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ENZYMATIC PEPTIDE SYNTHESIS IN FROZEN AQUEOUS SYSTEMS: USE OF N^{α}-UNPROTECTED UNUSUAL ACYL DONORS

Sabine Gerisch, Hans-Dieter Jakubke* and Hans-Jörn Kreuzfeld[1]

Universität Leipzig, Fakultät für Biowissenschaften, Pharmazie und Psychologie, Institut für Biochemie, Talstraße 33, D-04103 Leipzig, Germany

[1] Institut für Organische Katalyseforschung an der Universität Rostock e.V., Buchbinderstraße 5-6, D-18055 Rostock, Germany

Abstract: α-Chymotrypsin (EC 3.4.21.1) was used for catalyzing the reaction of various N^{α}-unprotected non-coded phenylalanine ester derivatives with H-Leu-NH_2 and H-Arg-NH_2 in frozen aqueous solution at -15 °C. Compared with reactions at room temperature, a significant yield increasing effect could be established. The kinetic parameters of ester hydrolysis show that most of the unusual acyl donors (compared with the coded phenylalanine methyl ester) are well accepted substrates for α-chymotrypsin.

Introduction

Unusual amino acids have been attracting attention because of their potential pharmacological utility[1]. Therefore, derivatives of non-natural amino acids are attractive objects for enzyme-catalyzed peptide synthesis. It has been shown in recent reports[2-6] that freezing the aqueous reaction mixture can drastically increase the yield of protease-catalyzed kinetically and thermodynamically controlled peptide synthesis reactions. As a basis for the understanding of this effect the 'Freeze-concentration model' has been discussed[7-9].

A simple strategy for peptide bond formation which cannot be achieved by chemical methods is the use of N-terminal free amino acid esters as acyl donors. It is known from recent papers that N^{α}-unprotected amino acid esters can act as carboxyl components in reactions catalyzed by elastase, carboxypeptidase Y and α-chymotrypsin[10,11]. The reactions catalyzed by the α-chymotrypsin were carried out only with the coded amino acid esters H-Phe-OEt and H-Tyr-OEt as acyl donors at room temperature. This strategy was also verified for various peptide syntheses in frozen solutions even using free amino acids as amino components[12-14].

Here we report the results of a comparative investigation of the α-chymotrypsin(CT)-catalyzed reaction of H-Phe-OMe and non-coded phenylalanine esters with H-Leu-NH_2 and H-Arg-NH_2, both at room temperature and

in frozen aqueous solution at -15 °C. It was the aim of these studies to check the acceptance of α-chymotrypsin for N^{α}-unprotected non-coded phenylalanine esters such as H-4-fluoro-Phe-OMe and H-4-nitro-Phe-OMe, and also for H-2-naphthyl-Ala-OMe and even β-phenyllactyl-OMe, bearing a hydroxy group instead of the α-amino group. Furthermore, the kinetic behaviour of H-4-methyl-Phe-OMe, H-4-trifluoromethyl-Phe-OMe and H-4-^{tert}butyl-Phe-OMe for α-chymotrypsin-catalyzed hydrolysis reactions have been compared

Results and Discussion

The kinetic parameters for CT-catalyzed hydrolysis of the substrates are collected in Table 1. Plots of the initial rates for H-Phe-OMe, H-4-fluoro-Phe-OMe, H-4-nitro-Phe-OMe and H-2-naphthyl-Ala-OMe are shown in Figure 1.

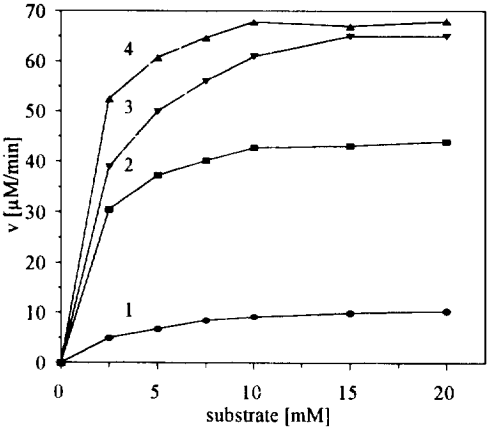

Figure 1 Rate Dependence of the CT-Catalyzed Hydrolysis of H-Phe-OMe (curve 3), H-4-fluoro-Phe-OMe (curve 4), H-4-nitro-Phe-OMe (curve 2) and H-2-naphthyl-Ala-OMe (curve 1) on the Substrate Concentration.

The data in Table 1 show that most of the ester derivatives investigated were hydrolyzed by CT. The reactivity of CT was considerably influenced by the substituents. It is remarkable that the K_m values for H-4-fluoro-Phe-OMe and H-4-nitro-Phe-OMe are smaller than that for natural H-Phe-OMe. These values might demonstrate that the substitution of a proton by fluorine or a nitro group in the *para* position of the benzene moiety of phenylalanine leads to a more specific P-S interaction in the binding pocket of CT. In the case of H-2-naphthyl-Ala-OMe, the k_{cat} value indicates a very slow reaction, whereas the hydrolysis rate of β-phenyllactyl-OMe was the highest.

