Tetrahedron Vol. 46, No. 24, pp. 8093-8102, 1990
Printed in Great Britain

0040-4020/90 $3.00+.00
Pergamon Press plc

ENZYME-CATALYZED PEPTIDE SYNTHESIS IN ICE

M. Schuster[a], A. Aaviksaar[b] and H.-D. Jakubke[a,*]

[a]Department of Biochemistry, Biosciences Division, Karl Marx University, Talstr.33, 7010 Leipzig, GDR
[b]Laboratory of Bioorganic Chemistry, Institute of Chemical Physics and Biophysics of the Estonian Academy of Sciences, P.O.Box 670, Tallinn 200026, USSR

(*Received in Germany* 13 *June* 1990)

Abstract: A new method for increasing product yield in kinetically controlled enzymatic peptide synthesis catalyzed by serine and cysteine proteases is proposed - freezing the reaction mixture. The use of the method in preparative synthesis has been demonstrated.

INTRODUCTION

It has been demonstrated by Grant and Alburn[1] that trypsin-catalyzed hydroxylaminolysis of amino acid ester substrates was faster in a frozen aqueous solution at -23°C than in a liquid aqueous solution at 1°C. From the data in Fig. 1 in their paper it can be concluded that freezing has also changed relative rates of the acyl-trypsin hydrolysis and hydroxylaminolysis in favour of aminolysis.

In order to test the conclusion and to evaluate the possible use of freezing in improving the product ratio in kinetically controlled peptide synthesis by proteases (for review of the method see ref.[2] and references therein) we have determined the yields of some peptide synthesis reactions catalyzed by serine and cysteine pro-

teases (α-chymotrypsin, EC 3.4.21.1; V8-protease, EC 3.4.21.9 - endoproteinase Glu-C from *Staphylococcus aureus* V8; papain, EC 3.4.22.2) in water at 25°C and in ice and studied the dependence of the peptide yields upon reaction conditions.

MATERIALS AND METHODS

Commercial preparations of papain (Boehringer, FRG), α-chymotrypsin (Leningrad Factory of Medical Preparations, USSR) and V8-protease (Pierce, USA) were used without additional purification. The sample of V8-protease was a generous gift of Prof. E. Munekata, Institute of Applied Biochemistry (University of Tsukuba, Japan). The nucleophiles used were from "Reanal" (Hungary) with exception of H-D-Leu-NH_2·HCl which was from our collection. Mal-Tyr-OMe (Mal = maleyl) and Z-Glu-OMe were synthesized as set out earlier[3,4]. Mal-Phe-Ala-OEtCl (-OEtCl = monochloroethylester) (C: calc. 54.49%, found 53.86%; H: calc. 5.33%, found 5.42%; N: calc. 7.06%, found 6.91%) was obtained by coupling Boc-Phe-OH with H-Ala-OEtCl by mixed anhydride method followed by exchange of the α-amino blocking group.

Acyl transfer reactions were performed in 0.3 ml polypropylene microcentrifuge tubes to allow rapid freezing and thawing of the reaction mixtures. Except the experiments at various protonation states, the nucleophiles were used in their half-protonated forms in order to use the buffer capacity of the amino group for holding pH = pK_a. Therefore, the 0.1 M stock solutions of H-Gly-Gly-OH, H-Gly-Gly-Gly-OH, H-Gly-Ala-OH, H-Ala-Ala-OH, H-D-Leu-NH_2·HCl, H-Leu-NH_2·HCl, H-β-Ala-Gly-OH were made in 50 mM NaOH and H-Arg-OH and H-Lys-OH - in 50 mM HCl. No additional buffer components were used.

Stock solutions of V8-protease and α-chymotrypsin were prepared in 1 mM HCl, of papain in 5 mM dithioerythritol (Reanal, Hungary).

Reactions in ice were performed as follows. The tube containing the appropriate acyl donor substrate and nucleophile in 200 µl of water was cooled to 0°C and 1-5 µl of enzyme stock solution was added (the enzyme concentrations were chosen to give the reaction times for 100% acyl donor substrate consumption of at least 240 min). The tube was rapidly shaken and inserted in liquid nitrogen. After five minutes it was transferred into a constant temperature cryostate

(MK70, MLW, GDR). At thawing (maximally 60 s) the reaction mixture was not allowed to reach 5°C. Chemical changes during freezing and thawing were found to be negligible.

