Biochimica et Biophysica Acta, 659 (1981) 244—248
Elsevier/North-Holland Biomedical Press

BBA 69275

THE EFFECTS OF SUBSTRATE FLOW-RATE ON IMMOBILIZED UREASE ASSAYS

FRANCIS N. ONYEZILI and AKINTUNDE C. ONITIRI

Department of Clinical Pathology, College of Medicine, University of Lagos, P.M.B. 12003, Lagos (Nigeria)

(Received October 28th, 1980)

Key words: Immobilized enzyme; Nylon tube; Substrate flow rate; Urease assay

Summary

Urease (urea aminohydrolase, EC 3.5.1.5) has been immobilized on *o*-alkylated nylon tubes in an enzymatically active form. The measured activity of the enzyme coil has been shown to be dependent on the flow rate of substrate through it. The apparent K_m decreased and V increased as flow rates were increased. It is suggested that diffusional control is responsible for these observations. Their implications in flow-through immobilized enzyme analyses are discussed.

Introduction

Interest in immobilized enzyme systems has been due both to the theoretical implications they suggest as biological models and to the practical applications they offer as less costly and more stable alternatives to soluble enzyme systems [1—3]. In particular, the utilization of immobilized enzymes in clinical chemistry is in rapid progress and it is now feasible for the small clinical laboratory to exploit the combined advantages of flow-through automation and the specificity of the enzyme assays [4—6]. One such system [7] involves immobilized urease, a potentially invaluable diagnostic tool for blood urea determination.

It was observed [8] that the activity of nylon tube-bound urease was altered by changing the flow rates of its substrates through the tube. Thus, the urease activity appeared to be dependent on the concentration of substrate available to the enzyme in its micro-environment rather than directly on the bulk substrate concentration in the system. This effect of the micro-environment on immobilized enzyme behaviour, and the limitations it imposes on the measurements of substrate levels in a given sample, has received relatively little attention. Here, we report further investigation of this phenomenon and discuss its implications in flow-through immobilized enzyme assays.

Materials and Methods

Wherever possible, Analar grade reagents were used without further purification. Jack bean urease (EC 3.5.1.5) was obtained as a freeze-dried powder from Miles-Seravac, Maidenhead, U.K. Its specific activity was approx. 100 I.U./mg at pH 7.0 and 25°C.

Nylon tube, extruded from Type 6 nylon, was obtained from Portex Limited, Hythe, Kent, U.K. For use, a $\frac{1}{2}$-m length of the tube, coiled round a glass tube and clamped at one end, was filled with dimethyl sulphate, clamped at the other end, and immersed in a boiling water bath for 3 min to allow *o*-alkylation. This reaction was stopped by immersing the tube in an ice bucket for 10 min and excess dimethyl sulphate was removed by perfusion with methanol. Amine arms were then incorporated into the tube by filling it with 0.2 M lysine, pH 9.0, and incubating it at 25°C for 3 h. Excess lysine was removed by perfusing the tube with 100 ml 1.0 M NaCl followed by 100 ml distilled water. Next, the tube was activated by filling it with 1.5% (w/v) glutaraldehyde in 0.2 M sodium borate buffer, pH 8.5, and incubating it at 25°C for 5 min. Excess glutaraldehyde was washed out with the borate buffer. Finally, the tube was filled with a solution (2 mg/ml) of urease in 0.05 M EDTA buffer, pH 7.0/1.0 mM mercaptoethanol, and the enzyme coupling reaction was allowed to proceed at 4°C for 4 h. Unbound urease was washed out with 1.0 M NaCl and distilled water sequentially. The immobilized enzyme derivative was stored at 4°C in the dry state.

Immobilized urease activity was determined by measuring the ammonia liberated by the enzyme-catalysed hydrolysis of urea [9]. The enzyme coil was immersed in a thermostatically controlled water bath at 37°C. Urea, in 0.05 M EDTA buffer, pH 7.0, was pumped through it at the required flow rate and the effluent was assayed for ammonia.

The initial velocity of the immobilized enzyme was measured by recycling 25 ml of the substrate solution in a closed system [10] and assaying aliquots pipetted from the mixing vessel for ammonia.

Results

It is evident, from results presented in Fig. 1A and B, that the measured activity of tube-bound urease is influenced by the flow rate at which substrate was pumped through the tube. Only 66% of the urease activity measured at a flow rate of 24 ml/min could be measured at a flow rate of 4 ml/min (Fig. 1A). Above a flow rate of 20 ml/min urease activity became independent of flow rates under the existing experimental conditions. When the bulk substrate concentration in the system was increased 5-fold, a higher percentage (84%) of the urease activity measured using a flow rate of 24 ml/min could be measured at a flow rate of 4 ml/min (Fig. 1B). At this higher substrate concentration, enzymatic activity became independent of flow rates at a slower speed (<10 ml/min). Thus, the flow rate effect was more severe when the initial bulk substrate concentration in the system was low.

