Production of a New \textit{\textalpha-}-Amino Acid Oxidase from the Fungus \textit{Fusarium oxysporum}

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The fungus \textit{Fusarium oxysporum} produced a \textit{\textalpha-}-amino acid oxidase (EC 1.4.3.3) in a medium containing glucose as the carbon source and ammonium sulfate as the nitrogen source. The specific \textit{\textalpha-}amino acid oxidase activity was increased up to 12.5-fold with various \textit{\textalpha-}amino acids or their corresponding derivatives as inducers. The best inducers were \textit{\textalpha-}alanine (2.7 \textmu kat/g of dry biomass) and \textit{\textalpha-}3-aminobutyric acid (2.6 \textmu kat/g of dry biomass). The addition of zinc ions was necessary to permit the induction of peroxisomal \textit{\textalpha-}amino acid oxidase. Bioreactor cultivations were performed on a 50-liter scale, yielding a volumetric \textit{\textalpha-}amino acid oxidase activity of 17 \textmu kat liter\(^{-1}\) with \textit{\textalpha-}alanine as an inducer. Under oxygen limitation, the volumetric activity was increased threefold to 54 \textmu kat liter\(^{-1}\) (3,240 \textmu U liter\(^{-1}\)).

\textit{\textalpha-}Amino acid oxidases (\textit{\textalpha-}A) (EC 1.4.3.3) catalyze the enantioselective oxidation of a broad variety of \textit{\textalpha-}amino acids to their corresponding \textit{\textalpha-}amino acids, which spontaneously hydrolyze to \textit{\textalpha-}keto acids and ammonium. These enzymes are useful in several areas of biochemistry and biotechnology (for a review, see reference 8). Most important are the applications in qualitative and quantitative analyses in either a soluble (12) or immobilized (11, 19) manner, the oxidation of cephalosporin C (4, 24, 27), the enantioselective conversion of \textit{\textalpha-}contaminated \textit{\textalpha-}amino acid solutions (6, 22) or racemic mixtures (15), and the production of keto acids (3, 5).

\textit{\textalpha-}A have been found in peroxisomes and microsomes of many eucaryotic cells or tissues (16, 28). However, up to now the yeasts \textit{Trigonopsis variabilis} (13, 17) and \textit{Rhodotorula gracilis} (23, 25) have seemed to be the only microbial \textit{\textalpha-}AO producers with satisfactory yields of \textit{\textalpha-}AO for commercial purposes. Although the gene of pig kidney \textit{\textalpha-}AO was expressed in \textit{Escherichia coli} recently (26) and the three-dimensional structure of this enzyme was solved by two groups (20, 21), pig kidney \textit{\textalpha-}AO is not appropriate for biotechnological processes due to its low binding constant for flavin adenine dinucleotide and its operational instability (8).

In a screening process for new microbial \textit{\textalpha-}AO, the fungus \textit{Fusarium oxysporum} was isolated from a soil sample in Lower Saxony (Germany) (10). It has been deposited at the Deutsche Sammlung von Mikroorganismen, Braunschweig, Germany, as DSM 12646. Its \textit{\textalpha-}AO accepts a broad range of \textit{\textalpha-}amino acids, including cephalosporin C, as substrates (7, 10). The enzyme was purified and characterized (9). In order to find the parameters important for \textit{\textalpha-}AO production, we tested different kinds of inducers, enantiomeric ratios, and inducer concentrations as well as the influence of oxygen supply during cultivation in a bioreactor.

\textbf{Culture conditions (shaking flasks).} \textit{F. oxysporum} was cultivated in 500-ml flasks with baffles at 30°C and 100 rpm. The standard medium (120 ml) contained glucose (18 g liter\(^{-1}\)), \textit{K}_2\text{HPO}_4 (4 g liter\(^{-1}\)), (NH\textsubscript{4})\text{SO}_4 (4 g liter\(^{-1}\)), yeast extract (4 g liter\(^{-1}\)), and metal salts (\textit{MgSO}_4\cdot7\text{H}_2\text{O} [1 g liter\(^{-1}\)], \textit{CaCl}_2\cdot2\text{H}_2\text{O} [0.5 g liter\(^{-1}\)], \textit{H}_3\text{BO}_3 [0.1 g liter\(^{-1}\)], \textit{NaMoO}_4 [0.04 g liter\(^{-1}\)], \textit{ZnSO}_4\cdot7\text{H}_2\text{O} [0.04 g liter\(^{-1}\)], \textit{CuSO}_4\cdot7\text{H}_2\text{O} [0.045 g liter\(^{-1}\)], \textit{FeSO}_4\cdot7\text{H}_2\text{O} [0.025 g liter\(^{-1}\)]). The \textit{pH} was set to 7.0 without further adjustment during cultivation.

