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Molecular Cloning, Sequencing, and Heterologous Expression of the 
vaoA Gene from Penicillium simplicissimum CBS 170.90 Encoding Vanillyl-Alcohol Oxidase*

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Jacques A. E. Benen‡, Paloma Sánchez-Torres‡, Matthé J. M. Wagemaker‡, Marco W. Fraaije§,
Willem J. H. van Berkel§, and Jaap Visser‡‡

From *the ‡Section Molecular Genetics of Industrial Microorganisms, Wageningen Agricultural University, Dreyenlaan 2, 6703 HA Wageningen, The Netherlands and the §Department of Biochemistry, Wageningen Agricultural University, Dreyenlaan 3, 6703 HA Wageningen, The Netherlands

The cDNA encoding vanillyl-alcohol oxidase (EC 1.1.3.7) was selected from a cDNA library constructed from mRNA isolated from Penicillium simplicissimum CBS 170.90 grown on veratryl alcohol by immunochromatographic screening. The vaoA-cDNA nucleotide sequence revealed an open reading frame of 1680 base pairs encoding a 560-amino acid protein with a deduced mass of 62,915 Da excluding the covalently bound FAD. The deduced primary structure shares 31% sequence identity with the 8α-(O-tyrosyl)-FAD containing subunit of the bacterial flavocytochrome p-cresol methyl hydroxylase.

The vaoA gene was isolated from a P. simplicissimum genomic library constructed using the vaoA-cDNA as a probe. Comparison of the nucleotide sequence of the vaoA gene with the cDNA nucleotide sequence demonstrated that the gene is interrupted by five short introns.

Aspergillus niger NW156 prfF pyrA leuA cspA transformed with the pyrA containing plasmid and a plasmid harboring the complete vaoA gene including the promoter and terminator was able to produce vaoA mRNA and active vanillyl-alcohol oxidase when grown on veratryl alcohol and anisyl alcohol. A similar induction of the vaoA gene was found for P. simplicissimum, indicating that similar regulatory systems are involved in the induction of the vaoA gene in these fungi.

Introduction of a consensus ribosome binding site, AGAAGGAG, in the vaoA-cDNA resulted in elevated expression levels of active vanillyl-alcohol oxidase from the lac promoter in Escherichia coli TG2. The catalytic and spectral properties of the purified recombinant enzyme were indistinguishable from the native enzyme.

Vanillyl-alcohol oxidase (EC 1.1.3.7) is a novel type of flavoprotein oxidase that was first isolated from Penicillium simplicissimum CBS 170.90 grown on veratryl alcohol (1). The enzyme is a homo-octamer with each 65-kDa subunit harboring an 8α-(N3-histidyl)-FAD (2). Vanillyl-alcohol oxidase has a broad substrate specificity. In addition to the conversion of vanillyl alcohol to vanillin (Equation 1), the enzyme catalyzes the conversion of a wide range of phenolic compounds bearing side chains of variable size at the para-position of the aromatic ring (3, 4). Due to its broad substrate spectrum, vanillyl-alcohol oxidase may be applied in the fine chemical industry (5). Based on induction experiments, 4-(methoxymethyl)phenol has been proposed to represent the physiological substrate (6). Recently, from rapid reaction kinetics conclusive spectral evidence was obtained that the vanillyl-alcohol oxidase-mediated oxidative demethylation of 4-(methoxymethyl)phenol proceeds through the formation of a quinone-methide product intermediate (4).

In the absence of oxygen, this intermediate is stabilized in the active site of the reduced enzyme. Upon flavin reoxidation, the quinone methide of 4-(methoxymethyl)phenol readily reacts with water, yielding 4-hydroxybenzaldehyde, methanol, and hydrogen peroxide as final products.

Recently, the three-dimensional structure of vanillyl-alcohol oxidase was elucidated (7). The crystallographic analysis corroborated earlier observations (2, 8) that the vanillyl-alcohol oxidase octamer can be described as a tetramer of tightly interacting dimers. Each vanillyl-alcohol oxidase monomer consists of two domains. The larger domain creates a binding site for the ADP moiety of the FAD, whereas the smaller cap domain covers the active site that is located between the two domains. Furthermore, from the structures of several vanillyl-alcohol oxidase inhibitor complexes, it could be deduced that the shape of the active site cavity controls substrate specificity (7).

