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Electrochemical assay of thrombin immobilized on carbon electrodes using an electrogenic and chromogenic substrate

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The enzymatic and electrochemical features of an immobilized thrombin chemically modified electrode, operating as a voltammetric biosensor, are compared with the availability of a substrate permitting both an electrochemical and spectrophotometric assay of thrombin. Such a device enables the detection of products issued from the enzymatic hydrolysis inside the diffusion layer of the stationary electrode.

1. Introduction

Enzymatic activities of blood coagulation proteases were assayed by electrochemical techniques using specific substrates [1–4]. Basically, these methods consisted in the voltammetric treatment of the current generated by the oxidation of a label released from a substrate through enzymatic hydrolysis. Because the platinum working electrode operates in contact with biological media (e.g. plasma or whole blood), an electrochemical pretreatment had to be applied to the electrode surface before its use.

The device under investigation here is aimed at restricting the electrochemical assay in the diffusion layer, instead of the bulk solution. Therefore, we used an unshielded carbon paste electrode which supports the protease being assayed. The enzyme is immobilized via irreversible adsorption. Owing to the heterogeneous nature of the kinetics, limiting mass transfer processes (diffusion towards the electrode for the substrate and backwards for the label after its oxidation) are expected to induce stationary currents. Results will be presented showing that such behaviour indeed occurs. A schematic diagram of the sequence of events that intervene in the system is given in Fig. 1.

2. Materials and methods

Tris buffer solutions (tris-HCl 50 mM, NaCl 125 mM, pH 8.5) were prepared with ultrapure water obtained after filtration on Multipore membranes. Pure lyophilized thrombin (E.C.3.4.21.5) (1230 NIH, lot Nr 9 × 155) was dissolved in the buffer in 50 NIH/ml aliquots. The specific substrate was D-H-Pro-phe-Arg-*p*-nitroaniline or S-2238. Other products were of analytical grade and used without purification. The conventional carbon paste electrode was prepared after thorough mixing of graphite powder with nujol in the proportion 30/9 (wt/wt).

Spectrophotometric assays of thrombin were realized with a MonoSpac 103 apparatus by monitoring at 405 nm the variation in the absorbance produced by the zero-order release of *p*-nitroaniline (*p*Na) through the enzymatic cleavage of the substrate. Preliminary experiments showed that following its adsorption, no further desorption of thrombin could be observed when the electrode surface was rinsed thoroughly under flowing water for 10 min.

Electrochemical measurements were carried out with a PAR 173 potentiostat monitored by a 175 PAR triangular wave generator, which applies the signal to a three-electrode system, including a saturated calomel electrode and a platinum counter-electrode. All vessels containing thrombin were in plastic (polystyrene or teflon) to avoid loss by adsorption.

A 1260 Multi-gamme counter equipped with a LKB computer is used to measure the superficial density of thrombin radiolabelled with ^{125}I on the electrode surface. By taking into account that one NIH unit of pure thrombin is equivalent to the activity of 8.9 $\times 10^{-12}$ mol [5, 6], calculation shows that, in a typical experiment, $1.3 \times 10^{+11}$ (or 2×10^{-13} mol cm^{-2}) of active thrombin molecules per cm^2 remain adsorbed at the end of the electrode preparation.

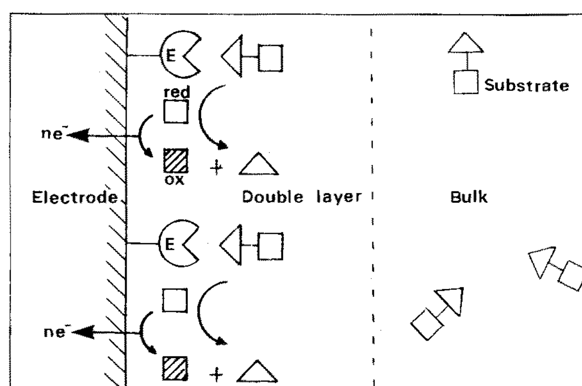

Figure 1 Diagram of the sequence of kinetic events occurring during the electrochemical detection.

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3. Results and discussion

3.1. Photometric assay of irreversibly adsorbed thrombin

First the enzymatic activity of thrombin in the immobilized state (adsorbed) is evaluated conventionally by a photometric procedure: the electrode which supports the protease is dipped in the assay cuvette containing the substrate solution and the measurement is carried out under forced convection. Assuming a Michaelis–Menten treatment of the kinetics, the Michaelis constant, K_m, and the maximum rate, V_m, are found equal to 27 μM and 5 nM s^{-1} (equivalent to 2.8×10^{-3} NIH/ml), respectively.

The conversion of this activity in terms of concentrations indicate that about 2×10^{-13} mol cm^{-2} thrombin are supported by the electrode surface, a value in coincidence with that calculated by direct radiomeasurements carried out independently. Otherwise, it should also be noted that the values of the kinetic parameters related to thrombin closely agree with those measured for a homogeneous solution of the enzyme (20 μM and $k_{cat} = 225$ s^{-1}). This suggests that the conformation of the enzyme remains unaffected by the adsorption.

