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Biochimica. — *Immobilization of mushroom tyrosinase on activated CH-Sepharose 4 B* (*). Nota di BRUNO DANISE, AMALIA VETROMILE e GIUSEPPE PROTA, presentata (**) dal Corrisp. A. RUFFO.

RIASSUNTO. — Nel quadro delle nostre ricerche sulla biochimica della melanogenesi, viene descritta l'immobilizzazione della tirosinasi da fungo su un derivato attivato del Sefarosio 4 B.

La determinazione del K_M apparente, insieme all'esame delle altre caratteristiche cinetiche, mostrano che l'enzima immobilizzato conserva inalterata la capacità di ossidare sia la tirosina che la dopa in un ampio intervallo di pH (5–8) con ritenzione della stereospecificità nei riguardi degli isomeri ottici tipica della tirosinasi. Da esperienze specifiche è risultato inoltre che l'enzima immobilizzato presenta in generale una maggiore stabilità che ne consente l'impiego per un certo periodo di tempo, anche se con diminuzione parziale dell'attività iniziale.

1. INTRODUCTION

Tyrosinases (o-diphenol : O_2 oxidoreductase E.C. 1.10.3.1.) are copper-containing enzymes which catalyse two different types of aerobic oxidation: the orthohydroxylation of monophenols to catechols, referred to as cresolase activity, and the dehydrogenation of catechols to o-quinones which is designated as catecholase activity [1]. These enzymes occur widely distributed in nature, and a number of them have been isolated from a variety of sources including mushroom [2], *Neurospora crassa* [3], potato tubers [4], broad beans [5], insect hemolymph [6], cephalopod ink [7] and mammalian melanomas [8].

Usually the occurrence of tyrosinase is associated with the production of melanins resulting from spontaneous polymerization of the generated o-quinones [9]. There are, however, a number of organisms which, although containing active tyrosinases, lack the ability to form melanins, suggesting that under certain biochemical conditions the catecholase activity of the enzyme may be effectively suppressed [10]. These examples are of particular interest in relation to the current efforts to utilize free or immobilized tyrosinases for a convenient synthesis of L-dopa from L-tyrosine, as well as for other pratical purposes.

In connection with our continuing studies on the biochemistry of melanogenesis we report here the immobilization of mushroom tyrosinase on activated CH-Sepharose 4 B and the kinetic characteristics of the immobilized enzyme.

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(**) Nella seduta del 26 giugno 1980.

2. Materials and methods

a) *Materials.*

The enzyme tyrosinase obtained from mushroom was purchased as a lyophilized powder (2230 enzyme units/mg.) from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

Activated CH-Sepharose 4 B was obtained from Pharmacia Fine Chemicals (AB Uppsala Sweden).

All other chemicals were of reagent grade and were supplied by Sigma, except D-tyrosine which was purchased from BDH Chemical (Ltd. Poole, England).

b) *Enzyme assays.*

Unless otherwise stated tyrosinase activity was determined spectrophotometrically at 25 °C by measuring the rate of formation of dopachrome using 1 mM L-dopa as substrate in sodium phosphate buffer pH 6.9. In calculating the enzyme units the molar extinction coefficient of dopachrome at 475 nm was taken as 3600 [11]. The enzyme unit was defined as the amount of enzyme required to produce 1 μmol of dopachrome/min under the above conditions.

Immobilized enzyme assays were carried out in batch as follows: identical amounts of the material were incubated in the assay solutions under the above conditions, with vigorous shaking, and at appropriate times aliquots of 1 ml. of the reaction mixture were withdrawn for spectrophotometric determination of the dopachrome formed.

All spectophotometric measurements were made on a Perkin-Elmer Mod. 550 double beam instrument.

c) *Immobilization procedure.*

In a typical experiment 4 g. of freeze-dried activated CH-Sepharose 4 B, swollen for 15 min. in 1 mM ice-cold HCl and washed on a glass filter with the same solutions (800 ml.), were coupled with 10 mg. of mushroom tyrosinase (3.3 dopachrome units/mg.) dissolved in 10 ml. of bicarbonate buffer (0.1 M containing 0.5 M NaCl pH 8) in a test tube and allowed to rotate end over end for 24 h. at 4 °C. After the coupling reaction, the gel was washed with 200 ml. of the same buffer to remove unattached enzyme and then treated with 10 ml. of Tris-HCl buffer (0.1 M containing 1 M NaCl, pH 8) for 24 h to block any remaining active group. The product was washed three times, alternately with acetate buffer (0.1 M pH 5) and Tris-HCl buffer (0.1 M pH 8), each containing 1 M NaCl, and stored in phosphate buffer (0.1 M pH 6.9) at 4 °C.

