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Detection of hydrogen peroxide by lactoperoxidase-mediated dityrosine formation

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Abstract
The aim of this work was to study the dityrosine-forming activity of lactoperoxidase (LPO) and its potential application for measuring hydrogen peroxide (H_2O_2). It was observed that LPO was able to form dityrosine at low H_2O_2 concentrations. Since dityrosine concentration could be measured in a simple fluorimetric reaction, this activity of the enzyme was utilized for the measurement of H_2O_2 production in different systems. These experiments successfully measured the activity of NADPH oxidase 4 (Nox4) by this method. It was concluded that LPO-mediated dityrosine formation offers a simple way for H_2O_2 measurement.

Keywords: *Lactoperoxidase, dityrosine, NADPH oxidase 4, hydrogen peroxide, reactive oxygen species*

Abbreviations: *LPO, lactoperoxidase; MPO, myeloperoxidase; EPO, eosinophil peroxidase; TPO, thyroid peroxidase; Nox4, NADPH oxidase 4; ROS, reactive oxygen species; HPLC, high performance liquid chromatography; MS, mass spectrometry; DT, dityrosine.*

Introduction

Peroxidases are heme-containing enzymes with highly conserved structure, serving diverse functions in the plant and animal kingdom [1]. Peroxidases catalyse the oxidation of various substrates in the presence of H_2O_2. Mammalian peroxidases have an important role in several physiological processes including host defense and hormone biosynthesis. The family of mammalian peroxidases consists of myeloperoxidase (MPO), eosinophil peroxidase (EPO), lactoperoxidase (LPO), thyroid peroxidase (TPO) and the mammalian peroxidasin [1]. MPO, EPO and LPO have antimicrobial activity and serve in the first line of host defense [2,3]. These enzymes oxidize halides using hydrogen peroxide. The reactions result in the formation of antimicrobial compounds such as hypochlorite (OCl^-) or hypothiocyanate ($OSCN^-$). The expression of TPO is restricted to the thyroid gland, where it has an essential role in hormone biosynthesis. TPO mediates the synthesis of reactive iodide species from iodide ions and H_2O_2. Peroxidases can also catalyse the formation of dityrosine from tyrosine. In lower species such as sea urchin and *C. elegans* the formation of dityrosine residues by peroxidases contributes to the formation of the extracellular matrix [4]. During the fertilization of sea urchin eggs, H_2O_2 is produced by the Udx1 NADPH oxidase [5]. Ovoperoxidase, a peroxidase

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which is present in high amount in sea urchin eggs, uses H_2O_2 to cross-link secreted proteins through dityrosine bridges [6]. This reaction has an important role in hardening the fertilization envelope, which prevents polyspermy. In *C. elegans* the formation of dityrosine bridges helps to stabilize the cuticle of the worm. Genetic evidence suggests that a Duox enzyme provides hydrogen peroxide for this reaction [4]. The exact nature of the peroxidase is still a matter of debate, since Duox enzymes contain a peroxidase-like domain suggesting that the ability to form and utilize hydrogen peroxide resides in one protein. This domain, however, lacks key residues which are present in all animal peroxidases and seem to be essential for heme binding. In an alternative model, we have previously suggested that *C. elegans* Duox may cooperate with a hitherto unidentified peroxidase in the formation of dityrosine cross-links, similar to sea urchin fertilization [7]. The ability to form dityrosine residues is not restricted to peroxidases of lower species. Mammalian peroxidases including MPO and LPO were both described to catalyse the formation of dityrosine [8,9]. The physiological significance of this reaction is still unclear, although the presence of dityrosine was detected in mammalian proteins under pathological conditions [10,11]. The mammalian peroxidase LPO is present in exocrine secretions, including saliva, milk, tear and bronchial secretion [3,12]. We have originally suggested that Duox enzymes provide H_2O_2 for LPO on mucosal surfaces, thus establishing the Duox-LPO host defense system [13]. This model has recently gained support from other groups as well [14,15]. In the antimicrobial reaction, thiocyanate (SCN^-) is the target of LPO-mediated oxidation, however LPO, similarly to other peroxidases, can also stimulate the formation of dityrosine [9]. Since dityrosine is an easily detectable fluorescent product, we were interested if the formation of dityrosine by LPO can be used for the detection of Nox-derived H_2O_2. In this report we show that detection of the LPO-formed dityrosine seems to be a simple and efficient method to detect the enzymatic activity of NADPH oxidases and other sources of H_2O_2.

