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Characterization of CIM monoliths as enzyme reactors

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Abstract

The immobilization of the enzymes citrate lyase, malate dehydrogenase, isocitrate dehydrogenase and lactate dehydrogenase to CIM monolithic supports was performed. The long-term stability, reproducibility, and linear response range of the immobilized enzyme reactors were investigated along with the determination of the kinetic behavior of the enzymes immobilized on the CIM monoliths. The Michaelis–Menten constant K_m and the turnover number k_3 of the immobilized enzymes were found to be flow-unaffected. Furthermore, the K_m values of the soluble and immobilized enzyme were found to be comparable. Both facts indicate the absence of a diffusional limitation in immobilized CIM enzyme reactors.

Keywords: Convective Interaction Media; Monoliths; Enzyme reactors; Organic acids

1. Introduction

Immobilized proteins are widely used in biocatalysis, bioprocessing, and affinity chromatography. The advantages of using immobilized enzymes instead of an enzyme solution include increased stability and an opportunity to work with a continuous system over a longer time. A wide range of supports have been utilized for immobilization among which particle-based supports are the most common. The type of the support used for immobilization is one of the key considerations in practical application due to different immobilization efficiency, ligand utilization and the mass transfer regime. The mass transfer between the mobile and the stationary phase has a pronounced effect on the enzyme kinetics or dynamic binding capacity. In the case of particulate porous supports, the substrate has to diffuse from the mobile phase into the pores in order to reach the catalytic sites of the immobilized enzyme. Since the diffusion is commonly slower than the kinetic process at the active site, the overall kinetic behavior of the immobilized enzyme in the packed bed column is governed by mass transfer.

In the last decade, several supports with improved hydrodynamic characteristics were introduced. Monolithic supports consist of a single monolithic unit containing flow-through pores [1]. In contrast with conventional supports, the only voids in a

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monolithic unit are the interconnected pores. Consequently, mass transfer is much faster due to the convective flow that becomes a dominant transport mechanism. This feature enables flow-unaffected separation efficiency and a dynamic binding capacity [2–4]. In conjunction with high mechanical and chemical stability [5], the methacrylate-based monoliths, commercialized under the trademark of CIM® (Convective Interaction Media), are prospective supports especially in the field of biomolecule separations. CIM supports were successfully used for the separations of proteins [6–10], DNA [11], and smaller molecules like oligonucleotides, peptides, steroids [12–14] and organic acids [4]. In these application areas, the chromatographic properties of the CIM supports were thoroughly examined. However, although there are several publications dealing with enzyme immobilization on CIM supports [7,15–19], no detailed investigation about kinetic properties has been performed.

In this work, CIM monolithic columns were used as a support for the immobilization of several enzymes: malate dehydrogenase (MDH), citrate lyase (CL), isocitrate dehydrogenase (ICDH) and lactate dehydrogenase (LDH). Reactions involving these enzymes are commonly used in analytical assays for determination of several organic acids in batch mode. All four enzymes have been studied immobilized on various supports as well. The characteristics of CL as a key enzyme for enzymatic analysis of citric acid, immobilized on controlled pore glass [20] and on a membrane [21] have been investigated. ICDH was used for determination of D-isocitric acid immobilized on controlled pore glass [20] and on Pt–Ir microelectrode [22]. Co-immobilized with the enzyme, isocitrate lyase was applied as an enzyme reactor in FIA determination of succinate [23]. Several studies of immobilized or co-immobilized LDH on different supports for the determination of lactate and pyruvate were reported [24–26]. Characteristics of the enzyme MDH, immobilized on controlled pore glass and co-immobilized on poly-(vinyl alcohol) have been studied [27,28].

In this article, the characteristics of the enzymes MDH, CL, ICDH and LDH immobilized on the CIM supports are described as well as the results of a mass transfer study examining the immobilized enzyme kinetics.

