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Mechanism of action of *Bacillus thuringiensis* insecticidal δ-endotoxin: interaction with phospholipid vesicles

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Bacillus thuringiensis **(Bt) crystal δ-endotoxin from three subspecies and the product of a cloned crystal protein gene were activated in vitro and their interaction with phospholipid liposomes studied. Despite their diverse spectrum of activity, all these toxins were found to cause a rapid increase in the light scattering of liposome suspensions, which reflects a morphological change in the lipid bilayer. When liposomes loaded with radioactive markers were incubated with *B. thuringiensis aizawai* IC1 toxin, a relatively rapid release of more than 70% of the trapped markers occurred after an initial lag. Activated Bta IC1 and *B. thuringiensis israelensis* toxins were shown to bind to phospholipid vesicles. Two of the five conserved domains (D1–D5) detectable in the sequence of a range of Bt toxins are predicted to be highly hydrophobic. It is suggested that these, together with an additional conserved hydrophobic region showing structural homology and two predicted amphiphilic helices, play a major part in the interaction of these toxins with target membranes.**

Introduction

B. thuringiensis (Bt) strains synthesise a cytoplasmic protein crystal during the sporulation phase of their development [1,2]. This crystal (δ-endotoxin) consists of one or more polypeptides of varying M_r that are toxic to insect larvae [3]. The native crystal δ-endotoxin is a protoxin which requires solubilisation and activation by proteolytic processing in the larval midgut [4,5]. For many of the toxins the proteolytic activation process involves removal of the C-terminal half of the protoxin [6–8]. The activation can be duplicated in vitro using alkaline buffers together with a combination of proteinases [4,9,10]. When the protoxin is activated in vivo in the larval midgut, it causes disruption of the gut epithelial cells which leads to larval mortality [11]. Recently a general model to explain the mechanism of action of Bt toxins has been proposed [12–14]. According to this model, the toxin first binds to a membrane receptor on the target cell. In a second step the toxin, either individually or in the form of oligomers, partitions into the membrane to create a pore/leakage channel in the membrane which ultimately leads to colloid osmotic lysis.

A 130 kDa crystal protein gene (*Cry* gene) has been cloned from Bta IC1 [15,16]. Overexpression of this *Cry* gene allowed the characterization of the toxicity and specificity of the cloned protein [16]. We have recently determined the complete nucleotide sequence of this gene and in the present investigation used this cloned toxin together with the activated toxins from three Bt subspecies with differing insecticidal activity, to investigate the interactions of these toxins with the lipid bilayers.

Materials and Methods

Bacterial strains and growth conditions. B. thuringiensis var. *kurstaki* (Bta) was obtained from Dr. H.D. Burges (Institute of Horticultural Research, England). *B. thuringiensis* var. *aizawai* IC1 (Bta IC1) has been described earlier [15]. *B. thuringiensis* var. *aizawai* HD-249 (Bta) was obtained from Dr. H.T. Dulmage (USDA, Brownsville, U.S.A.). *B. thuringiensis* var. *israelensis* IPS-78 (Bti) was from Professor H. de Barjac (Institut Pasteur, Paris).

Conditions for growth and synchronous sporulation of *B. thuringiensis* were as described by Stewart et al.

Abbreviations: Bta, *Bacillus thuringiensis aizawai*; Bti, *B. thuringiensis israelensis*; Btk, *B. thuringiensis kurstaki*; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; PC, phosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; PBS, phosphate-buffered saline.

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[17] for *Bacillus megaterium* KM. δ-Endotoxin crystals were separated from spores by discontinuous sucrose gradient ultracentrifugation as described by Thomas and Ellar [9] for (Btk, Bta and Bta IC1), and by sodium bromide gradients as described by Ward et al. [18] for Bti.

Solubilization and activation of δ-endotoxin. The methods for solubilization and activation have been described previously. The Bta, Bta IC1, Btk δ-endotoxins and the *Cry* gene product were first solubilised in carbonate buffer and then activated with trypsin [5]. The *Cry* gene product was purified and activated as described earlier [16,18].

Melittin (Sigma) was prepared as a 2 mg/ml stock solution in distilled water and stored at −20 °C.

Polyacrylamide gel electrophoresis. SDS-PAGE was performed as described earlier [19]. The staining and destaining procedures have been described earlier [5].

