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Crystal-Forming Proteins of *Bacillus thuringiensis*

THE LIMITED HYDROLYSIS BY ENDOGENEOUS PROTEINASES AS A CAUSE OF THEIR APPARENT MULTIPLICITY

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The crystals of the entomocidal protein of *Bacillus thuringiensis* are admixed with proteinases that in the course of their dissolution cause gradual degradation of the 'genuine' crystal-forming protein components (i.e. the primary biosynthetic products) to products of lower molecular weight. This phenomenon might explain at least partially the contradictory data on the molecular parameters of the crystal-forming proteins. Preliminary inactivation of the proteinases adsorbed on the crystals allowed us to eliminate this source of the artefacts and to gain more reliable data on the protein composition of the crystals formed by various strains of *B. thuringiensis*. It has been shown that the crystals formed by all serotypes of *B. thuringiensis*, with the exception of the serotype V, contain only one protein with a mol.wt. of 145000, 135000 or 130000, depending on the strain. The majority of the strains that belong to the serotype V form crystals consisting of two proteins with mol.wts. of 135000 and 130000, but some of them also have a third component with a mol.wt. of 65000.

Bacillus thuringiensis is known to produce a protein parasporal body at the time of sporulation (Rogoff, 1966). The parasporal body is a proteinaceous crystal released into the culture medium during cell lysis. The crystals are toxic to larvae of certain butterflies and moths (Lepidoptera).

14 serotypes of *B. thuringiensis* are known, characterized by specific H-antigens (flagellar antigens of vegetative cells). This classification obviously relies on criteria not directly related to the properties of crystal proteins. Nevertheless, taking in account the differences in the toxicity against various members of the Lepidotera shown by diverse serotypes, one may presume that some differences may exist also in the chemical properties of respective crystal-forming proteins. Hence the chemical characteristics of *B. thuringiensis* crystal-forming proteins deserve detailed study.

Contradictory results have been reported for the subunit structure of the entomocidal parasporal crystal of *B. thuringiensis* (Glatron *et al.*, 1972; Sales *et al.*, 1970; Akune *et al.*, 1971; Herbert *et al.*, 1971; Cooksey, 1968; Bulla *et al.*, 1977; Nagamatsu *et al.*, 1978). This discrepancy concerns both the number of polypeptides in the crystal and their molecular weights. It appears doubtful that these strikingly different results obtained by various techniques in different laboratories could be attributed to imperfection of the methods used or to experimental error. One suspects that there is some inherent cause behind these discrepancies, e.g. the variations in polypeptide pattern might reflect differences in the extent of crystal-protein post-translational modification.

One result of such modifications might be the action of endogeneous proteinases of *B. thuringiensis*, which, being adsorbed on the crystal surface or incorporated into its structure, could perform limited proteolysis.

The aim of the present work was to verify the presence of proteinases in the crystal preparations and to evaluate their effect on the crystal proteins. Another goal was to analyse the crystal proteins isolated from various *B. thuringiensis* serotypes under conditions specifically designed to exclude proteolysis.

Abbreviations used: SDS, sodium dodecyl sulphate; Z-Ala-Ala-Leu-*p*NA, benzyloxycarbonyl-L-alanyl-L-alanyl-L-leucine *p*-nitroanilide; N_2ph-Gly-Gly-Val-Lys, 2,4-dinitrophenylglycylglycyl-L-valyl-L-lysine; Leu-*p*NA, L-leucine *p*-nitroanilide; Bz-DL-Arg-*p*NA, benzoyl-DL-arginine *p*-nitroanilide.

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Preliminary data on the proteolysis of crystal proteins and its effect on the evaluation of protein composition of the crystals were published by us previously (Chestukhina *et al.*, 1978).

Materials and Methods

Materials

Pancreatin hydrolysate of casein, and β-mercaptoethanol, were purchased from Merck (Darmstadt, Germany). Yeast extract was the product of the Difco Laboratories (Detroit, MI, U.S.A.). Dithioerythritol was from Calbiochem (Los Angeles, CA, U.S.A.). EDTA (sodium salt), di-isopropyl fluorophosphate, phenylmethanesulphonyl fluoride, *p*-chloromercuribenzoate, *p*-chloromercuribenzenesulphonate, *N*-ethylmaleimide, iodacetamide, urea, SDS, guanidine, acrylamide, the standard proteins used as markers for electrophoresis (cytochrome *c*, myoglobin, ferritin, chymotrypsinogen A, aldolase, ovalbumin, catalase, bovine serum albumin), subtilisin BPN', thermolysin, leucine aminopeptidase and papain were purchased from Serva (Heildelberg, Germany). Chromogenic synthetic substrates (Z-Ala-Ala-Leu-*p*NA, N_2ph-Gly-Gly-Val-Lys, Leu-*p*Na and Bz-DL-Arg-*p*NA) were synthesized by Dr. Lyublinskaya in this laboratory (Lyublinskaya *et al.*, 1977).

