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Formation of cyanogen iodide by lactoperoxidase

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ABSTRACT

The haem protein lactoperoxidase (LPO) is an important component of the anti-microbial immune defence in external secretions and is also applied as preservative in food, oral care and cosmetic products. Upon oxidation of SCN^- and I^- by the LPO–hydrogen peroxide system, oxidised species are formed with bacteriostatic and/or bactericidal activity. Here we describe the formation of the inter(pseudo)halogen cyanogen iodide (ICN) by LPO. This product is formed when both, thiocyanate and iodide, are present together in the reaction mixture. Using ^{13}C nuclear magnetic resonance spectroscopy and gas chromatography–mass spectrometry we could identify this inter(pseudo)halogen after applying iodide in slight excess over thiocyanate. The formation of ICN is based on the reaction of oxidised iodine species with thiocyanate. Further, we could demonstrate that ICN is also formed by the related haem enzyme myeloperoxidase and, in lower amounts, in the enzyme-free system. As I^- is not competitive for SCN^- under physiologically relevant conditions, the formation of ICN is not expected in secretions but may be relevant for LPO-containing products.

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1. Introduction

Primarily isolated from bovine milk, the haem protein lactoperoxidase (LPO) is a crucial component of immune defence against pathogens [1]. Chemically and immunologically very similar proteins, here also referred to as lactoperoxidase, are found in secretions from human mammary, salivary, and lacrimal glands, and secretory glands of the upper airways, where they exert an anti-microbial activity together with hydrogen peroxide and thiocyanate [2]. Hydrogen peroxide activates ferric LPO to the oxo-ferryl species Compound I that oxidises thiocyanate to hypothiocyanite (^-OSCN) by abstracting two electrons. The rate of this oxidation is 2×10^8 M^{-1} s^{-1} at pH 7.0 and 15 °C [3]. This species is in equilibrium with hypothiocyanous acid (HOSCN) with a pK_a value of 5.3 [4]. The weak oxidant HOSCN/^-OSCN is able to permeate through cell membranes and penetrates into microorganisms of biofilms, where it preferentially oxidises low molecular weight thiols and inactivates enzymes with functional sulfhydryl groups [5,6]. This efficient anti-microbial activity is further supported by the high yield of SCN^- in secretions. In saliva, thiocyanate levels are around 0.5–4 mM, which strongly depend on diet, smoking habit, and time of day [7–9]. In upper airway fluids, about 400 μM SCN^- is present [10]. The LPO–H_2O_2 system also oxidises I^- with a high rate of 1.2×10^8 M^{-1} s^{-1} at pH 7.0 and 15 °C [3]. However, in secretions the iodide concentration is in the low micromolar range [9]. Thus, a significant contribution of iodide to immune defence in secretory epithelia is unlikely.

Due to the low price and easy availability, the anti-microbial LPO system is widely applied as preservative in food as well as in oral care, cosmetic and other products [2,11]. In these applications, LPO is used together with a hydrogen peroxide generating system and thiocyanate and/or iodide. The oxidation of I^- by Compound I of LPO results in the formation of hypoiodous acid (HOI), with a pK_a of 10.6 [12] and other oxidised iodine species such as I_2, I_3^-, ^-OI, and I_2OH^- [13]. In contrast to hypothiocyanite, oxidised iodine products are directed against a broader range of molecules: They target peptides and proteins with sulfhydryl and thioether moieties and incorporate iodine into tyrosine residues and oxidise NAD(P)H [12,14,15]. While the LPO–H_2O_2 system exhibits a bacteriostatic activity against *Escherichia coli* in the sole presence of SCN^-, this system acts bactericidal when I^- is used instead [16,17]. A further study demonstrated the antiviral activity of the LPO–H_2O_2–I^- system against the respiratory viral pathogens adenovirus and respiratory syncytial virus whereas the LPO–H_2O_2–SCN^- was ineffective [18].

