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In vitro activation, purification, and characterization of *Escherichia coli* expressed aryl-alcohol oxidase, a unique H_2O_2-producing enzyme

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Abstract

Aryl-alcohol oxidase (AAO), a flavoenzyme with unique spectral and catalytic properties that provides H_2O_2 for fungal degradation of lignin, has been successfully activated in vitro after *Escherichia coli* expression. The recombinant AAO (AAO*) protein was recovered from inclusion bodies of *E. coli* W3110 transformed with pFLAG1 containing the *aao* cDNA from *Pleurotus eryngii*. Optimization of in vitro refolding yielded 75% active enzyme after incubation of AAO* protein (10 μg/ml) for 80 h (at 16 °C and pH 9) in the presence of glycerol (35%), urea (0.6 M), glutathione (GSSG/GSH molar ratio of 2), and FAD (0.08 mM). For large-scale production, the refolding volume was 15-fold reduced and over 45 mg of pure active AAO* was obtained per liter of *E. coli* culture after a single anion-exchange chromatographic step. Correct FAD binding and enzyme conformation were verified by UV–visible spectroscopy and circular dichroism. Although the three enzymes oxidized the same aromatic and aliphatic polyunsaturated primary alcohols, some differences in physicochemical properties, including lower pH and thermal stability, were observed when the activated enzyme was compared with fungal AAO from *P. eryngii* (wild enzyme) and *Emericella nidulans* (recombinant enzyme), which are probably related to the absence of glycosylation in the *E. coli* expressed AAO.

Keywords: Aryl-alcohol oxidase; Flavoenzymes; *Escherichia coli* expression; In vitro activation; Hydrogen peroxide production; *Pleurotus eryngii*

White-rot fungi are the main organisms responsible for lignin degradation in nature [5]. Among this group of basidiomycetes, *Pleurotus eryngii* is able to remove lignin without a significant degradation of cellulose [25]. This selective degradation pattern is of biotechnological interest for paper pulp manufacturing and other industrial applications [18]. The ligninolytic system of *P. eryngii* has been characterized as being constituted by extracellular peroxidases [2,19,22], laccases [21], and aryl-alcohol oxidase (AAO) [1] [10,29].

Production of H_2O_2 by AAO through aromatic-alde hyde redox-cycling provides a continuous source of oxidative power for lignin degradation by *Pleurotus* species [9,11,12]. H_2O_2 participates in reactions catalyzed by ligninolytic peroxidases and acts as a precursor of hydroxyl-free radical (OH·). It has been suggested that OH· is involved in the initial attack of lignocellulose when peroxidases and other enzymes cannot penetrate the plant cell wall [6]. In addition to H_2O_2 production, AAO also prevents the repolymerization of

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[1] *Abbreviations used:* AAO, aryl-alcohol oxidase; AAO*, recombinant AAO; IPTG, isopropyl-β-D-thiogalactopyranoside; DTT, dithiothreitol; MALDI-TOF, matrix-assisted laser desorption and ionization-time of fly; CD, circular dichroism; GOX, glucose oxidase.

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products released from enzymatic degradation of lignin [20].

Pleurotus AAO is a unique enzyme due to both spectral and catalytic properties compared with other flavoenzyme oxidases. It is the only oxidase acting on non-phenolic aromatic alcohols, in contrast to vanillyl-alcohol oxidase that only oxidizes phenolic aromatic compounds (and has a covalently linked FAD, whereas AAO FAD is released during denaturation) [4]. AAO substrates include a variety of aromatic compounds (benzyl, naphthyl, and cinnamyl alcohols) together with aliphatic polyunsaturated alcohols that are oxidized to the corresponding aldehydes, which can be also slowly converted into acids [10]. It is intriguing how the active site of AAO, near the FAD cofactor, can successfully recognize and accommodate this wide range of substrates. Spectral evidences suggest that the FAD environment in AAO is different from that of most oxidases since it does not stabilize a semiquinone anionic intermediate during enzyme reduction to the flavin hydroquinone form [7]. The interest on AAO increased during recent years to elucidate the structural bases of its unique catalytic properties.

The first AAO gene was cloned from *P. eryngii* [29], and a very similar gene (95% amino acid identity) was then cloned from *Pleurotus pulmonarius* [27]. The corresponding cDNA has been expressed in the ascomycete *Emericella nidulans* (conidial state *Aspergillus nidulans*) resulting in extracellular active enzyme (AAO*) [28]. However, the AAO* yield from *E. nidulans* is comparatively low (< 4 mg/L), and the enzyme isolation process is tedious (due to both production and purification conditions). Simultaneously, crystallization of both wild AAO from *P. pulmonarius*, a natural AAO hyperproducer, and AAO* from *E. nidulans* has been investigated [27]. However, the quality of crystals was not good enough for solving the molecular structure of AAO by X-ray diffraction, most probably due to enzyme microheterogeneity as a consequence of differences in protein glycosylation degree by fungal hosts. Therefore, the availability of an alternative production system providing enough amounts of homogeneous AAO* represented a priority to continue the characterization of this interesting oxidase.

In this work, we describe a method for obtaining active AAO* from *Escherichia coli*. This is based on expression of the fungal cDNA and subsequent activation of the inactive AAO* protein (recovered from inclusion bodies) by its in vitro refolding under specific conditions in the presence of the cofactor. This method opens the possibility of a variety of structure–function studies in the near future including: (i) site-directed mutagenesis and production of enzyme variants; (ii) crystallization of homogeneous enzyme preparations; and (iii) investigation of catalysis mechanisms by stopped-flow spectrophotometry and reconstitution with flavin analogs. To verify the suitability of the in vitro-activated AAO*, its main physicochemical and kinetic properties were determined and compared with those of wild (from *P. eryngii*) and recombinant (from *E. nidulans*) fungal AAO.

