3. RESULTS
3.1 Immunological studies

3.1.1 Immunodiffusion

Immunodiffusion technique using bacteria induced haemolymph was carried out to identify antibacterial substances from housefly larvae. In such studies the haemolymph obtained from houseflies inoculated with *M. luteus* was collected and serially diluted. The centre well served as the antigen well and the bacteria treated with 0.05 mM EDTA and 1% SDS served as the antigen. Essentially the soluble proteins of the bacterial wall were assumed to act as antigen.

The results obtained by reaction of bacterial antigen with serially diluted haemolymph of *M. luteus* induced housefly larvae are shown in Fig. 2a. These results indicate that the soluble proteins of bacteria did react and form precipitin lines. It was also clear that this haemolymph had a broad spectrum specificity, as the precipitin line was not only obtained from *M. luteus* cell wall but *E. coli* (Fig. 2b) and *B. thuringiensis* (Fig. 2c) cell wall as well.

In an attempt to characterize the substances that might be possibly responsible for the immune, reaction the antibacterial serum was subjected to different treatments. These treatments included trypsin digestion of the haemolymph, heating of the haemolymph at 100°C in a boiling water bath and incubation of haemolymph with bacteria at 37°C for 1h. Haemolymph obtained from non-immunized housefly larvae served as control. The control antigen well was loaded with soluble protein from bacterial cell wall obtained by EDTA and SDS treatment. The peripheral wells were loaded with control, immunized and variously treated immunized haemolymph.

The results of such an immunodiffusion study is shown in Fig. 2d. Except the normal induced haemolymph which produced a precipitin line against the soluble proteins from the bacteria, the response from the control haemolymph
Fig. 2a, 2b, 2c: Double immunodiffusion pattern showing the reaction of serially diluted induced haemolymph viz., 100% (1), 50% (2), 25% (3), 12.5% (4), 6.25% (5) and 3.125% (6) against the antigens of *M. luteus* (2a), *E. coli* (2b) and *B. thuringiensis* (2c).

Fig. 2d: Double immunodiffusion pattern showing the reaction of *M. luteus* antigen (A) with immunized haemolymph (1), immunized haemolymph subjected to heat treatment (2), immunized haemolymph subjected to bacterial treatment (3), immunized haemolymph treated with trypsin for one hour (4) and control haemolymph (5).

Fig. 2e: Immunoelectropherogram showing the immunoprecipitin arc obtained against *M. luteus* antigen (A) with immunized haemolymph (IH).
and induced haemolymph treated with trypsin and bacteria remained negative. Nevertheless, heat treated induced haemolymph did produce a weak precipitin line indicating the possible thermostability of the substance. The fact that no precipitin arc was produced from trypsin treated induced haemolymph suggested the possibility of proteinaceous nature of the substances. The absence of precipitin line against the bacteria treated haemolymph indicated that possibly the bacterial wall proteins adsorbed the substance from the induced haemolymph and the substance \textit{per se} was not available for the immune reaction.

3.1.2 Immunoelectrophoresis

The soluble proteins of \textit{M. luteus} cell wall were subjected to agar gel electrophoresis as described in section 2.5.2 of materials and methods. An antiserum canal cut in the middle of the slide was filled with suitably diluted antibacterial haemolymph and incubated in a humid chamber overnight. A precipitin arc appeared as shown in Fig. 2e. The appearance of the precipitin arc at the anodic end suggested that the antigen is a negatively charged low molecular weight substance.

These immunological studies showed that inducible antibacterial substances are produced in the houseflies and these substances formed characteristic immunoprecipitin reactions when classical immunodiffusion and immunoelectrophoresis were performed. Further, the failure of diluted haemolymph to produce an immunoprecipitin reaction, suggested that a threshold concentration of the antibacterial substance was required for the normal antigen and antibody reaction.

3.2 SDS-PAGE

In order to determine the polypeptide subunits of bacteria induced larval haemolymph of housefly, the haemolymph of inoculated larvae was drawn at intervals and subjected to SDS-PAGE. Normal larval haemolymph served as
Fig. 3 : Electropherogram showing the protein profile of *M. luteus* induced haemolymph of *M. domestica* larvae.
Fig. 4: Densitometric scan of electropherogram obtained by SDS-PAGE gels of control haemolymph (Top) and *M. luteus* induced (12h) haemolymph (Bottom) of *M. domestica* larvae.
control. Fig.3 shows the protein profile of control and bacteria injected larval haemolymph. A total of 41 subunits were detected in the silver stained gels with the molecular weight ranging between 14 and 120 KD.

Table-1: Summary of the ultrascan data for bacteria induced proteins in the haemolymph of *M. domestica* larvae.

