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Direct immobilization of tyrosinase enzyme from natural mushrooms (*Agaricus bisporus*) on D-sorbitol cinnamic ester

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Abstract

Mushroom tyrosinase was immobilized from an extract onto the totally cinnamoylated derivative of D-sorbitol by direct adsorption as a result of the intense hydrophobic interactions that took place. The immobilization pH value and mass of lyophilized mushrooms were important parameters that affected the immobilization efficiency, while the immobilization time and immobilization support concentration were not important in this respect. The extracted/immobilized enzyme could best be measured above pH 3.5 and the optimum measuring temperature was 55 °C. The apparent Michaelis constant using 4-*tert*-butylcatechol as substrate was 0.38 ± 0.02 mM, which was lower than for the soluble enzyme from Sigma (1.41 ± 0.20 mM). Immobilization stabilized the extracted enzyme against thermal inactivation and made it less susceptible to activity loss during storage. The operational stability was higher than in the case of the tyrosinase supplied by Sigma and immobilized on the same support. The results show that the use of *p*-nitrophenol as enzyme-inhibiting substrate during enzyme extraction and immobilization made the use of ascorbic acid unnecessary and is a suitable method for extracting and immobilizing the tyrosinase enzyme, providing good enzymatic activity and stability.

Keywords: Mushroom tyrosinase; Immobilization; Cinnamic carbohydrate esters; Tyrosinase extraction; Enzyme kinetics; Tyrosinase stability

1. Introduction

Tyrosinase or polyphenol oxidase is a copper enzyme which catalyses the hydroxylation of monophenols to *o*-diphenols and the oxidation of the latter to *o*-quinones, using oxygen (Gómez-Fenoll et

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al., 2001). Because tyrosinase is an expensive enzyme, immobilising it is considered to be worthwhile so that it can be reused. Several authors have endeavoured to immobilize tyrosinase on different solid supports (Arica and Bayramoğlu, 2004; Durán et al., 2002; Munjal and Sawhney, 2002) using different immobilization methods (Durán et al., 2002; Klibanov, 1983). Attempts have been made to use immobilized tyrosinase for the commercial production of L-dopa (Seetharam and Saville, 2002), for the removal of phenolic compounds from wastewater (Karam and Nicell, 1997), as biosensors to detect pollutants in environmental samples (Wang et al., 2002), for the electrochemical determination of hydrogen peroxide (Hall et al., 1996) or for the analysis of thiol-containing compounds (Huang et al., 2002). In a previous study of ours, the polymeric support used to immobilize tyrosinase supplied by Sigma was the crosslinked totally cinnamoylated derivative of D-sorbitol (SOTCN) on glass beads (Marín-Zamora et al., 2005). In this work, we use the above support for the first time to directly immobilize the tyrosinase enzyme present in a mushroom extract containing *p*-nitrophenol (PNP). One of the reasons for developing this procedure was, besides the economic interest (the resulting enzyme would be cheaper than that provided by Sigma), to improve the operational, thermal and storage stability described in our previous work (Marín-Zamora et al., 2005).

Other authors have used ascorbic acid (AA) to prevent the browning of mushroom extracts in processes to purify the enzyme and for their storage (Núñez-Delicado et al., 1996; Espín and Wichers, 1999). However, for long storage intervals, the method is not feasible because large quantities of AA are needed, and these, in turn, will inactivate the enzyme (Andrawis and Kahn, 1990; Seo et al., 2003). In the present study, we use, for the first time, a substrate of the enzyme, *p*-nitrophenol, which has a high affinity constant for the enzyme and low transformation rate, to prevent browning during the enzyme extraction and immobilization processes and enzyme inactivation as a result of the consumption of the phenols present in the mushroom extract, thus rendering the use of others inhibitors unnecessary.

We study the parameters that influence optimization of the new extraction–immobilization process and the behaviour and stability of the samples in the face of different factors. We compare the results obtained in similar assay conditions with those shown by the enzyme supplied by Sigma.

2. Materials and methods

Natural mushrooms (*Agaricus bisporus*) were supplied by MERCADONA (Spain) and used to obtain fresh tyrosinase. Ascorbic acid (AA) was supplied by DOMCA (Spain), commercial tyrosinase (T-7755; EC 1.14.18.1 from mushroom, 6680 U mg^{-1} solid), 4-*tert*-butylcatechol (TBC) and *p*-nitrophenol (PNP) were purchased from Sigma (Spain). A stock solution of the phenolic substrate was prepared in 0.15 mM phosphoric acid to prevent autoxidation. All other chemicals were of analytical grade and supplied by Fluka (Spain), Panreac (Spain), J.T. Baker (Holland), Sigma (Spain) and Lab-Scan (Ireland). Ultrapure water from a Milli-Q system (Millipore Corporation) was used throughout this research.

Totally cinnamoylated derivative of D-sorbitol (SOTCN) was prepared by a modified version of the method proposed by Van Cleve (Marín-Zamora et al., 2005).

2.1. Tyrosinase extraction

Before use, the natural mushrooms were lyophilized and ground mechanically. The product obtained in this way was stored at −18 °C. To extract the fresh tyrosinase enzyme, 600 mg of lyophilized-ground mushroom was added to 16 ml of a 30 mM aqueous solution of PNP (pH 7.0) and magnetically stirred for 30 min at 4 °C. A pH value of 7.0 was used to avoid the extract browning (Núñez-Delicado et al., 1996). The extraction medium was then centrifuged at 6000 rpm for 5 min. The supernatant (9 ml) which contained the tyrosinase activity was collected and equilibrated to pH 5.5 by adding 1 ml of a 0.9 M aqueous solution of NaH_2PO_4 and 0.1 M of H_3PO_4. The elimination of all solids was attained by means of a second centrifugation.

2.2. Tyrosinase immobilization

Glass beads JM-50 of 1.7–2.4 mm diameter fabricated by SOVITEC IBERICA S.A. (Barcelona, Spain) were supplied by JAQUE (Murcia, Spain). Before use, the glass beads (i.e., the inert matrix) were washed and