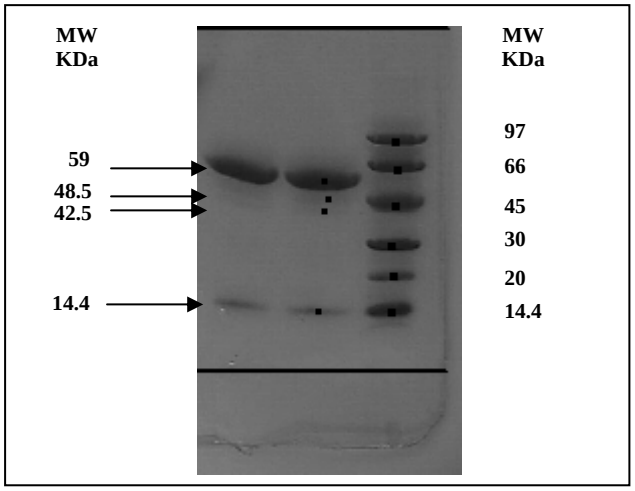
Preparation of human leucocyte and recombinant MPO

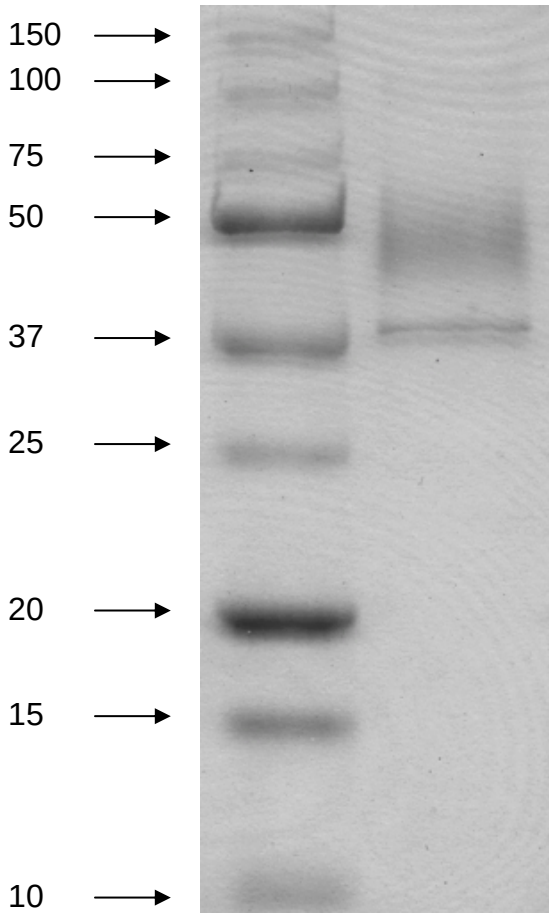
Dimeric leucocyte MPO- Briefly, polymorphonuclear cells were extracted from the supernatant after sedimentation in buffy coated tubes. This supernatant was gently centrifuged and the remaining blood cells in the sediment were lysed and eliminated after centrifugation by discarding the supernatant. The resulting material was suspended in a PBS buffer and activated with phorbol myristate acetate (PMA) before freezing at -80°C. The sample was defrosted and the granules were obtained after lysis of the cells with a cavitation bomb under nitrogen. The resulting solution was centrifuged to obtain a granule-rich sediment suspended in acetic acid before freezing. Releasing of protein was obtained after sonication and repeated cycles of freezing and thawing. The sample was stored overnight at 4°C in acetate buffer/Triton X100 buffer. Finally, the sample was centrifuged and the supernatant collected. The pellet was resuspended in an acetate/Triton X100 buffer. This procedure was repeated several times. After a filtration of the pooled supernatants, the solution was loaded onto a Source 15S column (GE Healthcare, Fairfield, CT, USA) equilibrated with acetate buffer, pH 4.5. Elution was performed with the same buffer supplemented with NaCl (6 %). Superdex 200 chromatochraphy (GE Healthcare, Fairfield, CT, USA) was used for further purification using the same buffer supplemented with NaCl (2 %) for loading and elution. Fractions absorbing at 430 nm were collected and submitted to ultrafiltration with an YM10 Amicon membrane in acetate buffer, pH 4.5. Finally, h-MPO was purified on Mono Q HR5/5 column (GE Healthcare, Fairfield, CT, USA) equilibrated with acetate buffer, pH 4.5, and eluted with the same buffer supplemented with NaCl (6%). Fractions absorbing at 430 nm were collected.

