CHAPTER III

RESULTS
RESULTS

Protease Activity During Larval Development:

The change in proteolytic activity at different larval ages after hatching from the eggs is shown in Fig. 1. Maximum activity was observed on the 9th day which declined subsequently till day 11. The pattern of changes in protein concentration in the gut was parallel to the protease activity. However, at 10 and 11 days, the decrease in proteolytic activity was slightly more in comparison to the decrease in protein concentration.

Protease Activity During Starvation:

The change in protease activity during starvation is shown in Fig. 2. There was an abrupt increase in enzyme activity at 4 hours of starvation, after which a continuous decrease in the activity was observed till 24 hours. The protein concentration of gut contents was found to decrease rapidly upto 4 hours of starvation followed by a slower decline till 24 hours. Thus, the specific activity was increased two-fold at 4 hours and subsequently declined on further starvation.

Effect of pH on Protease Activity of Crude Enzyme Solution:

The pH-activity curve is presented in Fig. 3. Since it was difficult to prepare casein solution below pH 6.0, or acid denatured
Figure 1: Protease activity during development of larvae of *Spodoptera litura*.

The protease activity and protein concentration of gut contents at different larval ages were determined as described in the text.

- Protease activity ●
- Protein concentration ○
The fifth instar larvae were starved for varying time intervals; the protease activity and protein concentration of gut contents were determined. See text for details.

Protease activity •
Protein concentration ○
Figure - 3 : Effect of pH on the protease activity of crude enzyme solution.

The reaction mixture in a total volume of 1 ml, contained 30 μg enzyme protein, 40 umoles buffer and 10 mg casein dissolved in buffer of desired pH. After incubation for 15 minutes at 40°C, the reaction was terminated by the addition of 0.5 ml of 20% TCA. The protease activity was determined as described in the text. Buffers used were: \( \text{KH}_2\text{PO}_4 \) - HCl (pH 2.0 - 3.0), \( \text{K}_2\text{HPO}_4 \) - HCl (pH 4.0 - 5.0), \( \text{KH}_2\text{PO}_4 \) - \( \text{K}_2\text{HPO}_4 \) (pH 6.0 to 8.0), \( \text{KH}_2\text{PO}_4 \) - NaOH (pH 9.0) and \( \text{K}_2\text{HPO}_4 \) - NaOH (pH 10.0 - 12.0).

Hydrolysis of acid denatured hemoglobin ▲

Hydrolysis of casein ●
haemoglobin solution above pH 6.0, haemoglobin was used as a substrate from pH 2.0 to 6.0 and casein was used between pH 6.0 to pH 12.0. No enzyme activity was observed below pH 4.0. The activity of the enzyme increased until pH 11.0, with a shoulder at pH 8.0, and declined sharply above pH 11.0.

Changes in Protease Activity During Incubation of Crude Enzyme Solution:

Crude enzyme solution was incubated at 37 ± 1°C for 55 hours and the specific activity was determined at different intervals (Table I). There was a sharp decrease in protein concentration until 22 hours of incubation without any significant change until 55 hours. The enzyme activity remained almost unaltered till 22 hours but subsequently a loss in activity was observed and by 55 hours, 37% of the activity was lost. Thus, incubation of the enzyme solution for 22 hours resulted in a three-fold increase in the specific activity which, however, declined on further incubation.

Effect of Dialysis on Protease Activity:

Crude enzyme solution was dialysed for 24 hours against 0.1M glycine-NaOH buffer, pH 11.0, at 4°C and 37°C. As shown in Table II, there was a 42% loss in enzyme activity when the enzyme solution was dialysed at 37°C, whereas only 10% loss in activity was observed at 4°C. In a separate experiment, it was observed that the enzyme activity was completely lost after 40 hours
TABLE - I

**PROTEASE ACTIVITY DURING INCUBATION OF CRUDE ENZYME SOLUTION**

Crude enzyme solution was incubated at 37°C, pH 11.0, and the aliquots were taken at different intervals of time to measure the enzyme activity by standard method as described in the text. Protein was determined according to the method of Khanna *et al.* as described in the text.

<table>
<thead>
<tr>
<th>Incubation period (hours)</th>
<th>Protein (mg/ml)</th>
<th>Activity (Units/ml)</th>
<th>Specific activity (Units/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>3.67</td>
<td>937</td>
<td>225</td>
</tr>
<tr>
<td>8</td>
<td>1.35</td>
<td>851</td>
<td>630</td>
</tr>
<tr>
<td>22</td>
<td>1.15</td>
<td>851</td>
<td>740</td>
</tr>
<tr>
<td>30</td>
<td>1.15</td>
<td>741</td>
<td>644</td>
</tr>
<tr>
<td>55</td>
<td>1.12</td>
<td>592</td>
<td>528</td>
</tr>
</tbody>
</table>
**TABLE - II**

**EFFECT OF DIALYSIS ON PROTEASE ACTIVITY**

Crude enzyme solution prepared in 0.1M glycin-NaOH buffer, pH 11.0, was dialysed at 37°C and 4°C for 24 hours. Suitable aliquots were taken to measure the enzyme activity by the standard method as described in the text.