<table>
<thead>
<tr>
<th>Sl. No</th>
<th>Induced protein subunit number</th>
<th>Molecular weight KD</th>
<th>Peak height (Au)</th>
<th>Area (Aux mm)</th>
<th>Relative area (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>con 12h</td>
<td>con 12h</td>
<td>con 12h</td>
</tr>
<tr>
<td>1</td>
<td>40</td>
<td>15</td>
<td>0.15 1.70</td>
<td>0.31 0.99</td>
<td>0.3 2.3</td>
</tr>
<tr>
<td>2</td>
<td>39</td>
<td>16</td>
<td>0.58 0.94</td>
<td>1.73 2.67</td>
<td>2.9 3.3</td>
</tr>
<tr>
<td>3</td>
<td>38</td>
<td>17</td>
<td>0.31 0.96</td>
<td>1.17 2.65</td>
<td>2.2 2.1</td>
</tr>
<tr>
<td>4</td>
<td>37</td>
<td>18</td>
<td>0.55 1.16</td>
<td>0.73 8.30</td>
<td>2.8 7.0</td>
</tr>
<tr>
<td>5</td>
<td>35</td>
<td>19</td>
<td>0.13 0.50</td>
<td>0.32 3.16</td>
<td>0.8 2.7</td>
</tr>
<tr>
<td>6</td>
<td>34</td>
<td>20</td>
<td>0.23 0.66</td>
<td>0.41 3.43</td>
<td>1.4 2.7</td>
</tr>
<tr>
<td>7</td>
<td>28</td>
<td>22</td>
<td>0.56 0.44</td>
<td>1.75 1.68</td>
<td>2.2 1.7</td>
</tr>
<tr>
<td>8</td>
<td>26</td>
<td>24</td>
<td>0.26 0.97</td>
<td>2.56 4.26</td>
<td>1.0 3.3</td>
</tr>
<tr>
<td>9</td>
<td>23</td>
<td>28</td>
<td>0.84 0.78</td>
<td>0.79 0.59</td>
<td>2.8 1.4</td>
</tr>
<tr>
<td>10</td>
<td>13</td>
<td>66</td>
<td>1.00 0.68</td>
<td>0.94 0.73</td>
<td>3.2 1.7</td>
</tr>
<tr>
<td>11</td>
<td>11</td>
<td>68</td>
<td>0.62 1.06</td>
<td>4.61 6.14</td>
<td>4.7 5.3</td>
</tr>
<tr>
<td>12</td>
<td>7</td>
<td>72</td>
<td>0.35 0.70</td>
<td>0.73 1.37</td>
<td>1.0 1.8</td>
</tr>
<tr>
<td>13</td>
<td>6</td>
<td>79</td>
<td>0.43 0.80</td>
<td>1.54 2.24</td>
<td>1.9 2.2</td>
</tr>
<tr>
<td>14</td>
<td>4</td>
<td>82</td>
<td>0.38 0.80</td>
<td>1.24 3.91</td>
<td>3.5 6.4</td>
</tr>
<tr>
<td>15</td>
<td>3</td>
<td>94</td>
<td>0.40 1.09</td>
<td>1.24 4.06</td>
<td>1.2 1.4</td>
</tr>
<tr>
<td>16</td>
<td>2</td>
<td>120</td>
<td>0.40 1.01</td>
<td>1.30 1.97</td>
<td>1.2 1.9</td>
</tr>
</tbody>
</table>

Con - Control haemolymph
12h - Induced haemolymph obtained after 12h of inoculation
Fig. 4. shows profile of the densitometer scan of the haemolymph proteins obtained using an ultrascanner. The haemolymph of control and bacteria inoculated (12h after inoculation) larvae were analysed for comparison. About 13 proteins increased in quantity due to bacterial injection. The 40th subunit of the induced haemolymph with molecular weight of 15 KD showed a seven fold increase in peak height and the subunit 37 with 18 KD showed an eleven fold increase in area as compared to the control haemolymph. Subunits 37 (18 KD), 35 (19 KD) and 26 (24 KD), showed three fold increase in peak height while the rest of the induced proteins showed two fold increase in both peak height and area. The proteins 28 (22 KD), 23 (28 KD) and 13 (66 KD) showed a slight decrease in injected flies compared to control (Table 1). About 50% of induced protein subunits were of low molecular weight ranging from 15 to 20 KD.

3.3 Antibacterial assay

3.3.1 Inhibition zone assay

The induction of antibacterial substances in housfly larvae due to inoculation of bacteria was further confirmed by IZA. The results of IZA obtained with *M. luteus* induced haemolymph against *M. luteus* cultures are shown in Fig. 5a, b and c.

It is obvious from the figure that there is a bacteria free zone surrounding the well in which the induced haemolymph was placed. With 2 µl of haemolymph that was placed in the well, the bacterial growth was inhibited to the extent of $11.83 \pm 0.61$ mm diameter

The induction of antibacterial substance could be achieved not only by inoculating the bacteria to housfly larvae but also by mere physical injury. Pricking the larvae with hypodermic needle did induce the synthesis of antibacterial substance. Fig.5c shows the inhibition of the bacterial growth surrounding the well that was loaded with induced haemolymph obtained after
Fig. 5: Inhibition zone assay showing the antibacterial activity of control (non-immunized haemolymph) (a), *M. luteus* immunized haemolymph (b) and haemolymph obtained by injuring the larvae (c).

