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# Activity and Stability of Native and Modified Subtilisins in Various Media

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**Abstract**—The activity and stability of native subtilisin 72, its complex with poly(acrylic acid), and subtilisin covalently attached to poly(vinyl alcohol) cryogel were studied in aqueous and organic media by hydrolysis of specific chromogenic peptide substrates. Kinetic parameters of the hydrolysis of Glp-Ala-Ala-Leu-pNA by native subtilisin and its complex with poly(acrylic acid) were determined. Based on the comparative study of stability of native and modified subtilisins in media of various compositions, it was established that covalent immobilization of subtilisin on poly(vinyl alcohol) cryogel is the most effective approach to improve enzyme stability in water as well as in mixtures with low water content.

*Key words*: immobilized subtilisin, poly(vinyl alcohol) cryogel, complex of subtilisin with poly(acrylic acid), organic solvents

Subtilisins are well-studied serine proteases [1]. Their widespread distribution, availability, stability, and broad substrate specificity make these enzymes useful as biocatalysts for various processes. During recent decades a new tendency has appeared—the use of subtilisins as synthases for obtaining biologically active compounds, especially peptidomimetics [2].

Applications of hydrolytic enzymes frequently require the addition of organic solvents to the reaction mixtures. When hydrolysis reactions catalyzed by proteases are studied, organic solvents are use to dissolve hydrophobic substrates. In synthesis reactions in organic media the shift of the equilibrium toward peptide bond formation is promoted and the risk of the secondary hydrolysis of the product is decreased [3]. For practical application of subtilisins as biocatalysts it is essential to study properties of these enzymes under different conditions, in particular in organic media.

It is known that addition of polar organic solvents in concentrations exceeding 20-30 vol. % often has negative influence on the catalytic properties of proteases [4]. To adapt enzymes for functioning in organic media and to improve the biocatalyst stability, various methods are used: chemical modification [5, 6], site-directed mutagenesis [7, 8], noncovalent complex formation of enzymes with detergents or polyelectrolytes [4, 9, 10], and covalent and noncovalent immobilization on different supports [4, 11]. However, as a rule the correlation of the influence of different modification methods on hydrolytic properties of an enzyme has not been done. The goal of this work was to compare activity and stability of native subtilisin 72, its noncovalent complex with poly(acrylic acid), and subtilisin covalently immobilized on poly(vinyl alcohol) cryogel in mixtures containing polar organic solvents.

## MATERIALS AND METHODS

In this work we used: the serine proteinase from *Bacillus subtilis* (strain 72 [12, 13]) isolated and purified according to Gololobov [14]; substrates Glp-Ala-Ala-Leu-pNA and Z-Ala-Ala-Leu-pNA obtained according to the procedures from Lublinskaya [15] and Yakusheva [16], respectively; HPLC grade acetonitrile (Lekbiofarm, Russia) containing no more than 0.01% water; analytically pure grade dimethylformamide (Reakhim, Russia) additionally purified according to a procedure [17]; poly(acrylic acid), citric acid, and $CaCl_2$ (Sigma, USA); Tris (ICN Biomedicals, USA); analytically pure grade NaOH and phosphoric acid (Lachema, Czech Republic).

*Abbreviations*: Glp) pyroglutamyl; Z) benzyloxycarbonyl; Bzl) benzoyl; pNA) *p*-nitroanilide; PAA) poly(acrylic acid); PVA) poly(vinyl alcohol).

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The optical absorbance was measured using a Specord UV VIS (Germany), Shimadzu UV-1601 (Japan), and Beckman-25 (USA) spectrophotometers.

Amino acid analysis was performed using a Hitachi-835 automatic amino acid analyzer (Japan) after samples hydrolysis in 5.7 M HCl at 105°C in evacuated ampoules for 24 or 48 h.

**Preparation of the subtilisin suspension in acetonitrile–dimethylformamide mixtures.** Lyophilized subtilisin 72 (1 mg, 35 nmol) was dissolved in 200 μl 0.05 M Tris-HCl buffer, pH 7.8, containing 1.5 mM $CaCl_2$, then 40 μl of this solution was added to 760 μl of acetonitrile–dimethylformamide mixture of the corresponding composition with stirring on a magnetic stirrer.

