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Formation of cyanogen iodide by lactoperoxidase

Denise Schlorke [a,*], Jörg Flemmig [a], Claudia Birkemeyer [b], Jürgen Arnhold [a]

[a] Institute for Medical Physics and Biophysics, Medical Faculty, University of Leipzig, Leipzig, Germany
[b] Institute of Analytical Chemistry, Department of Chemistry and Mineralogy, University of Leipzig, Leipzig, Germany

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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 $2 \times 10^8\ M^{-1}\ s^{-1}$ 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 $1.2 \times 10^8\ M^{-1}\ s^{-1}$ 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.

* Corresponding author at: Institute for Medical Physics and Biophysics, Medical Faculty, University of Leipzig, Härtelstr. 16-18, 04107 Leipzig, Germany.
E-mail address: denise.schlorke@medizin.uni-leipzig.de (D. Schlorke).

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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 ε_{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 ε_{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 (ε_{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

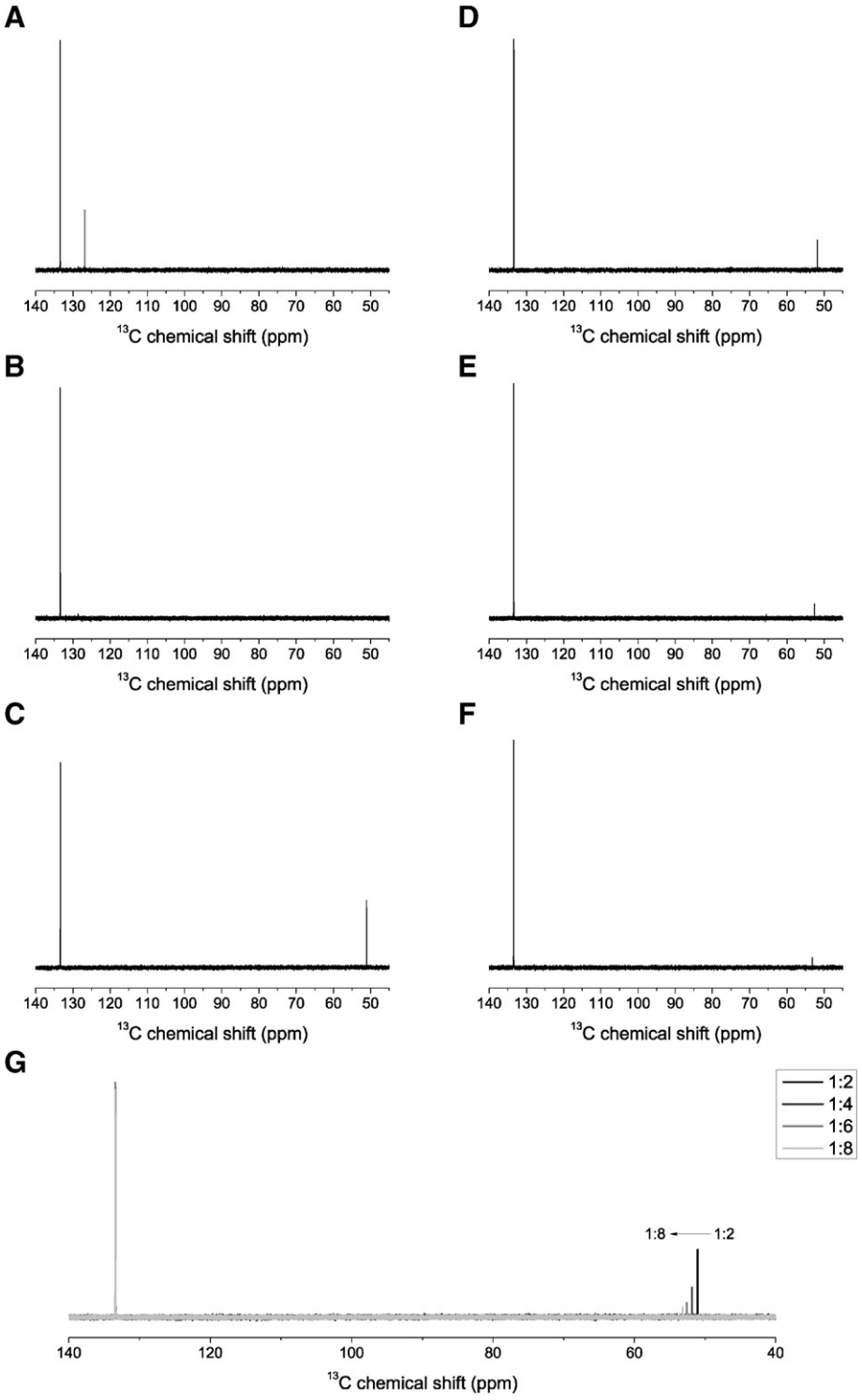

Fig. 1. ^{13}C NMR spectra of the LPO–H_2O_2–SCN^- reaction products in dependence on the I^- concentration. 