*Russian Journal of Bioorganic Chemistry, Vol. 29, No. 5, 2003, pp. 502–508. Translated from Bioorganicheskaya Khimiya, Vol. 29, No. 5, 2003, pp. 551–558.*


# Native and Modified Subtilisin 72 as a Catalyst for Peptide Synthesis in Media with a Low Water Content

**A. V. Bacheva*, O. V. Baibak**, A. V. Belyaeva*, E. N. Lysogorskaya*, E. S. Oksenoit*, V. I. Lozinskii**, and I. Yu. Filippova**[1]**

**Faculty of Chemistry, Moscow State University, Vorob'evy gory, Moscow, 119992 Russia*

***Nesmeyanov Institute of Organoelement Compounds, Russian Academy of Sciences, ul. Vavilova 28, Moscow, 117813 Russia*

Received October 31, 2002; in final form, December 6, 2002

**Abstract**—The catalytic efficiencies of native subtilisin, its noncovalent complex with polyacrylic acid, and the subtilisin covalently immobilized in a cryogel of polyvinyl alcohol were studied in the reaction of peptide coupling in mixtures of organic solvents with a low water content in dependence on the medium composition, reaction time, and biocatalyst concentration. It was established that, in media with a DMF content >80%, the synthase activity of modified subtilisins is higher than that of the native subtilisin. The use of *N*-acylpeptides with a free carboxyl group was found to be possible in organic solvents during the enzymatic synthesis catalyzed by both native and immobilized subtilisin. A series of tetrapeptide *p*-nitroanilides of the general formula Z-Ala-Ala-Xaa-Yaa-pNA (where Xaa is Leu, Lys, or Glu and Yaa is Phe or Asp) was obtained in the presence of immobilized enzyme in yields of 70–98% in DMF–MeCN without any activation of the carboxyl component and without protection of side ionogenic groups of polyfunctional amino acids.

Key words: *enzymatic peptide synthesis in organic solvents, immobilized subtilisin, subtilisin–polyacrylic acid complex, subtilisin–polyvinyl alcohol cryogel complex*

## INTRODUCTION

The use of organic solvents as a reaction medium for studying the synthase activity of proteases first of all allows the researcher to shift the thermodynamic equilibrium of the reaction toward synthesis, to minimize the side reaction of hydrolysis of the resulting peptide product, and to widen the range of substrates used [1].[2] Methodical studies of the biocatalytic ability of proteases as synthases could reveal their properties that are difficult to find in a context of the traditional ideas on these hydrolyzing enzymes.

The stability and activity of these proteases have more often been investigated by the examples of the reactions of hydrolysis of specific peptide substrates [2–4]. Synthase properties of these proteases in the media with a high content of organic solvents are little studied, and the information about the effect of various methods of their modification on their catalytic efficiency in the reactions of peptide synthesis is rather limited [5–8].

The goal of this study is the investigation of the influence of various methods of modification of subtilisin 72 on its ability to catalyze peptide synthesis in organic media of various compositions.

We compared the synthase activity of three preparations, the native subtilisin, PAA–subtilisin, and cryoPVA–subtilisin, in the media with a low water content. We used this approach as an example of noncovalent modification, because complexation with polyions is known to protect enzymes from inactivation in organic solvents [9]. We used the immobilization on an inert support as the known method of the enzyme stabilization against the unfavorable interactions with environment. The problem of difficult diffusion of substrates and products was solved by the application of the cryoPVA macroporous carrier for the immobilization. This carrier is prepared by freezing, keeping in the frozen state, and subsequent thawing of the concentrated aqueous solutions of polyvinyl alcohol [10].

## RESULTS AND DISCUSSION

Subtilisin 72 (EC 3.4.21.14) is an intracellular basic serine protease [11] produced by the *Bacillus subtilis* strain 72. This protease catalyzes various reactions of peptide bond formation and esterification [12–15]. This enzyme is accessible, can be easily isolated and purified, is stable toward unfavorable interactions, and has a wide substrate specificity.

[1] Corresponding author; phone: +7 (095) 939-5529; e-mail: irfil-ipp@genebee.msu.su

[2] Abbreviations: Abz, *o*-aminobenzoic acid; cryoPVA, cryogel of polyvinyl alcohol; subtilisin–cryoPVA, subtilisin immobilized in the polyvinyl alcohol cryogel; DeD, 2,4 dinitrophenylethylenediamine; PAA–subtilisin, complex of subtilisin with polyanionic polyacrylic acid; and pNA, *p*-nitroanilide. All the used amino acids belong to the *L*-series.

