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Continuous-flow step gradient mass spectrometry based method for the determination of kinetic parameters of immobilized mushroom tyrosinase in equilibrating conditions: comparison with free enzyme

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A mass spectrometry (MS)-based methodology for enzymatic assay in equilibrium conditions was designed and evaluated. This on-line assay involves the introduction of a continuous-flow step gradient (CFSG) of a substrate solution in the column containing immobilized enzyme and the simultaneous tracking of the product formation. We showed that the constant concentration of substrate in the entire bioreactor for an appropriate duration ensures the equilibration of the studied enzyme (mushroom tyrosinase). Under these conditions, it was demonstrated also that the kinetic and enzymatic parameters (Michaelis-Menten constant, K_M, the maximal specific activity, SA_{max}) are independent of the flow rate of the mobile phase. The feasibility of the mentioned approach for inhibitory tests was also investigated. The coupling of the mass spectrometer to the bio-reactor allows the selective monitoring of the enzymatic reaction products and increases their detection level. Very high sensitivity, 500 pmol/min/column, and selective monitoring of the products of the enzymatic reaction are allowed by MS detection. The methodology developed here constitutes a sensitive analytical tool to study enzymes requiring long equilibration times. Copyright © 2011 John Wiley & Sons, Ltd.

Immobilized enzymes are widely used on industrial scale[1] and on a laboratory scale, including analytical chemistry,[2,3] diagnostics,[4] drug screening,[5,6] etc. In many cases immobilized enzymes are used to determine enzymatic and kinetic parameters: maximal enzymatic velocity, V_{max}, Michaelis-Menten constants, K_M, for novel substrates or for the evaluation of the inhibitory properties of leading drug molecules.[7,8] The on-line kinetic assays of immobilized enzymes are normally conducted by injection of a known amount of substrate into an immobilized enzyme reactor (IMER) followed with detection of the product formed within the IMER.[7–11] In the single injection mode, both apparent K_M and V_{max} values depend drastically on the flow rate of the mobile phase[7–10]; in some cases, no specified trend of this relationship can be determined, even for the same enzyme.[9,10] A decrease in the flow rate leads to an increase in the contact time between enzyme and substrate. For very low flow rates the full conversion of given amount of substrate can be achieved that consequently leads to formation of plateau of the relation between product amount and flow rate.[11,12] Working within this range of flow rates makes it impossible to apply the Michaelis-Menten model (MM) to calculate kinetic parameters; thus the pre-optimization of the single injection assay is required. In the event of the use of long IMERs for single-injection assay (e.g. capillaries), enzymatic conversion of each single substrate portion gradually lowers its local concentration during its migration towards the outlet of the IMER. This affects the apparent V value for each substrate injection and causes deviation from the MM model. Another phenomenon that may influence the results of a kinetic assay in single injection mode occurs when the studied enzyme shows a delay ('lag phase') in substrate conversion.[13–15] To circumvent these limitations, in this paper we propose a continuous-flow step gradient method (CFSG) for on-line kinetic tests of immobilized enzymes using mass spectrometry (MS) for selective detection of the product formation. The presented method is based on stepwise increase of the substrate concentration in continuous-flow mode and ensures full equilibration of the bioreactor compared to single injection experiments. The CFSG approach that is described here for the first time combined a continuous flow bioreactor,[16] and a frontal analysis of ligand–protein binding methodology.[17,18] The mushroom tyrosinase, used as a model enzyme (EC 1.14.18.1), catalyzes the oxygen-based oxidation of monophenols to *o*-diphenols and their subsequent oxidation to *o*-quinones.[19] Thus, we demonstrate that the apparent V_{max} measured by the CFSG method is independent of the flow rate, show applicability of the CFSG method to inhibition studies and compare obtained values to free enzyme. In addition, we show a temperature dependence on the kinetic parameters of mushroom tyrosinase. The feasibility of this approach for inhibition studies was presented using kojic acid as model tyrosinase inhibitor.[20] Thanks to the application

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of MS it became possible to monitor all products of the tyrosinase activity and achieve an outstanding 500 pmol/min/column level of quantitation.

