RESULTS AND DISCUSSION
The inhibition of hemocyte aggregation was one of the first characteristic observations in hemolin from *B. mori*. However the study and analysis of present results *in vitro* have been hampered both by the difficulty of preventing non-specific aggregation once the hemocytes are collected. Therefore in order to prevent uncontrolled non specific aggregation, hemocytes are suspended in a 0.20 M NaCl solution containing DNAse. Hemocytes collected using this method remain disperse uniformly and increased viability (Fig. 1). Once non-specific clumping was prevented, we studied the conditions needed to induce the aggregation of hemocytes collected from normal healthy and immunized worms (Fig. 2). Cell aggregation in normal healthy worms were induced by 10 mM Ca\(^{2+}\) and 1 mM Ca\(^{2+}\) supported the aggregation with LPS, however 10 mM Mg\(^{2+}\) had no effect. These results indicate that LPS induced aggregation requires Ca\(^{2+}\) (Fig. 3). Microscopic studies revealed that cell clusters or aggregates showed a regular pattern. Granulocytes always observed in the core of the aggregates and surrounded by plasmocytes (Fig. 4).

Because the combination of 1mM Ca\(^{2+}\) and LPS induced best observable aggregation. Further experiments were done using hemocytes collected from pebrine infected silkworms. The effect of infection assayed without Ca\(^{2+}\) and LPS. Interestingly hemocytes resulted in a marked decrease in the aggregation. Absence of hemocyte aggregation was due to immune disfunction by pebrine
infection (Fig. 5). Because hemolymph has a cell adhesion molecules and recent results from Bettencourt and co-workers (1996) showed that hemolymph is also present in a membrane form on hemocytes. Present results had clearly indicates that the involvement of a hemolymph membrane form on hemocytic aggregation. During hemocyte aggregation some melanization was also observed in the experimental hemocytes (Fig. 6).

Hemocyte aggregation is clearly resembles to that of lymphocyte proliferation in vertebrate immune system. In this context we would like to furnish burnet clonal selection theory. A mature immune competent animals contain large number of antigen reactive clones of T and B lymphocytes. The specificity of each T and B lymphocytes is determined prior to its contact with antigen. Here the role of antigen becomes critical, when it interact with antigenitically committed T and B cells and stimulates a particular cell to undergo mitosis and develop into a clone of cells. Clonal selection provides a frame work for understanding three aspects of cellular immunity: specificity, memory and non-self recognition. However memory system in insects is not yet explored.

Further we have detected series of inducible immune proteins in fifth instar larvae of silkworm. During this response hemocytes from healthy. Silkworms have tendency to aggregate. The same phenomena was observed in humans with special reference to clonal expansion during immune up
regulation. Interestingly we had obtained non-aggregated hemocytes isolated from 3rd instar larvae of silkworms induced by *E. Coli* (Fig. 7). Immune response factors (IRF) may not be responsible for the intercellular aggregation. Since protein kinase-C is activated by LPS in invertebrates, (Ishikawa *et al.*, 1995; Geng *et al.*, 1993). Recently it has been found that LPS binds to granular hemocytes in *Hyalophora Cecropia* and *Galleria mellonella* (Muta and Iwanga, 1996). Further studies are necessary to understand intracellular pathways involved in hemocyte aggregation.

Because the combination of 1 mM Ca\(^{2+}\) and LPS induced the best aggregation, further experiments were done under following conditions. The effect of different immune response factors (IRF) from immunized silkworm hemolymph were assayed on LPS induced hemocyte aggregation. IRF shows a dose dependent capacity to prevent the aggregation of hemocytes (Fig. 2). IRF 100 \(\mu\)g/ml prevents almost 100% cell aggregation addition of IRF did not disaggregate hemocytes once the cluster had formed. Similar information of hemocyte aggregation explored by Bettencourt and his Coworkers (1996).

Pro-phenoloxidase of the silkworm was partially purified, and migrated as single band in SDS-PAGE system (Fig. 9, Lane 5) Corresponding to the protein markers of 35 KD, where as prophenoloxidase band is completely absent in cuticular protein samples from healthy normal controls. (Fig. 9, Lane 2).
Prophenoloxidase was converted into phenoloxidase, which was detected on the gel corresponding to 31 kD band (Fig. 9, Lane 3). Present results were clearly shown that the existence of prophenoloxidase polymorphism. One which migrates faster and formed band of 35 kD and the other show migrating type of 31 kD. Prophenoloxidase in fifth instar has shown and indicated that prophenoloxidase and phenoloxidase types were detected irrespectively of the developmental stage of the larvae from which hemolymph samples were collected.

_E. coli_ induced immune protein (IP) was purified from 50 ml of larval silkworm hemolymph. The purification procedure consisted of ammonium sulfate fractionation column chromatography on sepharose G-25. _E. coli_ induced IP could not be quantified in hemolymph and ammonium sulfate fraction. The yield of _E. coli_ induced IP in the first step could not be calculated, otherwise the amount of hemolymph protein was reduced to about 1/4000 in the sepharose G-25. After three chromatographic steps 56 μg of protein with _E. coli_ induced IP activity was obtained (Table I). Upregulation of immune response was detected in normal healthy controls and _E. coli_ induced hemolymph following the injection of _E. coli_. The immune response expression was significant in bacteria injected silkworms (Fig. 8).

Upregulation of immune transcription was previously observed in fat body as well as six other tissues, such as malphigean tubules, mid gut...
Table 1
Summary of *E. coli* induced immune protein

<table>
<thead>
<tr>
<th>Volume</th>
<th>Protein concentration mg/ml</th>
<th>Total protein mg</th>
<th>Total activity units</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hemolymph</td>
<td>50</td>
<td>19</td>
<td>4000</td>
</tr>
<tr>
<td>Ammonium sulfate</td>
<td>(B)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Sapharose G-25</td>
<td>20</td>
<td>0.0020</td>
<td>0.150</td>
</tr>
</tbody>
</table>

(A) Because hemolymph and ammonium sulfate precipitate contained unidentified factors which cause activation of prophenoloxidase in hemolymph - *E. coli* induced protein activity in these fractions could not be quantified.

(B) Not Determined.
epidermis, heart and pericardial cell complex, and muscle following injection of peptidoglycon fragments (Mulinix and Dunn, 1994). Insect immune upregulation is common after bacterial injection.

Lee and Brey (1994) were reported expression of immune response factor in silk glands and mid gut after injection of the bacteria into the hemocoel. In silkworms presently we have observed the induction and synthesis of another important insect immune factor (not identified) in the hemolymph of silkworms. Like majority of insect immune genes rapid upregulation of immune-response factor in \textit{E. coli} infected hemolymph was observed.

Immune response factor (1RF) 26 kD protein is present in significant amounts in the hemolymph of healthy silkworm injected with \textit{E. coli} (Fig. 8, Lane 2). Our results are not correlated with sun \textit{et al.}, (1990). They suggested that hemolin was a 48 kD protein present in hemolymph of \textit{Cecropia pupae} after injection with live bacteria. 1RF is completely absent in unimmunized silkworms (Fig. 8, Lane 1).

However, when the most heavily stained band was excised from the gel and separated further by SDS-PAGE it generated a protein band at 26 kD. Where as in \textit{E. coli} infected hemolymph, yielded three faint bands at 76, 96, and 105 kD (Fig. 8, Lane 3). These three bands are completely absent in
hemolymph of healthy silkworms, suggesting that formation of well characterized insect immune factors in *E. coli* induced silkworms.

Microflora of a variety of insects has attracted the attention of many insect physiologists with a view to explore the ecological interactions between the pathogenic and saprophytic micro-organisms on insect surface in the normal and diseased conditions. Specific composition of microbial flora depends on climatic conditions and also on developmental stage of insects having specific interrelationships between the micro-organisms. Immune functions altered by surface microflora in insects is not yet explored.