Preparation of liposomes. Liposomes were prepared from chromatographically pure lipids by the following modified procedure [9]; lipid-PC (egg yolk (type III, Sigma)), DPPC, cholesterol and dicetylphosphate (PC^- or stearylamine PC^+), were mixed in molar ratios of 2 : 1.5 : 0.5, respectively; the total lipid was 35 μM. The mixture was dried as a thin film in vacuo in a 100 ml round-bottomed flask and then resuspended with gentle shaking in 3 ml of phosphate-buffered saline (PBS, Oxoid). Further dispersion of the lipid was accomplished by brief treatment (2–5 min) in a sonic bath.

Turbidity assay for liposome–toxin interaction. Lipid vesicles scatter light to an extent that is dependent on their dimensions and aggregation state. Hence, the amount of light scattered by the lipid suspension is a sensitive measure of liposome aggregation, fusion and vesicularization that may be triggered by interaction of a protein with the lipid bilayer [20–23].

We have employed a simple photometric assay based on this principle to study the effect of Bt toxins on phospholipid vesicles. Liposomes were suspended in PBS in a spectrophotometer cell and toxin or control samples were added at room temperature. Turbidity change was detected as the difference in the amount of light transmitted before and after the addition of the test sample. Transmittance changes were measured at 334 nm [24], in the extinction mode of a Spectroplus (MSE) spectrophotofluorimeter connected with a Vitatron (MSE) recorder.

Release of radioactive markers from the liposomes. For these experiments, PC^+ liposomes were loaded with 50 μCi [^{3}H]uracil or [^{3}H]alanine (Amersham, 1 mCi/ml), respectively. The liposome suspension was then gel-filtered through Sephadex G-50 (Pharmacia) using PBS as the elution buffer to remove unincorporated label. Elution of the liposome fraction was monitored by measuring the transmittance of fractions at 334 nm. Aliquots were removed from these fractions and the radioactivity was measured in a Beckman scintillation counter using Optiphase-SAFE II (LKB) as scintillant.

Aliquots of the labelled liposome preparations were then incubated with Bt toxins for various time intervals at room temperature. After incubation the suspension was filtered through a Millipore filter (0.45 μm, type HA) and the filter washed with 2 ml PBS. Both filter and filtrate were used to determine the amount of radioactivity released at different time intervals. Spontaneous lysis was checked and normalised at each time point by following the same procedure except that no sample was added to the liposomes. Maximum lysis was measured by treating the liposomes with 0.1% Triton X-100. At each time point, the percent maximal lysis was calculated by the relationship described earlier [13] and plotted against time to obtain a curve.

Toxin binding to lipid vesicles. Different toxin preparations were incubated with liposomes (PC^+) for appropriate time intervals and filtered through a Millipore filter as described earlier. The filter was boiled for 10 min in gel sample buffer to obtain the bound fraction which was subjected to SDS-PAGE. The filtrate was precipitated with 50% trichloroacetic acid (TCA) at 0 °C for 30 min. The precipitated proteins were pelleted by centrifugation in a minifuge and the pellet was washed with acetone. The acetone-washed pellet was dried in vacuum for 30 min, dissolved in gel sample buffer and electrophoresed. In another set of experiments the liposomes were omitted to check nonspecific binding of the toxin to the filter.

Results and Discussion

B. thuringiensis δ-endotoxins consist of polypeptides of various masses depending on the strain [3]. Bta, Btk and Bta IC1 have a polypeptide doublet at 130–135 kDa and these are processed to 55 and 58 kDa proteins upon proteolytic activation [5,10] (Fig. 1). Bti crystal, however, is more heterogeneous and has a number of toxins [9,25,26]. The principle mosquitocidal toxins are, however, the 130, 67 and 27 kDa polypeptides in Bti [26].

Knowles and Ellar [13] have recently proposed a scheme for the molecular mode of action of Bt toxins. They postulated that the toxins interact with receptor(s) at the target cell membrane. This is followed by partitioning of toxin molecule into the membrane to form a pore which ultimately leads to death by colloid osmotic lysis. Their indirect experiments with insect cells provided evidence that intracellular markers of increasing size were released sequentially as a consequence of toxin action.

