Singlet-Oxygen Generation by Peroxidases and Peroxygenases for Chemoenzymatic Synthesis

Kim N. Ingenbosch,[a, b, c] Stephan Quint,[e] Melanie Dyllick-Brenzinger,[e] Dennis S. Wunschik,[a, b, c] Jan Kiebist,[f] Philipp Süß,[d] Ute Liebelt,[d, g] Ralf Zuhse,[e] Ulf Menyes,[d] Katrin Scheibner,[f] Christian Mayer,[c] Klaus Opwis,[b] Jochen S. Gutmann,[b, c] and Kerstin Hoffmann-Jacobsen[a]  

Singlet oxygen is a reactive oxygen species undesired in living cells but a rare and valuable reagent in chemical synthesis. We present a fluorescence spectroscopic analysis of the singlet-oxygen formation activity of commercial peroxidases and novel peroxygenases. Singlet-oxygen sensor green (SOSG) is used as fluorogenic singlet oxygen trap. Establishing a kinetic model for the reaction cascade to the fluorescent SOSG endoperoxide permits a kinetic analysis of enzymatic singlet-oxygen formation. All peroxidases and peroxygenases show singlet-oxygen formation. No singlet oxygen activity could be found for any catalase under investigation. Substrate inhibition is observed for all reactive enzymes. The commercial dye-decolorizing peroxidase industrially used for dairy bleaching shows the highest singlet-oxygen activity and the lowest inhibition. This enzyme was immobilized on a textile carrier and successfully applied for a chemical synthesis. Here, ascaridole was synthesized via enzymatically produced singlet oxygen.

Introduction

Reactive oxygen species (ROS) are involved in numerous metabolic activities, in intracellular signaling, regulating several kinases, transcriptional factors, in cytotoxicity and apoptosis.[1] ROS include the nonradicals hydrogen peroxide (H₂O₂), organic hydroperoxides (ROOH), hypochlorous acid (HOCl) and the radical species of medium lifetime such as superoxide (O₂⁻), the nitroxy radical (NO*) as well as short-lived diffusible entities such as hydroxyl (HO*), alkoxyl (RO*), peroxyl (ROO*), and singlet oxygen (O₅₂*). Among the family of ROS, those exhibiting very high reactivities, including *OH and *O₂, remain relatively underexplored. To combat ROS generated complications, cells have developed a complex enzymatic as well as a nonenzymatic antioxidant defense system in which peroxidases play an important role.[1]

Peroxidases are ubiquitous enzymes in all forms of life. In organisms, these enzymes serve not only the detoxification of reactive oxygen species but also the oxidation of numerous compounds by the use of ROS. For the oxidation process the co-substrate binds to the active site, which contains either an iron protoporphyrin IX (heme peroxidases), other metals like vanadium, manganese, halogens (non-heme peroxidases) or specific metal-free prosthetic groups.[3] Unspecific peroxides, peroxyge- nases (UPO) are a relatively new class of enzymes, which belong to the peroxidase family but differ in the reaction mechanism of electron transfer.[5]

Nowadays, chemists are becoming aware of the potential of peroxidases and peroxygenases in biocatalytic synthesis. Several peroxidases and peroxygenases are capable of activating inert or poorly activated C–H bonds and introducing oxygen functionalities into organic molecules by hydrogen peroxide.[6] These oxysterilization reactions are attractive green alternatives to transition metal catalysis.[7] The catalytic mechanisms of peroxidases, peroxygenases and catalases involve a joint intermediate compound 1 (Figure 1). Subsequently, peroxidases release two radicals from two one-electron oxidation steps, which undergo further coupling and disproportionation reactions. In contrast, peroxygenases can also perform direct oxygen transfer to the substrate in a two-electron oxidation.[8,9]
In catalases, however, compound I decomposes to oxygen and water without the concurrent oxidation of a second substrate.

It is well known that peroxidases as horseradish peroxidase are capable of forming hydroxyl radicals in the presence of hydrogen peroxide under suitable reaction conditions. Here, a Fenton type mechanism has been suggested, which is facilitated by the formation of an iron(II) oxygen complex in excess of hydrogen peroxide. Recently, this reaction path has been detected for UPOs, too. Yet, the synthetic use of hydroxyl radicals is scarce.

Conversely, singlet oxygen is a valuable and green but rare chemical reagent that can oxidize electron-rich organic compounds. Due to its low stability singlet oxygen has to be provided in situ. Typically, singlet oxygen is formed photocatalytically. The generation of singlet oxygen from sodium molybdate and hydrogen peroxide has also been described. Simply mixing hypochlorite with hydrogen peroxide will also produce 1O2. However, hypohalites can give rise to unwanted side reactions with organic substrates.