3. RESULTS

Immobilized mushroom tyrosinase was suitably prepared by reaction with a derivative of CH-Sepharose 4 B containing an active ester group for spontaneous covalent coupling with primary amino residues, in bicarbonate buffer. Typical batches so obtained had an initial activity of about 40–45 % compared to the free enzyme, with respect to both tyrosine and dopa oxidation (Fig. 1).

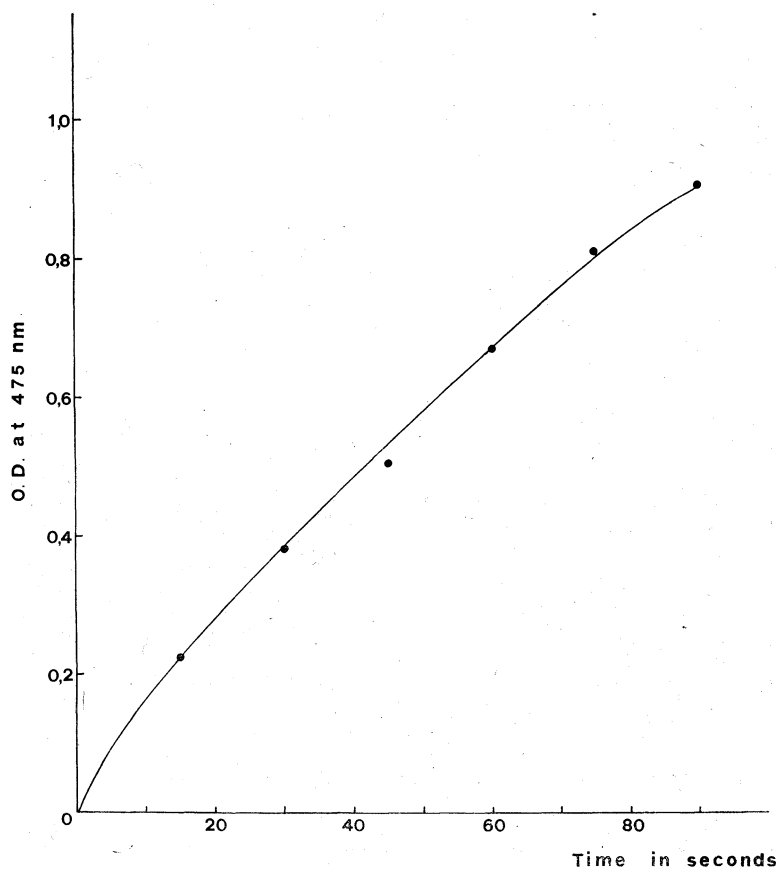

Fig. 1. – Oxidation of L-dopa (1 mM) by mushroom tyrosinase (0.4 μ/ml.) immobilized on activated CH-Sepharose 4 B, in 0.1 phosphate buffer pH 6.9 at 20 °C.

Determination of the apparent Michaelis-Menten constant (Fig. 2) using L-dopa as substrate gave a value of 6.9×10^{-4} M, close to that of free tyrosinase (3×10^{-4} M), indicating a limited change in the catalytic behaviour of the bound enzyme. A further characterization was obtained by measuring the reaction rates of the two optical forms of dopa, as well as of tyrosine, with immobilized tyrosinase. It can be seen from the data reported in Fig. 3 that the L-forms

of both substrates are oxidized faster than the corresponding D-isomers with retainment of the stereospecificity typical of tyrosinase.