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Activity (Units/ml)</th>
<th>% Inactivation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Undialysed</td>
<td>552</td>
<td>0</td>
</tr>
<tr>
<td>Dialysed at 4°C</td>
<td>497</td>
<td>10</td>
</tr>
<tr>
<td>Dialysed at 37°C</td>
<td>321</td>
<td>42</td>
</tr>
</tbody>
</table>
dialysis at 37°C. It is of interest to mention here that incubation of the enzyme solution for about 24 hours at 37°C caused very little inactivation.

Search for Zymogen:

Since many proteolytic enzymes from higher vertebrates (Boyer, 1971), lower vertebrates (Bradshaw et al., 1970; Reeck and Neurath, 1972), starfish (Camacho et al., 1970) and insects (Felsted et al., 1973; Ward, 1975) occur as inactive precursors, experiment was carried out to determine if any of the proteolytic enzymes in S. litura also occurred as inactive zymogens. Crude extract in 0.1M Tris buffer, pH 8.0 was incubated at 40°C for 1, 2, 3 and 4 hours and the residual activity was assayed by the caseinolytic method. Since no increase in enzyme activity was observed, it was concluded that the enzyme does not exist as zymogen.

ISOLATION AND PURIFICATION OF S. litura PROTEASES:

S. litura larvae were cultured according to the method described earlier. The fifth instar larvae were dissected and their intestines collected in an ice cold beaker. Their contents were squashed out by means of a glass rod in 0.1M Tris buffer, pH 8.0. The mixture was centrifuged in cold at 4000 r.p.m. for 20 minutes and the supernatant was used as crude extract.

Initially the enzyme was partially purified to 7-fold with 80% recovery in two steps comprising of incubation at 37°C for
22 hours and Sephadex G-75 permeation chromatography (Fig. 4).
The major active peak was, however, not homogeneous and gave
three sharp and well separated bands on PAGE electrophoresis.
The details have already been published (Zafeer et al., 1976).
The pH-activity profile of partially purified enzyme did not
show a shoulder at pH 8.0 which was present in the crude extract.
These observations suggested the presence of more than one
proteases in the gut of S. litura which might be obtained due to
proteolysis by incubation of crude extract at 37°C for 22 hours.
Therefore, the purification was modified from the previously
reported method (Zafeer et al., 1976). The yield and degree
of purity at each step of modified method of purification are
summarized in Table III.

(a) Acetone Fractionation:

Crude extract contains dark brown pigments and phenols.
Though, these pigments were removed at each step, acetone
fractionation was the major step. When the crude extract was
passed through the column, the pigments were strongly bound with
the gel and caused a retardation in the flow rate. Moreover,
to remove these pigments from the gel, continuous washing for
one week was required. Hence, to remove these pigments and
phenols, the enzyme was precipitated by the addition of a
three-fold chilled acetone prior to chromatography. It was left
for one hour at 4°C as such to ensure complete precipitation.
The acetone precipitated enzyme was collected by centrifugation
The column (2.0 x 35 cm) was equilibrated with 0.05M Tris buffer, pH 8.0. About 38 mg protein, obtained by incubation of crude extract at 37°C, was applied and 3 ml fractions were collected at a flow rate of 20 ml/hr. See text for details.

Protease activity ●
Protein concentration ○
<table>
<thead>
<tr>
<th>Purification Step</th>
<th>Total Protein (mg)</th>
<th>Total Activity (Units X 10⁻²)</th>
<th>Specific Activity (Units X 10⁻²/mg)</th>
<th>Yield (%)</th>
<th>Purification (Fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>348</td>
<td>128.08</td>
<td>0.33</td>
<td>100.0</td>
<td>1</td>
</tr>
<tr>
<td>Acetone fractionation</td>
<td>294</td>
<td>93.14</td>
<td>0.31</td>
<td>74.0</td>
<td>1</td>
</tr>
<tr>
<td>Chromatography on Sephadex G-75</td>
<td>130</td>
<td>85.00</td>
<td>0.65</td>
<td>67.4</td>
<td>2</td>
</tr>
<tr>
<td>Chromatography on DAE-Sephadex A-50</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P I</td>
<td>8.2</td>
<td>23.65</td>
<td>2.95</td>
<td>18.7</td>
<td>9</td>
</tr>
<tr>
<td>P II</td>
<td>14.8</td>
<td>25.97</td>
<td>1.75</td>
<td>20.5</td>
<td>5</td>
</tr>
<tr>
<td>P III</td>
<td>9.0</td>
<td>18.17</td>
<td>2.02</td>
<td>14.4</td>
<td>6</td>
</tr>
<tr>
<td>Rechromatography on DAE-Sephadex A-50</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P II</td>
<td>10.5</td>
<td>24.52</td>
<td>2.33</td>
<td>19.4</td>
<td>7</td>
</tr>
<tr>
<td>P III</td>
<td></td>
<td>17.25</td>
<td>2.61</td>
<td>13.6</td>
<td>8</td>
</tr>
</tbody>
</table>
at 4000 r.p.m. for 20 minutes. The precipitate was dissolved in 0.05M Tris buffer, pH 8.0.