In the past decades, occasional reports of singlet-oxygen formation by different enzymes have been released. Singlet oxygen production via hypochlorite formation by hydrogen peroxide/halide systems and chloroperoxidase, myeloperoxidase, lactoperoxidase, chloroperoxidase by chemoluminescence has been reported earlier as well as the synthetic use of a vanadium chloroperoxidase. Direct singlet oxygen production from hydrogen peroxide by horseradish peroxidase has been detected occasionally by chemoluminescence as well as spectroscopically via the formation of a hydroperoxy-dihydrofuran. Yet, no biocatalytic synthesis has been developed based on singlet oxygen production by iron peroxidases nor has a concise analysis of the enzymes capable of singlet-oxygen formation been performed after the pioneering works from the 1980s.

The direct enzymatic production of singlet oxygen requiring only hydrogen peroxide in suitable amounts should be ideally suited for providing singlet oxygen for synthetic chemistry without the need of photochemical equipment or metal catalysis. The present study aims to determine the iron enzyme classes that are capable of forming singlet oxygen from hydrogen peroxide. The kinetics of singlet-oxygen formation is elucidated in order to reveal relative activities and mechanistic differences of the enzymes under investigation. Different classes of heme proteins were analyzed: two heme peroxidases, two unspecific peroxygenases and three catalases were investigated in their ability to produce singlet oxygen by fluorescence spectroscopy. The commercial dye, singlet oxygen sensor green (SOSG), was used to trap singlet oxygen as a fluorescent SOSG endoperoxide. Finally, singlet oxygen is used in an exploratory chemical reaction, the synthesis of the natural terpene ascaridole.
Results and Discussion

Singlet-oxygen formation by different hydrogen peroxide-converting enzymes

The singlet-oxygen formation in the presence of hydrogen peroxide was analyzed by SOSG fluorescence. The SOSG endoperoxide is a fluorophore which can be excited at 475 nm and emits at 530 nm (Figure 2). SOSG is highly selective and can be used for the sensitive detection of singlet oxygen by fluorescence spectroscopy.[18] The dye is used frequently in the detection and visualization of singlet-oxygen formation in cellular environment.[19] Yet, quantitative analyses of \( ^1\text{O}_2 \) formation have only been carried out very rarely,[20] and a kinetic analysis is scarce.

In order to perform a quantitative analysis, the linearity of SOSG fluorescence intensity with singlet oxygen concentration was confirmed by photochemical synthesis of \( ^1\text{O}_2 \) in the presence of the photosensitizer Rose Bengal[21] (Figure S1 in the Supporting Information). Hence, at the constant detector voltage and the constant SOSG concentrations applied for the following measurements, the SOSG-EP fluorescence intensity is directly proportional to the concentration of \( ^1\text{O}_2 \). However, absolute \( ^1\text{O}_2 \) concentrations cannot be calculated due to the lack of an adequate calibration standard.

Singlet oxygen production by three different classes of enzymes accepting \( \text{H}_2\text{O}_2 \) as substrate are investigated: peroxidases, peroxygenases and catalases. Here, the commercial dye decolorizing peroxidase MaxiBright™ (from scordonanis) and the horseradish peroxidase, type I (HRP), as well as the unspecific peroxygenases from Marasmius rotula (MroUPO) and from Chaetomium globosum (CglUPO) were studied. The enzymatically produced singlet oxygen derived SOSG-EP intensity is depicted in Figure 3 for the peroxidases and peroxygenases at various hydrogen peroxide concentrations. The figure shows that all peroxidases and peroxygenases under investigation are capable of forming singlet oxygen in the presence of hydrogen peroxide. Yet, CglUPO produces the lowest \( ^1\text{O}_2 \) concentrations. The catalases under investigation originate from Aspergillus niger, Corynebacterium glutamicum and Micrococcus lysodeiticus. Remarkably, no singlet-oxygen formation was found with any catalase tested at any hydrogen peroxide concentration indicated.

Singlet-oxygen formation via peroxidases and peroxygenases is detected in a broad range of hydrogen peroxide concentrations. This is challenging for a fluorescence spectroscopic technique, as a quantitative analysis of fluorescence spectroscopic data requires low dye concentrations in the low-micromolar range in order to exclude inner filter and quenching effects. Hence, intensities in Figure 3 have to be analyzed with care as the maximum observable SOSG-EP intensity is limited by the SOSG concentration. Thus, the plateau detected for the peroxidases is an artifact arising mainly from SOSG scarcity and too slow \( ^1\text{O}_2 \) capturing. (Figure S2) Yet, it may be concluded, that peroxidases show a broader substrate concentration regime and an earlier onset of singlet oxygen production than the peroxygenases.