Reactions in solution were performed as described but without freezing and thawing procedures.

For HPLC analyses 100 µl aliquots were withdrawn from reaction mixtures. HPLC was performed using a Series 8800 gradient system (DuPont Instruments, USA) connected with a computing integrator SP 4100 (Spectra-Physics, USA). A 4.6·250 mm Silasorb L18 column with 7.5 µm particle size (Chemapol, Chechoslovacia) was used . Mixtures of 0.1 M phosphate buffer (pH 6.5) with methanole with varying volume ratios containing 10 mM tetrabutylammonium hydrogene sulfate as counter-ion have been used as eluents in isocratic elution. The substrate and product concentrations were detected at 255 nm. Since the hydrolysis and aminolysis products contain the same chromophoric system, the absorption coefficients were assumed to be equal.

All reactions were performed with at least 10 fold excess of the nucleophile free base over substrate ester. The product ratio was shown to be constant within the reaction time.

In accordance with the reaction scheme[5]

$$E + S \rightleftharpoons ES \xrightarrow{k_2} EA \begin{cases} \xrightarrow{k_3} E + P_2 \\ \xrightarrow{k_4[N]} E + P_3 \end{cases} \qquad (1)$$
$$\qquad\qquad\quad ES \xrightarrow{} P_1$$

we have used $[P_3] / ([P_3] + [P_2])$ in per cent for the yield of the peptide product and $[P_3] / [P_2]$ for the ratio of the initial rates of aminolysis and hydrolysis. No secondary hydrolysis of the peptide product was observed during the acyl transfer reactions and in the time intervals after ester substrate consumption until the product analysis.

RESULTS AND DISCUSSION

In Fig. 1 representative chromatograms of the reaction products in protease-catalyzed peptide synthesis via acyl transfer mechanism,

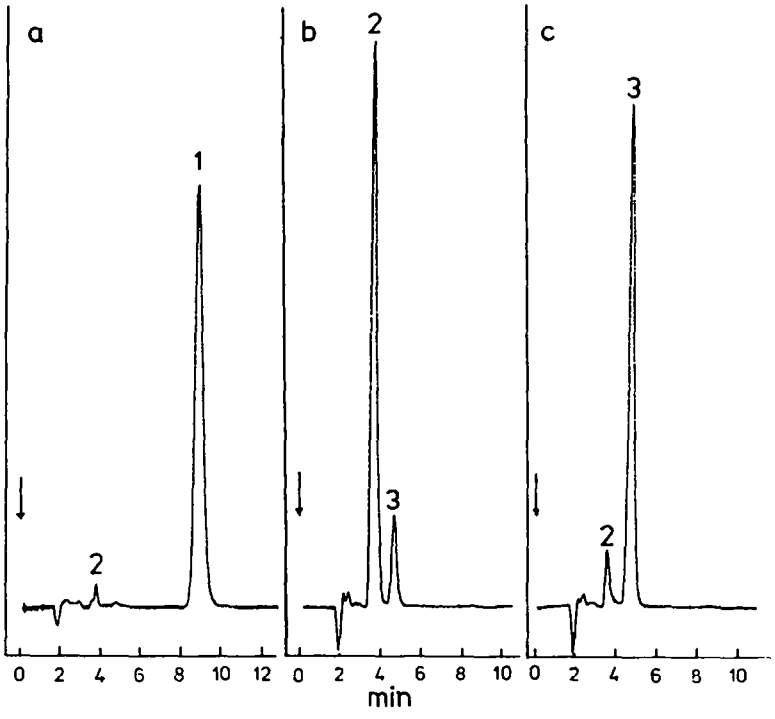

Fig. 1. HPLC-Analysis of the α-chymotrypsin-catalyzed reaction of Mal-Tyr-OMe with H-Ala-Ala-OH. a - Reaction mixture before enzyme addition; b - Reaction mixture after incubating it 20 min at 25°C in the presence of enzyme; c - Reaction mixture after incubating it 240 min frozen at -25°C in the presence of enzyme. 1 -Mal-Tyr-OMe; 2 - Mal-Tyr-OH; 3 - Mal-Tyr-Ala-Ala-OH.
[Mal-Tyr-OMe] = 2 mM, [H-Ala-Ala-OH] = 50 mM (25 mM as free base), [α-chymotrypsin] = 0.15 μM.

comparatively in water and ice, are shown. It can be seen that after total consumption of the ester substrate Mal-Tyr-OMe (peak 1) in its α-chymotrypsin-catalyzed reaction with water (the product Mal-Tyr-OH identified as peak 2) as well as with competing H-Ala-Ala-OH (the product given by peak 3) ,in the frozen solution at -25°C the product ratio has drastically improved in favour of the peptide compound

Table 1. Enzyme-catalyzed peptide synthesis in water and ice by serine (α-chymotrypsin, V8) and cysteine (papain) proteases.