40 mM $S^{13}CN^-$ was mixed with 0 mM (A), 40 mM (B), 80 mM (C), 160 mM (D), 240 mM (E) or 320 mM (F) I^- (corresponding to ratios of 1:1 (B) up to 1:8 (F)) and 4 μM LPO in 100 mM phosphate buffer, pH 7.0 at 22 °C. (Pseudo)halide oxidation was started by H_2O_2. Ten portions of H_2O_2 (final concentration 10 mM) were added every 30 s. ^{13}C NMR measurements were conducted at 6 °C. (G) is an overlay of the spectra shown in (C–F). Representative spectra of three independent experiments are given.

signals for $^-$OSCN (126.8 ppm) and $^-$OCN (128.2 ppm) were detected (Fig. 3A). Contrary, when two portions of I^- were applied at first together with LPO and H_2O_2, followed by addition of one portion $S^{13}CN^-$, the product with the peak at 51.0 ppm was formed (Fig. 3B). Thus, this product is derived from the interaction of oxidised iodine species with thiocyanate and a role of hypothiocyanite in this reaction can be excluded.

3.3. Formation of the product at 51–53 ppm by myeloperoxidase and in the enzyme-free system

Instead of LPO we next tested whether the haem protein MPO performs the similar reaction. This enzyme is also well known to oxidise both, I^- and SCN^- [24]. As with LPO, the MPO–H_2O_2 system did not

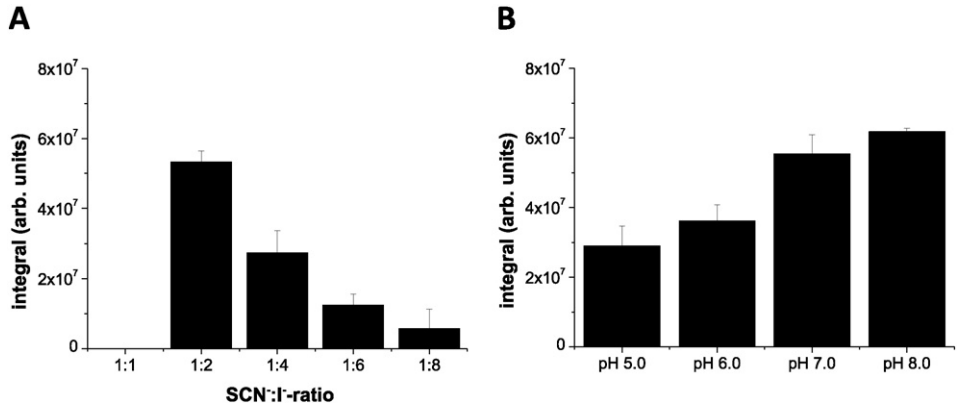

Fig. 2. Dependence of the intensity of the newly formed product at 51–53 ppm on the SCN^-/I^- ratio (A) and pH (B). (A) Samples were prepared as given in Fig. 1. In (B), the LPO-driven reaction was performed at pH values from 5.0 to 8.0 at a ratio of 1:2 SCN^- to I^-. Citrate-phosphate-buffer (200 mM/100 mM) at pH 5.0, or a 100 mM phosphate buffer at pH 6.0 to 8.0 was applied. Mean and standard deviation of three independent experiments are given.