As peroxidases are known to form superoxide in the presence of an excess of hydrogen peroxide,[22] which might be the source of singlet oxygen,[23] SOSG-EP fluorescence was investigated in the presence of superoxide dismutase (SOD), a superoxide scavenger.[24] As depicted in Figure 3C for Maxi-Bright, the SOSG-EP fluorescence intensity decreases with increasing amount of SOD. The same effect was observed with HRP (data not shown). SOSG-EP fluorescence is not quenched completely by a tenfold excess of SOD with respect to peroxidase. This shows that superoxide is a major source of singlet-oxygen formation by the peroxidases but it is not the

![Figure 2](https://example.com/figure2.png)

*Figure 2. Left: Singlet oxygen trapping by SOSG leads to the formation of the fluorescent endoperoxide (SOSG-EP). Right: Excitation and emission spectra of the SOSG dye and SOSG-EP.*
only source. Remarkably, SOD has only a minor effect on the SOSG-EP fluorescence generated by the peroxygenases indicating a smaller fraction of superoxide related singlet-oxygen formation (Figure 3D).

It is interesting to note that SOSG-EP fluorescence time traces of the peroxidases show intensity maxima. This results from SOSG-EP disintegration. The maxima vanish in the presence of SOD. No SOSG-EP degradation is observed in the presence of peroxygenases under any condition tested. This emphasizes the role of superoxide formation by peroxidases which is also involved in a destructive reaction scheme of the SOSG-EP chromophore.

**Analysis of the initial rate as observed by fluorescence spectroscopy**

The activity of the different enzymes for \(^1\text{O}_2\) formation was investigated by kinetic analysis. The initial rate of SOSG-EP formation does not suffer from artificial intensity leveling as it is acquired at (initial) low product concentrations. However, Figure 4 reveals a dependency of the initial rate of SOSG-EP formation on the SOSG concentration, which does not scale linearly with the dye content. This finding can be explained by incomplete \(^1\text{O}_2\) capture by SOSG. In competition to the reaction with SOSG, \(^1\text{O}_2\) can undergo spontaneous monomolecular decay to \(3\text{O}_2\). We will show that this parallel reaction scheme (Figure 5) of a monomolecular decay and second order capture reaction leads to the observed dependency of SOSG-EP formation on the SOSG concentration.
with $v_{\text{max}}$ being the maximum rate and $K_M$ the Michaelis constant.

By application of the steady state approximation the rate law for SOSG-EP formation is given by (Supporting Information):

$$\frac{d[SOSG-EP]}{dt} = \frac{k_j [SOSG]}{K_m + [S]} \cdot \frac{E [S]}{K_m + [S]}$$

(2)

with E being the enzyme and S hydrogen peroxide. Equation (2) shows that Michaelis-Menten type kinetics are observed at a constant SOSG concentration, where $k_{\text{cat}}$ is modified by the dimensionless factor $f$.

$$f = \frac{k_j [SOSG]}{k_{\Delta} K_m + [SOSG]}$$

(3)

Hence, a Michaelis-Menten analysis is feasible and gives correct Michaelis constants. Values of $k_{\text{cat}}$ differ from the real value by a constant factor (Table 1) and can thus be compared quantitatively with each other.

**Michaelis-Menten kinetics of singlet-oxygen formation**

The initial rate of SOSG-EP formation was determined over a broad range of hydrogen peroxide concentrations for the peroxidases and peroxygenases under investigation (Figure 6).
According to Michaelis-Menten kinetics, saturation behavior is expected. A qualitative assessment shows a strong deviation from Michaelis-Menten kinetics for all enzymes under investigation. Accordingly, the respective linearization plots fail to give classical Michaelis-Menten parameters. In contrast, both peroxidases show broad maxima in reaction rate, which indicate substrate inhibition.

It is well known that hydrogen peroxide is a substrate with a strong inhibitory effect on numerous enzymes as peroxigenases and peroxidases. The model by Yoshino et al.\textsuperscript{26} for uncompetitive substrate inhibition was used for kinetic data analysis. Here, substrate inhibition is displayed by the binding of an additional substrate to a non-catalytic site, which is characterized by the dissociation constant \( K_{S} \) leading to a reduced catalytic rate constant \( k' \) in the model (Figure 5). The initial rate is described by Equation (4).

\[
v = v_{\text{max}} \cdot \frac{\left(1 + \frac{[S]}{K_{S}}\right) [S]}{K_{m} + [S]\left(1 + \frac{[S]}{K_{S}}\right)}
\]