1068-1620/03/2905-0502$25.00 © 2003 MAIK "Nauka / Interperiodica"

The ability of the native and modified subtilisin to catalyze the peptide bond formation in triple acetonitrile–DMF–water systems of variable compositions was studied in the reaction:

$$\begin{array}{c} \text{Z-Ala-Ala-Leu-OH} \\ {\scriptstyle H_2O}\uparrow\downarrow \\ \text{Z-Ala-Ala-Leu-OCH}_3 + \text{E} \underset{CH_3OH}{\rightleftharpoons} \text{[Z-Ala-Ala-Leu-O-E]} \overset{\text{Phe-pNA}}{\rightleftharpoons} \text{Z-Ala-Ala-Leu-Phe-pNA.} \end{array} \quad (1)$$

This reaction was chosen, because the structures of starting components correspond to the subtilisin specificity. Previously, the catalysis of reaction (1) was comprehensively studied for the subtilisin Carlsberg in aqueous organic media [16] and for the suspension of subtilisin 72 in anhydrous organic solvents [17–19]. We used an equimolar ratio (30 mM) of the amino and the carboxyl components, and the molar [S]/[E] ratio of approximately 1000 : 1. In all the studied solvent systems, the reaction product was in solution and the enzyme was introduced into the reaction mixture as a suspension, which facilitated the separation of target product from the enzyme. The curves of dependence of the product yield on the reaction time are given in Fig. 1.

The yield of the product achieved its maximum value rather fast (approximately for 2–3 h) and then was practically unchanged when the reaction was carried out in 60% DMF and catalyzed by a suspension of native subtilisin (Fig. 1, curve *1*). Comparison of the curves of the product accumulation for the native and the modified subtilisin demonstrated that the maximal differences were observed in the first 2–5 h of the reaction. The reaction proceeded the most slowly in the case of PAA-subtilisin (Fig. 1, curve *3*). The maximal yield was achieved in all the cases after a day.

The rate of the tetrapeptide accumulation in the reaction mixture significantly decreased with the increase in the DMF content to 95%, but, within the first 2–3 h, we did not observe any significant differences in the yields of the reaction product under the catalysis by the three enzymatic preparations. The yield of Z-Ala-Ala-Leu-Phe-pNA in the reaction catalyzed by the native subtilisin decreased approximately threefold in comparison with the medium containing 60% DMF, and the product accumulation was even stopped after 3–5 h (curve *6*). The synthesis catalyzed by PAA-subtilisin did not stop after 3 h; it proceeded for at least 3 days. Cryo-PVA-subtilisin exhibited the highest operating stability; the synthesis did not stop even after 48 h (curve *4*) and the yield of the product was 74% after 3 days.

We analyzed the curves of dependence of the yield of Z-Ala-Ala-Leu-Phe-pNA on the DMF content in the reaction mixture (Fig. 2) and distinguished two ranges of the DMF concentrations (30–80% and 90–95%) between which the yields of the product were significantly reduced. The 80% DMF content was a threshold value for the synthase activity of both the native and the modified subtilisins.

Consequently, the unmodified enzyme quickly loses its activity in media with a high DMF content, while its modification (both covalent and noncovalent) preserves subtilisin from inactivation. Note that the stabilizing effect of covalent modification toward the denaturing action of DMF is higher than that of the complexation with PAA.

We compared the effects of various methods of the subtilisin immobilization on its synthase activity. Subtilisin was immobilized on cryoPVA by various methods: the treatment with glutaraldehyde (cryoPVA-A), epichlorhydrin (cryoPVA-E) [15], and with divinylsulfone (cryoPVA-VS). In addition, the enzyme was immobilized on the cryoPVA-A both in the presence of the reversible inhibitors of serine proteases Ac-Tyr-OH and Bzl-Tyr-$NH_2$ (subtilisin–cryoPVA-A + Ac-Trp-OH and subtilisin–cryoPVA-A + Bzl-Tyr-$NH_2$) and without them. The catalytic efficiency of various preparations of immobilized subtilisin was tested in the model reaction (1) in the mixture of 60% DMF and 40% acetonitrile (Fig. 3).

The samples of the catalyst prepared by the subtilisin immobilization on cryo-PVA-A exhibited the highest and practically equal synthesizing activity, and the most rapid accumulation of product was observed in the reaction catalyzed by the subtilisin immobilized on cryoPVA-A in the presence of Bzl-Tyr-$NH_2$ (the yield of Z-Ala-Ala-Leu-Phe-pNA was 66% for the 10-min reaction). The subtilisin immobilized on cryoPVA-VS was somewhat less effective in the peptide synthesis (64% yield of the product was achieved after 30 min). The synthesis catalyzed by subtilisin–cryoPVA-E still proceeded but with a lower rate, and the yield of the model peptide was 71% after 96 h, despite the fact that the enzyme immobilized on cryoPVA-E contained the lowest quantity of the enzyme and the [E]/[S] ratio was almost one order of magnitude lower ($1 : 10^4$).

