1. INTRODUCTION
One of the major reasons for the extraordinary evolutionary success the insects have achieved on the terrestrial environment appears to be, that besides their impermeable cuticle and reproductive potential, they have evolved mechanisms to defend themselves against a wide variety of pathogens including micro-organisms. The defense mechanisms of insects have some similarities but very many differences with those evolved by vertebrates in general and mammals in particular. The main difference in the humorally mediated immunity between insects and vertebrates is the lack of a specific immune response in the former and hence a clonal selection. Nevertheless, insects like vertebrates make use of synthesized proteins as a means to defend themselves from the invasion of foreign organisms. However, the insect defense proteins, more appropriately the peptides, are different from the vertebrate immunoglobulins both in structure as well as in the mechanism of action. Insects possess only a limited number of proteins to combat a wide range of micro-organisms and this property obviates the necessity for the specificity which is the hallmark of vertebrate immunity. Insects have evolved a cell mediated immune mechanism as well, and this mechanism also has its similarities and differences with the ones possessed by vertebrates. The present investigation is aimed at identifying and analysing certain aspects of the humoral mediated immunity in an economically important dipterous insect, the common housefly, \textit{Musca domestica}. As a prelude to such studies a survey of relevant literature was made, the more important of which is presented below.

Cell mediated immunity in insects has been the subject of study for several years and the role of haemocytes in protecting insects against invading organisms has been analysed in detail (Price and Ratcliffe, 1974; Nappi, 1975, 1981, 1984; Lackie, 1981; Ratcliffe et al., 1984; Rizki and Rizki, 1984; Ratcliffe, 1985; Brehelin, 1986; Mead et al., 1986; Nappi and Carton, 1986; Vass et al., 1993).

Insects are known to have evolved different mechanisms for combating different types of organisms; small invaders like bacteria and unicellular organisms are usually phagocytized and larger organisms such as the
aggregates of cells or nematode parasites are generally encapsulated with the aid of haemocytes. If the invasion assumes a greater proportion, then a coagulation process leading to the trapping of foreign cells or organisms is carried out. In all these functions the haemocytes play a significant role (Gupta, 1979). Of the several types of cells that are found in haemolymph, phagocytosis of invading organisms is carried out mainly by plasmatocytes and granulocytes. Plasmatocytes have a role to play in capsule and nodule formation as well. Granular cells release secretory products that form the core of nodule. There are at least six other types of cells which are involved in phagocytosis process. Due to a lack of clarity and uniformity in the nomenclature of these cells it has not been possible to clearly identify these cells and specify their functions. They could possibly be one of the plasmatocytes or granulocytes. (Ratcliffe et al., 1984; Nappi and Carton, 1986; Nappi, 1987).

Coagulation of haemolymph is accompanied by the activation of the enzyme phenol oxidase. The enzyme catalyses certain key steps in the formation of the black pigment melanin resulting in a dark layer around wounds and encapsulated parasite. In some cases, there is a darkening of haemolymph as well (Ratcliffe, 1985; Nappi and Vass, 1993). Such reactions also result in the hardening and pigmentation of cuticle. Phenol oxidase occurs as an inactive enzyme or as a proenzyme in haemolymph and the activation is achieved by a serine protease cascade, the details of which are relatively less understood in insects. There appears to be a similarity between serine protease cascade of insects and systems that activate complement and blood clotting in mammals (Ratcliffe, 1985; Nappi and Vass, 1993).

Humoral immunity in insects relates to the synthesis of bactericidal proteins within few hours of infection of bacteria. The main site of synthesis of these proteins is the fat body (Faye, 1978; Faye and Wyatt, 1980; Dunn et al., 1985; Samakovlis et al., 1990) which is essentially the site of intermediary metabolism in insects and is comparable to vertebrate liver in its functions. The fat body per se cannot synthesize the bactericidal proteins as there is no direct interaction between the site of synthesis and the injected bacteria.
Peptidoglycan fragments and lipopolysaccharide (LPS) moieties released from the ingested bacteria serving as the signal for protein synthesis is possible (Taniai et al., 1992).

The various antibacterial substances that have been isolated by challenging the insects with bacteria have proved to be peptides or polypeptides. Many such antibacterial substances have been identified, purified and characterized. The three dimensional structure of at least two types of antibacterial proteins has been well established. It is remarkable that the same class of compounds have been entrusted with the responsibility of protecting body from the attack of micro-organisms in both insects and vertebrates. In a short span of time a number of antibacterial molecules have been isolated from different species of insects and the micro-analytical methods developed in recent years have made possible the detection of more and more number of these molecules.

