4. DISCUSSION
Biosynthesis of small cationic protein molecules in response to bacterial infections have been reported in insects belonging to the orders Lepidoptera, (Faye et al., 1975; Hultmark et al., 1980, 1982, 1983; Abu-Hakima and Faye, 1981; Hoffmann et al., 1981; Boman, 1982; Merrifield et al., 1982; Qu et al., 1982, 1986; Shiba et al., 1983; Engstrom et al., 1984; Qi et al., 1984; Dunn et al., 1985, Spies et al., 1986; Hara and Yamakawa, 1996), Diptera (Okada and Natori, 1983, 1985; Keppi et al., 1986; Robertson and Postlethwait, 1986; Ando et al., 1987; Flyg et al., 1987; Kaaya et al., 1987; Bulet et al., 1995; Chalk et al., 1995; Lowenberger et al., 1995), Coleoptera (Spies et al., 1986; Lee et al., 1994, 1996; Miyanoshita et al., 1996; Moon et al., 1996) and also in an exopterygote order Hemiptera (de Azambuja et al., 1986; Chernysh, et al., 1996; Miura et al., 1996). These proteins are reported to possess pronounced antibacterial defense reaction.

There have been reports of insects relying on humoral defense reactions. Insects are capable of developing resistance to lethal doses of virulent bacteria once they are exposed to attenuated bacterial cultures. Several types of insect proteins that have distinct bactericidal effect have been reported. These mainly fall into four categories namely cecropins, all of which possess an amide group at their C-terminal ends, insect defensins, all of which have an α helical structure, proline-rich peptides mainly isolated from hymenopterans and glycine-rich peptides which come under the category of attacins (Cociancich et al., 1994). Although these different peptide molecules could be induced by bacterial inoculations, more interestingly insects can synthesize a large number of antibacterial peptides and polypeptides in response to injury. It is also true that from a single species of insect more than ten different antibacterial molecules have been isolated (Hoffmann et al., 1993).

In the present work at least three proteins with antibacterial activity have been identified and one of them was isolated and characterized from the induced haemolymph of M. domestica which possibly belongs to the family of cecropins. M. domestica, like many other insects, can be induced to synthesize
and release the antibacterial factors in response to bacterial inoculation or injury.

Injury to insects is known to stimulate several metabolic events, protein synthesis being one among them (Telfer and Williams, 1960; Barth et al., 1964; Marek, 1969; Cherbas, 1973). In *M. domestica* it is found that the immune response arising out of the injuring of insect with a hypodermic needle, is more or less similar to the one obtained by challenging the insects with specific bacteria. The difference in response could only be attributed to a more significant antibacterial activity in bacteria inoculated insects as against the relatively less specific activity exhibited by the injured insects. In *H. cecropia* a sham injection with sterile salt solution resulted in eliciting a lesser bactericidal activity in the haemolymph (Boman et al., 1981). In *S. peregrina* the injury caused by a hypodermic needle resulted in the induction of antibacterial proteins (Okada and Natori, 1983). Similarly, *P. terronovae* larvae could be induced to synthesize antibacterial proteins by the pricking process (Keppi et al., 1986).

Studies on comparisons in the immune response between the one obtained by inoculation of bacteria and the other by injury is not extensively available in literature. Nevertheless, in the pupae of cecropia moth Boman et al. (1981) reported that SDS-PAGE pattern of haemolymph proteins synthesized as a response to an injection and injury was more or less the same, although, a high molecular weight protein P5 was lower in concentration in the haemolymph obtained by injury. Further, comparing the immune response to infection and injury Boman et al. (1981) commented that injury could be the start of infection and hence the immune response also occurred in the same way. Keppi et al. (1986) commented that the similarity in response to injury and injection of high dose of bacteria was indicative of the existence of extremely subtle mechanism that could recognize stimuli in a broad sense. In contrast to these observations, in *D. melanogaster*, it was reported that the antibacterial activity was confined only in those flies that were inoculated with the bacteria.
Neither injection of saline nor injury by pricking showed antibacterial activity (Robertson and Postlethwait, 1986).

In a more interesting study *Galleria mellonella* larvae were injected with 1 μ diameter polystyrene beads at different doses. Some larvae received $1 \times 10^5$ living bacteria/larva initially, followed by a booster injection of $2 \times 10^6$ living bacteria/larva. Survival rate was very high among the latex injected larvae although the relative units of antibacterial activity was high in those larvae which received live bacteria. Nevertheless, the haemolymph of wax moths that received a high concentration of $3.6 \times 10^7$ latex beads exhibited a very high antibacterial activity (Wiesner, 1991).

