Isolation and Characterization of Multiple Forms of Rat Liver Lysozymes

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Multiple forms of lysozyme found in the rat liver were isolated and characterized from cellular organelles.

Isolation of the enzymes was achieved by Sephadex gel filtration and chromatography on CM cellulose-column. The purity of the preparations was examined by electrophoresis on polyacrylamide gel. The nuclear lysozyme moved as a single band indicating homogeneity, whereas other subcellular lysozymes appeared heterogeneous due to presence of more than one band, thus showing partial purity. Although the subcellular lysozymes were similar with respect to enzymatic properties, pH, buffer molarity optima and electrophoretic mobility, differences were observed in elution patterns, responses to nuclear inhibitor and heat sensitivity. Nuclear lysozyme was distinctly different by these criteria as compared to other subcellular lysozymes.

Earlier from our laboratory it was shown that lysozyme activity is distributed in all subcellular organelles in various vertebrate tissues, kidney, liver, spleen and others. Highest activity was located in nuclei followed by microsomes, mitochondria and supernatant.1~6) Thus it was found that, in rat kidney, lysozyme exists in multiple forms which could be distinctly distinguished by physicochemical, kinetic and thermostability characteristics.2~6) In addition tissue specific differences were detected. Although kidney and spleen lysozymes were similar in enzymatic properties they differed very widely compared to liver lysozyme. Besides, in liver nuclei lysozyme was present complexed with an inhibitor, a protein, and was immunologically unrelated to kidney nuclear lysozyme.7~8) Since liver nuclear lysozyme was found to be different from other subcellular lysozymes, particularly because of complexing with the inhibitor, existence of multiple forms of lysozyme in liver seemed natural as has been shown in the case of kidney.2) In this paper, therefore, isolation and enzymatic characterization of lysozyme from rat liver subcellular organelles is reported.

MATERIALS AND METHODS

Chemicals. Sephadex G-50 was purchased Pharmacia. CM-cellulose was obtained from Sigma Chemical Company. Chemicals used for polyacrylamide gel electrophoresis were from Canalco. Micrococcus luteus cells were purchased from Worthington Biochemical Corporation.

Animals. Female rats (wistar strain), bred in our animal house, weighing about 150~200 g were used for all experiments.

Assay of lysozyme activity. Lysozyme activity was assayed spectrophotometrically at 450 nm using lyophilized M. luteus cells as described previously.4~8) A cell suspension of the substrate (2 mg/ml) was made in 0.02 M phosphate buffer pH 5.4. The enzyme extract (0.1 ml) was added to 3.9 ml of the suspension and incubated at 37°C for 10 minutes. At the end of the incubation period, the turbidity of the solution was read at 450 nm. A substrate blank was simultaneously run without the enzyme. The difference between the blank reading and the experimental reading (ΔO.D.) was taken as the measure of enzyme activity. A change in turbidity of 0.001 OD/min was taken as one unit of enzyme activity. Both ΔO.D. and units are employed to represent enzyme activity.

Protein was estimated by the method of Lowry et al.9)
using bovine serum albumin as standard. Polyacrylamide gel electrophoresis was carried out at pH 4.5 using β-alanine acetic acid buffer, according to the method of Reisfeld et al.\textsuperscript{10} and at pH 2.7 using 0.9 M acetic acid by the method of Panyim and Chalkley.\textsuperscript{11}

**Preparation of subcellular fractions.** For each experiment for the preparation of the subcellular fractions, 30 g of liver was collected from 4 to 5 female rats. The subcellular fractions were prepared from the 10% homogenate by the method of Schneider and Hogeboom.\textsuperscript{12} The nuclear fraction was further purified by the method described by Chauveau et al.\textsuperscript{13}

The enzyme extract from organelle fractions was prepared as mentioned by Jolles.\textsuperscript{14} The subfractions were dialysed, lyophilized and stored at 4°C till next step. The method is detailed previously.\textsuperscript{7}

**Gel filtration on Sephadex G-50 column.** The lyophilized preparation of each fraction was suspended in 0.02 M acetic acid (3 ml) and filtered on a Sephadex G-50 column (49 × 9 cm) equilibrated and eluted with 0.02 M acetic acid. Thirty fractions containing 3 ml volume were collected and optical density of these fractions was read at 280 nm using Shimadzu QV-50 spectrometer.

