Korean J Biol Sci 1: 457-462, 1997

Purification and Characterization of Antibacterial Peptides, Spodopsin Ia and Ib Induced in the Larval Haemolymph of the Common Cutworm, *Spodoptera litura*

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Key Words:
Insect immunity
Antibacterial peptide
Spodopsin
Spodoptera litura

Antibacterial activity was induced in the haemolymph of the common cutworm, *Spodoptera litura* by the artificial injection of *E. coli* Ek132. Antibacterial peptides were purified from the immunized haemolymph by heat treatment, ion-exchange chromatography, gel filtration chromatography, and reverse phase FPLC, and their physicochemical characteristics were investigated. These purified antibacterial peptides designated as spodopsin Ia and Ib were named after *Spodoptera litura*. Spodopsin Ia and Ib had the apparent molecular masses of 3,823 Da and 3,886 Da, respectively, and about 20% of the sequences had basic amino acids, such as lysine and arginine but no cysteine. Also, spodopsin I was confirmed to be a new member of cecropin family having a similar amino acid sequence to cecropin of lepidopteran insects, such as *Bombyx mori* and *Hyalophora cecropia*. The purified spodopsin was active against gram-positive as well as gram-negative bacteria.

Although insects have no specific defence mechanism, such as the antibody of vertebrate animal, they effectively protect themselves from the infection by microorganisms due to cellular immunity and humoral immunity (Ratcliffe and Rowley, 1975; Dunn, 1986). Insect cellular immunity is accomplished by phagocytosis, encapsulation, and nodule formation (Horohov and Dunn, 1983; Chang et al., 1992; Kurihara et al., 1992). Insect humoral immunity depends on the amplification or production of a number of antibacterial proteins in response to the invasion of pathogens like bacteria (Boman and Hultmark, 1987; Boman et al., 1991). Insect immunity has been studied mainly in lepidoptera (Morishima et al., 1990; Boman et al., 1991; Yoe et al., 1995) and some in diptera (Ando and Natori, 1988; Dimarq et al., 1988) and in Hymenoptera (Casteels et al., 1990). These antibacterial proteins are low molecular weight proteins named cecropin, attacin, lysozyme, sarcotoxin, diptericin, apidaecin, abaecin, and hemolin (Hultmark et al., 1983; Okada and Natori, 1985; Dimarcq et al., 1988; Casteels et al., 1990; Sun et al., 1990). Of these antibacterial proteins, cecropin which was first purified in the haemolymph of *Hyalophora cecropia* pupae is an approximately 4 kDa molecular mass peptide consisting of less than 40 amino acids and has the most active antibacterial activity compared with those of other inducible antibacterial proteins of the infected insects (Hultmark et al., 1980; Steiner et al., 1981). To better understand the nature of major antibacterial peptides from the common cutworm, *Spodoptera litura*, which is one of severely harmful insects in Korea, we purified peptides which have strong antibacterial activity from the immunized haemolymph of the insects, and their characteristics were also examined.

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Materials and Methods

Insects

Larvae of the common cutworm, *Spodoptera litura* were reared on artificial diet at 27±1℃ and 75±5% R.H. with a photoperiod of 16L:8D. Last instar larvae were used in the experiment.

Bacteria, immunization, and collection of the Haemolymph

In order to check the antibacterial activity spectrum of purified peptides, the following pathogens were used: *Bacillus megaterium, B. subtilus, B. thuringiensis, B. sphaericus* PY 105, *Micrococcus luteus* KCTC 1056, *Streptococcus pneumoniae* KU 39 (gram-positive); *Acinetobacter calcoaceticus* KH 48, *Escherichia coli* Ek 132, *E. coli* Ek 112, *E. coli* K12, *Achromobacter spp.*, *Pseudomonas acidovarans, P. putida* KU 82, *Enterobacter cloacae* 1684, *Klebsiella pneumoniae* KU 7, *Salmonella typhimurium* IB 74, *Serratia marcescens* KU 9 (gram-negative); *Saccharomyces cerevisiae, S. diastaticus* (yeast); *Colletotrichum Gloeosporioides, Alternaria mali, Trichoderma hazianum* (fungi). All bacteria were grown in LB medium and fungi in potato dextrose medium. For immunization, larvae were injected with 1×10^6 viable log phase bacteria *E. coli* Ek 132 into

the abdomen. Haemolymph was collected at one day after immunization and put into cold test tube which contains small amounts of phenylthiourea. The immunized haemolymph was centrifuged at 6,500 g for 10 min to remove haemocytes and cellular debris and the supernatant was stored at -70℃ until used.

