JOURNAL OF APPLIED ELECTROCHEMISTRY 25 (1995) 508–511

Bioelectropolymerization of phenol

A. COURTEIX, A. BERGEL, M. COMTAT

Laboratoire de Génie Chimique, URA CNRS 192; Laboratoire de Génie Chimique et Electrochimie, Université Paul Sabatier, 118 route de Narbonne, 31062 Toulouse Cédex, France

Received 27 January 1993; revised 15 November 1993

1. Introduction

For some years, new electrochemical processes integrating biochemical syntheses have been appearing at an ever faster rate. The electrochemical process is used to continuously regenerate one of the species taking part in the biochemical reaction. In general, enzymatic catalysts are involved, examples being the production of gluconic acid by immobilized glucose oxidase with electrochemical regeneration of the electron acceptor [1], the production of acetaldehyde from ethanol with NAD^+ electrochemical regeneration [2], the electrochemical regeneration of NAD^+ in a plug flow reactor [3], the indirect electrochemical reduction of methemoglobin [4, 5] and the reduction of nitrate and nitrite in water catalysed by immobilized enzymes [6].

This work presents the foundation of a process combining the enzymatic catalysis of the oxidation of a substrate by hydrogen peroxide with the cathodic production of the latter by reduction of dissolved oxygen. The amount of peroxide in the reaction medium can thus be controlled by the electrochemical conditions to avoid the common phenomena of enzyme inhibition or degradation caused by excessively high hydrogen peroxide concentrations. Such a process has recently been proposed for the oxidation of dimethylaniline by horseradish peroxidase and electrogenerated peroxide [7]. This work deals with a more complex model reaction concerning the polymerization of phenol. It is of great interest for the detoxification of phenolic effluents, since the polyaromatic compounds formed are insoluble in aqueous media [8] and are therefore easily separable. Two types of biochemical catalyst were tested: horseradish peroxidase and hematin.

Peroxidases are hemic enzymes widely found in the animal and plant kingdoms. They catalyse numerous chemical reactions: additions [9], chlorinations [10], demethylations [11–15], hydroxylations [8, 16–18], as well as oxidations followed by polymerization of the phenolic compounds [19]. Using horseradish peroxidase (HRP) and hydrogen peroxide leads to the oxidation of phenol [20]. The phenoxy radical generated by the reaction can dimerize or react to the phenol and the polyphenols [21]. The mechanism of the catalysis involves a first two-electron oxidation of the enzyme by the hydrogen peroxide into the intermediate compound I (HRP-1) [20]:

$$\text{HRP} + H_2O_2 \longrightarrow \text{HRP-I} + H_2O \qquad (1)$$

This compound further oxidizes phenol into phenoxy radicals by two successive one-electron reactions involving a second enzyme intermediate (HRP-II):

$$\text{HRP-I} + \text{phenol} \longrightarrow \text{HRP-II} + \text{phenoxy radical} \qquad (2)$$

$$\text{HRP-II} + \text{phenol} \longrightarrow \text{HRP} + \text{phenoxy radical} + H_2O \qquad (3)$$

$$\text{phenoxy radicals} + \text{phenol} \longrightarrow \text{dimerization and polymerization} \qquad (4)$$

Hematin is a natural molecule, found in vivo in pathological cases, whose peroxidizing properties have recently been demonstrated [22]. It is a ferriprotoporphyrine and is similar to an HRP molecule without its polypeptide chain.

2. Material and method

The enzymes, protein and CAPSO buffer were supplied by Sigma, the horseradish peroxidase was of type VI. The other products were supplied by Merck. The buffers were: pH 5.1: 0.05 M sodium acetate and hydrochloric acid; pH 7.2: 0.2 M monopotassium and disodium phosphate; pH 11.3: 0.05 M CAPSO solution. The absorbances were measured with a Hewlett Packard spectrophotometer. An EGG potentiostat was used to perform the constant potential electrolyses and the scanning voltammetries. The measurement of HRP enzymatic activity corresponded to the initial oxidation rate of the guaiacol, 7 mM, by hydrogen peroxide, 8 mM, with 0.3 nM of enzyme in a 0.1 mM pH 7.0 phosphate medium.

