Synergism in mosquitocidal activity of 26 and 65 kDa proteins from *Bacillus thuringiensis* subsp. *israelensis* crystal

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Three major protein components, 130, 65 and 26 kDa of solubilized *Bacillus thuringiensis* subsp. *israelensis* crystal were separated by gel filtration. The isolated 26 kDa protein is inactive against mosquito larvae at 6.4 μg/ml protein. The 65 kDa protein is also inactive towards mosquito larvae at low concentrations and shows but weak activity at higher concentrations. However, 26 and 65 kDa proteins simultaneously present at low concentrations exhibit high mosquitocidal activity. Similar synergistic effects are observed between 26 and 130 kDa proteins. These results differ from the findings of other investigators who have reported that a single protein component is sufficient for mosquitocidal activity [(1984) FEBS Lett. 175, 377–382; (1985) Biochem. Biophys. Res. Commun. 126, 961–965]. Our data suggest that the simultaneous presence of at least two proteins is required for activity.

Synergism *Mosquitocidal activity* *Crystal protein*

1. INTRODUCTION

Bacillus thuringiensis subsp. *israelensis (B.th. israelensis)* produces parasporal crystals that are toxic to mosquito and blackfly larvae [1]. The World Health Organization regards the *B.th. israelensis* crystal to be the most effective bioinsecticide available against mosquitoes [2]. Which of the several proteins derived from the crystal are responsible for killing mosquito larvae has been the subject of intensive research in several laboratories [3–5]. Ward et al. [4] reported that the 26 kDa protein of solubilized *B.th. israelensis* crystal is required for mosquitocidal activity. This result was recently disputed by Hurley et al. [5] who reported that the 65 kDa protein, rather than the 26 kDa protein, is solely responsible for the activity. We have isolated three major components, 130, 65 and 26 kDa proteins from the solubilized *B.th. israelensis* crystal and studied their activity against larvae of *Aedes aegypti*. Contrary to the reports just mentioned [4,5] our results indicate that the simultaneous presence of at least two proteins is required for mosquitocidal activity.

2. MATERIALS AND METHODS

2.1. *Preparation of* B.th. israelensis *crystals*

B.th. israelensis was obtained from the Bacillus Genetic Stock Center, Columbus, Ohio. *B.th. israelensis* cells were grown in a GYS medium [6] at 30°C in a rotary shaker at 200 rpm for 5 days or until they autolysed. The lysates were centrifuged, the pellets were suspended in a solution containing 1 M NaCl and 0.01% Triton X-100 [7], and the suspension was dispersed by sonication. The sonicates were centrifuged on discontinuous renografin gradients of 75, 67, 62 and 55% at 20000 rpm for 2 h using a SW 41 rotor. Crystal protein banded at the interface between 55 and 62% renografin. The purified crystals were dialyzed against water, lyophilized, and stored in a −70°C freezer.

2.2. *Separation of proteins derived from solubilized crystals*

Crystals were dissolved overnight at 4°C in a solution consisting of 100 mM NaOH, 10 mM EDTA, and 5% 2-mercaptoethanol. The final pro-

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tein concentration was 2 mg/ml. Undissolved materials were removed by centrifugation. 1 ml of supernatant (protein concentration 0.5 mg/ml) was loaded onto a Sephacryl S-300 column (100 × 0.75 cm) and chromatographed in a solution containing 100 mM NaOH and 10 mM EDTA. Fractions (0.6 ml) were collected and either assayed immediately or stored at 0–4°C no longer than 1–2 days prior to assay.

2.3. *Protein determinations*

The protein concentration of each fraction (100 μl) was determined by the method of Bradford [8]. Similar results were obtained when the protein concentration was determined by the method of Schaffner and Weissmann [9].

2.4. *SDS polyacrylamide gel electrophoresis*

A 100 μl aliquot from each fraction was precipitated with trichloroacetic acid and washed twice with cold acetone. The pellet was dissolved in Laemmli electrophorsis buffer [10] and proteins were separated in a Laemmli system containing 10% acrylamide [10]. Gels were stained either with Coomassie blue or with a silver staining procedure (GELCODE).