(b) **Sephadex G-75 Chromatography**:

The enzyme solution obtained by acetone fractionation was now loaded on a G-75 column (2.16 x 56 cm), equilibrated and eluted with 0.05M Tris buffer, pH 8.0. 3 ml fractions were collected with a flow rate of 20 ml/hr. A suitable aliquot was taken for enzyme assay using casein as substrate. The chromatographic pattern for this gel filtration is shown in Fig. 5. Only two fold purification was achieved but a considerable amount of dark coloured impurities were removed at this step. Active fractions were pooled and dialysed against 0.02M Tris buffer, pH 8.0 at 4°C for 24 hours changing the buffer several times.

(c) **DEAE-Sephadex A-50 Chromatography**:

The dialysed solution was applied to a DEAE-Sephadex A-50 column (2.6 X 42 cm), equilibrated with 0.02M Tris buffer, pH 8.0. The column was first washed with 400 ml of the same buffer. The flow rate was 50 ml/hr and 8 ml fractions were collected. The column was then subjected to a salt gradient elution from 0.02M to 0.35M NaCl in the same buffer. 4 ml fractions were collected at a flow rate of 50 ml/hr. A suitable aliquot was taken for caseinolytic assay. The chromatographic profile is represented in Fig. 6. One of the protease which was
The column (2.16 x 56 cm) was equilibrated with 0.05M Tris buffer, pH 8.0. About 30 mg protein, obtained by acetone fractionation was applied and 4 ml fractions were collected at a flow rate of 20 ml/hr.

Protease activity
Protein concentration
Figure 6: DEAE-Sephadex Chromatography of *S. litura* alkaline proteases.

The column (2.6 X 42 cm) was equilibrated with about 2 litres of 0.02M Tris buffer, pH 8.0. About 130 mg protein in the same buffer was applied to the column. After washing the column with 200 ml 0.02M Tris buffer, pH 8.0, it was eluted with 0.02M – 0.085M NaCl and 4 ml fractions were collected at a flow rate of 50 ml/hr. ----------------, NaCl concentration in effluent.

Protease activity ●
Protein concentration ○
not absorbed to the gel and eluted without retardation was
designated as Protease I. Two other proteases which were
resolved by salt gradient elution were known as Protease II and
III. These two peaks were respectively eluted at 0.55 and 0.7M
salt concentration. Active fractions consisting of protease II
and III were separately pooled and dialysed for 24 hours against
0.02M Tris buffer, pH 8.0. These two proteases were rechromato-
graphed on DEAE-Sephadex column by the same procedure as above.
The chromatographic pattern is shown in Fig. 7 and Fig. 8. The
purification procedure was repeated 5 times. It was reproducible
with the exception that protease III was not detectable two times.

Homogeneity:

The purified preparations of alkaline proteases (P_I - P_III)
gave single band on polyacrylamide gel electrophoresis. Fig. 9A
represents the migration of protease I - III for 2 hours and
Fig. 9B represents the migration of protease I and II for 5 hours.

DETERMINATION OF MOLECULAR PARAMETERS OF S. litura PROTEASES:

The molecular weight of S. litura proteases were calculated
by gel filtration according to the procedure of Andrews (1964)
using Sephadex G-200 (2.16 X 48 cm) column, equilibrated with 20
mM Tris buffer, pH 8.0, containing 0.1M NaCl. The column was
calibrated with four marker proteins, cytochrome C, \( \alpha \)-chymotryps-
sinogen A, ovalbumin and bovine serum albumin. 5 - 10 mg of
Figure - 7: Rechromatography of Peak II on DEAE-Sephadex A-50 column.

Conditions were the same as in Figure 6.
Figure - 8 : Rechromatography of Peak III on DEAE-Sephadex A-50 column.

Conditions were the same as in Figure 6.
Figure - 9A: Polyacrylamide gel electrophoresis of purified *S. litura* alkaline proteases.

7.5% gels with Tris-glycine buffer system, pH 8.3, were used. Protease I; A; protease II; B and protease III; C. Migration took place from the top (-) to bottom (+) for 2 hours. See text for details.
Figure - 9B: Polyacrylamide gel electrophoresis of protease I and II.