Wiesner (1992) has used variously treated latex beads such as acid treated beads, beads loaded with proteins such as fibronectin from bovine plasma, BSA, poly-L-lysine and adjuvant peptides as inducers of antibacterial activity. The experiments of Wiesner suggested that inert evokators were powerful tool for eliciting an immune response in the insects.

As a prelude to isolation and characterization of the induced antibacterial substances in *M. domestica*, an attempt was made to establish an immunoidentity between the immune serum of housefly larvae and the soluble proteins of bacteria. It was interesting to note that immune serum of housefly larvae behaved almost like a vertebrate humoral factor and formed specific precipitin band on immunodiffusion plates. In the present work it was possible to characterize the immune serum as a heat-stable and trypsin digestable protein based on the double diffusion technique of Ouchterlony.

The immunodiffusion procedure also established that the precipitin reaction is concentration dependent, as the dilute serum formed precipitin line closed to antiserum well and concentrated serum close to the antigen well. In such experiments one could draw a parallel between the immune serum of insects and vertebrate antibody.
Periplaneta americana challenged by honeybee venom produced a humoral factor that behaved like an antibody molecule forming precipitin lines with homologous antigens on agar gel plates (Rheins and Karp, 1982). The true adaptive immune response said to be exhibited by the American cockroach appears to be an unique phenomenon. There are no other reports available on the occurrence of insect humoral factors capable of precipitating antigens on Ouchterlony gels. Although immune haemolymph of M. domestica larvae is capable of precipitating antigen on agar gel and its activity disappears on trypsin treatment, the immune response cannot fall under the category of possessing specificity. The immune haemolymph of M. domestica larvae can possess the bactericidal activity not only against M. luteus that was used for induction but against several Gram negative and Gram positive bacteria as well, indicating the non-specific nature of the immune response. Thus P. americana is the only instance to date of an insect exhibiting specific immune response and further experiments are needed to substantiate the hypothesis (Boman and Hultmark, 1987). Further, in the larvae of M. domestica the immunoelectropherograms showed that the precipitin arc was formed very close to one end of the microslide, suggesting that, as a low molecular weight protein, the substance was moving faster under the influence of an electric field.

The protein profile of induced haemolymph of SDS-PAGE has been demonstrated in several insects. Faye et al. (1975) compared E. cloacae injected pupal haemolymph of Samia cynthia and H. cecropia with respect to leucine labelled haemolymph proteins. They found that in both the pupae the leucine was distributed to nine protein bands, which were designated as P1 to P9. Among these proteins, the densitometer scan of gels showed two larger and four smaller peaks while the remaining three peaks were poorly separated. Further, Hultmark et al. (1980) identified P7 and P9 as lysozyme and cecropin respectively and P5 as attacin. Hughes et al. (1983) have reported five bacteria induced proteins in larvae and pupae of Manduca sexta. Among which M11 and M23 were comparable to P4 and P7 obtained from H. cecropia respectively.
Further, in *M. sexta*, Hulbert *et al.* (1985) demonstrated a larva-specific protein named as scolexin, formerly designed as M13. According to Spence *et al.* (1992) this protein was inducible and non-bactericidal one.

Administration of *B. sphaericus* spore to the larvae of *B. mori* resulted in the qualitative and quantitative changes in the haemolymph proteins (Madhavan and Velpandi, 1987). About 11 bacteria induced proteins have been detected in the larvae. Of the 11, only three proteins 50, 47 and 37.5 KD showed maximum synthesis. Robertson and Postlethwait, (1986) found that the bacteria inoculated *D. melanogaster* males induced eight new polypeptides which were called as antibacterial response (AR) polypeptides. The size of these polypeptides ranged between 5 KD and about 75 KD. AR10 was the most prominent band that appeared within 4h of inoculation. AR19, AR24 and AR75 increased with the time consistent course with the increase in antibacterial activity. The fractions AR19, AR22 and AR23 obtained from two dimensional electrophoresis were homologous in size and shape as compared to attacins of cecropia moth (Hultmark *et al.*, 1983).

According to Kaaya *et al.* (1986), the haemolymph of *Glossina morsitans morsitans* showed a remarkable increase of two proteins with molecular weight of approximately 17 and 70 KD after the injection of live *E. coli* and *E. cloacae*. Contrarily, the haemolymph induced with *M. luteus* and *B. subtilis* showed no response. Similarly, fly injected with the *Trypanosoma brucei brucei* showed no such response. The increase in the intensity of these proteins was recorded at approximately 18h after inoculation of bacteria. The injection of 60 µg/g of honeybee toxoid into female American cockroach revealed the appearance of two new haemolymph proteins with molecular weight 162 and 220 KD. Densitometer scans showed that proteins with 115, 102, 95 and 45 KD in increased quantities, as compared to those of control insects. Interestingly, the authors demonstrated that the 102 KD protein increased consistently in
quantity following immunization of both males and females with protein antigens (George et al., 1987).

