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Investigation of kinetics of immobilized liver esterase by flow calorimetry

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Abstract

Flow calorimetry (FC) was shown to be a powerful tool for investigation of the kinetics of phenyl acetate hydrolysis catalyzed by pig liver carboxyl esterase. The enzyme was immobilized in alginate gel particles that were placed in a calorimetric flow column and the heat effect of enzyme reaction was followed in single flow and total recirculation conditions. It was shown that the registered temperature change was proportional to molar amount of substrate transformed in the column. A mathematical model describing the enzyme reaction, mass transfer, and heat effects in the calorimetric system was developed and used for the kinetic data evaluation. By combining data from single flow and recirculation modes true kinetic parameters were evaluated by the proposed mathematical procedure based on the model solution and successive approximations.

The kinetic data for carboxyl esterase showed a slide substrate inhibition by phenyl acetate. The obtained kinetic parameters were as follows: Michaelis constant $K_m = 2\,\text{mmol}\,\text{dm}^{-3}$ and substrate inhibition constant $K_i = 42\,\text{mmol}\,\text{dm}^{-3}$. The method can be applied to kinetic study of immobilized enzymes directly in the flow calorimeter without any requirement of an independent analytical technique.

Keywords: Flow calorimetry; Kinetics; Pig liver carboxyl esterase; Immobilized enzyme; Reaction-diffusion system

1. Introduction

Immobilized biocatalysts (IMB)—enzymes or whole cells—are used in various areas of analytical, medical, and industrial applications. Independently on the scale of process using the IMB, ranging from analytical to industrial equipment, there is always need for accurate evaluation of kinetic parameters characterizing the enzyme reaction. Basically, enzyme kinetic parameters cannot be determined without accurate experimental data. For this purpose many experimental techniques can be used, that are more or less laborious and time consuming. In order to simplify the kinetic experiment a flow calorimetry (FC) was applied previously and it was proved to be sufficiently accurate, simple and fast for several enzyme systems [1–3].

In this work, kinetic properties of immobilized pig liver carboxyl esterase were studied by flow calorimetry. The work directly knots to the work [2] where kinetics of immobilized glucoamylase was investigated, using the "autocalibration" principle based on total recirculation of the reaction solution. Pig liver carboxyl esterase belongs to a large group of enzymes hydrolyzing ester bonds of carboxylic acids as carboxyl esterases, aryl esterases, lipases, acetyl esterases, cholin esterases and cholesterol esterases, which are widely distributed in animals, plants, and microorganisms and show wide substrate tolerance with preference for substrates in soluble state. Carboxyl esterases are usually stable and active even in organic solvents. Because of high regio- and stereo-specificity, they are attractive biocatalysts for the production of optically pure components. Esterases preferentially break ester bond of shorter chain fatty acid. Methanol and other weak acid alcohols are highly effective nucleophiles toward the acyl group in reactions catalyzed by esterases raising the reaction rate [4]. Probably, the most well-known application is the production of vanillin from ferulic acid released from plant cell wall polysaccharides such as pectin or xylan by carboxyl esterase [5].

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Reaction kinetics of carboxyl esterases depends strongly on the nature of substrate. The hydrolysis of different substrates may obey substrate activation [6] or inhibition [7], or it can follow the simple Michaelis–Menten kinetics [8]. In this work, the kinetic of phenyl acetate hydrolysis was investigated.

2. Experimental

2.1. Materials

Carboxyl esterase from porcine liver (EC 3.1.1.1, Sigma–Aldrich Co., St. Louis, MO, USA) with the declared activity 20 U mg^{-1} solid (one unit corresponds to hydrolysis of 1.0 μmol of ethyl butyrate to butyric acid and ethanol per minute at pH 8.0 at 25 °C) was used. Phenyl acetate of 99% purity was provided by Aldrich Chemical Company Inc. (Milwaukee, WI, USA). For enzyme immobilization Na-alginate, Protanal LF 10/40 RB (Pronova Biopolymer a.s.) containing up 40% of guluronic units was used.

