Tetrahedron: Asymmetry Vol. 4, No. 7, pp. 1559-1564, 1993
Printed in Great Britain

0957-4166/93 $6.00+.00
Pergamon Press Ltd

SINGLE-STEP SYNTHESIS OF KYOTORPHIN IN FROZEN SOLUTIONS BY CHYMOTRYPSIN

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(*Received in UK* 17 *March* 1993)

Abstract: A novel method of Kyotorphin (H-Tyr-Arg-OH) synthesis without protecting and deprotecting procedures is described - α-chymotrypsin-catalyzed aminolysis of H-Tyr-OEt by H-Arg-OH in frozen mixtures. In this one-step process yields of the peptide product above 80% have been obtained

INTRODUCTION

It has been found recently[1] that in frozen solution chymotrypsin is capable of catalyzing the aminolysis of N-acylated amino acid esters by free amino acids: Mal-Tyr-Arg-OH and Mal-Tyr-Lys-OH have been synthesized from Mal-Tyr-OEt and H-Arg-OH or H-Lys-OH, respectively, while no peptides were formed in these mixtures in liquid water.

In the present study we have shown that in ice one can synthesize the peptide bond between tyrosine and arginine making use of H-Tyr-OEt as the acyl donor and H-Arg-OH as the acceptor in the chymotrypsin-catalyzed acyl transfer leading to a new method for the single-step enzymatic synthesis of Kyotorphin with higher than 80% yields relative to H-Tyr-OEt.

MATERIALS AND METHODS

A commercial preparation of α-chymotrypsin from the St. Petersburg Factory of Medical Preparations (Russia) has been used without further purification. H-Tyr-OEt, arginine and leucine were from "Reanal" (Hungary); L-amino acids were used throughout the work.

Reactions were performed in 1 ml polypropylene tubes. The tubes containing the water solutions of H-Tyr-OEt and arginine, adjusted to appropriate pH by 1 M HCl, were cooled to 0°C and microliter quantities of freshly prepared solutions of α-chymotrypsin (10 mg/ml) in water were added. The tubes were rapidly shaken and inserted into liquid nitrogen. After about 3 minutes they were transferred into a freezer and kept at the appropriate temperature during the synthesis reaction. On thawing the reaction was stopped by adding 0.1 ml of 1 M HCl. Chemical changes during freezing and thawing were found to be egligible.

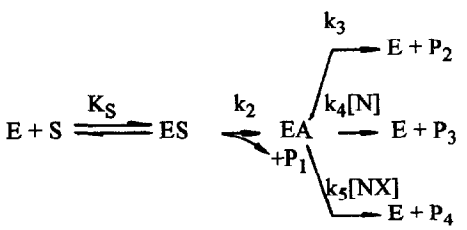
HPLC analyses were performed using a series 8800 gradient system (Du Pont Instruments, USA). A 4.6×250 mm Silasorb C_{18} column was used. Water/methanol mixture (20% v/v) in the presence of 0.1% trifluoro acetic acid was applied as an eluent. The substrate and products were detected with a UV detector at 225 nm considering tyrosine chromophore.

Amino acid analyses of the reaction products were carried out on a T339M amino acid analyzer (Czechoslovakia).

RESULTS AND DISCUSSION

In Fig.1 representative chromatograms of the products of the chymotrypsin-catalyzed reaction of H-Tyr-OEt with arginine in ice at -18°C are shown. In the chromatograms, peak 1 has been identified as H-Tyr-Arg-OH, peak 2 as tyrosine and peak 3 as H-Tyr-Tyr-Arg-OH by amino acid analyses. Peak 4 consisted of initial H-Tyr-OEt.

In calculating the relative yields of the products an operational reaction scheme (1) has been used



(1)

where S is H-Tyr-OEt, N is arginine, EA is the acyl-enzyme, P_1 is ethanol, P_2 is tyrosine, P_3 is Kyotorphin and P_4 is H-Tyr-Tyr-Arg-OH. The yield of the desired product P_3 is given as Y, % = $100\times[P_3]/([P_2]+[P_3]+[P_4]$.