EXPERIMENTAL

Instrumentation

MS experiments were performed on Bruker maXis UHR-Qq-TOF spectrometer (Bremen, Germany) with a Bruker sprayer in positive electrospray ionisation (ESI) mode, working in single-mass mode. The mass spectrometer was coupled in on-line mode with a Dionex UltiMate 3000 ultra-high-performance liquid chromatography (UHPLC) system (binary pump, standard thermostated column compartment and DAD detector) (Germering, Germany). For all experiments total ion chromatograms were recorded within the mass range of 110 to 400. Profiles of ions of interest (L-Tyr, *m/z* 182.081 and M + 2 isotopic peak of L-Tyr *m/z* 184.084; L-DOPA, 198.079 and dopachrome 194.047) were generated as 'extracted ion chromatograms' within the ±0.01 *m/z* range. Subsequently, average mass spectra were generated for the steady-state for each step gradient and used to obtain final values of ion responses.

Chemicals

Mushroom tyrosinase, L-tyrosine (>99%), (3-aminopropyl) triethoxysilane (APTES) (97%), glutaraldehyde (GLA) (25% in water) and kojic acid (KA) were purchased from Sigma Aldrich (Isle-d'Abeau, France), ammonia (28% solution in water) from Alfa-Aesar (Karlsruhe, Germany), formic acid (97%) and sodium hydroxide were obtained from Fluka (Isle-d'Abeau, France) and hydrogen peroxide (30%) from Fisher Scientific (Elancourt, France). Silica beads (Geduran Si 60, 40–63 μm) were purchased from Merck (Darmstadt, Germany). All experiments were conducted in ammonium formate buffer (11 mM, pH 6.8, verified before each experiment).

Preparation of the bioreactor

Non-porous silica beads (3 g) were purified and activated by incubation in a mixture of a 30% aqueous solution of NaOH/30% H_2O_2/H_2O: 1/1/4, v/v/v (48 mL) for 15 min. Then, silica beads were washed with distilled water and then incubated for 80 min at 80 °C in 48 mL of the following solution: 37% HCl/30% H_2O_2/H_2O: 1/1/4, v/v/v. Silica beads were then washed with distilled water and dried under vacuum.

To an amount of 2 g of activated silica beads 10 mL of toluene and 0.5 mL of APTES were added. The mixture was agitated under solvent reflux at 50 °C for 13 h. Silica beads were then washed with distilled water and kept in an oven at 100 °C for 1 h to promote the complete condensation reaction with APTES. To the resulting silica beads coated with APTES, 30 mL of glutaraldehyde solution (1.67 mL of aqueous solution of GLA (25%) per 1 mg of derivatised silica) prepared in sodium phosphate buffer (0.2 M, pH 7.5) was added. The resulting mixture was allowed to react at room temperature for 2 h. Silica-beads$_{APTES/GLA}$ were then dried under vacuum.

A volume of 1 mL of a solution of mushroom tyrosinase (0.91 mg/mL) was added to a flask containing 0.121 g of dry silica-beads$_{APTES/GLA}$. The mixture was allowed to react at 4 °C for 7 h. After completion of the reaction, the mixture was centrifuged, the supernatant was collected for assay purposes and the beads were packed into an empty HPLC column (30 mm length, 4.6 mm diameter). The empty space was filled with sand. The grafting yield was 90.2%.

Kinetic studies of the free enzyme

All kinetic tests were performed at room temperature (25 °C). Both buffers and samples were thermostated in the water bath. In addition, the measuring cell was also temperature controlled. Kinetic tests were based on several the 'time course' measurements of L-dopaquinone formation (at 475 nm, ε_{475nm} = 3716 L·mol/cm), produced during the enzymatic reaction.[21] The assay was conducted in a 1 mL quartz cuvette filled with tyrosinase solution (corresponding to 2.73 μg of enzyme) in 10 mM sodium phosphate buffer, pH 6.8. The reaction was started by addition of L-tyrosine solution in the same buffer (to obtain a final concentration between 70 and 600 μM). For the inhibition assay, the three following concentrations of kojic acid were used: 0, 11.57 and 21.21 μM. Values of specific activity (SA) for each L-Tyr concentration (Eqn. (1)) were fitted to the Michaelis-Menten (MM) model to obtain the kinetic parameters. Contrary to enzymatic velocity (V_{enz}), specific activity (SA) is an independent parameter describing enzymatic activity per unit of enzyme quantity; therefore, it will be used later on in this work.