In an attempt to gain information about the events preceding colloid osmotic lysis, we have studied the effect of activated Bt toxins on phospholipid vesicles (liposomes). Since we were interested to monitor physi-

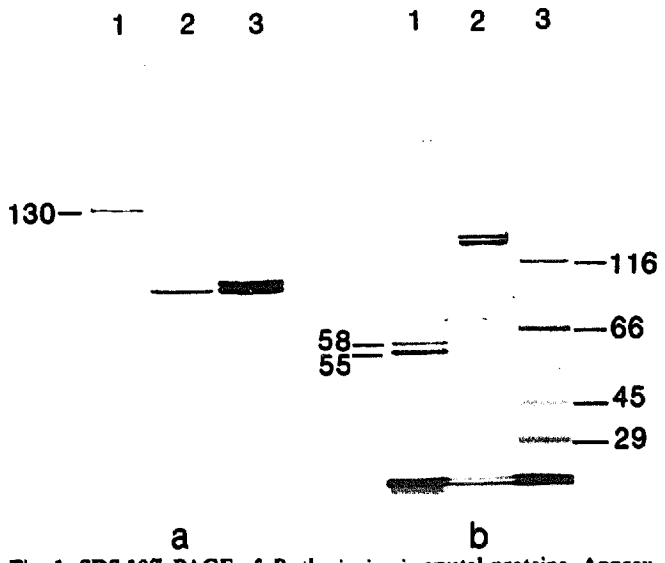

Fig. 1. SDS-10% PAGE of *B. thuringiensis* crystal proteins. Approx. 20–50 μg crystal proteins were solubilised in gel sample buffer, boiled for 5 min and loaded on the gel. Stain used was Coomassie blue. (a) lane 1, cloned 130 kDa protoxin; lane 2, trypsin-activated cloned toxin; lane 3, trypsin activated native Bta IC1 toxin; (b) lane 1, trypsin-activated Bta HD-249 toxin; lane 2, native Bta HD-249 protoxins; lane 3, M_r standards. The values on the sides are kDa.

cal changes in lipid bilayers rather than measuring absolute coefficients of permeability, we have used multilamellar liposomes. Changes in the light scattering of liposome suspensions have been monitored for four Bt toxins which vary in their insecticidal specificity. Addition of toxin to the liposome suspension leads to an immediate decrease in the amount of light transmitted (T) which reflects an increase in turbidity of the suspension (Figs. 2–3). When liposomes were treated with the non-ionic detergent Triton X-100 an initial decrease in T was followed by a sharp increase (Fig. 4). Triton X-100 causes disruption of the bilayer and the breakdown of vesicles lowers the turbidity and allows maximum light to be transmitted (as shown in Fig. 4).

The toxin-induced changes in the light transmittance are probably a consequence of the reorganization of lipid assemblies, which include vesicularisation and aggregation and/or fusion. Agents with known membrane-perturbing properties, like diphtheria toxin, bee venom toxin (melittin) and potassium ferricyanide, also cause various morphological changes in lipid vesicles, the nature of which depends on the experimental conditions [19,22,23,27,28]. Bt toxins appear to differ from the detergent Triton X-100 in that they do not completely clarify the liposome suspension (the transmittance remains low) even after prolonged treatment (not shown). This suggested that at the toxin–lipid ratio used the Bt toxins may have partitioned into the bilayer to form stable pore(s), which would allow the leakage of vesicle contents to the exterior. To test this, liposomes were loaded with [^{3}H]Ala (M_r 89) and [^{3}H]Ura (M_r 112) and treated with Bta IC1 toxin. Fig. 5 shows that addition of toxin caused a relatively rapid release of more than 70% of both markers. This was followed by a second prolonged slow release phase. The initial lag observed in these marker-release experiments might suggest a prior aggregation event in the bilayer, although other explanations are possible. Maddrell et al. [29] also observed a lag in the onset of fluid secretion when isolated insect Malpighian tubules were exposed to Bti 27 kDa toxin and, on the basis of this and other observations, they suggested that lytic pore formation depended upon the association of a critical number of monomers at one membrane site. Knowles and Ellar [13], however, observed no corresponding lag in the release of markers from insect cells. The absence of a detectable lag in these experiments may derive from the fact that the insect cells (CF1) possess membrane receptors specific for the three toxins studied by Knowles and Ellar [13]. Glycoprotein receptors have been identified in these cells for the Btk and Bta IC1 toxins [14,30] and preliminary results from site-directed mutagenesis indicate that these cells also contain a specific (non-phospholipid) receptor for the Bti 27 kDa toxin (Armstrong, Knowles, Ward and Ellar, unpublished data). The suggestion would be that in the liposomes and Malpighian tubules which lack specific receptors, pore formation is dependent upon the relatively slow association of toxin molecules by lateral diffusion, whereas in the insect cells the receptors, in addition to recognising and binding the toxins, may promote toxin aggregation, e.g., by binding more than one toxin molecule, and thus accelerate the process of pore formation.

