Korean J. Zool. 38:305-312, 1995

Induction and Purification of Antibacterial Proteins in Larval Haemolymph of Cabbage Butterfly, *Artogeia rapae*

Sung Moon Yoe, In Seok Bang, Byung Soo Chang[1], and Eun Jeong Cho

Department of Biology, Dankook University, Cheonan 330-714 and [1]Department of Clinical Pathology, Dongnam Health Junior College, Suwon 440-714, Korea

The injection of viable *Escherichia coli* K-12 with fifth instar larvae of cabbage butterfly, *Artogeia rapae*, induced at least five groups of proteins with the antibacterial activity against certain Gram-negative and/or Gram-positive bacteria in the haemolymph. These antibacterial proteins were separated and one was purified by different types of chromatography. The purified protein was heat-stable and basic peptide, and its molecular weight was approximately 4 kDa. We propose the name hinnavins for this antibacterial peptide.

KEY WORDS: Insect Immunity, Antibacterial Peptide, *Artogeia rapae*, Hinnavin

It is well known that insects have both humoral and cellular immunity to protect themselves from the infection of microorganisms (Boman and Hultmark, 1987). Insect cellular immunity is accomplished directly by phagocytosis, encapsulation, nodule formation (Ratcliffe *et al.*, 1985; Chang *et al.*, 1991, 1992), and indiretly by immunological factors, coagulation, prophenoloxidase-activating system, and poison detoxification mechanisms (Gupta, 1991). Insect humoral immunity depends on the production of a number of antibacterial proteins (Boman *et al.*, 1974; Yoe, 1993). Four groups of the antibacterial proteins have been described in insects (Hoffmann and Hetru, 1992), but no antibacterial protein has been identified in the butterflies.

In this paper, therefore, we report the induction and the purification of the antibacterial proteins in the larval haemolymph of the cabbage butterfly, *Artogeia rapae*, with injection of live bacteria.

Materials and Methods

Insects, immunization and collection of haemolymph

Cabbageworms, *Artogeia rapae*, used in the present study were reared on kail in greenhouse and fifth instar larvae were used throughout. For induction of antibacterial proteins, larvae were injected with 2 × 10^4 viable log phase *Escherichia coli* K-12 into the thorax. Haemolymph was collected by cutting leg of each larva and put into cold test tube which contains small amount of phenylthiourea. The haemolymph was centrifuged at 10,000 g for 10 min to remove haemocytes and the supernatant was stored at -70°C.

Assay of antibacterial activity

Two types of assay have been used to determine antibacterial activity. In the inhibition zone assay, we recorded the diameters of the zones of growth inhibition on thin agarose plates seeded with *E. coli* K-12 and *Bacillus megaterium* Bm11 (Hulmark *et al.*, 1983). A standard curve was made with known amounts of synthetic cecropin A, and 1,000 units were defined as an activity equal to that of 1 μg of cecropin A. Three μl of

