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SHORT COMMUNICATION

# Production of recombinant *Agaricus bisporus* tyrosinase in *Saccharomyces cerevisiae* cells

**Chiara Lezzi · Gianluca Bleve · Stefano Spagnolo · Carla Perrotta · Francesco Grieco**

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**Abstract** It has been demonstrated that *Agaricus bisporus* tyrosinase is able to oxidize various phenolic compounds, thus being an enzyme of great importance for a number of biotechnological applications. The tyrosinase-coding *PPO2* gene was isolated by reverse-transcription polymerase chain reaction (RT-PCR) using total RNA extracted from the mushroom fruit bodies as template. The gene was sequenced and cloned into pYES2 plasmid, and the resulting pY-*PPO2* recombinant vector was then used to transform *Saccharomyces cerevisiae* cells. Native polyacrylamide gel electrophoresis followed by enzymatic activity staining with L-3,4-dihydroxyphenylalanine (L-DOPA) indicated that the recombinant tyrosinase is biologically active. The recombinant enzyme was overexpressed and biochemically characterized, showing that the catalytic constants of the recombinant tyrosinase were higher than those obtained when a commercial tyrosinase was used, for all the tested substrates. The present study describes the recombinant production of *A. bisporus* tyrosinase in active form. The produced enzyme has similar properties to the one produced in the native *A. bisporus* host, and its expression in *S. cerevisiae* provides good potential for protein engineering and functional studies of this important enzyme.

C. Lezzi · C. Perrotta
Dipartimento di Scienze e Tecnologie Biologiche ed Ambientali, Università del Salento, via Prov. Lecce-Monteroni, 73100 Lecce, Italy

G. Bleve · S. Spagnolo · F. Grieco (✉)
Consiglio Nazionale delle Ricerche (CNR), Institute of Sciences of Food Production (ISPA), Operative Unit of Lecce, via Provinciale Lecce-Monteroni, 73100 Lecce, Italy
e-mail: francesco.grieco@ispa.cnr.it

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## Introduction

Tyrosinases (monophenol, *o*-diphenol:oxygen oxidoreductases, EC 1.14.18.1), often also referred to as polyphenol oxidases (PPO), are copper-containing metalloproteins widely distributed throughout the phylogenetic scale from bacteria to mammals [25]. Tyrosinases (PPO) and their corresponding genes have been characterized from various sources, including bacteria, fungi, plants, and mammals. Fungal tyrosinases were firstly characterized from the edible mushroom *Agaricus bisporus* [37], and recognized as responsible for browning during development and postharvest storage, which particularly decreases the commercial value of several vegetal commodities. The most thoroughly characterized fungal tyrosinases with regards to their structural and functional properties are the ones from *A. bisporus* [37] and *Neurospora crassa* [20]. Other microbial tyrosinase genes have been isolated from *Pseudomonas* [22], *Aspergillus*, *Chaetotomastia*, *Ascovaginospora* [1], *Trametes* [34], *Pycnoporus* [13], and *Trichoderma* [28] genera.

Comparison of the amino acid sequences of recently published tyrosinases reveals high heterogeneity concerning their length and overall identity. However, highly conserved regions can be found in the active site among all the studied tyrosinases [12, 21, 35].

Tyrosinases have traditionally been utilized in plant-derived food production, e.g., tea, coffee, raisins, and cocoa, where they contribute to the quality of the final product [30]. These enzymes have many interesting applications in food and nonfood processes, especially due to their ability to catalyze formation of covalent bonds between

