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PURIFICATION OF HUMAN GRANULOCYTE CATALASE IN CHRONIC MYELOID LEUKEMIA

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Summary

Human granulocyte catalase (hydrogen peroxide:hydrogen peroxide oxidoreductase, EC 1.11.1.6) was purified from chronic myeloid leukemia cells. The purification procedure included heat precipitation, ammonium sulphate fractionation, DEAE-Sephadex chromatography, gel chromatography on Sephadex G-200 and isoelectric focusing with an approximate yield of 30% and a 1000-fold purification. The molecular weight of the subunit obtained by sodium dodecyl sulphate electrophoresis was 65 800. $S^{\circ}_{20,w}$ was 11.6 ± 0.24. The pH-optimum was 6.6—6.7 and the spectrum showed a major peak at 405 nm and shoulders at 500, 540 and 625 nm typical for catalase. The electrophoretic mobility was towards the anode at pH 8.6 and identical to normal granulocyte and erythrocyte catalase. These three species of catalase gave the reaction of identity on immunodiffusion and crossed immunoelectrophoresis. The content of catalase and its activity of isolated granulocytes were approximately identical in normal and chronic myeloid leukemia granulocytes while the specific activity of leukemic catalase was higher than normal. No difference in catalase content was found between mature and immature leukemic granulocytes.

Introduction

During phagocytosis in granulocytes the oxygen consumption is increased several-fold [1]. The oxygen is almost quantitatively converted to superoxide anions and hydrogen peroxide due to the actions of oxidase and superoxide dismutase, respectively [2—4]. A major function of granulocyte catalase (hydrogen peroxide:hydrogen peroxide oxidoreductase, EC 1.11.1.6) is

Abbreviations: SDS, sodium dodecyl sulfate. CML, chronic myeloid leukemia.

probably to protect cytoplasmic structures from the noxious action of hydrogen peroxide. Granulocytes from several cases of chronic myeloid leukemia displayed a low intracellular ionination capacity during phagocytosis in combination with an abnormally high extracellular iodination [5], which could be an effect of defective catalase function. Nishimura et al. [6] have reported on an abnormal granulocyte catalase in chronic myeloid leukemia. We therefore attempted to isolate catalase from leukemic granulocytes in order to characterize enzymatic and physico-chemical properties.

Materials and Methods

Isolation of leukocytes. Leukocytes were obtained from the peripheral blood of a patient with chronic myeloid leukemia by the technique of repeated leukapheresis [7]. The yield was approximatley $4 \cdot 10^{11}$ leukocytes from 400 ml of blood. The erythrocyte to leukocyte ratio was 1 : 1000.

Purification of granulocyte catalase. The purification procedure is outlined in Table I.

Electrophoretic methods. Polyacrylamide gel electrophoresis was performed according to the Neville [8] in a discontinuous buffer system (pH 8.47 in lower gel) omitting the SDS and reducing agents. In some instances the electrophoresis was run in β-alanine buffer pH 4.5 as described by Reisfeld [9]. The complete Neville system with SDS and reducing agents (pH 8.47 in lower gel) was utilized for molecular weight determinations.

Protein determinations. The Folin method as described by Lowry et al. was used [10].

Analytical ultracentrifugation. Sedimentation analysis for determination of $S^{\circ}_{20,w}$ was done in a Spinco model E ultracentrifuge. A partial specific volume ($\bar{v}$) of 0.749 cm^{-3}/g was used.

Preparation of antiserum. Rabbits were immunized subcutaneously with 0.5 mg purified catalase in complete Freund's adjuvant. Booster injections of 0.5 mg catalase in incomplete Freund's adjuvant were given after 4 weeks and the rabbits were bled two weeks later.

Immunological methods. Double immuno-diffusion [11], and single radial immuno-diffusion [12], and crossed immunoelectrophoresis [13] were utilized.

Extraction of granulocyte catalase from individual cell specimens. The leukocyte-rich supernatant from dextran-sedimented blood was layered over a Hypaque-Ficoll solution, density 1.077 g/ml [14], and the tubes were centrifuged at $70 \times g$ for 10 min and $700 \times g$ for 15 min. Mature granulocytes were collected from the bottom while immature leukemic granulocytes were obtained from the interphase between plasma and Hypaque-Ficoll. Contaminating red cells were lysed in 0.87% ammonium chloride for 5 min. Leukocytes were washed twice in Krebs-Ringer phosphate buffer pH 7.4 and stored at -20°C in pellets before extraction by repeated freezing and thawing in 0.05 M phosphate buffer pH 7.4. Cell extracts were centrifuged at $1000 \times g$ for 10 min and the supernatants stored frozen at -20°C before analysis.