Fig. 7: Inhibition zone assay showing antibacterial activity of control, injured and heat treated larval and pupal haemolymph. Larval control haemolymph (a), Heat treated larval control haemolymph (b), Larval immunized haemolymph (c), Heat treated larval immunized haemolymph (d), Pupal control haemolymph (e), Heat treated pupal control haemolymph (f), Pupal immunized haemolymph (g), Heat treated pupal immunized haemolymph (h).
Fig. 6: Graph illustrating the linear relationship between concentration of antibacterial substance and the diameter of inhibition zones produced in agar plates seeded with *M. luteus*. Each value represents mean ± SD of 6 determinations.
pricking the housefly larvae. No such zone of inhibition appeared in the haemolymph obtained from non-immunized control larvae (Fig. 5a).

When the induced haemolymph was serially diluted and used as the source of antibacterial substance there was a proportionate reduction in the diameter of inhibition zones, indicating a linear relationship between the concentration of the antibacterial substance and the zone of inhibition (Fig. 6).

3.3.2 Thermostability of the antibacterial substance

Thermostability of antibacterial substance was tested by using heat treated induced haemolymph in IZA. In such studies, besides the larval haemolymph, the haemolymph of one day old pupae obtained by induction was also tested. The results of IZA carried out with induced and control haemolymph of larvae as well as pupae are shown in Fig. 7.

Inhibition zones were obtained around the wells that were loaded with the induced haemolymph both from larvae and pupae; on the contrary the haemolymph from uninduced larvae and pupae did not inhibit the bacterial growth. Further, heat treatment of larval and pupal induced haemolymph by placing them in a boiling water bath for 5 min, did not alter the inhibitory activity of the antibacterial substance suggesting that the substance is highly thermostable.

3.3.3 Lytic assay

Lytic assay procedure confirmed the growth inhibitory property of the antibacterial substance by causing lysis of bacteria. The results of lytic assay are shown in figures 8-12.

It could be observed that the cultures of M. luteus incubated with or without haemolymph of housefly larvae showed a characteristic growth curve consisting of an exponential and a stationary phase. Cultures treated with
Fig. 8: Antibacterial activity of immunized (against *M. luteus*) haemolymph with log-phase cultures of *M. luteus* (Δ) compared with culture containing control haemolymph (□) and culture without haemolymph (○).
Fig. 9: Antibacterial activity of immunized (against *M. luteus*) haemolymph with log-phase cultures of *P. aeruginosa* (Δ-) compared with culture containing control haemolymph (□-) and culture without haemolymph (○-).
Fig. 10: Antibacterial activity of immunized (against *M. luteus*) haemolymph with log phase cultures of *E. coli* (Δ—) compared with culture containing control haemolymph (□—) and culture without haemolymph (○—).
Fig. 11 : Antibacterial activity of immunized (against *M. luteus*) haemolymph with log phase cultures of *B. thuringiensis* (Δ) compared with culture containing control haemolymph (□) and culture without haemolymph (○).
Fig. 12: Antibacterial activity of immunized (against *M. luteus*) haemolymph with log phase cultures of *S. aureus* (△) compared with culture containing control haemolymph (○) and culture without haemolymph (∇).
immunized haemolymph did not exhibit normal growth as evidenced by a very low OD of the liquid medium (Fig.8). It appears that the bacteria induced haemolymph substance of the housefly larvae has potent bactericidal property.

Further, the lytic assay using *M. luteus* induced haemolymph was tested against the cultures of *P. aeruginosa, E. coli, B. thuringiensis* and *S. aureus*. *P. aeruginosa, E. coli* and *B. thuringiensis* cultures were susceptible to the presence of immunized haemolymph and did not exhibit normal growth (Fig.9, 10 and 11). Control cultures with or without normal haemolymph exhibited characteristic growth.

*S. aureus* appears to be resistant to the presence of immunized haemolymph in the medium. The bacteria did not undergo lysis and the growth curve was as normal as those of control cultures with or without haemolymph (Fig.12).

Statistical analysis of variance of the data on lytic assay in control and bacterial induced haemolymph of *M. domestica* larvae, provided a significant difference at *P* < 0.05, (Table A1). Addition of immunized haemolymph against *M. luteus* to the culture containing *P. aeruginosa, E. coli* and *B. thuringiensis* resulted in significant changes in OD at different hours of incubation (Tables A2, A3 and A4), whereas the culture containing *S. aureus* showed a non significant result (*P* > 0.05) during the treatments (Table A5).