2. Experimental

2.1. Enzyme immobilization

The enzyme solutions were prepared as follows: 2.5 mg/ml of malate dehydrogenase from pig heart mitochondria (E.C. 1.1.1.37) and 3 mg/ml of L-lactate dehydrogenase from rabbit muscle (E.C. 1.1.1.27) (both from Roche, Mannheim, Germany) were prepared by diluting the enzyme suspension with deionised water; 3 mg/ml of citrate lyase from *Enterobacter aerogens* (E.C. 4.1.3.6) and 3 mg/ml of isocitrate dehydrogenase from pig heart (E.C. 1.1.1.42) (both from Sigma, St. Louis, MO, USA) were prepared by dissolving a lyophilized enzyme in 0.5 *M* phosphate buffer, pH 7.8 and 0.1 *M* phosphate buffer, pH 7.0, respectively. The support for the enzyme immobilization was a CIM epoxy disk (BIA Separations, Ljubljana, Slovenia) with an I.D. of 12 mm, a thickness of 3 mm and a volume of 0.34 ml. For each enzyme, two immobilization procedures were tested: (a) the CIM epoxy disk was incubated in the enzyme solution (described above) at 4 °C for 24 h; (b) the enzyme solution was pumped through the CIM epoxy disk which was then incubated in residual solution for 24 h at 4 °C. After the immobilization was completed, the enzyme-modified CIM disk was thoroughly washed with the working buffer at room temperature. For co-immobilization of CL and MDH, a solution of 1.5 mg/ml of each enzyme was prepared in a 0.5 *M* phosphate buffer, pH 7.8 and the immobilization procedure (b) was performed. The amount of the enzyme coupled to the CIM support was determined by the material balance (the amount of enzyme in the supernatant before and after the immobilization) measured from the absorbance at 280 nm using a Knauer UV–Vis spectrophotometric detector (Knauer, Berlin, Germany).

2.2. Enzyme activity determination

The activity measurements were performed on an FI analyzer (ASIA, Ismatec, Zurich, Switzerland), composed of two peristaltic pumps, a six-way switching valve and an injection valve with an 80-μl injection loop. Spectrophotometric detection was made with a Knauer UV–Vis spectrophotometric

detector (Knauer) connected to an integrator with data processing software.

The determination of the isocitrate dehydrogenase, ICDH [29], L-lactate dehydrogenase, LDH [30], malate dehydrogenase, MDH, and citrate lyase, CL [31], activity was based on the following reactions:

$$\text{D-isocitrate} + \text{NADP}^+ \xrightarrow{\text{ICDH}} \alpha\text{-ketoglutarate} + \text{CO}_2 + \text{NADPH} + \text{H}^+ \quad (1)$$

$$\text{pyruvate} + \text{NADH} + \text{H}^+ \xrightarrow{\text{LDH}} \text{L-lactate} + \text{NAD}^+ \quad (2)$$

$$\text{citrate} \xrightarrow{\text{CL}} \text{oxaloacetate} + \text{acetate} \quad (3)$$

$$\text{oxaloacetate} + \text{NADH} + \text{H}^+ \xrightarrow{\text{MDH}} \text{L-malate} + \text{NAD}^+ \quad (4)$$

According to the reaction scheme, the concentrations of the reacted D-isocitrate, pyruvate, citrate and oxaloacetate were determined by monitoring the level of NADPH formed or NADH reacted, at a wavelength of 340 nm. For citrate determination, the sequence of reactions (3) and (4) was performed.

The substrate solutions of citric acid, *threo*-DL-isocitric acid (both three-Na salt), pyruvic acid (Na-salt), and oxaloacetic acid (all from Sigma) in diluting buffer were injected into the working buffer stream. The reagent solution stream carrying the second substrate of the reaction ($NADP^+$ or NADH) joined the working buffer stream before entering the CIM enzyme reactor. For oxaloacetic acid determination 0.1 *M* phosphate buffer, pH 7.8 was used as diluting and as working buffer, the reagent solution was 260 mg/l β-NADH (Sigma). For citrate determination 0.1 *M* phosphate buffer pH 7.8 was used as diluting buffer to which 1 m*M* $MgCl_2$ (Sigma) was added to get working buffer, the reagent solution was 520 mg/l β-NADH. For isocitrate determination, 0.05 *M* phosphate buffer pH 6.5 was used as diluting buffer to which 5 m*M* $MgCl_2$ was added to get working buffer, the reagent solution was 1 m*M* β-$NADP^+$ (Sigma). For pyruvate determination, 0.1 *M* Tris, pH 7.6 was used as diluting and as working buffer and the reagent solution was 160 mg/l β-NADH. In the experiment testing the immobilized enzyme time stability, reproducibility and flow-rate dependence, the organic acid concentrations were chosen to be 20 mg/l of oxaloacetate and citrate, 10 mg/l of isocitrate and 5 mg/l of pyruvate.