Materials and methods

Organisms and vectors

*Emericella nidulans biA*1, *metG*1, *ArgB*2 (IJFM A729) transformed with pALAAO was used for eukaryotic heterologous expression of *P. eryngii* AAO [28]. pALAAO was used for PCR amplification of the AAO cDNA, and pFLAG1 (International Biotechnologies) as *E. coli* expression vector. *E. coli* SURE (Stratagene) was used for subcloning plasmids and *E. coli* W3110 (ATCC 27325) as prokaryotic expression host.

Cloning aao into the E. coli expression vector

cDNA encoding the mature AAO protein of *P. eryngii* (GenBank Accession No. AF064069), without signal peptide, was amplified by PCR using *Pfu* polymerase (Stratagene), pALAAO as template, and the two oligonucleotide primers described below. Primer AAO-Nt (5′-GGGAATTCcatatgGCCGATTTTGACTACGTTGTCGTCGGGG-3′) corresponded to sequence encoding the N-terminus of mature AAO preceded by an *Nde*I restriction site (lowercase) that also provided the translational start codon. Primer AAO-Ct (5′-GGAagatctCTACTGATCAGCCTTGATAAGATC GG-3′) was reversed and complementary to sequence encoding the AAO C-terminus (including a stop codon) that was preceded by a *Bgl*II restriction site (lowercase). PCR amplification was carried out as follows: five cycles including denaturation at 94 °C for 2 min, annealing at 50 °C for 2 min, and extension at 74 °C for 8 min; 25 cycles including denaturation at 94 °C for 35 s, annealing at 60 °C for 1 min, and extension at 74 °C for 8 min; and a final extension period at 74 °C for 10 min. The amplified DNA was separated by electrophoresis on 0.8% agarose, purified (Geneclean BIO101 kit), digested with *Nde*I and *Bgl*II, and ligated on *Nde*I/*Bgl*II-digested pFLAG1. The sequence of the amplified DNA was confirmed in both orientations by automatic sequencing using an ABI 377 equipment and synthetic oligonucleotides. The construct, named pFLAG1-AAO, was used to transform *E. coli* W3110.

Expression of aao in E. coli and E. nidulans hosts and production of wild AAO

Overexpression in *E. coli* W3110 containing pFLAG1-AAO was carried out in 1 L flasks with 500 ml of Terrific Broth medium [23] supplemented

with 100 μg/ml of ampicillin. The medium was inoculated with an overnight culture and grown at 37 °C and 180 rpm. When an OD_{500} of 1 was attained, 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) was added as inducer, and the cultures were incubated for additional 4 h. The bacterial pellets obtained after centrifugation were stored at −20 °C till processed as described below. Production of AAO* in *E. nidulans* was according to Varela et al. [28]. Wild AAO from *P. eryngii* used for comparison was obtained as described by Guillén et al. [10].

*In vitro activation and purification of E. coli AAO**

The bacterial pellets were resuspended in 50 mM Tris–HCl buffer, pH 8.0, containing 10 mM EDTA and 5 mM dithiothreitol (DTT). The cells were ruptured by incubation with 2 mg/ml lysozyme for 1 h at 4 °C, followed by the addition of 0.1 mg/ml DNase and final sonication. The insoluble fraction was collected by centrifugation (30 min at 15,000 rpm and 4 °C in a Sorvall SS34 rotor) and the supernatant was discarded. The pellet was washed three times with 20 mM Tris–HCl buffer, pH 8.0, containing 10 mM EDTA and 5 mM DTT (each washing followed by centrifugation as described above). Tests on the solubility of AAO* protein forming the inclusion bodies were carried out in the above buffer containing 2 mM EDTA, 30 mM DTT, and different concentrations of urea in the range of 2–10 M. The pellet was solubilized under optimal conditions and subsequently centrifuged (15 min at 15,000 rpm and 4 °C in a Sorvall SS34 rotor). The supernatant was used as a stock solution for the in vitro activation experiments.

In preliminary experiments, the denatured AAO* solution (12 mg/ml) was diluted 100-fold in the above buffer containing 0.05 mM FAD, 1.5 mM GSSG, and 1 mM DTT (the latter providing a GSSG/GSH ratio of 1.5) and the mixture was incubated at 4 °C for a week (non-optimized conditions). To optimize the refolding process, small-scale assays were carried out in 8-ml reactions, where concentration of different components and reaction conditions were modified (following the order indicated below). These included glycerol (0–70%), urea (0.1–2.5 M), temperature (4–25 °C), time (0–173 h), protein (10–500 μg/ml), pH (7–10), GSSG/GSH molar ratio (0.2–4, obtained using 0.2–4 mM GSSG, and 1–5 mM DTT), and FAD (5–90 μM). Refolding efficiency was checked by activity estimation. Both conditions to attain the highest activation efficiency, independently of the refolding dilution, and conditions for large-scale activation, maintaining the refolding volume and FAD concentration as reduced as possible, were investigated.

For active AAO* purification, the refolding mixture was 40-fold concentrated (Filtron and Amicon, 3-kDa cut-off membranes) and dialyzed against 10 mM sodium phosphate buffer, pH 5.5. The soluble fraction after centrifugation (20 h at 15,000 rpm and 4 °C in a Sorvall SS34 rotor) was loaded into a 6-ml Resource-Q column (Pharmacia Biotech). AAO* was eluted with a linear NaCl gradient (0–0.5 M) in the above buffer, and the fractions containing activity were pooled, dialyzed against buffer, and stored at −80 °C.

Enzyme activity and kinetics

AAO* activity in refolding, purification, and stability studies was assayed as the transformation of 5 mM veratryl (3,4-dimethoxybenzyl) alcohol to veratraldehyde (3,4-dimethoxybenzaldehyde) in 0.1 M sodium phosphate buffer, pH 6.0 [10]. One activity unit was defined as the amount of enzyme oxidizing 1 μmol of substrate per minute.