Materials and methods

Dityrosine detection by fluorimetry

All reactions were carried out in a HEPES-based buffer (H medium) at 37°C [16]. After 30 min of incubation dityrosine production was detected in a fluorescence plate reader at excitation and emission wavelengths of 320 and 405 nm, respectively. The reaction volume was 100 μl.

A 2 mM stock solution was prepared from L-tyrosine (Sigma, St. Louis, MO, USA) and the final concentration in the reaction mixture was diluted to 1 mM. We used bovine LPO (Sigma) for the experiments in a final concentration of 0.5–5 μg/ml.

Preparation of Nox4 expressing cell line

Freestyle 293F (293F) cells (Invitrogen, Carlsbad, CA, USA) were transfected with pcDNA3.1 containing Nox4 cDNA. After incubation in 800 μg/ml Geneticin-supplemented media, surviving clones were selected. Nox4-expressing stable clones were identified by PCR and Northern blot analyses.

Measurement of H_2O_2 production in Nox4-tranfected cells

H_2O_2 productions of 100 000 cells were measured in triplicate. After 30 min, dityrosine concentration was detected in plate reader as previously described. As a control we used non-transfected cells and cells preincubated with 10 μM DPI at 37°C for 10 min. Standard calibration curve was prepared to quantify the H_2O_2 production.

Generation of H_2O_2 by the glucose/glucose oxidase system

H_2O_2 production was initiated by the addition of glucose oxidase (5 mU/ml) to H medium containing 5 mM glucose, 1 μg/ml LPO and 1 mM tyrosine. Catalase was applied at 500 U/ml.

Measurement of H_2O_2 production by the Amplex Red assay

H_2O_2 productions of 100 000 cells were measured in triplicate. Suspended cells were incubated in the presence of 50 μM Amplex Red and 0.1 U/ml horseradish peroxidase in H-medium. After 30 min incubation at 37°C, resorufin fluorescence was measured at 590 nm.

Measurement of cellular toxicity

We measured cellular toxicity using the Vialight Plus kit (Lonza, Basel, Switzerland) according to the manufacturer's instructions.

HPLC-MS measurements

HPLC-MS measurements were performed on an Applied Biosystems 3200QTrap hybrid mass spectrometer coupled to a Perkin-Elmer Series 200 HPLC system (consisting of two pumps and an autosampler). Purospher Star 55 × 2 mm, 3 μm particle size column (Merck, Darmstadt) was used for separation of components. Mobile phases were: A: 10 mM ammonium formate, B: Acetonitrile. Flow rate was 400 μl/min; 10 μl of each sample was injected to the column. A sensitive and selective tandem mass spectrometric method was developed for quantitative measurement of dityrosine. The instrument was scanned in multiple reaction monitoring (MRM)

mode. Two reactions were selected for each compound: 473→354 and 473→269 in the case of dityrosine. The acquisition time was 13 min. Dityrosine was obtained from VICHEM Ltd (Budapest, Hungary).

Results

In our experiments we first characterized the dityrosine formation by LPO, purified from bovine milk. As shown in Figure 1A, bovine milk LPO efficiently catalysed the formation of dityrosine in the presence of 10 μM H_2O_2 indicated by the increase of fluorescence detected at 405 nm. Figure 1B shows that LPO effectively catalysed the cross-linking of both L- and D-tyrosine. The molecular identity of the reaction