In the present investigation, more or less similar pattern of protein profile was observed in the induced haemolymph of *M. domestica* larvae. Haemolymph collected after different hours of injection of bacteria into *M. domestica* larvae, when subjected to SDS-PAGE showed an increase in the quantity of several proteins in general and proteins of low molecular weight ranging from 15 to 20 KD in particular. Totally 41 subunits were resolved with molecular weight ranging from 14 to 120 KD. While 13 subunits of the induced haemolymph showed an increase in quantity, three subunits showed a reverse trend. 50% of these induced subunits were of low molecular weight proteins ranging between 15 and 20 KD which may correspond to lysozyme and attacin of other dipterans and lepidopterans.

IZA has been routinely used for demonstrating the inducible humoral antibody response. In *M. domestica*, the haemolymph obtained from induced larvae exhibited a distinct antibacterial response when assayed for the formation of inhibition zones. These inhibition zones occur as clear, bacteria-free zones around the wells containing the immune serum. The diameter of the clear zones or the inhibition zones appear to increase proportionately to the concentration of the immune serum. Therefore, this assay can also be used to determine the minimum titres of antibacterial substances required to elicit an immune response.

IZA have revealed that in the larvae of *M. domestica*, 2 µl of induced haemolymph, free of haemocytes, inhibit the growth of bacteria to a diameter of nearly 12 mm. Four fold dilution of haemolymph reduced the diameter of the inhibition zone by about 75%. In other words, the inhibition zone was only 3.10 ± 0.52 mm. Since the well itself is 2 mm in diameter, the 25% of 2 µl that is 0.05 µl is the minimum concentration required to produce an inhibition zone.
In the milk weed bug, *Oncopeltus fasciatus*, the undiluted immune serum could elicit a 100% inhibition, a hundred fold dilution brought about about 15% inhibition and a thousand fold dilution, did not inhibit the bacterial growth. In all these cases the bacterial concentration was 2,800 cells in 0.1 ml (Gingrich, 1964). Interestingly, in milk weed bug the normal insect serum could by itself inhibit nearly 30% of bacterial growth. In the larave of *M. domestica* the normal serum did not elicit an immune response against the different species of bacteria tested except that in *P. aeruginosa*, a minimum inhibition of bacterial growth of 3 mm could be obtained.

Postlethwait et al. (1988) in a comparative IZA study of *D. melanogaster* and medfly *Ceratitis capitata* reported that the medfly adult males had four times the antibacterial activity as that of *D. melanogaster* adult males and the medfly larvae had four times higher activity than their adults. The authors attribute the difference in the activity to the size of the insects. Since the males of *C. capitata* which were about eight times as large as *D. melanogaster* males, antibacterial activity is about half as concentrated in the *C. capitata* haemolymph compared to *D. melanogaster* haemolymph. It is also reported that in medflies, one unit of antibacterial activity is required to form 1 mm inhibition zone around a well with 2.4 mm diameter.

In yet another comparative study it was found that the immune haemolymph from cecropia pupae showed that the activity in *tsetse* fly was about one-sixteenth of that in cecropia (Kaaya et al., 1987). *D. melanogaster* appears to have a potent antibacterial activity since as low as a concentration of 0.05 μl of haemolymph could effectively produce an antibacterial response (Robertson and Postlethwait, 1986).

In the present studies the antibacterial activity of the immune haemolymph of *M. domestica* larvae was assayed by several procedures. These procedures were useful for obtaining semi quantitative data on the efficacy of the antibacterial substance.
In lytic assay the survival of bacteria in liquid cultures treated with immune serum was estimated by measuring the OD of the medium over a period of 24h. The results suggested that the bactericidal effect of antibacterial substance was rather instantaneous in cultures treated with immune haemolymph whereas in control cultures the exponential growth was very evident. The OD measurement did not register any significant increase over 24h period. Similar results were obtained when the haemolymph immunized against *M. luteus* was included in the culture medium of *E. coli*, *P. aeruginosa* and *B. thuringiensis* suggesting the non-specific immune response of the antibacterial serum obtained from housefly larvae and a possible similarity in the mode of killing. *S. aureus* appears to be resistant to the immune serum obtained from housefly larvae as the cultures of the bacteria exhibited a normal exponential growth very similar to that obtained in control cultures.

The killing assay procedure established that the bactericidal action was more or less completed by about 4h after the introduction of the immune haemolymph into the medium. Bacterial growth curves generally indicate that during this period cells are in the lag phase and there is a limited growth. Analysis of the rate of mortality during the first 4h indicated that there was a progressive bactericidal activity. In other words, the bacterial numbers gradually decreased and by the end of 4h, the entire population was wiped out as evidenced by number of CFU that were obtained on the petriplates. Essentially the bacteria were killed over a period of time and not immediately after the addition of the immune haemolymph to the medium. Growth was normal in medium containing control haemolymph. Interestingly, in medium lacking nutrients the immune haemolymph did not cause mortality and the bacterial number remained constant. This observation over a period of 4h suggested that the bactericidal action affect the growth and multiplication of bacteria.

