

# Proteolytic cleavage of *Pseudomonas aeruginosa* exotoxin A in the presence of lipid bilayers of different composition

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**Abstract** *Pseudomonas aeruginosa* exotoxin A (ETA) must be proteolytically nicked by furin at Arg<sup>279</sup> before being translocated into the cytosol of target cells. A similar cleavage can also be obtained with trypsin. Using this assay we could show that the interaction with lipid bilayers can strongly influence the extent of nicking. We found that in the presence of vesicles containing negatively charged lipids ETA is cleaved into its two fragments A and B at enzyme concentrations ~50 times lower, or at pH values higher by 1.5 units, than in the absence of lipids. We suggest that the interaction with the lipid bilayer of the positively charged loop containing Arg<sup>279</sup> provides the energy for its partial unfolding and makes it more accessible for proteolysis.

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**Key words:** ADP-ribosylating toxin; Translocation; Enzymatic cleavage; pH dependence; Acidic phospholipid

## 1. Introduction

Exotoxin A (ETA) is the most potent toxin produced by *Pseudomonas aeruginosa* [1,2], an opportunistic microorganism responsible for severe infections in immunocompromised human hosts, e.g. patients with AIDS or cystic fibrosis [3]. Interestingly, it is also crucial for animal and even plant infection by this pathogen [4]. It causes apoptosis of the eukaryotic cell by blocking protein synthesis via ADP-ribosylation of elongation factor 2 [5]. Because of its potency, it has been intensively used for the construction of immunotoxins for cancer therapy [6]. ETA is a single polypeptide chain organized into three domains [2,7]: an amino-terminal domain that binds the  $\alpha_2$ -macroglobulin receptor [8], a carboxy-terminal domain carrying the enzymatic activity and a central domain that assists membrane translocation. ETA enters the endosomal pathway where it is cleaved by the membrane protease furin [9,10] at an exposed arginine-rich loop (between Arg<sup>274</sup> and Arg<sup>279</sup>) generating two fragments of 28 and 37 kDa, called B and A respectively. After reduction of the disulfide bond between Cys<sup>265</sup> and Cys<sup>287</sup> [11] fragment A, which includes the enzyme domain, is released into the cytoplasm where it can reach its target [12].

Intracellular translocation of ETA fragment A is a crucial step in cell intoxication, not yet completely understood. It

requires crossing a lipid membrane and, relevant to this, ETA strongly interacts with lipid vesicles, inducing permeabilization and aggregation [13–17], and with lipid monolayers, increasing their lateral pressure [18]. It shares these properties with other toxins of the A-B type, e.g. diphtheria toxin, tetanus and botulinum neurotoxins [19–21]. It is possible that the interaction with the lipid membrane of its central domain, which contains the above-mentioned arginine loop and disulfide bond, plays a role also in the processing of ETA. To investigate this we studied proteolytic nicking of this toxin by trypsin and its dependence on the interaction with lipid bilayers.

## 2. Materials and methods

### 2.1. Materials

ETA was purchased from the Swiss Vaccine Institute (Bern, Switzerland) and was more than 97% pure by HPLC. Trypsin was from Merck and PMSF from Sigma. Lipids used were: egg phosphatidylcholine (PC), from Calbiochem (La Jolla, CA), phosphatidic acid (PA), phosphatidylglycerol (PG), phosphatidylserine (PS) and phosphatidylinositol (PI) from Avanti Polar Lipids (Pelham, AL), asolectin from Fluka (Buchs, Switzerland). Purity was always more than 99% according to the manufacturer. The ratios reported for binary mixtures were calculated on a molar basis.

### 2.2. Vesicle preparation and fluorescence experiments

Small unilamellar vesicles (SUV) were prepared by pulsed sonication as described earlier [16]. Large unilamellar vesicles (LUV) loaded with calcein were prepared by the extrusion technique [22] through polycarbonate filters of 100 nm pore size, as previously described [23]. Calcein release was measured fluorimetrically exactly as we described earlier [16]. Intrinsic tryptophan fluorescence of ETA alone or in the presence LUV was measured as in [24].

