

## Investigation on Lysozyme, Protease and Hyaluronidase Activity in Extracts from Human Leukocytes

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Lysozyme activity was found in a crude extract of human leukocytes using glycolchitin as substrate. A low proteolytic activity was also found but no hyaluronidase activity. After gel filtration on Sephadex G-100 superfine two different lysozyme fractions were separated. These two fractions had the same maximum activity at pH 4.0-4.2. One of the lysozymes was activated by the addition of cysteine and glutathione but the other was only slightly activated by glutathione.

Fleming<sup>1</sup> in 1922 discovered an enzyme in nasal mucus, which he called lysozyme. Lysozyme breaks down the membranes of certain bacteria by a hydrolysis of  $\beta$ -1,4-links between *N*-acetylmuramic acid and *N*-acetylglucosamine.<sup>2</sup> Lysozyme is present in several human tissues and fluids with the highest concentration in cartilage, stomach and lower concentrations in kidney, liver, tonsil, brain, tears, saliva, milk, blood serum and spleen. Lysozyme is also present in secretions and in egg white from different animals, in some higher plants and microorganisms. Alderton *et al.*<sup>3</sup> isolated lysozyme in high yield and characterized it as a basic protein (isoelectric point between pH 10.5 and 11) with a low molecular weight. Detailed studies of the amino acid composition have been carried out.<sup>4-6</sup> These studies have established that egg white lysozyme is a single polypeptide chain of 131-133 amino acid residues with a molecular weight of 14 372-14 714.<sup>7</sup> The method generally used for determining the lysozyme activity is the bacteriolytic effect on suspensions of *Micrococcus lysodeikticus* or *Sarcina lutea*.<sup>3,8-12</sup> The optimum pH activity for bacteriolysis was found in the pH range 5.9-6.3.<sup>9</sup> Meyer and Hahnel<sup>13</sup> used a viscosimetric method to measure the depolymerisation of mucopolysaccharide extracts of susceptible bacterial cells by the action of lysozyme. Other substrates on which lysozyme has a lytic effect are chitin,<sup>14</sup> carboxymethylchitin,<sup>15</sup> and glycolchitin (hydroxyethylchitin).<sup>16-18</sup>

Fleming<sup>1</sup> first demonstrated the presence of lysozyme in leukocytes. Identification of particular populations of leukocytes containing lysozyme has

been the objective of several studies.<sup>19-20</sup> Osserman and Lawlor<sup>21</sup> found higher lysozyme activity in urine and serum from patients with monocytic leukemia.

Leukocytes are normally present in the oral cavity, saliva, in the dental plaque, the accumulation of microorganisms on the surfaces of the teeth, and in the crevicular fluid, the continuous outflow of fluid from the gingival pockets.<sup>22-26</sup> Egelberg<sup>24</sup> observed a greater number of leukocytes in the fluid from chronically inflamed gingivae than in fluid from clinically healthy gingival pockets.

Proteolytic activity also has been determined in leukocytes<sup>27</sup> as well as hyaluronidase activity.<sup>28</sup> These enzymes are thought to liberate cell substances which may stimulate the production of auto-antibodies which, in turn are conducive to a further discharge of lysosomal enzymes from bone causing an uncontrolled destruction of bone tissues.<sup>29</sup>

#### MATERIAL AND METHODS

*Leukocytes.* The mono- and polycytes were separated from about 4000 ml ACD-blood (O Rh-) by using a method devised by von Melen and Unger<sup>30</sup> and by Unger *et al.*<sup>31</sup> The leukocytes were resuspended in their own serum to 10.0 ml. The number of monocytes was 43 800 000 and polycytes 71 200 000 per ml. 1 ml of the leukocyte homogenate was diluted 1/15 for preliminary determination of enzyme activities. The leukocytes were stored at 4°C.