In *S. peregrina*, a study on the time course of loss of bacterial viability on incubation with the antibacterial substance showed that more than 50%
bacteria were killed in 10 min when incubated with antibacterial substance (Natori, 1977).

In *Rhodnius prolixus*, the immunized haemolymph caused a mortality very similar to the one observed in *M. domestica*. After 150 min of incubation in a peptone medium at 37°C viable bacterial number were dramatically reduced (de Azambuja et al., 1986). On the contrary, cells incubated at 4°C or in a non-growing medium did not show reduction in number. The results obtained in *M. domestica* and *R. prolixus* indicated that inducible antibacterial activity could be related to the defense mechanism of the insect against bacterial infection. Further, the fact that the antibacterial substance acts by causing a decrease in the number of CFU is indicative of its bactericidal action rather than the bacteriostatic action.

In an attempt to identify the basic proteins that are directly responsible for antibacterial activity acidic PAGE was carried out in combination with antibacterial assay on gel. The immune haemolymph of *M. domestica* larvae when subjected to acidic PAGE followed by an antibacterial assay on gels, showed two regions of antibacterial activity. The first region closer to the origin represented relatively high molecular weight proteins and the second region was resolved closer to the central region of the gel. Based on the width of inhibition zone it could be suggested that the second region probably consists of two proteins of relatively low molecular weight.

In the present work not much attention was paid to the high molecular weight immune proteins obtained from the housefly larvae. However, considering their location on the gel and pronounced antibacterial activity which they produce, the first region of antibacterial activity obtained on the gels could possibly be due to attacin-like molecules similar to the ones identified from *H. cecropia* (Hultmark et al., 1983). Subsequently, during the purification of antibacterial substance certain proteins that were eluted in the void volume did exhibit antibacterial activity. The proteins that exhibited antibacterial
activity on gel and the ones eluted in the void volume could be one and the same.

The antibacterial substances observed in the central region of the gel were the ones, that were further purified in the present studies. The fact that these basic proteins have relatively a faster electrophoretic mobility and therefore should be of relatively low molecular weight, coupled with the observation that they have potent bactericidal activity against Gram positive and Gram negative bacteria and inactive on eukaryotic cells, indicate that they are probably cecropin-like molecules.

In *H. cecropia*, two inducible bacteriolytic proteins very distinct from lysozyme fraction were identified and characterized. Both the proteins were shown to be potent against *E. coli* and several other Gram negative bacteria. They are heat stable and possessed more or less similar amino acid composition (Hultmark *et al.*, 1980). The two proteins designated as P9A and P9B and more commonly as cecropin A and cecropin B respectively were the first antibacterial proteins reported in cecropia moth and subsequently several authors reported the occurrence of cecropin-like molecule in other insects as well (Hoffmann *et al.*, 1981; Okada and Natori, 1983; Shiba *et al.*, 1983; Flyg *et al.*, 1987; Kaaya *et al.*, 1987; Keppi *et al.*, 1986; Spies *et al.*, 1986). In addition to the two proteins, another major antibacterial protein that was isolated and characterized was cecropin D. Minor cecropins C, E, F and G were also identified but not characterized in the major way. The identification of all these proteins was made by acidic PAGE followed by the seeding of gel with bacteria to locate the zones of non-growth of bacteria. The isolation and characterization of antibacterial proteins in other insects were made with reference to cecropins A, B and D of *H. cecropia* (Hultmark *et al.*, 1980, 1982).

Keppi *et al.* (1986) identified 5 heat-stable basic proteins in *P. terranovae* that exhibited antibacterial activity with *E. coli*. The identification of these five proteins was done by acidic electrophoresis followed by seeding the gel with *E. coli*. Hoffmann *et al.* (1981) identified two small basic proteins in
*G. mellonella* similar to cecropin A and cecropin B. They were separated by polyacrylamide gel at pH 4 and visualised later by seeding the gel with viable *E. coli*.

Kaaya *et al.* (1987) demonstrated both cecropin and attacin-like factors in the immune haemolymph from tsetse fly. The antibacterial activity of proteins belonging to these two families were detected on the gels. In *Antheraea pernyi*, Qu *et al.* (1982), identified two proteins from the induced haemolymph of diapausing pupae that corresponded more or less with cecropin B and D of *H. cecropia*. This analysis was again made by electrophoresis on acidic gels and the subsequent assay for antibacterial activity. Similarly in the present work the gel was sandwiched with an indicator bacteria and incubated overnight to identify cecropin and attacin-like proteins, as regions of non-bacterial growth.

