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Native Subtilisin Karlsberg and Modified Subtilisin 72 As Effective Catalysts of Peptide Bond Formation in Organic Media

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Abstract—The activity and stability of native subtilisin Karlsberg and subtilisin 72 and their complexes with sodium dodecyl sulfate (SDS) in organic solvents were studied. The kinetic constants of the hydrolysis of specific chromogenic peptide substrates Z-Ala-Ala-Leu-pNA and Glp-Ala-Ala-Leu-pNA by the subtilisins were determined. It was found that the subtilisin Karlsberg complex with SDS in anhydrous organic solvents is an effective catalyst of peptide synthesis with multifunctional amino acids in positions P_1 and P_1' (Glu, Arg, and Asp) containing unprotected side ionogenic groups.

Key words: *enzymatic peptide synthesis, organic solvents, SDS–subtilisin Karlsberg comple*

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INTRODUCTION

The use of proteases as catalysts of the peptide bond formation in polar organic media is of fundamental importance for the design of new methods of enzymatic synthesis.[2] The application of known enzymes to the catalysis of the peptide bond formation is often limited by their substrate specificity. Therefore, a search for new biocatalysts suitable for the purposeful enzymatic peptide synthesis is topical. It has earlier been shown in our laboratory that subtilisin 72 (both native and modified) is not only highly hydrolytically active, but can also catalyze the formation of peptide bond in organic media between various amino acids, including those that do not correspond the enzyme specificity in hydrolytic reactions [1]. In this work, we set a task to reveal, whether a high activity and wide spectrum of substrate specificities of subtilisin 72 are its unique properties or they are also inherent of subtilisin Karlsberg.

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[2] Abbreviations: Ded, *N*-[2-(2,4-dinitropenylamino)ethyl]amide; Glp, pyroglytamyl; pNA, *para*-nitroanilide; SDS, sodium dodecyl sulfate; and Z, benzyloxycarbonyl. All amino acids belong to the *L* series.

RESULTS AND DISCUSSION

Subtilisin Karlsberg is a widely distributed and commercially available enzyme with a wide substrate specificity; it is very close, but not identical to subtilisin 72 [2].

We chose two subtilisin-specific peptide substrates Z-Ala-Ala-Leu-pNA (**I**) [3] and Glp-Ala-Ala-Leu-pNA (**II**) [4] for a comparative analysis of the hydrolytic capacity of subtilisin 72 and subtilisin Karlsberg preparations. These substrates are close structural analogues, and they only differ in *N*-terminal residues, position P_4 according to the Schechter and Berger nomenclature [5]. The replacement of the bulky aromatic benzyloxycarbonyl group in (**I**) by a hydrophilic residue of pyroglutamic acid in (**II**) affected the substrate solubility: water–organic mixtures containing at least 20% DMSO or DMF should be used to dissolve (**I**), whereas (**II**) is much more soluble in water, which allowed us to decrease the concentration of organic solvents in system to 1–2%. Thus, a comparative use of substrates (**I**) and (**II**) permits us, first, to consider the effect of structurally various substituents in position P_4, which is crucial for the specificity of the whole subtilisin family, on the subtilisin activity and, second, to study the effect of organic solvents on the enzymatic activity.

Table 1. Kinetic constants of the subtilisin Karlsberg-induced hydrolysis of Z-Ala-Ala-Leu-pNA (**I**) and Glp-Ala-Ala-Leu-pNA (**II**)*

Enzyme	Substrate	Content of organic solvent (vol %)	K_m, mM		k_{cat}, s^{-1}		k_{cat}/K_m, s^{-1} mM^{-1}	
			DMF	DMSO	DMF	DMSO	DMF	DMSO
Subtilisin Karlsberg	Z-Ala-Ala-Leu-pNA (**I**)	20	0.19 ± 0.02	0.14 ± 0.01	70.9 ± 1.7	100 ± 1	373.2	714.3
	Glp-Ala-Ala-Leu-pNA (**II**)	2	0.18 ± 0.02	0.13 ± 0.01	64.3 ± 1.4	80 ± 2	357.2	615.4
Subtilisin 72	Z-Ala-Ala-Leu-pNA (**I**)	20	4.0 ± 1.0	0.25 ± 0.02	104 ± 19	77 ± 9	25.7	308.4
	Glp-Ala-Ala-Leu-pNA (**II**)	2	0.25 ± 0.02	–	69 ± 8	–	277.2	–

* A dash designates the lack of experimental data.