Organism and cultural conditions

The strains of *B. thuringiensis* used in the present study were obtained from Culture Collection of this Institute. The following strains of *B. thuringiensis* were used: 1223 (var. *thuringiensis*, serotype I); 199 and 1217 (var. *insectus*, serotype I); 1162 (var. *finitimus*, serotype II); 1225 (var. *alesti*, serotype III); 1226 (var. *kurstaki*, serotype III); 1227 (var. *dendrolimus*, serotype IV) 1228 (var. *kenyae*, serotype IV); 1252 (var. *morrisoni*, serotype VIII); 1253 (var. *tolworthi*, serotype IX); 1254 (var. *darmstadtiensis*, serotype X); 1255 (var. *toumanoffi*, serotype XI); 1166 (var. *thompsoni*, serotype XII); and several strains (612, 696, 561, 614, 615, 650, 223, 220, 449, 1167, 73, 16, 273) of var. *galleriae* (serotype V).

Cultures of *B. thuringiensis* were maintained in a freeze-dried state. Subcultures were grown on nutrient-agar slopes, but for the preparation of crystals, liquid cultures were used. All liquid cultures were grown for 48h at 28°C in 750ml flasks containing 100ml of medium and aerated by rotary agitation at 200rev./min.

The composition of nutrient medium was as follows: pancreatic hydrolysate of casein (0.5%), yeast extract (0.4%), glucose (0.2%), NaCl (0.5%), $MgSO_4,2H_2O$ (0.01%), $CaCl_2,2H_2O$ (0.05%), pH 7.4.

Spores and crystals were harvested by centrifugation (6000*g* for 10min). The precipitate was suspended in water (1g of the precipitate in 100ml of water) and kept for 18h at 37°C to allow the cells to lyse and release the spores and the crystals. The precipitate was then washed twice with water (4000*g* for 5min).

Isolation of the crystals from the spore/crystal mixture

The protein crystals were prepared from a wet paste (6g of the precipitate in 100ml of water) of spores and crystals by our modification (Chestukhina *et al.*, 1977) of the two-phase-distribution method using *p*-xylene (Pendleton & Morrison, 1966). The pure crystal preparations were stored at 4°C as a suspension in water, with a few drops of xylene added as a preservative. The pure crystal preparations contained less than 0.5% spores. This value was determined by counting the spores in stained smears (Smirnoff, 1962) of crystal preparation under an optical microscope.

Solubilization of the crystals

The solubilization was accomplished by the incubation of the crystals (1mg/ml) or of the spore/crystal mixture (2mg/ml) at 20 or 100°C (see the Results and Discussion section) in: (*a*) 0.05M-NaOH; (*b*) 10mM-Tris/HCl buffer, pH 8.5, containing 10mM-dithioerythritol; (*c*) 10mM-Tris/HCl buffer, pH 8.5, containing 10mM-dithioerythritol and 8M-urea or 6M-guanidine/HCl. For solubilization at 100°C, instantaneous heating of the mixture is essential.

Proteinase assay

Z-Ala-Ala-Leu-*p*NA was used as a substrate to determine subtilisin-like serine-proteinase activity (Lyublinskaya *et al.*, 1977). Crystals (1–2mg in 2ml of 50mM-Tris/HCl buffer, pH 8.5, containing 1mM $CaCl_2$) were incubated with 250μg of the substrate solution in 0.5ml of dimethylformamide for 2h at 37°C. After removal of the crystals by centrifugation, the A_{410} (released *p*-nitroaniline) was measured.

N_2ph-Gly-Gly-Val-Lys was used as a substrate for metalloproteinase assay (Lyublinskaya *et al.*, 1976). Crystals (1–2mg) in 1ml of 50mM-Tris/HCl buffer, pH 7.0, containing 1mM-$CaCl_2$, were incubated with 4ml of 0.2M-substrate solution in the same buffer for 20h at 37°C. After the addition of 0.2M-HCl to pH 1–2, the mixture was extracted with 5ml of ethyl acetate, containing 10% (v/v) ethanol. The upper phase was extracted with 4ml of 1% $NaHCO_3$ and the A_{360} (released N_2ph-Gly-Gly) was measured in the lower phase.

Leu-*p*NA was used as a substrate for the assay of

leucine aminopeptidase. Crystals (1–2mg) in 2.5ml of 50mM-Tris/HCl buffer, pH 8.5, containing 1mM-$CaCl_2$, were incubated with 0.5mg of the substrate in 0.5ml of 0.01M-HCl for 2h at 37°C and then the A_{410} was measured.

To reveal thiol-proteinase activity, Bz-DL-Arg-*p*NA was used as a substrate. Crystals (1–2mg) in 1ml of 100mM-phosphate buffer, pH 7.0, containing 1% of cysteine were activated for 1h at 37°C and then 0.5mg of the substrate in 0.5ml of dimethylformamide was added to the mixture. After 24h at 37°C the A_{410} was measured.

