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Biocatalytic Properties of Thermolysin Immobilized on Polyvinyl Alcohol Cryogel

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Abstract—Preparations with different contents of thermolysin were obtained by the immobilization of the enzyme on granulated polyvinyl alcohol cryogel. Their activity and stability in an aqueous medium and in mixtures of polar organic solvents of different composition were investigated. The catalytic properties of the preparations in reactions of peptide bond formation were studied, and the optimal amount of the biocatalyst, the concentrations of initial reagents, and the ratios of organic solvents and water necessary for effective enzymatic peptide synthesis catalyzed by immobilized thermolysin were determined. A series of peptides of the general formula Z-Ala-Ala-Xaa-pNA, where Xaa = Leu, Ile, Phe, Val, or Ala, were synthesized, and the immobilized enzyme was shown to retain substrate specificity in an organic medium.

Key words: polyvinyl alcohol cryogel, enzymatic peptide synthesis in water–organic mixtures, immobilized thermolysin

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INTRODUCTION

Thermolysin, a typical bacterial zinc-dependent neutral metalloprotease, is often used for the fragmentation of proteins and peptides in structural studies.[2] In the last few years, this enzyme has been widely employed as a catalyst at some stages of chemicoenzymatic synthesis in the production of peptide hormones, taste peptides, growth factors and their fragments, as well as in the industrial manufacture of the low-calorie sweetener aspartame [1–6]. ThL is involved only in thermodynamically controlled processes, affecting the rate of the attainment of equilibrium in the hydrolysis-synthesis reaction. In water, the equilibrium of the reaction is shifted toward hydrolysis. The shift of equilibrium toward synthesis is accomplished by (1) removing the product from the reaction through precipitation or extraction into another phase, (2) increasing the concentration of initial compounds, and (3) decreasing the water content in the reaction system in reactions conducted in organic solvent media.

It is known that the efficiency of peptide synthesis in polar organic solvents is substantially higher; however, a fast inactivation of protease restricts the process. One way of stabilizing the proteases to the inactivating effect of polar organic solvents is covalent immobilization [7–9]. We previously have shown that biocatalysts based on the serine protease subtilisin covalently immobilized on PVA cryogel [10] feature stability and a high synthase activity in anhydrous media [11]. PVA cryogel is an inert polymeric carrier, which is obtained by a simple procedure of freezing-thawing of a PVA solution [12]. A unique feature of PVA cryogels is the macroporous structure and stability in organic solvents. In addition, cryogels are distinguished by a high hydrophilicity, which enables them to hold water inside the polymeric matrix even in anhydrous medium of polar organic solvents. These properties make possible the immobilization of large enzyme molecules on the carrier, by minimizing the restrained diffusion of the substrate and the inactivation of the biocatalyst in organic solvent media.

The goal of this work was the synthesis of preparations of thermolysin covalently immobilized on PVA cryogel, the examination of their stability in aqueous medium and in media of polar organic solvents, includ-

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[2] Abbreviations: Dnp, 2,4-dinitrophenyl; pNA, *p*-nitroanilide; PVA, polyvinyl alcohol ThL, thermolysin; Z, benzyloxycarbonyl.

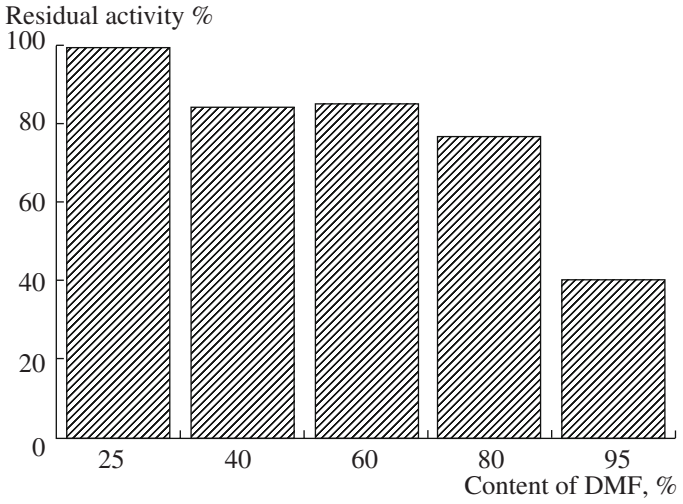

Fig. 1. Stability of immobilized ThL in DMF–MeCN of different composition (incubation 72 h).

