Kinetic Behaviour of Acid Phosphatase–Albumin Co-polymers in Homogeneous Phase and under Gel-Immobilized Conditions

By MARIA CANTARELLA,* MARIE-HÉLÈNE REMY,*† VINCENZO SCARDI,*
FRANCESCO ALFANI,‡ GABRIELE IORIO‡ and GUIDO GRECO, JR.‡

**Cattedra di Chimica delle Fermentazioni e Batteriologia Industriale, Facoltà di Scienze,
Università di Napoli, 80134 Napoli, Italy, and*

‡*Istituto di Principi di Ingegneria Chimica, Facoltà di Ingegneria,
Università di Napoli, 80125 Napoli, Italy*

(Received 21 August 1978)

1. An analysis of the kinetic behaviour of immobilized acid phosphatase (EC 3.1.3.2) layers, gelled on the active surface of an ultrafiltration membrane, was carried out. 2. Two possible forms of such immobilized-enzyme systems were dealt with, namely enzyme–polyalbumin co-gelation through an ultrafiltration process, and enzyme co-polymerization to the same albumin polymers and subsequent gelation. 3. A preliminary analysis was also performed on both the corresponding homogeneous-phase (soluble) systems to provide reference kinetics. 4. The main conclusions drawn are: (i) the enzyme–albumin co-polymers show a decrease in specific activity compared with the corresponding free enzyme in both soluble and immobilized forms; (ii) in the homogeneous phase a slight increase in the apparent Michaelis constant was measured for the co-polymerized enzyme compared with the free one, which suggests a decrease in affinity towards substrate; (iii) the activation energy in the immobilized phase is halved, compared with that in the homogeneous phase, which indicates that the combined mass-transfer/reaction step is rate-controlling.

One of the immobilization techniques more recently proposed consists essentially of the formation of a stable gel layer into which the enzyme is covalently bound and through which substrate permeation occurs (Cantarella *et al.*, 1977). The gel formation can be achieved by ultrafiltering an enzyme solution through an ultrafiltration membrane within an unstirred ultrafiltration cell. If the molecular-weight cut-off of the membrane is such as to reject the enzyme completely, a concentration-polarization phenomenon takes place in the ultrafiltration cell which eventually can lead to gelation on the active (upstream) surface of the membrane, provided that the protein is supplied in sufficient amount. The approximate concentration that leads to the formation of the gel layer ranges between 10 and 30% (w/v) for globular macromolecules (Blatt *et al.*, 1970). To decrease the amount of enzyme and still achieve gelation, an inert protein can be added to the enzyme solution. This procedure leads to a sort of enzyme entrapment with the gelled protein layer (co-gelation). As an alternative, the enzyme can be previously co-polymerized to the inert protein and the resulting soluble co-polymer gelled by the same technique (co-

polymerization/gelation). The latter method leads to obvious improvements of the system performance inasmuch as more permeable membranes (i.e. with higher molecular-weight cut-off) can be used owing to the larger macromolecules involved compared with the free enzyme ones, and hence higher flow rates can be obtained. On the other hand, owing to enzyme co-polymerization some decrease in specific activity can be expected with respect to the co-gelled enzyme, which should favourably compare with other immobilization techniques mainly because it does not involve chemical manipulation of the enzyme and therefore should not be associated with enzyme inactivation.

The purpose of the present paper is the comparison of the kinetic behaviour of co-gelled enzyme and co-polymerized/gelled enzyme. The enzyme considered here was acid phosphatase (EC 3.1.3.2), as its catalytic activity is easily measurable and, moreover, is not greatly affected by the chemical manipulations connected with covalent binding to a support; the inert protein was human serum albumin.

To achieve a better understanding of the kinetics in both immobilized-enzyme situations, systematic kinetic runs were also performed on the homogeneous (soluble) phase for both the free and the co-polymerized enzyme, yielding reference reaction kinetics.

† Present address: Laboratoire de Technologie Enzymatique, Université de Compiègne, Compiègne, France.

