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TYROSINASE ISOFORMS FROM THE FRUITBODIES OF *AGARICUS BISPORUS*

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Key Word Index—*Agaricus bisporus*; Agaricaceae; polyphenol oxidase; purification; tyrosinase.

Abstract—Two monomeric tyrosinases were isolated from fruitbodies of *Agaricus bisporus* strain U1. Both tyrosinases, with pIs *ca* 5.2 and 5.1, showed an M_r of *ca* 43 kDa under reducing and denaturing conditions and of *ca* 47 kDa under native conditions, and similar cresolase and catecholase activities of, respectively, 90–120 nkat mg^{-1} and 17–18 μkat mg^{-1}. The enzyme preparations were >95% homogeneous. Neither isoform appeared to be glycosylated or phosphorylated. Upon purification the isoforms showed a gradual decrease in pI from *ca* 5.4–5.6 to *ca* 5.1–5.2. Copyright © 1996 Elsevier Science Ltd

INTRODUCTION

Early studies on the tyrosinase (EC 1.14.18.1) from the common mushroom *Agaricus bisporus* suggested the enzyme to possess an M_r of 110–120 kDa [1] and to occur as a tetrameric protein composer of two subunits of M_r 43–48 kDa (H) and two subunits of M_r 13.4 kDa (L). The assumed H_2L_2 structure eventually yields a native M_r of *ca* 110 kDa. Importantly, the isolated H and L subunits were reported to possess no enzymatic activity [2].

Despite the generally recognized importance of colour as a quality parameter for mushrooms and the many papers that have appeared in particular on its physiological aspects, details on the molecular characteristics of *Agaricus*-tyrosinase, such as its purification to homogeneity, amino acid composition and sequence, are scarce [2]. With a view to eventually preparing polyclonal and monoclonal antibodies against mushroom tyrosinase to be used in molecular studies, tyrosinase was purified from *A. bisporus* fruitbodies.

In this paper, the isolation of two monomeric 43 kDa tyrosinases from the fruitbodies of *A. bisporus* U1 is reported. Evidence that structural modifications of the protein occur during purification is presented.

RESULTS AND DISCUSSION

Enzyme isolation and purification

A quantitative overview of the purification of tyrosinase from *A. bisporus* U1 fruitbodies is presented in Table 1.

Chromatographic purification of polyphenol oxidase (PPO) activity

PPO-activity was purified from a homogenate of *A. bisporus*-U1 fruitbodies by a series of sequential chromatographic steps. As a first step, the homogenate was

Table 1. Purification of tyrosinase from the fruitbodies of *A. bisporus* U1

	Protein (mg)	Tyrosinase (μkat)	Specific activity (μkat mg^{-1})	Purification factor
Homogenate	9260	1850	0.20	1
HAP-pool C	1030	850	0.83	4.15
Sephadex G-200	58.1	245	4.21	21.1
Mono-Q first run	16.7	154	9.24	46.2
Mono-Q second run:				
TyrosinaseAb1	2.6	44.2	17.0	85.2
TyrosinaseAb2	2.3	41.4	18.0	90.1
Yield (%)	0.05	4.6		