2.3. Determination of immobilized enzyme kinetic parameters

The kinetics of the enzymes was determined by an on-line frontal analysis experiment [32]. For the experiment with the immobilized LDH, seven solutions of β-NADH in an excess concentration of 1 m*M* and different concentrations of pyruvate in 0.1 *M* Tris, pH 7.6 were made. Pyruvate concentrations were changed in the range from 0.057 to 0.909 m*M*. Each solution was pumped through the LDH CIM disk and the reaction time of the substrate in the enzyme reactor was changed by altering the mobile phase flow-rate in the range from 6.24 to 10.1 ml/min. The level of the reacted β-NADH was monitored at 380 nm. Prior to the experiments, the calibration curve of the relationship between the absorbance and β-NADH concentration was made giving a high correlation coefficient of $R^2 = 0.9946$. All solutions were thermostated at 25 °C.

The kinetic parameters of the immobilized enzyme, Michaelis–Menten constant K_m and turnover number k_3, were calculated according to Eq. (5). This equation is usually used in calculating the apparent kinetic parameters, under steady state conditions, in packed-bed reactors where a plug flow assumption is made [33]:

$$F\kappa S_0 - FK_m \ln(1-\kappa) = k_3 E\varepsilon \quad (5)$$

where F is the volumetric flow-rate, κ is the conversion of substrate to product, E is the number of moles of the immobilized enzyme, ε is the porosity of the support ($\varepsilon = 0.6$ for CIM) and S_0 is the inlet substrate concentration. The plug-flow assumption is justified for the CIM disk since the estimated Peclet number is around 120 [34].

For these experiments, a HPLC pump (Knauer) instead of a peristaltic pump was used. To ensure the accurate measurements of mobile phase flow-rate, a validated digital flow meter (K-3773, Phase Separation, UK) was applied.

The determination of the turnover numbers of the immobilized enzymes, ICDH and MDH, utilized the same procedure as for the immobilized LDH. The solutions of oxaloacetate and isocitrate were pre-

pared in a suitable buffer in a concentration range from 0.038 to 0.61 m*M* of oxaloacetate and from 0.014 to 0.984 m*M* of isocitrate.

2.4. Determination of kinetic parameters of soluble enzyme

K_m and k_3 for soluble LDH were calculated from the Lineweaver–Burk linearization of the Michaelis–Menten curve. The initial velocities of the enzyme reaction were measured at different pyruvate concentrations ranging from 0.046 to 3.665 m*M* and with NADH in excess concentration. A UV–Vis Beckman DU 640 (Fullerton, USA) spectrophotometer was used for the measurement.

3. Results and discussion

Various factors that affect the enzyme reactor performance have been described in detail [35]. In general, the absolute value of the signal obtained depends on the enzyme loading, effective activity of the immobilized enzyme and the hydrodynamic characteristics of the support. To achieve a high enzyme loading, two different immobilization techniques were tested and the results for the enzymes MDH, ICDH and LDH are summarized in Table 1. The coupling yield was much higher when the CIM disk was immobilized in a flow-through mode. The immobilization via incubation shows about four times lower values for LDH, three times for MDH and two times for ICDH. The ratio between the amount of the enzyme immobilized via flow-through mode and via incubation increases with the molecular mass of the enzymes which are about 140 kDa for LDH, 70 kDa for MDH and 60 kDa for ICDH. These data indicate that diffusion is a limiting process. Results are in accordance with capacity determination in batch mode [36].

3.1. Characteristics of the immobilized enzyme

High activity of the immobilized enzyme comprises good sensitivity and an adequate linear response. To determine the linear response of the CIM enzyme reactors, the calibration curves for all four enzyme systems were prepared. For the LDH CIM enzyme reactor, the linear response was in the range from 1 to 20 mg/l with a correlation coefficient of $R^2 = 0.9992$. Data fitted the equation: $y = 5.1343x + 0.7734$. For the other enzyme reactors, a linear relationship was obtained in a range from 2 to 30 mg/l for the ICDH CIM disk ($R^2 = 0.9965$; $y = 0.9565x + 1.0196$), from 5 to 60 mg/l for MDH CIM disk ($R^2 = 0.9994$; $y = 2.2598x - 0.458$) and from 5 to 50 mg/l for the CL MDH CIM disk ($R^2 = 0.9905$; $y = 1.8792x - 7.2792$). The lowest sensitivity was measured at the immobilized ICDH where the amount of NADPH formed was monitored, while the highest sensitivity was determined for the LDH CIM enzyme reactor.