$$SA\left[\mu mol/min/mg\right] = \frac{\Delta Abs_{475nm}}{\Delta t \times \varepsilon_{475nm} \times C_{enz}} \times D \times 10^3 \quad (1)$$

where SA is specific activity; C_{enz} is the concentration of mushroom tyrosinase in the initial stock solution, mg/mL; ε is the absorption coefficient of dopaquinone at 475 nm, L/mol/cm; $\Delta Abs_{475nm}/\Delta t$ is the slope of the linear part of the time course curve, s^{-1}; D is the dilution factor of the enzyme stock solution.

Kinetic studies of immobilized enzyme by the CFSG method

The CFSG experiment requires a binary pump LC system to form a step gradient of two mobile phases: A contains the buffer solution and B: the substrate diluted into the buffer solution; this mobile phase is called the 'substrate phase'. By changing the ratio of A to B one may modify the effective substrate concentration in the bioreactor (Fig. 1).

Enzymatic specific activity (SA) is described as the amount of product generated per minute and per defined amount of enzyme (μmol/min/mg). If we consider the bioreactor as the place of residence of the constant mass of enzyme (mg), we can write:

$$SA\left[\mu mol/min/mg\right] = \frac{n_{pr}}{\tau \times m} \quad (2)$$

where τ is the time of contact between a substrate of a given concentration and enzyme (min); n_{pr} is the amount (μmol) of product generated within the time τ; m is the the total weight of immobilized enzyme in the bioreactor (mg).

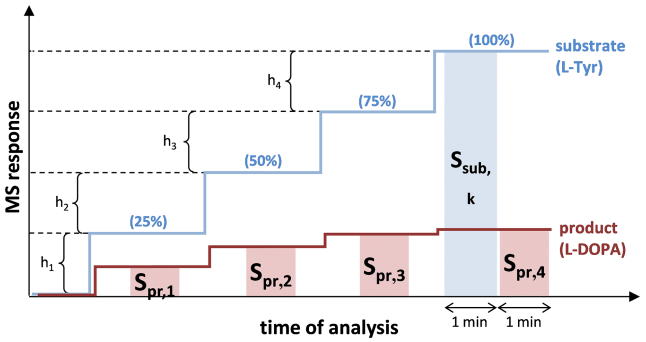

Figure 1. Schematic outlook of the methodology of continuous flow step gradient for analysis of enzymatic activity. Values in round brackets represent the contribution of the 'substrate phase' in the mobile phase. $S_{pr,i}/S_{sub,k}$ is the surface below the product/substrate profile for the time equivalent of 1 min; h_i is the growth of L-Tyr ion response between successive levels of step gradient.

In respect to the continuous-flow method, in which the product is continuously drained from the bioreactor with a given flow rate Q (mL/min), an average time of the residence of non-retained substrate in the bioreactor is given by the void volume of bioreactor (mL) divided by Q (Eqn. (3)):

$$\tau[\text{min}] = \frac{V_0}{Q} \tag{3}$$

Thus, the apparent enzyme activity may be described as:

$$\text{SA}[\mu\text{mol/min/mg}] = \frac{n_{pr} \times Q}{m \times V_0} = \frac{C_{pr} \times Q}{m} \tag{4}$$

where V_0 is the void volume of the bioreactor (mL), C_{pr} is the product concentration (µmol/mL) in the system after passing through the bioreactor.

C_{pr} is correlated to the amount of product of the enzymatic reaction (n_{pr}) found in volume equal to the void volume of the reactor (V_0) and generated within the time needed for V_0 to pass through the bioreactor.