### 3.3.4 Killing assay

Addition of immunized haemolymph to bacterial suspension and following up of the number of surviving bacteria by counting the number of colony forming units during the log phase of bacterial multiplication, showed that there was a drastic reduction in the number of viable bacteria. The number was reduced from $4 \times 10^5$ cells to around 90 CFU (Fig.13). The control cultures with non-immunized haemolymph in the medium exhibited normal growth.
Fig. 13: Viable count of *M. luteus* incubated with immunized haemolymph in LB medium (Δ—), phosphate buffer containing immunized haemolymph (—○—) and LB medium without immunized haemolymph (—□—). Number of colony forming units are expressed in log-concentration/ml.
Viability of bacteria suspended in a non-growth medium such as 0.1M phosphate buffer was unaffected.

Analysis of variance of the data on the viable count of *M. luteus* incubated with immunized haemolymph in LB medium, phosphate buffer containing immunized haemolymph and LB medium without haemolymph, showed no significant increase or decrease at different time intervals. But significant changes with a value of $P < 0.05$ was recorded between controls and immunized haemolymph (Table A6).

### 3.3.5 Acidic PAGE profile of antibacterial activity

Experiments performed thus far showed that the immunized haemolymph contained antibacterial activity. It was of interest to know which fraction(s) of haemolymph contained the immune property. Since the cellular fractions were removed immediately after the collection of haemolymph by centrifugation, it was obvious that the antibacterial activity was confined to the serum. The serum was therefore subjected to electrophoresis to identify those protein fractions that exhibited antibacterial activity.

The immunized haemolymph obtained from housefly larvae was subjected to acidic PAGE as described in section 2.8.5 under materials and methods. After the electrophoretic separation of proteins, the antibacterial activity was located by sandwiching of the gel with test bacteria as described under section 2.8.5.4 of materials and methods. The results of the study are presented in Fig. 14.

Two main antibacterial factors could be identified in immune haemolymph of housefly larvae. A slow moving fraction (I of Fig. 14) was confined to the top region of the gel and the second (II of Fig. 14) with relatively low molecular weight could be observed in the middle to bottom region of the gel. Protein fractions from heat treated immunized haemolymph also exhibited distinct antibacterial activity suggesting that heating of immunized
Fig. 14: Acidic-PAGE of immune haemolymph of *M. domestica* larvae. The gels were subjected to antibacterial assay with *M. luteus*. Control haemolymph (C) and heat treated control haemolymph (HL) showed no inhibition zone whereas immunized haemolymph (IH) and heat treated immuned haemolymph (HIH) showed extensive zones of inhibition.

Fig. 15: Inhibition zone assay showing the induction of antibacterial activity of immunized haemolymph of housefly larvae, in response to an injection of *M. luteus*. Haemolymph was collected from individuals after 2h (a), 3h (b), 4h (c), 8h (d), 12h (e), 18h (f), 24h (g) and 48h (h) of immunization. The antibacterial activity was determined by measuring the diameter of inhibition zones.
serum in a boiling water bath for 5 min did not alter the stability of the molecule. Antibacterial activity could not be located in the control or non-immunized haemolymph.

Since the low molecular weight proteins exhibited a much pronounced and intense antibacterial activity, further work relating to isolation, purification and characterization of the antibacterial factors were restricted to these fractions.

3.4 Induction kinetics

IZA revealed that the haemolymph of normal housefly larvae has no antibacterial activity under the assay conditions. However, haemolymph obtained from injured larvae and bacteria inoculated pupae exhibited a pronounced antibacterial activity 3h after the treatment. The activity showed enhancement with increase in time (between 5-18h) as revealed by higher diameter of inhibition zones in agar petriplates. Such an enhancement in activity could be observed upto 24h and after this time the activity stabilizes (Fig.15 and 16).

Further, the immune response of the haemolymph of injured larvae was relatively less as compared to the larvae immunized with bacteria, as revealed by relatively small diameter of inhibition zones (Fig. 16).

The two way analysis of variance of the data on inhibition zones recorded by the haemolymph of control, injured and *M. luteus* inoculated housefly larvae, collected at different time intervals, showed a value of P < 0.05. Further, significant changes were obtained at 1% level for the inhibition zones produced between control, injured and *M. luteus* injected haemolymph (Table A7).
Fig. 16: Induction kinetics of antibacterial activity as determined by IZA. Haemolymph from control (−□−), injured (−○−) and *M. luteus* injected (−Δ−) housefly larvae were collected at different time intervals. Each value represents the mean ± SD of 6 determinations.
3.5 Isolation and purification of antibacterial proteins

Earlier it was mentioned that the haemolymph collected from injured larvae or from larvae challenged with specific bacteria exhibited distinct antibacterial activity as demonstrated by IZA technique. Obviously the haemolymph of treated larvae did carry substances that cause a drastic inhibition of bacterial growth.

