THROMBIN ACTIVITY MEASURED WITH A NEW ELECTRIC METHOD

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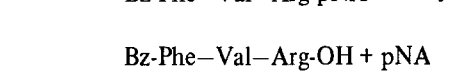
1. Introduction

There are several enzyme electrodes described in the literature. Most of them use ion-specific electrodes and immobilized enzymes [1,2]. They can be sensitive to the substrate, the products or the enzyme themselves. There are also spectrophotometrical methods, in which the enzymatic reaction produces or consumes chromogenic substances [3,4]. For the determination of thrombin activity the clotting time technique is often used.

In this communication we describe an electric method which makes it possible to determine enzymatic activity of serine proteases, e.g. thrombin and trypsin. The method uses a substrate with ability to adsorb on a metal surface. In presence of enzyme the substrate is hydrolyzed and the main part of it desorbes. The adsorbed substrate layer constitutes a (surface) capacitance, which is measured electrically [5]. The desorption rate is found to be proportional to the enzymatic activity in the solution. The technique is exemplified with a method for thrombin standardization and with an assay of antithrombin in plasma, using a synthetic chromogenic peptide substrate, sensitive to the enzyme studied. The substrate, S-2160, has the formula *N*-benzoyl-phenylalanyl–valyl–arginine-*p*-nitroanilide (Bz-Phe–Val–Arg-pNA) and is hydrolyzed through the catalysis of serine proteases according to:



The rate of *p*-nitroaniline (pNA) formation can be followed by its u.v. adsorption at 405 nm, which is proportional to the enzyme concentration.

Until now this system has only been used for spectrophotometric measurements [4,6].

2. Methods and results

The measurements are carried out in Tris-buffer with a pH of 8.2 and ionic strength of 0.15 M. The capacitance between two platinum electrodes in a test-tube containing buffer solution is measured with an impedance bridge. The electrolytic double-layer constitutes a capacitance, which together with the electrolyte resistance forms and electrical equivalent circuit as shown in fig.1. When the substrate, S-2160, is added it adsorbs on the electrodes and forms capacitancies, which electrically will be in series with

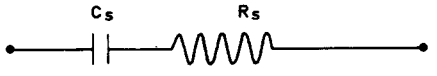


Fig.1. The electrical equivalent circuit of the electrode system. R_S is mainly the resistance of the electrolyte between the electrodes. In absence of substrate, C_S is due to electrode polarisation. The addition of substrate modifies C_S and this change is measured electrically.

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the double-layer capacitance. From the measured capacitance the equivalent coverage of the adsorbed substrate molecules can be calculated. Thus we can follow the decrease in coverage of adsorbed substrate molecules in presence of thrombin. The enzyme molecules hydrolyze the adsorbed substrate molecules on the electrode, and they also hydrolyze the substrate in solution causing some molecules to desorb to maintain the equilibrium between adsorbed substrate and substrate in solution. Thus, the measured decrease in coverage of adsorbed molecules in presence of thrombin is caused by two processes. The former dominates the decrease initially and for low enzyme concentrations. A consequence of having two simultaneous processes is that there exist an optimum in substrate concentration for maximal sensitivity. A low substrate concentration gives a small coverage of adsorbed molecules and for high concentrations most of the enzyme molecules will be occupied by substrate in solution.

The enzymatic activity is proportional to the initial rate of desorption and the test-equipment can be calibrated against solutions of known activity.

The presented results were obtained with a test-tube of Teflon containing two platinum electrodes. The substrate was dissolved in distilled water to a concentration of 1 mM. The substrate solution was added to 2 ml of buffer solution in the test tube. Fig.2

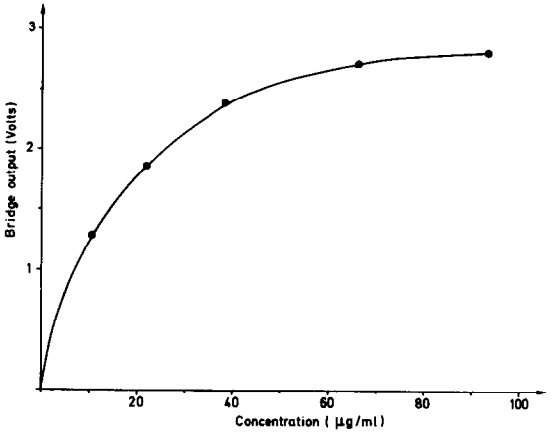

Fig.2. Steady-state change in voltage at 37°C as a function of substrate concentrations. The change in inverted series capacitance or the voltage shown, is related to the equivalent coverage of the adsorbed layer of substrate molecules. The results were obtained with a simple automatic capacitance bridge.

