Russian Journal of Bioorganic Chemistry, Vol. 31, No. 6, 2005, pp. 529–534. Translated from Bioorganicheskaya Khimiya, Vol. 31, No. 6, 2005, pp. 586–592.

Peptide Synthesis in Organic Media with the Use of Subtilisin 72 Immobilized on a Poly(Vinyl Alcohol) Cryogel

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Received January 14, 2005; in final form, April 14, 2005

Abstract—Subtilisin 72 serine protease (EC 3.4.21.14) immobilized on a poly(vinyl alcohol) cryogel was used as a catalyst in the syntheses of *N*-protected peptide *p*-nitroanilides of the general formulas Z(or Boc)-Xaa-Phe-pNA (Xaa = Leu or Ala), Z-Ala-Xaa-Yaa-pNA (Xaa = Leu or Ala; Yaa = Leu or Phe), and Z-Ala-Ala-Xaa-Yaa-pNA (Xaa = Leu, Arg, or Gly; Yaa = Phe, Leu, Gly, Asp, or Glu). The syntheses were carried out in DMF–acetonitrile mixtures. A number of protected di-, tri-, and tetrapeptides were prepared in yields up to 99%. The syntheses were found to retain stereoselectivity under the conditions studied. The activation of carboxyl group of the acylating component was shown to have a positive effect upon the coupling rate.

Key words: *enzymatic peptide synthesis, poly(vinyl alcohol) cryogel, subtilisin immobilized*

INTRODUCTION

It is known that the study of catalysis of the esterification/transesterification reactions can provide an important information on the properties of hydrolytic enzymes in organic solvent media [1–4].[2] In the case of proteolytic enzymes, this concerns the catalysis of cleavage and formation of peptide bonds. The serine protease-catalyzed kinetically controlled peptide synthesis [5] successfully proceeds in anhydrous or almost anhydrous media. A significant inactivation of the proteases can usually be prevented by their use in suspension or in an immobilized form [6, 7]. Covalent immobilization is one of the most effective methods of stabilization of these enzymes from the inactivating effect of mixtures of polar organic solvents with a low water content. In particular, the immobilization of the subtilisin 72 serine protease on PVA cryogel (macroporous gel formed after freezing–thawing of the aqueous solution of PVA [8]) was shown to effectively stabilize the enzyme for its action under such conditions [9]. The goal of this study is the search for the optimum conditions of the enzymatic peptide synthesis catalyzed by the immobilized subtilisin in organic medium, investigation of the influence of activation of the acylating agent carboxyl group on the rate of product formation under the chosen conditions, and widening of the range of compounds synthesized.

RESULTS AND DISCUSSION

Previously, we studied the effect of the DMF/MeCN ratio on the efficiency of the following model reaction:

$$\text{Z-Ala-Ala-Leu-OCH}_3 + \text{Phe-pNA} \xrightarrow{\text{Sbt}_{\text{im}}} \text{Z-Ala-Ala-Leu-Phe-pNA} + \text{CH}_3\text{OH}, \quad (1)$$

catalyzed by subtilisin 72 immobilized on PVA cryogel and found that this reaction most effectively proceeds at the DMF concentration from 30 to 80%. Therefore, all the syntheses catalyzed by Sbt_{im} in this study were carried out in a 60 : 40 v/v DMF–MeCN mixture (SMS), which was optimal for both the biocatalyst activity and the solubility of starting compounds. The reactions were carried out with equimolar ratio of the amino and carboxyl components (hereinafter, we consider the concentration of starting components as the partial concentrations of the amino and carboxyl components). The molar enzyme–substrate ratio was varied from 1 : 6000 to 1 : 25 600.

The effect of concentrations of the starting reagents on the peptide formation rate was studied in the model reaction (1) catalyzed by the immobilized subtilisin within the range of the substrate concentrations from 0.5 to 200 mM at the constant enzyme concentration (7.8 μM) (Fig. 1). One can see from the chart that the product formation rate increased with the increase in the concentrations of starting reagents. For example,

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[2] Abbreviations: Cam, carbamoylmethyl ($-CH_2CONH_2$); pNA, *p*-nitroanilide; PVA, poly(vinyl alcohol); Sbt_{im}, immobilized subtilisin; and SMS, a standard mixture of solvents, 60 : 40 v/v DMF–MeCN.