\( K_{S} \) is the dissociation constant of the inhibited enzyme substrate complex, \( k' \) depicts the respective rate constant and \( k_{cat} \) the rate constant of the native enzyme substrate complex. First, \( K_{S} \) and the apparent \( v_{\text{max}} \) were determined. All enzymes show Michaelis-Menten behavior in a limited regime at low substrate concentrations which varies from enzyme to enzyme. Michaelis-Menten parameters were estimated from the substrate concentration \([S]\) dependent reaction rate \( v \) data by fits to Equation (1) in this regime. These were used as initial parameters for the fit of the experimental substrate dependency of the initial rate to Equation (4). The Michaelis constants (Table 2) correspond with previous kinetic studies of peroxidases.\textsuperscript{27} The analysis reveals that MaxiBright shows the highest \( k_{cat} \) of the peroxidases. Peroxigenases have a remarkably lower singlet-oxygen formation activity represented in higher \( K_{S} \) values.

The inhibition model allows describing the Michaelis-Menten plots of singlet oxygen production by peroxigenases and peroxidases as illustrated in Figure 6. This gives strong evidence that substantial substrate inhibition by hydrogen peroxide occurs. Interestingly, the kinetic constant of the hydrogen peroxide bound enzyme substrate complex \( k' \) was found to approach zero for all enzymes. This represents complete inhibition upon binding of additional hydrogen peroxide and the model simplifies to

\[
v = v_{\text{max}} \cdot \frac{[S]}{K_{m} + [S]\left(1 + \frac{[S]}{K_{S}}\right)}
\]

The different enzymes vary in the response to hydrogen peroxide as described by the dissociation constant \( K_{S} \). The analysis shows that MaxiBright features the highest reaction rate and stability.

**Enzymatically produced singlet oxygen as chemical reagent**

Singlet oxygen is a valuable and green but rare chemical reagent. In an exploratory reaction, we performed the synthesis of the natural terpene ascaridole as depicted in Figure 7. Ascaridole is used as flavoring and as an antiparasitic agent. The synthesis of ascaridole is performed by the \( [4 + 2] \) cycloaddition of singlet oxygen to \( \alpha \)-terpinene.

The peroxidase MaxiBright was immobilized on a textile carrier as described recently for improved stability in organic solvent.\textsuperscript{28} Ethanol, acetonitrile and THF buffer mixtures were tested as solvents showing highest product yields in a buffer/acetonitrile mixture (1:10). The pH was varied in the range from 4 to 6. Keeping the pH below 5 achieves highest yields. The introduction of hydrogen peroxide via slow release from urea hydrogen peroxide proved to be a key step in increasing yields to a maximum of 13 %. Thus, a constant but low concentration of hydrogen peroxide is maintained. Moreover, urea hydrogen peroxide shows a higher solubility in organic media. Reaction times were varied from one to five days, with one day giving the highest yield of ascaridole. No reaction was observed in the absence of the enzyme.

The crude product was analyzed by \(^1\)H NMR spectroscopy (Figure S3). The comparative \(^1\)H NMR data were taken from the literature,\textsuperscript{29} as well as supported by chemical synthesis of ascaridole. The signal that is assigned to the hydrogen atoms of the double bond of ascaridole can be clearly identified. The respective section is given in Figure 8. The depicted coupling pattern coincides with the values given in the literature for chemically synthesized ascaridole.\textsuperscript{30} The conversion of \( \alpha \)-terpinene is 100 % as judged by NMR spectroscopy. The major side product (29%) was identified as cymene. It is readily formed by elimination and subsequent aromatization.

No product formation could be observed with any catalase under investigation. Moreover, the vanadium chloroperoxidase

---

**Table 2. Michaelis-Menten parameters \( K_{S} \) for singlet-oxygen formation, apparent \( v_{\text{max}} \) and \( k_{cat} \) for SOSG-EP formation and the inhibition constant \( K_{S} \).**

| Enzyme | \( K_{S} \) [\( \mu M \)] | \( v_{\text{max}} \) /Int. [a.u.] /s \(^{-1} \) | \( K_{S} \) [\( \mu M \)] | \( k_{cat} \) /Int. [a.u.] /s [\( \mu M \) \(^{-1} \)] |
|--------|-----------------|-----------------|-----------------|-----------------|
| HRP    | 0.24 ± 0.13     | 2.66 ± 0.27     | 620 ± 300       | 13 ± 1.3        |
| MaxiBright | 0.37 ± 0.16     | 12.42 ± 0.81    | 7000 ± 2100    | 83 ± 5.5        |
| MroUPO | 25 ± 20         | 3.61 ± 0.39     | 5000 ± 3800    | 21 ± 2.3        |
| CglUPO | 38 ± 27         | 1.99 ± 0.7     | 8600 ± 5300    | 7.0 ± 1.3       |

**Figure 7. Conversion of \( \alpha \)-terpinene to ascaridole by hydrogen peroxide (urea-H\(_2\)O\(_2\)-UHP) and the peroxidase MaxiBright.**
from *Curvularia inaequalis*, which is known to form singlet oxygen via the hypochlorite pathway,[30] did not give comparable product yields (ca. 1%) using bromide as cocatalyst.