Reaction components[a]		Enzyme[b]	Peptide yield, % 25°C	-25°C
Mal-Tyr-OMe	+H-Ala-Ala-OH	α-chymotrypsin	10.0	94.4
Mal-Phe-Ala-OEtCl	+H-Ala-Ala-OH	papain	42.2	78.9
Z-Glu-OMe	+H-Ala-Ala-OH	V8-protease	5.1	75.8

[a]Acyl donor substrates specific for the enzymes used have been chosen, [acyl donor] = 2 mM, [H-Ala-Ala-OH] = 50 mM (25 mM as free base). [b] [α-chymotrypsin] = 0.46 μM at 25°C and 0.15 μM at -25°C, [papain] = 0.15 mg/ml at 25°C and 0.1 mg/ml at -25°C, [V8-protease] = 7.5 μg/ml at 25°C and 5 μg/ml at -25°C. The concentrations were chosen to provide the reaction times for total acyl donor substrate consumption between 240-480 min at -25°C and 10-20 min at 25°C.

Table 2. α-Chymotrypsin-catalyzed peptide synthesis in water and ice using Mal-Tyr-OMe as acyl donor substrate and various peptides, amino acid amides and amino acids as nucleophiles[a].

Amino component of the reaction	Peptide yield, % 25°C	-25°C
H-Gly-Ala-OH	5.8	94.7
H-Gly-Gly-OH	2.6	95.4
H-Gly-Gly-Gly-OH	5.1	91.2
H-D-Leu-NH_2	9.9	73.0
H-Leu-NH_2	79.1	91.8
H-Arg-OH	< 2	32.7
H-Lys-OH	< 2	43.8
H-β-Ala-Gly-OH	< 2	78.8

[a][amino component] = 50 mM (25 mM as nucleophile free base), [Mal-Tyr-OMe] = 2 mM, [α-chymotrypsin] = 0.15 μM at 25°C and 0.3 μM at -25°C.

while the initial reaction mixtures in both cases were identical.

From the data in Table 1 it can be concluded that the yield-increasing effect of freezing in enzyme-catalyzed peptide synthesis is universal for serine as well as cysteine proteases.

The data in Table 2 demonstrate that in ice quite high yields can be achieved in the synthesis of peptides on the basis of Mal-Tyr-OMe and various "ineffective" amino components which are usually not considered applicable as nucleophiles in α-chymotrypsin-catalyzed peptide synthesis ("exceptional" H-Leu-NH_2 has been inserted in Table 2 for comparison with H-D-Leu-NH_2).

It should be noted that, since the experiments described in Table 1 and 2 were performed under conventionally chosen standard conditions, the observed peptide yields cannot be considered optimal. This is well demonstrated by data in Fig. 2 which shows the dependence of the yield of the α-chymotrypsin-catalyzed synthesis of Mal-Tyr-D-Leu-NH_2 and Mal-Tyr-β-Ala-Gly-OH in ice upon the relative amount of the nucleophile free base in the synthesis mixture (the yields in Table 2 correspond to the yields at $[N]_b / ([N]_a + [N]_b) = 0.5$ in Fig. 2).

In order to obtain more information about the possibilities and limitations of the peptide forming process in frozen solutions we have studied also the influence of temperature on the peptide yields as well as the influence of amino component concentration on the ratio of the initial rates of aminolysis and hydrolysis in α-chymotrypsin-catalyzed acyl transfer. The results of these studies are illustrated in Figures 3 and 4.

It can be seen in Fig. 4 that increase in the amino component concentrations in α-chymotrypsin-catalyzed reactions of Mal-Tyr-OMe with H-D-Leu-NH_2 and H-Arg-OH at -25°C above 40 mM (20 mM as free base) does not give any favourable effect in peptide synthesis. The reason may be that the amount of unfrozen water in ice at a given temperature is determined by the amount of amino component in a way to keep its concentration in the liquid phase of the frozen solution constant as discussed by Pincock and Kiovsky[6].