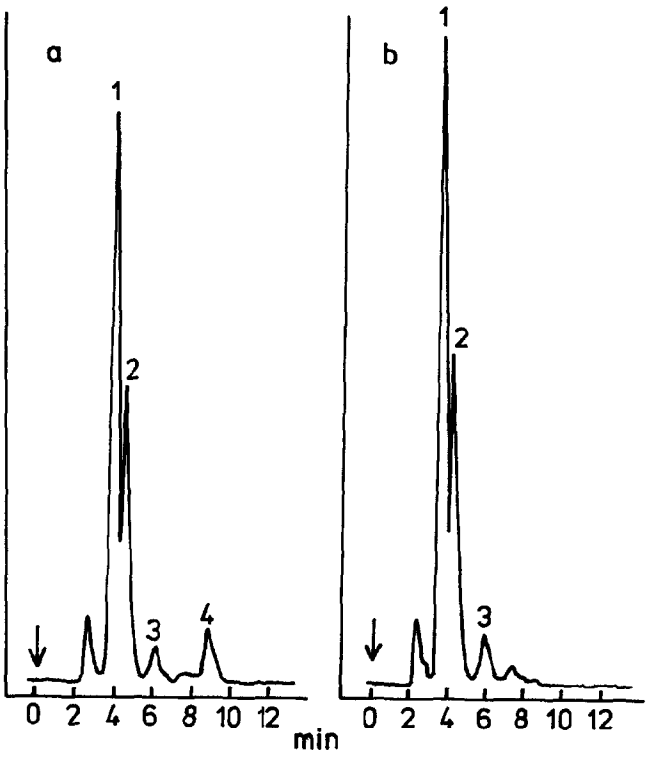


Fig.1. HPLC-Analysis of the products after 15 minutes (a) and 4 hour (b) of the incubation of the reaction mixture at -18°C, [H-Tyr-OEt]=5 mM [H-Arg-OH]=100 mM, [E]=2×10-6 M, pH 9.86 (before freezing). Peak 1 - H-Tyr-Arg-OH, 2 - H-Tyr-OH, 3 - H-Tyr-Tyr-Arg-OH, 4 - H-Tyr-OEt .

We have not studied the kinetic route of the appearance of H-Tyr-Tyr-Arg-OH in the reaction mixture. Nevertheless the following experiment has been performed to shed some light on this problem. Splitting of H-Tyr-OEt by α-chymotrypsin at -18°C and pH 10 in the presence of equimolar 5 mM concentration of Kyotorphin and 100 mM of leucine as buffer compound which did not give any peptide product, resulted in 0.5 mM of tripeptide H-Tyr-Tyr-Arg-OH which suggests that aminolysis of tyrosyl-chymotrypsin by the product Kyotorphin to give P_4 may be involved in the formation of tripeptide byproduct in the synthesis.

In Table 1, Kyotorphin yields at various concentrations of the substrate and arginine nucleophile are given; the influence of arginine concentration on the yield of P_3 depends on substrate concentration - the more of the substrate in the system the more arginine is needed to reach the higher yields of Kyotorphin.

Table 1.

Dependence of Kyotorphin yield on arginine nucleophile concentration at various substrate concentrations in the chymotrypsin-catalyzed synthesis of the peptide from H-Tyr-OEt and H-Arg-OH in ice at -18°C, [E]=2×10^{-6} M, pH 9.86 (before freezing), reaction time 4 h.

[H-Tyr-OEt] mM	[H-Arg-OH] mM	YIELD %	[H-Tyr-OEt] mM	[H-Arg-OH] mM	YIELD %
2.0	25.0	45	10.0	50.0	43
3.0	25.0	43	10.0	100	48
3.0	35.0	44	10.0	200	57
3.0	60.0	63	10.0	300	65
3.0	100	63	10.0	400	67
3.0	110	63	20.0	50.0	25
3.0	140	64	20.0	100	40
5.0	5.0	4	20.0	150	48
5.0	10.0	17	20.0	250	56
5.0	25.0	35	50.0	100	27
5.0	50 0	56	50.0	200	35
5.0	100	63	50.0	400	40
10.0	30.0	21	50.0	500	46

As shown in Fig.2, the yield of the dipeptide is determined by the [N]/[S] ratio in a form of "saturation" curve. From the practical standpoint it means that, whatever the substrate concentration is, a 20-fold excess of the arginine nucleophile over the substrate is needed to reach the highest (plateau) value of the dipeptide yield at a given reaction conditions - 65% in Fig.2.

The dependence of the product yield on [N]/[S] rather than on the nucleophile concentration may be an indication that the concentration of a solute in the liquid regions of the frozen solution is determined by the relative amounts of the solutes and the temperature and does not depend on their absolute concentrations in thawed solution as discussed by Pincock and Kiovsky[2].