Conditions were same as in Figure 9A except that the migration time was for 5 hours. A represents protease I and B represents protease II.
each protein was applied and eluted with the same buffer at a flow rate of 15 ml/hr and 2 ml fractions were collected. The proteins were determined by absorption either at 280 nm or at 660 nm using Lowry method. Blue dextran 2000 was measured at 625 nm. The enzyme activity for protease I - III was assayed by caseinolytic method. Elution profile for marker proteins and proteases is represented in Fig. 10 and 11 respectively. A plot of $V_e/V_o$ versus logarithm of molecular weight (Fig. 12) according to the procedure of Andrew gave a straight line. Blue dextran was used to determine void volume ($V_o$) and tyrosine was used to determine inner volume ($V_i$). The total volume ($V_t$) was determined directly with water. The void volume was occasionally checked during column chromatography of marker proteins. No detectable change in $V_o$ and $V_t$ was found.

However, the elution position of a protein during gel filtration is better revealed to the Stokes' radius than to the molecular weight (Seigel and Monty, 1966; Ackers, 1964) and therefore, the Stokes' radius of S. litura proteases were determined. The data were processed using the following expressions:

$$K_d = \frac{V_e - V_o}{V_i} \quad \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots (3)$$

$$K_{av} = \frac{V_e - V_o}{V_t - V_o} \quad \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots (4)$$

where $K_d$ is the distribution coefficient and $K_{av}$ is the available distribution coefficient.
Figure - 10: Gel filtration behaviour of standard proteins on Sephadex G-200 column.

About 5 - 10 mg of each standard proteins were chromatographed on the column (2.18 X 48 cm) which was equilibrated and eluted with 0.05M Tris buffer, pH 8.0 + 0.1M NaCl. 2 ml fractions were collected at a flow rate of 15 ml/hr.
Figure - 11: Gel filtration behaviour of *S. liture* alkaline proteases on Sephadex G-200 column.

The experimental conditions were the same as described in Figure 10. Proteolytic activity of the eluate giving a clear maximum curve was checked by the standard method as described in the text.
Figure 12: Estimation of the molecular weight of *S. litura* proteases by gel filtration on Sephadex G-200.

Elution data of Table IV were treated according to the method of Andrews (1964). Plot of $V_e/V_0$ vs. $\log M$ gave straight line by the method of least squares.
The gel filtration data (Table IV) were analyzed using the procedures of Porath (1963), Laurent and Killander (1964) and Ackers (1964) by least squares method (Figs. 13-15).

The molecular weight values calculated by Andrews method were 16980 for protease I, 21380 for protease II and 53700 for protease III. Values of Stokes' radii obtained by correlation of Porath, Laurent and Killander and Ackers are summarized in Table V. The average values were 1.89 nm for protease I, 2.16 nm for protease II and 3.08 nm for protease III. The diffusion coefficients were calculated from the Stokes' radii using the Stokes-Einstein equation (Siegel and Monty, 1966):

$$D = \frac{KT}{6\pi\eta a}$$

where K is the Boltzmann constant ($1.386 \times 10^{-16}$ erg/degree), T is the absolute temperature (303°C), and $\eta$ is the coefficient of viscosity of the medium (0.0100 p for water and dilute aqueous salt solutions at 20°C). Using the average values of Stoke's radii (Table V), diffusion coefficients for protease I - III were $11.79 \times 10^{-7}$, $10.32 \times 10^{-7}$ and $7.24 \times 10^{-7}$ cm²/Sec.

Determination of Molecular Weight by SDS Polyacrylamide Gel Electrophoresis:

The molecular weight of *S. Litura* proteases were also determined by SDS gel electrophoresis at pH 7.0 according to the
### GEL FILTRATION DATA OF *S. litura* ALKALINE PROTEASES AND STANDARD PROTEINS