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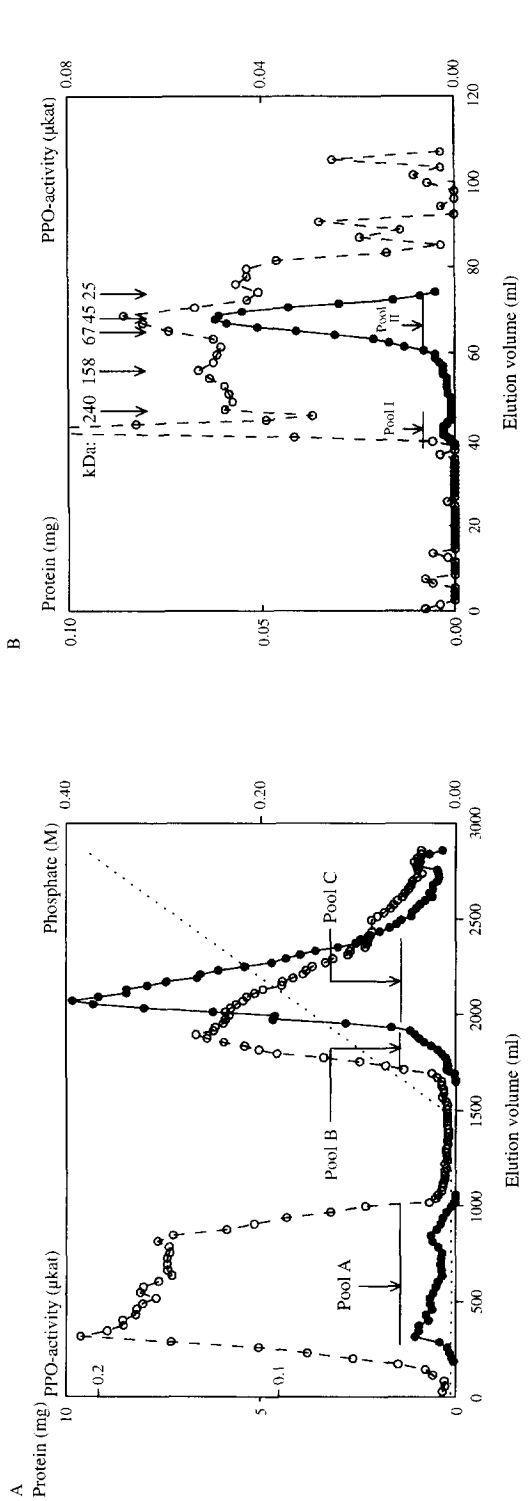

Fig. 1. Chromatographic purification of PPO-activity from *A. bisporus* U1 fruitbodies. ●—●, PPO-activity; ○----○, protein; ·····, K-PI (panel A) or NaCl gradient (panel C and D). Panel A–D: resp. HAP-, Sephadex G-200 and twice Mono-Q-HR-chromatography.

fractionated on hydroxyapatite (HAP) Biogel HT (Fig. 1A). A low concentration of tropolone, a general inhibitor of tyrosinase activity [3], was included in the elution buffers. Its concentration was such that no inhibition occurred after dilution of the sample in the tyrosinase assay. The major PPO-activity peak (pool C, eluting at *ca* 0.18 M K-Pi), was subsequently fractionated on Sephadex G-200 Superfine (Fig. 1B). Estimation of its M_r indicated an apparent value of 47 kDa for the major activity peak (pool II). This fraction was fractionated twice on Mono-Q-HR, eventually yielding two PPO-activity peaks eluting at respectively *ca* 0.10 M NaCl and at *ca* 0.12 M NaCl (Figs 1C and 1D). The second fractionation on Mono-Q-HR resulted in the removal of a 26 kDa protein impurity, after which the specific activity of the eventual purification product was increased a *ca* 2-fold (data not shown).

Characterization of PPO-activity from A. bisporus *U1 fruitbodies*

The two activity peaks from the second chromatography on Mono-Q-HR were analysed by SDS-PAGE under denaturing conditions, indicating these fractions to be apparently pure (<5% impurity indicated by density scanning). Both proteins showed an apparent M_r of 43 kDa (Fig. 2A). Both activity peaks possessed cresolase (resp. 119 and 90 nkat mg^{-1} using tyrosine as a substrate) and catecholase (resp. 17 and 18 μkat mg^{-1} using L-DOPA as a substrate) activity, and both were sensitive to inhibition by tropolone. This confirmed their classification as tyrosinases.

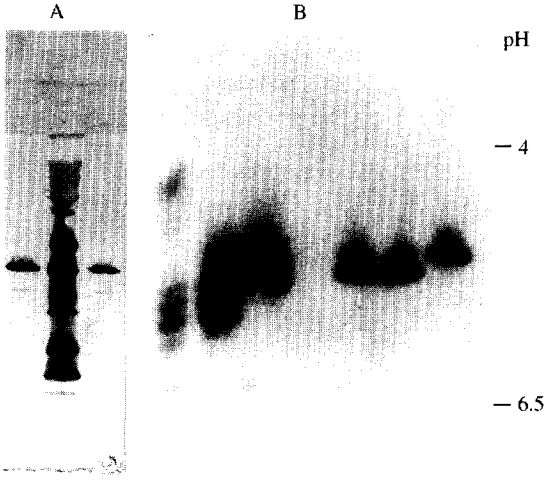

Fig. 2. Electrophoretic analysis of PPO-fractions. Panel A: SDS-PAGE (M_r of calibration proteins resp. 94 (top), 68, 43, 30, 20.1 and 14.4 kDa). From left to right: tyrosinaseAb1, calibration proteins, tyrosinaseAb2. Panel B: IEF. From left to right: homogenate; HAP Pool C; Sephadex G-200 pool II; lane with calibration proteins (do not stain); unbound fraction after rechromatography on Mono-Q-HR; tyrosinase Ab1; tyrosinase Ab2.

