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Biocatalytic properties of native and immobilized subtilisin 72 in aqueous-organic and low water media

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Abstract

Subtilisin 72 was immobilized on cryogel of poly(vinyl alcohol), the macroporous carrier prepared by the freeze-thaw-treatment of concentrated aqueous solution of the polymer. The obtained biocatalyst was active and stable in aqueous, aqueous-organic, as well as in low water media. The stability of immobilized biocatalyst was substantially higher than that of native enzyme in all mixtures especially in aqueous buffer containing 5–8 M Urea and in acetonitrile/60–90%DMF mixtures. The ability of native and immobilized subtilisin to catalyze peptide bond formation between Z-Ala-Ala-Leu-OMe and Phe-pNA was studied in non-aqueous media. Considerable enzyme stabilization in acetonitrile/90%DMF mixture, induced by the immobilization, resulted in higher product yield (57%) than in case of native subtilisin suspension (32%). Detailed study of synthesis reaction revealed that notable increase in product yield could be reached using increase in both substrate concentrations up to 200 mM.

Keywords: Subtilisin; Subtilisin immobilized on PVA cryogel; Low water media; Organic solvents; Enzymatic peptide synthesis

1. Introduction

Various organic reactions catalyzed by enzymes are up-to-date, basis of biotechnology [1]. Rational application of enzymes requires detailed investigations of their properties, such as substrate specificity, activity and stability under various conditions. Hydrolases, in particular lipases and proteases, i.e. enzymes, which catalyze the transfer of an acyl-donor to a water molecule, have been recently widely used in synthetic reactions [2,3]. The equilibrium shift is frequently provided by performing the reactions in non-aqueous environment. To ensure high enzyme activity, it is necessary to choose optimal enzyme stabilization technique among the multitude known nowadays [4]. There are a lot of articles dealing with enzyme stabilization, but only in few of them clear comparison of different stabilization ways has been accomplished [5–7]. In previous studies we compared in different media the catalytic properties of native subtilisin, its non-covalent complex with poly(acrylic acid) and subtilisin, immobilized on cryogel of poly(vinyl alcohol) [8]. It has been shown that the most efficient biocatalyst was the immobilized subtilisin. The aim of this work was to study hydrolytic and synthetic activities of native and immobilized subtilisin in different aqueous/organic mixtures in details.

2. Experimental

2.1. Materials

Serine proteinase from *Bacillus subtilis* strain 72 (subtilisin 72 [9,10], specific activity on chromogenic substrate Z-Ala-Ala-Leu-pNA 9 μmol/min per mg protein) was iso-

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lated and purified according to Gololobov et al. [11]. Acetonitrile was of HPLC grade (Lekbiofarm, Russia) containing no more than 0.01% water; dimethylformamide was of analytical grade (Reakhim, Russia) additionally purified according to a procedure [12]; substrates Glp-Ala-Ala-Leu-pNA (where Glp- is pyroglutamil residue) and Z-Ala-Ala-Leu-pNA were obtained according to the procedures described by Lyublinskaya et al. [13] and Yakusheva et al. [14], respectively; poly(vinyl alcohol) (PVA, MW 69,000) was from NPO "Azot" (Ukraine), other chemicals were of analytical grade.

2.2. Instrumentation

Reverse-phase HPLC analyses were performed on liquid chromatograph Altex Model 110A (United States) with column Microsorb-MV C8 (4.6 mm × 250 mm, Rainin Instrument Company, Inc., USA) at the elution by linear gradients of acetonitrile 10–70% for 26.2 min in 0.1% TFA at the rate of 1 ml/min and the detection at 220 and 280 nm.

Amino acid analyses were performed on Hitachi-835 amino acid analyzer (Japan) after samples hydrolysis in 5.7 M HCl at 105 °C in evacuated ampoules for 48 h. The results well agreed with those calculated for the product peptides.

The optical absorbance was measured on Specord UV–vis (Germany) and Shimadzu UV-1601 (Japan) spectrophotometers.

2.3. Methods

2.3.1. Preparation of subtilisin immobilized on poly(vinyl alcohol) cryogel

Beaded poly(vinyl alcohol) cryogel (cryoPVAG) were obtained as described elsewhere [15]; bead size was 0.8–1.0 mm in diameter.