In this paper we describe the cloning, sequencing, and expression of the gene encoding vanillyl-alcohol oxidase from P. simplicissimum CBS 170.90, providing the necessary amino acid sequence information which together with the three-dimensional structure establishes the basis for future protein engineering studies.

EXPERIMENTAL PROCEDURES

Strains and Media—Growth conditions for P. simplicissimum (CBS 170.90; ATCC 90172) have been described before (6). Aspergillus niger NW156 (prfF28, leuA6, pyrA1, cspA1) a derivative of A. niger N400 (CBS 120.49) from the laboratory collection was used for transformation and was grown in minimal medium (9), pH 6.0, containing 1% (mass/volume) fructose, 0.1% (mass/volume) yeast extract, 0.02% (mass/volume) leucine, 0.02% (mass/volume) uridine, and a trace element solution according to Ref. 10. Escherichia coli DH5α F’ [F 18F119 thiD1 supE44 thi-1 recA1 gyrA1 [NalR] relA1 ΔlacIZY- argF1U169 deor F80lacZ15 lacZ15 (11) was used for cloning throughout. λ phages were propagated in E. coli LE392 [F e4- (McrA-) hadR17 (rK mK) supE44 supF58 ΔlacZYA galK2 galT22 metB1 trpR55] (12). E. coli BB4 [F’ lacFZAM15 proAB Tn10 (tet)] and
E. coli SOLR (Δlac-mcrA ΔlacCB-hadSMR-mrr) 171 sbcC recB recA uvrC mutY::Tn5(kan) lac gyrA96 relA1 thrI endA1 λ^r [F′ lacIqZAM15 proAB Tn10 (tetR) Su^r] were used for cDNA cloning according to the suppliers recommendations (Stratagene Cloning Systems, La Jolla, CA). E. coli TG2 [Δ(lac-pro) thi supE recA (Res’ Mod’) k (k)] (traD386 proA B’ lacIqZAM15) (13) was used for expression of vanillyl-alcohol oxidase. E. coli strains were grown in Luria-Bertani medium supplemented with the appropriate antibiotic with the exception of E. coli LE392 which was grown in NZCYM medium. Media were solidified using 1.5% (mass/volume) bacter-agar. Top agar was prepared using NZCYM medium solidified with 0.7% agarose (electrophoresis grade).

Preparation of Cell Extracts—Cell extracts were prepared by sonication as described (6).

Enzyme Activity—Vanillyl-alcohol oxidase activity was determined at 25 °C in 50 mM potassium phosphate, 1 mM vanillyl alcohol, pH 7.5, by measuring the production of vanillin at 340 nm (ε280 vanillin = 14 mm^-1 cm^-1)

Protein Sequence Analysis—Protein sequence analysis of vanillyl-alcohol oxidase was performed by Edman degradation at Eurosequence, Groningen, The Netherlands.

Manipulation of DNA—Isolation of phage and plasmid DNA and other molecular manipulations were carried out essentially as described (12). A. niger and P. simplicissimum chromosomal DNA was isolated according to de Graaff et al. (14). Restriction enzymes were used as recommended by the supplier (Life Technologies, Inc., Gaithersburg, MD).

The nucleotide sequence was determined using either the Cy5TM AutoCycleTM Sequencing Kit (Pharmacia Biotech, Uppsala, Sweden) or the Cy5TM-dATP Labeling Mix (Pharmacia Biotech). The reactions were performed as recommended by the supplier (Stratagene Cloning Systems, La Jolla, CA). E. coli LE392 was precultured in medium described above using fructose (1%) as a carbon source. A. niger NW156-T10 was pregrown on complete medium (9) with 1% fructose as a carbon source. After 16 or 30 h of growth for A. niger and P. simplicissimum respectively, mycelia was harvested and processed for RNA extraction or preparation of cell extract as described above.

Northern and Southern Analysis—Northern blots and Southern blots were carried out as described previously (12). 32P(dATP-labeled vao-cDNA was used as a probe (see above). The membranes were stripped according to the instructions of the manufacturer and rehybridized with a 900-bp EcoRI fragment encoding part of the 3′ end of the 28 S rDNA of Agaricus bisporus (18) to provide a loading control.