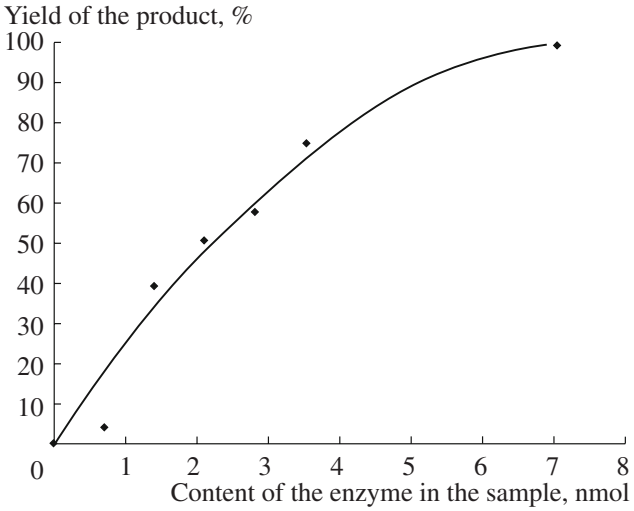

Fig. 2. Dependence of the yield of Z-Ala-Ala-Leu-pNA on the content of immobilized ThL in 300 μl of mixture DMF–MeCN 1 : 3 (1 h, [S] 40 mM).

ing the study of their catalytic properties in reactions of peptide bond formation.

RESULTS AND DISCUSSION

By the covalent fixation of the commercial ThL preparation on the glutaraldehyde-activated matrix of PVA cryogel, we obtained samples of the immobilized enzyme (subsequently referred to as biocatalyst) with a protein content of 2.4 (0.064 μmol), 4.4 (0.117 μmol), and 11.6 mg (0.309 μmol)/g carrier (samples 1, 2, and 3, respectively). The amount of the protein on the carrier was determined from the data of amino acid analysis after the hydrolysis of a biocatalyst sample by 5.7 N hydrochloric acid.

The activity of biocatalysts was measured from the hydrolysis of the chromophoric substrate Dnp-Gly-Gly↓Ile-Arg. The specific activity of samples 1, 2, and 3 per 1 g of carrier differed and were 0.13, 0.18, and 0.50 μmol/min. However, the specific activity values calculated per 1 mg of the enzyme were practically equal, 0.050, 0.048, and 0.043 μmol/min. The stability of the biocatalyst was estimated by measuring its specific hydrolytic activity after storage in media of different composition.

The biocatalyst had a high stability at +4°C in an aqueous 0.05 M Tris-HCl buffer, pH 7.2, containing 50 mM $CaCl_2$ (buffer A) and retained no less than 95% of initial activity even after 4 months of storage in this buffer (specific activity of the immobilized enzyme measured on the day of isolation was taken as 100%).

Of particular interest was the study of the stability of biocatalyst samples in practically anhydrous organic solvent media suitable for peptide synthesis [13]. Previously we have shown a high stability of ThL immobilized of PVA cryogel in MeCN [11]. A study of the stability of the immobilized enzyme in MeCN with different content of DMF showed that the activity of biocatalyst preparations did not change during the incubation for 72 h (Fig. 1). It was found that, during a more prolonged incubation for 3.5 days, the biocatalyst activity also remained unchanged. Keeping immobilized ThL in mixtures containing 40–80% DMF led to a slight inactivation of the enzyme; however, even after 10 days, the preparation retained no less than 60% of its activity (not shown in the figure). A more abrupt loss of activity was observed in 95% DMF/MeCN; however, in this case, too, the catalyst retained more than 40% of activity.

