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# Purification and Partial Characterization of an Induced Antibacterial Protein in the Silkworm, *Bombyx mori*

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**Injection of live *Escherichia coli* into larvae of the silkworm, *Bombyx mori*, induces antibacterial activity in the hemolymph. The major induced antibacterial activity was purified in two steps by CM-Sephadex C-50 and Sephadex G-100 column chromatography. After trypsin treatment, the purified antibacterial protein lost its activity and the antibacterial activity was found to be partially heat labile. The purified protein was a single polypeptide chain of molecular weight 16 kDa. The 20 N-terminal amino acid sequence of the protein was determined and this sequence showed homology with the N-terminal amino acid sequence of lysozymes reported in other species. The purified protein was found to have comparable antibacterial activity against both *E. coli* and *Micrococcus luteus*. The purification of antibacterial protein and the antibacterial properties of the purified protein are discussed.** 

KEY WORDS: Antibacterial activity; lysozyme-like protein; insect immunity; *Bombyx mori;* silkworm.

## INTRODUCTION

Insects are known to have both cellular and humoral immune systems which together form a potent defense against invading bacteria (see reviews by Boman and Hultmark, 1987; Gotz and Boman, 1985; Dunn, 1986; Kimbrell, 1991). In cellular immunity, mechanisms such as phagocytosis and encapsulation are operative (Boman and Hultmark, 1987; Dularay and Lackie, 1985; Ratcliffe *et al.*, 1985; Rizki and Rizki, 1984), while humoral responses mainly involve the production of a variety of antibacterial proteins that are induced or increased in response to infection. The antibacterial proteins identified in many insects operate cumulatively against a wide range of gram-positive and gram-negative bacteria. These antibacterial proteins can be categorized by homology as belonging to one of the several families, namely, the cecropins (Boman and Hultmark, 1987; Dickenson *et al.*, 1988; Hultmark *et al.*,1980; Kaaya *et al.*, 1987; Kanai and Natori, 1989; Kylsten *et al.*, 1990; Samakovlis, *et al.*, 1990, 1991), attacins (Casteels *et al.*, 1990; Hultmark *et al.*, 1983; Kockum *et al.*, 1984; Sun *et al.*, 1991; Wicker *et al.*, 1990), lysozymes (Engstrom *et al.*, 1985; Hultmark *et al.*, 1980; Jollès *et al.*, 1979; Mohrig and Messner, 1968; Powning and Davidson, 1976), defensins (Dimarcq *et al.*, 1990; Lambert *et al.*, 1989), and diptericins (Dimarcq *et al.*, 1990; Wicker *et al.*, 1990). The silkworm, *Bombyx mori*, an economically important insect, is a host for different pathogenic microorganisms (Chitra *et al.*, 1975). Different geographically differentiated genotypes of silkworm are known to show varying degrees of tolerance to different pathogens (Chitra *et al.*, 1975). Although earlier workers have characterized cecropin-like antibacterial proteins (Morishima *et al.*, 1990) and lysozyme (Powning and Davidson, 1973), to what extent they are involved in the humoral response in silkworm remains to be understood. In the present study we have examined the nature of antibacterial response in *B. mori* and we further show that the major protein involved in the antibacterial response of silkworm is of the lysozyme type.

## MATERIALS AND METHODS

### *Insects, Immunization, and Collection of Hemolymph*

*B. mori*, (strain $NB_{18}$) larvae were reared on fresh mulberry leaves under ambient conditions as per the standard rearing method (Krishnaswami, 1978). For studying induction kinetics of antibacterial activity, larvae reared on artificial diet were used. *Escherichia coli* strain JM103 and *Micrococcus luteus* were used for antibacterial assay. The 3-day-old fifth stadium larvae were injected with a 10μl suspension of log phase *E. coli* washed and suspended in saline (0.3 M NaCl, 0.005 M KCl) at $1 \times 10^7$ cells/ml. Hemolymph samples were collected after different intervals of injection into precooled tubes containing a few crystals of phenylthiourea, centrifuged at 10,000*g* at 4°C for 10 min, and

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stored at −20°C until use. Hemolymph from uninjected larvae was used as a control.