Assay of antimicrobial activity

Antibacterial activity was based on the inhibition zone assay on thin agarose plates seeded with bacteria (Hultmark et al., 1980). A standard curve was made from the known amount of synthetic cecropin A (Sigma), and one relative unit was defined as the activity in 1 ng of cecropin A (Steiner et al., 1981). Fractions after chromatography were lyophilized, concentrated, and used for assay. Also, hyphal extension-inhibition assay was used for antifungal activity (Borgmeyer et al., 1992). Fungi were cultured in PDA (potato dextrose agar) medium and actively growing fungal mycelium were harvested and placed in the center of petri dishes containing PDA medium. After 48 h culture at room temperature, 5 mm diameter holes were made or 10 mm sized sterile disc papers were laid on the agar surface in front of the mycelium growth region. Samples were treated to these holes or discs and then the plates were further cultured at 30℃ for 36 h for inhibition zone assay.

Electrophoresis

SDS-PAGE was carried out on a 15% seperating gel at 15 mA according to the procedure of Laemmli (1970). Tricine-SDS PAGE was carried out on 4% stacking gel, 10% spacer gel and 16.5% seperating polyacrylamide gel at 30 mA as described by Schägger and von Jagow (1987) with some modification. After electrophoresis, gels were stained with Coomassie brilliant blue R250.

Purification and protein determination

The cell-free haemolymph was subjected to heat treatment at 95℃ for 10 min and centrifuged at 10,000 g for 10 min. The supernatant was diluted by addition of an equal volume of 0.1 M ammonium acetate, pH 6.0. The sample was applied to a column (15 × 1.0 cm) on CM-52 cellulose (Whatman) equilibrated with the same buffer. After chromatography the antibacterial activity was determined and active fractions were pooled, freeze-dried, and dissolved in a small volume of water. Then the sample was subjected to gel filtration column (Sephadex G-50; Pharmacia) and eluted with 0.1 M ammonium acetate. Absorbance was monitored at 280 nm and the antibacterial activity was confirmed by inhibition zone assay. For further purification, these partially purified antibacterial peptides were applied to a reverse phase FPLC column of PepRPC HR 5/5 connected to a Pharmacia FPLC system. Elution was performed with a linear gradient of 25-35% solution B (0.1% trifluoroacetic acid in acetonitrile) in solution A (0.1% trifluoroacetic acid in water) at a flow rate of 0.6 ml/min. Ultraviolet absorption was monitored at 206 nm and the antibacterial activity was determined on aliquots of the fraction which had been vacuum-dried to remove acetonitrile. Protein concentration was determined at 595 nm by the method of Bradford (1976) based on the standard curve made with bovine serum albumin.

Amino acid composition analysis, sequencing, and mass spectrometry

Amino acid composition analysis, N-terminal sequencing, and mass spectrometry of purified peptides were conducted in Korea Basic Science Institute. For amino acid composition analysis, purified peptides were vacuum-dried, dehydrated with 6 N HCl for 24 h at 110℃, and then derivatized with PITC (phenylisothiocyanate).