The catalytic properties of ThL immobilized on PVA cryogel were studied in a model reaction of the synthesis of the chromogenic substrate of serine proteases Z-Ala-Ala-Leu-pNA (**I**) in mixtures of polar organic solvents DMF/MeCN and DMSO/MeCN:

$$\text{Z-Ala-Ala-OH + Leu-pNA} \underset{\textit{immobilized.ThL}}{\rightleftharpoons} \text{Z-Ala-Ala-Leu-pNA.} \quad (1)$$

The amount of ThL sufficient for the effective synthesis of tripeptide (**I**) (Fig. 2) was determined at the equimolar ratio of the amino and carboxyl components (experiments were carried out with sample 1). It is evident from Fig. 2 that initially (at ThL concentration up to 3 nmol) the yield of the product was linearly dependent on the amount of the enzyme in the biocatalyst sample introduced into the reaction mixture, following which it reached a plateau. A further increase in the amount of the enzyme did not lead to an abrupt increase in the yield of the product. Therefore, further experiments were carried out using biocatalyst preparations with a ThL content of 1.4 nmol. The effect of the concentrations of starting components on the yield of the target product is shown in Fig. 3. It is seen that, as the

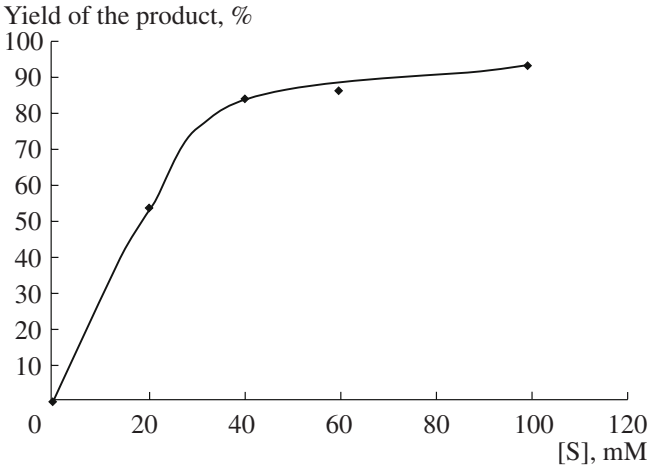

Fig. 3. Dependence of the yield of Z-Ala-Ala-Leu-pNA on the concentration of substrates in 300 μl of mixture DMF–MeCN 1 : 3 (incubation for 24 h, the amount of ThL 1.4 nmol)

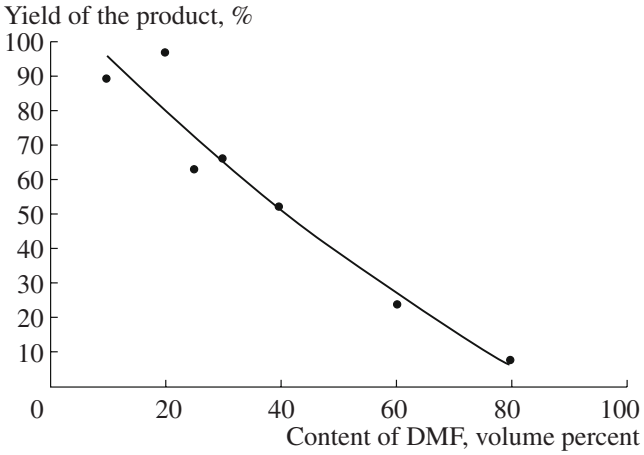

Fig. 4. Dependence of the yield of Z-Ala-Ala-Leu-pNA on the content of DMF in a mixture with MeCN (1 h, [S] = 40 mM, E : S = 1 : 5000).

concentration of starting components increased to 30 mM, the rate of synthesis rose sharply, and beginning from a concentration of 40 mM, it remained practically unchanged.

To determine how the DMF concentration in the reaction mixture affects the catalytic activity of immobilized ThL, the synthesis was conducted in DMF/MeCN mixtures containing from 10 to 80% of DMF (Fig. 4). It was shown that increasing the DMF concentration leads to a monotonous decrease in the synthase activity of the preparation throughout the range of solvent concentrations used. In further experiments, a mixture containing 20 volume percent of DMF was used.