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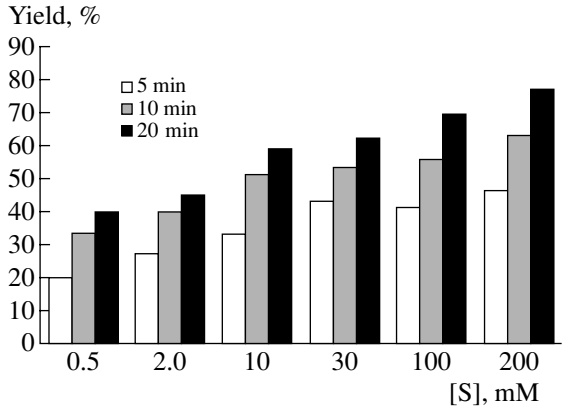

Fig. 1. The dependence of yield of Z-Ala-Ala-Phe-pNA on concentrations of the starting substances after 5, 10, and 20 min.

the difference in yields of the reaction after 5 and 20 min was 18 and 30% for the substrate concentrations of 2 and 200 mM, respectively. Hence, maximal substrate concentrations (the maximum values of the substrate concentrations are believed to be limited by the substrate solubility) are desirable for the most efficient syntheses.

One of the advantages of the enzymatic peptide synthesis is its strict stereoselectivity. However, some changes in the spatial structure of the binding region of the enzymatic active site are not ruled out in organic medium, and we chose to examine whether the stereospecificity of the reactions retained under our conditions. A number of cases of the formation of peptide bond between the amino acid residues one of which has *D*-configuration was described [11, 12]. For example, Cerovsky *et al.* demonstrated [11] that the coupling catalyzed by the Carlsberg subtilisin in organic medium is possible with the use of a large amino component excess containing *D*-amino acid.

The stereospecificity of enzymatic synthesis catalyzed by subtilisin immobilized on PVA cryogel was examined in the reactions similar to the above model reaction using the amino components and acyl components containing *D*-amino acid residues in positions P_1 and P_1' instead of the corresponding *L*-amino acids:

$$\text{Z-Ala-Ala-}D\text{-Leu-OCH}_3 + \text{Phe-pNA} \xrightarrow{\text{Sbt}_{\text{im}}} \ldots$$

$$\text{Z-Ala-Ala-Leu-OCH}_3 + D\text{-Phe-pNA} \xrightarrow{\text{Sbt}_{\text{im}}} \ldots$$

We observed no product in the reaction with *D*-amino acid *p*-nitroanilide even after three days, which suggested that the enzyme preserved its stereoselectivity when the starting reagents were taken at equimolar ratio in anhydrous organic medium.

A number of di- and tripeptides were synthesized with the use of the immobilized subtilisin under the conditions chosen for the model reaction (Table 1). The initial concentrations of starting reagents were 100 mM and, sometimes, 200 mM.

The influence of the length of acylating component (the number of amino acid residues in the acylating component) on the rate of formation of the reaction product was studied by the examples of syntheses of peptides (**1**)–(**5**). Actually, the length of acylating component considerably affected the efficiency of synthesis even at high concentrations of the substrate. For example, at the initial concentrations of the starting reagents of 100 mM, the analytical yield of Z-Ala-Phe-pNA (**1**) was 76% after 19 h, whereas the yield of Z-Ala-Leu-Phe-pNA (**4**) was 98% after 30 min. Dipeptide (**1**) was also synthesized at higher concentrations of the starting reagents (200 mM), and its yield was 77% after 2 h (data not given in the table). These facts indirectly prove the determining effect of the concentrations of starting reagents on the formation rate of the final product. The results are in a good agreement with the literature information on the peptide synthesis with the use of subtilisin absorbed on celite [13]. According to it, longer peptides are easily synthesized by subtilisin, which could be inferred from [14], where it was demonstrated that the enzyme had a rather large binding site. The subtilisin immobilized of the PVA cryogel retains the same properties.

The effect of carboxyl group activation on the reaction was studied in the syntheses of dipeptides (**1**) and (**2**). As expected, the reactions with the participation of carbamoylmethyl esters proceed more rapidly than those with the participation of methyl esters. The yield of dipeptide (**2**) prepared with the use of Boc-Leu-OCam as an acyl donor achieved 99% already after 30 min, whereas that of product (**1**) prepared with the use of methyl ester (Z-Leu-OMe) was 76% after 19 h. We carried out a control experiment: the synthesis with the carbamoylmethyl ester in the absence of the enzyme and failed to observe the product formation even after three days.