2.2. Immobilization of esterase

An amount of 49.8 mg of esterase and 0.3 g of natrium alginate were added to 10 ml of 1% suspension of bentonite in Tris buffer (0.05 mol dm^{-3}, pH 8.5) and the mixture was stirred to homogeneity. The mixture was then pumped through a double wall needle in which an air flow through a shell part was applied to form small droplets. The mixture was dropped into 0.2 mol dm^{-3} solution of $CaCl_2$ in Tris buffer (0.05 mol dm^{-3}, pH 8.5). The formed alginate particles were stirred in the solution for at least 3 h. Afterwards, they were suction-filtered and stirred for 1 min in 1% (v/v) of glutaraldehyde for the enzyme fixing. Finally, the particles were washed with water until the whole amount of unbound glutaraldehyde was removed and stored in 0.05 mol dm^{-3} solution of $CaCl_2$ in 0.05 mol dm^{-3} Tris buffer (pH 8). The mean particle radius was 0.48 mm.

2.3. Determination of phenol concentration

Phenol concentration was analyzed spectrophotometricaly. The method was based on the reaction of 4-aminoantipyrine with phenol in presence of potassium ferricyanide ($K_3Fe(CN)_6$). The analytical procedure was described in [9]. All chemicals for the analysis were provided by Sigma–Aldrich Co. (St. Louis, MO, USA).

2.4. Kinetic measurements in the flow calorimeter

Calorimetric measurements were performed by the flow calorimeter 3300 Thermal Assay Probe (Advanced Biosensor Technology AB, Lund, Sweden). The experimental set-up is depicted in Fig. 1. The main part consisted of the column (2.0 cm × 0.4 cm i.d.) with immobilized biocatalyst that was placed in thermostated aluminium jacket where the temperature was kept at 30 °C. The column was operated as a packed bed reactor. The temperature difference between the column input and output, ΔT, caused by enzyme reaction heat was measured by thermistors by means of Wheatstone bridge and registered by a personal computer.

Two techniques of measurement were applied: single flow mode and total recirculation mode. The single flow mode was performed with the switching valve 2 opened to the waste (Fig. 1). The system was stabilized by pumping buffer solution (0.05 mol dm^{-3} $CaCl_2$ in Tris 0.05 mol dm^{-3}; pH 8) at flow rate 1 ml min^{-1}. Then, the valve 1 (Fig. 1) was switched to the substrate solution reservoir (buffer solution with a given phenyl acetate concentration). When the system reached steady state (stable temperature signal), valve 1 was again switched to the buffer solution and the measurement was repeated with a new substrate concentration.

The second technique, total recirculation mode, was achieved by a slight modification of the single flow mode. The experiment started in the same way as in the case of single flow mode, but after reaching steady-state with the substrate solution valve 2 was switched to the recirculation loop. This way the system was closed and it behaved like a batch reactor system.

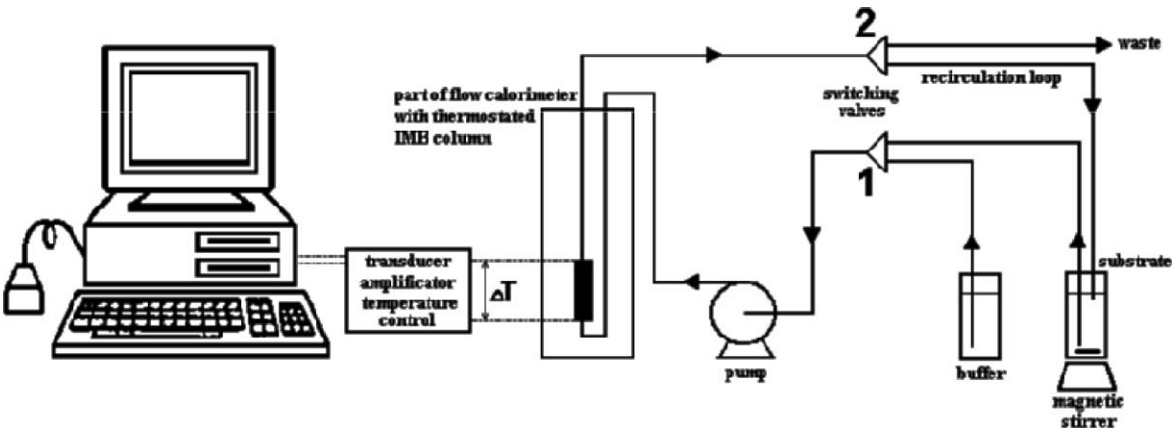

Fig. 1. Experimental set-up of flow calorimetry.