### 2.3. Protein cleavage experiments

ETA digestion was performed by incubating the toxin with trypsin and stirring for 90 min at 37°C in either MES 200 mM, EDTA 1 mM (for pH  $\geq$  6.0) or Na-acetate 200 mM, EDTA 1 mM (for pH  $\leq$  5.5). The pH was adjusted with NaOH. The reaction was stopped by adding 5 mM phenylmethylsulfonyl fluoride (PMSF) and thereafter boiling for 3 min in a denaturing solution: 10 mM Tris, 1 mM EDTA, 10% glycerol, 2.5% SDS pH 8.0 (plus 2.5% DTT in the case of reducing conditions). In the experiments with lipid vesicles the toxin was preincubated with SUV of variable lipid composition, for 15 min at room temperature.

### 2.4. Polyacrylamide gel electrophoresis

SDS gel electrophoresis was performed in the standard way [25] on precast polyacrylamide minigels purchased from Pharmacia (Uppsala, Sweden), using either a homogeneous polyacrylamide density of 20% or a density gradient ranging from 8 to 25%. A semi-automatic unit, PhastSystem by Pharmacia, was employed. Proteins were separated at 15°C in a buffer containing 0.5% SDS. Before running, the samples with lipid vesicles were supplemented with 1 mM Triton X-100 to better solubilize the lipid bilayer. Gels were stained first with Coomassie brilliant blue and then with silver. After two-dimensional densitometry, performed with a PhastImage densitometer (Pharmacia) and a band-pass filter at 546 nm, the amount of protein in each

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**Abbreviations:** ETA, *Pseudomonas aeruginosa* exotoxin A; SUV, small unilamellar vesicles; LUV, large unilamellar vesicles; PC, phosphatidylcholine; PA, phosphatidic acid; PG, phosphatidylglycerol; PI, phosphatidylinositol; PS, phosphatidylserine; PMSF, phenylmethylsulfonyl fluoride

band was quantitated as the optical volume of the band measured in  $\text{mOD}\cdot\text{mm}^2$ .

### 3. Results and discussion

ETA can bind to lipid vesicles and elicit their permeabilization (Fig. 1). This effect requires both low pH and the presence of acidic lipids in the membrane (Fig. 1A). This is consistent with previous results on liposomes [13,15–17] and lipid monolayers [18]. In this latter case, we have shown that the preference of ETA for negatively charged lipids is mediated by the negative surface potential they generate. This appears to be true also in the case of vesicles, as indicated by the effect of the ionic strength of the solution (Fig. 1B). Both the extent and the rate of ETA-induced permeabilization decrease at higher ionic strength, as a result of the screening of the surface potential by the accumulation of counterions at the interface.

We have previously shown that permeabilization of lipid vesicles by ETA involves stable binding of the protein to the lipid phase [16]; by studying its intrinsic tryptophan fluorescence we can show that this implies an unfolding of the protein (inset of Fig. 1B). In fact, tryptophan fluorescence quenching indicates a conformational change of ETA at low pH, leading to a state competent for lipid binding. Meanwhile, lipid vesicles can induce a much stronger quenching indicating further unfolding. Interestingly, while the quenching induced by acidic pH is very fast (it occurs in less than 1 min, as reported also by others [26]), the additional quenching induced by lipid has the same time course of permeabilization (not shown). We further observed that while the pH-induced quenching is quite reversible, the lipid-induced quenching is largely irreversible (Fig. 1B, inset).