In the present study attempts were made to isolate the antibacterial substances by standard protein purification procedures. Purification of antibacterial haemolymph of housefly larvae was achieved in 5 steps. They included an initial step of CM-cellulose cation exchange chromatography that would possibly eliminate all the anionic proteins and retained only the basic proteins. In the second step the thermostable property of antibacterial proteins was taken advantage of and by heating step I products to 100°C for 5 min in a water bath, it was possible to retain only the thermostable basic proteins while all the other thermolabile proteins were denatured. The third step involved gel permeation chromatography, in which the thermostable antibacterial proteins were resolved into a number of fractions. Among those fractions, the fraction or fractions that exhibited pronounced antibacterial activity and available in large quantities, were further purified by CM-cellulose chromatography. Such a step resolved the Sephadex G-50 purified fractions into two fractions. Finally the fraction that exhibited pronounced antibacterial activity was purified by hydroxyapatite column chromatography and the final purified product, as revealed by IZA, demonstrated a manifold increase in antibacterial activity.

3.5.1 Purification by CM-cellulose chromatography - Step I

About 30 ml of haemolymph collected from 9,000 housefly larvae were diluted with four volumes of 10 mM phosphate buffer pH 6.0. Prior to loading, the proteins in the haemolymph were estimated to be 570 ± 3.5 mg/ml. Specific antibacterial activity was found to be of the order of 0.9 units/mg of protein.
The total activity calculated was 15.4 KU (Kilounits). The total antibacterial activity at this stage was recorded as 100% (Table 2).

The diluted sample was loaded on a column of CM-cellulose that was previously equilibrated with 10 mM phosphate buffer. After complete loading, the column was extensively washed with 25 mM sodium chloride and the adsorbed material was eluted using 250 mM sodium chloride in buffer. Antibacterial activity was confined only to this fraction and could not be detected in the flow through or washed out fractions.

2 ml fractions were collected and activity could be recorded in 46 fractions, from fractions 5-51. From each fraction 5 μl was tested for antibacterial activity (Fig.17).

After the first step purification the protein concentration of the eluate was found to be 148 mg. There was almost a 100 fold increase in specific activity which was recorded to be 91 units/mg. Total antibacterial activity at this state of purification was 13.5 KU that yielded a recovery value of 87% (Table 2).

3.5.2 Purification by heat treatment - Step II

In the second step, the eluate collected from CM-cellulose chromatography was heated in a boiling water bath for 5 min to remove all the thermolabile proteins. Such a procedure resulted in the protein concentration of the step-I eluate to be reduced to 29.4 mg with a specific activity 380 units/mg. The total activity was 11.2 KU and the recovery percentage was 72 (Table 2). The denatured proteins were removed by centrifugation at 12,000 g for 30 min by repeating 4 times and the clear supernatant was used for further purification.
Fig. 17: Step I. Chromatographic separation of antibacterial protein from immune haemolymph of *M. domestica* larvae. 30 ml of haemolymph diluted four times with phosphate buffer and loaded on CM-cellulose column (3 cm x 4 cm) equilibrated with 10 mM phosphate buffer pH 6.0, washed with 25 mM NaCl and eluted with 250 mM NaCl. 2 ml fractions were collected and 5μl sample of each fraction was analysed for antibacterial activity.
Table 2: Summary of purification procedures, the total protein concentration at each step, specific activity measured and the percentage of recovery of antibacterial proteins from 30 ml haemolymph of *M. domestica* larvae.

<table>
<thead>
<tr>
<th>Steps in purification</th>
<th>Total Protein in mg</th>
<th>Specific Activity in units/mg</th>
<th>Total activity KU</th>
<th>Recovery in %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Haemolymph</td>
<td>17,140.00</td>
<td>0.90</td>
<td>15.400</td>
<td>100.00</td>
</tr>
<tr>
<td>Step I - After cation exchange (CM-cellulose) chromatography</td>
<td>148.00</td>
<td>91.00</td>
<td>13.500</td>
<td>87.00</td>
</tr>
<tr>
<td>Step II - After heat treatment</td>
<td>29.40</td>
<td>380.00</td>
<td>11.200</td>
<td>72.00</td>
</tr>
<tr>
<td>Step III- After Gel permeation through Sephadex G-50</td>
<td>pk I 4.00</td>
<td>22.00</td>
<td>0.088</td>
<td>0.57</td>
</tr>
<tr>
<td></td>
<td>pk II 12.00</td>
<td>890.00</td>
<td>10.700</td>
<td>69.00</td>
</tr>
<tr>
<td></td>
<td>pk III 3.50</td>
<td>18.00</td>
<td>0.063</td>
<td>0.41</td>
</tr>
<tr>
<td>Step IV - After cation exchange (CM-cellulose) chromatography of fractions of pk II</td>
<td>pk 1 6.70</td>
<td>7.20</td>
<td>0.048</td>
<td>0.31</td>
</tr>
<tr>
<td></td>
<td>pk 2 0.90</td>
<td>9000.00</td>
<td>8.100</td>
<td>53.00</td>
</tr>
<tr>
<td>Step V - After hydroxyapatite column chromatography</td>
<td>0.09</td>
<td>31000.00</td>
<td>2.790</td>
<td>18.00</td>
</tr>
</tbody>
</table>
3.5.3 Purification by Sephadex G-50 - Step III

Sephadex G-50 gel filtration chromatography was resorted to for further purification. The heat treated sample was freeze dried and dissolved in 2 ml of phosphate buffer pH 6.0. 2 ml fractions were collected and 5 μl of each fraction was used for IZA. The results of gel filtration chromatography are presented in Fig. 18.