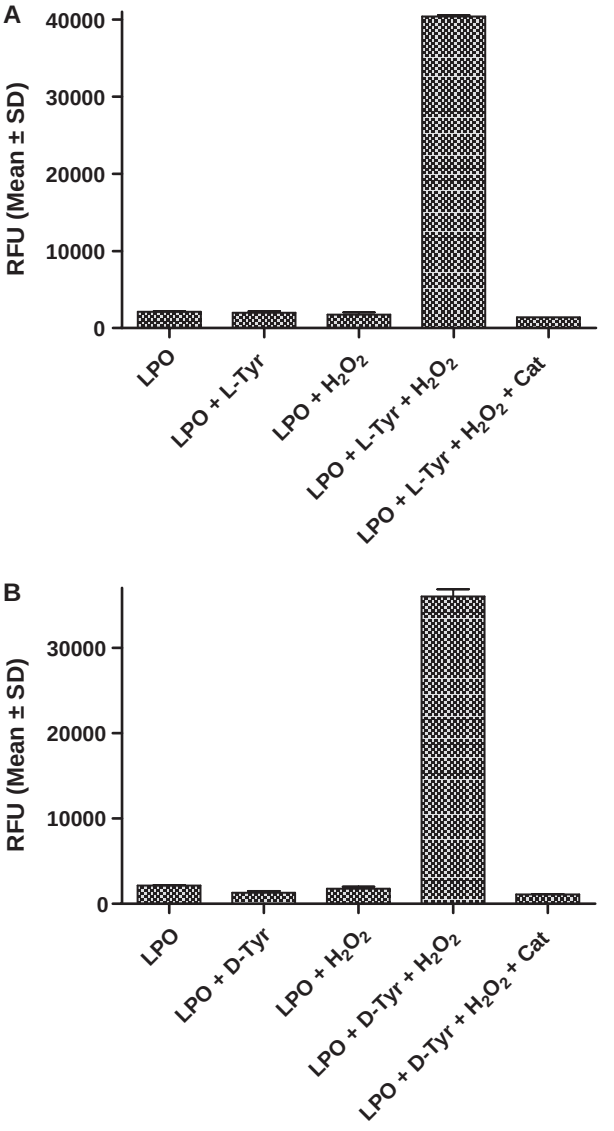

Figure 1. LPO-mediated dityrosine formation in the presence of H_2O_2. Dityrosine production was detected at excitation and emission wavelengths of 320 and 405 nm, respectively. We used 10 μM H_2O_2, 1 μg/ml LPO, 1 mM L-Tyr (A) or D-Tyr (B) and 1000 U/ml catalase in the reaction mixture.

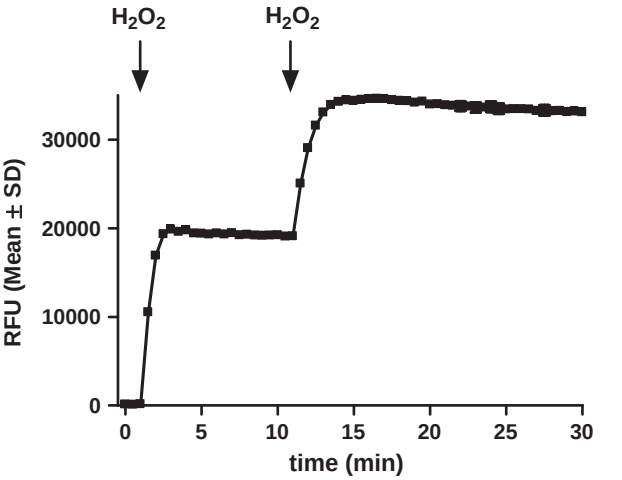

Figure 2. Kinetics of LPO-mediated dityrosine formation. The reaction was initiated by the addition of 5 μM H_2O_2, to a solution already containing 1 mM L-tyrosine and 0.5 μg/ml LPO. Changes of fluorescence were recorded continuously at excitation and emission wavelengths of 320 and 405 nm. After stabilization of the fluorescence, 5 μM H_2O_2 was added to the reaction mix at the time point indicated by the arrow.

products were also confirmed by HPLC analysis (data not shown). Omission of either component from the reaction mix resulted in no detectable change in fluorescence, indicating that the presence of LPO, H_2O_2 and tyrosine were all required for the reaction. Addition of catalase to the reaction mix prevented dityrosine formation (Figure 1A and B). We next characterized the kinetics of the LPO-catalysed dityrosine formation. Addition of 5 μM H_2O_2 to the medium already containing tyrosine and LPO induced a rapid increase in fluorescence, reaching its maximum within 5 min (Figure 2). Addition of H_2O_2 after the stabilization of the fluorescence caused further increase of the signal, indicating that consumption of H_2O_2 occurred in the reaction catalysed by LPO. H_2O_2 produced by the glucose/glucose oxidase system was also successfully detected by LPO-mediated dityrosine formation (Figure 3A and B). Addition of catalase to the reaction mix eliminated the increase in fluorescence.

