

## Crystalline Myeloperoxidase

KJELL AGNER

*Department of Clinical Chemistry, Serafimerlasarettet, Stockholm, Sweden\**

A modified method for the preparation of myeloperoxidase, MPO, from pus of infected dog uteri is described. The most significant improvement is the use of a chromatographic procedure where the MPO is adsorbed on kieselguhr and later eluted from it by ammoniacal ammonium formate solution.

The myeloperoxidase has been obtained as a crystalline preparation with an iron content of 0.074 %. The enzyme behaved as would be expected for a single component in chemical and physical tests. Two atoms of porphyrin bound iron per enzyme molecule are indicated by chemical splitting of the enzyme.

The isolation of myeloperoxidase, MPO, in quantities, that will suffice for studies of the structure and properties of the enzyme, requires a starting material enriched in leucocytes or methods and equipments for separation of leucocytes from large quantities of blood.

The peroxidase preparation, described 1941<sup>1</sup>, was isolated from empyema fluid from tuberculous patients. This starting material has become progressively more scarce because of general medical progress. The separation of large quantities of leucocytes from blood presents certain difficulties. It has therefore been found necessary to search for another suitable starting material.

Infected dog uteri have proved appropriate. These uteri have been obtained as surgical specimens from the dog clinic at the Veterinary Institute. They have contained hemorrhagic or sometimes green coloured pus in varying amounts up to 1.5 liter. It has been possible to use the earlier published method of preparation<sup>1</sup> as a fractionation procedure for the purification of the MPO from this initial material. However, certain modifications have proved appropriate. The most significant of these is the adsorption of the MPO to kieselguhr and eluting the enzyme by ammoniacal ammonium formate solution.

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\* Present address: Department of Clinical Chemistry, S:t Görans sjukhus, Stockholm, Sweden.

## PREPARATION PROCEDURE

Immediately after the operations the uteri have been taken to the laboratory and opened.

After step 3 in the preparation procedure given below the preparations usually have become purified to such an extent that it has been possible to determine the concentration of the MPO, the degree of purity, and the yield, by measurements of the absorbancy values at different wavelengths. At 430  $m\mu$  1.0 mg of the crystalline MPO per ml has an absorbancy value of 1.2. The values for the ratios

$$\frac{\text{absorbancy}_{430 \text{ m}\mu}}{\text{absorbancy}_{280 \text{ m}\mu}} \quad \text{and} \quad \frac{\text{absorbancy}_{430 \text{ m}\mu}}{\text{absorbancy}_{390 \text{ m}\mu}}$$

has been the guidance for an approximate evaluation of the degree of purity of the preparations. The values of these ratios for the crystalline peroxidase are 0.82 and 2.0, respectively. Lower ratio values imply an admixture of impurities — proteins, heme components, etc. The figures for the amounts of MPO given for each step in the preparative procedure relate to the over-all yield from 100 ml starting material. The values are means of determinations from seven separate preparations. The variations between these values for different preparations lie within  $\pm 35\%$ .

*Step 1. Addition of ammoniacal ammonium sulphate solution.* To every 100 ml of pus 10 ml of ammoniacal ammonium sulphate solution (800 ml of saturated ammonium sulphate solution + 100 ml ammonia) were added under stirring. At first there was a considerable gel-formation, which however, gradually disaggregated. The preparation was left at room temperature under stirring for 15–18 h.

*Step 2. Precipitation with barium acetate.* About 15 ml of saturated barium acetate solution were added for every 10 ml of the ammoniacal ammonium sulphate solution in the preceding step. The exact amount had to be checked for every preparation in order to avoid an excess of barium. After centrifugation the precipitate was discarded.

*Step 3. Precipitation with ethyl alcohol at +5°C.* The precipitate obtained at +5°C after addition of ethyl alcohol up to a concentration of 40 vol. % was discarded. The peroxidase was thereafter precipitated at about 65 vol. % ethyl alcohol. After centrifugation the precipitate was dissolved in saline.

Amount: 75 mg MPO per 100 ml of pus. Ratio:  $A_{430}/A_{280} = ca. 0.1$ .

