PROTEOLYTIC CLEAVAGE OF EXOTOXIN A FROM *PSEUDOMONAS AERUGINOSA*

Formation of an ADP-ribosyltransferase active fragment by the action of *Pseudomonas* elastase

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1. Introduction

Exotoxin A, produced by most clinical strains of *Pseudomonas aeruginosa* [1–3], is probably the virulent factor in the pathogenesis of *Pseudomonas* infections [4–8]. The action of this toxin is remarkably similar to that of diphtheria toxin [9]. Exotoxin A, like diphtheria toxin, also inhibits protein synthesis in mammalian cultured cells and some organs in whole animals [10–13] by catalyzing the transfer of the moiety of adenine 5′-diphosphate (ADP)-ribose from nicotinamide adenine dinucleotide (NAD) onto mammalian elongation factor 2 (EF-2). The structure–activity relationships of diphtheria toxin have been reviewed [14], but little definite information is available on pseudomonas exotoxin A. Although several studies suggest that by the action of contaminant protease, an enzymatically active fragment exists in partially purified exotoxin A preparations treated by repeated freezing and thawing [15] or in the culture supernatant [16] of strain PA 103, definite evidence is lacking. This study shows that an enzymatically active but non-toxic fragment can be formed from intact exotoxin A using elastase of *P. aeruginosa*.

2. Materials and methods

Exotoxin A from *P aeruginosa* was purified according to [17]. This toxin showed a homogeneous nature in polyacrylamide gel electrophoresis under non-denaturing conditions by the method below, and had minimal lethal dose (MLD) for mouse of 7500/mg toxin protein. Crystalline elastase from *P. aeruginosa*, IFO 3455, was prepared as in [18]. Highly purified EF-2 of pig liver was prepared by the method in [19]. LM cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD). LM is a mouse fibroblast cell line derived from L-929 cells. Cells were maintained in monolayers in medium 199 (Nissui Pharmaceuticals, Tokyo) with 0.5% bactopeptone (Difco, Detroit, MI). For the experiments, cells were seeded into multiwell tissue culture plates (Falcon 3088, Oxnard, CA) at 5×10^5 cells/well (8 cm^2) 18 h before use. Proteins were estimated by the Lowry method [20] using bovine serum albumin as standard. All other chemicals were of reagent grade.

Exotoxin A (0.5 mg/ml) was treated with elastase at 25°C in 50 mM Tris–HCl buffer (pH 7.5) with several molar ratios of elastase to exotoxin A. At selected times, samples were taken and diluted with cold saline solution to the suitable concentrations for assays of cytotoxicity for LM cells, ADP-ribosylation of EF-2 and gel electrophoresis.

ADP-ribosylation activity was assayed in 0.1 ml medium containing 0.1 M Tris–HCl buffer (pH 8.0), 40 mM dithiothreitol, 0.1 M ethylenediamine tetraacetate, 1.6 μM [U-^{14}C]NAD (280 mCi/mmol, Amersham-Searle) and 0.2 mg pig liver EF-2. Exotoxin A (5 μg) was used in this assay, depending on the specific activity of toxin. The reaction was initiated by the addition of toxin. Reaction mixtures were incubated at 25°C for 10 min, and the reaction was stopped by the addition of 0.25 ml 10% trichloroacetic acid. The radioactivity of the acid-insoluble material was measured with a liquid scintillation counter Beckman LS-230. A complete set of control tubes, with elastase and without toxin, was included in each experiment, and the amount of ADP-ribosyl EF-2 formed was determined by the difference

between the counts recovered from the incubation mixtures with and without toxin.

The cytotoxic activity of the toxin was expressed as the inhibition of protein synthesis in cultured whole cells, which was measured as in [13]. ^{3}H-Labelled amino acid mixtures were obtained from New England Nuclear (NET 250). The dose of the toxin used for the LM cells was 10 ng/well. Each assay was carried out in triplicate.