Substitution by a ^{tert}butyl residue at the *para* position results in almost extreme unreactivity. This is an unexpected result and not easy to explain by the size of the tert. butyl group alone. Possibly the hydrophobicity

plays a crucial role. The K_m value for H-4-trifluoromethyl-Phe-OMe is 3.5 times greater than that for H-4-methyl-Phe-OMe. The substituents are nearly identical so far as the steric parameters are concerned but quite different with respect to the electronic behaviour. The high electronegativity of the fluorine atoms is suggested to be the origin for the observed effect.

Table 1 Kinetic Constants of CT-Catalyzed Hydrolysis.[a]

Substrat	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($M^{-1} s^{-1}$)
H-Phe-OMe	2.26 ± 0.11	2.10 ± 0.03	9.30×10^2
H-4-fluoro-Phe-OMe	1.07 ± 0.08	2.09 ± 0.03	1.95×10^3
H-4-nitro-Phe-OMe	1.52 ± 0.16	1.38 ± 0.03	9.10×10^2
H-2-naphthyl-Ala-OMe	3.64 ± 0.28	0.35 ± 0.01	1.00×10^2
H-4-methyl-Phe-OMe	4.38 ± 0.30	1.55 ± 0.03	3.54×10^2
β-phenyllactyl-OMe	7.27 ± 0.62	14.45 ± 0.43	1.99×10^3
H-4-trifluoromethyl-Phe-OMe	15.19 ± 1.10	0.89 ± 0.03	0.59×10^2
H-4-^{tert}butyl-Phe-OMe	no enzymatic hydrolysis		

[a] The values refer to an active enzyme concentration of 5.9×10^{-4} mM based on the hydrolysis rate of nitrophenylacetate.

Following on from these results, peptide bond formation between the acyl donor esters described above and H-Leu-NH_2 and H-Arg-NH_2 as amino components was investigated. The results for the CT-catalyzed reaction of H-Phe-OMe with H-Leu-NH_2 at 25 °C compared with the peptide bond formation between H-4-fluoro-Phe-OMe, H-4-nitro-Phe-OMe, H-2-naphthyl-Ala-OMe and β-phenyllactyl-OMe and H-Leu-NH_2 as amino component are shown in Table 2.

Table 2 CT-Catalyzed Dipeptide Amide Synthesis Using N^{α}-Unprotected Acyl Donors and H-Leu-NH_2 as Nucleophile at 25 °C compared with -15 °C.

Substrate	Peptide	[CT]	Time (h)		Yield (%)	
		(μM)	25 °C	-15 °C	25 °C	-15 °C
H-Phe-OMe	H-Phe-Leu-NH_2	2	4	24	52	94
H-4-fluoro-Phe-OMe	H-4-fluoro-Phe-Leu-NH_2	2	4	7	47	90
H-4-nitro-Phe-OMe	H-4-nitro-Phe-Leu-NH_2	20	2	6	86	94
H-2-naphthyl-Ala-OMe	H-2-naphthyl-Ala-Leu-NH_2	50*	24	192	55	93
β-phenyllactyl-OMe	β-phenyllactyl-Leu-NH_2	2	5	24	78	98

Reaction conditions: [Acyl donor]=25 mM; [H-Leu-NH_2]=100 mM; without buffer; pH 9.0 adjusted with 1N NaOH before freezing; all results after complete ester consumption. * In this case the concentration of CT was 500 μM at -15 °C.

The hydrolysis results show that CT is capable of catalyzing peptide synthesis, with natural H-Phe-OMe and substituted phenylalanine esters, and even with a derivative in which the α-amino group is substituted by a hydroxy group, as carboxyl components. H-4-Nitro-Phe-OMe and β-phenyllactyl-OMe are particularly efficient as acyl donors. In order to study the influence of freezing on the reaction mixture, peptide bond formation was carried out at -15 °C using CT as catalyst (Table 2). It is obvious that a reduced rate of peptide synthesis was observed at -15 °C compared with 25 °C. But in all cases a significant increase of the peptide yield was observed. It is interesting that the peptide yields realized, higher than 90 %, do not depend on the type of acyl donor ester. In the frozen system relative rates of acyl enzyme hydrolysis and aminolysis changed in favour of the aminolysis. These results are in a good accordance with the 'Freeze-concentration model' and the elimination of secondary hydrolysis under the conditions used.

