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ScienceDirect
Journal of Biotechnology 131 (2007) 388–396
Journal of BIOTECHNOLOGY
www.elsevier.com/locate/jbiotec

Effects of the immobilization supports on the catalytic properties of immobilized mushroom tyrosinase: A comparative study using several substrates

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Received 30 November 2006; received in revised form 24 April 2007; accepted 8 May 2007

Abstract

Mushroom tyrosinase was immobilized from an extract onto glass beads covered with one of the following compounds: the crosslinked totally cinnamoylated derivatives of glycerine, D-sorbitol, D-manitol, 1,2-*O*-isopropylidene-α-D-glucofuranose, D-glucuronic acid, D-gulonic acid, sucrose, D-glucosone, D-arabinose, D-fructose, D-glucose, ethyl-D-glucopyranoside, inuline, dextrine, dextrane or starch, or the partially cinnamoylated derivative 3,5,6-tricinnamoyl-D-glucofuranose which was obtained by the acid hydrolysis of 1,2-*O*-isopropylidene-α-D-glucofuranose. The enzyme was immobilized by direct adsorption onto the support and the quantity of tyrosinase immobilized was found to increase with the hydrophobicity of the supports. The kinetic constants of immobilized tyrosinase acting on the substrates, 4-*tert*-butylcatechol, dopamine and DL-dopa, were studied. When immobilized tyrosinase acted on 4-*tert*-butylcatechol, the values of K_m^{app} were lower than these obtained for tyrosinase in solution while, when dopamine and DL-dopa were used, the K_m^{app} were higher. The order of the substrates as regards their ionizable groups, DL-dopa (two ionizable groups) > dopamine (one ionizable group) > 4-*tert*-butylcatechol (no ionizable group) coincided with the order of the K_m^{app} values shown by tyrosinase immobilized on the hydrophobic supports, and was the inverse of that observed for tyrosinase in solution. The K_m^{app} values of immobilized tyrosinase were in all cases higher than those of soluble tyrosinase and depended on the nature of the support and the hydrophobicity of the substrate, meaning that it is possible to design supports with different degrees of selectivity towards a mixture of enzyme substrates in the reaction medium.

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 al., 2001). Because tyrosinase is an expensive enzyme, its immobilization is considered worthwhile because it can be reused. Several authors have endeavoured to immobilize tyrosinase on different solid supports (Arslan et al., 2005; 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 (Yamada et al., 2005; Karam and Nicell, 1997), as biosensors to detect pollutants in environmental samples (Liu et al., 2005; Wang et al., 2002), for the electrochemical determination of hydrogen peroxide (Hall et al., 1996) and for the analysis of thiol-containing compounds (Huang et al., 2002).

In a previous work we optimized a new method for the direct immobilization of tyrosinase enzyme from natural mushrooms on the crosslinked derivate of D-sorbitol, and studied the

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0168-1656/$ – see front matter © 2007 Published by Elsevier B.V.
doi:10.1016/j.jbiotec.2007.05.004

behaviour and stability of this immobilized tyrosinase in the face of different factors (Marín-Zamora et al., 2006). The aim of the present work was to characterize the kinetic properties of tyrosinase immobilized by hydrophobic interactions on several immobilization supports using substrates with groups of a different ionizable nature and to clarify the kind of effects and interactions that affect the catalytic reaction of immobilized tyrosinase. Below we present an overview of this subject, which is discussed in the light of our results.

It is widely known that the kinetic constants of an enzyme can undergo substantial changes as a result of immobilization (Chibata, 1978). For example, in some earlier studies related to the adsorptive immobilization of native proteins on polymeric hydrophobic supports, higher catalytic activity, k_{cat}^{app}, was observed than that obtained with the free form (Hosseinkhani and Nemat-Gorgani, 2003; Azari et al., 1999). Furthermore, the K_m^{app} values for the immobilized enzymes depended significantly on the type of the matrix used and the k_{cat}^{app}/K_m^{app} values were also substantially greater than for free enzyme (Hosseinkhani and Nemat-Gorgani, 2003).

Most of the contributions concerning the effect of the support on the activity of tyrosinase immobilized by adsorption are ambiguous because they did not determine the quantity of enzyme immobilized in each case and so do not provide catalytic constants (k_{cat}^{app}) but only the values of V_{max}^{app}, from which it is impossible to determine whether the quantity of enzyme immobilized or its activity is greater. In the present study, the determination of these catalytic constants (k_{cat}^{app}) has enabled us to identify the supports on which the quantity of enzyme immobilized or its activity is greater.