<table>
<thead>
<tr>
<th>Proteins</th>
<th>Ve/Vo</th>
<th>Stoke's radii (nm)</th>
<th>K_d</th>
<th>(K_d)^1/3</th>
<th>K_av</th>
<th>(-log K_av)^1/2</th>
<th>erf^{-1}K_d</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytochrome C</td>
<td>2.75</td>
<td>1.64</td>
<td>0.763</td>
<td>0.914</td>
<td>0.730</td>
<td>0.37</td>
<td>0.21</td>
</tr>
<tr>
<td>Chymotrypsinogen A</td>
<td>2.50</td>
<td>2.24</td>
<td>0.654</td>
<td>0.868</td>
<td>0.626</td>
<td>0.46</td>
<td>0.32</td>
</tr>
<tr>
<td>Ovalbumin</td>
<td>2.12</td>
<td>2.73</td>
<td>0.490</td>
<td>0.788</td>
<td>0.469</td>
<td>0.58</td>
<td>0.49</td>
</tr>
<tr>
<td>Bovine serum albumin</td>
<td>1.89</td>
<td>3.55</td>
<td>0.390</td>
<td>0.708</td>
<td>0.373</td>
<td>0.65</td>
<td>0.61</td>
</tr>
<tr>
<td>Protease I</td>
<td>2.60</td>
<td>-</td>
<td>0.700</td>
<td>0.888</td>
<td>0.669</td>
<td>0.42</td>
<td>0.27</td>
</tr>
<tr>
<td>Protease II</td>
<td>2.48</td>
<td>-</td>
<td>0.645</td>
<td>0.864</td>
<td>0.608</td>
<td>0.47</td>
<td>0.33</td>
</tr>
<tr>
<td>Protease III</td>
<td>2.02</td>
<td>-</td>
<td>0.445</td>
<td>0.763</td>
<td>0.426</td>
<td>0.61</td>
<td>0.54</td>
</tr>
</tbody>
</table>
Figure - 13: Estimation of the Stokes' radius of *S. litura* proteases.

The gel filtration data of Table IV were treated according to the correlation of Porath (1963). Plot of $(K_d)^{1/3}$ vs. Stokes' radius gave straight line by the method of least squares.

Protease I  ○
Protease II ▲
Protease III △
1.0 -

n

0.9

0.8

CYTOCHROME-C

BOVINE SERUM ALBUMIN

0.7

X

0.6

X

0.5

0.4

0.3

0.2

0.1

0

STOKES RADIUS (n m)

( Kd )^1/3

CYTOCHROME-C

α-CHYMOTRYPSIN GEN A

OVALBUMIN

BOVINE SERUM ALBUMIN
Figure 1: Estimation of the Stokes' radius of *S. litura* proteases.

The gel filtration data of Table IV were treated according to the correlation of Laurent and Killander (1964). Plot of \((-\log K_{av})^{1/2}\) Vs. Stokes' radius gave the straight line by the method of least squares.

- Protease I ○
- Protease II ▲
- Protease III △
Figure - 15: Estimation of Stokes' radius of *S. litura* proteases.

The gel filtration data of Table IV were plotted according to the correlation of Ackers (1967). The straight line was drawn by the method of least squares.

Protease I  ○
Protease II ▲
Protease III △
0.6 - 0.5

^n OA

0.3

Bovine Serum Albumin

0.2

Ovalbumin

\( \alpha \)-Chymotrypsinogen-A

Cytochrome C

\textit{STOKES RADIUS (nm)}
method of Weber and Osborn (1969). The same marker proteins were used as in the calibration of Sephadex G-200 column described above. The relative mobilities of each marker proteins were determined and graphed against the subunit molecular weight (Fig. 16). A least square analysis of the data indicated a linear relation between log \( M \) and relative mobility (\( R_m \)).

\[
\log M = -1.94 R_m + 6.22
\]

(5)

*Helicoverpa* proteases gave single band in SDS polyacrylamide gel electrophoresis with the \( R_m \) values of 0.975, 0.96 and 0.785 which correspond the molecular weights of 18500, 22910 and 50000 respectively. The physical parameters of *Helicoverpa* proteases are summarized in Table V.

**ENZYMATIC PROPERTIES OF *Helicoverpa* PROTEASES:**

**Effect of Incubation Time:**

Each proteases were incubated for different intervals of time at 40°C and pH 9.0. The enzyme activity was assayed by the caseinolytic method. As shown in Fig. 17, *Helicoverpa* proteases showed a linear relationship between the enzyme activity and the incubation period upto 60 minutes.
Figure - 16: Molecular weight determination of *S. litura* alkaline proteases by SDS gel electrophoresis.

Plot of the relative mobilities (Rm) of the standard proteins Vs. logarithm of their subunit molecular weight gave straight line by the method of least squares. See text for details.
<table>
<thead>
<tr>
<th>Parameter</th>
<th>Determination</th>
<th>Values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Molecular weight</td>
<td>From gel filtration by Andrews method on Sephadex G-200.</td>
<td>Protease I: 16,980</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Protease II: 21,380</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Protease III: 53,700</td>
</tr>
<tr>
<td></td>
<td>From SDS gel electrophoresis.</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>18,500</td>
</tr>
<tr>
<td></td>
<td></td>
<td>22,910</td>
</tr>
<tr>
<td></td>
<td></td>
<td>50,000</td>
</tr>
<tr>
<td>Stokes' radius (nm)</td>
<td>According to Porath.</td>
<td>1.85</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2.10</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2.90</td>
</tr>
<tr>
<td></td>
<td>According to Laurent and Killander.</td>
<td>1.92</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2.21</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3.18</td>
</tr>
<tr>
<td></td>
<td>According to Ackers.</td>
<td>1.91</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2.18</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3.15</td>
</tr>
<tr>
<td></td>
<td>Average.</td>
<td>1.89</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2.16</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3.08</td>
</tr>
<tr>
<td>Diffusion coefficient, D</td>
<td>From average Stokes' radius.</td>
<td>11.79 × 10⁻⁷</td>
</tr>
<tr>
<td>(cm²/Sec.)</td>
<td></td>
<td>10.32 × 10⁻⁷</td>
</tr>
<tr>
<td></td>
<td></td>
<td>7.24 × 10⁻⁷</td>
</tr>
</tbody>
</table>
Figure - 17: Effect of incubation time on enzyme activity of *S. litura* proteases.