In Fig.3, the results of the pH and temperature optimization of Kyotorphin synthesis from H-Tyr-OEt and H-Arg-OH in ice are shown - at pH 10.5 and -26°C the maximum yield of 82% H-Tyr-Arg-OH was obtained; the amount of the tripeptide byproduct H-Tyr-Tyr-Arg-OH was about 2% in these conditions.

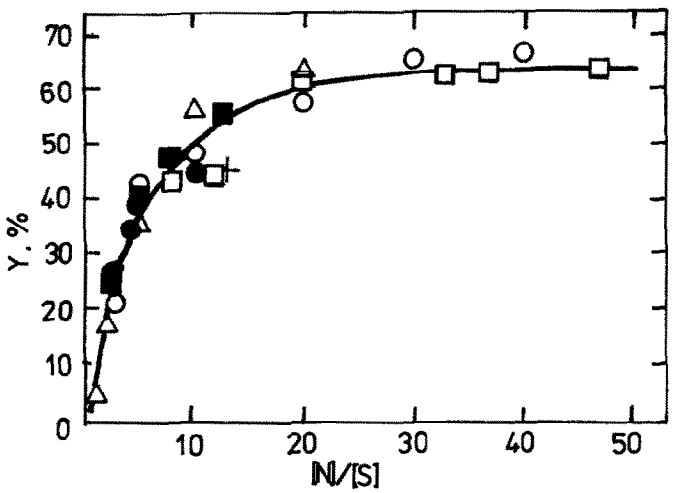


Fig.2. Dependence of Kyotorphin yield on the [N]/[S]-ratio in the chymotrypsin-catalyzed aminolysis of H-Tyr-OEt in ice at -18°C, [E]=2×10^{-6} M, pH 9.86 (before freezing), reaction time 4 h; substrate concentrations 2 mM (+), 3 mM (□), 5 mM (Δ), 10 mM (○), 20 mM (■) and 50 mM (●).

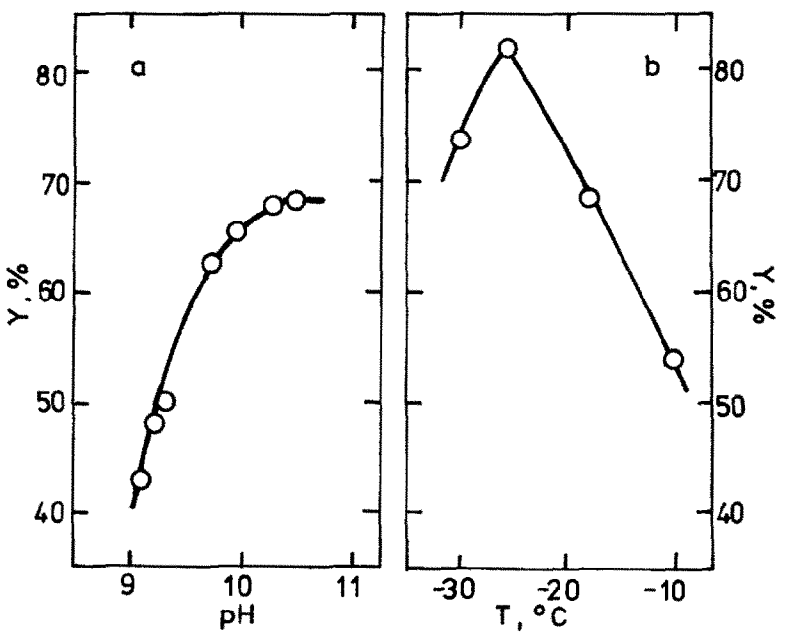


Fig.3. Dependence of the yield of the chymotrypsin-catalyzed synthesis of Kyotorphin in frozen solution upon pH before freezing at -18°C (a) and temperature at pH 10.5 (b); [E]=2×10^{-6} M, reaction time 4 h, [H-Arg-OH]=100 mM, [H-Tyr-OEt]=5 mM.

The synthesis is technically a single-step process with no protecting and deprotecting procedures involved and the high yield of the peptide product relative to the substrate ester makes it especially suitable for the synthesis of Kyotorphin with labelled tyrosine. Provided that an efficient chromatographic method is available for separating the products from the reaction mixture, the nucleophile which is in 20-fold excess can be used repeatedly.

Authors are thankful to Mrs. M. Raba for technical assistance.

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