Steady-state kinetic constants were calculated during the linear phase of oxidation of different alcohols to the corresponding aldehydes in the above buffer. The molar absorbances of benzaldehyde (ε_{250} 13,800 $M^{-1}\,cm^{-1}$), *m*-anisaldehyde (3-methoxybenzaldehyde; ε_{314} 2540 $M^{-1}\,cm^{-1}$), *p*-anisaldehyde (4-methoxybenzaldehyde; ε_{285} 16,980 $M^{-1}\,cm^{-1}$), and veratraldehyde (ε_{310} 9300 $M^{-1}\,cm^{-1}$) were obtained from Guillén et al. [10], and 2,4-hexadien-1-al (ε_{280} 30,140 $M^{-1}\,cm^{-1}$) was calculated by the authors. Apparent K_m and k_{cat} values were estimated from Lineweaver–Burk plots.

Protein characterization

Estimation of AAO* molar absorbance was based on heat denaturation [1]. AAO* (5–10 μM) was dissolved in 100 mM phosphate buffer, pH 6.0, and the absorbance at 463 nm was recorded. The sample was incubated at 75 °C for 10 min and centrifuged to remove the unfolded protein. The supernatant was recovered, and the free FAD concentration was estimated using a ε_{450} of 11,300 $M^{-1}\,cm^{-1}$. Then, the molar absorbance of AAO* from *E. coli* (ε_{463} 11,050 $M^{-1}\,cm^{-1}$) and *E. nidulans* (ε_{463} 10,280 $M^{-1}\,cm^{-1}$) was calculated considering a FAD/AAO molar ratio of 1 and used for estimation of enzyme concentration in subsequent studies. Total protein concentration, when required, was measured by the Bio-Rad assay using bovine serum albumin as a standard.

AAO* molecular mass was estimated by matrix-assisted laser desorption and ionization-time of fly (MALDI-TOF) mass spectrometry (Bruker). SDS–PAGE (using 5% mercaptoethanol) was performed in 7.5% polyacrylamide gels using high molecular mass standards from Bio-Rad. Isoelectric focusing was performed in 5% polyacrylamide gels with a thickness of 1 mm and a linear pH range of 3.5–5.0 (prepared with Amersham Biosciences Pharmalyte, mixing 87% from pH 2.5–5 and 13% from pH 3–10) using 1 M H_3PO_4 and 1 M NaOH in anode and cathode, respectively. Protein

bands in gels were stained with Coomassie blue R-250 and quantified by densitometric analysis using the ImageQuant program (Molecular Dynamics).

For circular dichroism (CD) analysis of secondary structure, purified AAO* (0.7–1.9 mg/ml) was diluted in 10 mM sodium phosphate buffer, pH 5.5. CD spectra (180–260 nm) were measured at 25 °C in a J-715 spectropolarimeter using a cell with 0.1 mm optical path length. Each spectrum was the average of two accumulations at a scanning speed of 20 nm/min using 1 nm bandwidth. The spectra were analyzed with the CDPro programs SELCON3 [24], CDSSTR [14], and CONTINLL [26].

Results

Escherichia coli expression of aao

For *E. coli* production of AAO, a 1.7 kb cDNA coding for the mature protein was amplified by PCR and subcloned into the pFLAG1 expression vector under the control of *tac* promoter. AAO* was overexpressed in *E. coli* W3110, the highest level being attained 4 h after IPTG induction. SDS–PAGE analysis of the cell extracts showed accumulation of AAO* in the insoluble fraction containing the inclusion bodies (Fig. 1). The AAO* band exhibited a slightly higher electrophoretic mobility than the wild AAO produced by *P. eryngii*. Densitometric analysis of stained gels showed that AAO* was the major protein in the insoluble fraction. When the inclusion bodies were washed to remove contaminating proteins, the AAO* yield attained 95%.

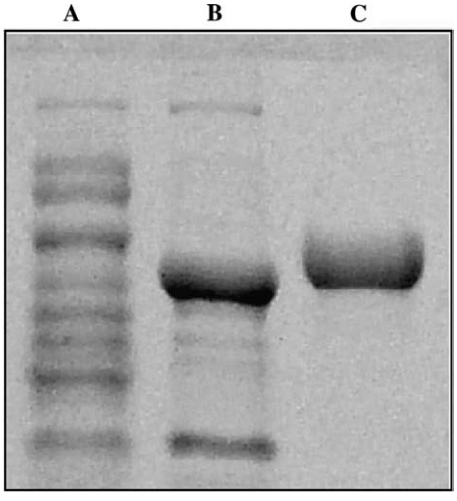

Fig. 1. SDS–PAGE showing AAO* accumulation in *E. coli* inclusion bodies: electrophoretic analysis of soluble (A) and insoluble fractions (B) from cell lysate, compared with glycosylated AAO purified from *P. eryngii* (C).

In vitro activation of AAO from inclusion bodies*

For in vitro activation, the washed inclusion bodies were first solubilized with urea in 20 mM Tris–HCl (pH 8) containing 2 mM EDTA and 30 mM DTT. Different concentrations of urea were tested (2 M urea resulted in 32% of the enzyme being solubilized, while 4 M urea solubilized 60%, 6 M urea solubilized 76%, and 8–10 M urea solubilized 100% of the recombinant enzyme). In consequence, 8 M urea, resulting in complete unfolding of AAO*, was used to solubilize the pellet containing the inclusion bodies. Then, activation conditions were established by successive optimization of different refolding parameters in small-volume reactions. The initial refolding conditions were based on those reported for glucose oxidase (GOX) [32] and included 100-fold dilution in 20 mM Tris–HCl buffer, pH 8.0, containing 1.5 mM GSSG, 1 mM DTT, and 0.05 mM FAD (in the presence or absence of 10% glycerol) and incubation at 4 °C for 1 week. AAO activity was detected only when glycerol was present in the refolding mixture. Therefore, glycerol concentration was the first parameter to be optimized.