In order to study toxin binding, the bound and filtered fractions were analysed by SDS-PAGE. Fig. 6 shows that the 55 kDa protein in the active Bta IC1 preparation was detected only in the bound fraction (track 2) and was absent from the filtrate (track 3, Fig. 6a); small M_r polypeptides are proteolysis products from the protoxin and have no role in toxicity [11]. The 55 kDa polypeptide has been shown to have the dual insecticidal toxicity while the role of 58 kDa protein in the overall toxicity is not clear [5,16]. When liposomes were omitted, no proteins were retained on the filter (track 4) which shows that the proteins retarded are bound to liposomes. Similar results were obtained when Bti 27K toxin was used (Fig. 6b), but in this case small amounts of the toxin were detectable in the filtrate. The ability of this toxin to bind to liposomes has also been demonstrated by a centrifugation binding assay [31].

A variety of evidence indicates that the N-terminal half of the high M_r (130–140 kDa) lepidopteran and dipteran toxins is fully toxic [7,8]. When the sequences of these segments and a coleopteran toxin sequence are aligned, five highly conserved domains can be seen after appropriate gaps have been inserted. In the Bta IC1 sequence (Haider and Ellar, unpublished data) these

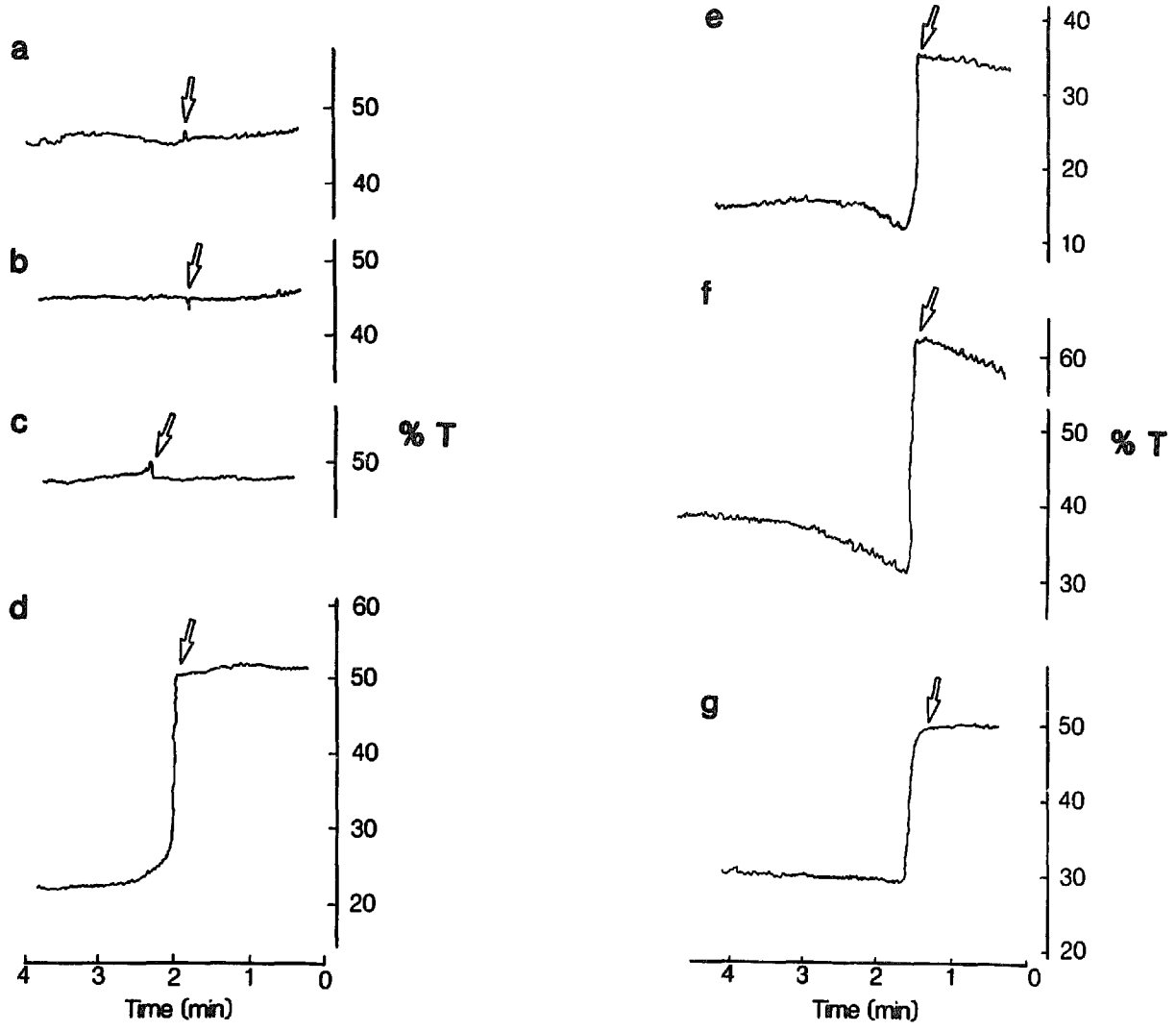