Protease I - III were incubated at 40°C for different time intervals and the proteolytic activity was determined as described in the text.

- Protease I
- Protease II
- Protease III
pH Optima:

The pH-activity curves of the alkaline proteases for hydrolysis of casein are presented in Fig. 18. The pH optima for protease I - III were 11.0, 10.5 and 9.0 respectively.

Temperature Optima:

The results presented in Fig. 19 show that the optimum temperatures for protease I - III obtained by 20 minutes reaction at pH 8.0 were 60°C, 55°C and 50°C respectively. The kinetic parameters are summarized in Table VI.

pH-Stability:

The effect of pH on the stability of proteases is shown in Fig. 20. While protease I and II were relatively more stable at pH 2.4 retaining more than 60% of the initial activity after 45 minutes at this pH, the protease III had only 10% activity. The protease I and II retained full activity at pH 6.0 but the protease III shows some denaturation even at this pH. At pH 10.0, protease I - III had about 90% of initial activity. There was no remarkable decrease in activity of protease I and II at pH 12 but only 50% activity remained in protease III.
The reaction mixture in a total volume of 1 ml contained 5-8 μg enzyme protein, 40 μmoles buffer and 10 mg casein dissolved in buffer of desired pH. After incubation for 20 minutes at 40°C, the reaction was terminated by the addition of 0.5 ml of 20% TCA. The proteolytic activity was determined as described in the text. Buffers used were: phosphate (pH 6.0 to 8.0) and glycine-NaOH (pH 9.0 to 12.0).

Protease I
Protease II
Protease III

○ △ x
Figure 19: Effect of temperature on the proteolytic activity of *S. litura* alkaline proteases.

The reaction mixture in a total volume of 1 ml contained 5-8 μg enzyme protein, 40 μmoles Tris buffer, pH 8.0 and 10 mg casein dissolved in the same buffer. After incubation at the indicated temperatures for 20 minutes, the reaction was terminated by the addition of 0.5 ml of 20% TCA. The proteolytic activity was determined as described in the text.

Protease I  O
Protease II  ▲
Protease III  X
Figure - 20 : Effect of pH on stability of *S. litura* alkaline proteases.

5.8 µg enzyme protein and 40 µmoles buffer in a total volume of 0.5 ml was incubated at the indicated pH values for 45 minutes at 35°C. The pH of reaction mixtures were adjusted to pH 9.0 with 0.1M glycine-NaOH buffer and a suitable aliquot was taken to determine the caseinolytic activity by standard procedure. The activity at pH 9.0 was taken as 100 per cent. Buffers used were: glycine-HCl (pH 2.0 to 3.0), acetate (pH 3.5 to 5.0), phosphate (pH 5.5 to 8.0) and glycine-NaOH (pH 8.5 to 12.5).

Protease I  ●
Protease II  ○
Protease III  ×
**Temperature Stability:**

Thermal stability measurements for each protease is given in Fig. 21. There was no remarkable difference in terms of inactivation of all the three proteases. The inactivation began at 45°C, and at 60°C, almost all the proteases lost full activity. At 50°C, the protease I - III respectively had 94, 85 and 80% activity.

**Lineweaver-Burk Plot:**

Linear double reciprocal plots were obtained with each of the protease using casein as the substrate (Fig. 22). The plots were obtained by the least square method and the values of Km were computed from the intercepts and slopes of the linear plots and listed in Table VI. These values are $5.7 \times 10^{-6}$ M for protease I, $2.9 \times 10^{-6}$ M for protease II and $2.1 \times 10^{-6}$ M for protease III.

**Effect of Guanidine Hydrochloride:**

Protease I - III were incubated with various concentrations of guanidine hydrochloride for 30 minutes at 35°C. Bovine pancreatic trypsin was also incubated under similar conditions for comparison. The residual peptidase activity was determined using BAPA as the substrate. Under the defined conditions, the denaturation effect of guanidine hydrochloride on proteases was
Figure - 21: Thermal inactivation of *S. litura* alkaline proteases.