**Thermal stability.** The enzyme extract of each subcellular fraction about 400 ~ 600 µg/ml in phosphate buffer 0.02 M pH 5.4 was heated at various temperatures 37~80°C in water bath for 5 min, and cooled immediately. Activity was determined in the supernatant after centrifugation, as stated above.

**Chromatography on CM cellulose column.** Active fractions of each subcellular lysozyme obtained on gel filtration were combined and adsorbed on CM cellulose-column (0.9 × 10 cm) equilibrated with 0.02 M acetic acid. The column was washed with 0.2 M phosphate buffer pH 6.2. The lysozyme and some other fractions which eluted together on Sephadex G-50 column stayed on the CM cellulose-column. Some of the adsorbed proteins were eluted in the buffer washings. The lytic enzymes were eluted from the column by stepwise elution with HCl, 10~50 mM.

**Qualitative detection of enzyme activity after electrophoresis.** The enzyme activity in the unstained gels was examined by cutting 1 mm sections of the gel and incubating the same with *M. luteus* cells (1.5 mg/10 ml) in 0.02 M sodium phosphate buffer, pH 5.4 at 37°C for 45 min. The O.D. was read at 450 nm.

**Absorption and fluorescence spectra.** The UV absorption spectra of rat liver nuclear lysozyme (500 µg) in 30 mM HCl was taken using a Perkin-Elmer double beam spectrometer against 30 mM HCl as the blank. Fluorescence spectrum of the enzyme (500 µg) in 30 mM HCl was taken manually using a Hitachi fluorescence spectrophotometer. Excitation wavelength was 280 nm.

**Effect of nuclear lysozyme inhibitor on the activity of subcellular lysozymes.** The nuclear lysozyme inhibitor was prepared according to the procedure detailed by Sidhan et al.\textsuperscript{7} The effect of the inhibitor was examined by assaying the enzyme activity in the presence and in the absence of the inhibitor. Other assay conditions were same as stated before.

### RESULTS

**Isolation and purification of multiple forms**

Elution pattern of lysozyme from each subcellular fraction on Sephadex G-50 is shown in Fig. 1. In all cases, a major protein peak was eluted followed by two minor protein peaks. The enzyme activity was found to be associated with the major protein peak in the case of nuclear enzyme, in 4 fractions (8~11); whereas lysozymes from other subfractions were eluted later. The mitochondrial enzyme was eluted in 9 fractions (14~22); microsomal enzyme in 6 fractions (9~14), and supernatant in 9 fractions (17~25) respectively.

The elution pattern of the enzymes of the four subcellular fractions obtained from CM cellulose-column is shown in Fig. 2. One protein peak each was eluted corresponding to 10 mM, 20 mM, 30 mM HCl eluant. Except microsomal enzyme, all other enzymes were eluted with 30 mM HCl. Microsomal enzyme was eluted with 20 mM HCl. The pattern was reproducible with every isolation experiment.

To check the purity of these preparations each was examined on electrophoresis on polyacrylamide gel as stated in methods. The pattern is shown in Fig. 3. The mobility of lysozymes from each fraction was more or less similar under identical conditions. The nuclear enzyme appeared to be a homogeneous preparation, whereas the others were found to be heterogeneous to some extent. The enzyme activity on gels was also identified in the unstained bands as stated in experimental section; only part of the unstained gel corresponding to the stained band showed lysis of *M. luteus* cells. The homogeneity of the nu-
Fig. 1. Gel Filtration Patterns of Rat Liver Lysozymes Obtained from Subcellular Organelles on Sephadex G-50 Column.
Crude extracts of each fraction containing the enzymes in 3 ml volume was loaded on the column equilibrated and eluted with 0.02 M acetic acid.