One can see from Table 1[3] that the K_m values for both substrates in the case of subtilisin Karlsberg are practically the same in the media containing 2 and 20% of organic solvents (DMF or DMSO). On the other hand, substantial differences were observed in K_m values for the hydrolysis of substrates (**I**) and (**II**) by subtilisin 72. The K_m value is approximately 4 mM for the hydrolysis of (**I**) in 20% DMF, whereas the K_m value is 0.25 mM for (**II**) when the DMF content decreased to 2%. In DMSO, this value decreased for substrate (**I**), but it still remains twice as high in 20% DMSO in comparison with subtilisin Karlsberg. This may be associated with a lower solvent–substrate competition for the enzyme binding site. In the case of subtilisin Karlsberg, the k_{cat} value is practically equal for both substrates and does not depend on the concentration of organic solvent in system. The pass from DMF to DMSO causes only insignificant increase in k_{cat}.

It seems interesting to compare proteolytic coefficients k_{cat}/K_m of subtilisins Karlsberg and 72 in DMF and DMSO. Obviously, in the case of subtilisin Karlsberg, their values insignificantly differ the two substrates both in DMF and DMSO, but, unlike DMF, the k_{cat}/K_m ratio in DMSO is twice as high. This fact supports a lower denaturing effect of DMSO for the enzyme. Unlike subtilisin Karlsberg, the k_{cat}/K_m ratio for subtilisin 72 differs in DMF by one order of magnitude for the two substrates.

A comparison of kinetic parameters of the substrate hydrolysis with subtilisin Karlsberg and subtilisin 72 indicates noticeable differences in enzymatic properties of these closely relative enzymes.

The preparation of noncovalent complexes of enzymes with detergents and polyelectrolytes is one of the most effective methods for the enzyme adaptation to functioning in polar organic solvents [6, 7]. We used this approach for the preparation of subtilisin Karlsberg–SDS complex. According to [8], such complexes must be highly stable in both aqueous media and in anhydrous organic solvents.

The subtilisin Karlsberg–SDS complex was obtained under the conditions earlier described for subtilisins BPN' and 72 [8, 9].

The activity of the subtilisin Karlsberg–SDS complex was monitored by the cleavage of the same chromogenic substrates (**I**) and (**II**) after the dilution of an aliquot of the complex in ethanol with a large volume of water. The values of specific activity of the resulting complex in DMF and DMSO are shown in Table 2.

An analysis of the results suggests that, according to the hydrolysis of (**I**) in 20% DMF, the activities of the native subtilisin Karlsberg and its SDS complex are equal. No stabilizing effect of complex formation was observed in DMSO, as the activity of the native subtilisin Karlsberg was two times higher than the activity of the complex. According to the hydrolysis of (**II**), the specific activity of subtilisin Karlsberg in the complex was rather high, although it was somewhat lower than that of the native enzyme.

We studied changes in the specific activity of the subtilisin Karlsberg–SDS complex during its storage with the goal to determine the effect of complex formation on the enzymatic stability. The activity of the native enzyme in 0.05 M Tris-HCl buffer, pH 8.2, containing 2 mM $CaCl_2$ (buffer A) in the reaction of (**II**) hydrolysis at the initial time point was taken as 100%. The subtilisin Karlsberg–SDS complex retained 75% of its activity when stored in ethanol at +4°C for

Table 2. Specific activity of the SDS-subtilisin Karlsberg complex in the presence of DMF and DMSO

Substrate	Content of organic solvent (vol %)	Specific activity*, μmol/OU_{280} min	
		DMF	DMSO
Z-Ala-Ala-Leu-pNA	20	14 (14)	33 (68)
Glp-Ala-Ala-Leu-pNA	2	52 (75)	57 (100)

* The activity of native enzyme is given in parentheses.

