Stabilization of D-Amino Acid Oxidase via Covalent Immobilization and Mathematical Model of D-Methionine Oxidative Deamination Catalyzed by Immobilized Enzyme

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doi: 10.15255/CABEQ.2015.2189

Original scientific paper
Received: February 8, 2015
Accepted: February 26, 2016

Porcine kidney D-amino acid oxidase was stabilized by covalent immobilization on spherical particles of Eupergit C because of its low stability in soluble form. The focus of this work was to evaluate operational stability of the immobilized enzyme. To evaluate D-amino acid oxidase's operational stability during process conditions, repetitive batch reactor experiments of D-methionine oxidation reaction were carried out with continuous aeration for oxygen supply at air-flow rates of 5 and 10 $dm^3\ h^{-1}$. Kinetic analysis of the immobilized enzyme was done as well. The mathematical model of D-methionine oxidative deamination catalyzed by the immobilized D-amino acid oxidase was developed and it described the data well. It enabled the estimation of operational stability decay rate constant. It was possible to achieve 100 % substrate conversion in all batch experiments.

Key words:
amino acid oxidase, enzyme immobilization, enzyme kinetics, mathematical model

Introduction

D-Amino acid oxidases (DAAOs) have an important role in several processes of interest in the fine chemical and pharmaceutical industry for drug discovery. They are used in the production of optically pure L-amino acids[1] and unnatural amino acids[2], and the production of 7-aminocephalosporanic acid (7-ACA)[2,3] which is a precursor of antibiotics with annual demand of approximately 100 t worldwide[3]. Therefore, DAAO stability is an important property for its industrial application.

Potential industrial use of enzymes in general is often limited by their instability under operating conditions. Consequently, enzyme properties have to be improved before their implementation at industrial scale[4]. Available stabilization strategies[5] are immobilization[6], protein engineering[4] or chemical modification[7], medium engineering by using additives, screening for enzymes from extremophiles and their isolation, as well as the production of stable enzymes in genetically manipulated mesophilic organisms.

Besides stabilization, enzyme immobilization enables several benefits for the industrial process, such as simplified product purification and easy enzyme recovery. It can also enable masking of sensitive enzyme residues against chemical reagents. This can be achieved by immobilization of enzyme on supports having large internal surfaces. In that case, the immobilized enzyme is in close contact with large support surface and may become less accessible to deleterious reagents[6]. This might also be useful for protection of oxidases against air bubbles[4] which may promote enzyme inactivation[8,9]. The literature data suggests that gas bubbles cannot inactivate the enzymes immobilized on porous support[10,11] and that is why such support was chosen for this work[12–14]. In our previous work[14] operational stability of soluble porcine kidney DAAO was investigated during D-methionine oxidative deamination (Fig. 1) in the presence and absence of aeration. It was found that DAAO's activity decreases during oxidation and that oxygen concentration in the reaction solution affects the rate of enzyme activity decay; even though aeration is crucial to achieve higher reaction rates and to shorten the reaction time. The operational stability decay was the result of the combined effect of shear stress at the air-liquid interface, which may induce an irreversible aggregation of proteins and a negative effect of oxygen, which may lead to oxidation of protein residues.[14] The topic of this work was therefore the investigation of the operational stability of immobilized porcine kidney DAAO. DAAOs of various origin were immobilized[15,16] on different supports with remarkable results. For example, a 600-fold

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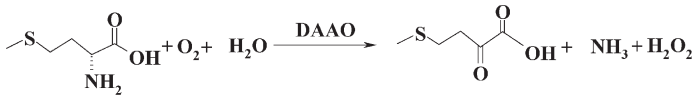
improvement of stability was achieved for *Trigonopsis variabilis* DAAO by immobilization that combined adsorption on a support coated with polyethylenimine and treatment with glutardialdehyde[15]. However, as noticed by other authors[16], a comprehensive analysis of operational stability of immobilized enzyme is most often missing. Thus, in this report the authors have focused on this topic, and not on the optimization of the immobilization procedure itself, although some groups of authors reported thorough studies on stability of immobilized enzyme[6,15,17,18].

DAAO was immobilized on Eupergit C in this work. Operational stability of immobilized enzyme at different aeration conditions was investigated in the reaction of D-methionine oxidative deamination (Fig. 1). Kinetic constants of immobilized enzyme were compared to that of soluble enzyme. Mathematical model of D-methionine oxidative deamination catalyzed by the immobilized enzyme was developed and validated in the batch reactor using estimated kinetic constants of immobilized enzyme. It enabled a more precise analysis of the experimental results and estimation of operational stability decay rate constant where necessary.



$$H_2O_2 \xrightarrow{\text{catalase}} 0.5\ O_2 + H_2O$$

Fig. 1 – *Reaction scheme*

Experimental

Chemicals

Catalase from *Micrococcus lysodeikticus* (dark brown solution, 65,000-150,000 units cm^{-3} towards hydrogen peroxide, E.C. 1.11.1.6), *o*-dianisidin and Eupergit C (150 μm spherical particles, binding capacity 50 $mg_{protein}$ per g of carrier, water binding capacity 4 cm^3 per g of carrier) were purchased from Fluka, Germany. D-Methionine, 2-oxo-4-methylthiobutyric acid, D-amino acid oxidase from porcine kidney (powder, ≥1.5 units mg^{-1} to D-alanine, E.C. 1.4.3.3) and peroxidase from horse radish (lyophilized powder, 250–330 units mg^{-1} solid using pyrogallol, E.C. 1.11.1.7) were purchased from Sigma Aldrich, Germany. Na_2HPO_4 and KH_2PO_4 were purchased from Kemika, Croatia. Nitrogen was purchased from Messer, Croatia.

HPLC analysis

D-Methionine and 2-oxo-4-methylthiobutanoic acid were determined by HPLC (Shimadzu) with a reverse phase C_{18} column (125 x 4 mm) – LiChrosorb® and UV detector at 210 nm. The mobile phase was water solution of perchloric acid pH 2.10–2.15[19] at the flow rate of 0.9 cm^3 min^{-1}. The analysis was performed at 30 °C. Standard solutions were prepared by dissolving appropriate masses of the D-methionine and 2-oxo-4-methylthiobutyric acid in redistilled water. Samples taken from the reactor were diluted with hydrochloric acid (0.1 mol dm^{-3}). Retention times of D-methionine and 2-oxo-4-methylthiobutanoic acid were 2.7 and 9.3 minutes, respectively.