Neither isoform appeared to be glycosylated (as is partly the case with the low pI isoform [4]), nor phosphorylated as tested by staining with Stains All (not shown).

The isoform eluting at 0.10 M NaCl, designated tyrosinaseAb1, showed a pI of *ca* 5.2, while the isoform eluting at 0.12 M NaCl (tyrosinaseAb2) showed a pI of *ca* 5.1. Although the enzyme bands after isoelectric focusing (IEF) and activity staining were rather broad, a shift in pI upon purification was apparent. pIs (calculated for the middle of the enzyme bands) shifted from *ca* 5.5 in the crude homogenate (showing a cluster of at least three isoforms), via *ca* 5.2–5.3 after HAP chromatography. The final pI values were estimated to be *ca* 5.1 and 5.2 after gel filtration (Fig. 2B).

The tyrosinases that were isolated in the present investigation showed catecholase activities at a similar order of magnitude for the α-, β-, γ- and δ-isoforms as described by Robb [5]. However, cresolase activities were somewhat lower than those reported by Robb [5]. No clear explanation for the apparent lack in activity that was assigned previously to the H-chains [2] can be given yet. Comparing the purification scheme that was used in the earlier study [2] with the present scheme indicates acetone pretreatments (for both substrate removal and protein purification) being the most important procedural difference that might have an impact on the structural properties of the purified enzyme. For instance, in a recent publication the effect of melanosomal lipids and of de-lipidization by non-ionic detergents on structure and electrophoretic behaviour of melanosomal tyrosinase was documented [6]. Possibly the isoforms isolated in the present study correspond to the 43–48 kDa H-chains that were described elsewhere [2]. It is noteworthy that in the present investigation a shift in pI upon purification was observed (Fig. 2), indicating structural modification of the enzyme during the purification procedure. Although only two isoforms were purified to homogeneity, at least five isoforms were detectable in crude homogenates (Fig. 2B). This suggests the *in vivo* occurrence of isoforms with different structural properties in addition to the ones isolated in this study. The *Agaricus*-tyrosinases isolated in the present study show similarity to the tyrosinase from *Neurospora crassa*. This enzyme occurs as a 46 kDa monomer that is formed upon proteolytic processing of a 75 kDa precursor and for which the complete coding sequence and primary amino acid sequence have been elucidated [7–9]. Further support for this similarity is provided by the cloning of a putative *Agaricus*-tyrosinase cDNA fragment encoding a *ca* 64 kDa protein [10]. For higher plant tyrosinases, post-translational processing of pre-tyrosinases into mature forms is well documented [11,12]. The present observations strengthen the concept of posttranslational events being involved in regulation of *Agaricus*-tyrosinase and the similarities between fungal and plant tyrosinases.

EXPERIMENTAL

Enzyme extraction. 350 g of lyophilized and powdered fruitbodies of *A. bisporus* strain U1, second flush, harvested in stage 3–4 ('closed-cup' to 'cup' stage) were homogenized in portions of *ca* 50 g each. Each portion was suspended in 1 l ice-cold 5 mM K-Pi buffer pH 6.5, containing 2% (w/v) charcoal, 3 μM tropolone and 1 mM phenyl(methylsulphonyl fluoride. The suspension was divided into 4 parts and each part was homogenized with a Sonifier for 5 min under cooling in ice. The homogenate was centrifuged for 30 min at 11 000 *g*, 4° and the supernatant was filtered through Whatman no. 1 filter paper.

Enzyme isolation. All buffers used in chromatography contained 1 μM tropolone. Chromatography on HAP and on Sephadex G-200 was carried out at 4°.