Elimination of proteinase mixture from the crystals

The suspension (2mg/ml) of the crystals (strain 199, var. *insectus*, or strain 612, var. *galleriae*) in 1M-NaCl was kept for 1h at 37°C, then centrifuged as described by Weber & Osborn (1969). Protein samples, containing 1% SDS, 2% (v/v) β-mercaptoethanol and 8M-urea were incubated at 100°C for 2min, then 10–100μl were applied to the 37°C and washed twice with water (6000*g* for 5min).

Polyacrylamide-gel electrophoresis.

Electrophoresis in 5% (w/v) polyacrylamide, containing 0.1% SDS and 8M-urea was performed as described by Weber & Osborn (1969). Protein samples, containing 1% SDS, 2% (v/v) β-mercaptoethanol and 8M-urea were incubated at 100°C for 2min, the 10–100μl were applied to the gels. The amount of protein applied to the gel varied from 5 to 50μg, being higher (up to 50μg) in the runs aimed to trace the minor components. Lower loadings (5–10μg) were used for molecular-weight determination, especially when two samples were run simultaneously on the same gel. The gels were stained for 5min at 100°C in a solution of 0.25% Coomassie Brilliant Blue R-250 in 7% (v/v) acetic acid. Destaining was performed with 7% (v/v) acetic acid at 100°C for 2h.

Results and Discussion

It was shown previously (Chestukhina *et al.*, 1977) that the crystals formed by strain 612 of *B. thuringiensis* var. *galleriae* contained two predominant components, A and B. The component B (85% of the total crystal protein) comprised two polypeptides with mol.wts. of 135000 and 130000; the component A (8–10% of the total protein) corresponded to the polypeptide with mol.wt. of 65000. The same weight ratio of A to B components was obtained when the crystals were dissolved by the application of denaturing agents or by the raising of the pH to 12.5.

The proteins corresponding to the B-component could be dissolved selectively at pH 8.5 in the presence of 10mM-dithioerythritol. If this solution was kept at 4–20°C, new bands, corresponding to polypeptides of mol.wts. in the 110000–65000 range, gradually appeared (Fig. 1). Stepwise degradation of 135000–130000-mol.wt. polypeptides could be measured also at 37°C. Under these conditions the polypeptides with mol.wts. of 110000 and lower appear within 45min. After the incubation of this solution for 48h at 37°C the polypeptides with mol.wts. of 65000 became predominant. Incubation of the crystal solutions at pH 12.5 gave comparable results. These data indicate the presence of proteinases in the crystals and their solutions. Their action was not observed, however, even after prolonged storage, when the crystals of *B. thuringiensis* strain 612 were dissolved in 8M-urea or 6M-guanidine hydrochloride. This might be explained by the inactivation of the proteinases in denaturing solvents.

The presence of the proteinases is not limited to *B. thuringiensis* strain 612. They were observed also in crystal preparations obtained from the strain 199 of var. *insectus* (serotype I). In this particular case the solutions of the crystals at pH 8.5 in 6M-guanidine hydrochloride in the presence of 10mM-dithioerythritol or at pH 12.5 were found to contain a polypeptide with a mol.wt. of 145000 as a predominant component alongside minor quantities of the polypeptides with mol.wts. of 95000–75000. After the treatment of the same crystal preparation with 8M-urea containing 10mM-dithioerythritol, pH 8.5 (Fig. 1), however, the solution contained roughly equal quantities of polypeptides with mol.wts. of 145000, 110000, 100000, 90000 and 75000. As with the case of *B. thuringiensis* strain 612 (discussed above), after 48h incubation of this solution, low-molecular-weight proteins (90000 and 75000) became predominant. In contrast, however, if solution was achieved by 2min treatment of the crystals with boiling 8M-urea containing 10mM-dithioerythritol, pH 8.5, essentially all the protein appeared after gel electrophoresis as one band corresponding to a mol.wt. of 145000.

These results might be rationalized as follows. The crystals contain the proteinase(s) that are relatively stable even in 8M-urea at room temperature, but lose their activity in the presence of 6M-guanidine hydrochloride or in boiling urea.

To characterize the proteinases found in crystal preparations, their effect on specific synthetic peptide substrates was investigated. As shown in Table 1, the suspension of crystals of both strains hydrolyse the substrates of serine proteinases (Z-Ala-Ala-Leu-*p*NA) and metalloproteinases (N_2ph-Gly-Gly-Val-Lys). Leucine aminopeptidase activity was also detected.