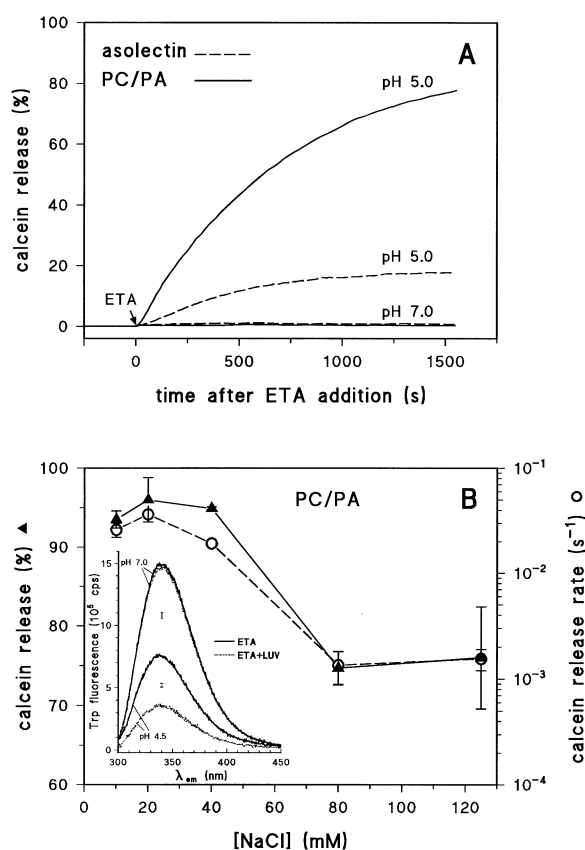
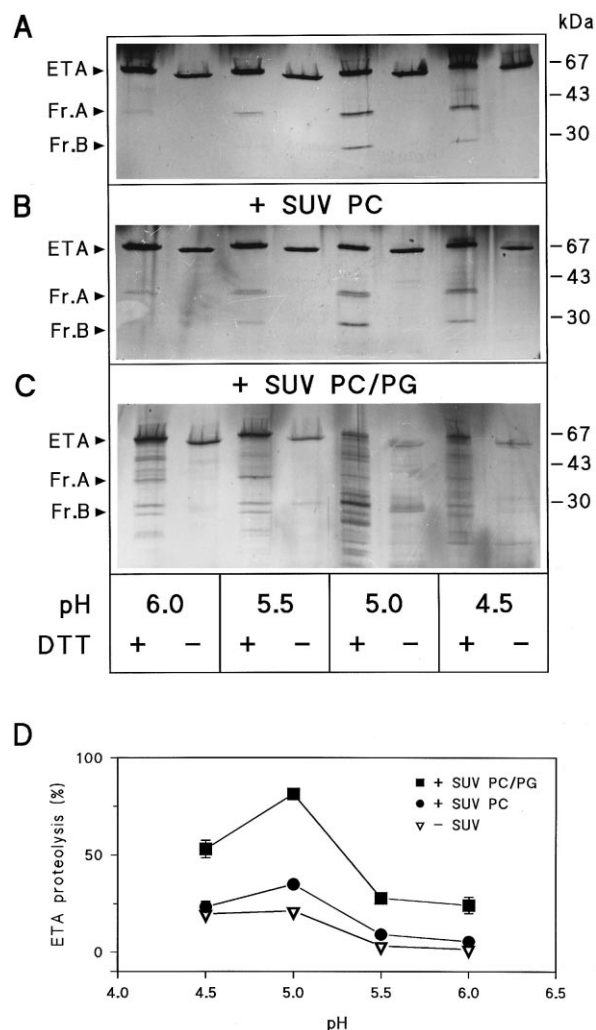


