Proteinases immobilized on poly(vinyl alcohol) cryogel: novel biocatalysts for peptide synthesis in organic media

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Covalent immobilization of subtilisin and thermolysin on cryogel of poly(vinyl alcohol) was carried out. The biocatalysts obtained are characterized by high stability in water and in DMF—MeCN mixtures of various compositions. The synthetic efficiency of immobilized subtilisin in the multiple iterative synthesis of the peptide Z—Ala—Ala—Leu—Phe—pNA was examined in organic mixtures of different solvent compositions. Immobilized subtilisin exhibits high synthetic activity in organic media. A series of *N*-acylated *p*-nitroanilides of tetrapeptides of the general formula Z—Ala—Ala—Xaa—Yaa—pNA (Z is benzyloxycarbonyl, Xaa = Leu, Lys, or Glu; Yaa = Phe or Asp; pNA = 4-NO_2—C_6H_4NH-) were synthesized in 70—98% yields using immobilized subtilisin as a biocatalyst without activation and protection of the ionogenic groups of polyfunctional amino acids. Immobilized thermolysin in a DMF—MeCN mixture catalyzed the formation of the peptide Z—Ala—Ala—Leu—pNA, which was obtained in 90% yield (during 1 h). It was demonstrated that the biocatalyst can be used repeatedly and that it retained activity after storage in an aqueous buffer during 6 months.

Key words: immobilized subtilisin and thermolysin, poly(vinyl alcohol) cryogel, peptide synthesis, organic solvents, proteinases.

The biological role of proteinases is to hydrolyze bonds between amino acid residues in polypeptides and proteins. Under certain conditions, proteolytic enzymes can also be used in the reverse reaction, *viz.*, in the synthesis of peptide bonds.[1] Obvious progress has been achieved in the field of the enzymatic peptide synthesis in recent years, which provides evidence that proteinases are promising catalysts of the peptide formation.[1,2] However, the scope of the enzymatic peptide synthesis in aqueous media is limited by low solubility of protected peptides and the possibility of undesirable hydrolysis of the products. The use of organic solvents instead of aqueous media promotes the shift of the thermodynamic equilibrium toward the formation of the target compounds and the enhancement of the solubility of protected peptides, but it is unfavorable for enzymes because organic solvents cause enzyme inactivation. In this connection, a search for systems, which ensure preservation of the functional properties of enzymes in organic solvents and protect them from inactivation over a long period, is an urgent problem. One of efficient approaches, which provides favorable conditions for both the functioning of the enzyme and solubility of peptides, involves immobilization of proteinases on appropriate insoluble supports.[3,4] Thus, it has been demonstrated[5] that the so-called poly(vinyl alcohol) cryogel (PVAC),[6] which was prepared by freezing-thawing of concentrated aqueous solutions of this polymer, has great potential for these purposes. After introduction of reactive groups, this support was used for covalent immobilization of trypsin,[5] α-chymotrypsin,[7] and pancreatic lipase.[8] The two last-mentioned types of biocatalysts have been successfully tested in enzyme catalysis in organic media with low water contents.

In the present study, we examined the properties of serine proteinase subtilisin-72 (SL) and metalloendoproteinase thermolysin (TL), which were covalently attached to PVAC granules. These immobilized biocatalysts (ISL and ITL, respectively) were used for the chemical enzymatic synthesis of peptides in media with high contents of polar organic solvents (MeCN and DMF).

Results and Discussion

The choice of the enzymes, which differ by the nature and the catalysis mechanism, for immobilization was dictated by their high synthetic potential as catalysts of the peptide formation.[1,2] The support and the procedure for immobilization of these enzymes should satisfy some specific requirements associated with the characteristic features of functioning of the immobilized biocatalysts in nonaqueous media. First, the support