The dependence of the product yield on the quantity of the subtilisin added was studied in the reaction catalyzed by subtilisin–cryoPVA-A in the mixture of 80% DMF and acetonitrile (Fig. 4). The curve of the dependence of the yield of Z-Ala-Ala-Leu-Phe-pNA on the concentration of the immobilized enzyme was somewhat lower than that of the suspended enzyme, but the possibility of the repeated use of the immobilized sub-

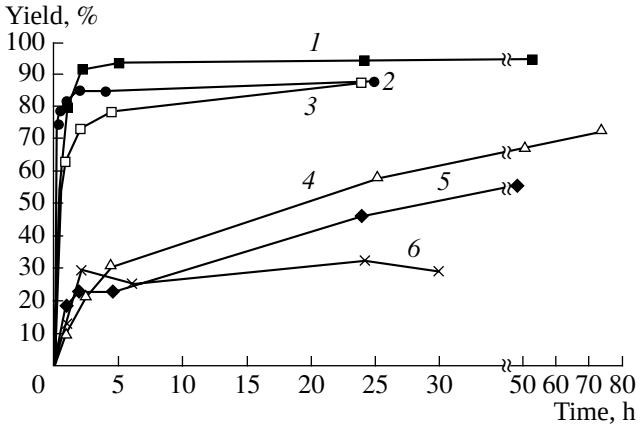


**Fig. 1.** The synthesis of Z-Ala-Ala-Leu-Phe-pNA in the MeCN–DMF mixtures with 5% $H_2O$ and various contents of DMF catalyzed with (*1*, *6*) the suspension of subtilisin, (*2*, *4*) subtilisin–cryoPVA-A, and (*3*, *5*) the suspension of PAA–subtilisin at the DMF content (*1*, *2*, *3*) 60 and (*4*, *5*, *6*) 95%.

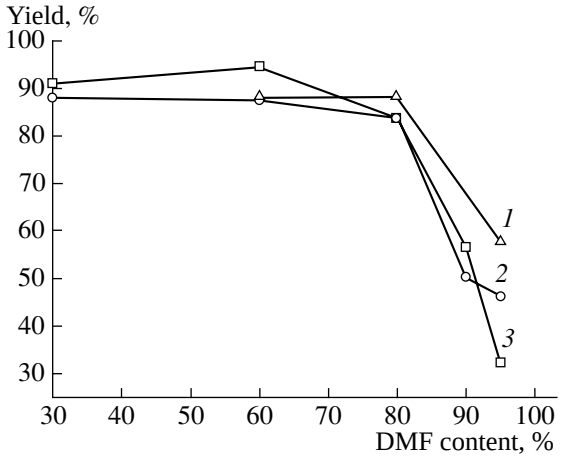


**Fig. 2.** The dependence of the Z-Ala-Ala-Leu-Phe-pNA yield in the MeCN–DMF mixtures with 5% $H_2O$ with various contents of DMF after 24-h reaction catalyzed with *1*, cryoPVA-A–subtilisin; *2*, suspension of PAA–subtilisin; and *3*, suspension of subtilisin.

tilisin after the synthesis [14, 15] and the easiness of its separation from the reaction mixture (which results in a decreased contamination of the peptide with the enzyme) gave the immobilized enzyme a significant advantage.

It is known [16] that an activation of carboxyl component in the peptide synthesis with the use of serine proteases is necessary for the achievement of good product yields for a relatively short time both in aqueous solutions and in water–organic mixtures. In this case, the reaction proceeds through the kinetically controlled pathway and the risk of the secondary hydrolysis of the product always remains. The reaction proceeds very slowly and with a low yield if the component with the free carboxyl group is used as an acyl donor.

We studied the effect of the structure of carboxyl component on the efficiency of the reaction (2) catalyzed by a suspension of native enzyme in the mixture of 60% DMF–acetonitrile–5% water and by cryoPVA-A–subtilisin in the mixture of 60% DMF–acetonitrile:

$$\text{Z-Ala-Ala-Leu-OR + Phe-pNA} \xrightarrow{\text{subtilisin}} \text{Z-Ala-Ala-Leu-Phe-pNA}, \quad (2)$$

where R = H or Me

The maximal difference in the rate of product accumulation was observed for the first 60 min. The yield of the product was 67% when the tripeptide methyl ester was used as the acyl donor and 32% when the peptide with free carboxyl group was used in the reaction catalyzed by the native subtilisin. The synthesis (2) proceeded similarly with the immobilized subtilisin. The yields were 60% (for Z-Ala-Ala-Leu-OMe) and 24% (for Z-Ala-Ala-Leu-OH) after the 30-min reaction. The difference in yields decreased to 10–15% after 2 h and disappeared after 3 h when the yield achieved 95% irrespective of the structure of acylating agent (Fig. 5).

