Mathematical modelling of amino acid resolution catalyzed by l-amino acid oxidases from Crotalus adamanteus and Crotalus atrox

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1. Introduction

Amino acids and α-keto acids in general are important pharmaceutical compounds [1]. They are widely used in industry as a starting material for various organic syntheses and as pharmacologically active compounds [1]. α-Amino acids in particular are important precursors for the production of antibiotics, as well as other pharmaceuticals [2]. They are employed in the production of food additives and agrochemicals as well [3]. Several methods for their production are available, which include chemical synthesis, fermentation and enzymatic methods [3]. Chemical synthesis is not suitable for the production of α-amino acids because of the low yield and high cost of the production [3]. As for fermentation methods are concerned, there are few successful examples [4], however, optical purity and productivity remain an issue [3]. Biotransformation [5] (by enzymes or whole cells) methods seem to be the most advantageous because of the optical purity and high productivity of the process [2,3,6–9]. Among them there are also those that employ l-amino acid oxidases for racemate resolution [6,10].

An amino acid produced in this work is d-methionine. This amino acid has already been synthesized before from a racemic mixture by degrading l-isomer. Parikh et al. [10] used l-amino acid oxidase (l-AAO) from C. adamanteus, and found it to be a success. These authors also tested this enzyme for various other amino acids. However, the system was not studied in detail, and the kinetics was not determined. Takahashi et al. [6] synthesized d-methionine in high optical purity from the corresponding raceme by using the cells of Proteus vulgaris IAM 12003. l-Amino acid oxidase activity was found to be responsible for this biotransformation.

l-Amino acid oxidases have certain advantages as catalysts. Namely, apart from some exceptions [11–18], they are relatively unspecific concerning the amino acid residue, which means that they accept most of the proteinogenic l-amino acids as substrates [17–21], and are therefore applicable as catalysts for many syntheses. Only a few l-amino acid oxidases are employed for racemate resolution [14,17]. There are also no industrially used l-amino acid oxidases yet, but this is not the case for d-amino acid oxidases, which found their important role in the synthesis of 7-aminocephalosporanic acid (7-ACA) and other precursors in the synthesis of β-lactam antibiotics [22–26]. Considering that the production of optically pure compounds is one of the most difficult and challenging problems in the field of specialty chemicals [27], these kinds of processes are very interesting. The industrial application of l-amino acid oxidases for the resolution of racemic mixtures of amino acids, and the oxidation of l-amino acids to keto acids are imaginable, but have not been studied extensively yet [28].

Besides for the production of enantiomerically pure amino acid from the corresponding racemates, amino acid oxidases can be
used for the production of \( \alpha \)-keto acids as well, since this compound is the product of amino acid oxidative deamination [29] and by-product of the amino acid oxidase (AAO) catalyzed racemate resolution (Fig. 1). These compounds are also products of interest in the pharmaceutical industry as precursors or starting material for various organic syntheses [9]. Therefore, if amino acid oxidases are used to act on amino acid racemate as a substrate, they oxidize one of the enantiomers (depending on their stereoselectivity) producing the corresponding \( \alpha \)-keto acid [29–34], while the other enantiomer stays untouched. There are various known methods for the separation of amino acids and \( \alpha \)-keto acids [26,35–38] which include chromatography, extraction and \( \alpha \)-keto acid derivatization.

Two \( \alpha \)-amino acid oxidases used in this paper were from snake venom origin: Crotalus adamanteus and Crotalus atrox [10,19–21,39–46]. Even though they are commercially available, their process activity and behaviour were not published before to our knowledge, except for \( \alpha \)-amino acid oxidase in our previous papers [41,47]. This enzyme was used in the synthesis of an \( \alpha \)-keto acid, which is an intermediate in the production of an (R)\(+\)-3, 4-dihydroxyphenyllactic acid, a pharmaceutically interesting compound found in plant Salvia miltiorrhiza, and also known as a traditional Chinese medicine (Danshensu) [48]. This compound is also an intermediate in the synthesis of rosmarinic acid [49] and an important anticancer drug rhabdossin [50].

