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Minimization of by-product formation during D-amino acid oxidase catalyzed racemate resolution of D/L-amino acids

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Abstract

D-Amino acid oxidase (DAAO, EC 1.4.3.3) was applied to biotransformations of D-methionine or D-phenylalanine to the corresponding α-keto acids starting with the racemic mixtures as substrates. Hydrogen peroxide is formed as by-product in the DAAO-catalyzed reaction. This reactive species is disadvantageous for the racemate resolution process due to the chemical decarboxylation of the α-keto acids as well as the inactivation of the DAAO. To immediately remove the generated H_2O_2 during the biotransformations three different commercially available catalases (bovine liver, *Aspergillus niger* or *Micrococcus lysodeikticus*) were tested. By external addition of catalase from *Micrococcus lysodeikticus* an α-keto acid yield of 100% was obtained after complete conversion of the D-phenylalanine. The space-time yield of the α-keto acid production increased up to 8.26 g l^{-1} h^{-1} compared to the process without addition of catalase (0.14 g l^{-1} h^{-1}). Another observation was a relationship between Δ-redoxpotential and H_2O_2 concentration which should be the basis for an automated catalase feeding in the amino acid resolution process using DAAO in the future.
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Keywords: D-Amino acid oxidase; Catalase; Racemate resolution; α-Keto acid; Redoxpotential

1. Introduction

The enantioselective flavoprotein D-amino acid oxidase (DAAO, EC 1.4.3.3) catalyzes the oxidative deamination of a wide range of D-amino acids to their corresponding imino acids, which undergo a non-enzymatic hydrolysis to the respective α-keto acids and ammonia. The re-oxidation of the coenzyme flavin adenine dinucleotide (FAD) depends on oxygen which acts as an electron-acceptor and is reduced to hydrogen peroxide during the reaction (Fig. 1).

DAAOs are widely spread in nature. The enzyme is found to be ubiquitous in eukaryotes and is detected in a number of microbial organisms [1]. Due to its enantioselectivity and its broad substrate spectrum DAAO is applied to diverse biotechnological processes. Generally, proteinogenic D-amino acids especially hydrophobic alkyl and aryl D-amino acids are accepted as substrates by yeast DAAOs [2]. The stereospecifity of DAAO is absolute, no activity versus L-amino acids was ever detected. The tightly bound cofactor favors the industrial application of DAAOs

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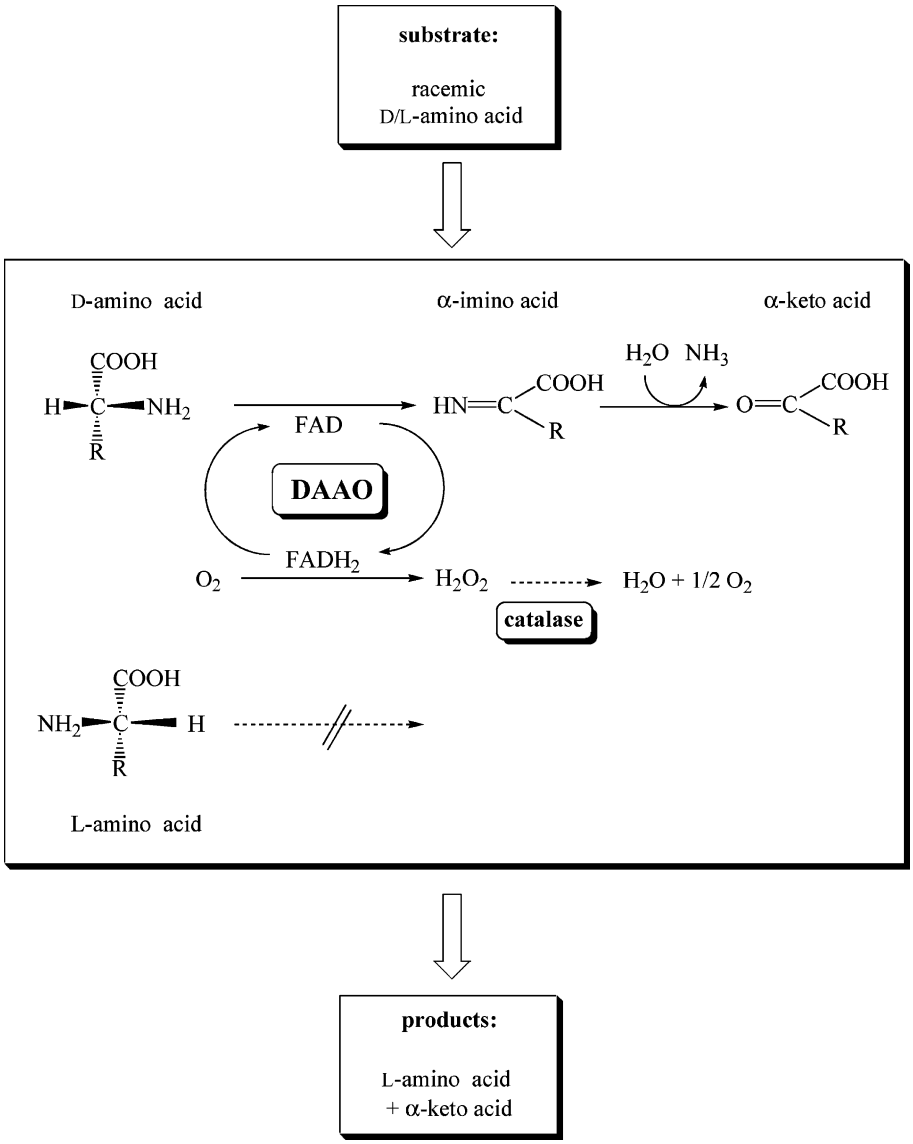