The present study was designated to explore quantitative and qualitative type of microbial flora present in healthy control and pebrine infected silkworms. We have observed equal number of bacterial colonies from healthy controls and from pebrine infected surface microbial flora of silkworms (Fig. 10). Interestingly we have observed more number of fungal colonies from pebrine infected surface of silkworms (Fig. 11). It was found that there was a significant variation in number of bacterial colonies in the hemolymph of pebrine infected silkworms than healthy control silkworms. The antagonism between bacterial species and silkworm immune factoral leads to development of more resistant towards pebrine disease.

We therefore assume that the immune system is to control the natural flora that silkworm meet. Microbial flora plays an important role in silkworm,
which have contaminated with pebrine infected mulberry form. An additional fact observed from present results considered that silkworms are dependent on intracellular microbiota to fight against pebrine disease. Present results demonstrated that fungal flora was completely absent in both hemolymph of healthy and pebrine infected silkworms.

It has been assumed that lepidopteran insects like silkworms may be synthesize various antifungal proteins in hemolymph. The microorganisms infecting insects are not restricted to bacteria. It was expected that insects are also contain proteins that suppress the growth of fungal flora in hemolymph.

Phenoloxidase activity increased along with the prophenoloxidase activity. Prophenoloxidase was incubated along with the silkworm cuticular melanin crude extract presently referred as activating cascade (AC) that had been previously treated with Ca$^{2+}$ (Fig. 12).

One of the factor responsible for prophenoloxidase activation in the AC extract was to be a specific protease shown because prophenoloxidase was converted to smaller polypeptide band in SDS-PAGE during incubation of prophenoloxidase fraction with Ca$^{2+}$ treated AC extract. After incubation the combinational system will refer as prophenol activating factor (PPAF).

Hemolymph, prophenoloxidase which is composed of two polypeptides with mobilities in SDS-PAGE corresponding to 35 kD and 31 kD (Fig. 9) but did not detect any polypeptide in the AC extract strongly support that the
presence of pro-phenoloxidase only in the phenoloxidase fraction. Further experiments were not demonstrated to confirm by using homogeneous hemolymph prophenoloxidase. Components other than prophenoloxidase in the cuticular prophenoloxidase fraction are not involved in appearance of phenoloxidase activity.

These results strongly suggested that prophenol activating factor (PPAF) is a serine proteinase that exists as a zymogen (equal to complement cascade in vertebrate immune system during microbial infection) in the AC extract. In the presence of Ca\(^{2+}\) the zymogen is slowly activated in the mixture of prophenoloxidase fraction and AC extract when incubated with ice (Fig. 12). During experimental system the mixtures were held on ice aliquots of 50 \(\mu\)l of the mixtures were used to assay phenoloxidase activity which is expressed A\(_{520}\) per 50 \(\mu\)l per 5 min at the indicated times (Fig. 12) The AC extracts were added to prophenoloxidase activity (50 \(\mu\)l of the mixture) was assayed.

If AC extract was not treated with Ca\(^{2+}\) no activity appeared. If however, the AC fraction was mixed with prophenoloxidase fraction and Ca\(^{2+}\) was added at the same time phenoloxidase activity gradually appeared (Fig. 12). It is true that melanization in insects is affected by age, nutritional and reproductive states of the silkworms. The weak response of susceptible silkworms to negatively charged beads may indicate that such surfaces are more closely resembles self and non-self, that render them invisible to the
Fig. 12. Effect of immune protein concentrations on hemocyte aggregation

Hemocytes were incubated in the presence of 50 μg/ml LPS with 1 mM Ca^{2+} different concentrations of immune protein

Percentage of hemocyte aggregation by following formula
% of hemocyte aggregation = 100 x \frac{\text{number of free cells}}{\text{number of input cells}}
immune recognition system Paskewitz and Riechle, (1994). The charged surface could provide a microenvironment, developed by surface microflora for enzyme activity the observed differences between healthy and infected silkworms reflected differential abilities during immune promotion and enzymes to function within the microenvironment.

It is evident that the biochemical nature of the factors involved in immunity to parasites is largely unknown, present results are tempting to speculate that appearance of pro-phenol activation is one of the immune factor in silkworms. Humoral antimicrobial peptides may play a role in the development of innate immune system in insects.

One of the distinguishing feature of Arthropods is their exoskeleton which provides them with a protective barrier against the aggressions of the external environment. It has been considered for the most part that the integument is only an inner physical barrier (Wigglesworth, 1972). However, in present study we demonstrated that integument can actively respond to minor injury in the presence of microbial cell wall components (LPS) and response to other pathogenic bacteria (Fig. 9).

Another common defense response is cuticular melanization. This response can be elicited by microbial invasion. It is well known from wealth of literature that melanin is synthesized from phenolic substances by the action of phenol oxidase Lai-fook (1966) and Barret (1991) suggested that cuticular
melanization resulted from injury by the activation of prophenoloxidase by the proteinaceous activator.

Present results clearly demonstrate that cuticular phenoloxidase is true zymogen form, which is activated through a limited proteolysis by the serine proteinase which is also in zymogen form. Hence, two distinct steps have been demonstrated in the activation of prophenoloxidase. Sass and his associates (1994) showed that cuticular peptides are synthesized by the epidermal cells and from hemocytes and be transported across the epidermis and enter into the cuticular matrix. This could explain present observations of prophenoloxidase activation both in hemolymph and from melanin extract. But the mechanism with which particular hemolymph protein is transported to cuticle remains unclear.

The insect tissues are literally surrounded by this enzyme zymogen and its cascade. Defense functions have already been attributed to be phenoloxidase cascade, but the information yielded during our experiments rise question whether prophenoloxidase was shown to be a protein homologous to arthropod hemocyanin as reported by Kawabata et al., (1995).

We have extended our experiments on the involvement of the immunoglobulin like molecules. Role of hemolin in insect immunity and its function in cellular defense reactions were investigated. To study the effect of *E. coli* injection on hemocytic responses we developed a simple method to
collect stable hemocytes. In our study we used lipophorin to prevent non specific aggregation. The mechanism of this molecule explained by (Coodan and Caveney, 1992) suggest that regulation of hemocyte adhesion involves a lipophorin receptor on the surface of hemocytes.

Once we had obtained non-aggregated hemocytes from normal healthy control silkworms and from \textit{E. coli} infected silkworms, aggregation was induced with LPS in the presence of Ca$^{2+}$. In nature insect hemocytes are capable of adhering at the site of break in the epidermis as well as to large foreign objects in the hemocoel. However, the hemocyte types and events involved in hemocyte adhesion activation have been difficult to identify, because \textit{in vitro} hemocytes rapidly adhere to different substrates in the apparent absence of activating factors like micro-sporidian infections in silkworms are due to mechanical injury. Therefore present model system demonstrated during our hemocyte associated experiments. Stimulation with LPS and other mitogens allows analysis of the effect of infection on aggregation and facilitates the study of factors and molecules involved in hemocyte aggregation.

Protein phosphorylation may be responsible for the intracellular aggregation. Since protein kinase C is activated by LPS and Ca$^{2+}$ in invertebrates (Ishikawa et al., 1995; Geng et al., 1993). Presently we have determined that \textit{E. coli} induced hemocyte aggregation may be involved in
cellular immune functions. During infection or in response to foreign object, a wound or other trauma, hemocytes tend to aggregate with each other and bind other tissues. It is possible that hemocytes attach to the basal membrane after the activation.

Several insect molecules have been found to modulate the cellular immune or defense reactions but they are not well characterized (Mohrig et al., 1979). Therefore present results clearly confirm that inducible protein in an silkworm hemolymph that is responsible for the control of a cellular immune responses against any infection or pathogenic action on silkworm.