2.3.1.1. Activation of cryoPVAG.

(a) Activation of cryoPVAG using glutaraldehyde: this was performed as described in [15].
(b) Activation of cryoPVAG by divinylsulfone: 0.6 ml of divinylsulfone and 2 ml of 0.1 M NaOH were added to 1 g of wet cryoPVAG beads. Reaction mixture was shaken vigorously for 1 h, then the granules were washed with dilute NaOH and excess amount of distilled water.
(c) Activation of cryoPVAG using epichlorohydrine: 1 g wet cryoPVAG was suspended in 1 ml of 2 M NaOH, then 1 ml of dioxane and 1 ml of epichlorohydrine were added. Reaction mixture was shaken at 40 °C for 24 h. After this the beads were washed with distilled water down to pH 7, then with 0.1 M Na-phosphate buffer pH 8.2 and again with distilled water.

2.3.1.2. Subtilisin immobilization on activated cryoPVAG.

Activated cryoPVAG (0.5–1 g) was suspended in 2–3 ml of 0.05 M Na-phosphate buffer pH 8.2 and stirred intensively for 30 min. Then solid support was filtered off, mixed with 5 ml of subtilisin solution in the same buffer (2 mg/ml) and incubated at constant shaking for 5 h. In the case of immobilization with competitive inhibitors, a 100-fold molar excess of Bz-Tyr-NH_2 or Ac-Trp-OH over enzyme was used. Thereafter the beads of immobilized biocatalyst were filtered, washed with 0.05 M Na-phosphate buffer pH 8.2 and treated with 3–4 ml of 0.05 M Tris–HCl buffer pH 8.2 containing 2 mM $CaCl_2$ for 1.5–2 h to block remaining CHO, vinyl or epoxy groups on cryoPVAG. The amount of immobilized protein was calculated from the data of amino acid analysis.

2.3.2. Determination of native subtilisin and immobilized subtilisin hydrolytic activity against Glp-Ala-Ala-Leu-pNA

Glp-Ala-Ala-Leu-pNA solution in DMF (50 μl, 5 mg/ml) was added to 2 ml of 0.05 M Tris/HCl buffer (pH 8.3) containing 1.5 mM $CaCl_2$, and the mixture was incubated at 20 °C for 10 min. Then, 10–20 μl of the enzyme solution (or 40 mg of the cryoPVAG-attached subtilisin) was added, and the mixture was incubated at 37 °C (at 20 °C for immobilized enzyme) and absorbance at 410 nm was measured. For native enzyme the reaction was stopped with 1 ml of 50% acetic acid. In control samples there was reverse order of addition of the enzyme solution and acetic acid.

2.3.3. Kinetic study of Z-Ala-Ala-Leu-pNA hydrolysis by native and immobilized subtilisin

(a) Dependence of initial hydrolysis rate on enzyme concentration: to 3 ml of substrate solution (1 mM) in 60% DMF/0.05 M Tris–HCl buffer pH 8.2 containing 2 mM $CaCl_2$ the subtilisin solution in the aqueous Tris–HCl buffer (0.05–0.3 mg/ml, 0.094–1.13 μM) was added. In the case of immobilized enzyme substrate solution was added to freshly filtered granules of immobilized biocatalyst (12–32 mg, 0.755–2.17 μM) and was quickly shaken. The mixture was incubated at room temperature and release of *p*-nitroaniline was followed. The initial rates of substrate hydrolysis were determined from the slopes of the straight lines of obtained optical absorbance at 410 nm versus time plots using molar extinction coefficient of *p*-nitroaniline in 60%DMF/buffer mixture (11,385 M^{-1} cm^{-1}).
(b) Dependence of initial hydrolysis rate on substrate concentration: the reactions were performed similarly (a) using 2.5–20 mM substrate solutions in 60% DMF/0.05 M Tris–HCl buffer pH 8.2 containing 2 mM $CaCl_2$ and 0.366 μM subtilisin solution in the aqueous Tris–HCl buffer or freshly filtered granules of immobilized biocatalyst (15 mg, 0.963 μM). The $V_{max}/K_M \times [E]$ values were determined from linear gradient of reaction rate against substrate concentration.