*Antibacterial Assay*

Antibacterial activity was assayed by measuring the zone of bacterial growth inhibition in thin agar plates with *E. coli, Serratia* species, *Bacillus thuringiensis, Pseudomonas aeruginosa,* and freeze-dried cells of *M. luteus* (Hoffman *et al.,* 1981). Serially diluted immunized and control hemolymph samples were applied into the wells on a thin agar plate seeded with bacteria. The zone of bacterial growth inhibition was measured and compared to that of control hemolymph. Another method used was bacteriolytic assay in which a fixed volume of control and test hemolymph samples was incubated with log phase *E. coli* suspended in 0.1 M phosphate buffer, pH 6.8, at 37°C, and reduction in absorbance at 570 nm was measured. A unit of lytic activity was defined as the amount of factor giving 50% reduction of absorbance at 570 nm compared to that of the control (Hultmark *et al.,* 1980).

*Electrophoresis*

Electrophoresis of immunized hemolymph and purified antibacterial protein was carried out in 15% polyacrylamide gel (PAGE), pH 4.3, using a discontinuous buffer system (Gabriel, 1971). The gel was run at 200 V until the tracking dye moved out of the gel. To localize the bands with antibacterial activity, the gel was incubated in rich bacterial medium (1% peptone in 0.1 M phosphate, pH 7.2) for 90 min and then overlaid with melted 0.6% agarose in rich bacterial medium containing about $1 \times 10^6$ viable *E. coli* cells. Bacterial inhibition zones were detected after incubating the gel at 37°C overnight. SDS–PAGE of the purified protein was carried out in 10% polyacrylamide using the discontinuous buffer system as described by Laemmli (1970). After electrophoresis gels were stained with Coomassie brilliant blue. Standard molecular weight markers (Sigma) were used for estimating the molecular weight.

*Purification of Antibacterial Activity*

*Step I.* Hemolymph was collected from 20–30 immunized larvae after 48 hr of vaccination. Hemolymph was diluted five times in 0.3 M ammonium acetate, pH 7.0, and applied to a CM-Sephadex C-50 (20 × 1 cm) (Pharmacia, Sweden) column equilibrated in 0.3 M ammonium acetate, pH 7.0, at a flow rate of 10 ml/hr. The column was washed with 5 column volumes of the same buffer and the bound proteins were eluted stepwise using 0.5 and 1 M ammonium acetate, pH 7.0. Fractions were followed by absorbance at 280 nm and bacteriolytic assay. The fractions having antibacterial activity were pooled and concentrated.

*Step II.* Antibacterial substance from the first step was applied onto a Sephadex G-100 column (55 × 1.5 cm) (Pharmacia) in 0.1 M phosphate buffer, pH 7.0, at a flow rate of 18 ml/hr. Fractions were followed by absorbance at 280 nm and bacteriolytic assay. The fractions showing antibacterial activity were pooled, concentrated by lyophilization, and dialyzed against the same buffer. Protein obtained from this step was used for further analysis. The elution volume of the protein was compared with that of standard molecular weight markers of cytochrome c, α-lactalbumin, trypsinogen, ovalbumin, and bovine serum albumin.

*Trypsin Treatment and Heat Treatment*

The immune hemolymph was diluted in 0.1 M phosphate buffer, pH 6.8, and incubated at 85°C for 10 min. For trypsin treatment hemolymph was diluted with an equal volume of 0.1 M Tris, pH 8.1, and trypsin (type 1, Sigma) was added to a final concentration of 250 μg/ml. After the mixture was incubated at 37°C for 30 min, the samples were stored at −20°C until use. The control was similarly treated except that trypsin was not added.

