PEPTIDE SYNTHESIS CATALYZED BY NATIVE PROTEINASE *K* IN WATER-MISCIBLE ORGANIC SOLVENTS WITH LOW WATER CONTENT*,**

Václav ČEŘOVSKÝ and Karel MARTINEK

Institute of Organic Chemistry and Biochemistry, Czechoslovak Academy of Sciences, 166 10 Prague 6

Received November 3, 1988
Accepted December 3, 1988

Reaction of Ac-Tyr-OEt with HBr.Gly-NH_2, catalyzed by free proteinase K in various water-miscible organic solvents in the presence of triethylamine and 5 vol. % of water, was studied. Some aliphatic alcohols and acetonitrile proved to be suitable solvents. The effect of water content (2%—20%) on the synthesis of Ac-Tyr-Gly-NH_2 was studied using acetonitrile as solvent. Lowering of the water content to 5% or 2% led to almost 100% yield of the desired dipeptide; higher water content accelerated the reaction, reducing at the same time the yield of Ac-Tyr-Gly-NH_2 due to the concurrent hydrolysis of the ester Ac-Tyr-OEt. No reaction was observed in the absence of base (triethylamine), whereas an excess of base only retarded the reaction. The enzyme is capable of catalyzing the peptide bond synthesis with N-acylamino acids or N-acyl peptides as acylating components, which may contain all types of L-amino acid residues (except Pro) in the P_1 position. However, the peptide bond synthesis depends strongly on the amino component composition, particularly on the amino acid residue in the P'_1 position. Only amides of glycine and of hydrophilic amino acids were acylated with Ac-Tyr-OEt; amides of hydrophobic amino acids enter the reaction only reluctantly or not at all. The presence of Leu or Phe in position P'_2 and Leu in position P'_3 has not so negative effect on acylation of the amino component as has its presence in the P'_1 position. The choice of protecting groups for the α-carboxyl of the amino component is restricted only to amide and in some cases its undesired enzymatic removal was observed. Unprotected peptides seem to be suitable amino components.

Potentialities of using proteolytic enzymes for the synthesis of peptides in organic solvents, containing low concentration of water, have been studied recently. Because of the still generally accepted opinion that higher concentration of organic solvents in the reaction medium strongly reduces the activity of native proteases[1], great attention has been paid to their stabilization, preferably by chemical modification. Polyethylene glycol-modified α-chymotrypsin[2] and thermolysin[3] were utilized for studying the peptide bond synthesis in benzene in the presence of acetone or methanol,

* Part CCXII in the series Amino Acids and Peptides; Part CCXI Collect. Czech. Chem. Commun. *54*, 266 (1989).

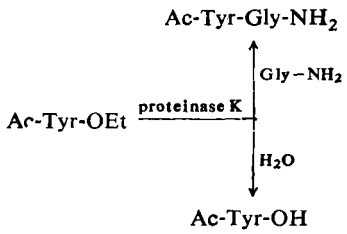
** A part of this paper was presented at the IUB Congress in Prague, 1988, as poster MO: 665.

the water concentration in these systems being lower than 0·1%. In both cases, the target peptides were obtained in considerably high yields. Another way how to stabilize proteases against organic solvents consists in their immobilization on insoluble carriers. Papain, immobilized on polyamide[4], was employed in an investigation of peptide bond synthesis in acetonitrile with water content lower than 0·5%; in 4-methylpentan-2-one as solvent the same enzyme was immobilized on poly(methylmethacrylate)[5]. α-Chymotrypsin, immobilized on polyvinyl alcohol[6] and chitin[7] was used in the synthesis of Ac-Tyr-Gly-NH_2* in ethanol and acetonitrile, respectively, the concentration of water being 5%–10%. The importance of enzyme immobilization for the synthesis in organic solvents was exemplified by a study[8] of α-chymotrypsin-catalyzed peptide bond synthesis in acetonitrile and its dependence on the water content in the system. A special method was applied to the synthesis of model peptides, catalyzed with a suspension of subtilisin[9] in tert-amyl alcohol. The enzyme was first lyophilized from an aqueous buffer and then employed in the reaction as an insoluble "powder" containing the necessary minimum amount of water. In one case, the peptide synthesis was achieved using stabilization by reversed micelles[10]; the reaction, catalyzed by α-chymotrypsin in reversed micelles, did not afford high yield of the desired peptide[11].

In our Laboratory we have focussed our attention on water-miscible organic solvents with low content of water as media for enzymatically catalyzed peptide synthesis. As concerns the solubility of the reaction components, use of such solvents is justified. In contrast to the above-mentioned studies we perform the peptide synthesis in these solvents with native enzymes, without any modification. Using the so-called kinetic approach, we have proven[12] that native α-chymotrypsin catalyzes the peptide bond synthesis in aliphatic alcohols (except methanol) with a low content of water, affording the desired product in fair yields. The medium was kept alkaline by addition of triethylamine and the optimum water concentration in the system was 2% or 5%. Native α-chymotrypsin was used as aqueous solution of a commercial preparation which was added to a solution of the reactants in aliphatic alcohols so as to achieve the desired concentration of water in the system. Conversion of the acylating component was complete within several minutes or hours, depending on the amount of water in the system and on the structure of the reaction components. No similar approach to the enzymatic synthesis of peptides has been used so far. The synthesis of peptide bonds was studied only under thermodynamic equilibrium conditions, using free proteolytic enzymes in organic solvents with varying water content[13]; Ac-Tyr-Gly-NH_2 was synthesized with free α-chymotrypsin[6] in ethanol containing 0% to 15% of water. The yields of products were not high.