peptides, proteins, and carbohydrates [2, 10, 12] and they have proved to be also applicable in promoting protein cross-linking to improve the texture of cereal-, dairy-, meat-, and fish-derived foods [5]. Use of tyrosinase in biocatalytic systems has recently been considered as an alternative to chemical *ortho*-hydroxylation of phenols, such as in production of L-DOPA for Parkinson's disease treatment [8]. Tyrosinase-based biosensors represent a promising tool for analysis of the total content of phenolics in order to determine the amount of phenolic compounds in wine [4], as well as to detect phenol compounds in other beverages, such as apple and citrus fruit juices [17], and to accomplish removal of phenols and aromatic amines from wastewaters [15]. *A. bisporus* tyrosinase is commonly found in the native host as a tetrameric complex with molecular mass of 120 kDa, composed of two subunits of ca. 43 kDa (H subunit, coded by a *PPO* gene) and two subunits of ca. 14 kDa (L subunit) [31]. Two different types of H chains have been identified: $H^a$ (48 kDa) and $H^b$ (45 kDa), giving origin to two different isoenzymes, $H^a_2L_2$ and $H^b_2L_2$ [26]. The H subunit is the tyrosinase domain, whereas the identity, function and origin of the L subunit remain as yet unknown [16]. Wichers and coworkers [37] purified two monomeric tyrosinases from *A. bisporus* fruit bodies, encoded by the *PPO1* and *PPO2* genes, respectively [38].

The *AbPPO1* and *AbPPO2* complementary DNAs (cDNAs) are able to code for tyrosinases of ca. 64 kDa, and both coded monomers were demonstrated to be fully active [37, 38].

Tyrosinase extracted from *A. bisporus* has been extensively used in several investigations as a tool in setting up enzyme model systems [18, 30]. However, it is extremely difficult to obtain a pure enzyme from a mushroom extract. In fact, it has been recently shown that commercial preparations contain proteins, carbohydrates, phenolic compounds and, moreover, other enzymes such as laccases, which may interfere with the determination of tyrosinase activity [9]. The *PPO2*, *PPO3*, and *PPO4* genes have already been expressed in *Escherichia coli*, but the polypeptides produced did not show any enzymatic activity [37, 38]. Herein, we present cloning and heterologous expression in *Saccharomyces cerevisiae* of the *PPO2* tyrosinase gene from *A. bisporus* and characterization of the biologically active recombinant protein is also reported. The results presented here describe the production of a functional *A. bisporus* tyrosinase in a heterologous expression system.

## Materials and methods

### Strains, media, vectors, and materials

Fruit bodies of the white-rot fungus *A. bisporus* (strain U1) were obtained from a local grower. The *E. coli* strain DH5α [*F*$^-$, *ϕ80dlacZΔM15, Δ(lacZYA-argF)*], used in all DNA manipulations, was grown in the Luria-Bertani medium (Sigma, USA) supplemented, when required, with ampicillin (50 μg/ml). The *S. cerevisiae* strain employed for heterologous expression was the CEN.PK2-1C strain (*MATa ura3-52 his3-Δ1 leu2-3,112 trp1-289 MAL2-8c SUC2*), and the plasmid used for protein expression in yeast was the pYES2 vector (Invitrogen, USA). Yeast cultures were grown using SDM medium (SDM = Synthetic Dropout Medium, with no uracil addition) [6]. Carbon sources for yeast cell growth were glucose (2 %, w/v) or galactose (2 %, w/v). Commercial mushroom tyrosinase preparation was obtained from Sigma-Aldrich (T3824).

### *PPO2* gene amplification and expression in *S. cerevisiae*

Isolation of total RNA from fungal mycelia and synthesis of first-strand cDNA were performed as previously described [3], using the primers PPO2_Eco.for (AAAGAATTCTCG CTGATTGCTACTGTCGGA) and PPO2.his_Not.rev (AAG CGGCCGCTCAATGGTGATGGTGATGATGACCACCA CCGTTAATAACATGCACC) (the nonfungal sequences are underlined). The PCR amplicon obtained was digested with *Eco*RI and *Not*I and ligated into pYES2, under the control of the galactose-inducible promoter *GAL1*. The produced recombinant plasmid, denoted pY-*PPO2*, was used to transform the *S. cerevisiae* strain CEN.PK2-1C [11].

