Purification of a Protein from *Bacillus thuringiensis* Toxic to Larvae of Lepidoptera

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The protein toxin of the parasporal body or crystal of *Bacillus thuringiensis* (Mattés isolate) has been purified severalfold by a combination of Sephadex G-200 gel filtration and ammonium sulphate precipitation. It has been shown that the use of highly alkaline conditions for dissolution of the crystals does not lead to serious artifacts. The crystal toxin has been shown to be quantitatively related to the crystal antigen. It is possible that there is a second distinct toxin present in the crystal and this too can be detected by its antigenic reaction. Purified toxic protein has been hydrolysed *in vitro* by regurgitated *Pieris brassicae* gut enzymes, chymotrypsin, trypsin and subtilisin. In each case the digest contained a product that was still antigenic, had mol.wt. about 40000 and was toxic to *P. brassicae* larvae. Smaller toxic molecules (mol.wt. approx. 10000) that did not react as antigens were also produced by proteolysis. It is possible that these smaller molecules were hydrolytic products of the larger digestion product.

Bacillus thuringiensis forms during sporulation a parasporal body that is a crystalline protein. This crystalline protein, which is released into the culture medium when the cell lyses, is toxic to larvae of many lepidopterous insects and is the basis of most of the commercial bacterial insecticides.

The protein crystals are toxic to susceptible larvae only by ingestion; they are not toxic by injection into the haemolymph of the insects. When the crystals are hydrolysed by caterpillar gut enzymes *in vitro*, the digest formed is toxic by injection (Lecadet, 1965a). This suggests that the protein crystal is a protoxin and that it is converted into the toxin in the caterpillar's gut.

To study the protein crystals it is necessary to separate them from the spores. They have been separated successfully by Angus (1959), by Lecadet (1965b) and by Murray & Spencer (1966), and were shown to be insoluble except at high pH or in the presence of reducing agents (Young & Fitz-James, 1959). The protein preparations obtained by dissolving the crystals in dilute alkali were, however, not homogeneous. Thus Lecadet (1966) detected four proteins by ultracentrifugation of an alkaline solution (pH 9.5) of crystals in 0.1M-sodium mercaptoacetate. Anderson & Rogoff (1966) found by paper electrophoresis five proteins in partially solubilized preparations of crystals. None of these workers reported on the toxicity of the separated protein fractions. The object of the present work was to examine freshly the properties of the

solubilized protein of the crystals and to define, if possible, the toxic substance.

MATERIALS AND METHODS

Materials. All chemicals were analytical reagent grade. Sephadex gel-filtration beads were purchased from Pharmacia Fine Chemicals A.B., Uppsala, Sweden. Triton X-100 detergent was the product of the Rohm and Haas Co., Philadelphia, Pa., U.S.A. Subtilisin and cytochrome *c* were obtained from Sigma Chemical Co., St Louis, Mo., U.S.A.; chymotrypsin and trypsin were from British Drug Houses Ltd., Poole, Dorset. Agar for immunodiffusion (Ionagar no. 2), peptone and Lab-Lemco broth were supplied by the Oxoid division of Oxo Ltd., London, E.C. 4. Polypropylene glycol was the product of the Shell Chemicals (U.K.) Ltd., London, S.E. 1. Dithiothreitol was obtained from Calbiochem, Los Angeles, Calif., U.S.A. Bovine plasma albumin was purchased from Armour Pharmaceutical Co. Ltd., Eastbourne, Sussex.

Growth of organisms. Cultures of *B. thuringiensis* were obtained from the Milstead Culture Collection (M.C.C.) and have been described previously by Norris (1964) and by Chapman & Norris (1966). All the work, except where specially stated, refers to the toxic parasporal body of the Mattés isolate (serotype 1; M.C.C. 9). The other organisms studied were: isolate IH-A (esterase type *galleriae*, serotype 7, M.C.C. 8), isolate T84-A (esterase type *sotto*, serotype 4A, M.C.C. 16), isolate Limassol (esterase type *entomocidus*, serotype 6, M.C.C. 25), isolate G2 (esterase type Morrison, serotype 8, M.C.C. 75) and isolate Tolworth (esterase type Tolworth, serotype 9, M.C.C. 13). The naming of these micro-organisms is reported here in some detail because of

the confused nomenclature that exists in the literature for organisms of this group.

Cultures of *B. thuringiensis* were maintained in the freeze-dried state. Subcultures were grown on nutrient-agar slopes, but for the preparation of spores and crystals liquid cultures were used. All liquid cultures were grown at 30° in a medium the composition of which is given below (g. of solid/l. of deionized water). Solution 1: KNO₃, 7.5g.; Na₂SO₄, 0.112g.; MgSO₄·7H₂O, 0.093g.; CaCl₂·6H₂O, 0.16g.; MnSO₄·4H₂O, 0.0017g.; Fe₂(SO₄)₃, 0.015g.; ZnSO₄·7H₂O, 0.011g.; peptone, 2.4g.; Lab-Lemco broth, 3.2g. Solution 2: KH₂PO₄, 10.2g.; KOH, 0.248g. Solutions 1 and 2 were sterilized separately by autoclaving, and were cooled and mixed aseptically. The pH of the medium was 6.1 before inoculation and 7.2 when sporulation and cell lysis was complete.

