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Subtilisin and α-Chymotrypsin Catalyzed Synthesis of Peptides Containing Arginine and Lysine *p*-Nitroanilides as C-Terminal Moieties

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Abstract—*p*-Nitroanilides of N-acylated di-, tri- and tetrapeptides with C-terminal arginine or lysine residues have been obtained, as a rule with good yields, via acylation of arginine or lysine *p*-nitroanilides by methyl esters of respective N-acylated peptides, catalyzed by subtilisin or α-chymotrypsin. The synthesis might be performed by two routes—by reaction in water–organic solvent mixtures, catalyzed by dissolved enzyme, or by condensation of the components in organic solvents with low water content in the presence of any enzyme distributed over a silica support surface. The second approach seems to be preferable due to suppression of hydrolytic side reactions and improved stability of an enzyme. Subtilisin 72 is especially effective as a catalyst for the acylation of *p*-nitroanilides by N-protected tripeptide methyl esters—the derivatives capable of occupying the S_1, S_2 and S_3 subsites of its extended binding site. Even dipeptide esters with D-amino acids in P_2 position can be applied for *p*-nitroanilide acylation. The efficiency of α-chymotrypsin as a catalyst for peptide synthesis is more limited due to restricted specificity of this enzyme.

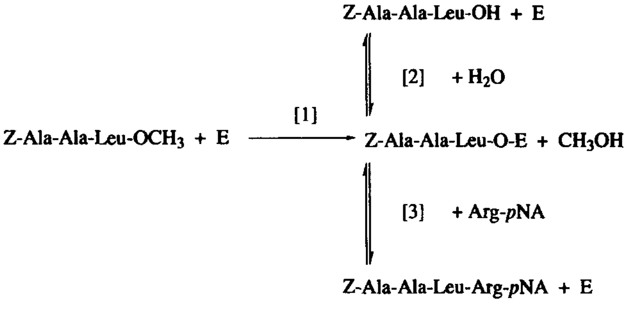
Introduction

Enzymatic peptide synthesis due to its regioselectivity is especially suitable for the preparation of peptides with unprotected functional groups in amino acid side chains. Several proteolytic enzymes effectively catalyze reactions of peptides or their derivatives with the α-amino groups of arginine or lysine esters.[1-5] We decided to apply this approach to obtain peptide derivatives that possess arginine or lysine *p*-nitroanilides as their C-terminal moieties. Peptides of this type are widely used as specific chromogenic substrates for the assay of proteolytic enzymes with trypsin-like specificity patterns, including those involved in hemostasis.[6] Their synthesis by conventional routes are handicapped by the presence of amino or guanidino groups in their side chains. This leads to the application of relevant protecting groups or other specifically designed, often rather complicated and labor-consuming procedures. Enzymatic peptide synthesis allows us to obtain these derivatives by acylation of arginine or lysine *p*-nitroanilides free α-amino groups with esters of N-acylated tri- or dipeptides, thus avoiding the problems connected with the functional groups protection and deprotection.

The aim of this paper was to compare two approaches to enzymatic synthesis of arginine or lysine containing peptide derivatives. The first one is based on the use of the enzyme in water–organic solvent mixtures, the second one previews the application of sorbed enzymes in organic solvents with very low water content.

Results

Two serine proteinases (E)-subtilisin 72[7]—an enzyme analogous to subtilisin Carlsberg—and bovine α-chymotrypsin have been selected as the catalysts for the synthesis of peptide *p*-nitroanilides. Consequently, methyl esters of N-protected peptides form O-acylated enzyme that further reacts with water or arginine *p*-nitroanilide according to the following scheme:



The process belongs to the so-called kinetically controlled peptide synthesis.[8] The peptide product, accumulated in the course of the reaction, is vulnerable to hydrolysis of the newly formed bond by the same

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Abbreviations: Z-, benzyloxycarbonyl-; Boc-, butyloxycarbonyl-; -*p*NA, *p*-nitroanilide; DMF, dimethylformamide; CH_3CN, acetonitrile; Tris, Tris(hydroxymethyl)aminomethane. The notation of enzyme binding subsites corresponds to the Schechter and Berger nomenclature.[25] If not otherwise stated, amino acid residues are of the L-configuration.