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and the procedure for the attachment of the enzyme must provide reliable fixation of protein macromolecules on a support in order to prevent enzyme leakage from catalyst granules. It should be noted that protein immobilization on insoluble supports through physical adsorption is not sufficiently efficient in the functioning of biocatalytic systems in polar organic media. This is associated with irreversible losses of the enzyme through its desorption into the reaction medium, high sensitivity with respect to the change in the medium composition and, as a consequence, with the need for the use of very large amounts of enzymes. Although the applicability of this approach to the enzymatic peptide synthesis in nonaqueous media was exemplified by the preparation of a number of peptides and peptide mimetics in organic media,[9—13] the covalent attachment of the protein to a support matrix seems to be preferable. Second, the support should be compatible both with the aqueous (at least at the stage of enzyme immobilization) and organic media. It is necessary to fulfill this condition for prevention of undesirable phenomena, such as collapse of polymeric materials or species agglutination on going from one medium to another. In this connection, a gentle procedure for immobilization based on the physical entrapment of the enzyme into a matrix of polymeric hydrogel can be used only with great caution due to subsequent strong collapse of such a support in polar dehydrating solvents. Finally, the support used should possess good performance characteristics, *i.e.*, mechanical, chemical, and biological stabilities. Poly(vinyl alcohol) cryogel, which was successfully used for the preparation of immobilized enzymes intended for functioning in nonaqueous media (see above), adequately satisfies the above-mentioned criteria.

Preparation of immobilized biocatalysts. Subtilisin was immobilized on two cryogel derivatives containing reactive aldehyde (PVAC-A) or epoxide (PVAC-E) groups.[5] The enzyme content on the support depended substantially on the mode of immobilization and varied from 0.1 (for PVAC-E) to 4.5 mg (for PVAC-A) of the protein per gram of the support. Immobilized thermolysin was prepared only with the use of PVAC-A; the amount of the immobilized enzyme was 3.5 mg g^{-1} (Table 1). In the subsequent experiments, the target peptides were synthesized with the use of the enzymes immobilized on PVAC-A.

The catalytic activity of ISL was estimated spectrophotometrically by hydrolysis of the highly specific chromogenic peptide substrate of subtilisin, *viz.*, Glp—Ala—Ala—Leu—pNA*.[14] The specific activity of ISL was 0.25—4% of the initial value. It should be noted that the results obtained correlate with the published data on immobilization of α-chymotrypsin (which is similar in properties to subtilisin) on PVAC-A. After immobilization, chymotrypsin retained approximately 10% of the activity exhibited by the native enzyme with respect to the highly specific substrate and more than 85% of the activity with respect to a less specific substrate.[7]

Table 1. Characteristics of ISL and ITL

Sample (support)	Enzyme content on the support/mg g^{-1}	α_{spec}*
ISL (PVAC-E)	0.1	2.2
ISL (PVAC-A)	3.7	0.37
	4.5	0.19
ISL (PVAC-A) + Ac—Trp—OH	2.0	0.65
ISL (PVAC-A) + Bzl—Tyr—NH_2	2.3	0.50
ITL (PVAC-A)	3.5	0.40

* α_{spec} is the specific activity per mg of the protein.

To reduce inactivation of subtilisin (protection of its active site during immobilization), the enzyme was attached to PVAC-A in the presence of reversible competitive inhibitors of serine proteinases, *viz.*, Ac—Trp—OH and Bzl—Tyr—NH_2. However, the specific activities of the resulting specimens were only insignificantly higher (see Table 1).

The enzyme activity of ITL was determined based on hydrolysis of the highly specific chromophoric peptide substrate of this enzyme, *viz.*, Dnp*—Gly—Gly—Ile—Arg—NH_2.[15] Immobilized thermolysin, unlike subtilisin, retained 60% of the initial activity, which indicates that thermolysin is more stable to unfavorable factors of immobilization.

Stability of immobilized enzymes. To ensure the successful use of enzymes in synthetic processes over a long period, it is necessary that the corresponding immobilized biocatalysts possess high stability both in the course of the reactions and upon storage. Hence, the stabilities of ISL and ITL were estimated in the course of their storage both in aqueous buffers and organic solvents.

The data on the time-dependent measurements of the enzyme activity of ISL in various media are presented in Fig. 1. The enzyme exhibited rather high stability in an aqueous buffer (curve *1*) and retained more than 50% of the initial activity even after storage in this medium for three months, whereas the activity of native subtilisin decreased to 40% upon storage under these conditions for only three days. After incubation in MeCN and DMF—MeCN mixtures of various compositions, ISL partially lost hydrolytic activity during two—three days (curves *2* and *3*), but about 30% of the initial activity was retained in the DMF—MeCN mixture (60/40 v/v) even after two weeks. The effect of the enhancement of the stability due to enzyme immobilization on PVAC-A was particularly pronounced as the

* Glp is the residue of pyroglutamic (L-5-pyrrolidone-2-carboxylic) acid, pNA = 4-$NO_2C_6H_4NH$-.

