An experimental method to determine the substrate protection of enzyme against deactivation in a reversible reaction

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The substrate protection effect on an enzyme in a reversible reaction was studied by using glucose isomerase immobilized in small particles (radius less than 100 μm). Deactivation of the enzyme at various substrate concentrations in Tris buffer, pH 8.25, at 62.1 °C was studied in eight-column reactor sets. At set times the immobilized enzyme in one of the eight reactors was taken out and rinsed thoroughly, and then its residual activity was determined. The conclusions are, first, that the protection by the reactant is equal to the protection by the product, and, secondly, that the half-life of the enzyme increases slowly at high sugar concentrations. Thus the experimental method described here appears to be a useful one for the determination of substrate protection of enzyme deactivation in reversible reactions.

INTRODUCTION

The chemical isomerization of glucose and fructose in alkaline solution was studied by Lobry de Bruyn-Alberda van Ekenstein (Fennema, 1976), and it is also well known that aqueous solutions of fructose are degraded by alkali (Shaw *et al.*, 1968). Therefore it is not possible to maintain constant concentrations of these two monosaccharides in alkaline solution without continuous replenishment.

The rate of deactivation of an enzyme is a function of temperature (Ollis, 1972; Cardoso & Emery, 1978), pH (Lee *et al.*, 1976), concentration (Sproull *et al.*, 1976; Ader *et al.*, 1979), reactant, product (Lee *et al.*, 1976; Verhoff & Golastein, 1982) etc. Generally, the effect of temperature is assessed independently of the other experimental variables. Because enzyme deactivation and reaction are likely to occur simultaneously, protection by the substrate is difficult to evaluate independently. Nevertheless, from an engineering point of view in the use of immobilized enzymes, it is worthwhile studying substrate protection of enzymes against deactivation.

We can divide the protective behaviour of substrate into two consecutive steps: (1) the so-called affinity of substrate (reactant and product) with enzyme; the reactant or product first has to be adsorbed by enzyme before reaction or protection; then (2) reaction and protection occur simultaneously. The substrate protection of glucose isomerase has been investigated previously (Lee *et al.*, 1976; Verhoff & Golastein, 1982). When the feed concentration of fructose was raised, Lee *et al.* (1976) found that the operational half-life of a packed-bed reactor was increased. This observation suggested the existence of substrate protection. Because deactivation and reaction of enzyme occur simultaneously, Verhoff & Golastein (1982) provided a theoretical discussion of the protection phenomena by mathematical analysis, but no experimentally obtained data were shown.

The present paper investigates the condition that the reactant–enzyme affinity is the same as product–enzyme affinity. Under this circumstance, with no differential affinity effects, I compare the protection by reactant with the protection by product, and calculate the enzyme half-life at various substrate concentrations.

MATERIALS AND METHODS

Materials and solutions

Glucose isomerase immobilized in small particles (radius less than 100 μm), with high stability in hot solution, was from Nagase Chemicals (Nishi-Ku, Osaka, Japan). Glucose was from Katayama (Osaka, Japan). Tris and fructose were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Acetonitrile (LC grade) was from Merck (Darmstadt, Germany). Tris buffer was prepared with 50 mM-Tris and 5 mM-MgSO₄, adjusted to pH 8.25 with HCl. Substrate solution was prepared with Tris buffer and the desired monosaccharide. The mobile phase for h.p.l.c. was prepared with acetonitrile/deionized water (17:3, v/v).

Determination of monosaccharide concentration

H.p.l.c. was used. A LiChrosorb-NH₂ (10 μm particle size) column from Merck was used to separate the mixture of glucose and fructose. The amount of each sugar component was detected by using a differential refractometer for measurement of the refractive index, and by comparison of the integrated area obtained by using the Data Modulus of Waters Associates with the standard curve the concentrations of the two sugars were determined.

The operational conditions of the h.p.l.c. were: flow rate of mobile phase, 1.1 ml/min; injected sample volume, 20 μl ; room temperature.

Determination of the enzyme activity

The particles of immobilized glucose isomerase, taken out from the column (see below), were rinsed thoroughly with Tris buffer, pH 8.25, at room temperature, and added to 60 ml of freshly made 2.0 M-glucose solution, pH 8.25, at 60 °C in a stirrer beaker for reaction. Samples (1 ml) of the reaction mixture were removed by pipette at 0, 20, 40 and 60 min, and each was diluted to 25 ml for determination of glucose and fructose concentrations by h.p.l.c. The enzyme activity was evaluated by calculating the initial reaction rate.