The induction of \textit{\textalpha-}AO was investigated by adding several \textit{\textalpha-}amino acids and \textit{\textalpha-}amino acid derivatives at concentrations of 5 and 30 mM, depending on whether the particular compound was a substrate for the \textit{\textalpha-}AO reaction (30 mM) or not (5 mM). The \textit{\textalpha-}AO activity of the cells was monitored during cultivation by taking samples periodically (every 3 h) and preparing a crude enzyme solution. For that, cells were harvested by centrifugation, suspended in buffer, and disrupted with a bead mill (13). After centrifugation, the clear supernatant was analyzed. \textit{\textalpha-}AO activity was determined by the peroxidase-\textit{\textalpha-}dianisidase assay (1, 7).

The highest \textit{\textalpha-}AO activities were usually measured at the beginning of the stationary growth phase. The data are summarized in Table 1 (means of two separate cultivations; the maximum deviation was 7%). The best inducers for \textit{\textalpha-}AO activity in \textit{F. oxysporum} were \textit{\textalpha-}alanine (yield of dry biomass, 12.2 g liter\(^{-1}\)) and \textit{\textalpha-}3-aminobutyric acid (yield of dry biomass, 10.5 g liter\(^{-1}\)). Compared to the activity in cells cultivated without any inducer, the specific \textit{\textalpha-}AO activity was increased about 13-fold. Hörner et al. showed that with \textit{N-carbamoyl-D-alanine} as an inducer instead of \textit{\textalpha-}alanine, the yeast \textit{T. variabilis} produced up to 4.2-fold more \textit{\textalpha-}AO (13). For \textit{F. oxysporum}, this inducer was not suitable (Table 1). In general, modification of the carboxy or amino group of \textit{\textalpha-}amino acids resulted in lower specific \textit{\textalpha-}AO activities than unmodified \textit{\textalpha-}amino acids. When we examined just the results obtained with modified amino acids, protection of the amino group resulted in higher levels than protection of the carboxyl group. When we examined protein-forming amino acids as inducers for \textit{\textalpha-}AO in \textit{F. oxysporum}, the best results were obtained with inducers having small hydrophobic groups. The yeast \textit{T. variabilis} showed a similar correlation between \textit{\textalpha-}AO induction and the character of the \textit{\textalpha-}amino acid residue (13, 18).

The dependence of \textit{\textalpha-}AO activity in \textit{F. oxysporum} on \textit{\textalpha-}alanine as well as \textit{\textalpha-}3-aminobutyric acid concentrations was investigated (range, 0 to 150 mM). With \textit{\textalpha-}alanine, the specific \textit{\textalpha-}AO activity increased continuously with increasing inducer concentration. Thus, the maximum \textit{\textalpha-}AO activity of approximately 4 \textmu kat g of dry biomass\(^{-1}\) was measured with 150 mM \textit{\textalpha-}alanine (\textit{K}_\text{inducer} = 26 mM). With \textit{\textalpha-}3-aminobutyric acid, the
TABLE 1. Compounds tested for induction of D-AO in _F. oxysporum_\(^a\)