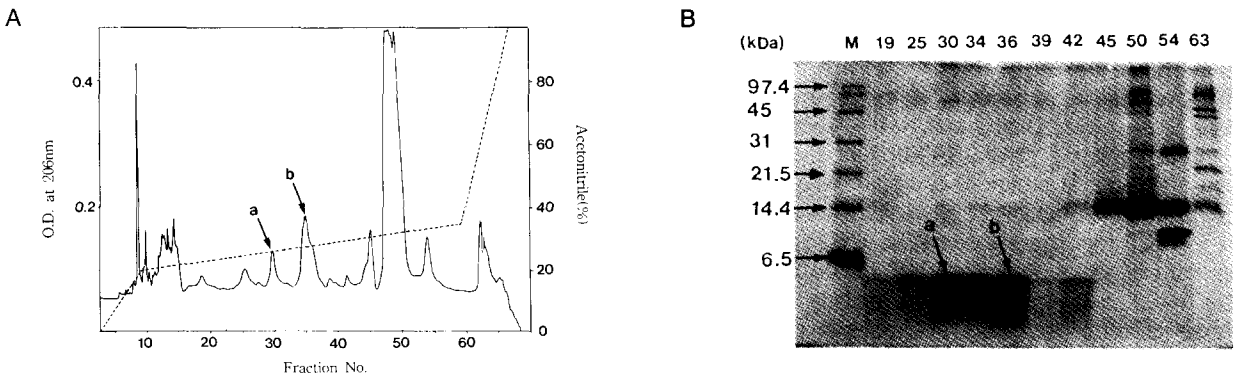
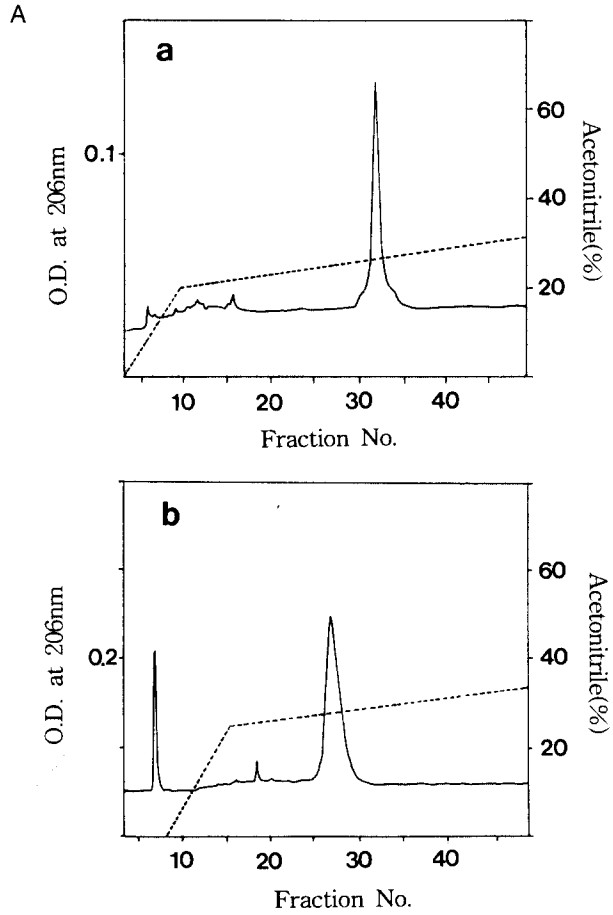


Fig. 1. (A), Reverse-phase FPLC of active fraction of gel filtration column chromatography. Chromatographic conditions were as follows: column, PepRPC HR 5/5 connected to Pharmacia FPLC system; solution A, 0.1% trifluoroactic acid in H_2O; solution B, 0.1% trifluoroacetic acid in acetonitrile; linear gradient of 20-35% solution B in solution A; flow rate, 0.6 ml/min. The absorbance at 206 nm (——) was monitored. ,·····, acetonitrile concentration; a, peak at 23% acetonitrile; b, peak at 25% acetonitrile (B), SDS-PAGE of fractions. M, molecular weight standard markers (phospholylase b, 97.4 kDa; ovalbumin, 45 kDa; carbonic anhydrase, 31 kDa; Soybean trypsin inhibitor, 21.5 kDa; lysozyme, 14.4 kDa; aprotinin, 6.5 kDa). Numbers indicate the fractions of RP-FPLC.