2.3.4. Stability of the native and immobilized subtilisin in the presence of urea

(a) Native subtilisin (1.4 mg) was dissolved in 70 μl Tris–HCl buffer pH 8.2 containing 1.5 mM $CaCl_2$, then 10 μl aliquots were taken and diluted with 190 μl of Tris–HCl buffer pH 8.2 containing 1.5 mM $CaCl_2$ and 0, 1, 3, 5 or 8 M urea. After 1.5 h storage at room temperature 10 μl aliquots from each solution were withdrawn for activity measurement as described in Section 2.3.2, but in this case the buffer used was the same as for incubation (i.e. containing 0, 1, 3, 5 or 8 M urea). The activity was calculated using molar extinction coefficients of *p*-nitroaniline in buffers used (8900, 8900, 9500 and 10,000 M^{-1} cm^{-1} in buffer containing 0, 1, 3, 5 or 8 M urea, respectively).

(b) The beads of immobilized biocatalyst (10 mg, 5 portions) were washed with Tris–HCl buffer pH 8.2 containing 1.5 mM $CaCl_2$ and 0, 1, 3, 5 or 8 M urea (3 × 2 ml), then 2 ml of corresponding buffer was added and mixture was incubated for 1.5 h at room temperature at constant shaking. After this the buffer was removed and activity of biocatalyst was measured as described in Section 2.3.2, but in this case the buffer used was the same as for incubation (i.e. containing 0, 1, 3, 5 or 8 M urea). The activity was calculated using molar extinction coefficients of *p*-nitroaniline in buffers used.

2.3.5. Preparation of subtilisin suspension in acetonitrile/DMF mixtures

Lyophilized subtilisin 72 (1 mg, 35 nmol) was dissolved in 200 μl 0.05 M Tris–HCl buffer pH 8.2 containing 1.5 mM $CaCl_2$, then 40 μl of this solution was added to 760 μl of the corresponding acetonitrile/DMF mixture with stirring on a magnetic stirrer.

2.3.6. Stability of native subtilisin suspension in non-aqueous media

Subtilisin suspension in mixture acetonitrile/DMF/water was incubated at 20 °C and aliquots (100 μl) were periodically withdrawn to determine the activity as described in Section 2.3.2.

2.3.7. Stability of immobilized subtilisin in non-aqueous media

A portion of immobilized biocatalyst was washed with acetonitrile (1 × 0.5 ml), with acetonitrile/DMF mixture of respective composition (70/30, 60/40 or 90/10) (2 × 0.5 ml), then 1 ml corresponding mixture was added and the resulting mixture was incubated at 20 °C for 24 h at constant shaking. After this, the solvent was decanted and the biocatalyst activity against Glp-Ala-Ala-Leu-pNA was measured as described in Section 2.3.2.

2.3.8. Synthesis of Z-Ala-Ala-Leu-Phe-pNA by native subtilisin suspension

To the solution of Z-Ala-Ala-Leu-OMe (5.1 mg, 12 μmol) and Phe-pNA (3.4 mg, 12 μmol) in the mixture of 240 μl dry DMF and 140 μl acetonitrile, 20 μl subtilisin solution (5 mg/ml, 2.4 nmol) in 0.05 M Tris–HCl buffer (pH 7.8) containing 1.5 mM $CaCl_2$ was added. The reaction mixture was stirred at 20 °C, and 10 μl aliquots were periodically withdrawn for HPLC analysis. The reaction in the mixture with 95% DMF was performed similarly. Retention time: Z-Ala-Ala-Leu-Phe-pNA 19 min.

2.3.9. Synthesis of Z-Ala-Ala-Leu-Phe-pNA by immobilized subtilisin

A portion of cryoPVAG-immobilized subtilisin (80 mg, 15 nmol of protein) was added to the solution of Z-Ala-Ala-Leu-OMe (5.1 mg, 12 μmol) and Phe-pNA (3.4 mg, 12 μmol) in the 380 μl of the mixture of organic solvents used. The reaction suspension was shaken at 20 °C, with 5 μl samples being periodically taken for HPLC analysis.