3.2. Electrochemical assay of adsorbed thrombin

3.2.1. Preliminary experiments

The enzymatic activity of thrombin is periodically evaluated via the measurement of the voltammetric response towards an oxidative potential sweep aimed at oxidizing the label released from the substrate by the enzyme. Because it is necessary to convert current amplitudes in concentrations, a calibration curve of the label was first established. On the bare electrode, the electrochemical oxidation of *p*NA generated an irreversible peak at about $E_p = 0.86$ V/ECS; after adsorption of thrombin, while E_p was anodically displaced to 0.95 V/SCE (Fig. 2), the slope of the calibration curve of *p*NA remains constant (0.43 A cm^{-1} M^{-1} at a sweep rate of 0.2 V s^{-1}). In the linear part of the calibration curve, in the μM–mM range, the relative experimental error is about 5%.

3.2.2. Kinetic measurements

After adsorption of thrombin, the "thrombinated" electrode was introduced into solutions containing

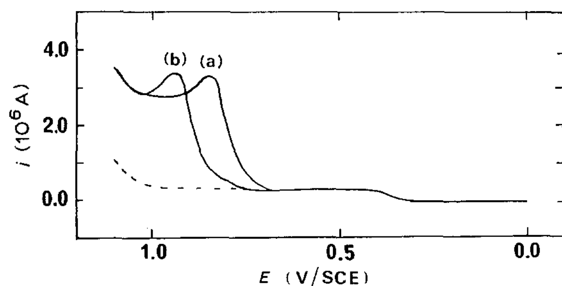

Figure 2 Voltammetric oxidation curves of *p*-nitroaniline on (a) a bare and (b) a modified electrode through thrombin adsorption, in an aqueous buffer solution (tris-HCl, pH 8.8, 25 °C) at 0.20 V s^{-1} sweep rate.

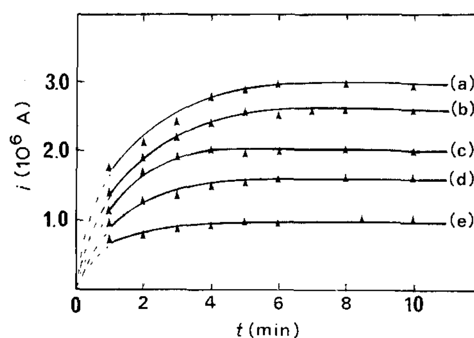

Figure 3 Amplitudes of the voltammetric peak current responses of the thrombin-modified carbon electrode as a function of the substrate concentration for increasing hydrolysis times.

the substrate at increasing concentrations, in order to measure the amplitude of the amperometric response, after various hydrolysis times. Each electrode surface operates only once. Results yield the homothetic curves represented in Fig. 3. Because the measurements require the electrode to be dipped in the solutions, the first measurements could not be performed before approximately 1 min, in order to avoid, as far as possible, convection around the electrode. As a result, the procedure described here does not enable short-term rate measurements to be made, at least in the order of time of conventional enzymatic kinetics. However, it will be seen later that such an inconvenience is only apparent and can easily be overcome by the global treatment of the curve profiles [7]. Under the diffusion-limiting conditions used, two separated regimes were observed: beyond an initial time-dependent variation, currents reached stationary values after 6 min, which could therefore be used with good precision for quantitative purposes.

It has been proved that the stationary currents can be linearized according to Equation 1, which appears to be an equivalent expression for the rate of a Michaelis–Menten behaviour applied to enzymatic kinetics in homogeneous conditions [8].

$$i(s) = i(\max)\,(S)/[K'_m + (S)] \qquad (1)$$

where i(s) is the stationary current, i(max) the maximum value of i(s), (S) the concentration of the

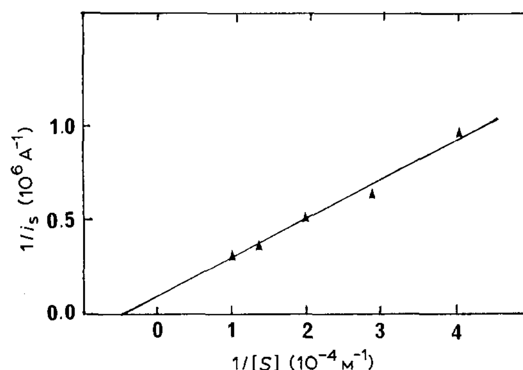

Figure 4 Equivalent Lineweaver–Burk representation applying for the voltammetric detection, and relative to the enzymatic kinetics occurring at the surface of the carbon electrode, in tris-HCl buffer, pH 8.5, 25 °C. Stationary currents, i(s), are the peak amplitudes recorded after an hydrolysis time of 10 min.

substrate and K'_m an apparent Michaelis constant. It must, however, be emphasized that the validity of Equation 1 had been tested under conditions of forced convection which prevail for rotating solid electrodes. Under our conditions which meet a fixed electrode and pure diffusional kinetic processes, the plot of the data according to Equation 1 also leads to a straight line, represented in Fig. 4; its intercepts with the axis yield an apparent $K'_m = 200\ \mu M$ and $i(\max) = 67 \pm 5\ \mu A\ cm^{-2}$.

4. Conclusion

Results show that the electrochemical (voltammetric) assay of thrombin adsorbed on a carbon electrode can be performed in stationary conditions with the same specific substrate which is used for conventional chromogenic assays. Moreover, it has been observed that the kinetic rate, expressed in terms of stationary currents instead of absorbance variation for the latter technique, fulfils the relationship established for heterogeneous rate measurements of immobilized enzymes operating under convective mass transfer.

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