Thus, we found that not only the Z-Ala-Ala-Leu tripeptide methyl ester but this tripeptide itself can be an effective acylating agent in the synthesis catalyzed by the unmodified subtilisin and by the immobilized subtilisin in organic medium. Such course of the reaction is probably associated with a low water content in the system and with a shift of equilibrium toward the formation of products. In addition, carboxyl group of

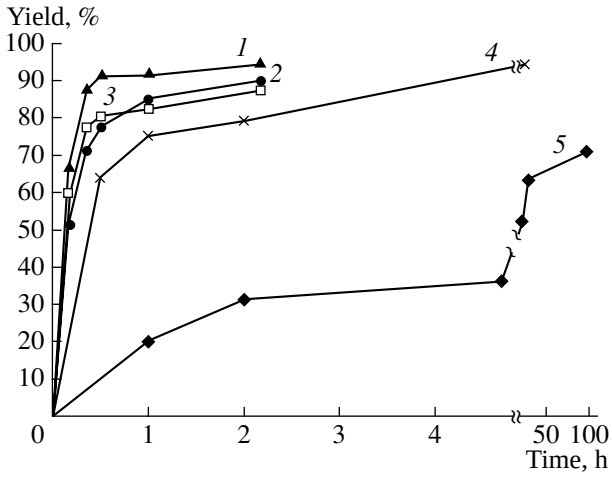


**Fig. 3.** The dependence of the Z-Ala-Ala-Leu-Phe-pNA yield on time for various samples of immobilized subtilisin: (*1*, subtilisin–cryoPVA-A + Bzl-Tyr-$NH_2$, [S]/[E] 1800 : 1; *2*, subtilisin–cryoPVA-A + Ac-Trp-OH, [S]/[E] 2000 : 1; *3*, subtilisin–cryoPVA-A, [S]/[E] 1100 : 1; *4*, subtilisin–cryoPVA-B, [S]/[E] 700 : 1; and *5*, subtilisin–cryoPVA-E, [S]/[E] 9000 : 1.

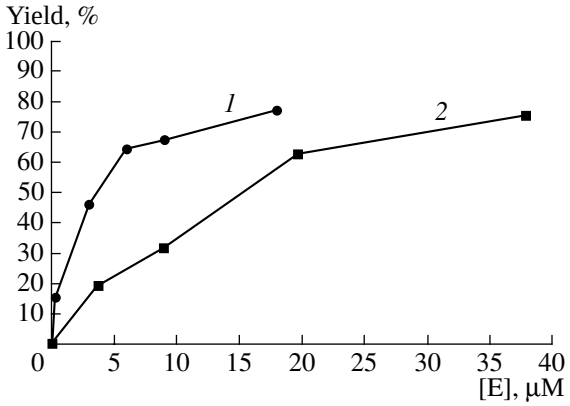


**Fig. 4.** The dependence of the Z-Ala-Ala-Leu-Phe-pNA yield for 2 h on the concentration of *1*, suspended subtilisin and *2*, immobilized cryoPVA-A–subtilisin.

the acylating component is not dissociated in our case and can effectively interact with the enzyme active site.

The greatest difficulty in the chemical peptide synthesis is the obtaining of peptides with polyfunctional amino acid residues due to the necessity of protection of trifunctional amino acids. In this case, the application of enzymes as catalysts decreases not only the number of stages but also the risk of racemization at each protection–deprotection step. We have previously demonstrated that the hydrophobic ion complex of subtilisin with SDS was able to catalyze the formation of Arg–Arg, Glu–Arg, and Lys–Glu bonds in the mixture of 30% DMSO and ethanol [19]. In this study, we found that the efficiency of synthesis of *N*-acylated tetrapeptides containing basic and acidic amino acid residues in $P_1$ and $P_1'$ positions can significantly increase by the application of subtilisin–cryoPVA-A in organic media with a low water content:

$$\text{Z-Ala-Ala-Xaa-OH + Yaa-pNA} \xrightarrow{\text{subtilisin–cryoPVA-A}} \text{Z-Ala-Ala-Xaa-Yaa-pNA}, \quad (3)$$

where Xaa = Leu, Lys, or Glu and Yaa = Phe or Asp

The reactions were carried out without any activation of carboxyl component and protection of the side functions of trifunctional amino acids (Fig. 6).

The *p*-nitroanilides of protected tripeptides were prepared in high yields, with only a single product being observed in all the reactions. The homogeneity of the synthesized peptides was confirmed by HPLC and amino acid analysis.

The successful use of Z-Ala-Ala-Lys-OH and Z-Ala-Ala-Glu-OH as acyl donors and Asp-pNA as an amino component in the mixtures of organic solvents significantly expands our ideas on the synthase potential of subtilisin immobilized on the PVA cryogel. We demonstrated the possibility of formation of the bond between the oppositely charged Lys and Asp amino acid residues (Fig. 6, curve *1*) and between the dicarboxylic amino acid residues Glu and Asp (Fig. 6, curve *3*), although the hydrophobic amino acid residues are known to be preferable for subtilisin in position $P_1$ [12, 20]. Such non-standard behaviour of the immobilized subtilisin in the synthesis could probably be explained by the fact that the side ionic groups of the amino acid residues are not charged in organic solvents. Moreover, one cannot exclude the influence of the cryoPVA matrix and the water attached to it on the distribution of the starting substances and the reaction products in the reaction mixture.