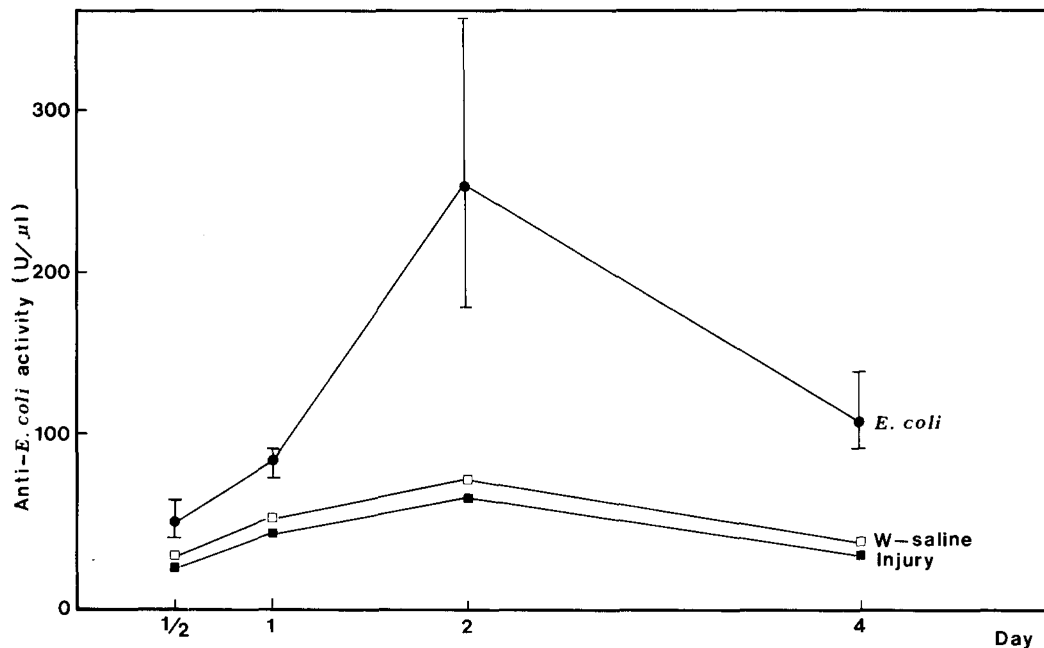

Fig. 1. Antibacterial activity of haemolymph from *Artogeia rapae*. ●, larva vaccinated with live *E. coli*; □, larva injected with an equal volume of sterile W-saline: ■, injured larva.

samples were used for each assay. In the lysis assay, log phase bacteria were suspended in ice-cold 0.1 M phosphate buffer pH 6.4, giving an absorbance of 0.3 to 0.5 at 570 nm. Twenty μl of haemolymph were mixed 1 ml of bacterial suspension and incubated at 37°C. After different times of incubation, the mixture was transferred back to an ice-bath and measured at 570 nm.

Electron microscopy

For transmission electron microscopy, *E. coli* K-12 suspension mixed with immune haemolymph (4:1) was prefixed in 2.5% paraformaldehyde in 0.01 M phosphate buffer, pH 7.2. After fixation for 2 hr at 4°C, specimens were rinsed with the same buffer three times and postfixed in 1% OsO_4 in the same buffer for 1 hr. The specimens were then rinsed in the same buffer, dehydrated in a grade ethanol series, and embedded in Epon-Araldite mixture. Thin sections were cut on a LKB-2088 ultramicrotome, picked up on a copper grid, stained with uranyl acetate and lead citrate, and examined with a JEOL 100CX-II

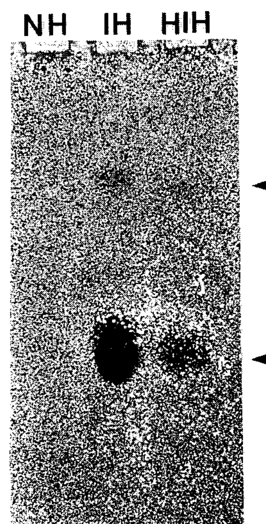

Fig. 2. Acidic PAGE of normal haemolymph, immune haemolymph and heated immune haemolymph from *A. rapae*. NH, normal haemolymph; IH, immune haemolymph; HIH, heated immune haemolymph.

