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Enhancing hypothiocyanite production by lactoperoxidase – mechanism and chemical properties of promotors

Jana Gau [a], Paul-Georg Furtmüller [b], Christian Obinger [b], Jürgen Arnhold [a,c], Jörg Flemmig [a,c,*]

[a] Institute for Medical Physics and Biophysics, Medical Faculty, University of Leipzig, Härtelstraße 16-18, 04107 Leipzig, Germany
[b] Department of Chemistry, Division of Biochemistry, University of Natural Resources and Life Sciences (BOKU) Vienna, Muthgasse 18, 1190 Vienna, Austria
[c] Translational Centre for Regenerative Medicine (TRM) Leipzig, University of Leipzig, Philipp-Rosenthal-Straße 55, 04103 Leipzig, Germany

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ABSTRACT

Background: The heme enzyme lactoperoxidase is found in body secretions where it significantly contributes to the humoral immune response against pathogens. After activation the peroxidase oxidizes thiocyanate to hypothiocyanite which is known for its microbicidal properties. Yet several pathologies are accompanied by a disturbed hypothiocyanite production which results in a reduced immune defense.
Methods: The results were obtained by measuring enzyme-kinetic parameters using UV–vis spectroscopy and a standardized enzyme-kinetic test system as well as by the determination of second order rate constants using stopped-flow spectroscopy.
Results: In this study we systematically tested thirty aromatic substrates for their efficiency to promote the lactoperoxidase-mediated hypothiocyanite production by restoring the native ferric enzyme state. Thereby hydrophobic compounds with a 3,4-dihydroxyphenyl partial structure such as hydroxytyrosol and selected flavonoids emerged as highly efficient promotors of the (pseudo-)halogenating lactoperoxidase activity.
Conclusions: This study discusses important structure-function relationships of efficient aromatic LPO substrates and may contribute to the development of new agents to promote lactoperoxidase activity in secretory fluids of patients.
Significance: This study may contribute to a better understanding of the (patho-)physiological importance of the (pseudo-)halogenating lactoperoxidase activity. The presented results may in future lead to the development of new therapeutic strategies which, by reactivating lactoperoxidase-derived hypothiocyanite production, promote the immunological activity of this enzyme.

1. Introduction

Lactoperoxidase (LPO) belongs to the immunological relevant chordata peroxidases [1]. While the related enzymes myeloperoxidase (MPO) and eosinophil peroxidase (EPO) are expressed in immune cells, LPO is secreted by mucosal glands to body fluids like milk, saliva, tears and airway secretions [2–4]. The immunological function of all three enzymes is mainly attributed to their (pseudo-) halogenating activity [3,5,6]. While all three peroxidases easily oxidize iodide and thiocyanate, bromide is only oxidized by EPO and MPO and at neutral pH and the oxidation of chloride is only known for MPO [7–9]. Considering low iodide (2 μM or less) and much higher thiocyanate concentrations (up to 6 mM in saliva) [10], in secretions the two-electron oxidation of SCN^- by LPO clearly dominates. Thereby hypothiocyanite (^-OSCN) is formed, which is well known for its microbicidal properties [10]. In fact, due to its lower reactivity as compared to e.g. hypochlorous acid (HOCl), hypothiocyanite is more efficient in entering bacterial biofilms [6]. Yet the ^-OSCN-production by LPO is known to be impaired under pathological conditions like cystic fibrosis or at neonatal pathologies, which results in a disturbed immune defense against pathogens [3,11].

Chordata peroxidases are known to catalyze two- and one-

Abbreviations: ABTS, 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid); DB, double bond; DTNB, 5,5′-dithiobis-(2-nitrobenzoic acid); EPO, eosinophil peroxidase; LPO, lactoperoxidase; MPO, myeloperoxidase; SB, single bond; ssp., subspecies; TNB, 5-thio-2-nitrobenzoic acid.

* Corresponding author at: Institute for Medical Physics and Biophysics, Medical Faculty, University of Leipzig, Härtelstraße 16-18, 04107 Leipzig, Germany. Fax: +49 341 9715709.
E-mail addresses: jana.gau@medizin.uni-leipzig.de (J. Gau), paul.furtmueller@boku.ac.at (P.-G. Furtmüller), christian.obinger@boku.ac.at (C. Obinger), juergen.arnhold@medizin.uni-leipzig.de (J. Arnhold), joerg.flemmig@medizin.uni-leipzig.de, joerg.flemmig@uni-leipzig.de (J. Flemmig).