3. Mathematical modeling

3.1. Apparent kinetics in flow calorimeter

The first step in the kinetic study by the flow calorimeter consisted of the measurement of the dependence of the steady-state thermometric signal, ΔT, on input substrate concentration, c_S. The experimental data were fitted by the following equation that was analogous to substrate inhibition kinetic model:

$$\Delta T = \frac{V'_m c_S}{c_S + K'_m + (c_S^2/K'_i)} \quad (1)$$

apparent kinetic parameters K'_m, K'_i and V'_m were calculated by non-linear regression and used as initial guesses for further parameter optimization which was based on the model including mass transfer, reaction kinetics, and heat balance in the calorimeter system.

3.2. Mathematical model of recirculation system

Determination of reaction rate from the steady-state data requires the knowledge of values of several quantities, like reaction enthalpy, fluid heat capacity and density, and packed-bed void fraction. In addition, the instrument has to be calibrated in order to avoid errors in the temperature measurement. These complications can be avoided by the proposed technique based on the total recirculation that, on the other hand, cannot be applied without some investments to mathematical modeling. For this purpose, the recirculation system (Fig. 1) was divided into four main parts forming a loop according to the scheme depicted in Fig. 2.

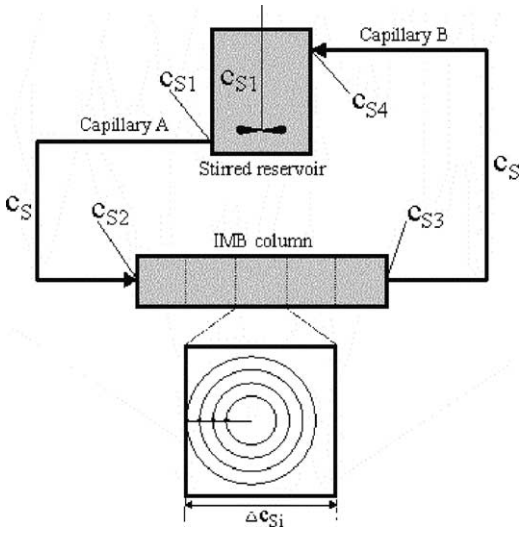

Fig. 2. Schematic diagram of recirculation system with notation of substrate concentrations. The bottom part represents one of five sections of IMB column including biocatalyst spherical particle with three internal collocation points.

Substrate balance equations for respective sections were formulated in the form of following expressions:

1. *Stirred reservoir*: perfect mixing was assumed:

$$\frac{\partial c_S}{\partial t} = \frac{\dot{V}}{V_{mix}}(c_{S4} - c_{S1}) \quad (2)$$

where $\dot{V}$ is the volumetric flow rate and V_{mix} is the volume of the stirred reservoir. Initial condition:

$$t = 0, \qquad c_{S1} = c_{S0} \quad (3)$$

where c_{S0} is the initial substrate concentration.

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

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

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

$$t = 0, \qquad c_S = c_{S0} \quad (5)$$

and boundary conditions:

$$l_A = \begin{cases} 0, & c_S = c_{S1} \\ L_A, & \dfrac{\partial c_S}{\partial l_A} = 0 \end{cases} \quad (6)$$

3. *Column with the IMB*: even though the biocatalyst amount in the column was very small, the substrate conversion could not be considered negligible. Therefore, for the purpose of mathematical modeling the column was divided into five sections (Fig. 2), modeled as a differential bed so that the spatial derivation of substrate concentration was replaced by differentiation through each section. The material balance for each section was derived from the assumption that the substrate consumption in the bulk phase was proportional to the reaction rate that was considered constant along the respective section. The overall reaction rate in the section was calculated from the substrate mass transfer rate across the particle surface:

$$\frac{\partial c_S}{\partial t} = \frac{\Delta c_{Si}}{\Delta l_i}\frac{w_D}{\varepsilon} - \frac{3(1-\varepsilon)D_e}{R_p\varepsilon^2}\left(\frac{\partial c_S}{\partial r}\right)_{r=R_p} \quad (7)$$