Next we sought to determine the dependence of the oxidation reaction on the concentration of hydrogen peroxide (Figure 4A). The fluorescent signal changed linearly as a function of H_2O_2 concentration up to 20 μM and as low as 500 nM already supported the formation of dityrosine. Above 60 μM, H_2O_2 inhibited LPO-mediated dityrosine formation suggesting that high levels of hydrogen peroxide inhibit the enzyme's catalytic activity (Figure 4B). The increase of fluorescence was also dependent on the tyrosine concentration, however because of solubility problems we routinely applied the concentration of 1 mM. To assess the specificity of the method we also tested whether LPO-mediated dityrosine formation is supported by other oxidants including superoxide

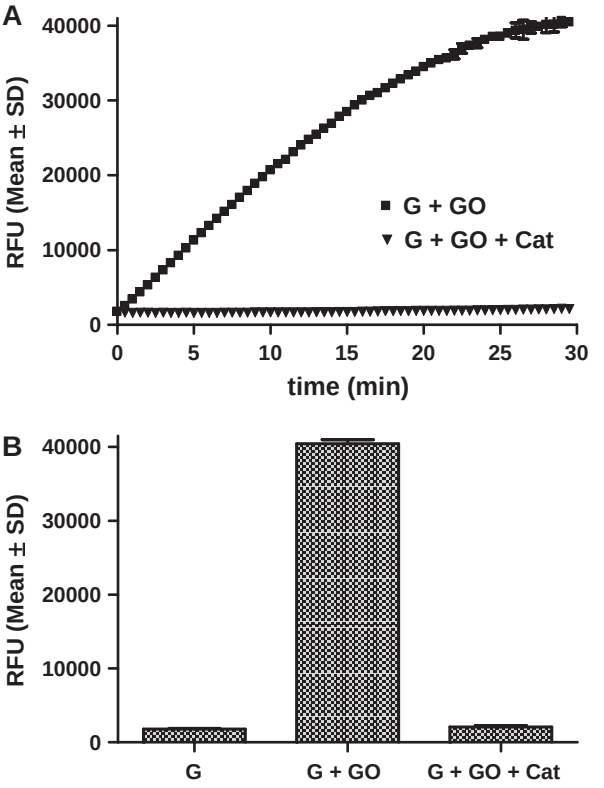

Figure 3. Detection of dityrosine formation in glucose oxidase system. (A) LPO catalysed dityrosine formation is suitable to follow the kinetic of the glucose oxidase produced H_2O_2. Adding 500 U/ml catalase into the reaction completely prevented the H_2O_2 generation of LPO. (B) The dityrosine fluorescence after 30 min incubation at 37°C. The concentration of the glucose oxidase in the reaction was 5 mU/ml.

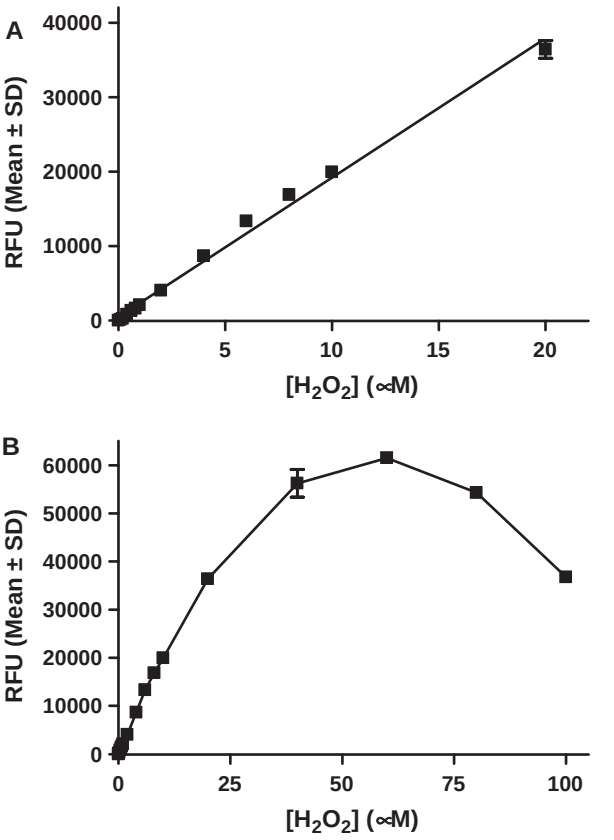

Figure 4. H_2O_2-dependence of LPO-catalysed dityrosine formation. (A) The fluorescence signal was a linear function of hydrogen peroxide concentration in the lower range (0–20 μM). Concentrations as low as 500 nM already supported the formation of dityrosine. (B) Above 60 μM H_2O_2 inhibited the dityrosine production of lactoperoxidase.

and peroynitrite. When superoxide was produced by the xanthine/xanthine oxidase system we observed an increase in dityrosine formation, however it was completely inhibited by catalase, indicating that the formation H_2O_2 was responsible for the signal. Peroxynitrite, either alone or in combination with lactoperoxidase, did not increase the formation of dityrosine (data not shown).