**Mechanism of singlet-oxygen formation**

The peroxidase mechanism involves the intermediates compound 0, I and II that are depicted in Figure 1. Excess of hydrogen peroxide leads additionally to the formation of compound III, which is an off-pathway intermediate.[31,32] These species give unique optical absorption spectra of the prosthetic group and can be identified by the Soret band (ca. 400 nm) and the position of the Q\(_\text{Q0}\) and Q\(_\text{Qv}\) band in the region from 500 to 700 nm.[33] HRP has been characterized in detail.[34] Therefor the analysis will be performed with this peroxidase.

The absorption spectra of the peroxidase HRP under assay conditions showing maximum singlet-oxygen formation rates are shown in Figure 9 (left). Immediately after hydrogen peroxide injection, changes in both regions are observed. The new absorption maxima at 419 nm and at 527 and 555 nm reflect compound II formation. The hydrogen peroxide concentration, where singlet oxygen production starts, coincides with the onset of new spectral features in the absorption spectra. At mM \(\text{H}_2\text{O}_2\) concentrations compound III is identified by maxima at 543 and 577 nm (Figure S4). Moreover, the inhibited species P670 is found in the concentration region where inhibition is observed in the kinetics. Similar spectra were obtained from MaxiBright (data not shown).

In contrast, the absorption spectra of the singlet oxygen producing *Mro*UPO do not reveal new bands upon hydrogen consumption (Figure 9, right) but partial bleaching at any concentration tested (Figure S4). Yet, it has been shown previously that the absorption spectra of *Mro*UPO species are less specific than the spectra of HRP.[11]

**Discussion**

Analysis shows that the peroxidases and *Mro*UPO peroxygenase form singlet oxygen without hypochlorite intermediates from hydrogen peroxide at least in micromolar concentrations and on the timescale of minutes. In particular, the peroxidases accept hydrogen peroxide as oxidation and reduction substrate over a broad concentration range. This is reflected by inhibitory constants in the millimolar range. The successful synthesis of ascaridole by MaxiBright emphasizes the synthetic amounts of
singlet oxygen produced even in the presence of organic solvents. The direct formation of singlet oxygen from hydrogen peroxide by peroxidases has only rarely been mentioned over the decades in literature and the synthetic potential of enzymatically produced singlet oxygen remains to be raised. In this exploratory study, α-terpinene was chosen as a substrate because it produces a stable endoperoxide (ascaridole). Generally, a variety of conjugates dienes are potential substrates for this enzymatically induced [2 + 4] cycloaddition.

The lacking singlet-oxygen formation by catalases is first surprising as oxygen formation by peroxidases and peroxygenase could be considered a catalase function. This shows that it is not compound I that is able to form singlet oxygen but compound II is involved. This idea is supported by our absorption spectroscopy analysis and the sequential H abstraction mechanism of catalases from hydrogen peroxide to oxygen.[35] It has already been shown for myeloperoxidases that hydrogen peroxide cannot mediate the direct reduction of compound I of peroxidases to the resting state but mediates the conversion to compound II.[36]

In the absence of a reducing substrate peroxidases and peroxygenases react with hydrogen peroxide in a complex set of reactions to compound II of the regular peroxidase cycle.[37-39] In the peroxidase cycle, compound II formation includes the release of a substrate radical (Figure 1). In the absence of substrate, superoxide radical formation by peroxidases was found.[36]

Superoxide can be oxidized to singlet oxygen.[40] It has been suggested previously that this superoxide decay can promote catalytic activities involving singlet oxygen.[41] Yet, the yield of singlet oxygen from superoxide is marginal (0.2 %) in solution.[42] A reaction with ferric peroxidase to oxygen and the resting state might increase the yield.[43] Thus, the catalytic cycle is closed. This path represents one possible origin of singlet-oxygen formation by peroxidases (Figure 1, red arrows). The suggested mechanism is in line with the observed dependence of the reaction rate of peroxidases on SOD concentration. Moreover, the low onset of hydrogen peroxide concentration required for 'O_2 formation by peroxidases and their optical absorption spectra in the micromolar range support the idea of compound II being the reactive species.