enzyme producing Z-Ala-Ala-Leu-OH and arginine *p*-nitroanilide (Reaction 4):

$$\text{Z-Ala-Ala-Leu-Arg-}p\text{NA} + H_2O \longrightarrow \text{Z-Ala-Ala-Leu-OH} + \text{H-Arg-}p\text{NA} \qquad [4]$$

Thus, the yield of the product should pass through its maximal value in the course of the reaction. We assessed the effectiveness of two approaches which might be applied to prepare peptide *p*-nitroanilides by enzyme-catalyzed synthesis. The first one envisaged the synthesis in a mixture of polar organic solvents, e.g. dimethylformamide and water, catalyzed by dissolved enzyme. The second one—the synthesis of these peptide derivatives in organic solvents with a very low water content with the hope in mind of controlling or even excluding hydrolysis reactions 2 and 4. To ensure the enzyme stability in organic solvents, subtilisin and α-chymotrypsin have been distributed on the surface of macroporous glass or silica.[9] The acylation of arginine or lysine *p*-nitroanilides with N-protected di- and tripeptide methyl esters in 20–40% (v/v) DMF proceeds as a typical kinetically controlled enzymatic peptide synthesis.[8] The product accumulation curve shows a characteristic maximum, then its yield drops as a result of Xaa–Arg bond hydrolysis, catalyzed by the same enzyme (Fig. 1).

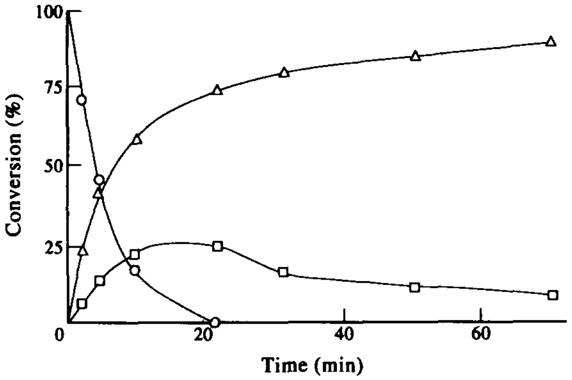

Figure 1. Time course of the reactions observed during peptide synthesis catalyzed by subtilisin in solution.
Conditions applied: DMF - 40%, H_2O - 60%, (v/v), pH 8.0; 37 °C; $[\text{Z-Ala-Ala-Phe-OCH}_3]_0$ = 38.8 mM, $[\text{Arg-}p\text{NA}]_0$ = 19.4 mM, subtilisin 72 - 230 nM. (O) – Z-Ala-Ala-Phe-OCH_3, (Δ) – Z-Ala-Ala-Phe-OH, (□) – Z-Ala-Ala-Phe-Arg-*p*NA.

The synthesis in water–organic solvent mixtures with high DMF concentration allowed us to suppress the hydrolytic reactions—the peptide ester saponification (Reaction 2) and the product splitting (Reaction 4)—due to the reduction of water activity in the reaction mixture. However, high DMF concentration caused a rather pronounced loss of the proteinase activity. Thus, 50% of subtilisin activity has been lost in 62% (v/v) DMF after 30 min and only 18% of the enzyme activity survived 30 min incubation in 92% DMF at 37 °C. Hence, the concentration of DMF in subtilisin-catalyzed reactions, as a rule, should not exceed 70–80%. It should be noted, that rather high relative amounts of both enzymes, approaching an enzyme: substrate 1:7000–1:9000 molar ratio, ought to be introduced into the mixture to compensate for their inhibition.

The yield of Z-Ala-Ala-Leu-Arg-*p*NA prepared in 70% DMF at the molar ratio Z-Ala-Ala-Leu-OCH_3:H-Arg-*p*NA : subtilisin 2:1:1 × 10^{-4} attained 84% (calculated on the basis of the product content in the reaction mixture as assayed by HPLC). The yield of the pure derivative isolated from the reaction mixture equaled 50%. The acylation of H-Arg-*p*NA with Z-D-Ala-Leu-OCH_3, catalyzed by subtilisin (molar ratio 4:1:1.4 × 10^{-4}) gave a 70% yield of Z-D-Ala-Leu-Arg-*p*NA as assayed in the reaction mixture and a 50% yield of the isolated product.