Fig. 2. Effect of *B. thuringiensis aizawai* IC1 toxin and the control samples on the turbidity of the phospholipid vesicles. The following samples were added to the PC^+ liposome suspension and the turbidity changes were measured as percentage decrease in transmittance (T) at 334 nm. (a) 50 mM sodium carbonate (pH 8.5) containing 10 mM dithiothreitol; (b) 50 μg bovine serum albumin; (c) 50 μg heat-inactivated (boiled for 5 min) Btk toxin; (d) 50 μg trypsin-activated Bta IC1 toxin, the arrows point to the time of addition of the sample; (e) trypsin-activated Btk; (f) trypsin-activated Bta HD-249; (g) trypsin-activated cloned Bta IC1 toxin, the arrows indicate the time of addition of the sample.

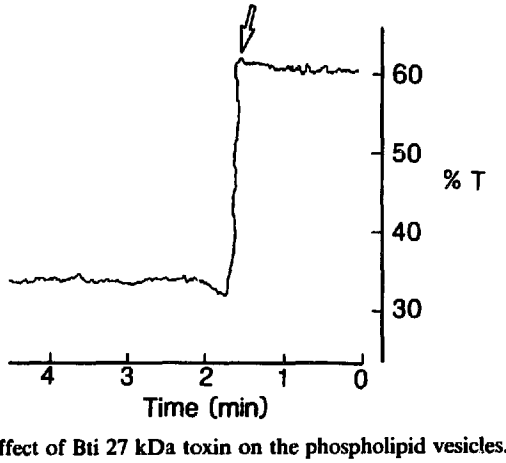

Fig. 3. Effect of Bti 27 kDa toxin on the phospholipid vesicles. 50 μg toxin was added to PC^+ liposomes and the turbidity change recorded.

occur between residues 153 and 183 (D1), 226 and 271 (D2), 455 and 498 (D3), 520 and 532 (D4) and 597 and 606 (D5). In the dual specificity P2 toxin from Btk [32] sequences corresponding to D1 and D3 are clearly conserved, D2 is absent, the D4 region shares few common residues with the other toxins and the C-terminus of this protein occurs before the expected position of the D5 domain. The conservation of these regions strongly suggests that they may be involved in determining the toxicity (i.e., cytolytic activity) of these proteins and that the specificity determinants may reside in the non-conserved regions of the molecule. Of these domains the ones closest to the N-terminus (D1 and D2) are predicted (Fig. 7) to include highly hydrophobic segments, which may therefore be capable of