2.3.10. Study of Z-Ala-Ala-Leu-Phe-pNA synthesis catalyzed by native and immobilized subtilisin

Z-Ala-Ala-Leu-OMe (75 mg, 0.16 mmol) and H-Phe-pNA (46 mg, 0.16 mmol) were dissolved in 400 μl 40% acetonitrile/60% DMF mixture then prepared mixture was diluted consecutively with 40% acetonitrile/60% DMF mix to obtain series of 2–200 mM substrates solutions. To immobilized biocatalyst granules (8 mg, 0.04 mg protein) freshly rinsed by 40% acetonitrile/60% DMF mixture (2 × 1 ml) 250 μl solution of substrates was added. Reaction mixture was shaken at room temperature and aliquots were periodically taken for HPLC analysis.

3. Results and discussion

3.1. Biocatalytic features of native and immobilized subtilisin in predominantly aqueous media

PVA cryogel is a very prospective and convenient support for enzyme immobilization due to its macroporosity, possibility of activation by different reagents and good physical and mechanical properties. Poly(vinyl alcohol) cryogels are hydrophilic gel matrixes with heterogeneous macroporous structure which is the result of gelation in two-phase media consisting of ice polycrystals and liquid microphase. This carrier was prepared by freezing, keeping in the frozen state, and subsequent thawing of the concentrated aqueous solutions of poly(vinyl alcohol). The characteristic feature of these supports is their ability to keep water inside the gel matrix even if it was placed into non-aqueous organic solvents [16]. Immobilization of several proteases and hog pancreas lipase on PVA cryogel was described in detail earlier [17–19]. In our work we used subtilisin, protease of serine type, which is a very promising catalyst for amide and es-

Table 1
Characteristics of immobilized subtilisin preparations

Immobilized biocatalyst	Sample number	Loading of enzyme on support (mg/g)	Activity, α_{spec}/mg protein (μmol min^{-1} mg protein^{-1})
cryoPVAG-ECH-subtilisin	1	0.1	2.2
	2	0.46	0.83
cryoPVAG-DVS-subtilisin	3	6	0.11
	4	1.43	1.3
cryoPVAG-GA-subtilisin	5	3.7	0.37
	6	4.5	0.19
	7	5.4	0.46
	8	13.7	0.041
cryoPVAG-GA-subtilisin + Ac-Trp-OH	9	2.0	0.65
cryoPVAG-GA-subtilisin + Bz-Tyr-NH_2	10	2.3	0.50

ter bond formations due to wide substrate specificity along with high stereo- and regioselectivity. Different activation reagents, such as epichlorohydrine (ECH), divinylsulfone (DVS) and glutaraldehyde (GA), have been employed to enzyme immobilization. Some characteristics of obtained biocatalysts are presented in Table 1.

Various samples of biocatalyst differed in loading (from 0.1 to 13.7 mg protein per g support) and in specific activity (α_{spec}), which was 0.1–5% of that shown by native enzyme (0.041–2.2 U/mg). Substantial loading increase greatly decreased specific activity of enzyme (e.g. samples 1 and 2, or 5 and 8). Low hydrolytic activity of immobilized enzyme in aqueous solution comparing to the native subtilisin could be explained by two different factors: (1) by the restriction of conformation mobility due to the covalent enzyme fixing and (2) partly by the limiting of substrate diffusion to those molecules of subtilisin which were attached to support near enzyme's active site. Diffusion of the substrate to enzyme environment inside pores cannot limit the hydrolysis rate because of macroporosity of PVA cryogel [20]. Immobilization in the presence of competitive inhibitors (Table 1, samples 9, 10) of serine proteases gave slightly more active and more stable biocatalysts probably due to bounding in the active site and fixing its conformation (active site protection).

Previously it was shown that immobilized subtilisin was effective in media with high DMF content. Based on these promising results we decided to study kinetics in 60% DMF/buffer mixture. Besides, the solubility of the substrate was moderate in DMF/aqueous mixtures with lower DMF concentration. The dependence of initial hydrolysis rate of the highly specific chromogenic substrate Z-Ala-Ala-Leu-pNA by native and immobilized subtilisin on the enzyme concentration was examined in Tris–HCl buffer containing 60% DMF (Fig. 1). Obtained dependences were linear over all the range studied. Concentrations of 0.366 μM for native subtilisin and of 0.963 μM for immobilized subtilisin were chosen as optimal as they were the minimum concentrations which provide reasonable and measurable hydrolysis rates. The concentration of immobilized biocatalyst was calculated according to amino acid analysis data on protein loading. Velocities of hydrolysis at the same enzyme concentration for native and immobilized subtilisin differed by 6.6 times. Note

