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Investigation of immobilized glucoamylase kinetics by flow calorimetry

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Abstract

Flow calorimetry was used for investigation of kinetic properties of glucoamylase covalently bound to controlled-pore glass particles. Maltodextrin hydrolysis was measured in steady-state (single flow mode) and in non-steady-state conditions (total recycling of the reaction solution). The experimental data were treated by mathematical modeling based on material and heat balances of the reaction system. The proposed technique enables to determine intrinsic kinetic parameters of enzyme reactions influenced by internal particle diffusion directly from calorimetric data.

Keywords: Flow calorimetry; Immobilized glucoamylase; Kinetic measurement; Pore diffusion; Intrinsic kinetics

1. Introduction

Immobilized biocatalysts (IMB) — enzymes or whole cells — are still in the interest of people working in different branches. They constitute principal parts of devices of very variable scale and application — starting from microgram amounts of IMB in special analytical devices up to industrial reactors with IMB loading of hundreds of kilograms. Independent of the application, there is one common point of the IMB use: their kinetic properties must be known. This is valid in the stage of IMB screening and design, as for the specification of operational conditions in which they should be used. Therefore, there is always the need coming with a new IMB to find sufficiently accurate, simple and fast experimental technique of investigation of their kinetic properties.

In the previous work [1], we described the principles and applications of flow calorimetry (FC) in the investigation of the IMB properties. Thanks to the versatility of the detection principle, the FC can be used practically for every enzyme–substrate system, under the condition that a sufficient reaction heat is produced and the substrate is in soluble form. One of the last improvements of this technique was the introduction of an ''autocalibration'' principle based on reaction solution recirculation enabling to determine true reaction rate of biocatalytic reaction without any requirement of an additional analytical technique [2]. The technique was demonstrated on the example of immobilized invertase bound on spherical particle surface. In that case, a simplified mathematical model (ideal batch stirred reactor) was used for

Abbreviations: CPG, controlled-pore glass; FC, flow calorimetry; IMB, immobilized biocatalyst(s); MDX, maltodextrin

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Nomenclature

c_S	substrate concentration
D_a	axial dispersion coefficient
D_e	effective diffusion coefficient
F	volumetric flow rate in the FC column
l	axial coordinate
L	length of the section
r	radial coordinate in IMB particle
R	radius of IMB particle
t	time
ΔT	temperature change in the column per gram of IMB
v	reaction rate
w	superficial flow rate

Greek symbols

α	transformation factor
ε	void fraction of IMB bed

data evaluation. In this work a more complex situation was analyzed:

- the enzyme — glucoamylase was immobilized in pores of the support particles meaning that the internal particle mass transfer had to be considered;
- the reaction mechanism was more complicated;
- as it will be explained below, the recirculation system required more complicated mathematical model for describing the reaction course.

Wide applications of glucoamylase in starch industry motivate research aimed in the improvement of the enzyme properties by methods of enzyme screening, molecular biology and enzyme engineering. The potential improvement of industrial process by glucoamylase immobilization still intrigues researchers. This research can be facilitated by developing suitable methods for the investigation of kinetic properties of immobilized glucoamylase. Measurement of the rate of the polymer hydrolysis can be a rather complicated deal. Comparing to many one-step enzyme reactions, the hydrolysis of macromolecular substrate follows more complicated, multi-step mechanism. Consequently, many molecular species appear in the reaction mixture and the question is how to analyze them and how to define the reaction rate. In this work, the task was simplified by reducing the experiment to the initial rate measurement in combination with the FC avoiding the requirement of a more complicated chemical analysis. For the purpose of the methodology development, the enzyme was immobilized in controlled-pore glass (CPG) particles and a well defined substrate — maltodextrin (MDX) — was used.