On the other hand, as seen in Fig. 2, the yield of the synthesis of Mal-Tyr-D-Leu-NH_2 at the same conditions in the presence of 50 mM total amino component concentration can be increased from 73% to more than 90% titrating H-D-Leu-NH_2 in initial reaction mixture

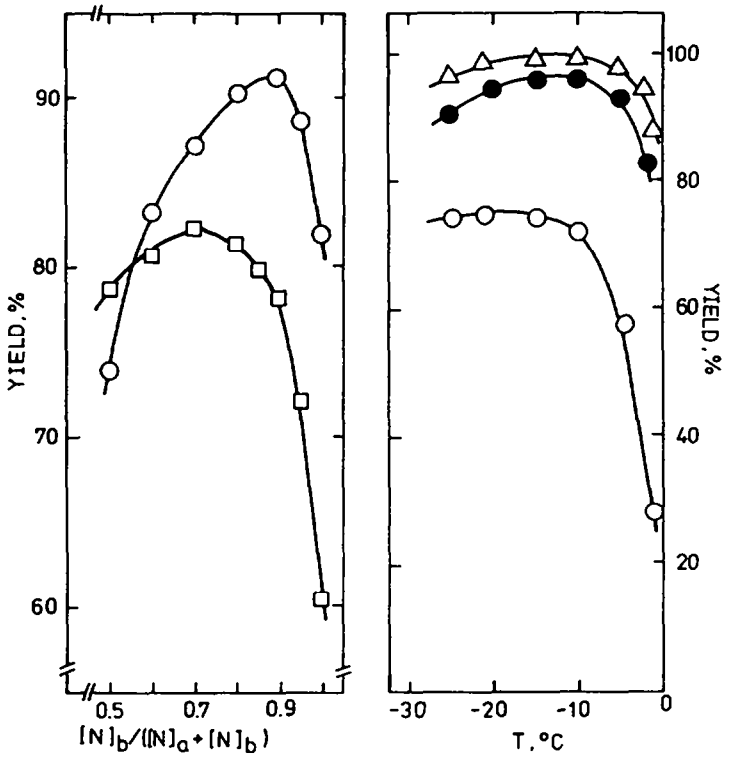

Fig. 2. Dependence of the yield of the α-chymotrypsin-catalyzed synthesis of the peptides Mal-Tyr-D-Leu-NH_2 (○) and Mal-Tyr-β-Ala-Gly-OH (□) in ice upon the relative amount of the nucleophile free base in the 50 mM solutions of the amino components. [Mal-Tyr-OMe] = 2 mM, [α-chymotrypsin] = 0.15 μM.

Fig. 3. Influence of temperature on the yield of the α-chymotrypsin-catalyzed peptide synthesis in ice using Mal-Tyr-OMe as acyl donor and H-D-Leu-NH_2 (○), H-Gly-Gly-Gly-OH (●) and H-Ala-Ala-OH (△) as amino components in 50 mM concentrations (25 mM as free bases). [Mal-Tyr-OMe] = 2 mM, [α-chymotrypsin] = 0.15 μM.