* Dnp is 2,4-dinitrophenyl.

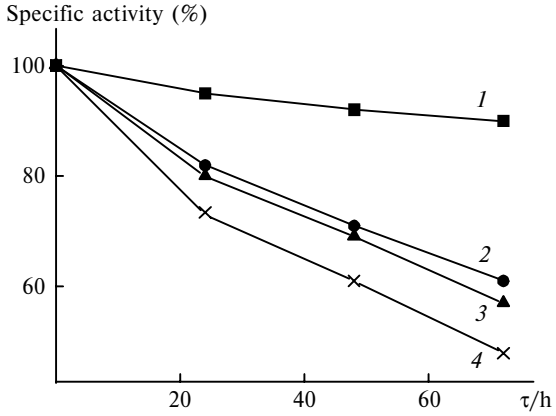

Fig. 1. Measurements of the specific activity of ISL upon incubation in media of various compositions: *1*, a 0.05 *M* Tris—HCl buffer (pH 8.3); *2*, MeCN; *3*, DMF—MeCN (60/40 v/v); *4*, DMF—MeCN (90/10 v/v); τ is the incubation time.

DMF content was increased. In the DMF—MeCN system (90/10 v/v), ISL retained 60% of the initial activity after two days (curve *4*). At the same time, powdered subtilisin suspended in the same mixture of organic solvents lost more than 90% of hydrolytic activity upon storage over the same period of time.[14] Apparently, the higher stability of ISL compared to that of suspended subtilisin arises from higher stability of the immobilized enzyme with respect to the denaturing action of polar organic solvents, for example, due to multisite attachment of subtilisin macromolecules at PVAC. Due to the highly organized supramolecular packing of polymer chains in the gel phase, which strongly holds a particular amount of water,[6] the support promotes the maintenance of the water balance required for the enzyme functioning. It was demonstrated by a special experiment that water is strongly bound to the support in the solvent systems under study. For this purpose, the ISL specimen was twice washed with MeCN and incubated with a DMF—MeCN mixture (60/40 v/v) for 48 h. It was established by Fischer titration that the concentration of water in the organic phase was only 0.16%.

The ITL specimens were characterized by somewhat higher stability upon storage in aqueous buffers compared to the ISL specimens. After storage in the 0.05 *M* Tris—HCl buffer* (pH 7.6), which contained 0.005 mol L^{-1} of Ca^{2+}, at +2 °C for four months, the specimen completely retained its activity. After storage in MeCN for one year, the specimen of the biocatalyst used in the synthesis showed 65% of the initial activity.

Hence, the results obtained are indicative of the efficiency of covalent immobilization of subtilisin and thermolysin on PVAC for the enhancement of stability of the biocatalysts both in aqueous buffers and organic solvents. However, in our opinion, the properties of the biocatalytic system in nonaqueous media cannot be unambiguously judged from the results of the determination of hydrolytic activity because the activities of proteinases were determined after the transfer of the enzymes from an organic medium to water. Consequently, the information obtained is circumstantial and reflects the ability of the enzyme to undergo reactivation rather than its true state in organic solvents. The valuable data on the properties of a biocatalyst can be obtained in studies of esterification and transesterification in organic media.[4,16—18] However, in this approach to the examination of the properties of proteinases in nonaqueous media, no consideration is given to an interesting function of proteolytic enzymes, *viz.*, to catalysis of the peptide bond formation. In our opinion, the properties of biocatalytic systems in organic solvents can be most adequately estimated using the data on the synthetic activities of proteolytic enzymes in the peptide formation. For this purpose, ISL and ITL were tested in the enzymatic peptide synthesis in media with high DMF and MeCN contents.