*N-Terminal Sequence Analysis*

The N-terminal sequence analysis was carried out on an Applied Biosystems 470A protein sequencer attached to an online ABI analyzer. The chemistry of sequencing is based on the method of Edman (Edman and Begg, 1967). The free amino group of the N-terminal residue reacts with phenyl isothiocyanate in alkaline medium (gaseous trimethylamine). Subsequent acid hydrolysis (gaseous TFA followed by TFA/$H_2O$) removes the N-terminal residue as the phenylthiohydantoin (PTH) which is then identified on 120A analyzer. During sequencing the protein is held on a porous glass fiber filter which is pretreated with biobrene. The PTH amino acid is removed and injected into the sequence analyzer and identified by comparison with a profile of standard mixture of PTH amino acids.

## RESULTS

*Induction of Antibacterial Activity*

Antibacterial activity was induced in the hemolymph of silkworm larvae by the injection of live *E. coli* (Fig. 1). The induction kinetics of antibacterial activity in the hemolymph of immunized larvae was determined by a bacteriolytic assay. The antibacterial activity was apparent only 6 hr after injection, which reached maximum at 48 hr followed by a slight decline at 72 hr after injection (Fig. 1). The number of colony forming bacteria in the hemolymph increased immediately after injection and decreased with the induction of antibacterial activity (data not shown). The higher

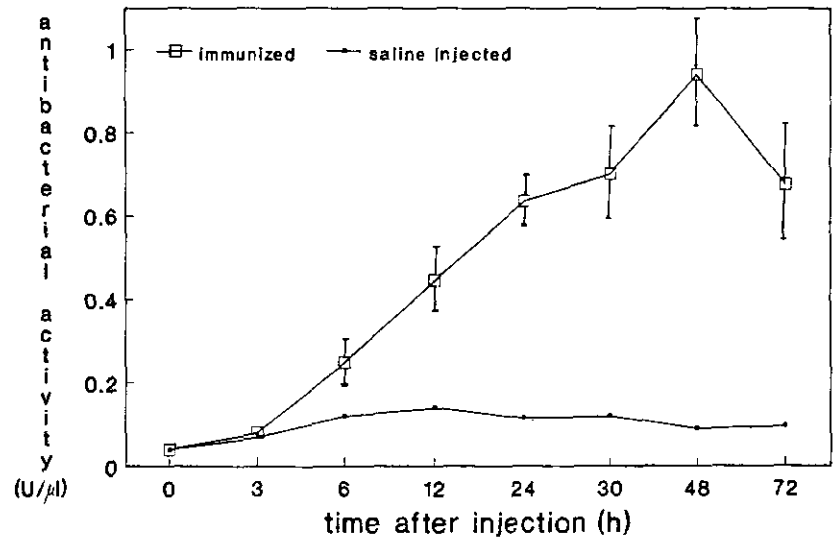


FIG. 1. Changes in the antibacterial activity in the hemolymph of silkworm larvae injected with 10 μl of *E. coli* ($1 \times 10^6$ cells) and saline. Each point represents the mean of duplicate assays for five individual larvae.

bacterial dose gave higher antibacterial response than the lower one. When the concentration of injected bacteria was over $1 \times 10^{10}$ cells/ml melanization of the hemolymph was observed and the inoculated larvae died 48–72 hr after inoculation. The hemolymph collected from the saline injected larvae also revealed very low but detectable levels of antibacterial activity (Fig. 1). The immune hemolymph collected 24 and 48 hr after infection was subjected to acidic PAGE and the antibacterial activity was detected by overlaying the gel with log phase bacteria in nutrient broth. The results showed that the immunized hemolymph contains antibacterial factors, which migrate toward the cathode at pH 4.5. Most of the activity was confined to the fast migrating band (Fig. 2A, lanes 2 and 3). However, the slow moving ones ($b_2$ and $b_3$) were detected only when the volume of immunized hemolymph loaded on the gel was increased (Fig. 2B, lane 1). No bacterial inhibition zone was detected in the lane charged with control hemolymph (Fig. 2A, lane 1). Coomassie brilliant blue staining of the parallel PAGE showed only a single induced band (Fig. 3, lane 2) in the lane loaded with immunized hemolymph corresponding to the major bacterial inhibition zone.