The activity of proteinases in preparations of crystals was relatively low (Table 1). The activity

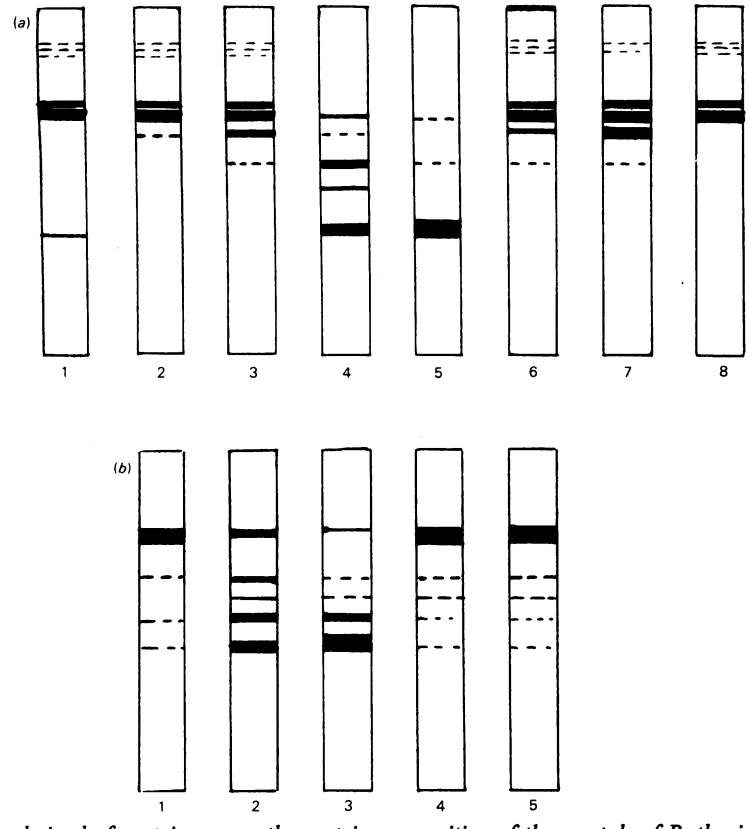

Fig. 1. *Effect of the admixed of proteinases on the protein composition of the crystals of B. thuringiensis var. galleriae strain 612 (a) and var. insectus strain 199 (b)*

Disc electrophoresis in 5% (w/v) SDS/polyacrylamide gels: (*a*) the crystals of var. *galleriae* strain 612 were treated as follows: (1) 10mM-Tris/HCl buffer (pH 8.5)/8M-urea/10mM-dithioerythritol, 37°C, 72h; (2–5) 10mM-Tris/HCl buffer (pH 8.5)10mM-dithioerythritol, 37°C, incubation time respectively 0, 45min, 17h, 48h. (6 and 7) 10mM-Tris/HCl buffer (pH 8.5) 10mM-dithioerythritol/1mM-phenylmethanesulphonyl fluoride (6) or 1mM-EDTA (7) 72h, 37°C; (8) 10mM-Tris/HCl (pH 8.5)/10mM-dithioerythritol after preliminary acid denaturation of proteinases (see the Materials and Methods section) and incubated for 72h at 37°C. (*b*) The crystals of var. *insectus* 199 strain were treated as follows: (1) 0.04M-NaOH, 1h, 20°C; (2) 10mM-Tris/HCl buffer (pH 8.5)/8M-urea/10mM-dithioerythritol, 1h, 20°C; (3) 10mM-Tris/HCl (pH 8.5)/8M-urea/10mM-diethioerythritol, 48h, 37°C; (4) 10mM-Tris/HCl buffer (pH 8.5)/8M-urea/10mM-dithioerythritol, 1mM-phenylmethanesulphonylfluoride, 48h, 37°C; (5) the same as 2 but 2min, 100°C.

Table 1. *Proteinase activity in the crystal preparations of B. thuringiensis*

The crystal preparations of the strain 612 (var. *galleriae*) and the strain 199 (*var. insectus*) were incubated in the reaction mixture containing synthetic substrates for subtilisin-like serine proteinase (Z-Ala-Ala-Leu-*p*NA), metalloproteinase (N_2ph-Gly-Gly-Val-Lys), leucine aminopeptidase (Leu-*p*NA) or thiol-group containing papain-like proteinase (Bz-DL-Arg-*p*NA). Details of the assay methods are given in the text. As a control, Z-Ala-Ala-Leu-*p*NA, N_2ph-Gly-Gly-Val-Lys, Leu-*p*NA and Bz-DL-Arg-*p*NA were each incubated with subtilisin BPN′, thermolysin, leucine aminopeptidase or papain. Results are expressed as a μmol of substrate hydrolysed/min at 37°C by 1mg of pure enzyme or crystals containing the adsorbed proteinases.

Preparation	Proteinase activity			
Substrate ...	Z-Ala-Ala-Leu-*p*NA	N_2ph-Gly-Gly-Val-Lys	Leu-*p*NA	Bz-DL-Arg-*p*NA
Crystals of strain 612 var. *galleriae*	0.2	0.00001	0.2	0
Crystals of strain 199 var. *insectus*	0.15	0.0008	0.46	—
Subtilisin BPN′	380			
Thermolysin		1.2		
Leucine aminopeptidase			60	
Papain				0.02

against the serine-proteinase substrate was comparable in both strains, whereas the activity of metalloproteinase in strain 199 (var. *insectus*) was 80 times as high as in the crystals of the strain 612 (var. *galleriae*). This distinction might be responsible for the difference observed in the hydrolysis of the respective proteins.