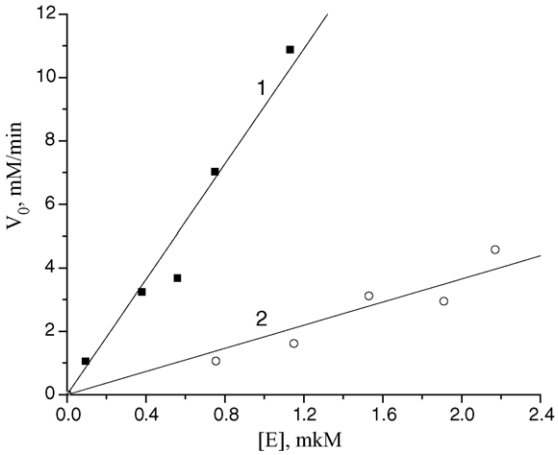


Fig. 1. Dependence of initial rate of hydrolysis of Z-Ala-Ala-Leu-pNA on enzyme concentration for native (1) and immobilized (2) subtilisin (sample 7) in 60% DMF/Tris–HCl buffer pH 8.2 containing 2 mM $CaCl_2$.

that specific activity measured under equal conditions (at 4% of DMF) differed more than in 100 times (α_{spec} were 45 and 0.46 U/mg protein for native and immobilized enzyme, respectively). Therefore, the immobilization considerably protected the enzyme against the inactivation by DMF.

In order to compare catalytic properties of native and immobilized subtilisin we calculated the ratios $V_{max}/[E]_{app} \times K_M$ which are presented in Table 2.

It was shown that there is practically no difference in $V_{max}/[E]_{app} \times K_M$ values for the hydrolysis of Z-Ala-Ala-Leu-pNA by native and immobilized subtilisin in buffer containing 60% of DMF. This fact proves our hypothesis that enzyme active site is not distorted upon immobilization stage. Moreover, to increase enzyme efficiency and to overcome diffusion limitations in hydrolysis reaction the substrate con-

Table 2
Kinetic constants of hydrolysis of Z-Ala-Ala-Leu-pNA by native and immobilized (sample 7) subtilisin in 60% DMF/40% 0.05 M Tris–HCl buffer pH 8.2 containing 2 mM $CaCl_2$

Enzyme	$V_{max}/[E]_{app} \times K_M$, s^{-1} M^{-1}
Native subtilisin	25 ± 2
Immobilized subtilisin	15 ± 1

centration should be increased. The decreased activity of biocatalyst could be explained by influence of immobilization on access of substrate to active pocket rather than on structure and conformation changes of active site. We believe that most of enzyme molecules quite probably have, to some extent, difficult access of substrates to active site due to random orientation of enzyme molecules and matrix flexibility. The last mentioned property of carrier could allow enzyme to immerse slightly into PVA cryogel.

Immobilized enzyme has superior stability (as function of enzyme activity on storage time) in water compared with native subtilisin. Since it has been demonstrated earlier that Ca^{2+} ions play crucial role and stabilize subtilisin against inactivation in aqueous media [8] we used Tris–HCl buffer containing 1.5 mM Ca^{2+} for storage experiments. Native subtilisin was shown to be inactivated 60% in the same buffer during 72 h of incubation [8]. For immobilized biocatalyst prepared using glutaraldehyde no leaching of enzyme and no substantial loss of its activity was observed during 10 months of storage (for all samples). Hydrolytic activity of immobilized biocatalyst was high for at least 2 years with only 40% of initial activity loss in that time, while being continuously incubated in buffer solution at +4°C [8,15,17]. Dependence of residual activity on storage time for samples 4, 7 and 10 is shown on Fig. 2. The most stable biocatalyst turned out to be the sample 10, when subtilisin was immobilized in the presence of Bz-Tyr-NH_2, inhibitor of serine proteases. This biocatalyst retained 75% of initial activity during 16 months storage. In the case of sample 4 prepared by DVS activation method the stability of enzyme was lower and loss of activity was about 60% during 3 months incubation. Nevertheless specific activity after 2 years storage (U/mg of protein) was equal or even exceeded activity of biocatalysts obtained using glutaraldehyde.

