The Lysozyme of Hevea brasiliensis Latex: Isolation, Purification, Enzyme Kinetics and a Partial Amino-Acid Sequence*

S.J. TATA, J.J. BEINTEMA* and S. BALABASKARAN**

Two basic proteins — a major and a minor basic protein — were isolated from the bottom fraction of ultracentrifuged Hevea latex and their lysozyme activities were assayed using Micrococcus lysodeikticus as the substrate. The two Hevea lysozymes were found to be less active than egg white lysozyme. Heparin, DNA, RNA and Ampholine were found to have more inhibiting effect on the activity of the Hevea lysozymes than on the avian lysozyme. The optimum pH and temperature for the two Hevea lysozymes were identical, but these values differed from the corresponding values for the avian lysozyme. The pH optimum and the molecular weight of the two Hevea lysozymes were closely similar to those cited in the literature for lysozymes from papaya and fucus.

The major basic protein (molecular weight approximately 26 000) was crystallised and the sequence of its first twenty-one amino acid residues was elucidated. This sequence was found to be entirely different when compared with published sequences of lysozymes from hen egg, duck egg, baboon milk and T4 phage.

It was previously observed that two basic proteins isolated from the sedimentable phase (the bottom fraction) obtained by ultracentrifugation of fresh latex from Hevea brasiliensis had lysozyme activity1. The enzyme activity was detected first by a zymographic technique and subsequently assayed using Micrococcus lysodeikticus as the substrate.

It is well known that the two basic proteins of Hevea latex are involved in the 'plugging phenomena' of latex vessels after tapping2, which ultimately results in the cessation of latex flow. These proteins are therefore important functional components of Hevea latex and deserve a detailed study. The present investigation was undertaken to isolate and purify the proteins, study some of the parameters affecting their enzyme activity and elucidate a partial amino acid sequence of the major basic protein (lysozyme).

EXPERIMENTAL

Isolation of Basic Proteins

Fresh latex was collected and B serum was prepared from the bottom fraction after ultracentrifugation of the latex as described earlier3. The serum was dialysed at 4°C against approximately fifty volumes of a volatile buffer containing 0.01M

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ammonium formate and adjusted to pH 9.5 with ammonia solution. The precipitate obtained on dialysis was centrifuged off and 25 ml of the supernatant was applied on a DEAE — cellulose column (15 X 2.5 cm) which had been previously equilibrated with the buffer. The column was eluted with the same buffer and 3 ml fractions were collected. The absorbance values at 260 nm and 280 nm of the fractions were plotted against the fraction numbers.

The fractions under the two peaks (Figure 1) were separately pooled and lyophilised to remove the volatile buffer components. The dry products from the two peaks were subsequently used for comparative studies. The protein peak which appeared first through the DEAE — cellulose column was referred to as the major basic protein and the second peak the minor basic protein. A commercially available sample of egg white lysozyme from SIGMA Chemicals, U.S.A. was also used for these investigations.

Characterisation of Basic Proteins

The two basic proteins were characterised by starch gel electrophoresis, followed by staining with naphthalene black to visualise the proteins as described previously.

Crystallisation of the major basic protein was carried out according to a modified method of Hash and Rothlauf. Samples of 30 mg of the amorphous protein were dissolved in 2 ml of 0.2M acetic acid and chromatographed on a Sephadex G — 100 column (50 X 2.0 cm). The column was eluted with 0.2M acetic acid and 3 ml fractions were collected. The absorbance values of the fractions at 280 nm were plotted against the fraction numbers and the fractions under the resulting peaks were pooled and lyophilised.

An aqueous solution of the lyophilised powder was precipitated with solid ammonium sulphate at 70% saturation. The precipitated protein was recovered and redissolved in a 45% saturated solution of ammonium sulphate. The solution was left to stand at 4°C for several days until crystals were observed. The crystals were redissolved and recrystallised at 4°C as described above and subsequently washed with 80% (volume/volume) acetone at 0°C. The homogeneity of the crystalline protein was confirmed by starch gel electrophoresis.
Detection of Lysozyme Activity on Zymograms

Lysozyme activities in both the basic proteins as well as in the B serum were detected by zymography as described previously\(^1\)\(^6\).

