

SOLUBILISATION AND PARTIAL PURIFICATION OF GUINEA PIG NEUTROPHIL NADPH OXIDASE

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INTRODUCTION

Phagocytosing neutrophils undergo a respiratory burst resulting in the production of oxygen centred radicals. These are the superoxide and hydroxyl radicals and hydrogen peroxide. Another important event is stimulation of the hexose monophosphate shunt.¹ The production of oxygen centred radicals is preceded by the activation of a membrane bound NADPH oxidase which is inactive in the resting state.² The enzyme catalyses the reaction:- $\text{NADPH} + 2\text{O}_2 \xrightarrow{2} \text{NADP}^+ + \text{O}_2^- + \text{H}^+$ (Ref.3) and is thought to be a complex consisting of a flavoprotein,⁴ a b-type cytochrome⁵ and possibly calmodulin.⁶

The purification and characterisation of the neutrophil NADPH oxidase has been the subject of numerous investigations. However, very little progress has been achieved. All the preparations described have resulted in the production of an extremely unstable enzyme complex with low yields.

MATERIALS AND METHODS

Materials. Cytochrome type VI, NADPH type III and phorbol-12-myristate-13 acetate (PMA) were obtained from Sigma Chemical Co. (St. Louis, Mo.,USA). Cyano[⁵⁷Co] cobalamin was purchased from The Radiochemical Centre, Amersham, England. Ultrogel Aca22 was obtained from LKB Products, Bromma, Sweden. Human copper/zinc superoxide dismutase was prepared from outdated blood. All the reagents were the highest grade available.

Assays. Assays were carried out using a Perkin Elmer 576 double beam spectrophotometer at 22°C. NADPH oxidase activity was quantitated as superoxide production by measuring the superoxide dismutase inhibition of the reduction of cytochrome c. Myeloperoxidase was measured by following the oxidation of guaiacol in the presence of H₂O₂, β-glucuronidase by monitoring phenolphthalein released from phenolphthalein B-glucuronide and 5'-nucleotidase by filtering adenosine released from AMP. Unsaturated vitamin B₁₂-binding capacity was determined by radioassay. Protein concentration was measured after trichloroacetic acid precipitation.

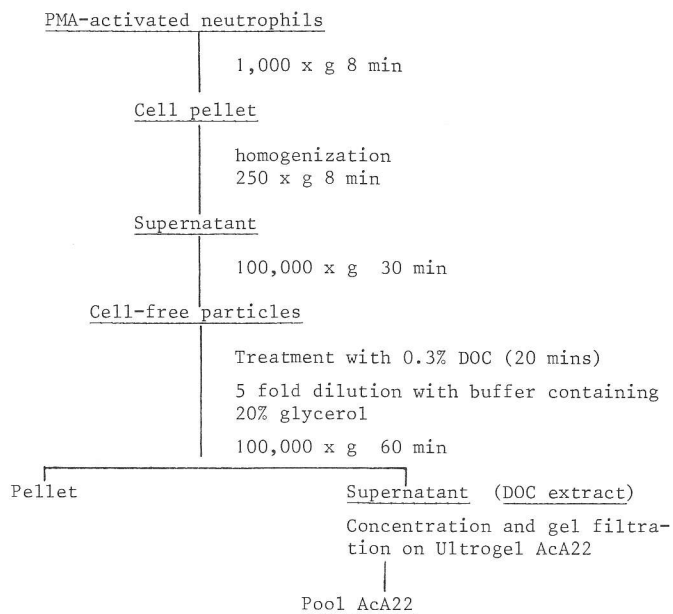
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RESULTS AND DISCUSSION

Neutrophils were raised in guinea pigs injected intraperitoneally with 1% sodium caseinate dissolved in 0.9% saline and collected by centrifugation at 100g of the peritoneal lavage. Precipitated cells were suspended in Krebs Ringer phosphate buffer pH 7.4 containing 5mM glucose and 0.5mM CaCl_2 and activated by incubation with PMA for 90 seconds at 37°C.