produce the new product at a 1:1-ratio of SCN^- and I^- (Fig. 4A). However, using a ratio of 1:2 between $S^{13}CN^-$ and I^-, a comparable strong signal at 51.0 ppm was detected as observed for LPO. Again, its intensity was reduced at a 1:4-ratio. Alternatively, this peak also appeared when first I^- was oxidised by the MPO–H_2O_2 system followed by addition of $S^{13}CN^-$, but not, when first $S^{13}CN^-$ and then I^- were added (data not shown). These results are also in line with those observed for LPO.

Similar experiments were also performed in the absence of LPO or MPO. The addition of H_2O_2 to a mixture of I^- and $S^{13}CN^-$ caused also the formation of this product between 50.6–53.2 ppm for all tested SCN^-/I^- ratios (1:1–1:8). However, in comparison to the enzyme system, the product was already formed when a 1:1-ratio of SCN^- and I^- was applied, but the yield of this product was generally lower (Fig. 4B). At a 1:2-ratio, its amount was by 73% lower in the enzyme free-system than in the LPO-dependent reaction. As in the enzyme-dependent reactions, we observed the highest yield at a SCN^-/I^- ratio of 1:2. At higher concentrations the production was reduced, yet, without concentration dependence.

Applying a rough quantitation comparing the integral intensities in NMR spectra, the product at 51–53 ppm has a concentration of 7.7 ± 0.3 mM (n = 3) for the MPO–H_2O_2 system at a 1:2 ratio between SCN^- and I^-. At this anion ratio, 1.5 ± 0.4 mM of this product were produced in the enzyme-free system.

3.4. Identification of the product at 51–53 ppm by ^{13}C NMR spectroscopy

Upon interaction of SCN^- with I_2, the formation of the inter(pseudo)halogen cyanogen iodide (ICN) was described [29,30]. Moreover, the ^{13}C NMR chemical shift of ICN was determined to be 50.39 ppm [31]. Hence, we analysed commercially available unlabelled ICN by ^{13}C NMR and actually found a small peak with a chemical shift at 50.1 ppm for the pure compound (Fig. 5). Interestingly, we observed the same pattern of downfield shifting upon addition of I^- as in experiments with LPO: additions of KI in two portions shifted the position of this peak to 51.1 ppm (ICN/I^- ratio 2.2:1) and further to 51.8 ppm (ICN/I^- ratio 1.1:1). The only difference to the LPO system was a broadening of the peak. This is apparently caused by using unlabelled ICN.

3.5. Identification of ICN by gas chromatography mass spectrometry as LPO product

In order to confirm the formation of ICN as reaction product of the LPO–H_2O_2–SCN^-–I^- system, we performed headspace GC–MS analysis (Fig. 6). Similar to ^{13}C NMR spectroscopy measurements, we did not observe any product formation when equimolar concentrations of SCN^- and I^- were applied (Fig. 6A). However, when we altered the SCN^-/I^- ratio to 1:2 (Fig. 6B), we detected a product whose identity was approved as ICN by comparing the retention time and mass spectrum with an authentic standard (not shown). A further proof was provided when we applied $S^{13}CN^-$ (Fig. 6C) and $S^{13}C^{15}N^-$ (Fig. 6D). In these corresponding spectra, the molecular ion of $ICN^{+\bullet}$ detected at an *m/z*-value of 152.9 shifted to *m/z* 153.9 and *m/z* 154.9 due to enhanced molecular masses of 1 u and 2 u, respectively, for the labelled molecular ions. The peaks at *m/z* 76.5 (Fig. 6B), 77.0 (Fig. 6C) and 77.5 (Fig. 6D) are suggested as the corresponding, doubly charged molecular ions (ICN^{2+}), confirmed by their isotopic pattern (not

