DETERMINATION OF ENZYME ACTIVITIES WITH THE ENZYME THERMISTOR UNIT

Bengt DANIELSSON and Klaus MOSBACH

Department of Biochemistry, Chemical Center, University of Lund, PO Box 740, 220 07 Lund 7, Sweden

Received 26 February 1979

1. Introduction

Practically all enzymic reactions can be followed by calorimetry. Important advantages of this technique are that the optical properties of the sample do not interfere with the measurements and that no auxiliary enzymic reactions are required such as those leading to changes in optical absorption that can be followed spectrophotometrically. The applicability of calorimetry for enzyme activity determination has been demonstrated in a number of articles [1–5]. In general, conventional microcalorimeters have been used except for one report we know of which describes the use of a simpler design; in this system the enzyme tested is added dropwise to a substrate solution present on the surface of a Peltier element [6].

The usefulness of the enzyme thermistor and similar devices in biochemical analysis has been demonstrated [7–11]. The enzyme thermistor is a simple device for measuring the heat generated when a sample solution is passed through a small column containing an immobilized enzyme which can convert the sample. The enzyme thermistor unit can also be used after a slight modification (as will be discussed here) for calorimetric determinations of enzyme activities. For such analysis the sample solution containing the enzyme is simply mixed with a solution containing excess of substrate. After an appropriate interval so as to allow sufficient conversion of the substrate by the enzyme, the temperature increase of the mixture is measured. This technique is very simple and rather sensitive. Alternatively, when dealing with very low enzymic activities present in a sample, a known amount of substrate can be 'preincubated' with the sample solution for a fixed period of time after which the remaining substrate concentration is determined by the enzyme thermistor containing the same enzyme in immobilized form.

2. Materials and methods

The following enzymes were purchased from Sigma Chem. Co. (St Louis, MO): amylase (EC 3.2.1.1) type II-A and III-A from *Bacillus subtilis*; catalase (EC 1.11.1.6) (cat. no. C-100, 1978) from beef liver; alkaline phosphatase (EC 3.1.3.1) type V from chicken intestinal mucosa; urease (EC 3.5.1.5) type III from Jack bean. The unit definitions employed by Sigma are used in this report. Amylase activity: 1 unit will hydrolyse 1.0 mg maltose from starch in 3 min at pH 6.9 at 20°C. Catalase activity: 1 unit will decompose 1 μmol H_2O_2/min at pH 7.0 at 25°C, while the H_2O_2 concentration falls from 10.3–9.2 μmol/ml reaction mixture. Alkaline phosphatase activity: 1 unit will hydrolyse 1.0 μmol *p*-nitrophenylphosphate/min at pH 10.4 at 37°C. Urease activity: 1 unit will liberate 1 mg ammonia nitrogen from urea in 5 min at pH 7.0 at 30°C.

Specific substrates used were for alkaline phosphatase 'Sigma 104' (from Sigma Chem. Co. St Louis, MO) which is the disodium salt of *p*-nitrophenylphosphate and for amylase amylose (Merck, Darmstadt) partially hydrolysed by treatment with 0.2 M HCl for 16 h at room temperature.

2.1. *Apparatus*

The experimental arrangement is illustrated schematically in fig.1. Pumps with negligible or little pulsation such as the peristaltic Varioperpex pump (LKB, Bromma) or infusion pumps were used. Usually substrate solutions of high concentration

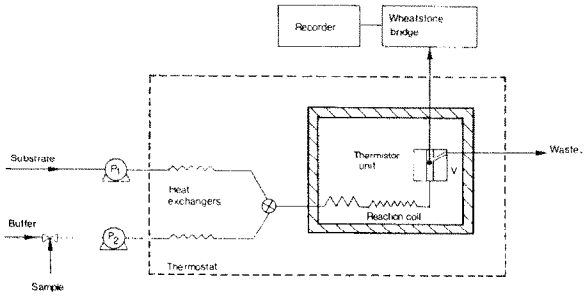

Fig.1. Arrangement of enzyme thermistor equipment used for enzyme activity determination. P1 and P2 designate the pumps. The reaction coil is teflon tubing preceeded by a short piece of metal tube that acts as a heat exchanger.

were used to guarantee substrate excess at the low flow rate used (0.1–0.5 ml/min) in order to minimise dilution; the sample/buffer was pumped through the apparatus at a 1–2 ml/min flow rate. The solutions were allowed to pass through two heat exchangers made of acid-proof steel tubes (50 cm long × 0.8 mm i.d.) that were placed in a waterbath. Thorough mixing of the solutions was accomplished by forcing them through a mixing chamber. The mixture was then rapidly passed through a short heat exchanger before entering a delay coil of teflon tubing (1.2 mm i.d.) in which the reaction was allowed to take place. The volume of this tubing and the flow rate through it were chosen for each enzyme system so that the temperature response of the reaction would be as high as possible; in most cases a 1 ml delay coil was used. The temperature of the mixture was measured with a thermistor placed at the outlet of the teflon tubing. The thermistor unit, indicated in fig.1, was identical with the split-flow enzyme thermistor apparatus described in [12], in which one of the columns was replaced by the delay coil. The temperature change was measured as in [8] employing a sensitive Wheatstone bridge and a potentiometric recorder. At the maximal sensitivity a full scale response of the recorder was obtained for a temperature change of 10^{-3} °C. The enzyme thermistor unit and the heat exchangers were placed in a waterbath and maintained at 30 ± 0.01°C.