In the darkling beetle *Eleodes*, Spies *et al.* (1985) demonstrated three zones of inhibition on polyacrylamide gel, of which one was attacin-like antibacterial protein and the other two were cecropin-like antibacterial proteins of *M. sexta*. In *D. melanogaster*, the acid electrophoresis in combination with antibacterial assay led to the identification of both cecropin and attacin-like components (Kaaya *et al.*, 1987). The attacin-like components of *D. melanogaster* had relatively faster mobility than attacins of cecropia. The acid electrophoresis-cum-antibacterial assay studies further showed that one of the antibacterial proteins of *D. melanogaster* had an electrophoretic mobility very close to that of cecropin A from cecropia moth (Flyg *et al.*, 1987).

In the medfly *C. capitata*, the inoculated *E. cloacae* as well as a sterile wound can induce antibacterial immune response. Four different factors both from larvae and adult medfly and an additional factor from larvae were shown to possess the antibacterial activity (Postlethwait *et al.*, 1988). Okada and Natori (1983) separated three antibacterial activity zones from the haemolymph of injured larvae of *S. peregrina* and one protein was purified to homogeneity.
Thus the acidic PAGE and subsequent antibacterial assay, very useful techniques for identifying the proteins with antibacterial activity, were used for the identification of antibacterial substances in *M. domestica* larvae and the substances were subsequently purified by suitable procedures.

The rapid inducibility of antibacterial activity has been demonstrated in several insect species. In the present studies the antibacterial activity makes it appearance 3h after the inoculation of the larvae of *M. domestica*. Presumably during this period the processing and sequestering of the bacteria through the fat body and secretion into the haemolymph occur. By about 24h maximum activity could be recorded. The activity recedes slowly by about 48h and could be observed until the second day of pupation. Haemolymph collection after 48h is rather difficult due to hardening of pupae.

Faye *et al.* (1975) vaccinated the diapausing pupae of *H. cecropia* and *S. cynthia* with viable *E. coli*, *E. cloacae* and *B. subtilis* and observed a lag period of about 10h before immune reaction and the commencement of antibacterial activity. The activity thereafter showed further increase and reached a maximum after two to four days. The antibacterial activity was demonstrated in *Locusta migratoria*, 4h after the immunization (Hoffmann, 1980). Enhanced activity could be observed from 4h onward. The activity decreased around 6-8 days after the inoculation and totally disappeared after 15 days. In *P. terranovae*, Keppi *et al.* (1986) observed the antibacterial activity after 5-6h of injection. Injured larvae also responded similarly.

In *G. mellonella* and *Pieris brassicae*, there was a lag period of 7h since the time of inoculation and appearance of antibacterial activity. This is also true of insects like *H. cecropia* and *S. cynthia*. In both these forms the activity persisted for several days and quantitative differences in the activity could be served between the insect species and such differences could be attributed as well to the inducer strain used (Jarosz, 1993). Casteels *et al.* (1989) reported
that in *A. mellifera* the activity appear in the haemolymph nearly after 8h of injection.

In *D. melanogaster*, rapid inducibility and long lasting antibacterial activity have been reported by Robertson and Postlethwait (1986). The activity appeared within 2h after inoculation and increased within 48h and persisted for about 65 days.

Studies on *M. domestica* as well as those cited in other insects suggest that induction of antibacterial activity is rapid once the insect is inoculated with specific bacteria. The activity lasted for a considerable length of time in most cases upto 24h, thereafter the activity weakened slowly.

Several workers have attempted successfully the isolation, purification and characterization of specific antibacterial proteins by using a mixture of cation exchange chromatography and gel filtration techniques. The cation exchange chromatography may also be used in several steps to obtain homogenous proteins.

Hultmark *et al.* (1982) outlined the scheme for the purification of antibacterial factors in haemolymph from immunized cecropia pupae. In this scheme, gel filtration technique lead to the separation of two pools of proteins, Pool I containing attacin-like substances and Pool II containing cecropin-like substances. Pool II was subjected to ion-exchange chromatography. This procedure led to the separation of minor and major antibacterial proteins. At least, two pools could be obtained from the CM-Sepharose chromatography, of which Pool II was treated with ammonium sulphate to precipitate the proteins which were subsequently passed through Phenyl-Sepharose column that removed the cecropin D molecule. Some fractions of this step as well as Pool II of cation exchange chromatography were passed through another column of CM-Sepharose to resolve various minor (cecropin D, E, F and C) and major (cecropin A and B) fractions. Lysozyme, a common lytic enzyme was also separated in pure form. In all these cases the active fractions were pooled,
freeze dried and dissolved in a small volume of water and were analysed for biological activity.