The kinetics of accumulation of the reaction product was thoroughly studied for the synthesis of tetrapeptide Z-Ala-Ala-Leu-Phe-pNA (**6**) (Fig. 2). The most significant differences in the rate of product accumulation were observed within the first 60 min of the reaction. For example, after 30 min, the yields with the use of Z-Ala-Ala-Leu-OH and Z-Ala-Ala-Leu-OMe were 52% and 85%, respectively; the difference in the yields was reduced to 10% after 1.5 h. Obviously, the use of esters as acylating agents provides for an increase in the rates of peptide synthesis in comparison with those achieved with the acylating agents bearing free carboxyl groups. These results agree well with the general concept of the catalytic mechanism of the peptide synthesis by serine proteases and correlates with the earlier published information [10] obtained for lower concentrations of the same substrates (30 mM) and another ratio ([E] : [S] = 1 : 1000).

Table 1. Peptides synthesized using subtilisin 72 immobilized on a PVA cryogel

Carboxyl component	Amino component	Reaction product		Reaction time, h	Content, %
		structure	number		
Z-Leu-OMe	Phe-pNA	Z-Leu-Phe-pNA	(**1**)	19	76
Boc-Leu-OCam	"	Boc-Leu-Phe-pNA	(**2**)	2	99
Boc-Ala-OCam	"	Boc-Ala-Phe-pNA	(**3**)	0.5	99
Z-Ala-Leu-OMe	"	Z-Ala-Leu-Phe-pNA	(**4**)	0.5	98
Z-Ala-Ala-OMe	Leu-pNA	Z-Ala-Ala-Leu-pNA	(**5**)	2	94
Z-Ala-Ala-Leu-OH	Phe-pNA	Z-Ala-Ala-Leu-Phe-pNA	(**6**)	1	63
Z-Ala-Ala-Leu-OMe	"	"	(**6**)	1	93
Z-Ala-Ala-Arg-OH	"	Z-Ala-Ala-Arg-Phe-pNA	(**7**)	24	26
Z-Ala-Ala-Arg-OMe	"	"	(**7**)	24	82
Z-Ala-Ala-Arg-OMe	Glu-pNA	Z-Ala-Ala-Arg-Glu-pNA	(**8**)	24	87
Z-Ala-Ala-Arg-OMe	Asp-pNA	Z-Ala-Ala-Arg-Asp-pNA	(**9**)	24	34
Z-Ala-Ala-Lys-OH	Phe-pNA	Z-Ala-Ala-Lys-Phe-pNA	(**10**)	2	90*
Z-Ala-Ala-Glu-OH	"	Z-Ala-Ala-Glu-Phe-pNA	(**11**)	2	98*
Z-Ala-Ala-Lys-OH	Asp-pNA	Z-Ala-Ala-Lys-Asp-pNA	(**12**)	2	98*
Z-Ala-Ala-Glu-OH	"	Z-Ala-Ala-Glu-Asp-pNA	(**13**)	4	74*
Z-Ala- Ala-Gly-OMe	Phe-pNA	Z-Ala-Ala-Gly-Phe-pNA	(**14**)	2	56
Z-Ala-Ala-Leu-OMe	Gly-pNA	Z-Ala-Ala-Leu-Gly-pNA	(**15**)	2	70

* Data were taken from [10]. The reaction conditions: 60 : 40 v/v DMF/MeCN; [E] 7.8 μM; [S] 100 mM for products (**1**)–(**4**), 200 mM for (**5**)–(**7**), 150 mM for (**8**) and (**9**), 30 mM for (**10**)–(**13**), and 50 mM for (**14**) and (**15**); 20°C.

The chemical synthesis of peptides containing polyfunctional amino acid residues is known to be difficult due to the necessity of protection of the side functional groups. One of the advantages of the application of enzymes for the synthesis of such peptides is the possibility both to decrease the number of stages and to avoid racemization at each step of protection or deprotection.

Previously, we demonstrated that the *N*-acylated tetrapeptides containing Lys, Asp, and Glu in P_1 and P_1' positions (**10**)–(**13**) can be effectively synthesized using the immobilized subtilisin in organic medium with a minimal water content without the protection of side ionogenic groups of polyfunctional amino acids [10]. The reactions were carried out without the activation of carboxyl components.