Several authors tested the activity of \( \alpha \)-AAO from C. adamanteus. They found that this enzyme is active to various amino acids like: \( \alpha \)-arginine, \( \alpha \)-valine, \( \alpha \)-leucine, \( \alpha \)-histidine, \( \alpha \)-tryptophan, \( \alpha \)-isoleucine, \( \alpha \)-alanine, \( \alpha \)-aspartic acid, \( \alpha \)-glutamic acid, \( \alpha \)-lysine, \( \alpha \)-methionine, \( \alpha \)-phenylalanine etc. [10,19,20,43]. The present work is focused on the racemate resolution of DL-methionine (Fig. 1). The activity and operational stability of the two snake venom enzymes were examined in the batch and repetitive batch experiments. Kinetic parameters were estimated from the independent experimental results measured by the initial reaction rate method. Mathematical model for \( \alpha \)-methionine oxidation was developed for the batch and continuously operated enzyme membrane reactor (Fig. 2).

### 2. Materials and methods

#### 2.1. Materials

##### 2.1.1. Chemicals

\( \alpha \)-Methionine, \( \alpha \)-methionine, 2-\( \alpha \)-oxo-4-methylthiobutyric acid, \( \alpha \)-amino acid oxidase from C. adamanteus (0.5 U mg\(^{-1}\) on L-phenylalanine as a substrate, E.C. 1.4.3.2), \( \alpha \)-amino acid oxidase from C. atrox (0.27 U mg\(^{-1}\) on L-phenylalanine as a substrate, E.C. 1.4.3.2), peroxidase from Arthromyces ramosus (E.C. 1.11.1.7), beef liver catalase (87,909 U mg\(^{-1}\), E.C. 1.11.1.6), o-dianisidine, KH\(_2\)PO\(_4\) and tris-hydroxymethylaminomethane were purchased at Fluka Chemie (Switzerland). Na\(_2\)HPO\(_4\), K\(_2\)HPO\(_4\) and hydrogen peroxide were purchased at Kemika (Croatia).

#### 2.2. Methods

##### 2.2.1. \( \alpha \)-AAO assay

The \( \alpha \)-AAO enzyme kinetics was measured according to the peroxidase-o-dianisidin assay [51]. The reaction mixture contained buffer–substrate solution, phosphate buffer (pH 7.6, 0.2 M), o-dianisidine, peroxidase (1796.5 mmol min\(^{-1}\) L\(^{-1}\)) suspended in ammonium sulphate solution (3.2 M) and enzyme \( \alpha \)-AAO solution in the final volume of 3 ml. The mixture was pre-incubated at 30 °C and the reaction started by adding the \( \alpha \)-AAO enzyme solution. Brown colouring was formed due to the reaction between hydrogen peroxide and o-dianisidine, which was measured via spectrophotometer (Shimadzu, UV 1601) at 436 nm. One unit (U) of \( \alpha \)-amino acid oxidase activity corresponds to 1 \( \mu \)mol of \( \alpha \)-methionine oxidized per minute at 30 °C and phosphate buffer pH 7.6.
2.2.2. HPLC

L-Methionine and 2-oxo-4-methylthiobutyric acid were followed by HPLC (Sykam, Shimadzu) with a reverse phase C18 column, Merck (125 × 4 mm) and UV detector at 210 nm. The mobile phase was water with the addition of perchloric acid—pH 2.10–2.15 [52] at a flow-rate of 0.5 ml min⁻¹. The analysis was performed at 30 °C. Standard solutions were prepared by dissolving appropriate masses of the standard in hydrochloric acid (0.1 M). Samples taken from the reactor were diluted with hydrochloric acid (0.1 M) as well. Retention times of l-methionine and 2-oxo-4-methylthiobutyric acid were 2.9 and 9.5 min, respectively.

