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Purification of Alpha-Toxin from *Staphylococcus aureus* and Application to Cell Permeabilization

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Crude alpha-toxin was produced by *Staphylococcus aureus*, strain Wood 46. The amount of exotoxin was monitored during growth and all subsequent purification steps by determination of its hemolytic activity against rabbit erythrocytes. The culture supernatant was treated with ammonium sulfate (75% saturation). The resulting precipitate was dialyzed and subjected to cation-exchange chromatography. The fractions containing the hemolytic activity were further purified by gel chromatography. The final product was enriched by a factor of 8.5 compared to the crude toxin. In sodium dodecyl sulfate–polyacrylamide gel electrophoresis the purified toxin exhibited one major band. It caused the release of $^{86}Rb^+$ and ATP from rat insulinoma (RIN A2) as well as pheochromocytoma cells (PC12) in culture, indicating efficient permeabilization of their plasma membranes for small molecules. © 1987 Academic Press, Inc.

KEY WORDS: *Staphylococcus;* alpha-toxin; purification; cell permeabilization.

The study of complex intracellular processes *in situ*, e.g., metabolic pathways, ion homeostasis, or exocytosis is hampered by the inaccessibility of the interiors of intact cells. The plasma membrane represents a permeability barrier that must be circumvented to afford access to the cytoplasm. Of the different techniques used for this purpose, the use of alpha-toxin produced by *Staphylococcus aureus* as the reagent appears to be ideal, since the alpha-toxin selectively permeabilizes the plasma membrane, leaving the intracellular organelles intact (1–4).

Alpha-toxin, in its monomeric form, is a water-soluble protein that hexamerizes in the target membrane, creating a stable transmembrane pore. The size of the pore formed by the alpha-toxin does not allow intracellular proteins to escape from the cells, whereas low-molecular-weight molecules are released and can thus be exchanged in permeabilized cell preparations (2–7,16,17).

Hitherto alpha-toxin has been used as a valuable tool to investigate the properties of the microsomal glucose-6-phosphatase system in hepatocytes (4) and the effect of cations and nucleotides on contraction of smooth muscle (8) as well as in the analysis of the minimal requirements for secretion by exocytosis in chromaffin cells (1–3).

Here we report on the production of crude alpha-toxin by *Staphylococcus aureus* strain Wood 46 and its subsequent purification and characterization. The efficiency of the permeabilization of secretory cells by the isolated toxin was determined by measuring $^{86}Rb^+$ efflux and ATP release from a rat pheochromocytoma (PC12) as well as a rat insulinoma (RIN A2) cell line.

MATERIALS AND METHODS

Preparation of crude toxin. Stock preparations of *Staphylococcus aureus* (strain Wood 46, ATCC 10832, DSM 20491) were kindly supplied by S. Bhakdi (Institute of Medical Microbiology, University of Giessen, FRG). Cultivation was performed aerobically using a 4% inoculum from an exponentially growing proculture in 100 ml of medium (tryptic

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soya broth, Difco, 30 g/liter, pH 7.2) in 0.5-liter indented Erlenmeyer flasks on a rotary shaker (120 rpm) at 37°C. At the times indicated growth was determined by measuring the absorbance of an appropriately diluted sample at 578 nm against the growth medium as a blank. The alpha-toxin content was assayed in the supernatant following centrifugation using the hemolytic titer (see below). After 18 h the bacteria were harvested by centrifugation at 4°C (20 min at 16,000*g*). Solid ammonium sulfate was added (75% saturation) to the supernatant, which was kept for 2 h in a cold room. The precipitate formed was collected by centrifugation at 4°C (15 min at 16,000*g*) and stored frozen as a stock of crude toxin.