where Δl_i is length of ith section, w_D is superficial flow rate in the section and ε was void fraction of the IMB bed. The substrate concentration gradient on the particle surface was calculated by solving the equation of substrate balance in the particle:

$$\frac{\partial c_{SP}}{\partial t} = D_e\left(\frac{\partial c_{SP}}{\partial r} + \frac{2}{r}\frac{\partial^2 c_{SP}}{\partial r^2}\right) - \frac{V_m c_{SP}}{K_m + c_{SP} + (c_{SP}^2/K_i)} \quad (8)$$

with initial and boundary conditions:

$$\begin{aligned} &t = 0, \quad 0 \le r \le 1, \quad c_{SP} = 0 \\ &t > 0, \quad r = 0, \quad \frac{\partial c_{SP}}{\partial r} = 0 \\ &\qquad r = R_p, \quad c_{SP} = c_S \end{aligned} \tag{9}$$

where c_{SP} is the substrate concentration in the particle. V_m, K_m, and K_i are kinetic parameters.

4. *Capillary B* (from column output to the stirred reservoir): convection transport with axial dispersion was considered:

$$\frac{\partial c_S}{\partial t} = D_{aB}\frac{\partial^2 c_S}{\partial l_B^2} - w_B\frac{\partial c_S}{\partial l_B} \tag{10}$$

where D_{aB} is the axial dispersion coefficient in capillary B and w_B is the superficial flow rate in capillary B. Initial condition:

$$t = 0, \qquad c_S = c_{S0} \tag{11}$$

and boundary conditions:

$$l_B = \begin{cases} 0, & c_S = c_{S3} \\ L_B, & \dfrac{\partial c_S}{\partial l_B} = 0 \end{cases} \tag{12}$$

the above mathematical model represents a set of partial and ordinary differential and algebraic equations. Spatial derivatives in balance equations for capillaries Eqs. (4) and (10) were discretized by the Crank–Nicholson scheme [9]. The spatial derivatives in particle balance equation (8) were solved by orthogonal collocation method [10] using three interior collocation points. The resulting set of ordinary differential and algebraic equations was solved using Athena Visual Workbench software package (Stewart and Associates Engineering Software).

3.3. Determination of kinetic parameters for immobilized esterase

If values of intrinsic kinetic parameters K_m and K_i in Eq. (8) were known, the maximum reaction rate V_m, could be obtained from the recirculation experiment data by one parameter mathematical optimization using the mathematical model of recirculation system. As K_m and K_i were unknown the task had to be solved by approximations. In the first step, the values of K_m and K_i were substituted by apparent parameters K_i' and K_m' (determined from Eq. (1) using steady-state data) and V_m was determined. Then, substrate concentration change, Δc_S was calculated from the mass balance of the whole column considering one passage of the substrate solution through the column. From this value the transformation parameter α was calculated as

$$\alpha = \frac{\Delta T}{\Delta c_S} \tag{13}$$

where ΔT was the value of thermometric signal at time zero. Afterwards, the steady-state thermometric signals were transformed into substrate concentration changes in the IMB column according to Eq. (13). Consequently, the data were used to recalculate kinetic parameters V_m, K_m and K_i by means of optimization based on solving material balances of the IMB column and IMB particle defined by Eqs. (7) and (8) and assuming steady-state conditions and zero accumulation. When new K_m and K_i were different from the initial guesses, V_m and α were calculated again from the recirculation mode using the obtained K_m and K_i. The whole procedure was repeated until the resulting parameter values remained unchanged. All computations were done by means of Athena Visual Workbench software package.

4. Results and discussion

The estimation of intrinsic kinetic parameters of phenyl acetate hydrolysis by immobilized pig liver carboxyl esterase by means of FC was made in two steps. The first step consisted in determination of dependence of steady-state heat response on phenyl acetate concentration achieved by the single flow measurement mode.

4.1. Single flow mode

The basic condition simplifying the evaluation of data obtained by flow calorimetry is a linear dependence between the amount of moles of substrate consumed in the column and the temperature change registered as thermometric signal. In the case of phenyl acetate hydrolysis catalyzed by pig liver esterase it was easy to determine spectrophotometrically the concentration of phenol as a reaction product and to calculate the substrate concentration change from material balance. In this way, the dependence showed in Fig. 3 was measured. The dependence measured in a broad range of concentration and temperature changes indicates very good linearity between the substrate concentration change and temperature signal measured (correlation coefficient for the regression straight line 0.9998) proving validity of Eq. (13).

