# STRUCTURAL STUDIES ON ε-PROTOTOXIN OF CLOSTRIDIUM PERFRINGENS TYPE D. LOCALIZATION OF THE SITE OF TRYPTIC SCISSION NECESSARY FOR ACTIVATION TO ε-TOXIN

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## SUMMARY

The mechanism of activation of ε-prototoxin to ε-toxin has been ascertained from partial amino acid sequences of both ε-prototoxin and ε-toxin. The activation of ε-prototoxin from Clostridium perfringens type D by brief exposure to trypsin is caused by scission of a peptide bond between $Lys_{14}$ - $Ala_{15}$. A small peptide (14 amino acid residues) is split from the $NH_2$-terminus of the ε-prototoxin to give the active ε-toxin.

## Introduction

A large number of biologically active proteins exist as inactive precursors that require limited proteolytic degradation for activation.

Whereas a considerable amount of work was done on the mechanism of activation of enzyme precursors (For review see ref. 1), very little work is available on the chemical and conformational changes accompanying the mechanism of activation of bacterial prototxins. The isolation of ε-prototoxin from culture fluids of Clostridium perfringens type D in a pure form[2,3] prompted us to undertake the present structural studies to identify the chemical changes accompanying the activation of the non-toxic ε-prototoxin to the powerful ε-toxin by short exposure to trypsin.

## Materials and Methods

Materials - Cloning, culture of Clostridium perfringens type D, pre-

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paration of crude ε-prototoxin and chromatography on DEAE-cellulose and CM-cellulose was as described previously in detail by Habeeb[2]. Pure ε-prototoxin was the major fraction F1,3 from CM-cellulose chromatography and will be referred to as ε-prototoxin. Trypsin-TPCK was obtained from Worthington Biochemical Corporation. Reagents and solvents for the sequencer were obtained from Beckman Instruments. All other chemicals were of analytical grade and used without further purification.

Activation of ε-Prototoxin - ε-Prototoxin (40 mg) was dissolved in 4 ml 0.01 M ammonium bicarbonate and brought to 40°C. Digestion was allowed to proceed for 30 min. with 0.4 mg trypsin-TPCK. After digestion, soybean trypsin inhibitor (0.5 mg) was added and inhibition of tryptic activity was confirmed by loss of activity on p-tosyl-L-arginine methyl ester hydrochloride[4] The solution was then freeze-dried.

Isolation of ε-toxin - The digestion product of ε-prototoxin was chromatographed on Sephadex G25 fine column (2.5 x 33 cm) and eluted with 0.01 M $NH_4HCO_3$ collecting fractions of 4.6 ml. The eluate was monitored for absorbance at 280 nm and at 235 nm for revealing the activation peptide. Fractions were pooled and freeze dried.

Reduction and alkylation of ε-toxin - ε-toxin or ε-prototoxin (30 mg) was dissolved in 2 ml Tris-glycine buffer pH 8 containing 0.5 mg EDTA/ml[5] and to the solution was added 2 ml 5 M guanidine in Tris glycine buffer followed by 1 ml 0.25 M β-mercaptoethanol. Reduction was allowed to proceed for 1 hr. followed by blocking the liberated sufhydryl groups by iodoacetamide (60 mg) at pH 8.4. After completeness of alkylation (15 min) the solution was dialyzed against 0.01 M $NH_4HCO_3$ and freeze dried.

Amino Acid Analyses - Amino acid analysis was performed in a Durrum D-500 amino acid analyzer on samples hydrlysed with 6N HCl at 110° for 20 hrs.

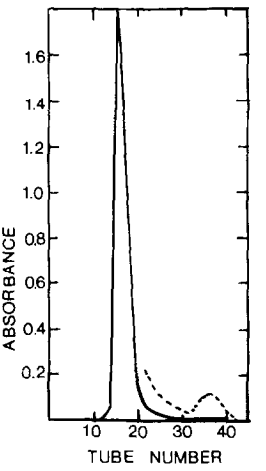


Fig. 1. Elution pattern of trypsin-treated ε-prototoxin on Sephadex G25. Absorbance at 280 nm ____; absorbance at 235 nm ___ ___.

Sequence determination of ε-Prototoxin and ε-Toxin - Sequential degradation of amino acids from the amino terminus of prototoxin (50-100 nm) and toxin (50-100 nm) was achieved in a Beckman 890C automated sequencer using Braner et al[6] program with slight modification, in which 0.1 M quadrol was replaced by 0.5M. PTH amino acids were identified on a Hewlett-Packard gas chromatograph #5830A, by thin layer chromatography on polyamide sheets[7] and also by back hydrolysis with 6N HCl or HI at 130° for 20 hrs to their parent amino acid.