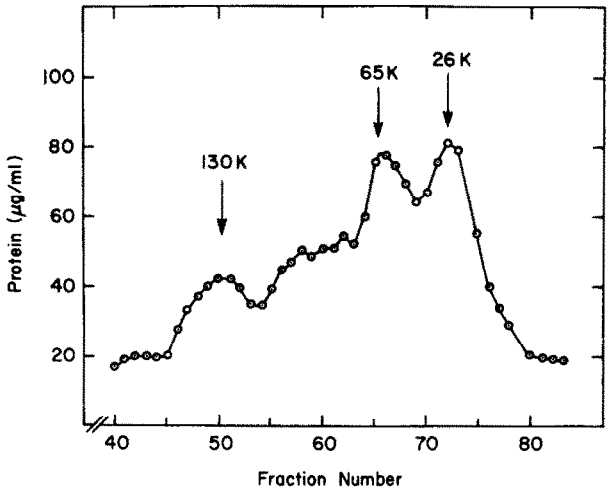

Fig.1. Sephacryl S-300 chromatography of dissociated *B.th. israelensis* crystals. 1 ml of supernatant of alkali solubilized crystal protein was loaded onto a Sephacryl S-300 column (100 × 0.75 cm) and chromatographed in a buffer solution containing 100 mM NaOH and 10 mM EDTA. Fractions (0.6 ml) were collected; protein concentrations were determined using the method of Bradford [8].

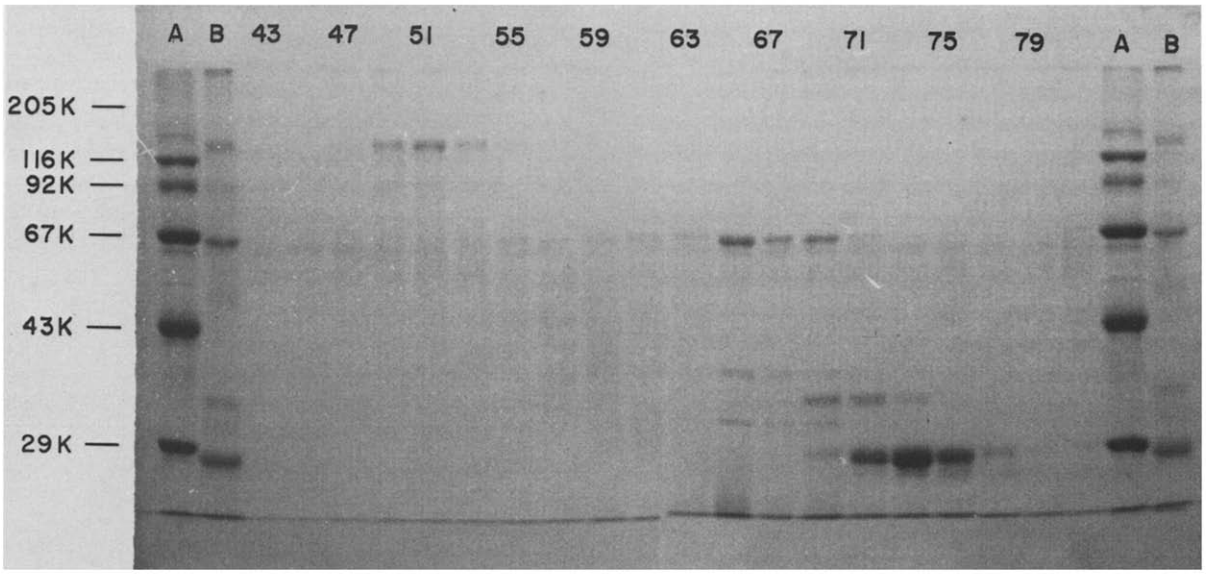

Fig.2. SDS-PAGE of fractions from Sephacryl S-300 chromatography of solubilized crystals. A 100 μl aliquot from each fraction in fig.1 was precipitated with trichloroacetic acid and washed twice with cold acetone. The pellets were dissolved in Laemmli buffer [10] and the electrophoresis was carried out in a Laemmli system containing 10% acrylamide [10]. Gels were stained with Coomassie blue. Lane A, M_r standards; lane B, alkali solubilized (unfractionated) *B.th. israelensis* crystals. Numbers on top indicate fraction number in fig.1.

