Gas diffusion electrode as novel reaction system for an electro-enzymatic process with chloroperoxidase

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Gas diffusion electrode as novel reaction system for an electro-enzymatic process with chloroperoxidase†

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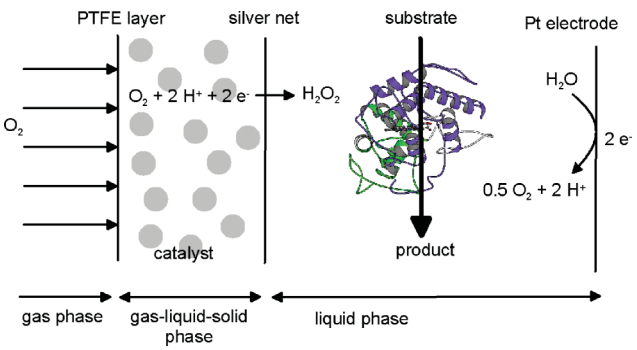
The versatile enzyme chloroperoxidase was used in a new reaction system, based on a gas diffusion electrode, for enzymatic chlorinations, sulfoxidations and oxidations. This is the first report on the combination of hydrogen peroxide production at a GDE with an enzymatic reaction.

Introduction

Electroenzymatic processes are interesting approaches due to the combination of the advantages of enzymes and electrochemical steps.[1,2] Redox enzymes have tremendous potential as catalysts for preparative organic chemistry.[3] Their usually high selectivity, combined with their catalytic performance, makes them important for synthesis of fine and bulk chemicals. Chloroperoxidase (CPO, EC 1.11.1.10) from the filamentous fungus *Caldariomyces fumago* is a versatile heme-dependent peroxidase requiring hydrogen peroxide and chloride, bromide or iodide for the halogenation of organic substrates suitable for electrophilic attack.[4] CPO catalyses the halogenation of different molecules such as aromatic hydrocarbons,[5,6] monoterpenes,[7] lignin structures[8] or flavanones.[9] In addition to halogenations CPO catalyses hydrogen peroxide-supported oxidation, the dismutation of hydrogen peroxide (catalase reaction), and some cytochrome P450 monooxygenase-like reactions.[10] Nevertheless, its use in preparative or industrial scale reactions has been hindered by instability towards the co-substrate hydrogen peroxide.[11] For instance, the half-life time in the presence of 2 mM H_2O_2 in phosphate buffer (pH 4.1) is only 5 min.[11] In order to avoid the irreversible inactivation, hydrogen peroxide can be generated electrochemically on a low but sufficient level for catalytic activity.[12,13] Electrogeneration of H_2O_2 is an attractive approach since it does not require additional chemicals, and electricity is readily available.[1] Furthermore, H_2O_2 is considered to be an environmentally friendly chemical, since it leaves no hazardous residues, such as other oxidants. The use of the electrons as "clean reagents" for enzymatic reactions provides several advantages over conventional, non-electrochemical techniques. In general, the main advantages of electrochemical processes are: versatility, energy efficiency, amenability to automation and cost effectiveness.[14,15] On the other hand, disadvantages arise from the fact that electrochemical reactions are heterogeneous and take place at the electrode-electrolyte interface in electrochemical reactors. Therefore, the cell performance often suffers from mass transport limitations depending on the size of the used electrode . Accordingly, new electrochemical reactors with high specific electrode surface areas and low investment and operation costs are needed for the industrial application of electroenzymatic processes.

Here we describe a gas diffusion electrode (GDE) as a new type of electrode material for electroenzymatic processes. GDEs are electrodes with a solid, liquid and gaseous interface and consist, besides the catalyst, of PTFE and additives. PTFE forms a hydrophobic matrix which binds the catalyst and the liquid attracting materials. For several years GDEs have been used in fuel cells, batteries and the chloralkali process. Scheme 1 shows a sketch of a GDE based reaction system for electro-enzymatic processes with CPO.



Scheme 1 Scheme of the reaction system. Oxygen is reduced to hydrogen peroxide at the gas-liquid-solid interphase of the GDE (cathode). Afterwards, the H_2O_2 diffuses into the liquid phase to the enzyme. Here the enzymatic conversion of the substrate takes place. At the anode, water is oxidized to form H^+ ions and oxygen gas.