As shown in Table I, the immobilized enzyme retains its activity over a broad range of pH with little variation even at pH 5 and pH 8 where mushroom tyrosinase shows a considerable loss of activity. The observed extension

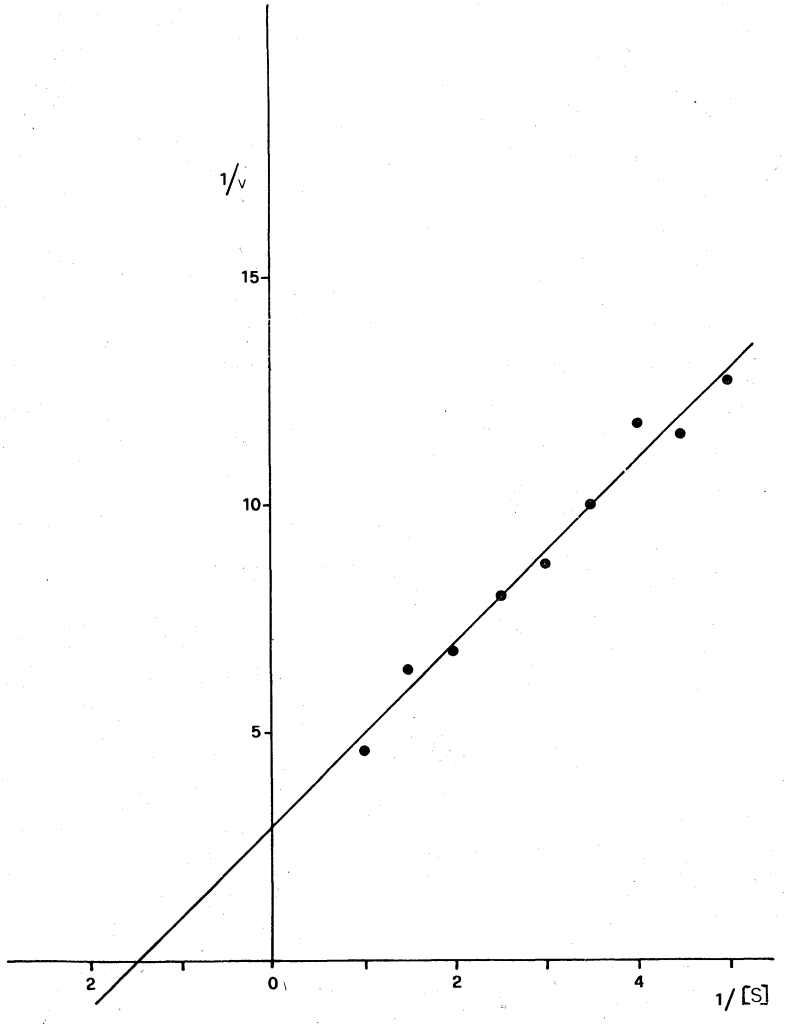

Fig. 2. – Lineweaver-Burk plot of immobilized mushroom tyrosinase with L-dopa as substrate. The reaction rates were determinated under standard assay conditions.

of the working pH range parallels the increased pH stability of immobilized tyrosinase as estimated by batch assays after 24 h. in 0.1 M phosphate buffers (Table II).

The feasibility of reusing the immobilized enzyme over a reasonable period of time was also examined. In this experiment 1 mM L-dopa was used