Fig. 1. ETA-induced permeabilization of LUV. A: Calcein-loaded LUV comprised of PC/PA 1/1 (50% charged lipids) or asolectin ( $\sim 25\%$  charged lipids, mainly PS) were exposed to ETA either at pH 7.0 or at pH 5.0. Calcein release from the interior of the vesicles was detected as an increase of the fluorescence measured with a spectrofluorimeter (Jasco FP550, Tokyo). The excitation wavelength was set at 495 nm and the emission wavelength at 520 nm (slit width 5 nm in both cases). The percent permeabilization was calculated as  $100 \times (F_{\text{fin}} - F_{\text{in}}) / (F_{\text{max}} - F_{\text{in}})$  where  $F_{\text{in}}$  is the value of fluorescence before the addition of the toxin,  $F_{\text{fin}}$  the value reached at equilibrium, and  $F_{\text{max}}$  the value obtained after addition of 1.4 mM Triton X-100. Permeabilization occurred only at the lower pH and was larger the higher the content of negatively charged lipids. Vesicles comprised of pure PC (100% neutral lipids) were not permeabilized at any pH (not shown). Other experimental conditions: ETA concentration was 40 nM; the solution was 125 mM NaCl, 1 mM EDTA and 20 mM of either HEPES or acetic acid for pH 7.0 or pH 5.0 respectively (adjustment with NaOH); lipid/toxin ratio was  $\sim 450$ ; room temperature. B: Ionic strength dependence of the kinetics of interaction of ETA with LUV of PC/PA (1/1). The rate of calcein release (right scale,  $\text{s}^{-1}$ ) was determined as the normalized slope of the curve at time zero:  $dF/dt|_{\text{in}} / (F_{\text{max}} - F_{\text{in}})$ . The solution contained 20 mM acetic acid, 1 mM EDTA and the reported amount of NaCl (pH 5.0). Other experimental conditions as in A. Points are averages  $\pm$  error of duplicate experiments. Inset: Conformational change of ETA at low pH. The intrinsic tryptophan fluorescence of 150 nM ETA in 125 mM NaCl, 1 mM EDTA, 20 mM HEPES was measured with a photon counting fluorometer (Spex Fluoromax) either in the absence (solid lines) or in the presence (dotted lines) of PC/PA (1:1) LUV (lipid concentration 30  $\mu\text{M}$ ). Fluorescence was excited at 295 nm to collect only Trp contribution [31] and is expressed in counts per second (cps). At pH 7.0 (upper two curves) no difference appears between the two samples. Dropping the pH to 4.5 by adding acetic acid (lower two curves) induced an immediate quenching of ETA fluorescence which was followed by an additional time-dependent quenching in the presence of LUV (the reported curve was obtained after approximately 10 min). At the end the reversibility of the quenching was tested by cycling back both the samples to pH 7.2, the values reached are indicated by error bars. The increased quenching and the reduced reversibility indicate that the interaction with lipid vesicles induces a new, unfolded, state of ETA. By following simultaneously tryptophan quenching and calcein release we could see that they have the same time course (not shown).

Because in vivo ETA is nicked by a membrane protease in an acidic environment [10], we investigated the role of pH and lipid interaction on its susceptibility to a proteolytic enzyme. The pH dependence of ETA cleavage by trypsin, either in the absence or in the presence of lipid vesicles, is shown in Fig. 2. Free ETA is cleaved by trypsin into two major fragments of MW  $39 \pm 2$  kDa and  $27.5 \pm 1.5$  kDa which are held together by a disulfide bond (Fig. 2A). By molecular weight, they correspond to the two fragments that are produced by furin (which cleaves after Arg<sup>279</sup> [12]) and accordingly will hereafter be called fragment A and B respectively. Extensive digestion occurs only at a pH lower than 5.5. In the presence of neutral vesicles (comprised of pure PC) the digestion follows a similar pattern and is only slightly more efficient (Fig. 2B). However, in the presence of SUV containing negatively charged lipids, digestion becomes exceedingly more extensive, and exhibits a more complicated, albeit still pH-dependent, pattern of fragmentation (Fig. 2C). The amount of digestion obtained is summarized in Fig. 2D. It varies sigmoidally with the pH with an apparent pK around 5.3. A lower percent of proteolysis at pH 4.5 is to be attributed to the drop of activity of trypsin at such low pH.

The massive digestion apparent in the presence of vesicles



containing negatively charged lipids suggests that they can trigger an unfolding of ETA which renders it more sensitive to proteolysis. Such unfolding is strongly suggested also by the drastic quenching of its tryptophan fluorescence and by its