Experimental

Materials

Acid phosphatase (from potato) and its artificial substrate, *p*-nitrophenyl phosphate (sodium salt), were from Boehringer Biochemia (Milan, Italy); human serum albumin was a gift of the Istituto Sieroterapico Italiano (Naples, Italy); glutaraldehyde (25%, w/v, solution) was from Serva Feinbiochemica (Heidelberg, Germany). Sepharose 6B was from Pharmacia (Uppsala, Sweden). All other chemicals were pure products from different commercial sources. The ultrafiltration membranes used, as enzyme-gel layer support were type DDS 600 from the Danish Sugar Corp. (Nakskov, Denmark).

Preparation of soluble polymers

The polymerization technique was substantially similar to that used by Paillot *et al.* (1974). To a solution of human serum albumin (40 mg/ml) in 0.02 M-sodium phosphate buffer, pH 6.8, glutaraldehyde was added to give a final concentration of 0.5% (w/v). The mixture was allowed to react at 4°C for 24 h, the polymerization process was stopped by addition of excess glycine, followed by dialysis against the buffer solution. To prepare the enzyme/albumin co-polymers, the same procedure was followed, starting from human serum albumin (40 mg/ml) and native enzyme (2 mg/ml) in the sodium phosphate buffer, the polymerization time being 10 h.

Chromatographic separation

This was performed on a Sepharose 6B column (2.6 cm × 34.5 cm) equilibrated with the sodium phosphate buffer (60 ml/h). The eluate was continuously monitored at 280 nm spectrophotometrically and 5 ml fractions were automatically collected.

Preparation of enzyme gels on membranes

The experiments with immobilized enzyme were carried out in an unstirred ultrafiltration cell (about 70 ml) equipped with a DDS 600 membrane (4 cm diameter, mol.wt. cut-off 20000), and connected to a pressurized feed vessel; temperature control was maintained within $\pm 0.2^\circ\text{C}$ by means of thermostatically controlled water circulating in the external cell jacket. To obtain enzyme immobilization, 10 ml of a solution containing either 20 mg of soluble human serum albumin polymers together with 0.5 mg of free acid phosphatase (co-gelled enzyme) or 20 mg of human serum albumin plus 1 mg of acid phosphatase co-polymers (co-polymerized/gelled enzyme) were introduced into the ultrafiltration cell and subsequently ultrafiltered at 100 kPa (1 atm). The total amounts added were, in any case, such as to ensure gel formation, which was attained by completely ultrafiltering the above solutions and by

keeping the ultrafiltration cell under nitrogen pressure overnight. The cell was then disconnected from the gas cylinder and kept at 4°C for 48 h.

Assay of acid phosphatase activity

This consisted of measuring spectrophotometrically, after alkalization of the sample with an equal volume of 1 M-NaOH solution, the *p*-nitrophenol liberated under the experimental conditions (0.05 M-sodium citrate/citric acid buffer, pH 5.6), as previously described (Drioli *et al.*, 1975). A molecular absorption coefficient of $18\,500\text{ litre} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$ at 405 nm was used.

Results and Discussion

Enzyme/albumin co-polymerization

The elution volume of the free enzyme was determined in a preliminary chromatographic run (Fig. 1) in which 2 ml of a solution containing human serum albumin polymers (20 mg) and free acid phosphatase (0.2 mg) was analysed. The enzyme elution volume was 112 ml. No enzyme entrapment by the soluble albumin polymers occurred. A second chromatographic run was performed on the enzyme/albumin co-polymers (Fig. 1). A close correspondence between enzyme activity and u.v. absorbance was observed, which allows us to state that all the acid phosphatase was co-polymerized to the inert protein. Furthermore, in both runs the elution volume of the soluble polymers and co-polymers suggests that the corresponding molecular weight exceeds 1 000 000.

Homogeneous-phase kinetics

Kinetic runs in the homogeneous phase were performed on both free enzyme and enzyme/albumin co-polymers at different temperatures and, at each temperature, at six different substrate concentrations. The initial specific reaction rate v' ($\mu\text{mol}/\text{min}$ per mg of enzyme) and the corresponding substrate concentrations $[S]$ (mM) were plotted in terms of $[S]/v'$ against $[S]$, i.e. according to the Hanes-Woolf correlation (Segel, 1975). The experimental data were fitted by means of a linear regression technique and hence the values of K_m were determined at each reaction temperature. Since no appreciable trend was observed, K_m was assumed to be constant within the temperature range explored (10–35°C).