It should be noted that in the course of tetrapeptide *p*-nitroanilide synthesis under the specified conditions after the exhaustion of the starting tripeptide ester - Z-Ala-Ala-Leu-OCH_3, its hydrolysis product - Z-Ala-Ala-Leu-OH became an acylating agent. As a result, the process was gradually transformed from a kinetically controlled to an equilibrium peptide synthesis (Reaction 5).

$$\text{Z-Ala-Ala-Leu-OH} + \text{H-Arg-}p\text{NA} \longrightarrow \text{Z-Ala-Ala-Leu-Arg-}p\text{NA} \qquad [5]$$

This conclusion was confirmed by the direct synthesis of Z-Ala-Ala-Leu-Arg-*p*NA from Z-Ala-Ala-Leu-OH and H-Arg-*p*NA under the same conditions, which gave a 44% yield after 22 h, whereas a 40% yield was attained after 5 min in Reaction 3, obviously proceeding at a much higher rate. On the contrary, we did not observe the direct acylation of arginine *p*-nitroanilide with Z-Ala-Ala-OH in the presence of dissolved subtilisin, apparently due to a weaker interaction of this dipeptide derivative with the enzyme.

Hence, subtilisin-catalyzed acylation of arginine or lysine *p*-nitroanilides in 70% (v/v) DMF turned out to be suitable for the synthesis of the peptide derivatives containing basic amino acid *p*-nitroanilides as their C-terminal moieties.[10] However, this procedure possesses substantial drawbacks—the intensive inhibition and irreversible inactivation of the enzyme, as well as the pronounced hydrolysis of the acylating peptide esters.

Further decrease of the water content in the reaction mixture, aimed to suppress the ester hydrolysis and to shift the reaction towards the synthesis, leads to almost complete enzyme inactivation. To overcome this problem, we turned to peptide synthesis in low water content organic solvents, catalyzed by proteolytic enzymes distributed over a support surface, which retained hydration water, thus protecting the enzyme from its inactivation by the organic solvent.[9,11-13] Although the role of various factors, including the nature of an organic solvent, water content and its distribution within the system, as well as enzyme–surface interaction, is far from being clear, the effectiveness of this procedure has been proven for water-miscible, as well as for water-unmiscible solvents, which contained only a low percentage of water.

Because of a poor solubility of arginine and lysine *p*-nitroanilide in acetonitrile, a solvent commonly used for enzymatic peptide synthesis, DMF was introduced into the mixture. The 28% of DMF in acetonitrile found empirically, allowed the achievement of the necessary concentrations of lysine and arginine *p*-nitroanilides.

Under these conditions the splitting of the synthesis product (Reaction 4) was negligible and the hydrolysis of the starting peptide ester (Reaction 2) was suppressed, therefore, rather high yields of tetrapeptide *p*-nitroanilides were achieved (Fig. 2). Thus, the reaction:

$$\text{Z-Ala-Ala-Phe-OCH}_3 + \text{H-Arg-}p\text{NA} \xrightarrow{\text{subtilisin}} \text{Z-Ala-Ala-Phe-Arg-}p\text{NA}$$

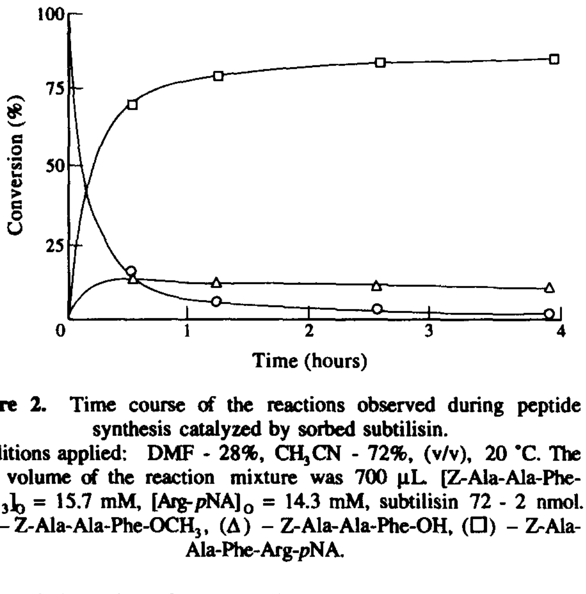

Figure 2. Time course of the reactions observed during peptide synthesis catalyzed by sorbed subtilisin.