Recombinant MPO - Briefly, the best producing clone was grown at 37 °C in αMEM supplemented with 5 % foetal calf serum, 2 mM L-glutamine and 25 nM methotrexate. At confluence, the culture medium was changed to medium containing with 1 % serum and maintained for 3-4 weeks. Culture supernatant was collected at 3-4 days intervals, filtered through 0.45 µm membranes and stored at 4 °C until purification. For purification, 2 L culture supernatant were passed through a Q-Sepharose fast-flow column (5 x 15 cm) equilibrated with 20 mM potassium phosphate, pH 7.5, at a flow rate of 400 mL/h. The unbound fraction was directly loaded on to a carboxymethyl-Sepharose fast-flow column (5 x 29 cm), equilibrated with the same buffer as above, supplemented with 100 mM

NaCl. After washing, r-MPO was eluted with 1.2 L of a linear NaCl gradient (100 mM - 500 mM) in the same buffer at a flow rate of 400 mL/h. The r-MPO containing fractions were pooled and applied to a chelating-Sepharose fast-flow column (1.7 × 30 cm) saturated with $CuSO_4$ and equilibrated with 20 mM Tris/acetate, pH 8.2, containing 500 mM NaCl. Recombinant MPO was eluted at a flow rate of 150 mL/h with 200 mL of a linear pH gradient of 20 mM Tris-acetate solution (pH 8.2-3.9) containing 500 mM NaCl.



Electrophoregram of 2 aliquots of h-myeloperoxidase showing 4 bands at 59 kDa, 48.5 kDa, 42.5 KDa and 15 kDa (arrows). The bands have been identified as the heavy chain (59, 48.5 & 42.5 Da) and the light chain (14.4 KDa)



Electrophoregram of an aliquot of r-myeloperoxidase showing 3 bands at 50 kDa, 38.5 kDa, 38 KDa (arrows). The bands have been identified as the heavy chain (50, 38.5 & 38 Da).
An analyse by proteomics of the batch has identified human myeloperoxidase.

Reduction, alkylation and trypsin hydrolysis- 500 µg of h-MPO or 1 mg of r-MPO were denatured by dissolution in 1 mL of 0.2 M Tris-HCl solution buffered at pH 8.8 containing 6 M guanidine hydrochloride. After addition of 100 mM dithiothreitol (DTT) (Biorad, Hercules, CA), samples were incubated at 50 °C for 2 h and, finally, 300 mM iodoacetamide (Biorad) were added. The reaction was then carried out in the dark at 37 °C for 2 h. After extensive dialysis of the reaction mixtures against 50 mM ammonium bicarbonate, samples were lyophilised. After reconstituting the lyophilized products in 1 mL of 50 mM ammonium bicarbonate, the proteolysis was performed by adding Tos-Phe-CH_2Cl-treated trypsin (Sigma Chemicals, St Louis, MO) at an enzyme-substrate ratio of 1:50 and the mixture was incubated at 37 °C for 24 h. The enzyme reaction was terminated by boiling at 100 °C for 15 min. The tryptic fragments obtained were then purified by C18 Sep-Pak cartridge, eluted with 80% acetonitrile in water containing 0.1% trifluoroacetic acid (TFA) and finally lyophilized. The purified peptides were ready to be separated either by affinity chromatography using lectin-coupled sepharose or by reverse-phase (RP) chromatography.