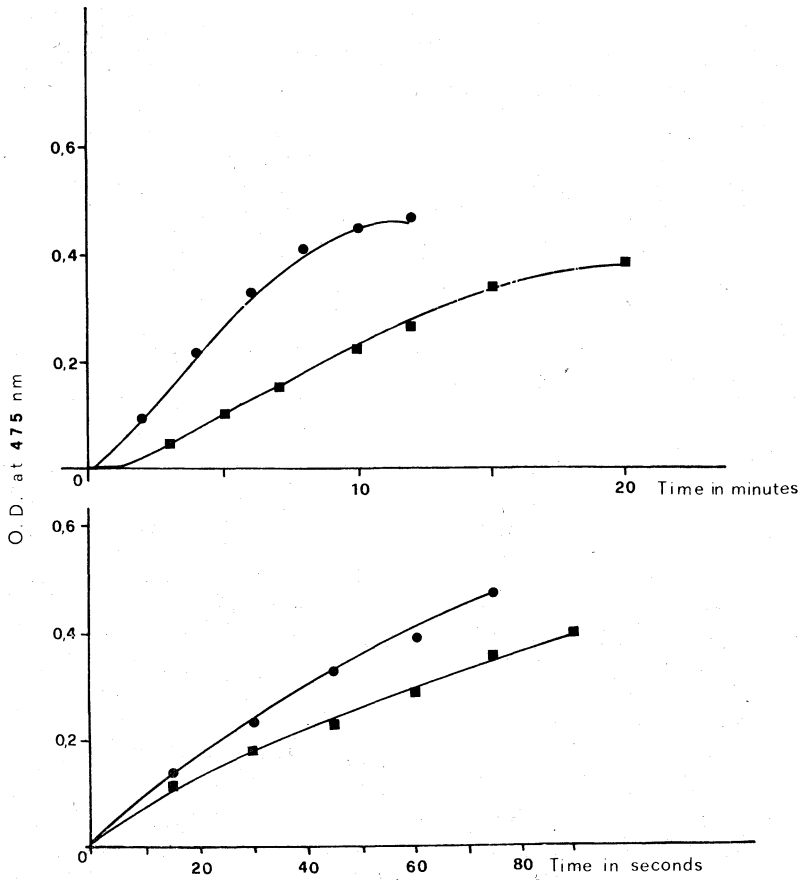

Fig. 3. – Reaction rates of L-tyrosine and D-tyrosine (a), L-dopa and D-dopa (b) with immobilized mushroom tyrosinase under standard assay conditions.

TABLE I.

Variation of the activity of immobilized tyrosinase (0.13 μ/ml.) *as a function of* pH.

pH	Activity (*)
5.00	0.12
6.00	0.12
7.00	0.13
8.00	0.14

(*) Expressed as mol of dopachrome formed per minute at 20 °C using 1 mM L-dopa as substrate in 0.1 phosphate buffer.

TABLE II.

pH *stability of immobilized tyrosinase.*

pH	% activity (*) recovered after 24 h. at 4 °C
4.00	68%
5.00	80%
6.00	100%
7.00	100%
8.00	100%
9.00	100%

(*) Expressed as mol of dopachrome formed after 60 sec. with 1 mM L-dopa as substrate at pH 6.9.

as the substrate and the reaction rates for the immobilized enzyme stored at 4 °C and 20 °C at day 1, day 10, and day 20 were assayed under standard conditions.

For the enzyme stored at 4 °C the activity decreased by 10 % after 10 days and 25 % after 20 days, while for that stored at 20 °C it decreased by 25 % after 10 days and 60 % after 20 days, compared to the activity of day 1.

4. Discussion

Tyrosinase has previously been immobilized on collagen membranes [12] and more recently in a polyacrylamide gel matrix [13] for possible use as a catalyst in the determination of phenolic pollutants in industrial effluents and surface waters [14].

A chemical immobilizzation of tyrosinase on diethylaminoethyl cellulose has been reported by Wykes *et al.* [15] to explore the possibility of implanting the immobilized enzyme in the blood stream of patients with Parkinson's disease to synthesize L-dopa *in situ*. Tyrosinase so immobilized exhibits the same pH optimum in the region of 6.5–7.5 as the free enzyme, but from experiments aimed at evaluating its stability in working conditions a loss of 75 % of activity after 24 h. was found, defeating possible applications in industrial processes.

The results reported in this study show that tyrosinase under suitable conditions may be reproducibly immobilized on activated CH-Sepharose 4 B with a good recovery of the initial units.

Determination of apparent K_M, together with assays of the other kinetic characteristics, show that the immobilized enzyme exhibits the usual ability of oxidising both tyrosine and dopa over a broad range of pH (5–8) with full retainment of the stereospecificity towards the L-isomers.

Unfortunately such a close similarity in the behaviour of immobilized tyrosinase is also extended to its stability under working conditions which, though much improved with respect to that of the free enzyme, is still too limited for pratical purposes.

Further studies are in progress to increase the stability of the immobilized enzyme by minimizing the catecholase activity which probably accounts for inactivation of tyrosinase by the generated quinones in the usual working conditions.

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