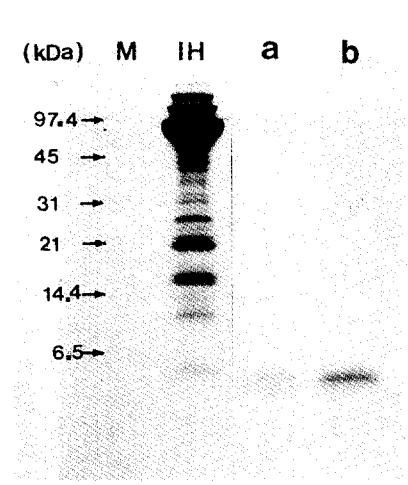


Fig. 2. (A), Second reverse-phase FPLC of active peaks, a and b of 1st FPLC. Chromatographic conditions were as follows: column, PepRPC HR 5/5 connected to Pharmacia FPLC system; solution A, 0.1% trifluoroactic acid in H_2O; solution B, 0.1% trifluoroacetic acid in acetonitrile; linear gradient of 20-30% (a), 25-35% (b) solution B in solution A for peak a and b of 1st FPLC, respectively; flow rate, 0.6 ml/min. The absorbance at 206 nm (--------) was monitored. ,·····, buffer concentration; a, spodopsin Ia; b, spodopsin Ib (B), SDS-PAGE of purified spodopsins; M, molecular weight standard markers; IH, immune haemolymph; a, spodopsin Ia; b, spodopsin Ib.

Resulting sample was analyzed by reverse phase HPLC using Waters PicoTaq System. Amino acid sequence was determined by automated protein sequencer (Milligen, model 6600B). For determination of more precise molecular mass of purified peptides, samples were analyzed by mass spectrometer using a Kratos Kompact MALDI 2. The mass spectrometer consisted of a nitrogen laser with 5 ns pulse at 337 nm. The laser was focused on a sample spot of about 30 μm diameter. Mass spectra shown were typically accumulated from 30 laser shots.

Results and Discussion

Purification of antibacterial peptides

Antibacterial activity fractions obtained after cation-exchange chromatography were applied to gel filtration column and aliquots of the eluted fractions were assayed against *E. coli*. Fractions having antibacterial activity were pooled and concentrated and then subjected to reverse phase FPLC (PepRPC HR 5/5). Antibacterial activity against *E. coli* was found in 25-35% acetonitrile (Fig. 1A). These peaks which exhibit antibacterial activity were electrophoresed, showing more than one band (Fig. 1B). Peaks a and b were rechromatographed on reverse phase FPLC (PepRPC HR 5/5). The result showed that two peaks were found in 23% and 25% acetonitrile, respectively (Fig. 2A). Purity of each peak was recognized by Tricine-SDS PAGE (Fig. 2B). Through these purification steps, 110 μg of peak a and 210 μg of peak b were obtained from approximately 10 ml of immunized haemolymph (Table 1). Peak a and peak b were designated spodopsin Ia and spodopsin Ib, respectively. Spodopsin is named after *Spodoptera litura*.

Molecular mass, amino acid composition, and N-terminal sequence

The purified peptide, spodopsin Ib was electrophoresed

Table 1. Summary of purifications of antibacterial peptides. About 10 ml of immune haemolymph of *Spodoptera litura* was used as starting material. Amounts of protein were determined by the method of Bradford with bovine serum albumin as a standard. One relative unit of antibacterial activity was defined as the activity in 1 ng of a synthetic cecropin A (Sigma)

Purification step	Total activity (KU)	Total protein (mg)	Specific activity (KU/mg)	Yield (%)
Haemolymph	2960	353	8.4	100
heated Haemolymph	2206	17.43	126.6	74.5
CM-Cellulose	444	2.68	166	15
Sephadex G-50	160	0.74	216	5.4
Pep RPC Ia	29.7	0.11	269	1.0
Ib	64.9	0.21	309	2.2