Western Analysis—Western blot analysis was done as described previously (6) using the same antibodies as used for the cDNA library screening. Bound antibodies were detected by an alkaline phosphatase based immunooassay.

UV Visualization of Vanillyl-Alcohol Oxidase—Vanillyl-alcohol oxidase was visualized after SDS-PAGE as described (6).

PCR Mutagenesis to Introduce a Shine-Dalgarno Sequence—To enhance the expression of the vao-cDNA a consensus Shine-Dalgarno sequence was introduced via PCR mutagenesis. This was done using the A. niger harboring plasmid pIM3970, taking advantage of the XbaI site at position 451 (numbering according to Fig. 1) and the KpnI site from the polylinker of the vector pBlueScript SK downstream of the vao-cDNA. Two oligonucleotides were used, primer 1, a 51-mer with the sequence 5′-GCGGAGCTCGTGGTTAACAGAGGATATA CATATGGCTAGACAGCAGAAGAATTC-3′, and primer 2, a 17-mer with the sequence 5′-CAGAAGTGGTTGGCCTC-3′. Primer 1 is a mutagenic oligonucleotide. The sequence shown in boldface is identical to the N-terminal coding sequence, and the italicized sequence represents the Shine-Dalgarno sequence. Primer 2 is complementary to the sequence from positions 587 to 603 in Fig. 1 downstream of the Shine-Dalgarno sequence as shown in Fig. 1). The fragment was labeled with [32P]dATP as described previously (16). Washing steps were carried out for 30 min at 65 °C in the following solutions: twice in 2× SSC, 0.5% (mass/volume) SDS, and once in 0.5× SSC, 0.5% (mass/volume) SDS. For secondary screening identical conditions were applied with the exception that phage dilutions were used yielding 200–300 plaques per plate allowing the selection of individual plaques. Phages were propagated in E. coli LE392 and phage DNA isolated as described (12).

Expression Studies in P. simplicissimum and A. niger—Expression studies were carried out via transfer experiments. P. simplicissimum was precultured in medium described above using fructose (1%) as a carbon source. A. niger NW156-T10 was pregrown on complete medium (9) with 1% fructose as a carbon source. After 16 or 30 h of growth for A. niger and P. simplicissimum respectively, mycelia was harvested and processed for RNA extraction or preparation of cell extract as described above.

Transformation of A. niger—A. niger NW156 was transformed as described previously, using 1 µg of the selection plasmid pGW635 and 20 µg of cotransferring plm3971. The copy number of the P. simplicissimum vao gene in A. niger NW156-T10 was determined by Southern blot analysis. 5 µg of SalI-digested P. simplicissimum chromosomal DNA and undiluted (5 µg) and serially diluted SalI-digested A. niger NW156-T10 chromosomal DNA were separated by agarose gel electrophoresis, blotted, and subsequently hybridized with [32P]dATP-labeled vao-cDNA.

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The PCR fragment generated with primers 1 and 2 was cloned into pGEM-T (Promega Corp., Madison, WI) and sequenced to check the orientation and to check for undesired mutations. Next, the fragment in the right orientation with respect to the polylinker-encoded PstI site was excised from pGEM-T by PstI/XbaI digestion and ligated into Pst/I/XbaI-digested pEMBL19. The gene was completed by cloning the XbaI/KpnI fragment isolated from plm3970 into the XbaI/KpnI-digested previous pEMBL construct yielding plasmid plm3972. The orientation of the gene is such that transcription takes place from the vector-encoded lac promoter.