Cultures of 25ml. in 100ml. shake-flasks were inoculated from an agar slope. While this culture was still in the exponential phase of growth, 10ml. of it was inoculated into 300ml. of medium in a 1l. shake-flask. The whole culture was used to inoculate 10l. of medium in a 14l. glass fermentation jar (New Brunswick Scientific Co., New Brunswick, N.J., U.S.A.; model no. FS314). The medium was agitated by an impeller turning at 600 rev./min. and aerated through a sparger at 6l. of air/min. After a 12 hr. period of growth, the content of this fermenter jar was used to inoculate 125l. of medium contained in a stainless-steel pilot-scale fermenter (manufactured by Taylor Rustless Fittings, Lower Wortley, Leeds, Yorks.). The culture in this vessel was aerated at 75l./min. and stirred with a six-bladed impeller (5.25 in. diam.) turning at 750 rev./min. Polypropyleneglycol (0.01%) was added to all liquid cultures to inhibit foaming.

Cultures not required for further transfer were harvested when the spores and crystals had been released from the cells. Volumes of culture less than 20l. were harvested by centrifuging at 2500g for 30 min. in a bucket centrifuge (MSE Mistral). Larger volumes were harvested in a continuous-flow centrifuge (Alfa-Laval Ltd., Brentford, Middlesex). The paste of spores and crystals obtained was stored without washing at -20°.

Biological assay of toxicity. The toxicity of protein preparations was measured by the inhibition of feeding of *Pieris brassicae* larvae. Protein solutions or suspensions of known protein concentration were serially diluted in 0.002% Triton X-100 in water. The solutions for assay were usually diluted over a 16-fold range. Leaf disks (4.7 cm. diam.) were cut with a brass punch from smooth leaves of *Brassica oleracea* (cabbage, variety Flowers of Spring). Triton X-100, a neutral detergent, was used as a wetting agent. The leaf disks were dipped into solutions of toxin, drained carefully on a piece of tissue and then air-dried. They were then placed in Petri dishes (4.8 cm. internal diam.) containing five caterpillars. The caterpillars were in the third instar and their average weight was 20mg. Untreated leaves or leaves treated with aq. 0.002% Triton X-100 were used as controls. After 48 hr. at 21° and 60% relative humidity in an illuminated room, the area of leaf eaten was measured with the aid of a photodensitometer, the scale of which had been calibrated to read directly in per cent of leaf area. The densitometer was designed and constructed in the Laboratory workshop. Assays were performed in triplicate and the arithmetic mean of the leaf area eaten at each dilution was used for calculation of the

relative toxicity. A linear logarithm of concentration-probit leaf-area relation was fitted by eye to obtain the concentration in µg. of protein/ml. required for 50% inhibition of feeding. The ED₅₀ value, in µg. of protein (dose required to give 50% inhibition), was obtained by dividing the value obtained in µg. of protein/ml. by 10, as each leaf was coated with 0.1 ml. of toxin solution.

Gut-juice enzyme preparation. Regurgitated gut juice from *P. brassicae* larvae was obtained by gently squeezing them. The few drops obtained from each larva were immediately frozen until needed.

Disc electrophoresis. The technique described by Davis (1964) was used in conjunction with the apparatus manufactured by the Shandon Scientific Instrument Co., London, N.W. 10. The gel system reported by Jovin, Chrambach & Naughton (1964), in which all the gels contain high concentrations of urea, was used in preference to that reported by Davis (1964), as it gave better resolution with the particular proteins being studied. One modification to the published formula of the sample gel was made: the stock solutions of this gel had twice the concentration recommended by Jovin *et al.* (1964). Thus the sample volume could then be one-half the total volume of the sample gel; the sample volume was normally 0.1 ml. in a total gel volume of 0.2 ml.

Preparation of antisera. Antisera were prepared by injecting solutions of partially purified protein toxin (see 'Purification of toxic protein', Results section), or suspensions of spores and crystals, into rabbits. Antigens were mixed with Freund's adjuvant (Freund, 1947) to give a concentration of 2mg. dry wt./ml. before injection. A portion (0.25 ml.) of this emulsion was injected intramuscularly into both hind legs of adult male rabbits. After an interval of about 1 month a further injection of 1 mg. was made. This injection was given in a suspension of aluminium phosphate suspended in 0.9% NaCl. Blood was withdrawn from the ear veins of the rabbit after a further 7, 9 and 12 days. Subsequently the rabbits were injected again and blood samples were taken at similar intervals. Serum was separated from the clotted blood and stored at -20° without added preservative. The sera are referred to below as partially-purified-protein antiserum, or spore-crystal antiserum.