Under further excess of hydrogen peroxide, peroxidases are known to form compound III, an oxysyme species.[37] This corresponds to the optical absorption spectra at the higher hydrogen peroxide concentrations probed herein. Compound III has been identified as the source of catalytic activities of peroxidases that are independent of the peroxidase cycle.[11,41] The nature of Fe-O_2 bonding in oxysyme has been a subject of active debate for decades. Pauling assigned a singlet state to dioxygen.[44] Weiss proposed a ferric-superoxide complex.[45] and McClure, Goddard and Olafson suggested the "ozone" model.[46] Theoretical investigations as well as spectroscopic analysis indicate that oxysyme is multiconfigurational and comprises singlet oxygen as minor species.[47-48] We suggest that compound III is the second source of singlet oxygen which might be either formed directly from Fe-O_2 or indirectly from superoxide (Figure 1, left side).[31,48] In contrast to the first path, this mechanism should not be disrupted by superoxide removal. The singlet oxygen production by MroUPO which only starts at high hydrogen peroxide concentrations and is less sensitive to SOD rather accords with this second mechanism. The partition between both mechanisms is supposed to depend on the hydrogen peroxide concentration and the enzyme type.

It has been reported that hydroxyl radicals are also formed by peroxygenases and peroxidases in the absence of electron donor substrate.[10,11] Concomitantly, a "catalase malfunction" was suggested involving the production of OH radicals by the Haber-Weiss reaction of compound III.[51] This supports the idea that peroxygenases form compound III in the absence of a second electron donor substrate. The present data indicate, that singlet oxygen is formed predominantly by compound III at intermediate hydrogen peroxide concentrations (µM-1 mM regime) whereas OH radicals are formed at higher concentrations (>1 mM regime).

The formation of different ROS in the reaction is supposed to be a major obstacle in the synthetic application of the presented enzymatic method as this will lead to side reactions. Future work should concentrate on the elucidation of the detailed mechanism and the means to control the reaction path.

Conclusion

In this study, we prove that peroxidases and peroxygenases are capable of forming synthetic concentrations of singlet oxygen in the presence of hydrogen peroxide. We can clearly exclude catalases from sharing this characteristic activity. The fluorescent trap SOSG allows a kinetic analysis after appropriate modeling of the reaction cascade leading to the fluorescent SOSG endoperoxide, revealing uncompetitive substrate inhibition. The commercial dye decolorizing peroxidase MaxiBright can be identified as the most active singlet oxygen-forming enzyme which is the least prone to substrate inhibition. The successful synthesis of ascaridole by MaxiBright catalysis shows that this enzyme can be used in chemo-enzymatic synthesis involving singlet oxygen as reagent.

Experimental Section

Materials: The commercially available dye decolorizing peroxidase MsP1 from _M. scordonius_ marketed as MaxiBright® and produced by _A. niger_ was a kind gift from DSM (Food Specialties B.V., Delft, Netherlands). Horseradish peroxidase type 1 was purchased from Sigma-Aldrich as lyophilized powder. The UPOs from the fungi _M. rotula_ (MroUPO) and _Chaetomium globosum_ (CgUPO) were isolated as described previously.[49] All enzymes were prepared as 1 % stock solutions in 50 mM sodium phosphate buffer pH 6 before use. SOSG was acquired from ThermoFisher. Vanadium chloroperoxidase from _C. inaequalis_ (CVHPO) was expressed in _Escherichia coli_.[50] Manganese superoxide dismutase from _E.coli_ (Sigma) was a kind gift from Prof. Katja Ferenz (University Hospital, Essen). All chemicals were purchased from Roth (Germany).

Singlet oxygen assay: 100 µg of SOSG was freshly diluted in 1 mL 3 % methanol in phosphate buffer solution, handled on ice and...
Fluorescence emission of the SOSG dye was measured with a Varian Cary Eclipse Fluorescence Spectrometer (λ<sub>ex</sub> = 475 nm, λ<sub>em</sub> = 530 nm) providing a time resolution of 50 ms at the beginning of the time traces. The detector voltage was 700 V for all data except the dye dependency. Here, it was 550 V for the variation of [SOSG] at 1 μM H<sub>2</sub>O<sub>2</sub> and 600 V for the combined variation of substrate and [SOSG].

'O<sub>2</sub> formation in the presence of SOD was analyzed at 1 μM hydrogen peroxide concentration. Peroxidase and peroxygenase concentration was 0.01 g/l, pre-mixed with 0.55 μM SOD and SOD, reaction was started with the injection of H<sub>2</sub>O<sub>2</sub>. SOD was introduced with different mass equivalents with respect to the peroxidase/peroxygenase.

**Ascaridole synthesis**: 10 mL acetonitrile were mixed with 1 mL citrate phosphate buffer, pH 5 (ca. 0.1 M), 0.39 mL α-terpene (2.4 mmol) were added. 1 cm<sup>2</sup> textile carrying MaxiBright was cut into four pieces and added. The mixture was stirred for 10 min before 2 g urea/H<sub>2</sub>O<sub>2</sub> (9.2 equiv.) were added. The reaction mixture was stirred overnight at room temperature.