Purification of Vanillyl-Alcohol Oxidase from E. coli TG2—E. coli TG2 cells carrying plasmid plm3972 were grown batchwise in 500 ml of
LB medium supplemented with 80 µg/ml ampicillin and 0.1 mM isopro- pyl-1-thio-β-D-galactopyranoside in 2-liter flasks in a rotary shaker set at 250 rpm at 37 °C. From 5-liter batch cultures cells were harvested by centrifugation and resuspended in 55 ml of 50 mM potassium phosphate buffer, 0.5 mM phenylmethylsulfonyl fluoride, 0.5 mM dithiothreitol, 0.5 mM EDTA, 0.5 mM MgSO₄, 10 mg of DNase I, pH 7.0. Cells were disrupted by passing three times through a precooled French pressure cell press operated at 10,000 p.s.i. Following centrifugation for 15 min at 15,000 × g to remove cellular debris, the supernatant was made 0.5% in protamine sulfate from a 2% mass/volume stock solution. Subsequently, the protamine sulfate aggregates were precipitated by centrifugation for 15 min at 15,000 × g. The supernatant was adjusted to 65% ammonium sulfate saturation. The precipitate was collected by centrifugation at 250 rpm at 37 °C. From 5-liter batch cultures cells were harvested by use of specific oligonucleotides. The vaoDNA was amplified to a titer of 1.4 × 10¹⁰ plaque-forming units was amplified to a titer of 1.4 × 10¹⁰ plaque-forming units/ml. The first screening of the amplified library revealed that approximately 4.5% of the phages reacted with the vanillyl-alcohol oxidase-specific antibodies. From the second screening five positive phages were selected, and the phagemid was excised. Restriction enzyme analysis showed that the five phagemids contained identical inserts. From two such phagemids the nucleotide sequence of the insert was determined over both strands by sequencing subclones and by the comparison of the cDNA sequence with the genomic sequence. The vaoDNA sequence and on sequences from the gene or by subclones generated. Fig. 1 shows the complete vaoDNA gene, sequenced over both strands, including 582-bp promoter sequence and 293 bp of downstream sequence.

Comparison of the cDNA sequence with the genomic sequence revealed that the coding region is interrupted by five introns. The intron sequences follow the rules for filamentous fungi as proposed earlier (19): they are short, 61, 60, 70, 52, and 75 bp for introns i, ii, iii, iv and v, respectively, and the introns have consensus 5′-3′ splice sites, GTpNUGPy and PyAG, respectively, and lariat sequences, NNCTpUAp (where P indicates purine and Py indicates pyrimidine), with only slight deviations.

The promoter region was analyzed for the presence of sequences involved in transcription regulation. The sequences 5′-GATA 3′ and 5′-GCCGAR 3′ were identified in intron 7 (20) and SpP regulation (21), respectively, were not present. The context independent CreA binding site of A. nidulans 5′-GYGGGG 3′ (22) which is probably also recognized in Penicillium (23) was found once (position −557 to −552 in Fig. 1). The 5′-CAAT 3′ sequence, shown to be involved in transcription activation in Saccharomyces and other fungi (24), was found at positions −38, −61, −112, and −373 (lowercase italic letters). No TATA box was found in the sequence immediately upstream of the start codon. However, CT-rich sequences thought to direct transcription initiation (19) were present.

Vanillyl-Alcohol Oxidase Induction in P. simplissium and A. niger—Earlier studies have shown that vaoA expression is gratuitously induced in P. simplissium when grown on veratryl alcohol (1) or anisyl alcohol (6). We have readdressed this issue in a transfer experiment in which veratryl alcohol was used as the sole source of carbon. Analysis was carried out by Northern and Western blotting and by vanillyl-alcohol oxidase activity determinations. In Fig. 2 the time course of induction is presented. Panel A shows the Northern blot probed with 32P-labeled vaoDNA. In panels B and C the Western blot probed with the vanillyl-alcohol oxidase antibodies and the relative vanillyl-alcohol oxidase activity are presented, respectively. The results clearly show that at the start of the transfer no vaoA-mRNA or vanillyl-alcohol oxidase enzyme is present. The vanillyl-alcohol oxidase activity and enzyme concentration follow the vaoA-mRNA concentration with a lag, i.e., while the mRNA is maximal at 36 h the maximal activity is observed between 48 and 72 h. Furthermore, when P. simplissium was grown on the combination of 0.1% (mass/volume) veratryl or 0.1% (mass/volume) anisyl alcohol and 1% (mass/volume) glucose no vanillyl-alcohol oxidase was produced (results not shown) indicating that the gene is carbon catabolite-repressed. This was also suggested by the presence of the context independent CreA binding site in the promoter region.