Alternatively, in a recent modification, the waterbath has been replaced by a thermostated aluminium block which facilitates handling of the instrument and improves its sensitivity.

2.2 *Procedures*

After thermal equilibration of the system, the samples were introduced in the buffer stream via a 3-way valve placed before the pump or via a sample injection valve placed after the pump. The height of the temperature peak recorded was used as a measure of the heat evolved in the reaction.

In the alternative, indirect method the sample was allowed to react for ⩽30 min at 30°C with a known amount of the appropriate substrate. Remaining substrate was immediately thereafter determined using the conventional enzyme thermistor technique [8].

3. Results

A linear relationship between different urease concentrations and temperature response was obtained in the range 0.01–30 units/ml. In fig.2 the heights of the temperature peaks obtained with different concentrations of urease in the interval 0.01–2 units/ml are illustrated. This plot was obtained with rather

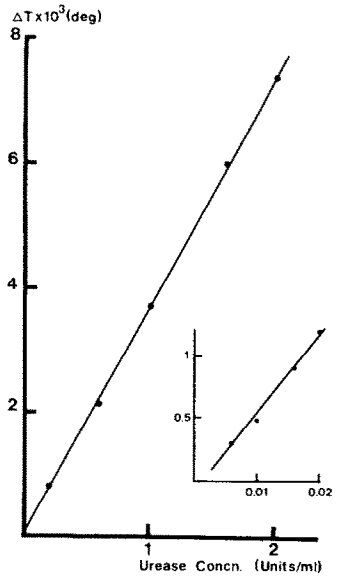

Fig.2. Temperature response obtained as a function of enzyme concentration. The urease samples (0.5 ml) were introduced at a 1.0 ml/min flow rate and mixed with substrate solution (0.2 M urea) introduced at 0.2 ml/min flow rate. The buffer used was 0.2 M KH_2PO_4/K_2HPO_4 (pH 7.0) containing 2 mM EDTA and 2 mM reduced glutathione. The insert shows the same relationship at lower enzyme concentrations. The pulse volume of the sample was increased to 1 ml.

short sample pulses (30 s), which are too short to give a 'thermal steady state'. Higher sensitivities can be attained by using longer sample pulses. With the system used here a pulse length of 3 min resulted in a steady state giving ~3-times higher temperature peaks than those shown in fig.2.

In the insert in fig.2, 1 min pulses of urease were applied and this diagram shows the lower part of the activity range studied. The precision in the determinations applying 0.01 unit/ml was 5% (C.V., coefficient of variation) and for 0.1 unit/ml was 1%.

The performance of the technique in crude solutions was studied by adding enzyme to various complex media. Urease was added to a fermentation solution used for penicillin production and to diluted human serum. No interference from these complex solutions could be observed in a 2-fold diluted fermentation solution or in 5-fold diluted serum. Undiluted serum, however, causes noticeable unspecific heat effects probably due to solvation effects that can be overcome by adding the proper buffer ions.

Catalase displayed similar temperature responses as urease, but in general much lower sensitivities were found for the other enzymes studied. For example, with partially hydrolysed–amylose as substrate amylase gave a temperature response of only 2×10^{-3} °C for 100 units enzyme. This low sensitivity could possibly be improved by using alternative substrates. The temperature responses obtained for alkaline phosphatase using *p*-nitrophenylphosphate as a substrate were higher as shown in fig.3.

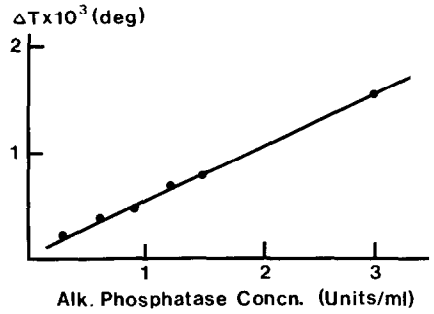

Fig.3. Relationship between temperature response and enzyme concentration. Alkaline phosphatase (1 min pulses) was pumped through the system at a 1.09 ml/min flow rate and mixed with 10 mM *p*-nitrophenylphosphate as substrate introduced by 0.1 ml/min. The buffer was 0.1 M glycine, (pH 9.0) containing 0.5 mM $MgCl_2$.