The corresponding mean values are: free enzyme, $K_m = 0.35\text{ mM}$, with a normalized variance about the mean of 18.6%; co-polymerized enzyme, $K_{m(\text{app.})} = 0.59\text{ mM}$, normalized variance 8.72%. A further linear regression was performed on the experimental data in which, on the basis of the averaged K_m previously determined, the values of k'_{cat} (i.e. maximal reaction rate, V , per mg of enzyme) were obtained for each reaction temperature, thus enabling Arrhenius plots to be drawn for both free and co-polymerized enzyme

Fig. 1 Chromatography of acid phosphatase/albumin co-polymers on Sepharose 6B column

A sample containing 20 mg of enzyme/albumin co-polymers was applied to the Sepharose 6B column (2.6 cm $\times$ 34.5 cm) equilibrated with 0.02 M-sodium phosphate buffer, pH 6.8, and eluted as described in the text. The flow rate was 60 ml/h, and the fraction volume 5 ml. The left-hand ordinate indicates protein content ($\bullet$) as A_{280} ; the right-hand ordinate indicates percentage of total enzyme activity (stippled bars). When a sample containing a mixture of albumin polymers (20 mg) and free native acid phosphatase was added to the column, a superimposable protein-elution profile was obtained (not shown in the Figure), the position of the free enzyme activity being indicated by the open bars.

Fig. 2. Arrhenius plot for both free and co-polymerized acid phosphatase in homogeneous phase
 $k'_{cat} = V/\text{mg of enzyme}$. Δ , Free native enzyme; $\blacktriangle$, enzyme-albumin co-polymers. For experimental details see the text.

Fig. 3. Hanes-Woolf plots for both free native acid phosphatase at different temperatures
 $\blacksquare$, 10°C; $\square$, 24°C; $\bullet$, 30°C; $\circ$, 35°C. For experimental details see the text.

Fig. 4. Hanes-Woolf plots for acid phosphatase-albumin co-polymers at different temperatures in the homogeneous phase
 ■, 10°C; □, 24°C; ●, 30°C; ○, 35°C. For experimental details see the text.

(Fig. 2). In the two cases, the calculated activation energy is 47 kJ/mol (11.2 kcal/mol). The solid lines in Fig. 2 were obtained by least-squares fit. On the basis of these smoothed correlations it was eventually possible to obtain values of k'_{cat} for each reaction temperature, which together with averaged values of the Michaelis constant previously determined were used to draw the lines in the Hanes-Woolf plots of Figs. 3 and 4. These Figures show that a satisfactory fit of the experimental data points was thus obtained.

The final kinetic expressions obtained in the homogeneous phase for the free and co-polymerized enzyme are respectively:

$$v' = \frac{5.10 \times 10^8 \exp(-1.19 \times 10^4/RT) [S]}{0.35 + [S]} \quad (1)$$

and

$$v' = \frac{3.44 \times 10^8 \exp(-1.19 \times 10^4/RT) [S]}{0.592 + [S]} \quad (2)$$

where R is the gas constant, and T the absolute temperature.

At this stage three main conclusions can be drawn: (i) the activation energy is the same for both free and co-polymerized enzyme, which implies that no mass-transfer resistance is introduced by the acid phosphatase molecule being co-polymerized to the inert protein of considerable molecular weight; (ii) owing to co-polymerization, the enzyme undergoes a considerable decrease in specific activity (k'_{cat} is decreased by 33%), as indicated by the Arrhenius plots of Fig. 2; (iii) the co-polymerized acid phosphatase shows a decreased affinity towards the substrate as compared with the free enzyme (70% increase in the apparent Michaelis constant), which could stem from a decrease in the degrees of freedom of the enzyme

molecule and/or from microenvironmental modifications.