The concentration of product for each of step substrate gradient, $C_{pr,i_{1\to4}}$, is directly proportional to the surface area under the product profile, $S_{pr,i_{1\to4}}$ (Eqn. (5), Figs. 1 and 2):

$$C_{pr,i_{1\to4}}[\mu\text{mol/mL}] = \beta_{pr} \times S_{pr,i_{1\to4}} \tag{5}$$

where index 'i' represents a given step of gradient; β is the response coefficient, which depends on the flow rate and settings of the mass spectrometer

From Eqns. (4) and (5), we obtain the following relationship describing the apparent enzyme activity for each concentration of substrate (Eqn. (6)):

$$\text{SA}_{i_{1\to4}}[\mu\text{mol/min/mg}] = \frac{\beta_{pr} \times S_{pr,i_{1\to4}} \times Q}{m} \tag{6}$$

The β coefficient depends highly on the current settings of the mass spectrometer such as ion transmission parameters, flow rate of drying gas, settings of the nebulizer, etc., and shows a linear relationship with the flow rate of the mobile phase. Therefore, an internal standard is required for the comparison of the subsequent analyses. If we introduce the ratio of product to substrate response coefficients, $\alpha_{pr/sub}$ (Eqn. (7)):

$$\alpha_{\frac{pr}{sub}} = \frac{\beta_{pr}}{\beta_{sub}} \tag{7}$$

and apply Eqn. (5) for the substrate, then insert it and Eqn. (7) into Eqn. (6), we obtain the final relationship describing the enzymatic activity in the continuous flow mode (Eqn. (8)):

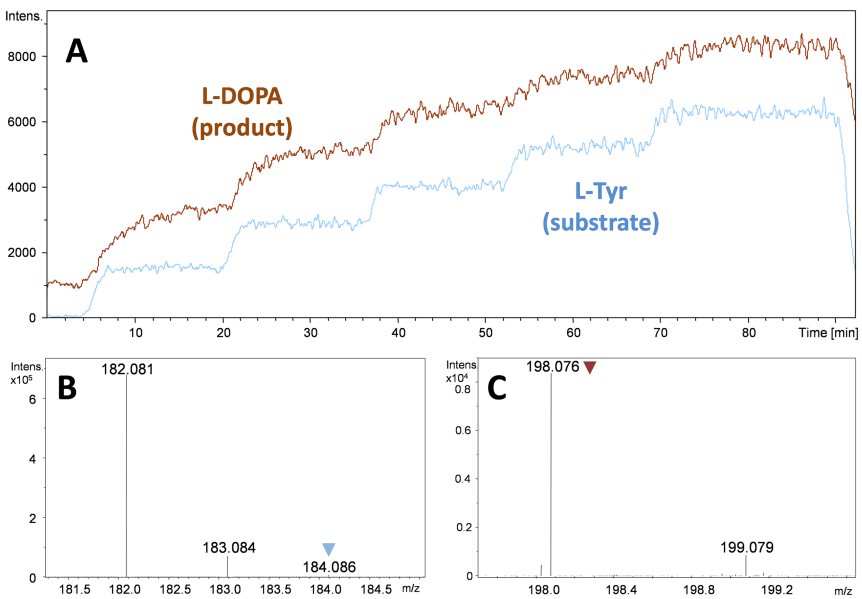

Figure 2. Practical profile of the substrate (L-Tyr) step gradient for exemplary assay. (A) The profile of ion intensities of L-Tyr (blue line, *m/z* 184.086, M + 2 isotopic peak) and L-DOPA (brown line, *m/z* 198.076, monoisotoic peak); the M + 2 isotopic ion is used for L-Tyr because the intensity of the monoisotopic ion of L-Tyr is too high to fit onto the same scale as L-DOPA. Mass spectra of (B) L-Tyr and (C) L-DOPA.

$$SA_{i_{1\to4}}\left[\mu mol/min/mg\right] = \frac{\alpha_{\frac{pr}{sub}} \times C_{sub_k} \times S_{pr,i_{1\to4}} \times Q}{S_{sub_k} \times m} \quad (8)$$

where the index '*k*' represents the reference level of the substrate gradient.

The response ratio (Eqn. (7)) may be determined experimentally on the basis of Eqn. (5) (for product and, analogically, for substrate) for mixtures of L-Tyr and L-DOPA measured together in the same buffer as applied during experiments with the bioreactor. The substrate can be considered as an internal standard only if its transformation during the enzymatic reaction is negligibly low (approximately 1–3%).