The enzymatic synthesis catalyzed by ThL is a thermodynamically controlled process, which is the reverse of the hydrolysis reaction [14]. Water (as a nucleophile) is immediately involved in the cleavage of the peptide bond, by attacking the hydrogen atom of the carbonyl group. In heterogeneous reactions of peptide bond formation, the amount of water in the reaction mixture should be sufficient to provide the maximum catalytic activity of the enzyme and, at the same time, it should not lead to the precipitation of poorly soluble hydrophobic compounds and the reaction product from the reaction mixture. Therefore, to find the conditions for the most efficient use of the biocatalyst, it was necessary to determine the amount of water in the reaction mixture at which the yield is maximal, and the product and starting substances are completely dissolved in the reaction mixture. Figure 5 shows the dependence of the yield of model peptide (**I**) on the water content in the reaction mixture. The concentration range used was restricted by the solubility of the reaction components (hydrophobic substrates and reaction product). As the volume of water in the reaction mixture increased from 0 to 7%, the yield of the product within 2 h first increased linearly; then it gradually reached some constant value at a water content of 10% and remained practically unchanged in the region corresponding to high concentrations of water. The small decrease in the yield observed at a 25% content of water is attributable to the exceeding of the threshold of reaction product solubility and the secondary hydrolysis of the resulting product. Thus, 10–15% of water is necessary for the occurrence of the maximum catalytic activity of immobilized ThL.

We studied the dependence of the yield of Z-Ala-Ala-Leu-pNA on the final concentration of added DMSO (in the range of 10–80% volume percent) in a system containing 10% aqueous buffer mixed with

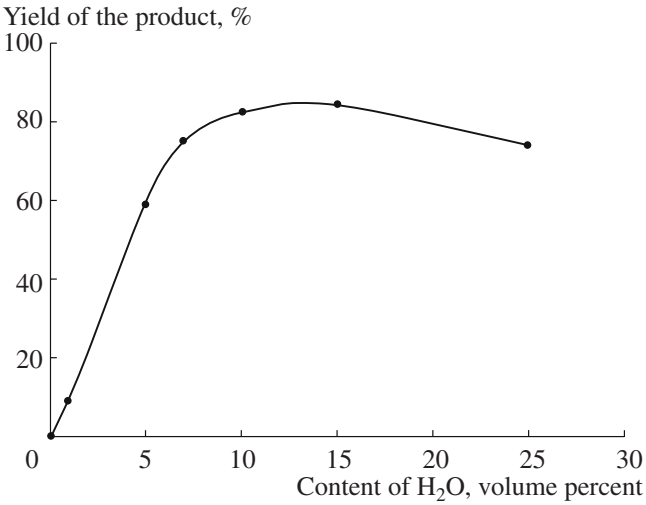

Fig. 5. Dependence of the yield of Z-Ala-Ala-Leu-pNA on the concentration of water in a mixture 20% DMF–55–75% MeCN–5–25% H_2O (2 h, [S] = 40 mM, E : S = 1 : 5000).

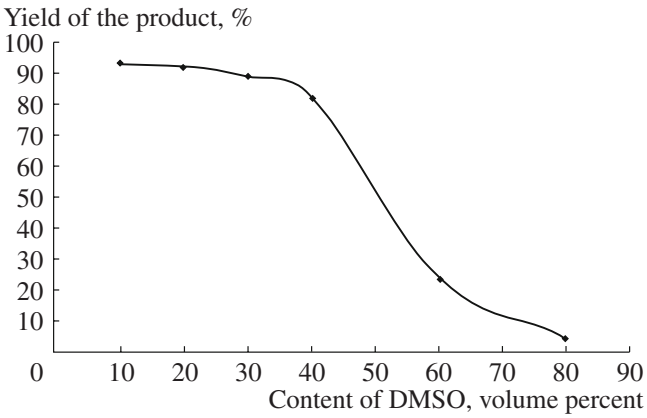

Fig. 6. Dependence of the yield of Z-Ala-Ala-Leu-pNA on the content of DMSO in a mixture 10% H_2O –10–80% DMSO –10–80% MeCN (2 h, [S] = 40 mM, E : S = 1 : 5000).

10–80% MeCN (Fig. 6). The high yields of the target peptide (80–90%) were observed in a mixture containing up to 40% of this solvent; a further increase in its concentration was accompanied by an abrupt decrease in the yield.

The replacement of DMF by DMSO, the experimental conditions being the same, did not substantially affect the yield of the reaction product.