When glycerol concentration was tested in the range of 0–70%, a sharp maximum of AAO* activity was obtained at a concentration around 35%. Refolding efficiency was strongly increased (50-fold) with respect to previous conditions using 10% glycerol. A second important factor for in vitro refolding was the dilution rate of the high molarity urea used for completely unfold the AAO* from inclusion bodies. Optimal concentration ranged in an interval between 0.3 and 0.7 M urea. Activation was slightly higher than under previous conditions (using 0.08 M urea) but it abruptly decreased at higher concentrations being negligible in the presence of 1.5 M urea. Refolding efficiency was also affected by temperature and time that were the next parameters to be optimized. The highest yield of active AAO* was obtained after 80–100 h incubation at 16 °C. Longer incubation periods did not increase significantly the refolding yield at low temperature (4–16 °C), and AAO* activation was hardly observed at 25 °C. Regarding protein concentration, the highest refolding efficiency, estimated as AAO* specific activity, was achieved at the lowest concentrations with a sharp decrease (from 40 to 15 U/mg of active protein) between 10 and 100 μg/ml. However, when the active AAO* yield was expressed per unit of refolding volume, the highest yield corresponded to a protein concentration of 400 μg/ml (see below). When the effect of pH was tested, alkaline conditions (over pH 8) were favorable for AAO refolding, and the maximum activity was obtained at pH 9.0. The refolding solution was supplemented with a mixture of GSSG and GSH, and the highest yield of active AAO* was found with a GSSG/GSH molar ratio of 2:1 (attained using 1 mM DTT and 2.5 mM GSSG). FAD addition was required for incorporation into AAO* during in vitro refolding. Over 90%

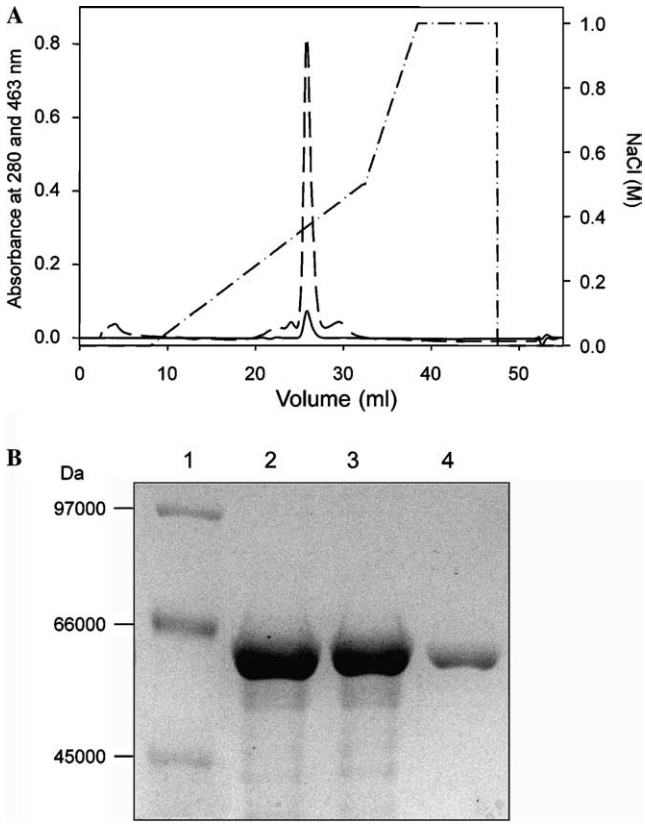

Fig. 2. Purification of refolded AAO*. (A) AAO* elution profile from Resource-Q column (OD at 280 and 463 nm are shown in dashed and continuous line, respectively, and the NaCl gradient in dash-point line). (B) SDS–PAGE at different stages of AAO* purification: washed inclusion bodies solubilized with 8 M urea (lane 2), refolded, concentrated/dialyzed, and centrifuged AAO* (lane 3), purified AAO* after Resource-Q chromatography (lane 4) (Bio-Rad molecular mass markers are shown in lane 1).

refolding efficiency was obtained with FAD concentrations in the range 0.02–0.10 mM (with a maximum at 0.08 mM FAD). Taking the above results into account, activation of 75% AAO* in the refolding mixture (10 μg/ml protein) was obtained after 80 h incubation at 16 °C in 20 mM Tris–HCl buffer, pH 9.0, containing 2.5 mM GSSG, 1 mM DTT, 35% glycerol, 0.6 M urea, and 0.08 mM FAD.

Due to the low protein concentrations used under the above optimized conditions, extremely high volumes need to be processed when high amounts of active AAO* are required (e.g., nearly 70 L of refolding mixture to process the AAO* from 1 L of *E. coli* culture obtaining 500 mg of active enzyme). Therefore, new conditions for large-scale activation were investigated, maintaining the refolding volume and the amount of FAD as reduced as possible. The refolding volumes required to obtain 100 mg of active AAO* at different protein concentrations and the corresponding efficiency of the process (calculated from specific activity) were estimated. Taking this information into account, an intermediate protein concentration of 150 μg/ml was selected for large-scale refolding because it provided good levels of active AAO* with a low refolding volume (100 mg of AAO* was activated in 4 L of refolding mixture). Moreover, it was found that a fourfold decrease of FAD concentration did not result in a significant decrease of AAO* activity, but enabled an important saving of this compound. Finally, refolding at 4 °C represents a lower contamination risk than 16 °C in long-term (80 h) incubation; therefore, the former temperature was chosen for large-scale refolding. According to the above results, the following conditions were used for large-scale refolding: 150 μg/ml protein, 0.02 mM FAD, and 4 °C incubation. This resulted in near 1500 U of active AAO* per liter of refolding reaction and enabled simultaneous processing of several liters of *E. coli* culture, and successful purification of a significant amount of activated enzyme.