* The following abbreviations are used: Ac acetyl, Z benzyloxycarbonyl, Boc tert-butyloxycarbonyl, OMe methyl ester, OEt ethyl ester, OPri 2-propyl ester, OBut tert-butyl ester, N_2H_2Ph phenylhydrazide. All the amino acids mentioned are of the L-configuration.

Our present study concerns the use of proteinase K (EC 3.4.21.14) as another representative of serine proteinases in the synthesis of peptides in water-miscible organic solvents with low content of water. The physicochemical properties of this enzyme are described in a producer's (Merck) brochure[14]. The question of specificity of proteinase K is hitherto not entirely solved. The enzyme has a very broad optimum pH region (7·2 – 10·5) for the cleavage of proteins and exhibits high activity in hydrolysis of esters and amides. Its ability to synthesize peptide bonds was studied in only one case[15], using the kinetic approach; the reaction was performed in an aqueous buffer containing 25% of dimethylformamide. Thanks to the high esterase activity of the enzyme, a great part of the acylating esters (Bz-Tyr-Ala-OEt and Bz-Tyr--Ala-Ala-OEt) was hydrolyzed, the yield of the synthesized peptides being at most 10%. Also our model synthetic reaction (*1*), carried out first in a mixture of an aqueous buffer (pH 9) and dimethylformamide (3 : 2) and analyzed by HPLC, is to a considerable extent accompanied by the undesired hydrolysis of Ac-Tyr-OEt.



(*1*)

The hydrolysis is somewhat suppressed by higher concentration of dimethylformamide; however, the reaction then stops as the result of loss of enzymatic activity (Table I). Therefore, in our further studies we used aliphatic alcohols with

TABLE I

Proteinase K-catalyzed reaction of Ac-Tyr-OEt with Gly-NH_2 in the mixture of 0·2M carbonate–bicarbonate buffer–dimethylformamide (pH 9·0—9·1). Concentration of Ac-Tyr-OEt was 0·1 mol l^{-1}, concentration of HBr.Gly-NH_2 was 0·2 mol l^{-1}, values of pH of the reaction mixtures were adjusted with 1M-NaOH

DMF vol. %	Time min	Ac-Tyr-Gly-NH_2 yield, %	Ac-Tyr-OH yield, %	Ac-Tyr-OEt unreacted, %
40	10	46·5	53·5	0
60	30	67	33	0
70	60	37	4	59
70	240	38·5	4·5	57

low water content (5 vol. %) as the reaction medium, analogously as in our previous work[12]. Data in Table II show that, like α-chymotrypsin, proteinase K is capable of catalyzing the peptide bond synthesis in the medium mentioned, affording the desired product in high yields. Although the reactions were somewhat slower than with α-chymotrypsin[12], the reaction time necessary for 100% conversion of Ac-Tyr--OEt was acceptable. Also in this case, methanol as well as ethylene glycol proved to be unsuitable. Transesterification reactions were observed only with propanols. In dimethylformamide and pyridine (both containing 5 vol. % of water) only the second solvent system gave rise to some (5%) Ac-Tyr-Gly-NH_2 after 1 h as well as 4 h. Beside aliphatic alcohols, also acetonitrile appears to be a good solvent which moreover dissolves better even longer peptides. The effect of water content in acetonitrile on the synthesis of Ac-Tyr-Gly-NH_2 and on the hydrolysis of Ac-Tyr-OEt is well shown by Figs 1*a*–1*d*. When the water concentration was decreased to 2% the cleavage of Ac-Tyr-OEt was almost suppressed, however, at the expenses of longer reaction time. (Under exactly the same reaction conditions, α-chymotrypsin catalyzed the synthesis of Ac-Tyr-Gly-NH_2 much more efficiently; complete conversion of Ac-Tyr-OEt was achieved already after 4 h and the dipeptide was obtained in 98% yield. The α-chymotrypsin-catalyzed reaction of Ac-Tyr-OEt with HCl. Leu--NH_2 in acetonitrile containing 5% of water afforded 91% of Ac-Tyr-Leu-NH_2 and 9% of Ac-Tyr-OH after 15 min.)

For our further studies on proteinase K catalyzed peptide synthesis in organic solvents, we have chosen 5% of water as the optimum concentration (relatively short reaction time and little undesired hydrolysis). The effect of other reaction conditions on the above-mentioned model reaction is shown in Table III. The "optimum" concentrations of the reaction components were selected empirically and are given in the first line of Table III. As seen, a lower concentration of the amino component retards the reaction and somewhat enhances the undesired hydrolysis. On the other hand, the hydrolysis was suppressed by an order of magnitude decrease in the enzyme concentration, with only slight retardation of the reaction. The reaction is substantially influenced by the presence of a base (triethylamine), which is necessary not only to neutralize the employed nucleophile hydrobromide but also to keep basic reaction of the raction medium. In the absence of base, the enzyme cannot catalyze the peptide synthesis and the cleavage of the starting Ac-Tyr-OEt is practically negligible. In acetonitrile with 5% water, Ac-Tyr-OEt was cleaved with proteinase K only in the presence of triethylamine. Importantly, although a great excess of the base retards the synthetic reaction, it has no substantial effect on the yield.

