with the following decreasing of enzyme activity in the mixture (Belyaeva, E.A. et al., 2002). Evidently such effects are connected with the hydrophobic interactions between the protein globule and organic solvent molecules (Martinek, K. et al., 1970).

Stimuli-sensitive polymers that respond to a change in temperature, pH, light, electric or magnetic fields, etc., were investigated as biocatalyst carriers recent decade. Such polymers and hydrogels undergo fast, reversible changes in microstructure from a hydrophilic to a hydrophobic state. Small changes in the environment lead to order-of-magnitude changes in the size and water content of hydrogel (Kokufuta, E., 1993). As the stimuli-sensitive behaviour occurs in aqueous solutions and at relatively low content of organic co-solvent in the mixture, these hydrogels are becoming increasingly attractive for biotechnology and medicine (Galaev, I.Y. et al., 1999). Transition from the hydrophilic to a hydrophobic state in the polymer results in increasing of hydrophobic interactions between the polymer and environmental species (Kanazawa, H. et al., 1998). In relation to immobilized biocatalyst such picture is very similar to an organic solvent action on enzyme.

Kinetical behaviour of α-chymotrypsin, immobilized into stimuli-sensitive poly-N-isopropylacrylamide gel, under the phase transition of a matrix caused by dimethyl sulfoxide content in the mixture and the temperature was investigated with the use of different substrates. It was shown that the hydrophobic interactions between the matrix and P2 sorption site of enzyme determine the observed kinetical picture.

Materials and methods.

α-Chymotrypsin was purchased from Samson Ltd (Russia). N-acetyl-L-tyrosine ethyl ester (ATEE), N-benzoyl-L-tyrosine ethyl ester (BTEE), N-benzoyl-L-tyrosine p-nitroanilide (BTNA), acryloyl chloride and N-isopropylacrylamide (NIPAA) were obtained from Sigma (USA). Acrylamide (AA), N,N'-methylene-bis-acrilamide (MBA), N,N,N',N'-tetramethylethylenediamine (TEMED) and ammonium persulfate were purchased from Reanal (Hungary). N-Acetyl-L-tyrosine p-nitroanilide (ATNA) was courteously supplied by N.A.Uskova (MSU, Faculty of Chemistry). Dimethyl sulfoxide (DMSO) and components of buffer solutions ("pure for analysis" grade) were obtained from Reakhim (Russia).

Synthesis of preparations of immobilized α-chymotrypsin. 200 mg of monomer (AA or NIPAA) and 0.36 mg of cross-linking agent (MBA) were added to 1 ml of 0.8 mM solution of acryloylated α-chymotrypsin (modification of α-chymotrypsin by acryloyl chloride was carried out according to

(Belyaeva, E.A. et al., 2002)). Block copolymerisation was started by adding 0.02 ml of aqueous 0.78 M ammonium persulfate solution and 0.01 ml of TEMED and was carried out at $0^{o}C$ for 1 h. For activity measurements, synthesized block copolymers were suspended in a homogenizer to get the particle size equal to 20-100 μkm. The suspension of gel particles was washed with 0.2 M NaCl solution until the absence of activity in washing water and equilibrated for 24 h in solution required to activity measurement.

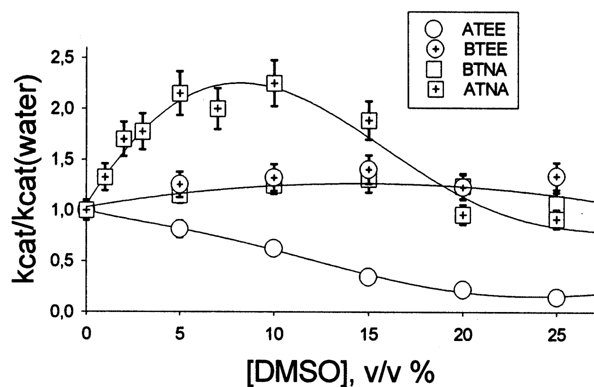
Enzyme activity assay. Activities of native and immobilized α-chymotrypsin were determined by recording of initial rates of substrates hydrolysis in pH-optimum (8.0 for the native enzyme and 9.0 for immobilized preparations). Catalytic constants for native enzyme and values of maximum velocity for immobilized preparations were calculated from Lineweaver-Burk plots (1/V – 1/[S]o). ATEE and BTEE hydrolysis (ionic strength was adjusted by 0.2 M NaCl; substrates concentrations were varied from 2 to 10 mM; $20^{o}C$) was investigated with a RTS822 pH-state (Radiometer, Denmark). ATNA and BTNA hydrolysis (0.05 M Na-phosphate buffer; substrates concentrations were varied from 0.02 to 0.3 mM) was investigated with an EPS-124 spectrophotometer (Hitachi, Japan). Molar absorbance of p-nitroaniline at 390 nm was measured independently for each DMSO concentration.