**Preparation of subtilisin–poly(acrylic acid) complex.** A solution of poly(acrylic acid) (56 mg/ml, 100 μl) in 0.05 M Tris-HCl, pH 8.2, was added to a solution of subtilisin 72 (1 mg, 35 nmol) in 100 μl 0.05 M Tris-HCl, pH 8.2, containing 1.5 mM $CaCl_2$, and the mixture was stirred for 1 min.

**Preparation of subtilisin immobilized on poly(vinyl alcohol) cryogel.** Preparation of reactive aldehyde-, epoxy-, and vinylsulfone-containing derivatives of poly(vinyl alcohol) cryogel and the covalent attachment of enzymes to them were carried out according to Filippova et al. [11]. The quantity of immobilized protein was estimated using amino acid analysis data.

**Determination of subtilisin hydrolytic activity against Z-Ala-Ala-Leu-pNA.** A solution of Z-Ala-Ala-Leu-pNA in dimethylformamide (0.5 mg/ml, 0.5 ml) was added to 2 ml of 0.05 M Tris-HCl buffer, pH 8.2. The mixture was incubated at 37°C for 10 min, then 10-20 μl of the enzyme solution was added and the mixture was incubated at 37°C until its absorbance at 410 nm reached 0.1-0.4. The reaction was stopped with 1 ml of 5% citric acid. In control samples there was reverse order of addition of the enzyme solution and citric acid. The specific activity was calculated according to the formula:

$$\frac{(A_{410} - A^{c}_{410}) \cdot V_0}{A_{280} \cdot t \cdot V_{enz} \cdot 8.9}, \qquad (1)$$

where $A_{410}$ is the absorption of the mixture at 410 nm, $A^{c}_{410}$ is the absorption of the control sample, $V_0$ is the total volume of the sample (ml), $A_{280}$ is the absorption of the enzyme solution at 280 nm, $t$ is the time of reaction (min), $V_{enz}$ is the volume of the subtilisin solution (ml), and 8.9 is the molar extinction coefficient of *p*-nitroaniline ($mM^{-1} \cdot cm^{-1}$).

An activity unit is defined as the amount of enzyme that liberates 1 μmol of *p*-nitroaniline per 1 min under the condition described above.

The activity of the subtilisin suspension was measured as for native enzyme by adding aliquots of the enzyme suspension (100 μl) in a mixture of organic solvents of the corresponding composition.

The activity of the poly(acrylic acid)–subtilisin complex was measured similarly to the activity assay of native subtilisin.

**Determination of the hydrolytic activity of subtilisin immobilized on poly(vinyl alcohol) cryogel against Glp-Ala-Ala-Leu-pNA.** A portion of immobilized subtilisin (30 mg, protein content 0.08 mg) was suspended in 2 ml 0.05 M Tris-HCl buffer, pH 8.3, containing 1.5 mM $CaCl_2$ and then a solution of Glp-Ala-Ala-Leu-pNA in dimethylformamide (5 mg/ml, 50 μl) was added. The resulting mixture was incubated with shaking at 20°C for 10 min; the absorption $A_{410}$ was measured at regular intervals. The specific activity was calculated according to the formula:

$$\frac{(A_{410} - A^{c}_{410}) \cdot V_0}{m_{ф} \cdot t \cdot 8.9}, \qquad (2)$$

where $A_{410}$ is the absorption of the mixture at 410 nm, $A^{c}_{410}$ is the absorption of the control sample, $V_0$ is the total volume of the sample (ml), $t$ is the time of reaction (min), $m_{ф}$ is the mass of immobilized subtilisin (mg), and 8.9 is the molar extinction coefficient of *p*-nitroaniline ($mM^{-1} \cdot cm^{-1}$).

**Study of stability of native and modified subtilisins.** The subtilisin suspension in a mixture acetonitrile–dimethylformamide–water was incubated at 20°C and aliquots (100 μl) were periodically taken to determine the activity.

Stability of the poly(acrylic acid)–subtilisin complex was determined analogously.

A portion of immobilized subtilisin was washed with acetonitrile (1 × 0.5 ml), an acetonitrile/dimethylformamide mixture of the corresponding composition (2 × 0.5 ml), then acetonitrile/dimethylformamide mixture of the corresponding composition (1 ml) was added and the resulting mixture was incubated at 20°C, and solvent was decanted and the activity against Glp-Ala-Ala-Leu-pNA was measured.