Purification of alpha-toxin. For purification of alpha-toxin the thawed precipitate was dialyzed overnight against 10 mM sodium acetate buffer, pH 5, containing 20 mM NaCl. Under these conditions the toxin becomes soluble. Other contaminating proteins that were still insoluble were removed by centrifugation (2 min at 12,000*g*). The clear supernatant was prefiltered through a 0.22-μm membrane filter (Millipore). Ion-exchange chromatography and gel filtration were done using the FPLC[1] technique (Pharmacia). Samples of about 12 mg of protein were first loaded onto a Mono S cation-exchange column (HR 5/5, Pharmacia) equilibrated with 10 mM sodium acetate, pH 5, containing 20 mM NaCl. Elution was carried out with a linear gradient (buffer A, equilibration buffer; buffer B, 10 mM sodium acetate, pH 5, containing 200 mM NaCl): 0–100% buffer B in 45 min with a flow rate of 60 ml/h. Protein in the effluent was detected at 280 nm. The fractions containing the alpha-toxin were collected, concentrated by ultrafiltration (Diaflo, 25 μM 10), and subjected to gel filtration in 200-μl samples (each about 3 mg of protein) on a Superose 12-HR 10/30 column (Pharmacia) equilibrated with 10 mM sodium phosphate, pH 7.0, containing 10 mM NaCl. SDS–PAGE and protein determination were done using standard techniques (9,10).

Alpha-toxin determination. Blood was obtained from the rabbit "Aurea" and immediately mixed with 4% sodium citrate. After three washes with 50 mM phosphate-buffered saline (pH 7.0), the erythrocytes were diluted to 2.5% in the same buffer. Fifty microliters of this suspension was mixed with 5 μl of appropriate dilutions of the toxin. After incubation for 40 min at 37°C under constant shaking the samples were again mixed followed by centrifugation (2 min at 12,000*g*). Hemoglobin was determined spectrophotometrically at 412 nm in 30 μl of the supernatant after addition of 1 ml of distilled water. Total hemolysis was determined after addition of SDS (0.2%, w/v, final). The dilution of toxin hemolyzing 50% of the red cells was determined and the reciprocal of the value obtained was taken as the number of hemolytic units (U) per milliliter of the undiluted toxin solution (11).

$^{86}Rb^+$ and ATP efflux from secretory cells. Rat pheochromocytoma cells (PC12) were grown as outlined previously (2) and insulinoma cells (RIN A2, kindly supplied by H. P. T. Ammon, Department of Pharmacology, Institute of Pharmaceutical Sciences, University of Tübingen, FRG) were cultured in RPMI medium containing 10% fetal calf serum. The permeability of the cells was assessed by measuring the release of $^{86}Rb^+$ (2) or of ATP (firefly assay, Boehringer Mannheim, FRG). PC12 cells on plates were washed twice in a medium containing (mmol/liter) NaCl (150), Pipes (10), EGTA (1), pH 7.2, and once with KG medium containing K^+ glutamate (150), Pipes (10), NTA (5), EGTA (0.5), pH 7.2, before suspending

[1] Abbreviations used: FPLC, fast protein liquid chromatography; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; Pipes, 1,4-piperazinediethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether) *N,N'*-tetraacetic acid; NTA, nitrilotriacetic acid; KG, medium containing 150 mmol/liter K^+ glutamate, 10 mmol/liter Pipes, 5 mmol/liter NTA, 0.5 mmol/liter EGTA, pH 7.2; LDH, lactic dehydrogenase.

them in the same buffer supplemented with 0.1% BSA. After preincubation (10 min at 37°C) the cells were permeabilized for 20 min at 37°C with either alpha-toxin or digitonin in KG medium. Then the cells were centrifuged (0.5 min at 12,000*g*). After the supernatant was removed, the cells were incubated for a further 10 min at 37°C in KG medium. In order to measure the ATP remaining in the cells, they were extracted with 250 μl Tris/acetate buffer (Tris, 50 mmol/liter, pH 7.8; Mg-acetate, 10 mmol/liter; EGTA, 1.5 mmol/liter) and immediately heated to 95°C for 5 min. ATP was measured in all supernatants and also in the final cell extract. Untreated cells at the beginning of the experiment contained 45 nmol of ATP/mg of protein (100%), which is in the range of values reported (12).