*Purification and characterization of active AAO**

In vitro-activated AAO* was purified in a single chromatographic step using an anion-exchange column (Fig. 2A) after refolding mixture concentration, dialysis, and 20-h centrifugation to remove incorrectly refolded proteins and excess of glycerol. The degree of purity of the enzyme was followed by SDS–PAGE that only showed minor contaminating proteins (< 5%) after solubilization of the inclusion bodies and in vitro refolding (Fig. 2B). The presence of a unique protein band at the end of the purification process revealed mass homogeneity. The results of the whole purification process after large-scale refolding conditions are summarized in Table 1, which show a very high purity degree at the end of the process (nearly 10-fold purification with respect to the refolding mixture). The purified AAO* had a specific activity of 96 U/mg (referred to veratryl alcohol oxidation) and a yield over 45 mg of active enzyme per liter of *E. coli* culture. *E. nidulans* AAO* and wild AAO from

Table 1
AAO* purification after in vitro activation using large-scale refolding conditions: total protein and activity, yield and purification factor (from one liter of *E. coli* culture)

	Protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Purification (fold)
Refolding mixture (80 h)	670	6500	10	100	1
Concentration/dialysis	354	5660	16	87	1.6
Centrifugation	197	5200	26	80	2.6
Resource-Q	47	4550	96	70	9.6

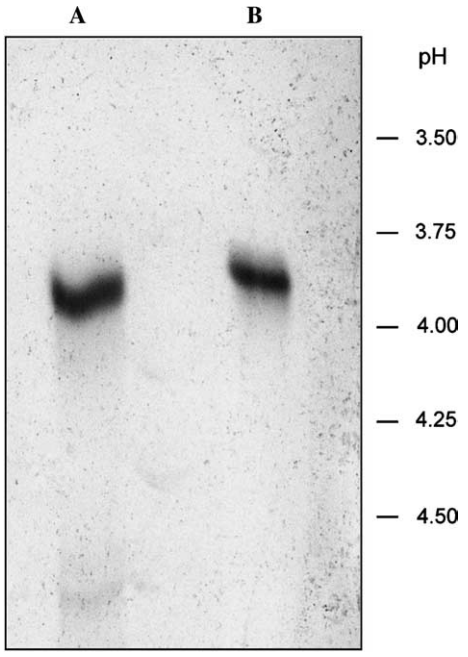

Fig. 3. Isoelectric focusing of purified refolded AAO* from *E. coli* (B) and AAO* from *E. nidulans* (A).

*P. eryngii*were produced and purified as described elsewhere [10,28].

The molecular mass of AAO* measured by MALDI-TOF was 61,847 Da, which was in good agreement with the theoretical mass (61,852 Da) calculated for the amino acid sequence plus the FAD group. The MALDI-TOF molecular masses of the wild AAO from *P. eryngii* and the AAO* from *E. nidulans* were 69,114 Da and 69,792 Da, respectively. Isoelectric focusing of the purified AAO* (Fig. 3) showed only one band of protein revealing charge homogeneity. Its p*I* was 3.85, a value slightly more acidic than those of the enzymes produced by *P. eryngii* and *E. nidulans* (p*I* 3.90–3.95).

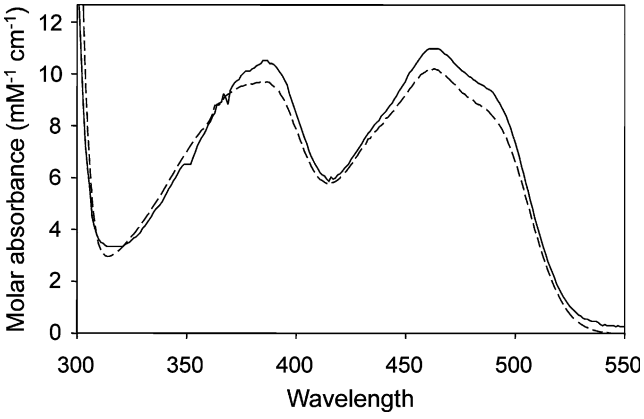

Fig. 4. UV–visible spectra of purified refolded AAO* from *E. coli* (—) and AAO* from *E. nidulans* (- - -). The spectra were recorded in 10 mM sodium phosphate buffer, pH 5.5, using 25 μM AAO* concentrations.

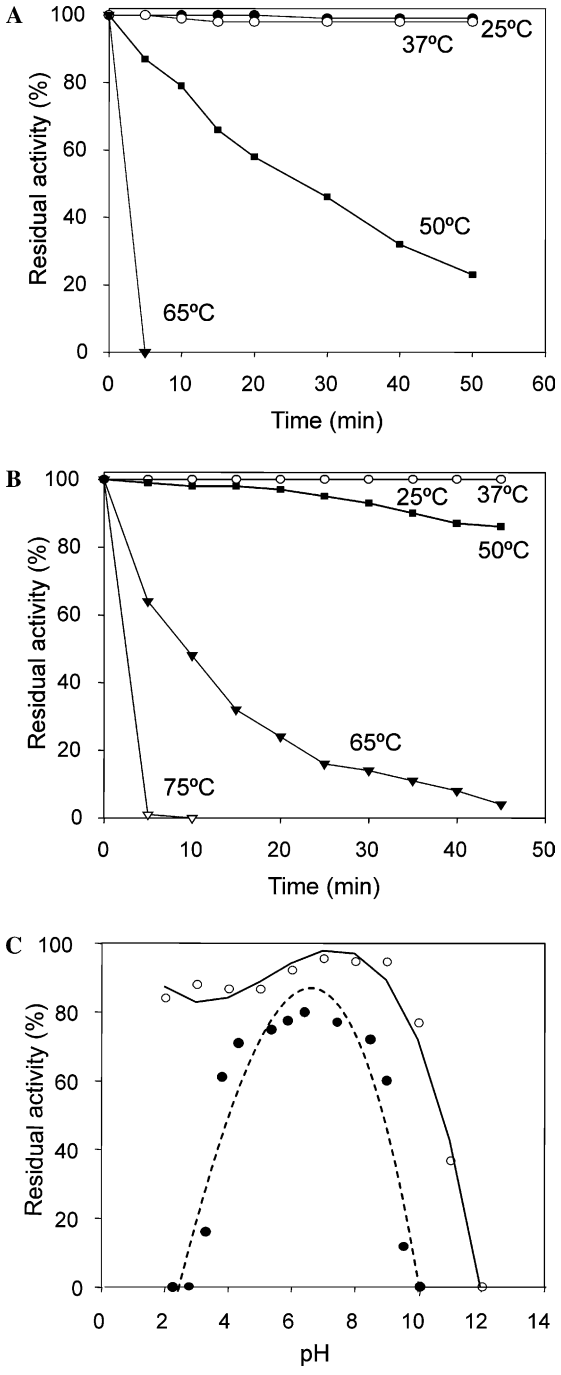

Fig. 5. Thermal and pH stability of AAO*: residual activity (%) during *E. coli* (A) and *E. nidulans* (B) AAO* incubation (1 h, in 10 mM sodium phosphate buffer, pH 6) at different temperatures; as well as after 24-h incubation (25 °C) of *E. nidulans* (-○-) and *E. coli* (-●-) AAO* at different pH values (in 10 mM citrate–phosphate–borate buffer) (C).