The reaction kinetics was monitored by measuring the absorbance variation at a wavelength of 470 nm, for which the product formed has the greatest absorbance. The oxidative polymerizations were accomplished with 25 cm^3 solution and an electrode area of 3 cm^2. Oxygen was bubbled through the buffered solution containing the phenol and the enzyme which was homogenized by stirring. In the presence of HRP, a pH of 5.0 was selected, which corresponds to the maximum activity of the enzyme. When oxidation was catalysed by the hematin, the pH was 11.3, an alkalin medium being necessary in order to solubilize the protein. The auxiliary electrode was a platinum grid. The reference electrode was a saturated calomel

0021-891X 1995 Chapman & Hall

electrode with respect to which all the potentials are expressed.

3. Results and discussion

On a mercury cathode, dissolved oxygen was reduced when the potential reached −50 mV. Hydrogen peroxide was only reduced when the potential was −800 mV. Electrolysis at a potential between these two values therefore allowed oxygen to be bi-electronically reduced to hydrogen peroxide according to the reaction:

$$O_2 + 2H^+ + 2e^- \longrightarrow H_2O_2 \qquad (5)$$

In the presence of peroxidase and phenol, the solution turned brown from the start of electrolysis at −400 mV. Brown streaks appeared on the surface of mercury. The colour of the solution no longer changed after a few minutes electrolysis. This was due to adsorption of the polymer on the surface of the electrode which no longer allowed the peroxidase to convert to compound I (HRP-I) from the hydrogen peroxide formed at the cathode. Furthermore, the enzymatic activity of HRP decreased by at least 30% after electrolysis for 60 min.

It has already been demonstrated that compounds I and II of horseradish peroxidase (HRP-I and HRP-II) can be obtained subsequent to the reduction of dissolved oxygen on a platinum cathode [23]. Mercury was replaced by a platinum grid, since the adsorption of phenol is weaker on platinum than on mercury. The current/potential curve recorded in the absence of peroxidase and phenol had only one wave. The reduction of dissolved oxygen took place directly with four electrons. The hydrogen peroxide is not a stable intermediate as it is on a mercury cathode. However, the possibility of obtaining the reaction intermediates of the peroxidase proves that the reduction takes place in two consecutive, but virtually simultaneous, steps.

$$O_2 + 2H^+ + 2e^- \longrightarrow H_2O_2 \qquad (5)$$

$$H_2O_2 + 2H^+ + 2e^- \longrightarrow 2H_2O \qquad (6)$$

The presence of a catalyst such as HRP, which is very reactive towards peroxide, allowed the hydrogen peroxide to be trapped. The current was recorded as a function of time during electrolysis at −300 mV. After a startup phase, the current stabilized (Fig. 1).

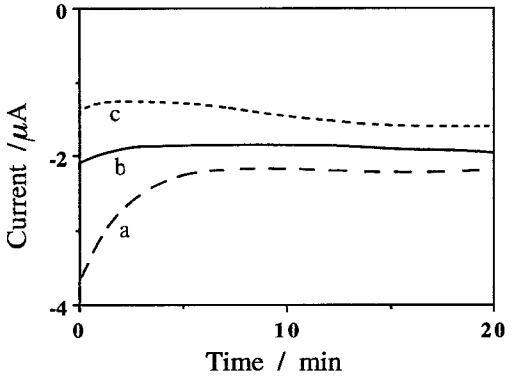

Fig. 1. Variation of the current as a function of time during an electrolysis at −300 mV vs SCE. Operating conditions: sodium acetate 0.05 M, pH 5.1; (a) buffer only; (b) buffer and phenol 7.4 mM; (c) buffer, phenol 7.4 mM and HRP 200 μM.

In the presence of phenol, but without peroxidase, the steady state current was always lower but the solution remained colourless. The current variation cannot, therefore, be due to the oxidation of the phenol by the hydrogen peroxide formed at the electrode. The drop in current by a factor of 15 to 20% was attributed to a change in the surface state of the electrode, due to phenol adsorption. Assuming that the current is directly proportional to the area of the electrode on which no species is adsorbed, the coverage, θ, of the electrode by adsorption is proportional to the quantity:

$$1 - \frac{\text{stable current after addition of phenol}}{\text{stable current with a clean electrode}} \qquad (7)$$

If the adsorption obeys a Langmuir law, θ, is linked to the phenol concentration in solution, C, by the relationship:

$$\theta = \frac{KC}{1 + KC} \qquad (8)$$

The values of θ were determined experimentally by successive additions of phenol under the same conditions as previously. The reproducibility of the results required very strict experimental conditions, especially relevant to the surface state of the electrode and the rate of oxygen bubbling into the solution. Nevertheless, it was possible to obtain identical results with very different electrodes areas (0.3 and 3 cm^2). Plotting $1/\theta$ as a function of $1/C$ (Fig. 2) gave a straight line intersecting the origin at a value of 1 that confirmed the Langmuir adsorption type.