Assay of Lysozyme Activity

This was carried out essentially according to the method of Shugar\(^7\): a suspension of dry cells of \(M.\) lysodeikticus was prepared in a 0.6M phosphate buffer of pH 6.2 to give an initial absorbance of 0.6–0.7 absorbance units at 450 nm in a 1 cm cell at 25\(^\circ\)C. The enzyme solution (0.3 mg per millilitre in the phosphate buffer) or whole B serum (diluted ten times with the buffer) was added to the above suspension to make up the final volume of the reaction mixture to 2.6 millilitres. The absorbance readings were taken at 30 s intervals for up to 120 seconds.

The lysozyme activity was calculated from the initial linear portion of the reaction rate.

One unit of activity will produce a \(\Delta A_{450}\) of 0.001 per minute at pH 6.25 at 25\(^\circ\)C using a suspension of \(M.\) lysodeikticus as substrate in a 2.6 ml reaction mixture and a 1 cm light path.

\(pH\) Optimum

The enzyme activities were assayed in 0.15\(M\) acetic buffer in the pH range 3.0–5.5 and in 0.06\(M\) phosphate buffer in the pH range 5.5–7.5.

Temperature Optimum

The activities of both the basic proteins from \(Hevea\) were assayed at pH 4.0 and that of the egg white lysozyme at pH 6.1. The assays were conducted between a temperature range of 20\(^\circ\)C–80\(^\circ\)C.

Effect of Inhibitors

Inhibition studies were carried out using heparin (sodium salt, 100 units per milligramme), RNA (yeast) and DNA (calf thymus) at a final concentration of 0.04% in the appropriate buffer while 'Ampholine' (pH range 3.5–10.0 obtained from LKB, Sweden) was used at a final concentration of 2.0% in the reaction mixture.

Determination of Isoelectric Point of the Major Basic Protein

Isoelectric focusing experiments were conducted on a solution of the lyophilised powder of the protein recovered from the Sephadex column (see above). The apparatus was the analytical electro-focusing column (LKB, Sweden) of 110 ml capacity. Use was made of a sucrose gradient of 0%–30% (weight/volume) and 'Ampholine' carrier ampholytes of pH range 3.5–10.0 at a final concentration of 1%. Initially, the current was 60 mA at 300 V which was increased after 2 h to 600 V at 50 mA. The voltage remained constant for 24 h but the current had dropped to 10 mA. The power supply was disconnected and 1.5 ml fractions were collected at the bottom of the tube to analyse for the protein content, enzyme activity and pH.

Molecular Weight Determination

Polyacrylamide gel electrophoresis in sodium dodecyl sulphate was performed essentially as described by Studier\(^8\) using 15% gel.

Amino Acid Analysis

Samples of the major basic protein were hydrolysed in 6\(M\) twice redistilled HCl in vacuo at 110\(^\circ\)C for 20–24 hours. The hydrolysates were analysed on a Kontron Liquimat III Analyser.

N-terminal Determination and Partial Sequencing

N-terminus was determined with dimethylaminonaphthalene sulphonyl
chloride (dansyl chloride) as described by Hartley, using 5 x 5 cm squares of polyamide sheet.

Automatic Edman degradation was performed in a Beckman spinning-cup Sequenator Model 890C, following the Beckman programme. The phenylthiohydantoin derivatives obtained were identified by high performance liquid chromatography.

RESULTS

As mentioned earlier, when a sample of B serum was dialysed and eluted through a DEAE - cellulose column, two protein peaks were detected (Figure 1). These included the major basic protein and the minor basic protein. Both the proteins appeared homogeneous by starch gel electrophoresis, each giving a single cationic band.