Conditions applied: DMF - 28%, CH_3CN - 72%, (v/v), 20 °C. The total volume of the reaction mixture was 700 µL. $[\text{Z-Ala-Ala-Phe-OCH}_3]_0$ = 15.7 mM, $[\text{Arg-}p\text{NA}]_0$ = 14.3 mM, subtilisin 72 - 2 nmol. (○) – Z-Ala-Ala-Phe-OCH_3, (△) – Z-Ala-Ala-Phe-OH, (□) – Z-Ala-Ala-Phe-Arg-*p*NA.

gave 82% yield of the product after 1.5 h. Under the same conditions, but in the absence of H-Arg-*p*NA, 19% of the ester was hydrolyzed to Z-Ala-Ala-Phe-OH. Although, the latter compound was still capable of interacting with H-Arg-*p*NA thus forming tetrapeptide *p*-nitroanilide, the reaction proceeded rather slowly. In a control experiment only 34% yield of Z-Ala-Ala-Phe-Arg-*p*NA from Z-Ala-Ala-Phe-OH and H-Arg-*p*NA was obtained after 24 h. It became clear that the latter reaction played a limited role under these conditions, whereas H-Arg-*p*NA acylation with the peptide ester dominated the process.

Subtilisin 72 sorbed on macroporous glass or silica (silochrom) has catalyzed the synthesis of a number of tetrapeptide *p*-nitroanilides of the general formula Z-Ala-Ala-Xaa-Yaa-*p*NA, where Xaa = Leu, Ala, Ile, Phe, Tyr, Trp, Met, His and Yaa = Lys or Arg (Table 1). Mostly, rather good yields of the products were achieved.

As a rule, three binding subsites of subtilisin—an enzyme especially sensitive to the length of a substrate—S_1, S_2 and S_3 have to be occupied by the protected peptide ester for an effective acylation of an amino component: amino acid *p*-nitroanilide, which is presumably bound at S_1' and (via *p*-nitroanilide moiety) at S_2' subsites. Therefore, N-protected tripeptide esters suit substantially better for the enzymatic condensation with the amino components, than the respective dipeptide ester derivatives. The lower reaction rates of the latter compounds can be compensated by an increase in the enzyme amount in the reaction mixture.

It appears that α-chymotrypsin performs the condensation of N-protected dipeptide esters more effectively, although pronounced specificity requirements of its S_1 subsite restrict the choice of the peptide esters suitable for acylation catalyzed by this enzyme (Table 2). We observed that a small amount of water (about 2%) should be added to the reaction mixture when α-chymotrypsin is used as a catalyst, apparently to provide the enzyme activity.[14] Subtilisin requirement in water addition is by far less pronounced, although it turned out to be useful for repeated application of the sorbed enzyme (see below).

Certain chromogenic peptide *p*-nitroanilides with C-terminal arginine or lysine residues, used as substrates to assay blood proteinases, contain a D-amino acid residue in the P_3 position, that improves their interaction with the enzymes S_3 subsites.[15–17] Therefore, we attempted to apply the N-protected peptide esters, which possessed D-amino acid residues, for acylation of arginine and lysine *p*-nitroanilides. It turned out that dipeptide esters with an N-terminal D-amino acid residue, e.g. Z-D-Ala-Leu-OCH_3 would not react with H-Arg-*p*NA in the presence of sorbed α-chymotrypsin. Instead, it was slowly converted into free Z-D-Ala-Leu-OH due to its spontaneous saponification. In the course of all other reactions studied this process was negligible.

The application of the sorbed subtilisin 72 allowed us to overcome this difficulty and to prepare the N-protected tripeptide *p*-nitroanilides with D-amino acid residues in the P_3 position via synthesis in an organic solvent. Z-D-Ala-Leu-OCH_3 and Z-D-Pro-Phe-OCH_3 successfully served as the acylating agents, provided the content of the catalyst in the mixture was substantially increased to compensate for unfavorable binding of dipeptide esters. Hence D-amino acid residues were acceptable at the S_2 subsite of subtilisin (Table 3). Thus, a two-fold excess of the peptide ester in the reaction:

$$\text{Z-D-Ala-Leu-OCH}_3 + \text{H-Arg-}p\text{NA} \longrightarrow \text{Z-D-Ala-Leu-Arg-}p\text{NA}$$

leads to a 70% yield of the product, whereas only 6% of the ester became hydrolyzed. A four-fold excess of the ester was needed to achieve a comparable yield of the same reaction catalyzed by subtilisin in DMF–water mixture.