The attribution of the proteolytic enzymes associated with the crystals of *B. thuringiensis* to the classes of serine- and metallo-proteinases was confirmed by the action of specific inhibitors (Table 2).

Bulla *et al.* (1977) also reported proteolytic activity associated with the protein crystals of *B. thuringiensis* (var. *kurstaki*). In their opinion this activity is inherent to the crystal protein itself and might be ascribed to a thiol proteinase on the basis of its inhibition by rather high concentrations of $HgCl_2$. In contrast, the activity of serine proteinase in our experiments was only partially inhibited by thiol-specific reagents (Table 2). We also failed to detect any papain-like activity against Bz-DL-Arg-*p*NA. These data did not allow us to accept the presence of thiol-proteinase activity in the crystals. The inhibition of proteolysis by $HgCl_2$ might depend on the modification of the substrate protein by the reagent. The same explanation might be valid for inhibition by Hg^{2+} and Cu^{2+} observed by Eppstein & Thoma (1975) for matrix protein of nuclear polyhedral viruses. These authors suggested that chymotrypsine-like proteinase (i.e. serine-type enzyme) was responsible for the cleavage of this protein.

The specific proteinase inhibitors prevent the degradation of the crystal proteins during their dissolution. Thus the components with mol.wts. of 75000–65000 did not appear during the dissolution of crystals in the presence of phenylmethanesulphonyl fluoride, di-isopropyl fluorophosphate (serine-proteinase inhibitors) or 1 mM-EDTA, although the latter did not prevent the degradation of the component B to polypeptides with mol.wts. about 100000. The presence of phenylmethanesulphonyl fluoride during the solubilization of the crystals isolated from the strain 199 of var. *insectus* at pH 8.5 in buffer containing 8 M-urea and 10 mM-dithioerythritol prevented the degradation of the major protein components (mol.wt. 145000) for at least 48 h at 37°C.

These experiments show that the preparations of *B. thuringiensis* crystals contain the proteinases of several types capable of hydrolysing the crystal proteins. The intensity of the proteolysis sharply increases during the crystal-protein solubilization under the conditions favourable for protein denaturation.

It is obvious that the extent of the proteolysis observed strongly depends on a number of factors: i.e. the content of the proteinases in the crystal formed by a given strain, the conditions used for the protein solubilization, the stability of the proteinases against the denaturing agents, etc. This largely explains the pronounced contradictions in the literature data on entomocidal proteins of *B. thuringiensis* (Table 3). It is necessary, therefore, to

Table 2. *Effect of inhibitors on the proteolytic activity of crystal preparations of B. thuringiensis*

Proteolytic activity was measured in the preparations strain 612 var. *galleriae* and strain 199 var. *insectus* crystals against Z-Ala-Ala-Leu-*p*NA (the substrate for the subtilisin-like proteinase), N_2ph-Gly-Gly-Val-Lys (metalloproteinase substrate) or Leu-*p*NA (leucine aminopeptidase substrate). The suspensions of crystals (1 mg/ml) in corresponding buffer were incubated for 2 h at 37°C in the presence of the inhibitor (10 mM), then the substrate was added and the activity was assayed as described in the text. Results are expressed as a percentage of the initial activity of non-treated preparations.

			Residual activity (%)					
			Z-Ala-Ala-Leu-*p*NA		N_2ph-Gly-Gly-Val-Lys		Leu-*p*NA	
Inhibitor	pH	Strain ...	612	199	612	199	612	199
Phenylmethanesulphonyl fluoride	8.5		3	0	—	54	100	—
Di-isopropyl fluorophosphate	8.5		1	0	—	67	—	—
EDTA	8.5		49	83	—	1	7	34
	7.0		—	—	0	1	—	—
p-Chloromercuribenzoate	9.4		23	6	—	—	—	—
p-Chloromercuribenzene-sulphonate	9.4		26	7	—	—	—	—
	7.0		11	—	—	—	—	—
Iodacetamide	9.4		100	—	—	—	—	—
N-Ethylmaleimide	9.4		134	147	—	—	—	—

Table 3. *Protein composition of crystals formed by various strains of B. thuringiensis (literature data)*

Variant	No. of protein components observed	Molecular weight or $s_{20,w}$	Reference
Berliner	1	80000	Glatron *et al.* (1972)
	4	5.5S; 7.8S; 11.1S; 13.45S	
	Several	13000 (predominant component)	Prasad & Shethna (1974)
	1	1000–1500	Sayles *et al.* (1970)
Matte's isolate	6		Cooksey (1968)
kurstaki	1	130000	Bulla *et al.* (1977)
dendrolimus	1	232000	Nagamatsu *et al.* (1978)
tolworthi	4	56000; 122000; 240000; 370000	Herbert *et al.* (1971)
sotto	1	25000, 373000 or 708000	Akune *et al.* (1971)

eliminate the action of proteinases on crystal-forming proteins in order to obtain reproducible data on their composition and properties.