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electron oxidation reactions [2]. Both reaction cycles are initiated by hydrogen peroxide-mediated oxidation of the ferric enzyme to Compound I (i.e. oxoiron(IV)porphyryl radical, $^{+\bullet}$Por-Fe^{IV}=O). In the halogenation cycle Compound I is directly reduced by halides or thiocyanate to the Fe(III) resting state, whereas in the peroxidase cycle Compound I is reduced in two one-electron reduction steps. Here, Compound II (i.e. oxoiron(IV), Por-Fe^{IV}-OH) is formed as an intermediate state which does not participate in the (pseudo-)halogenating activity [2,5,12]. Since the rate-limiting step within the peroxidase cycle is Compound II reduction to the ferric state, typically Compound II accumulates during reaction [3,5,13]. In addition to peroxidase substrate-mediated Compound I reduction to Compound II, in the absence of exogenous electron donors or at very low concentration of the substrates, the protein moiety of LPO can donate electrons thereby forming an alternative Compound I* (with remote radical site and Compound II-like UV–vis spectral signature) [10]. As a consequence, at pathological low SCN^- concentrations (e.g. at cystic fibrosis) Compound II and Compound I* will accumulate, with the latter leading to oxidative damage of LPO and, finally, to enzyme inactivation [11,14]. Formation of Compound I* and accumulation of Compound II of LPO (or MPO) can be overcome in the presence of good one-electron donors that efficiently react with both Compound I and Compound II [15,16]. This restores the ferric enzyme form and, thus, promotes the (pseudo-)halogenating enzyme activity. For LPO, we could recently show that substrates from olive tree (*Olea europaea* L.) leaves having a 3,4-dihydroxyphenyl partial structure are good re-activators of the $^-$OSCN production by LPO [12]. The same holds for a subsequent study on motherwort (*Leonurus cardiaca* L) [17]. Both plants were used in traditional medicine as a remedy against e.g. inflammatory diseases [18,19]. However, so far a systematic and detailed mechanistic investigation of kinetic parameters including the direct interaction of these molecules with all relevant catalytic redox intermediates of LPO was not performed.

This study describes the mechanism of re-activation of the (pseudo-)halogenating activity of LPO by benzoic acid derivatives carrying hydroxyl and methoxy groups at different positions of the aromatic ring as well as by selected phenylethanoids, cinnamic acid derivatives and flavonoids. Steady-state kinetic parameters for the peroxidase activity are presented and discussed with respect to chemical properties (e.g. hydrophobicity) of these molecules. Finally, second order rate constants for the interaction of selected aromatic substrates with LPO Compound I and II were determined by multi-mixing stopped-flow spectroscopy. Both, high degree of hydrophobicity and the presence of a 3,4-dihydroxyphenyl partial structure are important for efficient reduction of LPO Compound II and thus for promotion of its (pseudo-)halogenating activity of LPO.

2. Materials and methods

2.1. Materials

Lactoperoxidase from bovine milk was obtained as a lyophilized powder (≥ 200 U/mg) from Sigma-Aldrich, Steinheim, Germany. Aliquots of the enzyme (5 μM) were prepared in phosphate buffered saline pH 7.4 (PBS, Sigma-Aldrich) and stored at −25 °C. If not otherwise stated final enzyme concentrations of 5 nM (0.08 U/mg) were used.

Lactoperoxidase-mediated $^-$OSCN-formation was quantified by following the oxidation of 5-thio-2-nitrobenzoic acid (TNB) at 412 nm using a Varian Cary 50 UV/vis–spectrophotometer (Mulgrave, Australia). The final TNB concentration in the reaction mixture was 50 μM. Thiocyanate and hydrogen peroxide were used at final concentrations of 2 mM and 20 μM or 80 μM, respectively. Hydrogen peroxide working solution was freshly prepared each day from a 30% stock solution by dilution in Millipore water and stored at 4 °C until use. The concentration of H_2O_2 was determined at 230 and 240 nm (ε_{230}=74 M^{-1} cm^{-1} ε_{240} =43.6 M^{-1} cm^{-1}) [20]. Dibasic sodium phosphate, sodium citrate and sodium chloride (citrate phosphate buffer preparation), 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB, ≥ 98%) for TNB preparation, KSCN (≥ 99%), H_2O_2 and tryptophan (≥ 99%) were obtained from Sigma-Aldrich.