2.5. *Mosquitocidal activity assay*

The toxicity was determined following the procedure of Schnell et al. [11] using latex beads as a protein carrier (Sigma). Twenty 2nd or 3rd instar larvae of *A. aegypti* in 10 ml distilled water were exposed to various concentrations of *B.th. israelensis* protein and the mortality rate was determined after 4 or 16 h. In some experiments longer exposure times were used.

3. RESULTS AND DISCUSSION

The alkali solubilized *B.th. israelensis* crystal preparation contains three major proteins, 130, 65 and 26 kDa together with several minor components [11,12]. The mosquitocidal activity was localized to the 26 kDa protein by Ward et al. [4] but to the 65 kDa protein by Hurley et al. [5]. To resolve this discrepancy, we have separated the three major components of the solubilized crystals by gel filtration. Fig.1 shows the separation profile. The purity of the separated protein fractions was determined by slab gel electrophoresis in SDS; some representative fractions are presented in fig.2. Only the fractions located at the peak of each separated protein (fractions 49–51 for 130 kDa protein, fractions 65–67 for 65 kDa protein, and fractions 72–74 for 26 kDa protein) were used for bioassay. Although Coomassie blue staining indicates that both 26 and 65 kDa fractions are free from each other (fig.2), silver staining revealed that the 65 kDa protein fraction contains 2% of 26 kDa protein while the 26 kDa protein may contain 1% of 65 kDa protein (not shown).

The mosquitocidal activity of the isolated 130, 65 and 26 kDa proteins is shown in table 1. In our bioassay the proteins are bound to latex beads and mortality rates are determined at both 4 and 16 h exposure periods [11]. Longer exposure times were used when necessary. The LD_{50} of our unfractionated alkali-solubilized *B.th. israelensis* protein is approx. 0.2 μg/ml (4 h exposure period) in good agreement with the published value (0.19 μg/ml) of Schnell et al. [11]. It is evident from table 1 that the isolated 26 kDa protein is not active at 6.4 μg/ml even with long incubation periods (48 h). This result contradicts the finding of Ward et al. [4] who reported that the 26 kDa protein is mosquitocidal. The purified 65 kDa protein is not active at low concentrations and shows only weak activity at 1.6 μg/ml with a 16 h exposure period. This result contradicts the recent report by Hurley et al. [5] that the 65 kDa protein is very active and possesses the sole mosquitocidal activity. The purified 130 kDa protein, like the 65 kDa protein, is weakly active at high concentrations and long exposure periods. Even though both 26 and 65 kDa proteins are not active when tested alone, mixing small amounts of 26 kDa protein (0.4 μg/ml) with 65 kDa protein (0.1 μg/ml) results in a very substantial increase in the mosquitocidal activity (table 2). This experiment has been repeated several times with similar results (not shown). This synergism appears to be concentration dependent. When the concentration of the 26 kDa protein is lowered from 0.4 to 0.15 μg/ml a high concentration of 65 kDa protein is required for mosquitocidal activity (table 3). A similar synergistic effect

Table 1

The mosquitocidal activity of isolated 130, 65 and 26 kDa proteins

Protein (μg/ml)			Mosquitocidal activity (dead/total)			
26 kDa	65 kDa	130 kDa	4 h	16 h	30 h	48 h
0.4	–	–	0/20	0/20	0/20	0/20
0.8	–	–	0/20	0/20	0/20	0/20
1.6	–	–	0/20	0/20	1/20	0/20
6.4	–	–	0/20	0/20	0/20	1/20
–	0.2	–	0/20	0/20	0/20	1/20
–	0.4	–	0/20	1/20	2/20	2/20
–	0.8	–	0/20	4/20	8/20	13/20
–	1.6	–	0/20	10/20	15/20	19/20
–	4.0	–	4/20	13/20	19/20	20/20
–	–	0.15	0/20	0/20	0/20	0/20
–	–	0.3	0/20	0/20	0/20	1/20
–	–	0.6	0/20	0/20	2/20	3/20
–	–	1.2	3/20	4/20	8/20	19/20

Proteins were isolated as described in fig.1 and the peak fractions were selected for bioassay. The pH of the protein solution was lowered by adding 0.2 vols of 1 M Tris (pH 7.4); the final volume was adjusted to 0.5 ml with water. 10 μl of latex beads were then added and the mixtures were incubated at room temperature for 1 h. Mosquitocidal activity was determined after diluting the above mixtures to 10 ml with H_2O following the procedure of Schnell et al. [11]