*Step 4. Precipitation of impurities with cadmium.* Cadmium chloride solution (prepared from anhydrous powder) was added up to a concentration of about 0.02 M. The amount, which gave the optimum purification, had to be checked for every preparation. The precipitate formed was discarded after centrifugation.

Amount: 65 mg MPO per 100 ml of pus. Ratio:  $A_{430}/A_{280} = 0.22$ .

*Step 5. Ammonium sulphate precipitation.* The MPO was precipitated by ammonium sulphate at a concentration of 40–45 % saturation and thereafter dialysed against distilled water.

Amount: 55 mg MPO per 100 ml of pus. Ratio:  $A_{430}/A_{280} = 0.30$ .

*Step 6. Adsorption on kieselguhr.* Prior to its use, the commercial kieselguhr preparation was heated at 70–80°C in a large volume of 4 N hydrochloric acid solution, strained first with hot hydrochloric acid on a Büchner funnel until a negative qualitative test for iron in the wash water, and thereafter with distilled water until neutral reaction. The preparation was dried at 80°C.

For every 100 mg of MPO to be processed, 50–60 g of the dried kieselguhr powder were suspended in distilled water and packed in a chromatogram glass tube. The MPO preparation was then filtered through the column. In doing this the preparation was adsorbed on the powder in the two third upper parts of the column. The column was washed with saline and thereafter with an ammoniacal ammonium formate solution (prepared from a stock solution — equal amounts of 2 M ammonium formate and 1 M ammonium hydroxide — by adding 2 volumes of distilled water) until the eluate was free from substances absorbing at the wavelength of 280  $m\mu$ . Thereafter the MPO was eluated by the ammoniacal ammonium formate stock solution cited above.

The MPO in the eluate was precipitated with ammonium sulphate at 50 % saturation and thereafter dialysed against distilled water.



Fig. 1. MPO, recrystallized from ammoniacal ammonium sulphate solution. Dark field illumination. 300 × magnification.

Amount: 40 mg MPO per 100 ml of pus. Ratio:  $A_{430}/A_{280} = 0.80$ ;  $A_{430}/A_{390} = 1.95$ .

Step 7. *Crystallization from ammonium sulphate solution.* Ammoniacal ammonium sulphate solution (50 volumes of saturated ammonium sulphate solution + 1 volume of ammonia) was added to the MPO solution up to a degree of saturation of about 0.40. Thereafter very small quantities of the ammonium sulphate solution were added with intervals of 2–3 h until thixotropy was observed. After two or three days the greater part of the MPO had crystallized. The MPO-crystals separated as needles and displayed double refraction; Fig. 1.

#### ANALYSIS

The crystalline preparation obtained by the described procedure has been checked for homogeneity by ammonium sulphate solubility test, Fig. 2, electrophoresis at different pH-values, ultracentrifugation and diffusion. In

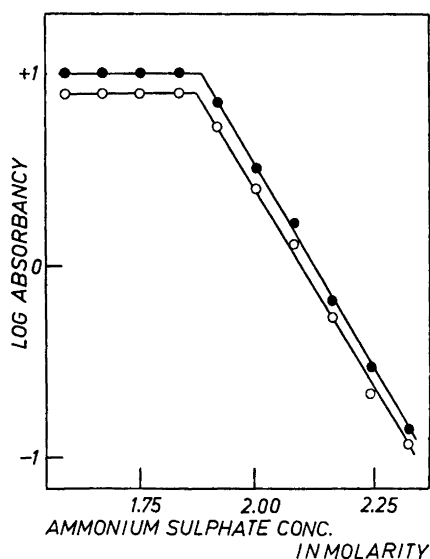


Fig. 2. Solubility of recrystallized MPO in solutions of increasing ammonium sulphate concentrations determined by spectrophotometric readings at 430 and 280 mμ. The MPO conc.: 7 mg/ml.