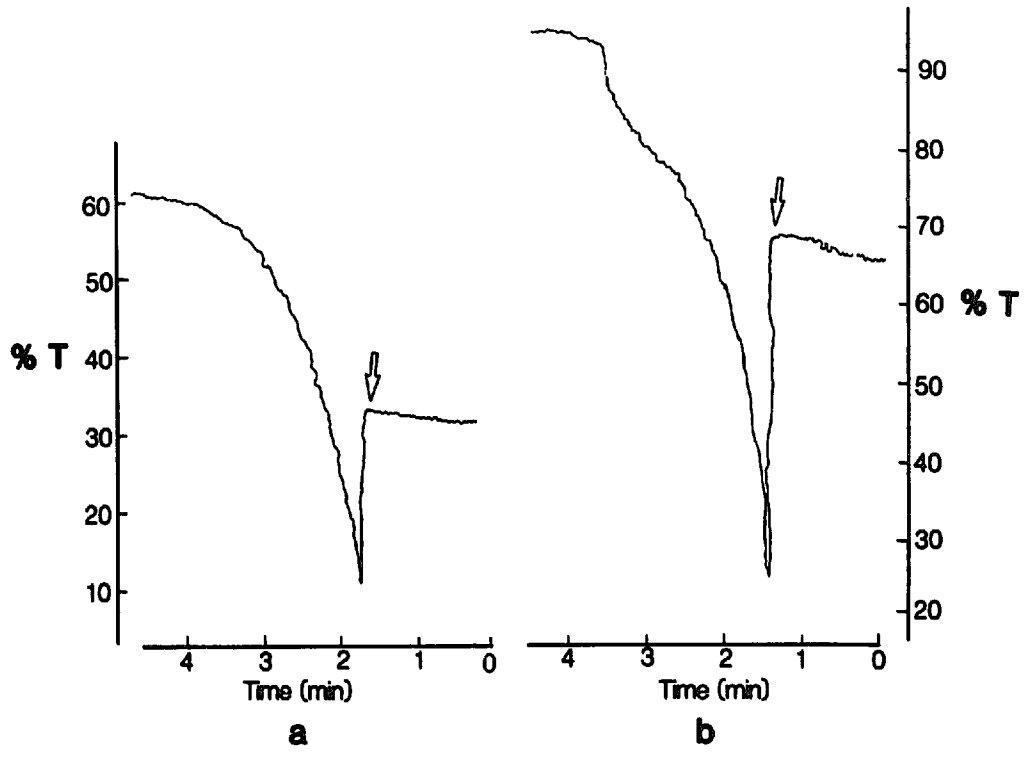

Fig. 4. Effect of mellitin and Triton X-100 on the PC^+ liposomes. For details of the experiments see Materials and Methods. Turbidity changes were measured as percentage decreases in transmittance at 334 nm. (a) Triton X-100; (b) melittin, arrows indicate time of sample addition.

interacting with lipid bilayers. Although not revealed by sequence comparisons, most of the toxins, including Bta IC1, the dual specificity Btk P2 toxin and the Bti 27 kDa toxin, are predicted to contain an additional conserved hydrophobic domain between residue 50 and 100 comprising a hydrophobic α-helical segment (residue 75–83 in Bta IC1) flanked on one or both sides by a hydrophobic β-segment. This segment would be even closer to the N-terminus of the activated form of these toxins, because of the removal of approx. 30 residues from the N-terminus during protoxin activation, and it may also be capable of binding lipids.

Helical wheel analysis [33] reveals that the membrane-active properties of the first 250 residues of the Bta IC1 toxin may be further augmented by the presence of two predicted amphiphilic helical segments [34]