[3] The results of active site titration of subtilisin Karlsberg and 72 were taken into account in the calculation of kinetic constants (see the Experimental section); the concentrations of active sites of subtilisins Karlsberg and 72 were 50 and 75%, respectively.

Table 3. The peptides synthesized using the SDS-subtilisin Karlsberg complex*

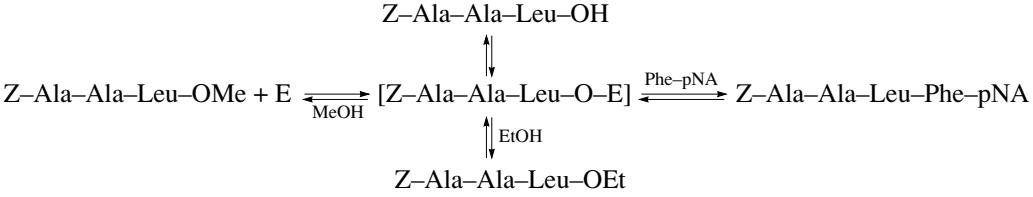
Acylating component	Amino component	Product	Time, h	Yield, %
Z-Ala-Ala-Leu-OCH_3	H-Phe-pNA	Z-Ala-Ala-Leu-Phe-pNA (**1**)	1	95
Z-Ala-Ala-Leu-OCH_3	H-Arg-pNA	Z-Ala-Ala-Leu-Arg-pNA (**2**)	1	90
Z-Ala-Ala-Leu-OCH_3	H-Asp-pNA	Z-Ala-Ala-Leu-Asp-pNA (**3**)	24	60
Z-Thr-Ala-Thr-OCH_3	H-Asp-pNA	Z-Thr-Ala-Thr-Asp-pNA (**4**)	1	98 (90)**
Z-Ala-Ala-Ile-OCH_3	H-Phe-pNA	Z-Ala-Ala-Ile-Phe-pNA (**5**)	24	0
Z-Ala-Ala-Arg-OCH_3	H-Arg-pNA	Z-Ala-Ala-Arg-Arg-pNA (**6**)	1	70
Z-Ala-Ala-Glu-OH	H-Arg-pNA	Z-Ala-Ala-Glu-Arg-pNA (**7**)	1	85
Z-Ala-Ala-Glu-OH	H-Phe-Phe-Ded	Z-Ala-Ala-Glu-Phe-Phe-Ded (**8**)	24	30
Z-Ala-Glu-OH	H-Phe-Phe-Ded	Z-Ala-Glu-Phe-Phe-Ded (**9**)	24	0
Z-Ala-Glu-OH	H-Phe-Ded	Z-Ala-Glu-Phe-Ded (**10**)	24	0
Z-Ala-Glu-OH	H-Phe-pNA	Z-Ala-Glu-Phe-pNA (**11**)	24	60

Notes: * Coupling conditions: 30 mM acylating and amino components, 2 μM SDS-subtilisin in ethanol, 20°C.
** The preparative yield of peptide is given in parentheses.

2 months, whereas the native subtilisin Karlsberg was completely inactivated in buffer A after one-day storage at room temperature. The stabilities of the native subtilisin 72 and its SDS complex are also shown in Fig. 1 for comparison. One can see that the stability of the SDS–subtilisin 72 complex was also higher than that of the native enzyme.

The subtilisin Karlsberg–SDS complex was tested in the reactions of enzymatic peptide synthesis in the media with a high concentration of organic solvents.

As a model reaction, we chose the reaction of Z-Ala-Ala-Leu-Phe-pNA formation from Z-Ala-Ala-Leu-OMe and Phe-pNA, which was thoroughly studied for the subtilisin 72–SDS complex [9]. It proceeded according to the scheme:



The structure and length of the starting compounds corresponded to the subtilisin specificity, which ensured their good binding to the enzyme active site. Equimolar amounts of amino and carboxy components were introduced into the reaction. The [E]/[S] molar ratio was $1 : 5 \times 10^3$. The reaction proceeded in anhydrous polar organic solvents: DMF or DMSO and ethanol. The dependence of the product yield on the enzyme concentration was studied in the model reaction of Z-Ala-Ala-Leu-Phe-pNA (**1**) formation in the presence of the subtilisin Karlsberg–SDS complex in DMF (Fig. 2, curve *1*). The maximal yield (98%) was achieved after 0.5 h at the enzyme concentration exceeding 5 μM. For a comparison, the data on the efficiency of the use of subtilisin 72–SDS complex in a similar reaction are shown in this figure (Fig. 2, curve *2*) [9]. The comparison demonstrates that the synthase activity of the subtilisin Karlsberg–SDS complex was slightly higher in this reaction than that of a similar complex of subtilisin 72.