Early attempts to establish \textit{in vitro} systems for the study of insect immunity were hampered by the inability to maintain permanent cultures of hemocytes and fat-body. Presently we have demonstrated the silkworm hemocytes used as suitable system for \textit{in vitro} studies on the induction of silkworm immune response. This response can be triggered by known elicitors of insect immune reactions like LPS and other pathogenic bacteria. The effects of the tested chemical and biological macromolecules appear to be specific. However we have not been able to measure any biochemical endogenous reactions after induction with LPS.

The primary function of any immune system is the protection against the natural flora of microorganisms (Boman, 1991). To induction of silkworm immune factor we have tested various tissues such as cuticular epidermis,
hemocytes and hemolymph. The upregulation of immune factor was observed in hemolymph, following injection of Gram +ve bacteria. Immune protein expression in other tissues was insignificant in both bacteria injected and control silkworms (Fig. 8). Immune factor upregulation in insects was explored well by Hultmark (1996). Expression of the cecropin on antibacterial factor was detected in several tissues, including epidermis following bacterial injection (Dickinson et al., 1988).

It is well known that a wide variety of foreign objects irrespective of abiotic or biotic components recognized as non-self by insect hemocytes. However biotic materials generally provoke more intense encapsulation reactions than that of abiotic materials (Gotz, 1986). Insects seems to have mechanisms to specifically recognize molecules on the surface or molecules released from invading organisms to amplify the recognition signal to mobilize defense mechanism and to prevent invasion. Recently β-1, 3-glucan specific lectin 38 kDa protein was reported to be present on the surface of plasmatocytes of the great Wax moth Galleria mellonella (Matha et al., 1990). The function of the lectin has not been known. However, the authors speculate that the lectin plays an important role in the recognition of fungi as non-self.

In silkworms we have observed the induction and synthesis of important immune protein from hemolymph (Fig. 9, Lane 5). On the other hand (Daffre, et al., 1994) showed that lysozyme constitutively expressed in the adult
salivary glands and midgut was expressed rather than activated after injection of bacteria into the hemocoel.

Melanization through the action of phenoloxidase is an important aspect of insect defense against microorganisms and Eukaryotic parasites (Gotz, 1976; Nappi and Vass, 1993). The inactive precursors of prophenoloxidase is converted into the active form by serine proteases which themselves in response to various elicitors including components of bacteria and fungi. The cascade results in the production of melanin and associated toxic metabolic biproducts which may kill encapsulated parasites because of potential toxicity of the ultimate products. Phenoloxidase activation is highly localized and increased phenoloxidase activity associated with melanotic encapsulation of eukaryotic parasites.

Phenoloxidase synthesized in insect fat body, the circulating hemocytes and midgut epithelium may become localized to the parasite surface as part of the innate immune recognition system (Paskewitz et al., 1985; Brey et al., 1995). Ham et al., (1995) speculated that phenoloxidase may serve as another immune function in black flies by binding to parasite surface as a recognition factor for hemocytes and this suggestion is based on the comparison of the amount of phenoloxidase that can be detected in the hemolymph of black flies infected with eukaryotic parasites.
During the course of evolution invertebrates and vertebrates have maintained common defense or signaling molecules have been found in the blood circulation of vertebrates and in the hemocytes and hemolymph of insects. These molecules are act as a immunomodulators. In vertebrates release occurs during stress or against pathogens, which triggers the hypothalmo-hypophysical adrenal (HPA) axis. Similarly these neuropeptides are used as messengers to initiate and stimulate the immune response in invertebrates. In this regard naturally occurring antibacterial peptides have been found in both vertebrates and invertebrates reflecting the presence of common immune response. Further more, since invertebrates arose first during evolution, we surmise this innate immune mechanisms first arose in these primitive animals and then were retained during evolution. During our project, we demonstrate for the first time in silkworm the presence of an antibacterial peptide pro-enkaphalin A (PEA). This supports the hypothesis that these PEA molecules first evolved in simpler animals and these immune signaling molecules may lead to enhance the release of PEA derived defense complex IL-1 and phenoloxidase. In this scenario antimicrobial peptides would stimulate immunocyte chemotaxis and phagocytosis as well as production of classical cytokines.

We have demonstrated the effects of the lipopolysaccharides (LPS) on the release of antimicrobial peptides and implication of these yielded products
on the nonspecific immune response in silkworms. It is our expectation that these studies can provide insights into same processes that occur in more complex organisms.

Silkworms were used for these experiments and were reared under laboratory conditions. Silkworm life is divided into 6 stages. These experimental silkworms in stage 5 were used which is characterized by apoptosis of the salivary glands and an empty midgut. Moreover it is easier to collect hemocytes at this stage of life-span. LPS at different concentrations (0.1, 1 and 10 μm) were injected (10 μl) into the hemocoel of the silkworm.

Hemocytes were collected at 5 min intervals up to 4 h of LPS injection. Electrophoresis was performed on a 10-25% PAGE. Antibacterial test on solid medium allowed to detect rapidly in presence of the antibacterial peptides in the hemocyte lysates. The antibacterial activity of the material was assayed by a solid growth inhibition assay using Gram +ve bacteria *M. luteus* and Gram -ve bacteria *E. coli*. Bacteria were grown on luria broth (LB) overnight at 37°C under agitation. They were then diluted 250 μl in 50 ml of LB agar placed in petri dishes and stored in 4°C, 5 to 0.5 μl sample were plated on the nutrient agar containing bacteria and incubated at 37°C overnight. Antibacterial activity was recognized by the lack of growth on the deposit.

Analysis of PEA fragments in hemocyte lysate was performed to sustain the hypothesis of the release of peptide B after LPS stimulation. LPS not
treated hemocyte lysate treated as control and LPS treated hemocyte lysate were subjected to electrophoresis. The results shown that a single protein having molecular mass of 18.5 kDa absent in control experiment (Fig. 13, Lane b). Confirming the above results, PEA is consequently present in LPS stimulated hemocytes (Fig.13, Lane a and c). This molecule correspond to the silkworm hemocyte lysate PEA. We further studied the time course of PEA derived. Secondary molecules release following LPS injection. The hemocytes were collected at different times after injection (15-30 min), fifteen minutes of post-injection PAGE revealed a single protein of 13 kDa. This suggests either a quick process of PEA in hemocyte lysates or a massive release of mature PEA fragment of 13 kDa molecule stored in hemocytes (Fig. 13, Lane d).

Hemocyte lysate has marked activity (MIC < 20 mM) against Gram +ve bacteria *M. luteus* (100-200 nm). The invertebrate immune factor like the mammal material in a concentration dependent manner seemed to contribute to the release of prophenoloxidase or interleukin-1 (1L-1) similar to vertebrate peptide B (Fig. 14). More over the LPS in a dose dependent manner seemed to contribute to the release of peptide B.

Presently we do not know if the effects were related to the LPS or to the injection itself, the results showed that the increase in concentrations of injected LPS provoked a higher release of invertebrate peptide B in hemocyte
Fig. 14. Biological activity of Peptide-B/prophenoloxidase

*M. luteus* is incubated with increasing concentrations of LPS stimulated lysate in bacteria liquid medium

Microbial growth is assessed by measuring the increase in A. 620 after incubation at 37°C for 24 hr to control
lysates of silkworm (Fig. 15). These results have clear evidence that upon stimulation by LPS the levels of PEA derived peptides i.e. peptide B the 13 kDa fragment is significantly increased. As the PEA is present in the hemocytes, we suggested two possible processing pathways, an intracellular and/or an extracellular processing.