Catalase activity. Catalase was measured with a Clark electrode (Eschweiler and Co., Kiel, Germany, model A4) at 37°C according to the procedure of Goldstein [15]. Hydrogen peroxide (approximately 30%) was diluted 1:800 in

TABLE I

OUTLINE OF THE PURIFICATION PROCEDURE

Catalase activity was determined with a Clark electrode as described in Material and Methods.

Procedure and material		Protein	Total activity (mmol O_2/min)	Specific activity (μmol O_2/min per mg)	Yield (%)
Homogenized leukocytes					
300 × g nuclei					
8000 × g granules	8000 × g Supernatant (I)	82 g	420	5.13	100
heat precipitation 55°C 10 min					
precipitate	Supernatant (II)	52 g	360	6.92	86
salting out $(NH_4)_2SO_4$ 55% saturation					
supernatant	Precipitate (III)	9.2 g	245	26.6	58
dialysis					
precipitate	Supernatant	2.6 g	232	89.2	55
DEAE-Sephadex					
	Pooled fractions (IV)	150 mg	196	1307	47
Sephadex G-200					
	Pooled fractions (V)	100 mg	147	1470	35
isoelectric focusing					
	Highest purity fractions pooled (VI)	25 mg	134	5360	32

0.05 M phosphate buffer pH 7.4 [19]. The incubation chamber (2.24 ml) was filled with hydrogen peroxide-phosphate buffer, and then 10—20 μl of the cell extracts or column fractions were introduced. The formation of oxygen was linear with time for 30—60 s.

Erythrocyte catalase. Catalase from normal human erythrocytes was partially purified according to the method described by Aebi et al. [17] and used in electrophoretic and immunochemical analysis for comparison with granulocyte catalase.

Results

Catalase purification

An average yield of 30% was achieved for the overall isolation procedure

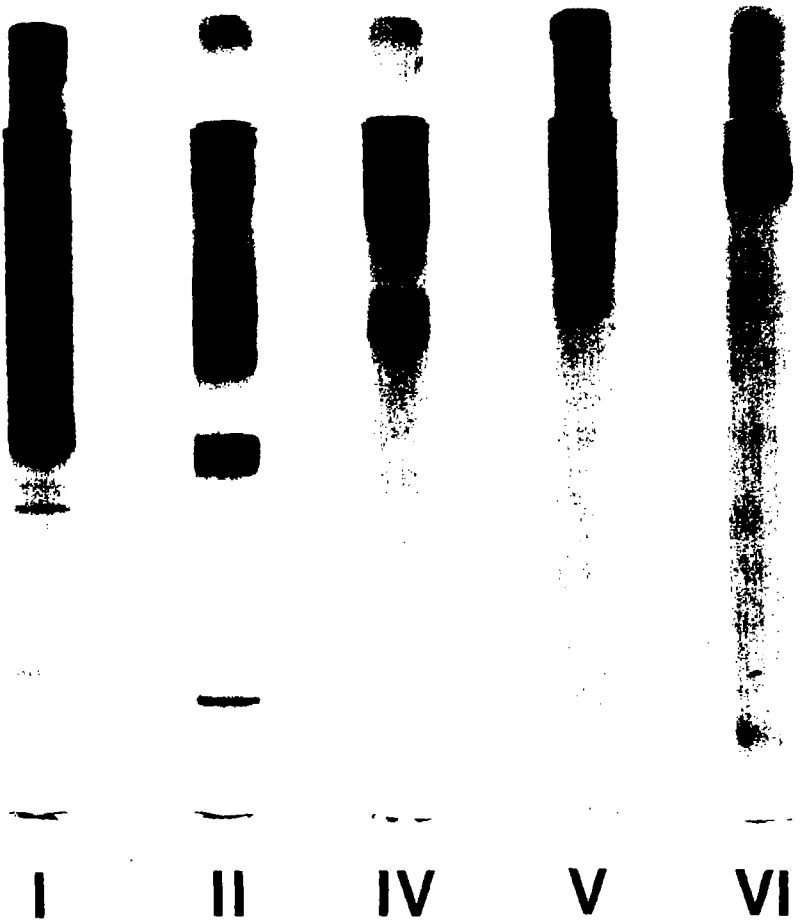

Fig. 1. Polyacrylamide gel electrophoresis patterns at pH 4.5 of the material obtained at the different purification steps. The roman numerals apply to the numerals given in Table I. I, 8000 × *g* supernatant; II, supernatant after heat precipitation; IV, catalase peak of ion exchange chromatography; V, catalase peak of gel chromatography; VI, catalase peak of isoelectric focusing. Anode at top.

(Table I and Fig. 1). The purification was more than 1000-fold.

The catalase was eluted from a DEAE-Sephadex column with 0.09 M Tris in 0.1 M NaCl as a single peak appearing as a brownish green band on the column (Fig. 2).

In the next purification step, the catalase was eluted as a single peak from a Sephadex G-200 column (Fig. 3). The calculated K_{av} was 0.23.