Examination of protein solutions for antigenic reactions. The double-diffusion technique of Ouchterlony (1958) was used. Pits in the agar gel were made with Feinberg cutters (Shandon Scientific Instrument Co.). Immuno-electrophoresis was performed on lantern-slide cover glasses (3½ in. × 3½ in.) covered with 12 ml. of 1% (w/v) agar containing sodium azide (1%, w/v) as a preservative and buffered at pH 8.6 with veronal-acetate (0.05 M). The technique was essentially that of Grabar & Williams (1955). Antigen pits and antiserum wells were cut in standard patterns with an agar cutter (Buchler Instruments Inc., Fort Lee, N.J., U.S.A.). The electrophoresis was performed in an apparatus similar to that described by Kohn (1960).

Disc immunoelectrophoresis. A further method for the examination of the antigen composition of a protein mixture was based on the high resolution of the disc-electrophoresis method, and the antigen-antibody reaction. Two samples of a protein solution to be examined were separated on polyacrylamide-gel rods as described above. One gel was stained with Naphthalene Black in the normal manner. The other gel rod was removed from the glass tube, blotted

with a paper tissue and placed on a glass lantern slide that had been covered with gel-diffusion agar. Antiserum trenches were cut 7 mm. each side of the gel rod and filled with antiserum. The precipitin lines formed, after 24 hr. incubation in a water-saturated atmosphere at 30°, as arcs around the separated protein bands. After development of the precipitin arcs the unstained gel rod was replaced by the stained one. The antigen-antibody reaction could then be related to a particular protein band. Seto & Hokama (1964) have reported a similar method. If the gel rod had swollen during the destaining procedure it could be reduced in size by washing in aq. ethanol.

Quantitative immunoassay. Quantitative immunoanalyses were made in a manner similar to that described by Mancini, Vaerman, Carbonara & Heremans (1963). The actual method used here was developed by H. M. Chapman, W. H. Kingham & J. R. Norris (unpublished work) in this Laboratory. Antigen solutions were diffused from a pit cut in 1% (w/v) agar in water, containing antiserum (5%, v/v). Molten agar and antiserum were mixed at 50°, before filling circular wells ($\frac{5}{16}$ in. diam. $\times$ $\frac{1}{16}$ in. deep) in a Perspex tray. The tray was conveniently made from $\frac{1}{16}$ in. Perspex sheet with $\frac{5}{16}$ in. holes at intervals, cemented to a base plate of $\frac{1}{8}$ in. thickness. Holes $\frac{1}{8}$ in. in diameter were cut in the centre of the agar circles with a sharpened stainless-steel tube and antigen solutions added to fill the holes (approx. 10 μ l.). The diameters of the precipitin circles formed, after 48 hr. incubation at 30° in a damp atmosphere, were measured with a micrometer eyepiece in a dissecting microscope. The diameter was found to be proportional to the logarithm of the antigen concentration when the diameters of the circles fell between 6.0 and 10.0 scale divisions (5.7 mm. and 9.6 mm.). It is possible to compare the antigen content of two protein solutions (or any number) by plotting the diameter of precipitin circles obtained with a range of protein concentrations against the logarithm of the protein concentration used for each circle. The ratio of one antigen concentration to another is then found by comparing the reciprocal of the slopes obtained from the graphs.

Preparation of crystals from spore and crystal mixture. Protein crystals were prepared from a wet paste of spores and crystals by a two-phase method with chloroform and water (Murray & Spencer, 1966). The method of Pendleton & Morrison (1966) worked well for strains other than the Mattés isolate of *B. thuringiensis*. The purer preparations of crystals usually contained less than 1% of spores. This content was determined by counting spores in stained smears of crystal preparations (carbolfuchsin).

Determination of protein and concentration of protein solutions. Protein was determined by the method of Lowry, Rosebrough, Farr & Randall (1951); crystalline bovine plasma albumin was the reference standard. For disc electrophoresis and column chromatography, dilute protein solutions were concentrated to 2–3 mg./ml. with an ultra-filtration apparatus (Sartorius Membranfilter, obtained from Hudes Merchandising Co. Ltd., London).

Column chromatography. Sephadex columns were prepared and run as recommended by Pharmacia Fine Chemicals A.B., Uppsala, Sweden. All column separations were made at 4°.

Spectrophotometry. Measurements in the visible region of the spectrum were made with a Unicam SP.600 spectrophotometer. A Zeiss PQ11 or a Unicam SP.800 instrument was used for u.v. spectrophotometry.

RESULTS

Preparation of soluble protein toxin. For purposes of comparison by electrophoresis, three types of preparation were made: (a) a partially purified protein toxin from a spore-crystal mixture made by the method described further under 'Purification of toxic protein' (below); (b) a preparation made by dissolving 6 mg. of protein crystals, separated from spores by the method of Murray & Spencer (1966), in 3 ml. of 0.04N-sodium hydroxide. After dissolution of the crystals (60 min. at 30°) the pH of the solution was lowered to 4.4 by the addition of N-acetic acid, and the amorphous protein precipitate was sedimented by centrifuging at 10000g for 15 min. The precipitate was dissolved in a small volume of 0.05M-sodium carbonate-sodium hydrogen carbonate buffer, pH 9.5; (c) a preparation made by dissolving 10 mg. of crystals in 0.05M-sodium carbonate-sodium hydrogen carbonate buffer, pH 9.5, containing 8M-urea and 0.1M-2-mercaptoethanol. The urea and mercaptoethanol were removed by dialysis against several changes of 0.05M-sodium carbonate-sodium hydrogen carbonate buffer, pH 9.5.