The preparation of substrates for proteases of various classes is one of the areas of practical application of the enzymatic peptide synthesis. We studied the synthe-

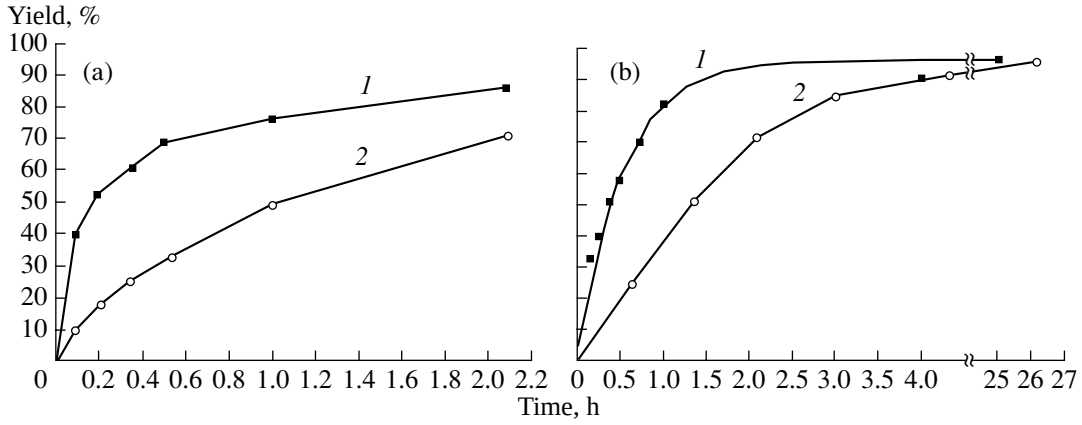


**Fig. 5.** The synthesis of Z-Ala-Ala-Leu-Phe-pNA in DMF–MeCN–$H_2O$ mixture with 60% DMF catalyzed by (a) subtilisin suspension and (b) immobilized cryoPVA-A–subtilisin at the use of *1*, Z-Ala-Ala-Leu-OMe and *2*, Z-Ala-Ala-Leu-OH carboxylic components; [S] = 30 mM.

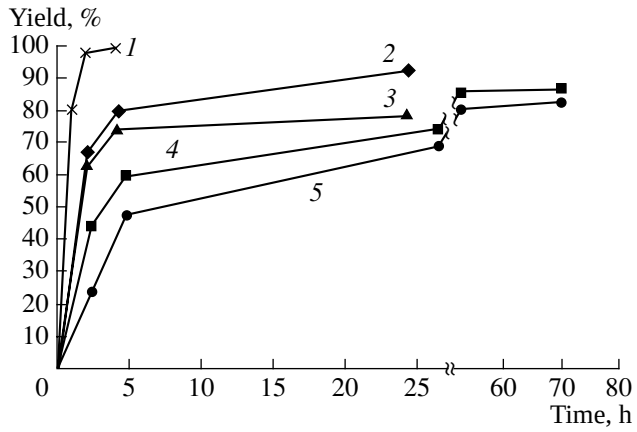


**Fig. 6.** The catalysis by cryoPVA-A–subtilisin of the synthesis in the 6 : 4 DMF–MeCN of the Z-Ala-Ala-Xaa-Yaa-pNA tetrapeptides where *1*, Xaa = Lys and Yaa = Asp; *2*, Xaa = Leu and Yaa = Asp; *3*, Xaa = Glu and Yaa = Asp; *4*, Xaa = Glu and Yaa = Phe; and *5*, Xaa = Lys and Yaa = Phe; [S]/[E] = 800 : 1; [S] = 30 mM.

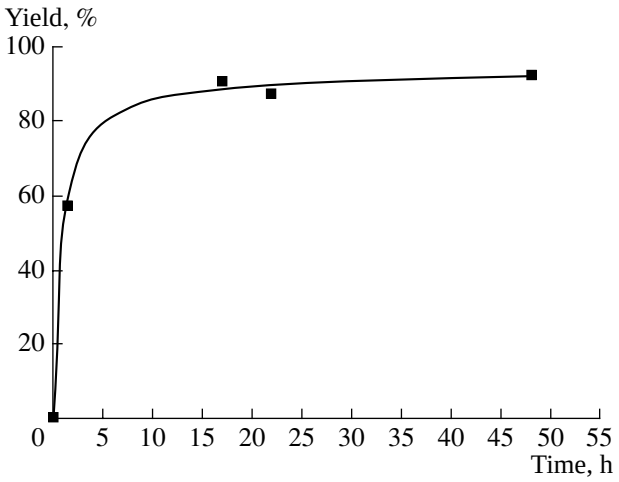


**Fig. 7.** The dependence of the Abz-Ala-Ala-Phe-Phe-Ala-Ala-DeD yield on time in the 6 : 4 DMF–MeCN system at the cryoPVA-A–subtilisin catalysis; [S]/[E] = 1000 : 1; [S] = 30 mM.