The UV–visible spectrum of the refolded AAO* (Fig. 4) was typical of a FAD-containing protein and presented absorption maxima at 386 and 463 nm with a shoulder at 490 nm, and valleys at 315 and 414 nm. The A_{280}/A_{463} ratio (around 11) was similar to that of AAO* from *E. nidulans* (around 10). The molar absorbance of

Table 2
Steady-state kinetic constants of recombinant (from *E. coli* and *E. nidulans*) and wild AAO (from *P. eryngii*) [a]

	AAO* from *E. coli*	AAO* from *E. nidulans*	AAO from *P. eryngii*[b]
Benzyl alcohol			
K_m	873	758	840
k_{cat}	30	32	59
k_{cat}/K_m	35	47	70
m-Anisyl alcohol			
K_m	269	293	220
k_{cat}	21	18	34
k_{cat}/K_m	80	65	155
p-Anisyl alcohol			
K_m	37	28	40
k_{cat}	134	149	239
k_{cat}/K_m	3870	5230	5970
Veratryl alcohol			
K_m	541	592	410
k_{cat}	99	119	143
k_{cat}/K_m	172	210	349
2,4-Hexadien-1-ol			
K_m	120	92	n.d
k_{cat}	184	144	n.d
k_{cat}/K_m	1540	1270	n.d

[a] Mean K_m (μM), k_{cat} (s^{-1}), and k_{cat}/K_m ($s^{-1}\,mM^{-1}$) values (n.d, not determined). All confidence limits were below 20% of mean values.
[b] Guillén et al. [10].

the refolded AAO*, ε_{463} 11,050 $M^{-1}\,cm^{-1}$, was higher than that of the AAO* from *E. nidulans*, estimated as ε_{463} 10,280 $M^{-1}\,cm^{-1}$.

CD spectroscopy showed that the refolded AAO* is an ordered protein with a secondary structure similar to that of recombinant AAO* from *E. nidulans*. The percentages of α-helix, β-strand, and turns or non-ordered structures were the following: 21 ± 2% α-helix, 27 ± 1% β-strand, and 52 ± 1% turns/non-regular structures for *E. coli* AAO*; and 24 ± 6% α-helix, 28 ± 3% β-strand, and 48 ± 5% turns/non-regular structures for *E. nidulans* AAO*.

The in vitro-refolded AAO* showed the same optimal temperature (50–55 °C) and a higher range of optimal pH (4–8) than the AAO* from *E. nidulans* (pH 5–6). However, the thermal and pH stabilities of the activated *E. coli* AAO* were lower than those of the glycosylated enzyme from *E. nidulans*. Figs. 5A and B shows the thermal stability of *E. coli* AAO*, that was stable up to 37 °C but resulted is 100% inactivation after 5 min at 65 °C, compared with that of the *E. nidulans* enzyme that still retained 65% activity after 5 min at 65 °C. Moreover, the refolded AAO* was partially stable after 24 h at pH 5.0–7.5, whereas the *E. nidulans* AAO* was almost completely stable between pH 2 and pH 10 (Fig. 5C).

The catalytic properties of the activated *E. coli* AAO* were analyzed, and the steady-state kinetic constants obtained were compared with those of AAO* from *E. nidulans* and wild AAO from *P. eryngii* (Table 2). All the enzymes oxidized the five AAO substrates selected for this study, including four aromatic and one aliphatic-polyunsaturated primary alcohols. In all cases, benzyl alcohol was worse substrate than the three methoxy-substituted alcohols. *p*-Anisyl appeared as the best AAO substrate with high efficiency values. However, the present study also showed 2,4-hexadien-1-ol as a very good substrate of this oxidase, the k_{cat} value of the *E. coli* AAO* (184 s^{-1}) being the highest among the different substrates tested.

Discussion

We have optimized high yield production of active *P. eryngii* AAO using *E. coli* expression, as an alternative to the heterologous expression in *E. nidulans* reported previously [28]. The latter expression system is not suitable for some studies because of tedious isolation, low yield, and microheterogeneity of the AAO* obtained. In vitro activation after *E. coli* expression will permit to overcome these problems.

In vitro activation

AAO* production per liter of *E. coli* culture was over 1000-fold than that produced by *E. nidulans* [28], although the enzyme was inactive and accumulate in inclusion bodies. Formation of inclusion bodies has been also observed for other recombinant flavoenzymes expressed in *E. coli* [3,32]. Isolation and washing of the inclusion bodies (95% AAO* purity) was the only step required before in vitro refolding. The initial conditions [32] yielded low levels of active AAO*. However, after optimizing the refolding parameters (including glycerol concentration that had a key effect) up to 75% activation was attained at the lowest protein concentration.

Addition of glycerol (and FAD) was absolutely necessary to obtain active AAO*. Glycerol plays a chaperone-like role in inhibiting protein aggregation [8]. The activation yield was also increased at alkaline pH promoting thiolate anion formation, as well as in the presence of an excess of GSSG providing oxidizing conditions. Both factors stimulate disulfide bridges [17] and seem to contribute to the only disulfide bridge expected in AAO* [30]. At high protein concentrations intermolecular interactions led to aggregation and precipitation, and low protein concentrations were required to attain high AAO* activation yields, as found for some other proteins [17].

