STUDIES ON LYSOZYMES III

ISOLATION AND CHARACTERIZATION OF HUMAN MILK AND LEUCOCYTES LYSOZYMES

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Human milk and leucocytes lysozymes had prepared from human milk and leucocytes by a simple method. They were biochemically compared with hen egg white lysozyme.

Human lysozymes were different from hen egg white lysozyme in regard to the biochemical properties. Both of human lysozymes were recognized to be similar about enzymic activity, chemical composition and physical properties.

INTRODUCTION

The lysozyme (N-acetylmuramid glycanohydrolase; EC 3.2.1.17) has been considered as one of the biological materials which make a contribution to the bactericidal and fungicidal function of living cells.

This enzyme occurs in many of species of various vertebrates and invertebrates; the presence of lysozyme has also been established in plant. Human lysozymes are found in many tissues and secretions.

Jolles et al (1, 2) obtained a number of lysozymes from normal human tissues and secretions, i.e. lysozymes from milk (3, 4), tear (4), saliva (5), placenta (6), spleen, serum, and leucocytes (7). Human milk lysozyme has also been isolated by Parry, Chandan and Shahani (8). Osserman and Lawlor (9) have purified human serum and urinary lysozyme in monocytic and monomyelocytic leukemias. The researchers for human lysozymes have pointed out that all these human lysozymes exhibit very similar biochemical properties.

Further work on human lysozymes is necessary to clarify physiological role in living cells. Such studies are interesting also from the standpoint of the prevention of infection and the effects of various chemicals on the protection mechanism of living cells. It may be possible to use effectively the purified human lysozymes for the biochemical and immunochemical studies of many toxic chemicals.

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The present paper indicates to isolate much of human milk and leucocytes lysozymes (HML and HLL) and to examine their biochemical characteristics.

MATERIALS AND METHODS

(i) Human milk

Human milk was obtained through cooperation of several doctors from local hospitals. It was stored at 2°C. Individual samples were pooled until sufficient materials had been collected.

(ii) Human leucocytes

Human leucocytes were prepared from buffy coat of fresh human blood or preserved human serum.

(iii) Preparations of HML and HLL

Human milk was defatted by centrifugation and subsequently dialyzed against distilled water. It was mixed with the carboxymethylcellulose-powder (Brown Co., 0.8–0.9 meq.) which was previously equilibrated with 0.02 M phosphate buffer, pH 6.8. The mixture was stirred overnight. After standing, the powder precipitated was washed with 0.02 M phosphate buffer, pH 6.8 and followed by the washing by batch method with 0.02 M phosphate buffer, pH 6.8, contained 0.1 M NaCl. The CM-cellulose which absorbed lysozymes was transferred to a column. The lysozymes were desorbed from the CM-cellulose with 0.02 M phosphate buffer, pH 6.8, contained 0.5 M NaCl. The active fractions were pooled and then lyophilized. It, dissolved in minimum volume of 0.9% NaCl–1% acetic acid, was fractionated on a Sephadex G-50 column, using the same solution as the effluent. The active fractions were collected. The purified enzyme was desalted on Bio-Gel P-2#(50-100 mesh) column using 1% acetic acid as the effluent. The salt-free lysozymes were lyophilized.

Human leucocytes were prepared from buffy coat of fresh human blood. After the addition of equal volume of 5% Dextran 250 (Pharmacia C., M₀=250,000) –4% EDTA-2Na salt–1% Tween 80 solution, the buffy coat was stirred gently for 30 minutes. And then, it was centrifuged at 4,000 rpm for 5 minutes. The white buffy coat was washed with 0.01 M phosphate, pH 7.0, contained 0.9% NaCl by the centrifugation (6,000 rpm for 5 minutes). The leucocytes rich fraction was washed with distilled water for the hemolysis, and followed by the centrifugation (10,000 rpm for 10 minutes). The leucocytes was homogenized with 0.1 M acetic acid–0.9% NaCl by a Waring blender for 1 minute. The homogenate was stirred overnight. Then, it was centrifuged at 10,000 rpm for 15 minutes. The supernatant was dialyzed against distilled water. HLL was adsorbed on CM-cellulose powder by a batch method. It was isolated by the same procedure as the preparation of HML.

All treatments were carried out in a cold room. (4°C)

Hen egg white lysozyme (EWL) was purchased from Seikagaku Kogyo Co.,
(iv) Lysozyme activity

Lytic activity was assayed as previously reported in this journal (10). The method involved the measurement of the change in percentage of transmittance ($\Delta T$) of a suspension of Micrococcus lysodeikticus cells at 540 m$\mu$. One unit of lytic activity is equivalent to the $\Delta T$/min obtained from one microgram of EWL under the same condition of the assay.