We also synthesized a number of tetrapeptides (**7**)–(**9**) containing Arg in P_1 position. The concentrations of starting reagents were 200 mM and 150 mM for peptide (**7**) and peptides (**8**) or (**9**), respectively (Table 1). Peptide (**7**) was prepared using the tripeptide with the free carboxyl group and its ester as carboxyl components. The compound with free carboxyl reacted considerably worse than its ester analogue: after a day, yields of the resulting product were 26 and 82%, respectively.

We succeeded in the synthesis of tetrapeptides (**8**) and (**9**) containing differently charged amino acid residues in positions P_1 and P_1' (Arg and Glu or Asp, respectively). After one day, yields of the products were 87 and 34%, respectively. The possibility of such syntheses can be explained by the specificity of the biocatalyst system. One can expect the absence of charge on the ionogenic groups in anhydrous organic medium and, consequently, an effective binding of electroneu-

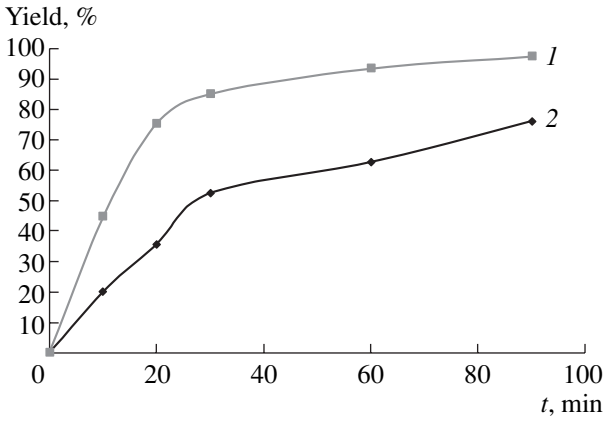

Fig. 2. The synthesis of Z-Ala-Ala-Leu-Phe-pNA (*1*) from Z-Ala-Ala-Leu-OMe or (*2*) Z-Ala-Ala-Leu-OH as the carboxyl components at a starting concentration of 200 mM catalyzed by the immobilized subtilisin in SMS at the [E] : [S] = 1 : 25 600.

Table 2. Amino acid analysis of the peptides synthesized using subtilisin 72 immobilized on a PVA cryogel

Peptide	Amino acid analysis, nmol							Retention time, min (gradient)
	Ala	Leu	Gly	Arg	Asp	Glu	Phe	
Z-Leu-Phe-pNA (**1**)		2.4					2.3	21.9 (A)
Boc-Leu-Phe-pNA (**2**)		1.6					1.8	14.1 (A)
Boc-Ala-Phe-pNA (**3**)								13.5 (A)
Z-Ala-Leu-Phe-pNA (**4**)	1.1	1.1					0.8	21.5 (A)
Z-Ala-Ala-Leu-pNA (**5**)	13.9	6.7						16.8 (A)
Z-Ala-Ala-Leu-Phe-pNA (**6**)	10.4	5.5					5.2	19 (C)
Z-Ala-Ala-Arg-Phe-pNA (**7**)	3.3			1.4			1.3	17.9 (C)
Z-Ala-Ala-Arg-Glu-pNA (**8**)	4.5			2.2		2.1		17 (C)
Z-Ala-Ala-Arg-Asp-pNA (**9**)	43			24	23.5			17 (C)
Z-Ala-Ala-Gly-Phe-pNA (**14**)	2.3		1.6				1.0	24.5 (D)
Z-Ala-Ala-Leu-Gly-pNA (**15**)	2.7	1.3	1.9					15.5 (D)

tral hydrophobic amino acid residues in the active site of subtilisin. Our results also demonstrate that the Arg residue in position P_1 of the substrate negatively affects the efficiency of the synthesis of the corresponding peptides in comparison with Lys and Glu [10] (cf. the syntheses of peptides (**7**)–(**9**) and (**10**)–(**13**), Table 1).

Tetrapeptides (**14**) and (**15**) containing Gly in positions P_1 and P_1' were also synthesized. Note that the reactions proceeded more effectively for the peptides with Gly in position P_1'. After 2 h, the yield of (**14**) and (**15**) was 70%, whereas the yield of (**14**) was only 56%. Therefore, we demonstrated the ability of subtilisin immobilized on a PVA cryogel to catalyze the synthesis of peptides containing Gly in positions P_1 and P_1'. At the same time, an attempt to prepare a peptide containing Pro in position P_1' by this method was unsuccessful; we observed no reaction product even after three days from the beginning of the reaction (data not given).