The use of the enzyme sorbed on the support, as well as the high conversion of amino acid *p*-nitroanilides into the products, substantially facilitated their isolation and allowed the recycling of the enzyme (Table 3). To compensate for certain activity losses, caused by enzyme leakage from the support and its partial inactivation, the incubation period ought to be gradually increased on each cycle.

The N-protected acylpeptide *p*-nitroanilides thus obtained might be used as substrates for trypsin-like proteinases. We determined the kinetic parameters of trypsin hydrolysis for some of these compounds (Table 4). Our results are consistent with those of Pozsgay *et al.*[18] indicating that bulky amino acid residues like Phe, in the P_2-position were unfavorable for the substrate, increasing respective K_m values.

Discussion

We have found that the condensation of N-protected peptide esters with amino components, catalyzed by subtilisin or α-chymotrypsin, is a convenient method for the preparation of peptide derivatives that contain arginine or lysine *p*-nitroanilides as their C-terminal moieties. The choice between two options—the use of an enzyme dissolved in the water–organic solvent mixture or the application of a proteinase sorbed on a solid support surface to catalyze the reaction in low water content organic solvents—depends on the specific features of the peptide to be prepared. In general, the use of the sorbed enzyme appears to be preferable.

Whereas both procedures require comparable amounts of an enzyme (taking into account the possibility of the sorbed catalysts re-utilization), the application of the sorbed enzyme in the mixture of organic solvents with very low water content presents substantial advantages, being more versatile than the synthesis in the presence of an enzyme in solution. Thus, it often overcomes the limitations resulting from the poor solubility of the starting compounds, a rather common problem afflicting reactions in water–organic solvent mixtures. It also

Table 1. Synthesis of peptide *p*-nitroanilides from N-protected peptide esters ($RCOOCH_3$) and arginine or lysine *p*-nitroanilides (NH_2B) catalyzed by subtilisin 72 (E), sorbed on macroporous glass

Peptide derivative	Reactants molar ratio $NH_2B:RCOOCH_3:E$	$RCOOCH_3$ concentration mM	Conversion of NH_2B %	Yield of the isolated product %
Z-Ala-Ala-Leu → Arg-*p*NA	$1:1.1:1.9 \times 10^{-4}$	17.1	97	70
Z-Ala-Ala-Phe → Arg-*p*NA	$1:1.1:1.9 \times 10^{-4}$	17.1	99*	85
Z-Ala-Ala-Ala → Arg-*p*NA	$1:1.5:8.4 \times 10^{-4}$	21.4	93	83
Z-Ala-Ala-Trp → Arg-*p*NA	$1:1.5:7.5 \times 10^{-4}$	42.1	100*	78
Z-Ala-Ala-Tyr → Arg-*p*NA	$1:1.5:7.5 \times 10^{-4}$	42.8	100*	79
Z-Ala-Ala-Met → Arg-*p*NA	$1:1.5:3.8 \times 10^{-4}$	42.8	100	83
Z-Ala-Ala-His → Arg-*p*NA	$1:1.5:3.8 \times 10^{-3}$	42.8	83**	-
Z-Ala-Ala-Ile → Arg-*p*NA	$1: 2 :1.6 \times 10^{-3}$	41.7	17**	-
Z-Ala-Ala-Ile → Lys-*p*NA	$1: 2 :3.8 \times 10^{-3}$	57.2	48**	-
Z-Ala-Ala-Phe → Lys-*p*NA	$1:1.1:3.8 \times 10^{-3}$	34.2	95	77
Z-Ala-Ala-Leu → Lys-*p*NA	$1:1.1:3.8 \times 10^{-3}$	34.2	85	-
Z-Ala-Ala-Trp → Lys-*p*NA	$1:1.5:3.8 \times 10^{-4}$	42.8	76	-
Z-Ala-Ala-Tyr → Lys-*p*NA	$1:1.5:3.8 \times 10^{-4}$	42.8	75	-
Z-Ala-Ala-His → Lys-*p*NA	$1:1.5:3.8 \times 10^{-3}$	42.8	40**	-

*Reaction time 4 h.

**Reaction time 72 h.

Conditions: 28% DMF, 72% CH_3CN (v/v), 20 °C. Reaction time: 24 h. Arrow shows the peptide bond formed. See also Table 5.