In large-scale AAO* production and refolding, the amount of protein is not a limiting factor due to the high levels of enzyme produced in *E. coli*. By contrast, the refolding volume to be handle and the cost of reagents to be used could be the limiting factor. For this reason, a protein concentration higher than optimized for

small-scale refolding and a lower FAD concentration were used under these conditions. Although the refolding yield was reduced to around 10%, the new conditions resulted in twofold to threefold more active enzyme for the same refolding volume.

Purification of refolded AAO* was reduced to a single chromatographic step, compared with the two steps necessary to purify wild AAO [10] and AAO* from *E. nidulans* [28]. The main difference between the three AAO expression systems concerns the levels of pure active enzyme obtained, which were 0.8, 3.4, and 45–500 mg/L of *P. eryngii*, *E. nidulans*, and *E. coli* cultures, respectively.

Comparison with fungal (wild and recombinant) AAO

In vitro-activated AAO* from *E. coli* and AAO* from *E. nidulans* showed the same spectral characteristics, including typical FAD peaks and a A_{280}/A_{463} ratio nearly 10, which basically matched the spectrum described by Guillén et al. [10] for the wild enzyme. The presence of 1 mol of FAD per mol of AAO* was shown by the MALDI-TOF molecular mass of the in vitro-refolded AAO*. This molecular mass matched (with < 0.01% error) the theoretical value and differed from the molecular masses of the enzymes from *P. eryngii* and *E. nidulans* that are both glycosylated. The carbohydrate contents were estimated as 10.5 and 11.4% for the wild AAO and the AAO* from *E. nidulans*, respectively, and constitute a more accurate estimation than reported previously [28]. CD spectra of the in vitro-refolded AAO* and the AAO* from *E. nidulans* showed similar percentages of α-helix, β-strand, and non-regular structure in both proteins. This fact together with the similar FAD environment suggest correct folding under the in vitro conditions used.

The in vitro-activated AAO* exhibited kinetic constants similar to those of the wild and *E. nidulans* AAO*. However, the k_{cat} on some substrates was slightly lower for the two recombinant enzymes. Moreover, the in vitro-activated AAO* showed the same temperature optimum than the wild and recombinant fungal enzymes, but its pH optimum was wider. The carbohydrate moiety in the fungal preparations seems to be responsible for some of the differences in physicochemical properties. The p*I* of the *E. coli* AAO* was slightly more acidic than those of the glycosylated fungal enzymes, probably due to the absence of basic sugars or to a higher exposition of acidic residues. Moreover, differences in UV–visible spectra are most likely also due to the lack of glycosylation of the *E. coli* AAO* resulting in 8% higher ε_{463} than *E. nidulans* AAO*. Finally, a positive effect of glycosylation on protein stability has been described for other enzymes including GOX [16,31] and could explain the most important difference between the bacterial and fungal AAO preparations. This concerned pH and temperature stability that was significantly higher in the glycosylated enzymes from *P. eryngii* [10] and *E. nidulans* than in the non-glycosylated AAO* from *E. coli*.

AAO from fungal systems is difficult to be crystallized, probably due to microheterogeneity of the carbohydrate moiety [13,15], and attempts of complete deglycosylation under non-denaturing conditions were unsuccessful. Crystals from glycosylated [27] and partially deglycosylated AAO diffracted at low resolution, being not suitable for determination of the molecular structure. Crystallization of the in vitro-activated AAO* from *E. coli*, which shows mass and charge homogeneity, will confirm whether glycosylation is the main problem to obtain crystals diffracting to high resolution.

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References

[1] A. Aliverti, B. Curti, M.A. Vanoni, Identifying and quantitating FAD and FMN in simple and in iron–sulfur-containing flavoproteins, Methods Mol. Biol. 131 (1999) 9–23.
[2] S. Camarero, S. Sarkar, F.J. Ruiz-Dueñas, M.J. Martínez, A.T. Martínez, Description of a versatile peroxidase involved in natural degradation of lignin that has both Mn-peroxidase and lignin-peroxidase substrate binding sites, J. Biol. Chem. 274 (1999) 10324–10330.
[3] H. Connaris, J.B. Chaudhuri, M.J. Danson, D.W. Hough, Expression, reactivation, and purification of enzymes from *Haloferax volcanii* in *Escherichia coli*, Biotechnol. Bioeng. 64 (1999) 38–45.
[4] E. de Jong, W.J.H. van Berkel, R.P. van der Zwan, J.A.M. de Bont, Purification and characterization of vanillyl alcohol oxidase from *Penicillium simplicissimum*. A novel aromatic alcohol oxidase containing covalently bound FAD, Eur. J. Biochem. 208 (1992) 651–657.
[5] K.-E.L. Eriksson, R.A. Blanchette, P. Ander, Microbial and Enzymatic Degradation of Wood Components, Springer-Verlag, Berlin, 1990.
[6] C.S. Evans, M.V. Dutton, F. Guillén, R.G. Veness, Enzymes and small molecular mass agents involved with lignocellulose degradation, FEMS Microbiol. Rev. 13 (1994) 235–240.
[7] P. Ferreira, M. Medina, F. Guillén, M.J. Martínez, W.J. van Berkel, A.T. Martínez, Spectral and catalytic properties of aryl-alcohol oxidase, a fungal flavoenzyme acting on polyunsaturated alcohols, Biochem. J. (in press), doi:10.1042/BJ20041903.
[8] K. Gekko, S.N. Timasheff, Mechanism of protein stabilization by glycerol: preferential hydration in glycerol–water mixtures, Biochemistry 20 (1981) 4667–4676.