<table>
<thead>
<tr>
<th>Inducer</th>
<th>Inducer concn (mM)</th>
<th>D-AO activity (μkat/g of dry biomass)</th>
<th>Induced activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0.2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>D-Alanine</td>
<td>30</td>
<td>2.7</td>
<td>100</td>
</tr>
<tr>
<td>DL-3-Aminobutyric acid</td>
<td>10</td>
<td>2.6</td>
<td>96</td>
</tr>
<tr>
<td>DL-α-Aminobutyric acid</td>
<td>30</td>
<td>1.8</td>
<td>64</td>
</tr>
<tr>
<td>DL-Alanine methyl ester</td>
<td>10</td>
<td>1.3</td>
<td>44</td>
</tr>
<tr>
<td>D-Methionine</td>
<td>30</td>
<td>1.3</td>
<td>44</td>
</tr>
<tr>
<td>DL-Alanine-glycine</td>
<td>10</td>
<td>1.0</td>
<td>32</td>
</tr>
<tr>
<td>D-Tyrosine</td>
<td>30</td>
<td>1.0</td>
<td>32</td>
</tr>
<tr>
<td>D-Histidine</td>
<td>30</td>
<td>0.9</td>
<td>28</td>
</tr>
<tr>
<td>D-Arginine</td>
<td>30</td>
<td>0.9</td>
<td>28</td>
</tr>
<tr>
<td>D-Valine</td>
<td>30</td>
<td>0.8</td>
<td>24</td>
</tr>
<tr>
<td>DL-Alanyl-DL-alanine</td>
<td>20</td>
<td>0.8</td>
<td>24</td>
</tr>
<tr>
<td>DL-Alanine ethyl ester</td>
<td>10</td>
<td>0.7</td>
<td>20</td>
</tr>
<tr>
<td>D-Glutamine</td>
<td>30</td>
<td>0.7</td>
<td>20</td>
</tr>
<tr>
<td>DL-3-Aminoisobutyric acid</td>
<td>10</td>
<td>0.6</td>
<td>16</td>
</tr>
<tr>
<td>D-Proline</td>
<td>30</td>
<td>0.6</td>
<td>16</td>
</tr>
<tr>
<td>L-Alanine</td>
<td>30</td>
<td>0.2</td>
<td>0</td>
</tr>
</tbody>
</table>

\(^a\) Conditions: 120 ml of standard medium (for composition, see the text), 500-ml shaking flask, 30°C, and 100 rpm.

The influence of zinc ions on D-AO induction. Without additional thoxypropane, D-cysteine, D-serine, maximum D-AO activity (approximately 3 μkat g of dry biomass−1) was obtained at about 25 mM (K\(_{\text{inducer}}\) = 2.7 mM).

An interesting finding regarding specific D-AO induction by D-alanine was obtained when various D-alanine/L-alanine ratios were used to supplement the standard medium. The concentration of D-alanine at the beginning of the cultivations was always kept at 30 mM, while the concentration of added L-alanine was varied from 0 to 100 mM. The highest production of D-AO was measured at a D-alanine/L-alanine ratio of 3:1. Without L-alanine and at a 1:1 ratio (racemate), the specific D-AO activities assayed were nearly the same and were about 50% the value obtained at a 3:1 ratio. If the L enantiomer was present in excess relative to the D enantiomer, the production decreased in inverse proportion to the excess L enantiomer. With pure L-alanine, no D-AO induction was recognized (Table 1). However, the increased D-AO production obtained in the presence of D-alanine/L-alanine ratios of >1:1 is surprising and is difficult to explain. For economic reasons, further cultivations were carried out with racemic inducers, as was done by others (13, 25).

As with the yeast _T. variabilis_ (13), there was a significant influence of zinc ions on D-AO induction. Without additional zinc sulfate in the standard medium containing glucose, ammonium sulfate, and D-alanine, no induction of D-AO was detected, although the growth of the suspension culture was as good as with additional zinc sulfate. The zinc ion concentration necessary for D-AO induction was tested in the range of 20 to 420 μM. A zinc sulfate concentration of >70 μM resulted in a constant maximum specific D-AO activity (a concentration of >280 μM caused a decrease in growth).

**Bioreactor cultivations.** _F. oxysporum_ was cultivated in a 50-liter bioreactor (Braun-Diesel Biotech, Melsungen, Germany) at 30°C with an air stream of 0.4 volume per volume of liquid per min (v/vm). The stirrer used was a three-stage Rushton turbine (400 rpm). The standard medium (see above) plus the inducer DL-alanine (80 mM) (Fig. 1) or DL-3-aminobutyric acid (20 mM) was used. Determination of the inducer concentration of the medium was done by capillary gas chromatography (CGC) analysis with a CGC-Chirasil-α-Val column (Chrompack, Frankfurt, Germany) after derivatization of the samples (6).