The filtrate (*ca* 900 ml, containing *ca* 1 mg ml^{-1} of protein) was applied to a column (2.6 × 90 cm, column vol. *ca* 480 ml) of HAP Bio-Gel HT (BioRad), previously equilibrated with 5 mM K-Pi buffer pH 6.5. The column was eluted with 1400 ml 5 mM, 1.5 l of a linear gradient of 5–400 mM and 500 ml 1 M K-Pi buffer pH 6.5 (flow rate of 30 ml h^{-1}). Frs of *ca* 10 ml were collected. The conductivity of the eluate was determined with a CDM 80 conductivity meter (Radiometer Copenhagen). Three PPO-activity frs eluted from the column and were labelled HAP pool A, B and C, respectively. The corresponding frs were pooled, dialysed against 4 × 6 l 10 mM NH_4OAc buffer pH 6.5 and lyophilized. A Sephadex G-200 Superfine column (1.6 × 45 cm) was equilibrated with 25 mM Bis-Tris buffer pH 6.5 (buffer A). 15 mg of HAP pool C were dissolved in 15 ml buffer A, centrifuged for 15 min at 11 000 *g*, 4° and the supernatant was applied to the column. The column was eluted with buffer A, at 2.6 ml h^{-1}, and 90 frs of 0.9 ml, followed by 60 frs of 1.5 ml, were collected. 2 Peaks containing PPO-activity were labelled I and II and the fractions of these peaks were pooled. To determine the apparent native M_r of the PPO-activity, the column was calibrated with catalase, aldolase, bovine serum albumin, chicken albumin and chymotrypsin A (M_rs of 240, 158, 67, 45 and 25 kDa, respectively).

Pool II (containing the bulk of the enzyme) was applied to a Mono Q HR anion exchange column (0.5 cm × 5 cm, column vol. *ca* 1 ml) (Pharmacia) previously equilibrated with buffer A. The column was eluted with 25 ml buffer A, a linear gradient of 0.1–0.3 M NaCl in 15 ml of buffer A and 10 ml 1 M NaCl in buffer A. PPO-activity, eluting after 30–34 ml at *ca* 0.16 M NaCl, was pooled, dialysed against buffer A and rechromatographed on Mono Q with 10 ml buffer A, followed by a linear gradient of 0–0.2 M NaCl in 40 ml of buffer A, a linear gradient of 0.2–0.3 M NaCl in 10 ml buffer A, and 10 ml buffer A containing 1 M NaCl. Equilibration and runs were carried out at room temp. at a flow rate of 0.5 ml min^{-1} and 0.5 ml fractions were collected.

Enzyme assays. L-DOPA oxidizing activity and tyrosine hydroxylating activity were measured spectrophotometrically as previously described [4]. 0.1% (w/v) SDS was included in the assays. The sensitivity of the purified enzymes for inhibition by tropolone was assessed by using 0.5 mM tropolone in the DOPA-oxidizing assay.

Protein assay. Protein was determined by a modified Bradford method [13]. To 50 μl sample soln 950 μl H_2O and 1 ml 0.06% (w/v) Coomassie Brilliant Blue G250 in 3% (w/v) $HClO_4$ were added. The *A* at 595 nm was read after 15 min.

Isoelectric focusing, SDS gel electrophoresis and staining methods. Isoelectric focusing was performed on a PhastGel system (Pharmacia) using gels with a gradient of pH 4 to 6.5, according to the manufacturer's instructions Samples were dissolved in H_2O and centrifuged for 5 min at 11 000 *g* prior to application.

SDS-PAGE was carried out on the PhastGel system using gels with a gradient of 8 to 25% polyacrylamide. Samples were dissolved in 50 mM Tris–HCl buffer pH 6.5 containing 2% (w/v) SDS, 5% (v/v) 2-mercaptoethanol and 0.01% (w/v) bromophenol blue. They were denatured for 5 min at 100°, cooled in ice and centrifuged for 10 min at 11 000 *g* prior to application.

Gels were stained for protein using a standard silver staining method. For the estimation of the molecular mass, the M_r range 14–94 kDa (Pharmacia) was used. In the case of IEF, L-DOPA oxidizing activity was localized by incubating the gels in 100 mM NaPi buffer pH 6.5 containing 10 mM L-DOPA. Glycoproteins were stained with periodic acid/Schiff's stain as previously described [4]. Tyrosinase were tested for phosphorylation using the Stains All reagent [14].

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