We also carried out a similar model reaction in the medium containing DMSO with the goal to determine the effect of the nature of organic solvents on the efficiency of the peptide bond formation catalyzed by the subtilisin Karlsberg–SDS complex. The yield of Z-Ala-Ala-Leu-Phe-pNA at the complex concentration of 1 μM achieved 90% in DMSO, whereas it was only 50% in DMF.

The study of the synthase activity of the subtilisin Karlsberg–SDS complex by the example of the model reaction showed that it is a rather effective catalyst of the peptide bond formation. With Z–Ala–Ala–Leu–OCH_3 as an acylating component and the SDS–subtilisin complex as a catalyst, we synthesized tetrapeptides containing residues of unprotected ionogenic multifunctional acids, Arg and Asp, in position P_1' (Table 3). A high efficiency of the complex was observed in the synthesis of peptide (**2**), whose content in the reaction mixture was 90% after 1 h, whereas only 60% of peptide (**3**) was obtained after 24 h. At the same time, the

synthesis of peptide (**4**) with Asp-pNA as an amino component proceeded quantitatively already after 1 h. Apparently, the replacement of Leu by a Thr residue in position P_1 affected the efficiency of the synthesis. On the other hand, the replacement of Leu by a β-branched Ile residue in position P_1 did not result in the formation of Z-Ala-Ala-Ile-Phe-pNA (**5**) even after 2 days. This agrees well with the results on the subtilisin Karlsberg specificity in the hydrolysis of the substrates, in which amino acid residues preferable in position P_1 can be arranged in the following order: Leu > Phe > Val >Ile [10].

By the example of the peptide (**6**) synthesis, we showed an ability of effective use of the subtilisin Karlsberg–SDS complex as the catalyst for the peptide bond formation between two Arg residues. The yield of the target product achieved 70% after 1 h, whereas it did not exceed 30% after 17-h reaction catalyzed by the subtilisin 72–SDS complex [11].

We synthesized tetrapeptide (**7**) containing oppositely charged amino acid residues Glu and Arg in positions P_1 and P_1' in 85% yield for 1 h. The fundamental possibility of this synthesis can be explained by a specificity of the biocatalytic system: anhydrous organic solvents allow the avoidance of the charge appearance on ionogenic groups, whereas electroneutral hydrophobic amino acid residues are good bound by the subtilisin active site.

The synthesis of peptide (**8**) was much less successful: the peptide yield was only 30% after 24 h. This may be explained by the use of strongly hydrophobic and sterically voluminous dipeptide Phe-Phe-Ded as an amino component. The use of the same amino component and a truncated dipeptide analogue of Z-Ala-Ala-Glu-OH did not result in product (**9**) even after 3 days. Probably, the presence of a 2,4-dinitrophenylethylenediamine residue in the substrate molecule dramatically affect the substrate–enzyme binding. The syntheses of peptides (**10**) and (**11**) support this hypothesis: the yield of Z-Ala-Glu-Phe-pNA was 60% for a day, whereas no formation of Z-Ala-Glu-Phe-Ded was observed.

We carried out a preparative synthesis of Z-Thr-Ala-Thr-Asp-pNA (**4**), a chromogenic highly specific substrate for the caspaselike protease of the plant origin [12], for the demonstration of catalytic capacity of the tested modified enzyme in peptide synthesis.