### *Thermal Stability and Proteinaceous Nature of Antibacterial Activity*

Trypsin treatment of the 48-hr immunized hemolymph completely destroyed the antibacterial activity (Fig. 4, lane 4), whereas the heat treatment destroyed the induced antibacterial activity partially (Fig. 4, lane 3).

### *Purification of the Antibacterial Activity*

The antibacterial activity was purified in two steps using CM-Sephadex C-50 and Sephadex G-100 column chromatography. Application of undiluted immunized hemolymph to CM column severely reduced the flow rate, necessitating 5-fold dilution of the sample. Under the experimental conditions used the majority of the hemolymph proteins was not adsorbed to the column and appeared as a single protein peak in the flow-through fractions. The unbound proteins did not show antibacterial activity under the conditions of assay. The 0.5 M ammonium acetate eluted out majority of the bound proteins which did not have antibacterial activity (Fig. 5). As shown in Fig. 5, a protein peak which showed antibacterial activity was eluted out with 1 M ammonium acetate. Fractions having antibacterial activity were pooled, concentrated, and applied to a Seph-

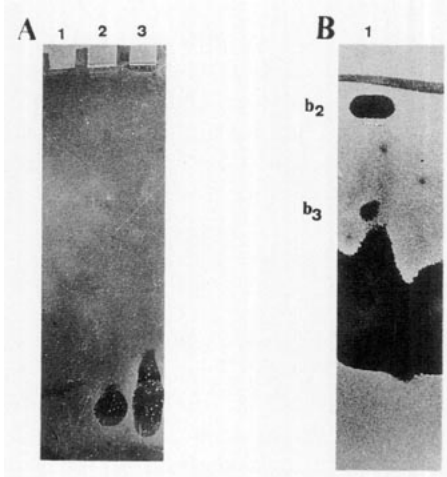


FIG. 2. Acidic PAGE of immunized and control hemolymph. Antibacterial activity was visualized as dark bands after overlaying *E. coli*. (A) Lane 1, 10 μl hemolymph of uninjected larvae; lanes 2 and 3, 10 μl each of immunized hemolymph collected 24 and 48 hr after injection with *E. coli*, respectively. (B) Lane 1, 30 μl of hemolymph collected after 48 hr injection.

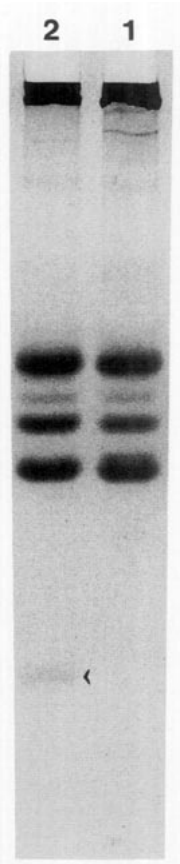


FIG. 3. Acidic PAGE of control and immunized hemolymph. Protein bands are detected by Coomassie brilliant blue staining. Lanes 1 and 2, 3 μl of control and immunized hemolymph collected 48 hr after injection, respectively. The arrow indicates the induced protein band.

adex G-100 column. The antibacterial activity was eluted as a single peak on gel filtration (Fig. 6) and the protein obtained from this step was found to be homogeneous electrophoretically and was active against bacteria (Fig. 4, lane 2). The purification procedure was quite reproducible and antibacterial activity of the protein purified from the hemolymph contributed to 40% of the total antibacterial activity of the immunized hemolymph with 500-fold increase in specific activity (Table 1).