Immobilized-enzyme kinetics

Kinetic tests were also performed under immobilized-enzyme conditions. Once the co-gelled or the co-polymerized/gelled acid phosphatase layer had been formed on the ultrafiltration membrane surface by the procedure described in the Experimental section, the ultrafiltration cell was connected to the feed vessel and pressure applied. Fractions of solution passing through the membrane were automatically collected at fixed time intervals and the product concentration (i.e. *p*-nitrophenol) was determined. The volumetric flow rate was measured with a capillary flow meter. The specific reaction rate can be obtained by the overall steady-state substrate mass-balance equation, which yields:

$$v' = \frac{Q[P]}{w} \times 10^3 \quad (3)$$

where Q is the volumetric flow rate (ml/min), $[P]$ the product concentration and w the enzyme amount (mg). For eqn. (3) to hold, care had to be taken that a steady state had actually been attained by the system. Therefore the outlet product concentration $[P]$ was measured at sufficiently long reaction time (6h for the experimental conditions adopted; Cantarella *et al.*, 1977).

Kinetic runs were performed both by varying the feed substrate concentration at constant reaction temperature (30°C) and by varying the reaction temperature at the same substrate concentration (2 mM) for both immobilized-enzyme conditions. The results are reported in Table 1, in comparison with the corresponding homogeneous-phase reaction rates calculated by means of eqns. (1) and (2) respectively. The immobilized-enzyme reaction rates are much lower than the corresponding homogeneous-phase ones. In fact, if one assumes that Michaelis-Menten kinetics still hold for both types of immobilized enzyme, one could evaluate by a least-squares fit of the experimental data at 30°C the following kinetic parameters: $k'_{cat} = 0.45 \mu\text{mol/min per mg}$ and $K_{m(app.)} = 2.70 \text{ mM}$ for the co-gelled enzyme; $k'_{cat} = 0.347 \mu\text{mol/min per mg}$ and $K_{m(app.)} = 3.5 \text{ mM}$ for the co-polymerized/gelled enzyme. The corresponding homogeneous-phase values are: $k'_{cat} = 1.328 \mu\text{mol/min per mg}$ and $K_{m(app.)} = 0.35 \text{ mM}$ for the free enzyme; $k'_{cat} = 0.896 \mu\text{mol/min per mg}$ and $K_{m(app.)} = 0.592 \text{ mM}$ for the co-polymerized enzyme [see eqns. (1) and (2) respectively].

A substantial variation seems therefore to occur in the kinetic parameters owing to immobilization. However, both k'_{cat} and $K_{m(app.)}$ obtained in the ultrafiltration cell actually describe the intrinsic kinetics of the immobilized enzyme provided that the rate-controlling step is the enzymic reaction itself.

Table 1. Comparison between experimental reaction rates for the two immobilized acid phosphatase conditions and the corresponding homogeneous values

Data for the co-gelled enzyme and the co-polymerized/gelled enzyme are compared with the reaction rates of the native enzyme and the co-polymerized enzyme in free solution, respectively, evaluated by means of eqns. (1) and (2) respectively, at the same reaction temperature and substrate (*p*-nitrophenyl phosphate) concentration. For experimental details see the text.

Temperature (°C)	Substrate concn. (mM)	Reaction rate ($\mu\text{mol}/\text{min}$ per mg of enzyme)			
		Co-gelled enzyme	Native enzyme in free solution	Co-polymerized/ gelled enzyme	Co-polymerized enzyme in free solution
30	0.5	0.067	0.782	0.047	0.414
30	1.0	0.134	0.984	0.071	0.563
30	1.5	0.155	1.080	0.099	0.643
30	2.0	0.191	1.130	0.134	0.692
15	2.0	0.146	0.404	0.086	0.247
22	2.0	0.190	0.662	0.116	0.405
35	2.0	0.253	1.560	0.163	0.952

Fig. 5. Arrhenius plot for both the immobilized forms of acid phosphatase

v' ($\mu\text{mol}/\text{min}$ per mg) is the overall specific reaction rate at equal substrate concentration. ●, Co-gelled enzyme; ■, co-polymerized/gelled enzyme. For experimental details see the text.