### Tyrosinase production and purification

The tyrosinase-expressing yeasts were grown into SDM medium supplemented with 2 % glucose and incubated at 30 °C. When the turbidity of the culture reached optical density of 0.8 at 600 nm, cells were harvested by centrifugation. Yeast cells were then washed once with water and suspended at concentration of 0.6 $OD_{600}$/ml in 1 l SDM medium, supplemented with 1 mM $CuSO_4$ and 2 % galactose as carbon source in order to induce the *GAL1* promoter. Yeasts were grown at 18 °C for 2 days and then harvested by centrifugation (5,000×*g* for 10 min). The sediment was suspended in lysis buffer (50 mM $NaH_2PO_4$, 150 mM NaCl, 30 mM imidazole, 10 % glycerol, 1 mM phenylmethylsulfonyl fluoride, pH 8) to final volume of 90 ml. Cells were lysed in a French Press (Thermo Electron Corporation, USA) and the homogenate was centrifuged at 4 °C for 20 min at 30,000×*g* to remove cell debris. The filtered crude fresh extract was applied to a 5-ml Ni–NTA Superflow Agarose (Qiagen, USA), gravity-fed column at 0.5 ml/min flow rate. Cell lysate was loaded onto the column equilibrated in lysis buffer and the column was washed with 40 column volumes of wash buffer (50 mM $NaH_2PO_4$, 150 mM NaCl, 30 mM imidazole,

20 % glycerol, 0.1 % Triton × 100, 1 mM phenylmethylsulfonyl fluoride). The tyrosinase protein was eluted from the column using 5 volumes of elution buffer (50 mM $NaH_2PO_4$, 150 mM NaCl, 250 mM imidazole, 1 mM phenylmethylsulfonyl fluoride).

### Tyrosinase activity assay

Tyrosinase activity was monitored spectrophotometrically using 50 mM potassium phosphate buffer pH 6.5 and either L-tyrosine ($\varepsilon_{280} = 1.290\ cm^{-1}\ M^{-1}$), L-DOPA (L-3,4-dihydroxyphenylalanine; $\varepsilon_{475} = 3.600\ cm^{-1}\ M^{-1}$), pyrocatechol ($\varepsilon_{420} = nm = 2.040\ cm^{-1}\ M^{-1}$), and pyrogallol ($\varepsilon_{430} = 2.600\ cm^{-1}\ M^{-1}$) as substrates [29]. All reactions were carried out at 20 °C. Enzyme activity was expressed as international units (IU). Enzyme activity measurements were performed on a Beckman DU-600 spectrophotometer (Beckman, USA). Protein concentration was determined using the Protein Assay dye reagent (Bio-Rad, USA) with bovine serum albumin as standard. Kinetic parameters were determined using a spectrophotometric method for all substrates. $K_{cat}$ values were calculated using total protein concentration in the enzyme solution. Control measurements were carried out without enzyme addition to correct for any chemical oxidation of the substrates at 20 °C. Tyrosinase was incubated with increasing concentrations of substrates in reaction buffer, and the reaction product formed was measured every 30 s along 10 min. Lineweaver–Burke analysis was used to analyze reaction kinetic parameters. All determinations were carried out in triplicate.

### Polyacrylamide gel electrophoresis and Western blot assays

The polyacrylamide gel electrophoresis assay was performed at 140 V and 25–30 mA in sodium dodecyl sulfate (SDS) Tris/glycine buffer in BioRad MiniProtean II gel electrophoresis equipment [27]. Silver staining procedure was performed as previously described by Heukeshoven and Dernick [14]. Protein concentration was determined using the Protein Assay dye reagent (Bio-Rad, USA) with bovine serum albumin as standard. Western blots were performed as previously described [3], by using respectively a sheep anti-mushroom tyrosinase polyclonal (Serotec, UK) and a rabbit anti-sheep IgG conjugated with peroxidase (Zymed Laboratories, USA), as primary and secondary antibodies. Tyrosinase activity in gels was stained by L-DOPA as follows: protein samples were suspended into loading sample buffer (prepared without $\beta$-mercaptoethanol addition) and then directly loaded onto a 10 % polyacrylamide native gel [27]. Electrophoretic analysis was carried out at 50 mA/gel at 4 °C. The gels were then washed in distilled water for at least 15 min and stained with 10 mM L-DOPA solution at room temperature.