### *Antibacterial Activity*

Purified protein (1 μg) was tested for its antibacterial activity against *E. coli, Serratia* spp., *B. thuringiensis, P. aeruginosa,* and *M. luteus*. The protein was not active against *P. aeruginosa,* and *B. thuringiensis* and showed very little activity against *Serratia* spp. (data not shown). The protein was found to have comparable activity against *E. coli* and *M. luteus* (Fig.7, row 1). Further, activity of the purified protein against *M. luteus* was sevenfold higher than that of the chicken lysozyme (Fig. 7, row 2).

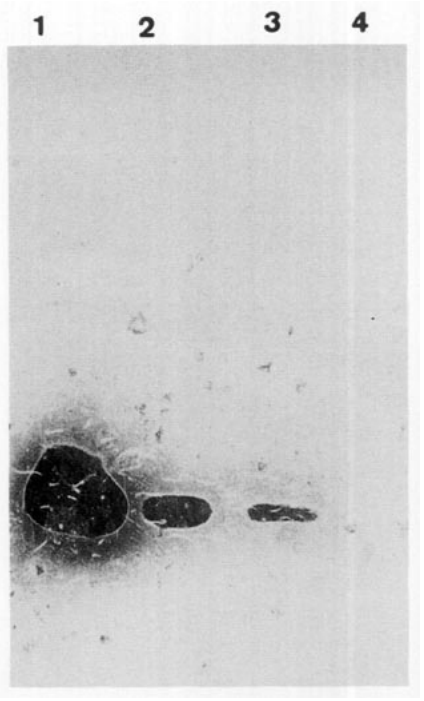


FIG. 4. PAGE of heat- and trypsin-treated immune hemolymph. PAGE overlaid with *E. coli*. Lane 1, 5 μl of immunized hemolymph collected after 48 hr of injection; lane 2, 5 μg of the purified antibacterial protein; lane 3, 5 μl hemolymph equivalent of heat-treated sample; and lane 4, 5 μl hemolymph equivalent of trypsin-treated sample.

### *Molecular Weight*

The purified protein was eluted after cytochrome c on the gel filtration column and a molecular weight of 11.5 kDa was determined by extrapolation of the elution volume of the standard proteins (data not shown).

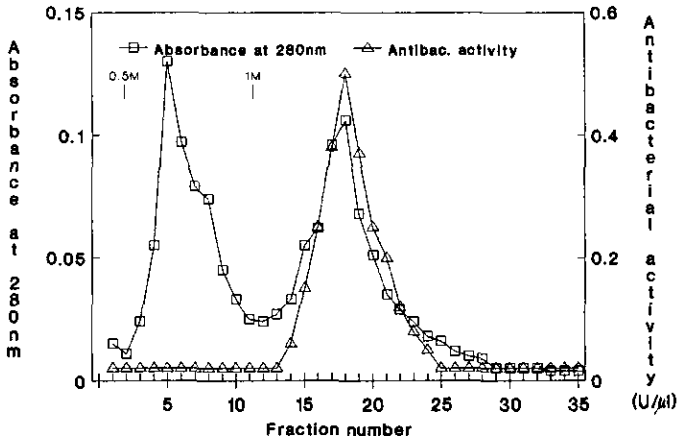


FIG. 5. Step I in the chromatographic purification of antibacterial protein. Five milliliters of immunized hemolymph after dilution was applied to a column of CM-Sephadex C-50, which after washing was eluted with step gradient of 0.5 and 1 M ammonium acetate, pH 7.0, as described under Materials and Methods. The fractions were assayed for antibacterial activity against *E. coli* and absorbance at 280 nm. The fractions having antibacterial activity were pooled.