Determination of oxygen concentration in the reactor

Oxygen concentration was monitored by using the Clark oxygen electrode (Ingold, model 34,100,3002 Metler Toledo). The oxygen saturation level, shown as a percentage, was recalculated to mmol dm^{-3} of oxygen by using literature data[20]. Conductivity of the reaction solution was determined for that purpose (20.4 mS cm^{-1}) at 30 °C. The reaction solution consisted of buffer, substrate, product and immobilized enzymes. The electrode was immersed in the solution during the experiments. Oxygen concentration at saturation was 0.219 mmol dm^{-3}. The reactor set-up is described in detail in section entitled Reactor experiments.

Determination of protein concentration

Protein concentration in soluble DAAO and catalase samples was determined according to Bradford[21]. Albumin from bovine serum was used as standard. The method was used to determine the amount of protein in the initial sample prepared for immobilization, as well as the amount of proteins after immobilization.

D-Amino acid oxidase activity assay for the soluble enzyme

The DAAO enzyme kinetics of the soluble enzyme was measured according to the peroxidase-*o*-dianisidin assay[22]. The reaction mixture contained buffer-substrate solution (10 mmol dm^{-3} D-methionine), phosphate buffer (pH 8.0, 0.2 mol dm^{-3}), *o*-dianisidin (23.2 mmol dm^{-3}), peroxidase (20 mg cm^{-3}) suspended in ammonium sulphate solution (3.2 mol dm^{-3}), and DAAO solution (0.022 mg cm^{-3}) to the final volume of 1 cm^3. The mixture was pre-incubated at 30 °C for 5 min, and the reaction was started by adding the DAAO enzyme solution. Brown coloring had formed due to the reaction between hydrogen peroxide and *o*-dianisidin, which was measured *via* spectrophotometer (Shimadzu UV 1601) at 436 nm. The enzyme assay was used to determine the enzyme kinetic parameters of the

soluble enzyme at pH 8, and to compare them with the immobilized enzyme. One unit of soluble D-amino acid oxidase activity was defined as the amount of enzyme necessary to perform oxidative deamination of 1 μmol of D-methionine per minute in 0.2 mol dm^{-3} phosphate buffer pH 8.0 and at 30 °C. The details of the experiments are described in our previous work done with the soluble porcine kidney DAAO, and are therefore not presented here[14].

Procedure for immobilization of DAAO

D-Amino acid oxidase from porcine kidney and catalase from *Micrococcus lysodeikticus* were covalently immobilized on Eupergit C[23]. The enzymes were immobilized on the carrier according to the typical procedure, as follows: 10.1 mg of porcine kidney DAAO powder was mixed with 200 mg of Eupergit and 2.5 cm^3 of activation buffer (0.2 mol dm^{-3} phosphate buffer pH 8.0) for 68 h at 100 rpm and at 25 °C in a closed vessel. Similarly, 1.38 cm^3 of *Micrococcus lysodeikticus* catalase was mixed with 200 mg of Eupergit and 1.5 cm^3 of the activation buffer for 68 h at 100 rpm and at 25 °C in another closed vessel. The immobilized DAAO and catalase were used in batch reactor experiments carried out with and without aeration. The immobilized enzymes were washed with phosphate buffer (pH 8.0, 0.01 mol dm^{-3}) and filtered through a filter paper (grade 1), and the protein concentration in the filtrate was measured according to the Bradford method[21]. The overall percentage of protein immobilization in all cases was calculated according to the immobilized protein concentration divided by the initial protein content. Immobilization efficiency was defined as the ratio of actual enzyme activity measured for the immobilized enzyme and the theoretically expected value (activity of the same amount of soluble enzyme). Actual enzyme activity is the activity of the immobilized enzyme determined by the initial reaction rate method, while the theoretically expected value corresponds to the activity of the same amount of soluble enzyme. The immobilization efficiency, as well as the overall percentage of protein immobilization are given in the results for each experiment, as well as in the figure legends for each experiment, and are in good agreement regarding reproducibility of the immobilization procedure.

Reactor experiments

D-Methionine oxidative deamination experiments were carried out in a 50-cm^3 batch reactor supplied with a magnetic stirrer and thermostated at 30 °C (Fig. 2) without aeration, and with aeration at zero, 5, and 10 dm^3 h^{-1} air flow rates. The reactor was a round-bottom Schott Duran glass bottle with 100 cm^3 capacity, 56 mm diameter, height 105 mm, and DIN-Thread GL 45. The air flow rate was adjusted by using the air flow meter. The reaction media was 0.2 mol dm^{-3} phosphate buffer pH 8.0. The oxygen concentration in the 50 cm^3 reactor was monitored by using the Clark oxygen electrode (Ingold, model 34,100,3002 Metler Toledo). The reactor was placed on the magnetic stirrer at 600 rpm. Aeration, respectively the oxygen supply, was performed by using an air compressor (Super Silent Profi 66, Rheinland Elektromaschinen Group, Germany). The defined amount of fresh immobilized DAAO and catalase were added to the reactor to start the reaction. Concentrations of D-methionine, 2-oxo-4-methylthiobutyric acid and oxygen were monitored by the previously described methods. Detailed reaction conditions are presented in the legends of Figs. 3–5. The same reactor set-up was used to determine the overall oxygen volume transfer coefficient. These experiments were carried out at zero, 5, 10, and 15 dm^3 h^{-1} flow rates. Air was supplied through the perforated plastic ring air distributor of diameter 3 cm containing 15 uniformly distributed holes with a diameter 0.7 mm as shown in Fig. 2.