Fig. 1. Reaction scheme for the production of pure L-amino acids and α-keto acids applying D-amino acid oxidase.

from yeasts, since it is not necessary to add FAD during biotransformation processes. The availability of large amounts of DAAO by fermentation is another advantage of yeast DAAOs in comparison with DAAOs from non-microbial sources such as porcine kidney. In industrial processes either whole, permeabilized and crossed-linked cells or immobilized DAAO are used since these biocatalysts are re-usable, their operational stability is increased and downstream processing of the products is simplified [3].

To date the most important industrial application of DAAO is the production of 7-aminocephalosporanic acid (7-ACA) from cephalosporin-C (Ceph-C) [4]. DAAO serves in this two-step enzymatic process as the first biocatalyst and converts Ceph-C to α-keto-adipyl-7-ACA which is chemically decarboxylated

by the DAAO-generated H_2O_2 to give glutaryl-7-ACA. Subsequently in the enzymatic second step glutaryl-7-ACA is deacylated by glutaryl-7-ACA acylase and 7-ACA is produced. Another application of DAAO is the production of pure L-amino acids through racemate resolution [2]. Chemo-enzymatic processes (DAAO plus sodium borohydride) were also examined in case of the production of L-proline and L-pipecolate [5]. Optically pure L-amino acids are utilized for instance as starting material for pharmaceuticals or as feed additives. The racemate resolution via DAAO has the advantage of the simultaneously production of α-keto acids which are also useful products as food additives for people with chronic uremia [6].

As mentioned above another product of the DAAO-catalyzed reaction is hydrogen peroxide (see Fig. 1). In vivo H_2O_2 is converted to water and molecular oxygen by catalase (E.C. 1.11.1.6), which co-exists with DAAO in the peroxysomes or microsomes of cells and tissues. The industrial used DAAO-preparations possess only traces of catalase activities in order to allow the necessary chemical decarboxylation reaction for the production of 7-ACA (see above). Whilst the formation of hydrogen peroxide is desired in the process of 7-ACA production [7], this reactive side product is absolutely disadvantageous for the process of L-amino acid and α-keto acid production mainly due to decarboxylation of the α-keto acids leading to the corresponding carboxylic acids. Additionally, hydrogen peroxide has strong denaturating effects on proteins and therefore influences the operational stability of the DAAO.

Besides chemical methods to remove H_2O_2 [8], or cost-intensive co-immobilization of catalase [6], which both are not efficient from the applied point of view, addition of free catalase might be the method of choice, since catalase is a technical enzyme which is available in large quantities [9,10]. In our studies, bioconversion experiments with and without external addition of catalase were performed with a DAAO-immobilizate from *Trigonopsis variabilis* CBS 4095. The influence of different commercially available catalases on the degree of H_2O_2 degradation and α-keto acid yield was investigated. Additionally, the process stability of the examined catalases was compared. An appropriate sensor method was seeked in order to establish an automated process for the L-amino acid and α-keto acid production in future.

2. Experimental

2.1. Materials

2.1.1. Chemicals

All chemicals were commercially available and reagent grade. The substances were purchased from Fluka Chemie (Switzerland), Sigma–Aldrich (Germany), Roche (Germany) or Merck (Germany). 3-Methylthiopropionic acid was acquired from Chemos GmbH, (Germany).