Fig. 2. pH-dependent proteolysis of ETA by trypsin in the presence or in the absence of lipid bilayers. The susceptibility of ETA to trypsin was evaluated by SDS-PAGE for either free toxin (A) or toxin in the presence of uncharged SUV comprised of PC (B) or of negatively charged SUV comprised of PC/PG 1/1 (C). Concentrations during incubation were: ETA 200  $\mu\text{g/ml}$ ; trypsin 1.25  $\mu\text{g/ml}$ ; lipid, when present, 600  $\mu\text{g/ml}$ . Solutions are described in Section 2. Gels with a polyacrylamide gradient from 8 to 25% were used. The positions of molecular weight standards are indicated next to each panel on the right. The whole molecule and the two major fragments (called fragment A and B) are indicated on the left. Their MW was  $66 \pm 1$  kDa,  $39 \pm 2$  kDa and  $27.5 \pm 1.5$  kDa respectively (average  $\pm$  S.E.M. of 18, 11 and 10 lanes from three different gels). The two fragments are held together by a disulfide bond since they are separated only under reducing conditions (2.5% DTT). The apparent lower intensity of the bands without DTT is due to the fact that in the presence of this reducing agent ETA is stained better. D: To calculate the extent of protein digestion by trypsin, the optical density of all the bands separated by SDS-PAGE in the presence of DTT was read and their volume (in  $\text{mOD}\cdot\text{mm}^2$ ) was determined by integrating over the area. The percent is given as 100 times the ratio between the sum of the volumes of all the fragments and the sum of all the bands present in the lane (including intact ETA). Error bars indicate uncertainty in the densitometric determination.

ability to permeabilize lipid vesicles (Fig. 1 and [16]) as well as forming channels in planar lipid bilayers [27]. A titration of the amount of digestion versus trypsin concentration is shown in Fig. 3. In the presence of negatively charged SUV ETA is digested at an enzyme concentration which is  $\sim 50$  times smaller than that necessary for the toxin in free solution. Furthermore, at these lower concentrations of enzyme (0.15  $\mu\text{g/ml}$ ) it is evident that also with vesicles we obtain mainly the two fragments A and B and only a few smaller fragments. If a slightly higher trypsin concentration is used (0.3  $\mu\text{g/ml}$ ), we can split the toxin into the two main fragments even at pH 6.5 if it is bound to lipid vesicles, but only at pH 5.0 if it is free in solution (Fig. 4).

The dependence of the extent of ETA digestion upon the kind and the amount of acidic lipid present in the lipid vesicles was finally evaluated (Fig. 5). Clearly, several negative lipids could be used, but PG and PA were the most active (Fig. 5A). We have previously shown that these two phospho-

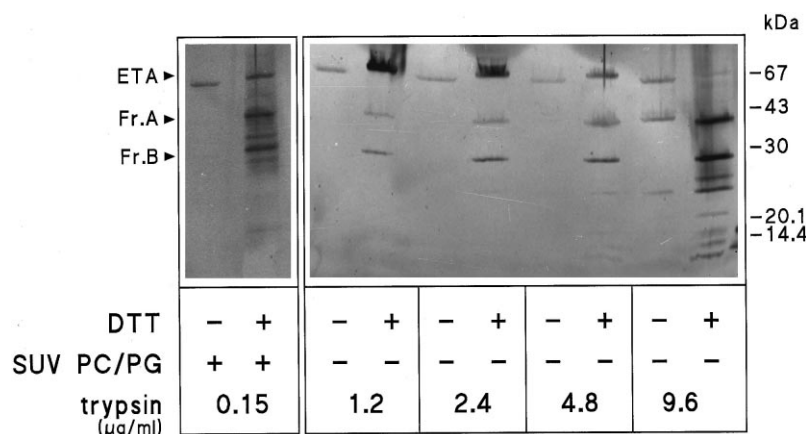


Fig. 3. Relative susceptibility of ETA to trypsin digestion in the presence or in the absence of lipid bilayers at pH 5.0. Experimental conditions are the same as in Fig. 2 except that trypsin concentration was varied as indicated, SUV (when present) were PC/PG 1/1 at a concentration of 300  $\mu\text{g/ml}$  and the pH was constantly 5.0. Molecular weight standards and ETA fragments are indicated as in Fig. 2. When the percent of protein digested by trypsin was quantitated as in Fig. 2D, a linear dependence of the extent of digestion on the amount of enzyme was observed (not shown). From this we could extrapolate that the extent of proteolysis observed with 0.15  $\mu\text{g/ml}$  trypsin in the presence of SUV would have required an enzyme concentration of 8.6  $\mu\text{g/ml}$  in their absence.