5-8 μg enzyme protein dissolved in 40 umoles Tris buffer, pH 8.0, was preincubated at the indicated temperatures for 20 minutes, immediately cooled to 0°C and the residual activity was assayed by the standard assay procedure. The activity at 40°C was taken as 100 per cent.

- Protease I
- Protease II
- Protease III
Figure - 22: Lineweaver-Burk plots of *S. litura* alkaline proteases with casein as a substrate.

The assay mixture in a total volume of 1 ml contained 5-8 µg enzyme protein. The substrate concentration was varied in the range $0.012 \times 10^{-4}$ to $0.3 \times 10^{-1}$M, pH 9.0, 40°C. The enzyme activity was determined by the standard method as described in the text.

Protease I
Protease II
Protease III
<table>
<thead>
<tr>
<th>Enzyme</th>
<th>pH Optima</th>
<th>Temperature Optima (°C)</th>
<th>$K_m$ ($M^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protease I</td>
<td>11.0</td>
<td>60</td>
<td>$5.7 \times 10^{-6}$</td>
</tr>
<tr>
<td>Protease II</td>
<td>10.5</td>
<td>55</td>
<td>$2.9 \times 10^{-6}$</td>
</tr>
<tr>
<td>Protease III</td>
<td>9.0</td>
<td>50</td>
<td>$2.1 \times 10^{-6}$</td>
</tr>
</tbody>
</table>
almost similar to trypsin except in case of protease III which was relatively less susceptible to guanidine hydrochloride (Fig. 23).

Effect of Metal Ions:

Effect of various metal ions on the \textit{S. litura} proteases are summarized in Table VII. Even at relatively higher concentrations, no significant effect was observed with Mg$^{++}$, K$^+$, Ca$^{++}$ and Co$^{++}$. The enzymes were markedly inhibited by Zn$^{++}$ and Cu$^{++}$. $10^{-2}\text{M} \text{Hg}^{++}$ completely inactivated all the three proteases.

Substrate Specificity (Protease and Peptidase Activity):

All the proteases were found to hydrolyse BAPA and BAEE but not BTEE. The proteolytic and peptidase activities by each of the proteases were determined under identical conditions. The results are summarized in Table VIII. It can be seen that the protease I is most active against casein as well as BAPA. Though protease III was more active than protease II against casein, the situation was reverse for BAPA as substrate.

Effect of Various Substances:

To understand more about the nature of the enzymes, the effect of various substances on protease activity was studied (Table IX). Metal chelating agent, EDTA, did not show any inhibitory effect on the proteolytic activity of each proteases at
Figure - 23: Effect of guanidine hydrochloride on the peptidase activity of trypsin and *S. litura* alkaline proteases.

5-8 μg enzyme protein in a total volume of 1.0 ml contained requisite amount of guanidine hydrochloride in 0.1M Tris buffer, pH 8.0, to final molarity as indicated. After incubation for 30 minutes at 35°C, the residual activity was determined against BAPA as described in the text.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Symbol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trypsin</td>
<td>●</td>
</tr>
<tr>
<td>Protease I</td>
<td>○</td>
</tr>
<tr>
<td>Protease II</td>
<td>▲</td>
</tr>
<tr>
<td>Protease III</td>
<td>△</td>
</tr>
</tbody>
</table>
TABLE - VII

EFFECT OF VARIOUS METAL IONS ON THE PROTEOLYTIC ACTIVITY OF *S. litura* ALKALINE PROTEASES.

The reaction mixture in a total volume of 1 ml contained 30 umoles Tris buffer, pH 8.0, 5-8 µg enzyme protein and 10 mg casein. After incubation at 40°C for 15 minutes, the caseinolytic activity was determined as described in the text. 10 umoles metal ions were added prior to the addition of casein and pre-incubated for 60 minutes at 35°C.

<table>
<thead>
<tr>
<th>Additions</th>
<th>Relative Activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Protease I</td>
</tr>
<tr>
<td>None</td>
<td>100</td>
</tr>
<tr>
<td>Mg²⁺</td>
<td>108</td>
</tr>
<tr>
<td>K⁺</td>
<td>100</td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>107</td>
</tr>
<tr>
<td>Mn²⁺</td>
<td>103</td>
</tr>
<tr>
<td>Co²⁺</td>
<td>100</td>
</tr>
<tr>
<td>Hg²⁺</td>
<td>0</td>
</tr>
<tr>
<td>Cu²⁺</td>
<td>58</td>
</tr>
<tr>
<td>Zn²⁺</td>
<td>40</td>
</tr>
</tbody>
</table>
**TABLE - VIII**

**ACTIVITY OF THREE ALKALINE PROTEASES OF *S. litura***

<table>
<thead>
<tr>
<th>Proteases</th>
<th>Specific activity against casein. (Units/mg)</th>
<th>Specific activity against BAPA. (Units/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P_I</td>
<td>150</td>
<td>1.08</td>
</tr>
<tr>
<td>P_II</td>
<td>12</td>
<td>0.11</td>
</tr>
<tr>
<td>P_III</td>
<td>29</td>
<td>0.013</td>
</tr>
</tbody>
</table>
TABLE IX

