Volume 329, number 1,2, 40–42 FEBS 12854 August 1993

Peptide synthesis by chymotrypsin in frozen solutions

Free amino acids as nucleophiles

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Received 1 June 1993; revised version received 29 June 1993

Nucleophilic efficiency of free amino acids in chymotrypsin-catalyzed acyl transfer in ice at −18°C using ethyl esters of *N*-maleyl-L-tyrosine and L-tyrosine as the acyl group donors has been studied. Although the amino acids did not act as acyl acceptors in liquid water, the high yields of peptides were obtained in frozen solutions at pH 10.5 (before freezing). The efficiency of amino acids in the formation of the corresponding dipeptides depended on the substrate used, and decreased in the order Ser,Thr,Gln>Lys>Cit>Ala>Gly>Asn>Arg>Glu>Val>Orn>Asp (with no peptide formed with His, Leu, Ile and Pro) for *N*-maleyl-L-tyrosine ethyl ester and Ser>Lys>Orn>Arg,Cit>Gln>Thr>Asn>Ala>Gly (with no peptide formed with Glu, Val, Asp, His, Leu, Ile and Pro) for L-tyrosine ethyl ester.

α-Chymotrypsin; Nucleophile specificity; Frozen solution

1. INTRODUCTION

It has been shown recently that freezing of the reaction mixture in the protease-catalyzed acyl-transfer changed the relative rates of the acyl-enzyme hydrolysis and aminolysis in favor of aminolysis [1]. In the reactions of chymotrypsin with Mal-Tyr-OEt or H-Tyr-OEt in the presence of free arginine in frozen solution the appropriate dipeptides have been synthesized in remarkably high yields while no peptides were formed in these mixtures in liquid water [1,2].

The possibility of using free amino acids as nucleophiles may significantly increase the synthetic potential of protein-catalyzed acyl transfer as a method of preparing peptides since the use of the nucleophiles in the form of amides, as is possible in water solutions, generates the rather inconvenient problem of removing amide blocking from the peptide synthesized, and the use of amino acid esters as nucleophiles is not effective because of their low nucleophilic activity and the liability of ester bond to enzymatic cleavage which leads to the appearance of undesired side-products.

In this paper we report on successful dipeptide synthesis in the reactions of α-chymotrypsin with H-Tyr-OEt and Mal-Tyr-OEt in ice using various free amino acids as acyl acceptors. The relative nucleophilic efficiencies of the amino acids have been compared with those of the respective amides in water [3–5] and organic solvents [4,5].

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Abbreviations: HPLC, high performance liquid chromatography; OEt, ethyl ester; OMe methyl ester; Ac, acetyl; Mal, maleyl; Bz, benzoyl; Cit, citrulline; Orn, ornithine; X, amino acid residue. Amino acids were of L-configuration.

2. MATERIALS AND METHODS

α-Chymotrypsin (EC 3.4.21.1.) from the St. Petersburg Factory of Medical Preparations (Russian Federation) was used without further purification.

H-Tyr-OEt and amino acids were from 'Reanal' (Hungary). Mal-Tyr-OEt was synthesized as described elsewhere [6].

Reactions were performed in 1 ml polypropylene tubes. The tubes containing the water solutions of substrate and amino acid adjusted to appropriate pH by 1 M HCl or NaOH, were cooled to 0°C and microliter quantities of the freshly prepared enzyme solution (10 mg/ml) in water were added. The tubes were rapidly shaken and inserted into liquid nitrogen. After about 3 min, they were transferred into a freezer and kept at −18°C 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 negligible.

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 containing 0.1% of trifluoro acetic acid was used as an eluent. The substrate and products were detected at 225 nm considering tyrosine chromophore. Amino acid analyses of the reaction products were carried out on a T 339 amino acid analyzer (Czechoslovakia).

In accordance with the operational reaction scheme **1**

$$E + S \underset{}{\overset{K_s}{\rightleftarrows}} ES \xrightarrow{k_2} EA \begin{cases} \xrightarrow{k_3} E + P_2 \\ \xrightarrow{k_4[H\text{–}X\text{–}OH]} E + P_3 \end{cases} \quad (1)$$

where S is H-Tyr-OEt or Mal-Tyr-OEt, H-X-OH is the amino acid

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nucleophile, EA is the acyl-enzyme, P_1 is ethanol, P_2 is H-Tyr-OH or Mal-Tyr-OH, P_3 is H-Tyr-X-OH or Mal-Tyr-X-OH, we have used the ratios of the peak areas $[P_3]/[P_2]+[P_3]$ in per cent for the yield of desired peptide product.

