# HUMAN EOSINOPHIL PEROXIDASE: A NOVEL ISOLATION PROCEDURE, SPECTRAL PROPERTIES AND CHLORINATING ACTIVITY

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Received 12 November 1980

## 1. Introduction

Like neutrophils, eosinophils contain a high concentration of a peroxidase that may have enzymic activity which is associated with the antiparasitic function of these cells [1]. In neutrophils it has been demonstrated that the peroxidase (myeloperoxidase) is involved in the killing of micro-organisms (reviewed in [2]). This antimicrobial activity of myeloperoxidase is probably due to the ability of this enzyme to oxidize $Cl^-$ with $H_2O_2$ to the reactive hypochlorous acid (HOCl) [3–5]. It is not known whether human eosinophil peroxidase can catalyse the same reaction. It was recently shown [6], however, that eosinophil peroxidase partially purified from guinea pig eosinophils is bactericidal when combined with $H_2O_2$ and $Cl^-$. Studies on human eosinophil peroxidase are hampered since eosinophils can only be obtained in significant amounts from blood from patients with eosinophilia. However, in order to be able to purify the peroxidase from these eosinophils and to study the enzyme in some more detail, sufficient patient blood must be available, which is generally not the case. In addition, it is conceivable that the properties of patient eosinophils, and the enzymes in these cells, differ from those normally found in blood circulation.

As shown in [7] the amount of haem iron derived from the peroxidase in an eosinophil is ~4-times higher than the concentration of myeloperoxidase in a neutrophil. Since white blood cells of normal donors contain 3–5% eosinophils, 12–20% of the total peroxidase (on the basis of haem iron) in a homogenate of white cells must be due to eosinophil peroxidase. From 100 l human blood 200 mg pure myeloperoxidase can be obtained [8]; it should therefore be possible also to isolate the eosinophil peroxidase. Reduced eosinophil peroxidase is distinctly different from myeloperoxidase in its spectral properties [7], having a Soret peak at 448 nm, whereas the Soret peak of reduced myeloperoxidase is at 472 nm. This difference in peak position makes it possible to monitor spectrophotometrically the purification of the peroxidase during the isolation procedure. This paper describes a novel method to isolate human eosinophil peroxidase from normal leucocytes. In addition, it is shown that the enzyme catalyses the peroxidative chlorination of monochlorodimedon at a rate which is similar to that of myeloperoxidase from neutrophils.

## 2. Materials and methods

The halogenating activity of eosinophil peroxidase was measured in the assay system with monochlorodimedon as in [9]. $H_2O_2$ solutions were prepared by dilution of a 30% stock solution of perhydrol (Merck, Darmstadt). The concentration of $H_2O_2$ was determined spectrophotometrically with an absorption coefficient of 43.6 $M^{-1} . cm^{-1}$ at 240 nm [10]. Cetyltrimethylammonium bromide was obtained from Fluka AG (Switzerland). Sulphopropyl–Sephadex (C-50) was obtained from Pharmacia (Uppsala), Ultrogel, AcA-34 from LKB (Bromma) and monochlorodimedon from Sigma (St Louis). Protein was determined by the method in [11] with bovine serum albumin as standard.

All spectrophotometric measurements were done on a Cary-17 recording spectrophotometer.

### 2.1. *Purification of eosinophil peroxidase*

Buffy coats from outdated blood were centrifuged

at 1000 X *g* for 15 min. The buffy coat was washed twice with a hypotonic solution of 155 mM $NH_4Cl$, 10 mM $KHCO_3$, 0.1 mM EDTA to lyse remaining red cells. After centrifugation at 800 X *g* for 15 min the white cells were resuspended in 0.3 M sucrose, 100 mM potassium phosphate (pH 7.3) and homogenized in a Waring Blendor at 14 000 rev./min for 1 min. The homogenate was centrifuged at 20 000 X *g* for 25 min and the supernatant was discarded. The pellet was homogenized in 0.5% cetyltrimethylammonium bromide, 0.1 M $Na_2SO_4$ and 0.1 M potassium phosphate (pH 7.3). After centrifugation at 20 000 X *g* for 25 min, the supernatant was discarded; the pellet was rehomogenized twice in the buffer (with 1% cetyltrimethylammonium bromide). After centrifugation the green supernatants were combined. An ammonium sulphate precipitation was carried out at pH 7.3. The yellow–green precipitate at 40–60% saturation was dissolved in 0.1 M potassium phosphate (pH 7.3) and 0.5% Tween 80. The precipitate at 60–65% was used for the purification of myeloperoxidase [8]. The solution was dialyzed overnight against 0.1 M potassium phosphate (pH 7.3) and 0.5% Tween 80 and centrifuged to remove denatured material. A sulphopropyl–Sephadex (C-50) column was equilibrated with 0.1 M potassium phosphate (pH 7.3) and 0.5% Tween 80 and the dialyzed solution applied to it. The column was washed with 0.25 M potassium phosphate (pH 7.3) and 0.5% Tween 80 to remove myeloperoxidase and other contaminants. Eosinophil peroxidase, which remained bound as a brown band on top of the column, was eluted with 0.5 M potassium phosphate (pH 7.3) and 0.5% Tween 80.