Fig. 1. Apparatus of eight-column reactor sets for enzyme-deactivation experiments

Substrate (monosaccharide) was in Tris buffer, pH 8.25, at 62.1 °C. The flow rate in each tube was about 2 ml/h. Key: 1, reservoir; 2, pump; 3, valve; 4, deactivation reactor; 5, stirrer, heater and controller; 6, output reservoir; 7, water bath; 8, immobilized glucose isomerase; 9, inert glass beads.

Determination of the effect of substrate on enzyme deactivation

The apparatus for investigation of enzyme deactivation is shown in Fig. 1. The monosaccharide was pumped into the eight columns, which contained packed immobilized glucose isomerase. The monosaccharide concentration was 0.2, 0.93 or 2.2 M. The stirrer, heater and controller were used to maintain the desired temperature. The output solutions were collected for monitoring of the constant concentration that was necessary for this work. The flow rate of substrate solutions was about 2 ml/h for replenishment. At set times the particles of immobilized glucose isomerase were removed, rinsed thoroughly, and the residual activity was determined as described above.

RESULTS AND DISCUSSION

Definition and symbols

The symbols used are defined as follows: D_a , diffusivity in particles (cm^2/h); E , concentration of enzyme (g/l); EF and EG , concentrations of glucose-enzyme and fructose-enzyme complexes respectively (g/l); F , G and G_0 , concentration of fructose, glucose and total monosaccharide respectively (M); k_t , deactivation constant of enzyme unbound by reactant or product (h^{-1}); k_d , constant defined in eqn. (6) (h^{-1}); K_m , modified Michaelis constant (M); k_{+1} and k_{-1} , rate constants of forward and reverse reaction respectively (h^{-1}); K_{mf} and K_{mr} , dissociation constants of the forward and reverse reactions respectively (M); K_{mi} , either K_{mf} or K_{mr} ; R , radius of immobilized enzyme particle (cm); $t_{1/2}$, half-life of enzyme (h); V_m , modified maximum reaction rate (M/h); α and β , protection coefficients of glucose and fructose respectively (dimensionless); ψ , activity coefficient of enzyme (i.e. enzyme activity at any given time/initial enzyme activity).

Fig. 2. Temperature-dependences of K_{mf} and K_{mr} of immobilized glucose isomerase

Reactions were performed in Tris buffer, pH 8.25. ●, K_{mf} ; ○, K_{mr} .

Affinity effect of substrate

When the Thiele Modulus ($\phi = R\sqrt{V_m/D_a \cdot K_m}$) is less than 0.9, the internal mass-transfer resistance can be neglected (Lin, 1984). In this work, the radius of the particles of immobilized glucose isomerase used is less

than 100 μm ($\phi = 0.79$), and so the mass-transfer resistance can be neglected. Also, the reaction rate is faster than the enzyme deactivation rate. In other words, the intrinsic kinetic constant K_m can be determined at various temperatures from initial-velocity estimates by using the Lineweaver-Burk-plot method.

The temperature-dependences of the intrinsic kinetic constants K_m of the forward and reverse reactions with Tris buffer, pH 8.25, are shown in Fig. 2. The temperature-dependences of K_m values can be written in an Arrhenius form. The dissociation constants K_m will equal each other at 62.1 °C and pH 8.25, i.e. $K_{mf} = K_{mr} = K_{mi}$ in eqn. (1).

The reversible reaction scheme of glucose isomerase with substrate protection can be given by (Verhoff & Golastein, 1982):

where E, G and F represent enzyme, glucose and fructose respectively. Glucose and fructose are degraded or isomerized chemically (Shaw *et al.*, 1968; Fennema, 1976) in alkaline solution, and so the substrate must be replenished continuously, as described in the Materials and methods section. The apparatus was designed for substrate replenishment only, not for enzyme activity assay. Therefore it is assured that the enzyme deactivation was continuing at a constant substrate concentration. At set times its residual activity was determined as described in the Materials and methods section.

Figs. 3 and 4 show the time courses of log(enzyme activity) at various substrate concentrations and with buffer only. All log(enzyme activity) values are approximately linear functions of time, and so it can be realistically assumed that they obey first-order decay form, i.e.:

$$\frac{dE}{dt} = -k_t \cdot E; \frac{d(EG)}{dt} = -\alpha k_t \cdot EG; \frac{d(EF)}{dt} = -\beta k_t \cdot EF \quad (2)$$

In accordance with these assumptions, I obtained:

$$\frac{d\psi}{dt} = \frac{-k_t \psi (1 + \alpha G/K_{mf} + \beta F/K_{mr})}{1 + G/K_{mf} + F/K_{mr}} \quad (3)$$

When the temperature is 62.1 °C, then $K_{mf} = K_{mr} = K_{mi}$, and eqn. (3) can be reduced to:

$$\frac{d\psi}{dt} = \frac{-k_t \psi (K_{mi} + \alpha G + \beta F)}{K_{mi} + G_0} \quad (4)$$

where $G_0 = G + F$ is the total monosaccharides.