The stronger bactericidal effect of the LPO–H_2O_2 system in the presence of I^- as compared to SCN^- is also demonstrated by experiments where both ions were applied together: Increasing amounts of thiocyanate reduced or abolished the bactericidal effect of I^- [19–21]. In contrast, the addition of 2.5 mM potassium iodide to a mixture of LPO–H_2O_2–saliva (containing 0.3–2.5 mM SCN^-) strongly reduced the viability of *Fusobacterium nucleatum* (60 ± 22%) compared to LPO–H_2O_2–saliva alone (89 ± 2%), but was only slightly more effective than an enzyme-free mixture (KI–H_2O_2–saliva, 66 ± 18%) [22]. A supplementation with iodide was also more effective in the case of *Candida*

Abbreviations: GC–MS, gas chromatography–mass spectrometry; HS, headspace; LPO, lactoperoxidase; MPO, myeloperoxidase; NMR, nuclear magnetic resonance.

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albicans, *Escherichia coli* and *Staphylococcus aureus* as compared to the sole LPO–H_2O_2–SCN^- system [23].

It remains unknown whether the results of the mixed application of SCN^- and I^- with the LPO–H_2O_2 system can solely be explained by competitive effects between these ions for Compound I, or whether in addition to hypothiocyanite and oxidised iodine species some yet unknown inter(pseudo)halogens are formed that may contribute to the cytotoxic effect. Therefore, we analysed in the present work the species formed by the LPO–H_2O_2–SCN^-/I^- system by ^{13}C nuclear magnetic resonance (NMR) spectroscopy and gas-chromatography–mass spectrometry (GC–MS). We could identify and characterise the inter(pseudo)halogen cyanogen iodide (ICN) as a yet unknown lactoperoxidase product. This product was also formed by applying the haem protein myeloperoxidase (MPO) or an enzyme-free system.

2. Materials and methods

2.1. Materials

Lactoperoxidase from bovine milk was purchased from Sigma-Aldrich (Deisenhofen, Germany). 800 μM aliquots of LPO were prepared in 100 mM potassium phosphate buffer and stored at −25 °C. Enzyme purity was 0.8 (absorbance ratio 412 nm/280 nm). Its concentration was determined using $\varepsilon_{412} = 112{,}000\ M^{-1}\ cm^{-1}$ [3]. Human myeloperoxidase was obtained from Planta Natural Products (Vienna, Austria) at a purity of 99.5% (according to the absorbance ratio 430 nm/280 nm). Its concentration was determined using $\varepsilon_{430} = 91{,}000\ M^{-1}\ cm^{-1}$ per haem [24]. ^{13}C-enriched (^{13}C 95–99%) and ^{13}C–^{15}N-enriched (^{13}C 99%, ^{15}N 98%) potassium thiocyanate was obtained from Cambridge Isotope Laboratories (Tewksbury, USA) at a purity of 95%. Unlabelled cyanogen iodide (97.5% purity) was purchased from Acros Organics (Geel, Belgium). Deuterium oxide, hydrogen peroxide as a 30% solution, potassium iodide (99% purity) and all other chemicals were purchased from Sigma-Aldrich (Deisenhofen, Germany). The concentration of a stock solution of hydrogen peroxide was spectroscopically determined immediately prior to use ($\varepsilon_{230} = 74\ M^1\ cm^{-1}$, [25]).

2.2. Peroxidase-dependent and -independent oxidation of SCN^- and I^-

KSCN (40 mM) was mixed with KI in different ratios (1:1–1:8) in 0.1 M phosphate buffer (pH 7.0), at 22 °C in the presence or absence of 4 μM LPO. Ten portions of hydrogen peroxide (final concentration 10 mM) were added every 30 s to initiate (pseudo-)halide oxidation. Afterwards, the samples were measured immediately. In experiments concerning the pH dependence of the reaction, 100 mM phosphate buffer (pH 6.0–8.0) or 200 mM/100 mM citrate-phosphate buffer (pH 5.0) was used.

In samples in which LPO should be removed, the solution was filtered through a filter with a cut-off of 30 kDa (VWR International, Darmstadt, Germany) by centrifugation (8 min, 15,000 ×*g*). In selected experiments, the LPO–H_2O_2-reaction was performed in the sole presence of SCN^- or I^-. After filtration, either I^- (to oxidised SCN^-) or SCN^- (to oxidised I^-) was added to yield a final ratio of 1:2 between SCN^- and I^-.

In similar experiments, MPO was used instead of LPO. Selected control experiments were also performed in the absence of peroxidases. If mentioned in the text, ^{13}C- or ^{13}C^{15}N-labelled thiocyanate was used.