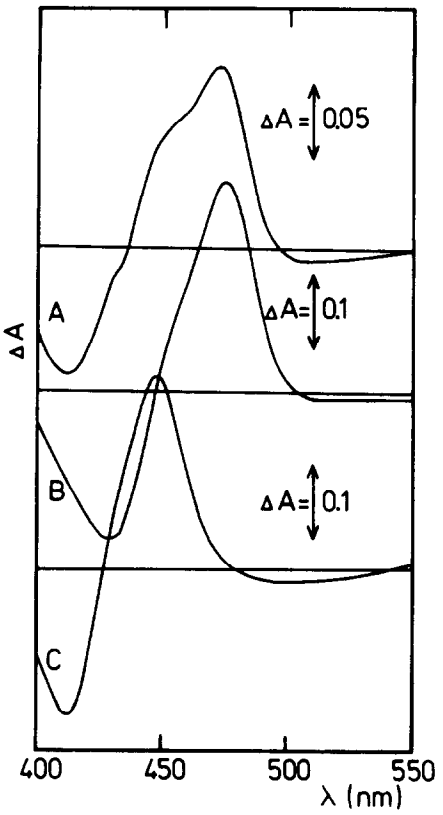


Fig.1. Difference spectra (reduced–oxidized) of the detergent extract of leucocytes, purified myeloperoxidase and eosinophil peroxidase: (A) Detergent extract of leucocytes, 2.65 mg protein/ml in 1% cetyltrimethylammonium bromide, 0.1 M $Na_2SO_4$ and 0.1 M potassium phosphate (pH 7.3); (B) Myeloperoxidase, 3.7 μM in 100 mM potassium phosphate (pH 7.3); (C) Eosinophil peroxidase, 2.3 μM in 100 mM potassium phosphate (pH 7.3) and 0.5% Tween 80.

## 3. Results

Fig.1, trace A shows the reduced–oxidized difference spectrum of the green supernatant obtained from extracts of white blood cells (200 buffy coats) with 1% cetyltrimethylammonium bromide. It is clear from comparison with purified myeloperoxidase (fig.1, trace B) that a substantial shoulder is present in the spectrum at 450 nm. As shown in [7], this shoulder is due to the eosinophil peroxidase. To calculate the amount of eosinophil peroxidase present in this extract, the absorption coefficient of eosinophil peroxidase is needed. This value is, however, not yet known and therefore the absorption coefficient of lactoperoxidase which has a spectrum similar to eosinophil peroxidase [7] was used. The absorption coefficient of lactoperoxidase is 110 $mM^{-1} . cm^{-1}$ at 413 nm [12] and, assuming that this value is the same for eosinophil peroxidase, it is possible to calculate from fig.2 that the absorption coefficient at 449 nm (reduced–oxidized) is 78 $mM^{-1} . cm^{-1}$. On the basis of this value and after correction for the contribution of myeloperoxidase it was found that the detergent extract (fig.1A) contained 0.71 μmol haem iron derived from eosinophil peroxidase (table 1).

The amount of myeloperoxidase present in the same extract is substantial (1.2 μmol). It is possible to obtain a preparation of eosinophil peroxidase that is nearly free of myeloperoxidase by ammonium sulphate fractionation between 40–50% saturation, which

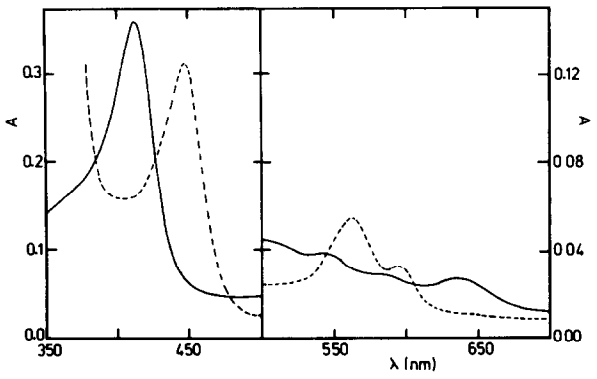


Fig.2. Absorption spectra of oxidized (——) and reduced (– – – –) eosinophil peroxidase (3.3 μM) in 100 mM potassium phosphate (pH 7.3) and 0.5% Tween 80. The protein was reduced with a slight excess of $Na_2S_2O_4$.