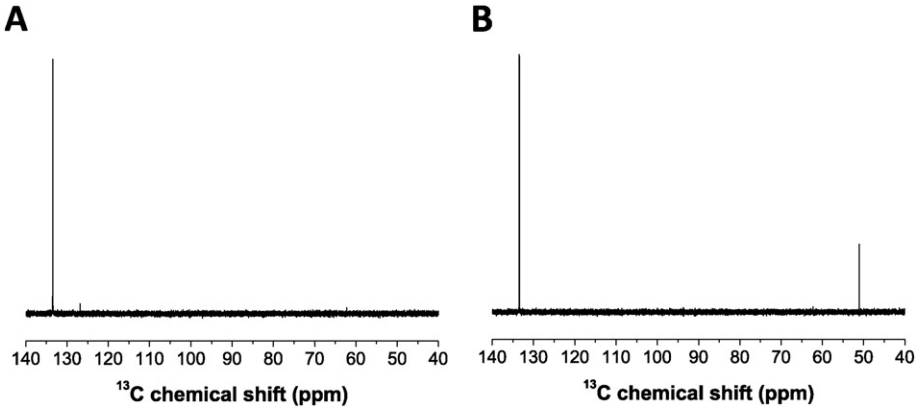

Fig. 3. Effects of the sequential addition of SCN^- and I^- on the formation of the novel product. (A) ^{13}C NMR spectra were taken after the addition of I^- to the ultrafiltrate of the LPO–H_2O_2–$S^{13}CN^-$ system. In (B), $S^{13}CN^-$ was added to the ultrafiltrate of the LPO–H_2O_2–I^- system. In both cases, a ratio of 1:2 between SCN^- and I^- was applied. Representative spectra of three independent experiments are given.

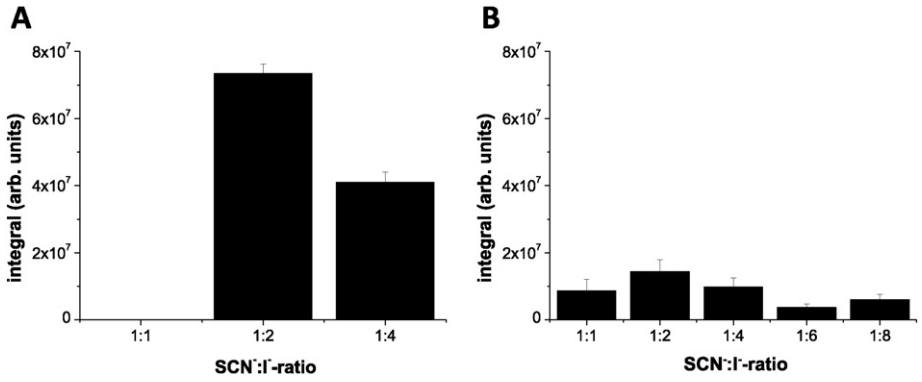

Fig. 4. Formation of the new product in the presence of MPO and in the enzyme-free system. (A) 40 mM $S^{13}CN^-$ was mixed with 40–160 mM I^- and 4 μM MPO in 100 mM phosphate buffer, pH 7.0 at 22 °C. Upon addition of H_2O_2 (final concentration 10 mM), the reaction was started. The intensity of the signal at 51–53 ppm is given in arbitrary units. (B) H_2O_2 (10 mM) was incubated with I^- (40–320 mM) and $S^{13}CN^-$ (40 mM). Mean and standard deviation of three independent experiments are given.

shown). Furthermore, we could observe a fragmentation pattern with an I^+ in all spectra at *m/z* 127.0 as well as *m/z* 139.0 for IC^+ (*m/z* 140.0 for $I^{13}C^+$) derived from the cleavage of nitrogen. The latter species formed for SCN^- samples at *m/z* 139.0 and at *m/z* 140.0 for the $S^{13}CN^-$ and $S^{13}C^{15}N^-$ samples.