From several column chromatography experiments the yield of the major basic protein was ten times more than that of the minor basic protein. Because of the sufficient yield of the former, only this protein fraction was crystallised.

Following the procedure described in the text the major basic protein could be crystallised into prismatic crystals as shown in Figure 2. When the crystals were isolated, solubilised and electrophoresed on starch gel, a single protein band identical with that from the amorphous protein was detected. This implied that the crystals isolated were not (NH$_4$)$_2$SO$_4$ crystals.

While the crystals were being photographed on a glass slide, some of the ammonium sulphate solution adhering to the crystals dried up and appeared as tiny specks of the salt as shown in Figure 2. Attempts to remove them by washing with 80% (volume/volume) acetone at 0°C were not successful.

The lysozyme activity appeared on zymograms as clear zones in a translucent acrylamide gel overlay containing $M. lysodeikticus$ (Figure 3). The optimum pH for both the Hevea lysozymes was pH 4.0 while that for egg white lysozyme was pH 6.1 (Figure 4). It appears from this figure that while the avian lysozyme had an activity of 33 000 units per milligramme at its optimum pH, the Hevea lysozymes had only 5300 units (for the major basic protein) and 4000 units (for the minor basic protein) per milligramme at their optimum pH.

The order of the enzymic reaction was determined by plotting $-\log$ absorbance at 450 nm and also the reciprocal of the absorbance against the time of reaction. Figure 5 shows a linear relationship between the reciprocal of the absorbance and the reaction times indicating that the reaction followed second-order kinetics.

Maximum activity of egg white lysozyme was observed at 55°C and that of the two Hevea lysozymes at 50°C (Figure 6). A phosphate buffer of 60 mM concentration and pH 6.1 was found to give the maximum activity of egg white lysozyme. The Hevea enzymes had their maximum activities in 150 mM sodium acetate buffer of pH 4.0 (Figure 7).

In the present investigations, heparin, RNA and DNA were all found to be inhibitors of the two Hevea lysozymes. Their inhibitory effect was much greater on the Hevea lysozymes than on egg white lysozyme (Table 1).

During the determination of the isoelectric point (pI) 'the 'Ampholine' was found to inhibit the lysozyme activity of the major basic protein in the recovered fractions from the column. The full activity was restored after dialysis to remove the 'Ampholine'. In a separate experiment 'Ampholine' at a final concentration of 2% was found to have a strong inhibiting effect on the enzymes when added to the reaction mixture (Table 1).

The pI of the major basic protein was found to be pH 9.0 (Figure 8). Unfor-
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Figure 2. Crystals of the major basic protein recovered from the first peak in Figure 1. The tiny white dots are ammonium sulphate. Magnification 70X.

Unfortunately this could not be determined for the minor basic protein as the quantity of this enzyme available was too small.

SDS—polyacrylamide gel electrophoresis gave a single band for the major basic protein corresponding to an approximate molecular weight of 26 000 (Figure 9). Hevamine B\textsuperscript{11} which also has lysozyme activity\textsuperscript{1}, has been found to have a similar molecular weight and electrophoretic behaviour on starch gel\textsuperscript{6}.
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Figure 3. Photograph of an acrylamide gel containing M. lysodeikticus which was overlaid on a starch gel in which the basic proteins had been subjected to electrophoresis. Lysozyme activity was detected as clear zones in the translucent acrylamide gel. The samples were (from top) the major basic protein, the minor basic protein and whole B serum. The zymogram was photographed against a black background. The clear zones have appeared as black bands (see also Reference 1).

The UV absorption curve for the major basic protein showed no unusual features. The absorption maximum was at 278 nm and minimum at 252 nm (Figure 10). The $E_{278\text{nm}}^{1\%}$ was calculated to be 20.6 which agrees well with a previous value of 20.5 published by Archer$^{11}$.

Only a single N-terminus of the major basic protein was detected. This was identified as glycine. The protein therefore has a single peptide chain. This was further confirmed by paper electrophoresis of the performic acid - oxidation product of the protein which showed only one band. (Using the same techniques, the minor basic protein was also found to have glycine at the N-terminus in a single peptide chain).