Two approaches might be used to exclude the action of crystal-associated proteinases on the proteins: the first consists in the elimination of the proteinases from the crystals before their dissolution; the second consists in the dissolution of the crystals under the conditions providing complete and instantaneous inactivation of the proteinases.

Acid denaturation of the proteinases was found to be the most effective way to eliminate from the crystals the mixture of proteinases. Thus, in the solution of the component B obtained from the crystals of the strain 612 var. *galleriae* treated by this procedure, protein hydrolysis was not observed even after 72h at 37°C (Fig. 1).

In order to inactivate the proteinases during the solubilization of the crystal, we used another method. The crystals were solubilized in pH 8.5 buffer containing 8M-urea and 10mM-dithioerythritol for 10min at 100°C ('hot extraction').

Fig. 2 displays the results of SDS/polyacrylamide-gel electrophoresis of proteins obtained by the dissolution of the crystals (*a*) at pH 8.5 and 100°C in 8M-urea/10mM-dithioerythritol ('hot extraction'), (*b*) at pH 8.5 and 20°C in 8M-urea and 10mM-dithioerythritol, or (*c*) at pH 8.5 and 20°C in 8M-urea and 10mM-dithioerythritol after preliminary acid denaturation of the proteinases. Both procedures effectively prevented the proteolysis.

The 'hot-extraction' procedure avoids the hydrolysis of the crystal protein, not only during the solubilization of the pure crystals, but also of the crystal/spore mixtures. In the latter case the major components found in the solution are the crystal proteins and protein(s) with mol.wts. about 13000 (Fig. 3), the latter presumably being one of the spore components. Proteins with the same molecular weights (13000) were found during the 'hot extraction' of intact-spore preparations (var. *kenyae*, var. *galleriae*). Insofar as other proteins did not appear in substantial quantities under the conditions of the spore/crystal-mixture dissolution, the method of 'hot extraction' might be of use for rapid analysis of the crystal proteins formed by various strains.

If the crystals of different strains are solubilized under the conditions excluding the action of admixed proteinases, the protein composition of the crystals formed by various serotypes of *B. thuringiensis* appears to be more simple.

This shows that the multiplicity of protein species observed after the dissolution of *B. thuringiensis* crystals depends on the action of the endogenous proteolytic enzymes adsorbed on the surface or entrapped in the matrix of the crystal. Obviously the limited proteolysis of the crystals proteins is greatly enhanced by the conditions of dissolution, which favour the partial or complete denaturation of the proteins. Comparable data on the limited proteolysis of the matrix protein of nuclear polyhedral viruses by matrix-associated proteinases were obtained previously (Kozlov *et al.*, 1973; Eppstein & Thoma, 1975). The proteolytic enzyme content in the crystal preparations is relatively low. Nevertheless, this amount is apparently sufficient for the limited proteolysis of the macromolecular substrate. One also has to take in account rather high local concentration of these enzymes during the crystal dissolution.

It has been established that the crystals formed by all the analysed strains (those of serotypes I–IV and VIII–XII) are built from one polypeptide chain, and only few strains of var. *galleriae* (serotype V) possessed crystals composed of two or three proteins.

The molecular weight of the main protein component found in crystals of different strains varied from

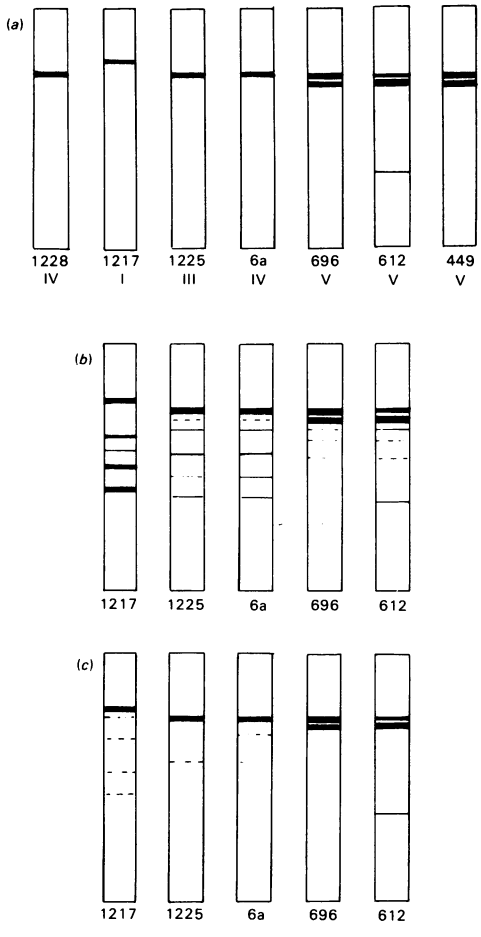

Fig. 2. *Protein composition of B. thuringiensis crystals after different procedures of crystal dissolution*

The numbers under the gels correspond to the strain; the roman numerals refer to the serotype. Pure crystal preparations obtained from different strains of *B. thuringiensis* were dissolved under the following conditions: (*a*) 10mM-Tris/HCl buffer (pH 8.5)/8M-urea/10mM-dithioerythritol, 10min, 100°C; (*b*) the same as in (*a*), but 60min, 20°C; (c) the same as in (*b*), but after preliminary acid denaturation of proteinases.