**Kinetic study of Glp-Ala-Ala-Leu-pNA hydrolysis by subtilisin and by the poly(acrylic acid)–subtilisin complex.** To 2.5 ml 0.05 M buffer of required composition and pH in a thermostatically controlled quartz cuvette (37°C), the solution of Glp-Ala-Ala-Leu-pNA (50 μl, 2.75-42.5 mM) in dimethylformamide was added. The final concentration of the substrate in the sample varied from 0.055 to 0.85 mM. Then the enzyme solution in the same buffer (20 μl, 2 μM) was added to the sample. The initial rate of the substrate hydrolysis was measured spectrophotometrically at 410 nm by *p*-nitroaniline liberation. The catalytic constant of the enzymatic reaction $k_{cat}$ and Michaelis constant $K_m$ were determined from the reaction rate dependence on the substrate concentration in Lineweaver–Burk double-reciprocal coordinates.

## RESULTS AND DISCUSSION

The complex of subtilisin 72 with the polyanion poly(acrylic acid) (PAA) was obtained under conditions described for an analogous complex with α-chymotrypsin [18]. Protein–polyelectrolyte complexes form spontaneously in aqueous solutions due to the electrostatic interactions between oppositely charged groups of protein and polyelectrolyte. According to [4], such complexes possess high stability both in aqueous solutions and in organic solvents.

The initial PAA–subtilisin ratio was 0.14 mol/mol, which is equal to 10-fold excess of negative charges of poly(acrylic acid) with respect to the number of subtilisin amino groups (in subtilisin 72 there are nine Lys residues and four Arg residues).

For estimation of the effect of complex formation on enzyme stability, the change in relative activity of PAA–subtilisin complex during its storage was studied. The activity of native enzyme measured against Z-Ala-Ala-Leu-pNA in 0.05 M Tris-HCl buffer, pH 8.2, containing 1.5 mM $CaCl_2$ at the starting point was taken as 100%. After dissolving PAA in 0.05 M Tris-HCl buffer, its pH value decreased from 8.4 to 5.2. The activity of the complex at pH 5.2 without $Ca^{2+}$ dropped very quickly (Fig. 1). When 1.5 mM $Ca^{2+}$ was added to the buffer, the stability of enzyme was increased. The complex being incubated in buffer with pH 8.1, which corresponds to optimum of native subtilisin activity, the overall tendency of activity decrease remained, but the value of the activity was higher than at pH 5.2. The stability of native subtilisin during storage in 0.05 M Tris-HCl (pH 8.2) was also studied (Fig. 1). The native enzyme activity decreased by half in 1 day, and then it remained practically unchanged. Thus, it was shown that the stability of subtilisin in aqueous buffer was equal for the native enzyme and for the enzyme in complex with PAA, and $Ca^{2+}$ was needed to maintain the activity of subtilisin.

It is known [19] that the pH optimum of enzyme activity changes after the enzyme–polyelectrolyte complex formation. Therefore, we tested the dependence of specific enzyme activity of native subtilisin and its complex with PAA on pH (Fig. 2).

As shown in the figure, for the PAA–subtilisin complex the broadening of the pH optimum and its slight shift to more basic region were observed.

Though Z-Ala-Ala-Leu-pNA binds with the active site of subtilisin 72 very effectively ($K_m$ = 25 μM [20]), measuring the kinetic parameters of the hydrolysis of this substrate using subtilisin is hindered by the low solubility of the substrate in water as well as by the need to add dimethylformamide (more than 20 vol. %), which severely decreases the catalytic activity of the enzyme. Thus, the pH-dependence of $k_{cat}$ both for the native subtilisin and for the PAA–subtilisin complex against another peptide substrate Glp-Ala-Ala-Leu-pNA having higher solubility

