Short Communication

SYNERGISTIC EFFECTS OF THE 65- AND 25-KILODALTON PROTEINS OF *BACILLUS THURINGIENSIS* STRAIN PG-14 (SEROTYPE 8A:8B) IN MOSQUITO LARVICIDAL ACTIVITY

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Parasporal inclusion (PI) proteins produced by *Bacillus thuringiensis* strain PG-14 (serotype 8a:8b) has an extremely high mosquito larvicidal activity similar to that of the type strain of *B. thuringiensis* subsp. *israelensis* (serotype 14) (1–3). In our previous study (3), we purified two distinct proteins with different biological activity from protease-treated PIs of the strain PG-14. The results clearly demonstrated that the 65-kD protein was the mosquitocidal entity and the 25-kD protein was the hemolytic principle. Under natural conditions, however, mosquito larvae ingest these two proteins simultaneously together with other components contained in PIs. So it was worthwhile to determine whether there is any combined effect in the mosquitocidal activity among these PI proteins. In this study, we conducted the combined dosage studies with the 65-kD and 25-kD proteins of the strain PG-14.

The strain PG-14 used in this study was reported in our previous study (1) and has been maintained in this institute. It was grown on nutrient agar plates, pH 7.2, at 27°C for 7 days. PIs of the strain PG-14 were purified from the sporulated culture by the method described previously (3). Purified PIs were solubilized with 0.1 M NaOH at 27°C for 30 min. Undissolved materials were then removed by centrifugation at 4,000 × *g* for 20 min at 4°C. The supernatant containing the soluble proteins was further treated with 0.1 M NaOH supplemented with larval gut juice of the silkworm *Bombyx mori* at a ratio of 1:100 (v/v) at 27°C for 2 hr according to the method of Tojo et al. (4), with minor modification. The 65- and 25-kD proteins were purified using antibody-mediated affinity chromatography and CM-cellulose chro-

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matography, respectively (YU et al., submitted for publication). The solubilized and silkworm gut juice-treated PI proteins were applied to the immunoaffinity column and the 65-kD protein bound to the column was eluted with 1 M propionic acid, pH 2.5. The eluted protein fractions were pooled and dialyzed against 0.1 M phosphate-buffered saline (PBS), pH 7.2. The 25-kD protein was purified from a flow-through fraction of affinity chromatography by a CM-cellulose column chromatography and was dialyzed against PBS. Purity of the protein preparations was monitored by sodium dodecyl sulfate-polyacrylamide gel electrophoresis(3). The protein concentration was determined by the method of LOWRY et al. (5) using bovine serum albumin as the standard.

For analysis of the combined effects of the two proteins in mosquitocidal activity, we used the following experimental design: (1) assay of the 65-kD protein toxicity in the presence of the 25-kD protein at sublethal concentrations and (2) assay of the 25-kD protein toxicity in the presence of the 65-kD protein at sublethal concentrations. Both tests were carried out using increasing 5-fold (or 10-fold) serial dilutions of the former protein and a constant amount of the latter protein. A mixture (10 ml) of the two protein solutions was prepared in distilled water in a test tube (2 × 18 cm). Ten 4-day-old *Aedes aegypti* (Liverpool strain) larvae were then introduced into this mixture. The LD₅₀ was calculated by the method of REED and MUENCH(6) after the larvae were starved at 25°C for 48 hr. Table 1 shows the combined effects of the two proteins in mosquitocidal activity. The LD₅₀ values of the 65- and 25-kD proteins for *A. aegypti* larvae were 0.42 µg/ml and 50 µg/ml, respectively. When sublethal concentrations of the 25-kD protein was added to the

Table 1. Combined effects of the 65-kD and the 25-kD proteins in the mosquito larvicidal activity.

Protein supplemented (µg/ml)	Mosquitocidal activity			
	65-kD protein		25-kD protein	
	LD ₅₀ (µg/ml)	Enhancement (times)	LD ₅₀ (µg/ml)	Enhancement (times)
25-kD protein				
0	0.42			
0.01	0.32	1.3		
0.1	0.074	5.7		
1	0.048	8.8		
10	0.003	140.0		
65-kD protein				
0			50.0	
0.00064			27.6	1.8
0.0032			6.92	7.2
0.016			3.16	15.8
0.08			0.15	333.3

Table 2. Hemolytic activity in the mixture of the 65-kD and the 25-kD proteins against sheep red blood cells.

25-kD protein ($\mu\text{g/ml}$)	65-kD protein ($\mu\text{g/ml}$)										
	1,000	250	125	63	31	16	8	4	2	1	0
50	+	+	+	+	+	+	+	+	+	+	+
25	+	+	+	+	+	+	+	+	+	+	+
13	+	—	—	—	—	—	—	—	—	—	—
6	+	—	—	—	—	—	—	—	—	—	—
0	+	—	—	—	—	—	—	—	—	—	—

+: Hemolysis was observed, —: No hemolysis was observed.

assay system of the 65-kD protein, a dosage-dependent increase in the toxicity appeared. The addition of the 25-kD protein at concentrations of less than 1 $\mu\text{g/ml}$ resulted in a moderate enhancement of the toxicity (5.7–8.8 times), and a marked increase (140 times) in the toxicity was observed when 10 $\mu\text{g/ml}$ was added. Similarly, when a sublethal dose (0.08 $\mu\text{g/ml}$) of the 65-kD protein was added to the assay system of the 25-kD protein, the toxicity of the 25-kD protein was enhanced more than 300 times.

We then examined various combined dosages of the 65-kD and 25-kD proteins in hemolyzing sheep red blood cells (RBC). A mixture (100 μl) of the two proteins in PBS was added with an equal volume of 2% (v/v) sheep RBC suspension prepared in PBS. Hemolysis was observed after incubation at 25°C for 18 hr. As shown in Table 2, the 25-kD protein was 40 times more hemolytic than the 65-kD protein. When the 25-kD protein was supplemented with the 65-kD protein, there was no alteration in the hemolytic activity. Similarly, the hemolytic activity of the 65-kD protein did not change upon addition of the nonhemolytic amount of the 25-kD protein.

Our results clearly show that there is a synergism in mosquito larvicidal activity but not in hemolytic activity between the two purified proteins of the strain PG-14. Similar synergistic effects of PI proteins in mosquitocidal activity were also reported in *B. thuringiensis* subsp. *israelensis* (7, 8). However, the degree of synergism under natural conditions is uncertain since in both strains the ratio of the two proteins in PIs is unknown at present.

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