*Gel filtration.* Sephadex G-75 and G-100 superfine (Pharmacia, Uppsala, Sweden) were used in the gel filtration experiments and the gels were treated according to the instructions from the manufacturer. The elution medium consisted of 0.005 M Tris-(hydroxymethyl)aminomethane-HCl buffer at pH 8.1 in 0.5 M NaCl and 2 % butanol as bactericidal agent. The elutions were made at room temperature and the changes in extinctions were followed continuously using a UV absorptiometer (LKB, Stockholm, Sweden). The optical density of the fractions was measured at 260 and 280 nm in a Beckman spectrophotometer model DU. In order to compare the enzymes found in the leukocytes to proteins of known molecular weights after separations on columns of different sizes the  $K_{av}$ <sup>32</sup> was determined where  $K_{av} = (V_e - V_0)/(V_t - V_0)$ .  $V_e$  = the elution volume,  $V_0$  = the void volume,  $V_t$  = the total bed volume. Laurent and Killander<sup>32</sup> found a correlation between the  $K_{av}$  and the logarithm of the molecular sizes.

*Non-enzymatic protein molecular weight markers.* Bacitracin, cytochrome C (horse heart), myoglobin cryst. (sperm whale) salt free, chymotrypsinogen A (beef pancreas) 6 × cryst., salt free, ovalbumin (2 × cryst.), albumin (bovine) cryst., gamma globulins (human), apo-ferritin (horse), amorphous salt free (Mann research lab. inc. New York, N.Y., U.S.A.).

*Hen's egg lysozyme.* Lysozyme (muramidase) from egg white, Grade I 3 × crystallized, dialyzed and lyophilized. (Sigma Chem. St. Louis, Mo.). Blue dextran (Pharmacia) was used for determining the void volume.

*Measurement of the lysozyme activity.* The lysozyme activity was determined by the viscosimetric method of Lundblad and Hultin,<sup>18</sup> using a procedure similar to that of Nord *et al.*<sup>33</sup> Glycolchitin (hydroxyethylchitin) was used as substrate and was synthesized according to a method by Senju and Okimasu.<sup>34</sup>

Chitin (Calbiochem, Los Angeles, U.S.A.) was dissolved in 42 % NaOH solution at 0°C by stirring. This solution was diluted to 13 % and stored at 0°C for 48 h with stirring. At the end of the stirring period an equal amount of ethylene oxide (Hopkin and Williams, Great Britain) was added to the solution and the solution was kept at constant temperature (33°C) for 7 h. The solution was neutralized with 1 N HCl and filtered. The glycolchitin was then precipitated by ethanol and lyophilized. A 1.0 % solution of glycolchitin in 0.2 M sodium acetate buffer (pH 5.1), 0.3 % NaCl, was used as substrate stock solution.

1.0 ml of the supernatant from the leukocyte suspension was mixed with 3.0 ml substrate stock solution in Ostwald viscosimeters. The outflow times of the enzyme-

substrate mixtures were measured after different periods of incubation time in the viscosimeters at 37°C in a waterbath. The lysozyme activity was calculated from the formula of Hultin.<sup>35-37</sup>

*Measurement of the proteolytic activity.* The gelatinase activity was determined by the Hultin viscosimetric method<sup>35-37</sup> at 37°C. Gelatin (U.S.P. granular, Fischer, Sc. Co., N.J. U.S.A.) was used as a 4% stock solution in 0.1 M Tris-HCl, pH 8.1, and 0.1 M acetic acid-sodium acetate buffer pH 5.5.

*Measurement of the hyaluronidase activity.* The hyaluronidase activity also was determined using the viscosimetric method (Hultin<sup>35,36</sup>). Hyaluronic acid, sodium salt (Sigma Grade-11S from human umbilical cord) was used; 3.0 ml of a 0.2% stock solution and 1.0 ml of the leukocyte extract in 0.1 M imidazole-HCl, pH 6.5.

For determination of the pH dependence of activity and stability the following buffers were used: 0.4 M sodium acetate-acetic, pH 3.6-5.8, 0.4 M sodium arsenate-HCl pH 5.8-8.2.

### EXPERIMENTS AND RESULTS

*Proteolytic and hyaluronidase activity in crude extract of leukocytes.* The leukocyte extract was diluted 1/15 for the determination of proteolytic activity at pH 8.1 and 5.5. The activity was within the limits of error of this method. No hyaluronidase activity was detected in the diluted leukocyte extract at pH 6.5.

