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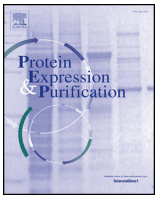

Efficient purification of a highly active H-subunit of tyrosinase from *Agaricus bisporus*

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ABSTRACT

A highly-active tyrosinase (H subunit) isoform has been purified from a commercial crude extract of *Agaricus bisporus* by a specific, two step-hydrophobic chromatography cascade process based on the differential adsorption of the proteins from the extract to hydrophobic-functionalized supports.

At first, commercial, crude tyrosinase from *Agaricus bisporus (AbTyr)* dissolved in aqueous media was added to octadecyl-Sepabeads matrix at 25 °C. Under these conditions, the support specifically adsorbed a protein with a molecular weight of 47 kDa which showed no tyrosinase activity. The known H subunit of tyrosinase from *Agaricus bisporus* (45 kDa, H-*Ab*Tyr) and another protein of 50 kDa were present in the supernatant. Sodium phosphate buffer was added to adjust the ionic strength of the solution up to 100 mM and Triton X-100 was added (final concentration of 0.07% v/v) to control the hydrophobicity effect for both proteins. This solution was offered again to fresh octadecyl-Sepabeads support, immobilizing selectively the H-*Ab*Tyr and leaving exclusively the 50 kDa protein as a pure sample in the supernatant. This tyrosinase isoform of 50 kDa was almost 4-fold more active than the known H-Tyr*Ab*, with a specific tyrosinase activity of more than 38,000 U/mg.

1. Introduction

Tyrosinases (polyphenol oxidase, EC 1.14.18.1) are ubiquitous copper-containing metalloenzymes described in a wide range of species including bacteria, fungi, plants and animals. Mushroom *Agaricus bisporus* tyrosinase (*Ab*Tyr) is commonly used for investigation purposes due to its affordability and homology with other tyrosinases like Human tyrosinase. Recently, six tyrosinase genes of *Agaricus bisporus* mushroom (PPO1-PPO6) have been identified and at least two of them (*ab*PPO3 and *ab*PPO4) were isolated from fruiting bodies, the main source of the commercial preparations of “mushroom tyrosinase” [1–3].

The crystal structure of *ab*PPO3 showed a hetero-tetrameric protein around 120 kDa consisting of two large (H) subunits of around 45 kDa each and two smaller (L) subunits of 14 kDa each [4,5].

The H subunit is the only one that presents catalytic activity due to the presence of two copper atoms coordinated in the active site with histidine residues (Fig. 1) for years it has only been considered active when paired with the L subunit but it has been proven that the monomeric H subunit presents catalytic activity by itself [6]. *Ab*Tyr has two different catalytic activities: monophenolase or cresolase activity causing the specific hydroxylation of monophenols to *o*-diphenols- and diphenolase or catecholase activity -catalyzing the oxidation of *o*-diphenols to *o*-quinones- [7,8]. Translating that to the melanogenic pathway, the enzyme catalyzes the hydroxylation of L-tyrosine to L-DOPA (L-3,4-dihydroxyphenylalanine) (cresolase activity) which is then oxidized to DOPA-quinone (catecholase activity) that undergoes either spontaneous or enzymatic reactions causing its polymerization and formation of the pigment melanin. Melanin plays important roles in lower species (camouflage, thermal regulation in amphibians, etc.) and in human skin for photoprotection against UV radiation, presenting up to 30–40 fold higher risk of skin cancer in lighter skins over darker ones [9]. Tyrosinase malfunction can be caused by mutations of its genes as in albinism where the patient does not produce melanin, or by autoimmune responses as some cases of vitiligo where the patient presents progressive depigmentation of its skin in patches as melanocytes are being destroyed [10].

Commercial extracts of *Ab*Tyr are mostly used for investigations about tyrosinase because of the difficult availability of the human one. However, these preparations do not contain pure enzyme and the presence of contaminant enzymes deserves serious consideration [11,12]. Laccases, quinone reductase and β-glucosidase have been identified in these preparations which can either react with the same phenolic substrates used by tyrosinase or with other substances (like β-glucosides) producing tyrosinase inhibitors [13]. In addition, different tyrosinase isoforms have been described depending on the source or even the mushroom area used for extraction [14].

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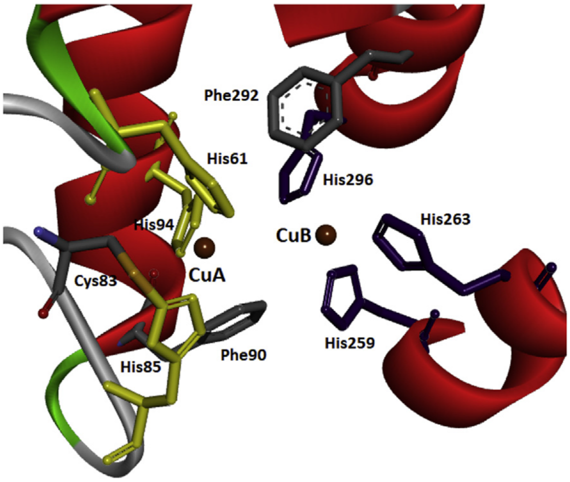

Fig. 1. Geometry of the binuclear copper-binding site of *Ab* Tyrosinase. Figure was drawn using Biovia Discovery Studio 2016.