Indeed, if substantial mass-transfer resistances take place under the experimental conditions adopted, the kinetic parameters thus obtained are only apparent, the overall reaction rate being affected by an effectiveness factor, i.e. the ratio of the observed reaction rate to that of the free enzyme at the same substrate concentration (Denbigh & Turner, 1971), less than unity. That this is actually the case can be seen by inspection of the set of experimental data at various reaction temperatures and constant substrate concentration (2mM) also reported in Table 1. The Arrhenius plots obtained by correlating the logarithm (base e) of the overall specific reaction rate at equal substrate concentration, $\ln v'$, against reciprocal absolute temperature (Fig. 5) actually indicate an activation

energy of 23 kJ/mol (5.5 kcal/mol) for both co-gelled and co-polymerized/gelled enzyme, i.e. the activation energy is halved compared with that in the homogeneous phase. This clearly shows that the rate-controlling step is a diffusion/reaction stage, and hence the conclusion has to be drawn that the previously determined kinetic parameters for the immobilized-enzyme systems do not depict the intrinsic kinetics (Engasser & Horvath, 1976). In any case, it has to be pointed out that a decrease in intrinsic activity is exhibited by the co-polymerized/gelled enzyme compared with the co-gelled one, which is of the same order of magnitude as that measured in the corresponding homogeneous-phase runs.

The major conclusions that can be drawn from the experimental results in the present work are: (i) the polymerization technique adopted is such as to ensure that all the acid phosphatase is co-reticulated to the inert albumin polymers; (ii) the resulting co-polymers in the homogeneous phase show a decrease in specific activity (decrease in k'_{cat}) and in affinity towards the substrate (increase in $K_{\text{m(app.)}}$) compared with the free enzyme; (iii) when used in the immobilized state within an unstirred ultrafiltration cell, a decrease occurs in k'_{cat} , together with an increase in $K_{\text{m(app.)}}$ for both co-gelled and co-polymerized/gelled enzyme as compared with the corresponding homogeneous-phase results; (iv) this result does not necessarily imply that a modification in intrinsic kinetics took place in the gelled enzyme, since the kinetic parameters experimentally determined are only apparent ones. Indeed, the activation energy evaluated for the immobilized enzyme is halved compared with that in homogeneous phase. This in turn implies that the rate-controlling step is a combined mass-transfer/reaction stage and that the overall rates are therefore affected by an effectiveness factor less than unity.

It has to be pointed out that the problem of evaluating these effectiveness factors cannot be dealt with in terms of the usual effectiveness factor against Thiele modulus diagrams (Denbigh & Turner, 1971) for Michaelis-Menten kinetics that have been evaluated on the basis of simple substrate diffusion within an immobilized enzyme. In fact, in the configuration adopted both substrate diffusion and convection occur within the enzyme-gel layer. An adequate mathematical model of the phenomenon is required in order to identify the actual intrinsic kinetics of the immobilized enzyme.

This work was supported in part by a grant (CT 76/1084/03) of the Italian C.N.R., Rome. We are grateful to Dr. E. Romano (I.S.I., Naples) for generously giving the human serum albumin.

References

- Blatt, S. F., David, A., Michaels, A. S. & Nelson, L. (1970) in *Membrane Science and Technology* (Flynn, F., ed.), pp. 47-97, Plenum Press, New York
- Cantarella, M., Gianfreda, L., Palescandolo, R. & Scardi, V. (1977) *Biochem. J.* **167**, 313-315
- Denbigh, K. G. & Turner, J. C. R. (1971) *Chemical Reactor Theory*, 2nd edn., pp. 140-145, Cambridge University Press, Cambridge
- Drioli, E., Gianfreda, L., Palescandolo, R. & Scardi, V. (1975) *Biotechnol. Bioeng.* **17**, 1365-1367
- Engasser, J.-M. & Horvath, C. (1976) *Appl. Biochem. Bioeng.* **1**, 127-220
- Paillot, B., Remy, M. H., Thomas, D. & Broun, H. (1974) *Pathol. Biol.* **22**, 491-495
- Segel, I. H. (1975) *Enzyme Kinetics*, 1st edn., p. 220, J. Wiley and Sons, New York