*The lysozyme activity in the crude leukocyte extract.* The leukocyte extract was diluted 1/100 and the lysozyme activity was determined at pH 5.1 1 ml

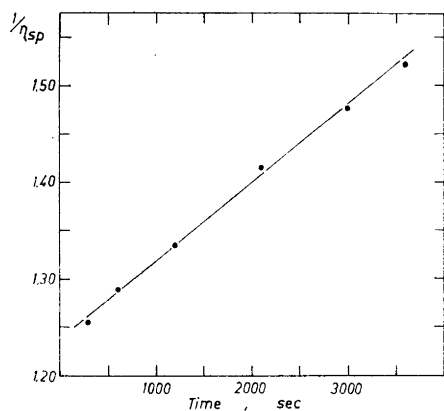


Fig. 1. The relationship between time of incubation and viscosity. The lysozyme activity was calculated from the slope of the line.

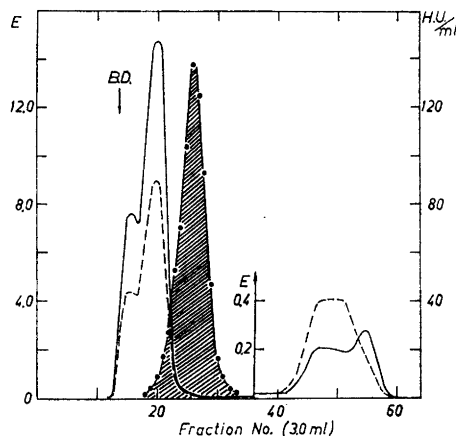
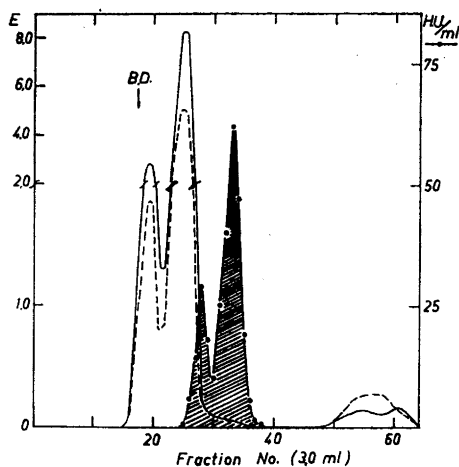


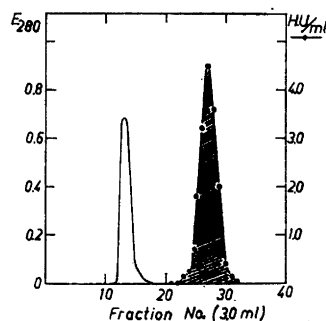
Fig. 2. Gel filtration of 2.75 ml of a leukocyte supernatant on a 2.0 × 51 cm Sephadex G-75 column. The leukocyte preparation was centrifuged at 20 000 *g* for 15 min. Eluent was 0.005 M Tris-HCl, 0.5 M NaCl, pH 8.1, with 2% butanol. The flow rate was 40 ml/h at room temperature. ● = lysozyme activity expressed in H.U./ml. The arrow indicates the effluent volume of blue dextran (BD) = *V*<sub>0</sub>. The scale changed for extinction (*E*) after 36 fractions. — Extinction at 280 nm, - - - Extinction at 260 nm.

of the leukocyte preparation was mixed with 3.0 ml of a 1.0 % substrate solution. As can be seen from Fig. 1 the change in the reciprocal of the specific viscosity was directly proportional to the time of reaction. The plot is used for determining the enzymatic activity. The slope of the line was  $0.82 \times 10^{-4} \text{sec}^{-1}$ . The lysozyme activity in this crude material was 1840 H.U./ml.