Subtilisin immobilized on cryoPVAG also showed higher stability and residual activity than native enzyme in the presence of denaturing reagent – urea (Fig. 3). This reagent can be used in some cases for better solubility of substrates or reactants [21]. Activity of cryoPVAG-GA-subtilisin was about 67% from initial after 1.5 h storage in 8 M urea, whereas native enzyme almost completely lost its activity already in 5 M urea during 1.5 h incubation. Note that activity was measured in the same buffer, where biocatalyst has been incubated for 1.5 h.

3.2. Biocatalytic features of native and immobilized subtilisin in low water media

Application of water-miscible organic solvents as the reaction media to favor synthesis over hydrolysis and to prevent side processes in reaction (1) is rather routine procedure.

$$R_1\text{-}(Xaa)_n\text{-}OH + H_2N\text{-}Yaa\text{-}R_2 \rightarrow R_1\text{-}(Xaa)_n\text{-}Yaa\text{-}R_2 + H_2O \quad (1)$$

Usually enzymes in a non-aqueous solvent are in form of suspension and quite often quickly lose their activity. Various ways of stabilization of structure and activity of enzymes in such environment were developed during last 35–40 years and in many cases profound stabilization was claimed [22,23]. Enzyme stability and efficiency in non-aqueous media can be improved using suitable additives or chemical modification. Besides that the enzyme immobilization is one of the common strategies to achieve stable and active biocatalyst.

PVA cryogel is the hydrophilic matrix and can concentrate water that enables subtilisin to act effectively in non-aqueous environment. Immobilized biocatalyst was prepared in aqueous media and to remove the excess of water we thoroughly washed the biocatalyst by acetonitrile/DMF mixture (water-miscible solvents) prior to use in non-aqueous media. Obviously, organic solvents cannot strip all water molecules from support and immobilized enzyme as activity of biocatalyst remains at high level (Fig. 4). No extra water was added to acetonitrile/DMF mixture for immobilized subtilisin. The accurate measurement of water content in immediate vicin-

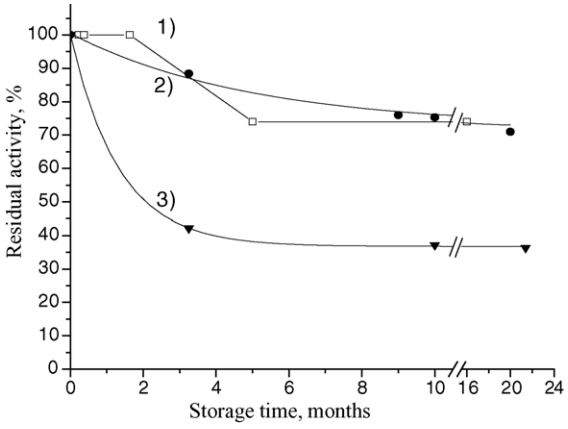


Fig. 2. Residual activity of immobilized subtilisin: (1) sample 10; (2) sample 7; (3) sample 4 after storage in Tris–HCl buffer pH 8.2 containing 1.5 mM Ca^{2+}.

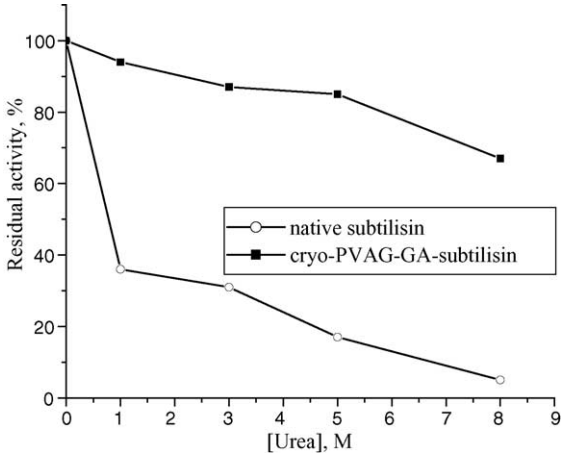


Fig. 3. Residual activity of native and immobilized subtilisin (sample 7) after 1.5 h storage in Tris–HCl buffer pH 8.2 containing various concentrations of urea.