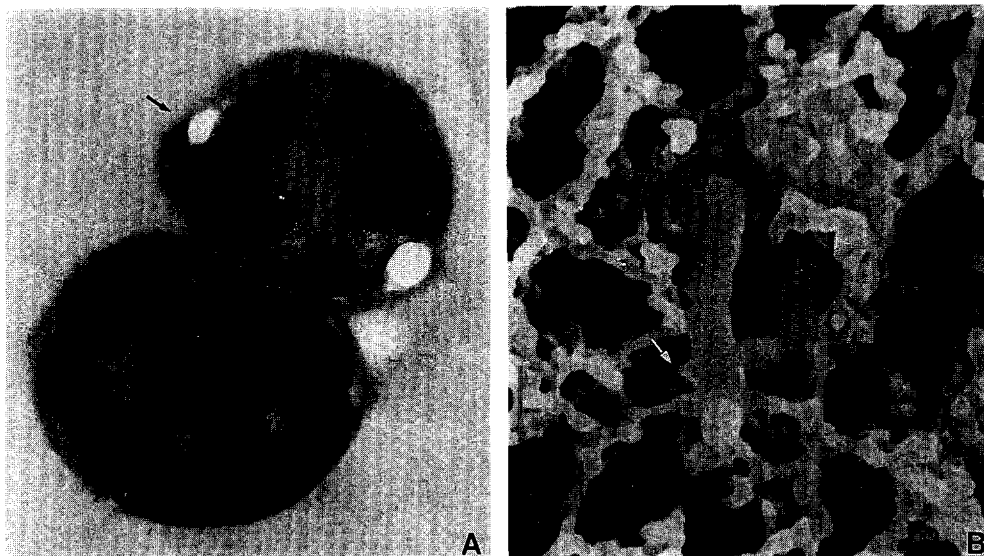

Fig. 3. Electron micrographs of *E. coli* treated immune haemolymph of *A. rapae*. A, transmission electron micrograph of bacteria 8 hr after treatment of immune haemolymph (× 76,000); B, scanning electron micrograph of bacteria 2 hr after treatment of immune haemolymph (× 22,800). Arrows indicate unusual projections.

transmission electron microscope.

For scanning electron microscopy, above *E. coli* K-12 suspension was incubated for 2 hr. The cells were collected on a Millipore filter (0.25 μm pore size) by filtration. Specimens were fixed, dehydrated by above methods, and critical-point dried. Dried specimens were coated (20 nm thick) with a gold and examined under a JSH-840A scanning electron microscope.

Polyacylamide gel electrophoresis (PAGE)

Electrophoresis of native protein was carried out in 15% polyacrylamide gels pH 4, using a discontinous buffer system (Gabriel, 1971) with modification as described (Hultmark *et al.*, 1980). To localize bands with activity against *E. coli*, the gels were incubated in a rich bacterial medium containing 0.2 M phosphate buffer, pH 7.4 and then overlaid with 5 ml of the same medium containing 0.6% agar and 2 × 10^5 viable *E. coli* cells and incubated at 37°C overnight as described (Hultmark *et al.*, 1980). Tricine SDS gel was performed using the method of Schägger and von Jagow (1987).

Purification and protein determination

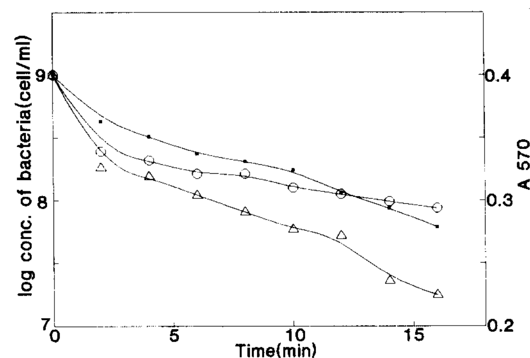

Fig. 4. Lysis of bacteria by immune haemolymph from *A. rapae*. Lysis was followed as absorbance at room temperature in a spectrophotometer, -○-, *E. coli* K12; -■-, *E. coli* K12, D21; -△-, *B. megaterium* Bm11.

The cell-free haemolymph was subjected to heat treatment at 100°C for 5 min and then heat-treated haemolymph was centrifuged for 10 min at 10,000 g. 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) with CM-Sepharose (Pharmacia) equilibrated with the same buffer. After application of the sample, the antibacterial proteins were eluted with a linear gradient of 0.1-