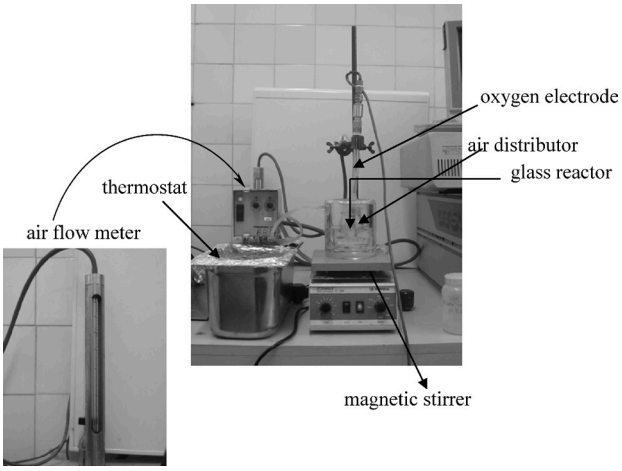


Fig. 2 – *Experimental set-up of the batch experiments with and without continuous aeration*

Determination of the overall oxygen volume transfer coefficient (k_La') by the integral method

The overall oxygen volume transfer coefficient (k_La') was estimated from the measurements carried out at zero air flow rate, and 5, 10 and 15 dm^3 h^{-1}, respectively in the previously described experimental set-up. The integral method based on the oxygen mass balance in the batch reactor (Eq. 1) was used for that purpose. The following procedure was used: the oxygen was completely removed from the solution by purging with nitrogen. After its concentration had dropped to zero, the aeration started at the

selected air flow rate. The concentration of oxygen started to rise and from its time dependence, the oxygen volume transfer coefficient was estimated. The reaction solution contained 400 mg of enzyme carrier (without enzyme) to closely replicate the reaction conditions. No air flow was used to determine the $k_L a'$ at zero flow rate. The oxygen was removed from the reaction solution at the beginning, and then the process of oxygen saturation in the solution was monitored in time. By using the oxygen balance (Eq. 1), $k_L a'$ at zero flow rate was estimated.

$$\frac{\mathrm{d}c_{O_2}}{\mathrm{d}t} = k_L a' \cdot (c^*_{O_2} - c_{O_2}) \tag{1}$$

Kinetics of the immobilized D-amino acid oxidase

The kinetics of the immobilized enzyme was measured according to the initial reaction rate method. The reaction rate was determined with 10 mmol dm^{-3} D-methionine as a substrate. The substrate concentration was monitored by HPLC in the initial part of the reaction (conversion less than 10 %). The specific activity of immobilized enzyme was determined from these data according to the Eq. 2. For the purpose of kinetics determination, the influence of substrates (D-methionine and oxygen) and product (2-oxo-4-methylthiobutyric acid) on the initial reaction rate was evaluated. To determine the influence of each compound on the initial reaction rate, one set of experiments was carried out. In each set of experiments, the initial concentration of one compound (D-methionine, oxygen or 2-oxo-4-methylthiobutyric acid) was changed and the reaction was monitored in its initial part (substrate conversion less than 10 %). The amount of immobilized DAAO used in these experiments was 10 mg. The concentration of D-methionine was varied in the first series of batch experiments up to 10.319 mmol dm^{-3} with 7 experimental points. The batch experiments were carried out for 30 minutes, and all were done in triplicate. The experimental error was below 10 %. Prior to the initialization of experiments, the solution containing the buffer and substrate was saturated with air. When measuring the influence of 2-oxo-4-methylthiobutyric acid on the initial reaction rate in the second series of experiments, the initial concentration of D-methionine was 10.002, and of oxygen 0.219 mmol dm^{-3}, respectively. 2-Oxo-4-methylthiobutyric acid was varied up to 10.021 mmol dm^{-3} with 8 experimental points. The specific activity of immobilized enzyme was calculated from the initial linear slope of the product concentration *vs* time curve by using the Eq. 2.

$$\textit{specific activity} = \frac{\Delta c_{2-\mathrm{oxo}}}{\Delta t} \cdot \frac{V_{\mathrm{reactor}}}{m_{\mathrm{enzyme}}} \tag{2}$$

The oxygen concentration in the reaction solution was varied in the third series of batch experiments up to saturation i.e., 0.219 mmol dm^{-3}. The initial concentration of D-methionine in these experiments was 10.002 mmol dm^{-3}. The oxygen concentration was adjusted by using nitrogen (nitrogen was used to lower the oxygen concentration and air was used to increase it) and air compressor. After the initial concentration of oxygen was set, the reaction was started by the addition of 10 mg of immobilized DAAO. The oxygen concentration was monitored in the initial part of the experiment, to determine the initial reaction rate. From the concentration change during that time, the value of the initial reaction rate was estimated. Eq. 3 was used for that purpose. The equation indicates that oxygen dissolves in the reaction solution during the reaction ($k_L a_0' \cdot (c^*_{O_2} - c_{O_2})$), and it is spent at the same time due to the enzyme catalyzed reaction ($-r_0$).

$$\frac{\mathrm{d}c_{O_2}}{\mathrm{d}t} = k_L a_0' \cdot (c^*_{O_2} - c_{O_2}) - r_0 \tag{3}$$

D-Methionine oxidative deamination in the batch reactor

D-Methionine conversion experiments were carried out in a reactor, which worked as single and repetitive batch. The details of the experimental set-up of the single batch are described in section entitled Reactor experiments. After the initial D-methionine concentration was spent in the repetitive batch experiments, the reaction solution was filtered and the immobilized enzyme separated, washed with 10 mmol dm^{-3} phosphate buffer pH 8.0, and subsequently reused in the next batch experiment. The enzyme was added to the newly prepared D-methionine solution in buffer, i.e. second or third batch experiment in the same reactor. The concentrations of substrate are presented in detail in the legends of Figs. 4 and 5.

Mathematical model and data processing

The overall reaction rate of D-methionine oxidation catalyzed by DAAO in the presence of catalase was described with a simplified double substrate Michaelis-Menten equation (the reaction rate r – Eq. 4). Considering that the estimated value of Michaelis constant for oxygen is quite high in comparison to the concentration of saturation, a simplification of the kinetic model expressed by Eq. 5 was done. Kinetic equation 6 presents the reaction rate of hydrogen peroxide degradation. Hydrogen peroxide evolves in the reaction of D-methionine oxidative deamination, which is described by the reaction rate r, and it is degraded in the cat-

alase catalyzed reaction described by the reaction rate r_1.