In this study we have investigated the chlorination of monochlorodimedone, the sulfoxidation of thioanisole and the oxidation of indole. The following equations describe the electrode reactions.

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† Electronic supplementary information (ESI) available: Figure of the long-term productivity of hydrogen peroxide. See DOI: 10.1039/c1gc15391a

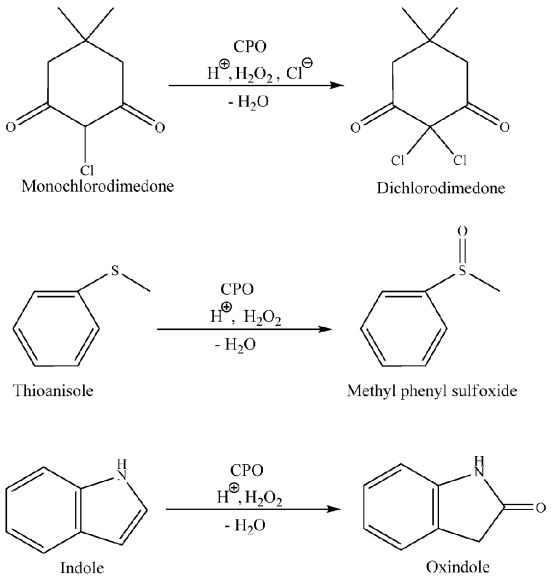
Cathode reaction:

$$O_2 + 2H^+ + 2e^- \rightarrow H_2O_2$$

Anode reaction:

$$H_2O \rightarrow 2e^- + \tfrac{1}{2}O_2 + 2H^+$$

Scheme 2 shows the enzymatic conversion of mono-chlorodimedone (MCD) to dichlorodimedone, thioanisole to methyl phenyl sulfoxide and indole to oxindole.



Scheme 2 Conversion of monochlorodimedone, thioanisole and indole.

Experimental

The production and purification of the CPO with *C. fumago* is described elsewhere.[16] The CPO activity was measured with the MCD assay under standard conditions (25 °C, 100 mM citric acid buffer, pH 2.75, 100 µM mono-chlorodimedone, 20 mM NaCl and 2 mM H_2O_2). CPO activity was photometrically detected by measuring absorption at 278 nm. The concentration of CPO was photometrically determined at 400 nm (ε = 75300 M^{-1} cm^{-1}).[17]

The reactor consists of an anode and a cathode enclosed in a poly(methyl methacrylate) case. The gas diffusion cathode (surface area = 5.5 cm^2 or 16.5 cm^2) was a commercial electrode supplied by Gaskatel (Kassel, Germany) and the volume of the cell was 8 mL. The cathode consists of a support net to which a catalyst mixture is attached and of a polytetrafluoroethylene foil which serves as the electrolyte barrier. Platinum is used as the anode which has the same surface area as the cathode. The reactors casing has three parts, the middle part forming the flow channel. The platinum anode is placed to one side part, the gas diffusion cathode is placed to the second one. The distance between the anode and the cathode was 1 cm. Oxygen from the air can diffuse through a notch to the reverse side of the GDE. A potentiostat (Gamry Reference 600, Warminster, USA) was used to characterize the two electrode system. For the substrate conversions a current generator is used to realize a more process-oriented set-up. All experiments were performed in citrate or sodium acetate buffers. The reactor was applied in the bypass of a 50 mL reservoir. A flow of 63 mL min^{-1} was pumped through the reactor cell. 4 mL samples were taken periodically from the reactor and the H_2O_2 was quantified using the NANOCOLOR® Peroxid 2 test kit (Macherey-Nagel, Düren, Germany). MCD concentration in the reaction system was determined photometrically (ε = 12.2 mM^{-1} cm^{-1}). Further reaction products were determined with GC-MS (GC17A (Shimadzu, Kyoto, Japan), Valcobond VB-5 column, l = 30 m; ID = 0.25 mm (VICI, Schenkon, Switzerland), temperature program 100 °C (2 min) then 15 °C min^{-1} to 260 °C (2 min), injector temperature: 250 °C, GC-MS interface temperature: 290 °C, scan range MS: 40–400 *m*/*z*. The substrate concentration was 5 mM. Thioanisole was added during the reaction to prevent substrate limitations. The retention times of the reaction products methyl phenyl sulfoxide and oxindole were 11.2 min and 7.7 min, respectively. The products were quantified using 1-octanol as internal standard.