Besides the wide linear response range, the essential characteristics of the enzyme reactor are long-term stability and reproducibility. The results of the long-term stability experiments for the enzymes MDH, LDH and ICDH immobilized on CIM supports are presented in Fig. 1. MDH CIM enzyme reactor and LDH CIM enzyme reactor show excellent time stability by exhibiting an unchanged response during the entire measuring period of over 100 days. On the other hand, the activity of the immobilized ICDH decreased in a few days to about half of the initial value and afterwards declined slowly to 10% of the starting value in 50 days.

A special example regarding long-term stability is immobilized enzyme CL. As already stated, enzymes

Table 1
Comparison of two immobilization procedures for enzymes MDH, LDH and ICDH. (A) Immobilization in flow-through mode; (B) immobilization via incubation

	MDH		ICDH		LDH	
	A	B	A	B	A	B
Coupling yield (%)	62.3	20.7	20.9	11.5	42.4	11.1
Amount of bound enzyme (mg)	1.58	0.52	0.63	0.34	1.27	0.33

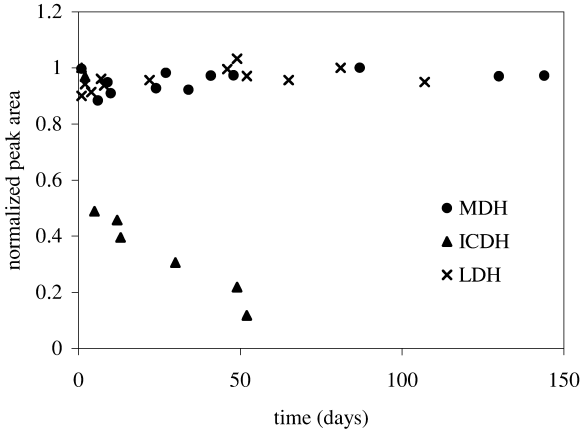

Fig. 1. Long-term stability of enzymes MDH, ICDH and LDH, immobilized on CIM support.

CL and MDH have to be applied for citrate determination where in the first reaction the oxaloacetate formed enters the second reaction catalyzed by MDH (see reactions (3) and (4)). Thus, enzymes CL and MDH were immobilized separately, each on one CIM disk, and the disks were then incorporated consecutively into the FI system, according to a CLC principle [7,37]. From Fig. 2, where the results of the time stability for this system are presented, it is evident that the response is maintained only for about five injections and starts to decrease steeply afterwards. Since immobilized MDH exhibits excellent time stability (Fig. 1), it was clear that the reason for this behavior was due to the poor stability

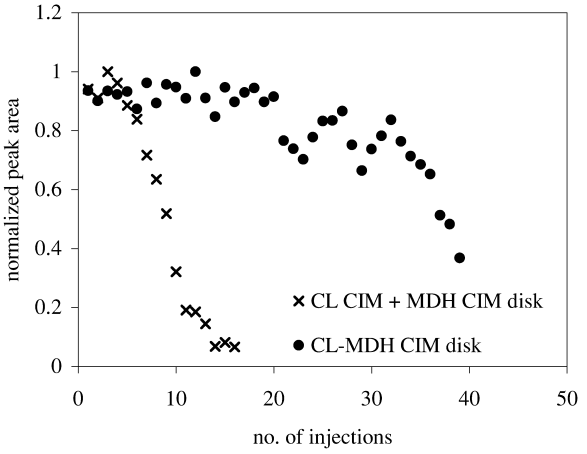

Fig. 2. Stability of the CL, immobilized on CIM support.

of the CL enzyme. This fact is well known from the literature. Marked loss of CL activity practically with repeated injections of citric acid is a bottle-neck in developing FIA enzymatic methods with immobilized CL for citric acid determination. The problem was mainly omitted by applying a CL solution instead of immobilized enzyme [31,38,39], however, a few attempts at improving the stability of the CL enzyme reactor were presented as well [20,21].