The peptide synthesis using the so-called thermodynamic approach was much slower. In the reaction (*2*),

$$\text{Boc-Tyr-OH} + \text{Gly-NH}_2 \overset{\text{proteinase K}}{\rightleftharpoons} \text{Boc-Tyr-Gly-NH}_2 + \text{H}_2\text{O} \qquad (2)$$

TABLE II

Composition of reaction mixtures from proteinase K-catalyzed reaction of Ac-Tyr-OEt with Gly-NH_2 in aliphatic alcohols and acetonitrile containing 5 vol. % of water

Ac-Tyr-Gly-NH_2 %	Ac-Tyr-OH %	Ac-Tyr-OEt %	Ac-Tyr-OR %	Solvent	Time min
0	0	100	0	methanol	5 to 4 h
10·5	2·5	87	—		15
26	4	70	—	ethanol	60
51	5	44	—		240
74·5	6·5	19	—		24 h
20	2·5	70·5	7		15
50	6	30	14		60
71	10	5	14	1-propanol	240
86·5	13·5	0	0		24 h
25	3·5	71	0·5		15
54	6	38	2		60
82	8·5	6	3·5	2-propanol	240
90	9·5	0	0·5		24 h
9	0·5	90·5	0		15
25	2	73	0		60
50·5	3·5	46	0	1-butanol	240
83	7	10	0		24 h
27·5	5	67·5	0		15
57	8·5	34·5	0		60
83	9·5	7·5	0	tert-butanol	240
89	11	0	0		24 h
12	1	87	0		15
44	3	53	0		60
86·5	6·5	7	0	1-pentanol	240
92	7·5	0·5	0		24 h
1·5	12[a]	86·5	?[a]		8 h
2·5	22·5	75	?		24 h
3·5	32	64·5	?	1,2-ethane-diol	48 h
6	46·5	47·5	?		96 h
32·5	1·5	66	—		15
65	2·5	32·5	—		60
92·5	3	4·5	—	acetonitrile	240
95·5	3·5	1	—		24 h

[a] Ac-Tyr-OH and Ac-Tyr-O-CH_2-CH_2-OH have the same retention time in the given HPLC system.

performed in 2-propanol, containing triethylamine and 5% of water, about 5% of the dipeptide was detected only after 48 h; the formation of Ac-Tyr-OPr^i was not observed.

In our further investigation we studied other potentialities of utilizing proteinase K in the peptide synthesis which are given by its specificity. As follows from Tables