Experimental data were fitted to the MM model to obtain kinetic parameters and displayed as Lineweaver-Burk plots. The L-Tyr ion was monitored in the extracted ion chromatogram (*m/z* 182.081, $[M + H]^+$, Fig. 2(B)) and L-DOPA as 198.079, $[M + H]^+$, Fig. 2(C) (see next section). MS parameters were optimized to minimize formation of adducts of L-tyrosine and L-DOPA and fragmentation of these compounds.

RESULTS AND DISCUSSION

Practical description of the methodology

The experimental profile obtained from CFSG experiments (Fig. 2(A)) is very similar to the theoretical profile (Fig. 1). Differences between these profiles give information on enzyme behaviour. Indeed if the observed slope for the substrate profile can be attributed to apparatus dead volume, the long equilibration time observed on the product is the outcome of the complex enzyme cycle. The cycle of tyrosinase activity includes regeneration of enzyme from the most reduced (E_{deoxy}) to fully oxidized form (E_{oxy}). This comprises other products like dopaquinone or dopachrome.[13,14] Both dopaquinone (*m/z* 196.063, $[M + H]^+$) and dopachrome (*m/z* 194.047, $[M + H]^+$) ions were observed during the experiments but at negligible levels, approximately 9.5% of the total ion response for L-DOPA, within the range of 4–12% for the lowest step of gradient for dopachrome, and less than 1% for dopaquinone. Such a conversion of L-DOPA increases K_M and lowers observed SA_{max} values and is in all cases smaller than 10%. One should note that, in the same way, transformation of dopaquinone (into leukodopachrome and dopachrome) occurs also during assays of tyrosinase activity, both immobilized and in solution and is never taken into account during the data analysis.[8,9,15,19,20,24] This phenomenon is absent for enzymes catalysing one defined reaction. Full equilibration of the enzymatic system is only achievable by the CFSG approach. This shows the utility of the proposed methodology compared to the conventional single injection method.[7–10]

In the present work, L-Tyr was used as internal standard. Consumption of L-Tyr is on average at a level of 1.7% (0.3–3.3%). This means that the relationship between the substrate ion intensity (M + 2 isotopic peak of L-Tyr: *m/z* 184.084, $[M + 2 + H]^+$) and its concentration both before and after passing through the bioreactor is linear ($h_1 = h_2 = h_3 = h_4$, Figs. 1 and 2(A)). This linearity is also maintained for the second isotope of L-Tyr (its response is comparable to standard product ion response). In addition, the use of L-Tyr as internal standard for L-DOPA is justified by the structural similarity of these compounds.[22,23] Due to linearity of the relationship of L-Tyr ion response to L-Tyr concentration, selection of the reference 'level' of the step gradient (Eqn. (8)) is fully unrestricted, and in the rest of this work will be called '*k*'.

The response ratio of L-DOPA/L-Tyr (Eqn. (7)) was found to be 1.16 ± 0.07 (n = 5; measured for different ratios of L-DOPA/L-Tyr concentrations ranging from 0.3 to 2.3).

Influence of the flow rate on the kinetic parameters of immobilized enzyme

Subsequent experiments for three flow rates at 25 °C (Fig. 3 and Table 1) showed lack of significant dependence of the maximal specific activity (SA_{max}) and K_M on the flow rate (*Q*) (Student's t-test, $\alpha = 0.05$). Moreover, the error was homogeneous (according to Cochran's test). The non-significant variations obtained for these parameters are much smaller than the ones observed in the literature. For example, for tyrosinase: deviations of K_M and V_{max} are found up to 70%, 30% respectively for $\Delta Q = 0.1$ mL/min and for acetylcholinesterase: K_M and V_{max} are up to 21%, 27% respectively for $\Delta Q = 0.2$ mL/min.[7–10] The lack of influence of the flow rate on enzymatic parameters for the CFSG method is the result of reaching an equilibrium state