In the experiments where u-methionine was used as a substrate, a chiral HPLC column was used: CROWNPAK CR (+). The samples were analyzed at 210 nm and 30 °C. The mobile phase was water solution of HClO₄—pH 1.0. The flow-rate was 0.4 ml min⁻¹. The retention time of l-methionine, 2-oxo-4-methylthiobutyric acid and u-methionine were 9.4, 12.6 and 17.8 min, respectively.

2.2.3. Conversion experiments

Batch and repetitive batch experiments were carried out in the 25 ml glass reactor. In the repetitive batch the same enzyme was used three times for the reaction (in our case, three batches). This is the method to test enzyme activity during the repetitive use, i.e., operational stability. After all substrate was oxidized, a new amount was added in the reactor in the repetitive batch experiments. The product was not charged out of the reactor, which differs these experiments from the one described by other authors [53]. There was no significant change in the reaction volume during the experiment since 50–100 µl samples were taken. Small losses due to the sampling were compensated in the next cycle change in the reaction volume during the experiment since 50–100 µl samples were taken. Small losses due to the sampling were compensated in the next cycle.

In the repetitive batch the same enzyme was used three times for the reaction solution that was pumped in the reactor was 4 mm) and UV column was used: CROWNPAK CR (+). The samples were analyzed at 210 nm and the retention time of D-methionine, 2-oxo-4-methylthiobutyric acid were 2.9 and 9.5 min, respectively. At the beginning of each cycle fresh catalase was added. The reactor was thermostated at 30 °C and stirred on the magnetic stirrer. Samples were taken and immediately analyzed by HPLC.

The experiments in continuously operated enzyme membrane reactor (EMR) (Bioengineering AG, Wald, Switzerland) (which operates as continuous stirred tank reactor) [34–36] were carried out in 10 ml reactor volume. The reactor set-up is carried out in 10 ml reactor volume. The reactor set-up is described in Section 4.1). The linear part of the concentration vs. time during the non-stationary continuously operated enzyme membrane reactor experiments. The initial parameter values for the optimization procedure deactivation of the first-order was also assumed in this experiment (Eq. (6), Table 1). Eqs. (10) and (11) (Table 1) represent mass balance equations for l-methionine and 2-oxo-4-methylthiobutyric acid in the continuously operated enzyme membrane reactor.

3. Mathematical modelling

3.1. Mathematical model

Overall reaction rate of the l-methionine oxidation catalyzed by l-amino acid oxidase from C. adamanteus was described using Michaelis–Menten equation with competitive substrate (2-oxo-4-methylthiobutyric acid) inhibition (Eq. (1), Table 1) [41,55–58]. The parameters kₘ, m and Kₑ were estimated from the initial reaction rate experiments by non-linear regression analysis and optimized by using simplex and least-squares method implemented in SCIENTIST software [60]. They were evaluated by fitting the mathematical model to the experimental data. A non-linear least-square fitting was performed using a modified Powell simplex method may provide a useful means of locating a

Mathematical model of L-methionine oxidative deamination catalyzed by l-amino acid oxidases from C. adamanteus and C. atrox

Kinetic equations

\[
\begin{align*}
\frac{d[\text{L-Met}]}{dt} & = \frac{V_{\text{cat, Met}}[\text{L-Met}][\text{O2}]}{K_{\text{oxo, Met}} + [\text{O2}]} - \frac{V_{\text{cat, Met}}[\text{L-Met}][\text{O2}]}{K_{\text{oxo, Met}} + [\text{O2}]} \\
\frac{d[\text{O2}]}{dt} & = \frac{V_{\text{cat, Met}}[\text{L-Met}][\text{O2}]}{K_{\text{oxo, Met}} + [\text{O2}]} - \frac{V_{\text{cat, Met}}[\text{L-Met}][\text{O2}]}{K_{\text{oxo, Met}} + [\text{O2}]} \\
\frac{d[\text{H2O}]}{dt} & = \frac{V_{\text{cat, Met}}[\text{L-Met}][\text{O2}]}{K_{\text{oxo, Met}} + [\text{O2}]} - \frac{V_{\text{cat, Met}}[\text{L-Met}][\text{O2}]}{K_{\text{oxo, Met}} + [\text{O2}]} \\
\end{align*}
\]