The reaction mixture was extracted with ethyl acetate after the addition of 5 mL water. The combined organic phases were washed with 10% HCl and dried with Na<sub>2</sub>SO<sub>4</sub>. After filtration and concentration 200 mg crude product was obtained.

**Conflict of Interest**

The authors declare no conflict of interest.

**Keywords**: chemo-enzymatic synthesis · fluorescence · peroxidase · peroxygenase · singlet oxygen · singlet oxygen sensor green

---

[1] a) K. Nomura, H. Imai, T. Kounuma, Y. Nakagawa, *Biol. Signals Recept.* 2001, 10, 81–92; b) S. Camandola, G. Poli, M. P. Mattsson, *J. Neurochem.* 2000, 74, 159–168.

[2] a) P. Popskiöll, *Biochim. Biophys. Acta Bioenerg.* 2009, 1787, 1151–1160; b) M. Yoshino, K. Murakami, *SpringerPlus* 2014, 3, 1–18; c) F. Passardi, G. Theiler, M. Zamocky, C. Cosio, N. Rousier, F. Teixera, M. Margis-Pinheiro, V. Ioannidis, C. Penel, L. Falquet, C. Dunand, *Photochemistry* 2007, 68, 1605–1611.

[3] a) R. Rowiński, M. Kozakiewicz, K. Kędziora-Kornatowska, E. Hübner-Woźniak, J. Kędziora, *Exp. Gerontol*. 2013, 48, 1141–1146; b) J. A. Imlay, *Nat. Rev. Microbiol*. 2013, 11, 443–454.

[4] a) P. L. Thomas, *Curr. Opin. Biotechnol*. 1993, 4, 484–489; b) N. C. Veitch, *Phytochem.* 2004, 64, 1–18; c) F. Passardi, G. Theiler, M. Zamocky, C. Cosio, N. Rousier, F. Teixera, M. Margis-Pinheiro, V. Ioannidis, C. Penel, L. Falquet, C. Dunand, *Photochemistry* 2007, 68, 1605–1611.

[5] a) M. Hofrichter, R. Ulrich, *Curr. Opin. Chem. Biol*. 2014, 19, 116–125.

[6] a) T. Martin, J. R. Kanofsky, L. D. Finch, M. Tovborg, O. M. Helm-Jürgens, M. Hofrichter, *Biotechnol. Adv.* 2017, 35, 813–831.

[7] a) E. Roduner, W. Kaim, S. Sarkar, V. B. Uralche, J. Pleiss, R. Gläser, W.-D. Einicke, G. A. Spugger, U. Beifuß, E. Klemm, C. Liebner, H. Hironymus, S.-F. Hsu, B. Plettker, S. Laschat, *ChemCatChem* 2013, 5, 82–112.

[8] a) N. C. Veitch, A. Smith, *Adv. Inorg. Chem.* 2001, 51, 107–162; b) M. Gajidehe, D. J. Schaller, A. Henriksen, A. T. Smith, T. L. Pouloat, *Nat. Struct. Biol.* 1997, 4, 1032–1038.

[9] a) M. Hofrichter, H. Kellner, M. J. Pecyna, R. Ulrich in *Monoxygenase, Peroxidase and Peroxygenase Properties and Mechanisms of Cytochrome P450*, Springer, 2015.

[10] a) S.-X. Chen, P. Schopfer, *Eur. J. Biochem.* 1999, 260, 726–735.

[11] a) A. Karich, K. Schembier, R. Ulrich, M. Hofrichter, *J. Mol. Catal. B* 2016, 134, 238–246.

[12] a) R. Page, *Pure Appl. Chem.* 1975, 41, 535–558; b) O. Schenck, K. Ziegler, *Naturwissenschaften* 1944, 32, 157; c) R. Pr. Ogilby, *Chem. Soc. Rev*. 2010, 39, 3181–3209.

[13] a) J.-M. Aubry, S. Bouttemy, *J. Am. Chem. Soc.* 1997, 119, 5286–5294.

[14] a) A. M. Held, D. J. Halko, J. K. Hurst, *J. Am. Chem. Soc.* 1978, 100, 5732–5740.

[15] a) J. R. Kanofsky, *J. Biol. Chem.* 1983, 258, 5991–5993; b) J. R. Kanofsky, *J. Biol. Chem.* 1984, 259, 5596–5600; c) J. R. Kanofsky, J. Wright, G. E. Miles-Alrston, A. L. Tauber, *J. Clin. Invest.* 1984, 74, 1489–1495.