The peptide was synthesized by the kinetically controlled reaction from two fragments using the subtilisin Karlsberg–SDS complex in ethanol.

$$\text{Z-Thr-Ala-Thr-OCH}_3 + \text{H-Asp-pNA} \longrightarrow \text{Z-Thr-Ala-Thr-Asp-pNA}.$$

The starting compounds were dissolved in DMSO up to the concentration of 30 mM; the [E] : [S] was 1 : 5000. The enzyme was used as the complex with SDS dissolved in ethanol. The analytical peptide yield achieved 98% for 1 h. The peptide was purified by precipitation with ether from DMF solution. When the whole reaction mixture was dissolved in DMF, the subtilisin Karlsberg–SDS complex was precipitated and, therefore, was easily separated by centrifugation. The

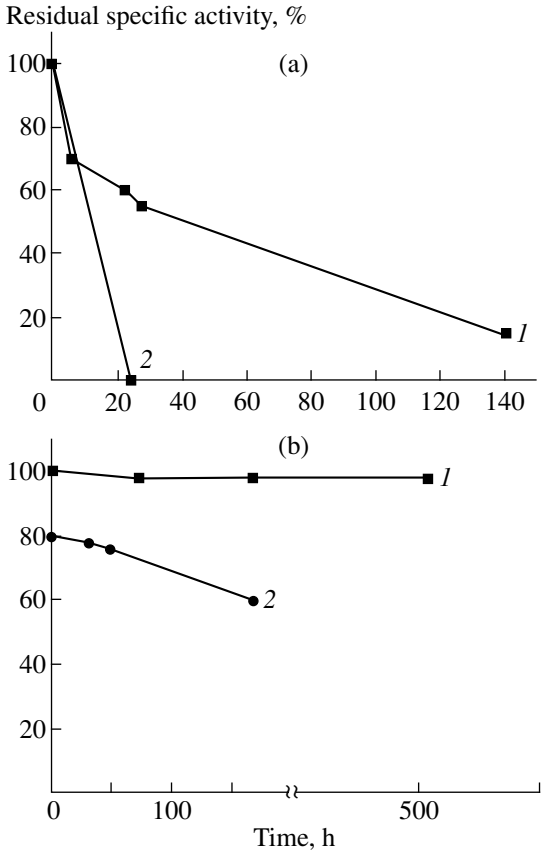


Fig. 1. The time dependence of activities of (a) native (*1*) subtilisin Karlsberg and (*2*) subtilisin 72 in Tris-HCl buffer (pH 8.2) and (b) of their SDS complexes at +4°C.

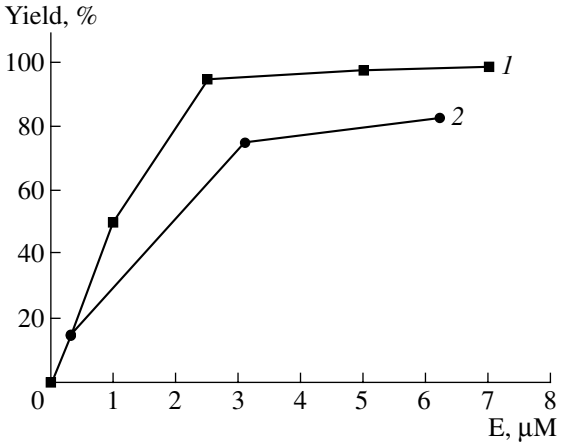


Fig. 2. The yields of Z-Ala-Ala-Leu-Phe-pNA in the syntheses catalyzed by SDS complexes of (*1*) subtilisin Karlsberg and (*2*) subtilisin 72 in ethanol. The synthetic conditions: [S] was 30 mM, 3 : 7 DMF–ethanol (vol), 0.5 h.

yield of the target peptide was 90%. The product was characterized by HPLC and amino acid analysis.

We demonstrated a fundamental possibility of using the resulting substrate for testing the activity of the caspaselike protease from tobacco leaves in preliminary experiments.

To summarize, according to the analysis of results, the subtilisin Karlsberg–SDS complex is an effective catalyst of peptide formation in a mixture of anhydrous organic solvents.