sis of a fluorogenic substrate for the aspartyl proteases catalyzed by subtilisin immobilized on cryoPVA-A:

$$\text{Abz-Ala-Ala-Phe-OMe + Phe-Ala-Ala-DeD} \xrightarrow{\text{subtilisin–cryoPVA-A}} \text{Abz-Ala-Ala-Phe-Phe-Ala-Ala-DeD} + \text{MeOH}. \quad (4)$$

The reaction was carried out in 6 : 4 (vol/vol) DMF–acetonitrile mixture at the equimolar ratio of the amino and carboxyl components and the [E]/[S] ratio of 1 : 1000. The Abz residue (fluorophore) is situated in $P_4$ position of carboxyl component. Abz is a bulky hydrophobic substituent that meets the subtilisin specificity requirements in this position. The amino component contained a DeD residue (fluorescence quencher) on its *C*-end. The curve of product accumulation is given in Fig. 7.

A considerable rate of the product accumulation (90% yield for 17 h) and the absence of the side reaction of secondary hydrolysis are the prerequisites for the preparative synthesis of this practically important substrate.

We demonstrated the possibilities of the enzymatic synthesis in mixtures of organic solvents by the example of preparative synthesis of Z-Ala-Ala-Leu-Phe-pNA catalyzed by the suspension of native subtilisin in a 6 : 13 : 1 DMF–MeCN– $H_2O$ mixture at equimolar ratio of amino and carboxylic components (30 mM) and the [S]/[E] ratio of 5000 : 1. After a day, the enzyme was separated by centrifugation and the yield of the target product was 73%. In addition, a preparative synthesis of Z-Ala-Ala-Lys-Asp-pNA was carried out in the 6 : 4 DMF–MeCN mixture at the equimolar ratio of amino and carboxylic components at the [S]/[E] ratio of 1000 : 1 under the catalysis by subtilisin–cryoPVA-A. According to HPLC, the product content in the reaction mixture was approximately 100% after a day, and no additional purification was necessary. The preparative yield was 93%.

Thus, we showed that the native subtilisin 72 in the form of suspension, PAA–subtilisin, and subtilisin–cryoPVA can catalyze the reactions of peptide bond formation in mixtures of organic solvents with a low water content. The immobilized enzyme has a number of advantages in comparison with the native and the PAA–subtilisin. The stability under storage, the possibility of multiple use, and the ability to form bonds between the peptide derivatives and the amino acids containing ionogenic groups without any protection of side chain functions make the immobilized subtilisin a prospective biocatalyst for the creation of peptide bonds in media with a high content of organic solvents.

## EXPERIMENTAL

In this work, we used the serine protease from *Bacillus subtilis* strain 72 isolated from the commercial preparation of cultural fluid and purified according to the procedure [20]; acetonitrile for HPLC of the special purity grade (Lekbiofarm, Russia) containing no more that 0.01% water; and DMF and TEA of the analytical purity grade (Reakhim, Russia) additionally purified according to the procedure [21]. Derivatives of amino acids and peptides were synthesized in our laboratory by the standard procedures [22]. All the other reagents were of the analytical purity grade.

Peptides were analyzed on a liquid chromatograph Altex Model 110A (United States) under the following conditions: (1) column Microsorb-MV $C_8$ (4.6 × 250 mm, Rainin Instrument Company, Inc., United States) at the elution by linear gradients of acetonitrile

(A) 20–100% for 35 min and (B) 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 and (2) column Nucleosil $C_{18}$ (4.6 × 250 mm, Biokhimmak, Russia) at the elution by linear gradients of acetonitrile (C) 20–80% for 35 min and (D) 10–80% for 42 min in 0.1% TFA at the rate of 1 ml/min. When calculating the compositions of reaction mixtures, no correction for the difference in the molar absorption coefficients of components was applied.

The amino acid analysis was performed on an automatic amino acid analyzer Hitachi-835 (Japan) after the hydrolysis by 5.7 M HCl at 105°C in evacuated ampoules for 24 and 48 h.

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

**Subtilisin immobilized on cryoPVA.** The preparation of reactive aldehyde-, epoxy-, and divinylsulfone-containing derivatives of cryoPVA and the covalent attachment of enzymes to them were carried out by the procedure [15]. The quantity of immobilized protein was estimated using amino acid analysis.

### *Syntheses under the Catalysis by Native Subtilisin Suspension*

**Coupling of Z-Ala-Ala-Leu-OMe with Phe-pNA.** A solution of subtilisin in 0.05 M Tris-HCl buffer (pH 7.8) containing 1.5 mM $CaCl_2$ (5 mg/ml, 20 μl) was added to a mixture of 200 mM solution of Z-Ala-Ala-Leu-OMe in DMF (60 μl), 200 mM solution of Phe-pNA in DMF (60 μl), and MeCN (260 μl). The reaction mixture was stirred at 20°C when occasionally taking samples (10 μl) for HPLC. The retention time of Z-Ala-Ala-Leu-Phe-pNA is 30 min (gradient A).