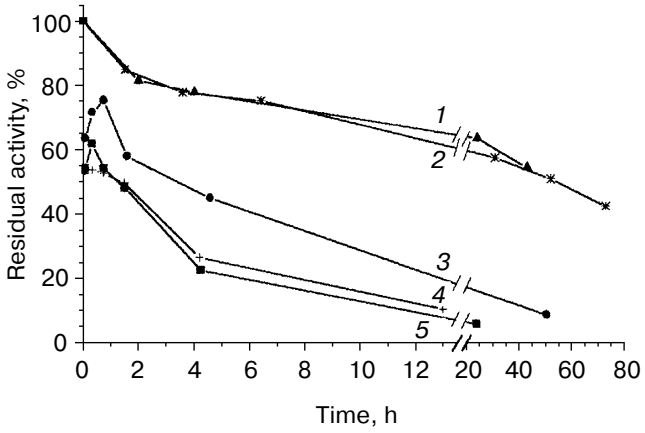


**Fig. 1.** Dependence of residual activity of subtilisin and of PAA–subtilisin complex measured against Z-Ala-Ala-Leu-pNA on storage time in 0.05 M Tris-HCl buffer: PAA–subtilisin, pH 8.1, 1.5 mM $CaCl_2$ (*1*); subtilisin, pH 8.2, 1.5 mM $CaCl_2$ (*2*); PAA–subtilisin, pH 5.2, 1.5 mM $CaCl_2$ (*3*); subtilisin, pH 5.2 (*4*); PAA–subtilisin, pH 5.2 (*5*). Activity of native enzyme in 0.05 M Tris-HCl buffer, pH 8.2, containing 1.5 mM $CaCl_2$ at the starting point was taken as 100%.

was studied. In these kinetic experiments, the dimethylformamide content in the reaction mixture was lower than 2%. The data is shown on Fig. 3.

As the figure indicates, the $k_{cat}$ values for native and modified subtilisin had no significant differences over the investigated pH range, although the maximum of the activity of the PAA–subtilisin complex shifted to the basic region by 0.3 pH compared to the native enzyme.

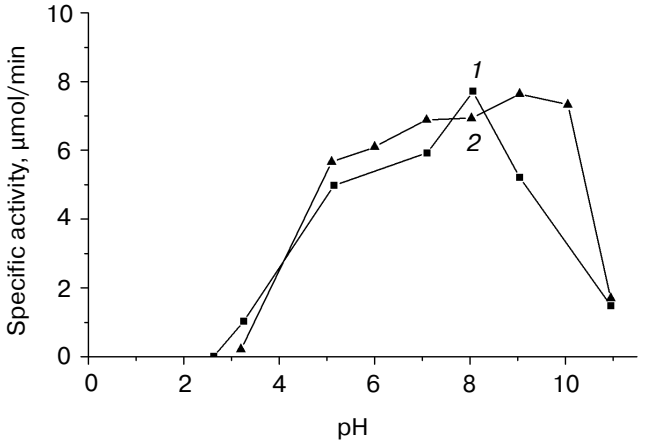


**Fig. 2.** Dependence of specific activity of native subtilisin (*1*) and PAA–subtilisin complex (*2*) on pH in buffer with 1.5 mM $CaCl_2$ containing 20% dimethylformamide, by hydrolysis of Z-Ala-Ala-Leu-pNA.

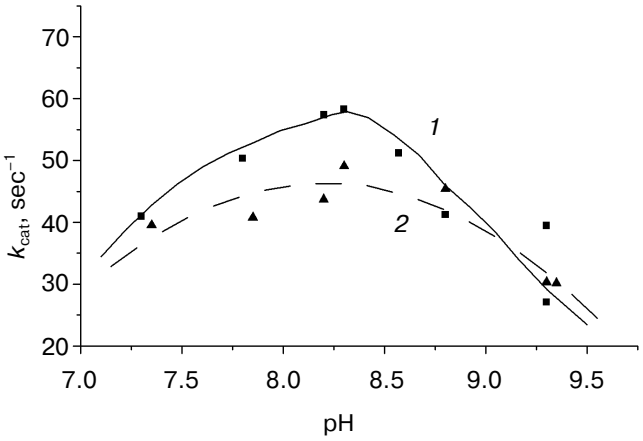


**Fig. 3.** Dependence of $k_{cat}$ of subtilisin (*1*) and PAA–subtilisin complex (*2*) on pH measured against Glp-Ala-Ala-Leu-pNA in buffer with 1.5 mM $CaCl_2$ containing 2% dimethylformamide.

The kinetic constants for native subtilisin and the PAA–subtilisin complex were obtained for the hydrolysis reaction of Glp-Ala-Ala-Leu-pNA in 0.05 M Tris-HCl buffer, pH 8.2, containing 1.5 mM $CaCl_2$ (table).

