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Short Communication

Electroenzymatic process to overcome enzyme instabilities

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ABSTRACT

The versatile enzyme chloroperoxidase was used in a reaction system, based on a gas diffusion electrode, for enzymatic chlorinations. Due to an adjusted and continuous electro-generation of the co-substrate hydrogen peroxide a *ttn* up to 1,150,000 for the CPO was achieved. Space time yields were dependent on the electrochemically produced H_2O_2 and reached up to 52 g L^{-1} d^{-1}. The ratio of hydrogen peroxide production per added enzyme unit can be used as a dimensionless parameter for process characterization and a knowledge based process design.

1. Introduction

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 [1]. CPO catalyzes the halogenation of different molecules such as aromatic hydrocarbons [2,3], monoterpenes [4,5], lignin structures [6] or flavanones [7]. In addition to halogenations CPO catalyzes hydrogen peroxide-supported oxidation, the dismutation of hydrogen peroxide (catalase reaction) and some cytochrome P450 monooxygenase-like reactions [8].

Nevertheless, its use on preparative or industrial scale reactions has been hindered by instability towards the co-substrate hydrogen peroxide [9]. In order to avoid the irreversible inactivation, hydrogen peroxide can be generated electro-chemically on a low, but sufficient level for catalytic activity [10,11]. Electrogeneration of H_2O_2 is an attractive approach since it does not require additional chemicals and electricity is readily available [12]. In general, such electroenzymatic processes are interesting systems due to the combination of the advantages of enzymes and electrochemical steps [12–14]. The applications of redox enzymes have tremendous potential as catalysts for preparative organic chemistry [15–17]. Their usually high selectivity, combined with their catalytic performance, makes them important for synthesis of fine and bulk chemicals. The main advantages of electrochemical processes are: versatility, energy efficiency, amenability to automation and cost effectiveness [18,19].

The use of the electrons as "clean reagents" for enzymatic reactions provides several advantages over conventional, non-electrochemical techniques. Furthermore H_2O_2 is considered to be an environmentally friendly chemical, since it leaves no hazardous residues, such as other oxidants. Krieg et al. described the use of a gas diffusion electrode (GDE) as a new type of electrode material for electroenzymatic processes [20]. GDEs are electrodes with a solid, liquid and gaseous interface and consist, besides the catalyst, of polytetrafluoroethylene (PTFE) and additives. PTFE forms a hydrophobic matrix which binds the catalyst and the liquid attracting materials.

In the present study the GDE-based system was used to investigate the operational stability of the CPO in a model reaction, the oxidative chlorination of monochlorodimedone (MCD), a common substrate of haloperoxidases, to dichlorodimedone (Scheme 1). One important indicator of lifetime biocatalyst productivity is the dimensionless total turnover number (*ttn*) [21]. Starting point for the investigation was a published *ttn* of 203,000 for the conversion of MCD in a GDE-based reaction system [20]. An ideal catalyst would have an infinite turnover number, because it would not ever be consumed, but in practice the stability of all biocatalysts is limited. It is a challenge in process development to identify suitable conditions to improve catalyst lifetime. As a benchmark the highest reported *ttn* for a chloroperoxidase catalyzed reaction was used (860,000 for the conversion of oxidation of indole) [22]. Furthermore the space time yield as well as the productivity related to the electrode surface should be optimized in the present study.

2. Experimental

The production and purification of the CPO with *C. fumago* was described elsewhere [23]. The CPO activity was measured with the MCD assay under standard conditions (25 °C, 100 mM citric acid buffer,

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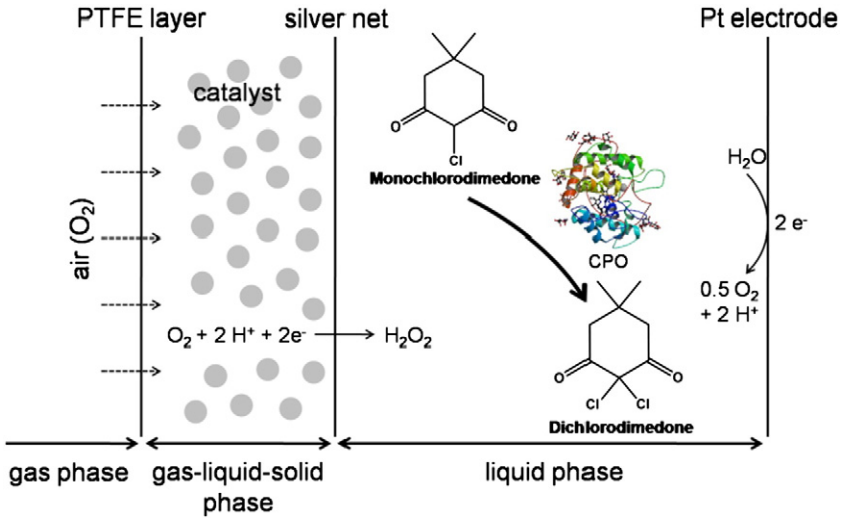