The N-terminal sequence of the first twenty-one amino acid residues of the major basic protein is presented in Figure 11 which also includes the first twenty-one residues of lysozymes from hen egg, duck egg, baboon milk and T4 phage.

DISCUSSION

*Hevea brasiliensis* latex has long been known to have lysozyme (EC 3.2.1.17) activity$^{12}$. This activity is concentrated in the ‘bottom fraction’$^{13}$ (obtained by ultracentrifugation) which consists largely of the lutoid particles. These particles have been referred to as microvacuoles$^{14}$ and have been shown to be lysosomes$^{15}$. Matile$^{14}$ has proposed that vacuoles and lysosomes are equivalent in plant cells. The lutoids from which the *Hevea* lysozymes were isolated have thus a special function in the *Hevea* tree.

In the present study two basic proteins having lysozyme activity were isolated from *Hevea* latex. Compared with egg white lysozyme the two *Hevea* lysozymes are much less active at their pH optimum.
This is in agreement with Meyer et al.\textsuperscript{16} who observed that in general plant lysozymes have less bacteriolytic activity than animal lysozymes. Lysozymes from papaya\textsuperscript{17,18} and fig\textsuperscript{19} have also been reported to be less active than egg white lysozyme.

The reaction of the two lysozymes from Hevea as well as the egg white lysozyme followed second-order kinetics.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure4.png}
\caption{The optimum pH for Hevea and egg white lysozymes.}
\end{figure}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure5.png}
\caption{Kinetics of lysozyme activity in the proteins recovered from the first and second peaks (in Figure 1) and egg white lysozyme.}
\end{figure}
Figure 6. The optimum temperatures for the lysozymes.

Prasad and Litwak also reported that the reaction of egg white lysozyme followed second-order kinetics while Smith et al. reported the same for papaya lysozyme.

The pH optimum of egg white lysozyme obtained in this investigation (pH 6.1) agrees with the previously reported pH range 5.9-6.3. This is, however, different from the pH optimum for the two Hevea lysozymes (pH 4) found in this study.

Among the lysozyme inhibitors cited in the literature are heparin, hyaluronic acid, RNA, DNA, and polyglutamic acid. Some of these have also been found to inhibit the two Hevea lysozymes. 'Ampholine' which also inhibits enzyme activity is a mixture of polyamino acids. Similar compounds have been reported to have inhibitory activity on the avian lysozyme.

The two Hevea lysozymes have the same molecular weight - approximately 26,000 - in this study and approximately 25,000 in a previous investigation. The
Amino acid sequences of more than a dozen lysozymes are available in the literature. All these originated from animals, birds or bacteria. As far as the present authors are aware no plant lysozyme has yet been sequenced fully and the result presented in Figure 11 is a start on the elucidation of the sequence of Hevea lysozyme. In this figure, the sequences of the three vertebrate lysozymes are closely similar. These are higher vertebrate homologous enzymes and they all begin with lysine at the N-terminus. Substitutions of the amino acid residues occur only at a few positions within the first twenty-one residues. The sequences of the Hevea lysozyme (the present study) and the T4-phage lysozyme are entirely different from each other and from the vertebrate lysozymes.

It would be interesting to compare the sequence of Hevea lysozyme with that of a lysozyme from another angiosperm, but the only directly relevant information seems to be the N-terminal sequence of papaya lysozyme determined by Howard and Glazer: Gly-Ile-Lys-Ile and the N-terminus of fig lysozyme which is Gly. The other similarities between Hevea, papaya and fig lysozymes are suggestive, however. Like Hevea lysozyme, the papaya and fig enzymes both consist of a single peptide chain. The molecular weights