Table 2

Synergism in mosquitocidal activity of isolated 26, 65 and 130 kDa proteins

Experiment	Protein (μg/ml)			Mosquitocidal activity	
	26 kDa	65 kDa	130 kDa	4 h	16 h
1	0.4	–	–	0/20	0/20
2	0.8	–	–	0/20	0/20
3	–	0.1	–	0/20	0/20
4	–	0.2	–	0/20	0/20
5	–	0.4	–	0/20	2/20
6	–	–	0.1	0/20	0/20
7	–	–	0.2	0/20	0/20
8	–	–	0.4	0/20	2/20
9	0.4	0.1	–	2/20	10/20
10	0.4	0.2	–	5/20	12/20
11	0.4	–	0.1	0/20	7/20
12	0.4	–	0.2	1/20	13/20
13	–	0.2	0.4	0/20	0/20
14	–	0.2	0.4	0/20	8/20
15	0.4	0.2	0.2	8/20	14/20
16	0.8	0.4	–	13/20	17/20
17	0.8	–	0.4	9/20	16/20

The assay conditions were the same as in table 1

Table 3

Concentration dependent synergism in mosquitocidal activity of 26, 65 and 130 kDa proteins

Protein (μg/ml)			Mosquitocidal activity			
26 kDa	65 kDa	130 kDa	4 h	16 h	30 h	48 h
0.15	–	–	0/20	0/20	0/20	0/20
0.15	0.1	–	0/20	0/20	4/20	11/20
0.15	0.2	–	0/20	2/20	5/20	14/20
0.15	0.4	–	6/20	13/20	14/20	16/20
0.15	0.8	–	12/20	16/20	17/20	17/20
0.15	1.6	–	15/20	19/20	19/20	19/20
0.15	–	0.1	0/20	0/20	2/20	2/20
0.15	–	0.15	0/20	0/20	0/20	0/20
0.15	–	0.3	1/20	1/20	1/20	1/20
0.15	–	0.6	1/20	3/20	5/20	5/20
0.15	–	1.2	11/20	15/20	15/20	17/20

The assay conditions were the same as in table 1

is observed between 26 and 130 kDa protein (table 2).

The weak mosquitocidal activity observed with the isolated 65 kDa protein (1.6 μg/ml) at exposure periods over 16 h deserves comment. We believe that this activity is due to the synergistic action of a small amount (approx 2%) of contaminating 26 kDa protein (detected by silver staining) in our 65 kDa protein preparation. The 26 kDa protein has a very high affinity for 65 kDa protein; to separate them we had to use 0.1 M NaOH in the presence of 10 mM EDTA. When the pH of the solution is lowered, association of 26 kDa protein with 65 kDa protein occurs. Since Hurley et al. [5] used a pH 7.0 buffer to separate their 65 kDa protein, it is likely that their 65 kDa fraction contains 26 kDa protein. A synergistic effect of the contaminating 26 kDa protein would explain their report that 65 kDa protein is active against mosquito larvae. Similar reasoning would explain the report of

Ward et al. [4] that the 26 kDa protein is mosquitocidal because only small amounts of contaminating 65 kDa protein are needed for the synergistic activity (table 2).

The requirement for both 26 and 65 kDa proteins to exhibit mosquitocidal activity may have a biological basis. The 26 kDa protein appears to contain cytolytic activity [3,5,13] which may be necessary for killing mosquito larvae. Alternatively, during our purification of 65 and 26 kDa proteins we may have partially inactivated one of the proteins so that the other protein is necessary to restore bioactivity. This possibility seems unlikely because we can restore activity by mixing small amounts of the isolated components under conditions when each component is inactive when presented alone (table 2). If one of the components turns out to be labile then it maybe protected by the other protein in the native state.

The synergism between the 26 and 130 kDa proteins may have a different explanation. 130 kDa protein may be degraded to 65 kDa protein which then shows bioactivity. However, we cannot completely rule out the possibility that regions of the 130 kDa protein other than the 65 kDa region are responsible for the synergistic effect. There are several minor proteins (e.g. 98 kDa protein) that are present in solubilized *B.th. israelensis* crystal preparation at low concentration. We are isolating these minor protein components to determine whether or not they can exert a synergistic effect on 26 kDa protein or other proteins.

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