A. niger NW156-T10, a plm3971 multicopy transformant harboring 25–80 vaoA copies, was used to study vanillyl-alcohol oxidase expression. Southern analysis using the vaoDNA as a probe demonstrated that A. niger NW156 does not contain a vaoA gene with sufficient homology with the P. simplissium vaoA gene to be detected under the conditions applied. In transfer experiments with A. niger NW156-T10 the inducing properties of veratryl alcohol, anisyl alcohol, vanillyl alcohol, veratric acid, vanillie acid, and 4-hydroxybenzoic acid were assessed 3 h after transfer (see Table I and Fig. 3). The highest level of vanillyl-alcohol oxidase activity was obtained with veratryl and anisyl alcohol, whereas no activity was seen when A. niger NW156-T10 was grown on fructose and when an untransformed A. niger strain was transferred to veratryl or anisyl alcohol (not shown). Both 4-methoxybenyl alcohol were used alone and in combination with fructose to study the

RESULTS

Cloning and Sequencing of the vanA Gene—By using methods and conditions described under “Experimental Procedures” a genomic library of P. simplissium was constructed and subsequently screened with the [32P]dATP-labeled vaoDNA as a probe. Two pools of 10,000 phages resulted in four positive phages that were characterized by restriction enzyme analysis and Southern blot analysis (results not shown). It was concluded that the entire gene should be located on an 8-kilobase pair SalI fragment which was subsequently subcloned into SalI-digested pUC18 yielding plm3971. plm3971 was used for sequencing either using specific oligomers based on the

vanA-cDNA sequence and on sequences from the gene or by subclones generated. Fig. 1 shows the complete vaoA gene, sequenced over both strands, including 582-bp promoter sequence and 293 bp of downstream sequence.

Comparison of the cDNA sequence with the genomic sequence revealed that the coding region is interrupted by five introns. The intron sequences follow the rules for filamentous fungi as proposed earlier (19): they are short, 61, 60, 70, 52, and 75 bp for introns i, ii, iii, iv and v, respectively, and the introns have consensus 5′-3′ splice sites, GTpNUGPy and PyAG, respectively, and lariat sequences, NNCTpUAp (where P indicates purine and Py indicates pyrimidine), with only slight deviations.

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time course of vanillyl-alcohol oxidase induction (results not shown). With anisyl alcohol already after 3 h strong induction was observed both by Northern and Western analysis, whereas after 6 h the induction decreased. When fructose was included the induction was weaker both at 3 and 6 h. With veratryl alcohol induction was retarded when compared with anisyl alcohol. Fructose alone or a mixture of veratryl alcohol and fructose resulted in no detectable \( \text{vao} \) mRNA, and no vanillyl-alcohol oxidase was found by Western analysis.

**Expression of vao-cDNA in E. coli TG2 and Purification of Recombinant Vanillyl-Alcohol Oxidase**—Although vanillyl-alcohol oxidase could easily be detected with the vanillyl-alcohol oxidase-specific antibodies during screening of the cDNA library, the expression of the gene was quite low in *E. coli* (less than 0.5% of total protein, based on the specific activity of vanillyl-alcohol oxidase). In addition to the codon usage (see “Discussion”), the apparent lack of a good ribosome binding site may be the cause of this. To enhance the expression level of vanillyl-alcohol oxidase, a consensus Shine-Dalgarno sequence was introduced via PCR mutagenesis as described under “Experimental Procedures.” Cell extracts of *E. coli* TG2 harboring pIM3972 showed a 7-fold increase of expression of vanillyl-alcohol oxidase when compared with *E. coli* TG2 transformed with pIM3970.

Recombinant vanillyl-alcohol oxidase was purified from *E. coli* TG2 harboring pIM3972 in a two-column procedure (Table II). The recombinant enzyme migrated as a single band in SDS-PAGE (Fig. 4) and was identical with vanillyl-alcohol oxidase.

**Fig. 1.** Nucleotide sequence and deduced amino acid sequence of the vaoA gene and vaoA-cDNA of *P. simplicissimum* encoding vanillyl-alcohol oxidase. Coding sequence is shown in capital letters. Introns are indicated by lowercase letters. Promoter sequence is shown in lowercase letters with negative numbering. The downstream sequence of the gene is also represented by lowercase letters. The deduced amino acid sequence is indicated above the coding sequence. Amino acids shown in boldface were obtained by automated peptide sequencing via Edman degradation. The context independent CreA binding site is shown in boldface lowercase letters. The transcription activation sequence CAAT is indicated with lowercase italic letters. Polyadenylation was found at positions indicated with an asterisk above the nucleotide.
oxidase from *P. simplicissimum* (1) in all aspects tested: spectral properties (250–520 nm), steady state kinetic parameters for vanillyl alcohol, and the association into octamers.