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**TABLE 1. EFFECT OF INHIBITORS ON LYSOZYME ACTIVITY**

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Concentration of inhibitor in reaction mixture (%)</th>
<th>Egg white lysozyme</th>
<th>Percentage inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heparin</td>
<td>0.04</td>
<td>17.8</td>
<td>84.7</td>
</tr>
<tr>
<td>DNA</td>
<td>0.04</td>
<td>11.1</td>
<td>72.4</td>
</tr>
<tr>
<td>RNA</td>
<td>0.04</td>
<td>6.7</td>
<td>13.5</td>
</tr>
<tr>
<td>'Ampholine' (pH 3.5 - 10.0)</td>
<td>2.00</td>
<td>34.7</td>
<td>88.3</td>
</tr>
</tbody>
</table>

Figure 8. The isoelectric point of the major basic protein on a sucrose gradient (0%–30%) with 'Ampholine' carrier ampholytes of pH range 3.5–10.0.

molecular weight calculated from the amino acid composition (Table 2) agrees well with these figures.
are similar: Hevea — 25 800 (Table 2); papaya — 25 000$^{18}$ and fig — 29 000$^{19}$. Their amino acid compositions are similar$^{6}$, and so are their optimum pH values: Hevea — pH 4.0; papaya — pH 4.65$^{17}$ and fig — pH 4.5$^{19}$.

It may be anticipated that the sequence of Hevea lysozyme would show homology with those of papaya and fig but this is, of course, a speculation until the sequences of all the three lysozymes are fully elucidated.

The basic proteins of Hevea latex are involved in the physiology of latex flow$^{2,6,31-34}$. This involvement is partly due to their high isoelectric point and their consequent ability to flocculate the negatively charged rubber particles in latex. It is not known whether their lysozyme activity is also important in this connection. It is, however, obvious that the enzyme might have an important function in the defence of the trees against invading bacteria in the event of an injury such as that caused by tapping to obtain latex. Moreover, in view of the fact that the same basic proteins also
Figure 11. Amino acid sequences of twenty-one residues of the N-terminal peptides of lysozymes from four different sources (—X— is an unidentified residue).
TABLE 2. AMINO ACID COMPOSITION OF THE MAJOR BASIC PROTEIN

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Residues per molecule</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asp</td>
<td>31</td>
</tr>
<tr>
<td>Thr</td>
<td>11</td>
</tr>
<tr>
<td>Ser</td>
<td>25</td>
</tr>
<tr>
<td>Glu</td>
<td>15</td>
</tr>
<tr>
<td>Pro</td>
<td>13</td>
</tr>
<tr>
<td>Gly</td>
<td>29</td>
</tr>
<tr>
<td>Ala</td>
<td>19</td>
</tr>
<tr>
<td>Cys(^a)</td>
<td>3 or 4</td>
</tr>
<tr>
<td>Val</td>
<td>10</td>
</tr>
<tr>
<td>Met</td>
<td>2</td>
</tr>
<tr>
<td>Ile</td>
<td>17</td>
</tr>
<tr>
<td>Leu</td>
<td>18</td>
</tr>
<tr>
<td>Tyr</td>
<td>14</td>
</tr>
<tr>
<td>Phe</td>
<td>7</td>
</tr>
<tr>
<td>Lys</td>
<td>14</td>
</tr>
<tr>
<td>His</td>
<td>2</td>
</tr>
<tr>
<td>Arg</td>
<td>7</td>
</tr>
<tr>
<td>Trp</td>
<td>4</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>241 or 242</strong></td>
</tr>
</tbody>
</table>

| Approx. molecular weight | 25 800 (242 residues)\(^a\) |

\(^a\) Because no free -SH group was discovered it was assumed that there were 4 Cys residues.

exhibited chitinase activity (EC 3.2.1.14) on zymograms\(^6\), it could imply that the enzyme may play a defensive role against fungi and even conceivably insects. In this connection it is interesting to note that lysozymes from fig and papaya have also been found to have marked chitinase activity\(^17,19,30\).

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10. BECKMAN INSTRUMENTS INC. U.S.A. Quadrol Double – Cleavage Programme No. 072172C for the Beckman Sequanator Model 890C.