Comparison of kinetic constants of the peptide substrate hydrolysis by subtilisin and PAA–subtilisin complex under the same conditions did not reveal significant difference between the native and modified subtilisin. Thus the complex formation of subtilisin with poly(acrylic acid) had almost no influence on its kinetic characteristics in aqueous solution and probably had no effect on enzyme structure, a fact corresponding with data on other protein–polyelectrolyte complexes [4].

Covalent immobilization on different supports is an effective approach to stabilize enzymes in polar organic solvent mixtures with low water content. For investigation of this approach we tested subtilisin covalently immobilized on poly(vinyl alcohol) cryogel [21].

Poly(vinyl alcohol) cryogels are hydrophilic gel matrixes with heterogeneous macroporous structure. The characteristic feature of these supports is their ability to keep water inside the gel matrix even if it was placed into nonaqueous organic solvents [22]. The enzymes immobilized on cryogels are able to contact both organic solvent, containing the substrate, and hydrophilic support matrix, which is functioning as a reservoir, providing water for the maintenance of the enzyme efficiency. For the preparation of biocatalyst portions, three cryogel reactive derivatives were used—aldehyde- (cryoPVAG-A), epoxy- (cryoPVAG-E) [11], and vinylsulfone-containing (cryoPVAG-VS).

Kinetic constants of hydrolysis of Glp-Ala-Ala-Leu-pNA by native subtilisin and subtilisin–poly(acrylic acid) complex

| Enzyme | $K_m$, mM | $k_{cat}$, $sec^{-1}$ | $k_{cat}/K_m$, $M^{-1}\cdot sec^{-1}$ |
|---|---|---|---|
| Subtilisin | 0.25 ± 0.01 | 69.3 ± 3.5 | 277 240 |
| PAA–subtilisin | 0.18 ± 0.01 | 51 ± 2.6 | 282 099 |

The amount of the protein on the support was estimated using amino acid analysis data after the acid hydrolysis of the immobilized biocatalyst specimen. It should be noted that an estimation of amount of protein $NH_2$-groups contacting the support could be given in specimens prepared using divinylsulfone. It was found that about six-to-seven of nine Lys residues of the subtilisin molecule participate in immobilization. Besides, a pH stable C–N σ-bond is formed by reaction with vinylsulfonic anchor groups of the support.

Subtilisin content was 0.1-13.7 mg protein per 1 g of the support. The dependence of the subtilisin specific activity on protein content in the support is shown in Fig. 4. Hydrolytic activity calculated per 1 mg protein exponentially decreased in as protein content increased. The dependence of activity normalized per 1 g of the support was more complicated. First, the activity value sharply increased, then it reached a maximum, and then it gradually decreased. This means that for the conditions of immobilization used here the optimal protein content is 2-4 mg of enzyme per 1 g of the support. Further increase of the protein content on the support not only failed to increase, but actually diminished the total biocatalyst activity. It should be noted that an analogous dependence of the specimen activity on the content for subtilisin BPN′ immobilized on polymeric polyester matrix covered by poly(acrolein), was obtained [23].

The immobilization of subtilisin was accompanied by a decrease in its hydrolytic activity by 4-10 times. One should note that the addition of competing inhibitors of serine proteases (e.g., Bzl-Tyr-$NH_2$) to protect the active site of subtilisin on immobilization did not bring about considerable activity increase of the resulting samples [11]. Therefore, it seems that the most probable cause of decrease in the subtilisin activity is not the structural distortion of its active site, but difficulty of substrate access to the enzyme active site. Such diffusion limitations could be caused by formation of multipoint contacts in aqueous medium, both due to H-bonds between OH-groups of the matrix and protein molecule and as a result of hydrophobic interactions between poly(methylene) chain of the polymer and hydrophobic amino acid residues of subtilisin.

The stability of samples of immobilized subtilisin was rather high. After 6-12 months of storage the enzyme possessed more than 50% of its initial activity (Fig. 5), whereas native subtilisin lost 60% of its activity in 3 days under the same conditions.