One obvious advantage of immobilized enzyme preparations in the peptide synthesis, as compared with native enzymes, is the possibility of their repeated utilization. Previously in our laboratory we have shown that serine protease preparations retain their catalytic properties after at least three synthesis cycles with the use of one and the same sample [11].

The catalytic properties of immobilized ThL on repeated utilization were studied by conducting consecutive syntheses with one and the same sample of a biocatalyst without its intermediate reactivation. For this purpose, between the synthesis cycles (reaction time 2 h), the preparation was washed with a 20% DMF/80% MeCN mixture containing no water. The amount of water required for the synthesis was introduced to the reaction mixture by the addition of the corresponding aliquot of 0.05 M Tris-HCl buffer, pH 7.2, containing 50 mM $CaCl_2$ (buffer A). Even after six cycles, the yield of the product remained high.

Thus, immobilized ThL preparations can be repeatedly used as effective catalysts in the reactions of peptide bond formation.

The potentialities of this immobilized biocatalyst were exemplified by the preparative synthesis of Z-Ala-Ala-Leu-pNA.

The reaction was conducted in a system 10% DMSO/10% H_2O/MeCN at the equimolar ratio of the amino and carboxyl components (E : S ~ 1 : 5000). The analytical yield of the product was 100%. After purification, the yield was 71%. The product was characterized by the data of HPLC and amino acid analysis; it was also successfully tested as a substrate for subtilisin Carlsberg.

Using immobilized ThL under the above-described conditions, we synthesized a series of peptides of the general formula Z-Ala-Ala-Xaa-pNA, where Xaa = Ile, Phe, Val [peptides (**II**)–(**IV**), respectively] with a yield of 70–80% (table). The synthesis of Z-Ala-Ala-Ala-pNA [peptide (**V**)] proceeded somewhat worse. Immobilized ThL did not catalyze the formation of a peptide bond between residues Pro [peptide (**VIII**)], Gly [peptide (**IX**)], and those of dicarboxylic amino acids [peptides (**X**) and (**XI**)]. These data suggest that ThL in organic medium retained the substrate specificity inherent in this enzyme in hydrolytic reactions [15]. It should be noted that this regularity has been observed earlier in studies of peptide bonding catalyzed by ThL in water–organic media [16].

It was found in the synthesis of Z-Ala-Ala-Leu-NH_2 [peptide (**VI**)] that substituting amide for *p*-nitroanilide did not influence the efficiency of peptide condensation: peptide (**VI**) was obtained with a quantitative yield. The possibility of using Leu-OCH_3 and Phe-OCH_3 as amino components was exemplified by the synthesis of Z-Ala-Ala-Leu-OCH_3 [peptide (**VII**)] and Z-Asp-Phe-OCH_3 (**XII**), a precursor of aspartame; the yield of these peptides was 33 and 49%, respectively.

Thus, we showed that ThL covalently immobilized on PVA cryogel is highly reactive and stable in media of different composition. Optimal conditions for the peptide synthesis catalyzed by immobilized ThL were determined, and it was found that the biocatalyst for the most part retains the primary substrate specificity of native ThL in organic medium and can also be used for the synthesis of esters and amides of peptides that are the precursors of peptides of a more complicated structure. We showed that one biocatalyst sample can be repeatedly used in the synthesis reaction.

EXPERIMENTAL

The following preparations were used: ThL with *M* 37500 Da from *Bacillus thermoproteoliticus* (Fluka Chemie AG, Switzerland, EC 3.4.24.27); PVA 16/1 (*M* 69000) (NPO Azot, Severodonetsk, Ukraine); SP Sephadex C-25 (Pharmacia, Sweden); acetonitrile for HPLC (extra pure grade, Lekbiofarm, Russia) containing no more than 0.01% of water; DMF (analytical grade, Reakhim, Russia) additionally purified as described in [17]; trifluoroacetic acid (analytical grade, Fluka Chemie AG, Switzerland); acetic acid (Reakhim, Russia); $CaCl_2$ (Sigma, United States); Tris (ISN Biomedicals, United States); triethylamine (analytical grade, Reakhim, Russia) additionally distilled as described [17]; Leu-pNA (Sigma, NOA), and Phe-pNA (Serva, Germany). The derivatives of other amino acids and peptides were synthesized by standard methods [18].