Hydrolytic activity was assayed as previously reported in this journal (10). The method involved the measurement of the change of viscosity (min/cm) of a solution of glycol chitin. One unit of hydrolytic activity is equivalent to the min/cm obtained from one microgram of EWL under the same condition of the assay.

(v) Polyacrylamide gel electrophoresis

All lysozymes isolated were characterized by polyacrylamide gel electrophoresis (11).

(vi) Determination of molecular weight by gel filtration

The gel filtration behavior of a series of protein (myoglobin, lysozymes and cytochrome C) was compared on Sephadex G-50 with 0.9% NaCl—1% acetic acid

![Flow sheet diagram of the procedure used for the isolation of human milk lysozyme.](image-url)
as the effluent. The volume of elution can be plotted as the log. of molecular weight of these proteins (12)

(vii) Amino acid analyses

The amino acid analyses were conducted on a Hitachi amino acid analyzer (Model 034). The analyses were carried out on the CM-lysozymes (13) and the lysozymes treated with performic acid. Protein samples were hydrolysed with 6N HCl in evacuated sealed tube for 24 hours at 110°C. Calibration runs were carried out with a standard calibration amino acid mixture (Ajinomoto. Co.) and with the hydrolysate of CM-EWL. The relative molar ratios were calculated on the basis of two phenylalanine residues per molecule of lysozyme.

RESULTS

Isolation of purification of HML and HLL

The HML was isolated from human milk. The flow diagram of the procedure is given in Fig. 1. The average yield was 10-20 mg of the purified HML from one liter of human milk. The overall yield reached 10-20 per cent for the starting lysozyme activity of the original milk.

The HLL was isolated from human buffy coat of blood. The flow diagram of the procedure is given in Fig. 2. The average yield was 10-20 mg of the purified

Blood (Buffy coat) (5 liters)
- Added equal volume of 5% Dextran 250–4% EDTA-2Na salt–1% Tween 80. Stirred for 30 min. Centrifuged at 2°C, 4,000 rpm. 5 min.
- Buffy coat
  - Washed with 0.01 M phosphate, pH 7.0, contained 0.9% NaCl. Centrifuged at 2°C, 6,000 rpm, 5 min.
- Buffy coat
  - Added distilled water for hemolysis. Centrifuged at 2°C, 10,000 rpm, 10 min.
- Leucocytes
  - Extracted with 0.1 M acetic acid–0.9% NaCl by a blending for 1 min. Stirred overnight at 2°C. Centrifuged at 2°C, 10,000 rpm, 15 min.

Fig. 2. Flow sheet diagram of the procedure used for the isolation of human leucocytes lysozyme.
HLL from one liter of human buffy coat. The overall yield could not be calculated as the original activity could not be assayed.

**Homogenity of HML and HLL**

The homogenity of lysozymes was examined by Disc electrophoresis at various pH values (pH 4.3, 7.0 and 8.3). It was found that HML and HLL migrated as a single band toward the cathode, indicating them to be basic protein. Both of lysozymes were similarly run. At all pH values tested, a single band was observed in all preparations.

**Molecular weight**

The minimal molecular weight was calculated by the following experiments: (a) by the dialysis with calibrated cellophane membranes (14) and (b) by the gel filtration on Sephadex G-50. The molecular weight of HML and HLL was indicated 14,000–15,000.

**Quantitive amino acid analyses**

The amino acid composition of HML and HLL are quite comparable (see Table 1. Amino acid composition of hen egg white and human lysozymes.

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Human Lysozyme</th>
<th>Hen Egg White Lysozyme</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Milk</td>
<td>Leucocytes</td>
</tr>
<tr>
<td>Lys</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>His</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Arg</td>
<td>14</td>
<td>14</td>
</tr>
<tr>
<td>Asx</td>
<td>18</td>
<td>17–18</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thr*</td>
<td>(4)</td>
<td>(4)</td>
</tr>
<tr>
<td>Ser*</td>
<td>(5)</td>
<td>(4)</td>
</tr>
<tr>
<td>Glx</td>
<td>8–9</td>
<td>8–9</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pro</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Gly</td>
<td>11</td>
<td>11</td>
</tr>
<tr>
<td>Ala</td>
<td>12–14</td>
<td>12–14</td>
</tr>
<tr>
<td>1/2Cys**</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>Val</td>
<td>8</td>
<td>7–8</td>
</tr>
<tr>
<td>Met</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Ile</td>
<td>4–5</td>
<td>4–5</td>
</tr>
<tr>
<td>Leu</td>
<td>7–8</td>
<td>7–8</td>
</tr>
<tr>
<td>Tyr</td>
<td>5–6</td>
<td>5–6</td>
</tr>
<tr>
<td>Phe</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Trp</td>
<td>5</td>
<td>5</td>
</tr>
</tbody>
</table>

Molar ratios of amino acid residues of HML and HLL are calculated as 2 phenylalanine residues per one molecule and the values of EWL are calculated as 12 alanine residues per one molecule. Thr and Ser of HML and HLL are not corrected for the destruction during the hydrolysis. Trp are measured optically. 1/2 Cys; see text.
1). The dissimilarities between human lysozymes and egg white lysozyme are evident in their amino acid composition.