*Purification of lysozyme from human leukocytes.* The crude leukocyte suspension was centrifuged at 20 000 *g* at 4°C for 15 min and the supernatants were applied to a Sephadex G-75 column and a G-100 superfine column with 0.005 M Tris-HCl buffer, 0.5 M NaCl, and 2 % butanol, pH 8.1, as eluent (Figs. 2 and 3). The presence of lysozyme activity was tested at pH 5.1. After gel filtration on Sephadex G-75, lysozyme activity was found as one broad peak in fractions 20 to 30. However, the supernatant filtered through Sephadex G-100 superfine (Fig. 3) contained two well separated peaks of lysozyme activity, the first small lysozyme peak ( $L_1$ ) in fractions 25 to 30 and the second in fractions 30 to 38 ( $L_2$ ). Fig. 3 shows one of three similar separations. The maximum activity for  $L_1$  was found in fraction 28 ( $K_{av}=0.29$ ) with an activity of 30 H.U./ml and  $L_2$  in fraction 33 ( $K_{av}=0.40$ ) with an activity of 62 H.U./ml.  $L_1$  was still partly within the protein peak but  $L_2$



*Fig. 3.* Separation of the two lysozymes from human leukocytes. 1.0 ml of the supernatant after centrifugation at 20 000 *g* for 15 min was applied on a  $1.93 \times 55.6$  cm Sephadex G-100 superfine column. Eluent was 0.005 M Tris-HCl, 0.5 M NaCl, pH 8.1, with 2 % butanol. The flow rate was 11.5 ml/h. ● = lysozyme activity expressed in H.U./ml. — = Extinction at 280 nm, - - - Extinction at 260 nm. The arrow indicates the effluent volume of blue dextran (BD) =  $V_0 = 55.5$  ml,  $V_t = 168$  ml,  $V_c(L_1) = 101$  ml,  $V_c(L_2) = 87$  ml,  $K_{av}(L_1) = 0.29$ ,  $K_{av}(L_2) = 0.40$ .



*Fig. 4.* Reseparation experiment. Fractions 32, 33, and 34 from Fig. 3 were pooled, and 2.0 ml of the pool was applied on a  $1.93 \times 51.5$  cm column of Sephadex G-100 superfine. The eluent was 0.005 M Tris-HCl, 0.5 M NaCl, pH 8.1 with 2 % butanol. The flow rate was 7.5 ml/h. ● = lysozyme activity expressed in H.U./ml. — = extinction of blue dextran at 280 nm.  $V_0 = 39$  ml,  $V_t = 150$  ml,  $V_c = 83$  ml,  $K_{av}(L_2) = 0.40$ .

was between the high molecular weight protein peaks and the low molecular peak. The proteins were separated in three peaks, two of high molecular and one of low molecular weight. The recovery of the activity after gel filtration was about 80 %.

*Reseparation the lysozyme L<sub>2</sub>.* After separation on Sephadex G—100 superfine the fractions 32, 33, and 34 (Fig. 3) containing lysozyme L<sub>2</sub> were pooled and a 2 ml sample was fractionated again. The distribution of activity from this second fractionation is shown in Fig. 4. The  $K_{av}$  value of the lysozyme was the same in this experiment as in the first separation ( $K_{av}=0.40$ ), even though the elution volume ( $V_e$ ) and the size of the column had changed.

*Influence of pH on the lysozymes L<sub>1</sub> and L<sub>2</sub>.* The Sephadex G—100 superfine fractions containing the first lysozyme L<sub>1</sub> (25—27) and the fractions containing the second lysozyme L<sub>2</sub> (31—35) were pooled separately and the influence of pH on glycolchitin hydrolysis was investigated (Fig. 5). The optimum activity of both lysozymes was found between pH 4.0 and 4.2. The different buffers used in the experiments are given in Material and methods.