Results and discussion

The electrochemical behaviour of the GDE cathode was characterized by linear sweep voltammetry with a scan speed of 2 mV s^{-1} (Fig. 1). For the estimation of the hydrogen peroxide production rates different constant cell voltages were applied and the resulting H_2O_2 concentration in the electrolyte was measured.

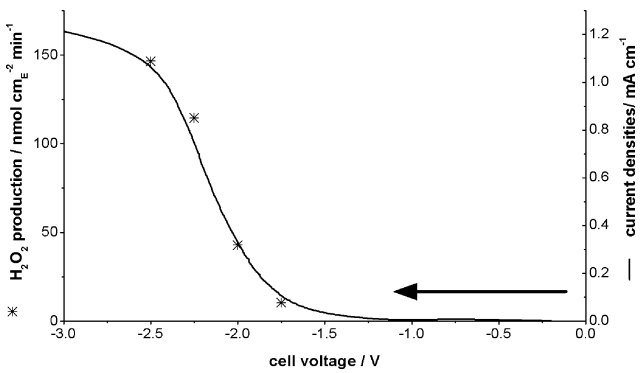


Fig. 1 Hydrogen peroxide production rate and current densities (*j* in mA cm^{-2}) as a function of applied potential (potentiostat as cell voltage generator, $A_{cathode}$ = 5.5 cm^2, 100 mM citrate buffer pH 2.75 with 10 mM sodium chloride, *V* = 150 mL). Each value for the H_2O_2 productivity is a mean of triple measurements with an error smaller than the symbol. The arrow shows the direction of the linear sweep.

Cell voltages between 0 and −1.5 V cause only low current densities. From −1.5 V an increase of the current densities can be measured. The hydrogen peroxide concentration in the electrolyte indicates that the production rate depends on the applied cell voltage. Thus, the production of H_2O_2 can be easily controlled by the applied cell voltage or current. In a blind test without an applied potential no conversion takes place. To investigate the long-term productivity of the GDE hydrogen peroxide was produced continuously at a constant current. In this case the reaction medium ($A_{cathode}$ = 5.5 cm^2, 100 mM citrate buffer pH 3.5 + 10 mM NaCl, *j* = 5.5 mA cm^{-2}) was pumped only once from the reservoir through the reaction cell and was

removed afterwards without any recirculation. After a short run-in period the hydrogen peroxide production rate was constant for more than 18 h. The hydrogen peroxide concentration in the effluent of the cell was 165 ± 12 μM.

The current efficiency (C.E.) is an important parameter that determines how much energy is consumed for the production of hydrogen peroxide or for the formation of side products. C.E. of hydrogen peroxide production was calculated using the equation:

$$C.E. = \frac{nF[H_2O_2]V}{Q} \times 100$$

Where n is the stoichiometric number of transferred electrons (2), F is the Faraday constant (96 500 C mol^{-1}), [H_2O_2] is the H_2O_2 concentration in the electrolyte in mol L^{-1}and V is the reaction volume in L. The total charge (Q in C) was calculated by integrating the current over the time. The C.E. is basically the ratio between the amount of produced hydrogen peroxide and the total amount of consumed electrons. Fig. 2 shows the current efficiencies, the hydrogen peroxide production rates and the resulting cell voltages at different current densities produced with a current generator. The hydrogen peroxide production rates show a linear relation to the applied current densities (slope 13.5 ± 0.17, R^2 = 0.998).

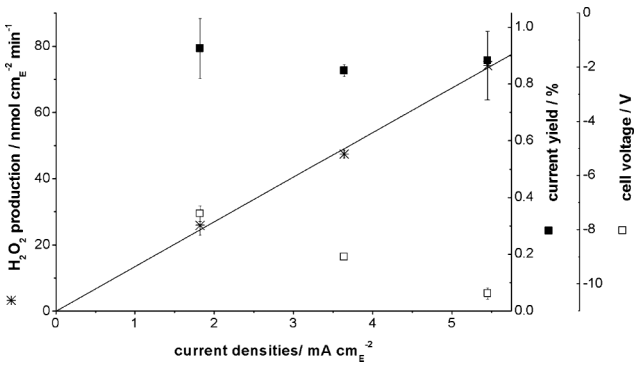


Fig. 2 Hydrogen peroxide production rate, current efficiency and cell voltage as a function of applied current densities ($A_{cathode}$ = 16.5 cm^2, 100 mM sodium acetate buffer pH 5 + 50 mM Na_2SO_4). The presented data correspond to mean values of two independent experiments.