There were three peaks of antibacterial activity and these corresponded to fractions 1-7, 40-53 and 95-103, obtained from Sephadex column. Essentially first peak was obtained in the void volume and appears to be of a high molecular weight antibacterial substance. The specific activity of peak I was 22 units/mg and the protein concentration was 4 mg/ml. The recovery percentage was 0.57. Peak III had a low specific activity of 18 units/mg with a protein concentration of 3.5 mg/ml and recovery percentage of 0.41. It was only fractions that were responsible for the second peak that exhibited considerable antibacterial activity of the order of 890 units/mg with protein concentration of 12 mg/ml. Total activity amounted to 10.7 KU and the recovery was 69% (Table. 2). Fractions responsible for peak I and III which exhibited low specific activity were not purified further. It was only the fractions of peak II with relatively high protein concentration and significant antibacterial activity, which was further purified.

3.5.4 Purification by CM-cellulose - Step IV

Fraction II obtained from gel filtration chromatography was further fractionated in CM-cellulose column, by diluting the sample with 4 volumes of 10 mM phosphate buffer pH 6.0. After washing the column extensively, the adsorbed material was eluted using two different concentrations of eluent. Fig. 19 shows two distinct zones of antibacterial activity corresponding to two peaks that were eluted. Peak I consisted of fractions between 15-26 and peak II from 50-69.
Fig. 18: Step III Chromatogram of antibacterial activity on a column of Sephadex G-50 (1.5 cm x 6 cm). 2 ml of heat treated, freeze dried sample from step II was fractionated through gel permeation and eluted with 130 mM NaCl. 2 ml fractions were collected at a rate of 40 ml/h and the activity was assayed with 5μl of each fraction using inhibition zone assay.
Fig. 19: Step IV. Cation exchange chromatogram of antibacterial proteins on a column of CM-cellulose (2 cm x 4 cm). Pooled peak II fractions from Sephadex G-50 of step III were diluted 4 times, eluted stepwise with 130 mM and 260 mM NaCl. 3 ml fractions were collected at a rate of ml/min and assayed using 10 µl of each fraction by inhibition zone assay.
Fractions of peak I activity were designated as muscinIIA (MIIA), M standing for antibacterial protein derived from Musca. The Roman numeral II indicated that it is a second fraction obtained from Sephadex column and 'A' referring to that it is the first fraction obtained in the penultimate step (Fig. 19). MIIA fraction had a protein concentration of 6.7 mg and a low specific activity of 7.2 units/mg of protein. The total activity amounted to 0.048 KU and there was a low recovery of 0.31% (Table 2).

The second CM-cellulose fraction was designated as MIIB where as the 'B' referred to the second fraction in this step of purification. Protein content of the second fraction was 0.9 mg and it possessed very high biological activity of 9000 units/mg. The total activity was 8.1 KU and recovery was 53%. Obviously MIIB is the most potent antibacterial protein in houseflies produced by induction (Table 2).

3.5.5 Purification by Hydroxyapatite chromatography - Step V

Further, purification of MIIB was attempted on a column of hydroxyapatite. 60 ml fractions of MIIB was diluted with ten fold phosphate buffer and applied to the hydroxyapatite column, that was previously equilibrated with same buffer. The adsorbed material was washed extensively at 50 mM phosphate buffer and then the active fractions were eluted with 100 mM phosphate buffer (Fig. 20).

A single distinct peak that spanned between fraction 19 and 25 showed a significant biological activity (Fig. 20). Although the protein recovered was only 0.09 mg, the specific activity was 31,000 units/mg amounting to a total activity of 2.79 KU. The final percentage of recovery was 18. It is a homogenous protein as evidenced in the electropherogram (Table 2).
Fig. 20: Step V. Hydroxyapatite column chromatogram of antibacterial protein MIIB of *M. domestica* larvae. MIIB fractions obtained from step IV were diluted ten times with phosphate buffer and loaded to a column (2cm x 2cm) of hydroxylapatite. The column was washed in 50 mM phosphate buffer pH 6.0 and fractions were eluted with 100 mM phosphate buffer. 6 ml fractions were collected and 5 µl of each fraction assayed for zone of inhibition with *M. luteus.*
3.6 Electrophoretic profile of antibacterial proteins and their activity at each step of purification

3.6.1 Acidic PAGE-antibacterial assay

The antibacterial proteins were assayed after they were purified at each step. The heat treated sample that consisted of only thermostable proteins, fraction II obtained from Sephadex column, fractions MIIA and B obtained by cation exchange chromatography and the single homogenous fraction obtained by hydroxyapatite chromatography were subjected to acidic PAGE, followed by sandwiching of the gel between two agar layers, the bottom of which was inoculated with bacteria. The results are presented in Fig.21.