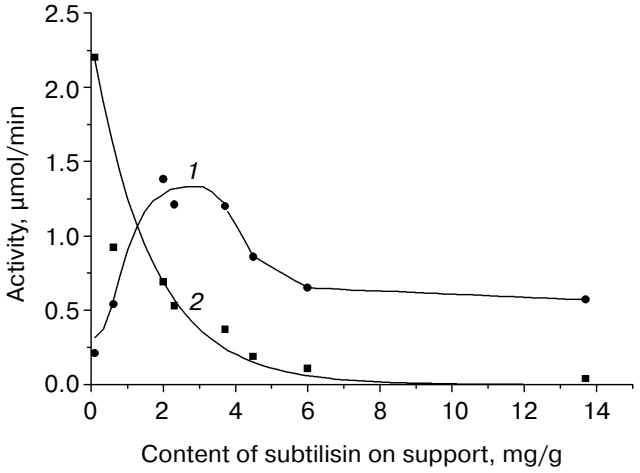


**Fig. 4.** Dependence of specific activity of subtilisin immobilized on cryoPVAG-A on the enzyme content on the support: activity per 1 g of the support (*1*); activity per 1 mg enzyme (*2*). The activity was determined by hydrolysis of Glp-Ala-Ala-Leu-pNA.

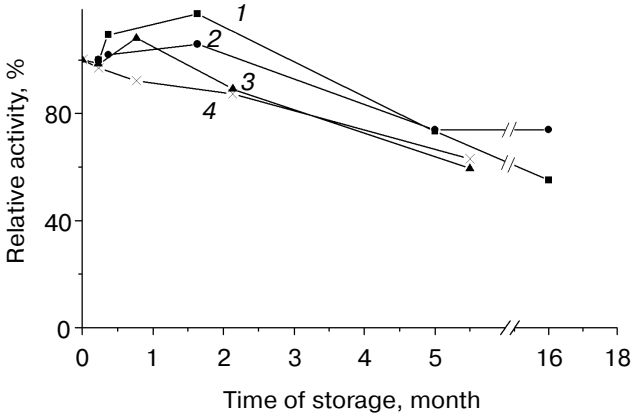


**Fig. 5.** Dependence of specific activity of portions of immobilized subtilisin on storage time in 0.05 M Tris-HCl buffer, pH 8.2, containing 1.5 mM $CaCl_2$ at 4°C: subtilisin–cryoPVAG-A + Ac-Trp-OH (*1*), subtilisin–cryoPVAG-A + Bzl-Tyr-$NH_2$ (*2*), subtilisin–cryoPVAG-A (*3*), subtilisin–cryoPVAG-E (*4*). Activity of the specimen right after immobilization was taken as 100%. The activity was determined based on hydrolysis of Glp-Ala-Ala-Leu-pNA.

The enzyme immobilized in the presence of Bzl-Tyr-$NH_2$ was the most stable of all the studied samples (loss of activity was just ~30% in 16 months of storage at 4°C in 0.05 M Tris-HCl buffer, pH 8.2, containing 1.5 mM $CaCl_2$). Thus, the immobilization on insoluble support greatly augmented the enzyme stability on storage in a buffer solution.

In the next stage, the behavior of native and modified subtilisin in organic solvents mixtures with low water content was studied. Investigations were carried out in ternary acetonitrile–dimethylformamide–water mixtures of various compositions. The dimethylformamide content varied from 30 to 95%, the water content being 5% in most cases.

Native enzyme as an aqueous solution in Tris-HCl buffer, pH 8.2, containing 1.5 mM $CaCl_2$ was added to acetonitrile–dimethylformamide mixture with stirring. It is known that the most enzymes, including subtilisin, do not dissolve in media with high content of organic solvents, forming a suspension. Besides, direct suspension of lyophilized powdery powdered enzymes in organic solvents can lead to inaccessibility of the enzyme for substrate interactions and to diffusion limitations [24]. In the studied acetonitrile–dimethylformamide–water system subtilisin also formed a suspension.

The enzyme activity was estimated by following the hydrolysis of the peptide substrate Z-Ala-Ala-Leu-pNA after transferring an aliquot of the enzyme suspension into aqueous buffer solution. The subtilisin activity in 0.05 M Tris-HCl buffer (pH 8.2) was taken as 100%. Subtilisin retained high activity for 24 h in acetonitrile–dimethylformamide–water mixtures (5%) containing up to 70 vol. % dimethylformamide (Fig. 6). Under higher concentrations of dimethylformamide the relative enzyme activity decreased more quickly, but nevertheless, the enzyme was not fully inactivated, retaining some residual activity after 1 day. It is possible to note the following regions of dimethylformamide concentrations that are greatly differ-

