# Chymotrypsin modified with polyethylene glycol catalyzes peptide synthesis reaction in benzene

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Chymotrypsin was modified in the zymogen form with 2,4-bis(*O*-methoxypolyethylene glycol)-6-chloro-*s*-triazine (activated $PEG_2$), followed by activation with trypsin. The modified enzyme was soluble in benzene and retained its enzymic activity. Acid-amide bond formation by the modified enzyme proceeded efficiently in benzene: *N*-benzoyltyrosine butylamide was made from *N*-benzoyl-L-tyrosine ethyl ester and *n*-butylamine, and benzoyltyrosine(oligo)phenylalanine ethyl esters were formed from *N*-benzoyl-L-tyrosine ethyl ester and L-phenylalanine ethyl ester.

*Chymotrypsin* *Polyethylene glycol* *Peptide synthesis*

## 1. INTRODUCTION

A chemical modification of proteins and enzymes with polyethylene glycol (PEG) has become an approach applicable to the solution of various problems in biological sciences. The production of IgE caused by protein allergens such as ovalbumin and ragweed pollen was suppressed by the treatment with respective proteins modified with PEG [1]. Modification of *E. coli* asparaginase [2], yeast uricase [3] and snake venom batroxobin [4] with PEG decreased their immunoreactivity towards antibodies against respective proteins. We have demonstrated that horseradish peroxidase modified with PEG is soluble in benzene and retains its enzymic activity [5]. A similarly modified lipase was also soluble in various organic solvents and had an ability to catalyze ester synthesis and aminolysis in benzene [6,7].

Here we report that chymotrypsin becomes soluble in benzene by the modification with PEG and that the modified enzyme catalyzes the aminolysis of esters to form peptide bonds.

## 2. MATERIALS AND METHODS

Crystallized $\alpha$-chymotrypsinogen (EC 3.4.21.1) was prepared from bovine pancreas as in [8]. Monomethoxypolyethylene glycol ($M_r$ 5000) was obtained from Polyscience and amino acid derivatives were obtained from Peptide Research Foundation. Other reagents used were of analytical grade. 2,4-Bis(*O*-methoxypolyethylene glycol)-6-chloro-*s*-triazine (activated $PEG_2$) was synthesized from monomethoxypolyethylene glycol and cyanuric chloride as in [2,4].

### 2.1. *Preparation of modified chymotrypsin*

Since the terminal amino groups newly exposed by the activation play an important role in the enzymic activity of chymotrypsin [9], the modification with activated $PEG_2$ was performed with its zymogen as follows prior to the activation: to 100 ml of chymotrypsinogen solution (1.4 mg/ml) in 40 mM sodium tetraborate buffer (pH 10.0) was added 14 g of activated $PEG_2$, followed by gentle stirring at 37°C for 1 h. The reaction mixture was subjected to ultrafiltration using an Amicon Diaflo PM-30 membrane to remove unreacted activated $PEG_2$. The degree of modification of amino groups in the chymotrypsinogen molecule was 83%, which was estimated using the trinitrobenzenesulfonate method [10]. The modified chymo-

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trypsinogen was activated with trypsin to obtain modified chymotrypsin. The modified enzyme was dialyzed against cold water and lyophilized. The yield of the modified enzyme was 96% in terms of recovered protein. The modified chymotrypsin retained 57% of the esterolytic activity in aqueous solution when *N*-acetyl-L-tyrosine ethyl ester was used as a substrate.

### 2.2. *Synthesis of* N-*benzoyltyrosine butylamide (Bz-Tyr-NHBu)*

To 200 $\mu$l benzene containing 0.7 mM benzoyl-L-tyrosine ethyl ester (Bz-Tyr-OEt) and 50 mM *n*-butylamide ($BuNH_2$) were added 100 $\mu$l benzene containing the modified chymotrypsin (1 mg protein/ml). The transparent mixture (300 $\mu$l) was incubated at 37°C and an aliquot (10 $\mu$l) was removed from the reaction mixture at a given time. The sample was subjected to analysis with a Shimadzu LC-3A high-performance liquid chromatograph using a Shodex Silicapack E-411 column with 2.5% methanol in chloroform. The elemental analysis of the product was made by a Perkin-Elmar elemental analyzer model 240.

### 2.3. *Synthesis of* N-*benzoyltyrosine oligophenylalanine ethyl ester (Bz-Tyr-(Phe)$_n$-OEt)*

To 950 $\mu$l benzene containing 1.24 mM Bz-Tyr-OEt, 10 mM L-phenylalanine ethyl ester (Phe-OEt) and 1% diethyl ether were added 50 $\mu$l of the modified chymotrypsin in benzene (0.3 mg protein/ml) at 1-h intervals over a period of 7 h. The modified enzyme in benzene was stabilized in the presence of a trace amount of ethyl ester as was observed with $PEG_2$-modified lipase (unpublished). The mixture was incubated at 37°C in the presence of Molecular Sieves 3A (100 mg) and 10 $\mu$l of the mixture were taken out at a given time. The amount of products was assayed with a Shimadzu chromatograph mentioned above. Elution was conducted with 0.1% acetic acid and 1% methanol in chloroform. The molecular mass and amino acid composition of each product were determined by liquid chromatography with a Shodex GPC, KF-802 column equilibrated with tetrahydrofuran and by amino acid analysis with a Hitachi model 835 amino acid analyzer, respectively.