shows that the peroxidases differ in their solubility. However, a considerable part of eosinophil peroxidase is not precipitated at 50% saturation and therefore 60% $(NH_4)_2SO_4$ was used to precipitate the eosinophil peroxidase. This precipitate still contains a considerable amount of myeloperoxidase (0.56 μmol, table 1). In line with [13] it was found that eosinophil peroxidase, like intestinal peroxidase [14], was only slightly soluble in water; hence, a detergent was added to the buffer in the subsequent steps to keep the enzyme in solution. Since the cationic detergent cetyltrimethylammonium bromide is strongly bound to sulphopropyl–Sephadex the non-ionic detergent Tween 80 was used. Both peroxidases were absorbed on a sulphopropyl–Sephadex column equilibrated

Table 1
Purification of eosinophil peroxidase from human leucocytes

| Fraction | Protein (mg) | Myeloperoxidase (μmol) | Eosinophil peroxidase (μmol) | Recovery (%) |
|---|---|---|---|---|
| Cetyltrimethyl–ammonium bromide extracts | 2464 | 1.2 | 0.71 | 100% |
| $(NH_4)_2SO_4$ precipitate after dialysis | 754 | 0.56 | 0.31 | 44% |
| Eluate from SP–Sephadex column | 6.3 | 0.0 | 0.06 | 8% |

with 0.1 M potassium phosphate and 0.5% Tween 80, but the myeloperoxidase could be washed from the column with 0.25 M potassium phosphate and 0.5% Tween 80, whereas the eosinophil peroxidase remained bound under these conditions. This shows that eosinophil peroxidase is a more basic protein than neutrophil myeloperoxidase.

Fig.1C shows the difference spectrum (reduced–oxidized) of the purified eosinophil peroxidase. It is clear that spectrophotometrically no myeloperoxidase is detectable in the eluate. On the basis of haem iron the yield of eosinophil peroxidase (table 1) is 0.06 μmol (6.3 mg protein) with an $A_{413}/A_{280}$ of 0.7.

From the last line of table 1, it is possible to estimate a relative molecular mass ($M_r$) of eosinophil peroxidase of 105 000 based on the assumption that 1 molecule of enzyme contains 1 haem iron group. This value, determined indirectly, poses only an upper limit to the molecular weight since the enzyme may not be completely pure. Furthermore, the amount of haem iron is also determined indirectly.

The absorbance spectra of the oxidized and the reduced enzyme are shown in fig.2. The oxidized enzyme is characterized by absorption maxima at 413 nm and 640 nm with shoulders at 470 nm, 500 nm, 550 nm and 580 nm. The enzyme reduced by dithionite shows maxima at 449 nm, 563 nm and a distinct shoulder at 597 nm. The reduced enzyme forms a carbon monoxide compound with a Soret maximum at 437 nm (not shown).

Myeloperoxidase from neutrophils is microbicidal and cytotoxic in combination with $H_2O_2$ and $Cl^-$ due to oxidation of $Cl^-$ to hypochlorous acid. Since eosinophils have an antiparasitic function and discharge their granules onto the parasite surface [15], it is likely that eosinophil peroxidase has an enzymic activity directed against parasites. It was of interest therefore to determine whether human eosinophil peroxidase was also able to oxidize $Cl^-$ to the reactive hypochlorous acid. Indeed, as is found for myeloperoxidase, incubation of eosinophil peroxidase with $H_2O_2$, chloride and monochlorodimedon results in a rapid decrease in the absorbance of monochlorodimedon at 290 nm, due to the formation of dichlorodimedon [9] (not shown).