The antibacterial proteins that have been identified and characterized thus far essentially belong to four different families. They are, two families of 4 KD peptides, the cecropins and the defensins; and two other classes designated as proline-rich and glycine-rich peptides. The glycine-rich peptides more commonly known as attacins and are essentially large peptides with a molecular weight that may range between 8 and 20 KD. More than one type of antibacterial substance may be present in the same insect (Cociancich et al., 1994).

All these inducible antibacterial peptides share the following common features:

1) all are heat stable;
2) they are inactivated by trypsin;
3) they are small sized, strong basic proteins;

4) they are absent in non-immunized flies;

5) they appear in the haemolymph few hours after the bacterial challenge and their concentration reaches high plateau after 24h;

6) they are transient and lost in a short period of few days;

7) they are induced by a variety of stimuli like injury, biological antigenic substances starting from peptidoglycan fragment to live bacteria and also by a variety of physical factors and chemical substances ranging from ultrasound to polyanions;

8) the cationic peptides are selectively synthesized in fat body and most of them are produced as preproproteins and modified post-translationally;

9) most of these peptides show polymorphism and occur in multiple forms in the same insect with slight differences in amino acid sequence;

10) they are either bacteriolytic or bacteriostatic affecting either the outer or inner cell membrane or the cell wall of bacteria;

11) most of them have broad spectrum of antibacterial activity and a few showing activity only either with Gram positive or Gram negative bacteria;

12) generally they also lyse a variety of artificial liposomes;

13) they are active only on prokaryotic cells and are chemically inert to eukaryotic cells like yeast and red blood corpuscle, although there are reports of their toxicity against protozoan parasites; and

14) as they are highly non-specific, can be used as the drugs of choice in future.
The following account provides a description of each type of antibacterial substance.

**Cecropins**

Cecropins can be regarded as the first of different classes of antibacterial substances that have been isolated, fully characterized (Hultmark et al., 1980, 1982; Steiner et al., 1981) and identified in cecropia moth, *Hyalophora cecropia*. Cecropins were obtained from diapausing pupae challenged with bacteria. Subsequently cecropins have been isolated from induced haemolymph of different groups of insects such as lepidopterans (Hoffmann et al., 1981; Qu et al., 1982, 1986; Spies et al., 1986; Teshima et al., 1986) and dipterans (Okada and Natori., 1983; Keppi et al., 1986; Ando et al., 1987; Flyg et al., 1987; Kaaya et al., 1987).

Cecropins are known to be active against both Gram negative and Gram positive bacteria (Hultmark et al., 1982). Early studies indicated that eukaryotic cells were totally resistant (Steiner et al., 1981). However, at concentrations ≥ 100 μM eukaryotic parasites like *Trypanosoma*, *Plasmodium* and *Leishmania* are also killed (Jaynes et al., 1988; Wade et al., 1990) and it has been possible to cure malarial parasite infected mosquitoes by repeated injections of cecropin (Gwadz et al., 1989). They are synthesized as 62-64 amino acid residue precursors and post-translational processing cleaves 24-26 residues at the amino terminal (N-terminal) end to produce mature proteins. The final 25-39 residue cation protein has amidated peptide at its carboxyl terminal (C-terminal) end and assumes a helix bend helix structure (Merrifield et al., 1982; Steiner, 1982; Holak et al., 1988). The N-terminal end of cecropin is amphipathic while C-terminal end is hydrophobic.

The mechanism of functioning of cecropin appears to be that it forms channels across the membrane. These voltage dependent anionic channels cause changes in the membrane property and subsequent lysis of bacteria (Wade et al., 1990; Boman, 1991). The only cecropin-like substances identified in
vertebrates are cecropin P1 (Lee et al., 1989) and PR-39 (Agerbeth et al., 1991) from the small intestine of a pig. Cecropins occur in multiple forms. Two years after the discovery of cecropin A and B, cecropin D was isolated along with four minor components E, F, C and G (Hultmark et al., 1982).

**Insect defensins**

Insect defensins are relatively small cationic peptides with a molecular weight of 4 KD that contain 29-34 amino acid residues with 3 intramolecular disulphide bonds. They are more active against Gram positive bacteria and are also shown to be cytotoxic against eukaryotic cells. Like cecropins they are produced from the preproproteins of 93-94 residues. First identified in embryonic cell line of a dipterus insect *Sarcophaga peregrina* as sapecins (Matsuyama and Natori, 1988) and later in *Phormia terronovae* (Lambert et al., 1989), these antibacterial proteins have no similarity with mammalian defensins (Lehrer et al., 1991) and found to be distributed in hymenopterans, odonatans, coleopterans, trichopterans and hemipterans (Hoffmann and Hetru, 1992). To date defensins have not been reported from Lepidoptera.