SCHEME 1

EXTRACTION AND FRACTIONATION OF NEUTROPHIL SUPEROXIDE-GENERATING ACTIVITY



NADPH oxidase was isolated from activated neutrophils as detailed in Scheme 1. Cells were homogenized in 10mM Tris-HCl pH 7.0 containing 6.34M sucrose and 2mM phenyl methyl sulfonylfluoride and centrifuged for 8 mins at 250g to remove nuclei and cells. The supernatant obtained was centrifuged at 100,000g for 30 mins. The resulting pellet was suspended in 5mM phosphate buffer, pH 8.0 and the NADPH oxidase was extracted with sodium dioxycholate (DOC). Optimal extraction was at 0.3% detergent concentration. The activity of the extracted enzyme was decreased by 90% after 18 hours. The activity could, however, be stabilised when the DOC concentration was brought to 0.05%

by dilution with buffer and by the addition of 20% glycerol. At -70°C the enzyme was found to be stable indefinitely.

Chromatography on Ultrogel AcA22 was carried out on the deoxycholate extract following concentration by ultrafiltration using an Amicon XM-300 membrane. NADPH oxidase activity was found to coincide with the main protein peak which eluted in the void volume. Pooled fractions contained 38% of the proteins loaded in the column and 31.4% of the NADPH oxidase activity.

TABLE 1
PROTEINS, ENZYMATIC ACTIVITIES, UNSATURATED B_{12} -BINDING CAPACITY (UBBC) AND CYTOCHROME B CONTENT OF CELL-FREE PARTICLES AND OF DEOXYCHOLATE EXTRACT FROM PMA ACTIVATED GUINEA PIG NEUTROPHILS

	Cell-free particles	Deoxycholate extract		Pool AcA22	
	Total ^a	Total ^a	Recovery %	Total ^a	Recovery %
Protein (mg)	21.3	6.9	32.3	2.3	38
NADPH oxidase ^b	4149	1193	28.7	292.6	31.4
Myeloperoxidase ^c	64.1	4.9	7.6	0.27	7.9
β -glucuronidase ^d	32.4	22.7	70.1	0.25	1.1
5'-nucleotidase ^e	2268	573	25.2	324.0	58.7
UBBC ($\mu\text{g B}_{12}$)	1034	968	93.6	8.6	2.0
Cyt.b (pmoles)	2718	1027	37.8	566.0	92.7

a = Values are given for proteins or activity obtained from 10^9 PMNL.

b = nmoles O_2^-/min .

c = μ moles tetraguaiacol/min.

d = μ moles phenolphthalein/4 hours.

e = μ moles adenosine/min.

Table 1 reports the protein concentration, NADPH oxidase activity, b-type cytochrome concentration and the activity of the enzyme markers in the cell free particles, deoxycholate extract and the pool from the Ultrogel AcA22 column. The activity of the markers was minimal after the gel filtration set. The specific activity of the NADPH oxidase did not increase after chromatography but that of the b-type cytochrome almost doubled. The low recovery of NADPH oxidase is probably due to the loss of activity during the time necessary for the chromatographic procedure. This is shown by the fact that the specific activity decreases from 153.9 to 64.4 nmoles superoxide/min/mg protein when

maintained for 30 hours at 4°C. If the activity of the NADPH oxidase eluted from the column (31.4%) is corrected for the spontaneous loss in activity, then the recovery amounts to 75%. When this factor is taken into account the ratio of NADPH oxidase to cytochrome b concentration remains virtually unchanged indicating co-purification.

Gradient polyacrylamide gel electrophoresis of the cell free particles, deoxycholate extract and pool of Ultrogel AcA22 is shown in Figure 1. The multitude of bands decreases with each step. Interestingly, however, an enrichment of a band around 35,000 dalton is seen with purification. The significance of this result is as yet not clear.