The data obtained for cultivation with DL-alanine as the inducer are shown in Fig. 1 (means for at least two separate samples; the maximum deviation was 5%). After 13 h of bioreactor cultivation with DL-alanine, glucose was consumed and L-alanine and D-alanine were taken up into the cells to serve as energy sources. L-Alanine was metabolized first, but D-alanine uptake had already started when about 25 mM L-alanine (ca. 60%) was still present. When D-alanine uptake began, the induction of D-AO started and continued until all the D-alanine was consumed. The specific D-AO activity reached its maximum after approximately 16 h and, after 18 h, a volumetric D-AO activity of approximately 18 μkat liter−1 was present (yield of dry biomass, 14.2 g/liter; maximum growth rate, 0.11 h−1). Compared to the results obtained in the shaking-flask experiment (51 μkat liter−1), only 35% of the volumetric activity was reached.

A bioreactor cultivation with DL-3-aminobutyric acid (20 mM) as the inducer was also performed under otherwise identical conditions (data not shown). In this case, the fungus took up the inducer after approximately 6 h, when more than 80% of the glucose was present. As expected, the induction of D-AO started at the same time. After 20 h (yield of dry biomass, 10.5 g/liter; maximum growth rate, 0.11 h−1), the maximum volumetric D-AO activity determined was 15 μkat liter−1, corresponding to 55% the value obtained in the shaking-flask experiment (27.3 μkat liter−1). **Bioreactor cultivations with limited oxygen supply.** The bioreactor cultivations resulted in lower volumetric activities than did the shaking-flask experiments with the same medium. Therefore, one major difference between the systems, oxygen supply, could have been responsible. The influence of a limited oxygen supply on the induction of D-AO was investigated by repeating the bioreactor cultivation shown in Fig. 1 with DL-alanine (80 mM). The changed parameters for the cultivation were a decrease of the stirrer speed from 400 to 300 rpm and a decrease of the gas flow rate from 0.4 to 0.2 v/vm. After 12 h, the pO2 in the medium was decreased to <10% until the stationary phase was reached, after approximately 18 h. Again, when D-alanine was taken up by the fungus, D-AO induction occurred (after approximately 14 h) and volumetric D-AO activity increased rapidly up to a maximum of 54 μkat liter−1 (after approximately 18 h). At this time, the yield of dry biomass was 14.1 g liter−1. Due to the lower oxygen supply, a lower maximum growth rate of 0.09 h−1 was estimated, compared to that in the previous cultivation (Fig. 1).

The influence of oxygen concentration on D-AO production was significant. Under oxygen limitation conditions, threefold-higher specific D-AO activity was achieved, compared to that achieved in a cultivation without this limitation. Because reoxidation of the D-AO cofactor flavin adenine dinucleotide depends on oxygen concentration, the fungus might compensate for the slower turnover of D-amino acids in the presence of low oxygen content by increasing the total production of D-AO. Thus, the “bottleneck” for growth would be widened.

The volumetric activity of 54 μkat liter−1 gained on a 50-liter scale in a stirring bioreactor under oxygen limitation is one of the highest reported for bioreactor productions of D-AO in the literature. Higher D-AO activity has only been reported for the yeast _T. variabilis_ (77 μkat liter−1) (13). The data reported by Huber et al. (14) and Biopure Corporation (2) indicated about
112 μkat liter⁻¹ (T. variabilis CSB 4095) and about 250 μkat liter⁻¹ (mutant strain of T. variabilis), respectively, but in both cases, the assays were done under conditions significantly different (substrate and oxygen concentrations) from our conditions. Thus, a comparison is not fruitful (7, 8).

The results described here establish that excellent production of D-AO from F. oxysporum is possible by bioreactor cultivation, thus enlarging the arsenal of microbial D-AO available to serve as useful tools in analysis and biotechnology.

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