Disc gel electrophoresis under non-denaturing conditions was performed as in [21]. Sodium dodecyl sulphate (SDS)–polyacrylamide gel electrophoresis was performed following [22]. Sample loads were ~25 μg protein/slot. Marker proteins used for M_r estimation were: pig liver EF-2 (100 000); ovalbumin (43 000); *P. aeruginosa* alkaline protease (49 000); *Bacillus subtilis* neutral protease (34 000); bovine pancreatic chymotrypsinogen (25 000); and soybean trypsin inhibitor (22 000). Gels were stained for protein with Coomassie blue G-250 in 3% perchloric acid. To determine the enzymic activity in various bands, parallel running gels were sliced and soaked in the assay solutions for 5 h at 25°C, then ADP-ribosyl EF-2 was measured as above.

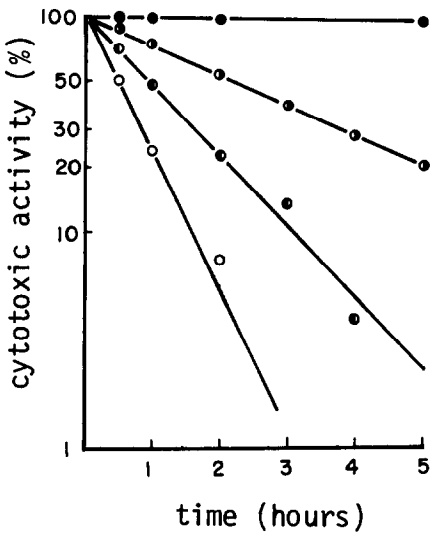

Fig.1. Kinetics of inactivation of cytotoxicity of exotoxin A by *Pseudomonas* elastase. Exotoxin A (0.5 mg/ml) was incubated at 37°C with *Pseudomonas* elastase at molar ratios (elastase:exotoxin A) of 1:10 (○), 1:20 (◔), 1:50 (◑) and exotoxin A only (●). Radioactivity incorporated with LM cells monolayer without exotoxin A was 63 000 cpm and elastase at these concentrations did not affect the radioactivity uptake by LM cells.

3. Results and discussion

The inactivation of the cytotoxicity for mouse LM cells of exotoxin A by *Pseudomonas* elastase follows pseudo-first order kinetics (fig.1). However, ADP-ribosyltransferase activity (ADP-ribosylation of EF-2) is considerably more stable towards the action of elastase (fig.2). After 5 h treatment of exotoxin A with elastase, enzymic activities were to a considerable extent retained intact. When samples of an incubation mixture containing exotoxin A and elastase were subjected to SDS–polyacrylamide gel electrophoresis, the band corresponding to intact exotoxin A (M_r 72 000) gradually disappeared and several fragments including one large one appeared as the incubation time increased (fig.3). The large proteolytic fragment had M_r 48 000. Among the new fragments, only this fragment had enzymic activity (fig.4), although it had lost its cytotoxicity for LM cells at the level of 1 μg of this fragment/well. Interestingly, exotoxin A treated with elastase at a molar ratio of 1:20 (elastase: toxin) for 2 h showed a single band in disc gel electrophoresis under non-denaturing conditions (fig.5).

Two possibilities are postulated from these results:

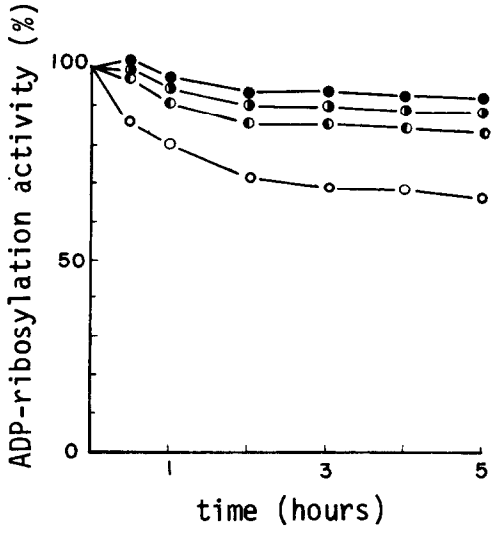

Fig.2. Effects of *Pseudomonas* elastase on ADP-ribosylation activity of exotoxin A. Proteolysis conditions were described in text and symbols were the same as in fig.1. Radioactivities incorporated to acid-insoluble materials by the action of intact exotoxin A under no treatment had 4250 cpm.