The three dimensional structure of defensins of *P. terronovae* has been elucidated in detail. The molecule shows a N-terminal loop, a central α helix and a C-terminal twisted antiparallel β sheet. The three domains are linked by three disulphide bridges. *Phormia* defensin is shown to act on Gram positive bacteria by forming voltage dependent channels in membrane (Hoffmann and Hetru, 1992).

There is no similarity in the structure and organization of insect defensins and mammalian defensins as the latter lack α helix and consists of β sheet (Pardi et al., 1988; Hill et al., 1991). Contrarily there is a structural similarity between the insect defensin and charybdotoxin synthesized by the venom glands of scorpion (Miller et al., 1985).
Proline-rich peptides

A third class of bactericidal peptides are predominantly isolated from the hymenopterans (Casteels et al., 1989, 1990). Their presence is recorded in Diptera (Lowenberger et al., 1995) as well as Hemiptera (Chernysh et al., 1996; Miura et al., 1996). An unique property of these peptides is that nearly one-third of their structure has proline in them. The proline-rich peptide isolated from *Apis mellifera* belongs to a family called apidaeins (Casteels et al., 1989), a 18 amino acid residue peptide that has a O-glycosylated substitute. Another peptide, with 34 amino acids of which ten of them are proline, is abaecin (Casteels et al., 1990). The N-terminal end of abaecin resembles apidaeins. Both apidaeins and abaecins are active against Gram negative bacteria. Apidaeins are generated from a large single precursor protein containing nearly 12 copies of apidaecin isoform (Casteels et al., 1993).

Yet another proline-rich peptide is drosocin (Bulet et al., 1993) isolated from *Drosophila melanogaster* and is shown to be active against Gram negative bacteria. It is a 19 amino acid residue peptide, one-third of which comprises proline residues. The 11th amino acid threonine carries a disaccharide N-acetylgalactosamine-galactose. In the absence of the disaccharide, activity is much lower. Such a O-glycosylated proline-rich small sized antibacterial peptide has also been isolated from hemipterans *Pyrrhocoris apterus* (Cociancich et al., 1994).

Attacins and attacin-like substances

Attacins are relatively high molecular weight peptides isolated from a number of immunized insects. They were first isolated from *H. cecropia* (Hultmark et al., 1983; Engstrom et al., 1984; Kokum et al., 1984). At least four basic and two acidic attacins with an 80% sequence of homology are known. In cecropia moth the attacins are synthesised as preproattacins and are post-translationally modified to yield four basic and two acidic attacins. Attacins have a molecular mass higher than 20 KD with nearly 184-186
Amino acids. They are highly active against Gram negative cells but some times activity extends to Gram positive bacteria as well.

Attacin-like substances have been isolated from other insects. For instance sarcotoxin II isolated from S. peregrina (Ando et al., 1987) has two glycine-rich domains, G domain in the C-terminus and a proline-rich domain at their N-terminus. The proline-rich domain is not characteristic of attacins. Besides sarcotoxin II, the dipterinc-A isolated from P. terronovae (Dimarcq et al., 1988) as well as D. melanogaster (Wicker et al., 1990) are also attacin-like substances but have separate P and G domains as in sarcotoxin II. Dipterin have 18 residue proline-rich N-terminal domain and 64 residue glycine-rich C-terminal domain. The P domain is homologous to apidaecins and G domain to the attacins.

Another attacin-like substance that has been isolated from immune honeybees is termed hymenoptericin (Casteels et al., 1993). The 93 residue peptide has 19% glycine and has a certain sequence homology with dipterin. The peptide is active against both Gram negative and Gram positive bacteria. The mechanism of action is by sequential permeabilization of outer and inner membranes.

Another glycine-rich strongly basic polypeptide shown to kill rapidly Gram negative bacteria is termed coleoptericin. It has no sequence homology with other attacin-like molecules (Bulet et al., 1992).

**Mode of action of antibacterial substances**

Different classes of antibacterial proteins appear to have different modes of antibacterial action. The highly amphipathic small molecular weight cecropins are known to interact with lipid membranes causing voltage dependent ion channel of variable size in bacteria. They probably break down the permeability barrier of cell membrane. Their bacteriolytic action against
Gram negative bacteria suggests that the substance could penetrate the outer membrane of the bacteria.