## 3. RESULTS AND DISCUSSION

The modified chymotrypsin prepared by the activation of modified chymotrypsinogen with trypsin had an $M_r$ far greater than that of the non-modified enzyme ($M_r$ 25000) [9]. This is due to the attachment of polyethylene glycols to the enzyme molecule. In fact, 12 out of the total of 15 amino groups in the chymotrypsinogen molecule were coupled with activated $PEG_2$. The modified enzyme was soluble in organic solvents such as benzene, toluene, dimethylformamide, dioxane and ethanol and was able to catalyze hydrolysis and aminolysis in benzene.

Our attention was focused on aminolysis of esters to form peptide bonds. Fig.1 represents the peptide bond formation with the modified chymotrypsin in benzene. Synthesis of Bz-Tyr-NHBu proceeded efficiently with increasing incubation time when Bz-Tyr-OEt and $BuNH_2$ were used as substrates (curve A). In contrast, the amount of Bz-Tyr-OEt decreased markedly with incubation time as shown by curve B. The final amount of product closely agreed with the initial amount of the substrate, Bz-Tyr-OEt. In the absence of the modified chymotrypsin (curve C), Bz-Tyr-NHBu was not synthesized at all. These results indicate that peptide bond formation by

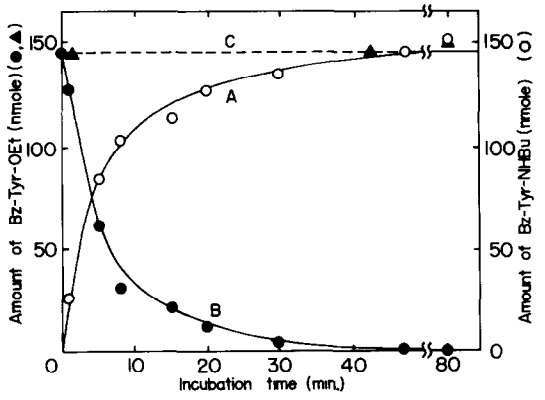


Fig.1. Synthesis of *N*-benzoyltyrosine butylamide (Bz-Tyr-NHBu) in benzene at 37°C catalyzed by the modified chymotrypsin when Bz-Tyr-OEt (0.49 mM) and $BuNH_2$ (33.3 mM) were used as substrates. (A) Amount of Bz-Tyr-NHBu synthesized; (B) amount of one of the substrates, Bz-Tyr-OEt; (C) amount of Bz-Tyr-OEt without modified chymotrypsin. Modified chymotrypsin, 13.3 $\mu$M.

Table 1

Synthesis of *N*-benzoyltyrosine-oligophenylalanine ethyl ester (Bz-Tyr-(Phe)$_n$-OEt) catalyzed by modified chymotrypsin in benzene at 37°C

| Incubation time (h) | Relative amount (%) | | |
|---|---|---|---|
| | Bz-Tyr-OEt | Bz-Tyr-(Phe)$_n$-OEt | |
| | | $n = 1$ | $n = 2$ |
| 0 | 100 | 0 | 0 |
| 1 | 74 | 17 | 0 |
| 2 | 43 | 37 | 0 |
| 3 | 11 | 53 | 2 |
| 4 | 10 | 60 | 7 |
| 5 | 0 | 60 | 11 |
| 6 | 0 | 48 | 12 |

[Bz-Tyr-OEt] = 1.24 mM, [Phe-OEt] = 10 mM. $n$, number of phenylalanine residues per molecule (estimated from the $M_r$ and amino acid composition)

aminolytic reaction in benzene occurs quantitatively without any ester hydrolysis. This conclusion was confirmed by elemental analysis of the product. The ratio of nitrogen and carbon atoms (N/C) of the product was 0.118 which is in good agreement with the theoretical value for Bz-Tyr-NHBu (0.116). In the synthesis mentioned above, *N*-acetyl-L-tyrosine ethyl ester and *N*-acetyl-L-phenylalanine ethyl ester were also good substrates, while L-phenylalanine ethyl ester, phenylacetic acid ethyl ester, $\beta$-phenylpropionic acid cholesterol ester and cinnamic acid ethyl ester were not. Aliphatic amines such as *n*-butylamine, caprylamine, laurylamine and myristylamine were all preferred amines for the reaction.

The next series of experiments concerned the synthesis of Bz-Tyr-(Phe)$_n$-OEt with the modified chymotrypsin in benzene (table 1). Incubation of a mixture of Bz-Tyr-OEt and Phe-OEt in benzene gave rise to peptide bond formation. The amount of one of the substrates, Bz-Tyr-OEt, decreased with incubation time, accompanied by an increase in the amount of Bz-Tyr-Phe-OEt. The formation of Bz-Tyr-(Phe)$_2$-OEt was noted after several hours of incubation. Products with much higher $M_r$ values, probably oligopeptides, were obtained after extensive incubation (not shown). Peptide synthesis reaction with chymotrypsin has been extensively studied in either aqueous [11] or biphasic aqueous organic systems [12]. The problem of hydrolysis is always associated with these techniques. This problem was obviated by the present technique.

This communication describes the first successful attempt to form a peptide bond by aminolysis in benzene, using chymotrypsin modified with polyethylene glycol. It is of note that the enzyme retains its activity and hence its proper conformation even in benzene by the modification with an amphipathic polymer, polyethylene glycol.

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