*Thermostability of the lysozymes L<sub>1</sub> and L<sub>2</sub>.* The fractions containing L<sub>1</sub> and L<sub>2</sub> were pooled and samples were adjusted to pH 4.3, pH 6.5 (L<sub>2</sub> only) and pH 7.5. Aliquots from these samples were heated for 30 min at 20°C, 37°C, 50°C, 60°C, 65°C, and 75°C (Fig. 6A-B). The lysozyme activity was measured at pH 4.3. When L<sub>1</sub> was kept at 37°C and pH 4.3 the activity increased about

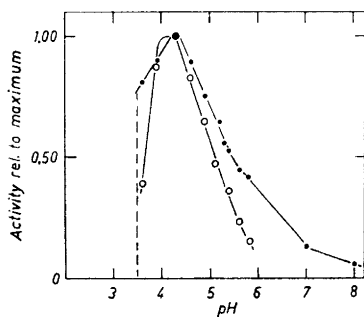


Fig. 5. The influence of pH on the hydrolysis of glycolchitin by lysozyme L<sub>1</sub> from human leukocytes. The fractions 25—27 (Fig. 3) were used and the different pH's were obtained with buffers mentioned in Material and methods. L<sub>1</sub>=○. The fractions 31—35 containing lysozyme L<sub>2</sub> from a similar experiment were pooled and the influence of pH was determined. L<sub>2</sub>=●.

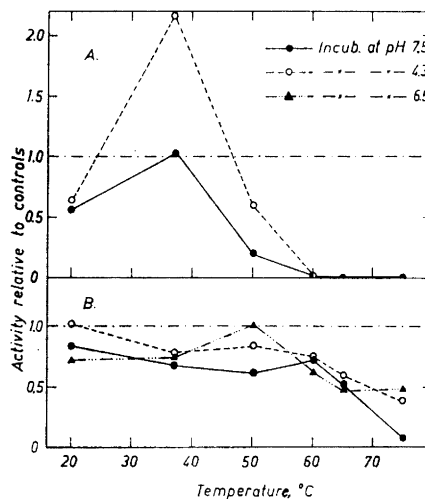


Fig. 6 A—B. Activity of lysozyme L<sub>1</sub> and L<sub>2</sub> separated by gel filtration on Sephadex G—100 superfine. The enzymes were kept at different temperatures in buffered solutions for 30 min. A: L<sub>1</sub> at pH 4.3 and 7.5. B: L<sub>2</sub> at pH 4.3, 6.5 and 7.5. The enzyme activity was measured at pH 4.3. — · · · — Activity before incubation. For data see Fig. 3.

100 % but reached the initial value at pH 7.5. All activity was lost at 60°C. (Fig. 6A). The activity of L<sub>2</sub> decreased at pH 6.5 and pH 7.5 about 20 % of the initial. At pH 4.3 there was no change in activity. At 37°C the activity decreased about 20 % for all the pH's used, and at 50°C the activity at pH 6.5 again reached the initial value. At 75°C there was still about 50 % of the activity left at pH 4.3 and 6.5 but none at pH 7.5 (Fig. 6B).

*The effect of various substances on the lysozyme activity.* The influence of different substances on the two lysozyme fractions showed that at concentrations of 10<sup>-3</sup> and 10<sup>-2</sup> M, KI, Na<sub>2</sub>SO<sub>3</sub>, and sodium citrate had no effect on the enzyme activity. However, there was a 100 % increase in activity of L<sub>1</sub> upon the addition of 10<sup>-3</sup> and 10<sup>-2</sup> M cysteine-HCl and a 75 % increase in activity when 10<sup>-3</sup> or 10<sup>-2</sup> M glutathione was added. L<sub>2</sub> was not influenced by 10<sup>-3</sup> and 10<sup>-2</sup> M cysteine-HCl and only a 20 % increase in activity was found upon addition of 10<sup>-3</sup> and 10<sup>-2</sup> M glutathione.

*A comparison of the lysozymes to known non-enzymatic protein molecular weight markers and hen's egg white lysozyme.* Table 1 shows the K<sub>av</sub> values

*Table 1.* The values of molecular weight (M) and K<sub>av</sub> of non enzymatic protein molecular weight markers, hen's egg white lysozyme, and the lysozymes L<sub>1</sub> and L<sub>2</sub> found in human leukocytes. The K<sub>av</sub> value for the HEW lysozyme is too low because of the retardation of the enzyme in the gel. The retardation factor is 1.65; corrected with this factor the molecular weights of L<sub>1</sub> and L<sub>2</sub> are 59 000 and 38 000, respectively.