The protein concentration of each preparation was determined and equivalent weights of each preparation were subjected to disc electrophoresis. The electrophoretic patterns obtained (Fig. 1) show only minor differences, indicating that the protein of the crystals can be extracted from a spore-crystal mixture with 0.04N-sodium hydroxide without serious contamination by protein extracted from the spores. The proteins extracted with

Fig. 1. Disc-electrophoresis separation of the protein components of: (a) partially purified protein toxin prepared from spore-crystal mixture by the method described under 'Purification of toxic protein' (Results section); (b) partially purified toxin prepared from purified crystals; (c) protein crystals dissolved in 8M-urea buffered at pH 9.5 and made 0.1M with respect to mercaptoethanol (solution dialysed against 0.05M-Na₂CO₃-NaHCO₃ buffer, pH 9.5, before electrophoresis).

0.04N-sodium hydroxide did not differ greatly, as shown by electrophoresis, from the proteins extracted by the carbonate buffer (preparation c).

Isoelectric precipitation of toxin. A solution of toxic protein was made by extracting 8g. wet wt. of spore-crystal mixture with 200ml. of 0.04N-sodium hydroxide for 2hr. The spores were removed by centrifugation at 20000g for 10min. The supernatant protein solution was then titrated in steps with N-acetic acid to pH 7.5, 5.4, 5.0 and 4.4. The precipitate formed after each step was removed by centrifuging and dissolved in 0.01N-sodium hydroxide; the protein content and biological activity of each solution was then determined. As shown in Table 1, toxic protein was precipitated between all pH ranges, and hence this method offered no satisfactory enrichment of the toxin.

Purification of toxic protein. A spore-crystal mixture (40g. wet wt.) was stirred for 1hr. with 1l. of each of the following solutions in succession:

Table 1. *Isoelectric precipitation of protein toxin from alkaline solution*

A spore-crystal mixture (8g. wet wt.) was extracted for 2hr. with 200ml. of 0.04N-NaOH. The supernatant liquid, after centrifuging, was titrated in steps with acetic acid to pH 4.4. Precipitates that formed were removed by centrifuging and dissolved in 0.01N-NaOH. Their protein content and biological activity were determined.

Fraction no.	pH range of fraction	Protein in fraction (% of total protein)	Toxin in fraction (% of total toxin)
1	12.0-7.5	1.9	2
2	7.5-5.4	55.8	74
3	5.4-5.0	22.2	12
4	5.0-4.4	7.6	11
5	Supernatant solution from fraction 4	12.5	1

0.1M-sodium phosphate buffer, pH 6.0, M-sodium chloride and water. The spore-crystal mixture was recovered by centrifuging at 5000g for 20min. after each washing. The pellet obtained after the last washing was suspended in 1l. of 0.04N-sodium hydroxide and stirred for 2hr.

After centrifuging, the supernatant liquid was titrated to pH 4.4 by slow addition of acetic acid with continuous mixing. After 30min. at 4°, the precipitate that had formed was recovered by centrifuging at 5000g for 15min. It was redissolved in the minimum volume of 0.04N-sodium hydroxide and the pH of this solution adjusted to 9.5 with M-sodium hydrogen carbonate, after which it was frozen. The toxicity of the preparation at this stage was stable for at least 4 months. Toxic protein at this stage of purification is referred to elsewhere in the text as 'partially purified toxin' or 'partially purified protein'; it was used to immunize rabbits.

Portions of partially purified toxin (5ml.), made denser than the buffer with 1g. of sucrose, were placed on a Sephadex G-200 column (3.5cm. x 27cm., total volume, V_t , 260ml.) equilibrated with 0.05M-sodium carbonate-sodium hydrogen carbonate buffer, pH 9.5, containing dithiothreitol (0.5mM). The void volume (V_0) of this column was 82ml. The fractions eluted from the column from K_{av} 0.1 to K_{av} 0.3 were pooled and frozen.

The values for K_{av} were derived from $(V_e - V_0)/(V_t - V_0)$, where V_e is the elution volume, V_0 is the void volume of the gel-filtration column and V_t is the total volume of the gel bed. Successive portions of partially purified protein were separated on this column and the active fractions pooled.

Saturated ammonium sulphate solution ($\frac{1}{2}$ vol.), neutralized with aq. ammonia solution, was added to the pooled fractions eluted from the Sephadex G-200 column. The precipitate formed was dissolved in 7M-urea containing dithiothreitol (0.5mM) and 0.05M-sodium carbonate-sodium hydrogen carbonate buffer, pH 9.5. The precipitate was

Table 2. *Purification of protein toxin*

A spore-crystal mixture (40g. wet wt.) was extracted with 0.04N-NaOH. Protein was precipitated from this solution at pH 4.4. The precipitate was redissolved in 0.04N-NaOH, and the pH of the solution was adjusted to 9.5. It was then chromatographed on Sephadex G-200 columns. Toxicological assay was performed as described in the text.