EXPERIMENTAL

Serine proteases from *Bacillus licheniformis*, subtilisin Karlsberg (EC 3.4.21.62, *M* 23 000 Da), and $CaCl_2$ were purchased from Sigma (United States). Subtilisin 72 was isolated from a commercial preparation of the cultural fluid of *Bacillus subtilis*, strain 72 (*M* 27500 Da) and purified as described in [2]. Acetonitrile for HPLC of special purity grade containing no more than 0.01% water was from Lekbiopharm (Russia); DMF of the analytical purity grade was additionally purified as described in [2]. Acetic acid was from Reakhim (Russia), and triethylamine of the analytical purity grade (Reakhim, Russia) was additionally distilled as described in [13]. Trifluoroacetic acid of the analytical purity grade was from Fluka Chemie (Sweden).

Amino acid and peptide derivatives were synthesized in our laboratory by standard procedures [14]. The Z-Ala-Ala-Leu-pNA (**I**) and Glp-Ala-Ala-Leu-pNA (**II**) substrates were synthesized by the procedures [3] and [4], respectively.

Spectrophotometric measurements were carried out on a Specord UV VIS (Germany), a DW-2000_{TM} a UV VIS SLM Aminco (United States), or a Pharmacia LKB Ultrospec III (Sweden) spectrophotometer.

Amino acid analysis was obtained on an automatic amino acid Hitachi 835 analyzer (Japan) after acidic hydrolysis with 5.7 M HCl at 105°C in vacuumated ampoules for 24 and 48 h.

Peptides were analyzed on an Altex Model 110 A liquid chromatograph (United States). The elution with 0.1% CF_3COOH in a linear gradient of acetonitrile (from 10 to 70% for 26 min, A) at a flow rate of 1 ml/min on a Microsorb-MV C_8 column (4.6 × 250 mm, Rainin Instrument Company, Inc.) and in a linear gradient of acetonitrile (from 10 to 50% for 26 min, B) on a Nucleosil C_{18} (4.6 × 250 mm, Biochimmak, Moscow) column. The detection was carried out at 220 and 280 nm. The compositions of reaction mixtures were calculated without corrections to the differences in molar absorption coefficients of components.

Titration of the subtilisin Karlsberg active sites. A solution of *N-trans*-cinnamoylimidazole in acetonitrile (2 mg/ml, 25 μl) was added into a quartz cuvette containing 0.1 M acetate buffer (3 ml), pH 5.05. Changes in optical absorption of the solution at 310 nm were registered (a nonenzymatic substrate hydrolysis). A subtilisin solution in 0.1 M acetate buffer (pH 5.05, 100 μl, 7.6 mg/ml of protein) was added to the resulting solution, and the fall in optical absorption was registered. The active site concentration in the subtilisin preparation was calculated using the *N-trans*-cinnamoylimidazole molar absorption $\varepsilon^{310} = 23\ 800\ M^{-1}\ cm^{-1}$.

The preparation of the subtilisin Karlsberg–SDS complex. A 7 mM solution of sodium dodecyl sulfate in water (0.5 ml) was added to a solution of the subtilisin Karlsberg preparation (2 mg, 70 nmol of protein) in 1 mM $CaCl_2$ (1 ml, pH 5.5). The reaction mixture was stirred for 5 min, and kept for 30 min at room temperature. The resulting precipitate was separated by centrifugation for 15 min at 12 000 rpm, dissolved in ethanol (1 ml), and the optical absorption of supernatant was measured at 280 nm.

The determination of subtilisin enzymatic activity by hydrolysis of Glp-Ala-Ala-Leu-pNA in DMF and DMSO. A 20 mM substrate solution in anhydrous DMF (or DMSO) (10 mg/ml, 50 μl) was added to a 0.05 M Tris-HCl buffer (pH 8.2, 2.5 ml), and the solution was incubated for 10 min at 37°C. A solution of the tested enzyme (0.1 mg/ml, 10 μl) was added, and the mixture was incubated at 37°C until the absorption value achieved 0.1–0.4 at 410 nm. The reaction was quenched by addition of 50% acetic acid (0.5 ml) and A_{410} was measured. The order of the enzyme and acetic acid addition was reverse in reference samples. The specific activity was calculated by the following formula:

$$\frac{(A_{410} - A_{410}^{c})V^{sample}}{A_{280}tV_E \times 8.9},$$

where A_{410}^{c} is the absorption of reference solution at 410 nm; A_{410}, the absorption of the tested solution at 410 nm; A_{280}, the absorption of enzyme solution at 280 nm; V^{sample}, the sample volume (ml); t, the reaction time (min); V_E, the volume of the enzyme sample (ml); and 8.9, the millimolar absorption coefficient of *p*-nitroaniline ($mM^{-1}\ cm^{-1}$).