When the current was recorded in the presence of peroxidase and phenol, the solution turned brown within a few tens of seconds. This confirms that it is possible to catalyse oxidation reactions in contact with a platinum cathode performing the reduction of dissolved hydrogen. The current decreased by

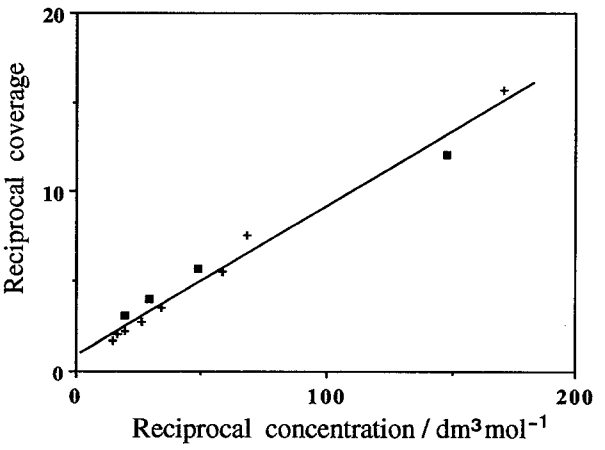

Fig. 2. Reciprocal coverage of the platinum electrode as a function of the reciprocal phenol concentration. Operating conditions: sodium acetate 0.05 M, pH 5.1; oxygen bubbling; electrolysis at −300 mV vs SCE; (■) platinum grid electrode of 0.3 cm^2 area; (+) platinum grid electrode of 3 cm^2 area.

approximately 15% with respect to the measurement made with the control solution containing phenol only. The reduction of hydrogen peroxide at the electrode (Reaction 6) and the oxidation of HRP into HRP-I by H_2O_2 (Reaction 1) were in competition. In the control experiments, without peroxidase, the current was relative to the reduction of O_2 (Reaction 5) for one half and to the reduction of H_2O_2 (Reaction 6) for the other half. When peroxidase was added, the decrease in current therefore indicated that 30% of the hydrogen peroxide was consumed in the oxidation reaction.

The curves recorded with a far higher [phenol]/[HRP] ratio confirmed this hypothesis: the current/time curve recorded with a smaller amount of enzyme differed only by 5% from that obtained with phenol alone. The solution turned a less intense shade of brown. The extent of oxidation was lower since, because the amount of enzyme close to the electrode was smaller, the amount of HRP-I was lower and the cathodic reduction of hydrogen peroxide was encouraged.

To avoid this competing electrochemical reaction, an electrode on which the hydrogen peroxide reduces in two distinguishable steps must be used. Various materials were examined by recording a voltammogram in buffered solutions suitable for enzymatic catalysis. Among all the materials taken under analysis: copper, nickel, molybdenum, carbon, zirconium, tantalum, tungsten and gold, only copper and gold allowed a reduction of oxygen in two stages. With a neutral pH, the two waves obtained on a copper cathode were only about 100 mV apart, but the hydrogen peroxide was stable over more than 300 mV with a gold electrode.

A gold cathode allowed a better oxidation of phenol in the presence of HRP: in a few tens of seconds the solution turned a more intense shade of brown than with platinum, while remaining identical in other aspects. After a few minutes, streaks of precipitate appeared in suspension in the solution, however, the reaction rapidly came to a halt. This cannot be attributed to the denaturation of the enzyme by the phenoxy radicals because controlled experiments performed with the same concentrations, in which the hydrogen peroxide was not electrochemically produced but added in excess at the beginning, gave greatly higher transformation rates. The rate decrease was due to the adsorption of the polyphenolic products on the electrode which appeared blackened. The formation of highly adhesive and very hydrophobic films during the oxidation of phenol derivatives on metallic surfaces has already been described [24, 25]. These films, responsible for passivation phenomena, can provide protection against corrosion. All the methods tried to clean the electrode surfaces were fruitless.