Since the mechanism of the enzyme activity decay was not studied, the simplest first-order kinetic model was used to describe the enzyme activity decay (Eq. 7). Mass balances for D-methionine and 2-oxo-4-methylthiobutyric acid in the batch reactor are presented by Eqs. 8 and 9. The mass balance for oxygen is presented by the Eq. 10, and it can be divided into three separate parts; oxygen is spent in the enzyme catalyzed reaction; oxygen dissolves in the reaction solution and the rate of the process is described by the oxygen volume transfer coefficient; and oxygen evolves in the reaction of hydrogen peroxide degradation. The mass balance for hydrogen peroxide is presented by the Eq. 11.

$$r = k \cdot c_{O_2} \cdot \frac{c_{D-met}}{K_m^{D-met} + c_{D-met}} \quad (4)$$

$$K_m^{O_2} >> c_{O2} \quad \frac{V_m}{K_m^{O_2} + c_{O_2}} \approx \frac{V_m}{K_m^{O_2}} = k \quad (5)$$

$$r_1 = k_{cat} \cdot c_{H_2O_2} \quad (6)$$

$$\frac{dV_m}{dt} = -k_d \cdot V_m \quad (7)$$

$$\frac{dc_{D\text{-met}}}{dt} = -r_1 \quad (8)$$

$$\frac{dc_{2\text{-oxo}}}{dt} = r_1 \quad (9)$$

$$\frac{dc_{O_2}}{dt} = -r + k_L a' \cdot \left(c_{O_2}^* - c_{O_2}\right) + 0.5 \cdot r_1 \quad (10)$$

$$\frac{dc_{H_2O_2}}{dt} = r - r_1 \quad (11)$$

The model parameters were estimated by non-linear regression analysis and optimized by using Simplex and Least Squares method implemented in SCIENTIST software[24]. They were evaluated by fitting the mathematical model to the experimental data. A non-linear least squares fitting was performed using a modified Powell algorithm to find a minimum of the sum of squared deviations between observed data and model calculations. The expression for calculating the sum of squared deviation is presented by the following equation: $\sum_{i=1}^{n}\left(y_i - \bar{y}\right)^2$ where n is the number of data points and $\bar{y}$ is the algebraic mean of the y data column.

The "Episode" algorithm implemented in SCIENTIST software was used for simulations. Kinetic parameters K_m, V_m and K_i were estimated by performing different initial reaction rate experiments, and by using the non-linear regression: K_m and V_m were estimated from the initial reaction rate *vs* substrate concentration, K_i from the initial reaction rate *vs* product concentration. The linear part of the concentration *vs* time curve was taken to calculate the initial reaction rate. Operational stability decay rate constant, k_d, was estimated directly from the batch experiments in which activity decay was present (Fig. 3, third batch; Fig. 4, fourth batch). Otherwise, the mathematical model of the system was used only for simulation of experimental data. The goodness of fit is evaluated by the calculation of statistical data such as: R^2, correlation, coefficient of determination, standard deviation of data, model selection criteria etc., which are all available and calculated by the SCIENTIST software. These values are presented in the results for each experiment in the batch reactor carried out with and without aeration.

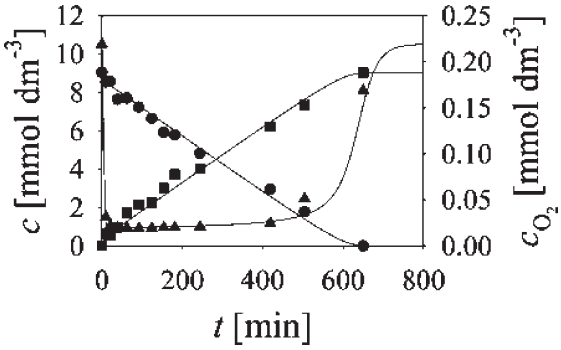


Fig. 3 – *D-Methionine oxidative deamination in the batch reactor without aeration catalyzed by immobilized D-amino acid oxidase from porcine kidney and catalase ($V_{reactor}$ = 50 cm³, $c_{D\text{-met}}$ = 9.03 mmol dm⁻³, 30 °C, 0.2 mol dm⁻³ phosphate buffer pH 8.0, n = 600 rpm). Immobilization efficiency and the overall percentage of protein immobilization were 12.0 and 96.0 %, respectively for DAAO. 10.1 mg of DAAO was weighed for immobilization. Overall percentage of protein immobilization for 1.38 cm³ catalase was 84.1 %. Legend: circles – D-methionine concentration, squares – 2-oxo-4-methylthiobutyric acid concentration, triangles – oxygen concentration.*

Results and discussion

Overall oxygen volume transfer coefficient

The overall oxygen volume transfer coefficients were estimated from the independent experimental data in the absence of enzymatic reaction at different air flow rates, and are presented in Table 1. $k_L a$' is an important parameter used in simulations of the experiments carried out in the batch reactor. The estimated values show that the system is suitable for D-methionine oxidation by DAAO.

Table 1 – *Estimated oxygen volume transfer coefficients at different aeration conditions*

q_v [dm^3 h^{-1}]	$k_L a'$ [min^{-1}]
0	0.0189 ± 0.0003
5	0.2600 ± 0.0078
10	0.3800 ± 0.0537
15	0.4466 ± 0.0047

D-Amino acid oxidase kinetics – Soluble *vs* immobilized enzyme

Table 2 presents the estimated kinetic parameters for the soluble and immobilized enzyme. The kinetic parameters of soluble enzyme estimated in this work at pH 8 are compared with the parameters obtained for immobilized enzyme. The efficiency of protein immobilization was about 96 % and DAAO retained approximately 11.3 % of initial activity of soluble enzyme (immobilization efficiency). This was expected due to the formation of covalent bonds, which are non-selective and may destroy the active site of the enzyme[25]. Immobilized enzyme had 5.8 fold higher apparent Michaelis constant for D-methionine, which implies that it had lost its affinity towards this substrate. As for oxygen, it was found that the dependence of specific activity on oxygen concentration can be described by the linear relationship, i.e. first-order kinetic model, and that is why there is no Michaelis constant for oxygen in Table 2. The kinetic constant k represents the reaction rate constant of the first order. The active site of enzyme probably became less available to substrates (due to immobilization), and that is the reason for lower affinity towards O_2.[25] It was also found that the immobilized enzyme was not inhibited by 2-oxo-4-methylthiobutyric acid. The literature confirms that immobilization can minimize or prevent enzyme inhibition[4], which in our case is probably due to small distortion of the enzyme active site after immobilization. By knowing these parameters, it can be concluded that air (oxygen) saturation concentration will not be enough to reach maximum reaction rate in the reactor, and aeration is necessary. Considering the gathered data, a kinetic model has been proposed for oxidative deamination of D-methionine in the form of simplified double substrate Michaelis-Menten kinetics (Eq. 4).