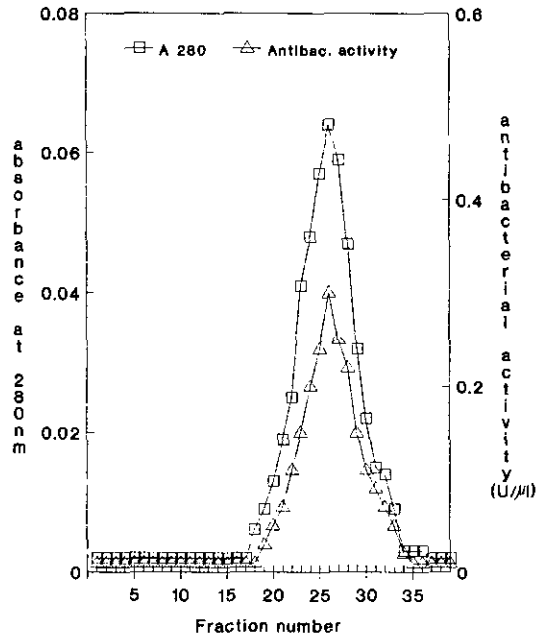


FIG. 6. Step II in chromatographic purification of antibacterial protein. Antibacterial peak collected from step I was applied to a Sephadex G-100 column as described under Materials and Methods. The fractions were assayed for antibacterial activity against *E. coli* and absorbance at 280 nm.

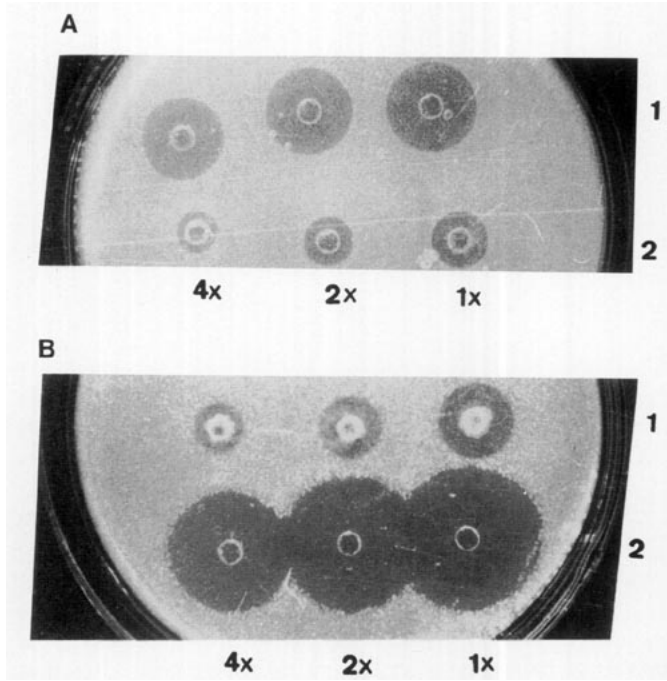


FIG. 7. Comparison of the antibacterial activity of the purified protein with that of chicken lysozyme by zonal inhibition assay. (A) Against *E.coli;* (B) *against M. luteus.* Rows 1x, 2x, and 4x are 1, 0.5, and 0.25 μg of purified antibacterial protein; rows 2, 1x, 2x, and 4x are 50, 25, and 12.5 μg of chicken lysozyme, respectively.

The subunit molecular weight of the purified protein was determined by SDS-PAGE. As shown in Fig. 8, lane 2, a single band whose molecular weight was estimated to be 16 kDa was observed.

### *Amino Terminal Amino Acid Sequence*

From the sequenced 20 N-terminal residues it appears that the purified protein resembles the other reported lysozymes purified from different animal species. The first residue is lysine. Histidine occurs at the 10th and 15th positions (Fig. 9). While lysine is found in all the other reported lepidopteran species and many of the vertebrates (Jollès and Jollès, 1984), the 10th histidine residue seems to be a characteristic feature of this protein.