Taken together, the data comprising ^{13}C NMR spectra and headspace GC–MS analysis reveal that cyanogen iodide is indeed a reaction product formed by LPO and MPO when SCN^- and I^- are both present in the reaction mixture with H_2O_2.

4. Discussion

The anti-microbial lactoperoxidase–hydrogen peroxide–I^-/SCN^- system is able to form the inter(pseudo)halogen species cyanogen iodide ICN as revealed by ^{13}C NMR spectroscopy and confirmed by headspace GC–MS. Thereby, an oxidised iodine species, most likely hypoiodous acid (HOI) or I_2, reacts with SCN^- as shown by sequential addition of (pseudo-)halide ions. In contrast, the addition of I^- to preformed hypothiocyanite did not generate ICN. The formation of ICN was also observed by replacing LPO by MPO and in the absence of these haem enzymes. In the latter case, however, a considerably lower amount of ICN was formed. Thus, while hydrogen peroxide itself is also able to oxidise I^- [32] and, consequently, leads to the formation of ICN in the presence of SCN^-, the LPO–H_2O_2 system (and also the MPO–H_2O_2 system) strongly accelerates the I^- oxidation, resulting in a more efficient ICN formation if SCN^- is present.

In the chemical literature, the reaction between iodine and SCN^- has been known for more than a hundred years [29,30,33], whereby the following equation was given for the reaction in neutral and slightly basic media:

$$4I_2 + SCN^- + 4H_2O \rightarrow SO_4^{2-} + 7I^- + 8\,H^+ + ICN.$$

Under strong acidic conditions, a protonated complex (HI_2SCN) between I_2 and SCN^- is formed, from which the inter(pseudo)halogen ISCN is derived [34,35]. However, under our neutral experimental conditions, we did not detect any other interhalogen species than ICN.

The slight downfield shift of the ^{13}C NMR signal for ICN at increasing I^- concentrations can be explained by the reversible formation of the complex ion I_2–CN^- resulting from the reaction between I^- and ICN [36]. In our LPO experiments, the ICN signal shifted from 51.0 to 53.2 ppm with increasing iodide. Applying commercial ICN, this range was 50.1 (without iodide addition) to at least 52.9 ppm (1:2 ratio of ICN to KI, data not shown). The low value for pure ICN of 50.1 ppm is apparently caused by the fact that no iodide was present in this sample. As all LPO (and MPO) samples contained a certain amount of iodide, it is not surprising that chemical shifts were not observed lower than 51.0 ppm under these conditions. In ICN, iodine is slightly positively charged as the electron pair of the iodine-cyanide bond is pulled closer to the cyanide group [37]. This property favours the formation of the above mentioned complex with I^-.

In ^{13}C NMR experiments with the LPO–H_2O_2 system, the highest yield of ICN was observed applying a SCN^-/I^- ratio of 1:2. The decrease in ICN formation at higher I^- concentrations might have several reasons. Increasing I^- concentrations can affect binding of hydrogen peroxide to the active site of LPO and can disturb, thus, LPO activity similarly