Table 2 – *Comparison of estimated kinetic parameters of soluble and immobilized enzyme*

Parameter	Unit	Soluble enzyme	Immobilized enzyme
V_m	U mg^{-1}	4.260 ± 0.127	0.481 ± 0.044
K_m^{D-met}	mmol dm^{-3}	0.275 ± 0.064	1.604 ± 0.396
K_i^{2-oxo}	mmol dm^{-3}	0.201 ± 0.046	–
$K_m^{O_2}$	mmol dm^{-3}	0.096 ± 0.010	–
k	min^{-1}	–	1.437 ± 0.135

D-Methionine oxidative deamination in the batch reactor catalyzed by DAAO from porcine kidney

Experiment without aeration

In our previous work, soluble DAAO was completely inactivated in the batch experiment without aeration after approximately 18 hours[14]. This time was significantly shorter when the oxygen concentration was increased above 0.1 mmol dm^{-3} (approx. 50 % of oxygen saturation)[14], which normally happens when amino acid concentration lowers (near the end of reaction), and the reaction rate decreases. The dissolution of oxygen from air continues and oxygen cannot be spent because the other substrate is almost gone. Thus, it was shown by our previous work[14] that the concentration of oxygen in the reactor is an important factor for DAAO stability. Consequently, the enzyme was immobilized and its operational stability was tested at similar conditions. The efficiency of protein immobilization for DAAO and catalase was found to be 96 % and 84 %, respectively. The immobilization efficiency of DAAO was 12 %. The results presented in Fig. 3 show that the oxygen concentration dropped very fast at the beginning of the experiment, and remained low until D-methionine was almost spent. It took 10 hours to reach 100 % D-methionine conversion. The reaction rate is limited by oxygen concentration, and its dissolution in the reaction solution. Therefore, aeration is necessary to increase the reaction rate. The mathematical model (kinetic equations 4 and 6, mass balance Eqs. 8–11) simulated the data well. This was confirmed by the statistical data calculated by the software presented in Table 3. Operational stability decay was not included in the mathematical model, as it did not occur. Under conditions with no oxygen (air) supply, the dissolved oxygen concentration increased after approximately 500 min (Fig. 3). This was expected, since oxygen is no longer spent in the reaction.

Table 3 – *Statistical data for the developed mathematical model – experiment without aeration*

Experiment – 0 dm^3 h^{-1}	1st batch
R^2	0.982
Correlation	0.990
Coefficient of determination	0.964
Standard deviation of data	0.563
Model selection criteria	3.260

Experiments with continuous aeration at air flow rate of 5 dm³ h⁻¹

DAAO from porcine kidney is an enzyme extremely sensitive to aeration and oxygen concentration in the reaction solution, and the enzyme operational stability decay rates are so high that, in some cases, complete D-methionine conversion cannot be achieved[14]. Immobilized DAAO and catalase were used in the repetitive batch experiment of D-methionine oxidative deamination with continuous aeration at 5 dm³ h⁻¹. The overall percentage of immobilized proteins for DAAO and catalase was 96 % and 84 %, respectively. The immobilization efficiency for DAAO was 10.2 %. The results are presented in Fig. 4. Oxygen concentration drops at the beginning of the experiment but not as low as in the experiment without aeration. The initial reaction rates were calculated from the experimental data (Fig. 4) in each batch, and are presented in Table 4. There was no significant difference between these values for the immobilized enzyme, so it can be stated that the enzyme had stabilized. However, if the initial reaction rates in this experiment are compared with the one estimated from the batch experiment carried out with soluble enzyme (Table 4), it can be concluded that the enzyme activity was significantly lowered by the immobilization procedure. Nevertheless, the immobilization procedure improved enzyme stability against damage made by gas/liquid interface[14]. The experiments were simulated with the developed mathematical model (kinetic equations 4 and 6, mass balance equations 8–11), and the model described the experimental data well in the first two batches. However, to simulate the third batch, enzyme operational stability decay of the first order (Eq. 7) was introduced, and such mathematical model described the data well. The statistical data for the model are presented in Table 5, and the difference in goodness of fit in the cases without deactivation and with deactivation can be observed. The operational stability decay can be also observed from the duration of batches, since the initial concentration of D-methionine was similar in all experiments. In the first and the second batch, it took about 220 minutes to convert all D-methionine to 2-oxo-4-methylthiobutyric acid. In the third batch, this time was somewhat prolonged to 240 minutes (Fig. 4). The enzyme operational stability decay rate constant was estimated, and is presented in Table 6. Compared to the inactivation of the soluble enzyme, the enzyme activity decay of immobilized enzyme was 3.5 slower in the third experiment[14]. It has to be emphasized here that the immobilized en-

Table 4 – *Comparison of estimated enzyme specific activities during repetitive use of immobilized enzyme in the experiments with continuous aeration at 5 dm³ h⁻¹ with the initial reaction rate in the experiment carried out with soluble enzyme*

Enzyme form	*SA* [U mg^{-1}]
Soluble	4.12
Immobilized –1st batch	0.42
Immobilized –2nd batch	0.39
Immobilized –3rd batch	0.39