More or less a similar approach was followed in isolating and purifying the antibacterial proteins from *M. domestica* larvae, except that the step I was the cation exchange chromatography followed by heat treatment of fractions collected in the step I to retain only the basic proteins. The next step was the gel filtration technique that separated the molecules based on their size. Three distinct peaks of activity were obtained in this step. Fractions of peak I were collected in the void volume and exhibited a pronounced antibacterial activity. Although, the fractions of the peak I were not further processed, it could be reasonably argued that they constituted attacin-like substances. In general the attacins are high molecular weight substances and are therefore collected in the void volume during gel filtration.

Peak II also showed marked antibacterial activity and as determined later consisted of proteins with molecular weight close to 8 KD. The last peak in the Sephadex G-50 column had relatively a low antibacterial activity and possibly had a low molecular weight as well. Since only a low antibacterial activity was obtained, peak III fractions was also not processed further.

Peak II was further purified using CM-cellulose. This step IV purification yielded once again two peaks of activity which were respectively termed as MIIA and MIIB. In other words, the lytic fraction II of haemolymph of *M. domestica* larvae obtained through Sephadex G-50 column resolved into two fractions A and B on CM-cellulose. MIIB was subjected to last step purification by passing it through the cationic column of hydroxyapatite. A homogenous fraction of MIIB in purified form was obtained with a very high degree of biological activity.

Following isolation and purification of cecropins from *H. cecropia*, Shiba *et al.* (1983), reported on the occurrence of another self-defense substance from silkworm, the lepidopteran. The purification procedure was more or less
similar to one developed by Hultmark et al. (1982), except that the ammonium sulphate was used to precipitate the proteins directly from the haemolymph and the proteins were gel filtered.

Okada and Natori (1983) isolated three antibacterial proteins from the induced haemolymph of *S. peregrina* and the purification procedures consisted of initial passing of haemolymph through a cationic exchange column followed by gel filtration using Sephadex G-50. The fraction G II obtained from the latter step was further fractionated, on column of CM-cellulose that yielded two fractions of antibacterial activity. Fraction C II which showed a high degree of antibacterial activity was further purified on a column of hydroxyapatite to obtain the homogenous protein that was termed as sarcotoxin I.

Following these pioneering studies, the list of antibacterial proteins isolated and purified has been ever increasing. A new procedure was followed in the isolation and purification of an inducible antibacterial protein named tenicin I from the larvae of coleopteran *Tenebreo molitor*. Moon et al. (1994) followed a two step purification procedure, the first one being a reverse phase (C$_{18}$) open column chromatography of the heat treated crude haemolymph solution, followed by reverse phase high performance liquid chromatography (RP-HPLC). Similarly in *Holotrichia diomphalia*, Lee et al. (1994) identified an antibacterial protein holotricin II using the procedure that involved a CM-cellulose and Sephadex G-50 column chromatography followed by RP-HPLC.

In the isolation and purification of the antibacterial peptides from honeybees, Casteels et al. (1989) resorted to analytical chromatographic procedures using RP-HPLC. The study led to the identification of a new family of inducible peptide antibiotics, the apidaecins that possess a bacterostatic rather than the lytic capability. Subsequently, Casteels et al. (1990) also isolated a proline-rich antibacterial protein, abaecin that was purified repeatedly in HPLC columns. Thus in the purification of several antibacterial
proteins from different insects, the chromatographic procedures were extensively used. While the earlier workers depended on gel filtration and ion-exchange chromatography for the isolation and purification procedures, recent workers have switched over to more powerful and sensitive technique such as RP-HPLC.

Cecropins kill Gram positive and Gram negative bacteria, but ineffective against eukaryotic cells. They are small cationic peptides with a molecular weight of 4 KD. Insect defensins are known to be active only against Gram positive bacteria and the proline-rich peptides are sensitive only against Gram negative bacteria. Glycine-rich polypeptides, the attacins, have a molecular mass higher than 20 KD and although they are active against Gram negative cells, the activity spectrum sometimes includes Gram positive cells also.

MIIB, an antibacterial protein from *M. domestica* larvae is active against both Gram positive and Gram negative bacteria but is apparently inactive on eukaryotic cells, a property which is common to all cecropins. The molecule is slightly larger than the cecropins as the latter are all 4 KD cationic peptides. MIIB has an apparent molecular weight close to 8000. Based on these limited available similarities MIIB could be characterized as belonging to family of cecropins.

The various steps followed in the purification procedures of antibacterial proteins in *M. domestica* larvae led to a progressive decrease in the recovery of the protein but resulted in an increased specific activity of the molecule. When the haemolymph was used as direct source, the specific activity against bacteria was 0.9 units/mg, but there was a 34000 fold increase in the specific activity of final purified product. The sarcotoxin I obtained from flesh fly *S. peregrina* also showed 30,000 fold increase in the specific activity compared with the original haemolymph (Okada and Natori, 1983).