It could be observed that there were distinct zones of bacterial lysis in the regions of the gel where the antibacterial proteins were present. The fact that bacterial lytic zones were resolved to a specific region was indicative that the presence of antibacterial protein was well confirmed by procedures followed in this study.

3.6.2 SDS-PAGE

At the end of each step of purification SDS-PAGE was done and the results are shown in Fig.22. The step II electrophoretic profile was of several fractions being resolved on the gel. Fraction I obtained from Sephadex G-50 column was relatively a high molecular weight protein with a low electrophoretic mobility on 15% gel. The second fraction of Sephadex column showed two fractions, viz., MIIA and MIIB on the CM-cellulose column. MIIB was subsequently detected as a single homogenous fraction in the hydroxyapatite column.
Fig. 21: Electrophoresis and subsequent antibacterial assay of bacteria induced haemolymph proteins of *M. domestica* larvae obtained at various steps of purification. Step II fractions after heat treatment (a), peak II fractions from Sephadex G-50 (b), MIIA (c), MIIB (d) and MIIB from hydroxyapatite (e).

Fig. 22: SDS-PAGE showing the antibacterial proteins of housefly larvae at various steps of purification. Step II fractions after heat treatment (a), pooled peak I fractions (b) and pooled peak II fractions (c) from Sephadex G-50, MIIA (d), MIIB (e) and molecular weight standards (f).
3.7 Characterization of MIIB

From about 30 ml of haemolymph consisting of 17.14 g of protein, only 90 μg of purified MIIB could be obtained but the specific activity of the purified protein was nearly 34,000 times higher than the one obtained in the haemolymph (Table 2).

3.7.1 Thermostability

The antibacterial protein exhibited activity over a wide range of temperatures, at which it was stored, ranging from -20°C to 100°C. MIIB was retrieved at different temperatures at which it was stored, as outlined in section 2.13.1 of materials and methods, and IZA was performed. The diameter of the inhibition zones produced by the protein stored at different temperatures was more or less similar (Fig. 23) suggesting that the molecule is highly thermostable.

3.7.2 Susceptibility to trypsin

MIIB was susceptible to proteolytic activity as evidenced by the non-inhibition of the growth of bacterial cultures when trypsin digested antibacterial protein was used in IZA.

3.7.3 Molecular weight determination

An attempt was made to determine the approximate molecular weight of MIIB isolated and purified in five steps from the haemolymph of housefly larvae by gel permeation chromatography. Four different proteins of known molecular weight were used to obtain a plot of molecular weight versus Ve/Vo ratio. Cytochrome c, aprotinin, insulin and glucagon which ranged in molecular weight between 12,400 and 3,500 were the proteins used as standards (Table 3). Under the experimental condition the Ve/Vo ratio of MIIB was calculated to be 1.35 and molecular weight was approximately 7,690 (Fig. 24).
Fig. 23: Bar diagram showing thermostability of MIIB subjected for several weeks at -20°C and 20°C, for a day at 33°C, for 30 min at 60°C and 5 min at 100°C.
Fig. 24: Plot of ratio of elution volume (Ve) to void volume (Vo) against log molecular weight of standard proteins on Sephadex G-50 column.
Table 3: Molecular weights and Ve/Vo ratio of standard proteins used in gel permeation chromatography

<table>
<thead>
<tr>
<th>S.No</th>
<th>Standards</th>
<th>Molecular weight</th>
<th>Ve/Vo</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Cytochrome c</td>
<td>12,400</td>
<td>2.80/37.00 = 0.076</td>
</tr>
<tr>
<td>2</td>
<td>Aprotinin</td>
<td>6,500</td>
<td>61/37.00 = 1.65</td>
</tr>
<tr>
<td>3</td>
<td>Insulin</td>
<td>5,734</td>
<td>68/37.00 = 1.84</td>
</tr>
<tr>
<td>4</td>
<td>Glucagon</td>
<td>3,500</td>
<td>106/37.00 = 2.86</td>
</tr>
</tbody>
</table>

Note: Ve/Vo of MIIB is 1.35 and molecular weight is about 7690.

3.7.4 Bactericidal effect

3.7.4.1 Killing assay

Bacterial effect of MIIB was assayed, as described in section 2.13.5.1 by incubating \(4 \times 10^8\) log phase *M. luteus* with 10 µl of MIIB. Two control cultures one in MIIB free *M. luteus* inoculated LB medium and the other in MIIB containing LB medium with bacteric incubated at 4°C were simultaneously maintained and assayed for CFU.

Fig. 25 Shows that after the incubation there was a significant reduction in number, from \(4 \times 10^8\) cells to less than 90 CFU within 1h. Control cultures maintained in MIIB free medium showed an increase in number of bacteria to \(5 \times 10^8\) cells and cultures maintained at 4°C were static.