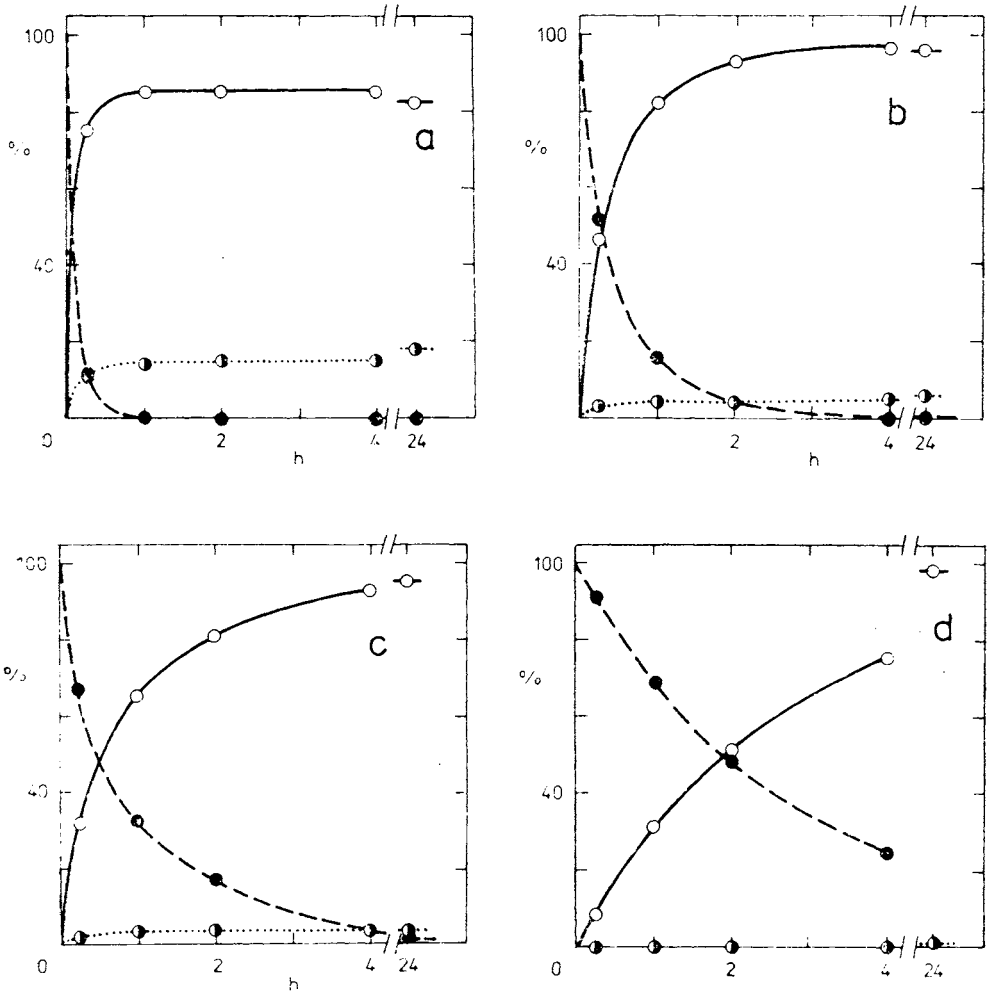


FIG. 1

Composition of reaction mixtures for proteinase K — catalyzed reaction of Ac-Tyr-OEt with Gly-NH_2 in acetonitrile containing varying amount of water (vol. %): a 20, b 10, c 5, d 2. ○ Ac-Tyr-Gly-NH_2, ◐ Ac-Tyr-OH, ● Ac-Tyr-OEt

IV and V, the enzymatically catalyzed reaction with Gly-NH_2 converted practically all acylating components into the desired product of synthesis in fair yields. It is apparent that proteinase K may be employed for peptide bond synthesis, starting from amino acid and peptide derivatives with any amino acid residue, except Pro, in the position P_1. The structure of an amino acid moiety in position P_2 or P_3 should have no substantial effect on the synthesis. The broad specificity of proteinase K for the P_1 amino acid residues was also ascertained by Morihara[16] for cleavage of esters of synthetic substrates; however, larger differences occured in the k_{cat}/K_M values between the individual substrates, with preference for aromatic amino acids in P_1 and for longer peptides. A similar preference has been proven in the cleavage of *p*-nitroanilides of N-protected amino acids and peptides[17,18]. We have observed no undesired cleavage of peptide bonds. The use of acetonitrile instead of 2-propanol is more advantageous not only because of its better solvating properties but also because it excludes the transesterification which with 2-propanol occurs to a considerable extent and which would unfavourably influence isolation of the desired peptides.

Much more complicated is the effect of the amino component structure on the peptide bond formation. The results of synthesis of dipeptides Ac-Tyr-Xaa-NH_2 from amino acid amides (Xaa-NH_2) of various stucture are given in Table VI. The dipeptides were obtained in satisfactory yields only with amides of hydrophilic amino acids and Met-NH_2. The reaction with Tyr-NH_2 was relatively rapid but gave low yield of the dipeptide. It is known from the literature that the oxidized

TABLE III

Proteinase K-catalyzed reaction of 0·1M Ac-Tyr-OEt with Gly-NH_2 in acetonitrile containing 5 vol. % of water under various reaction conditions

[HBr.Gly-NH_2] mol l^{-1}	[Base] mol l^{-1}	[Enzyme][a] mmol l^{-1}	Ac-Tyr-Gly-NH_2 yield, %		Ac-Tyr-OH yield, %		Ac-Tyr-OEt unreacted, %	
			1 h	24 h	1 h	24 h	1 h	24 h
0·2	0·33	0·02	65	95·5	2·5	3·5	32·5	1
0·1	0·11	0·02	26·5	73	0·5	5	73	22
0·2	0·22	0·002	30	96	0	1	70	3
0·2	0·4	0·02	28	91	0·5	4·5	71·5	4·5
0·2	0	0·02	0·2	0·2	1·3	1·3	98·5	98·5
0	0	0·02	—	—	2·5	7	97·5	93
0	0·02	0·02	—	—	44·5	72	55·5	28

[a] Concentration 0·02 mmol l^{-1} corresponds to 0·6 mg of proteinase K per 1 ml of reaction mixture.

TABLE IV

Proteinase K-catalyzed reaction of acyl components with Gly-NH_2 in 2-propanol containing 5 vol. % of water

Acyl component	Product of synthesis yield, % (time, h)	Product of hydrolysis yield, % (time, h)	Product of transesterification yield, % (time, h)
Boc-Tyr-OMe	Boc-Tyr-Gly-NH_2	Boc-Tyr-OH	Boc-Tyr-OPr^i
	19·5 (1) 84 (24)	1·5 (1) 5·5 (24)	2 (1) 6 (24)
Boc-Trp-OMe	Boc-Trp-Gly-NH_2	Boc-Trp-OH	Boc-Trp-OPr^i
	24 (1) 87 (24)	2·5 (1) 5 (24)	2 (1) 6 (24)
Boc-Met-Gly-Trp-OMe	Boc-Met-Gly-Trp-Gly-NH_2	Boc-Met-Gly-Trp-OH	Boc-Met-Gly-Trp-OPr^i
	39 (1) 91 (24)	6·5 (1) 8·5 (24)	0·5 (1) 0·5 (24)
Z-Glu-OMe	Z-Glu-Gly-NH_2	Z-Glu-OH	Z-Glu-OPr^i
	33 (1) 75 (48)	3·5 (1) 11 (48)	2 (1) 5 (48)
Z-Ala-OMe	Z-Ala-Gly-NH_2	Z-Ala-OH	Z-Ala-OPr^i
	61·5 (1) 87 (8)	4 (1) 7 (8)	3·5 (1) 6 (8)
Bz-Arg-OEt	Br-Arg-Gly-NH_2	Br-Arg-OH	Bz-Arg-OPr^i
	35 (4) 65 (48)	10 (4) 19 (48)	3 (4) 7 (48)
Z-Gly-Gly-OMe	Z-Gly-Gly-Gly-NH_2	Z-Gly-Gly-OH	Z-Gly-Gly-OPr^i
	57 (3) 74 (24)	6 (3) 10 (24)	12 (3) 16 (24)