Fig.3 illustrates the pH dependence of this chlorination reaction. In line with the bactericidal effect, which is found at slightly acid condition [6], at 100 mM $Cl^-$ and 0.1 mM $H_2O_2$ the reaction shows a pH optimum at 4.6. However, the pH optimum shifts to

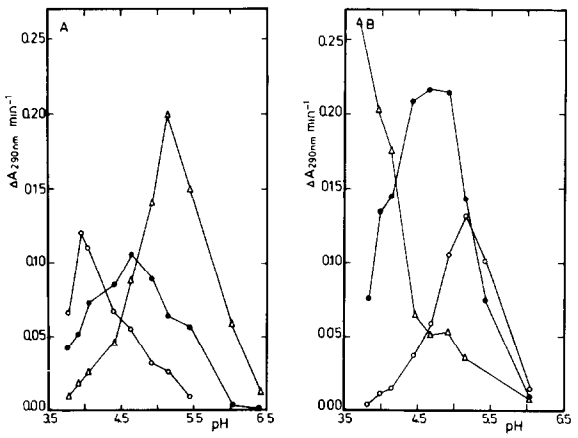


Fig.3. (A) The pH dependence of the chlorination reaction as a function of $[Cl^-]$. The reaction was started by the addition of $H_2O_2$ (100 μM) to the sample which contained 0.2 M potassium sulphate, 0.1 M sodium acetate (pH 3.8–5.5) or 0.1 M sodium phosphate (pH 5.6–6.5), 60 μM monochlorodimedon and 12 nM eosinophil peroxidase: (○——○) 10 mM KCl; (●——●) 100 mM KCl; (△——△) 500 mM KCl. (B) The pH dependence of the chlorination reaction as a function of the $H_2O_2$ concentration: $[Cl^-]$ 250 mM; (○——○) 25 μM $H_2O_2$; (●——●) 250 μM $H_2O_2$; (△——△) 1 mM $H_2O_2$.

higher values upon increasing the $Cl^-$ concentration. In contrast, when the $Cl^-$ concentration is fixed and the $H_2O_2$ concentration is increased, the pH optimum shifts to lower pH values (fig.3B). Similar kinetics were also observed for myeloperoxidase of neutrophils [15]. It is interesting to note that the pH optima at the substrate concentrations used are shifted by 0.8 pH unit to more acid pH compared to those of myeloperoxidase [16].

The rate of chlorination of monochlorodimedon at its optimum pH of 5.2 (fig.3A) is 42 nmol/min. Since $3 \times 10^{-1}$ nmol eosinophil peroxidase were present, this corresponds to a turnover rate of 23 $s^{-1}$. From [16] it follows that the turnover value for myeloperoxidase calculated from the maximal velocity at pH 4.5 is 68 $s^{-1}$. This demonstrates that the enzymic activities of the two peroxidases are similar.

## 4. Discussion

Up to now eosinophil peroxidase has been partially purified only from rat [17] and guinea pig eosinophils [13]. Human eosinophil peroxidase has not been isolated before and had been studied only in extracts or homogenates of eosinophils obtained from patients with eosinophilia. As noted by various authors there is no certainty that these cells are comparable to normal circulating eosinophils. The simple isolation procedure of eosinophil peroxidase from normal human leucocytes reported here makes it possible to obtain sufficiently purified enzyme for spectral and kinetic studies. At present, it is not known whether the enzyme is completely pure. Attempts to purify the enzyme further by gel chromatography on AcA-34 failed since most of the peroxidase was adsorbed by this material. However, the $A_{413}/A_{280}$ of 0.7 found by us is higher than the value of 0.4 reported for partially purified eosinophil peroxidase from rat [16].

The optical spectra of the human enzyme are remarkably similar to those of the enzyme from rat eosinophils [17], lactoperoxidase [12] and intestinal peroxidase [14]. Also, the reduced–oxidized difference spectrum of eosinophil peroxidase is nearly identical to that observed in homogenates of pure patient eosinophils [7]. Thus, in this respect eosinophils from patients with eosinophilia do not differ from those normally present in the circulation.

It was reported in [18] that the eosinophil peroxidase was unable to affect bacterial viability in the presence of 0.1% gelatine when $Cl^-$ was used as a substrate. However, in [6] using more purified eosinophil peroxidase from guinea pig, it was demonstrated that eosinophil peroxidase in the presence of $H_2O_2$ and $Cl^-$ had a considerable bactericidal activity. This activity was inhibited completely by albumin, a well-known scavenger for HOCl, and by gelatine. This demonstrates that extraneous proteins should not be added to the assay. In line with [6] this paper demonstrates that eosinophil peroxidase is indeed able to oxidize $Cl^-$ to the bactericidal hypochlorous acid at a rate which is comparable to that of myeloperoxidase from neutrophils.

## Acknowledgements

The authors wish to thank Dr D. Roos for his interest and criticism. This study was in part supported by grants from the Netherlands Organization for the Advancement of Pure Research (ZWO) under the auspices of the Netherlands Foundation for Chemical Research (SON).

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