**Coupling of Z-Ala-Ala-Leu-OH with Phe-pNA** was carried out similarly.

**Preparative synthesis of Z-Ala-Ala-Leu-Phe-pNA.** A solution of subtilisin in 0.05 M Tris-HCl buffer (pH 7.8) containing 1.5 mM $CaCl_2$ (5 mg/ml, 100 μl) was added to a solution of Z-Ala-Ala-Leu-OMe (25.3 mg, 60 μmol) and Phe-pNA (17.1 mg, 60 μmol) in a mixture of DMF (1.6 ml) and MeCN (0.3 ml). The reaction mixture was stirred on a magnetic stirrer for 24 h at 20°C, the insoluble protein was removed by centrifugation for 10 min at 16000 *g*, and the supernatant was added dropwise at rigorous stirring to 0.5 M HCl (12 ml). The resulting precipitate was separated by centrifugation, dissolved in DMF (0.4 ml), and the procedure of precipitation with 0.5 M HCl was repeated. The resulting precipitate was centrifuged, washed with water, and dried in a vacuum over NaOH. The product yield was 30 mg (73%); amino acid composition (nmol): Ala (10.4), Leu (5.4), and Phe (5.2); *RT* (A) 30 min.

**The synthesis of Z-Ala-Ala-Leu-Phe-pNA catalyzed by the suspension of PAA–subtilisin** was carried out similarly to the procedure used for native enzyme, using 20 μl of a solution of PAA–subtilisin complex in 0.05 M Tris-HCl buffer containing 1.5 mM $CaCl_2$.

### *Syntheses under the Catalysis by Immobilized Subtilisin*

**Coupling of Z-Ala-Ala-Leu-OMe with Phe-pNA.** MeCN (160 μl), DMF (120 μl), a 200 mM solution of Z-Ala-Ala-Leu-OMe in DMF (60 μl), and 200 mM solution of Phe-pNA in DMF (60 μl) were added to a preparation of immobilized subtilisin (80 mg, protein content 0.3 mg) preliminarily washed with MeCN (1 × 1 ml) and a MeCN–DMF mixture of the corresponding composition (2 × 1 ml). The reaction mixture was shaken on an orbital shaker at 20°C, with an occasional taking off 5-μl samples for HPLC; Z-Ala-Ala-Leu-Phe-pNA *RT* (B) 25.5 min; amino acid composition (nmol): Ala 10.4, Leu 5.5, and Phe 5.2.

**Coupling of Z-Ala-Ala-Leu-OH with Phe-pNA** was carried out similarly.

**Z-Ala-Ala-Lys-Phe-pNA, Z-Ala-Ala-Glu-Phe-pNA, Z-Ala-Ala-Lys-Asp-pNA, Z-Ala-Ala-Glu-Asp-pNA, Z-Ala-Ala-Leu-Asp-pNA, and Abz-Ala-Ala-Phe-Phe-Ala-Ala-DeD** were obtained similarly.

**Z-Ala-Ala-Lys-Phe-pNA,** *RT* (C) 25.2 min; amino acid composition (nmol): Ala 7.8, Lys 3.8, and Phe 3.9.

**Z-Ala-Ala-Glu-Phe-pNA,** *RT* (C) 27.0 min; amino acid composition (nmol): Ala 8.3, Glu 4.0, and Phe 4.1.

**Z-Ala-Ala-Leu-Asp-pNA,** *RT* (C) 26.0 min; amino acid composition (nmol): Ala 9.6, Leu 4.9, and Asp 4.7.

**Z-Ala-Ala-Lys-Asp-pNA,** *RT* (D) 24.0 min; amino acid composition (nmol): Ala 6.4, Lys 3.1, and Asp 3.3.

**Z-Ala-Ala-Glu-Asp-pNA,** *RT* (D) 26.0 min; amino acid composition (nmol): Ala 8.8, Glu 4.1, and Asp 4.5.

**Abz-Ala-Ala-Phe-Phe-Ala-Ala-DeD,** *RT* (C) 19.7 min; amino acid composition (nmol): Ala 6.4 and Phe 3.0.

**Preparative synthesis of Z-Ala-Ala-Lys-Asp-pNA.** DMF (540 μl), MeCN (800 μl), and 1 M TEA in DMF (60 μl) were added to a solution of Z-Ala-Ala-Lys-OH · HCl (28 mg, 60 μmol) in dry DMF (300 μl) and a 200 mM solution of Asp-pNA · HCl (60 μmol) in dry DMF (300 μl). The resulting solution was added to cryoPVA-A–subtilisin (405 mg, protein content 1.8 mg), and the reaction mixture was shaken for 24 h at 20°C. The biocatalyst was separated from the reaction mixture and washed with 4 : 6 (v/v) MeCN–DMF mixture (4 × 3 ml). The reaction mixture and washings were combined and evaporated on a rotary evaporator. The oily residue was crystallized under acetone at +4°C; yield 39 mg (93%); amino acid composition (nmol): Ala 10.6, Lys 5.4, and Asp 4.4; *RT* (C) 21 min; MS, *m*/*z*: $[MH]^+$ 658.4, calculated 657.7.