Wealth of literature in vertebrates shown that PEA is processed in the intracellular compartment (Breslin et al., 1993). Moreover presence of enkaphalin in human monocytes suggesting it's involvement on the PEA processing in these silkworm hemocytes.

Consequently after LPS stimulation, a quick release of PEA derived products occurred. These peptide fragments are due to pro-phenoloxidase action before stored in silk glands or in hemocytes. On the other hand we demonstrated that the peptide B is released in silkworm hemocyte lysate after 15 min of LPS stimulation, suggesting a processing of 13 kDa fragment in the hemocytes. These invertebrate peptide B was found to process a strong antibacterial towards Gram +ve bacteria. We surmise that processing and liberation of antibacterial peptide represent a unified neuroimmune protective response to immediate threat to the organism regardless of the form the stimulation takes place.

Taken together, these results suggest that this type of innate immunity is conserved and that derived immune complex factors play an important role in
Fig. 15. The effect of LPS dose on the concentration of peptide B / prophenol oxidase after 15 min injection.
activation of such non specific immune response. In the process of immune
defense or neural activation, a bactericidal compound peptide B may be
released along with other processed peptides (Solzet and Stefano, 1997).

The reason for the evolving relationship between opioid peptides and
immune process now appears quite simple, associated with anti-infections/
anti-inflammatory process. This combination would provide a high degree of
survival benefit to any organism since it would ensure appropriate behaviour to
meet not only noncognitive challenges, but also cognitive ones.

In part, the significance of these observations is upheld by neuropeptide
processing. Antibacterial peptides present evolutionary diverse immunocytes,
are found with their processing enzymes. When silkworm larvae were induced
with LPS, there were no significant influence on survival and cocoon quality.
However, the increase of hemocyte density (Fig. 16) were clearly restrain and
the functional disorder of the fat body was resulted, more over a kind of
hemolymph protein was discovered when it disappeared after local injection of
LPS. This protein may be related with the function of hemocytes of silkworm
and is suggested to be an important clue in clarifying the function of unknown
hemocytes.

It has long been known that insect hemolymph contains phenoloxidase,
which oxidizes endogenous phenols on removal from the hemocoel (Wyatt and
Pann, 1978). The blackening is suppressed \textit{in vivo}, since phenoloxidase exists
The silkworms were induced with LPS at the 4th instar premolting stage.

The hemocyte densities of LPS induced larvae had hardly increased during the earlier half stage of the fifth instar. Hemocyte density began to increase at a latter stage. But were found to be significantly lower than those of the Non LPS control.

0 - LPS induction

□ - No LPS induction

Fig. 16. Changes of hemocyte densities during the fifth instar
as on inactive precursor pro-phenoloxidase. Pro-phenoloxidase in hemolymph has been demonstrated in many insects (Crossley 1975). In the silkworm it was demonstrated that activation of hemolymph Pro-phenoloxidase by cuticular PPAE involves limited proteolysis resulting in release of a peptide having a molecular weight of about 5000 (Ashida et al., 1974 and Ashida and Dohke, 1980).

It is unclear, whether the activator present in hemolymph is the same proteolytic enzyme as in cuticle and how the activation of Pro-phenoloxidase is inhibited \textit{in vivo} and initiated on removal of hemolymph from the hemocoel. To examine the role of this phenoloxidase system and the mechanism of suppression of Prophenoloxidase, activation a method for obtaining hemolymph without perturbing these entities was required. Presently by we have demonstrated that cane sugar factor and the activation of Pro-phenoloxidase in silkworm hemolymph, will be described.

Invertebrates contain an opioid precursor, proenkephalin. Enkelylin, an antibacterial peptide is found in invertebrate proenkephalin, exhibiting 98\% sequence identity with mammalian enkephalin. Stein (1995) surmise that the function of enkelytin is to attack bacteria and allow time for the immunocyte stimulating capacity of the opioid peptides to emerge. Bacterial products such as LPS or stress for example cut, can induce the simultaneous release of peptide B induces chemotaxis, hemocyte activation, cytokine production and
phenoloxidase cascade peptide B exerts an antibacterial action (Fig. 17). Silkworm hemocytes expressing the proenkephalin molecule process and release its derived peptides upon appropriate stimulation.

In the process of immune defence or neural activation, a bacteriocidal compound, enkelytin, may be released along with the opioid peptides (Zadina et al., 1997). The organism must be capable of the allosteric regulation of the immune response to avoid immunologic dissonance which can lead to death from disorders such as systemic inflammatory response syndrome. An increase in the stress alert signal to corticotrophin releasing hormone would contribute to the immune response. Eventually, the decreased threat would elicit a decreased stress signal and the immune system would gain be in balance, primed to respond to another threat.

In part, the significance of the observation from Fig. 17 is upheld by neuropeptide processing. Opioid precursors present in evolutionary diverse immunocytes, McEwen (1998) are found with their processing enzymes. In some cases, the actual inactive products may act as a competitive inhibitors to further limit the activity of the prime enzyme, adding the dimension of microenvironmental control.

Association of opioid peptides and enkelytin in proenkephalin suggest that evolutionarily the opioid pentapeptides have originated as immune
Fig. 17: Immune action of peptide B released from silkworm. *Bacterial products* (LPS) and Stress induce simultaneous release of peptide B from hemocytes, induces hemocyte chemotaxis and other signaling molecules.
signaling molecules. The significance of these process is revealed by their intact presence in organisms 500 million years divergent in evolution.

When physiological saline containing 20 mM cane sugar (CS) was injected into silkworm larvae and hemolymph was collected after 20 minutes. Hemolymph did not develop the suppression of development of phenoloxidase activity. The suppressive functions depend on the concentration of cane sugar in the saline and also on the intervals between injection and collection of hemolymph (Fig. 18). At least 20 minutes were required for complete suppression of the activation of Prophenoloxidase when saline containing 20 mM cane sugar was administered.

Many samples of CS-hemolymph showed no phenoloxidase activity after incubation for several hours at 25°C and remained clear yellow without forming a brown pigment of clotting. Even after overnight incubation some CS hemolymph samples were still clear yellow and phenoloxidase activity was undetectable.

Effect of various sugars on development of phenoloxidase activity was examined (Table 2). Surprisingly among sugars tested only CS was effective, whereas other mono- and di-saccharides were practically ineffective. Apparently sucrose itself is not the active principle. Phenoloxidase activity and clotting were not detectable in CS-hemolymph. In addition, the total hemocyte count was much decreased when it was examined using
Fig. 18. Suppressing effect of physiological saline containing cane sugar on development of phenol oxidase activity in hemolymph

Physiological saline was injected into haemocoel of silkworm larvae 48 hrs after 4th molt and hemolymph collected

a. PO activity of the haemolymph was assayed immediately after bleeding
b. PO activity after incubation for 20 min at 25°C.
haemocytometer (Table 3). Even taking into consideration dilution of hemolymph due to injection of physiological saline alone reduced total hemocyte count as shown in Table 3. But the effect was less than that of injection of saline containing CS. Hemocytes observed in CS-hemolymph were all round shaped.

A method for obtaining hemolymph (CS-hemolymph) of the silkworm larvae without triggering the activation of prophenoloxidase was described. A cane sugar factor (CSF) seems responsible for retention of prophenoloxidase in CS-hemolymph. CSF was only effective in vivo. This method was also applicable to full grown wax-moth larva (Galleria mellonella) from which hemolymph with properties similar to CS-hemolymph was obtained (Pye, 1974). Absence of CSF in beet sugar seems to suggest that CSF originates from cane (Table 2). It may be desirable to larvae extracts of cane sugar and other plants to find better source of CSF.

