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Physical-Chemical Characterization of Myeloperoxdase (MPO) and Related Degradation Products

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ABSTRACT

The goal of this study was to render porcine MPO a well-characterized biopharmaceutical via physical-chemical characterization. MPO was purified to near homogeneity, formulated, and evaluated for stability using reversed phase HPLC (RP-HPLC), UV spectrophotometry (UV), size exclusion chromatography (SEC), Refractive Index (RI) detection, Laser Light Scattering (LLS) detection, and Mass Spectrometry (MS) and an enzyme activity assay using guaiacol as the substrate. Sequencing of porcine DNA was performed as an aid in peptide map identification by LC/MS/MS. Intact MPO was found to be too heterogeneous for direct mass spectrometric characterization. MPO was first denatured, reduced, and blocked then separated into its respective heavy and light subunits by RP-HPLC followed by tryptic mapping. The light chain analyzed by LC/MS, had a m/z value of approximately 12,600. Deglycosylation of the heavy subunit was achieved using three successive enzymatic digestion steps with neuraminidase, O-glycosidase, and glycopeptidase-F, in order to remove sialic acid, O- and N-linked carbohydrates prior to LC/MS. Stability studies on intact MPO revealed that more than 75% of the protein activity, and RP-HPLC peak area were retained after storage at 40°C for up to 1 year. SEC with LLS detection showed no differences between samples stored at 2-8°C, 25°C/60% RH, or 40°C/75% RH; however, the UV-VIS spectrum of samples of MPO stored at 40°C/75% RH showed a significant shift in the secondary maximum. These results indicate that MPO is remarkably stable and that its quaternary structure is consistent with published data.

What is Myeloperoxidase?
What does it do? How does it do it?

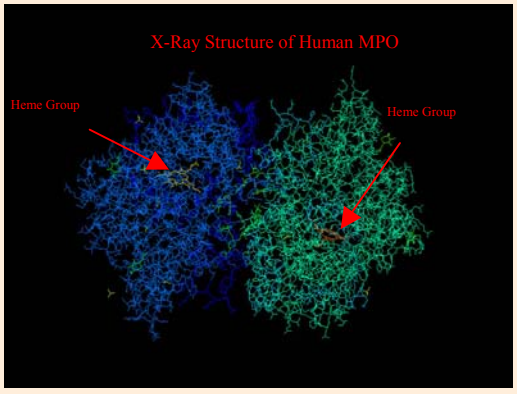
MPO is a heme containing protein found in granules in neutrophils and monocytes.

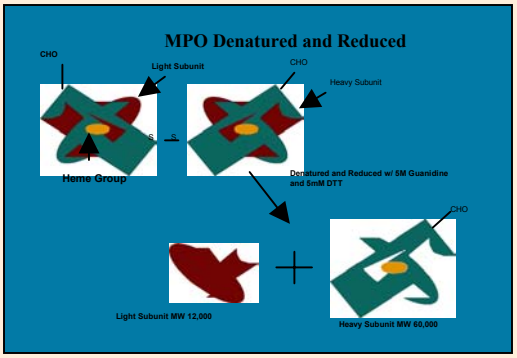
MPO is an enzyme with potent antimicrobial and tumoricidal activity.

MPO catalyzes the production of hypochlorous acid. This is BLEACH!!!!!

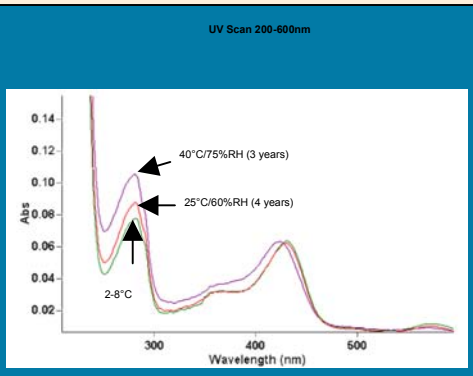
The reaction catalyzed by MPO is:

$$H_2O_2 + Cl^- + H_3O^+ \quad HOCl + 2H_2O$$

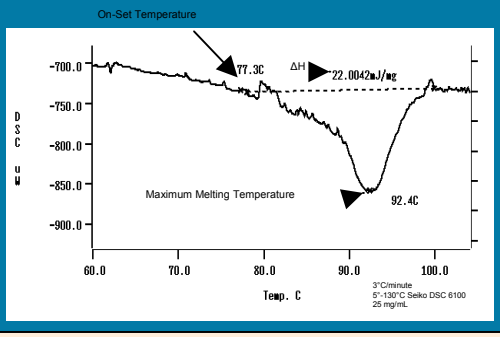




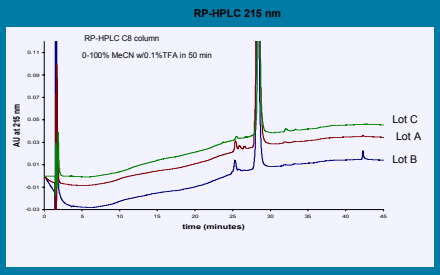
Characterization of MPO by UV, DSC, and RP-HPLC



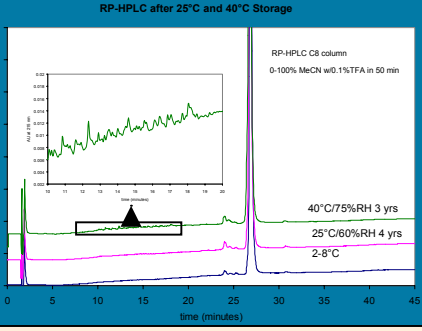
The heme absorbance of MPO (430 nm) is red-shifted relative to other peroxidses due to a sulfonium ion linkage. The blue shift observed in degraded MPO may be related to breaking the bond between the heme group and Met_{243}.



High Melting Temperature (92.4°C) is characteristic of stable proteins.



RP-HPLC can be used for final product characterizaton of MPO



Observation of degradation products after prolonged storage.

MPO is stable to prolonged storage at 40°C/75%RH or 25°C/60%RH.

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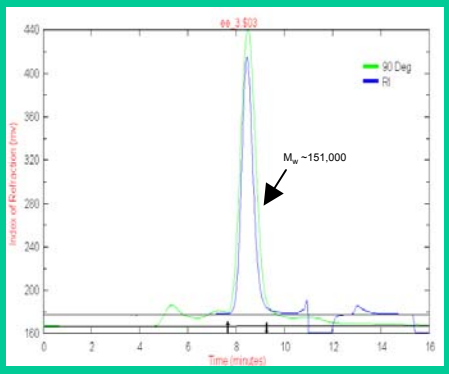
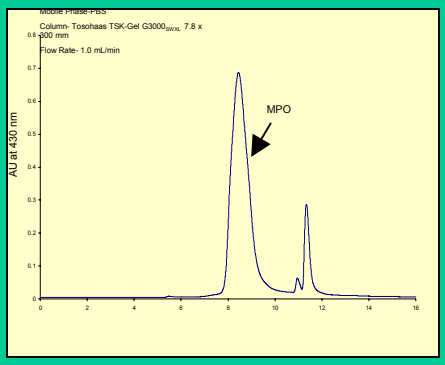
Characterization of size and mass of MPO by SEC with UV, RI and LLS

Native MPO LLS and RI



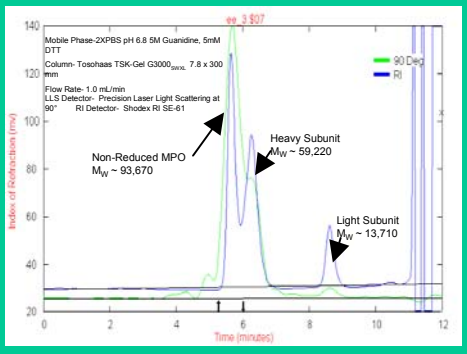
Molecular weight of Native MPO (151,000) determined by LLS is consistent with expected molecular weight.

Native MPO UV at 430 nm



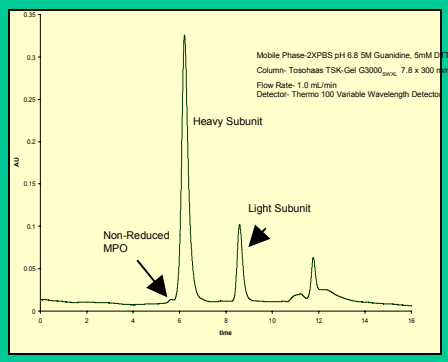
MPO is a Heme containing protein with significant absorbance at 430nm.