Fig. 2 is a double-reciprocal plot [11] using data from experiments in which the initial velocity of the immobilized urease was measured at various substrate

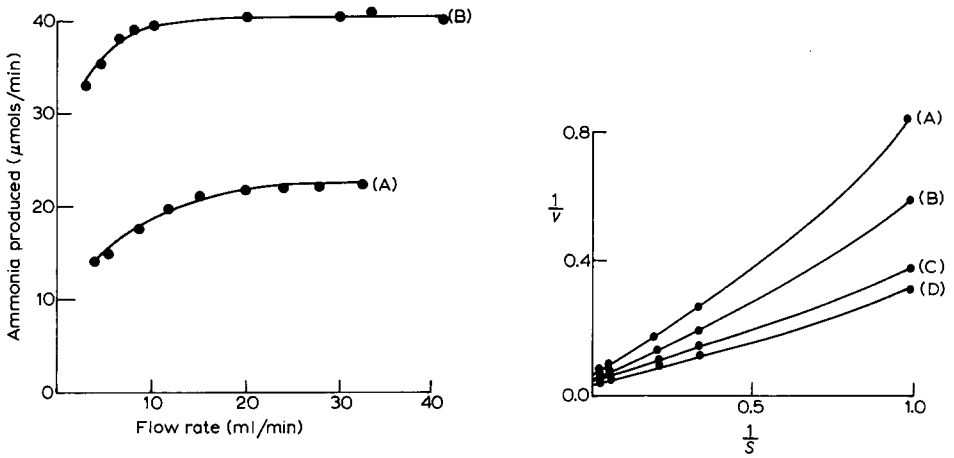

Fig. 1. The measured activity of nylon tube-urease at various substrate flow rates. Urease was immobilized on a 0.5-m length of nylon tube. The assay of enzyme coil was performed in the presence of 50 mM urea (A) and 250 mM urea (B).

Fig. 2. Double-reciprocal plots of initial velocities of nylon tube-urease at various concentrations of substrate. Assays were performed at substrate flow rates of 3 ml/min (A); 6 ml/min (B); 15 ml/min (C) and 36 ml/min (D). v was expressed as μmol urea hydrolysed per min and S was in mM units.

flow rates. An intercept could not be obtained on the $1/S$ axis, because of the curved nature of the plots, and the K_m was therefore calculated by reading off the substrate concentration at $\frac{1}{2}V$; ($1/V$ being the intercept on the $1/v$ axis). The K_m value for immobilized urease was calculated to be 20.0 mM when

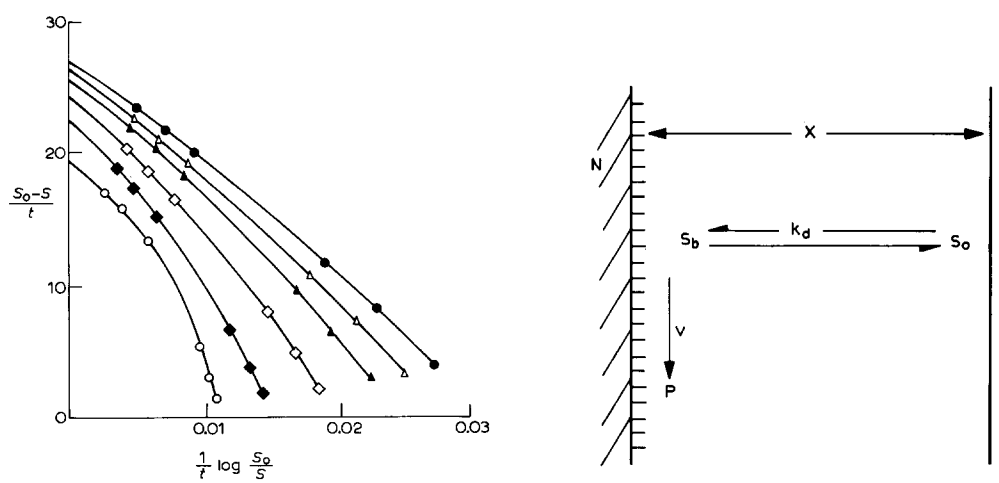

Fig. 3. Integrated Michaelis-Menten plots from data of initial velocities of nylon tube-urease at various concentrations of substrate. Assays were performed at substrate flow rate of 30 ml/min (●——●); 20 ml/min (△——△); 15 ml/min (▲——▲); 10 ml/min; 6 ml/min (■——■) and 3 ml/min (○——○). S_o = initial urea concentration (mol) in assay mixture. S = urea concentration at time, t. t = 1 min.