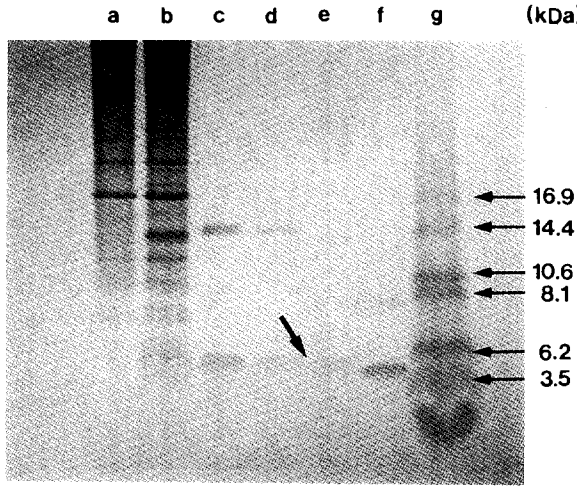


Fig. 3. Tricine-SDS PAGE of antibacterial protein from *Spodoptera litura* at different steps of purification. The gel was stained with Coomassie brilliant blue. a, normal haemolymph; b, immune haemolymph; c, active fraction of CM-cellulose chromatography; d, active fraction of gel filtration chromatography; e, spodopsin Ib; f, synthetic cecropin A (Sigma); g, molecular weight standard markers (myoglobin, 16.95 kDa; myoglobin I+II, 14.4 kDa; myoglobin I+III, 10.6 kDa; myoglobin I, 8.1 kDa; myoglobin II, 6.2 kDa; glucagon, 3.5 kDa).

with samples from each purification step, showing that spodopsin Ib has a molecular mass of 4 kDa which is similar to that of cecropin (Hultmark et al., 1980; Morishima et al., 1990) or sarcotoxin (Okada and Natori, 1985) (Fig. 3). For a more precise determination, spodopsins were subjected to mass spectrometer, indicating that spodopsin Ia and Ib have molecular masses of 3,823 Da and 3,886 Da, respectively (Fig. 4). About 20% basic amino acids, such as lysine and arginine, constitutes both spodopsin Ia and Ib but no cysteine residue was found (Table 2). Also, N-terminal sequence analysis showed that spodopsin has the characteristics of cecropin family (Fig. 5). That is, tryptophan in second residue and lysine in 3rd, 6th, 7th and glutamic acid in 9th and arginine in 16th residue are characteristics of cecropin. Especially, purified spodopsins Ia and Ib showed high similarity to the cecropin of lepidopteran insects such as *Bombyx mori* and *Hyalophora cecropia* (Cociancich et al., 1994). However, spodopsin's amino acid sequence showed some differences from those of other cecropin-like peptides. Though amino acid in 1st residue of spodopsin is arginine like *B. mori,* cecropin of *H. cecropia* has lysine in its 1st residue. Amino acid in 15th residue of spodopsin Ia is valine like cecropin-like peptide from *Manduca sexta,* not isoleucine which is 15th amino acid of spodopsin Ib, *B. mori* and *H. cecropia* (Cociancich et al., 1994). It was known that positive charge due to the presence of basicity undergoes electrostatic attraction with negative charge on the surface of the bacteria (Christensen et al., 1988; Gabay, 1994). In fact, it was reported that most of antibacterial proteins have basicity and some bacterial proteins appear to act as cationic detergents (Bevins and Zasliff, 1990; Hoffmann and Hetru, 1992; Cociancich et al., 1994; Gabay, 1994; Vaara and Vaara, 1994). It can be inferred that the basicity of N-terminal region of spodopsin (40% of 15 amino acid residues at N-terminal) makes binding of spodopsin to bacteria easier and is consequently contributed to antibacterial activity.

Antimicrobial spectrum of spodopsin

Six kinds of gram-positive and 11 kinds of gram-negative bacteria, yeasts, and spore-type fungi were used to determine antimicrobial activity of spodopsin Ia and Ib. The antimicrobial spectrum of spodopsins was compared to that of synthetic cecropin A (Sigma). Results showed that spodopsin represents antibacterial activity against gram-negative bacteria including *E. coli*

Table 2. Amino acid composition of spodopsin Ia and Ib from *spodoptera litura.* Experiments were done three times and averaged