Table 2. Synthesis of tripeptide *p*-nitroanilides catalyzed by α-chymotrypsin (E) sorbed on macroporous glass, from N-protected (di) peptide esters ($RCOOCH_3$) and arginine or lysine *p*-nitroanilides (NH_2B)

Peptide derivative	Reactants molar ration $NH_2B:RCOOCH_3:E$	Conversion of NH_2B %	Yield of the isolated product %
Z-Ala-Phe→Arg-*p*NA	$1:2:3.8 \times 10^{-3}$	97*	75
Z-Phe→Arg-*p*NA	$1:2:7.5 \times 10^{-3}$	97	60
Boc-Gly-Phe→Arg-*p*NA	$1:2:7.5 \times 10^{-3}$	98	44
Boc-Gly-Phe→Lys-*p*NA	$1:2:3.8 \times 10^{-3}$	53	-
Z-Ala-Phe→Lys-*p*NA	$1:2:3.8 \times 10^{-3}$	63	-

*Yield after 6 h.

Conditions: 28% DMF, 2.0% H_2O), 70% CH_3CN (v/v), 20 °C. Concentrations of arginine *p*-nitroanilide: 14.0 mM, lysine *p*-nitroanilide: 28.8 mM. Reaction time: 24 h. Arrow shows the peptide bond formed.

Table 3. Repeated use of subtilisin 72 (E), sorbed on macroporous silica, to prepare the peptide derivatives from N-protected peptide esters ($RCOOCH_3$) and arginine or lysine *p*-nitroanilides (NH_2B)

Peptide derivative	Reactants molar ratio NH_2B:$RCOOCH_3$:E	Cycle	Reaction time h	NH_2B conversion %
Z-D-Ala-Leu→Arg-*p*NA	1:2:5.4 × 10^{-3}	1	39	96
		2	52	96
		3	69	93
	1:2:8.5 × 10^{-3}	1	24	93
		2	42	96
		3	46	95
		4	89	94
Z-D-Pro-Phe→Arg-*p*NA	1:2:1.4 × 10^{-2}	1	24	83
		2	26	70
		3	84	61
Z-Ala-Ala→Arg-*p*NA	1:1.5:4.1 × 10^{-2}	1	1.5	90
		2	1.5	89
		3	20	87
Z-Ala-Ala→Lys-*p*NA	1:1.5:3.4 × 10^{-3}	1	1.5	99
		2	2	93
		3	19	91
		4	72	89

Conditions: 27.6% DMF, 3.4% H_2O, 69% CH_3CN, 20 °C. Concentration of arginine *p*-nitroanilide: 17.2 mM, lysine *p*-nitroanilide: 74mM. Arrow shows the peptide bond formed.

Table 4. Kinetic parameters of the chromogenic substrates hydrolysis with bovine trypsin

Substrate	V_m mmol min^{-1}	K_m mM	k_{cat}/K_m s^{-1}
Z-Ala-Ala-Phe-Arg-*p*NA	6.50 × 10^{-2}	1.8 × 10^{-1}	2.5 × 10^{5}
Z-Ala-Phe-Arg-*p*NA	7.90 × 10^{-2}	9.2 × 10^{-2}	5.4 × 10^{5}
Z-Phe-Arg-*p*NA	2.50 × 10^{-2}	4.0 × 10^{-1}	4.4 × 10^{4}
Z-Ala-Ala-Arg-*p*NA	1.35 × 10^{-1}	1.7 × 10^{-2}	7.2 × 10^{6}
Z-Ala-Ala-Ala-Arg-*p*NA	1.48 × 10^{-1}	3.3 × 10^{-2}	3.8 × 10^{6}
Z-Ala-Ala-Met-Arg-*p*NA	1.38 × 10^{-1}	1.1 × 10^{-1}	7.5 × 10^{5}
Z-Ala-Ala-Trp-Arg-*p*NA	9.30 × 10^{-2}	1.6 × 10^{-1}	4.3 × 10^{5}

Conditions: 0.05 M Tris–buffer (pH 8.15), 1–5% of DMF (v/v), 20 °C. The kinetic parameters were obtained by a non-linear regression of the progress curves. The data represent the arithmetic mean of the values (3–5) lying within a 15% range.