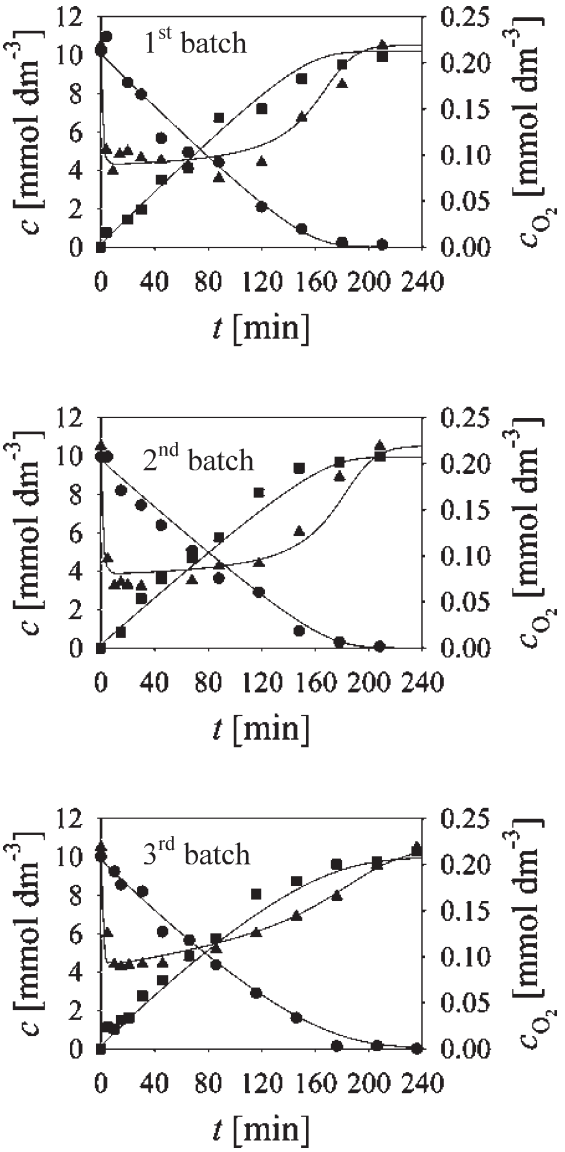


Fig. 4 – *D-Methionine oxidative deamination in the batch reactor with continuous aeration at 5 dm³ h⁻¹ catalyzed by separately immobilized D-amino acid oxidase from porcine kidney and catalase ($V_{reactor}$ = 50 cm³, $c_{D\text{-}met}$ = 10.20 (1st batch); 9.96 (2nd batch); 10.00 (3rd batch) mmol dm⁻³, 30 °C, 0.2 mol dm⁻³ phosphate buffer pH 8.0, n = 600 rpm). Immobilization efficiency and the overall percentage of protein immobilization were 10.0 % and 96.1 %, respectively for DAAO. 10.2 mg of DAAO was weighed for immobilization. Overall percentage of protein immobilization for 1.38 cm³ catalase was 84.3 %. Legend: circles – D-methionine concentration, squares – 2-oxo-4-methylthiobutyric acid concentration, triangles – oxygen concentration.*

zyme started to deactivate after more than 440 minutes of operation in the reactor (repetitive batch experiment, Fig. 3, after the second batch), while the operational stability decay of the soluble enzyme occurred instantaneously after the beginning of the experiment[14].

Table 5 – *Statistical data for the developed mathematical model – experiment at 5 dm³ h⁻¹*

Experiment – 5 dm³ h⁻¹	1st batch	2nd batch	3rd batch	3rd batch model with deactivation
R^2	0.993	0.976	0.993	0.997
Correlation	0.994	0.980	0.995	0.997
Coefficient of determination	0.987	0.959	0.987	0.994
Standard deviation of data	0.423	0.760	0.452	0.283
Model selection criteria	4.323	3.126	3.968	5.037

Table 6 – *Enzyme decay rate constants of the immobilized DAAO estimated from the batch experiments carried out with continuous aeration*

Experiment	k_d [min⁻¹]
3rd batch – 5 dm³ h⁻¹	0.003587 ± 0.000428
4th batch – 10 dm³ h⁻¹	0.005833 ± 0.000593

Experiments with continuous aeration at air flow rate of 10 dm³ h⁻¹

The results of the conversion experiment are presented in Fig. 5. Overall percentages of immobilized proteins used in this experiment were 98 % and 55 % for DAAO and catalase, respectively. Immobilization efficiency of DAAO was 12.3 %. Four consecutive experiments were carried out with these immobilized enzymes. The immobilized enzyme was found to be stable according to the initial reaction rates estimated from the experimental data presented in Table 7. In four consecutive experiments, the initial enzyme activity was constant.

The mathematical model (kinetic equations 4 and 6, mass balance equations 8–11) was used to simulate the data. It was successful in the first three batches. During the fourth batch, experiment enzyme activity decay of the first order (Eq. 7) had to be included in the model to describe the experimental data well. The statistical data related to the goodness of fit are presented in Table 8, and the difference between the model with and without enzyme activity decay can be observed. This shows that, even though the initial reaction rates were similar, a slight operational stability decay occurred. This op-