The cecropin A has over 80,000 units/mg of specific activity, much higher than the ones obtained from the bulk immune haemolymph. Similarly,
cecropin B is over 60,000 times more potent than the original haemolymph from which it was isolated (Hultmark et al., 1980).

Okada and Natori (1984) in explaining the mode of action of sarcotoxin I obtained from S. peregrina found that this protein disrupted the membrane potential by blocking the uptake of tetraphenylphosphonium ions and proline. To determine the bacterial activity of MIIB, it was subjected to killing assay. The results of such an assay showed a dramatic reduction of viable M. luteus from $10^5$ cells to 50 CFU within 1h. Similar results have been reported by Hultmark et al. (1980) for P9B of cecropia pupae. When 8 units/ml of cecropin was incubated with $10^8$ viable cells of E. coli D31, a drastic reduction to less than $10^5$ cells was observed, within a min. Dimarcq et al. (1988) recorded a dramatic drop in the number of viable count after 60 min when E. coli D31 was incubated with the semipurified diptericin A. As opposed to this, the viability of bacteria suspended in saline was not affected by diptericin A. Okada and Natori (1984) observed that the viability rapidly decreased irrespective of growth state of bacteria and the binding of sarcotoxin I to bacteria is temperature dependent.

Bactericidal effects of a partially purified antibacterial substance and a completely purified MIIB are different. Antibacterial substance obtained after the step II purification when incubated with M. luteus caused the complete lysis of the bacteria. In contrast, MIIB did not cause such a profound effect but the number of bacteria in a bacterial culture was reduced. These two observations do suggest differences in the mechanism of action between MIIB and step II purification product. Step II purification product was shown to contain attacin-like large molecular weight substance and a cecropin-like low molecular weight substance. Both these substances are able to kill the bacteria when used in microquantities. Attacins are known to affect dividing cells of E. coli and other Gram negative bacteria, and are known to cause them to grow in long chains (Hultmark et al., 1983; Ishikawa et al., 1992). It is further suggested that the attacin blocks the synthesis of major protein of outer membrane in E. coli (Hultmark, 1993). In the present studies there is no much evidence to
suggest the specific role of attacin, but the step II purification product which contains both attacin-like and cecropin-like antibacterial substances could cause the lysis of the bacteria possibly by a synergistic action. MIIB also kills the bacteria but not as rapidly as it would occur in the presence of attacin-like substances.

It has been reported that in *P. aeruginosa* when treated with immune haemolymph obtained from *G. mellonella*, there was a rapid *in vitro* bactericidal action effected by extensive cell breakdown. The damage was directed towards the cell wall leading to total loss of cellular integrity (Chadwick *et al.*, 1982). The loss of viability in *E. coli* cells that were treated to sarcotoxin I, a cecropin-like substance, was also shown to begin with the membrane breakdown. It is suggested that the sarcotoxin I binds to bacterial membrane. More significantly the inner membrane was affected on treatment with sarcotoxin I and there is a loss of membrane potential (Okada and Natori, 1984). In explaining the selective toxicity of sarcotoxin I to bacteria, Nakajima *et al.* (1987) suggested that the absence of cholesterol in bacterial membrane could be one of the reasons for the sensitivity of bacterial cells to the presence of antibacterial substance. In eukaryotic cells, cholesterol the intrinsic component of cytoplasmic membranes may possibly prevent the biological activity of sarcotoxin IA.

Steiner *et al.* (1988) studied the mechanism of action of cecropin using liposomes as a model system. The bilayer was efficiently destroyed if the liposome net charge was zero or negative. He further suggested that N-terminal end was essential for binding. Shortening of N-terminal helix by one residue had no effect, whereas removal of two residues lowered the lytic activity drastically. Further studies using radioactive cecropin on binding and bacterial viability suggested that there was no differential binding, rather different bacterial species resisted different amounts of bound cecropin before they underwent lysis. Mammalian erythrocytes are not affected by cecropin since they avoid the binding of antibacterial substance.
In a detailed study involving three cecropins and their synthetic analogues it was shown that the antibacterial substances form large time variant and voltage dependent ion channels in planar lipid membrane. Cecropin A and D together formed effective channels and are the most potent antibacterial peptides against several bacteria (Christensen et al., 1988).

Further, a tentative model for the interaction of cecropin with a lipid bilayer membrane was also suggested that the cecropin first adsorbs to the bilayer by electrostatic forces and inserts its hydrophobic segment into membrane core and by applying voltage, it undergoes a major conformational rearrangement, resulting in a channel formation. Sarcotoxin-I (Okada and Natori, 1984) and megainins (Zasloff, 1987, 1992) were the two antibacterial peptides related to cecropin work in a similar fashion.