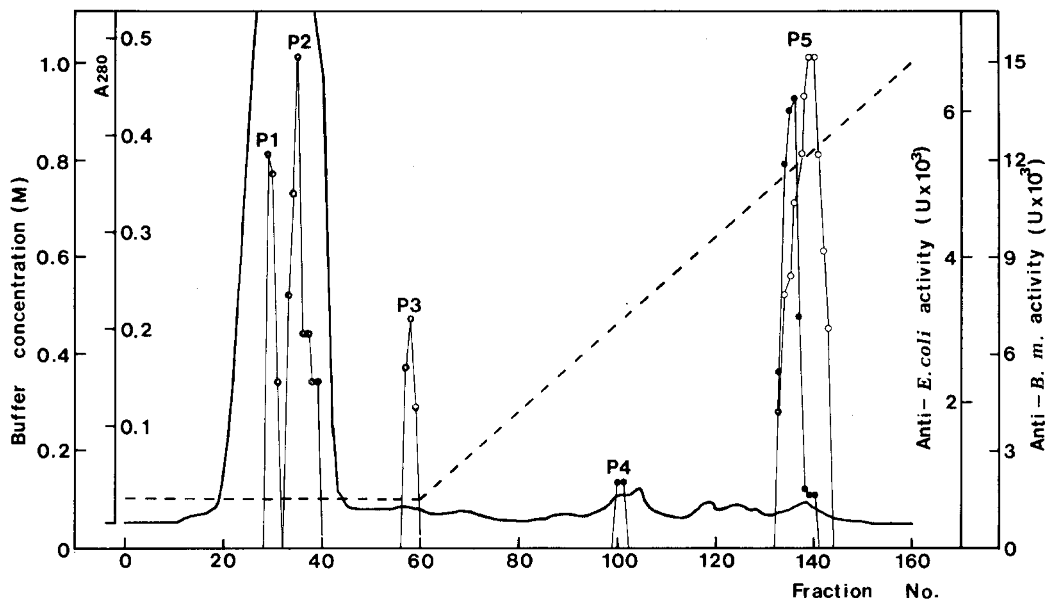

Fig. 5. CM-Sepharose cation exchange chromatography of cell free, heat treated immune haemolymph of *A. rapae*. —, A_{280}; ···, ammonium acetate concentration; ●---●, anti-*E. coli* activity; ○---○, anti-*B. megaterium* activity.

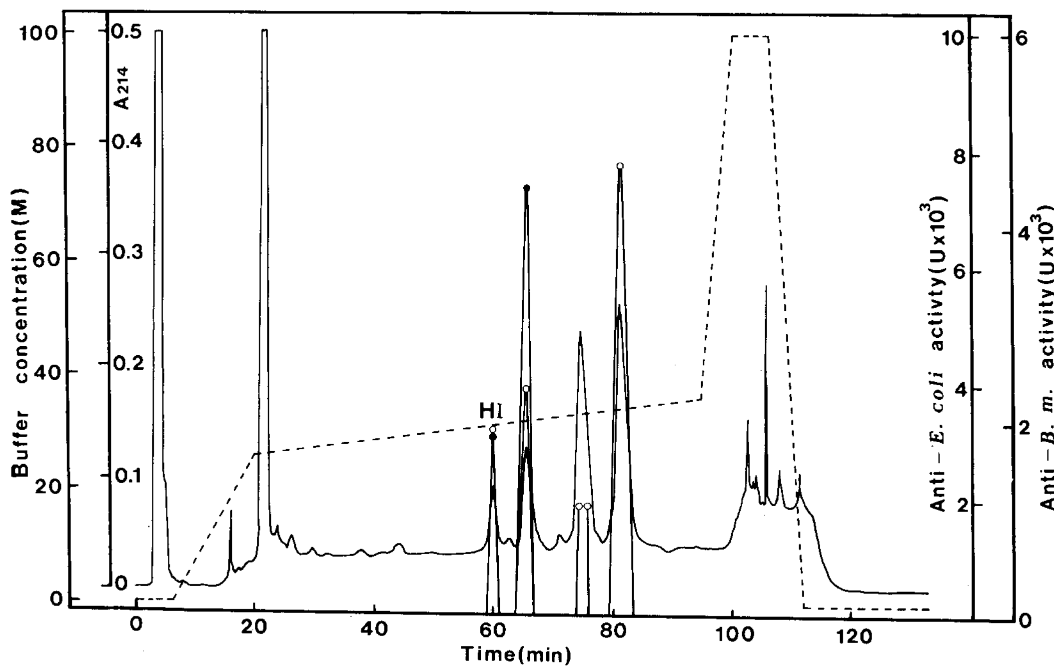

Fig. 6. Reversed-phase FPLC of hinnavin I from P5 of CM-Sepharose column. —, A_{214}; ···, acetonitrile concentration; ●---●, anti-*E. coli* activity; ○---○, anti-*B. megaterium* activity.