Enzymatic peptide synthesis catalyzed by ISL and ITL. The synthetic activity of ISL was examined in DMF—MeCN systems with the DMF concentration varying from 60 to 95 vol. % using a model reaction*:

$$\text{Z—Ala—Ala—Leu—OMe + Phe—pNA} \xrightarrow{\text{ISL}} \text{Z—Ala—Ala—Leu—Phe—pNA.} \quad (1)$$

Reaction (1) was carried out using the equimolar ratio of the amino and carboxyl components and the molar enzyme : substrate ratio of 1 : 800. The ISL-catalyzed synthesis proceeded most rapidly in a medium containing 60% of DMF (Table 2). Even after 2 h, the yield of the target peptide was 95%. The rate of accumulation of the product decreased as the concentration of DMF was increased. However, the reaction in 95% DMF did not cease even after 48 h**. After three days, the yield of the product was 74%, whereas the synthesis, which was carried out in an analogous system and catalyzed by suspended subtilisin, was terminated after 3 h due to complete enzyme inactivation.[14] Therefore, noticeable stabilization of the enzyme with respect to the denaturing action of DMF was achieved by immobilization of subtilisin on PVAC-A.

To elucidate the possibility of the use of immobilized proteinases in organic media of various compositions, a series of successive syntheses of Z—Ala—Ala—Leu—Phe—pNA were carried out using the same ISL sample in DMF—MeCN mixtures with increasing DMF content (60, 80, and 95%, respectively). After each cycle, ISL beads were washed with the 0.05 *M* Tris—HCl buffer (pH 8.3). After three cycles, the synthetic ability of the biocatalyst was tested

* Tris is 2-amino-2-(hydroxymethyl)propane-1,3-diol.

* Z is benzyloxycarbonyl.

** Each experiment was carried out with the use of a new portion of the biocatalyst.

Table 2. Synthesis of Z—Ala—Ala—Leu—Phe—pNA catalyzed by the same ISL specimen in MeCN—DMF media of various compositions

Sequence of experiments	DMF—MeCN (vol. %)	Reaction time/h	Yield of the product (%)
1	60/40	0.25	74
		2	95
2	80/20	0.25	25
		2	76
		25	88
3	95/5	2.5	22
		25	57
		51	67
		72	74
4	60/40, repeatedly*	0.25	6
		2	41
		24	84
		72	95

* See comments in the text.

once again in a DMF—MeCN mixture (60/40 v/v). After 72 h, the final yield of the product was equal to that obtained in the first cycle after 2 h (95%).

It was found that ISL is also rather efficient in the synthesis without intermediate rehydration, at least within several cycles (Fig. 2). The synthetic efficiency of one portion of the immobilized biocatalyst was examined in three successive synthetic cycles in DMF—MeCN (60/40 v/v) without intermediate washing with an aqueous buffer. After each cycle, the granules of the catalyst were twice washed with a mixture of organic solvents to extract the starting compounds and the products. The reaction product was obtained in high yield (see Fig. 2). Hence, the water quantity provided for the enzyme by the supramolecular PVAC matrix is sufficiently large for the biocatalyst to exhibit high synthetase activity in spite of functioning in polar dehydrating organic solvents. Compared to the results of investigations of the ISL stability in organic media, the last-mentioned data even better reflect the high biocatalytic potential of the enzyme and provide evidence that covalent immobilization on hydrophilic cryogels is an efficient procedure for adapting proteinases to functioning in nonaqueous media.

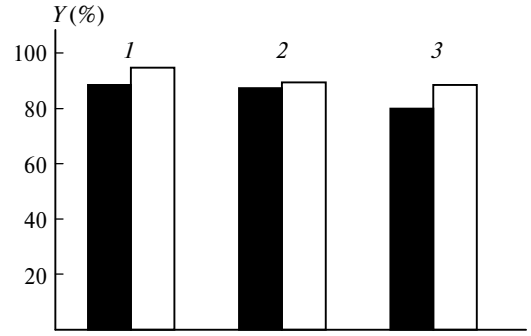

Fig. 2. Yield (*Y*) of Z—Ala—Ala—Leu—Phe—pNA in successive cycles (*1*—*3*) of the synthesis catalyzed by the same ISL specimen in DMF—MeCN (60/40 v/v) without intermediate rehydration of the biocatalyst. The yields after 1 and 2 h are colored black and white, respectively.