## DISCUSSION

The results obtained in this study show that *Bombyx* larvae respond to injection of live *E. coli* by secreting antibacterial molecules into the hemolymph. This response is rapid as the hemolymph shows marked antibacterial activity 5–6 hr after injection. It is induced not only by injection of live bacteria but also by injection of sterile saline. The results are consistent with the observations of Dunn (1986) and Powning and Davidson (1973) who have reported the induction of anti-

TABLE 1
Purification of Antibacterial Protein from Silkworm Hemolymph

| Fractions | Volume (ml) | Total protein (mg) | Total lytic activity (U) | Recovery (%) | Specific lytic activity (U/mg) |
|---|---|---|---|---|---|
| Hemolymph | 5 | 460.00 | 20,250 | 100.0 | 44 |
| Purified protein | | | | | |
| Step I | 5 | 1.58 | 10,050 | 49.6 | 6578 |
| Step II | 2 | 0.85 | 7,890 | 38.9 | 9257 |

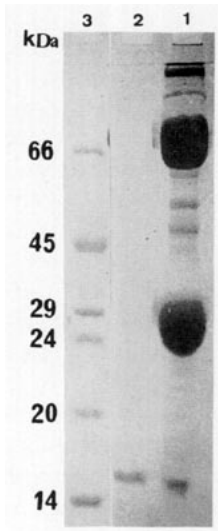


FIG. 8. SDS–PAGE of the purified antibacterial protein and immunized hemolymph. Lanes 1, 2, and 3, 3 μl of immunized hemolymph, 5 μg of purified antibacterial protein, and standard molecular weight markers, respectively.

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *Bombyx mori* | K | T | F | T | R | C | G | L | V | H | E | L | R | K | H | G | F | E | E | N |
| *Antheraea mylitta* | K | R | F | T | R | C | G | L | V | Q | E | L | R | R | Q | G | F | D | E | * |
| *Spodoptera littoralis* | K | Q | F | T | R | C | G | L | V | Q | E | L | R | R | Q | G | F | D | E | D |
| *Galleria mellonella* | K | T | F | T | R | C | G | L | V | Q | A | L | R | R | Q | G | F | D | E | A |
| *Hyalophora cecropia* | K | P | F | T | R | C | E | L | V | Q | E | L | R | R | R | G | F | D | E | T |
| *Chicken* | K | R | F | G | R | C | G | L | A | A | A | M | K | R | H | G | L | D | N | Y |
| *Human* | K | V | F | E | R | C | G | L | A | R | T | L | K | R | L | G | M | D | G | Y |

FIG. 9. N-terminal amino acid sequence of the purified antibacterial protein. Comparison of this sequence with the corresponding lysozyme sequence of other lepidopteran species, chicken, and human. Similar sequences are given in boxes.

bacterial substance in response to injection of saline. However, Morishima *et al.* (1988) failed to detect any antibacterial activity against *E. coli* in the saline injected silkworm larvae. The slight induction of antibacterial activity by the injection of sterile saline is presumed to be due to injury or to contamination of sterile solution with suspended particles (Boman and Hultmark, 1981; Keppi *et al.*, 1986; Okada and Natori, 1983; Trenczek, 1988).