Step	Description of preparation	Total protein (mg.)	Protein recovered (% of total protein)	No. of bands by disc electrophoresis	ED ₅₀ (μg. of protein)
1	Alkaline extraction	1150	100	13	0.063
2	pH 4.4 precipitate ('partially purified toxin')	1000	87	13	0.060
3	First Sephadex G-200 column fraction, K_{av} 0.1-0.3	259	22.5	3 + traces of others	0.032
4	Subsequent Sephadex G-200 column fraction, K_{av} 0.1-0.3	188	16.5	3	0.032

difficult to dissolve without the use of urea. Portions (5ml.) of this solution were placed on the same Sephadex G-200 column described above and the same fraction was collected, i.e. having *K* 0.1–0.3. The protein at this stage of purity was frozen and used for further experiments. A typical purification is shown in Table 2. Analyses of the protein by disc electrophoresis at each stage of purification (Fig. 2) show that the protein solution after precipitation at pH 4.4 contains at least 13 protein bands, but that after purification this number is reduced to three bands. The electrophoretic method detects only proteins that stain with Naphthalene Black dye and that are anionic at pH 9.5, which is the running pH of the gel (Ornstein, 1964). Fig. 2 also shows the results of examination of the purified toxin by combined disc electrophoresis and immunotoxin by combined disc electrophoresis and immunodiffusion. The toxic protein at purification

step 2 could be adsorbed on ECTEOA-cellulose and DEAE-cellulose, but it could not be eluted from either. DEAE-cellulose columns equilibrated with 7*M*-urea have been used without success. It can be shown by Ouchterlony double-diffusion immuno-analysis that the preparation at step 4 contains two antigens. I have called these antigens A and B (see section on antigenicity of the protein toxin).

Relation between the toxic and antigenic activities of the protein crystal. A solution of partially purified toxin containing 7.5mg. of protein/ml. in 0.05*M*-sodium carbonate–sodium hydrogen carbonate buffer, pH 9.5, was made 5*M* with respect to urea and then titrated to pH 7.5 with 0.2*M*-sodium dihydrogen phosphate. The volume of the solution was adjusted with water to give a protein concentration of 2.5mg./ml. To this solution $\frac{1}{3}$ vol. of saturated ammonium sulphate, pH 7.5, was added with gentle stirring. The precipitate that formed (fraction 1) was removed by centrifuging. Solid ammonium sulphate (95g./l.) was added slowly to the supernatant solution. The precipitate was removed by centrifuging (fraction 2). Finally 288g. of ammonium sulphate was added to each litre of this supernatant solution and the precipitate was collected by centrifuging (fraction 3). The precipitates were dissolved separately in the minimum quantity of 0.01*N*-sodium hydroxide and dialysed against 0.05*M*-sodium carbonate–sodium hydrogen carbonate buffer, pH 9.5, overnight at 4°. The fractionation was performed at 20°. The presence of urea in the original solution is necessary to extend the range of ammonium sulphate saturation over which the protein is precipitated. In the absence of urea, a large fraction of the protein is precipitated below 10% saturation. The protein concentration, toxicity and antigenicity of each fraction were measured. The results of this experiment are given in Table 3.

It can be seen that the toxic component in the three fractions is related quantitatively to the antigenic component. It is therefore probable that the antigenic site and the toxically active centre are on the same molecule. In the quantitative immuno-assay, two concentric precipitin circles were

Fig. 2. Disc electrophoresis of toxin solutions at several stages of purification. Samples of protein were examined by disc electrophoresis at the following stages in purification: (a) step 2, pH 4.4 precipitation; (b) step 3, *K* 0.1–0.3, fraction from Sephadex G-200 column; (c) step 3, fraction eluted subsequent to protein shown in gel (b); (d) step 4, purified toxin; (e) step 4, purified toxin with precipitin lines associated with Naphthalene Black-stained protein bands. These precipitin lines were obtained by the technique of disc immunoelectrophoresis described in the text.

Table 3. Comparison of antigenicity and toxicity of protein fractions obtained by ammonium sulphate fractionation

A solution of partially purified toxin was fractionated with ammonium sulphate. The toxicity and antigenicity of each fraction was then determined. Fraction 3 was used as a reference preparation for both toxicological and immunological comparison (last two columns). The results of Ouchterlony immunodiffusion examination are shown in Fig. 4.

Fraction	Saturation with (NH ₄) ₂ SO ₄ (%)	Toxicological assay (ED ₅₀ /mg. of protein)	ED ₅₀ /mg. of protein, related to fraction 3	Antigenic response, related to fraction 3
1	0–25	5920	1.2	1.1
2	25–40	14100	2.8	2.3
3	40–75	5050	1.0	1.0

Fig. 3. Immunodiffusion pattern obtained with the toxic protein from several isolates of *B. thuringiensis*. Partially purified toxin (see the text) was dissolved in 0.05 M- Na_2CO_3 - NaHCO_3 buffer, pH 9.5, and allowed to diffuse from the outer pits to react with antiserum (7) prepared against partially purified toxin from Mattés isolate of *B. thuringiensis*. Toxins are from isolates: 1, Mattés; 2, Limassol; 3, Tolworth; 4, T84-A; 5, 1-HA; 6, G2 (see the Materials and Methods section for detailed naming of bacteria).