## Results

Chromatography of the product of activation of ε-prototoxin on Sephadex G25 -

Separation of ε-toxin from the split peptide was achieved on a Sephadex G25 column (Fig. 1). The first peak (tubes 14-22) comprised ε-toxin while the second peak (tubes 32-42) contained the split peptide.

Amino acid analysis of prototoxin, and toxin is reported in Table I, while the sequence is presented in figure 2.

Amino Acid Sequence - As shown in Fig. 2, the first 20 residues from

Prototoxin-

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                    5                   10                  15                  20
Lys-Glu-Ile-(Cys(Cm)-Asx-Pro-Val-Ser-Tyr-Glu-Met-Ser-Tyr-Lys-Ala-Ile-Tyr-Asx-  ?-Val-
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Toxin-

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                                                    5                   10
                                          Ala-Ile-Tyr-Asx-Asx-Val-Leu-Asx-Pro-Leu-Ile-Glx-  ?-Ty
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Fig. 2. - Amino acid sequence of prototoxin and toxin. Amino acids not confirmed are in parenthesis and those not identified are shown as ?.

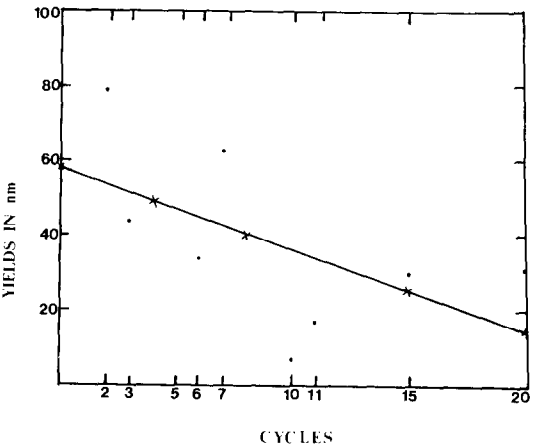


Fig. 3. Yields of stable PTH amino acids ar various cycles of ε-prototoxin.

the $NH_2$-terminal end of ε-prototoxin have been ascertained except cysteine at position 4 and a blank at position 19. Cysteine at position 4 was identified by gas chromatography but could not be confirmed. However, its position has been tentatively assigned based on the reasoning that ε-prototoxin looses its only cysteine residue[3] during its activation. Similarly, the first 13 residues of ε-toxin have been determined but for position 12 which could not be identified. The yields of PTH amino acids at different cycles are shown in Fig. 3. Theinitial yield was 60% and repetitive yields were 96% between two consecutive cycles. Alignment of the sequenced ε-prototoxin and toxin (Fig. 2) shows that both have a common sequence Ala-Ile-Tyr-Asx-Asx-Val. That this sequence commences the $NH_2$-terminal end of ε-toxin is evidence that the process of activation of ε-prototoxin by trypsin involves the scission of one peptide bond between $Lys_{14}$-$Ala_{15}$. Moreover the presence

Table I. AMINO ACID COMPOSITION OF ε-TOXIN AND FRAGMENT (RESIDUES PER MOLE)

| | ε-Toxin | | | Split Fragment | | Prototoxin |
|---|---|---|---|---|---|---|
| | | | Integral | Calculated[b] | Sequence[c] | (ref. 3) |
| Asp | 49.3 | 51.0 | 51 | 1 | 1 | 52 |
| Thr | 28.7 | 30.1 | 31[a] | 0 | 0 | 31 |
| Ser | 19.9 | 19.9 | 22[a] | 3 | 2 | 25 |
| Glu | 27.6 | 28.6 | 28 | 0 | 2 | 28 |
| Pro | 10.7 | 11.1 | 11 | 1 | 1 | 12 |
| Gly | 16.6 | 17.5 | 17 | 0 | 0 | 17 |
| Ala | 13.1 | 14.1 | 14 | 0 | 0 | 14 |
| Val | 25.7 | 26.5 | 26 | 2 | 1 | 28 |
| Met | 4.4 | 4.6 | 5 | 0 | 1 | 5 |
| Ile | 11.9 | 12.4 | 12 | 3 | 1 | 15 |
| Leu | 18.4 | 18.8 | 18 | 0 | 0 | 18 |
| Tyr | 16.4 | 16.6 | 17 | 0 | 2 | 17 |
| Phe | 9.0 | 9.1 | 9 | -1 | 0 | 8 |
| Lys | 27.6 | 26.8 | 28 | 3 | 2 | 31 |
| His | 2.3 | 2.9 | 3 | -1 | 0 | 2 |
| Arg | 4.0 | 4.1 | 4 | 1 | 0 | 5 |
| SCM-Cysteine | | | 0 | 1 | 1 | 1 |
| | | | | 13 | 14 | |

[a]Values corrected for losses during hydrolysis by extrapolation to zero time of hydrolysis.