The above result demonstrates that the temperature difference corresponds very closely to the reaction rate in the column. Therefore, kinetic properties of carboxylesterase were further investigated by measurement of the influence of substrate concentration on temperature change. The resulting dependence showed in Fig. 4 indicates the inhibitory effect of substrate on the enzyme reaction. Each experimental point was obtained from two parallel measurements, mean standard deviation throughout the measured dependence was 3.7%.

The data were described by Eq. (1) giving apparent kinetic parameters $V_m' = 12.4 \pm 1.3$ mK, $K_m' = 7.7 \pm 1.3$ mmol dm^{-3}, $K_i' = 21 \pm 4$ mmol dm^{-3}. The kinetic parameters obtained in this manner are so called apparent, as the kinetic behavior of immobilized enzyme is

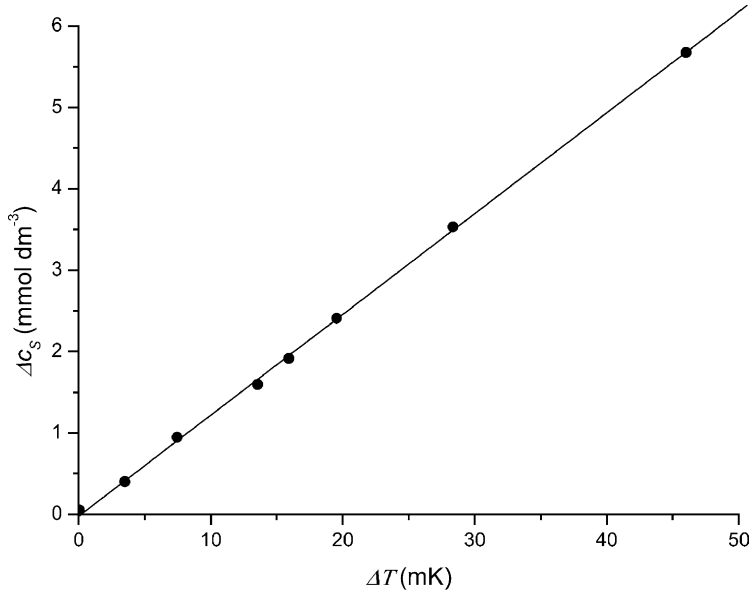

Fig. 3. Relation between substrate concentration change in the column with carboxyl esterase determined by chemical analysis and thermometric signal in the flow calorimeter measured at constant flow rate 1 ml min^{-1}.

influenced by particle mass transfer. In order to determine true kinetic parameters experimental data of real substrate concentration change are needed and a mathematical model including mass transfer has to be solved. In the following part, we propose the experimental and mathematical procedure enabling to achieve such data.

4.2. Total recirculation mode

When there is a small amount of enzyme immobilized in the column, the concentration changes are small as well and it may not be possible to determine the substrate concentration change by chemical analysis. Then, the need of analysis can be avoided by applying the proposed procedure based on a total recirculation system. The data obtained by this procedure described above are shown in Fig. 5. In initial experiments two pumps, HPLC and peristaltic, were tested. As the data obtained with the HPLC pump were rather floating the peristaltic pump was used for the further work.

The thermometric data were transformed to substrate concentrations by solving the equation:

$$c_S^2 + c_S\left(K_i' - \frac{V_m' K_i'}{\Delta T}\right) + K_m' K_i' = 0 \quad (14)$$