Two reasons for poor stability of immobilized CL are stated in the literature. Firstly, it has been reported that the CL enzyme is inhibited by an oxaloacetate–Mg^{2+}-complex [40,41]. For its catalytic activity, CL needs Mg ions as enzyme activators [41,42] which are at the same time inhibitory in the complex with the product of the reaction. Matsumoto and Tsukatani [20] tried to solve this problem by the co-immobilization of two enzymes in order to rapidly convert oxaloacetate, produced in the first reaction, to further non-inhibitory products. Applying this idea, we co-immobilized CL and MDH on the same CIM disk. As a result, the time stability of the CL MDH CIM enzyme reactor was prolonged to about 20 injections, but afterwards the enzyme activity decreased rapidly again (Fig. 2). Despite this partial success, the deactivation of CL seems to be also subject to a second possible cause. Namely, the CL enzyme undergoes inactivation during the course of the reaction which it catalyses, which is an uncommon behavior in the world of biocatalysts [42,43]. In vivo, the subsequent activation takes place with the help of another enzyme, CL ligase [44,45]. This stabilization was, however, not tested.

Finally, reproducibility of the immobilized enzyme reactors was examined by performing 10 consecutive injections of a specific organic acid solution into the corresponding enzyme system. Highly reproducible results were obtained, showing the RSD values of 1.2% for the immobilized MDH, 3.0% for the MDH CL CIM enzyme reactor, 0.88% for the ICDH CIM disk and 3.3% for the LDH CIM disk.

3.2. Kinetic characteristics of the immobilized enzyme

To investigate how the hydrodynamic features of

the CIM material influence the characteristics of the immobilized enzyme, the kinetic constants of immobilized LDH, Michaelis–Menten constant K_m and turnover number k_3, were calculated for different flow-rates. Obtaining these data one can evaluate to what extent the enzyme process is subject to mass transfer limitations. As already stated, the diffusional limitations in monoliths proved to be negligible for different separation modes, however, the immobilized enzyme active site can be oriented in a way that access is restricted due to the random orientation. This might result to some extent as diffusional limitation.

The overall reaction rate for the LDH CIM disk was measured dependent on the inlet pyruvate concentration and liquid flow-rate. The results are presented as Michaelis–Menten curves in Fig. 3. To calculate the values of K_m and k_3, Eq. (5) was used. The relationship κS_0 vs. ln $(1-\kappa)$ at different liquid flow-rates is presented in Fig. 4. The linear plots of the data were found to be in parallel which reflects in flow-unaffected K_m values, calculated from their slopes. The flow-rate dependence of the calculated Michaelis–Menten constant K_m and the turnover number k_3 is presented in Fig. 5. It is evident that the kinetic parameters for the LDH immobilized on the CIM support are independent of the flow-rate leading to the conclusion that the mass transfer of the substrate in immobilized CIM enzyme reactor is not diffusional limited. The average values of the Michaelis–Menten constant and turnover number for

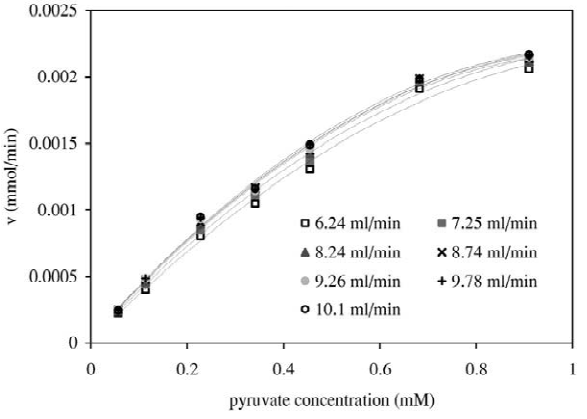

Fig. 3. Michaelis–Menten curves for LDH CIM enzyme reactor at different flow-rates.

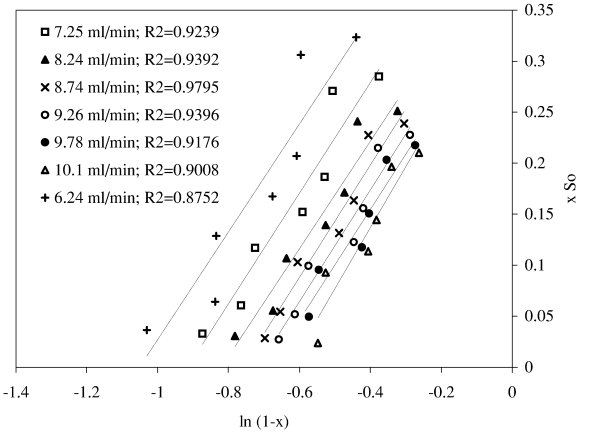

Fig. 4. Relationship between κS_0 and ln $(1-\kappa)$ for the conversion of pyruvate by LDH CIM enzyme reactor.