Table 3 shows the results for the reaction described above with H-Arg-NH_2 as amino component at 25 °C compared with the reaction in frozen aqueous solution at -15 °C. Because of the P_1'-specifity of CT for arginine the peptide yields are very high already at room temperature[15]. In all cases the peptide bond formation at -15 °C was increased compared with 25 °C. No secondary hydrolysis was observed after a 24 h reaction time.

Table 3 CT-Catalyzed Dipeptide Amide Synthesis Using N-Terminal Free Acyl Donors with H-Arg-NH_2 as Nucleophile at 25 °C compared with -15 °C.

Substrate	Peptide	[CT]	Time (h)		Yield (%)	
		(μM)	25 °C	-15 °C	25 °C	-15 °C
H-Phe-OMe	H-Phe-Arg-NH_2	2	2	7	79	98
H-4-fluoro-Phe-OMe	H-4-fluoro-Phe-Arg-NH_2	2	1	6	73	95
H-4-nitro-Phe-OMe	H-4-nitro-Phe-Arg-NH_2	20	2	4	90	98
β-phenyllactyl-OMe	β-phenyllactyl-Arg-NH_2	2	7	24	92	100

Reaction conditions: [Acyl donor]=25 mM; [H-Arg-NH_2]=100 mM; without buffer; pH 9.0 adjusted with 1N NaOH before freezing; all results after complete ester consumption.

In conclusion, it was established that protease-catalyzed peptide bond formation can be carried out both with natural amino acid esters and unusual amino acid derivatives as acyl donors. The protection of the α-amino group of the acyl donor ester is not necessary in CT-catalyzed peptide synthesis. These findings improve significantly the strategy of enzymatic peptide synthesis, since protection and deprotection steps in chemical peptide synthesis are time-consuming and accompanied with unwanted side reactions. Furthermore, it was shown that freezing the reaction mixture leads to a significant increase of peptide yields in protease-catalyzed peptide synthesis.

Experimental

Specificity Studies

The specifity constants for the substrates were determined potentiometrically using a pH-stat Video-Titrator VIT 90 (Radiometer, Copenhagen). In the titrimetric assay similar conditions without buffer were used, controlling pH 9.0 with 0.02 M NaOH containing 0.2 M NaCl. The reactions were carried out at 25 °C. The total volume was adjusted to 2 ml. The substrate concentration was varied from 0.5 mM to 30 mM. The reaction was started by addition of enzyme solution.

The kinetic parameters K_m and V_{max} were obtained by nonlinear curve fitting of the data (ENZFITTER, Elsevier Biosoft, Cambridge, UK). The k_{cat} values were determined as a value of $V_{max}/[E_0]$.

Peptide Synthesis Reactions

Peptide synthesis reactions were performed in 1.5 ml polypropylene tubes at a total sample volume of 1 ml for room temperature and 0.1 ml at -15 °C. After addition of the reaction components and adjusting the pH to 9.0 using 1N NaOH, the tube was shaken and placed in liquid nitrogen for 20 s to achieve shock-freezing. Then it was transferred for the time of reaction into a constant temperature cryostate (Haake, Germany). After definite reaction time the reaction was stopped by adding 0.3 ml of a mixture of 50 % methanol and 2.5 % trifluoroacetic acid (TFA) in water. Chemical changes during freezing and thawing were found to be negligible.

The reactions at room temperature were performed in the same manner as described but without the freezing and thawing procedure.

HPLC-Analysis

The samples were analyzed by RP-HPLC. Analysis were performed using an isocratic system (Shimadzu LC 6A, Shimadzu, Japan). A Lichrosorb RP18 column with 7μm particle size (Merck, Germany) and an Impaq RP18 column with 10μm particle size (Bischoff, Germany) were used. Acetonitrile/water mixtures containing 0.1 % TFA in various volume ratios were chosen as eluents in isocratic elution. Substrate and peptide ratio were detected at 254 nm. Since the hydrolysis and aminolysis products contain the same chromophoric groups, the molar extinction coefficients were assumed to be equal.

Chemicals

Amino acid amides were used from BACHEM (Switzerland). α-Chymotrypsin was a product of Serva (Germany). It was used without additional purification.

The non-coded amino acid esters were isolated as hydrochlorides in very pure form after deprotection of the N-Boc derivatives and recrystallisation from MeOH/ether.

H-4-fluoro-L-Phe-OMe.HCl: $C_{10}H_{13}ClFNO_2$ (233.7), calcd. C 51.40 H 5.61 N 5.99; found C 51.33 H 5.75 N 6.07, m. p. 195-199 °C, $[\alpha]_D^{24}$ +32.2 (c 1, EtOH), 99.4 % ee by HPLC.

H-4-nitro-L-Phe-OMe.HCl: $C_{10}H_{13}ClN_2O_4$ (260.7), calcd. C 46.07 H 5.03 N 10.75; found C 46.11 H 5.12 N 10.71, m. p. 221-224 °C, $[\alpha]_D^{24}$ +34.2 (c 1, EtOH), 99.6 % ee by HPLC.