2. Experimental

2.1. Materials

Glucoamylase from *Aspergillus niger*, under the brand name Spezyme GA 300 N (EC 3.2.1.3, 1,4-α-Δ-glucan glucohydrolase), as a product of GENENCOR International with the declared activity 300 SGU/ml (one SGU will liberate 1.0 g of glucose from soluble starch in 60 min at pH 4.3 at 60°C) was supplied by Gammazym Slovakia (Bratislava). Aminopropyl glass particles (AMP00350A, particle radius 76 μm, pore diameter 32.4 nm, pore volume 1.36 cm^3/g) were provided by CPG Inc. (Lincoln Park, NJ) and activated by glutaraldehyde (Fluka, Buchs, Switzerland) before binding the glucoamylase. MDX MALDEX 180 (obtained by enzyme hydrolysis of potato starch, M.W. 13100, as determined by size-exclusion HPLC chromatography) was provided by Amylum Slovakia, s.r.o. (Boleráz, Slovak Republic). All other chemicals were obtained from Lachema (Brno, Czech Republic).

2.2. Immobilization of glucoamylase

One gram of aminopropyl glass particles was stirred in 50 cm^3 of 6.25% (v/v) glutaraldehyde solution in 0.1 M acetate buffer, pH 6.0 at 4°C for 24 h. Activated glass particles were suction-filtered and washed successively with buffer solution until the whole amount of unbound glutaraldehyde was removed. The washed particles were stirred with 1 ml of glucoamylase in 50 ml of 0.1 M acetate buffer, pH 6.0 at 4°C for 24 h. Particles with covalently bound glucoamylase were washed with 0.1 M acetate buffer, pH 4.7 and thus ready for measurements.

2.3. Kinetic measurement in the flow calorimeter

The calorimetric measurements of immobilized enzyme kinetics were performed in a flow calorimeter

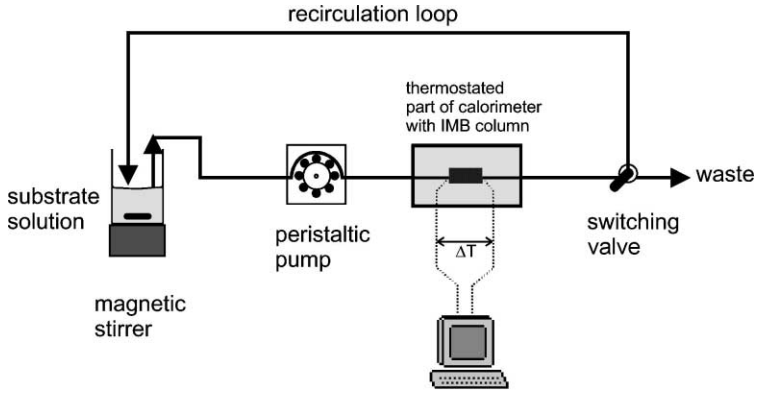

Fig. 1. Experimental calorimetric recirculation system.

(3300 Thermal Assay Probe, Advanced Biosensor Technology AB, Lund, Sweden). The experimental set-up used for the measurements is depicted in Fig. 1. The main part of the system is a thermostatic cell with the immobilized enzyme column. The column is operated as a small packed bed reactor. The temperature difference between the column input and output, ΔT, is measured by thermistors and registered by a personal computer.

For the kinetic measurement, 12.2 mg of glass particles with immobilized glucoamylase were packed in the Delrin column (2.0 cm × 0.3 cm i.d.) between two layers of inert glass beads and placed in the flow calorimeter. The system was kept at temperature of 303.15 K, while the buffer solution was continuously pumped through the column with constant flow rate of 1 ml/min. The experiment was started by replacing the buffer solution by the substrate solution containing 1–200 g/l of MDX in 0.1 M acetate buffer (pH 4.7). The method of measurement was described previously [2]. Two measurement techniques were applied: single flow mode and mode with total recirculation of the reaction mixture. In the single flow mode (switching valve opened to waste), steady-state temperature signals were measured in the dependence on the input substrate concentration. In the recirculation mode (closed waste), the signal change due to the substrate consumption was measured in the dependence on time.