3. RESULTS AND DISCUSSION

The amount of the peptides formed in the chymotrypsin-catalyzed acyl transfer in frozen solutions has been shown to depend on pH of the reaction mixture before freezing [1,2]. The pH-dependence of the peptide yield in the case of Mal-Tyr-OEt as substrate is given in Fig. 1. The peptide yield increased with increasing pH up to about 10.5 which corresponds to the almost complete deprotonization of the α-ammonium group of the nucleophiles.

It is interesting to note that changing the dissociation equilibrium of the ammonium group is the only possibility of increasing the effective concentration of the amino component in the reaction medium since the experiments were carried out in a 20-fold excess of the nucleophiles over the substrate where an apparent saturation with the nucleophile is achieved [2]. In these conditions the volume of liquid microinclusions in ice in the reaction mixture is determined by the total amount of the excess nucleophile in a way that keeps its concentration in the unfrozen microinclusions constant as discussed in [7].

Further increase in the initial pH value of the reaction mixture decreased the peptide yield. This observation may indicate dissociation of an ionizable group of the enzyme with pK_a value over 11 which has been shown to affect the rates of hydrolysis of various acyl-chymotrypsins [8].

Fig. 2 shows that with H-Tyr-OEt as acyl donor the pH-dependencies of the peptide yield are similar to those with Mal-Tyr-OEt. This allows us to use the 'maximum' peptide yields at pH 10.5 as the comparable parameters for describing the efficiency of amino acids as acyl acceptors in frozen solutions. The data are listed in Table I. No peptide formation was observed in the reference experiments in liquid solutions at 2°C and at room temperature.

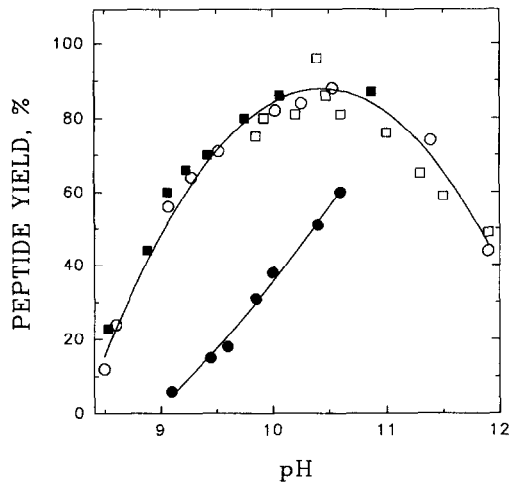

Fig. 1. Dependence of the yield of the chymotrypsin-catalyzed synthesis of dipeptides Mal-Tyr-X-OH in frozen solution upon pH at −18°C; $[E]\approx 2\times10^{-6}$ M, reaction time 4 h, [H-X-OH] = 100 mM, [Mal-Tyr-OEt] = 5 mM; X = Gln (■), Thr (□), Glu (●), Lys (○).

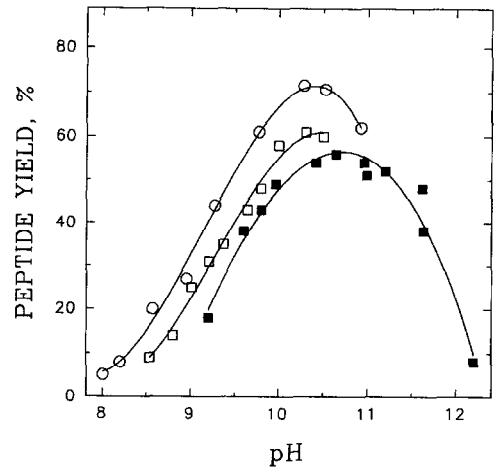

Fig. 2. Dependence of the yield of the chymotrypsin-catalyzed synthesis of H-Tyr-X-OH in frozen solution upon pH at −18°C; $[E]\approx 2\times10^{-6}$ M, reaction time 4 h, [H-X-OH] = 100 mM, [H-Tyr-OEt] = 5 mM; X = Lys (○), Thr (■), Arg (□).

The efficiency of amino acids as acyl acceptors was different for the two substrates used: with the exceptions of arginine and ornithine the peptide yields were higher

Table I

Dipeptide yields in the α-chymotrypsin-catalyzed acyl-transfer in frozen solutions at −18°C using H-Tyr-OEt and Mal-Tyr-OEt as the acyl donors and free amino acids as nucleophiles at pH 10.5 (before freezing).