B-chain of insuline is cleaved with proteinase K preferentially at the -Leu-Tyr-bond[19]; preferred cleavage of the -Ala-Ser-bond of RNase[20] and glucose dehydrogenase[21] was also observed. Phe-NH_2 was acylated not at all, Leu-NH_2 and Ala-NH_2 only in low yield with subsequent cleavage of the arising amides Ac-Tyr-Leu-NH_2 and Ac-Tyr-Ala-NH_2. Similar results were obtained in a study of interactions of proteinase K and synthetic peptide substrates[22]. With respect to k_{cal}/K_M values, small amino acid residues such as Ala and Gly were favoured in position P_1'. Peptides with bulky residues such as Phe or Leu in P_1' were hydrolyzed to a lesser extent. The enzymatic cleavage of amide group in N-protected peptides is described in ref.[16], an N-protected amino acid amide has not been cleaved.

Table VII shows the effect of structure of peptide amino components on their acylation with Ac-Tyr-OEt with respect to the peptide chain length, the presence of a bulky amino acid residue in positions P_1', P_2' and P_3', and the type of the protecting group on the α-carboxyl. Because most of the studied peptides were sparingly soluble in acetonitrile, the water content in the reaction medium had to be increased to 20 vol. %. As seen, for amino components consisting exclusively of glycine residues

TABLE V

Proteinase K-catalyzed reaction of acyl components with Gly-NH_2 in acetonitrile containing 5 vol. % of water

Acyl component	Product of synthesis yield, %		Product of hydrolysis yield, %	
	1 h	24 h	1 h	24 h
Z-Ala-OMe	Z-Ala-Gly-NH_2		Z-Ala-OH	
	45·5	95·5	2	4·5
Z-Leu-Ala-OMe	Z-Leu-Ala-Gly-NH_2		Z-Leu-Ala-OH	
	42	94	2·5	4
Z-Gly-Leu-OMe	Z-Gly-Leu-Gly-NH_2		Z-Gly-Leu-OH	
	48	97·5	1·5	2·5
Z-Pro-Leu-OMe	Z-Pro-Leu-Gly-NH_2		Z-Pro-Leu-OH	
	24	82·5	0·5	3·5
Z-Thr-OMe	Z-Thr-Gly-NH_2		Z-Thr-OH	
	9·5	52	1	4
Boc-Met-OMe	Boc-Met-Gly-NH_2		Boc-Met-OH	
	25	87	2	7
Z-Ala-Gly-Pro-OMe	Z-Ala-Gly-Pro-Gly-NH_2		Z-Ala-Gly-Pro-OH	
	0	0	0	0

increasing peptide chain length retards slightly the reaction without affecting the final extent of the synthesis. A reversed dependence has been observed[22] for cleavage of synthetic peptides composed of the $(Ala)_n$-NH_2 ($n = 1-3$) moiety at the C-terminal side of the scissile bond. Also in the case of peptide amino component, a bulky amino acid in position P'_1 hinders the acylation; its shift into position P'_2 or P'_3 makes the acylation possible, however, the negative influence of the bulky moiety on the

TABLE VI

Proteinase K-catalyzed reaction of Ac-Tyr-OEt with amino acid amides in acetonitrile containing 5 vol. % of water

Amino acid amide[a] [Triethylamine], mol l^{-1}	Product of synthesis yield, % (time, h)	Ac-Tyr-OH yield, % (time, h)
	Ac-Tyr-Ala-NH_2	
Ala-NH_2	11·5 (4) 27·5 (96)	2·5 (4) 36·5 (96)
0·02	Ac-Tyr-Ala-OH	
	0 (4) 15·5 (96)	
	Ac-Tyr-Leu-NH_2	
HCl.Leu-NH_2	2 (4) 1·5 (24)	51 (4) 72·5 (24)
0·22	Ac-Tyr-Leu-OH	
	12·5 (4) 20 (24)	
TFA.Met-NH_2	Ac-Tyr-Met-NH_2	
0·22	37 (15 min) 64 (4)	13 (15 min) 33 (4)
HCl.Phe-NH_2	Ac-Tyr-Phe-NH_2	
0·22	0 (4) 0 (24)	36 (4) 55 (24)
Ser-NH_2	Ac-Tyr-Ser-NH_2	
0·02	41 (15 min) 75 (4)	5 (15 min) 25 (4)
Thr-NH_2	Ac-Tyr-Thr-NH_2	
0·02	15 (15 min) 41 (24)	23 (15 min) 55·5 (24)
TFA.Tyr-NH_2	Ac-Tyr-Tyr-NH_2	
0·22	9 (15 min) 28 (4)	61 (15 min) 72 (4)
$(TFA)_2$.Arg-NH_2	Ac-Tyr-Arg-NH_2	
0·44	21·5 (15 min) 68 (4)	3·5 (15 min) 32 (4)
HBr.Glu-NH_2[b]	Ac-Tyr-Glu-NH_2	
0·44	12 (15 min) 19 (4)	35 (15 min) 58 (4)
	Ac-Tyr-Glu-OH	
	0 (15 min) 3·5 (4)	