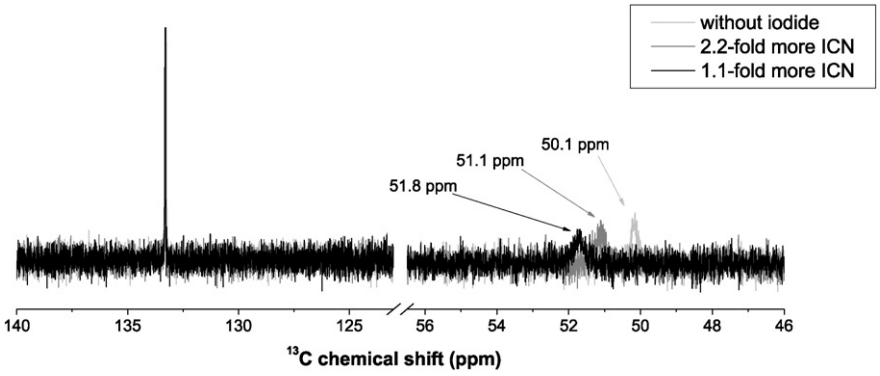

Fig. 5. ^{13}C NMR overlay spectrum of unlabelled ICN in the presence of KI. The addition of I^- to a mixture of unlabelled ICN and $S^{13}CN^-$ caused a downfield shift of the peak for ICN from 51.0 ppm to 51.8 ppm. ^{13}C NMR spectra were taken at 6 °C under the following conditions: 178 mM ICN + 10 mM $S^{13}CN^-$ (light grey spectrum); 165 mM ICN + 9.3 mM $S^{13}CN^-$ + 74 mM KI (dark grey spectrum); and 153 mM ICN + 8.6 mM $S^{13}CN^-$ + 138 mM KI (black spectrum). All measurements were done in 100 mM phosphate buffer pH 7.0. Selected spectra of three independent experiments are shown.

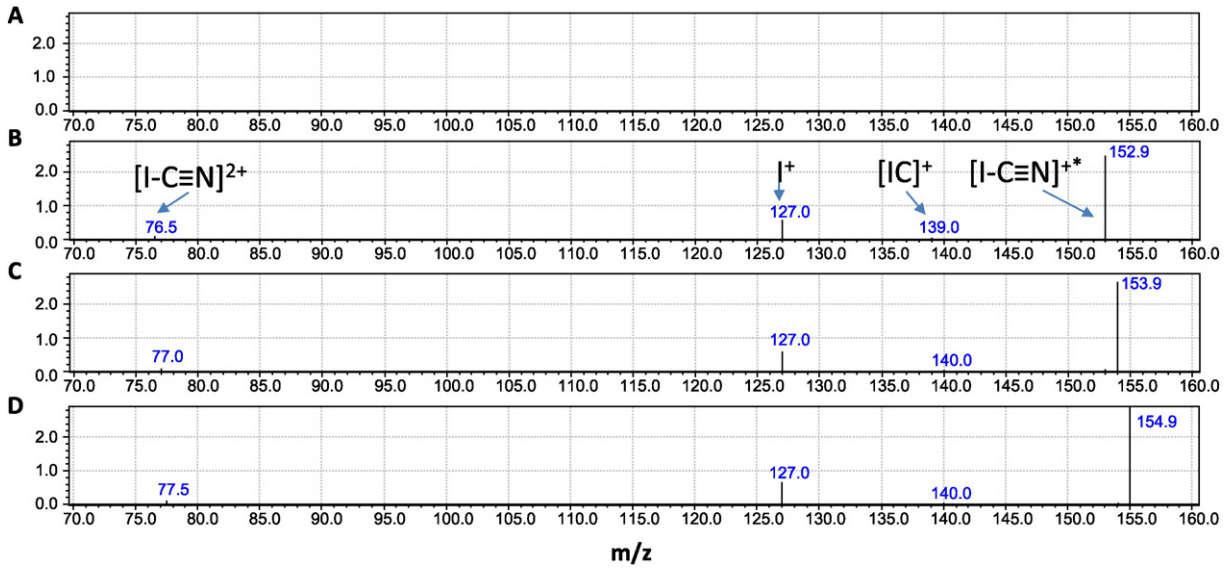

Fig. 6. Headspace GC–MS analysis of the reaction products of the LPO–H_2O_2–SCN–I^- system. Mass spectra at 3.55 min retention time in the corresponding chromatograms are given for the reaction of 4 μM LPO with 10 mM H_2O_2 and 40 mM I^- in the presence of (A) 40 mM SCN^-, (B) 80 mM SCN^-, (C) 80 mM $S^{13}CN^-$ and (D) 80 mM $S^{13}C^{15}N^-$. Selected chromatograms of three independent experiments are shown.