It should be noted that activation of the carboxy group is required for efficient catalysis of the peptide bond formation under the action of serine proteinases in aqueous-organic mixtures.[1] We found that not only ester of the tripeptide Z—Ala—Ala—Leu—OMe but also its analog containing the free carboxy group, *viz.*, Z—Ala—Ala—Leu—OH, can serve as an efficient acylating agent in the ISL-catalyzed synthesis in a nonaqueous medium:

$$\text{Z—Ala—Ala—Leu—OH + Phe—pNA} \xrightarrow{\text{ISL}} \text{Z—Ala—Ala—Leu—Phe—pNA.} \quad (2)$$

As can be seen from the data in Table 3, the yields were virtually identical in both cases. Apparently, this is determined by the fact that in nonaqueous organic media, the carboxy group of the acylating component remains nonionized and efficiently interacts with the enzyme active site.

The above-mentioned characteristic feature of catalysis of the peptide bond formation by ISL in an organic medium with the minimum water content was demonstrated by the synthesis of *N*-acylated tetrapeptides containing basic and acidic amino acid residues. The reactions were carried out without activation of the carboxy component and protection of the side ionogenic groups of polyfunctional amino acids:

$$\text{Z—Ala—Ala—Xaa—OH + Yaa—pNA} \xrightarrow{\text{ISL}} \text{Z—Ala—Ala—Xaa—Yaa—pNA,} \quad (3)$$

where Xaa = Leu, Lys, or Glu; Yaa = Phe or Asp.

p-Nitroanilides of protected tetrapeptides were prepared in high yields, the only reaction product being obtained in all cases (see Table 3). The purities of the peptides synthesized were confirmed by the data from HPLC and amino acid analysis.

The synthetic activity of ITL was examined in DMF—MeCN (25/75 v/v) using the following reaction as an example:

$$\text{Z—Ala—Ala—OH + Leu—pNA} \xrightarrow{\text{ISL}} \text{Z—Ala—Ala—Leu—pNA.} \quad (4)$$

The reaction, which was carried out with the equimolar ratio of the amino and carboxyl components and with the enzyme : substrate ratio of 1 : 750 for 1 h, afforded the product in 90% yield. Immobilized thermolysin, which has been twice used in the synthesis and stored in a buffer for six months, catalyzed the synthesis of Z—Ala—Ala—Leu—pNA in organic solvents in 80% yield. These data are indicative of the high efficiency of ITL as a catalyst of the peptide synthesis in organic media.

To summarize, new biocatalysts, *viz.*, PVAC-attached subtilisin and thermolysin, were obtained. These immo-

Table 3. Peptides synthesized under the action of ISL in organic media

Acylating component	Amino component	Product	DMF/MeCN (vol. %)	t /h[a]	Y (%)[b]	τ_{HPLC} /min[c]	Amino acid composition/nmol[d]
Z—Ala—Ala—Leu—OMe	Phe—pNA	Z—Ala—Ala—Leu—Phe—pNA	60/40	2	90	25.5[e]	Ala 10.4 (2), Leu 5.5 (1), Phe 5.2 (1)
Z—Ala—Ala—Leu—OMe	Phe—pNA	Z—Ala—Ala—Leu—Phe—pNA	80/20	25	90	25.5[e]	
Z—Ala—Ala—Leu—OMe	Phe—pNA	Z—Ala—Ala—Leu—Phe—pNA	95/5	74	72	25.5[e]	
Z—Ala—Ala—Leu—OH	Phe—pNA	Z—Ala—Ala—Leu—Phe—pNA	60/40	2	90	25.5[e]	
Z—Ala—Ala—Lys—OH	Phe—pNA	Z—Ala—Ala—Lys—Phe—pNA	60/40	2	90	25.2[f]	Ala 7.8 (2), Lys 3.8 (1), Phe 3.9 (1)
Z—Ala—Ala—Glu—OH	Phe—pNA	Z—Ala—Ala—Glu—Phe—pNA	60/40	2	98	27.0[f]	Ala 8.3 (2), Phe 4.1 (1), Glu 4.0 (1)
Z—Ala—Ala—Lys—OH	Asp—pNA	Z—Ala—Ala—Lys—Asp—pNA	60/40	2	98	24.0[f]	Ala 6.4 (2), Lys 3.1 (1), Asp 3.3 (1)
Z—Ala—Ala—Glu—OH	Asp—pNA	Z—Ala—Ala—Glu—Asp—pNA	60/40	4	74	26.0[f]	Ala 8.8 (2), Glu 4.1 (1), Asp 4.5 (1)
Z—Ala—Ala—Leu—OH	Asp—pNA	Z—Ala—Ala—Leu—Asp—pNA	60/40	24	92	31.5[f]	Ala 9.6 (2), Leu 4.9 (1), Asp 4.7 (1)