Another characteristic feature of CS hemolymph is the great decrease in total hemocytes (Table 3). Although the reason for the lowered hemocyte count is unknown, CSF seems to cause selective transition of hemocytes from the free to the tissue found state, because disruption of hemocytes was not observed, and only hemocytes with spherical shape similar to proleukocyte according to the classification of the Nittono (1960), were detectable in CS-hemolymph. Since silkworm hemocytes usually aggregate in hemolymph
Table 2
Effect of various sugars on phenoloxidase in hemolymph

<table>
<thead>
<tr>
<th>Sugar</th>
<th>Po activity A$_{490}$/5 min</th>
<th>Developmental stage of silkworms used hours after fourth moult</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose + Fructuose</td>
<td>0.193 ± 0.047</td>
<td>42</td>
</tr>
<tr>
<td>Mannose</td>
<td>0.214 ± 0.045</td>
<td>150</td>
</tr>
<tr>
<td>Maltose</td>
<td>0.290 ± 0.042</td>
<td>66</td>
</tr>
<tr>
<td>Trehalose</td>
<td>0.330 ± 0.029</td>
<td>66</td>
</tr>
<tr>
<td>Control (without sugar)</td>
<td>0.407 ± 0.033</td>
<td>72</td>
</tr>
</tbody>
</table>

Injection of saline containing sugar collection of hemolymph and assay of Po activity at 20 min after bleeding. Concentration of each sugar in the saline was 20 mM.
Table 3
Total Hemocyte count of Hemolymph

<table>
<thead>
<tr>
<th>Hemolymph</th>
<th>Total hemocyte count/number of hemocyte/mm³ of hemolymph</th>
<th>Total hemocyte count corrected for dilution of hemolymph</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal hemolymph</td>
<td>10353 ± 313</td>
<td>10353</td>
</tr>
<tr>
<td>Hemolymph obtained after injection of saline solution</td>
<td>808 ± 204</td>
<td>3551</td>
</tr>
</tbody>
</table>

Hemolymph was collected from fifth instar larvae at 96 hr after fourth molt
collected by conventional methods, the absence of associated hemocytes may reflect some changes of surface properties may be due to the remaining of a kind of hemocytes without ability to associate each other.

Pay (1974) reported a method for obtaining hemocytes without phenoloxidase activity by immunization of larvae of *G. mellonella* with lipopolysaccharide B from *Shigella flexner*. The immunized hemolymph and CS-hemolymph share the same property in that phenoloxidase was induced by incubation with Zymosan or by mechanical agitation. However, they differ in the total hemocyte count: in brief; immunized hemolymph contains many hemocytes as normal hemolymph, while the total hemocytes of CS-hemolymph are reduced to only 9.3% of the normal levels. The mechanism by which CSF produces hemolymph without phenoloxidase activity is apparently different from that by which LPS does, since the action was rapid whereas LPS required 16 hr before the effect become apparent.

Phenoloxidase activity in cray fish (*Astacus astacus*) hemolymph was enhanced by laminarin (Unestam and Soderhall, 1977). The activation reaction of Pro-phenoloxidase in CS-plasma was also triggered by laminarin. In this case however, a 200 fold higher concentration was required. This may indicate that the affinity of substances to the glucan are different between the silkworm and *cray fish*. 
Phenoloxidase catalyzes melanin formation from phenolic substances (Lerner, 1953). The occurrence of latent phenoloxidase of Prophenoloxidase has been reported in both animals (Ashida, 1971; Barisas, 1974) and plants (Bailey 1961). Activation of Prophenoloxidase is thought to play a potentially important role in the regulation of pigment formation. The pro-enzyme has been shown to be activated either by protein denaturants such as anionic detergents (Yamaguchi et al., 1970). Presently we have demonstrated phenoloxidase free from PPAE at three pH values i.e. 6.5, 7.5 and 9.0. Pro-enzyme was activated by PPAE in 0.01 M phosphate buffer pH 6.5 and 7.5 or 0.01 M Tris-HCl buffer pH 9.0 at 0°C. In the reaction mixture concentration of pre-enzyme and PPAE were 200 µg/ml respectively. After incubation for 60 min at 0°C. Phenoloxidases thus prepared at pH 6.5, 7.5 and 9.0.

The pro-enzyme was activated by PPAE at various pH values. The specific activity of phenoloxidase activated at pH 9.0 is very low when compare with pH 6.5 and 7.5 (Table 4). It's lower specific activity is not a result of inactivation of the enzyme, since the activity did not change during activation.

PPAE activity increased when the pH was increased from 5 to 8 and then remained constant at pH 10 whereas with the pro-enzyme, the pH optimum was around 7.5. The discrepancy between the two pH activity
Table 4
Specific activities of phenoloxidase activated at different pH values

<table>
<thead>
<tr>
<th>pH of activation reaction</th>
<th>Incubation time (min)</th>
<th>Amount of phenoloxidase recovered (µg/protein/ml)</th>
<th>Phenoloxidase activity (Units/ml)</th>
<th>Specific activity (Units/µg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.5</td>
<td>30</td>
<td>7.5</td>
<td>520</td>
<td>69.3</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>7.5</td>
<td>560</td>
<td>74.7</td>
</tr>
<tr>
<td>7.5</td>
<td>30</td>
<td>12.6</td>
<td>1000</td>
<td>74.9</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>12.5</td>
<td>970</td>
<td>77.6</td>
</tr>
<tr>
<td>9.0</td>
<td>3</td>
<td>11.5</td>
<td>390</td>
<td>33.9</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>10.7</td>
<td>380</td>
<td>35.5</td>
</tr>
</tbody>
</table>
profiles of PPAE with the pro-enzyme as substrate seems to be due to
difference of procedures for obtaining the profiles.

Lysozymes of phenoloxidase have been found in various organisms,
some of them were reported to have only diphenolase activity (Belitz, 1977). Phenoloxidase formed from the pro-enzyme seems to offer an ideal material for testing how association and dissociation of an identical subunit may affect substrate specificity. Since phenoloxidase (pH 9.0) being rich in smaller aggregates had less mono-phenolase activity than phenoloxidase (pH 6.5 and pH 7.5) which were composed mainly of larger aggregates, the degree of association or resulting conformation seems to affect substrate specificity of silkworm hemolymph phenoloxidase.

Insects have effective defense mechanisms against microorganisms such as bacteria and fungi. The major defense mechanisms in the hemocoel are either cellular (Phagocytosis) and formation of multicellular hemocytic capsules (Lackie, 1988) or humoral effected by immune proteins and lectins and the prophenoloxidase cascade (Johansson and Soderhall, 1989). Recognition of microorganisms as non-self is apparently involved when the insect defense mechanisms are set in motion (Janeway, 1989). Clonally selected recognition molecules like immunoglobulins in higher vertebrates have not evolved in insects, but molecules for non-clonal pattern recognition play a central role in discrimination of self from non-self. Thus a number of
molecules with affinity to particular structures of bacteria or fungal cell walls have been reported from insect hemolymph, and they have thought to be potential recognition molecules for foreignness (Sun et al., 1990). Lipopolysaccharides and peptidoglycons have been suggested as recognition molecules. However, the biological activities of these proteins are far from being fully understood (Komono and Natori, 1985).

We have already predicted the presence of two kinds of hemolymph molecules which have specific affinity for peptidoglycan (PG) (bacterial cell wall component). This molecule was supposed to have ability to trigger prophenoloxidase cascade upon binding to their respective ligands. We proposed the name peptidoglycon recognition protein (PGRP). PG has been shown to have various biological functions both in mammals (Kotani et al., 1986) and in insects (Dunn, 1986).