Protein	Molecular weight (M)	log M	K <sub>av</sub>
Apo-ferritin	480 000	5.681	0
γ-Globulins	160 000	5.204	0.032
Albumin	67 000	4.826	0.134
Ovalbumin	45 000	4.653	0.234
Chymotrypsinogen A	25 000	4.398	0.383
Myoglobin	17 800	4.250	0.475
Cytochrome C	12 400	4.093	0.555
Bacitracin	1 450	3.160	0.963
Hen's egg white Lysozyme (HEW)	15 000	4.176	0.64
Lysozyme L <sub>2</sub>	38 000 corr.	4.36	0.40
Lysozyme L <sub>1</sub>	59 000 corr.	4.56	0.29

of the lysozymes from human leukocytes, non-enzymatic protein molecular weight markers, and hen's egg white lysozyme (HEW) after separation on Sephadex G-100 superfine. 1 ml of each protein (1 mg/ml) was applied to the column in order of decreasing molecular weight with 3 fractions difference between each protein. Hen's egg white lysozyme was measured first by the amount of protein applied to the column by adsorbance at 280 nm and second by the splitting effect on glycolchitin after the gel filtration. As can be seen from the table the hen's egg white lysozyme had a K<sub>av</sub> of 0.64 and was clearly separated from the lysozymes from human leukocytes. The K<sub>av</sub> of lysozymes L<sub>1</sub> and L<sub>2</sub> was 0.29 and 0.40, respectively.

## DISCUSSION

In contrast to Goggins *et al.*<sup>28</sup> we did not find any hyaluronidase activity in the leukocyte extract we used. The proteolytic activity was small and could not be responsible for the proteolytic activity in the dental plaque material<sup>38</sup> as  $10^8$  leukocytes/mg dry weight would be needed to reach the activity of dental plaque material. The lysozyme activity found in human serum<sup>18</sup> could possibly originate from the leukocytes.<sup>39</sup> Lundblad and Hultin,<sup>18</sup> however, found a higher pH optimum of pH 5.0–5.1; the same optimum was found for the lysozyme activity in dental plaque.<sup>33</sup> Our findings are in agreement with those of Hayashi, Hamaguchi and Funatsu<sup>40</sup> who found a pH optimum of 4.0–4.5 for hen's egg white lysozyme with the same kind of substrate. Wardlaw,<sup>41</sup> however, found a pH optimum of 8.3–8.5 and Jollés and Jollés<sup>42</sup> found maximum activity at pH 6.8 for lysozyme from human leukocytes. They used *M. lysodeikticus* as substrate. They also estimated the molecular weight to be about 14 000. Our experiments, however, indicated that two lysozymes were present. The first had a  $K_{av}=0.29$  and the second with  $K_{av}=0.40$ . The  $K_{av}$  for the hen's egg white lysozyme used was 0.64. These enzymes could be easily separated from each other in a Sephadex G-100 superfine column. The enzymes possibly could be di- or tri-mers or the enzymes could be conjugated to other proteins. The elution volume ( $V_e$ ) possibly was too high since the lysozyme as a basic protein would be retarded in the column.<sup>43</sup> This fact is clearly visible with the hen's egg white lysozyme used in the experiments (Table 1).

The two enzymes,  $L_1$  and  $L_2$ , possibly were not of the same origin since  $L_1$  was strongly activated by cysteine-HCl and glutathione in agreement with Azari,<sup>44</sup> but  $L_2$  was activated only by glutathione.  $L_2$  was more thermostable than  $L_1$  over a pH range of 4.3–7.5. The thermolability of  $L_1$  possibly could be due to the presence of other proteins in the fraction which might be coagulated at lower temperature and the apparent difference in thermostability between the two enzymes may be an artefact. The question of whether the enzyme is elaborated exclusively by a specific group of cells responsible for the activity is still not clearly resolved. This failure may be explained by the difficulties in obtaining pure cell populations for the study and the fact that the precise interrelationship between monocytes and polymorphs is not clearly defined. Briggs *et al.*<sup>20</sup> found lysozyme activity in monocytes, polymorphs, and polymorph precursors but no activity in lymphocytes, plasma cells, basophils, mast cells, or marrow reticuloendothelial cells.

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