On isoelectric focusing, the catalase peak is eluted at pH 6.5—6.9 (Fig. 4). The ratio between absorbance at 405 nm and 280 nm is given as a measurement of purity [17]. In several cases, an R_z above 1.0 was achieved in fractions with the highest enzyme activity.

Characterization of granulocyte catalase

A molecular weight of 65 800 (mean of 21 determinations) was calculated

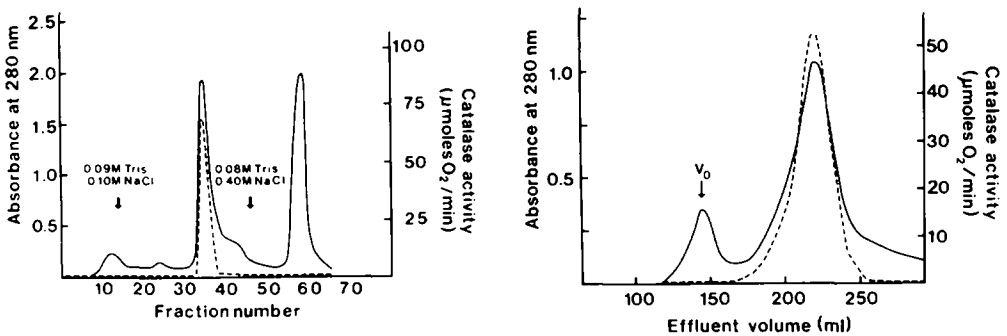

Fig. 2. Ion exchange chromatography on DEAE-Sephadex at pH 8.0. The column was eluted by stepwise increasing the buffer salt concentration as indicated in the figure. The protein concentration was measured at 280 nm (unbroken line) and catalase activity measured by the Clark electrode after proper dilution (broken line).

Fig. 3. Gel chromatography on Sephadex G-200 eluted by 0.05 M Tris, 0.05 M sodium acetate in 0.10 M NaCl pH 7.4. Flow rate 14 ml/h. Protein was measured at 280 nm (unbroken line) and catalase activity by the Clark electrode after proper dilution (broken line).

from SDS electrophoresis. Analytical ultracentrifugation showed a single symmetrical peak with $S^{\circ}_{20,w} = 11.06 \pm 0.24$ (2 × S.D.).

Crossed immune electrophoresis in 0.05 M barbital buffer pH 8.6 showed normal and leukemic catalse to have identical electrophoretic mobility towards the anode. Granulocyte catalase had the same electrophoretic mobility as erythrocyte catalase. Normal and leukemic granulocyte catalase, as well as erythrocyte catalase, showed complete immunological identity (Fig. 5). Small pieces of the agarose gel were cut out parallel to the strip of agarose that was used for the crossed immune electrophoresis for assay of the catalase activity after elution in 0.05 M phosphate buffer pH 7.0. All catalase activity from both leukemic and normal granulocyte extracts is confined to the region occupied by catalase-anti-catalase precipitate.

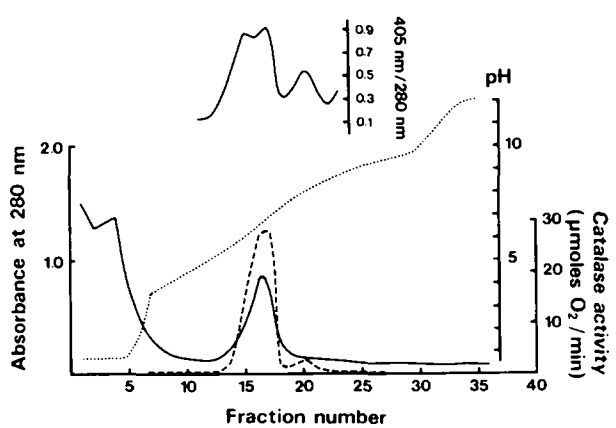

Fig. 4. Isoelectric focusing of catalase in a pH gradient 3.5—10.0 after a 48-h run. Anode solution 0.4 M H_2SO_4, cathode solution 0.3 M ethanolamine. The unbroken line shows the absorbance at 280 nm, the dotted line the pH gradient, and the broken line catalase activity measured by the Clark electrode. In the upper part of the figure, the absorbence quotient 405 nm/280 nm is given as a measure of purity (R_Z).