PGRP was purified from 250 ml of larval silkworm hemolymph. The purification procedure consisted of ammonium sulphate fractionation, column chromatography on peptidoglycon-sepharose 4B. As PGRP could not be quantified in hemolymph and the ammonium sulphate fraction, the yield of PGRP in the first steps could not be calculated (Table 5). In SDS-PAGE under reducing conditions purified PGRP migrated as a single band to the position corresponding to that of the 19 kDa polypeptide (Fig. 19, Lane a). Similar molecular mass (16.5 kDa) in the SDS-PAGE (Fig. 19, Lane b).
Table 5
Summary of purification of PGRP<sup>C</sup>

<table>
<thead>
<tr>
<th></th>
<th>Volume ml</th>
<th>Protein concentration mg/ml</th>
<th>Activity Units/ml</th>
<th>Specific activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hemolymph</td>
<td>250</td>
<td>78</td>
<td>A</td>
<td>A</td>
</tr>
<tr>
<td>Ammonia Sulfate</td>
<td>B</td>
<td>B</td>
<td>B</td>
<td></td>
</tr>
<tr>
<td>Peptido glycon Sepharose 4B</td>
<td>120</td>
<td>0.0075</td>
<td>2.5</td>
<td>0.333</td>
</tr>
</tbody>
</table>

A = Because hemolymph and ammonium sulfate precipitate contained unidentified factor which causes activation of prophenoloxidase in the hemolymph - PG in the absence of peptidoglycon.

B = Not determined.

C = Peptidoglycon recognition protein.
The restoration of reactivity of the pro-phenoloxidase cascade to PG in hemolymph-PG-supplemented with varied amounts of purified PGRP is shown in (Fig. 20). A decreasing lag period was observed as the concentration of PGRP increased. Once activation of prophenoloxidase is initiated, however the rate of conversion of prophenoloxidase seems to be independent of the amount of PGRP added.

Silkworm hemolymph prophenoloxidase cascade includes a PGRP which have specific affinity to PG. These molecules were proposed to trigger the cascade upon binding to their respective ligands. The PGRP preparation was shown to be homogeneous by SDS-PAGE. The molecules are capable of restoring the reactivity of the pro-phenoloxidase. Cascade to PG in hemolymph-PG which is assumed to contain all components of prophenoloxidase cascade except for PGRP (Fig. 20). These results indicate that the purified protein is PGRP. The assay for PGRP activity by using hemolymph-PG enabled us to detect PGRP at concentrations as low as 90 ng/ml (Fig. 20). However, prophenoloxidase in hemolymph-PG can be activated without PG by unknown factors, in hemolymph of the ammonium sulfate fraction. Such non-specific activation of prophenoloxidase was experienced in the assay of βGRP and the reason for it discussed (Ochiai and Ashida, 1988). To ensure that we assayed PGRP, the effect of given sample on the pro-phenoloxidase cascade in hemolymph-PG was examined both with and
Fig. 20. Effect of supplement peptide glycan recognition protein (PGRP) on activation of the prophenol-oxidase cascade

(a) Each reaction mixture consisted of 10 μl of serially diluted PGRP solution, 110 μl of hemolymph PG containing 10 mM CaCl₂

(b) The reaction mixtures were incubated at 25°C and at intervals an aliquot was assayed for phenol oxidase activity to monitor the activation of prophenol oxidase cascade
without PG and a given sample was judged to contain PGRP only when it could trigger the prophenoloxidase cascade in hemolymph PG with, but not without PG.

The chitinous exoskeleton of the insect is a non-living matrix of carbohydrate and protein secreted from a monolayer of epidermal cells, which cover the entire surface of the insect, including respiratory trachea, the anterior and posterior portion of the digestive tract, and reproductive ducts (Wigglesworth, 1972). The cuticle serve as a protective barrier between the internal tissues and the external environment. Injury causes the cuticle to darken or melanize around the damaged zone. Pasteur (1870) while studying silkworm diseases noted that the cuticles of healthy silkworms were often topically scratched and as a result, the cuticle darkened around the injured area. Furthermore, he observed that silkworms infected with *Nosema bombysis* often manifested a cuticular melanization reaction. Cuticular melanization is thought to be an important defense reaction of insects because it is involved in wound healing and sequestration of pathogen (Vey and Gotz, 1986).

Lai-Fook (1966) studied the mechanism of the cuticular melanization reaction with regard to injury. She speculated that phenoloxidase was responsible for melanization and was present in a latent form, which in turn was activated by a proteinaceous activator upon injury. The regulation mechanism of activation of prophenoloxidase and the site of it’s synthesis have
remained important unresolved questions in cuticular biochemistry (Barrett, 1991). Our experiments extended to demonstrate the presence of the prophenoloxidase cascade in the cuticle of the silkworm.

Phenoloxidase activity increased with time when the prophenoloxidase fraction was incubated with the activating cascade (AC) fraction that had been previously treated with Ca$^{2+}$. It was demonstrated that Ca$^{2+}$ was necessary for the AC fraction to acquire the activity to activate prophenoloxidase in the mixture of both fractions (Fig. 21). Hemolymph prophenoloxidase, which is composed of two polypeptides with mobilities in SDS-PAGE corresponding to 71 kDa and 70 kDa (Fig. 22, Lane c), but did not detect any polypeptides in the activated cascade fraction, indicating the presence of prophenoloxidase only in the prophenoloxidase fraction.

Among the proteins extracted from cuticles with 5% SDS, 3% 2-mercaptoethanol and 5M urea, no polypeptide other than a polypeptide doublet of prophenoloxidase cross reacted with anti-hemolymph prophenoloxidase (Fig. 22, Lane b). The prophenoloxidase polypeptides were calculated to be 71 kDa and 70 kDa respectively. The mobilities of the prophenoloxidase polypeptides in the pro-phenoloxidase fraction were similar to those in the SDS/urea cuticular extract (Fig. 22, Lane c). If the prophenoloxidase fraction is incubated with the AC fraction in the absence of Ca$^{2+}$, no difference in mobility could be detected (Fig. 22, Lane e). If however
Fig. 21. Activation of hemolymph associated prophenoloxidase

(a) Fraction resulted in the phenol oxidase activity
(b) Fraction no phenol oxidase activity appeared
the prophenoloxidase fraction was incubated with the Ca\textsuperscript{2+} treated AC fraction as 4-kDa decrease in molecular mass of both polypeptides could be observed (Fig. 22, Lane f).

One of the distinguishing features of arthropods is their exoskeleton, which provides them with protective armour against the aggressions of external environment. It has been considered for the most part the integument is only an inert physical barrier (Wigglesworth, 1972). Another common injury of defense response is cuticular melanization. This response can be elicited by a mechanical scratch or microbial invasion.

Present results clearly demonstrate that cuticular phenoloxidase is truly in zymogen form which is activated through a limited proteolysis by the serine proteinase which is also in a zymogen form. Hence two distinct steps have been elucidated in the activation of prophenoloxidase. Ca\textsuperscript{2+} is necessary for the conversion of pro-PPAE to PPAE, however, we do not know if Ca\textsuperscript{2+} is involved in this specific reaction or if it is involved in cascade events prior to this step. The mechanism by which injury or pathogen invasion set the cuticular cascade into motion remain unknown during our demonstrations.

Sass and his colleagues (1994) showed not only that cuticular peptides are synthesized by the epidermal cells but also that they can also originate from hemocytes and be transported across the epidermis and enter into the cuticular matrix. This could explain our observation of prophenoloxidase localization
along the basal surface (hemocoel side) of the epidermal basement membrane of the tracheal and body wall integument.

Present results demonstrate the ubiquity of prophenoloxidase throughout the insect body i.e. hemolymph and cuticle. The insect tissues are literally bathed in or surrounded by this enzyme zymogen and its activating cascade. Defense functions have already been attributed to the prophenoloxidase cascade, but basic question is whether prophenoloxidase could fulfill other functions, such as an oxygen transport like hemocyanin or hemoglobin because silkworm prophenoloxidase was shown to be a protein homologous to arthropod hemocyanin (Kawabata et al., 1995). One could speculate that prophenoloxidase picks up oxygen from both the vast body surface and tracheal system and transports it through the hemolymph to the tissues.