Two way analysis of variance of the data on bactericidal effect of MIIB on viable count of *M. luteus* in LB medium incubated at 37°C and at 4°C showed no significant differences at different time intervals (Table A8), but exhibited significant differences on the viable count between controls and experiment.
Fig. 25: Bactericidal effect of MIIB on viable count of *M. luteus*. $4 \times 10^5$ cells suspended in LB medium incubated with MIIB (10 µg/ml) at 37°C (---), at 4°C (△) and *M. luteus* suspended LB medium without MIIB at 37°C (□).
3.7.4.2 Observation under phase contrast microscope

Three different bacterial species as well as one eukaryotic yeast species were used as test organisms to study the bactericidal effect of MIIB.

In this study the antibacterial substance obtained from step II purification procedure as well as the final purified product from step V were separately added to the cultures and observations were made under a phase contrast microscope. The effect of adding step II purification product to the bacterial culture was dramatic as there was an instant lysis of bacterial cells. Cultures to which MIIB was added only show a reduction in number of cells rather than a complete lysis of all bacterial cells. However, the antibacterial substances from housefly larvae did not have any effect on eukaryotic yeast cells (Fig.26a, b and c).

Fig.27a, b, c show *B. thuringiensis* cells. These cells had undergone lysis in the presence of step II fractions (Fig.27b). Reduction in the number of cells in the presence of MIIB is shown in Fig.27c. Similar results were obtained for *E. coli* (Fig.28a, b and c) and *M. luteus* (Fig.29a, b and c).

3.7.5 Bacterial specificity of MIIB

The bacterial specificity of MIIB was tested with various bacteria in liquid cultures. Two Gram positive species, *M. luteus* and *S. aureus* and eight Gram negative species, *P. aeruginosa, E. coli K12 strain E. cloacae, B. thuringiensis, B. subtilis, B. polymyxa, B. megaterium* and *E. coli* from human source were used as test organisms. MIC required for each of the bacterial species was determined and results presented in Table 4.

*M. luteus* which was used for obtaining the immune serum was the most sensitive organism to MIIB and a concentration of 0.1 µg/ml was sufficient to bring about 50% mortality.
Fig. 26: Phase contrast microphotographs of yeast species without antibacterial proteins (control) (a). Yeast incubated with pooled fractions from step II (b) and with MIIB (c). Yeast is not affected by MIIB at a concentration of 1 mg/ml. (650X)

Fig. 27: Phase contrast microphotographs of B. thuringiensis Control cultures (a). Cultures incubated for 10 min with step II fractions (b) showing lysis indicated by cellular debris and with MIIB (c) showing drastic reduction both in size and number. (650X)
Fig. 28: Phase contrast microphotographs of control *E. coli* culture (a), *E. coli* suspended in bacterial saline treated with step II fractions (b), within few minutes bacteria become spherical, thin walled swollen and enlarged leading to cell lysis and *E. coli* cultures with MIIB (c) showing drastic reduction in number and cellular lysis. (650X)

Fig. 29: Phase contrast microphotographs of control *M. luteus* cultures (a), *M. luteus* treated with step II fractions (b) and the same with MIIB (c). Drastic reduction and lysis were observed. (650X)
Table 4: Antibacterial spectrum of MIIB

<table>
<thead>
<tr>
<th>S.No</th>
<th>Bacterial strain</th>
<th>Minimum inhibitory concentration µg / ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>M. luteus</td>
<td>0.1</td>
</tr>
<tr>
<td>2</td>
<td>P. aeruginosa</td>
<td>0.2</td>
</tr>
<tr>
<td>3</td>
<td>E. coli K12 strain</td>
<td>0.4</td>
</tr>
<tr>
<td>4</td>
<td>S. aureus</td>
<td>1.5</td>
</tr>
<tr>
<td>5</td>
<td>E. cloacae</td>
<td>1.5</td>
</tr>
<tr>
<td>6</td>
<td>B. thuringiensis</td>
<td>0.2</td>
</tr>
<tr>
<td>7</td>
<td>B. subtilis</td>
<td>0.2</td>
</tr>
<tr>
<td>8</td>
<td>B. polymyxla</td>
<td>1.5</td>
</tr>
<tr>
<td>9</td>
<td>B. megaterium</td>
<td>1.5</td>
</tr>
<tr>
<td>10</td>
<td>E. coli from human source</td>
<td>1.5</td>
</tr>
</tbody>
</table>

P. aeruginose B. thuringiensis and B. subtilis were also relatively sensitive and underwent lysis at MIC of 0.2 µg/ml. E. coli K12 strain was more sensitive than E. coli from human source. E. cloacae, S. aureus, B. polymyxla, B. megaterium and E. coli from human source required higher MIC to bring about 50% mortality. In general both Gram positive and Gram negative bacteria were affected by MIIB (Table 4).