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Identification and discrimination between some contaminant enzyme activities in commercial preparations of mushroom tyrosinase

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Abstract

Tyrosinase is a copper-enzyme involved in important biological processes. Many studies investigating these topics have relied on commercial preparations of mushroom tyrosinase as a tool or use as a model system. In this study, several commercial preparations of tyrosinase have been examined with regard to their composition and purity. Enzyme activities different from tyrosinase were found. Laccase, β-glucosidase, β-xylosidase, and xylanase activities were found in almost all samples examined. In particular, laccase and β-glucosidase were investigated for their ability to affect tyrosinase activity under certain experimental conditions. Laccase activity was variable in different commercial preparations and its presence could lead to misinterpretation of results ascribed to tyrosinase activity. In fact, it could hide the inhibitory effect of tropolone and kojic acid in relation to tyrosinase activity. β-Glucosidase released the aglycon moiety from the glucoside esculin which in turn could become a tyrosinase inhibitor or substrate. SDS–PAGE of commercial mushroom tyrosinase showed a complex pattern of unidentified proteins with at least four major and many minor protein staining bands. Taken together, these findings confirm that investigators should use caution in interpreting data relying on commercial sources of mushroom tyrosinase.

Keywords: Mushroom tyrosinase; Commercial preparations; Laccase; β-Glucosidase; Enzymic contaminant

1. Introduction

Tyrosinase, a copper-enzyme belonging to type-3 copper proteins, is involved in the initial step of melanin biosynthesis and it is widespread throughout microrganisms, plant and fungal species, invertebrates, and vertebrates [1–5]. This enzyme catalyses the *o*-hydroxylation of monophenols to the corresponding catechols (monophenolase or cresolase activity) and the oxidation of such catechols to the corresponding *o*-quinones (diphenolase or catecholase activity). Tyrosinase, also called polyphenol oxidase (PPO), is involved in other important biological processes, such as enzymic browning easily observed in vegetables [6], betalain biosynthesis [7], sclerotization of insect cuticle [8,9], and defense responses in arthropods [10], plants [11], and also in fungi [12]. Also studies on enzyme immobilisation for biotechnological purposes make use of mushroom tyrosinase [13].

Most of investigations, which make use of tyrosinase, have relied on commercial sources of the enzyme from the champignon mushroom, *Agaricus bisporus*. Unfortunately, these commercial preparations do not contain purified enzyme. In this regard, we would like to clarify that suppliers of commercial tyrosinase do not claim the preparations be homogeneous. In the past, some investigators [14,15] suggested the possibility that the presence of contaminant enzyme activity had to be taken in serious consideration in interpreting data using commercial sources of mushroom enzyme. These investigators, beyond the shadow of a doubt, demonstrated the presence of significant amount of laccase in such commercial preparations. Recently, the presence of contaminant laccase activity in tyrosinase commercial preparations was also confirmed [16].

Moreover, it is well-known that constitutive forms of laccase are present in white rot fungi and also in the *Agaricus* genus [17–22]. Recently, purification and characterisation of both a tyrosinase and a laccase from the same bacterium species was achieved [23].

Laccases are copper-enzymes, containing four copper ions, that oxidize phenolic compounds (but also aromatic amines)

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with poor specificity by means of electron abstraction thus forming a radical [3]. Further studies reached the conclusion that white rot fungi invest part of their metabolic energy to produce laccase for the purpose of degrading lignin. To do this, they also use additional enzymes [24]. Among them, quinone reductase, a membrane bound flavo-enzyme that binds FAD as the prosthetic group and in turn reduces quinones produced by laccase back to phenolic compounds. This redox cycle leads to the release of reactive oxygen species that take part in lignin degradation. When performing in vitro investigations relying on the enzymic properties of tyrosinase, the presence of laccase should be accurately excluded, otherwise, it could lead to ambiguous results as laccases share several substrates in common with tyrosinase, i.e. *o*-diphenols and *o*-aminophenols [25,26].

In addition, other contaminant enzymes could bring about misleading results. For example, β-glucosidase in the presence of a suitable β-glucoside could release either phenolic substrates or inhibitors utilized by tyrosinase. Formally, a glycoside is any molecule in which a sugar group is bonded through its anomeric carbon to another group *via* a glycosidic bond. Many authors often suggest that the sugar be bonded to a non-sugar for the molecule to qualify as a glycoside. The sugar group is referred to as the glycone and the non-sugar group (often containing a phenolic group) as the aglycone or genin part of the glycoside. In nature, a huge number of glycosides are known. Among these glycosides, many are β-glucosides and they behave as substrates for β-glucosidases. After hydrolysis of β-glucosides by β-glucosidases, the product may potentially interact interact with tyrosinase and laccase.

Based on these considerations, we have investigated the purity of several lots of commercial tyrosinase that were purchased in the course of the last several years for our research needs. The results of this investigation are reported and some considerations about the need to examine commercial tyrosinase preparations more carefully are given.