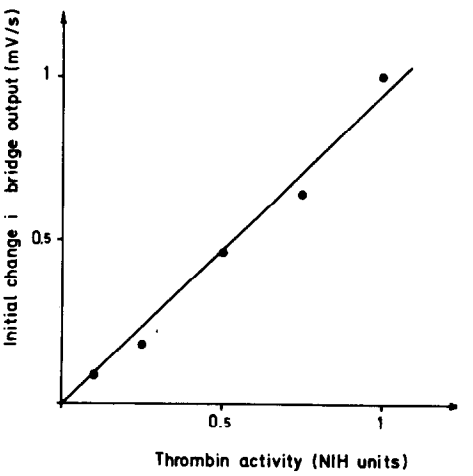

Fig.3. Initial rate of desorbtion of the substrate molecules at room temperature as a function of total enzyme content in the test tube given in NIH-units per ml. dV/dt shown is proportional to the initial rate of change of $1/C_S$. The points were obtained from measurements on different enzyme solutions and without temperature control.

shows the equilibrium equivalent coverage of adsorbed molecules as a function of substrate concentration. The following measurements were made at a substrate concentration of 30 µg/ml. The enzyme powder (Topostasin, Roche) was dissolved in the buffer solution to an activity of 10 NIH per ml. Different amounts of this solution were added to the test tube with the electrodes inserted. The initial rate of desorbtion of the substrate molecules is shown in fig.3. This figure can be used as a calibration curve for the present method.

To test the technique in a physiological system some attempts were made to determine the antithrombin content in blood plasma. Plasma which had been kept at −20°C was diluted with buffer solution (the same as above). 0.4 ml of this solution was prewarmed for 4 min at 37°C and after that 0.1 ml of thrombin solution (30 NIH per ml) was added. After an incubation time of 5 min the remaining thrombin-activity was measured. Measurements were made at different plasma dilutions and the antithrombin activity or thrombin inhibiting capacity can be calculated from fig.4.

For comparision literature results obtained with the spectrophotometrical method [6] utilizing the

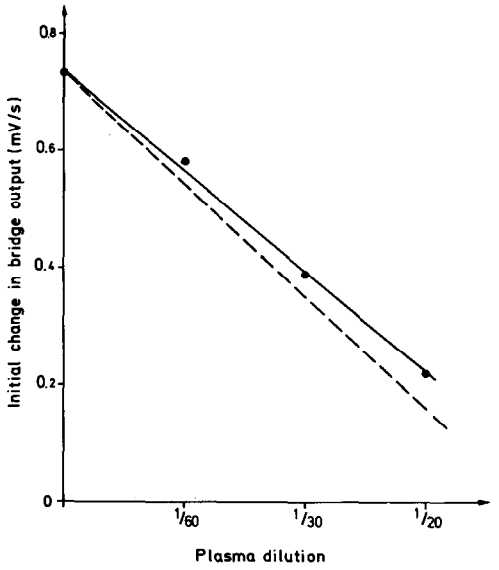

Fig.4. The points show the measured remaining enzyme activity in diluted plasma samples after incubation with a given amount of thrombin. The dashed line is normalized literature results obtained with S-2160 using a spectrophotometric method [6].

same substrate are also shown in fig.4. To decrease the incubation time and prevent clotting some measurements were made with diluted plasma solutions containing heparin. This requires the presence of a heparin neutralizing agent, such as polybrene or protamin, in the test tube. Polybrene caused an undesirable adsorption on the electrodes. Protamine also gave some adsorption but measurements were still possible. In the spectrophotometrical method heparin and polybrene are used. In our electric method it is not necessary to use these agents. In fact the simplification of excluding them is to prefer to avoid undesirable adsorption.

3. Discussion

The method is well suited for automation. The electrodes can be made of less expensive metals than platimum. With preadsorbed throw-away electrodes the present method can be a simple, fast and cheap method for determination of enzyme activities.

The substrate (S-2160) is also hydrolyzed by other serine proteases e.g. plasmin, brinase and trypsin. Some studies with our method have been made on trypsin. The method works well also in this case.

Of course this technique can be used also with other substrates and enzymes, provided that the substrates adsorb on the surface of the electrodes. Generally the technique may be used in any reaction causing an electrode adsorption or desorption of molecules.

Acknowledgement

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References

[1] Gough, D. A. and Andrade, J. D. (1973) Science 180, 380.
[2] Christian, G. D. (1971) Advan. Biomed. Eng. Med. Phys. 4, 95.
[3] Data sheets S-2160. AB Bofors, Nobel Division, Peptide Research, Mölndal, Sweden.
[4] Svendsen, L., Blombäck, M. and Olsson, P. (1972) Thromb. Res. 1, 267.
[5] Arwin, H. and Lunström, I., New electric method for measurements of enzymatic activity, submitted for publication.
[6] Odegård, O. R., Lie, M. and Abildgaard, U., (1975) Thromb. Res. 6, 287.