EFFECT OF VARIOUS SUBSTANCES ON THE PROTEOLYTIC ACTIVITY OF *S. litura* ALKALINE PROTEASES

The reaction mixture in a total volume of 1 ml contained 30 umoles Tris buffer, pH 8.0, 5-8 ug enzyme protein and 10 mg casein. After incubation at 40°C for 15 minutes the caseinolytic activity was determined as described in the text. Various substances, as indicated, were added prior to the addition of casein and pre-incubated for 30 minutes at 35°C.

<table>
<thead>
<tr>
<th>Additions</th>
<th>Final Concentration</th>
<th>Relative Activity (%)</th>
<th>Protease I</th>
<th>Protease II</th>
<th>Protease III</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EDTA</td>
<td>$2 \times 10^{-3} M$</td>
<td>110</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>L-Cysteine</td>
<td>$10^{-3} M$</td>
<td>105</td>
<td>110</td>
<td>110</td>
<td>112</td>
</tr>
<tr>
<td>Monoiodoacetic Acid</td>
<td>$10^{-2} M$</td>
<td>111</td>
<td>95</td>
<td>95</td>
<td>116</td>
</tr>
<tr>
<td>L-Ethylmaleimide</td>
<td>$10^{-2} M$</td>
<td>106</td>
<td>106</td>
<td>106</td>
<td>110</td>
</tr>
<tr>
<td>B-Mercaptoethanol</td>
<td>$2 \times 10^{-3} M$</td>
<td>100</td>
<td>105</td>
<td>96</td>
<td></td>
</tr>
<tr>
<td>PCMB</td>
<td>$10^{-3} M$</td>
<td>101</td>
<td>91</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>N-Bromosuccinimide</td>
<td>$10^{-3} M$</td>
<td>12</td>
<td>26</td>
<td>33</td>
<td></td>
</tr>
<tr>
<td>N-Bromosuccinimide</td>
<td>$10^{-2} M$</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>
the concentration of $10^{-2}$M. The activity of each protease remained unaltered after the treatment with the thiol specific reagents, iodoacetic acid, N-ethylmaleimide, PCMB, β-mercapto-ethanol and cysteine hydrochloride. All the proteases were completely inhibited by $2 \times 10^{-3}$M N-bromosuccinimide.

**Effect of Specific Inhibitors:**

In order to determine the amino acid residues present at the active site of the proteases, the enzymes were incubated with various inhibitors for 30 minutes at 35°C. The residual activity was measured using BAPA as substrate. Table X shows the comparison of the inhibition characteristic of the *S. litura* proteases and bovine pancreatic trypsin. PMSF, which is known to react specially with functional serine residue, inhibited all the alkaline proteases of *S. litura*. Protease I and II were most susceptible to PMSF and protease III was as sensitive as trypsin. The specific inhibitor of trypsin, TLCK, showed pronounced effect on all the proteases and inhibition was similar to trypsin. The chymotrypsin specific inhibitor, TPCK, had no inhibitory effect on any of the enzymes. $10^{-5}$M SBTI caused about 70% inhibition of each protease in comparison to complete inhibition of trypsin. Each of the proteases were also inhibited by $10^{-4}$M LBTI though the inhibition was only 30-50%. Ovomucoid only inhibited the protease I and II.
TABLE - X

EFFECT OF SPECIFIC INHIBITORS ON *S. litura* ALKALINE PROTEASES

The reaction mixture in a total volume of 1 ml contained 0.0010 - 0.0015 umoles enzyme protein and the requisite amount of inhibitors in 0.1M Tris buffer, pH 8.0, to final molarity as indicated. After incubation for 30 minutes at 35°C, the residual activity was determined against BAPA as described in the text.

<table>
<thead>
<tr>
<th>Additions</th>
<th>Concentration</th>
<th>Inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Trpsin</td>
</tr>
<tr>
<td>PMSF</td>
<td>$5 \times 10^{-3}M$</td>
<td>58</td>
</tr>
<tr>
<td>TLCK</td>
<td>$4 \times 10^{-3}M$</td>
<td>96</td>
</tr>
<tr>
<td>TPCK</td>
<td>$10^{-3}M$</td>
<td>0</td>
</tr>
<tr>
<td>SBTI</td>
<td>$10^{-5}M$</td>
<td>100</td>
</tr>
<tr>
<td>LBTI</td>
<td>$10^{-4}M$</td>
<td>100</td>
</tr>
<tr>
<td>Ovomucoid</td>
<td>$10^{-4}M$</td>
<td>100</td>
</tr>
</tbody>
</table>