The essential rule for kinetic measurements was to keep a differential amount of IMB preparative in the column. This was ensured by the method described previously [1]. In principle, the IMB amount in the column was set so low that the steady-state signals obtained with fresh substrate solution were approximately the same as those with the solution used repeatedly after one passing through the column. This means that the reaction mixture composition was not changed significantly during the first passage and the reaction rate in the column could be considered constant.

2.4. Mathematical treatment of experimental data

First of all the thermometric signals, ΔT, at steady-state were plotted against MDX concentration, c_s (Fig. 3) and the data was fitted by equation that is an analogy to substrate inhibition model (analogous to Eq. (17))

$$\Delta T = \frac{p_1 c_S}{c_S + p_2 + p_3 c_S^2} \quad (1)$$

Parameters p_i were calculated by non-linear regression.

Eq. (1) was used as a calibration for the treatment of data obtained by the recirculation experiment that was necessary for the determination of the relation between the thermometric signal and true reaction rate in the flow calorimeter column. The typical reaction course in the recirculation system is shown in Fig. 4. The principle of the evaluation method published previously [2] is based on the assumption of a pseudo-steady-state in the FC column: when a differential bed of immobilized enzyme is used and the rate of enzyme reaction is not too high, the actual substrate concentration at an arbitrary time is the same as that in steady-state for the same value of the

thermometric signal. In order to prevent possible product inhibition effects exclusively, the data from the initial reaction period were used. Substrate concentrations corresponding to the thermometric signals were calculated from Eq. (1). The ΔT–time registrations (Fig. 4) were transformed into substrate–time dependencies and the resulting dependencies were evaluated by mathematical modeling explained below.

2.4.1. Mathematical model of recirculation system

The reaction course was modeled through the system of material balance equations for different sections of the recirculation system. For the purpose of mathematical modeling, the recirculation system depicted in Fig. 1 was divided into sections according to the scheme in Fig. 2. The pump and switching valve were omitted with the aim of simplification.

Substrate balance equations for the four sections in non-steady-state conditions were as follows:

1. Stirred reservoir — perfect mixing was assumed:

$$\frac{\partial c_{S1}}{\partial t} = F(c_{S4} - c_{S1}) \tag{2}$$

where F is the volumetric flow rate. Initial condition

$$t = 0, \quad c_{S1} = c_{S0} \tag{3}$$

2. For capillary A (from the stirred reservoir to the column input) convection transport with axial dispersion was considered:

$$\frac{\partial c_S}{\partial t} = D_{aA}\frac{\partial^2 c_S}{\partial l_A^2} - w_A\frac{\partial c_S}{\partial l_A} \tag{4}$$

Initial condition

$$t = 0, \quad c_S = c_{S0} \tag{5}$$

and boundary conditions

$$l_A = 0, \quad c_S = c_{S1}, \qquad l_A = L_A, \quad \frac{\partial c_S}{\partial l_A} = 0 \tag{6}$$

where D_{aA} is the axial dispersion coefficient, w_A the superficial flow rate, l_A the axial coordinate in the capillary A.

3. Column with IMB — differential bed was considered with negligible volume and accumulation:

$$w_{DR}\frac{\partial c_S}{\partial l_{DR}} = -v \tag{7}$$

where w_{DR} is the superficial flow rate in the IMB column, v the reaction rate.

Conditions at the column boundaries

$$l_{DR} = 0, \quad c_S = c_{S2} \tag{8}$$

$$l_{DR} = L_{DR}, \quad c_S = c_{S3} \tag{9}$$

An acceptable simplification was to describe the IMB packed bed as a differential bed reactor with very small volume. In such conditions, the reaction rate was constant along the reactor and Eq. (7) could be integrated giving the following equation:

$$c_{S3} = c_{S2} - v\frac{L_{DR}}{w} \tag{10}$$

that was combined with the system of balance equations describing other sections.