●—●—● log absorbancy, 280 mμ  
○—○—○ log absorbancy, 430 mμ

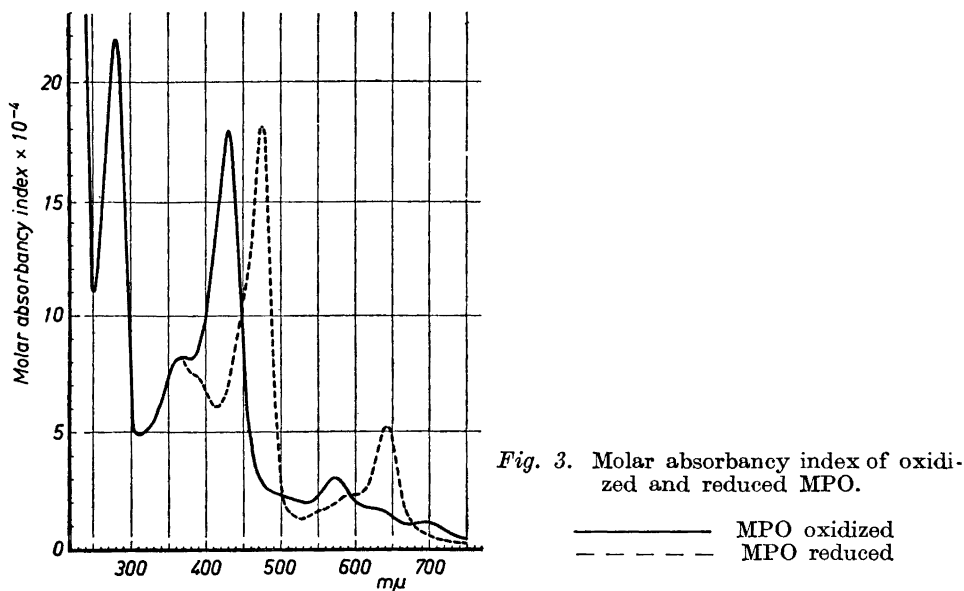


Fig. 3. Molar absorptivity index of oxidized and reduced MPO.

all these experiments the preparation behaved as expected for a single component. The results from the ultracentrifugation and diffusion experiments, as well as the estimation of the molecular weight, 149 000, will be described in a separate paper<sup>2</sup>.

The molar absorptivity index values<sup>3</sup>,  $A_{\mu}$ , for the MPO at different wavelengths have been calculated from the absorptivity readings obtained in a Beckman spectrophotometer, model DU and are summarized in Fig. 3.

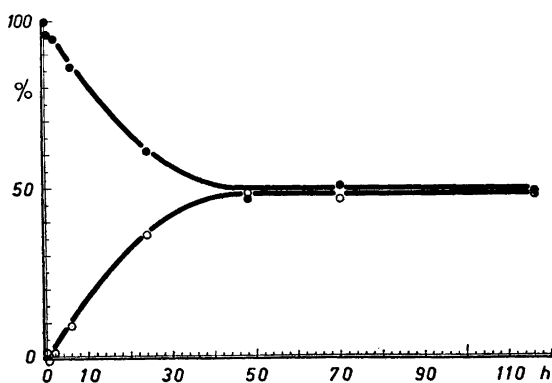


Fig. 4. Distribution of the iron after fractionation by methyl alcohol from 50 % pyridine solution after varying time.

○—○—○ percentage of iron, "soluble in methanol"  
●—●—● percentage of iron, "insoluble in methanol"

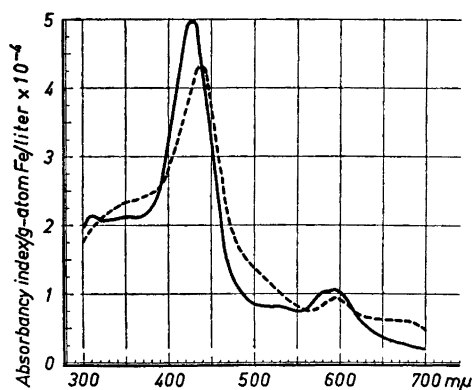


Fig. 5. Absorbancy index/g-atom Fe/liter for  
 the iron compound "soluble in methanol" —————  
 » » » "insoluble in methanol" - - - - -

The iron content of the MPO has been analysed by a modification of the method described by Lorber <sup>4</sup>. It was found to be  $0.74 \mu\text{g}$  per mg of dry weight.