## ACKNOWLEDGMENTS

This work was supported by the Russian Foundation for Basic Research, project nos. 00-03-32803a, 02-04-06281, and 00-04-48455, and in part by the INTAS grant 01-673.

## REFERENCES

1. Klibanov, A.M., *Nature*, 2001, vol. 409, pp. 241–246.
2. Gill, I., Lopez-Fandino, R., Jorba, X., and Vulfson, E.N., *Enzyme Microb. Technol.*, 1996, vol. 18, pp. 163–183.
3. Dordick, J.S., *Biotechnol. Prog.*, 1992, vol. 8, pp. 259–267.
4. Wong, C.-H., *Trends Biotechnol.*, 1992, vol. 10, pp. 378–381.
5. Novick, S.J. and Dordick, J.S., *Biotechnol. Bioeng.*, 2000, vol. 68, pp. 665–671.
6. Dordick, J.S., Khmelnitsky, Y.L., and Sergeeva, M.V., *Curr. Opin. Microbiol.*, 1998, vol. 1, pp. 311–318.
7. Wang, P., Sergeeva, M.V., Lim, L., and Dordick, J.S., *Nat. Biotechnol.*, 1997, vol. 15, pp. 789–793.
8. Blanco, R.M., Bastida, A., Cuesta, C., Alvaro, G., Fernandez-Lafuente, R., Rosell, C.M., and Guisan, J.M., *Biomed. Biochim. Acta*, 1991, vol. 50, pp. 110–113.
9. Kudryashova, E.V., Gladilin, A.K., Vakurov, A.V., Heitz, F., Levashov, A.V., and Mozhaev, V.V., *Biotechnol. Bioeng.*, 1997, vol. 55, pp. 267–277.
10. Lozinskii, V.I., *Usp. Khim.*, 1998, vol. 67, pp. 641–655.
11. Rudenskaya, G.N., *Bioorg. Khim.*, 1994, vol. 20, pp. 475–484.
12. Stepanov, V.M., *Pure Appl. Chem.*, 1996, vol. 68, p. 1335.
13. Stepanov, V.M., Terent'eva, E.Y., Voyushina, T.L., and Gololobov, M.Y., *Bioorg. Med. Chem. Lett.*, 1995, vol. 3, pp. 479–485.
14. Bacheva, A.V., Plieva, F.M., Lysogorskaya, E.N., Filippova, I.Y., and Lozinsky, V.I., *Bioorg. Med. Chem. Lett.*, 2001, vol. 11, pp. 1005–1008.
15. Filippova, I.Yu., Bacheva, A.V., Baibak, O.V., Plieva, F.M., Lysogorskaya, E.N., Oksenoit, E.S., and Lozinskii, V.I., *Izv. Ross. Akad. Nauk, Ser. Khim.*, 2001, no. 10, pp. 1811–1816.
16. Voyushina, T.L., Lyublinskaya, L.A., and Stepanov, V.M., *Bioorg. Khim.*, 1985, vol. 11, pp. 738–744.
17. Getun, I.V., Filippova, I.Yu., Lysogorskaya, E.N., Oksenoit, E.S., Anisimova, V.V., Kolobanova, S.V., Bacheva, A.V., and Stepanov, V.M., *Bioorg. Med. Chem. Lett.*, 1997, vol. 7, pp. 2691–2696.
18. Getun, I.V., Filippova, I.Yu., Lysogorskaya, E.N., Kolobanova, S.V., Oksenoit, E.S., Anisimova, V.V., and Stepanov, V.M., *Bioorg. Khim.*, 1998, vol. 24, pp. 306–312.
19. Getun, I.V., Filippova, I.Y., Lysogorskaya, E.N., and Oksenoit, E.S., *J. Mol. Catal., B: Enzymatic*, 2001, vol. 15, pp. 105–110.
20. Gololobov, M., Morozova, I.P., Voyushina, T.L., Timokhina, E.A., and Stepanov, V.M., *Biokhimiya* (Moscow), 1991, vol. 56, pp. 230–239.
21. Gordon, A.J. and Ford, R.A., *The Chemist's Companion: A Handbook of Practical Data, Techniques and References*, New York: Wiley, 1972. Translated under the title *Sputnik khimika*, Moscow: Mir, 1976.
22. Gershkovich, A.A. and Kibirev, V.K., *Khimicheskii sintez peptidov* (Chemical Synthesis of Peptides), Kiev: Naukova Dumka, 1992.