At 5.5 mA cm^{-2} 74 nmol hydrogen peroxide was produced per minute and cm^2 of the cathode. The resulting cell voltages increase with higher current densities (slope –0.81 ± 0.04, R^2 = 0.999). The measured current efficiency in sodium acetate buffer (pH 5) with addition of 50 mM sodium sulphate or 100 mM citrate buffer (pH 2.75) with addition of 10 mM sodium chloride as electrolyte was 88 ± 4% or 55%, respectively. Panizza *et al.* used a comparable GDE to reduce oxygen to hydrogen peroxide in 0.05 M Na_2SO_4 as electrolyte with air feed.[18] The highest current efficiency was 53%. Dissolved oxygen can be electrochemically reduced to hydrogen peroxide at glassy carbon particles with a high current efficiency up to 95% when using sodium acetate buffer with sodium sulphate addition.[12] By using a graphite felt electrode and 0.1 M phosphate buffer (pH 5.5) as electrolyte the maximum current efficiency was 63%.[1] The current efficiencies measured in our study indicate that the production of hydrogen peroxide at a GDE is environmentally benign as obviously only few side reactions occur. The most important reactions negatively influencing the current efficiency are the self-decomposition of H_2O_2 to O_2 and the further reduction of hydrogen peroxide to water.[18,19] Besides lowering the current efficiency this side products have only a slight impact on the whole process. To illustrate the broad range of applications of the GDE-CPO-system halogenation of monochlorodimedone, sulfoxidation of thioanisole and oxidation of indole were investigated. Fig. 3 exemplarily shows the kinetics of MCD conversion in the electrochemical cell.

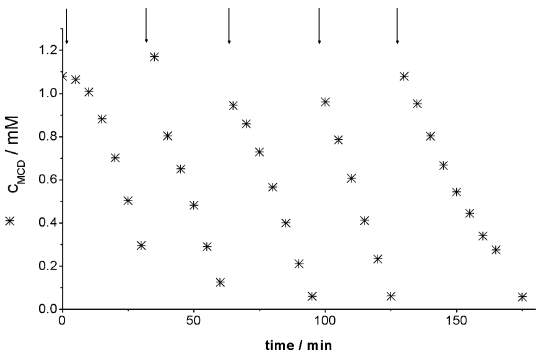


Fig. 3 Conversion of MCD in an electro-enzymatic process ($A_{cathode}$ = 5.5 cm^2, 100 mM citrate buffer pH 2.75 with 10 mM sodium chloride, 30 nM CPO). Due to low solubility MCD was added five times (arrows). The applied potential was –2.5 V with a potentiostat as cell voltage generator; the resulting current density was 3.6 mA cm^{-2}.

At the beginning the substrate conversion rate is nearly constant (36.2 ± 3.4 μmol min^{-1}). In this phase the generation of H_2O_2 is the limiting step in this reaction. During the first 120 min no H_2O_2 can be detected in the solution (data not shown). After the fifth addition the conversion rate decreases to 24.9 μmol min^{-1}. After a further substrate addition no conversion takes place and H_2O_2 can be detected in the solution. In the absence of the enzyme no product formation was measured excluding a non-selective oxidation at the electrode. A reaction system with a 3-fold higher electrode surface of 16.5 cm^2 was tested. Table 1 shows the conditions and results for the conversion of different substrates. The reaction system can be described by the total turnover number (ttn) as quotient of the moles of formed product and added enzyme, the space-time-yield (STY) as the mass of a product formed per volume of the reactor and time unit and by the electrode surface time yield (ETY) as the mass of a product formed per electrode surface area and time unit. The ttn values were calculated at the end of a process where no further substrate conversion can be measured.