The insect defensins which include sapecins (Matsuyama and Natori, 1988) of Diptera, royalisins (Casteels et al., 1993) of Hymenoptera and defensins (Bulet et al., 1991) of Coleoptera appear to resemble, the potassium channel blocking scorpion toxin namely charybdotoxin. Insect defensins kill the bacteria relatively slowly and the mechanism could be different from the one found in cecropins. Cociancich et al. (1993) showed that the venom disrupts the permeability barrier of the membrane of Micrococcus luteus, resulting in a loss of cytoplasmic potassium, a partial depolarization of the inner membrane, a decrease in cytoplasmic ATP and an inhibition of respiration. The defensin oligomers form channels in giant liposomes.

Attacin and attacin-like substances affect the dividing cells of Gram negative bacteria causing them to grow in a chain like fashion. The major outer membrane proteins are inhibited from being synthesized leading to the breakdown of integrity of outer membrane (Hultmark et al., 1983; Ishikawa et al., 1992).

It is well known that the immune system of insects is non-specific both in terms of induction and response. Induction can be achieved by a variety of agents. They may include cellular debris, polyanions, reactive oxygen intermediates and even an aseptic wound (Hoffmann and Hetru, 1992). Nevertheless, the maximum response is to the inoculation of living bacteria. Breakdown products of bacteria such as LPS of the outer membrane of Gram negative bacteria, peptidoglycan of cell walls of bacteria (Dunn et al., 1985; Yoshida and Ashida, 1986) and β-1,3 glucan of cell walls of fungus (Samakovlis et al., 1990) appear to be good inducers of immune response to insects. The molecules that recognize the inducing substances and which in turn trigger the immune response have not yet been identified, except in the case of activation of phenol oxidase pathway (Nappi and Vass, 1993; Vass et al., 1993). Two soluble proteins from silkworm haemolymph have been purified (Ashida and
Yamazaki, 1990); one of them recognizes the peptidoglycans of bacterial cell wall and the other recognizes the \( \beta-1,3 \)-glucans of fungal cell wall. When the appropriate inducing substance binds its specific recognition molecule, the phenol oxidase system is activated. In the king crab *Limulus* a recognition molecule termed as factor C that binds to LPS is well characterized (Iwanaga *et al.*, 1992). The factor C is shown to be a complement related serine protease and on binding to LPS, it is autocatalytically activated. There are suggestions that the recognition molecule for antibacterial substances could be membrane bound receptors. If that were to be true, then different mechanisms would exist for the induction of phenol oxidase and antibacterial peptides. Since washed cells of certain cell lines can be induced to produce antibacterial substances, the receptors of antibacterial peptides may not resemble soluble recognition molecule involved in phenol oxidase activation.

**Insect and mammalian immunity**

It was earlier said that the insect and mammalian immune systems have a few similarities, but very many differences. However, a number of antibacterial peptides of insects are being detected in higher vertebrates. Cecropin-like molecule in pig intestine, mammalian defensins and magainins (Zasloff, 1992) are some of the molecules that appear to be common to both groups. Six hundred million years of divergent evolution has made insects and mammals very distinct and could very well explain the differences in the structure and functioning of immune system. Insects at the same time have to confront the problems posed by microbes and evolve mechanisms to overcome their harmful effects. There could be certain similarities between insects and higher vertebrates in the recognition mechanisms of potential pathogens. Such mechanisms have not been well characterized in insects, but well understood in mammalian system. Janeway (1989, 1992) has put forth the idea that the recognition receptors have been selected over evolutionary time to provide broad spectrum recognition of various pathogens. In other words, it is hypothesized that the adaptive immune response involving the development of clonally
distributed receptors should have evolved later than the recognition system. The occurrence of LPS and β-1,3-glucan binding proteins in insects are often cited as examples of early evolution of recognition systems. Further, identification of recognition molecules of insect would throw more light on basic similarities in the immune system of insects and mammals.

In the present work an attempt has been made to identify, isolate, purify and characterize the antibacterial substance(s) in a dipterous insect *M. domestica*, the common housefly. As a prelude to such studies immunodiffusion and immunoelectrophoretic techniques were used to confirm the presence of antibacterial substances in the immune haemolymph of housefly larvae. An elaborate purification regime involving CM-cellulose, Sephadex G-50 and hydroxyapatite chromatography were followed in a five step purification procedure. Characterization was based on appropriate experiments. Specific activity at each step of purification and the bacterial spectrum against which the isolated protein termed as muscinIIB (MIIB) could be active, were determined. The bactericidal activity of the isolated factor was also tested against chosen bacterial species.