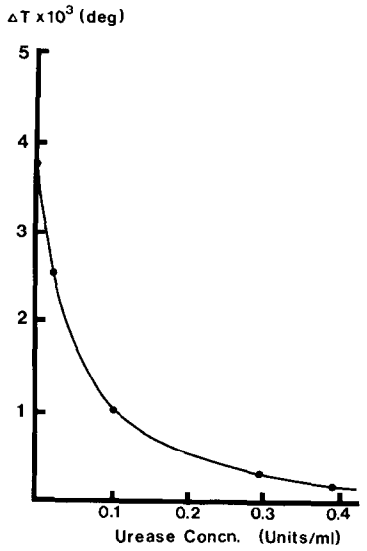

Fig.4. Substrate solution (5 ml 5 mM urea) was preincubated for 30 min at 30°C with samples containing various amounts of urease. The temperature response obtained when the reaction mixture was assayed for urea by an enzyme thermistor (containing immobilized urease) was plotted as a function of the urease concentration in the sample. The buffer used was 0.1 M NaH_2PO_4/Na_2HPO_4 (pH 7.0).

A higher sensitivity can be gained by the indirect method in which the sample is reacted with a known amount of substrate than with the above method. Figure 4 exemplifies this technique for urease activity determination. A 5 mM urea solution was incubated at 30°C for 30 min with different amounts of urease. The remaining substrate concentration was determined with an enzyme thermistor charged with immobilized urease. As is seen, the lower the amount of enzyme present, the higher the final heat response and sensitivity. The working range of this technique is affected by the substrate concentration and the incubation time and much lower enzyme concentrations than those given here can be determined.

4. Discussion

This report describes a simple and general method for enzyme activity determination based on calorimetry. It employs the rugged and uncomplicated enzyme thermistor device [8,9] in a slightly modified version.

In spite of its simplicity the results obtained with, e.g., urease determination compare favourably with those obtained with conventional microcalorimeters [1,2]. The sensitivity of calorimetric procedures in comparison with spectrophotometric assays is, however, generally much lower. Nevertheless, calorimetry may be attractive in situations where existing photometric methods are less reliable or too laborious or where the enzymic reaction cannot be easily 'coupled to' reactions leading to changes in optical absorption. An obvious advantage of calorimetry is its insensitivity to the optical properties of the sample and consequently it can be applied directly to crude solutions.

Improved sensitivity can be achieved using the above indirect procedure or by enrichment of the enzyme under study on an affinity column with the latter mounted either in the enzyme thermistor or used as precolumn [13]. After loading the column with the enzyme sample the activity can be determined by introducing an excess of substrate into the flow. The enzyme can be subsequently desorbed and the column reused as has been shown for instance with concanavalin A–Sepharose [13]. Alternatively, depending on the type of affinity system applied, the enzyme may immediately be eluted on addition of substrate.

Potential fields of application of the technique described here may be found in clinical chemistry, i.e., for the measurement of various enzymes such as amylase and alkaline phosphatase in body fluids. Furthermore, the course of an enzyme purification procedure could be monitored. In biotechnology the production of extracellular enzymes could be followed continuously.

Acknowledgements

This work has been supported by grants from the Swedish Board for Technical Development. Skillful technical assistance was given by Mrs Birgitta Rapp and Miss Maj-Britt Larsson.

References

[1] Monk, P. and Wadsö, I. (1969) Acta Chem. Scand. 23, 29–36.
[2] Beezer, A. E., Steenson, T. I. and Tyrrell, H. J. V. (1974) Talanta 21, 467–474.
[3] Yourtee, D. M., Brown, H. D., Chattopadhyay, S. K., Phillips, D. and Evans, W. J. (1975) Anal. Lett. 8, 41–49.
[4] Rehak, N. N., Everse, J., Kaplan, N. O. and Berger, R. L. (1976) Anal. Biochem. 70, 381–386.
[5] O'Farrell, H. K., Chattopadhyay, S. K. and Brown, H. D. (1977) Clin. Chem. 23, 1853–1856.
[6] Pennington, S. N. (1976) Anal. Biochem. 72, 230–237.
[7] Mosbach, K. and Danielsson, B. (1974) Biochim. Biophys. Acta 364, 140–145.
[8] Danielsson, B., Gadd, K., Mattiasson, B. and Mosbach, K. (1976) Anal. Lett. 9, 987–1001.
[9] Danielsson, B., Gadd, K., Mattiasson, B. and Mosbach, K. (1977) Clin. Chim. Acta 81, 163–175.
[10] Bowers, L. D. and Carr, P. W. (1976) Clin. Chem. 22, 1427–1433.
[11] Schmidt, H.-L., Krisam, G. and Grenner, G. (1976) Biochim. Biophys. Acta 429, 283–290.
[12] Mattiasson, B., Danielsson, B. and Mosbach, K. (1976) Anal. Lett. 9, 867–889.
[13] Danielsson, B., Mattiasson, B. and Mosbach, K. (1978) 1st Eur. Congr. on Biotechnology, 25–29 Sept., Interlaken, Switzerland, DECHEMA, pt 2, 1/3, 107.