4. Capillary B (from the column output to the stirred reservoir):

$$\frac{\partial c_S}{\partial t} = D_{aB}\frac{\partial^2 c_S}{\partial l_B^2} - w_B\frac{\partial c_S}{\partial l_B} \tag{11}$$

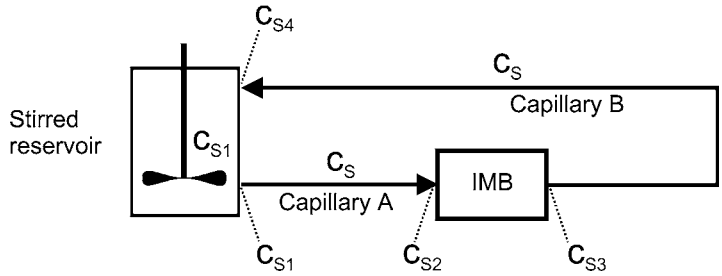

Fig. 2. Model part of the experimental system with notation for substrate concentrations.

Initial condition:

$$t = 0, \quad c_S = c_{S0} \tag{12}$$

and boundary conditions:

$$l_B = 0, \quad c_S = c_{S3}, \qquad l_B = L_B, \quad \frac{\partial c_S}{\partial l_B} = 0 \tag{13}$$

The above mathematical model was used for calculation of the reaction rate, v, in the initial stage of the reaction. The model represents a set of partial and ordinary differential and algebraic equations. Spatial derivatives in balance equations for capillaries (4) and (11) were discretized by finite difference Crank–Nicholson scheme. The resulting set of ordinary differential and algebraic equations was solved using Athena Visual Workbench software package (Stewart and Associates Engineering Software).

Once the reaction rate was known, the transformation parameter α was calculated from the relation

$$\alpha = \frac{v}{\Delta T} \tag{14}$$

where the calculated initial reaction rate and the thermometric signal registered at time zero in the recirculation system were introduced. It was shown that in conditions of FC measurement, the parameter α is a constant [3] that enabled to transform thermometric signals in Fig. 3 into steady-state reaction rates by means of Eq. (14).

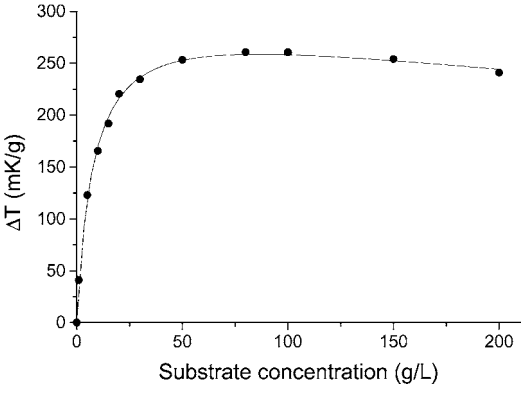

Fig. 3. Thermometric measurement of kinetic properties of immobilized glucoamylase in the steady-state mode.

Thus, a true kinetic data depicted in Fig. 5 were obtained that were used for evaluation of kinetic parameters for the immobilized enzyme.

2.4.2. Determination of kinetic parameters for immobilized glucoamylase

Assuming glucoamylase was immobilized in porous particles the reaction–diffusion mathematical model was applied for calculation of the reaction rate. The steady-state material balance for the spherical biocatalyst particle gives

$$D_e\left(\frac{d^2 c_S}{dr^2} + \frac{2}{r}\frac{dc_S}{dr}\right) - v_r = 0 \tag{15}$$

with boundary conditions:

$$r = 0, \quad \frac{dc_S}{dr} = 0, \qquad r = R, \quad c_S = c_{Sb} \tag{16}$$

where r is the particle radial coordinate, R the particle radius, and D_e the substrate (MDX) effective diffusion coefficient. The needed value of effective diffusion coefficient for MDX ($D_e = 9.4 \times 10^{-9}\,dm^2\,s^{-1}$) in CPG pores was estimated on the base of literature data as explained in Appendix A. The reaction rate, v_r, was calculated from the substrate inhibition model

$$v_r = \frac{V_m c_S}{K_m + c_S + c_S^2/K_i} \tag{17}$$

in which V_m, K_m and K_i are kinetic parameters.