In our next experiments we were interested in whether cellular sources of H_2O_2 can support LPO-mediated dityrosine formation. We have established cell lines which stably expressed Nox4. For this purpose, we used a 293 cell line, since these cells have endogenous $p22^{phox}$ expression, which was shown to form a complex with non-phagocytic Nox proteins as well [17]. Nox4 mRNA expression of the stable clones was initially checked by PCR, then we further analysed Nox4 expression level by Northern blot analysis (Figure 5A). Cell lines showing the highest level of Nox4 were expanded and their H_2O_2 production was measured through LPO-mediated dityrosine formation. As shown in Figure 5B, Nox4-expressing cells produced H_2O_2 which was reflected in a continuous increase in dityrosine fluorescence. This pattern of H_2O_2 production was similar to that reported previously by others [17,18]. The production of H_2O_2 was also confirmed by using the Amplex red H_2O_2 detection assay (Figure 5C). Specificity of the measurements were confirmed by the inhibitory effect of catalase (Figure 5B and C). We also attempted to measure superoxide production by these cells, however we could not detect superoxide using the cytochrome c assay (data not shown). Since the enzymatic reaction catalysed by NADPH oxidases yields superoxide, it is likely that Nox4 is located on internal membranes and H_2O_2 is formed through the intracellular dismutation of superoxide. Although several aspects of the non-phagocytic NADPH oxidases are being studied currently, little is known about the ROS-producing capacity of the non-phagocytic systems. We therefore determined the rate of H_2O_2 production by the Nox4-expressing cells, which was 8.6 nmol/10^6 cells/h. This amount is lower than that reported by Serrander et al. [18]. Since the LPO-based H_2O_2 detection assay is composed of components which are naturally occurring in the mammalian organism, we expected that the system may be suitable for the long-term measurement of H_2O_2 production. In fact we could measure

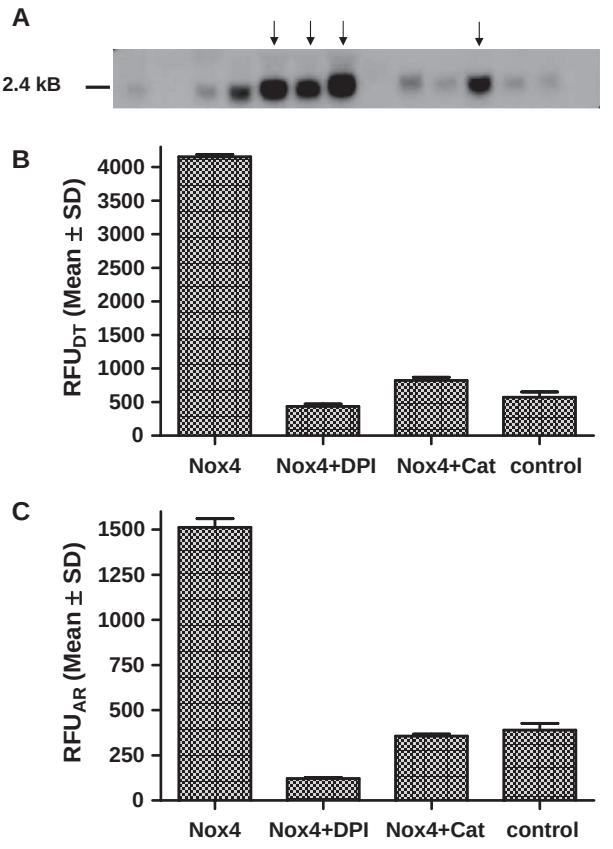

Figure 5. Detection of Nox4 activity by the measuring LPO-catalysed dityrosine formation and by using the Amplex Red assay. (A) Detection of Nox4 expression in stably-transfected cell lines by Northern blot analysis. Arrows indicate the highest expression levels which were also confirmed by real time PCR. (B) Nox4 expressing cells were incubated in the presence of 1 mM tyrosine and 0.5 μg/ml LPO for 30 min at 37°C in H-medium. Dityrosine production was detected at excitation and emission wavelengths of 320 and 405 nm, respectively. (C) Cells were assayed for Nox4 activity by the Amplex Red assay. The fluorescent product, resorufin fluorescence was measured at 590 nm. Addition of catalase (1000 U/ml) to the reaction mixture decreased the fluorescence in both assays.