Table 1. Purification of Hinnavin I from immune haemolymph of *A. rapae*

Fraction	Volume (ml))	Total protein (mg)	Total activity (kU)	Recovery activity (kU/mg)	Specific
Immune haemolymph	6	139.92	726	100	5.19
Heat treatment	8.2	17.71	396.88	54.67	22.40
CM-Sepharose	0.7	1.16	52.25	7.20	45.04
PepRPC HR 5/5	1	0.35	3.87	0.53	11.06

Amounts of protein were measured by the method of Bradford (1976) using BSA as the standard. Antibacterial activity against *E. coli* K12 was determined in absolute units (U) as described by the method of Hultmark *et al.* (1982).

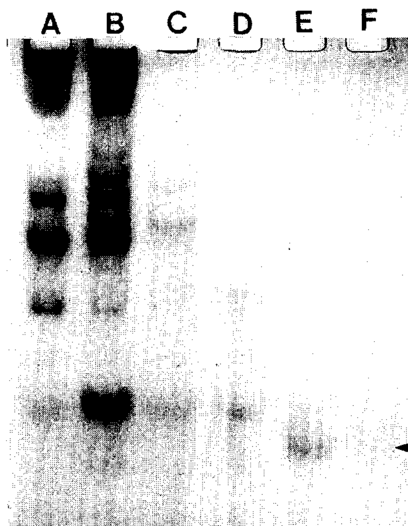

Fig. 7. Acidic PAGE of antibacterial proteins during the different purification steps. Gel was stained for proteins with Coomassie brilliant blue. A, normal haemolymph: B, immune haemolymph; C, heated immune haemolymph; D, P5 of CM-Sepharose chromatography; E, first peak of the reversed-phase FPLC; F, hinnavin I of the second reversed-phase FPLC.

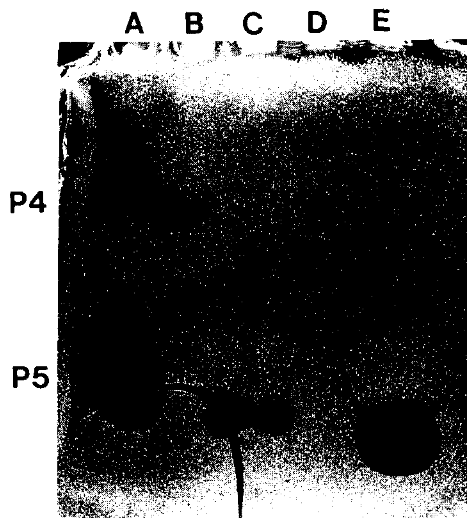

Fig. 8. Acidic PAGE of purified antibacterial proteins from *A. rapae*. A, immune haemolymph; B, P4 of CM-Sepharose chromatography; C, P5 of CM-Sepharose chromatography; D, hinnavin I of the second reversed-phase FPLC; E, 1 μg of synthetic cecropin A.

1.0 M ammonium acetate, pH 6.0 and the antibacterial activity was determined as above. Active fractions were pooled, freeze-dried and dissolved in a small volume of water.

For further purification, the isolated P5 was subjected to reversed-phase FPLC on a PepRPC HR5/5 column (Pharmacia) equilibrated with 0.1% trifluroacetic acid. Elution was performed with a linear gradient of 25-35% acetonitrile in water. Ultraviolet absorption was monitored at 214 nm and the antibacterial activity was determined on aliquots of the fraction which had been vacuum-dried to remove acetonitrile.

Protein concentration was determined by the method of Bradford (1976) and on cation-exchange chromatography monitored at 280 nm.