Peptides synthesized at the catalysis by ThL immobilized on PVA cryogel

Carboxyl component	Amino component	Reaction product	Reaction time, h	Yield, %	Retention time, min*
Z-Ala-Ala-OH	H-Leu-pNA	Z-Ala-Ala-Leu-pNA **(I)**	24	91	27
	H-Phe-pNA	Z-Ala-Ala-Phe-pNA **(II)**	24	73	27.5
	H-Val-pNA	Z-Ala-Ala-Val-pNA **(III)**	24	80	26.0
	H-Ile-pNA	Z-Ala-Ala-Ile-pNA **(IV)**	24	82	27.5
	H-Ala-pNA	Z-Ala-Ala-Ala-pNA **(V)**	24	30	22.1
	H-Leu-NH_2	Z-Ala-Ala-Leu-NH_2 **(VI)**	24	100	17.0
	H-Leu-OCH_3	Z-Ala-Ala-Leu-OCH_3 **(VII)**	24	33	22.6
	H-Pro-pNA	Z-Ala-Ala-Pro-pNA **(VIII)**	72	0	
	H-Gly-pNA	Z-Ala-Ala-Gly-pNA **(IX)**	72	0	
	H-Asp-pNA	Z-Ala-Ala-Asp-pNA **(X)**	72	0	
	H-Glu-pNA	Z-Ala-Ala-Glu-pNA **(XI)**	72	0	
Z-Asp-OH	H-Phe-OCH_3	Z-Asp-Phe-OCH_3 **(XII)**	48	49**	22.5

Notes: Reaction conditions: 20% DMF/10% buffer A/MeCN, [S] = 40–58 mM, +20°C, E : S = 1 : 5000.
*HPLC for all peptides in system M; for (**XII**) in system N (see the Experimental section).
** Reaction was carried out in *tert*-butanol containing 5% H_2O.

The preparation of granulated (1–1.5 mm) PVA cryogel and its reactive aldehyde-containing derivative and the covalent linking of ThL to it were carried out in the laboratory of cryochemistry of biopolymers (Institute of Organoelement Chemistry, Russian Academy of Sciences) as described [11, 19]. Three samples of the biocatalyst were obtained (2 g each). The biocatalyst was stored in 0.05 M Tris-HCl buffer, pH 7.2, containing 50 mM $CaCl_2$ (buffer A) The amount of ThL immobilized on PVA cryogel was estimated from the amino acid analysis data to be 2.4 (0.064 μmol), 4.4 (0.117 μmol), and 11.6 mg (0.309 μmol)/g carrier (samples 1, 2, and 3, respectively).

The peptides were analyzed on an Altex Model 110A liquid chromatograph (United States). The elution was with 0.1% CF_3COOH in a linear gradient of acetonitrile concentration from 10 to 70% (flow rate 1 ml/min) for 30 min using Microsorb-MV C_8 columns (4.6 × 250 mm) (Rainin Instrument Company, Inc.; United States) (system M) and Nucleosil C_{18} column (4.6 × 250 mm; Biokhimmak, Moscow) (system N). The detection of eluted peptides was at 220 and 280 nm.

The composition of a reaction mixture consisting of initial components and a target peptide was calculated without any correction for the difference of molar absorption coefficients of the components.

Spectrophotometric measurements were carried out on Specord UV VIS (Germany) and Genesys 10-S UV spectrophotometers (Spectronics, United States).

The amino acid analysis was carried out on a Hitachi 835 automatic amino acid analyzer (Japan) after acid hydrolysis by 5.7 N HCl at 105°C in vacuumated ampoules for 24 and 48 h.

Enzymatic activity of immobilized ThL in the hydrolysis of Dnp-Gly-Gly-Ile-Arg. A solution of substrate Dnp-Gly-Gly-Ile-Arg (1 ml, 0.5 mg/ml) in 0.05 M Tris-HCl buffer A was added to a biocatalyst (10–15 mg) containing the enzyme (0.6–4.6 nmol), and the reaction mixture was incubated at 37°C. After 30–40 min, 900 μl of the solution was taken, 400 μl of 50% acetic acid was added, the solution was stirred, and the mixture was applied to Sephadex SP-25 columns (volume 3 ml), eluted with 2 ml of 0.1 N acetic acid, and the optical absorption of the eluate (Dnp-Gly-Gly) was measured at 360 nm.