LDH, immobilized on the CIM support, are $K_m = 0.54$ mM and $k_3 = 53.94\ s^{-1}$.

The obtained results confirm the already described hydrodynamic features of CIM supports, studied when they have been used as separation units, where flow unaffected dynamic binding capacity and separation quality of different sized molecules have been reported [3,4,37,46]. Because of that, a higher flow-rate results in higher conversion rates and shorter reaction times. Thus, the productivity in the CIM enzyme system is limited by the enzyme kinetics itself.

The influence of the enzyme's catalytic features on the enzyme reactor performance can be seen in Fig. 6 where the effect of the flow-rate on the response of

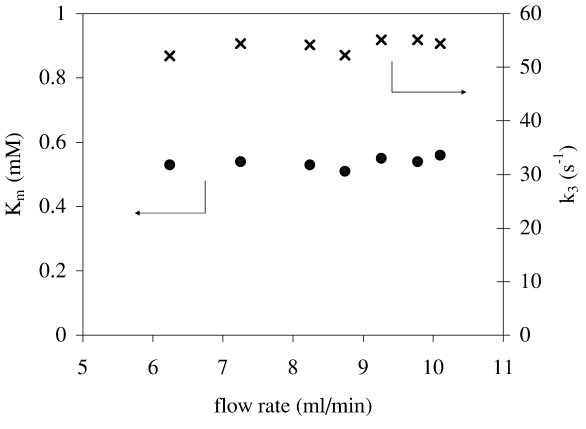

Fig. 5. Kinetic constants K_m and k_3 in dependence on flow-rate.