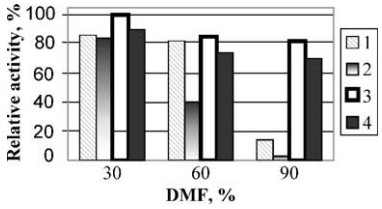


Fig. 4. Relative activity of native and immobilized subtilisin (sample 6) in acetonitrile/DMF mixtures with different DMF content after 2 and 24 h of incubation; (1) native subtilisin, 2 h; (2) native subtilisin, 24 h; (3) immobilized subtilisin, 2 h; (4) immobilized subtilisin, 24 h.

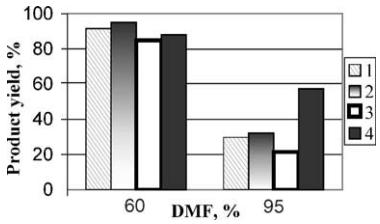


Fig. 5. The dependence of the Z-Ala-Ala-Leu-Phe-pNA yield on DMF concentration in acetonitrile/DMF mixtures after 2 and 24 h of synthesis catalyzed by native and immobilized subtilisin (sample 6): (1) native subtilisin, 2 h; (2) native subtilisin, 24 h; (3) immobilized subtilisin, 2 h; (4) immobilized subtilisin, 24 h.

ity of immobilized enzyme molecule cannot be provided but amount of water in reaction volume was not more than 0.2% measured using Karl Fischer water titration technique [15].

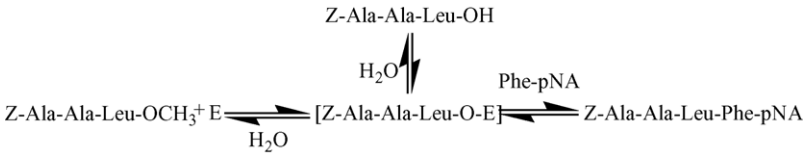
Native enzyme could be mixed with reaction medium with low water content by different ways. It has been shown by several independent research groups [24,25] that the form, in which enzyme is introduced into the non-aqueous solvent, is of great importance. Addition of the enzyme as a concentrated aqueous solution with suitable pH value to a mixture of organic solvents usually provides higher reaction rates rather than direct suspending (by stirring or swirling) of lyophilized powder of catalyst [24]. Both methods give enzyme suspension, but size of obtained solid particles and conformation of enzyme molecules could be different. Since in aqueous environment enzyme molecules have optimal hydration and ionization level we chose this approach and introduced enzyme in the preformed catalytically active state. The water concentration is quite crucial parameter and in the case of low water media regulates the compromise between low synthetic/hydrolytic process ratio and high enzyme activity. The narrow range of water concentrations, which is necessary to retain catalytic activity, is the enzyme- and solvent-dependent. Previously it was reported that subtilisin stability and activity are greatly increased when the final mixture DMF/acetonitrile/water contained about 5% of water [26].

Activity of native or immobilized biocatalyst in Tris–HCl buffer pH 8.2 containing 1.5 mM $CaCl_2$ was assumed as 100%. In the mixture of 30% DMF/acetonitrile both native and immobilized subtilisin possessed high activity (more than 80%) and stability (Fig. 4). Native enzyme in 60%DMF/acetonitrile had moderate stability retaining about 40% of initial activity during 24 h of incubation. In the mixture with 90%DMF content the unmodified subtilisin maintained about 15% activity after 2 h of incubation, still keeping some residual activity (~5%) after 24 h in this media. Unlike these data the immobilized subtilisin showed extremely high stability not only in 60%DMF/acetonitrile, where activity was about 80% during 24 h storage, but even in 90% DMF/acetonitrile mixture, where biocatalyst loosed less than 30% of initial activity after the same time of incubation. Since stability of native and immobilized enzyme in low water media was high we decided to test activity of subtilisin in the reaction of peptide bond formation under these conditions.

3.3. Synthetic properties of biocatalysts in low water media

The ability of enzyme to realize biotransformations in low water solvents is more valid evaluation of its biocatalytic properties in such an environment than hydrolytic activity measured after transfer of enzyme from organic to aqueous media. In addition, the synthesis of hydrophobic compounds, which are not soluble in aqueous media, is of our particular interest. Synthesis of Z-Ala-Ala-Leu-Phe-pNA (Scheme 1) was chosen as a typical example and studied in detail, because carboxyl- and amino components fit well in terms of enzyme specificity. This peptide (Z-Ala-Ala-Leu-Phe-pNA) precipitates in the mixture DMF/acetonitrile/5% water with DMF content less than 60% because of its moderate solubility. In case of immobilized enzyme the precipitate can soil pores of support, so in this work we used mixtures containing 60% DMF and more.