Insects respond to the entry of bacteria into the hemocoel with a series of cellular and humoral defensive reactions (Dunn, 1986). These include immediate response by circulating hemocytes and induction of the synthesis of several antibacterial proteins such as cecropin, attacin, diptericin and lysozyme (Boman and Hultmark, 1987). The antibacterial protein synthesis is a specific response to the presence of bacteria or bacterial cell wall components in the hemocoel. The response is not elicited by injection of saline or non-bacterial molecules.
We have demonstrated that bacterial PG fragments are recognized by the fat body and elicit cecropin synthesis in silkworm larvae. It was been suggested that the PG fragments are released from invaded bacteria by the action of hemolymph lysozyme and to act as a signal molecule for activation of a series of antibacterial genes (Dunn and Dai, 1990). *Lepidopteran* insects contain relatively high levels of lysozyme as a normal component of the hemolymph. The exact roles of the high levels of lysozyme in normal hemolymph and the further increase of the amount of in response to invading bacteria have not been elucidated.

Silkworm larvae were injected with soluble peptidoglycon (SPG) from *B. megaterium* and time course of elevation of cecropin activity in the hemolymph was determined. The results in Fig. 23 show that cecropin activity was induced in parallel after the injection of elicitors. Cecropin activity increased significantly 7 hrs after injection of SPG, but in the larvae injected with saline alone the elevation of the activities was slightly delayed and become detectable 9 hrs after injection.

To determine the specificity of elicitor necessary for antibacterial protein synthesis silkworm larvae were injected with various bacterial cell wall components of structurally related glucons and LPS. The cecropin activity in the hemolymph was measured 24 hrs after the injection (Table 6). High activities of cecropin was induced specifically by SPGs of *Bacillus* and *E. coli,*
Table 6
Specific induction of cecropin by various biological agents

<table>
<thead>
<tr>
<th>Materials injected</th>
<th>Dose (μg/larvae)</th>
<th>Cecropin activity Units/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td></td>
<td>Not detected</td>
</tr>
<tr>
<td><em>Escherichia coli</em> (SPG)</td>
<td>10</td>
<td>252 ± 22</td>
</tr>
<tr>
<td><em>Micrococcus luteus</em></td>
<td>10</td>
<td>20 ± 12</td>
</tr>
<tr>
<td>LPS</td>
<td>10</td>
<td>324 ± 64</td>
</tr>
<tr>
<td><em>Bacillus subtilis</em></td>
<td>10</td>
<td>Not detected</td>
</tr>
</tbody>
</table>
Fig. 23. Induction of Cecropin activities by an injection of the silkworm larvae soluble peptidoglycon (SPG) from *Bacillus* Sps. 1 μg in 10 μl saline (a) Control silk worms were similarly injected with 10 μl of saline(b)

Hemolymph was collected at intervals and assayed for cecropin activities
but slight activities by SPG of *M. luteus*. LPS was also an effective elicitor for cecropin. Cecropin activity in the hemolymph increased in parallel with the decrease of saline alone injected larvae with SPG from *Bacillus*. The elevation of the cecropin activities in the immunized hemolymph is attributed to the induction of the gene expression in the fat body.

The elevation of lysozyme activity was not a non-specific reaction for wounding of injection of foreign materials but a specific response for the presence of some bacterial cell wall components as observed in *Manduca sexta* (Kanost *et al.*, 1988). The structural requirements as an effective elicitor for lysozyme induction were identical to that for cecropin induction. It is important to note that SPG from *Bacillus* or *E. coli* was a strong elicitor for cecropin induction, although SPG from *M. luteus* was not a strong cecropin inductor. The hemolymph lysozyme is suggested to play an important role in degrading the cell wall and producing the signal molecule necessary for the induction of antibacterial protein synthesis.

Insects possess an innate immune system composed cellular and humoral mechanisms that promptly respond to invasion by microorganisms and other parasites. The cellular mechanisms include, phagocytosis, nodule formation and encapsulation (Lackie, 1988). The humoral response activated within hours generates a diverse set of broad-spectrum antibacterial proteins and peptides such as cecropins and lysozymes (Faye and Hultmark, 1993).
The most significant and intriguing difference between the insect and vertebrate systems are that, there is no evidence for clonal selection mechanisms in insects, no immunoglobulins have yet been observed in insects. Some insect proteins (fasciclin and neuroglian) which contain immunoglobulin (Ig) like domains play a role during the development of the insect nervous system.

Hemolin is so far the only invertebrate member of the immunoglobulin super-family found to be up regulated and released upon infection. Hemolin is present at low amounts in the hemolymph of naive insects and after bacterial infection the concentration increases 18-fold in *Hyalophora cecropia* (Sun *et al.*, 1990).

The *in vitro* experiments in present research project show that hemolin is a potent regulator of cellular responses and intracellular signaling pathways. The inhibition of hemocyte aggregation was one of the first characteristics observed in hemolin from *M. sexta* (Ladendorff and Kanost, 1991). However, the study and analysis of these results *in vitro* have been hampered both by the difficulties of preventing unspecific aggregation once the cells are collected and by the necessity of a quantitative assay. Therefore, in order to prevent uncontrolled aggregation we developed a simple method in which the hemocytes are resuspended in a 0.20 M NaCl solution containing DNAase.
Hemocytes collected using this technique remain disperse without clumping or decreased viability.

Once the non-specific clumping was prevented, we studied the conditions needed to induce the aggregation (Fig. 24). PHA and LPS failed to induce hemocyte aggregation in Ca\(^{2+}\) free buffer. Hemocyte aggregation was induced by 10 mM Ca\(^{2+}\) and 1 mM Ca\(^{2+}\) supported the aggregation with LPS and PHA, however 1 or 10 mM Mg\(^{2+}\) had no effect. These results indicate that PHA or LPS induced hemocyte aggregation requires Ca\(^{2+}\).

Because the combination of 1 mM Ca\(^{2+}\) and PHA or LPS induced the best aggregation the next experiments were done under similar conditions. The effect of different hemolin concentrations were assayed on PHA induced cell aggregation. Hemolin shows a dose dependent capacity to prevent the aggregation of hemocytes (Fig. 25). Hemolin 100 \(\mu g/ml\) prevents almost 100% cell aggregation. Previous methods used to obtain hemocytes without clumping or aggregation were not sufficient to obtain stable hemocytes from \textit{H. cecropia} (Horonov and Dunn, 1982).

In our study we used DNAase to prevent non-specific aggregation. Once we had obtained non-aggregated hemocytes we induced aggregation with PHA and LPS in the presence of Ca\(^{2+}\). In nature insect hemocytes are capable of adhering at the site of the break in the epidermis as well as to large foreign objects in the hemocoel. However, the hemocyte types and events involved in
Fig. 24. Effect of various compounds on the induction of hemocyte aggregation. Cells were incubated

1. 1 mM Ca^{2+}
2. 10 mM Ca^{2+}
3. 50 mM µg/ml LPS
4. 50 µg/ml PHA
5. 50 µg/ml LPS with 1 mM Ca^{2+}
6. 20 µg/PHA with 1 mM Ca^{2+}
Fig. 25. Effect of different hemolin concentrations on cell aggregation. Cells were incubated in the presence of 50 μg/ml PHA with 1 mM Ca²⁺ and different concentrations of hemolin.
hemocyte adhesion activation have been difficult to identify, because *in vitro* hemocytes rapidly adhere to different substrates in the apparent absence of activating factors. Therefore, this model system of hemocyte stimulation allows analysis of the effect of hemolin on aggregation and facilitates the study of factors and molecules involved in both aggregation and nodule formation.