Enrichment of glycopeptides by affinity chromatography on immobilized lectin- The peptide digest, corresponding to 1 mg of r-MPO, was loaded onto a Concanavalin A (Con A)-Sepharose column (Amersham, Pharmacia Biotech, Uppsala, Sweden) (1 mL) equilibrated with 50 mM Tris-HCl solution, pH 7.4, containing 100 mM NaCl, 1 mM $CaCl_2$, 1 mM $MnCl_2$ and 1 mM $MgCl_2$ (Buffer B). After washing the column with 10 mL of buffer B, the elution was sequentially performed by 5 mL of 0.01 M and 0.5 M α-methyl-D-glucoside in buffer B, in order to obtain low and high affinity fractions, respectively. The lectin-captured fractions were desalted on a C18 Sep-Pak cartridge according to the manufacturer's instructions and then freeze-dried before RP-HPLC fractionation.

RP-HPLC separation of trypsin-digested peptides- The tryptic digest was applied to a C18 3.5 µm XTerra column (2.1 x 150 mm) (Waters, Milford, MA) equipped with a guard column Optiguard 1 mm C18 (Interchim, Montluçon, France) and equilibrated with 0.1 % TFA in water. The elution was obtained by a gradient mode with a 0.1 % TFA solution applied for 15
min, followed by a linear gradient of acetonitrile from 0 to 40 % over 90 min, then from 40 to 70 % over 30 min at a flow rate of 0.150 mL/min. Eluates were monitored at 220 nm. Glycopeptide-

containing fractions were characterized by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS).

Matrix-assisted laser desorption/ionisation mass spectrometry- MALDI-MS experiments were carried out on a Voyager Elite DE-STR Pro instrument (Perseptive Biosystems, Framingham, MA) in reflectron mode with delayed extraction. Samples were prepared by mixing a 1 µL aliquot (5-10 pmoles) with 1 µL of matrix solution (2,5-dihydroxybenzoic acid, DHB), concentrated at 10 mg/mL in a methanol/water solution (1:1, v/v). The MALDI analysis of the in-gel proteolysed tryptic (glyco)-peptides was performed by spotting the eluate directly on to the MALDI sample plate. External calibration was performed using calibration standard kit (Sigma, St-Louis, MO).

Deglycosylation- Recombinant-MPO (~2 mg/mL) was deglycosylated according to the standard protocol of a glycoprotein deglycosylation Kit (Calbiochem). One ml of MPO was incubated during 5 days at 37 °C with 100 µL of buffer, 5 µL of endo-α-N-acetylgalactosaminidase, 5 µL of β1,4-galactosidase and 5 µL de β-N-acetylglucos-aminidase. The sample was purified on QAE Sepharose fast flow micro-column. The column was equilibrated with 20 mM phosphate buffer, pH 7.5, (Buffer A) and the sample was then added. The column was washed twice with buffer A. MPO was finally eluted with the buffer A supplemented with 450 mM of NaCl. The column was washed with water followed by 5 volumes of 200 mM phosphate buffer, pH 7, and 20% of ethanol (MPOdegly). Another sample from the same batch was treated similarly but in the absence of any glycosidase (MPO37°). The protein concentration of each sample was measured by the Lowry assay (27).

In order to assess the deglycosylation, the three samples were alkylated, reduced, digested and glycopeptides enriched according to the protocols described above. The peptides were analysed by LC/ESI/(MS)MS with the QTOF6520 (Agilent, Palo Alto, CA, USA).

Liquid chromatography coupled to electrospray tandem mass spectrometry (LC/ESI/MSMS)- Samples enriched on the ConA sepharose column were deglycosylated, purified by SepPak, and finally dissolved in 50 µl of HCOOH 0.1 %. Ten µl were injected in the LC system and through a Zorbax Eclipse Plus C18 50 x 2.1 mm, 1.8 µm (600 bars). The following gradient was applied:

Time (min.)	B (%)	Flow rate (ml/min)	Max. Press. (bar)
0	1	0.2	400
5	1	0.2	400
15	5	0.3	400
25	5	0.5	400
80	30	0.5	400
83	30	0.5	400
93	90	0.5	400
95	90	0.5	400
99	1	0.5	400
101	1	0.2	400
105	1	0.2	400