**Specific activity**

The lytic activity against *Micrococcus lysodeikticus* and the hydrolytic activity against glycol chitin were determined. As shown in Table 2, the human lysozymes exhibited three times much of lytic and hydrolytic activities than the egg white lysozyme.

Table 2  Relative activities of Hen Egg White and Human Lysozymes.

<table>
<thead>
<tr>
<th>lysozyme</th>
<th>lytic activity (ML-cell)</th>
<th>viscosity (glycol chitin)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hen egg white</td>
<td>100%</td>
<td>100%</td>
</tr>
<tr>
<td>Human milk</td>
<td>230</td>
<td>230</td>
</tr>
<tr>
<td>Human leucocytes</td>
<td>190</td>
<td>300</td>
</tr>
</tbody>
</table>

**pH optimum**

The pH optimum of the purified HML and HLL was investigated. Assays were run in 0.1 M Tris-Maleic acid buffer (adjusted pH from 4.5 to 8.5). The HML and HLL had doublet pH optimum near 6 and 8, as presented in Fig. 3. The EWl had also similar doublet pH optimum in this buffer system.

Fig. 3. pH dependence of lytic activity of hen egg white and human lysozymes.

Lytic activity is expressed as change in percentage of transmittance of a suspension of *Micrococcus lysodeikticus* cells at 540 m	ext{\textmu} (lysozyme; 1–3 γ). Buffers; 0.1 M Tris (hydroxymethyl) aminomethane was mixed with 0.1 M maleic acid to make solutions of pH 4.5 to 8.5.
**Effect of ionic strength**

The dependency of ionic strength for the activity of lysozymes were investigated. The HML and HLL exhibited a wide range of NaCl concentration of 0.01 M–0.1 M with maximum activity, as presented in Fig. 4. The EWL indicated the same tendency for the effect of ionic strength.

**Heat activation**

The thermal stability of HML was examined, as compared with that of EWL. HML was dissolved in 0.1 M acetate buffer, pH 5.5 (0.5% of concentration). The solution was incubated at 30, 40, 60 and 80°C during a coarse time. Then it was assayed for the enzymic activity. These results are shown in Fig. 5. The human lysozymes seem to be more unstable for heat activation than egg white lysozyme.

**DISCUSSION**

The purification procedure for human lysozymes, HML and HLL, used in this study is a modified method of Jollès et al. (4). The simple technique gave more convenient preparation than the original.

The enzymic activity, chemical composition and physical property (heat stability) of human lysozymes are different from them of EWL, but HML and HLL isolated from human different sources do not differ markedly in regard to their

![Graph](image_url)

**Fig. 4.** Effect of NaCl concentration on hen egg white and human lysozymes.

The mixture of 1.0 ml of NaCl and 3.0 ml of Micrococcus lysodeikticus cells suspension (aqueous, 30 mg per 100 ml) was preincubated at 37°C for 3 minutes. 0.1 ml of lysozyme aqueous solution (2–3γ) was added to the mixture. The turbidity of the reaction mixture was measured at 540 m\u00b4 during a time coarse of 1, 3, and 5 minutes. The ΔT was plotted to the concentration of NaCl (final; 0 to 0.15 M).
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Lysozymes are found in saliva, sputum and nasal secretions which play a role for the prevention of bacterial infection. Significant activity is also found in skin, lung and kidney. Skin is the tissue which contacts with the outside of human body and lung is related to a breath. Kidney is connected with an excretion. The enzymic function of lysozyme has an important meaning for the protection of infection. Lysozyme found in leucocytes may be responsible for lysis of bacteria and fungi during phagocytosis.

HML and HLL could be utilized as the representative of human lysozymes for various studies, such as immunochemical experiments and researches for the prevention mechanism of infection.

Abbreviations. HML; Human milk lysozyme, HLL; Human leucocytes lysozyme, EWL; Hen egg white lysozyme. CM-cellulose; Carboxymethylcellulose.

Note §; Pharmacia Co., medium size.
Note #; Calbio Chem.

REFERENCES

M. KIMURA, N. OTAKI, K. MURAYAMA, H. OGAWA AND S. KOBAYASHI