RESULTS AND DISCUSSION

Staphylococcus aureus strain Wood 46 exhibited exponential growth in the first 6 h when grown aerobically on tryptic soy broth (Fig. 1). Toxin production also started during this period, and even when the bacteria reached the stationary phase toxin release into the growth medium continued. The

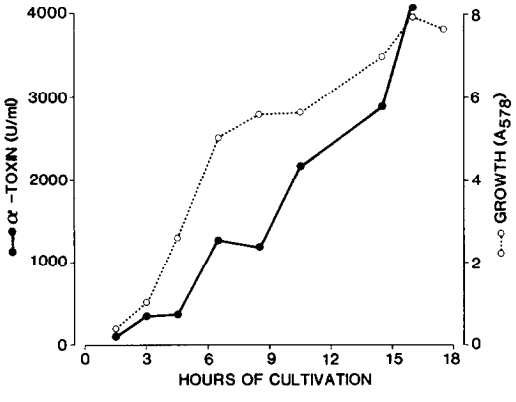

FIG. 1. Growth and production of alpha-toxin by *Staphylococcus aureus* (Wood 46). Hemolytic units (U) of alpha-toxin per milliliter of supernatant (left) were determined using rabbit erythrocyte suspensions, and growth was monitored by measuring the absorbance at 578 nm (see Materials and Methods).

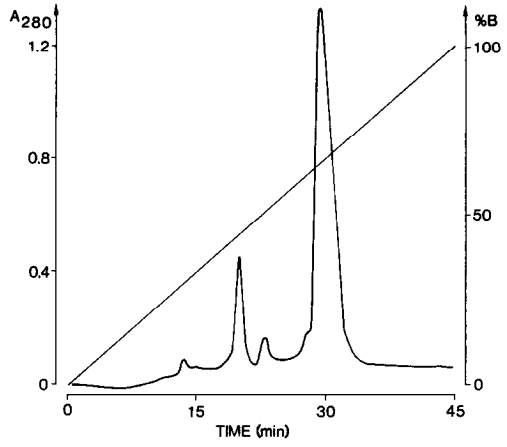

FIG. 2. Cation-exchange chromatography of crude alpha-toxin. Separation of alpha-toxin was achieved on a Mono-S column using a sodium chloride gradient. Eluants: A, 10 mM sodium acetate, pH 5, containing 20 mM NaCl; B, 10 mM sodium acetate, pH 5, containing 200 mM NaCl. Hemolytic activity was found exclusively in the main protein peak appearing after 30 min. Detection of protein was carried out at 280 nM.

crude toxin harvested after 18 h and precipitated with ammonium sulfate (see Materials and Methods) contained 3600 ± 1000 units/mg of protein (n = 6). After dialysis and removal of insoluble proteins by centrifugation the toxin was subjected to cation-exchange chromatography on a Mono-S column. The alpha-toxin-containing fractions (see main peak in Fig. 2) were eluted after 30 min with 170 mM NaCl present in the column buffer (10 mM sodium acetate, pH 5). Cation-exchange chromatography resulted in a 6.5-fold enrichment of the toxin. The enrichment of the toxin and removal of most protein bands was also shown by SDS–PAGE (Fig. 3). However, at this stage contaminating proteins of molecular weights lower than that of the alpha-toxin monomer (34 kDa) were present. These proteins were successively removed by gel chromatography (see Materials and Method and Fig. 4) which yielded the final product. Also, SDS–PAGE indicated a further purification (Fig. 3) after this step. The alpha-toxin isolated by the procedures described above contained