Solvent A: Formic acid 0.1% / Solvent B: Acetonitrile 90 : 10 formic acid 0.1%

The analyses were carried out in positive mode with MS and autoMSMS acquisitions mode at 4 GHz with a slope of 3.6 V/100Da and an offset at -4.8 V/100Da for the autoMSMS. The source temperature was at 300 °C, the drying gas at 11 l/min, the capillary voltage at 4500 V and the fragmentor at 175 V. The chromatograms in MSMS were finally analysed with Spectrum Mill® software® (Agilent, Palo Alto, CA, USA) in order to identify peptides and the MS chromatogram were analyzed with Qualitative Analysis software® (Agilent, Palo Alto, CA, USA).

Liquid chromatography coupled to electrospray mass spectrometry (LC/ESI/MS)- Samples enriched on the ConA sepharose column were deglycosylated, purified by SepPak, and finally dissolved in 50 µl of HCOOH 0.1 %. The peptides eluted during the enrichment were purified by SepPak and dissolved in 50 µl of HCOOH 0.1 %. Ten µl were injected in the LC system and through a Zorbax Eclipse Plus C18 50 x 2.1 mm, 1.8 µm (600 bars). The following gradient was applied:

Time (min.)	B (%)	Flow rate (ml/min)	Max. Press. (bar)
0	5	0.5	400
15	30	0.5	400
18	90	0.5	400
22	5	0.5	400

Solvent A: Formic acid 0.1% / Solvent B: Acetonitrile; stop time at 25 min.

The analyses were carried out in positive mode with MS mode at 1 GHz. The source temperature was at 300 °C, the drying gas at 11 l/min, the capillary voltage at 4500 V and the fragmentor at 175 V. The chromatograms in MS were finally analysed with Qualitative Analysis software® (Agilent, Palo Alto, CA, USA) in order to identify peptides and integrated corresponding peaks.

Measurement of MPO activity- The measurement of MPO activity has been carried out according to a standard protocol that follows chlorination of monochlorodimedone (MCD) (20). Fifty µL of

glycosylated or non-glycosylated r-MPO were diluted 25 times and added to 2850 µL of phosphate saline buffer, pH 5.5, (0.1 M phosphate, 0.3 M NaCl) with 50 µL of a stock solution of MCD (2 mM). The reaction was started by the addition of 50 µl of 10 mM hydrogen peroxide. The activity was calculated by linear fitting of the initial (4-90 s) absorbance decrease at 290 nm. The specific activity was expressed as units (i.e. ΔAbs_{290}/min) per mg of protein.

Lipoprotein MPO dependent oxidation- The assay was performed as described by Moguilevsky et al. (7) in a NUNC maxisorp plate (VWR, Zaventem, Belgium): wells were coated with 200 ng/well of LDL overnight at 4 °C in sodium bicarbonate pH 9.8 buffer (100 µl). The plate was then washed with TBS 80 buffer and saturated during 1h at 37 °C with the PBS buffer containing 1 % of BSA (150 µl/well). After two TBS 80 washes, the monoclonal antibody Mab AG9 (200 ng/well) obtained according to a standard protocol as previously described (7), was added as a diluted solution in PBS buffer with 0.5 % of BSA and 0.1 % of Polysorbate 20. After incubation during 1 h at 37 °C, the plate was washed 4 times with the TBS 80 buffer. Alkaline phosphatase coupled Ig G anti-mouse Alkaline Phosphatase (Promega, Leiden, The Netherlands) was then added at a 1/3000 dilution in the same buffer (100 µl/well). The wells were washed again 4 times and 150 µl/well of a revelation solution containing 5 mg of paranitrophenyl phosphate in 5 ml of diethanoamine buffer were added and allowed to incubate for 30 min. at room temperature. The reaction was stopped with 60 µl/well of NaOH 3 N solution. The measurement of the absorbance was performed at 405 nm with a background correction at 655 nm with a Bio-Rad photometer for a 96-well plate (Bio-Rad laboratories, CA, USA). Results were expressed as the mean ± SD of the percentage of LDL oxidation for 6 independent measurements.