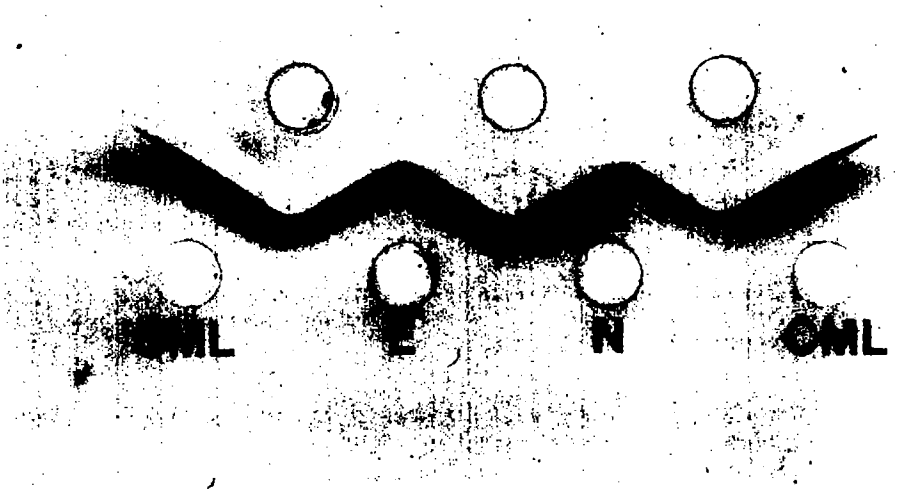

Fig. 5. Double immuno-diffusion in agarose gel showing complete immunochemical identity between normal (N) and leukemic (CML) granulocyte catalase and erythrocyte catalase (E). Antiserum against granulocyte catalase was applied in the three upper wells.

The spectrum of leukemic catalase showed, besides the protein peak at 280 nm, several additional optima, a large peak at 405 nm, a minor peak at 625 nm and shoulders at 500 and 540 nm. This is in accordance with earlier reports on other catalase species [18].

The pH optimum of granulocyte catalase was at pH 6.6—6.7.

TABLE II

CATALASE CONTENT AND ACTIVITY IN NORMAL AND CML GRANULOCYTES

Isolated granulocytes were extracted in 0.05 M phosphate buffer, pH 7.4 by repeated freezing and thawing and disruption through a thin needle. Catalase was quantified by single radial immunodiffusion and enzyme activity measured by a Clark electrode as the evolution of oxygen from hydrogen peroxide containing 0.05 M phosphate buffer pH 7.4. N.S., not significant. Immature CML granulocytes consisted mainly of myelocytes and promyelocytes (60—70%) and a few per cent blasts; the rest of the cells were mainly lymphocytes.

	$\mu g/10^7$ cells	$\mu mol\ O_2/10^7$ cells per min	$\mu mol\ O_2/\mu g$ per min
CML granulocytes (n = 26)	9.76 ± 3.57	14.62 ± 5.42	1.52 ± 0.30
CML immature (n = 6)	11.07 ± 5.34	16.31 ± 8.20	1.54 ± 0.36
Normal (n = 10)	13.16 ± 7.28	13.19 ± 5.74	1.10 ± 0.35
	N.S.	N.S.	$p < 0.01$

Table II shows that there are no significant differences in enzyme content between normal and leukemic granulocytes. The specific activity, however, was somewhat higher for leukemic than for normal catalase. No difference was found for catalase content and activity between mature and immature chronic myeloid leukemia granulocytes. Enzyme activity was completely inhibited by 30 mM aminotriazole.

Discussion

The catalase purified from chronic myeloid leukemia leukocytes showed only one band on SDS electrophoresis with a molecular weight of 65 800. The native catalase molecule consists of 4 subunits of equal molecular weight [19]. The molecular weight of the native tetrameric granulocyte catalase would be 263 000, which is somewhat higher than the molecular weight of erythrocyte and bovine liver catalase (240 000) when analyzed in identical systems.

The leukemic catalase showed immunologic identity with normal erythrocyte and granulocyte catalase. Crossed immune-electrophoresis at pH 8.6 showed identical mobility towards the anode for leukemic and normal granulocyte catalase as well as for erythrocyte catalase. Several leukemic extracts all showed the catalase to have an anodal mobility at pH 8.6. Furthermore, both normal and leukemic catalase showed the same distribution on isoelectric focusing with a peak at pH 6.5—6.9 indicating that the electrophoretic mobility at pH 8.6 should be anodal. The finding of identical properties for normal and chronic myeloid leukemia catalase is in disagreement with the report by Nishimura et al. [6]. These authors used antiserum against human liver catalase, which cross-reacted with granulocyte catalase, and found a cathodal mobility for normal catalase and an anodal mobility for leukemic catalase at pH 8.6. Normal as well as acatalasemic leukocyte catalases have strong anodal mobility at pH 8.4 [20]. These considerations question the earlier reported differences between normal and chronic myeloid leukemia catalase.

Chronic myeloid leukemia have been reported to show increased catalase activity [21]. In the present report, 15 cases on 26 occasions did not show either catalase content or enzyme activity of isolated granulocytes different from normal granulocytes. Leukemic catalase had, however, a slightly higher specific activity than normal. No differences were found between mature and immature chronic myeloid granulocytes in terms of catalase content, activity or electrophoretic mobility.

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