2.3. ^{13}C NMR measurements

^{13}C NMR measurements were performed at a Bruker Avance III 600 MHz spectrometer (Bruker Biospin GmbH, Rheinstetten, Germany) equipped with a 5 mM broad-band probe. 10% D_2O was used as frequency lock. All measurements were performed at 6 °C. Chemical shifts were referenced to trimethylsilyl propanoic acid, which was set to −3.1 ppm. The Bruker software Topspin™ 2.1 was used for data analysis.

2.4. Headspace gas chromatography–mass spectrometry

Headspace (HS) GC–MS analysis was carried out on a Shimadzu QP-2010 GC-EI-quadrupole-MS equipped with a DB5-MS capillary column (J&W Fisher, 30 m, 0.25 mM id, 0.25 μM film) connected to an HS-20 headspace injector (all modules from Shimadzu, Kyoto, Japan). Headspace injection was done in loop mode (0.5 min load time and 1 min injection time) with helium as carrier gas at a column flow of 0.95 mL/min and a split of 10:1. The HS oven was set to 60 °C, sample and transfer line to 160 °C with a pressurising gas pressure of 90 kPa and 1 min pressurising time. The GC programme started at 35 °C, held for 1 min and ramped with 10 °C/min to 75 °C. The electron impact ion source operated at 200 °C and 70 eV scanning from *m*/*z* 45–300.

3. Results

3.1. Analysis of the LPO–H_2O_2–SCN–I^- reaction by ^{13}C NMR spectroscopy

Mixed preparations of SCN^- and I^- together with the LPO–H_2O_2 system exhibits stronger and broader anti-microbial effects than the sole application of thiocyanate [19–21,23]. In order to analyse whether a new oxidation product is formed in this system, we used ^{13}C NMR spectroscopy to identify the nature of prospective ^{13}C-labelled products. ^{13}C-labelled thiocyanate (133.4 ppm) was oxidised by the LPO–H_2O_2 system to hypothiocyanite ($^-$OSCN) with a chemical shift of 126.8 ppm (Fig. 1A). There was also a small peak for cyanate at 128.2 ppm. The formation of $^-$OSCN and $^-$OCN by the LPO–H_2O_2–SCN^- system agrees well with literature data [26]. Chemical shift data given for $^-$OSCN/HOSCN range from 126.5 ppm to 127.7 ppm and depend apparently on pH [26–28].

In the additional presence of iodide (Fig. 1B-F), the peak for $^-$OSCN completely vanished and a new, singlet signal appeared with a chemical shift of approximately 51.0 ppm. With increasing iodide concentration, the position of this signal shifted slightly downfield from 51.0 ppm (SCN^-:I^- = 1:2) to 53.2 ppm (SCN^-:I^- = 1:8). In Fig. 1G an overlay of the spectra shown in (B–F) is given, illustrating again the dependence of the position of the new product on the applied SCN^-/I^- ratio.

The direct comparison of the spectra indicates also different product yields with increasing iodide. In fact, as shown in Fig. 2A, in case of a 1:1 ratio for SCN^-/I^- neither $^-$OSCN nor the signal at 51–53 ppm was detected, whereas a maximum intensity of the 51–53 ppm signal was observed at a 1:2 ratio. At higher ratios, its generation decreased. This signal did not change its intensity over 2 h upon storage of samples at 6 °C (data not shown), indicating a considerable stability of the corresponding new product under the chosen experimental conditions. By comparing the integral intensities of signals in ^{13}C NMR spectra, and using the signal at 133.6 ppm for SCN^- (40 mM) as reference, the product at 51–53 ppm has a concentration of 5.6 ± 0.4 mM (n = 3) at a 1:2 ratio between SCN^- and I^-.

We further analysed which influence the pH value has on the formation of the 51–53 ppm product (Fig. 2B). A decrease in pH to 5.0 and 6.0 reduced the amount of detectable product as compared to pH 7.0 while an increase to 8.0 slightly increased the product formation. The higher values observed under more alkaline conditions may indicate a more efficient product formation or its higher stability.

3.2. Formation of the product at 51–53 ppm by the LPO–H_2O_2–SCN–I^--system is based on iodide oxidation

Next, we analysed whether the oxidation of SCN^- or I^- is required for the formation of the new product. The lactoperoxidase reaction was run first with S^{13}CN$^-$. After removing the enzyme by ultrafiltration, the double amount of I^- was added. In the ^{13}C NMR spectrum, only the

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