145000 to 130000 (as determined by SDS/polyacrylamide-gel electrophoresis). To ascertain that these relatively small differences in protein molecular weights were significant, we performed SDS/electrophoresis of the proteins in question applied as a mixture on to the same gel. The appearance of the double bands in such experiments was considered as a proof of difference in molecular weights of the proteins in question.

It has been established that the crystals of serotype I (var. *insectus* and var. *thuringiensis*) had been built from polypeptides with a mol.wt. of 145000 (Table 4). The proteins with a mol.wt. of 135000 are characteristic for the crystals of serotypes III (var. *alesti*), IV (var. *kenyae* and var. *dendrolimus*) and X (var. *darmstadtiensis*). The polypeptides with a mol.wt. of 130000 were found in the crystals of the following serotypes: II (var. *finitimus*), III (var. *kurstaki*), IX (var. *tolworthi*), XII (var. *thompsoni*), and in one strain (1167) of var. *galleriae* (belonging to the serotype V). The presence of two proteins with mol.wts. of 135000 and 130000 was found to be typical for most strains of var. *galleriae* studied in our experiments. Several strain of this serotype have a third protein with mol.wt. of 65000.

Although identical values for molecular weight of the crystal proteins formed by various strains can not be considered as a definite proof of their identity, taking into account the similarity of the amino acid composition of the crystals (and, hence, of respective proteins), one might presume that these proteins are largely similar from the structural point of view. Therefore it might be suggested that all the strains of *B. thuringiensis* form initially one crystal protein with mol.wt. of 145000. It does not imply, of course, that all the proteins synthesized by the various strains are identical. On the contrary, the differences found in the entomocidal activity indicate variations in the chemical structures of the proteins, e.g. in their amino acid sequences. We assume only that each strain synthesizes primarily one protein of a structural type common for all the strains. Before or during the formation of the crystal this protein may be exposed to secondary modification by limited hydrolysis (Chestukhina *et al.*, 1979).

Nevertheless it is impossible to exclude the possibility that the appearence of two polypeptides in the crystal (e.g. in that of var. *galleriae* serotype V) might depend on the action of two closely related structural genes. The appearance of the protein with mol.wt. of 65000 in some strains of the same serotype is most likely the result of the limited proteolysis during the formation of the crystal.

The appearance of this protein might be explained by the proteolysis of the high-molecular-weight components. Formation of the fragment with mol.wt. of 70000 as a result of tryptic digestion of *B. thuringiensis* crystal-forming proteins has been described by Lilley & Somerville (1975). We studied analogous conversion of the proteins with mol.wts. 145000–130000 into the fragments with mol.wts. of 75000–65000 under the action of trypsin, chymotrypsin, subtilisin, extracellular serine proteinase of *B. thuringiensis*, intracellular serine proteinase of *B. subtilis* and the proteolytic enzymes of *Galleria mellonella* (wax moth) gut (L. I. Kostina,

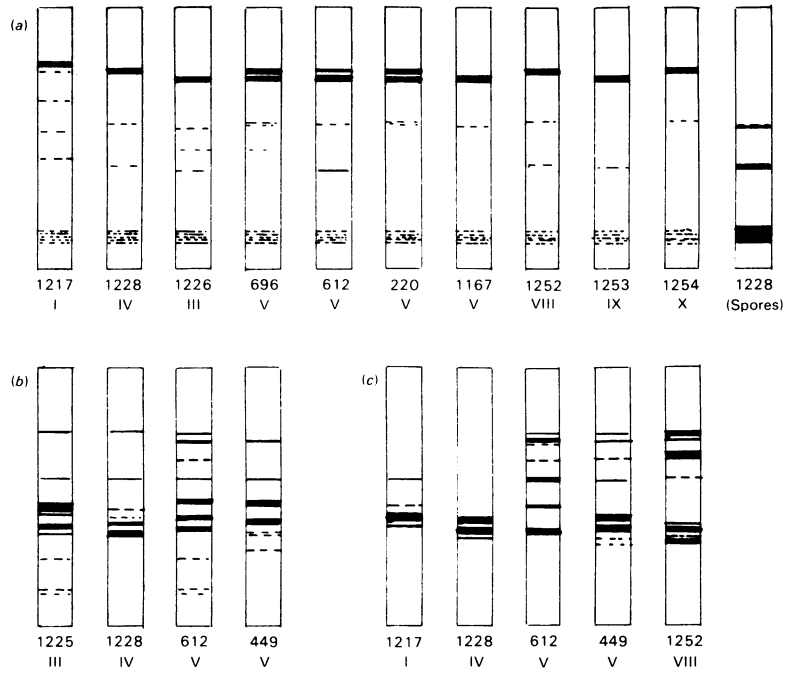

Fig. 3. *Protein pattern observed after extraction of B. thuringiensis spore and crystal mixtures*
Arabic and Roman numerals refer to strains and serotypes respectively. The mixtures of spores and crystals of different *B. thuringiensis* strains were treated as follows: (*a*) 10mM-Tris/HCl buffer (pH 8.5)/8M-urea/10mM-dithioerythritol, 10min, 100°C; (*b*) 0.04M-NaOH, 60min, 20°C; (*c*) 10mM-Tris/HCl buffer (pH 8.5)/8M-urea/10mM-dithioerythritol, 60min, 20°C.