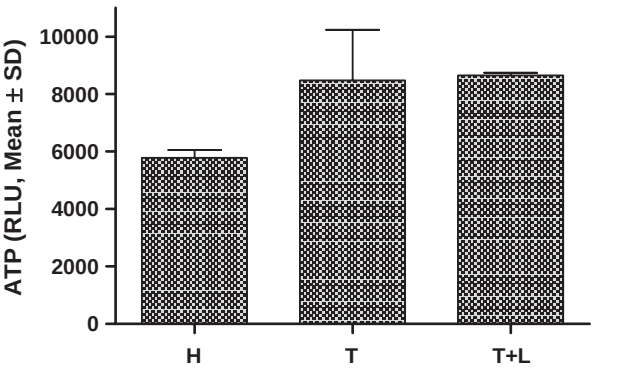

Figure 6. Assessment of toxicity of LPO-catalysed dityrosine formation. Nox4-expressing 293F cells were incubated for 6 h at 37°C in H-medium (H) in the presence of tyrosine (T) or in the presence of tyrosine and LPO (T+L). ATP content of the cells was measured by a luciferase-based cell viability assay.

the continuous production of H_2O_2 for several hours without any sign of toxicity on the cells (Figure 6). These results suggested that the LPO-based H_2O_2 measurement is a simple and efficient way of measuring reactive oxygen species (ROS) release from cellular sources.

We have also attempted to measure H_2O_2 production by stimulated neutrophils, however stimulated granulocytes synthesized dityrosine in the absence of LPO, indicating that an endogenous peroxidase, presumably MPO, substituted LPO in the reaction [8].

Discussion

In this work we characterized the dityrosine-forming activity of LPO. We found that LPO purified from bovine milk efficiently catalysed the formation of dityrosine at low (submicromolar) concentrations of H_2O_2. The ability to form dityrosine seems to be a general feature of peroxidases; enzymes of lower species and mammalian peroxidases were previously described to catalyse this reaction [4,6,8,9].

Since we observed that submicromolar concentrations of H_2O_2 already support this reaction, we decided to examine if this reaction is suitable for the detection of H_2O_2 produced by Nox4-expressing cells. We chose to work with Nox4-expressing cells because Nox4 was reported to produce primarily H_2O_2 when it was expressed in different cell lines [1,18]. We found that using this system we could measure H_2O_2 production from Nox4-expressing cells. With the help of this system we determined the rate of ROS production of Nox4-containing cells, which was 8.6 nmol/10^6 cells/h. In agreement with the observations of previous studies [17,18], we detected only H_2O_2 production by these cells. The absence of superoxide release from Nox4-expressing cells might be a consequence of intracellular dismutation during diffusion, however it is also possible that some hitherto unknown enzymatic mechanism is responsible for the selective production of H_2O_2. Measuring H_2O_2 production through the formation of dityrosines represents one of the simplest methods of H_2O_2 detection described so far, with several potential advantages. Due to the low cost of the components, the method seems particularly suitable for high-throughput screening studies. We also showed that the system had no toxicity on the tested cell lines, which is well explained by the fact that the components of this system naturally occur in mammalian organisms. It is important to note that our method is primarily suited for measurement of H_2O_2 production from intact cells or in cell-free systems in the absence of reducing agents, because the presence of NADH, NADPH or reduced glutathione interfered with LPO-mediated dityrosine formation (data not shown). Interference by NADH and reduced

glutathione was also reported for the Amplex Red system [19].

LPO is present in several exocrine secretions including milk, saliva, tears and bronchial secretions. The antimicrobial action of LPO is thought to be a consequence of its ability to turn thiocyanate into hypothiocyanate in the presence of H_2O_2. Hypothiocyanate has antimicrobial activity against a broad spectra of micro-organisms, including bacteria and viruses [3], however its exact importance in host defense processes is still unknown. Since LPO is present in high concentrations in exocrine secretions it is possible that the enzyme can catalyse the formation of dityrosine residues *in vivo*. The possibility of such activity requires further investigation.

In summary, we conclude that the dityrosine-forming activity of LPO is a promising tool for measuring H_2O_2 in different cellular systems, and this activity of the enzyme might have broader significance in mammalian organisms.

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