The conversion of MCD showed the highest ttn (over 203 000). The GDE-based reaction system (V = 50 mL) showed a STY up to 23.0 g L^{-1} d^{-1} for the conversion of thioanisole and an ETY over 0.87 g cm^{-2} d^{-1} for the conversion of MCD. Seelbach *et al.*[17] measured a ttn of 53 410 and a STY of 9.3 g L^{-1} d^{-1} for the conversion of indole with CPO. The GDE-CPO-system generated comparable values. After 120 min up to 98% of thioanisole were converted to the product. Further improvements of the ttn and STY are possible by using a cosolvent such as *tert*-butanol.[12,17] After addition of 10% (v/v) *tert*-butanol Lütz *et al.*[12] measured a STY of 104 g L^{-1} d^{-1} and a ttn of 145 000 for the conversion of thioansiole. With the given

Table 1 Comparison of different substrate conversions with CPO in the GDE-based reaction system (conversion of MCD: 100 mM citrate buffer pH 2.75 + 10 mM sodium chloride; conversion of thioanisole: 100 mM sodium acetate buffer pH 5 + 50 mM Na_2SO_4; conversion of indole: 100 mM citrate buffer pH 2.75 + 10 mM sodium chloride)

Substrate	[CPO]/nM	Electrode surface area/cm^2	Current densities/ mA cm^{-2}	ttn/mol product mol enzyme^{-1}	STY/g L^{-1} d^{-1}	ETY/g cm^{-2} d^{-1}
MCD	10	5.5	5.5	203 100	9.5	0.870
Thioanisole	100	5.5	5.5	48 200	12.2	0.112
Thioanisole	100	16.5	1.8	83 600	19.8	0.061
Thioanisole	600	16.5	5.5	3 120	23.0	0.070
Indole	500	5.5	5.5	9 500	3.3	0.300
Indole	100	16.5	1.8	39 000	8.3	0.025

electrode surface of 1350 cm^2 the ETY can be calculated to 0.007 g cm^{-2} d^{-1}. Table 1 shows that an increased electrode surface leads to improved ttn and STY. The increase is not a linear function of the electrode surface. The most important parameter influencing the ttn and STY is probably still enzyme inactivation. Our results demonstrate that the ttn, STY and ETY in a process with electrogeneration of hydrogen peroxide at a GDE and comparable reaction conditions are similar to processes with sensor-controlled dosing of diluted hydrogen peroxide solutions,[17] *in situ* generation of H_2O_2 by oxidizing glucose with glucose oxidase[20] or other electrochemical methods, *e.g.* fixed bed electrodes. 3-fold higher current densities lead to a decrease of ttn through a faster inactivation of CPO by an excess of H_2O_2. Furthermore, the denaturation of CPO at the electrode can negatively influence the catalytic performance of the reaction system.[21] Therefore, the combination of electrogeneration of hydrogen peroxide at the GDE with suitable CPO immobilization techniques[22,23] thereby avoiding direct contact of the enzyme with the electrode could further improve the performance of the reaction system. Several authors have already shown that the immobilization of CPO improves the operational stability of the enzyme.[22–24] According to the values presented in Table 1 high electrode surfaces at low enzyme concentrations and low current densities resulted in optimized ttn and STY. The GDE-peroxidase-system should be further improved in regard to the applied current, the distance between the electrodes, the enzyme concentration and substrate delivery. Furthermore, the electrode material should be adapted to electrolytes containing organic solvents. This may lead to further advancements of STY and ttn as shown in a similar approach with a fixed bed electrode.[12]

By electrogenerating hydrogen peroxide no second enzyme is needed and therefore the formation of by-products is avoided. Furthermore, the most important advantages of the gas diffusion electrode system are the easy technical set-up, elimination of mass transfer limitations and the feasibility of high specific electrode surface areas. The combination of hydrogen peroxide electrogeneration with enzyme immobilization could further improve the performance of the reaction systems.

Conclusions

We showed for the first time that the production of hydrogen peroxide at a GDE can be successfully combined with an enzymatic reaction. Our data illustrate that an electro-enzymatic process with a reaction system based on a GDE is possible. We found high current efficiencies and substrate conversion rates and yields comparable to published data. The use of a GDE has several advantages: the hydrogen peroxide production is easily controllable, the GDE system is easily scaleable, the operation of the system is cheap and environmentally friendly and the use of immobilized CPO in the reaction system is possible. The GDE-peroxidase-system presented is a promising tool for applications in white biotechnology and for the production of chiral pharmaceuticals.

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