[a] t is the reaction time.
[b] Y is the yield.
[c] τ is the retention time.
[d] The number of the amino acid residues is given in parentheses.
[e] HPLC on column *1* using the gradient A (see the Experimental section).
[f] HPLC on column *2* using the gradient B (see the Experimental section).

bilized enzymes were tested for synthetase activity, which demonstrated that covalent immobilization of proteinases on hydrophilic cryogels is a promising procedure for adaptating the enzymes to functioning in nonaqueous media. The biocatalysts obtained are characterized by high stability in water and polar organic solvents. The advantages of new biocatalysts are their ability to efficiently catalyze the synthesis of peptides, which are soluble in organic media, without activation and protection of ionogenic groups of polyfunctional amino acids, the possibility of the repeated use of biocatalysts without essential loss of their activity, and the fact that they can be readily removed from the reaction mixture.

Experimental

The experiments were carried out with the use of serine proteinase from *Bacillus subtilis* (strain 72; subtilisin 72, EC 3.4.21.14), which was isolated from a culture liquid according to a known procedure,[14] thermolysin (EC 3.4.24.1) (Fluka, Switzerland), MeCN for HPLC (Lekbiopharm, Russia), DMF (analytically pure grade, Reakhim, Russia), which was additionally purified according to a procedure reported previously,[19] trifluoroacetic acid (TFA; analytically pure grade, Fluka Chemie AG, Switzerland), Phe—pNA (Serva, Germany), and Asp—pNA (Bachem Bioscience Inc., USA). Other derivatives of amino acids and peptides were synthesized according to standard procedures.[20]

Granules of poly(vinyl alcohol) cryogel (PVAC) approximately 1 mm in diameter were prepared from poly(vinyl alcohol) 16/1 (NPO Azot, Severodonetsk, Ukraine) according to a known procedure.[21] The preparation of reactive aldehyde- and epoxide-containing PVAC derivatives and the covalent attachment of the enzymes were carried out according to procedures, which involved the treatment of granules of the initial cryogel with glutaraldehyde in an acidic medium and epichlorohydrin in an alkaline medium followed by incubation of the activated support with an enzyme solution.[5] The amount of the immobilized enzyme was estimated based on the data from amino acid analysis.

Peptide analysis by HPLC was carried out on an Altex Model 100A liquid chromatograph (USA) equipped with Microsorb-MV C_8 (4.6×250 mm; Rainin Instrument Company, Inc., USA) (*1*) and Nucleosil C18 (4×250 mm; Biochemmack, Russia) columns (*2*). Elution was performed using a linear gradient of MeCN in water, which contained 0.1% TFA, from 10 to 70% during 26 min (A) and from 20 to 80% during 35 min (B). The elution rate was 1 mL min^{-1}. The detection was carried out at 220 and 280 nm. In the calculations of the compositions of the reaction mixtures (based on absorption at 220 nm), the difference between the extinction coefficients of the components was ignored.

Amino acid analysis was performed on an automated Hitachi-835 analyzer (Japan) after acid hydrolysis of peptides and specimens of the immobilized enzymes in 5.7 *M* HCl at 105 °C for 48 h.

The optical absorption measurements for the solutions under study were carried out on Specord UV VIS (Germany) and Shimadzu UV-1601 (Japan) spectrophotometers.