We also tried to synthesize the peptides containing the Gly-Gly fragment, which often occurs in physiologically active peptides. Both the fragment with a free carboxyl group and various esters (Boc-Met-Gly-Gly-OH, Boc-Gly-Gly-OCam, and Z-Trp-Ala-Gly-Gly-PhCl) were used as acylating components. However, no reaction product was observed after three days in all the cases. This phenomenon is probably associated with the impossibility of the substrate to be bound with the enzyme due to the absence of bulky side radicals.

The potential of the biocatalytic system studied was demonstrated by the example of preparative synthesis of Z-Ala-Ala-Leu-pNA, a chromogenic peptide substrate for serine proteases. The Z-Ala-Ala-Leu-pNA peptide was synthesized by a kinetically controlled coupling of two fragments catalyzed by immobilized subtilisin:

$$Z\text{-Ala-Ala-OCH}_3 + \text{Leu-pNA} \xrightarrow{\text{Sbt}_{\text{im}}} Z\text{-Ala-Ala-Leu-pNA} + \text{CH}_3\text{OH}.$$

The starting compounds were dissolved in SMS. Initial concentrations of the reagents in the reaction mixture were 200 mM. The analytical yield of the reaction product achieved 100% for 2 h at an equimolar ratio of the amino and carboxyl components at the [E] : [S] = 1 : 5300. After one day, the biocatalyst was removed from the reaction mixture and washed with SMS. All the washings were combined and evaporated on a rotary evaporator. The product was characterized by HPLC and amino acid analysis and tested as a substrate for the determination of the hydrolytic activity of the native subtilisin 72; the test was positive.

Thus, a number of *p*-nitoranilides of di-, tri-, and tetrapeptides, including the peptides with basic and acidic amino acid residues, were synthesized without any protection of the side chains of polyfunctional amino acids using subtilisin 72 immobilized on a PVA cryogel in SMS in yields of 26–99% (Table 2). We found that the application of the active carboxyl components facilitate the formation of reaction product. Our studies proved that subtilisin immobilized on a PVA cryogel is a promising catalyst of the stereoselective formation of peptide bond between various amino acid residues under the chosen optimum conditions of the synthesis in the practically anhydrous organic medium.

EXPERIMENTAL

The serine protease from *Bacillus subtilis* strain 72 (molecular mass 27 500 Da) used in this study was isolated from a commercial preparation of the cultural medium and purified according to the procedure [15]. Polyvinyl alcohol 16/1 (PVA, M_r 69000, NPO "Azot,"

Severodonetsk, Ukraine); acetonitrile for HPLC of the os. ch. (high purity) grade (Lekbiofarm, Russia) containing no more than 0.01% of water; and calcium chloride (Sigma, United States), Tris (ICN Biomedicals, United States), trifluoroacetic acid (Fluka Chemie AG, Switzerland), Phe-pNA (Serva, Germany), and Leu-pNA (Bachem Bioscience Inc., United States) of the analytical grade quality were used in this study. Triethylamine and DMF of ch. d. a. (analytical grade) quality (Reakhim, Russia) were additionally purified according to the procedure [16]. The Cam-esters of amino acids and peptides were kindly presented by Yu.V. Mitin (Institute of Protein Research, Russian Academy of Sciences, Pushchino). Other amino acid and peptide derivatives were synthesized in our laboratory according to standard procedures [17].

The reactions were monitored and the peptides were analyzed by HPLC on an Altex Model 110A liquid chromatograph (United States) equipped with the following columns: a Microsorb-MV C_8 (4.6 × 250 mm) (Rainin Instrument Company, Inc., United States) column [elution with a linear gradient of acetonitrile in 0.1% aqueous solution of trifluoroacetic acid (A) from 20 to 100% for 35 min or (B) from 10 to 70% for 26.2 min] and a Nucleosil C_{18} (4.6 × 250 mm, Biokhimmak, Russia) column [elution with a linear gradient of acetonitrile in 0.1% trifluoroacetic acid (C) from 20 to 80% for 35 min or (D) from 10 to 80% for 42 min]. In all the cases, flow rate was 1 ml/min, and the substances were detected at 220 and 280 nm. The amino acid analysis was carried out on an automatic Hitachi-835 amino acid analyzer (Japan) after the acidic hydrolysis of peptides and proteins in 5.7 M HCl at 105°C in vacuum-sealed ampoules for 24 and 48 h.

Preparation of the subtilisin immobilized on a PVA cryogel (Sbt_{im}). Preparation of the reactive aldehyde-containing PVA derivatives and covalent attachment of the enzyme to them were carried out according to the procedure [6]. The quantity of the immobilized protein was determined by amino acid analysis and proved to be 5.4 mg of protein per g of the carrier.