The enzyme amount necessary for the cleavage from substrate of 1 μmol of *p*-nitroaniline for 1 min under the described conditions is taken as 1 activity unit.

The determination of subtilisin enzymatic activity by the Z-Ala-Ala-Leu-pNA hydrolysis in DMF and DMSO. A 0.05 M Tris-HCl buffer (pH 8.2, 2.0 ml) was added to a thermostatted at 37°C solution of Z-Ala-Ala-Leu-pNA in DMF or DMSO (0.5 mg/ml, 0.5 ml). A solution of the enzyme tested (0.5 mg/ml of protein, 10 μl) was then added and the mixture was incubated at 37°C until the development of yellow color. The reaction was quenched by addition of 50% acetic acid (0.5 ml), and A_{410} was measured. The order of the enzyme and acetic acid addition was reverse in refer-

ence samples. The specific activity was calculated by the above formula.

The activity of the subtilisin Karlsberg–SDS complex dissolved in ethanol was similarly measured by the addition of the solution of subtilisin Karlsberg–SDS complex in ethanol (0.66 mg/ml of protein) to the reaction mixture.

Kinetic studies of the subtilisin Karlsberg-induced hydrolysis of Glp-Ala-Ala-Leu-pNA. Solutions of 0.05 M Tris-HCl buffer (pH 8.2) containing 2 mM $CaCl_2$ (2.5 ml) and Glp-Ala-Ala-Leu-pNA in DMF or DMSO (50 μl) were placed into a thermostatted cell (37°C) to a final substrate concentration of 0.1–0.75 mM, and the enzyme solution in Tris-HCl buffer (pH 8.2, 50 μl) was added. The initial reaction rate was determined spectrophotometrically at 410 nm according to the *p*-nitroaniline formation. The catalytic constant k_{cat} and Michaelis constant K_m were determined by analyzing the reaction rate–substrate concentration dependence using double reverse Lineweaver–Burk coordinates.

Peptide Synthesis

Z-Ala-Ala-Leu-Phe-pNA (conventional procedure). Ethanol (65 μl) and a solution of the subtilisin Karlsberg–SDS complex in ethanol (60 μl, 0.66 mg/ml of protein) were added to a solution of Z-Ala-Ala-Leu-OMe (5.5 μmol) and Phe-pNA (1.4 mg, 5.5 μmol) in DMF (50 μl). The reaction mixture was shaken on an orbital shaker, when periodically taking aliquots (10 μl each) and analyzing them by HPLC in gradient A. RT of **Z-Ala-Ala-Leu-Phe-pNA** (**1**) was 18.4 min. The amino acid analysis (nmol): Ala 10.2, Leu 5.6, and Phe 5.4.

Z-Ala-Glu-Phe-pNA (11) was synthesized in a similar way. RT of **Z-Ala-Glu-Phe-pNA (11)** in gradient B was 26 min. The amino acid analysis (nmol): Ala 3.9, Phe 4.1, and Glu 4.0.

Z-Thr-Ala-Thr-Asp-pNA (4), Z-Ala-Ala-Ile-Phe-pNA (5), and Z-Ala-Ala-Arg-Arg-pNA (6) were synthesized in a similar manner using DMSO to dissolve starting reagents. The HPLC RTs of the peptides (**4**) and (**6**) in gradient A were 16 and 24.5 min, respectively. The amino acid analysis (nmol) for peptide (**4**): Thr 14.8, Asp 7.0; Ala 6.8; and for peptide (**6**): Ala 4.6, Arg 4.4.