Fig. 4. Immunodiffusion pattern obtained from ammonium sulphate fractions. Pits 1 and 4, partially purified toxin from Tolworth isolate; 2, 3 and 5, fractions 1, 2 and 3 respectively from ammonium sulphate fractionation in the presence of urea; 6, partially purified toxin from which fractions 1, 2 and 3 were obtained (Mattés isolate); 7, antiserum against 6.

obtained in each diffusion test, but only the stronger precipitin circle (which also had the larger diameter) was measured. The reason for this is discussed below.

When partially purified toxins from several isolates of *B. thuringiensis* were examined with the aid of the Ouchterlony double-diffusion technique it was found that they contained a common

Fig. 5. Immunodiffusion reactions of antigen B. Pits 1 and 2, untreated antigen B preparation eluted from Sephadex G-200 column; 3, antigen B solution treated at pH 12.0 for 2 hr.; 4, partially purified Mattés toxin; 5, antigen B solution, pH 9.5, treated with 0.05 M-mercaptoethanol for 2 hr.; 6, partially purified toxin from Tolworth isolate; 7, antiserum to Mattés partially purified toxin.

Fig. 6. Immunoelectrophoresis of antigens from Mattés spore-crystal mixture. Immunoelectrophoresis was performed in agar at pH 8.6 with 0.05 M-veronal-acetate buffer. (1) Antigen B, after separation on Sephadex G-200 column and concentration by ultrafiltration, is compared with (2), purified Mattés toxin; (a) partially purified Tolworth toxin; (b) and (c), antiserum to partially purified Mattés toxin. Antigens were dialysed against 0.05 M-veronal-acetate, pH 8.6, at 4° for 12 hr. before electrophoresis.

antigen. Fig. 3 shows the pattern obtained with isolates of the indicated strains, tested against Mattés partially-purified-toxin antiserum. The identity of the two precipitin lines obtained from the Mattés isolate, when reacting with homologous antiserum, can be established by placing partially purified Tolworth toxin (for example) adjacent to it. Thus Fig. 4 shows that the major antigen in the ammonium sulphate fractionation (Table 3) is the A antigen and not the B antigen.

Extraction of B antigen from spore-crystal mixture. A spore-crystal mixture was washed as described under 'Purification of toxic protein' (above). The washed spore-crystal mixture (40g. wet wt.) was then extracted with 1l. of 0.1M-sodium carbonate-sodium hydrogen carbonate buffer, pH 8.9, at 30°. Although carbonate is a poor buffer at pH 8.9, it was used in preference to tris-hydrochloric acid or to sodium borate adjusted with boric acid because it gave higher concentrations of antigen B in the extract than either of these buffers. There is no explanation for this at present. The spores and partially solubilized crystals were removed by centrifuging at 10000g for 20min. and the supernatant liquid obtained was titrated to pH 4.4 with acetic acid. The precipitate that formed was dissolved in 0.04N-sodium hydroxide. After adjustment to pH 9.5 with M-sodium hydrogen carbonate, the solution was fractionated on a Sephadex G-200 column as described under 'Purification of protein toxin'. The fractions that gave a reaction of identity with Tolworth partially purified toxin, when tested against antiserum to partially purified Mattés toxin, were pooled and frozen. This antigen is smaller in size than antigen A as it is eluted later from the Sephadex G-200 column. In some experiments, the extract at pH 8.9 was concentrated by ultrafiltration and fractionated directly on a Sephadex column.

Attempt to modify B antigen. Portions (5ml.) of a

solution of antigen B in 0.05M-sodium carbonate-sodium hydrogen carbonate buffer, pH 9.5, that had been separated on a Sephadex G-200 column without prior precipitation at pH 4.4 and re-solution in 0.04N-sodium hydroxide, were treated as follows: (a) mercaptoethanol was added to give 0.05M solution; (b) N-sodium hydroxide was added to give pH 12.0; (c) was untreated to act as a control.

The solutions were incubated at 30° for 2hr. The antigen reaction of each solution was tested by a double-diffusion test. In (b) the pH was adjusted to 9.5 with M-sodium hydrogen carbonate before the immunoanalysis. The results showed (Fig. 5) that the untreated preparation contained traces of the antigen A, but that antigen B was present in a much higher concentration. Antigen B does not change its antigenic property when exposed to reducing conditions or to conditions of high pH. Fig. 6 shows an immunoelectrophoresis of untreated antigen B and purified Mattés toxin. Antigen B has a different pK value from the major antigen of the toxin preparation. This is evident from the opposite migration of the antigen B from the toxin. A comparison of the toxicity of antigen B with partially purified toxin gave an ED₅₀ value of 0.34 µg. of protein for antigen B and 0.054 µg. of protein for the partially purified toxin. It should be noted that the preparation of antigen B is not entirely free of antigen A.