In this part we compared the product yields in the synthesis performed by native subtilisin suspension and immobilized biocatalyst in reaction media contained 60 and 95% of DMF (Fig. 5). Concentration of substrates was 30 mM. For native subtilisin suspension high reaction rates and maximum of peptide yield can be reached in triple DMF/acetonitrile/water



Scheme 1. Synthesis of Z-Ala-Ala-Leu-Phe-pNA catalyzed by subtilisin.

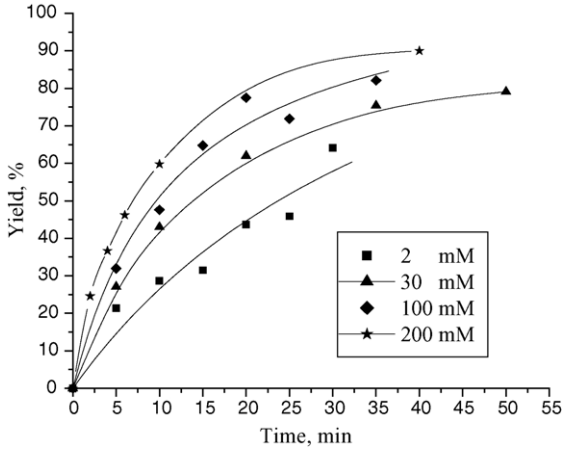


Fig. 6. The time dependence of Z-Ala-Ala-Leu-Phe-pNA yield in reaction catalyzed by cryoPVAG-subtilisin (sample 7), with different substrate concentrations in 60% DMF/40% acetonitrile mixture; [*E*] = 8.8 μM.

mixtures with DMF content up to 60–70% and enzyme concentration 6 μM (and higher) [26]. In the mixture with 60% DMF content the 95% yield of product was obtained after 2 h reaction. In the same mixture the immobilized enzyme gave 80–90% yield in 2 h reaction with 20 μM enzyme concentration for all samples (Fig. 5). Despite the great difference in hydrolytic activity of native and immobilized subtilisin, time needed to achieve the maximal product yield using both enzymes' forms was almost the same.

The most significant difference in reaction progress was observed in the mixture with 95% DMF. In this media reaction catalyzed by native subtilisin suspension was shown to stop at 30% product yield after 2–3 h [26]. Unlike this, the immobilized biocatalyst was capable to perform peptide synthesis in 95%DMF/5% acetonitrile mixture and product yield achieved 58% after 24 h reaction and 74% in 72 h. In last case excellent stability of immobilized subtilisin resulted in the prolonged functioning of enzyme.

The effect of substrates concentration on degree of their conversion in 60% DMF/40% acetonitrile mixture was studied using equimolar amounts of amino- and acyl components. The course of synthesis was monitored using RP HPLC. Fig. 6 shows the dependences of the product yield on the reaction time in the range of substrate concentrations from 2 to 200 mM.

The overall tendency is obvious: the higher the substrates concentrations, the higher the synthesis rate. For example, the product yield after 2 h was 26% at [*E*]/[*S*] = 1/22,700 ([*S*] = 30 mM), whereas when the substrate concentration was 200 mM the yield of Z-Ala-Ala-Leu-Phe-pNA was 64% after 10 min.

4. Conclusions

It has been shown that immobilization of subtilisin in PVA-cryogel beads gives considerably enhanced stability in aqueous media during long-term storage, as well as in buffers containing denaturing reagent in high concentration. Immobilized biocatalyst was extremely stable in low water media and the most profound effect was observed in mixtures with high DMF content. Synthetic activity of native and immobilized subtilisin in non-aqueous media was found to be comparable in spite of valuable difference in hydrolytic activity of these two enzyme's forms. In the solvent mixtures containing 95% DMF peptide synthesis performed by immobilized subtilisin run significantly better than with native enzyme suspension. To conclude, the immobilized enzyme was found to be most effective biocatalyst in synthetic reactions in low water media.

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