[b]Obtained by difference between amino acid composition of ε-prototoxin and ε-toxin.

[c]Obtained from sequence of N-terminal fragment of ε-prototoxin.

of only one amino acid at each cycle during sequencing of either ε-prototoxin or ε-toxin indicates the purity of the two proteins and that during the period

of activation (30 min) only $Lys_{14}$ is susceptible to cleavage by trypsin. Therefore activation of ε-prototoxin to toxin is accompanied by a release of low molecular weight (14 amino acid residues long) peptide from the $NH_2$-terminal end of prototoxin.

Amino acid analysis - Table I gives the amino acid compositions of ε-toxin, ε-prototoxin and the split peptide. The difference in the amino acid compositior between ε-prototoxin and ε-toxin gives the calculated amino acid composition of the split peptide. Although the calculated amino acid composition of the split peptide does not compare exactly with the residues identified in its sequence, this anomaly is not totally unexpected. The loss of one or two residues is hard to quantitate on amino acid residues which exist in high concentrations. Therefore the amino acid composition of the split peptide derived from sequence analysis is to be considered as the most representative.

## Discussion

The biological activity of various proteins is dependent on activation of a precursor by a brief enzymic exposure as shown with several proteolytic enzymes, clotting factors, components of the complement systems, and various prototoxins. This brief exposure to a proteolytic enzyme results in scission of a susceptible peptide bond resulting in small conformational changes and the protein may still maintain its compact structure. In some cases a longer exposure to proteolytic enzyme may result in further cleavage at newly exposed sites and progressive fragmentation of the biologically active protein results in concomitant loss of activity. Activation of chymotrypsin A involves an initial scission of $Arg_{15}$-$Ile_{16}$ bond followed by scission at $Leu_{13}$-$Ser_{14}$, $Try_{148}$ and $Asp_{147}$-$Ala_{148}$[8]; and of trypsin, the bond between $Lys_6$-$Ile_7$ is split[9]. However, during activation of pepsinogen an $NH_2$-terminal fragment consisting of 40-44 amino acid residues[10] was split which was latter found to involve an initial scission to liberate a fragment $Leu_1$-$Leu_{16}$[11].

Various bacterial toxins are secreted in the culture fluids as inactive prototoxins which are subsequently activated by proteolytic enzymes elaborated by proteolytic strains of the bacteria e-g. ε-prototoxin of C-perfringens type D and botulinum toxin from C-botulinum. To study the mechanism of activation of a bacterial prototoxin, the isolation of a pure prototoxin free of any contaminating toxin and other bacterial exoantigens is necessary. By use of the proper bacterial strain and culture conditions[2] it was possible to isolate ε-prototoxin from C-perfringens type D free of ε-toxin (less than 0.10%) and free of contaminating exoantigens. It showed only one $NH_2$-terminal lysine residue and one COOH-terminal lysine residue[3]. Activation of ε-prototoxin resulted in a toxin which was more electronegative on polyacrylamide gel than the parent molecule[2]. Moreover, the activation was not accompanied by a large decrease in molecular weight[3] nor by a loss in immunochemical reactivity[2] since both prototoxin and toxin showed a reaction of complete immunochemical identity in agar double diffusion with antiprototoxin antiserum[2]. Based on these observations it was suggested that the activation of ε-prototoxin to ε-toxin involves the removal of a small peptide with concomitant conformational changes detected by optical rotatory dispersion and circular dichroism measurements[12]. The present study sheds some light on the mechanism of activation of ε-prototoxin and identifies $Lys_{14}$ as the site of tryptic attack. Therefore, the process of activation of ε-prototoxin is similar to that of enzyme precursors. It is not clear whether the observed conformational change(s) that occur on removal of the split peptide $Lys_1$-$Lys_{14}$ are necessary pre-requisites for toxicity or whether they are simply the irrelevant by-products of the cleavage process.

In contrast to the activation of ε-prototoxin by trypsin, tetanus toxin (mol. wt. 160,000) is split into two fragments (mol. wt. 53,000 and 167,000) by mild trypsinization and reduction with loss of toxicity [13,14]. On reoxidation the two fragments associate to form the toxin. Diphtheria

toxin (mol. wt. 60,000) requires activation by a brief tryptic digestion followed by reduction which splits the molecule into two fragments (fragment A, mol. wt. 24,000 and fragment B, mol. wt. 38,000). Only fragment A is active when released from B after reduction of the disulfide bond. However fragment B is required for toxicity in test animals, and it appears to be necessary for attachment of toxin to cells[15,16].

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