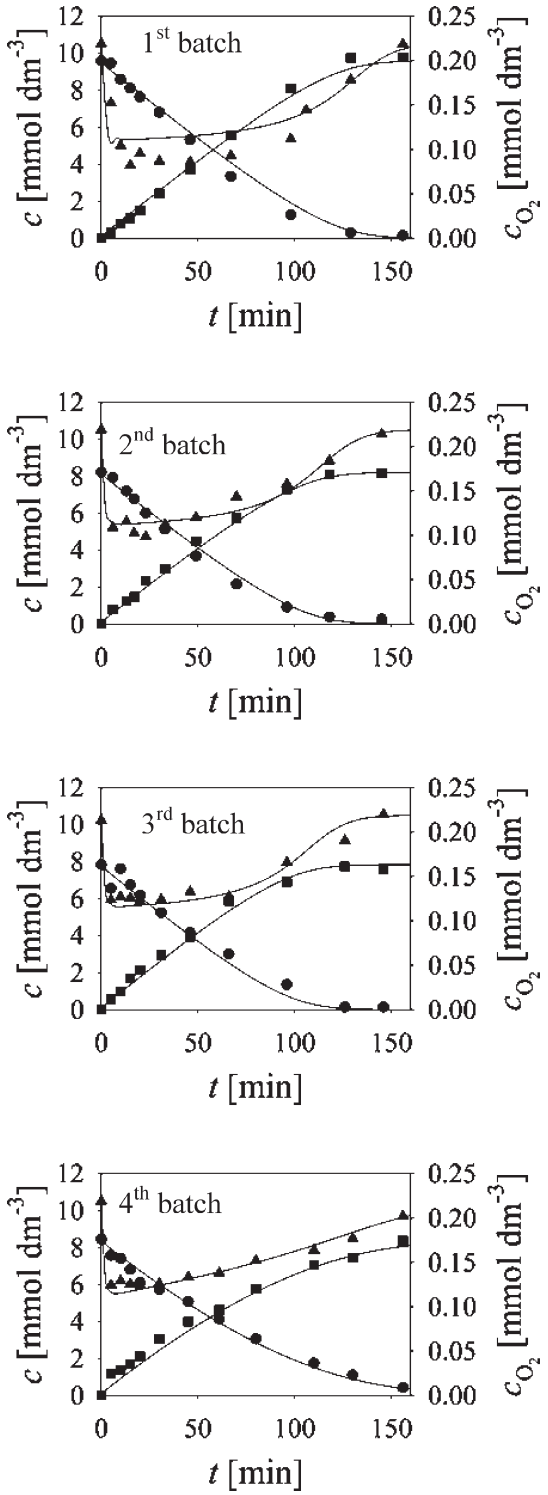


Fig. 5 – *D-Methionine oxidative deamination in the batch reactor with continuous aeration at 10 dm³ h⁻¹ catalyzed by separately immobilized D-amino acid oxidase from porcine kidney and catalase ($V_{reactor}$ = 50 cm³, $c_{D\text{-}met}$ = 9.58 (1st batch); 8.20 (2nd batch); 7.84 (3rd batch); 8.45 (4th batch); mmol dm⁻³, 30 °C, 0.2 mol dm⁻³ phosphate buffer pH 8.0, n = 600 rpm). Immobilization efficiency and the overall percentage of protein immobilization were 12.3 % and 98 %, respectively for DAAO. 10.1 mg of DAAO was weighed for immobilization. Overall percentage of protein immobilization for 1.38 cm³ catalase was 85.0 %. Legend: circles – D-methionine concentration, squares – 2-oxo--4-methylthiobutyric acid concentration, triangles – oxygen concentration.*

Table 7 – *Comparison of estimated specific enzyme activities during repetitive use of immobilized enzyme in the experiments with continuous aeration at 10 dm³ h⁻¹ with the initial reaction rate in the experiment carried out with soluble enzyme*

Enzyme form	*SA* [U mg^{-1}]
Soluble	4.47
Immobilized –1st batch	0.55
Immobilized –2nd batch	0.53
Immobilized –3rd batch	0.51
Immobilized –4th batch	0.55

Table 8 – *Statistical data for the developed mathematical model – experiment at 10 dm³ h⁻¹*

Experiment – 10 dm³ h^{-1}	1st batch	2nd batch	3rd batch	4th batch	4rd batch model with deactivation
R^2	0.997	0.998	0.994	0.937	0.944
Correlation	0.998	0.998	0.996	0.993	0.988
Coefficient of determination	0.995	0.996	0.989	0.874	0.888
Standard deviation of data	0.280	0.206	0.338	1.055	0.997
Model selection criteria	4.954	5.359	4.117	2.018	2.132

erational stability decay can also be observed from the time necessary to convert all D-methionine. In the first three batches, it took approximately 150 minutes for this to occur, while in the fourth batch, a longer time was necessary. The operational stability decay rate constant is presented in Table 6. If it is compared to the enzyme operational stability decay rate constant of the soluble enzyme at the same conditions, the constant for the immobilized enzyme is 4.1-fold lower[14]. Besides that, enzyme operational stability decay was noticed after approximately 10 hours of continuous use.

Table 9 – *Volume productivities in the experiments*

Air flow rate [dm³ h^{-1}]	Volume productivity [mmol dm^{-3} h^{-1}]
0	0.83
5	2.75
10	3.32

Comparison of volume productivities in the conducted experiments

From the data presented in Figs. 3–5, the volume productivities were calculated by dividing the mmoles of the synthesized α-keto acid by the reaction volume and the time necessary to achieve full substrate conversion. The results presented in Table 9 show that aeration increased the reactor productivity, and the highest was achieved when the air flow rate was at 10 dm³ h^{-1}.

Conclusion

It was shown that immobilization of porcine kidney DAAO on particles of Eupergit C can be used to stabilize DAAO against inactivation on the air/liquid interface. The enzyme's affinity towards substrates (D-methionine and oxygen) had been lowered after immobilization. The developed mathematical model described the data well. It enabled the estimation of the operational stability decay rate constants. The operational stability decay occurred with the prolonged use of the immobilized enzyme. However, it was significantly lower than it was in the case of soluble enzyme.

ACKNOWLEDGEMENTS

This work was supported by the Croatian Ministry of Science, Education, and Sports (Contract Grant Number 125-1252086-2793). Technical assistance of Mrs. Nataša Car is greatly acknowledged.

List of symbols

c – concentration, mmol dm^{-3}
c^* – saturation concentration, mmol dm^{-3}
k – reaction rate constant of the first order, min^{-1}
k_d – operational stability decay rate constant of the first order, min^{-1}
$k_L a'$ – oxygen volume transfer coefficient, min^{-1}
K_i – inhibition constant, mmol dm^{-3}
K_m – Michaelis constant, mmol dm^{-3}
m_{enzyme} – mass of enzyme, mg
q_v – air flow rate, dm³ h^{-1}
r – reaction rate, mmol dm^{-3} min^{-1}
r_0 – initial reaction rate, mmol dm^{-3} min^{-1}
SA – specific activity of enzyme, U mg^{-1}
V_m – maximal specific activity of enzyme, maximal reaction rate, U mg^{-1}
$V_{reactor}$ – reactor volume, cm³
t – time, min

List of abbreviations

D-met – D-methionine
DAAO – D-amino acid oxidase
cat – catalase
2-oxo – 2-oxo-4-methylthiobutyric acid