Denatured and Partially Reduced MPO LLS and RI



SEC with LLS is a very useful characterization tool for MPO.

Fully Denatured and Reduced MPO, UV at 280 nm



Heavy and Light subunit can be easily separated by SEC.

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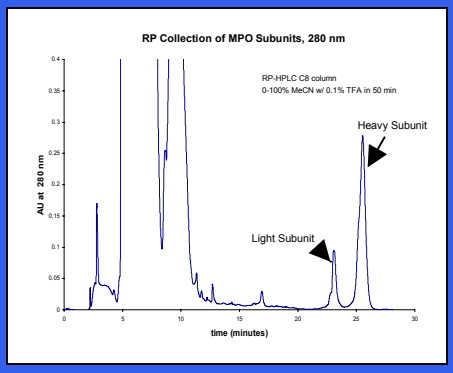
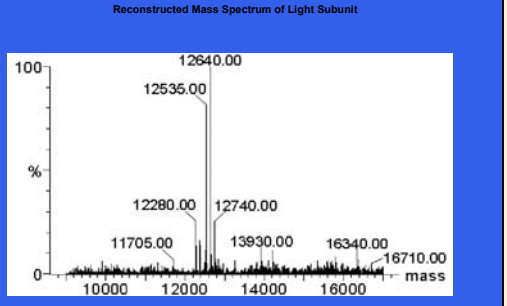
Characterization of MPO subunits by MS, and tryptic digest by LC/MS/MS

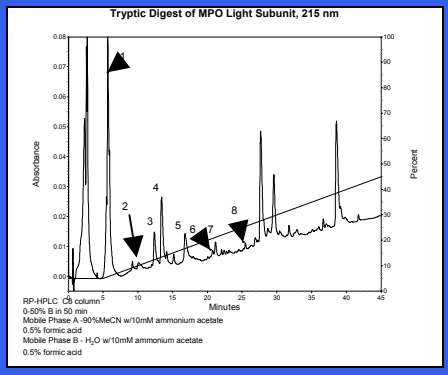


Enzymatic digestion coupled with LC/MS/MS in an extremely powerful tool that can pinpoint the exact areas of the sequence where a change occurs (e.g. degradation).



Expected Mass 12,510

The majority of the light subunit sequence can be verified by LC/MS/MS.



Identification of Tryptic Peptides by MS and MS/MS

Peaks on UV	Sequence	Observed Avg. Mass (Da)	Expected Avg. Mass (Da)
1	43-52	1058.1	1058.2
	26-33	1038.0	1038.2
	44-52	902.2	902.0
2	90-95	658.8	658.8
	88-95	829.0	829.0
3	44-52	900.0	900.0
4	71-77	743.9	743.9
5	96-107	1460.7	1460.6
	82-95	1462.6	1462.7
6	155-160	652.0	651.8
7	79-87	970.3	970.1
8	71-77	743.9	743.9

The heavy subunit is green upon collection confirming the presence of heme.

Summary/Conclusion

- The heavy and light subunits of Porcine MPO are easily resolved by SEC and RP-HPLC. The heavy subunit contains covalently bound heme, as expected.
- MPO is unusually stable to prolonged storage at up to 40°C and also to thermal denaturation. DSC shows that the thermal transition for MPO occurs around 90°C, an unusually high temperature for any protein.
- Degradation of MPO upon storage is accompanied by a blue shift in the heme absorption spectrum. This represents a change in the environment surrounding the heme.
- Intact MPO gives a complex mass spectrum. After the subunits have been separated the light subunit gives well resolved MS peaks and the subunit can be fragmented into tryptic peptides which are amenable to MS. The heavy subunit requires deglycosylation and separation of tryptic peptides in order to return an interpretable signal. Interpretation of the MS data was facilitated by sequencing of pDNA to provide protein sequence information.
- Porcine MPO has been characterized by SEC using UV detection, LLS and RI. Results show that the molecule has the MW expected for an A_2B_2 tetramer in solution, as expected. Porcine MPO has little tendency to aggregate as judged by SEC-LLS.
- Results of physical chemical characterization of Porcine MPO are consistent with expected structures based on published historical data.
- Further work on the characterization of the heavy subunit and its carbohydrate moiety are on-going
- SDS-PAGE and bioactivity data (not shown here) are consistent with these conclusions.