Scheme 1. Electroenzymatic reaction system. Oxygen is reduced to hydrogen peroxide at the gas–liquid–solid interphase by a carbon catalyst of the GDE (cathode). Afterwards the H_2O_2 diffuses into the liquid phase to the enzyme. Here the enzymatic chlorination of monochlorodimedone takes place. At the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

pH 2.75, 100 μM monochlorodimedone, 20 mM NaCl and 2 mM H_2O_2). CPO activity was photometrically detected by measuring absorption at 278 nm [24]. The concentration of CPO was photometrically determined at 400 nm ($\varepsilon = 75{,}300\ M^{-1}\ cm^{-1}$, [22]). The electrochemical 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) was a commercial electrode supplied by Gaskatel (Kassel, Germany) and the volume of the cell was 8 mL. Platinum is used as anode which has the same surface area as the cathode (for further details please check [20]). Oxygen from the air can diffuse through a notch to the reverse side of the GDE. For the substrate conversions a current generator is used. All experiments were performed in citrate buffer (0.1 M, pH 2.8 or 3.5). The reactor was applied in the bypass of a reservoir. A flow of 50 mL min^{-1} was pumped through the reactor cell. MCD concentration in the reaction system was determined photometrically at 278 nm. 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).

3. Results and discussion

First of all the electrogeneration of H_2O_2 was characterized. The knowledge of the H_2O_2 productivity is necessary for later coupling of the electrochemical to the enzymatic step. Fig. 1 shows the produced H_2O_2 at different currents applied. It is obvious that up to 20 mA, corresponding to 3.6 mA cm^{-2}, the productivity linearly depends on the applied current. The hydrogen peroxide productivity can be calculated from Eq. (1) ($R^2 = 0.97$):

$$P\left[\mu mol\ \ min^{-1}\ \ cm^{-2}\right] = 0.027 \cdot I[mA]. \quad (1)$$

A higher current did not result in a further increase in the productivity. Most probably the diffusion of the oxygen in the reaction system was the limiting parameter. In a blind test without electric current applied no production of H_2O_2 occurred. 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 given elsewhere [20]. The C.E. is basically the ratio between the amount of produced hydrogen peroxide and the total amount of consumed electrons. In the chosen buffer and applied electrochemical system the C.E. was 50%.

The result shows that the hydrogen peroxide productivity can be easily controlled by an electrochemical parameter. In the next step the electrochemical H_2O_2 production was coupled with the enzymatic chlorination of monochlorodimedone. In these reactions the concentrations of CPO (5–30 nM), the applied current (15–30 mA) as well as the ratio of apparent electrode surface to reaction volume (0.11–0.32 $cm^2\ mL^{-1}$) were varied in the given ranges while the substrate concentration was kept constant. The operational stability of the enzyme was characterized by the ratio of product molecules formed per enzyme. These *ttn* values were calculated at the end of a process where no further substrate conversion could be measured. In Fig. 2 this *ttn* is shown as a function of the ratio of electrochemically produced H_2O_2 in μmol min^{-1} and the amount of CPO added which is expressed in enzyme units. One enzyme unit is defined as the activity that catalyzes the chlorination of 1 μmol of MCD per minute under non-electrochemically driven standard assay conditions. The new dimensionless parameter was named specific hydrogen peroxide production and is defined as the ratio of electrochemically produced H_2O_2 per minute per enzyme unit of CPO.

At low ratios of produced H_2O_2 and added CPO units the *ttn* is low. In the range between 0.03 and 0.1 μmol H_2O_2 min^{-1} per CPO unit the *ttn* is most likely not limited by an inactivation of hydrogen peroxide. Further limitations such as thermal instability or interactions with buffer components such as salts may influence the operational stability of CPO at low co-substrate concentrations. At high specific hydrogen productions

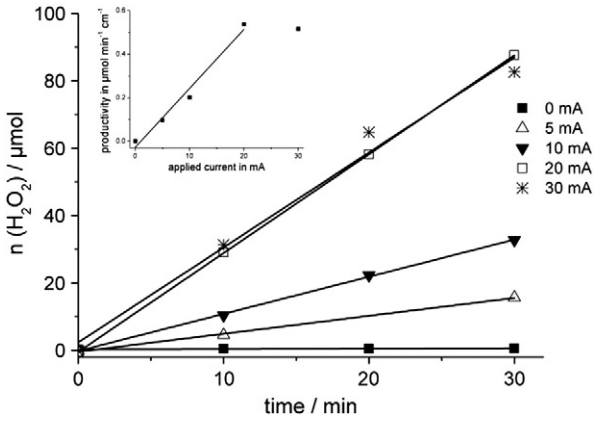

Fig. 1. H_2O_2 formation in a GDE-based flow-through reaction system at different electric currents applied (apparent electrode area 5.5 cm^2, citrate buffer 0.1 M, 10 mM NaCl, T = 30 °C, V = 50 mL, flow rate 50 mL min^{-1}). The insert shows the calculated productivities as a function of the current applied.