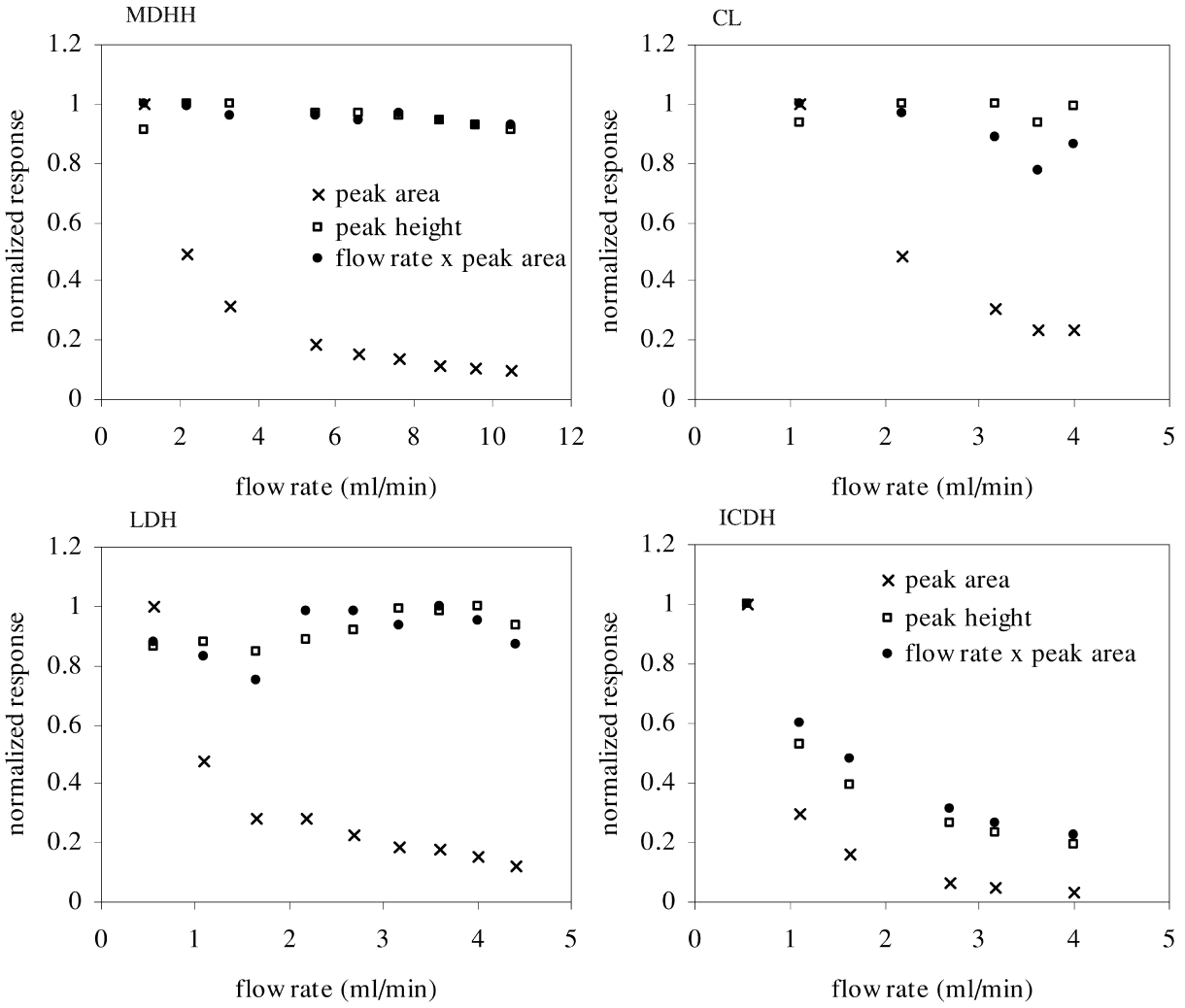

Fig. 6. Response of MDH, CL-MDH, ICDH and LDH CIM enzyme reactors in the dependence on flow-rate.

the enzyme system for immobilized MDH, CL, ICDH and LDH is presented. In this experiment, the organic acid solution was injected into the FIA system at different flow-rates and the response was measured and presented in three ways: as peak area, as peak height and as the product of peak area and the flow-rate at which the reaction was performed. As expected, the peak area in all of the enzyme systems decreases with flow-rate because of the monitoring system. Thus, for actual comparison of the rate of organic acid conversion at different flow-rates, each peak area has to be multiplied by the corresponding flow-rate. The product of the peak area and corresponding flow-rate was found to be flow-unaffected for MDH, CL-MDH, and LDH enzyme reactors in the entire flow-rate measuring range. The same was observed for peak height. On the other hand, the results for the ICDH enzyme reactor show that the peak height and the product of peak area and flow-rate decrease with flow-rate through the enzyme reactor (Fig. 6). This behavior of the immobilized ICDH can be explained by its low turnover number which was measured to be 0.17 s^{-1}. The measured turnover numbers of immobilized

MDH ($k_3 = 53.9\ s^{-1}$) and LDH ($k_3 = 25.3\ s^{-1}$) were more than 100 times higher indicating that the decreasing response at the higher flow-rates for ICDH CIM enzyme reactor is a consequence of the kinetic features of the immobilized ICDH.

To evaluate the extent that the immobilization procedure affects the enzyme activity, the kinetic constants for immobilized and soluble LDH were compared. As already mentioned, the Michaelis–Menten constant and turnover number of immobilized LDH were measured to be $K_m = 0.54$ m*M* and $k_3 = 53.9\ s^{-1}$ while the kinetic values of the soluble LDH, measured under the same conditions, are $K_m = 0.44$ m*M* and $k_3 = 2500\ s^{-1}$. It can be seen that the K_m values of the immobilized and free enzyme are comparable, again indicating the absence of diffusional limitation. On the other hand, the turnover number for the immobilized LDH is about 50 times lower which indicates a decreased rate of conversion of the enzyme–substrate complex into product. From this comparison, it is evident that the immobilization procedure decreases the activity of the enzyme. This behavior is well-known from the literature and is due to the conformational and steric effects that are invoked by random immobilization, the chemical and physical stress during the immobilization procedure as well as due to the micro-environmental effects on the active site and the mass transfer effects [15,35,47]. Since the mass transfer effects in CIM material can be neglected, the reason for the decreased activity of the immobilized LDH can probably be assigned to the conformational influences and random immobilization.

4. Conclusions

In general, CIM enzyme reactors exhibit good reproducibility, a wide range of linear responses, and long-term stability. The flow-unaffected kinetic parameters of the enzymes immobilized on CIM supports confirm that mass transfer in CIM monoliths is not diffusional limited enabling the application of higher flow-rates which result in higher conversion rates and shorter reaction times. All of these features make CIM monoliths competitive supports for enzyme immobilization and application as enzyme reactors in analytical systems. Furthermore, due to their good hydrodynamic characteristics, CIM supports can represent a useful tool for the kinetic studies of immobilized enzymes.

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