Determination of hydrogel swelling. Thoroughly washed block copolymers were cut into 5 mm x 5 mm x 2 mm slices. The slices of gel were equilibrated for 24 h at selected temperature and DMSO content in 0.05 M Na-phosphate buffer, pH 9.0, gently blotted by filter paper and weighted. Swelling ratio was expressed as a per cent ratio between sample weight at selected conditions and sample weight at $20^{o}C$ in the absence of DMSO (pure buffer).

Results and discussion.

Non-specific action of water-miscible organic solvent on the enzymes consists in denaturation of the protein and related loss of enzyme activity when the critical content of organic solvent is reached (for α-chymotrypsin the critical content of DMSO is 30 vol.% (Khmelnitsky, Yu.L. et al., 1991)). But at non-denaturating DMSO concentrations in the mixture native α-chymotrypsin demonstrates different kinetical behaviour when the different substrates are used for the determination of residual activity of the enzyme, and such difference is determined by the structure of N-acyl substituent of the substrate (Fig.1). Evidently, that such behaviour of α-chymotrypsin is a result of the specific interactions between the DMSO and protein globule in the sorption site of N-acyl substituent of substrate. It is known that the decrease in the level of standard free energy of the enzyme-substrate complex with the change of N-acetyl to N-benzoyl derivatives of aminoacids is equal to 2,7-2,9 kcal/mol (Klyosov, A.A. et al., 1977). So, we can suppose that more hydrophobic N-benzoyl group is able to displace DMSO molecule from the protein globule and structures of such complexes are identical both in water and in water/DMSO mixtures. More hydrophilic N-acetyl group can't displace DMSO molecule from the active site of a-chymotrypsin and in this case hydrolytic reaction of substrate takes place from some structural modification of the enzyme active site.

Immobilization of enzymes obviously protects the protein structure against conformational changes. Fig.2 shows that, really, multipoint covalent immobilization of α-chymotrypsin into poly-AA gel sufficiently increases the working range of biocatalyst in water/DMSO mixtures – the matrix preserve the enzyme against non-specific denaturating process. Much more interesting that such immobilized preparation demonstrates the similar residual activity from DMSO content with the different substrates. The more – this dependence is practically identical to the dependence of N-benzoyl derivatives hydrolysis by native enzyme (compare Figs.1 and 2). So, we can make a conclusion that: i) specific interactions between DMSO and protein globule really lead to some



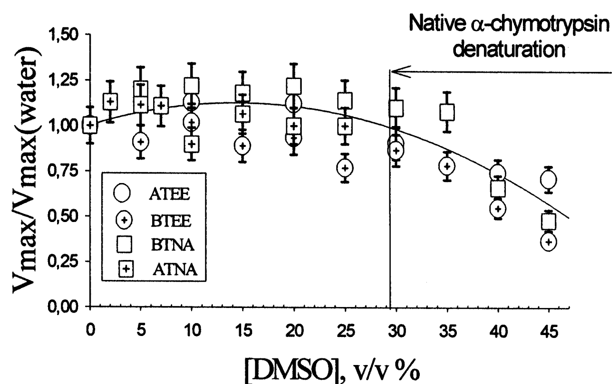
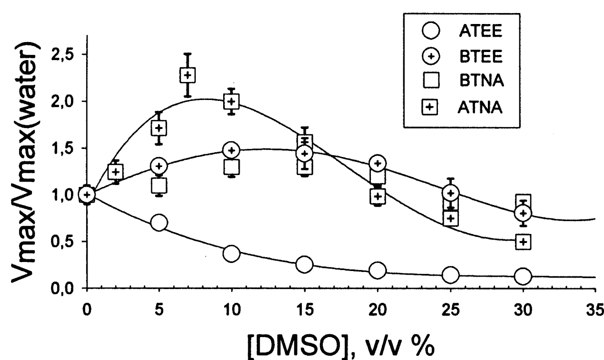


Fig.1. Relative activity of native α-chymotrypsin in water/DMSO mixtures with different substrates.