2.1.2. Enzymes

DAAO-immobilizate (*Trigonopsis variabilis* CBS 4095) was a gift from BC Biochemie GmbH (Austria). Catalases from bovine liver and *Micrococcus lysodeikticus* were purchased from Sigma–Aldrich (Germany). Catalase from *Aspergillus niger* was a gift from Genencor International (USA). Peroxidase from horseradish was obtained from Roche (Germany).

2.2. Enzyme assays

2.2.1. DAAO assay

The activity of DAAO was determined according to a slightly modified peroxidase-*o*-dianisidine assay [11]. The reaction mixture contained three solutions: solution 1 consisting of 6.62 mM *o*-dianisidine 2 HCl, 30 kU/l peroxidase and 50% (v/v) glycerol, solution 2 contained 50 mM D-amino acid in potassium phosphate buffer (50 mM, pH 8) and solution 3 contained 50 mM potassium phosphate buffer (pH 8, oxygen saturated). A mixture of solution 1 (250 μl), solution 2 (350 μl) and solution 3 (390 μl) was pre-incubated at 30 °C for 5 min in a thermomixer (Thermomixer comfort, Eppendorf, Germany). The reaction was initiated by adding 10–20 mg wet DAAO-immobilizate. After 1 min, the reaction was stopped by adding 500 μl H_2SO_4 (30%) and the mixture was centrifuged (Biofuge pico, Heraeus, Germany) for 3 min at 13,000 rpm. The clear supernatant was measured spectrophotometrically at 540 nm (Ultrospec 3000, Amersham Pharmacia, BioTec, Germany) and DAAO-activity was calculated by using a H_2O_2 calibration curve. In order to determine the dry-weight of the immobilizate, aliquotes were dried overnight at 95 °C. One nkat of DAAO corresponds to the formation of 1 nmol H_2O_2 per second.

2.2.2. Catalase assay

The pre-incubated reaction mixture contained 100 μl of 0.1 mM H_2O_2 and 630 μl of 50 mM potassium phosphate buffer (pH 8, oxygen saturated). The reaction was started by adding 20 μl enzyme solution. After 1 min 250 μl of solution 1 (see DAAO assay) was added to the reaction mixture. The reaction mixture was acidified with 500 μl H_2SO_4 (30%) and the formed dye was detected as mentioned above.

2.3. Determination of hydrogen peroxide

Hydrogen peroxide was determined with the above described peroxidase-*o*-dianisidine assay (see DAAO assay) using a H_2O_2 calibration curve (0–0.1 mM).

2.4. Determination of D/L*-amino acids, α-keto acids and carboxylic acids*

The progress of the bioconversion reactions was followed by RP-HPLC (Thermoquest GmbH, Germany) and proved by standards. Measurements of amino acids and corresponding α-keto acids and carboxylic acids were performed on a 3 μm Grom-Sil ODS-3 column (125 mm × 4.6 mm i.d., Grom GmbH, Germany) at a flow-rate of 1 ml min^{-1} and a fixed wavelength of 210 nm. Mobile phase A, consisting of 20 mM KH_2PO_4 was titrated to pH 4.0 with phosphoric acid. Mobile phase B was methanol. After injection the sample for determination of D/L-methionine, 4-methylthio-2-oxobutyric acid and 3-methylthiopropionic acid, the column was equilibrated with phase A for 1 min, then A decreased to 80% at 3 min and held up to 8 min. The gradient was returned to 100% A at 9 min and the initial conditions were restored for the following 6 min. A slightly modified gradient system was used for the analysis of D/L-phenylalanine, phenylpyruvic acid and phenylacetic acid: 80% of phase A was held up to 5 min, then decreased to 60% at 10 min and held up to 12 min. The gradient was returned to 100% A at 15.5 min and the primary conditions were restored for the next 1.5 min. Aliquotes of 20 μl of the samples (see Section 2.5) were injected. Retention times of D/L-methionine, 4-methylthio-2-oxobutyric acid and 3-methylthiopropionic acid were 2.3, 5.1 and 7.4 min. Retention times of D/L-phenylalanine, phenylpyruvic acid and phenylacetic acid were 6.9, 9.6 and 14.1 min.