References

1. *Pollegioni, L., Molla, G.*, New biotech applications from evolved D-amino acid oxidases, Trends Biotechnol. **29** (6) (2011) 276. http://dx.doi.org/10.1016/j.tibtech.2011.01.010
2. *Kostova, D. D., Petrova, V. Y., Kujumdzieva, A. V.,* Over-expression of DAAO and catalase in *Kluyveromyces marxianus* through media optimization, permeabilization and GA stabilization techniques, Enzyme and Microbial Technology **42** (2008) 113. doi: http://dx.doi.org/10.1016/j.enzmictec.2007.08.010
3. *Ma, X., Deng, S., Su, E., Wei, D.*, One-pot enzymatic production of deacetyl-7-aminocephalosporanic acid from cephalosporin C via immobilized cephalosporin C acylase and deacetylase, Biochem. Eng. J. **95** (2015) 1. doi: http://dx.doi.org/10.1016/j.bej.2014.11.015
4. *Mateo, C., Palomo, J. M., Fernandez-Lorente, G., Guisan, J. M., Fernandez-Lafuente, R.,* Improvement of enzyme activity, stability and selectivity via immobilization techniques, Enzyme Microb. Technol. **40** (2007) 1451. doi: http://dx.doi.org/10.1016/j.enzmictec.2007.01.018
5. *Iyer, P. V., Ananthanarayan, L.*, Enzyme stability and stabilization – Aqueous and non-aqueous environment, Proc. Biochem. **43** (2008) 1019. doi: http://dx.doi.org/10.1016/j.procbio.2008.06.004
6. *Fernandez-Lafuente, R., Rodriguez, V., Mateo, C., Fernandez-Lorente, G., Arminsen, P., Sabuquillo, P.,* Stabilization of enzymes (D-amino acid oxidase) against hydrogen peroxide via immobilization and post-immobilization techniques, J. Mol. Cat. B-Enzym. **7** (1999) 173. doi: http://dx.doi.org/10.1016/S1381-1177(99)00040-5
7. *Lopez-Gallego, F., Montes, T., Fuentes, T., Alonso, N., Grazu, V., Betancor, L., Guisan, J. M., Fernandez-Lafuente, R.,* Improved stabilization of chemically aminated enzymes via multipoint covalent attachment on glyoxyl supports, J. Biotechnol. **116** (2005) 1. doi: http://dx.doi.org/10.1016/j.jbiotec.2004.09.015
8. *Bommarius, A. S., Karau, A.*, Deactivation of formate dehydrogenase (FDH) in solution and at gas-liquid interfaces, Biotechnol. Prog. **21** (2005) 1663. doi: http://dx.doi.org/10.1021/bp050249q
9. *Caussette, M., Gaunand, A., Planche, H., Lindet, B.,* Enzyme inactivation by inert gas bubbling, Prog. Biotechnol. **15** (1998) 393. doi: http://dx.doi.org/10.1016/S0921-0423(98)80057-9
10. *Bes, T., Gomez-Moreno, C., Guisan, J. M., Fernandez-Lafuente, R.,* Selective oxidation: Stabilisation by multipoint attachment of ferredoxin $NADP^+$ reductase, an interesting cofactor recycling enzyme, J. Mol. Cat. **98** (1995) 161. doi: http://dx.doi.org/10.1016/1381-1169(95)00019-4
11. *Bolivar, J. M., Wilson, L., Ferrarotti, S. A., Fernandez-Lafuente, R., Guisan, J. M., Mateo, C.*, Stabilization of a Formate Dehydrogenase by Covalent Immobilization on Highly Activated Glyoxyl-Agarose Supports, Biomacromolecules **7** (2006) 669. doi: http://dx.doi.org/10.1021/bm050947z
12. *Ludwig, R., Ozga, M., Zamocky, M., Peterbauer, C., Kulbe, K. D., Haltrich, D.*, Continuous Enzymatic Regeneration of Electron Acceptors Used by Flavoenzymes: Cellobiose Dehydrogenase-Catalyzed Production of Lactobionic Acid as an Example, Biocatal. Biotransform. **22** (2004) 97. doi: http://dx.doi.org/10.1080/10242420410001692787
13. *Findrik, Z., Vrsalović Presečki, A., Vasić-Rački, Đ.,* The influence of aeration on activity and operational stability of two snake venom amino acid oxidases, Biochem. Eng. J. **60** (2012) 91. doi: http://dx.doi.org/10.1016/j.bej.2011.10.008
14. *Findrik, Z., Valentović, I., Vasić-Rački, Đ.,* A mathematical model of oxidative deamination of amino acid catalyzed by two D-amino acid oxidases and influence of aeration on enzyme stability, Appl. Biochem. Biotechnol. **172** (6) (2014) 3092. doi: http://dx.doi.org/10.1007/s12010-014-0735-3
15. *Lopez-Gallego, F., Betancor, L., Hidalgo, A., Alonso, N., Fernandez-Lorente, G., Guisan, J. M., Fernandez-Lafuente, R.*, Preparation of a robust biocatalyst of D-amino acid oxidase on sepabeads support using the glutaraldehyde crosslinking method, Enzyme Microb. Technol. **37** (2005) 750. doi: http://dx.doi.org/10.1016/j.enzmictec.2005.04.016
16. *Dib, I., Nidetzky, B.*, The stabilizing effects of immobilization in D-amino acid oxidase from *Trigonopsis variabilis*, BMC Biotechnology **8** (2008) 72. doi: http://dx.doi.org/10.1186/1472-6750-8-72
17. *Betancor, L., Hidalgo, A., Fernandez-Lorente, G., Mateo, C., Rodriguez, V., Fuentes, M., Lopez-Gallego, F., Fernandez-Lafuente, R., Guisan, J. M.*, Use of physicochemical tool to determine the choice of optimal enzyme: Stabilization of D-amino acid oxidase, Biotechnol. Prog. **19** (2003) 784. doi: http://dx.doi.org/10.1021/bp025761f
18. *Fernandez-Lafuente, R., Rodriguez, V., Guisan, J. M.*, The coimmobilization of D-amino acid oxidase and catalase enables the quantitative transformation of D-amino acid (D-phenylalanine) into α-keto acids (phenylpyruvic acid), Enzyme Microb. Technol. **23** (1998) 28. doi: http://dx.doi.org/10.1016/S0141-0229(98)00028-3
19. *Lian, H. Z., Mao, L., Ye, X. L., Miao, J.,* Simultaneous determination of oxalic, fumaric, maleic and succinic acids in tartaric and malic acids for pharmaceutical use by ion-suppression reversed-phase high performance liquid chromatography, J. Pharmaceut. Biomed. **19** (1999) 621. doi: http://dx.doi.org/10.1016/S0731-7085(98)00101-0
20. U.S. Geological Survey TWRI Book 9, 4/98, 27-38. doi: http://water.usgs.gov/owq/FieldManual/Chapter6/Archive/Section6.2.pdf, last accessed February 5th 2015.
21. *Bradford, M. M.,* A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, Anal. Biochem. **72** (1976) 248.
22. Biochemica information I, Boehringer Manheim GmbH, Biochemica, Germany, 1973, pp. 41.
23. *Katchalski-Katzir, E., Kraemer, D. M.,* Eupergit C, a carrier for immobilization of enzymes of industrial potential, J. Mol. Cat. B-Enzym. **10** (2000) 157. doi: http://dx.doi.org/10.1016/S1381-1177(00)00124-7
24. SCIENTIST handbook, Salt Lake City: Micromath® 1986–1995.
25. *Cao, L.*, Carrier-bound Immobilized Enzymes, Wiley-VCH, 2005, pp. 169–293.