[a] 0·2M Amino acid amide, 0·1M Ac-Tyr-OEt; [b] in acetonitrile containing 20 vol. % of water.

synthesis is still apparent. Unprotected amino acids cannot be used for the synthetic reaction (no acylation was detected with Gly, Leu, Phe), suitable amino components are free peptides. Similarly, it has been found[16] that proteinase K does not cleave

TABLE VII

Proteinase K-catalyzed reaction of Ac-Tyr-OEt with peptides in acetonitrile containing 20 vol. % of water

Peptide[a] [Triethylamine], mol l^{-1}	Product of synthesis yield, %			Ac-Tyr-OH yield, %		
	15 min	1 h	4 h	15 min	1 h	4 h
$HBr.Gly\text{-}NH_2$	Ac-Tyr-Gly-NH_2					
0·22	75	85	85	12	14·4	15
$HCl.Gly\text{-}Gly\text{-}NH_2$	Ac-Tyr-Gly-Gly-NH_2					
0·22	50	72·5	83	7·5	13·5	16·5
$HCl.Gly\text{-}Gly\text{-}Gly\text{-}NH_2$	Ac-Tyr-Gly-Gly-Gly-NH_2					
0·22	39·5	71·5	83	8·5	13·5	17
Gly-Gly	Ac-Tyr-Gly-Gly					
0·22	62·5	81·5	82	11	16	18
	Ac-Tyr-Gly-Leu					
Gly-Leu	51·5	55	55	21·5	29·5	31
0·22	Ac-Tyr-Gly-Leu-Gly-Leu					
	5	15	14			
	Ac-Tyr-Gly-Gly-Leu					
Gly-Gly-Leu	66	68·5	67	22	26·5	27·5
0·22	unidentified side-product					
	1·5	5	5·5			
	Ac-Tyr-Gly-Phe-Leu					
Gly-Phe-Leu	38	43·5	41	40	51·5	55
0·22	unidentified side-product					
	6	4	4			
Leu-Gly	Ac-Tyr-Leu-Gly					
0·22	1	2	2	80	96·5	98
Gly-Gly-OBu-[b]	Ac-Tyr-Gly-Gly-OBu-					
0·02	4	8	12	13	23	52
Gly-Gly-Gly-N_2H_2Ph	Ac-Tyr-Gly-Gly-N_2H_2Ph					
0·02	31	32	29	50	65	71

[a] 0·2M Peptide, 0·1M Ac-Tyr-OEt; [b] in acetonitrile containing 5 vol. % of water.

Gly from Z-Gly-Leu-Gly but cleaves Gly-Gly from Z-Gly-Leu-Gly-Gly. From the preparative viewpoint it is very unfavourable that α-carboxyl protecting groups, commonly used in the enzymatic synthesis of peptides (tert-butyl ester, phenylhydrazide) make the acylation of a given amino acid completely impossible (attempts to acylate Gly-OBu^t, Gly-N_2H_2Ph and Leu-OBu^t were unsuccessful). A greater separation of the protecting group from the position of the synthesized bond does not improve the situation very much. The preparation of Ac-Tyr-Gly-Leu using Gly-Leu as the amino component was accompanied by formation of Ac-Tyr-Gly-Leu-Gly-Leu as the side-product. The mechanism of its formation is not clear because in the thermodynamically controlled reaction (Eq. (*2*)) the peptide bond synthesis is much slower. Also with Gly-Gly-Leu and Gly-Phe-Leu we detected side-products, but in lesser amounts. Reaction of Ac-Tyr-OEt with Gly-Leu-Gly-NH_2 afforded several side-products which were identified as Ac-Tyr-Gly-NH_2 (predominant), Ac-Tyr-Gly-Leu and Ac-Tyr-Gly-Leu-Gly-Leu by HPLC-comparison with standards. The desired Ac-Tyr-Gly-Leu-Gly-NH_2 was found only as the least populated product.

We used the obtained findings in the preparative synthesis of model peptides. The protected C-terminal oxytocin tripeptide Z-Pro-Leu-Gly-NH_2 was synthesized from Z-Pro-Leu-OMe and HBr.Gly-NH_2 in acetonitrile with 5% of water.