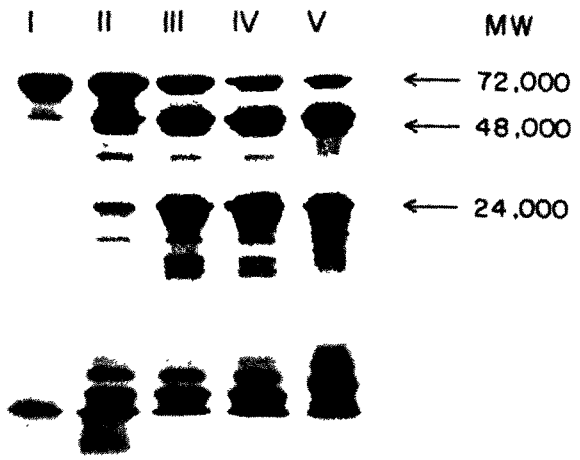

Fig.3. SDS–polyacrylamide gel electrophoresis of exotoxin A treated with *Pseudomonas* elastase. The molar ratio (elastase:exotoxin A) was 1:20. The incubation time intervals were 0 h (I), 0.5 h (II), 1 h (III), 2 h (IV) and 5 h (V), respectively. After digestion, each reaction mixture was treated for SDS electrophoresis (10% gel, pH 8.3) according to [22]. The direction of migration was toward the bottom of the photo graph.

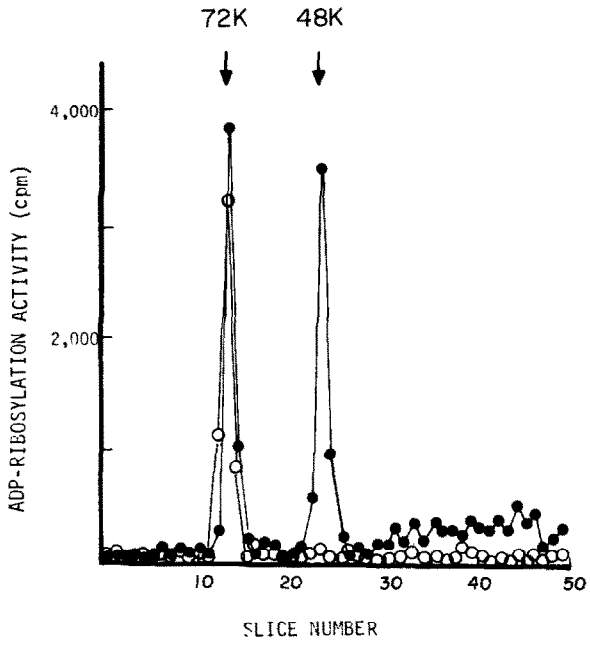

Fig.4. ADP-ribosylation activities of exotoxin A and its products by the action of *Pseudomonas* elastase. After SDS electrophoresis described in text, gel was sliced (3 mm thick) and ADP-ribosylation activity of each slice was measured as in text. Molar ratio (elastase:exotoxin A) was 1:20 and incubated for 2 h at 37°C. (○) Intact exotoxin A; (●) modified exotoxin A with elastase.

Fig.5. Polyacrylamide disc-gel electrophoresis of exotoxin A treated with *Pseudomonas* elastase at molar ratio of 1:20 (elastase:exotoxin A) for 2 h.

(1) Exotoxin A is split into several fragments by the action of *Pseudomonas* elastase, and the fragments have the same mobilities as that of intact exotoxin A in disc gel electrophoresis under non-denaturing conditions. One of the fragments, corresponding to M_r 48 000, possesses ADP-ribosylation activity but no cytotoxic activity.

(2) Exotoxin A is nicked with *Pseudomonas* elastase but fragments are not liberated due to the presence of intra-chain disulfide bonds.

The nicked exotoxin A shows ADP-ribosylation activity but no cytotoxic activity. A fragment (M_r 48 000) possessing the enzymic activity can be released from the nicked exotoxin A by SDS treatment in the presence of a reducing agent. The latter possibility may

be more plausible because it would be unusual for all the proteolytic fragments to show the same patterns in disc gel electrophoresis under non-denaturing conditions.

This study is the first to show that an enzymatically active non-toxic fragment of exotoxin A is produced by the action of *Pseudomonas* elastase followed by SDS treatment. A similar fragment found in [15,16] was believed to be formed by the action of contaminant protease, but its M_r of 27 000 was considerably smaller than that observed in this study. Further work is needed to clarify the conflict.

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