The electrophoresis of immunized hemolymph samples followed by gel overlay with *E. coli* showed a major fast migrating antibacterial band and two minor bands (Fig. 2B). Since the major inhibition zone turned out to be a lysozyme type of protein (see following), we believe that the slow migrating electrophoretic bands belong to the attacin type of proteins as observed in most of the reported attacins of insects (Kaaya *et al.*, 1987). The antibacterial activity was purified by CM-Sephadex and Sephadex G-100 column chromatography. The molecular weight of the purified antibacterial protein was 16 kDa and is compatible with the molecular weight of lysozyme purified from different insect sources (Jollès *et al.*, 1979). However, the purified protein was active against both gram-negative and gram-positive bacteria, unlike lysozymes, which are reported to attack only gram-positive bacteria (Hoffman *et al.*, 1981). The amino terminal amino acid sequence of the purified protein has shown 75% homology with the corresponding amino acid sequence of lysozyme from *Antheraea mylitta* (Nagaraju *et al.*, 1992), 65% homology with those of *Hyalophora cecropia* (Engstrom *et al.*, 1985) and *Spodoptera littoralis* (Jollès *et al.*, 1979), 60% homology with that of *Galleria mellonella* (Jollès *et al.*, 1979), and 35% homology with chicken (Canfield, 1963) and human lysozymes (Jollès and Jollès, 1972). The 20 residue N-terminal sequence determined in this study is in complete agreement with the corresponding sequence of lysozyme of *B. mori* (Jollès and Jollès, 1984). An induced antibacterial protein of 15.5 kDa having activity against both gram-negative and gram-positive bacterial strains has been detected in the female reproductive accessory glands of medfly, *Ceratitis capitata* (Marchini *et al.*, 1991). Xylander and Neverman (1990) have reported the presence of an antibacterial substance other than lysozyme which is active against *M. luteus* and *Enterobacter cloacae* in the hemolymph of myriapods. The induced protein purified in this study belongs to the above-mentioned group with respect to its antibacterial property, but the molecular weight and partial primary structure put the protein unambiguously with the lysozyme group. The understanding of high activity of the protein purified in this study against *E. coli* as well as *M. luteus* requires further analysis of the three-dimensional structure and active sites. Lysozymes, in general, have been reported to be heat stable (Dunn, 1986; Powning and Davidson, 1976). However, the protein purified in this study is partially heat labile and is similar to the one purified from *H. cecropia* with respect to its thermal stability (Hultmark *et al.*, 1980).

Results of this study call for several comments. First, earlier studies (Morishima *et al.*, 1988, 1990) have purified cecropin-like peptides from the silkworm larvae immunized with *E. coli*. We have been unable to monitor, with the same purification protocols, the appearance of molecules showing properties of cecropins in the same insect. Morishima *et al.* (1990) failed to detect any lysozyme activity bound to CM-Toyopearl 650M at pH 7.0 in 0.3 M ammonium acetate. The plausible explanation for our inability to purify the cecropin type polypeptide may be due to either the difference in the ion exchange matrix (CM-Sephadex C-50 instead of CM-Toyopearl 650M) or the difference in the bacterial strain (*E. coli* JM 103 instead of *E. coli* K12M11) or both. The recent report shows that cecropin B gene expression in *B. mori* reaches maximum level 8 hr after injection of *E. coli* and decreases thereafter (Kato *et al.*, 1993). In light of this observation it is quite probable that we did not detect cecropin B in the induced hemolymph sample which was collected 48 hr after infection. Second, what is more significant is that Morishima *et al.* (1988) chromatographically separated two

antibacterial peaks, one active against *E. coli* and the other active against *M. luteus*. The latter fractions which were considered to be lysozyme showed no antibacterial property against *E. coli* under their assay conditions. On the other hand the antibacterial peak which we obtained gave high bacteriolytic activity against *E. coli* like most of the insect source cecropins (Boman, 1991; Kimbrell, 1991; Steiner *et al.*, 1981). It is very unlikely that the cecropin contaminations in our preparation would have given rise to this difference since the purified protein was found to be highly homogeneous on silver staining of SDS–PAGE (data not shown) and on sequencing. The lysozyme-like protein purified in this study accounts for more than 40% of the total antibacterial activity against *E. coli* in the immunized hemolymph under our assay conditions. This, in addition to its bacteriolytic activity against both gram-positive and gram-negative bacteria, shows that the highest level of lysozyme-like protein is produced in response to injection of bacteria and it is one of the major components of an extensive antibacterial response that includes cecropin type proteins in *B. mori* (Teshima *et al.*, 1986). This is in contrast to *H. cecropia* in which cecropins constitute main antibacterial factors in the hemolymph and the lysozyme functions as a clearing agent which takes care of debris left after cecropin action (Boman and Hultmark, 1981). In light of the current observations, more critical analysis of the biochemical and bactericidal nature of the antibacterial proteins is required to understand the role of lysozyme in the *B. mori* defense mechanism.

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