Amino acid residue	Result[1]		Mol %	
	Spodopsin Ia	Spodopsin Ib	Spodopsin Ia	Spodopsin Ib
Cya[2]	0.00	0.00	0.00	0.00
Asx[3]	389.52	176.59	6.62	5.89
Glx[3]	424.30	192.42	7.21	6.42
Ser	170.52	121.25	2.90	4.04
Gly	889.76	429.93	15.13	14.34
His	53.09	0.00	0.90	0.00
Arg	417.26	230.50	7.10	7.69
Thr	113.66	10.96	1.93	0.37
Ala	676.20	368.91	11.50	12.31
Pro	197.72	89.56	3.36	2.99
Thr	21.33	0.00	0.36	0.00
Val	593.92	267.75	10.10	8.93
Met	23.93	48.51	0.41	1.62
Ile	479.41	271.66	8.15	9.06
Leu	378.18	162.79	6.43	5.43
Phe	259.27	142.85	4.41	4.77
Trp	163.51	87.94	2.78	2.93
Lys	629.40	395.97	10.70	13.21
Total	5880.98	2997.59	100.00	100.00

[1] Each number is expressed as pmol per 30 μl injection vol.
[2] Cya means cysteic acid.
[3] Asx and Glx mean the sum of asparagine and aspartic acid and glutamine and glutamic acid, respectively.

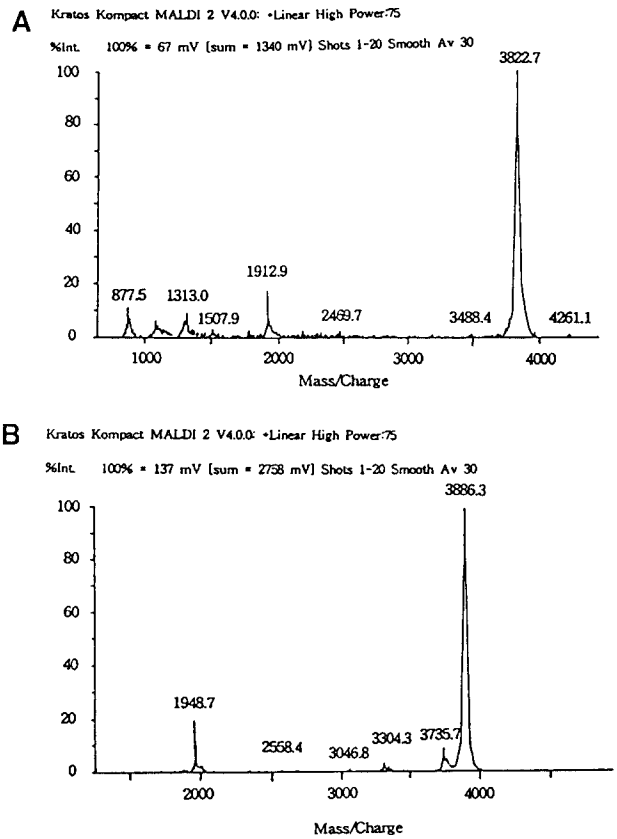


Fig. 4. Electrospray ionization mass spectrum of purified protein from immunized haemolymph of *S. litura*. Mass spectrometric identification of purified antibacterial peptide; (A), spodopsin Ia; (B), spodopsin Ib.

and gram-positive bacteria including *M. luteus* (Table 3). This spectrum of antibacterial activity was very similar to that of cecopin A. Cecropin A and about 3.5 fold concentrations of spodopsins were found to induce similar sensitivity. But we could not identify the antifungal activity against fungi including yeast by hyphal extension-inhibition method (Borgmeyer et al., 1992). It was inferred that no activity against spore was ascribed to the thick cell wall, whose property protect the organisms under unfavorable living condition. *E. coli* and *M. luteus* were the most sensitive ones to antibacterial peptides and *Bacillus* showed a little higher resistence among gram-positive bacteria (Steiner et al., 1981; Morishima et al., 1990). It was inferred that spodopsins composed of basic proteins represent antibacterial activity against various gram-positive and gram-negative bacteria and are kinds of cecropin bearing a lot of similarity in amino acid composition to that of cecropin.

Acknowledgements

This research was supported by grants (1992-1995) from the Ministry of Education, Republic of Korea.