Nucleophile H-X-OH	Peptide yield, %	
	H-Tyr-X-OH	Mal-Tyr-X-OH
Arg	68	65
Lys	71	94
Orn	70	55
His	<2	<2
Cit	68	92
Gln	61	96
Asn	39	80
Ser	78	96
Thr	50	96
Asp	<2	29
Glu	<2	60
Gly	11	85
Ala	15	87
Val	<2	58
Leu	<2	<2
Ile	<2	<2
Pro	<2	<2

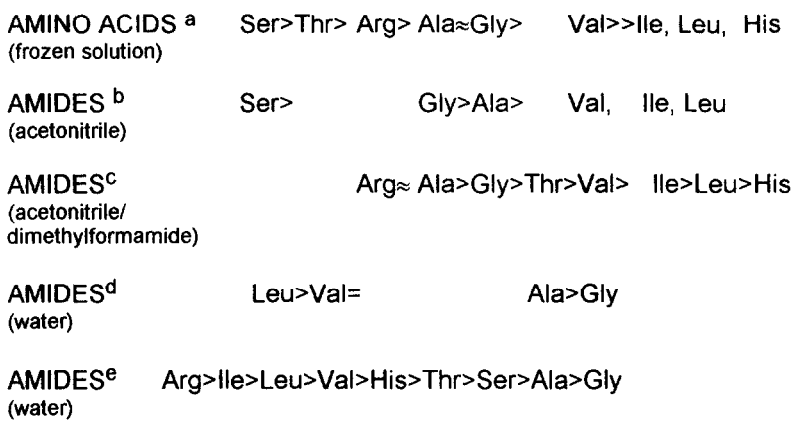

Fig. 3. Relative reactivities of free amino acids and the corresponding amides with acyl-chymotrypsins. [a]Amino acids are ordered by the descending of the sum of the maximum peptide yields in the reactions with H-Tyr-OEt and Mal-Tyr-OEt. [b]Data from [4], Ac-Tyr-OEt as substrate. [c]Data from [5], Bz-Tyr-OEt as substrate. [d]Data from [11], Ac-Tyr-OEt as substrate. [e]Data from [3,5], Mal-Ala-Ala-Phe-OMe as substrate.

with Mal-Tyr-OEt. Amino acids with negatively charged side chains gave peptides only with this substrate. The lower peptide yields in the case of H-Tyr-OEt could not be caused by a competing aminolysis of the tyrosyl-enzyme by H-Tyr-OEt since the amount of H-$(Tyr)_n$-OH formed during the synthesis was found to be negligible. The efficiency of amino acid nucleophiles was also different from that with Mal-Phe-OMe as acyl donor [9]. In water, on the other hand, a good correlation between the nucleophilic reactivities of amides has been found for different acyl enzymes [10].

The relative nucleophilic efficiencies of free amino acids in frozen solutions in comparison with the reactivities of the corresponding amides towards various acyl-chymotrypsins in water and in two organic solvent systems are shown in Fig. 3. The specificity of the tyrosyl-chymotrypsin aminolysis by free amino acids in frozen solution is substantially different from that by the corresponding amides in water. The high activity of serine and threonine in ice is especially noteworthy.

Favorable hydrophobic interactions in the active site of chymotrypsin have been found to be responsible for the comparatively high reactivities of amides with nonpolar side chains in water [3,5]. In the frozen solution the amino acids with bulky hydrophobic side chains did not yield any detectable amount of peptide.

As seen from Fig. 3 the relative nucleophilic efficiency of amino acids in peptide synthesis in frozen solutions is similar to that of the corresponding amides in organic solvents.

It is interesting to note that arginine nucleophiles occurred to be effective in all systems. In liquid water, the high efficiency of arginine-containing nucleophiles is believed to result from a favorable ionic interaction of the cationic charge of the Arg residue with a negatively charged residue in the enzyme surface near the S′-substitute [6]. However, as the amino acids containing hydrophilic hydroxyl or amide groups in the side chain, as well as the spatial uncharged analog of arginine, citrulline, showed also the high nucleophilic efficiencies, the yield-determining step of the acyl transfer reaction in frozen solutions seems not to involve these electrostatic interactions.

In conclusion, the possibility of using free amino acids as nucleophiles and their characteristic efficiency in the frozen solutions considerably increase the synthetic potential of the chymotrypsin-catalyzed acyl-transfer reaction as the method for the preparation of peptides.

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