2.5. Conversion experiments

Biotransformations were performed in a continuously stirred tank reactor (B. Braun, Germany, modified) with 320 ml operating volume. The pH-controlled reaction mixture (racemic amino acid, at saturating concentration; methionine: 270 mM, phenylalanine: 100 mM) was mechanically stirred (400 rpm), aerated with pure oxygen (3.1 vvm) and kept at 30 °C. In order to start the reaction, immobilized DAAO (40 g wet immobilizate corresponding to 3200 nkat for biotransformation of methionine and 20 g wet immobilizate corresponding to 1390 nkat for conversion of phenylalanine) was added and pH was automatically adjusted to 8.0 by adding 12.5% NaOH (w/v). Samples (0.5–1 ml) were filtered and analyzed by RP-HPLC in order to determine substrate and product concentrations. Additional samples were taken and their H_2O_2 concentration was quantified immediately. Furthermore, pO_2 (oxygen-electrode, Mettler-Toledo, Switzerland) and redoxpotential (redox-electrode InLab 501, Reference system Ag/AgCl, Mettler-Toledo) were monitored during the biotransformations. After complete substrate conversion the solution was filtered and the immobilized enzyme was washed with phosphate buffer. Enzyme activities of the DAAO-immobilizate and the catalase (if existent) were compared before and after every experiment.

3. Results and discussion

3.1. Biotransformations without external addition of catalase

Biotransformations of methionine and phenylalanine with DAAO-immobilizate in absence of external catalase were performed under standard conditions (see Section 2). The results are shown in Table 1.

By applying the two substrates a quantitative difference concerning by-product formation was observed during biotransformations. After complete D-methionine conversion 20% of the maximum possible α-keto acid yield was detected. The further product represented mainly the corresponding carboxylic acid (3-methylthiopropionic acid). The yield of the corresponding α-keto acid with phenylalanine

Table 1
Bioconversion-experiments of methionine or phenylalanine *without* addition of catalase and methionine-biotransformations *with* addition of different quantities of catalases from *Aspergillus niger*

Parameter	Substrate			
	D/L-Methionine			D/L-Phenylalanine
Initial relative catalase-activity in relation to DAAO-activity	–	1.4	9.7	–
α-Keto acid (%)	20[a]	52[a]	73[a]	5[b]
By-product (%)	80[c]	48[c]	27[c]	95[d]
Space-time yield of α-keto acid ($g\,l^{-1}\,h^{-1}$)	1.45	3.39	4.77	0.14
Total conversion time (min)	205	190	150	120
Residual catalase-activity after bioconversion (%)	–	70	81	–
Residual DAAO-activity after bioconversion (%)	100	94	96	100

Further conditions: pH 8, 30 °C, 400 rpm, $V = 320$ ml, aeration with pure oxygen (3.1 vvm) in a CSTR. Initial concentrations: D/L-methionine: 270 mM, D/L-phenylalanine: 100 mM.
[a] 4-Methylthio-2-oxobutyric acid.
[b] Phenylpyruvic acid.
[c] Mainly 3-methylthiopropionic acid.
[d] Phenylacetic acid.

as substrate was only 5%. Therefore, the nature of the amino acid side chain seems to influence the non-enzymatic α-decarboxylation reaction by H_2O_2.

Although hydrogen peroxide was detectable throughout the entire biotransformations with both substrates, DAAO-activity of the immobilizate remained constant during the reactions. When whole permeabilized and stabilized cells from *Trigonopsis variabilis* DSM 70714 were used, a loss of 20% of their DAAO-activity after one biotransformation-cycle with methionine as substrate was observed under otherwise similar conditions [12].

3.2. Biotransformations of methionine with external addition of catalase

Conversions of methionine with DAAO-immobilizate in presence of different quantities of catalase from *Aspergillus niger* were carried out (see Table 1). With 1.4-fold excess of catalase-activity in relation to DAAO-activity the α-keto acid yield was increased up to 52% which was in comparison with the yield reached in absence of catalase (20%) a 2.6-fold enhancement. A further improvement of the α-keto acid yield by 21% was reached with 9.7-fold excess of catalase. The resulting space-time yields of the α-keto acid production were 3.39 and 4.77 $g\,l^{-1}\,h^{-1}$, respectively. After total substrate consumption, a residual DAAO-activity of 94–96% was observed.