This led us to envisage replacing the HRP with hematin which offers many advantages: although hematin has catalytic properties, it is not an enzyme but a ferriprotoporphyrin, i.e. a smaller molecule ($633.5\ g\ mol^{-1}$) more stable than the horseradish peroxidase; it is cheaper than the horseradish peroxidase; it is only soluble in alkaline media, at pHs where the polyphenolic compounds are also soluble,

Control experiments, performed at pH 11.3 without electrochemistry by direct addition of hydrogen peroxide into the solution, showed no precipitation of polyphenols. Only a latter addition of acid caused precipitation and the appearance of the brown colour. It is therefore possible to conceive an electrochemical process in which the separation is accomplished after electrolysis by acidifying the medium. The polyphenolic compounds, soluble under the electrolysis conditions, should not foul the electrode surface. Experiments performed with a gold electrode at pH 11.3 with hematin confirmed that there was no precipitation after electrolysis for 1 h. Furthermore, the current only decreased slightly, indicating that the electrode was not fouled by the products. After the electrolysis was stopped, the solution was acidified and centrifuged to remove the precipitate. Spectrophotometric measurements indicated that at least 60% of the phenol was transformed.

4. Conclusion

These preliminary tests allowed very fast progress in the description of a process for electroenzymatic polymerization of phenol. It was verified that the association of HRP with the cathodic reduction of dissolved oxygen lead to polymerization. Phenol adsorption on the electrode was identified and did not pose any problem. On the other hand, the products deposited on the electrode were a serious problem. It can be solved by replacing HRP with hematin. The catalytic properties of this molecule have recently been demonstrated and the reaction mechanism has yet to be established. These first results indicate that it is possible to monitor an enzymatic catalysis of oxidation by hydrogen peroxide by means of an electrochemical reactor performing the cathodic reduction of dissolved oxygen.

References

[1] C. Bourdillon, R. Lortie and J. M. Laval, *Biotechnol. Bioeng.* **31** (1988) 553.
[2] J. M. Laval, J. Moiroux and C. Bourdillon, *ibid.* **38** (1991) 788.
[3] A. Fassouane, J. M. Laval, J. Moiroux and C. Bourdillon, *ibid.* **35** (1990) 935.
[4] P. Labrune, A. Bergel and M. Comtat, *ibid.* **36** (1990) 323.
[5] P. Labrune and A. Bergel. *Chem. Eng. Sci.* **47** (1992) 1229.
[6] R. B. Mellor, J. Ronnenberg, W. H. Campbell and S. Diekmann, *Nature* **355** (1992) 717.
[7] J. K. Chen and K. Nobe, *J. Electrochem. Soc.* **140** (1993) 299; *ibid.* **140** (1993) 304.
[8] J. S. Dordick, M. A. Marletta and A. M. Klibanov, *Biotechnol. Bioeng.* **30** (1987) 31.
[9] J. R. Kanofsky, *Biochem. Biophys. Res. Commun.* **159** (1989) 1051.
[10] W. D. Hewson and L. P. Hager, *J. Biol. Chem.* **254** (1979) 3175.
[11] B. W. Griffin and P. L. Ting, *Biochemistry* **17** (1978) 2206.
[12] G. L. Kedderis and P. F. Hollenberg, *J. Biol. Chem,.* **258** (1983) 8129.
[13] B. W. Griffin, *FEBS Lett.* **74** (1977) 139.

[14] G. Meunier, C. Paoletti and B. Meunier, *C. R. Acad. Sc. Paris* **299** (1984) 629.
[15] G. Meunier and B. Meunier, *J. Biol. Chem.* **260** (1985) 10576.
[16] J. W. Daly and D. M. Jerina, *Biochem. Biophys. Acta* **208** (1970) 340.
[17] A. M. Klibanov, Z. Berman and B. N. Alberti, *J. Am. Chem. Soc.* **103** (1981) 6263.
[18] J. S. Dordick, A. M. Klibanov and M. A. Marletta, *Biochemistry* **25** (1986) 2946.
[19] J. L. Michot, A. Virion, D. Deme, S. de Prailaune and J. Pommier, *Eur. J. Biochem.* **148** (1985) 441.
[20] B. L. Saunders, A. G. Holmes–Siedle and B. P. Stark, 'Peroxidase', Butterworths London (1964).
[21] A. M. Klibanov, T. N. Tu and K. P. Scott, *Science* **221** (1983) 259.
[22] Z. Genfa and P.K. Dasgupta, *Anal. Chem.* **64** (1992) 517.
[23] H. Durliat, A. Courteix and M. Comtat, *Bioelectrochem. Bioenerg.* **19** (1988) 413.
[24] F. Bruno, M. C. Pham and J. E. Dubois, *Electrochim Acta* **22** (1977) 451.
[25] M. C. Pham, P. C. Lacaze and J. E. Dubois, *J. Electroanal. Chem.* **86** (1978) 147.