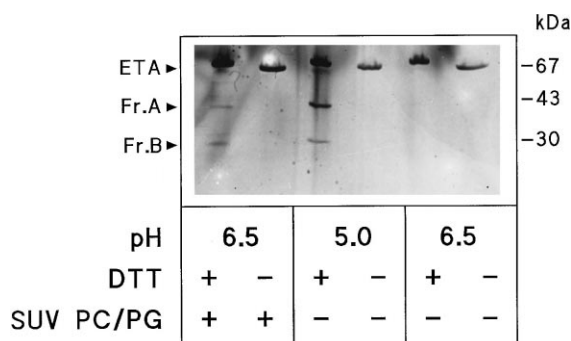


Fig. 4. Different pH dependence of ETA proteolysis by trypsin in the presence or in the absence of lipid bilayers. Experimental conditions are the same as in Fig. 3 except that here the ETA concentration was 400  $\mu\text{g/ml}$ , the trypsin concentration was 0.3  $\mu\text{g/ml}$ , and the pH was varied as indicated. Molecular weight standards and ETA fragments are indicated as usual. The extent of proteolysis observed at pH 6.5 in the presence of SUV is comparable only to that obtained at pH 5.0 in their absence.

lipids bind ETA best [16]. If PA is used, a significant digestion is obtained starting at about 20% of negative charges present (Fig. 5B). This corresponds to a surface potential of around  $-20$  mV, if it is evaluated according to the Guy-Chapman-Stern theory (see [18]). The finding that the unfolding effect is mainly due to the surface charge of the lipid film and not the

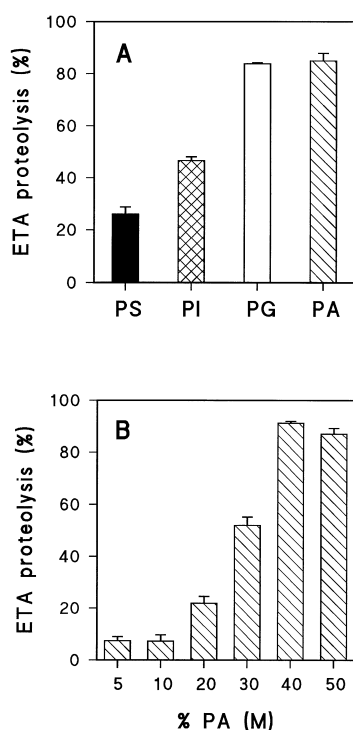


Fig. 5. ETA susceptibility to trypsin digestion in the presence of lipid membranes of different compositions. Experimental conditions are similar to those in Figs. 2 and 3: ETA concentration was 200  $\mu\text{g/ml}$ ; trypsin concentration was 0.15  $\mu\text{g/ml}$ ; lipid concentration was 600  $\mu\text{g/ml}$ ; pH was 5.0. Lipid was either SUV comprised of PC/PS, PC/PI, PC/PG and PC/PA, all in a 1/1 ratio (A) or SUV of PC/PA containing the indicated amount of PA (B). The percentage of digestion was determined as described in Fig. 2D except that here gels used for electrophoresis had a homogeneous polyacrylamide density of 20%.

particular lipid composition used is of importance. In fact, eukaryotic cell membranes possess only a small amount of acidic phospholipids (mainly PA and PI) on their outer leaflet [28–30]. Nevertheless, they can reach similar surface potential values by the contribution of other negatively charged components, such as glycoproteins and glycolipids. It appears thus likely that, as soon as the acidification of the endocytic compartment begins, ETA can partially unfold and bind to the lipid surface in a step which is preliminary to proteolytic nicking and eventually translocation. Interestingly, the loop that has to be proteolytically processed by the membrane protease furin, encompassing residues 274–279, contains four positively charged residues (three arginines and one histidine) and is therefore strongly attracted by the negative surface potential of the membrane.

Taken together our results suggest that the interaction with the lipid membrane, by providing the energy for partially unfolding the protein at the susceptible loop, might be paramount for the processing of ETA by furin *in vivo*. Furthermore, this step might occur very early in the endocytic pathway, as soon as the pH in the vesicles drops to around 6.5.

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