Effect of substrate on enzyme deactivation

First, it was of interest to determine how the total monosaccharide concentration affects the enzyme deactivation rate. The time courses of enzyme activity at various ratios of glucose and fructose with the same amount of total sugar are shown in Fig. 3, at 62.1 °C, pH 8.25. Unexpectedly, the enzyme deactivation rate was independent of the glucose/fructose ratio. These results led to the conclusion that $\alpha = \beta$, i.e. that the protections of glucose isomerase by glucose and fructose are the same as each other. This may result from the similarity of their

Fig. 3. Time courses of log(enzyme activity) at various ratios of glucose and fructose, but with the total monosaccharide concentration kept at 2.2 M

Reactions were performed in Tris buffer, pH 8.25, at 62.1 °C. ○, $G = 2.2$ M, $F = 0.0$ M; △, $G = 1.0$ M, $F = 1.2$ M; □, $G = 0.0$ M, $F = 2.2$ M.

Fig. 4. Time courses of log(enzyme activity) at various total monosaccharide concentrations

Reactions were performed in Tris buffer, pH 8.25, at 62.1 °C. ○, $G_0 = 2.2$ M; □, $G_0 = 0.93$ M; △, $G_0 = 0.2$ M; ●, $G_0 = 0$ M (buffer only).

stereochemical structure. In accordance with the conclusion above, eqn. (4) can be rearranged to:

$$\frac{d\psi}{dt} = \frac{-k_t (K_{mi} + \alpha G_0)}{K_{mi} + G_0} \quad (5)$$

$$= -k_d \cdot \psi \quad (6)$$

Then:

$$\ln \psi = -k_d \cdot t \text{ or } t_{\frac{1}{2}} = \ln 2 / k_d \quad (7)$$

$$\alpha = [k_d (K_{mi} + G_0) / k_t - K_{mi}] / G_0 \quad (8)$$

From eqns. (6) and (8) and the data in Fig. 4, the α values at various total monosaccharide were calculated and they are shown in Table 1. The averaged α and β values are equal to 0.2. This means that the protection efficiency against enzyme decay by both of the two substrates reaches 80%.

Table 1. Half-life ($t_{1/2}$) and α values for immobilized glucose isomerase at various concentrations of total monosaccharides

The average value for α is 0.2.

G_0 (M)	$10^3 \times k_d$ (or $10^3 \times k_t$) (h ⁻¹)	α	$t_{1/2}$ (days)
0.0	2.1	—	13.8
0.2	1.4	0.18	20.6
0.93	0.85	0.22	33.0
2.2	0.6	0.19	48.1

Effect of total monosaccharide concentrations on enzyme half-life ($t_{1/2}$)

In accordance with eqn. (5), two special cases are obtained by theoretical analysis.

First, when G_0 equals zero, the enzyme deactivates in buffer only. Eqn. (5) can be integrated to:

$$\ln \psi = -k_t \cdot t, \text{ when } G_0 = 0 \quad (9)$$

Then:

$$t_{1/2} = \ln 2 / k_t, \text{ when } G_0 = 0 \quad (10)$$

From eqn. (9) the k_t value shown in Table 1 can be determined by the method described in the Materials and methods section. Let us return to eqn. (1): the enzyme in these conditions is unbound by reactant or product, and so its deactivation rate constant is k_t .

Secondly, when $G_0 \rightarrow \infty$, the situation is not a practicable one in real conditions, because high monosaccharide concentrations result in high viscosity and saturation. Nevertheless in mathematical analysis eqn. (5) can be integrated to:

$$\ln \psi = -\alpha k_t \cdot t, \text{ when } G_0 \rightarrow \infty \quad (11)$$

Then:

$$t_{1/2} = \ln 2 / \alpha k_t, \text{ when } G_0 \rightarrow \infty \quad (12)$$

Let us return to eqn. (1) again: most of the enzyme now would be in the form of enzyme-substrate complex at high monosaccharide concentration, and so its deactivation rate constant is weakly dependent on monosaccharide concentration (approximate to αk_t or βk_t). This conclusion explains why the half-life ($t_{1/2}$) of the enzyme

Fig. 5. Half-life ($t_{1/2}$) of immobilized glucose isomerase plotted versus the total monosaccharide concentration

Reactions were performed in Tris buffer, pH 8.25, at 62.1 °C. —, Calculated from eqn. (7); O, experimental values from Fig. 4.

increases slowly at high monosaccharide concentrations, as shown in Fig. 5. It also can realistically explain the results obtained by Lee *et al.* (1976).

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