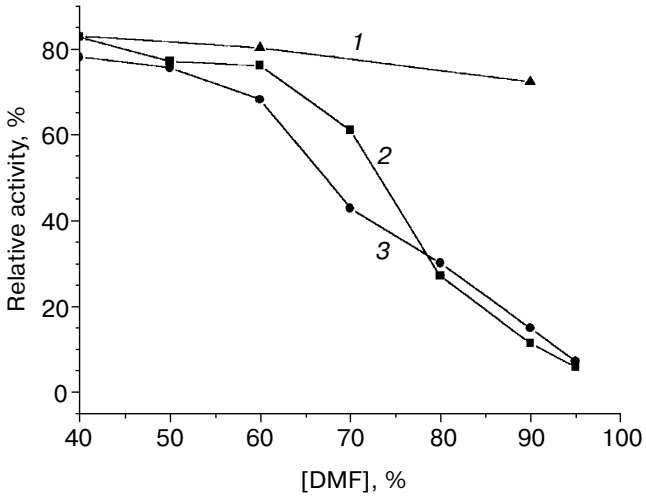


**Fig. 6.** Effect of dimethylformamide (DMF) content on the relative activity of subtilisin after 24 h incubation in acetonitrile–dimethylformamide–water (5%) mixtures in 0.05 M Tris-HCl buffer, pH 8.2, with 1.5 mM $CaCl_2$: subtilisin–cryoPVAG-A (*1*), subtilisin (*2*), and PAA–subtilisin (*3*). The activity for native and PAA–subtilisin was determined by hydrolysis of Z-Ala-Ala-Leu-pNA; for subtilisin–cryoPVAG-A it was determined by hydrolysis of Glp-Ala-Ala-Leu-pNA. Activity of enzyme portions in 0.05 M Tris-HCl buffer (pH 8.2) containing 1.5 mM $CaCl_2$ was taken as 100%.

ent in activity from each other: *1*) 30-70%; *2*) 80%; *3*) 90-95% (Fig. 6). The data show that 70% concentration of dimethylformamide corresponds to a threshold value above which the enzyme is inactivated quite fast.

When aqueous solution of PAA–subtilisin complex was added to the organic solvents mixture as in the case of native enzyme, formation of a finely dispersed precipitate was observed. In acetonitrile–dimethylformamide mixture the activity of the complex depending on the dimethylformamide concentration was determined (40-95%) (Fig. 6). However, the PAA–subtilisin complex remained highly stable. With the increase in dimethylformamide concentration in the mixture, the enzyme activity decreased, but total loss of activity was not observed even after 25 h long incubation in mixture containing 95% dimethylformamide and 5% water. Comparison of the data shows that PAA–subtilisin complex under described conditions is comparable with native enzyme, and stabilization of subtilisin complexed with PAA with respect to the inactivating influence of dimethylformamide was not observed.

The stability of subtilisin samples immobilized in cryogel was estimated as the change in the enzyme activity measured against the peptide substrate Glp-Ala-Ala-Leu-pNA in aqueous buffer after keeping the enzyme in acetonitrile–dimethylformamide mixtures of various compositions. Activity of the samples right after the immobilization was taken as 100%. The data on activity of immobilized subtilisin after 24 h in media containing various quantities of dimethylformamide is shown in Fig. 6. The curve of the dependence of the immobilized enzyme activity lies above those for the native and PAA–subtilisin, which means a greater stability to the inactivating effect of dimethylformamide.

Thus, the native and modified subtilisins are highly stable in polar organic solvent media with low water content; the activity of native subtilisin and PAA–subtilisin complex in acetonitrile–dimethylformamide–water (5%) mixtures containing up to 60% dimethylformamide was found to be higher than in aqueous buffer.

The data show that the covalent immobilization of enzyme on poly(vinyl alcohol) cryogel is the most effective approach for increasing subtilisin stability both in aqueous media and in organic solvents. Subtilisin immobilized on poly(vinyl alcohol) cryogel proved to be the most stable in all the studied mixtures, the stabilization effect being the most remarkable in aqueous buffer and in acetonitrile–dimethylformamide mixtures containing more than 60% dimethylformamide. In addition, it should be noted that the immobilized form of the enzyme was found to be the most effective biocatalyst in syntheses of organic-soluble peptides [11, 25].

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