For the purpose of kinetic data treatment, a material balance in the packed bed is needed. In steady state, this gives the relation for calculation of the reaction rate corresponding to the reaction rate in Eq. (10):

$$v = \frac{3(1-\varepsilon)D_e}{R\varepsilon}\left(\frac{dc_s}{dr}\right)_{r=R} \tag{18}$$

The value of derivative in Eq. (18) was calculated by solving Eq. (15). Using the described mathematical model, kinetic parameters in Eq. (17) were determined by non-linear regression from kinetic data in Fig. 5. All computations were done by means of Athena Visual Workbench software package.

3. Results

The investigation of kinetics of MDX hydrolysis catalyzed by immobilized glucoamylase was effectuated

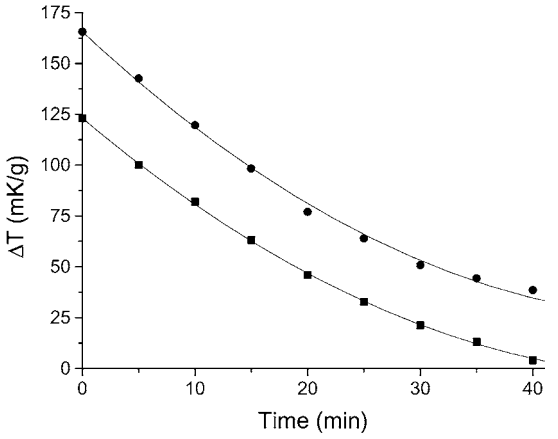

Fig. 4. Signal variation during the measurement in the recirculation system. Solid lines were obtained by polynomial regression and used for calculation of initial rates from the line slopes at time zero. Initial MDX concentrations: 10 g dm^{-3} (●); 5 g dm^{-3} (■).

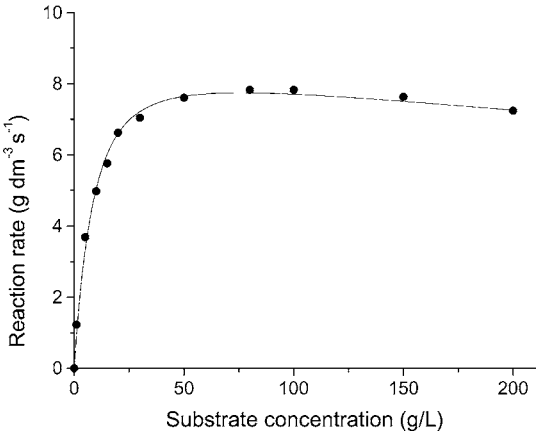

Fig. 5. Kinetic data for immobilized glucoamylase obtained from the data depicted in Fig. 3. The solid line was obtained by mathematical modeling.

in two steps based on the FC. The first step consisted in determination of dependence of steady-state heat response on MDX concentration (single flow measurement). The typical result of such study is depicted in Fig. 3.

The second step of the kinetic experiment was based on the registration of the thermometric signal during the reaction in non-steady-state conditions in the closed system (total flow recirculation). The typical registrations for two initial MDX concentrations are shown in Fig. 4.