Table 4. *Molecular weight of proteins forming the crystals of various strains of B. thuringiensis*

Mol. wt. ... 145000	135000	130000	130000 135000	135000 130000 65000
var. *thuringiensis* (I) Strain 1223 var. *insectus* (I) Strain 1217 Strain 199	var. *alesti* (III) Strain 1225 var. *kenyae* Strain 1228 var. *dendrolimus* (IV) Strain 1227 var. *morrisoni* (VIII) Strain 1252 var. *darmstadtiensis* (X) Strain 1254	var. *finitinus* (II) Strain 1162 var. *kurstaki* (III) Strain 1226 var. *galleriae* (V) Strain 1167 var. *tolworthi* (IX) Strain 1253 var. *thompsoni* (XII) Strain 1166	var. *galleriae* (V) Strain 696 Strain 449 Strain 273 Strain 220 Strain 561 Strain 16 Strain 73	var. *galleriae* (V) Strain 612 Strain 614 Strain 615 Strain 650 Strain 223

A. L. Mikhailova, I. A. Zalunin, S. P. Katrukha & G. C. Chestukhina, unpublished work).

It is not yet clear whether the presence of proteolytic enzymes during the course of the crystal formation is simply fortuitous or whether these proteinases may play a definite role in the processing of crystal proteins. The possibility of participation of these proteinases in the hydrolysis of *B. thuringiensis* protein within the insect gut also cannot be excluded.

References

Akune, S., Watanabe, T., Mukai, J. I., Tsutsi, R. & Abe, K. (1971) *Jpn. J. Med. Sci. Biol.* **24**, 57–59

Bulla, L. A., Jr., Kramer, K. J. & Davidson, L. I. (1977) *J. Bacteriol.* **130**, 375–384

Chestukhina, G. G., Kostina, L. I., Zalunin, I. A., Kotova, T. S., Katrukha, S. P., Kuznetsov, Yu. S. & Stepanov, V. M. (1977) *Biokhimiya* **42**, 1660–1667

Chestukhina, G. G., Zalunin, I. A., Kostina, L. I., Kotova, T. S., Katrukha, S. P., Lyublinskaya, L. A. & Stepanov, V. M. (1978) *Biokhimiya* **43**, 857–864

Chestukhina, G. G., Kotova, T. S., Zalunin, I. A. & Stepanov, V. M. (1979) *Biokhimiya* **44**, 796–802

Cooksey, K. E. (1968) *Biochem. J.* **106**, 445–454

Eppstein, D. A. & Thoma, J. A. (1975) *Biochem. Biophys. Res. Commun.* **62**, 478–484

Glatron, M-F., Lecadet, M. M. & Dedonder, R. (1972) *Eur. J. Biochem.* **30**, 330–338

Herbert, B. H., Gould, H. J. & Chain, E. B. (1971) *Eur. J. Biochem.* **24**, 366–375

Kozlov, E. A., Levitina, T. L., Radavski, Yu. L., Sogulyaeva, V. M., Sidorova, N. M. & Serebryani, S. B. (1973) *Biokhimiya* **38**, 1014–1019

Lilley, M. & Somerville, H. J. (1975) *Proc. Soc. Gen. Microbiol.* **3**, 10

Lyublinskaya, L. A., Lastovetskaya, L. V., Shekhvatova, G. V., Vaganova, T. I. & Stepanov, V. M. (1976) *Khim. Prir. Soedin.* **1**, 75–80

Lyublinskaya, L. A., Yakusheva, L. D. & Stepanov, V. M. (1977) *Bioorg. Khim.* **3**, 273–279

Nagamatsu, Y., Tsutsui, R., Ichimaru, T., Nagamatsu, M., Koda, K. & Hayashi, K. (1978) *J. Invertebr. Pathol.* **32**, 103–109

Pendleton, I. R. & Morrison, R. B. (1966) *Nature (London)* **212**, 728–729

Prasad. S. S. V. & Shethna, G. I. (1974) *Biochim. Biophys. Acta* **363**, 558–566

Rogoff, M. N. (1966) *Adv. Appl. Microbiol.* **8**, 291–313

Sayles, V. B., Aronson, J. N. & Rosenthal, A. (1970) *Biochem. Biophys. Res. Commun.* **41**, 1126–1133

Smirnoff, W. A. (1962) *J. Insect Pathol.* **4**, 384–386

Weber, K. & Osborn, M. (1969) *J. Biol. Chem.* **244**, 4406–4412