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DIAFILTRATION IN THE PRESENCE OF ASCORBATE IN THE PURIFICATION OF MUSHROOM TYROSINASE

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Key Word Index—*Agaricus bisporus*; Agaricaceae; mushrooms; purification; tyrosinase; ascorbate.

Abstract—In the present paper a method for the extraction of *Agaricus bisporus* tyrosinase, based on the use of a diafiltration procedure in the presence of ascorbate, is proposed. During the extraction of tyrosinase the oxidation of mushroom phenolics was avoided, keeping phenolic compounds in their reduced form throughout the extraction step. Their complete removal by means of a diafiltration apparatus was achieved. The method here described represents a useful way to obtain an enzymic solution of tyrosinase, devoid of phenolics and melanic compounds.

INTRODUCTION

Tyrosinase (monophenol, *o*-diphenol: oxygen oxidoreductase, EC 1.14.18.1) is a binuclear copper protein [1–3] which has received considerable attention in the last 20 years as an indispensable tool to perform studies over a wide range of topics [4–7]. Tyrosinase found in mushrooms (*Agaricus bisporus*) has been utilized in the majority of these studies since it is readily obtainable in relatively large quantities. Commercial preparations of this enzyme are available. Unfortunately, a further purification is needed because of the enzyme tanning and the presence of some other enzymes which could affect interpretation of experimental data [8]. On the other hand, purification of *A. bisporus* tyrosinase is difficult owing to the oxidation of phenolics localized in the mushroom tissues [9, 10]. It is well known that tyrosinase oxidizes these phenolic compounds to quinones, followed by coupling reactions or by oxidation of protein functional groups by the quinones, which are powerful electrophilic agents. Besides, tyrosinase could be inactivated as the result of irreversible reaction between a product of the enzyme-catalysed reaction and the active site of the enzyme [11]. Quinones also tend to polymerize yielding melanins which in turn hinder the application of chromatographic procedures and irreversibly damage chromatographic resins. Although several methods have been proposed in order to solve this problem [12–14], colourless enzyme preparations were rarely obtained. We carried out the first step of the purification of *A. bisporus* tyrosinase with a simplified method, which avoided the formation of melanins.

RESULTS AND DISCUSSION

We describe an extraction procedure for tyrosinase from *A. bisporus* which requires the use of sodium ascorbate to keep phenolics present in the homogenate in the reduced state. Browning of a mushroom homogenate, due to the continued oxidation of phenolics by tyrosinase, could be avoided by means of both a quick washing of mushrooms and their homogenization in the presence of ascorbate [14, 15].

A further step consists of the removal of phenolics while still in the reduced state. Conventional dialysis against ascorbate seems a convenient solution to this problem. However, the complete removal of phenolics requires several days and continuous changes of ascorbate solution. Moreover, the ascorbate has to be in turn removed prior to the application of conventional chromatographic procedures. Therefore, we propose a method of extraction of *A. bisporus* tyrosinase, which employs a diafiltration system based on the crossflow technique in the presence of ascorbate. This procedure avoided the oxidation of phenolics by keeping them in the reduced form throughout the extraction phase until their removal by means of diafiltration. This was very fast in comparison with conventional dialysis. The removal of phenolic compounds from the mixture could be determined by monitoring the A_{280} of the filtrate. The presence of ascorbate allowed an increased of homogenization time compared with conventional extraction techniques, so that larger amounts of enzyme could presumably be released from the mushroom tissues. A pale yellow solution was obtained at the end of the

diafiltration step, and the browning of this solution was not observed even after 24 hr of air exposure at 4°. The solution, whose specific activity was 1700 U mg^{-1}, could be further purified with the chromatographic procedures previously described [11–13].

EXPERIMENTAL

Materials. Freshly packed white mushrooms (*A. bisporus*) were supplied by a local mushroom grower and stored at 4° until use. The carpophores (1 kg) were gently cleaned to remove earthy residues and washed with cooled 50 mM Na ascorbate, pH 5.4 (soln A). All other chemicals were of reagent grade and used without further purification.

Extraction. Carpophores were homogenized with soln A (150 ml per 100 g) for 2 min in a Waring blender. The resulting suspension was then centrifuged at 26 000*g* for 20 min at 4°, the ppt. was discarded and the supernatant filtered through Whatman filter paper *in vacuo*. The enzyme soln was then subjected both to a concn and diafiltration procedure in a Sartocon Mini Crossflow System (Sartorius AG, Germany) equipped with 3 polysulphone modules (nominal M_r cut-off = 10 000) and a membrane pump. The entire filter area was 0.3 m^2. Throughout this operation, a total vol. of 8 l of 20 mM Na ascorbate (pH 5.4) was added to the enzyme soln. The reservoir containing enzyme soln was immersed in a cooled bath at 4°, and the diafiltrating vol. was kept close to 300 ± 150 ml; then, 3 l 30 mM K-Pi buffer (pH 7) was added with same procedure to remove ascorbate, and the final enzyme soln was withdrawn in a final vol. of *ca* 350 ml. The overall diafiltration and concn process took *ca* 3 hr.

Enzyme assay. Tyrosine hydroxylase activity was measured using 0.325 mM tyrosine as final concn of substrate. The standard assay system contained 100 mM K-Pi buffer (pH 6.5) and 130 μl daily prepd tyrosine soln. The reaction was started by addition of 50 μl enzyme soln in a final vol. of 1 ml. One unit of enzyme activity was defined as amount of enzyme which caused an increase in A_{280} of 0.001 min^{-1} at 25° [13].

Protein analysis. Proteins were estimated by the method of ref. [16] using BSA as standard.

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