**DISCUSSION**

In this paper we have described the cloning and sequencing of the gene encoding vanillyl-alcohol oxidase from *P. simplicissimum*, the first 8a-(N²-histidyl)-FAD containing enzyme of known three-dimensional structure (7). The gene is strongly induced in *P. simplicissimum* when the fungus is grown on 4-methoxybenzyl alcohols (6). This high amount of enzyme is reflected in the abundance of 4-methoxybenzyl alcohols (6). Apart from 4-(methoxymethyl)phenol, which may represent the natural substrate, the flavin is bound to the phenolic oxygen of Tyr-384 (25). Furthermore, as can be seen from Fig. 5, Tyr-384 of p-cresol methylhydroxylase is shifted 8 residues toward the N terminus compared with His-422 of vanillyl-alcohol oxidase.

The crystal structure of vanillyl-alcohol oxidase shows that each monomer is composed of two domains (7). The larger domain (residues 6–270 and 500–560) binds the ADP part of flavin and the data presented in Fig. 5, it is clear that the most conserved parts of the sequence (101–147, 178–219, and 245–271) concern residues that are located in the FAD binding domain. Several active site residues are also conserved. These include Tyr-108, Tyr-503, and Arg-504 that are involved in binding the phenolic moiety of the substrate. In vanillyl-alcohol oxidase, these residues facilitate substrate deprotonation upon binding (3). Asp-170, which is thought to play a crucial role in catalytic mechanism of vanillyl-alcohol oxidase (7), is not conserved (Fig. 5). This might explain the different reactivities of both enzymes toward p-cresol (8). However, a detailed comparison of the active sites of vanillyl-alcohol oxidase and p-cresol methylhydroxylase requires the completion of the crystallographic refinement of the p-cresol methylhydroxylase structure. Besides the 31% sequence identity with MurB, a flavoenzyme involved in the biosynthesis of the bacterial cell wall (29). Moreover, it has been suggested that this unusual FAD-binding fold is shared by other flavoprotein oxidoreductases (30, 31).
transformed with 25–30 copies of the vao gene, strong expression of the gene was observed with these methoxybenzyl alcohols. With 4-hydroxybenzoic acid, ferulic acid, vanillic acid, and vanillyl alcohol vanillyl-alcohol oxidase was detected as well. This indicates that the vaoA gene in A. niger NW156-T10 is rendered under the control of at least one regulator involved in regulation of genes involved in the metabolism of aromatic compounds. Furthermore, the vaoA gene is both in A. niger NW156-T10 and P. simplicissimum under the control of carbon catabolite repression. Since the vaoA gene is expressed from its own promoter, this means that both in A. niger NW156-T10 and P. simplicissimum similar regulation mechanisms must be operative. In both organisms a surprisingly high expression level of vanillyl-alcohol oxidase is observed in the presence of veratryl and anisyl alcohol. This may be explained by assuming that these compounds, or one of their metabolites, have a high affinity for the common aromatic pathway regulator(s), most likely repressor(s). The affinity must be much higher than the

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Specific activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>units/mg</td>
</tr>
<tr>
<td>Fructose</td>
<td>0.000</td>
</tr>
<tr>
<td>Anisyl alcohol</td>
<td>0.484</td>
</tr>
<tr>
<td>Ferulic acid</td>
<td>0.079</td>
</tr>
<tr>
<td>4-Hydroxybenzoic acid</td>
<td>0.124</td>
</tr>
<tr>
<td>Vanillic acid</td>
<td>0.175</td>
</tr>
<tr>
<td>Vanillyl alcohol</td>
<td>0.136</td>
</tr>
<tr>
<td>Veratric acid</td>
<td>0.209</td>
</tr>
<tr>
<td>Veratryl alcohol</td>
<td>0.346</td>
</tr>
</tbody>
</table>

Fig. 3. Effect of various carbon sources on P. simplicissimum vaoA expression in A. niger NW156-T10. A. niger NW156-T10 transformed with 25–30 copies of the P. simplicissimum vaoA gene pregrown on fructose was transferred to the following substrates: fructose (lane 2), anisyl alcohol (lane 3), ferulic acid (lane 4), 4-hydroxybenzoic acid (lane 5), vanillic acid (lane 6), vanillyl alcohol (lane 7), veratric acid (lane 8), and veratryl alcohol (lane 9) and analyzed 3 hours after transfer. Extracts were prepared by sonication. P. simplicissimum grown on veratryl alcohol (lane 10) served as a control. Lane 1, marker. Panel A, 10% SDS-PAGE gel stained with Coomassie Brilliant Blue. Panel B, UV-visualized vanillyl-alcohol oxidase. Panel C, Western blot screened with vanillyl-alcohol oxidase-specific antibodies.