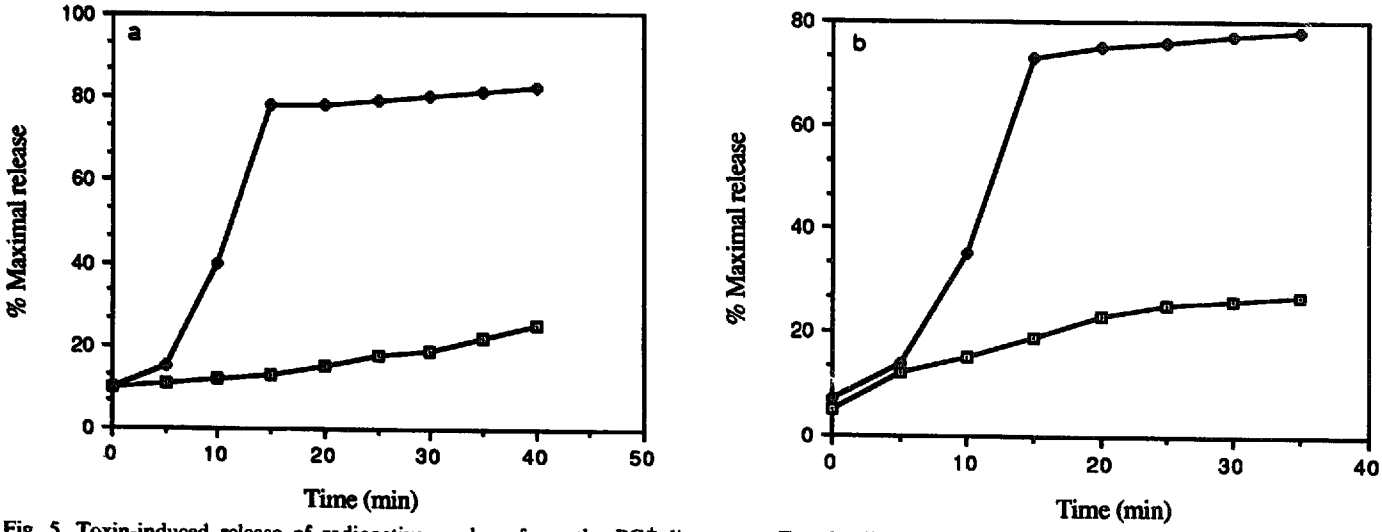

Fig. 5. Toxin-induced release of radioactive markers from the PC^+ liposomes. For details of the experimental procedure see Materials and Methods. (a) 50 μg Bta IC1 toxin was added to the liposomes loaded with [^{3}H]uracil, which were incubated for various time intervals and the free activity was separated by filtration through a Millipore filter; (b) the same sample as in (a) was added to liposomes loaded with [^{3}H]alanine.

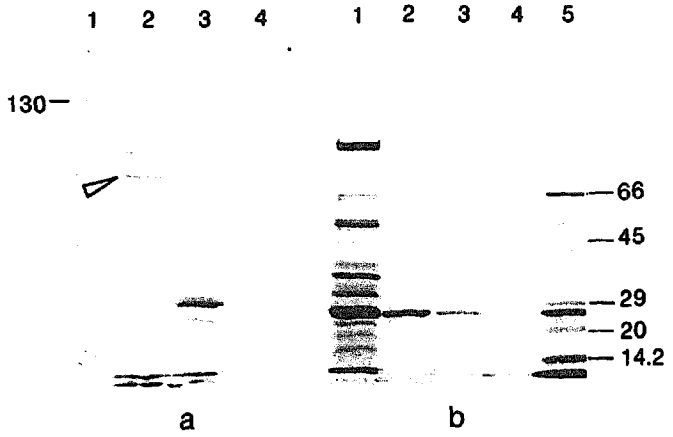

Fig. 6. Toxin binding to phospholipid vesicles. The following samples were boiled in gel sample buffer for 5 min and loaded on a 13% SDS polyacrylamide gel. Stain used was Coomassie blue. (a) Lane 1, 10 μg Bta IC1 protoxins; 50 μg trypsin-activated native Bta IC1 toxin was added to liposomes for 10 min at room temperature, which were then filtered through Millipore filters. The filter was washed with PBS and used as the bound fraction (lane 2). The proteins in the filtrate were precipitated with 50% TCA, and used as the filtered (unbound fraction) lane 3. Lane 4, toxins were filtered in the absence of liposomes. The arrowhead denotes the 55 kDa protein referred to in Results and Discussion. (b) Exactly the same procedure was used as in (a) but with 27 kDa Bti toxin. Lane 5 contains molecular mass markers with the values shown in kDa.

between residues 75 and 96 and 126 and 134 (data not shown). The membrane affinity of this N-terminal Bta IC1 toxin segment has been demonstrated in experiments in which the protein product of a deletion mutant encoding only the first 241 residues was found to perturb the structure of lipid bilayers (Haider et al., FEMS Microbiol. Lett., in press).