<table>
<thead>
<tr>
<th>Table 1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mathematical model of l-methionine oxidative deamination catalyzed by l-amino acid oxidases from C. adamanteus and C. atrox</td>
</tr>
</tbody>
</table>

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especially for the estimation of $V_{\text{m}}$, $K_{\text{m}}$, and $K_i$ from the initial reaction rate experiments, since these kind of equations (Michaelis–Menten kinetic model) do not present a problem for neither of the optimization methods used. As for others are concerned (reactor model with differential equations included), problems for the least-squares method may occur if the initial parameter value is far from its optimum, however, this can be solved by using the simplex method which converges to the optimum.

4. Results and discussion

4.1. L-AAO kinetics

The kinetic properties of two L-amino acid oxidases from snake venom (C. adamanteus and C. atrox) were compared in a series of experiments. The reaction studied was L-methionine oxidation. Kinetic measurements were carried out by the initial reaction rate method. All experiments were carried out in 0.2 M phosphate buffer pH 7.6 and 30 °C. The results are presented in Fig. 3 and the estimated kinetic parameters in Table 2. Fig. 3A compares the dependence of the initial reaction rate on the concentration of L-methionine for both enzymes used. It was found that the enzyme from C. atrox is inhibited by L-methionine, which was not the case for the enzyme from C. adamanteus. Kinetic constants ($V_{\text{m}}$, $K_{\text{m}}$ and $K_i$) were estimated from these experimental data. They are presented in Table 2. Both enzymes are equally as active ($V_{\text{m}}$) towards L-methionine as a substrate, and they also have quite similar affinity ($K_{\text{m}}$). Another difference between these two enzymes is presented in Fig. 3B where dependence of the initial reaction rate on the concentration of D-methionine was examined. It was found that the enzyme from C. adamanteus is inhibited by the presence of D-substrate. Inhibition constant ($K_i$) was estimated (Table 2). The enzyme from C. atrox was not inhibited by D-methionine. Product inhibition was also studied (Fig. 3C), and it was found that 2-oxo-4-methylthiobutyric acid as a reaction product inhibits both enzymes. Inhibition is somewhat higher for L-AAO from C. adamanteus which can be seen from the estimated inhibition constant ($K_{ii}^{\text{oxo}}$) presented in Table 2. Keto acids in general are competitive inhibitors [57,61,62], which was also shown in this case by the additional measurements presented in Fig. 3D where 2-oxo-4-methylthiobutyric acid inhibition type was investigated.

4.2. Batch reactor experiments with L-methionine as a substrate

Batch reactor experiments of L-methionine oxidation catalyzed by L-amino acid oxidases from C. adamanteus (Fig. 4A and C) and C. atrox (Fig. 4B and D) were carried out with and without catalase in the 0.2 M phosphate buffer pH 7.6 and 30 °C. The purpose of catalase addition is degradation of hydrogen peroxide into water and oxygen. If catalase is not added, hydrogen peroxide reacts with 2-oxo-4-methylthiobutyric acid (α-keto acids in general). The corresponding carboxylic acid (3-methylthiopropionic acid), as well as carbon dioxide (Fig. 1) are formed in this reaction of oxidative decarboxylation. The results are presented in Fig. 4. Experiments of L-methionine oxidative deamination carried out without catalase are presented in Fig. 4A and B. Fig. 4A presents the