The data shown in Fig. 4 were used for determination of the transformation factor, α, according to the procedure described above. The values of transformation factor obtained for two different initial substrate concentrations are introduced in Table 1. The mean value of $\alpha = 3.33 \times 10^{-2}\,\mathrm{g\,dm^{-3}\,s^{-1}/mK\,g^{-1}}$ was taken for conversion of thermometric data depicted in Fig. 3 into reaction rate values using Eq. (14). Thus, the steady-state reaction rates were calculated resulting in the kinetic dependence that is shown in Fig. 5. This is a conventional type of kinetic dependence that can be evaluated by usual approaches to the evaluation of enzyme kinetic data. Considering the CPG pore diameter and the size of glucoamylase molecule, it was reasonable to assume that the enzyme was immobilized inside the pores. This assumption was confirmed by a dramatic decrease of the specific activity of IMB particles when a CPG with smaller pores was used (data not shown). Therefore, the internal diffusion was included in the mathematical model represented by Eqs. (15)–(18). The kinetic parameter values calculated by non-linear regression were (with 95% confidence interval in parentheses): $V_m = 4.0\,(3.8;\ 4.2)\ \mathrm{g\,dm^{-3}\,s^{-1}}$, $K_m = 0.56\,(0.15;\ 0.96)\ \mathrm{g\,dm^{-3}\,s^{-1}}$, $K_i = 1190\,(470;\ 1910)\ \mathrm{g\,dm^{-3}\,s^{-1}}$. The model fitted the experimental data with correlation coefficient 0.997.

Table 1
Determination of transformation factor α

Initial MDX concentration, c_{S0} (g dm^{-3})	Initial thermometric signal, ΔT (mK g^{-1})	Initial reaction rate[a], v (g dm^{-3} s^{-1})	Transformation factor, $\alpha = v/\Delta T$ (g dm^{-3} s^{-1}/mK g^{-1})
5	123	4.450	3.61×10^{-2}
10	166	5.031	3.04×10^{-2}

[a] Calculated according to Eq. (10) by procedure described in paragraph 2.5.

4. Discussion

Application of FC for investigation of IMB kinetics using the same instrument as reported in this paper was demonstrated several years ago [4]. The kinetic data were obtained by single flow measurement in steady state. In such case, only kinetic parameters in denominator of enzyme kinetics equations can be determined. In order to determine parameters in the nominator (V_m), the reaction rate should be measured. For the reaction rate measurement, the continuous flow mode must be combined with the recirculation one [2]. Then, the reaction rate in the system is calculated from the rate of the signal decrease due to the substrate consumption. The necessary condition of obtaining correct data is using sufficiently small amount of the IMB in the column so that the substrate concentration change in the column for one passing of the reaction mixture is negligible and the IMB bed in the column can be considered as a differential one. However, if a very small amount of IMB is used, the rate of substrate conversion in the recirculation mode is too low and prolongs the measurement time. In such case, the total volume of the reaction mixture in the system must be reduced in order to reduce the initial substrate amount. As a consequence, if the recirculation rate is not sufficiently high, reactant concentrations are spatially distributed and the recirculation system cannot be approximated by a perfectly mixed reactor model used previously [2]. Therefore, the mathematical model completed with the axial distribution of substrate in connecting capillaries was used in this work.

The main advantage of the described technique is the possibility to determine the reaction rate directly from the thermometric data without any independent calibration technique. Thus, the kinetic data can be treated by mathematical modeling enabling to determine intrinsic kinetic parameters of enzyme reaction. All kinetic study can be performed with a very small amount of the immobilized enzyme preparation reaching the order of several milligrams.

Acknowledgements

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Appendix A. Estimation of effective diffusion coefficient of MDX

The value of diffusion coefficient of MDX in water was estimated from the following relation [5]:

$$D = 9.40 \times 10^{-16} \frac{T}{\mu_w M_{\mathrm{MDX}}^{1/3}} = 1.51 \times 10^{-10}\ \mathrm{m^2\ s^{-1}}$$

Then, the effective diffusion coefficient for MDX transport in CPG pores was determined from [6]:

$$D_e = D\left(1 - 2\frac{d_h}{d_p}\right) = 9.4 \times 10^{-11}\ \mathrm{m^2\ s^{-1}}$$

where d_p is the pore diameter (it was 32.4 nm for used CPG). The hydrodynamic diameter of MDX molecule, d_h, was calculated from [7]

$$d_h = 0.0542 M_{\mathrm{MDX}}^{0.498} = 6.09\ \mathrm{nm}$$

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