as has been shown for SCN^- and H_2O_2 [38]. Another aspect concerns the complex chemistry of oxidised iodine species [39]. Increased I^- concentrations may favour the formation of I_3^- from I_2 and I^- and decrease, thus, the concentration of active oxidised iodine intermediates.

Although the formation of ICN from I_2 and SCN^- has been well known for more than 100 years, its production was never described before in connection with haem peroxidases. The formation of ICN by LPO and MPO is not expected under physiologically relevant conditions as iodide concentration is usually rather low in biological fluids [9,40]. Considering millimolar concentrations of SCN^- in saliva [7–9] or around 400 μM SCN^- in upper airway secretions [10], thiocyanate is preferentially oxidised by the LPO–H_2O_2 system with hypothiocyanite as the dominating product. Thus, I^- is not competitive as LPO substrate for SCN^- under these conditions [41]. Although MPO is normally not released into secretions, this enzyme can occur in these fluids under inflammatory conditions [42]. In contrast to LPO, MPO oxidises also chloride and bromide with significant rate [24]. Considering (pseudo-)halide concentrations and reaction rates, SCN^- is the preferred target for MPO in saliva, while in plasma both Cl^- and SCN^- will be oxidised to a comparable extent by the enzyme [43].

However, in numerous applications of LPO as anti-microbial agent, conditions exist favouring the formation of ICN. In our experiments, the most intense formation of ICN was found at a 1:2-ratio between SCN^- and I^-. This agrees well with the second order rate constants of LPO Compound I with SCN^- and I^-. At 15 °C and pH 7.0, the rate of LPO Compound I with SCN^- is about 1.7 times higher than for I^- [3]. Another factor is the competition between SCN^- and I^- for the binding near the haem. As iodide oxidation is the prerequisite for ICN formation, this product will be formed under conditions where the binding of I^- dominates over the binding of SCN^-.

Furthermore, at a given ratio between both ions, we found an increased production of ICN by LPO with increasing pH. At pH values lower than 7.0, the yield of ICN was reduced. We suggest that this might be mainly caused by the pH dependence of the reaction between HOI or I_2 and SCN^- [34,35] since the (pseudo-)halogenating activity of LPO depends on the concentration ratio between SCN^- and H_2O_2 [38] and should have an optimum activity around pH 5 under our experimental conditions, where this ratio is <10.

It is generally believed that ICN is a very toxic agent [44]. However, it remains unknown to what extent ICN will contribute to the anti-microbial activity of LPO-derived oxidants. Mechanistic data about toxic reactions of ICN are very scarce. ICN induces a rapid and irreversible swelling of the gram-negative bacterium *Pseudomonas aeruginosa* and modifies sulfhydryls in these cells [45,46]. Cultures of the marine diatom *Nitzschia cf pellucida* produce cyanogen bromide and cyanogen iodide in a haloperoxidase-dependent reaction and use them to control the growth of competing microalgae [47]. Thus, at present we can only speculate about any microbicidal activity of ICN in LPO applications. Due to its low boiling point, ICN is volatile [44] and can exert its activity also in close neighbourhood of its production place.

Cyanogen chloride (ClCN) and cyanogen bromide (BrCN) are related inter(pseudo)halogens to ICN. Their formation has been observed after reaction between the corresponding hypohalous acid and cyanide [31,48]. Cyanogen chloride is also formed upon exposure of HOCl to cyanocobalamin [49]. However, the roles of ClCN and BrCN in haem-dependent oxidation reactions and in the anti-microbial system have to be determined as well.

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