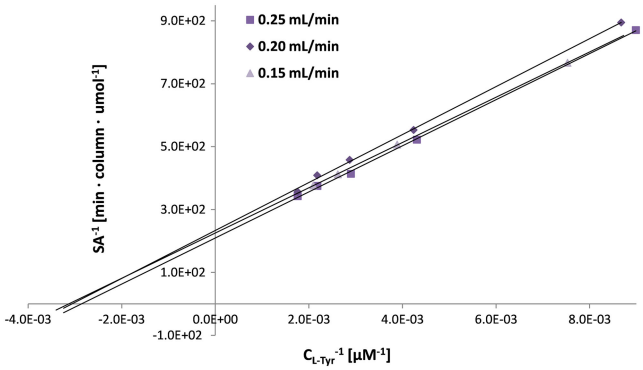

Figure 3. Influence of the flow rate on the Lineweaver-Burk plots representing experiments at 25 °C.

Table 1. Kinetic parameters of immobilized tyrosinase for various flow rates. For each flow rate one series consisting of four or five points (indicated in the table) was recorded.

Flow rate [mL/min]	SA_{max}[a] [nmol/min/column]	K_M[a] [µM]
0.25 (n = 5)	4.60 ± 0.13	324 ± 19
0.2 (n = 5)	4.43 ± 0.24	350 ± 39
0.15 (n = 4)	4.46 ± 0.06	323 ± 9
collective (n = 14)	4.49 ± 0.21	331 ± 32

[a]Non-significant difference between all values of a row checked by Student's t-test ($\alpha = 0.05$). Deviations of SA_{max} and K_M are homogenous according to Cochran's test ($\alpha = 0.05$).

and keeping the substrate concentration constant within the bioreactor. For all flow rates, the apparent substrate concentration for a given step in gradient is the same and is not subjected to dilution or diffusion as for the single-injection approach. An average K_M constant for immobilized enzyme (331 ± 32 μM) is higher than for the free enzyme (125.8 ± 11.9 μM) which is consistent with the literature.[24–26] In addition this results from the steric constraints caused by covalent attachment of the enzyme to the support. Day-to-day repeatability of K_M and SA_{max} is satisfactory and the standard deviation does not show any statistical significance (Student's t-test, α = 0.05). For example, an experiment with two values of flow rate, 0.20 and 0.15 mL/min (both n = 5 points per run) conducted one day before the experiments summarized in Table 1, yielded collective K_M and SA_{max} 366 ± 46 μM and 4.38 ± 0.28 nmol/min/column, respectively. RSD% values for K_M and SA_{max} are 7% and 1%, respectively; however, we observed a gradual drop in enzyme activity, especially after aggravating tests at higher temperatures.

To confirm these results, a single switch of 'substrate phase' was conducted as follows: L-Tyr, ×μM (%): 0 (0) – 557 (100) – 0 (0) for five various flow rates. The observed growth of product/substrate amount ratio upon the decrease in flow rate is caused by longer contact time between substrate and enzyme. According to expectations, due to correction by the flow rate, SA_{app} (calculated by Eqn. (8) for one, fixed concentration of L-Tyr for all values of flow rate) remains unchanged despite the growth in relative product amount (Fig. 4). When the speed of product drainage from the IMER is the same as or higher than the conversion rate, product/substrate amount ratio is expected to reach a plateau, because the product dilution phenomenon is eliminated after the solvent evaporation in the ion source of the mass spectrometer.

Influence of the temperature on the kinetic parameters of immobilized enzyme

The relation between SA_{max} and temperature of enzymatic reaction is fully compliant with Arrhenius' equation (Fig. 5). The SA_{max} value may be used to plot the Arrhenius relationship, because the total amount of enzyme is unchanged during subsequent experiments. The activation energy of L-DOPA formation by immobilized tyrosinase calculated on the basis of the Arrhenius equation is 110.4 kJ/mol.

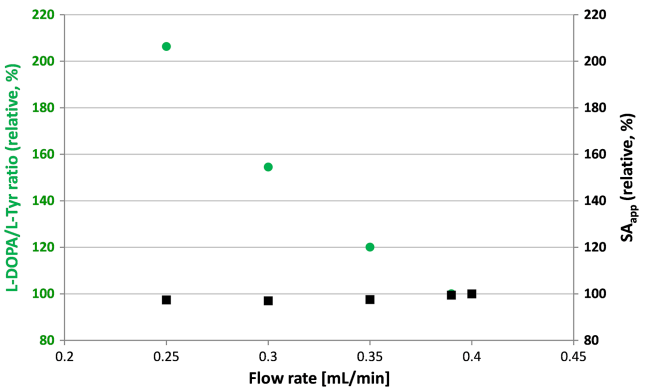

Figure 4. Comparison of L-DOPA/L-Tyr (product/substrate ratio (dots, ●) and apparent SA (squares, ■, calculated on the basis of Eqn. (8)) for fixed L-Tyr concentration.