Phenoloxidase is a potentially dangerous enzyme to insects, because it oxidizes phenolic compounds to the corresponding quinones. Quinones are very reactive and therefore harmful to organisms invading the insect body and at the same time to the insect cells. This seems to be one of the reasons why all insect phenoloxidases are synthesized as inactive precursors, prophenoloxidases.

Precursors of granular phenoloxidase and laccase are programmed to be activated at defined times of development. However, precursors of wound phenoloxidase and hemolymph phenoloxidase must be readily activated in response to accidental cuticular wounds and invasion of microorganisms into the hemocoel. Although it has become more and more evident that all insect prophenoloxidases are activated through a limited proteolysis, the regulation mechanisms for the activation are limited proteolysis, the regulation mechanisms for the activation are totally unknown except for that in hemolymph prophenoloxidase.
Whatever mechanisms insects have exploited, it is obvious that their malfunction would seriously jeopardize insect survival. For example, undesired systemic activation of hemolymph prophenoloxidase in the silkworm would result in blackening of hemolymph in a matter of seconds and would lead to the death of the insect. In this context, insects have harnessed the destructive potential of phenoloxidase by developing complex regulation mechanisms for its activation. In this respect, regulation mechanisms for the activation of cuticular prophenoloxidases and pro-laccase could be as complicated as that for prophenoloxidase in hemolymph, as is suggested by the occurrence of many mutations affecting body colour or colour pattern of *Drosophila*.

It is fascinating to learn how extensively insects have exploited the various potential functions of phenoloxidases (defense against non-self, wound healing, sclerotization, and coloration). Other living organisms, both plants and animals possess phenoloxidases, but none have taken advantage of the multifaceted functions of phenoloxidases like the insect. Phenoloxidase could be said to be a substance characterizing the physiology of insects.

The antibacterial activities of the isolated and synthetic peptides against various bacteria were compared by the growth of inhibition zone assay (Hultmark *et al.*, 1982). Peptide 1, 2, 3 showed antibacterial activity against the
two gram negative bacteria *Acinetofacter sp* and *E. coli* (Table 7). Peptide 3 had the highest activity against the two bacteria followed by peptide 2.

As shown in Table 3, the antibacterial activity of the novel peptides was much lower than that of cecropin. It was possible that the antibacterial activity of the novel peptides was inhibited by salt as in the case for abaecin, a proline rich antibacterial peptide from honeybee (Casteels *et al.*, 1997).

In preliminary experiments, the peptides at a concentration of the order of 10 μg/ml showed bactericidal activity against *E. coli* when incubated in water. However they showed only slight activity when incubated in the presence of a salt such as NaCl, KCl and MgCl₂. Sucrose did not have much effect on the antibacterial activity of the peptides suggesting that the decrease in activity caused by salt was not due to osmotic stabilization of the bacteria but was due to inhibition of the adsorption of the peptides to the bacterial surface which is mediated by the ionic interactions.
### Table 7

**Antibiotic activity of hemolymph peptides**

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>Amount (n mol/well)</th>
<th>Inhibition zone diameter (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td><strong>Acinetobacter sp</strong></td>
<td>2</td>
<td>2.3 ± 0.6</td>
</tr>
<tr>
<td><strong>E. coli</strong></td>
<td>2</td>
<td>2.5 ± 0.3</td>
</tr>
<tr>
<td><strong>Pseudomonas fluorescens</strong></td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td><strong>Staphylo coccus aureus</strong></td>
<td>2</td>
<td>2</td>
</tr>
</tbody>
</table>

The growth inhibition zone assay was performed on assay plates of nutrient medium. Well diameter was 2 mm and the value 2 mm indicates the formation of no inhibition zone.

Cecropin isolated from *B. mori* was used as a control antibacterial peptide.
Fig. 1 Viable hemocytes from healthy silkworms (Bombyx mori L.)
Stages: 4th instar larvae collected from selected Sericulture farm.

Fig. 2. Light photograph of E. coli strain: MTCC 443 induced hemocyte aggregation in normal healthy silkworms (Bombyx mori L.)
Fifth instar larvae were artificially immunized by inter-hemocoelic injection of 10 μl late log phase E. coli to induce immune function.

Fig. 3. LPS induced hemocyte aggregation in normal healthy silkworms (Bombyx mori L.)
LPS + 10 mM Ca2+

Fig. 4. Light photograph of healthy silkworm hemocyte development
Aggregation of granulocytes surrounded by plasmocytes.
Fig. 5. Inhibition of hemocyte aggregation with infection without Ca2+ and LPS.

Fig. 6. Light photograph of pebrine (Nosema bombysis) induced hemocyte aggregation in pebrine infected silkworms.

Fig. 7. Non-aggregation of hemocytes from E. coli induced silkworm of 3rd instar larvae.

Fig. 8. In vitro immune response function assay. The gels were stained with Coomassie brilliant blue after SDS-PAGE separation of the following:
Lane 1: The acid extract of hemolymph of healthy silkworm.
Lane 2: The acid extract of hemolymph of healthy silkworm injected with E. coli. The position of immune protein 26 kDa was separated as single band.
Lane 3: Pebrine infected hemolymph injected with E. coli appearance of three faint bands 76, 96 and 105 kD as formed antipebrine protein factors an early stage of pebrine infection.
Lane 4: Control extracted with late log phase of E. coli with PBS buffer.
Lane 5: Pebrine infected hemolymph alone
Lane 6: Molecular mass markers indicated on the right side are in kilodaltons.
Fig. 9. Detection of prophenoloxidase and phenoloxidase by SDS-PAGE.
Lane 1: Protein molecular mass markers.
Lane 2: SDS-PAGE pattern of cuticular protein samples from healthy normal control fifth instar larvae.
Lane 3: Phenol oxidase fraction the 31 kDa band is marked by arrow.
Lane 4: Cuticular protein samples from pebrine infected silkworms. Appearance of prophenoloxidase as 35 kDa band is marked by arrow.
Lane 5: Pebrine induced protein fraction as 35 kDa band is marked by arrow.

Fig. 10. (A) Bacterial colonies isolated from cuticular surface of pebrine infected silkworms.
(B) Bacterial colonies isolated from cuticular surface of healthy silkworms.

Fig. 11.
(A) Fungal colonies isolated from cuticular surface of pebrine infected silkworms.
(B) Fungal colonies isolated from cuticular surface of healthy silkworms.
PLATE 4

Fig. 13. PAGE analysis of pro-enkephalin process in silkworm hemocyte lysate injected with LPS (lane a & c) and control (lane b) hemocyte lysate collected 15 min post injection (lane d).

Fig. 19. SDS-PAGE of peptidoglycon recognition peptides (PGRP) about 3.5 mg of protein was applied to each lane.
(a) PGRP
(b) Silkworm lysozyme
(c) Egg white lysozyme
(d) Marker proteins

Fig. 22. Detection of prophenoloxidase and phenoloxidase by SDS-PAGE.
SDS-PAGE pattern of hemolymph associated protein samples stained by Coomassie brilliant blue.
Lane a: Marker proteins.
Lane b: SDS-urea extract of hemolymph.
Lane c: Prophenoloxidase fraction from hemolymph.
Lane d: AC fraction (Activating cascase).
Lane e: Mixture of (1:1 V/V) of the prophenoloxidase fraction and the AC fraction incubated on ice for 30 min.
Lane f: Mixture of (1:1 V/V) prophenoloxidase fraction and Ca+ treated AC fraction incubated for 30 min. on ice.
Lane g: Marker proteins.