## Results and discussion

Tyrosinases are present in vertebrates, invertebrates, plants, and microbes and they play an important role in several biological processes [25]. Although several microorganisms produce tyrosinases, we chose the enzyme from *A. bisporus* as the object of this study, based on its potential in biotechnological and environmental applications [12]. In addition, *A. bisporus* tyrosinase has already been investigated at the molecular level by Ismaya et al. [16], who reported the first available 2.3-Å-resolution crystal structure of a full fungal polyphenoloxidase. In the present investigation, the *PPO2 A. bisporus* gene was cloned and expressed in *S. cerevisiae*. The recombinant tyrosinase was purified and was demonstrated to exhibit PPO activity.

To isolate the tyrosinase-coding *PPO2* gene from *A. bisporus*, an RNA-PCR-based strategy was used. Isolation of the gene was carried out by RT-PCR with the aim of adding six histidine codons to its 3′-terminus. By using total RNA extracted from *A. bisporus* fruit bodies as template, the expected 1.6-kbp fragment was amplified and cloned in the pYES2 vector. The subsequent nucleotide sequence analysis showed that the cloned tyrosinase gene was identical to the *PPO2* gene (AJ223816) previously described by Wichers et al. [38] (not shown). The sequence analysis results were confirmed by sequencing five independent

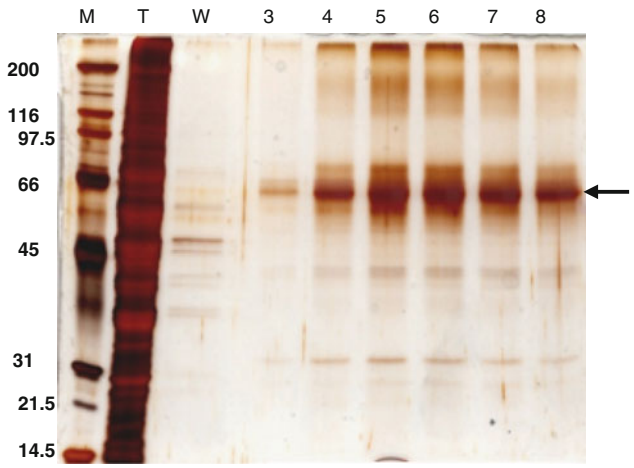


**Fig. 1** SDS-PAGE analysis of the recombinant mature tyrosinase produced in *S. cerevisiae* and purified by affinity chromatography by using phosphate buffer containing 250 mM imidazole and 0.15 M NaCl. The tyrosinase band is evident (*arrow*) in the samples obtained after elution with low salt. *M* molecular markers (expressed in kDa); *T* cell lysate; *W* portion of cell lysate not bound to the affinity resin; *lanes 3–9*, fractions 3, 4, 5, 6, 7, and 8 eluted from the affinity column

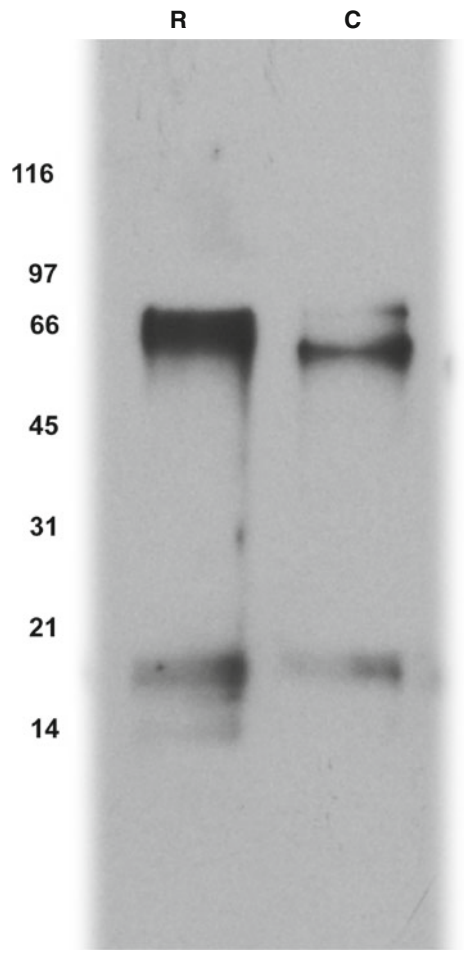


**Fig. 2** Western blot analysis of recombinant (*R*) and commercial (*C*) tyrosinase probed against a commercial antityrosinase antiserum. The results are representative of four independent experiments