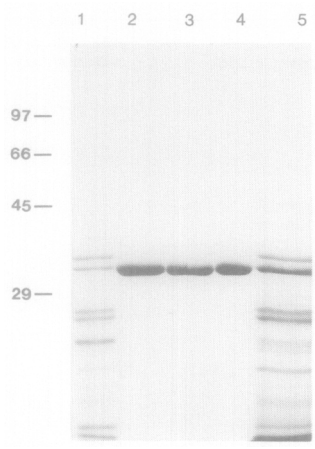

FIG. 3. Analysis of the alpha-toxin fractions by SDS–PAGE. The samples (lanes 1–4, 1.5 μg; lane 5, 15 μg protein) were separated in 12% acrylamide gels. The indicated molecular weight values (kDa) were determined by running standards in parallel (carbonic anhydrase 29; ovalbumin 45; albumin 66; phosphorylase *b* 97). The gel was stained in Coomassie blue. The ammonium sulfate precipitate of the culture supernatant prior to (sample 5) and after (sample 1) dialysis plus removal of insoluble proteins by centrifugation differ greatly from the alpha-toxin fractions eluted during cation-exchange chromatography (samples 2 and 3). Following gel chromatography (sample 4) only one protein band is observed.

31,100 ± 4500 units/mg of protein (n = 6), i.e., the purification achieved was 8.5 times that of the crude toxin.

The specific activity of the purified alpha-toxin compared well with other preparations carried out with less advanced protein purification methods (cf. (13)). One advantage of the procedure described here is that, starting with the dialyzed ammonium sulfate precipitate of the culture supernatant, purified alpha-toxin can be obtained within 2 h. The purified toxin was stable for several weeks at 4°C after the addition of ammonium sulfate (75% saturation). When the toxin was rapidly frozen in liquid nitrogen and kept at −20°C no loss of toxicity was observed for several months.

Alpha-toxin causes hemolysis of erythrocytes of various species. The channels formed do not allow the passage of myoglobin (17 kDa) or Dextran 4 (4 kDa) (5,6). Thus it is highly unlikely that the native alpha-toxin molecules (34 kDa) are able to cross such channels and gain access to the intracellular space. This is an important feature for its application to selective permeabilization of the plasma membranes of secretory cells in order to study the requirements for exocytosis under controlled conditions with respect to ions, nucleotides, and other small molecules.

Alpha-toxin elicits the release of $^{86}Rb^{+}$, reflecting the effective permeabilization to ions when applied to pheochromocytoma cells (2), chromaffin cells in primary culture (3), and insulinoma cells (Fig. 5) in a dose-dependent manner. In this respect it resembles digitonin, which has been used by several groups for the permeabilization of chromaffin cells (cf. (3,14,15)). In contrast with digitonin, alpha-toxin does not cause the escape of LDH (150 kDa) from either pheochromocytoma cells or chromaffin cells in primary culture (2,3), a fact which is in accordance

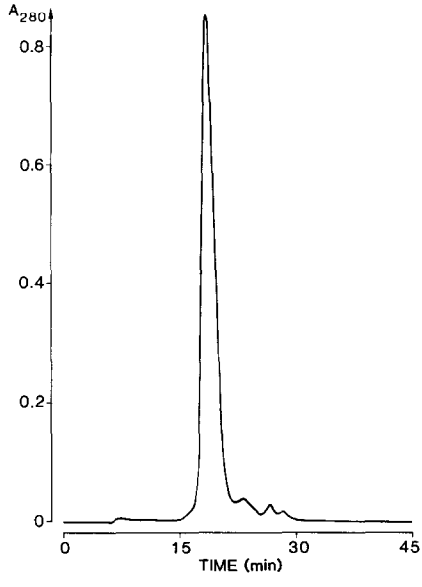

FIG. 4. Gel filtration of the alpha-toxin fraction eluted from the cation exchanger. Protein (3 mg) of the toxin-containing fractions recovered from the cation-exchange column (see Fig. 3) was separated on a Superose 12-HR 10/30 column. Alpha-toxin was present in only the main peak (detection of protein was carried out at 280 nm).

with findings observed with erythrocytes (see above).

The investigation of the intracellular requirements for exocytosis by secretory cells is the main interest of the authors' laboratory. Ca^{2+}- and Mg^{2+}-ATP are important factors for the final step in exocytosis in permeabilized chromaffin cells in primary culture (3). On the other hand, the rat pheochromocytoma cells, when permeabilized by alpha-toxin, do not depend on added Mg^{2+}-ATP (1,2). The reason for this observation certainly is not the ineffectiveness of the perturbant. Here we clearly demonstrate that the holes generated by alpha-toxin are sufficient to deplete PC12 cells of their cytoplasmic ATP at a level comparable in magnitude to digitonin (Fig. 6). Release of small molecules of similar size (e.g., α-amino acids) by alpha-toxin has been also observed in fibroblasts (7).