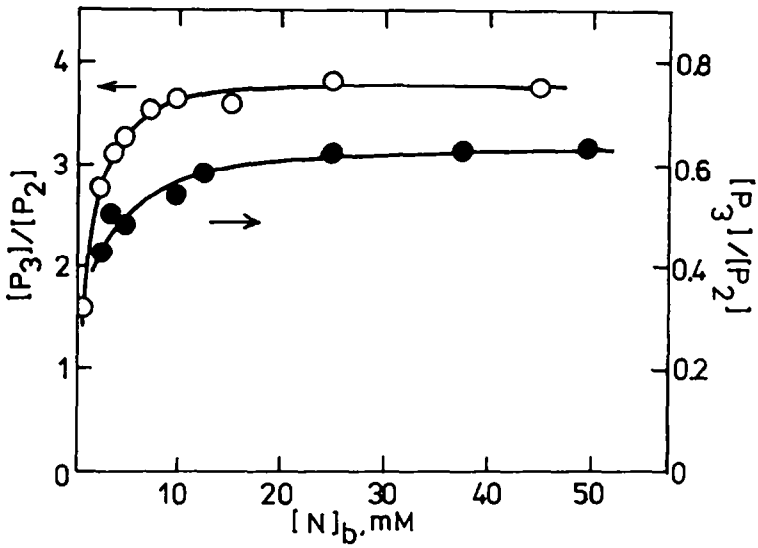

Fig. 4. Dependence of the ratio of the initial rates of aminolysis and hydrolysis of the α-chymotrypsin-catalyzed reactions of Mal-Tyr-OMe with H-D-Leu-NH_2 (○) and H-Arg-OH (●) at -25°C in ice upon the concentration of the amino component (the nucleophile free base concentrations of the amino components, $[N]_b$, were $[N]_{tot}/2$ as given in Materials and Methods).
[Mal-Tyr-OMe] = 0.2 mM, [α-chymotrypsin] = 0.015 μM

by NaOH to $[N]_b/([N]_a + [N]_b) = 0.9$. The decrease in the peptide yields at $[N]_b/([N]_a + [N]_b) > 0.9$ (Fig. 2) may be the result of a sharp increase in pH concidering the low buffer capacities of amino compounds at the pH values much above their pK_a (pK_a values for the amino groups in H-β-Ala-Gly-OH and H-Leu-NH_2 are 9.59 and 7.89, respectively[7]). An influence of the high pH on the enzyme causing a pH-optimum of the synthesis reaction cannot be excluded.

As can be seen in Fig. 3 the yields of the studied α-chymotrypsin-catalyzed syntheses in ice show the trend to decrease at temperatures above -10°C.

During the last decade, protease-catalyzed peptide synthesis via acyl transfer reactions has become a valuable addition to conventional synthetic methods in protein chemistry. Several ways of increasing peptide yields in the kinetically controlled systems of competing hydrolysis and aminolysis of acyl enzymes have been proposed (for reviews see ref.[8]). In this context freezing of reaction mixture can be considered a new method of increasing the peptide yields in their protease-catalyzed synthesis. In order to demonstrate the practical applicability of the method we present an example of preparative synthesis by α-chymotrypsin in ice at -13°C.

3 mmol (0.88 g) Mal-Tyr-OMe and 6 mmol (1.14 g) H-$(Gly)_3$-OH were suspended in 15 ml water in a polypropylene centrifuge tube. The suspension was cooled to 5°C and 8.5 ml 1 M NaOH solution was added under stirring until clear solution was obtained. After addition of 40 µl α-chymotrypsin stock solution (2 mg/ml) the tube was rapidly shaken and inserted in liquid nitrogen. After about 5 minutes it was transferred into a cryostate at -13°C for 5 hours. By this time all the substrate ester was consumed with 94% analytical yield of the peptide product (by HPLC).

After thawing the whole mixture, pH of the solution was adusted to 1 with 10% HCl, 30 ml ethyl acetate was added and the tube was vigorously shaken until the peptide product precipitated as a white solid. It was filtered off, recristallyzed from water/ethyl acetate and dried in vacuum to give 1.05 g Mal-Tyr-$(Gly)_3$-OH (78% of theoretical yield). Amino acid analysis (for 1 mmol peptide): 0.96 mmol Tyr; 2.87 mmol Gly. M_p = 175-178°C; $[\alpha]_D^{25}$(c=2/DMF) = -8.6°.

REFERENCES

1. Grant, N.H.; Alburn, H.E. *Nature* 1966, 212, 194.
2. Jakubke, H.-D.; Kuhl, P.; Könnecke, A. *Angew. Chem. Int. Ed.* 1985, 24, 85.
3. Schellenberger, V.; Jakubke, H.-D. *Biochim. Biophys. Acta* 1986, 869, 54.
4. Schellenberger, V.; Schuster, M.; Jakubke, H.-D. *Biocatalysis* (in press)
5. Bender, M.L.; Clement, G.E.; Gunter, C.R.; Kezdy, F.J. *J. Am. Chem. Soc.* 1964, 86, 3697.
6. Pincock, R.E.; Kiovsky, T.E. *J. Chem. Educ.* 1966, 43, 358.
7. Schellenberger, V. unpublished results.
8. Kasche, V.; Haufler, U.; Riechmann, L. *Methods in Enzymology* 1987, 136, 280.