Z-Ala-Ala-Glu-Arg-pNA (7). A 1 M triethylamine solution in DMF (7.5 μl) was added to a 400 mM HCl · Arg-pNA in DMF (18.8 μl), the mixture was stirred for 15 min, and the resulting precipitate was separated by centrifugation. The supernatant was poured into 400 mM Z-Ala-Ala-Glu-OH solution (18.75 μl), DMF (30 μl), ethanol (154 μl), and the subtilisin–SDS complex in ethanol (0.66 mg/ml, 20.5 μl) were added. The reaction mixture was stirred at 20°C, and aliquots (10 μl) were taken out at certain intervals and analyzed by HPLC in gradient B. The RT of peptide (**7**) was 29.1 min. The amino acid analysis (nmol): Ala 8.2, Glu 4.0, and Arg 3.9.

Z-Ala-Ala-Leu-Arg-pNA (2), Z-Ala-Ala-Leu-Asp-pNA (3), and Z-Ala-Ala-Glu-Phe-Phe-Ded (8) were synthesized similarly. The HPLC RTs of the peptides (**2**), (**3**), and (**8**) in gradient B were 21.9, 24, and 27.7 min, respectively. The amino acid analysis (nmol) for peptide (**2**): Ala 4.1, Leu 1.9, and Arg 2.0; for peptide (**3**): Ala 6.4, Leu 3.0, and Asp 3.3; and for peptide (**8**): Ala 6.1, Glu 3.1, and Phe 6.2.

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REFERENCES

1. Bacheva, A.V., Baibak, O.V., Belyaeva, A.V., Filippova, I.Yu., Lysogorskaya, E.N., Oksenoit, E.S., Lozinskii, V.I., and Velichko, T.I., *Biokhimiya* (Moscow), 2003, vol. 68, pp. 1567–1574.
2. Akparov, V.Kh., Belyanova, L.P., Baratova, L.A., and Stepanov, V.M., *Biokhimiya* (Moscow), 1979, vol. 44, pp. 886–891.
3. Lyublinskaya, L.A., Voyushina, T.L., and Stepanov, V.M., *Bioorg. Khim.*, 1982, vol. 8, pp. 1620–1624.
4. Lyublinskaya, L.A., Khaidu, I., Balandina, G.N., Filippova, I.Yu., Markaryan, A.I., Lysogorskaya, E.N., Oksenoit, E.S., and Stepanov, V.M., *Bioorg. Khim.*, 1987, vol. 13, pp. 748–753.
5. Schechter, I. and Berger, A., *Biochem. Biophys. Res. Commun.*, 1967, vol. 27, pp. 157–162.
6. Gladilin, A.K. and Levashov, A.V., *Biokhimiya* (Moscow), 1998, vol. 63, pp. 408–421.
7. Meyer, J., Kendryck, B., and Matsuura, J., *Int. J. Pept. Protein Res.*, 1996, vol. 47, pp. 177–181.
8. Wangikar, P.P., Michels, P.C., Clark, D.S., and Dordick, J.S., *J. Am. Chem. Soc.,* 1997, vol. 119, pp. 70–76.
9. Getun, I.V., Filippova, I.Yu., Lysogorskaya, E.N., Bacheva, A.V., and Oksenoit, E.S., *Rus. J. Bioorg. Chem.*, 1999, vol. 25, pp. 526–531.
10. Perona, J.J. and Craik, C.S., *Protein Sci.*, 1995, vol. 4, pp. 337–360.
11. Getun, I.V., Filippova, I.Yu., Lysogorskaya, E.N., and Oksenoit, E.S., *J. Mol. Catalysis, B: Enzymatic*, 2001, vol. 627, pp. 1–6.
12. Chichkova, N.V., Kim, S.H., Titova, E.S., Kalkum, M., Morozov, V.S., Rubtsov, Yu.P., Kalinina, N.O., Taliansky, M.E., and Vartapetian, A.B., *Plant Cell*, 2004, vol. 16, pp. 157–171.
13. Gordon, A.J. and Ford, R.A., *The Chemist's Guide, New York: J. Wiley and Sons*, 1974.
14. Gershkovich, A.A. and Kibirev, V.K., *Khimicheskii sintez peptidov* (Chemical Synthesis of Peptides), Kiev: Naukova Dumka, 1992.