Fig.2. Relative activity of α-chymotrypsin immobilized into poly-AA gel with different substrates.

conformational changes in the active site of α-chymotrypsin and ii) both high sorption energy of N-benzoyl substituent of the substrate and "rigidation" of the protein structure by its multipoint covalent immobilization protects the "native" conformation of the enzyme active site (enzyme-substrate complex) from the specific action of DMSO.

Preparations of α-chymotrypsin immobilized into poly-NIPAA gel were obtained in much the same way as poly-AA preparations (see Materials and Methods). The amount and modification degree of enzyme, the concentration of the monomers and cross-linking density are the same in both cases. For each used substrate the activity of poly-AA and poly-NIPAA preparations in water are equal. At the same time residual activity of poly-NIPAA preparation in water/DMSO mixtures measured with the use of different substrates principally differs from poly-AA samples (compare Figs.2 and 3) and below 30 vol.% of DMSO content is quite similar to the native enzyme (compare Figs.1 and 3). The explanation of this fact, for our mind, lays in the properties of poly-NIPAA matrix. Stimuli-sensitivity of poly-NIPAA is determined by hydorphobization of polymeric chains in response to increase in temperature or organic solvent content. The increasing in polymer hydrophoby can make the interactions of poly-NIPAA with the immobilized protein more energetically advantageous than the α-chymotrypsin-water interactions. The influence of poly-NIPAA on enzyme in this case must be similar to DMSO action (i.e. cause the structural modification of the enzyme active site) and such similarity we see in kinetical behaviour of native and immobilized into poly-NIPAA gel α-



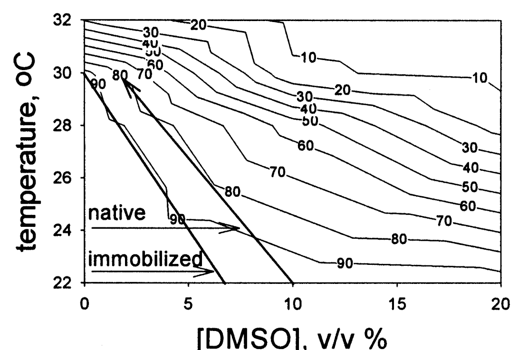


Fig.3. Relative activity of α-chymotrypsin immobilized into poly-NIPAA gel with different substrates

Fig.4. Swelling degree of poly-NIPAA gel and cross-sections of peaks of ATNA hydrolysis by native and immobilized into poly-NIPAA gel α-chymotrypsin.

chymotrypsin in non-denaturating water/DMSO mixtures. Such hypothesis may be confirmed by a comparative analysis of residual activity of poly-NIPAA preparation measured by the rate of ATNA hydrolysis and water content of this gel matrix during its collapse under the simultaneous increasing in temperature and DMSO content (Fig.4). The increase in temperature leads to increase in flexibility of enzyme globule. Conformational changes become possible in smaller concentrations of DMSO, and the peak of the native enzyme activity shifts to lower concentrations of DMSO with the increasing of temperature. At the same time the analogous cross-section for immobilized into poly-NIPAA gel α-chymotrypsin passes at smaller concentrations of DMSO in comparison to native enzyme. So, hydrophobization of polymeric chains during the collapse of the matrix really causes polymer-protein interactions and, as a result, structural modification of the enzyme active site.

Conclusions

Transition from the hydrophilic to a hydrophobic state in stimuli-sensitive polymeric hydrogels causes specific hydrophobic interactions between the polymer and protein immobilized in it. Structural modification of the enzyme active site takes place as the result of such interactions – that is why the residual activity of immobilized preparation in water/organic solvent mixtures becomes to be dependent from the substrate used. It means that for practical usage of biocatalysts immobilized into stimuli-sensitive hydrogels the checking of their activity must be done with a great accuracy, i.e. in conditions maximally closed to proposed process.

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