Using the two representatives of serine proteinases, α-chymotrypsin and proteinase K, we have proven in the present and the preceding[12] communication that in native form these enzymes are capable of catalyzing the peptide bond synthesis in water-miscible organic solvents with a low water content. Both the reaction rate and the yield can be influenced by the amount of water in the system. The yields further depend first of all on the character of the reaction components (specificity of the enzymes). In these media, the synthetic potency of both the enzymes is retained for several days and thus no stabilization by immobilization, chemical modification or other pretreatment is necessary. In the case of proteinase K, organic solvents with low water content represent the only reaction medium in which the synthesis of peptide bonds can be achieved in satisfactory yields. Thanks to its low specificity for amino acid residues in the P_1 position, this enzyme can catalyze the peptide bond synthesis practically with all types of L-amino acids in the P_1 positions of the acyl components used. However, its restricted specificity for amino acid residues in the P_1' position, together with the very limited possibility of using common groups for protection of the α-carboxyl in the amino components, make its more general use impossible.

EXPERIMENTAL

Proteinase K and α-chymotrypsin were Serva (F.R.G.) products, activity 9·3 DMC-U/mg and 45 U/mg, respectively, Ac-Tyr-OEt.H_2O was purchased from Koch–Light Laboratories, Ltd., other derivatives of amino acids and peptides were synthesized in our Laboratory by standard

procedures. Their purity was checked by TLC, HPLC or high-voltage paper electrophoresis. The physicochemical properties of known substances were compared with the published data. Thin-layer chromatography (TLC) was carried out on Silufol sheets (Kavalier, Czechoslovakia) in the following systems: 2-butanol–98% formic acid–water (75 : 13·5 : 11·5) (S1), 2-butanol–25% aqueous ammonia–water (85 : 7·5 : 7·5) (S2), 1-butanol–acetic acid–water (4 : 1 : 1) (S3), 1--butanol–pyridine–acetic acid–water (15 : 10 : 3 : 6) (S4). Electrophoreses were performed in moist chamber on a Whatman 3MM paper (20 V/cm) in 1M acetic acid (pH 2·4) and in a pyridine–acetate buffer (pH 5·7). Spots were detected with ninhydrine or by the chlorination method. Melting points were determined on a Kofler block and are uncorrected. Amino acid analyses were done on an AAA 339 analyzer (Mikrotechna, Czechoslovakia), optical rotations were determined on a Perkin–Elmer 141 MCA polarimeter. High performance liquid chromatography (HPLC) was performed on a Spectra Physics SP 8700 instrument equipped with an SP 8400 UV detector and SP 4100 integrator. Analytical HPLC was carried out on a 15 × 0·4 cm column packed with Separon SIX C-18 (7 μm); flow rate 42 ml/h, detection at 280 nm or 254 nm, mobile phase methanol with 0·05% trifluoroacetic acid. For the values of k' the subscript denotes the amount of methanol (%) in the mobile phase used. Preparative HPLC was carried out on a Vydac C_{18} column (20 × 1 cm); flow rate 180 ml/h, gradient elution using the same mobile phase. Stock solutions were prepared by dissolving the commercial proteinase K (0·6 mg) in 0·01M-$CaCl_2$ (50 μl), or the commercial α-chymotrypsin (0·5 mg) in water (50 μl). All the enzymatically catalyzed reactions were executed at 30°C.

Enzymatically Catalyzed Synthesis of Peptides in Organic Solvents Containing 5 vol. % of Water

To a solution of the amino component salt (0·02 mmol) in the given organic solvent (95 μl) was added triethylamine (3 μl, 0·022 mmol) (when using free amino component, only 0·002 mmol of triethylamine was added; amino components with free carboxyl required another equivalent of triethylamine), followed by the acylating component (0·01 mmol). The basicity of the medium was checked by dropping the mixture on a moist pH-indicator paper. Stock solution of the enzyme (5 μl) was added and the mixture was incubated for the time specified in the tables. At regular time intervals aliquots (1 μl) were withdrawn, which were injected into 50% aqueous methanol, containing 0·1% of trifluoroacetic acid (100 μl) and then analyzed by HPLC.

Benzoyloxycarbonylglycyl-glycyl-glycine Amide

Triethylamine (60 μl, 0·44 nmol) was added to a solution of benzyloxycarbonylglycyl-glycine methyl ester (56 mg; 0·2 mmol) and glycine amide hydrobromide (62 mg; 0·4 mmol) in 2-propanol (1·9 ml). A solution of proteinase K (1·2 mg; 100 μl) was added and the mixture was incubated for 24 h. The precipitate was filtered, washed with water and 2-propanol and dried, affording 45 mg (70%) of the product, m.p. 220—223°C. R_F 0·43 (S1), 0·25 (S2), 0·41 (S3), 0·62 (S4), $k'_{40} = 1·97$. For $C_{14}H_{18}N_4O_5$ (323·3) calculated: 52·17% C, 5·63% H, 17·38% N; found: 52·23% C, 5·62% H, 17·51% N.