H-4-methyl-L-Phe-OMe.HCl: $C_{11}H_{16}ClNO_2$ (229.7), calcd. C 57.52 H 7.02 N 6.10; found C 57.65 H 6.89 N 6.14, m. p. 193-196 °C, $[\alpha]_D^{24}$ +29.9 (c 1, EtOH), 98.4 % ee by HPLC.

H-4-trifluoromethyl-L-Phe-OMe.HCl: $C_{11}H_{13}ClF_3NO_2$ (283.7), calcd. C 46.57 H 4.62 N 4.94; found C 46.73 H 4.69 N 5.00, m. p. 192-194 °C, $[\alpha]_D^{24}$ +24.0 (c 0.5, EtOH), 99.5 % ee by HPLC.

H-4-tert.butyl-L-Phe-OMe.HCl: $C_{14}H_{22}ClNO_2$ (271.8), calcd. C 61.87 H 8.16 N 5.15; found C 61.76 H 8.03 N 5.39, m. p. 143-152 °C, $[\alpha]_D^{24}$ +21.1 (c 1, ETOH), 98.8 % ee by HPLC.

H-2-naphthyl-L-Ala-OMe.HCl: $C_{14}H_{16}ClNO_2$ (265.7), calcd. C 63.28 H 6.07 N 5.27; found C 62.83 H 6.14 N 5.30, m. p. 181-185 °C, $[\alpha]_D^{24}$ +24.6 (c 1, EtOH), 99.2 % ee by HPLC.

H-Phe-Leu-NH_2. m. p. 108-110 °C, m/z (M+1) 278

^{1}H-NMR (DMSO): 0.84-0.89 (m, 6H, 2xCH_3); 1.45-1.48 (t, 2H, CH_2); 1.58-1.60 (m, 1H, CH); 2.89-2.96 (dd, 1H, CH_2); 3.07-3.14 (dd, 1H, CH_2); 4.03-4.07 (q, 1H, CH); 4.22-4.30 (q, 1H, CH); 7.02 (d, 2H, NH); 7.21-7.34 (m, 5H, C_6H_5); 8.60-8.63 (d, 1H, NH).

H-4-fluoro-Phe-Leu-NH_2. m. p. 172-177 °C

^{1}H-NMR (DMSO): 0.84-0.88 (t, 6H, 2xCH_3); 1.42-1.60 (m, 3H, CH_2, CH); 2.80 (m, 2H, CH_2); 4.23 (m, 1H, CH); 4.50-4.52 (m, 1H, CH); 6.98-7.25 (m, 5H, 4-F-C_6H_4, NH); 7.99-8.07 (m,1H, NH).

H-4-nitro-Phe-Leu-NH_2. m. p. 184-189 °C, m/z (M+1) 323

^{1}H-NMR (DMSO): 0.82-0.88 (m, 6H, 2xCH_3); 1.45 (m, 2H, CH_2); 1.49-1.56 (m, 1H, CH); 2.69-2.72 (m, 1H, CH_2), 2.92-2.98 (m, 1H, CH_2); 4.22-4.24 (m, 1H, CH); 4.63-4.65 (m, 1H, CH); 6.99 (d, 1H, NH); 7.30-7.51 (m, 4H, 4-NO_2-C_6H_4); 8.07-8.09 (m, 2H, NH).

H-2-naphthyl-Ala-Leu-NH_2. m. p. 122-123 °C, m/z (M+1) 328

^{1}H-NMR (DMSO): 0.78-0.80 (d, 6H, 2xCH_3); 1.39-1.41 (t, 2H, CH_2); 1.85-1.87 (m, 1H, CH); 2.83-2.86 (dd, 1H, CH_2): 3.08-3.10 (dd, 1H, CH_2); 3.53-3.57 (q, 1H, CH); 4.23-4.25 (m, 1H, CH); 6.98 (s, 1H, NH); 7.36-7.47 (m, 4H, $C_{10}H_7$); 7.71 (s, 1H, NH); 7.79-7.92 (m, 4H, $C_{10}H_7$).

β-phenyllactyl-Leu-NH_2. m. p. 148-149 °C, m/z (M+1) 279

^{1}H-NMR (DMSO): 0.81 (s, 6H, 2xCH_3); 1.36 (m, 3H, CH_2, CH); 2.75-2.78 (dd, 1H, CH_2); 2.94 (dd, 1H, CH_2); 4.12 (m, 1H, CH); 4.24 (m, 1H, CH); 5.69 (d, 1H, OH); 7.02 (s, 1H, NH); 7.20-7.24 (m, 5H, C_6H_5); 7.36 (s, 1H, NH); 7.50 (d, 1H, NH).

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