Specific activity was calculated by the formula:

$$\frac{(A_{360} - A^{c}_{360}) \cdot V_{tot}}{\varepsilon \cdot t \cdot m \cdot V_{samp}},$$

where A_{360} and A^{c}_{360} is the absorption of an experimental and control solution at 360 nm; V_{tot} is the total volume of the eluate, ml; ε is the molar absorption coefficient of the Dnp-group, 15 mM^{-1} cm^{-1}; t is the reaction time, min; m is the mass of a sample of the immobilized enzyme, mg; V_{sapm} is the volume of the sample applied to the column, ml. The specific activity was expressed in μmol/mg × protein min.

Stability of immobilized ThL in aqueous buffer. A biocatalyst preparation was stored under the layer of buffer A at +4°C. At regular intervals, biocatalyst samples (15–20 mg) were taken, and the activity of the preparation was determined. The activity of a biocatalyst preparation obtained immediately after immobilization was taken to be 100%.

Stability of immobilized ThL in organic solvent mixtures was determined in a similar way by incubating a biocatalyst sample (15–20 mg) in an MeCN/DMF mixture of the corresponding composition. Before activity determination, the organic solvent was decanted, and the preparation was washed by buffer A (3 × 1.5 ml).

Synthesis of Z-Ala-Ala-Leu-pNA (general method). A solution (300 μl) of 40 mM Z-Ala-Ala-OH and Leu-pNA in the corresponding mixture of solvents was added to 20 mg of a biocatalyst preparation [sample 2, content of ThL 0.09 mg (2.4 nmol)], and the mixture was shaken on an orbital shaker at 20°C. At regular intervals, samples (5 μl) were taken for HPLC; elution was in a gradient of M.

Dependence of the yield of Z-Ala-Ala-Leu-pNA on the amount of immobilized ThL was studied after the synthesis carried out by the general method in system MeCN–DMF 75 : 25 (volume percent). Different biocatalyst samples (sample 1) (10, 20, 30, 45, 60, and 110 mg; content of ThL 0.6–7.0 nmol) were introduced into the reaction.

Dependence of the yield of Z-Ala-Ala-Leu-pNA on the concentration of starting components was studied after the synthesis carried out by the general method in system MeCN/DMF 75/25 (volume percent) at the equimolar ratio of starting components (Z-Ala-Ala-OH and Leu-pNA) in the concentration range of 20–200 mM. The content of the enzyme was 1.4 nmol.

Dependence of the yield of Z-Ala-Ala-Leu-pNA on the content of DMF was studied in syntheses carried out by the general method in MeCN–DMF mixtures containing from 10 to 80% DMF.

Dependence of the yield of Z-Ala-Ala-Leu-pNA on the content of water in reaction mixture was studied in syntheses carried out by the general method in mixtures 20% DMF/55–75% MeCN/5–25% Tris-HCl buffer, pH 7.2, and 50 mM $CaCl_2$. Biocatalyst granules were preliminarily washed with MeCN (3 × 1.5 ml) and a mixture MeCN–DMF 80 : 20 (volume percent) (2 × 2.5 ml). Samples were taken and analyzed according to the general method after 1, 2, and 24 h.

Dependence of the yield of Z-Ala-Ala-Leu-pNA on the content of DMSO was studied in syntheses carried out by the general method in mixtures 10-80% DMSO/10–80% MeCN/10% Tris-HCl buffer, pH 7.2, and 50 mM $CaCl_2$. Biocatalyst granules were preliminarily washed with MeCN (3 × 1.5 ml) and a mixture MeCN–DMSO 90 : 10 (volume percent) (2 × 2.5 ml). Samples were taken and analyzed according to the general method after 1, 2, and 24 h.

Possibility of repeated use of immobilized ThL samples in the synthesis of Z-Ala-Ala-Leu-pNA, was tested by the general method in a mixture MeCN–DMF–H_2O (70/20/10 per cent by volume), was studied using one sample of immobilized ThL. After each synthesis, the reaction mixture was separated (by a pipette), and biocatalyst granules were washed with a MeCN–DMF mixture 80 : 20 volume percent (4 × 200 μl, 15 min) and introduced into the next synthesis of this peptide, adding the solution of starting components.