Fig. 4. Schematic illustration of diffusional control of reaction velocity of a nylon tube-supported enzyme. N, nylon surface; X, diffusion layer; S_o, 'apparent' substrate concentration; S_b, 'effective' substrate concentration; k_d, diffusion-controlled rate; v, measured enzyme reaction velocity; P, product.

substrate flow rate was 3 ml/min. At a flow rate of 36 ml/min, the K_m value was reduced to 10.5 mM.

The V of the immobilized urease, the vertical intercept of plots of an integrated form of the Michaelis-Menten equation (Fig. 3), increased as the flow rate at which the substrate was perfused was increased. This V was 18.0 μmol urea hydrolysed per min at a flow rate of 3 ml/min and was 29.0 μmol at a flow rate of 36 ml/min.

It is concluded from these observations that K_m and V values decreased and increased, respectively, as the flow rate substrate through the immobilized enzyme coil was increased.

Discussion

The situation inside a nylon tube-supported urease coil during assay may be illustrated as shown in Fig. 4. In such a system, v, the measured rate of urease activity is dependent on S_b which, in turn, is dependent on how long it takes the substrate (S_o) to diffuse across the layer, x. Thus, the measured urease activity is critically dependent on the thickness of the diffusion layer, x. At slow flow rates, when the agitation and turbulence inside the tube are minimal, the diffusion layer is thicker and the diffusional effect is therefore greater. S_b will be much less than S_o under these conditions and the measured K_m will be higher than would be the case in the absence of a diffusion layer. As the flow rate of substrate is increased, agitation and turbulence inside the tube increase and, as the diffusion layer becomes thinner, the difference between S_o and S_b becomes smaller and K_m decreases towards its true value. The curvature of the plots obtained (Figs. 2 and 3) may itself be indicative of the extent of diffusion control, this curvature increasing at the lower substrate concentration when the conversion per glass of substrate to products is greater.

The observed increase in V at increased flow rates would suggest that nylon tube-bound urease cannot function at its maximum velocity under the assay conditions used in present-day flow-through autoanalyser systems, where the gross liquid flow is usually less than 3.0 ml/min. The rate K_d (Fig. 4) cannot be greater than the rate of enzyme catalysis (v) because, if so, no diffusional effects should have been observed. Also, v cannot be greater than K_d since it is dependent on the availability of S_b which is limited by the rate K_d. Thus, the measured enzymic activity is only as high as the diffusion layer permits. This becomes a significant consideration when the diffusion is a relatively slow process with respect to substrate consumption at the enzyme surface. Since different enzyme immobilization techniques yield derivatives with varying enzymic activity, it is suggested that a quantitation of the extent of diffusion control in each derivative is a pre-requisite for improving the sensitivity of nylon tube-supported enzymes.

Acknowledgement

The authors are grateful to Dr. W.E. Hornby for his advice.

References

1 Silman, I. and Katchalski, E. (1966) Annual Review of Biochemistry 35, Part II, 873
2 Goldman, R., Goldstein, L. and Katchalski, E. (1971) in 'Biochemical aspects of reactions on Solid Supports' (Stark, G., ed.) Academic Press, London
3 Bihari, V.S. and Ghosh, P. (1973) Chemical Age of India 24, 419—429
4 Joseph, M.D.; Kasprazak, D.J.; and Crouch, S.R. (1977) Clin. Chem. 23, 1033—1036
5 Sundaram, P.V., Blumenberg, B. and Hinsch, W. (1979) Clin. Chem. 25, 1436—1439
6 Wease, D.F., Anderson, Y.J. and Ducharme, D.M. (1979) Clin. Chem. 25, 1346—1347
7 Sundaram, P.V., Igloi, M.P., Wassermann, R., Hinsch, W. and Koche, K.J. (1978) Clin. Chem. 24, 234—239
8 Onyezili, F.N. (1974) M.Sc. Thesis, University of St. Andrews, U.K.
9 Chaney, A. and Marbach, E. (1962) Clin. Chem. 8, 130
10 Ford, J. (1972) Biotech. Bioeng. Symposium 3, 267—284
11 Lineweaver, H. and Burk, D. (1934) J. Am. Chem. Soc. 56, 685