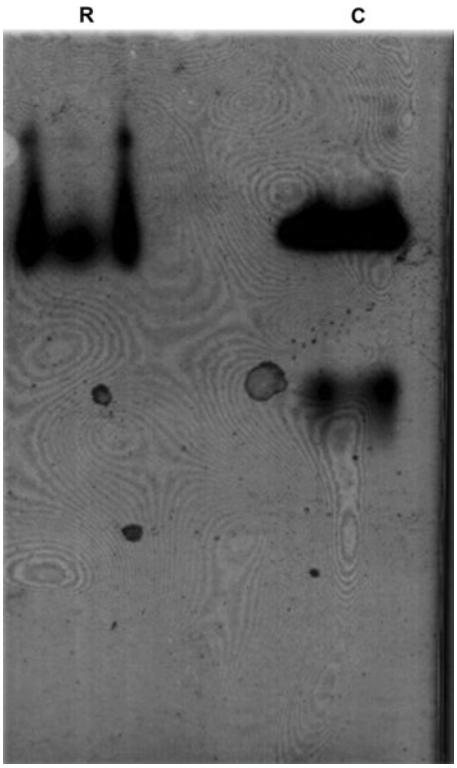


**Fig. 3** Identification of PPO activity in recombinant (*R*) and commercial (*C*) tyrosinase samples by staining a polyacrylamide native gel with L-DOPA. The commercial tyrosinase consisted of a mixture of *A. bisporus* proteins that used DOPA as substrate. The results are representative of four independent experiments

clones. Yeast cells transformed with the pY-*PPO2* vector were grown under induction condition (galactose as unique carbon source) and then lysed by using a French Press. The enzyme activity was directly tested on the fresh extract, resulting as 1.43 ± 0.12 U/g (grams of yeast cells). Tyrosinase purification was carried out by affinity chromatography by initially using a phosphate buffer containing 0.5 M NaCl. However, the protein purified using this saline concentration did not show any enzymatic activity and the SDS-PAGE purification profile evidenced the presence of several contaminant proteins co-eluted with tyrosinase (data not shown). A phosphate buffer containing an increased imidazole concentration (250 mM) under low salt conditions (0.15 M NaCl) was instead adopted for protein elution, thus allowing purification of recombinant tyrosinase in an active form and free from other contaminant polypeptides (Fig. 1). Enzyme total activity of 4.93 ± 0.24 U was achieved, corresponding to 0.33 ± 0.02 U/ml and to specific activity of 27.4 ± 3.4 U/mg of total protein recovered. The protein samples obtained after purification were analyzed by Western blot assay using a commercial antiserum raised against tyrosinase. By this assay, two bands, of 65 kDa and 21 kDa, respectively, corresponding to the same bands evidenced in the commercial tyrosinase, were detected in the recombinant protein preparation (Fig. 2).

Tyrosinase activity was detected on gels by loading purified enzyme samples onto polyacrylamide native gel; after electrophoresis, the gels were stained with L-DOPA solution. To check the sensitivity of the staining method, different concentrations of the recombinant enzyme and of the commercial mushroom tyrosinase (Sigma, USA) were loaded and tested for enzyme staining (not shown). After 3–10 min, a brown band appeared in the gel, thus indicating that the produced recombinant protein possessed clear PPO activity (Fig. 3, lane R).