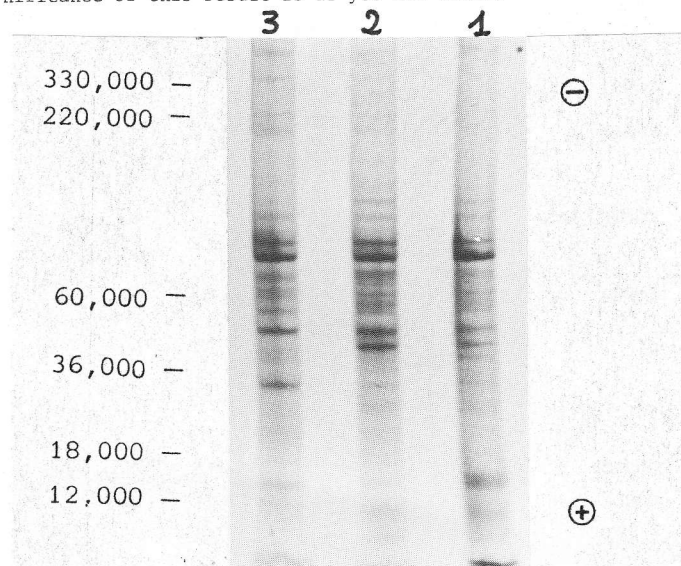


Fig. 1. Gradient gel electrophoresis (4-30%) of (1) cell-free particle, (2) deoxycholate extract and (3) pool of Ultrogel AcA22 column. All samples were incubated overnight in 1% SDS at room temperature prior to electrophoresis.

The results obtained describe a procedure for the extraction of a very active and stable NADPH oxidase activity from stimulated guinea pig neutrophils. Gel filtration indicated a high molecular weight complex. Whether this complex forms as a result of the extraction procedure or whether this is the true physiological state of the enzyme is not clear and remains to be elucidated.

The purified complex exhibited a higher affinity for NADPH ($K_m = 15.8\mu\text{M}$) than for NADPH ($K_m = 434\mu\text{M}$) and a neutral pH optimum. This is in agreement with the characteristics for the particle associated enzyme.

Isolated enzyme in the presence of NADPH produces solely superoxide radicals. This was observed using epr spin trapping techniques. The characteristic of the signal observed in Figure 2 (top) in the presence of the spin trap, 5,5-dimethyl-1-pyrroline-N-oxide (DMPO) are identical with those previously reported for the superoxide spin adduct DMPO, 5,5-dimethyl-2-hydroperoxypyrrolidino-1-oxyl (DMPO-OOH). This leaves open the question of the production of hydroxyl radicals. Various workers have postulated a Haber-Weiss reaction.⁷⁻⁹ After the addition of transferrin (a non-haem iron protein) and hydrogen peroxide to the enzyme and NADPH, a second spin adduct appears (Figure 2, bottom). This was found to have the same characteristics as those of the hydroxyl spin adduct of DMPO, 5,5-dimethyl-2-hydroxypyrrolidino-1-oxyl (DMPO-OH).

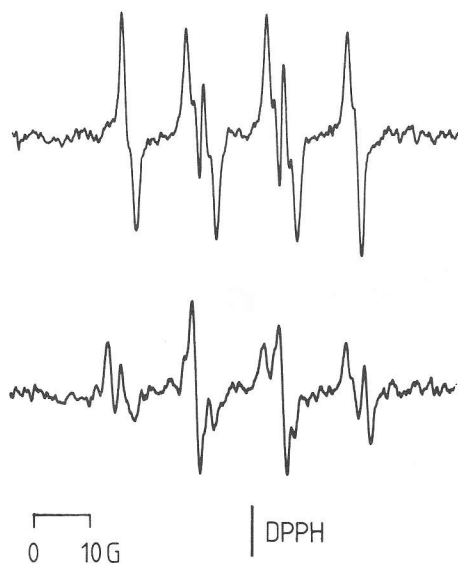


Fig. 2. Spin trapping of superoxide production (top spectrum) by NADPH oxidase in the presence of NADPH and DMPO and of superoxide and hydroxyl radicals (bottom spectrum) by a mixture of NADPH/NADPH oxidase/transferrin/hydrogen peroxide and DMPO.

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