Benzyloxycarbonylglycyl-leucyl-glycine Amide

Triethylamine (150 μl; 1·1 mmol) was added to a solution of benzyloxycarbonylglycyl-leucine methyl ester (169 mg; 0·5 mmol) and glycine amide hydrobromide (155 mg; 1 mmol). After addition of a solution of proteinase K (3 mg; 250 μl), the mixture was incubated for 24 h, cooled, diluted with water (5 ml) and filtered through a column of Dowex (10 ml) in 50% aqueous methanol. The eluate was concentrated under diminished pressure and filtered through a column

of Amberlite IR-4B (10 ml) in the same solvent. The eluate was taken down and the dry residue crystallized from ethyl acetate–light petroleum. Yield 170 mg (90%) of the product, m.p. 113 to 114°C. R_F 0·65 (S1), 0·54 (S2), 0·67 (S3), 0·71 (S4), $k'_{60} = 1·75$, $[\alpha]_D$ −23·2° (*c* 0·3, methanol). Amino acid analysis: Gly 2·05, Leu 1·00. For $C_{18}H_{26}N_4O_5.0·5\ H_2O$ (387·4) calculated: 55·81% C, 7·03% H, 14·46% N; found: 55·58% C, 6·66% H, 14·35% N.

Benzyloxycarbonylprolyl-leucyl-glycine Amide

Triethylamine (150 µl; 1·1 mmol) was added to a solution of benzyloxycarbonylprolyl-leucine methyl ester (189 mg; 0·5 mmol) and glycine amide hydrobromide (155 mg; 1 mmol) in acetonitrile (4·75 ml). After addition of a solution of proteinase K (3 mg; 250 µl), the mixture was incubated for 48 h. The product was purified by filtration through columns of ion exchangers as described in the preceding experiment. The residue was triturated with ether and crystallized from ethyl acetate, affording 184 mg (88%) of the product, which was chromatographically identical with the compound prepared previously[23]. R_F 0·64 (S1), 0·60 (S2), 0·65 (S3), 0·70 (S4); $k'_{60} = 2·63$. M.p. 162–163°C and $[\alpha]_D$ −67° (*c* 0·3, ethanol) agreed with the reported values[23,24].

Acetyltyrosine-glycyl-leucine

Triethylamine (30 µl; 0·22 mmol) was added to a solution of acetyltyrosine ethyl ester (27 mg; 0·1 mmol) and glycyl-leucine (38 mg; 0·2 mmol) in a mixture of acetonitrile (0·8 ml) and water (0·15 ml). After addition of a solution of proteinase K (0·6 mg; 50 µl), the mixture was incubated for 4 h, cooled, diluted with water (0·6 ml) and filtered through a column of Dowex 50 (2 ml) in 50% aqueous methanol. The eluate was concentrated and the mixture was separated by preparative HPLC using gradient elution. After evaporation of methanol, the pertinent fractions were freeze-dried and the lyophilizates were triturated with hexane. Ac-Tyr-Gly-Leu was obtained in 46% yield (18 mg), m.p. 118–119°C. R_F 0·77 (S1), 0·14 (S2), 0·73 (S3), 0·60 (S4), $k'_{50} = 1·49$. $[\alpha]_D$ +29° (*c* 0·2, methanol). Amino acid analysis: Gly 0·99, Leu 1·08, Tyr 1·00. For $C_{19}H_{27}.N_3O_6.H_2O$ (411·5) calculated: 55·46% C, 7·10% H, 10·22% N; found: 55·28% C, 6·77% H, 9·96% N. Mass spectrum: 394 (M^+).

Ac-Tyr-Gly-Leu-Gly-Leu as the side-product (4·5 mg) had m.p. 133–134°C, R_F 0·79 (S1), 0·19 (S2), 0·75 (S3), 0·62 (S4), $k'_{50} = 5·03$. Amino acid analysis: Gly 1·99, Leu 1·96, Tyr 1·00. For $C_{27}H_{41}N_5O_8.H_2O$ (581·7) calculated: 55·75% C, 7·45% H, 12·04% N; found: 55·58% C, 6·96% H, 11·75% N. Mass spectrum: 564·5 (M^+).

Acetyltyrosyl-threonine Amide

Triethylamine (6 µl; 0·044 mmol) was added to a solution of acetyltyrosine ethyl ester (27 mg; 0·1 mmol) and threonine amide (24 mg; 0·2 mmol) in acetonitrile (0·95 ml). After addition of proteinase K (0·6 mg; 50 µl), the mixture was incubated for 24 h. The product was purified by filtration through columns of ion-exchangers as described above. The residue was triturated with ether, affording 11 mg (34%) of product, m.p. 85–86°C. R_F 0·46 (S1), 0·28 (S2), 0·39 (S3), 0·56 (S4); $k'_{40} = 0·31$, $[\alpha]_D$ +20° (*c* 0·4, methanol). Amino acid analysis: Thr 1·09, Tyr 1·00. For $C_{15}H_{21}N_3O_5.0·5\ H_2O$ (332·4) calculated: 54·20% C, 6·67% H, 12·64% N; found: 53·87% C, 6·33% H, 12·53% N.

The authors are indebted to Mrs Z. Ledvinová for the optical rotation measurements and to Mr J. Zbrožek for the amino acid analyses. Elemental analyses were carried out in the Analytical Laboratory of this Institute (Dr V. Pechanec, Head), mass spectra were measured in the Laboratory of Mass Spectrometry of this Institute (Dr K. Ubik, Head).

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Translated by M. Tichý.