		1			5					10			
S. litura	Ia	Arg	Trp	Lys	Val	Phe	Lys	Lys	Ile	Glu	Lys	Val	Gly	Arg
S. litura	Ib	-	-	-	-	-	-	-	-	-	-	Met	-	-
B. mori	A	-	-	-	Leu	-	-	-	-	-	-	-	-	-
B. mori	B	-	-	-	Ile	-	-	-	-	-	-	Met	-	-
H. cecropia	A	Lys	-	-	Leu	-	-	-	-	-	-	-	-	Gln
H. cecropia	B	Lys	-	-	-	-	-	-	-	-	-	Met	-	

		15					20					25		
S. litura	Ia	Asn	Val	Arg	X	Gly	Ile	Ile	X	Ala	Gly	Pro	Ala	Ile
S. litura	Ib	-	Ile	-	Asp	-	-	-	Lys	-	-	-	-	Val
B. mori	A	-	Ile	-	Asp	-	Leu	-	Lys	-	-	-	-	-
B. mori	B	-	Ile	-	Asp	-	-	Val	Lys	-	-	-	-	-
H. cecropia	A	-	Ile	-	Asp	-	-	-	Lys	-	-	-	-	Val
H. cecropia	B	-	Ile	-	Asn	-	-	Val	Lys	-	-	-	-	-

		30									
S. litura	Ia	Gly	Val	Leu	X	Gln	Ala	X	Ala	Leu	
S. litura	Ib	Glu	-	-	Gly	Ser	-	X	-	-	
B. mori	A	Ala	-	Ile	Gly	-	-	Lys	Ser	-	
B. mori	B	Glu	-	-	Gly	Ser	-	Lys	-	Ile	
H. cecropia	A	Ala	-	Val	Gly	-	-	Thr	Gln	Ile	Ala	Lys
H. cecropia	B	Ala	-	-	Gly	Glu	-	Lys	-	-		

Fig. 5. Comparison of N-terminal amino acid sequence of 6 cecropin-like antibacterial peptides from *Spodoptera litura, Bombyx mori,* and *Hyalophora cecropia* (From Cociancich et al., 1994). Identical amino acid residues to those of *S. litura* Ia are indicated by dashes. X represents the absence of identifiable residue.

Table 3. Activity spectrum of synthetic cecropin A and antibacterial peptides from *Spodoptera litura*

Bacterial strains[1]	Cecropin A	Ia	Ib
Gram (+) bacteria			
Bacillus Sphaericus PY105	+ +	+	+
B. megaterium	+ +	+ +	+
B. subtilus	+	+	+
B. thurengiensis	-	-	-
Micrococcus luteus KCTC 1056	+++	+++	+++
Streptococcus pneumoniae KU39	+ +	+ +	+
Gram (-) bacteria			
Achromobacter spp.	+ +	+ +	+ +
Acinetobacter calcoaceticus KH48	+ +	+	+ +
Escherichia coli Ek132	+++	+++	+++
E. coli Ek112	+++	+++	+++
E. coli K12	+++	+++	+++
Enterobacter cloacae 1684	+ +	+ +	+ +
Klebsiella pneumoniae KU7	+ +	+ +	+ +
Pseudomonas acidovorans KU82	+	+ +	+ +
P. putida	+ +	+ +	+ +
Salmonella typhimurium IB74	+ +	+ +	+ +
Serratia marcescens KU9	+	+	+
Yeast			
Saccharomyces cerevisiae	-	-	-
S. diastaticus	-	-	-
Fungus spores			
Colletotrichum Gloeosporioides	-	-	-
Alternaria mali	-	-	-
Trichoderma hazianum	-	-	-

[1]Bacteria (about 1×10^5 cells) was plated on agar medium and equal volumn (3 μl) of protein solution was applied to each well. The diameter of growth inhibition were recorded after 24 h incubation at 30℃ or 37℃. The concentration of cecropin A and spodopsin Ia, Ib was 0.1 mg/ml and 0.34, 0.38 mg/ml, respectively. Expression is noted as follows. -, no inhibition; +, clear zone of less than 6 mm; ++, clear zone of 6 to 8 mm; +++, clear zone of more than 8 mm. Well diameter 3 mm.

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[Received May 15, 1997; accepted July 10, 1997]