Fig. 2. Elution Patterns of Rat Liver Lysozymes from Subcellular Organelles on CM Cellulose-column.
The pooled fractions containing enzyme, obtained on gel filtrations, were adsorbed on CM cellulose-column. They were eluted in the sequential order as described in the experimental section.
Fig. 3. Electrophoretic Patterns of Lysozymes from Subcellular Organelles Obtained from CM Cellulose-column.

Electrophoresis was done at pH 4.5 using β-alanine acetic acid buffer. The current applied was 6 mA/tube for 15 minutes. The enzyme concentration of each lysozyme preparation in µg was as follows: nuclei (NUC), 15; mitochondria (MIT), 100; microsomes (MIC), 60; supernatant (SUP), 30.

clear preparation was further confirmed by electrophoresis at pH 2.7.

In Table I the protocol of the purification of liver nuclear lysozyme is shown. By gel filtration on Sephadex G-50 the specific activity, units/min/mg protein, of the enzyme rose to 18.75 x 10²; thus in this single step a 10 fold purification was achieved. By chromatography on CM cellulose-column the specific activity of the enzyme rose to 4.4 x 10³, and a 25 fold purification was achieved.

**UV absorption and fluorescence spectra of nuclear lysozyme**

The absorption and the fluorescence spectra of purified nuclear lysozyme were also examined. Maximum absorption was observed at 275 nm and minimum at 245 nm. The ratio of the maximum to minimum was 1.7. The fluorescence maximum was at 340 nm, Fig. 4; which is typically characteristic of tryptophan fluorescence in proteins.¹⁵)

**Characterization of the subcellular lysozymes**

Figures 5 and 6 show activity—pH and activity—buffer molarity profiles of lysozymes from four subfractions; these appeared quite similar. They were all found to be maximally active at pH 5.4. The molarity optimum was also similar, around 0.02 M and a hump appeared in 0.06~0.08 M range in phosphate buffer.

**Effect of nuclear inhibitor on the activity of subcellular lysozymes**

As shown in Table II except the nuclear lysozyme the activity of the other subcellular lysozymes was not affected under identical

<table>
<thead>
<tr>
<th>Purification steps</th>
<th>Total activity (units)</th>
<th>Total protein (mg)</th>
<th>Specific activity* (units/min/mg protein)</th>
<th>Purification fold</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole homogenate</td>
<td>206,584</td>
<td>1125</td>
<td>183</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>Purified nuclear fraction</td>
<td>14,000</td>
<td>20</td>
<td>700</td>
<td>3.8</td>
<td>7</td>
</tr>
<tr>
<td>After filtration through Sephadex G-50 column</td>
<td>11,250</td>
<td>6</td>
<td>1,875</td>
<td>10.2</td>
<td>5</td>
</tr>
<tr>
<td>After elution from CM-cellulose column</td>
<td>3,100</td>
<td>0.725</td>
<td>4,444</td>
<td>24.8</td>
<td>2</td>
</tr>
</tbody>
</table>

* Enzyme activity was determined in 0.02 M sodium phosphate pH 5.4.
Multiple Forms of Liver Lysozyme

Fig. 4. Fluorescence Spectrum of RLN-lysozyme (500 µg) in 30-mM HCl. The excitation wavelength was 280 nm.

Fig. 5. pH Optima of Crude Preparations of Lysozymes from Various Subcellular Fractions of Rat Liver in 0.02 M Phosphate or Glycine-NaOH Buffers at Different pH Values. Other assay conditions were same as used elsewhere.

conditions at the same concentration of the inhibitor.

**Thermostability**

The thermal inactivation pattern of subcellular lysozymes is shown in Fig. 7. The nuclear lysozyme apparently was more heat labile as compared to the others which showed similar relative heat sensitivity.

Fig. 6. Buffer Molarity Optima of Crude Preparations of Lysozyme from Various Subcellular Fractions of Rat Liver in Varying Concentrations of Sodium Phosphate. Other assay were same as used elsewhere.

Fig. 7. Temperature Stability of Lysozymes. Enzyme extracts were obtained from various subcellular fractions as described in Experimental. Temperature exposure and assay conditions are mentioned in Experimental.
Table II. Specificity of Rat Liver Nuclear Lysozyme Inhibitor

The enzyme and inhibitor were mixed 1:1 (vol/vol) and kept for one hour at 0°C. Then 0.2 ml of the mixture was added into the assay system with appropriate enzyme and inhibitor control.