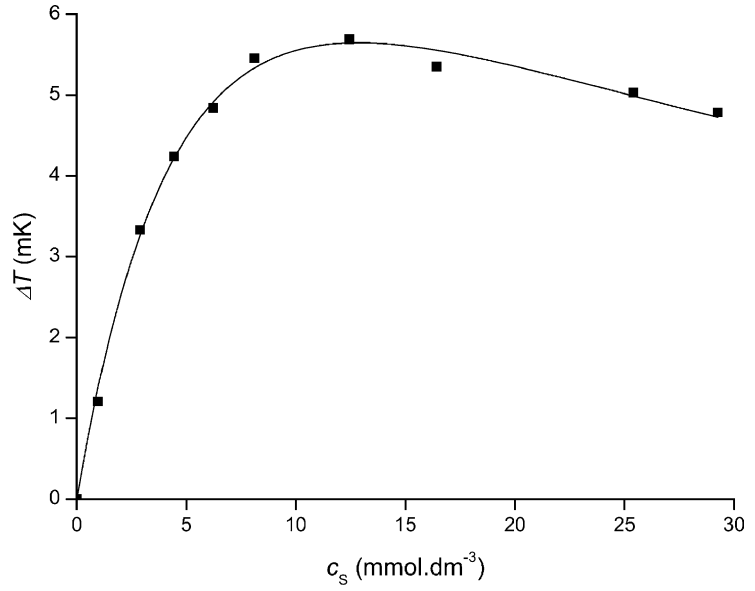

Fig. 4. Thermometric single flow measurement of kinetic properties of immobilized carboxyl esterase in the steady-state mode.

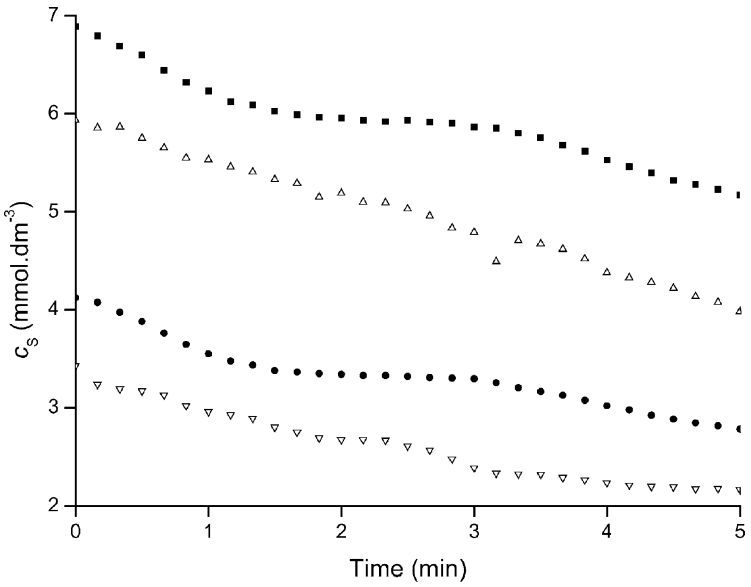

Fig. 5. Reaction course of substrate consumption during the measurement in the recirculation system. Two different pumps (HPLC, peristaltic) and four initial phenyl acetate concentrations were used: HPLC pump—6.89 mmol dm^{-3} (■), 4.12 mmol dm^{-3} (●) and peristaltic pump 5.93 mmol dm^{-3} (△) and 3.43 mmol dm^{-3} (▽).

derived from Eq. (1) while apparent kinetic parameters obtained from the single flow mode were used. Typical registrations for two different initial phenyl acetate concentrations–time dependences are depicted in Fig. 6.

Solid lines in Fig. 6 were calculated from the mathematical model represented by Eqs. (2)–(12). These data were used to determine the transformation factor α according to the procedure described in Section 3.3. The values of transformation factor obtained for two different initial substrate concentrations are introduced in Table 1.

4.3. Experimental data treatment

Values of the transformation factor were used for converting temperature signals measured by the single flow measurement (Fig. 2) into values of substrate concentration change in IMB column using Eq. (13). From the resulting dependence of substrate concentration change on the input substrate concentration depicted in Fig. 7 kinetic parameters K_m, K_i were determined by regression using the mathematical model of the calorimetric column described above.