Table 5. Characteristics of peptides obtained by enzymatic synthesis

Peptide	HPLC retention time (min)	$[\alpha]^{20}_{D}$ /c=1, DMF/	Amino acid composition (nmol)
Z-Ala-Ala-Leu Arg-*p*NA	19.3	−2.9°	Ala 24.6, Leu 11.2, Arg 10.3
Z-Ala-Ala-Phe-Arg-*p*NA	20.5	−17.3°	Ala 7.1, Phe 3.5, Arg 3.2
Z-Ala-Ala-Trp-Arg- *p*NA	20.5	−13.3°	Ala 9.3, Trp 4.5, Arg 4.2
Z-Ala-Ala-Tyr-Arg- *p*NA	14.1	−17.5°	Ala 21.0, Tyr 10.5, Arg 9.7
Z-Ala-Ala-Met-Arg- *p*NA	17.5	−16.7°	Ala 7.3, Met 3.5, Arg 3.3
Z-Ala-Ala-Ala-Arg- *p*NA	13.4	−19.1°	Ala 12.7, Arg 3.8
Z-Ala-Phe-Arg- *p*NA	22.3	−17.4°	Ala 5.5, Phe 5.3, Arg 5.0
Z-Phe-Arg- *p*NA	23.1	−1.8°	Phe 6.7, Arg 6.3
Z-Ala-Ala-Arg- *p*NA	11.9	−11.8°	Ala 10.9, Arg 5.2
Z-Ala-Ala-Lys- *p*NA	12.2	−11.9°	Ala 20.5, Lys 12.5
Z-Ala-Ala-Phe-Lys- *p*NA	20.1	−16.1°	Ala 11.5, Phe 5.8, Lys 5.3
Boc-Gly-Phe-Arg-*p*NA	21.2	−2.0°	Gly 3.1, Phe 3.8, Arg 3.3
Z-D-Pro-Phe-Arg- *p*NA	22.5	+13.0°	Pro 8.8, Phe 8.8, Arg 8.3
Z-D-Ala-Leu-Arg- *p*NA	18.8	−3.4°	Ala 6.6, Leu 6.6, Arg 6.3

provides a satisfactory level of enzyme stability, excludes its co-precipitation with the product[19] and suppresses the hydrolytic side reactions, thus increasing the yield and providing more favorable conditions for product isolation. For the most part, a substantially lower excess of N-protected peptide ester is needed when the sorbed enzyme is used. This also simplifies the separation of the product from the reaction mixture.

Contrary to the opinion expressed in the literature,[20] subtilisin, due to its rather broad specificity, could be used for the preparation of various peptide derivatives that contain amino acids with non-protected functions in their side chains, as well as those which usually present difficulties in the course of synthesis, such as tryptophan, methionine, histidine, etc.

Experimental

General

α-Chymotrypsin was purchased from Serva. Serine proteinase from *Bacillus subtilis* strain 72 (subtilisin 72), purified by affinity chromatography in this laboratory[21] possessed specific activity of 33.3 μmol mg^{-1} min^{-1} against Z-Ala-Ala-Leu-*p*NA. Macroporous glass CPG-10 (120–200 mesh, specific surface 27.6 m^2 g^{-1}, average pore diameter 1000 $Å^2$ was supplied by Serva. Macroporous silica (silochrom C-80), particle size 0.35–0.5 mm, average pore diameter 450 Å was obtained from Reachim (Russia). *p*-Nitroanilides of arginine[22] and lysine[23] were prepared as described, as well as methyl esters of N-protected di- and tripeptides.[24] Optical rotation was measured with a Perkin Elmer 241 polarimeter. Amino acid analyses were performed on a Biotronic LC-5001 amino acid analyzer after the hydrolysis of the samples with 5.7 M HCl at 105 °C for 24 h. The mixture contained 0.1% of phenol, when Tyr-containing peptides were hydrolyzed. Met was determined as methionine sulphone after the previous oxidation by an H_2O_2:HCOOH (1:9, v/v) mixture. The hydrolysis of tryptophan derivatives was accomplished with 4 M methanesulfonic acid in the presence of 0.2% 3-(2-aminoethyl)indole.