<table>
<thead>
<tr>
<th>Parameter (unit)</th>
<th>Crotalus adamanteus</th>
<th>Crotalus atrox</th>
</tr>
</thead>
<tbody>
<tr>
<td>$V_{\text{m}}$ (mmol min$^{-1}$ g$^{-1}$)</td>
<td>0.79 ± 0.02</td>
<td>0.78 ± 0.03</td>
</tr>
<tr>
<td>$K_{\text{m}}^{\text{L-met}}$ (mM)</td>
<td>0.19 ± 0.02</td>
<td>0.25 ± 0.02</td>
</tr>
<tr>
<td>$K_{\text{m}}^{\text{D-met}}$ (mM)</td>
<td>0.83 ± 0.06</td>
<td>2.04 ± 0.20</td>
</tr>
<tr>
<td>$K_{\text{i}}^{\text{L-met}}$ (mM)</td>
<td>–</td>
<td>19.27 ± 1.26</td>
</tr>
</tbody>
</table>

Fig. 3. L-AAO kinetics ($\gamma$-AAO (Crotalus adamanteus) = 0.100 g L$^{-1}$; $\gamma$-AAO (Crotalus atrox) = 0.033 g L$^{-1}$). Initial reaction rate dependence on the concentration of (A) L-methionine, (B) D-methionine ($c_{\text{D-met}}$ = 4.60 mM), (C) 2-oxo-4-methylthiobutyric acid ($c_{\text{oxo}}$ = 4.60 mM). Experimental results: black circles, Crotalus adamanteus; white triangles, Crotalus atrox; lines, the line corresponds to the best fitting to the proposed model. (D) 2-oxo-4-Methylthiobutyric acid inhibition type L-AAO from C. adamanteus. Black circles: 0.047 mM 2-oxo; white circles: 0.186 mM 2-oxo; black squares: 0.372 mM 2-oxo; white squares: 0.651 mM 2-oxo; black triangles: 0.930 mM 2-oxo.

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results of l-methionine oxidative deamination catalyzed by l-AAO from C. adamanteus \( (\gamma_{l-AAOO} \text{C. adamanteus}) = 0.073 \text{ g L}^{-1} \), and Fig. 4B the results of the experiment catalyzed by l-AAO form C. atrox \( (\gamma_{l-AAOO} \text{C. atrox}) = 0.081 \text{ g L}^{-1} \). The concentration of 2-oxo-4-methylthiobutyric acid was quite low in these experiments due to the reaction of \( \alpha \)-keto acid with hydrogen peroxide. This reaction was simulated by the first-order kinetics (Eq. (3)). The kinetic constant \( (k_1) \) was estimated and presented in Table 3. The model parameters (kinetic Eqs. (1) or (2)) that were independently estimated earlier (from the results in Fig. 3, Table 2) were kept constant during this optimization procedure (for \( k_1 \) estimation). Since the proposed model (kinetic Eqs. (1) or (2) and (3); mass balance equations: (7) and (8), Table 1) described the data well, it could be concluded that there was no additional inhibition. Hence, it can be concluded that hydrogen peroxide present in the reaction system did not inhibit the enzymes. The developed mathematical model for this system (kinetic equations: (1) (C. adamanteus) or (2) (C. atrox); mass balance equations: (7) and (8), Table 1) describes the data well. The kinetic parameters used for the simulation are presented in Table 2 and were estimated from the initial reaction rate experiments.

4.3. Batch reactor experiments with \( \alpha \)-methionine as a substrate

Enzyme kinetics determination revealed that \( \alpha \)-methionine inhibits l-AAO from C. adamanteus. Since the inhibition constant is relatively high \( (K_{i}^{\text{D-met}} = 1.41 \text{ mM}) \), this inhibition is not of crucial concern. However, it was not neglected in mathematical modelling (Eq. (4), Table 1) since inhibition will depend on initial concentration of DL-methionine. It was expected that there would

![Fig. 4.](image-url) | l-Methionine oxidation in the batch reactor. (A) Without catalase: l-AAO from C. adamanteus; (B) without catalase: l-AAO from C. atrox; (C) repetitive batch: l-AAO from C. adamanteus; (D) repetitive batch: l-AAO from C. atrox. Black circles: l-methionine; white triangles: 2-oxo-4-methylthiobutyric acid; line: the line corresponds to the best fitting to the proposed model.