Tyrosinase activity was biochemically characterized in liquid by assays in 50 mM phosphate buffer pH 6.5, at 20 °C using L-tyrosine, L-DOPA, pyrocatechol, and pyrogallol as substrates, with respect to the commercial purified *A. bisporus* tyrosinase enzyme (Table 1). Although the affinity values for all the tested substrates ($K_m$) were similar for the recombinant Ppo2 protein and the commercial tyrosinase, catalytic constants were generally higher for Ppo2 than for commercial tyrosinase. Indeed, the $K_{cat}$

**Table 1** Kinetic parameters of commercial and recombinant *A. bisporus* tyrosinases in the presence of different substrates

| Substrate | Commercial | | | Recombinant | | |
|---|---|---|---|---|---|---|
| | $K_m$ (μM) | $K_{cat}$ ($min^{-1}$) | $K_{cat}/K_m$ ($\mu M^{-1}$ $min^{-1}$) | $K_m$ (μM) | $K_{cat}$ ($min^{-1}$) | $K_{cat}/K_m$ ($\mu M^{-1}$ $min^{-1}$) |
| L-Tyrosine | 0.375 | 175.9 | 469.1 | 0.302 | 683.6 | 2,263.8 |
| L-DOPA | 1.33 | 934.6 | 702.78 | 1.22 | 8,459 | 6,713.7 |
| Pyrocatechol | 1.26 | 303.6 | 240.96 | 1.38 | 857 | 625.5 |
| Pyrogallol | 0.865 | 513.25 | 600.29 | 0.885 | 4,776.1 | 5,399 |

The commercial tyrosinase consisted of a mixture of *A. bisporus* proteins that used DOPA as substrate. Commercial tyrosinase was purchased from Sigma-Aldrich (USA)

values of Ppo2 showed an increase ranging from 9.3-fold (pyrogallol) to 2.8-fold (pyrocatechol) when compared with the values obtained for the commercial tyrosinase (Table 1). These results may be related to the purity level of the recombinant tyrosinase, which was purified to homogeneity from the yeast *S. cerevisiae*, whereas commercial tyrosinase preparations are commonly contaminated by other proteins [9].

According to literature data, very few reports of heterologous production of active fungal tyrosinases are available. The *Aspergillus oryzae* tyrosinase gene (*melO*) has been recently expressed in *Yarrowia lipolytica*, resulting in production of an active tyrosinase [23]. The tyrosinase Tyr2 of another *Ascomycetes*, the fungus *Trichoderma reesei*, was produced in *Pichia pastoris*, and the recombinant enzyme was shown to be biologically active [36]. The only *Basidiomycetes* tyrosinase produced by a heterologous expression system is that of the white-rot fungus *Pycnoporus sanguineus*, which was expressed in *Aspergillus niger* and secreted in the extracellular medium, under the control of glucoamylase pre-pro-sequence of *A. niger* [13]. The maturation process was effective in the heterologous host and the recombinant enzyme was fully active.

The yeast *S. cerevisiae* has been effectively and widely used for expression of a variety of heterologous proteins [7]. To achieve production of active tyrosinase, the yeast eukaryotic expression system was chosen, because it appeared to be the most efficient to ensure the posttranslational processing usually occurring in fungal cells during protein synthesis. In the case of *A. bisporus* tyrosinase, a chymotrypsin posttranslational cleavage in the C-terminus was postulated to occur at the tyrosine at position 381 [38]. This protein modification does not occur in the prokaryotic expression system and this is likely to be the reason for the failure to produce biologically active fungal PPOs in *E. coli* [38, 39].

The majority of studies on tyrosinase are based on use of commercial sources of the enzyme from the same mushroom, but unfortunately commercial preparations contain not only the purified enzyme but also a variety of contaminating compounds and other fungal enzymes [24]. The availability of a source of pure tyrosinase will be significant for all investigators dealing with physiological processes in which this enzyme takes part in order to avoid misinterpretation of obtained data due to the presence of contaminants [19, 32, 33].

In the present study, for the first time to our knowledge, a biologically active *A. bisporus* tyrosinase was produced in a heterologous host and then purified to homogeneity. Even though further experiments are needed to improve the enzyme yield and stability, for instance *PPO2* gene codon optimization and/or evaluation of other yeast expression vectors, the data reported herein indicate that the described heterologous system represents a promising procedure suitable for scale-up to large-scale tyrosinase production.

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