[16] a) R. Renirie, C. Pleriot, J.-M. Aubry, A. F. Hartog, H. E. Schoemaker, P. L. Alsters, R. Wever, *Adv. Synth. Catal.* 2003, 345, 849–858.

[17] a) J. F. Platt, A. S. Cheema, P. J. O'Brien, *FEBS Lett.* 1977, 74, 251–254; b) A. R. Pereira, R. S. da Costa, L. Yokoyama, E. M. Alhadeff, L. A. C. Teixeira, *Appl. Biochem. Biotechnol.* 2014, 174, 2741–2747; c) M. Kretner, G. Allth, H. Koren, S. Loew, R. Ebermann, *Anal. Biochem.* 1993, 212, 63–67.

[18] a) H. Wu, Q. Song, G. Ran, X. Lu, B. Xu, *TrAC Trends Anal. Chem.* 2011, 30, 133–141.

[19] a) C. Flors, M. J. Fryer, J. Waring, B. Reeder, U. Bechtold, P. M. Mullineaux, S. Nonell, M. T. Wilson, N. R. Baker, *J. Exp. Bot.* 2006, 57, 1725–1734; b) A. Prasad, M. Sedlálová, P. Pospíšil, *Sci. Rep.* 2018, 8, 13685.

[20] a) H. Lin, Y. Shen, D. Chen, L. B. Wilson, B. S. Xie, *J. Fluoresc.* 2013, 23, 41–47.

[21] a) D. C. Neckers, *Photochem. Photobiol.* 1989, 47, 1–29.

[22] a) H. Warrishi, M. H. Gold, *J. Biol. Chem.* 1990, 265, 2070–2077.

[23] a) A. Khan, *Science* 1970, 168, 476–477.

[24] a) J. M. McCord, I. Fridovich, *J. Biol. Chem.* 1969, 244, 6049–6055.

[25] a) S. Y. Egorov, V. F. Kamalov, N. I. Koroteev, A. A. Krasnovsky Jr, B. N. Toleutaev, S. V. Zinukov, *Chem. Phys. Lett.* 1989, 163, 421–424.

[26] a) M. Yoshino, K. Murakami, *SpringerPlus* 2015, 4, 292.

[27] a) M. Gilabert, L. G. Fenoll, F. García-Molina, P. A. García-Ruiz, J. Tudela, F. García-Cánovas, J. N. Rodríguez-López, *Biol. Chem.* 2004, 385, 1177–1184.
[28] D. S. Wunschik, K. N. Ingenbosch, P. Süss, U. Liebelt, S. Quint, M. Dyllick-Brenzinger, R. Zuhse, U. Menyes, K. Hoffmann-Iacobsew, K. Opwis, J. S. Gutmann, Enzyme Microb. Technol. 2020, 136, 109512.

[29] F. Lévesque, P. H. Seeberger, Org. Lett. 2011, 13, 5008–5011.

[30] A. Maetzke, S. J. K. Jensen, Chem. Phys. Lett. 2006, 425, 40–43.

[31] A. N. P. Hiner, J. Hernández-Ruiz, J. N. Rodríguez-López, M. B. Arnao, R. Varón, F. García-Cánovas, M. Acosta, JBC J. Biol. Inorg. Chem. 2001, 6, 504–516.

[32] R. Nakajima, I. Yamazaki, J. Biol. Chem. 1987, 262, 2576–2581.

[33] D. Metodiewa, M. P. de Melo, J. A. Escobar, G. Cilento, H. B. Dunford, Arch. Biochem. Biophys. 1992, 296, 27–33.

[34] G. I. Berglund, G. H. Carlsson, A. T. Smith, H. Szöke, A. Henriksen, J. Hajdu, Nature 2002, 417, 463–468.

[35] M. Alfonso-Prieto, X. Biarnés, P. Vidossich, C. Rovira, J. Am. Chem. Soc. 2009, 131, 504–516.

[36] L. A. Marquez, J. T. Huang, H. B. Dunford, Biochemistry 1994, 33, 1447–1454.

[37] S. A. Adediran, A.-M. Lambeir, Eur. J. Biochem. 1989, 186, 571–576.

[38] J. Vlasits, C. Jakopitsch, M. Bernroitner, M. Zamocky, P. G. Furtmüller, C. Obinger, Arch. Biochem. Biophys. 2010, 500, 74–81.

[39] H. B. Dunford, Peroxidases and Catalases: Biochemistry, Biophysics, Biotechnology and Physiology, Wiley, 2010.

[40] W. H. Koppenol, Nature 1976, 262, 420–421.

[41] M. J. Davies, J. Clin. Biochem. Nutr. 2010, 48, 8–19.

[42] L. A. MacManus-Spencer, K. McNeill, J. Am. Chem. Soc. 2005, 127, 8954–8955.