<table>
<thead>
<tr>
<th>Type of reaction</th>
<th>LAAO source</th>
<th>Parameter (unit)</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Keto-acid degradation by ( \text{H}_2\text{O}_2 )</td>
<td>C. adamanteus</td>
<td>( k_1 ) (min(^{-1}))</td>
<td>0.0596 ± 0.0025</td>
</tr>
<tr>
<td>Keto-acid degradation by ( \text{H}_2\text{O}_2 )</td>
<td>C. atrox</td>
<td>( k_1 ) (min(^{-1}))</td>
<td>0.0573 ± 0.0089</td>
</tr>
<tr>
<td>Spontaneous l-methionine degradation (EMR experiment)</td>
<td>C. adamanteus</td>
<td>( k_1 ) (min(^{-1}))</td>
<td>0.0012 ± 0.0003</td>
</tr>
<tr>
<td>Enzyme deactivation in EMR with catalase (50 kDa)</td>
<td>C. adamanteus</td>
<td>( k_2 ) (min(^{-1}))</td>
<td>0.0459 ± 0.0141</td>
</tr>
<tr>
<td>Enzyme deactivation in EMR with catalase (5 kDa)</td>
<td>C. atrox</td>
<td>( k_2 ) (min(^{-1}))</td>
<td>0.0159 ± 0.00087</td>
</tr>
<tr>
<td>Enzyme deactivation in EMR without catalase (5 kDa)</td>
<td>C. atrox</td>
<td>( k_4 ) (min(^{-1}))</td>
<td>0.0061 ± 0.00048</td>
</tr>
<tr>
<td>Enzyme deactivation in EMR without catalase (5 kDa)</td>
<td>C. atrox</td>
<td>( k_4 ) (min(^{-1}))</td>
<td>0.0237 ± 0.00253</td>
</tr>
</tbody>
</table>
be no problem if racemate is used as a substrate. The results are presented in Fig. 5. The experiments were carried out in 0.2 M phosphate buffer pH 7.6 and 30 °C. Fig. 5A represents the results of the reaction catalyzed by l-AAO from C. adamanteus (\(Y_{L-AAO(C.\text{adamanteus})} = 0.106 \text{ g L}^{-1}\), \(Y_{\text{catalase}} = 0.12 \text{ g L}^{-1}\)), and Fig. 5B the results of the reaction catalyzed by l-AAO from C. atrox (\(Y_{L-AAO(C.\text{atrox})} = 0.122 \text{ g L}^{-1}\), \(Y_{\text{catalase}} = 0.12 \text{ g L}^{-1}\)). The substrate was added two times and products were not charged out of the reactor. In both experiments (for both enzymes) similar results were obtained and 100% conversion was achieved. The developed mathematical model described the data well. Kinetic parameters presented in Table 2 were used for the simulation. By using amino acid oxidases as catalysts it was possible to obtain pure D-methionine from the corresponding racemate.