Benzoic acid (≥ 99.5%), 2-hydroxybenzoic acid (≥ 99%), 3-hydroxybenzoic acid (≥ 99%), 4-hydroxybenzoic acid (≥ 99%), 2,3-dihydroxybenzoic acid (≥ 99%), 2,4-dihydroxybenzoic acid (≥ 97%), 2,5-dihydroxybenzoic acid (≥ 99.5%), 2,6-dihydroxybenzoic acid (≥ 97.5%), 3,4-dihydroxybenzoic acid (≥ 97%), 3,5-dihydroxybenzoic acid (≥ 97%), 3-hydroxy-4-methoxybenzoic acid (≥ 97%), 4-hydroxy-3-methoxybenzoic acid (≥ 97%), 3,4-dimethoxybenzoic acid (≥ 99%), 2,3,4-trihydroxybenzoic acid (≥ 97%), 2,4,6-trihydroxybenzoic acid monohydrate (≥ 90%) 2,4,5-trihydroxybenzoic acid, 3,4,5-trihydroxybenzoic acid (≥ 98.5%), cinnamic acid (≥ 99%), *o*-coumaric acid (≥ 97%), *m*-coumaric acid, (≥ 99%), *p*-coumaric acid (≥ 98%), caffeic acid (≥ 8%), ferulic acid (≥ 99%), isoferulic acid (≥ 97%), sinapic acid (≥ 98%), 3,4-dimethoxycinnamic acid (≥ 99%) *m*-tyrosol (≥ 99%), apigenin (≥ 98%), eriodictyol (≥ 95%), naringenin (≥ 98%), luteolin (≥ 98%), (−)-epicatechin (≥ 90%), taxifolin (≥ 85%), chrysin (≥ 98%) and quercetin (≥ 95%) were purchased from Sigma-Aldrich. Hydroxytyrosol (≥ 98%) was obtained by Tokyo Chemical Industries Co., Ltd., Tokyo, Japan, and tyrosol (≥ 98%) from Biopurify Phytochemicals Ltd.

Apigenin, naringenin were dissolved in DMSO (Sigma-Aldrich). The final DMSO concentration in the test system did not exceed 1% (v/v). Control measurements showed no effect of such a solvent concentration on the enzyme activity. All other test substances were dissolved in cold or hot PBS (pH 7.4), depending on their solubility. Stock solutions of 1–20 mM were prepared. Final concentrations up to 5 mM were tested.

2.2. Hypothiocyanite-dependent TNB degradation

All enzyme-kinetic measurements were performed in PBS pH 7.4 at 37 °C using a microplate reader Tecan Infinite 200 PRO (Männedorf, Switzerland). Control measurements in the absence of SCN^- or in the sole presence of H_2O_2 were performed to determine the contribution of the direct LPO-mediated TNB degradation and the H_2O_2-mediated oxidation of TNB, respectively. From these data the initial LPO-mediated $^-$OSCN formation rate was calculated by considering the molar absorption coefficient of TNB (ε_{412}=14.100 M^{-1} cm^{-1}) [21], the length of the optical pathway (0.73 cm) and the stoichiometry of the $^-$OSCN-mediated TNB oxidation [12]. For all tested substances a clear dependence between substrate concentrations and $^-$OSCN formation rate was found, often showing a Michaelis-Menten-like correlation. Thus, Lineweaver-Burk plots were used to determine enzyme kinetic parameters (V_{max}, K_m, k_{cat}, specificity constant).

All measurements were performed at least in triplicate. Before calculating the LPO-mediated $^-$OSCN formation rate the initial TNB degradation in the absence of thiocyanate (only LPO and H_2O_2) was subtracted from the TNB degradation rate determined for the whole LPO–H_2O_2–SCN^- system. This was necessary to consider the degradation of TNB by the LPO–H_2O_2 system in the absence of SCN^- [12]. Thereby while the mean values were subtracted the corresponding standard deviations were calculated according to the law of error propagation. Significance was tested using two tailed *t*-test. The indicated stars correspond to *p* values below 0.05 (*), 0.01 (**) and 0.001 (***), respectively. As the K_m and V_{max} values of the tested substances were determined via a