In addition they have found that the positively charged phospholipids and cholesterol decrease cecropin channel formation by 5 and 60 respectively. Since eukaryotic membranes are rich in cholesterol, this may explain in part the selectivity of cecropin for bacterial cells, which lack cholesterol and have very low levels of positively charged phospholipids.

Whereas a spectrum of bacteria was affected in varying degrees in *in vitro* preparations using the antibacterial substance from housefly larvae, eukaryotic cells such as the yeast remained totally unaffected at a concentration of 1 μg/ml. This seems to be the property of various antibacterial substances that have been reported so far. Considering the fact that there are reports of antifungus substances (Natori, 1994), it appears that the antibacterial substance have limited specificity in that their action and the activity is confined against only bacteria.

Despite the specificity of antibacterial substances particularly cecropins to the bacteria (and not to eukaryotic cells) there appears to be a variation in the degree of specificity to various species of bacteria. This essentially means that some species of bacteria are more sensitive to the antibacterial substance than certain others. For instance lepidopteran, an antibacterial substance, from
B. mori, when tested against a spectrum of bacteria showed that E. coli, Aerobacter aerogenes, Shigella sonnei, Salmonella enteritidis required a MIC of 4 μg/ml for complete lysis. S. aureus required double the concentration and species of Pseudomonas and Streptococcus required four times the MIC required for E. coli (Shiba et al., 1983). Similarly, the purified antibacterial protein from S. peregrina was required in concentration as low as 0.08 μg/ml for the lysis of S. sonnei and B. megaterium and 0.3 μg/ml was required for species such as Proteus vulgaris. Corynebacterium bovis and M. flavus. P. aeruginosa and Sarcina lutea were resistant even at a high concentration of 1 μg/ml (Okada and Natori, 1983).

H. cecropia pupae when incubated with various species of bacteria at a concentration of 10 units/ml of purified proteins, cecropin A and B, showed that only < 0.25 min were required to kill E. coli, 12 and 15 min respectively of cecropin A and B for E. cloacae and more than 120 min for Serratia marcesens (Hultmark et al., 1980).

Cecropin A, B and D also showed differences in MIC required for killing the various bacterial species. 0.19 μM of cecropin A was sufficient for E. coli, whereas 0.32 and 0.43 μM of cecropin B and D respectively were required for the same species. In another example, 0.9 μM of cecropin B was sufficient to cause the lysis of P. aeruginosa a concentration of 4.8 moles and 100 moles of A and D respectively were required to carryout the lysis (Hultmark et al., 1982). Similar reports are also available for the cecropins from A. pernyi (Qu et al., 1982).

Different bacterial species show differences in sensitivity to the presence of MIIB in the medium. M. luteus is lysed at MIC as low as 0.1 μg/ml. P. aeruginosa, B. subtilis and B. thuringiensis require twice the quantity. E. coli strain is lysed at four times the MIC required for M. luteus. Other species such as B. polymyxa, B. megaterium E. cloacae, S. aureus and E. coli
from human source require 15 times greater concentration when compared to \textit{M. luteus}.

Like most cecropins MIIB is equally effective against Gram negative and Gram positive bacteria. In general it appears that the coliform bacteria \textit{E. cloacae, S. aureus} and \textit{E. coli} from human source appear to be more resistant to MIIB.

Interestingly the antibacterial peptides from honeybees collectively called apidaecins are known to be active against a wide range of plant associated bacteria as well as human pathogen and the mode of action appears to be bacteriostatic rather than lytic. The MIC required to neutralize some of the plant bacteria ranged between 0.02 and 0.50 |\mu|g/ml, whereas concentrations required against other species of bacteria ranged between 50 and 200 |\mu|g/ml. Although the induced antibacterial substances are known to arise as a result of a nonspecific immune response, there appears to be certain limited specificity as it is known that some antibacterial substances act against the Gram positive bacteria, some against Gram negative bacteria and some are active against both. Insects like \textit{A. mellifera} which are associated more with plants by way of collecting pollen and nectar when induced produce antibacterial substances that are highly specific to plant bacterial species (Casteels \textit{et al.}, 1989).

In the present work there remains certain gaps that need to be filled in future investigations. The purity of the isolated MIIB can further be checked. The amino acid composition and primary sequencing of amino acids need to be done. Future studies may concentrate on the potency of MIIB as a drug in lysing the cancer cells as has been reported for cecropins and its analogues (Moore \textit{et al.}, 1994). A molecular approach to the problem would include the isolation and characterization of cDNA of MIIB using the appropriate probes. Such analyses would indicate the possible identity of MIIB with the cecropins isolated from other species of insects.