[9] F. Guillén, C.S. Evans, Anisaldehyde and veratraldehyde acting as redox cycling agents for H_2O_2 production by *Pleurotus eryngii*, Appl. Environ. Microbiol. 60 (1994) 2811–2817.
[10] F. Guillén, A.T. Martínez, M.J. Martínez, Substrate specificity and properties of the aryl-alcohol oxidase from the ligninolytic fungus *Pleurotus eryngii*, Eur. J. Biochem. 209 (1992) 603–611.
[11] F. Guillén, A.T. Martínez, M.J. Martínez, C.S. Evans, Hydrogen peroxide-producing system of *Pleurotus eryngii* involving the extracellular enzyme aryl-alcohol oxidase, Appl. Microbiol. Biotechnol. 41 (1994) 465–470.
[12] A. Gutiérrez, L. Caramelo, A. Prieto, M.J. Martínez, A.T. Martínez, Anisaldehyde production and aryl-alcohol oxidase and dehydrogenase activities in ligninolytic fungi from the genus *Pleurotus*, Appl. Environ. Microbiol. 60 (1994) 1783–1788.
[13] J. Hendle, H.J. Hecht, H.M. Kalisz, R.D. Schmid, D. Schomburg, Crystallization and preliminary X-ray diffraction studies of a deglycosylated glucose oxidase from *Penicillium amagasakiense*, J. Mol. Biol. 223 (1992) 1167–1169.
[14] W.C. Johnson, Analyzing protein circular dichroism spectra for accurate secondary structures, Proteins 35 (1999) 307–312.
[15] H.M. Kalisz, H.J. Hecht, D. Schomburg, R.D. Schmid, Crystallization and preliminary X-ray diffraction studies of a deglycosylated glucose oxidase from *Aspergillus niger*, J. Mol. Biol. 213 (1990) 207–209.
[16] H.M. Kalisz, J. Hendle, R.D. Schmid, Structural and biochemical properties of glycosylated and deglycosylated glucose oxidase from *Penicillium amagasakiense*, Appl. Microbiol. Biotechnol. 47 (1997) 502–507.
[17] H. Lilie, E. Schwarz, R. Rudolph, Advances in refolding of proteins produced in *E. coli*, Curr. Opin. Biotechnol. 9 (1998) 497–501.
[18] A.T. Martínez, S. Camarero, F. Guillén, A. Gutiérrez, C. Muñoz, E. Varela, M.J. Martínez, J.M. Barrasa, K. Ruel, M. Pelayo, Progress in biopulping of non-woody materials: chemical, enzymatic and ultrastructural aspects of wheat-straw delignification with ligninolytic fungi from the genus *Pleurotus*, FEMS Microbiol. Rev. 13 (1994) 265–274.
[19] M.J. Martínez, F.J. Ruiz-Dueñas, F. Guillén, A.T. Martínez, Purification and catalytic properties of two manganese-peroxidase isoenzymes from *Pleurotus eryngii*, Eur. J. Biochem. 237 (1996) 424–432.
[20] L. Marzullo, R. Cannio, P. Giardina, M.T. Santini, G. Sannia, Veratryl alcohol oxidase from *Pleurotus ostreatus* participates in lignin biodegradation and prevents polymerization of laccase-oxidized substrates, J. Biol. Chem. 270 (1995) 3823–3827.
[21] C. Muñoz, F. Guillén, A.T. Martínez, M.J. Martínez, Laccase isoenzymes of *Pleurotus eryngii*: characterization, catalytic properties and participation in activation of molecular oxygen and Mn^{2+}oxidation, Appl. Environ. Microbiol. 63 (1997) 2166–2174.
[22] F.J. Ruiz-Dueñas, M.J. Martínez, A.T. Martínez, Molecular characterization of a novel peroxidase isolated from the ligninolytic fungus *Pleurotus eryngii*, Mol. Microbiol. 31 (1999) 223–236.
[23] J. Sambrook, D.W. Russell, Molecular Cloning, CSHL Press, Cold Spring Harbor, New York, 2001.
[24] N. Sreerama, R.W. Woody, A self-consistent method for the analysis of protein secondary structure from circular dichroism, Anal. Biochem. 209 (1993) 32–44.
[25] M. Valmaseda, G. Almendros, A.T. Martínez, Substrate-dependent degradation patterns in the decay of wheat straw and beech wood by ligninolytic fungi, Appl. Microbiol. Biotechnol. 33 (1990) 481–484.
[26] I.H. van Stokkum, H.J. Spoelder, M. Bloemendal, R. van Grondelle, F.C. Groen, Estimation of protein secondary structure and error analysis from circular dichroism spectra, Anal. Biochem. 191 (1990) 110–118.
[27] E. Varela, B. Böckle, A. Romero, A.T. Martínez, M.J. Martínez, Biochemical characterization, cDNA cloning and protein crystallization of aryl-alcohol oxidase from *Pleurotus pulmonarius*, BBA Protein Struct. Mol. Enzym. 1476 (2000) 129–138.
[28] E. Varela, F. Guillén, A.T. Martínez, M.J. Martínez, Expression of *Pleurotus eryngii* aryl-alcohol oxidase in *Aspergillus nidulans*: purification and characterization of the recombinant enzyme, Biochim. Biophys. Acta 1546 (2001) 107–113.
[29] E. Varela, A.T. Martínez, M.J. Martínez, Molecular cloning of aryl-alcohol oxidase from *Pleurotus eryngii*, an enzyme involved in lignin degradation, Biochem. J. 341 (1999) 113–117.
[30] E. Varela, M.J. Martínez, A.T. Martínez, Aryl-alcohol oxidase protein sequence: a comparison with glucose oxidase and other FAD oxidoreductases, Biochim. Biophys. Acta 1481 (2000) 202–208.
[31] C. Wang, M. Eufemi, C. Turano, A. Giartosio, Influence of the carbohydrate moiety on the stability of glycoproteins, Biochemistry 35 (1996) 7299–7307.
[32] S. Witt, M. Singh, H.M. Kalisz, Structural and kinetic properties of nonglycosylated recombinant *Penicillium amagasakiense* glucose oxidase expressed in *Escherichia coli*, Appl. Environ. Microbiol. 64 (1998) 1405–1411.