Results and Discussion

Induction of antibacterial activity in the haemolymph

To understand the mechanisms that induce the antibacterial response of *Artogeia rapae*, we

assayed antibacterial activities in the haemolymph by various stimuli. As shown in Fig. 1, antibacterial activity increased with time, reaching a maximum after 2 days, and then decreased rapidly. Actually an infection may often start with an injury. It was, therefore, necessary to investigate whether the injury and an injection of physiological salt solution induced any antibacterial activity. The activities by the injury and the injection were always significantly lower than that by the injection of live bacteria. In the case of some Diftera, however, a marked antibacterial activity was induced when the body wall of larvae was injured with hypodermic needle (Okada and Natori, 1983; Keppi *et al.*, 1986).

To see how many antibacterial proteins were induced, we collected hamolymph 2 days after injection of live *E. coli*, subjected it to electrophoresis, and located antibacterial activity by the method of Hultmark *et al.* (1980). Two spots of material inhibiting bacterial growth were found in the immune haemolymph and the immune haemolymph with heat, respectively, but none in the normal hemolymph as control (Fig. 2). The lower spot migrated fastest under these electrophoretic conditions had the most antibacterial activity against *E. coli*. Thus at least two groups of antibacterial proteins seemed to be induced on injection with live bacteria.

Hoffmann *et al.* (1981) observed multiple antibacterial proteins in the immune haemolymph from different Lepidoptera species and the presence of cecropin-like protein in all of them. Okada and Natori (1983) also found three antibacterial proteins in the immune haemolymph of injured *Sarcophaga peregrina* larvae. Therefore it is important to identify whether *A. rapae* larvae have cecropin-like protein from the viewpoint of comparative immunology.

Mode of action of antibacterial protein

In order to know mode of action of the antibacterial protein we observed bacterial shape treated with immune haemolymph by transmission and scanning electron microscopies. Bacteria treated with the immune haemolymph showed clear morphological changes after 2 and 8 hrs, respectively (Fig. 3). These bacteria have unusual

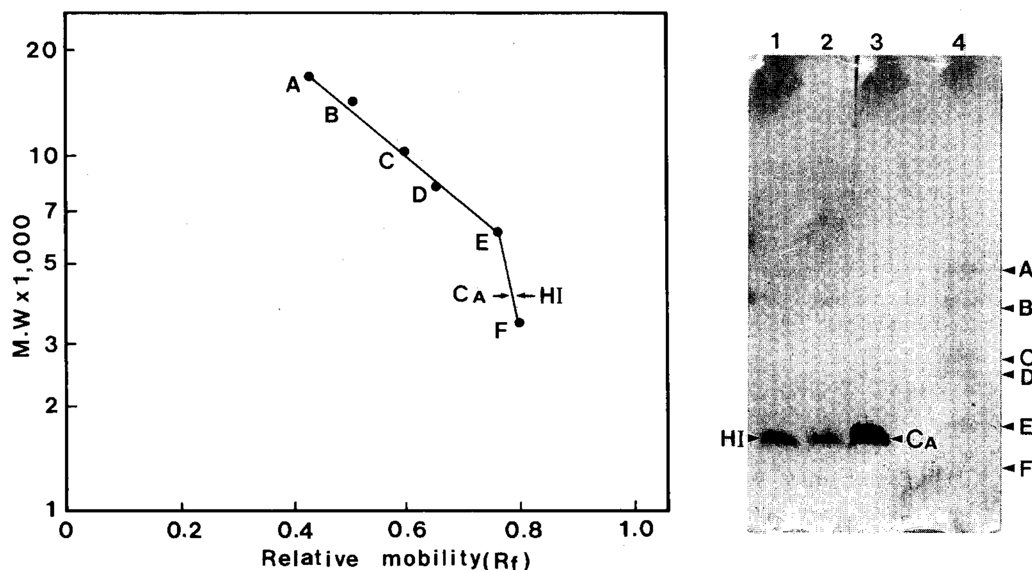

Fig. 9. Determination of molecular weight of hinnavin I was carried out as described (Schägger and von Jagow, 1987). Lanes 1 and 2, 30.1 - 30.7 % and 30.9 - 31.8 % acetonitrile concentration of reversed-phase FPLC column; lane 3, cecropin A; lane 4, standard markers. Standard molecular weight markers were used A, myoglobin (polypeptide backbone 1-153, 16,950); B, myoglobin (I+II, 1-131, 14,440); C, myoglobin (I+III, 56-131, 10,600); D, myoglobin (I, 56-131, 8,160); E, myoglobin (II, 1-55, 6,210); F, glucagon (3,480); C_A, cecropin A; HI, hinnavin I.

projections, or bulges, on their membrane surface. These bulges may be formed by inhibition of cell wall synthesis by the antibacterial proteins.