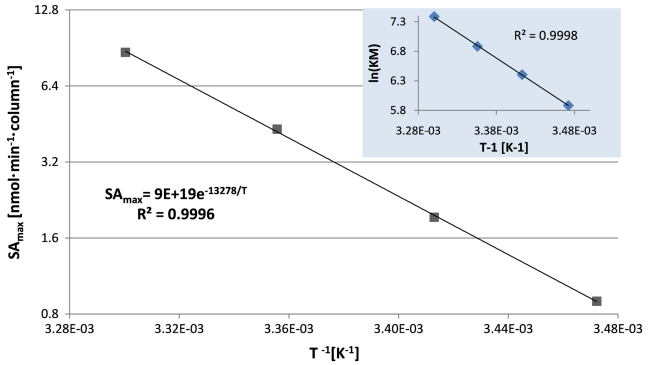

Figure 5. Arrhenius relation $SA_{max}(T^{-1})$, logarithmic scale was applied. Inset: relationship between K_M and reversed temperature.

The linear relationship of $\ln(K_M)$ versus inversed temperature (Fig. 5, inset) is consistent with the van't Hoff equation and shows that the Michaelis-Menten constant may be attributed to dissociation constant of the L-Tyr from the enzyme-substrate complex.[27]

Inhibitory studies of tyrosinase by the CFSG method

Kojic acid (KA) was selected as a model inhibitor of tyrosinase. It was shown to be a competitive inhibitor of monophenolase activity of mushroom tyrosinase towards L-Tyr.[20] For CFSG experiments, the concentration of inhibitor was maintained constant in the bioreactor during entire gradient duration. In the case of the free enzyme assay, the inhibition constant (K_I) was found to be 3.56 ± 0.44 μM, while for the immobilized enzyme it was 3.10 ± 0.05 μM (Lineweaver-Burk plots are shown in Fig. 6).

CONCLUSIONS

The continuous-flow step gradient method for studying enzyme activity in on-line mode with MS detection is a very useful technique for bioreactors showing very low activity (e.g. 500 pmol/min/bioreactor, which is obtained for the highest concentration of KA recorded for this study). This is

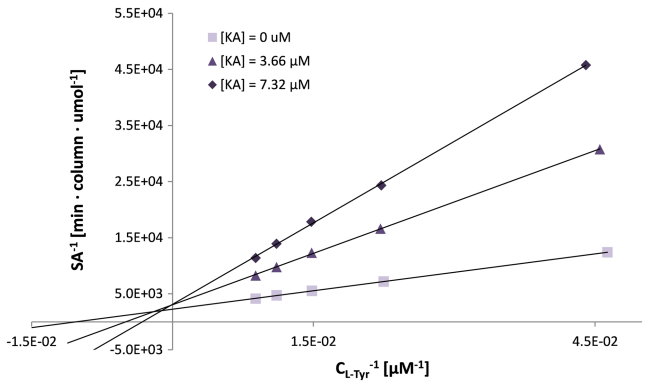

Figure 6. Lineweaver-Burk plots representing inhibitory properties of kojic acid towards immobilized tyrosinase.

especially valuable for screening purposes, where in many cases human enzymes have to be applied to avoid false positive or false negative results during the use of their animal or bacterial equivalents. Amounts of human-derived enzymes are usually very limited thus rendering the need to apply extremely sensitive analytical methods. The CFSG method eliminates the influence of flow rate on the kinetic parameters (activity, K_M) and enables on-line inhibition assays. Simultaneously, application of a mass spectrometer as the detector enables the specific and sensitive monitoring of enzymatic products, which is often not possible in the case of UV-VIS spectroscopy.

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