Fig. 7. Elution profile of products obtained from purified toxin when digested with *P. brassicae* gut enzymes. Purified toxic protein was digested at pH 9.7 and 30° with regurgitated gut enzymes from *P. brassicae* larvae. The whole digest was chromatographed on a Sephadex G-200 column equilibrated with 0.05M-Na₂CO₃-NaHCO₃ buffer, pH 9.7. Antigen reaction (●) was measured by the radial diffusion method described in the text. Where no precipitin reaction was detected, a figure of 3.0 scale divisions was plotted, as this was the size of the diffusion pit in scale divisions. Toxicity (▲) was assayed by measuring the area of leaf eaten after 48hr. The numbers are % of leaf-area eaten when fractions diluted 50-fold were tested as described in the text. ○, E₂₈₀, measured in a Unicam SP.800 spectrophotometer.

Fig. 8. Elution profile of products obtained from purified toxin when digested with chymotrypsin. Purified toxic protein was incubated with chymotrypsin at pH 9.7 and 30° for 4 hr. The whole digest was chromatographed on a Sephadex G-200 column equilibrated with 0.05M-Na₂CO₃-NaHCO₃ buffer, pH 9.7. Antigen reaction (●) was measured by the radial diffusion method described in the text. Where no precipitin reaction was detected, a figure of 3.0 scale divisions was plotted, as this was the size of the diffusion pit. Toxicity (▲) was assayed by measuring the area of leaf eaten after 48 hr. The figures are % of leaf area eaten when fractions diluted 50-fold were tested as described in the text. ○, E_{280} , measured on a Unicam SP.800 spectrophotometer.

Enzymic hydrolysis of purified toxic protein. (a) Digestion with gut enzymes of *P. brassicae*. A solution of purified protein toxin was concentrated by ultrafiltration. Regurgitated gut juice from *P. brassicae* larvae was centrifuged at 27000g for 20 min. to remove undigested debris from the larval gut before incubation with the purified protein. The incubation mixture consisted of 23 mg. of purified toxin in 0.05M-sodium carbonate-sodium hydrogen carbonate buffer, pH 9.7, and 0.035 ml. of gut-juice enzyme solution in a total volume of 3.5 ml. After 1 hr. at 30°, a further 0.035 ml. of gut juice was added. This procedure was repeated twice more. After a total of 4 hr. incubation, the digest was made 10% (w/v) with respect to sucrose and chromatographed on a Sephadex G-200 column (42.0 cm. × 2.3 cm.; V_t 175 ml., V_0 51 ml.) equilibrated with the incubation buffer. The flow rate was approx. 9 ml./hr. The increased density of the sample permitted it to be applied to the column without interrupting the flow of buffer from the column outlet. The extinction at 280 mμ of each fraction was measured, together with the antigenic reaction and toxicity. Fig. 7 shows the results of a typical elution profile. Crystalline bovine plasma albumin and cytochrome *c* were separated on the same column to obtain an approximate molecular weight corresponding to the elution volume (V_e) of the digested toxin that still gave a precipitin reaction. Fig. 7 shows the elution positions of these

protein standards. The molecular weight corresponding to K_{av} 0.35, which is the elution position of the digested antigen, is approx. 40000. This value was obtained by assuming that the elution volume of a compound is proportional to the logarithm of the molecular weight (Whitaker, 1963).

(b) Digestion with purified proteolytic enzymes. Purified toxin (16.8 mg.) was digested with 1 mg. of crystalline chymotrypsin, trypsin or subtilisin in a total volume of 3 ml. The experimental conditions were the same as those used in (a), although no further additions of enzyme were made. The digests were frozen until they could be chromatographed on a Sephadex G-200 column as described in (a). The elution profile for these digestions is shown in Fig. 8. The graphs obtained after hydrolysis with chymotrypsin, trypsin and subtilisin were very similar; therefore only the profile after chymotrypsin digestion is shown.

DISCUSSION

Hannay & Fitz-James (1954) found that the crystalline protein inclusion bodies of *B. thuringiensis* could be dissolved at pH 11.8 to produce a solution that was toxic to *Bombyx mori* larvae. It was further shown by Hannay & Fitz-James (1954) that the protein that resulted from dissolution of crystals could be precipitated by lowering the pH of the solution to 4.4. It was

shown that the crystals were partially soluble at pH 9.5 in the presence of a reducing agent (Anderson & Rogoff, 1966). It has been found that soluble toxic protein, after precipitation at pH 4.4, was very soluble in 8M-urea at pH 9.0. At pH 12 it was possible that some alkali-labile linkages in a protein were broken. However, Lecadet (1966) found the same number of *N*-terminal amino acids in her preparations whether the protein was extracted at pH 9.5 under reducing conditions or at pH 12 without a reductant. To facilitate the production of large quantities of amorphous toxic protein for this and other studies of a more applied nature, conditions of high pH have been used to extract toxin from a spore-crystal mixture, rather than from purified crystals. It has been shown that this protein is electrophoretically similar to the toxic protein extracted from the crystals by the same method, and to protein extracted from crystals by a much milder treatment. It was therefore probable that labile linkages that would give rise to smaller protein fragments were not broken, although there was no information about permanent damage to the structures on which the biological activity depended.