3.3. Kinetics and influence of different catalases on racemate resolution of D/L-phenylalanine

In order to further enhance the α-keto acid yield catalases from *Aspergillus niger*, *Micrococcus lysodeikticus* and bovine liver were examined in biotransformations of phenylalanine. The results are presented in Table 2. DAAO and catalase were added in a constant activity ratio of 1:10 before starting the conversion. The catalases from *Aspergillus niger* and bovine liver showed a similar behavior regarding phenylpyruvic acid yield (about 80%) and space-time yield (6.35–6.45 $g\,l^{-1}\,h^{-1}$). Using the catalase from *Micrococcus lysodeikticus* the formation of by-product could be completely avoided and 100% phenylpyruvic acid yield was achieved. This was confirmed by a constant massbalance by HPLC analysis. The superior result using the *Micrococcus*-catalase is probably due to a lower K_m for hydrogen peroxide in comparison to the other both catalases tested. This favours the industrial application of the immobilized DAAO plus free catalase from *Micrococcus lysodeikticus* in the racemate resolution of amino acids, since this high degree of purity decreases the costs of down-stream processing of the α-keto acid enormously. The space-time yield of the phenylpyruvic acid was increased 60-fold (8.26 $g\,l^{-1}\,h^{-1}$) in comparison to the biotransformations without external addition of catalase (0.14 $g\,l^{-1}\,h^{-1}$).

Table 2
Bioconversion-experiments of phenylalanine with catalases of different origin

Parameter	Origin of catalase		
	Bovine liver	*Aspergillus niger*	*Micrococcus lysodeikticus*
Phenylpyruvic acid (%)	81	83	100
Phenylacetic acid (%)	19	17	0
Space-time yield of α-keto acid ($g\,l^{-1}\,h^{-1}$)	6.05	6.23	8.26
Total conversion time (min)	60	70	55
Residual catalase-activity after bioconversion (%)	66	88	55

Relative catalase-activity in relation to DAAO-activity was 10:1. Conditions: see legend of Table 1.

The residual catalase activities after complete substrate conversion (Table 2) indicated that the enzyme from the fungus *Aspergillus niger* showed the highest operational stability (88% residual catalase-activity after bioconversion) which corresponds to reported literature data [13].

3.4. Relationship between Δ-redoxpotential and H_2O_2 concentration

In order to investigate whether the redoxpotential could be an appropriate control parameter for an automatic feeding of catalase in the future, this parameter was monitored during all biotransformations. The profiles of the redoxpotential and the hydrogen peroxide formation of a phenylalanine biotransformation are depicted in Fig. 2. In this experiment, catalase was added 20 min after DAAO. The hydrogen peroxide concentration raised after addition of the immobilizate. After catalase was added to the reaction mixture, both hydrogen peroxide concentration and redoxpotential decreased immediately. Thus, a relationship of the Δ-redoxpotential and the H_2O_2 concentration/catalase-activity is recognizable. Current investigations will prove whether the redoxpotential, as a complex parameter, is suitable for controlling an automated feeding of catalase.

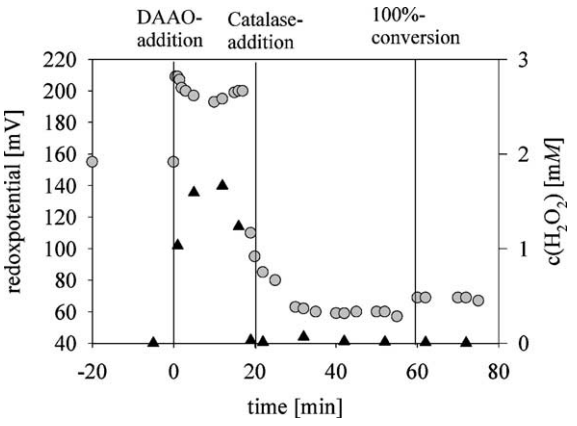

Fig. 2. Bioconversion of phenylalanine *with external addition* of catalase from bovine liver (10-fold excess in relation to DAAO-activity). Catalase was added 20 min after the addition of DAAO. Redoxpotential (●) and H_2O_2 concentration (▲).

4. Conclusions

The immobilized form of DAAO is an appropriate biocatalyst for industrial applications due to its high operational stability. In combination with catalase from *Micrococcus lysodeikticus* by-product formation can be completely avoided in the process of racemate resolution of D/L-amino acids and, in relation to the amount of D-amino acid, a 100% of the α-keto acid yield was obtained. The resulting mixture of L-amino acids and α-keto acids can easily be purified and thus, two commercially attractive products arise in this racemate resolution process.

Furthermore, a process optimization through an automatic feeding of catalase based on the redoxpotential as control parameter by applying a physico-chemical electrode (redoxelectrode) is possibly feasible and also profitable with regard to avoid excess of external added catalase.

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