Earlier investigators have reported lytic activities both in the haemolymph (Boman *et al.*, 1974) and in the gut of insects (Zacharuk, 1973). We therefore investigated whether immune haemolymph from *A. rapae* larvae vaccinated with live *E. coli* could also lyse *E. coli* and *Bacillus megaterium*. In both cases we found lytic action. Fig. 4 shows that 2% of the immune haemolymph gave 50% reduction of viable count while the absorbance dropped 13%.

Purification of antibacterial protein from immune haemolymph

To isolate the antibacterial proteins in the immune haemolymph, we used different types chromatography, such as cation-exchange chromatography, first reversed-phase, and second reversed-phase FPLC. Cell-free haemolymph obtained after heat treatment was applied to a column with CM-Sepharose under coditions as described, and aliquots of the eleuted fractions were assayed against *E. coli* and against *B. megaterium*. At the first step, antibacterial activity was separated into five peaks and material in the fifth peaks, named fraction P5, was found activity both against *E. coli* and against *B. megaterium* (Fig. 5). As the next step, solution of the fraction P5 was used for FPLC with a reversed-phase column. The final step was rechromatography on the same column. The antibacterial activity after the last step was separated into four peaks and of these peaks the first peak was named as hinnavin I (HI), as shown in Fig. 6. Hinnavin I has higher activity against *E. coli* than *B. megaterium*. The last step was performed with about 0.35 mg of proteins and then recovery was very low (Table 1).

Electrophoresis

Samples at each purification step of the immune haemolymph were examined with acidic PAGE, and gels were stained for protein, or overlaid with *E. coli*. Fig. 7 shows that isolated hinnavin I was homogeneous and gave a single band on the gel of acidic PAGE. We next examined whether it really had antibacterial activity. After acidic electrophoresis of the samples and 1 μg synthetic cecropin A, gel was overlaid with viable *E. coli* to detect antibacterial activity. As shown in Fig. 8, two spots of protein inhibiting growth of bacteria were found in the immune haemolymph and the lower spot must correspond to hinnavin I. The mobility of this peptide was almost similar to that of synthetic cecropin A. The molecular weight of hinnavin I was estimated to be about 4 kDa by tricine SDS-PAGE (Fig. 9).

Thus an antibacterial protein purified in this study from the immune haemolymph of *Artogeia rapae* larvae has several aspects. First, it is inducible. Second, it is heat-stable. Third, it is basic 4 kDa peptide. Fourth, it possesses higher antibacterial activity against Gram-negative than against Gram-positive bacteria tested (data not shown). Hinnavin I, therefore, is an antibacterial protein belonging to the cecropin family.

Acknowledgements

This work was supported in part by grant (#91-05-00-14, S. M. Yoe) from the Korea Science and Engineering Foundation, Republic of Korea.

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(Accepted April 24, 1995)

배추흰나비의 유충 혈림프로 부터 항균단백질의 유도와 분리

여성문 · 방인석 · 장병수[1] · 조은정(단국대학교 자연과학대학 생물학과,
[1]동남보건전문대학 임상병리과)

배추흰나비 5령 유충의 복부에 *E. coli* K-12를 주입한 다음 면역혈림프로 부터 다섯 종류의 항균단백질을 유도하였으며, 이러한 단백질들은 그람 양성균과 그람 음성균에 대해 항균성이 있거나 혹은 그람 양성균에 대해서만 항균성이 있었다. 유도된 단백질 가운데 열에 안정되고 염기성인 4 kDa 정도의 분자량을 갖는 peptide를 분리하였으며, 이것을 hinnavin이라고 명명하였다.