Another effect studied was the influence of the hydrophobicity of the support, concerning which the bibliography provides many contradictory affirmations, for example, Iborra et al. (1977) indicated that a hydrophobic support gave the best enzyme activity and stability, whereas Estrada et al. (1991) results showed that the hydrophilicity of the support played an important role in influencing the activity of the tyrosinase. It has been reported that moderate hydrophobicity is necessary to maintain optimum tyrosinase activity (Kim and Lee, 2003) and that maximum tyrosinase activity critically requires the optimum hydrophobicity of the immobilization matrix. A more hydrophilic or more hydrophobic support is detrimental to the retention of the activity of tyrosinase (Zhang et al., 1999) (due to a lower quantity of immobilized enzyme or activity of the same).

The microenvironment of immobilized tyrosine molecules is a very important factor in enzyme behaviour. For example, in studies of organic phase enzyme electrodes using organic solvents, the enzyme active site microenvironment can be manipulated to obtain an enhanced sensor response compared with the responses obtained in the aqueous phase (Iwuoha and Smyth, 1997). When latent tyrosinase was activated with benzyl alcohol (Espín and Wichers, 1999) similar values of K_m^{app} were obtained to those we obtained with our immobilized fresh tyrosinase, values which can partly be explained by changes in substrate concentration in the enzyme microenvironment (Rijiravanich et al., 2004).

Moreover, the higher k_{cat}^{app} values obtained for non-covalent tyrosinase complexes with polybrene in water-organic solvent mixtures than for free enzyme have also been attributed to changes in the microenvironment of the enzyme molecules (Shipovskov and Levashov, 2003). Such variations in the apparent catalytic constants have been related with partially unfolded forms of the enzyme that give rise to greater hydrophobic interactions which, in some cases, these higher hydrophobic interactions make the adsorption process virtually irreversible. These unfolded forms are present in denaturalisation–renaturalisation processes and may contribute to the greater degree of thermal stability of enzymes immobilized in this way (Hosseinkhani and Nemat-Gorgani, 2003; Ulbrich-Hofmann et al., 1999; Park et al., 2003). Covalently immobilized tyrosinase also shows significant changes in the kinetic parameters due to structural changes in the enzyme molecule (Arica, 2000).

Previous contributions (Marín-Zamora et al., 2005, 2006) have pointed to the intense hydrophobic interactions that take place after tyrosinase immobilization on SOTCN (evidenced by the marked improvement in thermal stability and because the process is virtually irreversible). If, moreover, it is borne in mind that mushroom tyrosinase has a structurally very stable nature and that during unfolding with different denaturating mechanisms, the enzyme passes through intermediate states until the completely unfolded state is reached (Park et al., 2003), it is very probable that the changes observed in the catalytic constants are due to the conformational changes to more unfolded states (Manjon et al., 1984) provoked by the strong hydrophobic interactions that take place during immobilization. In an aqueous solution the enzyme would find itself in a state in which the ionic groups are in contact with the solution and the innermost hydrophobic part would remain protected; on being immobilized on the hydrophobic supports, the enzyme would open out, binding itself to the support by means of the hydrophobic part, while the ionic part remains in contact with the solution. The substrate would then be able to reach the active centre of immobilized tyrosinase more easily than the active centre of free enzyme, explaining the higher k_{cat}^{app} values in the first case, this accessibility being greater for ionic substrates (k_{cat}^{app} higher by one order of magnitude—see below) than for hydrophobic substrates. The fact that tyrosinase immobilized on hydrophilic supports, such as hydroxyaluminium–montmorillonite complexes, undergoes unfavourable conformational changes that result in a decrease in the catalytic activity lends weight to the above reasoning (Naidja et al., 1995, 1997). A fall in catalytic activity has also been observed during the immobilization of other enzymes on hydrophilic supports (Bellezza et al., 2004; Yashi et al., 2005).

2. Materials and methods

Natural mushrooms (*Agaricus bisporus*) were supplied by MERCADONA (Spain) and used to obtain fresh tyrosinase. Dopamine, DL-dopa, 4-*tert*-butylcatechol (TBC) and *p*-nitrophenol (PNP) were purchased from Sigma (Spain).