### Table I

<table>
<thead>
<tr>
<th>Step</th>
<th>Protein Activity</th>
<th>Specific activity</th>
<th>Yield</th>
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<tr>
<td>Cell extract</td>
<td>2640</td>
<td>46</td>
<td>1.7 × 10⁻²</td>
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<tr>
<td>Protamine sulfate</td>
<td>1648</td>
<td>42</td>
<td>2.5 × 10⁻²</td>
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<tr>
<td>Ammonium sulfate treatment</td>
<td>801</td>
<td>37</td>
<td>4.6 × 10⁻²</td>
</tr>
<tr>
<td>Phenyl-Sepharose chromatography</td>
<td>88</td>
<td>38</td>
<td>0.4</td>
</tr>
<tr>
<td>Hydroxyapatite chromatography</td>
<td>24</td>
<td>35</td>
<td>1.5</td>
</tr>
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</table>

Fig. 4. SDS-PAGE of purification steps of recombinant vanillyl-alcohol oxidase from E. coli TG2 transformed with pIM3972 harboring the P. simplicissimum vaoA-cDNA.
affinity of 4-hydroxybenzoic acid, ferulic acid, and vanillyl alcohol or their metabolites. These latter compounds induce vanillyl-alcohol oxidase expression in *A. niger* NW156-T10 but not in *P. simplicissimum*. The discrepancy in the level of vanillyl-alcohol oxidase expression between *A. niger* NW156-T10 and *P. simplicissimum* during growth on 4-hydroxybenzoic acid, ferulic acid, and vanillyl alcohol may be accounted for by the following: (i) in *A. niger* NW156-T10 25–30 copies of the *vao* gene are present versus 1 copy in *P. simplicissimum*, and (ii) in the present study *A. niger* NW156-T10 mycelia were harvested 3 or 6 h following transfer, whereas in the studies with *P. simplicissimum* mycelia were allowed to grow 2 days after transfer which may have caused degradation of vanillyl-alcohol oxidase due to toxic effects and/or the poor carbon sources these aromatic compounds represent (6).

Although expression of *vaoA*-cDNA in *E. coli* TG2 cells was evident, since specific antibodies could be used to select the cDNA, the expression level was low. It was previously observed that *E. coli* TG2 cells are capable of producing relatively high amounts (>50 mg/liter of culture) of recombinant enzymes from multicopy plasmids like pBlueScript and pUC under the direction of the plasmid-encoded *lac* promoter (32, 33). Inspection of the *vao* cDNA sequence revealed two possible explanations for the low expression. The first reason may be the codon usage. Codons that are considered modulator codons in *E. coli*, suppressing high expression (34), occur with a relatively high frequency in the cDNA. The second reason may be the apparent absence of a good ribosome binding site. The

![FIG. 5. Sequence alignment between vanillyl-alcohol oxidase and the flavoprotein subunit of *p*-cresol methylhydroxylase. Vertical lines represent identical amino acids; dots indicate conserved residues. Gaps introduced for optimal alignment are indicated with dashes; residues forming the covalent bond with FAD are indicated with arrows, and active site residues are in boldface and are underlined.](image)
introduction of a consensus E. coli ribosome binding site at the correct distance from the start codon increased the expression level only 7-fold indicating that the low expression is related to the codon usage.

Finally, this study has clearly established that expression of the vanOA gene in a prokaryotic or eukaryotic host results in active, fully covalently flavinylated enzyme. This suggests that the flavinylation is an autocatalytic process as shown for 6-hydroxy-\(\alpha\)-nicotine oxidase (35, 36). However, for \(p\)-cresol methylhydroxylase it was shown that autocatalytic flavinylation only occurred after binding of the cytochrome subunit (28).

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REFERENCES