Chromatography

HPLC was performed on a Gilson 704 chromatograph equipped with an Ultrasphere ODS Beckman column 4.6 x 250 mm. 5% CH_3CN (v/v) in water (solution A) and 90% CH_3CN (v/v) in water (solution B) contained 0.05% (v/v) CF_3COOH and 0.05% (v/v) triethylamine. A linear gradient of B from 30 to 72% in 35 min with a 1.5 mL min^{-1} elution rate was used to elute the column. Peptide *p*-nitroanilides were detected at 315 nm, other peptide derivatives at 215 nm. Thin layer chromatography on Silufol (Kavalier, Czechoslovakia) silica sheets was performed in *n*-butanol: pyridine:water:acetic acid (10:15:12:3) with detection in UV, ninhydrine and Cl_2/KI.

Catalyst

Weighed amounts of the enzymes, dissolved in 0.1 M phosphate buffer, pH 8.1 (for subtilisin 72) or pH 7.6 (for α-chymotrypsin), were added to CPG-10 macroporous glass or silochrom to moisten the dry powder, mixed and left to dry in a desiccator over P_2O_5 at 20 °C, then kept at 4 °C.

Synthesis in organic solvents

The analytical experiment. Z-Ala-Ala-Phe-OCH_3 (5.0 mg, 11 μmol) and H-Arg-*p*NA.HBr (3.75 mg, 10 μmol) were dissolved in DMF (200 μL), then CH_3CN (500 μL) and CPG-10 (12 mg) containing 2 nmol of subtilisin 72 were added. The mixture was gently shaken at 20 °C. Periodically 6 μL aliquots of the reaction mixture were taken, diluted with 200 μL CH_3OH, centrifuged and applied onto the HPLC column.

The preparative experiment. Water (300 μL) and CPG-10 (500 mg) containing α-chymotrypsin (22.5 mg, 0.9 μmol) were added to H-Arg-*p*NA•HBr (75 mg, 200 μmol) and Z-Ala-Phe-OCH_3, (154 mg, 400 μmol) dissolved in DMF (4 mL) and CH_3CN (10 mL). The mixture was shaken for 24 h at 20 °C, then the catalyst was filtered off, rinsed with a mixture of DMF and CH_3CN, the filtrates combined, evaporated *in vacuo* to a minimal volume and then treated with dry ethyl acetate. The residue thus obtained was suspended in a small volume of 0.1 M HCl to dissolve co-precipitated H-Arg-*p*NA. Then the suspension was extracted with *n*-butanol, the organic layer separated, concentrated and Z-Ala-Phe-Arg-*p*NA was precipitated by ethyl ether, which gave a 75% yield.

The catalysts reutilization. Z-D-Ala-Leu-OCH_3 (88.3 mg, 250 μmol) and H-Arg-*p*NA•HBr, (46.8 mg, 125 μmol) were dissolved in DMF (2 mL) and CH_3CN (5 mL). To this solution water (250 μmol) and silochrom (400 mg) that contained subtilisin 72 (30.8 mg) were added. After shaking for 24 h, the catalyst was filtered off, rinsed with a mixture of 5 mL acetonitrile, 2 mL DMF and 250 μL water, then added to the solution of the next portion of starting materials. The synthesis was then repeated with increased shaking duration (see Table 3). Combined filtrates were concentrated *in vacuo*. Precipitation with ethyl acetate gave a residue which crystallized gradually. The crystals were filtered off, rinsed with ethyl ether and dried. The total yield of Z-D-Ala-Leu-Arg-*p*NA was equal to 76%.

Synthesis in water–dimethylformamide mixture. Z-Ala-Ala-Leu-OCH_3 (9.09 mg, 20 μmol) and H-Arg-*p*NA•HBr (3.75 mg, 10 μmol) were dissolved in DMF (100 μL) and 0.1 M NH_4HCO_3 (400 μL) pH 8.4, then subtilisin 72 (3.6 mg, 0.12 nmol) in H_2O (7 μL) were added. The mixture was agitated at 20 °C. Periodically 6 μL aliquots of the reaction mixture were taken, diluted with 200 μL of CH_3OH, centrifuged and applied onto the HPLC column.

Tryptic hydrolysis

An aliquot of the peptide *p*-nitroanilide solution in DMF (10–15 μL, concentration 10 mg mL^{-1}) was added to 1 mL of 0.05 M Tris–buffer pH 8.15 that contained 0.15 M NaCl. The mixture was incubated at 37 °C for 5 min, then 10–50 μL of the enzyme solution (with a concentration of 1.2 μM) was added to initiate the reaction. The absorbance increase was measured at regular time intervals. The kinetic constants were calculated from the analysis of the progress curves.

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