Isoelectric precipitation was unsuitable as a purification procedure for the toxin since the protein precipitated at various pH values had very similar toxicities. The technique was of use, however, for the concentration of protein solutions by precipitation and re-solution in a smaller volume of solvent.

There have been several studies of the parasporal bodies as antigens (Krywienczyk & Angus, 1960; Monro, 1961; De Barjac & Lecadet, 1961; Pendleton & Morrison, 1966), but only Krywienczyk & Angus (1960) attempted to show a relation between the crystal as an antigen and as a substance toxic to insects. They showed that, although the gross compositions of the crystals of three strains of *B. thuringiensis* were serologically different, there was a common toxic component. Thus antiserum to the crystals of one strain could precipitate the toxin of another strain. Fig. 3 showed that antigen B was common to the toxic protein from several isolates, although toxicological assay suggested that this antigen was less toxic than antigen A. It was possible that Krywienczyk & Angus (1960) were not dealing with the same protein as antigen A, as this antigen is not common to all the isolates studied here. It was found to be important during studies on the purification of the soluble toxin to compare the toxicity and antigen reaction of a preparation. During several fractionation procedures, such as gel filtration, preparative electrophoresis and isoelectric precipitation, all toxic preparations obtained reacted with spore-crystal antiserum or partially-purified-protein antiserum. The experiments re-

ported show that the quantitative immunoassay, which is relatively easy to perform, could replace toxicological assay as a means of assessing the relative amount of toxin in a soluble protein preparation. This technique was of particular use when looking for active fractions, e.g. from chromatographic columns.

The purification procedure was not completely effective, as usually the purified product contained three proteins. On occasion, the small protein band shown in Fig. 2(e) was barely visible, although the toxicity of these preparations was not significantly altered. This suggested that either or both of the main bands of protein seen in Fig. 2(e) were responsible for the toxicity of the preparation.

The protein toxin was not soluble in the operating pH range of CM-cellulose, so that this ion-exchange method could not be used. The toxin was strongly bound to DEAE-cellulose at pH 8.6 in the presence or absence of 7M-urea or a reducing agent. It was possible to elute some protein from DEAE-cellulose with the buffer containing M-sodium chloride, but this had the same disc-electrophoresis pattern as the material applied to the DEAE-cellulose column.

The criterion for heterogeneity of the purified protein toxin was the ability to demonstrate at least two components by disc electrophoresis, a method capable of very fine resolution. It would appear that the only means of further resolution of the purified protein would be to utilize this principle in a preparative manner. It was possible that both proteins shown in Fig. 2(d) were toxic and that they differed by one or two amino acids in their primary structure. The production of several related toxins by microbes is not unusual. For example, Berheimer & Schwartz (1963) showed that a preparation of staphylococcal α -toxin could be resolved by density-gradient electrophoresis into four fractions, all of which were biologically active.

The extraction and partial purification of antigen B showed that there may be another distinct toxic protein in the parasporal body. The results did not show conclusively that the toxicity of this preparation was due to antigen B, for immunodiffusion indicated that the preparation was not free of antigen A. In Fig. 5, for instance, the precipitin line due to the antigen A in pit 4 was not straight, but curved slightly towards pit 3, which demonstrated the presence of a small quantity of antigen A in pit 3. A contamination of about 20% of antigen A would account for the toxicity of the antigen B preparation. Only further purification of antigen B will settle this question.

It was thought possible that antigen A may be derived from antigen B by an artifact of extraction. The antiserum was usually produced with partially purified toxin, which contained both antigens A

and B. As antigen A was only demonstrable in extracts made at pH 12, or at pH 9.5 in reducing conditions, it was thought worth while to check that these same conditions did not convert antigen B into antigen A. The results in Fig. 5 showed the presence of a small amount of antigen A in antigen B, but not a significant amount. The amount was not sufficient to give rise to a separate precipitin line.

Proteolysis of the toxin with chymotrypsin, trypsin, subtilisin and *P. brassicae* gut-juice enzymes yielded similar products, as seen by exclusion chromatography on Sephadex G-200. All the proteolytic enzymes gave hydrolysis products that still reacted as antigens and had molecular weights of approx. 40000. The specificities of the major proteolytic enzymes in *P. brassicae* larval gut juice have been shown to be similar to those of chymotrypsin and trypsin (Lecadet & Dedonder, 1966), so that this result was to be expected. Subtilisin has a different specificity from these, however, yet it too gave rise to an elution profile similar to that of the other proteolytic enzymes. The resolution of a Sephadex G-200 column is not sufficient to make much of this fact, however. All four enzyme preparations yielded products that no longer gave precipitin reactions in agar, but that were still toxic to *P. brassicae* larvae. The molecular weight of these compounds, as judged by their elution position from the Sephadex column, was approx. 10000. It was also evident that the gut-juice hydrolysis gave more of these smaller molecules than the single-enzyme hydrolyses, which is in keeping with the fact that the unpurified gut juice contained at least two proteolytic activities (Lecadet & Dedonder, 1966). Probably further digestion of the molecule(s) of molecular weight 40000 produced the smaller toxic components. The active toxin therefore could have a molecular weight as low as 10000.

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