The activity of subtilisin was determined according to a procedure described previously.[14] The activity of thermolysin was determined according to a known procedure.[15]

Determination of the activity of ISL. An ISL specimen (20—30 mg; the protein content was 0.08 mg) was suspended in a 0.05 *M* Tris—HCl buffer (pH 8.3, 2 mL) containing $CaCl_2$ (1.5 mmol L^{-1}) and then a solution of Glp—Ala—Ala—Leu—pNA in DMF (50 μL, 5 mg mL^{-1}) was added. The resulting mixture was incubated with stirring at ~20 °C for 10 min. The absorption was measured at 410 nm (A_{410}) at regular intervals. The specific activity (μmol (min mg of the protein)$^{-1}$) was calculated according to the formula

$$\alpha_{spec} = (A_{410} - A^{c}_{410})V^{o}/8.9m^{e}t,$$

where A_{410} is the absorption of the mixture at 410 nm, A^{c}_{410} is the absorption of the reference solution, V^{o} is the total volume of the sample (mL), t is the reaction time (min), m^{e} is the weight of immobilized subtilisin (mg), and 8.9 is the molar absorption coefficient of *p*-nitroaniline (mmol L^{-1} cm^{-1}).

Determination of the activity of ITL. An ITL specimen (35—50 mg; the protein content was 0.16 mg) was suspended in a 0.05 *M* Tris—HCl buffer (pH 7.3, 1 mL) containing Dnp—Gly—Gly—Ile—Arg—NH_2 (0.5 mg). The resulting mixture was incubated with stirring at 37 °C for 30—60 min. Then the solution was decanted from granules of the biocatalyst and 50% AcOH (0.2 mL) was added. The resulting solution was passed through a column with a SP Sephadex C-25 ion-exchanger (3 mL) and the column was eluted with 0.5 *M* AcOH (2 mL). The absorption of the eluate was measured at 360 nm (A_{360}). The specific activity (μmol (min mg of the protein)$^{-1}$) was calculated according to the formula

$$\alpha_{spec} = (A_{360} - A^{c}_{360})\ V^{o}/15m^{e}t,$$

where A_{360} is the absorption of the mixture at 360 nm, A^{c}_{360} is the absorption of the reference solution, V^{o} is the total volume of the sample, t is the reaction time (min), m^{e} is the weight of the immobilized thermolysin (mg), and 15 is the molar absorption coefficient of 2,4-dinitrophenylethylenediamine (mmol L^{-1} cm^{-1}).

Synthesis of Z—Ala—Ala—Leu—Phe—pNA (typical procedure). An ISL specimen (80 mg; the protein content was 0.3 mg, 15 nmol), which has been preliminarily washed with MeCN (1 mL) and then twice washed with a MeCN—DMF mixture (1 mL) of the corresponding composition, was added to a solution of Z—Ala—Ala—Leu—OMe (5.1 mg, 12 μmol) and Phe-pNA (3.4 mg, 12 μmol) in a MeCN—DMF mixture (400 μL). The reaction mixture was stirred at 20 °C and the aliquots (5 μL) were taken at regular intervals and analyzed by HPLC. After completion of the synthesis, the reaction mixture was separated and the cryogel granules were washed with either the buffer or a DMF—MeCN mixture (40/60 vol. %).

Syntheses of Z—Ala—Ala—Lys—Phe—pNA, Z—Ala—Ala—Glu—Phe—pNA, Z—Ala—Ala—Lys—Asp—pNA, Z—Ala—Ala—Glu—Asp—pNA, and Z—Ala—Ala—Leu—Asp—pNA were carried out analogously to the synthesis of Z—Ala—Ala—Leu—Phe—pNA.

Synthesis of Z—Ala—Ala—Leu—pNA. An ITL specimen (100 mg; the protein content was 0.35 mg, 9.3 nmol), which has been preliminarily washed with MeCN (1 mL) and then twice washed with a DMF—MeCN mixture (25/75 vol. %; 1 mL), was added to a solution of Z—Ala—Ala—OH (2.1 mg, 7 μmol) and Leu—pNA (1.8 mg, 7 μmol) in a DMF—MeCN mixture (25/75 vol. %; 300 μL). The reaction mixture was stirred at 20 °C and the aliquots (5 μL) were taken at regular intervals and analyzed by HPLC. After completion of the synthesis, the reaction mixture was separated and the cryogel granules were washed with the 0.05 *M* Tris—HCl buffer (pH 7.3), τ_{HPLC} = 28 min using the gradient B on a Microsorb-MV C_8 column.

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