<table>
<thead>
<tr>
<th>Source of lysozyme</th>
<th>Enzyme activity in units</th>
<th>Enzyme concentration (μg)</th>
<th>Inhibitor concentration (μg)</th>
<th>% Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat liver</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nuclei</td>
<td>100</td>
<td>60</td>
<td>50</td>
<td>48.0</td>
</tr>
<tr>
<td>Mitochondria</td>
<td>100</td>
<td>100</td>
<td>50</td>
<td>5.0</td>
</tr>
<tr>
<td>Microsomes</td>
<td>70</td>
<td>80</td>
<td>50</td>
<td>0.0</td>
</tr>
<tr>
<td>Supernatant</td>
<td>80</td>
<td>30</td>
<td>50</td>
<td>2.0</td>
</tr>
<tr>
<td>Rat kidney nuclei</td>
<td>120</td>
<td>4</td>
<td>60</td>
<td>0.0</td>
</tr>
<tr>
<td>Hen egg white</td>
<td>100</td>
<td>2</td>
<td>60</td>
<td>0.0</td>
</tr>
<tr>
<td>Human (urine)</td>
<td>100</td>
<td>1.5</td>
<td>60</td>
<td>0.0</td>
</tr>
</tbody>
</table>

Discussion

The elution pattern of these subcellular enzymes on Sephadex G-50 and their chromatographic behaviour on CM cellulose-column indicates separate subcellular localization of the four forms of these enzymes. On gel filtration, the nuclear enzyme was eluted along with the void volume followed by microsomal enzyme. The mitochondrial and supernatant enzymes were eluted after the microsomal enzyme. These differences in gel filtration profile were further investigated, as it appeared that these differences could arise due to different subcellular location of lysozymes; where these enzymes could be bound with different proteins, which could lead to some alteration in their elution pattern. This possibility seemed more likely because, earlier we had shown that nuclear lysozyme was complexed with a protein in the nuclei, which turned out to be its inhibitor. However, similar inhibitor protein was not found in liver mitochondria, microsomes or supernatant; but the possibility of other proteins, which alter the elution pattern, thus affecting size but not affecting activity, complexing with these lysozymes could not be ruled out. However, differences were also seen in the elution order on CM cellulose-column. This indicated differences in the overall charge of the molecule. Since microsomal lysozyme was eluted with 20 mM HCl it would appear that preparation was less basic as compared to other enzymes. But except nuclear lysozyme others were electrophoretically heterogeneous. It would mean that these were complexed with other subcellular proteins, which in some way influenced their elution pattern on CM cellulose-column and supported our conclusion drawn from the observation of the elution pattern on gel filtration discussed above.

With respect to the response of subcellular lysozymes to the nuclear inhibitor, nuclear lysozyme was specifically inhibited, whereas the other three preparations were not affected. This set nuclear lysozyme apart from others. This was further supported by the heat stability profile which showed nuclear lysozyme more heat sensitive than other subcellular lysozymes. In this respect behavior of liver nuclear lysozyme resembles closely that of kidney nuclear lysozyme.

It is clear from the data presented above that except for heat sensitivity and response to the nuclear inhibitor, all subcellular lysozymes appeared very similar in general in other respects. All showed maximum activity at pH 5.4 and required low concentration of the buffer (0.02 M) for the activity. The pH-activity profile was studied in detail in buffers containing different species of ions, even below pH 5.0. Enzyme is still active at pH 2.0. Details would be discussed elsewhere. In this respect, although, they differ from kidney subcellular lysozymes, they resemble T₄ and plant lysozymes, which similarly are found to be active in low concentration of buffer around
acidic pH.

At the present state of studies therefore, although liver nuclear lysozyme can be distinguished by its positive response to nuclear inhibitor, others can be proved to be distinct or otherwise, only from their amino acid composition and immunological homologies after further purification of the mitochondrial, microsomal and supernatant lysozymes. These are in progress.

REFERENCES