After addition of concentrated sulphuric acid and perchloric acid (0.2 ml of each) the preparation (2–8 mg) was combusted in test tubes ( $140 \times 15 \text{ mm}$ ) at about  $250^\circ\text{C}$ . The heating was extinguished when the white heavy vapour had disappeared. The sample was diluted, 0.2 ml of a 20 % solution of sulphosalicylic acid was added, followed by ammonia until a yellow colour was produced. After dilution of the sample up to 5 ml the colour intensity was determined at  $455 \text{ m}\mu$  in a Beckman spectrophotometer, model B, equipped with a special adapter for 50 mm long and 4 mm wide cuvettes. Absorbancy = 0.100 per  $\mu\text{g}$  of iron in the sample.

Procedures described for a fission of the linkages between the iron-porphyrin structures and their protein moieties by treatment with acid acetone or with some metals, e.g. silver ions, have been found to be unsuitable for breaking the bonds between the iron-porphyrin compound and the protein of the MPO. The effect of some other substances upon the MPO has been studied. It has been found that MPO splits into two parts in 50 % pyridine solution. The separation of the two fragments has been achieved by precipitation of one of them by adding 4 volumes of methyl alcohol. The second fragment is soluble in methyl alcohol. Fig. 4 demonstrates the velocity of this reaction at room temperature (about  $22^\circ\text{C}$ ).

The reaction is a rather slow one at room temperature. The velocity increases at higher temperature. At  $70^\circ\text{C}$  the fission was completed within 60 min.

The absorbancy index values per g-atom of iron per liter have been determined for the two split products dissolved in 50 % pyridine solution, Fig. 5.

#### DISCUSSION

The crystalline MPO obtained from dog leucocytes by the procedure described above has proved to behave as would be expected for a homogeneous substance in ammonium sulphate solubility tests, ultracentrifugation, and

electrophoretic experiments at different pH-values. The molecular weight for the dog MPO calculated from the sedimentation and diffusion constants and the value for the specific volume is found to be 149 000<sup>2</sup>. The iron content of the enzyme is 0.074 %. The theoretical value for the iron content, when assuming two atoms of iron per molecule of MPO, is 0.075 %. The experimental results suggest that the preparation is homogeneous and can be characterized as »pure».

The absorption spectrum and other properties investigated are very similar to those found for the preparations from the human starting material in 1941. The figure for the iron content given for the earlier preparation (1941) from human leucocytes was 0.1 %. However, according to the analyses, mentioned above, the iron content is found as 0.074 %. The molar absorptancy index calculated for the MPO from dog leucocytes is about 25 % higher than the values found for the preparation in 1941\*. These differences may reflect a diversity in characteristics of the MPO from the two different species. However, it cannot be excluded that the explanation for the differences might be a misinterpretation of the iron content in the previous experiments due to a shortage of material.

When acid acetone is added to hemoglobin, myoglobin, catalases, horse radish peroxidase, cytochrome a and b, the prosthetic group is released from the protein part which precipitates, while the prosthetic group remains in the dissolved state. Likewise the cysteine-porphyrin bonds in cytochrome c have been found to be broken by treatment with AgNO<sub>3</sub> and some other metal salts. If these procedures be applied to the MPO the whole molecule is precipitated without fission; therefore, the linkages between the porphyrin and the protein are more stable in this respect. By treatment with pyridine the MPO molecule is split into two parts. Each of them contain almost exactly 50 % of the original amount of iron and, thus, one atom of iron. The two components, when dissolved in 50 % pyridine solution, have absorption spectra, which indicate that both of the iron atoms are incorporated in porphyrin structures.

At present it is not possible to ascertain whether the two prosthetic groups are linked to the protein differently or if the findings are due to a splitting of the protein moiety into two parts.

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\* Recalculated from the values expressed as the absorption coefficients cm<sup>2</sup>/g-atom of iron (logarithmic base e).