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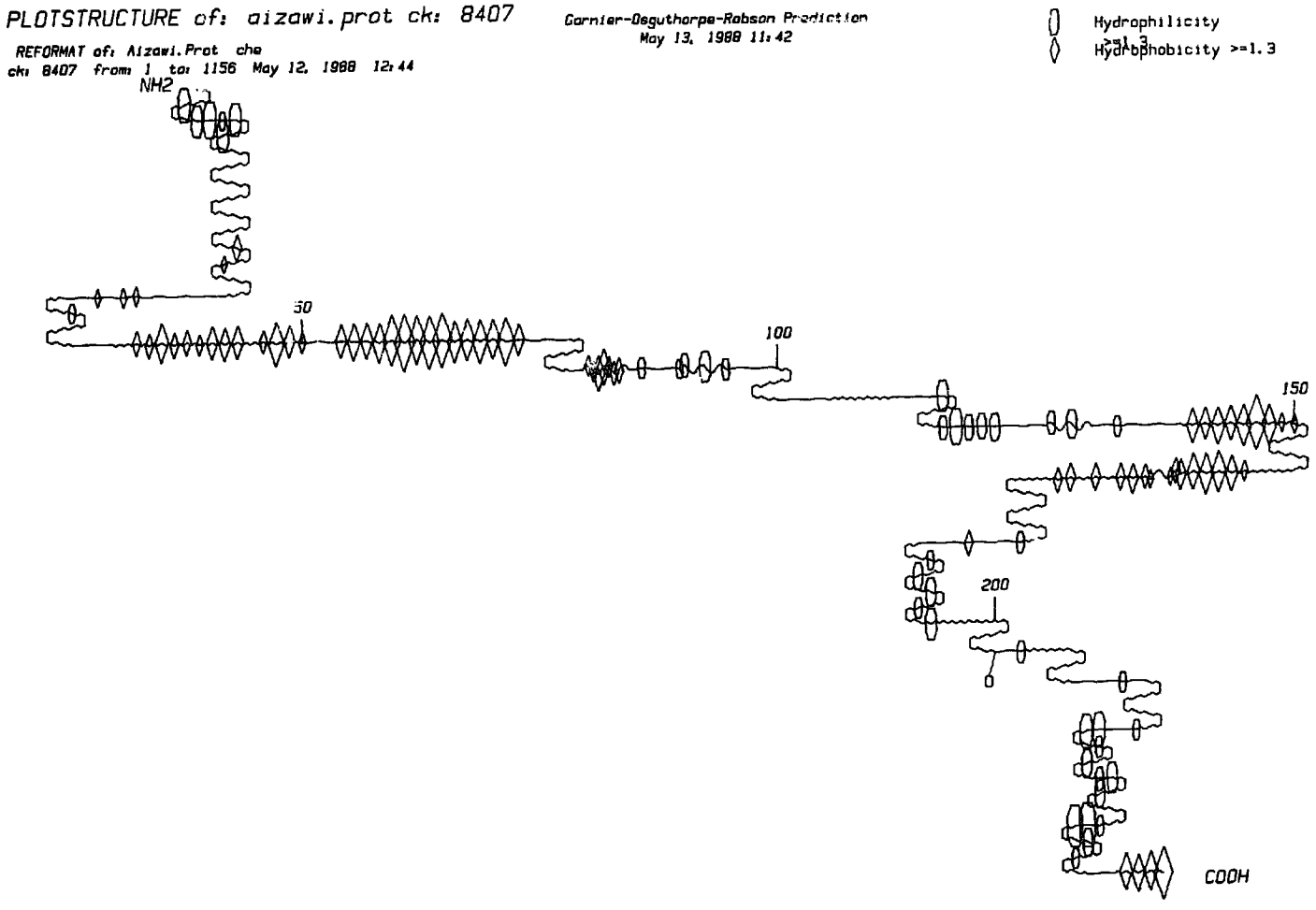

Fig. 7. Secondary-structure prediction of the conserved hydrophobic regions of the Bta IC1 *Cry* gene using the algorithm of Garnier et al. [36]. The plot was generated using the Peptidestructure and Plotstructure programmes of the GCG package [35]. A hydrophilicity threshold of 1.3 was used.

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