2. Materials and methods

2.1. Preparation of commercial tyrosinase

Six commercial preparations (purchase in the last eight years) were under investigation for the purposes of this study. Two of them (lot 17H9557, lot 112H9580, in the following named as S1- and S2-lot, respectively) were purchased from Sigma–Aldrich (Milan, Italy); three (lot 421497/1, lot 1179697, lot unreadable, in the following named F1-, F2- and F3-lot) were purchased from Fluka (Milan, Italy); the sixth (lot 36E8802, named WMT-1) was from Worthington (Lakewood, NJ, USA). The contents of each bottle were weighed and dissolved in 50 mM potassium phosphate buffer (pH 6.5), in accordance with the instructions of the manufacturer, to obtain 1 mg powder mL^{-1} as the final concentration. For electrophoresis experiments, an aliquot of powder was suspended in the same buffer to obtain 2 mg powder mL^{-1} concentration. Each sample was centrifuged at 9000 × *g*, portioned out into 2 mL aliquots, and stored at −80 °C until used.

In addition, another commercial preparation of mushroom tyrosinase (WMT-2, lot 33H6588Q) from Worthington was used for Western blotting experiments.

2.2. Enzyme and protein assays

Tyrosinase (E.C. 1.14.18.1) assays of commercial preparations were carried out according to supplier's instructions, but the unit definition of tyrosinase activity was designated differently with respect to the manufacturer's instructions. In this paper, one tyrosinase unit corresponds to the amount of enzyme which increases the absorbance at 305 nm by 0.001 per minute in the assay conditions. Laccase activity was measured spectrophotometrically by monitoring absorbance increases at 525 nm when 50 μM syringaldazine was present in a final volume of 1 mL of a buffered (50 mM potassium phosphate, pH 6.0) solution. One laccase unit is defined as the amount of the enzyme capable of forming one micromole of product per minute [27].

Enzymatic assays for β-xylosidase (E.C. 3.2.1.37), peroxidase (E.C. 1.11.1.7), xylanase (E.C. 3.2.1.8), and pectinase (E.C. 3.2.1.15) were carried out as described in Sigma–Aldrich Enzyme Assay database [28]. β-Glucosidase (E.C. 3.2.1.21), and NAD(P)H (quinone acceptor) oxidoreductase (E.C. 1.6.99.2) assays were carried out as described in [29] and [30], respectively. All enzyme activities were done in duplicate and the results are a mean of the data. Protein content was estimated by means of the Bio-Rad Protein Assay kit, based on the Bradford method [31].

2.3. HPLC analysis

Before performing HPLC analysis, protein-containing samples were deproteinized by phosphoric acid (about 0.05 M in final concentration) and centrifuged at 10,000 × *g* for 10 min. The resulting supernatant was then filtered through a 0.45 μm pore size membrane filter (Millipore).

Identification of the products of enzymatic activity was carried out with a Beckman System Gold apparatus equipped with an UV-Vis detector module. The column used for chromatographic separations was a Phenomenex, Luna, RP-C18 (250 mm × 3 mm i.d., 5 μm,) purchased from Chemtek Analytica (Bologna, Italy). Separations of the compounds were achieved with 0.085% phosphoric acid in water, v/v (solvent A) and 95% acetonitrile in 0.085% phosphoric acid (solvent B) as mobile phases. Chromatographic conditions: initial isocratic elution, 5 % B for 5 min, followed by a gradient phase, 5 → 90% B, in 10 min at 0.6 mL min^{-1} flow rate. The detector was set at 280 nm.

2.4. Electrophoresis and Western blotting

Experiments with Sigma–Fluka tyrosinase were performed with the Mini Protean III apparatus (Bio Rad Italiana, Milan, Italy), following the manufacturer's instructions for gel preparation. SDS-gel electrophoresis was carried out in 4% stacking gel and 10% separating polyacrylamide gels (1.5 mm thickness) and gels were stained with Bio-Safe Coomassie (Coomassie G250 stain) purchased from Bio-Rad. Native-PAGE was also carried out in 4% stacking gel and 10% separating gel (1.5 mm thickness).

When electrophoresis was completed, the gel was subjected to activity staining for tyrosinase activity as described elsewhere [32] or for β-glucosidase activity as described in [33]. Western blotting, native- and SDS–PAGE of WMT-2 were carried out using methods described earlier by Chen and Flurkey [34].

2.5. Preparative SDS–PAGE and protein sequencing

Approximately 2 mg of WMT-2 powder were resuspended in 10 mM phosphate buffer pH 6.5 and boiled for 2 min. One-fourth volume of 4x Laemmli sample buffer was added and the solution boiled again. Samples were applied to a 1 mm thick (10 cm × 10 cm) 11% SDS–PAGE gel prepared by the authors and subjected to SDS–PAGE electrophoresis. After electrophoresis was terminated the gel was soaked in transfer buffer (10 mM CAPS, 10% methanol, pH 11) for 10 min before electrophoretic transfer onto ABI Protoblott PVDF membranes using the same buffer. After transfer, the membrane was stained briefly with Coomassie Blue R-250 and destained extensively. N-terminal protein sequencing of individual bands present on the PVDF membranes was carried out by the Michigan State University Macromolecular Structure Facility using standard Edman degradation procedures. Internal protein sequences of some bands on the PVDF membranes were determined after tryptic digestion of the band(s), HPLC separation of peptides, and N-terminal protein sequencing of selected peptides from the HPLC separation. The identities of the proteins in the SDS–PAGE bands subjected to protein sequencing were determined by conducting BLASTP searches against the National Center for Biotechnology Information (NCBI) nr database.