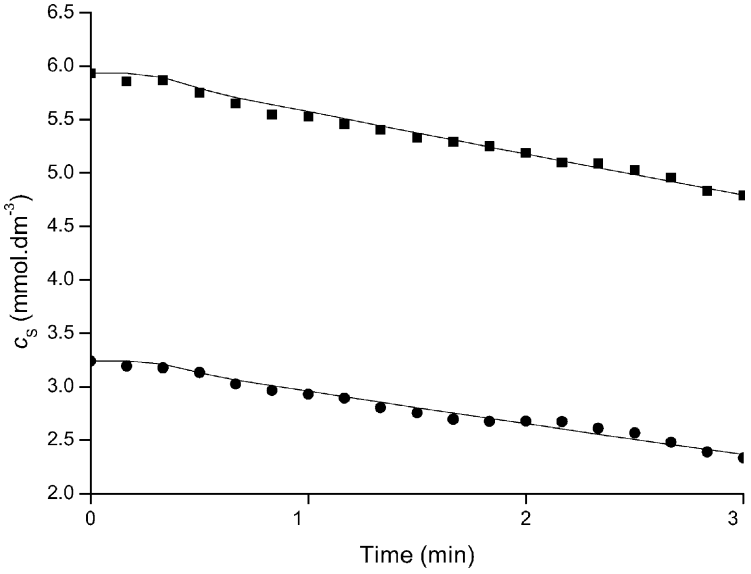

Fig. 6. Substrate concentration variation during the measurement in the recirculation system. The solid lines were obtained using the non-linear regression of the data for two different initial substrate concentrations: 3.43 mmol dm^{-3} (●); 5.93 mmol dm^{-3} (■).

Table 1
Determination of the transformation factor α

Initial phenyl acetate concentration c_{S0} (mmol dm^{-3})	Initial thermometric signal ΔT_0 (mK)	Initial change of substrate concentration, Δc_{S0} (mmol dm^{-3})	Transformation factor α (mK mmol^{-1} dm^3)
3.240	3.70	0.347	10.658
5.932	4.77	0.442	10.797

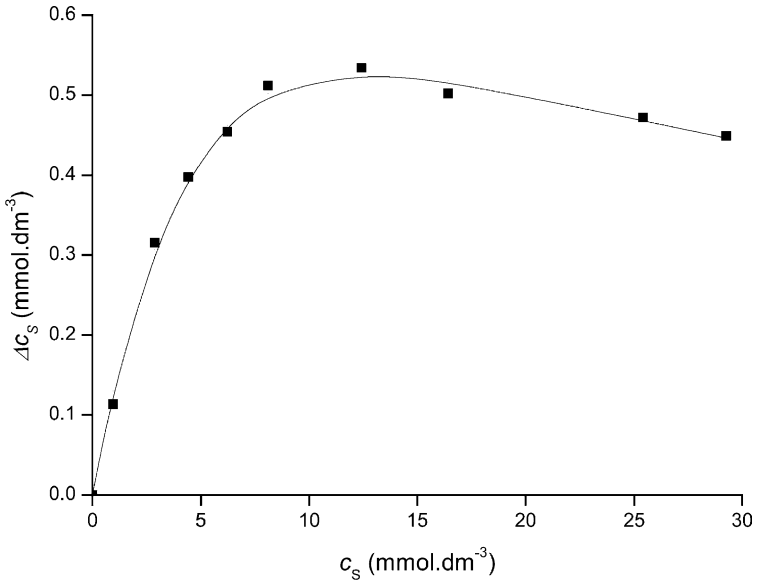

Fig. 7. Single flow measurement of kinetic properties of immobilized carboxyl esterase in the steady-state mode, after transformation into dependence of substrate concentration change on input substrate. Transformation parameter $\alpha = 10.658$ mK mmol^{-1} dm^3 was used.

Table 2
Intrinsic kinetic parameters according to different values of transformation factor α introduced in Table 1

α	Optimal estimate	95% Confidence intervals	
		Lower value	Upper value
10.658 mK mmol^{-1} dm^3			
K_i (mmol dm^{-3})	42.042	26.209	57.875
K_m (mmol dm^{-3})	2.0436	1.6990	2.3882
10.797 mK mmol^{-1} dm^3			
K_i (mmol dm^{-3})	41.928	26.156	57.699
K_m (mmol dm^{-3})	2.0590	1.7112	2.4069

Kinetic parameter values were calculated by non-linear regression using the model, which fitted the experimental data with correlation coefficient 0.9940. The results from least-square estimation are summarized in Table 2.

5. Conclusion

The reported results demonstrate the possibility to use the flow calorimetry to investigate kinetics of enzymes immobilized in porous particles considering particle mass transfer. Compared to previous works, in this work a slight deviation from differential bed behavior was included into the mathematical model taking into the account non-negligible substrate conversion in the calorimeter column.

Acknowledgements

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