COMPUTERIZED MICROACIDIMETRIC DETERMINATION OF β LACTAMASE MICHAELIS–MENTEN CONSTANTS

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1. Introduction

Measurement of Michaelis–Menten constants is a fundamental step in the study of enzymes. The simplest kinetics correspond to the monomolecular decomposition of a reversible enzyme substrate complex [1]:

$$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} E\text{–}S \overset{k_3}{\rightleftharpoons} E + P$$

with $K_m = \frac{K_2 + K_3}{K_1}$ and V_{max} = maximum velocity.

In spite of their sensitivity the microiodometric [2] hydroxamate [3] and other methods of characterisation of β lactamases [4] do not allow precise measurement of K_m, although V_{max} is obtained most accurately. Automation effects some improvement in the precision but reports of K_m values in the literature remain sparse [5].

In the 1950's a pHmetric determination of penicillin was proposed [6] but, nevertheless because of its low accuracy and sensitivity it was not developed to any great extent [7–11].

. In the study of the enzyme produced by a strain of *Escherichia coli* resistant to ampicillin, penicillin G and V, we were able to increase the sensitivity of the pHmetric technique up to 1 μg/ml of ampicillin, pHstat neutralisation of the penicilloic acid liberated by hydrolysis of penicillin provided an accurate measure of the degree of reaction at any instant. Computer analysis of the curve obtained from a single titration gave values of K_m and V_{max}.

The kinetics of β lactamase fits the Michaelis–Menten equation $(V_{max}/v) - (K_m/[S]) = 1$ where v = velocity and [S]: substrate concentration. A statistical treatment is obtained with the quantification $tn = n\Delta t$; [Sn] is the corresponding substrate concentration and the velocity is $vn = [(Sn+1) - (Sn-1)]/2\Delta t$. As vn is the main source of error it is possible to fit the equation $1/S = -1/K_m + (V_{max}/K_m \cdot 1/v)$ to a modified linear regression program for a Wang 600 computer. The correlation constant indicates the precision of the experiment and, eventually if the kinetics are more complex.

Although a variety of publications have dealt with enzyme kinetics [12] the conclusions are highly generalized to cover a large variety of kinetic problems and are not very easy to apply to a simple case.

2. Material and methods

2.1. *Bacterial strain*

E. coli used was K_{12} and resistant to ampicillin, penicillin G and penicillin V. Preparation of the crude enzymatic extract:

The *E. coli* K_{12} were grown in 250 ml yeast extract tryptone medium, harvested in the late logarithmic phase of growth by centrifugation and washed with 0.05 M sodium chloride solution. After another centrifugation the pellets are resuspended in I .05 M sodium chloride solution then disrupted by sonication, the cell debris being removed by an additional centrifugation at 20 000 rpm for 30 min. The supernatant fluid is collected and used in the subsequent experiments.

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Table 1
Influence of the time quantum on the accuracy of the method.

Δt	K_m (μM)	V_{max} (rel)	Correlation	Number of values
10 sec	17.1	28.2	0.72	45
20 sec	25.8	32.5	0.96	23
30 sec	26.2	32.3	0.96	15
1 min	27.5	33.0	0.98	7
2 min	28.25	32.5	0.99	3

A correlation better than 0.97 corresponds to reproducible results.

2.2. *Typical experiment*

The cell, thermostatically controlled to 37°C, is filled with 9.5 ml of a solution of 500 μg ampicillin in water containing 5 g/ℓ NaCl; the pH is adjusted to 7.00. The enzyme solution is also adjusted to pH 7.00 and 0.5 ml of it added to the cell. The enzyme must be diluted in order to get a reaction half time of about 3 min. The titration is performed with a $5 \cdot 10^{-3}$ M sodium hydroxide solution in water. The volume of sodium hydroxide is measured at periods of 1 min giving an average of 6 to 10 values which are subsequently fed into the computer. The pHstat (Mettler DK serial) must regulate the pH with an accuracy greater than 0.02 pH units, in order to get K_m with a standard deviation less than 3%.

3. Results

3.1. *Influence of the time quantum*

In the experiments conducted with ampicillin Δt = 1 min is a good optimum. A longer time interval also gives a good correlation, but there are too few points for the results in this case to have much meaning. The results of varying Δt are summarized in table 1.

3.2. *Reproducibility*

From 10 experiments with ampicillin under the same conditions Δt = 1 min, we obtained the average values: K_m = 27.5 μM (minimum = 26.0, maximum = 29.6) and V_{max} = 40.8 (minimum = 39.7, maximum = 41.7) (relative values); which show a very small margin of error.

Table 2
Determination of K_m and V_{max} for the β lactamase studied.

Substrate	K_m (μM)	V_{max}	Correlation
Ampicillin	26	34.8	0.99
Penicillin G	19.4	35.9	0.99
Penicillin V	12.3	25.8	0.99
Corbenicillin	11.8	4.1	0.99
Cephaloridin	250	25.4	0.98
Cefalotin	114	2.3	0.95

3.3. *Determination of the β lactamase constants*

The analysis of the curve obtained from a single pHstat titration of different penicillin and cephalosporins gives the K_m and V_{max} values as shown in table 2. Iodine potassium iodide solution gives a rapid irreversible total inhibition at concentrations as low as 10^{-4} M in respect to iodine.

4. Discussion

It is evident from the results that this new method is a simple and efficient one for studying enzymes involved in penicillin and cephalosporin resistance.

Its use appears to be very broad and experiments aimed at extending this technique to other enzymes are now under way.

5. Conclusion

One can consider that the improvements of the acidimetric titration of penicillin related in this paper leads to a new, simple accurate and reproducible determination of β lactamase Michaelis constants.

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