The effects of detergents like digitonin and saponin are difficult to control due to their lack of specificity and the detergents have the disadvantage of lysing all cellular membranes, thereby disintegrating the whole cell. The attack by alpha-toxin, however, is restricted to the plasma membrane and even at very high concentrations it leaves the secretory vesicles intact, as seen by the retention of catecholamines and of chromogranin A within the cells (2,3).

Thus, alpha-toxin allows the control of intracellular cations and anions without impairing the exocytotic machinery (1–3). This opens up the possibility of directly studying fusion of secretory vesicles and the plasma membrane *in situ*. Permeabilization with alpha-toxin can also be used to follow muscle contraction (7) and complex enzymatic reac-

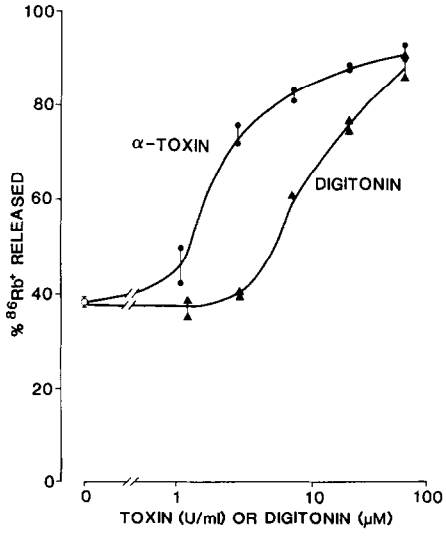

FIG. 5. $^{86}Rb^+$ release from alpha-toxin- and digitonin-permeabilized rat insulinoma cells. The cells were treated with alpha-toxin or digitonin and the percentage of $^{86}Rb^+$ released was determined after 20 min. Both substances caused a dose-dependent release of $^{86}Rb^+$ from the cells. The abscissa gives the final concentration of toxin in hemolytic units per milliliter or of digitonin in micromoles per liter.

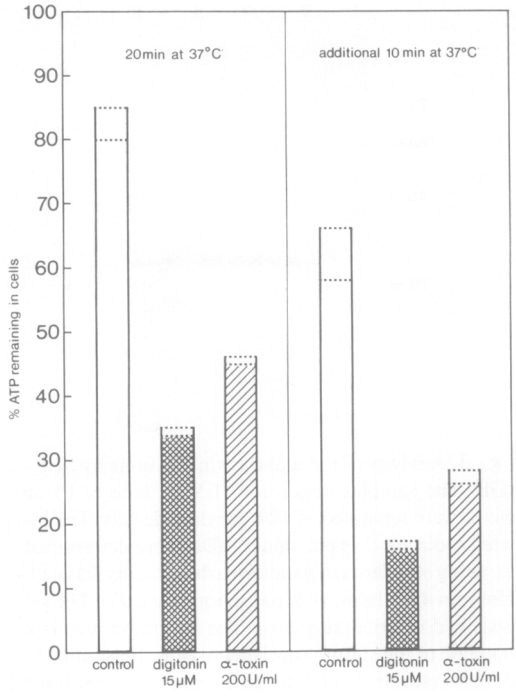

FIG. 6. Release of ATP from rat pheochromocytoma cells treated with alpha-toxin or digitonin. PC12 cells grown as described (2) contain 45 nmol of ATP/mg of protein (100%). When incubated for 20 min at 37°C with alpha-toxin or digitonin they release ATP. An additional incubation for 10 min resulted in a further reduction of cell-associated ATP (see also Materials and Methods). The dotted lines represent single values.

tions (4) within different cells, i.e., processes which require a precise structural array of components for their proper function.

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