4.4. Continuously operated enzyme membrane reactor experiments

The results of l-methionine oxidation in the continuously operated enzyme membrane reactor are presented in Fig. 6. All experiments were carried out at the constant residence time of 176.5 min (the highest possible with the experimental set-up used), 30 °C and in 0.2 M phosphate buffer pH 7.6. The reactor set-up is presented in Fig. 2. The main difference between the batch reactor and the continuously operated enzyme membrane reactor is that this reactor-type works under pressures higher than atmospheric and that a membrane separates the enzyme from the product and also retains the enzyme in the reactor during the experiment. In both cases magnetic stirring is used to ensure the homogeneity of the reaction mixture. Fig. 6A and B presents the results of l-methionine oxidation catalyzed by l-AAO from C. adamanteus in the continuously operated enzyme membrane reactor (\(c_{\text{l-met,0}} = 9.99 \text{ mM}, Y_{\text{catalase}} = 0.2 \text{ g L}^{-1}\) every 2 h, \(t = 2.97 \text{ h}\)). (A) 50 kDa membrane, \(Y_{L-AAO} = 0.11 \text{ g L}^{-1}\) (white circles: l-amino acid oxidase activity). (B) 5 kDa membrane, \(Y_{L-AAO} = 0.066 \text{ g L}^{-1}\). l-Methionine oxidation catalyzed by l-AAO from C. atrox in the continuously operated enzyme membrane reactor (\(c_{\text{l-met,0}} = 9.39 \text{ mM}, t = 2.97 \text{ h}, 5 \text{ kDa membrane}) (C) \(Y_{L-AAO} = 0.12 \text{ g L}^{-1}\), \(Y_{\text{catalase}} = 0.2 \text{ g L}^{-1}\) every 2 h. (D) \(Y_{L-AAO} = 0.04 \text{ g L}^{-1}\), without catalase addition. Black circles: l-methionine; white triangles: 2-oxo-4-methylthiobutyric acid; line: the line corresponds to the best fitting to the proposed model.
oxidation with l-AAO from C. adamanteus as catalyst. Two different membranes were considered: cut-off 50 and 5 kDa. Both enzymes are homodimers with the molecular weight 110 kDa (C. atrox [63,64]) and 140 kDa (C. adamanteus [42,64]). Besides substrate and product concentration, enzyme activity was also followed during the experiments. Excess catalase was added every 2 h in the reactor to avoid 2-oxo-4-methylthiobutyric acid degradation and possible protein damage caused by hydrogen peroxide. During the first 77 min of the experiment (Fig. 6A) 2-oxo-4-methylthiobutyric acid concentration increases. After that it starts to drop. This was due to the enzyme deactivation which was proven by the activity measurements (Fig. 6A). Enzyme deactivation was described by the kinetic model of the first-order. The deactivation was practically complete after 23rd hour. It was assumed that the deactivation might be happening due to the washing out of FAD. FAD is a coenzyme which is non-covalently bound to the structure of l-amino acid oxidase [42]. To test this hypothesis FAD was added to the reaction solution. Nevertheless, there was no positive change in the conversion. Evidently, washing out of FAD was not the problem. Fresh enzyme was added to the reactor after 26 h. Once again, besides concentrations of reactant and product, enzyme activity was measured simultaneously and independently by the enzyme assay. The enzyme sample was taken directly from the reactor through the injection septum (Fig. 2). It was found that the enzyme was very active at the beginning (26th hour), but its activity rapidly dropped during the next 2 h. Even though the enzyme was found to be very active, there was no change in the product concentration. l-AAO deactivation constant was estimated from this experimental data and is presented in Table 3 ($k_d$). Besides the deactivation constant, l-methionine spontaneous degradation was considered. Even though the reaction solution was kept on ice during the experiment, slow degradation occurs. It was assumed that it occurs by the kinetics of the first-order and the kinetic constant was estimated ($k_g$, Table 3). To rule out enzyme dissociation a smaller cut-off membrane (5 kDa) was used in the other experiment (Fig. 6B). Since enzyme deactivation occurred in this experiment as well, it was concluded that the membrane was not an issue, even though the deactivation rate appears to be lower with the lower cut-off membrane ($k_d = 0.0159 \pm 0.0007 \text{ min}^{-1}$). The developed mathematical model (kinetic model: 1, 5, 6, mass balances: 10, 11, Table 1) described the data very well. The kinetic parameters presented in Table 2 were kept constant, while the enzyme deactivation constant ($k_d$) and methionine degradation constant ($k_g$) were estimated.

Fig. 6C and D presents the results of l-methionine oxidation catalyzed by l-AAO from C. atrox. The first experiment (Fig. 6C) was carried out with catalase (added every 2 h). The similar enzyme behaviour was observed as in the case of l-AAO from C. adamanteus (Fig. 6A). After the third hour of the experiment maximum l-methionine conversion was achieved ($\approx 40\%$). The product concentration started to drop after that. Complete enzyme deactivation occurred after approximately 18 h. The second addition of l-AAO (the same concentration as in the first part of the experiment) resulted in the increase of product concentration up to 17%, which is much less than after the first enzyme addition. Complete l-AAO deactivation was faster ($k_d = 0.0226 \pm 0.00179 \text{ min}^{-1}$) in this case and it occurred after 9 h (Fig. 6C).