Peptide Syntheses with the Use of Immobilized Subtilisin

Determination of the concentration effect of the starting reagents on the rate of synthesis. Solutions of the starting components of 0.5–200 mM concentrations were prepared by successive dilutions of a solution of Z-Ala-Ala-Leu-OMe (75 mg, 0.16 mmol) and Phe-pNA (46 mg, 0.16 mmol) in a MeCN–DMF mixture (40 : 60 v/v, SMS, 400 μl). A solution of Z-Al-Ala-Leu-OMe and Phe-pNA (200 μl) of the corresponding concentration was added to a preparation of immobilized subtilisin (8 mg with the protein content of 0.043 mg) preliminarily washed with acetonitrile (1 × 1 ml) and SMS (2 × 1 ml). The reaction mixture was shaken on an orbital shaker at 20–22°C, and samples (5–10 μl) were occasionally taken for an HPLC analysis. The samples were diluted with the starting eluent to the final concentration of 0.5–0.7 mM before the application onto the column. The retention time of Z-Ala-Ala-Leu-Phe-pNA was 19.0 min in gradient (B). The amino acid analysis of Z-Ala-Ala-Leu-Phe-pNA: Ala 10.4 nmol, Leu 5.5 nmol, and Phe 5.2 nmol.

Condensation of Z-Ala-Ala-Leu-OMe or Z-Ala-Ala-Leu-OH with Phe-pNA. The 400 mM solutions of Z-Ala-Ala-Leu-OCH_3 (Z-Ala-Ala-Leu-OH) (125 μl) and Phe-pNA (125 μl) in SMS were added to an immobilized subtilisin (10 mg with the protein content of 0.054 mg) preliminarily washed with acetonitrile (1 × 1 ml) and SMS (2 × 1 ml). The reaction mixture was shaken on an orbital shaker at 20°C, and samples (5 μl) were taken for HPLC analysis at regular intervals. The samples were diluted with the starting eluent (20% solution of acetonitrile in water, 1.7 ml) before the application onto the column.

The peptides **Z(Boc)-Xaa-Phe-pNA (Xaa = Leu and Ala), Z-Ala-Xaa-Yaa-pNA (Xaa = Leu and Ala; Yaa = Leu and Phe), Z-Ala-Ala-Arg-Phe-pNA, Z-Ala-Ala-Gly-Phe-pNA, and Z-Ala-Ala-Leu-Gly-pNA** were similarly prepared.

The coupling of Z-Ala-Ala-Arg-OMe with Glu-pNA. A solution of Z-Ala-Ala-Arg-OMe (19.1 mg, 37.5 μmol) in SMS (100 μl) and a solution of HCl × Glu-pNA (37.5 μmol) and triethylamine (37.5 μmol) in SMS (150 μl) were added to the immobilized subtilisin (10 mg with the protein content of 0.05 mg) preliminarily washed with MeCN (1 × 1 ml) and SMS (2 × 1 ml). The reaction mixture was shaken at 20°C on an orbital shaker. The samples for HPLC (5 μl) were taken from the reaction mixture at regular intervals. The retention time of the Z-Ala-Ala-Arg-Glu-pNA product was 17.0 min in gradient C.

A preparative synthesis of Z-Ala-Ala-Leu-pNA (5). A solution (625 μl) of Z-Ala-Ala-OMe (88 mg, 250 μmol) and Leu-pNA (63 mg, 250 μmol) in SMS was added to the subtilisin immobilized on a PVA cryogel (245 mg with the protein content of 1.3 mg). The reaction mixture was shaken on an orbital shaker at 37°C for one day. The samples for HPLC (5 μl) were taken from the reaction mixture at regular intervals. The biocatalyst was separated and washed with SMS (4 × 0.8 ml). The reaction mixture and all the washings were combined and evaporated on a rotary evaporator. The resulting solution was dropwise added to 0.1 M HCl (3 ml) upon a careful stirring and diluted with cool water (10 ml). The precipitated solid was washed with water on a glass filter and dried in a vacuum over NaOH. The yield of Z-Ala-Ala-Leu-pNA was 98 mg (74%); RT 16.8 min in gradient B.

ACKNOWLEDGMENTS

This study was supported by the Russian Foundation for Basic Research, project no. 03-03-32847 and by INTAS, project no. 01-0673.

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