Therefore, purification techniques are critical in order to obtain a specific and pure tyrosinase activity. Several reports about the purification of this enzyme have been described in literature, although these strategies involve several column chromatography steps, which are long and tedious processes that result in moderate overall yields [15], or constitutes really expensive procedures [16].

Therefore, the development of efficient and economic strategies for the obtainment of high amounts of purified enzyme is mandatory.

In this work, we have developed an effective and simple method to purify a highly active tyrosinase isoform from a commercial preparation of mushroom tyrosinase by an easy two-step hydrophobic chromatography cascade process.

2. Materials and methods

2.1. Materials

Tyrosinase from mushroom lyophilized powder, L-tyrosine, L-3,4-dihydroxy-phenylalanine (L-DOPA), Bradford's reagent, bovine serum albumin, Triton™ X-100, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and *N*-Hydroxysuccinimide (NHS) were from Sigma-Aldrich (Spain). Agarose was from AgaroseBeads. Octyl-Sepharose (C8) was from GE Healthcare. Octadecyl-Sepabeads® (C18) was a gift of RESINDION, Mitsubishi Chemical Corporation. PEI-agarose was prepared according to Mateo et al. [17]. Boronate-agarose was prepared as previously described [18].

2.2. Tyrosinase activity assay

Enzyme activity was tested in the presence of 2 mL of 1 mM L-DOPA in 0.1 M sodium phosphate buffer pH 7 at room temperature using a V-730 spectrophotometer (Jasco), measuring the increase in absorbance of the aminochromes at 475 nm caused by 40 μL of enzyme solution, and taking the initial rate, between 10 and 70 s of the reaction [7,19]. In the presence of L-Tyrosine, the linear portion after the initial lag period is taken [20]. An enzyme activity Unit (U) was defined as the amount of enzyme causing an increase of absorbance by 0.001/min at 25 °C [21].

Enzyme activity was tested varying the pH (acetate buffer at pH 5 and sodium phosphate buffer at pH 7), ionic strength of the medium (from 10 mM to 1 M of sodium phosphate buffer at pH 7), in the presence of 1,4-Dioxane (15% v/v) and detergent Triton™ X-100 (up to a concentration of 3%).

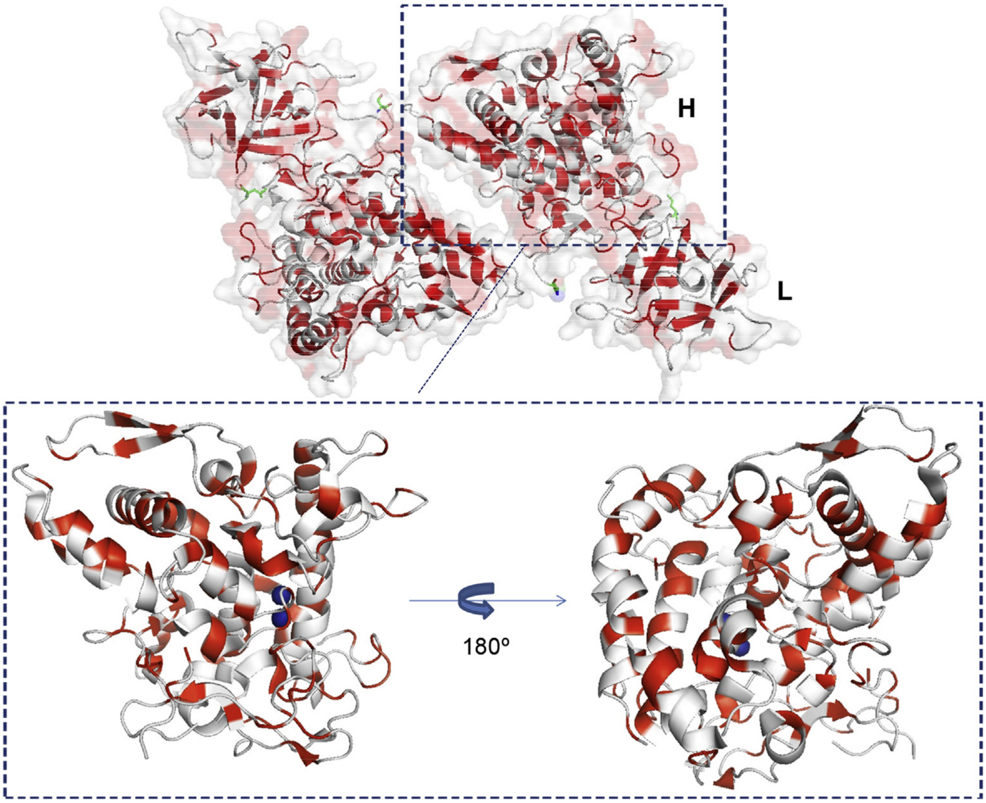

Fig. 2. Representations of the crystal structure surface of *Agaricus bisporus* tyrosinase. Hydrophobic residues distribution (red). Copper atoms (blue). Structure was obtained from PDB data bank with following codes: 2Y9W.Figure was drawn using Pymol 0.99 program. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)