An experiment presented in Fig. 6D was carried out without the addition of catalase. Lower enzyme concentration was used than in the experiment presented in Fig. 6C, however, the enzyme deactivation constants can be compared between these two experiments. It was found that the absence of catalase causes quicker deactivation (nearly fourfold). The enzyme deactivation constants are presented in Table 3.

The most common problem with CSTR’s which was discussed by various authors and in different reaction systems [55,56,58] is high enzyme consumption. It is therefore necessary to consider other reactor types, like repetitive batch, or fed-batch. Another possibility is to stabilize the enzyme by some of the genetic engineering methods, or by immobilization. With immobilized enzyme the column reactor as continuously operated reactor could be considered. It is known [29] that l-AAOs are enzymes that require oxygen for their activity. Low-oxygen concentration in the completely closed continuously operated enzyme membrane reactor (reactor set-up presented in Fig. 2) could be the explanation for enzyme deactivation in this reactor type, since this reactor type does not allow continuous oxygen supply. Hence, the continuously operated enzyme membrane reactor with the continuous oxygen supply should be also considered.

5. Conclusions

The results show that both l-amino acid oxidases from snake venom (C. adamanteus and C. atrox) exhibit similar behaviour. Namely, they were found to be equally as active ($V_{\text{m}}$) and equally as specific ($K_{\text{m}}$) to l-methionine as a substrate. Nevertheless, they also have certain differences in their inhibition properties. l-Amino acid oxidase from C. adamanteus was found to be inhibited by o-methionine, which was not the case for l-AAO from C. atrox. l-AAO from C. atrox was found to be inhibited by substrate (l-methionine), which was not the case for l-AAO from C. adamanteus. The product inhibition was somewhat higher in the case of l-AAO from C. adamanteus.

Both studied enzymes were found to be equally successful in the reaction of l-methionine oxidation and no enzyme deactivation occurred in the repetitive batch experiment. Batch experiments that were carried out without the addition of catalase showed that there was no hydrogen peroxide inhibition in the observed reaction time. Amino acid oxidases were found to be successful in the process of racemate resolution (repetitive batch experiments with racemate). 100% l-Methionine conversion was achieved in the repetitive batch experiments where racemate was used as a substrate. Continuously operated enzyme membrane reactor was not appropriate reactor type for this kind of reaction due to the high deactivation rate of the enzymes. Since repetitive batch experiments showed good results, it seems that it would be appropriate reactor type for amino acid oxidation and racemate resolution with soluble enzymes. It could be possible that the experiments in continuously operated column reactor with immobilized enzymes could match the results of the repetitive batch experiments.

Acknowledgment

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Appendix A. List of symbols

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>$c$</td>
<td>substrate and products concentration (mM)</td>
</tr>
<tr>
<td>$\omega$</td>
<td>o-methionine</td>
</tr>
<tr>
<td>$K_{\text{m}}$</td>
<td>Michaelis–Menten constant (mM)</td>
</tr>
<tr>
<td>$K_i$</td>
<td>inhibition constant (mM)</td>
</tr>
<tr>
<td>$r$</td>
<td>reaction rate (mmol min$^{-1}$ L$^{-1}$)</td>
</tr>
<tr>
<td>$V_{\text{m}}$</td>
<td>maximal reaction rate (mmol min$^{-1}$ g$^{-1}$)</td>
</tr>
<tr>
<td>$\gamma$</td>
<td>concentration (g L$^{-1}$)</td>
</tr>
<tr>
<td>2-oxo</td>
<td>2-oxo-4-methylthiobutyric acid</td>
</tr>
</tbody>
</table>

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