Preparative synthesis of Z-Ala-Ala-Leu-pNA. A solution (3.75 ml) containing Z-Ala-Ala-OH (55.9 mg, 190 μmol) and Leu-pNA (47.7 mg, 190 μmol) in a mixture MeCN–DMSO–H_2O (80/20/10 volume percent) was added to a biocatalyst sample (350 mg, content of the protein 1.54 mg). The reaction mixture was shaken on an orbital shaker at 20°C, and 5 μl samples were taken at regular intervals for the analysis by HPLC. After 24 h, the reaction mixture was separated from the biocatalyst by decantation, and biocatalyst granules were washed with a MeCN–DMSO 90 : 10 (volume percent) mixture (5 × 2 ml). The reaction mixture and all washings were combined and evaporated on a rotor evaporator. A solution of 0.1 N hydrochloric acid (1.5 ml) was added dropwise to the residue (a yellowish precipitate occurred), the solution was stirred, and cold water (8 ml) was introduced in small portions. The yellowish precipitate was transferred to a filter, washed with cold water, and dried over NaOH in a vacuum exsiccator. Yield: 71 mg (71%).

Amino acid analysis of Z-Ala-Ala-Leu-pNA: Ala 16.7 nmol, Leu 9.9 nmol.

Condensation of Z-Ala-Ala-OH and Phe-pNA. MeCN (100 μl), DMF (50 μl), a 250 mM solution of Z-Ala-Ala-II (100 μl) and a 250 mM solution of Phe-pNA in DMF were added to a biocatalyst preparation (20 mg, the content of ThL 0.05 mg). The reaction mixture was shaken on an orbital shaker at 20°C, and 5 μl samples were taken at regular intervals for the analysis by HPLC. Before being applied to a column, samples were diluted with a starting eluent (20% solution of MeCN in water; 1.7 ml). The retention time of Z-Ala-Ala-Phe-pNA in gradient (M) was 27.5 min.

The amino acid analysis of Z-Ala-Ala-Phe-pNA: Ala 3.4 nmol, Phe 2.2 nmol.

The condensation of **Z-Ala-Ala-OH** and **Ile-pNA**, **Z-Ala-Ala-OH** and **Val-pNA**, **Z-Ala-Ala-OH** and **Ala-pNA**, **Z-Ala-Ala-OH** and **Leu-OMe**, **Z-Ala-Ala-OH** and **Leu-NH_2** was carried out similarly to the condensation of **Z-Ala-Ala-OH** and **Phe-pNA**.

Retention time in system I, min: **Z-Ala-Ala-Ile-pNA**—27.5, **Z-Ala-Ala-Val-pNA**—26.0, **Z-Ala-Ala-Ala-pNA**—22.1, **Z-Ala-Ala-Leu-OMe**—22.6, and **Z-Ala-Ala-Leu-NH_2**—17.0.

Amino acid analysis, nmol: **Z-Ala-Ala-Ile-pNA**: Ala 3.7, Ile 2.4; **Z-Ala-Ala-Val-pNA**: Ala 1.0, Val 0.53; **Z-Ala-Ala-Ala-pNA**: Ala 17.9; **Z-Ala-Ala-Leu-OMe**: Ala 17.7, Leu 7.5; and **Z-Ala-Ala-Leu-NH_2** Ala 2.4, Leu 1.2.

Condensation of Z-Asp-OH and Phe-OMe. Z-Asp-OH (50 μmol) and HCl · Phe-OMe (100 μmol) were suspended in *tert*-butanol (500 μmol), then TEA (14 μl, 100 μmol) was added, and the solution was stirred for 20 min. A biocatalyst preparation (20 mg, the content of the protein 0.2 mg) and 50 mM $CaCl_2$ (30 μl) were added to the resulting suspension. The reaction mixture was shaken on an orbital shaker at 20°C, and 5 μl samples were taken at regular intervals for the analysis by HPLC. Before being applied to a column, samples were diluted with a starting eluent (10% solution of MeCN in water; 200 μl). The retention time of Z-Asp-Phe-OMe in system N was 22.5 min.

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