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USE OF AFFINITY CHROMATOGRAPHY FOR PURIFICATION OF TYROSINASE

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Abstract. The possibility of using affinity chromatography for the purification of tyrosinase was explored. When purified mushroom tyrosinase was employed, almost all tyrosinase became bound to Sepharose containing 3-iodotyrosine, an inhibitor of tyrosinase. When crude extracts from B16 melanoma were employed, partial purification of tyrosinase and separation of two or possibly three forms of tyrosinase could be obtained. The results indicate that affinity chromatography could possibly be employed for the purification of tyrosinase during the final stages of the purification procedures.

Key words: Melanoma; Tyrosinase; Diphenol oxidase; Chromatography; Pigmentation

Tyrosinases have been purified from mushrooms and a few types of melanomas and some micro-organisms (1–5, 7–12). These procedures involve several stages and the yields are usually fairly small. In recent years, affinity chromatography has been successfully employed for the purification of several enzymes, often with good yield and requiring relatively shorter procedures.

This communication reports the results of our attempts to purify tyrosinase using the technique of affinity chromatography.

MATERIALS AND METHODS

Mushroom tyrosinase (polyphenoloxidase) Grade III (approximately 3 600 units per mg) was obtained from Sigma Chemical Company. Tyrosine-3, 5-^{3}H (specific activity approximately 30 Ci/mmole) was obtained from Amersham/Searle. CNBr-activated Sepharose-4B was obtained from Pharmacia Fine Chemicals. Protein was determined according to the method of Lowry et al. (6).

Sepharose 4B coupled to 3-iodotyrosine, an inhibitor of tyrosinase, was employed for the affinity chromatography of mushroom tyrosinase. CNBr-activated Sepharose 4B was gradually added to a solution of 0.1 M 3-iodo-tyrosine at pH 11 and the slurry was stirred for 18 hours at 4°C. The Sepharose was treated with 1 M ethanolamine at pH 8 for 2 hours, and then washed alternately with 0.1 M acetate buffer (pH 4) and 0.1 M borate buffer (pH 8.5), both buffers containing 1 M NaCl. The washing procedure was repeated 4 times. A column of the washed product (1 × 12 cm) was equilibrated with 0.1 M PO_4 buffer (pH 6.2). 0.5 ml of purified mushroom tyrosinase (5 mg/ml) in 0.1 M PO_4 buffer (pH 6.2) was added to the column. The column was washed with 30 ml of the PO_4 buffer and then 30 ml of the same buffer containing 1 mM L-tyrosine. The chromatographic procedure was carried out at 4°C. The flow rate was approximately 0.1 ml per min. 2 ml fractions of the eluate were collected. The fractions containing tyrosine were dialysed against 2 litres 0.1 M PO_4 buffer (pH 6.2) for 4 hours, changing the buffer after 2 hours. Protein contents and tyrosinase activities of each fraction were determined.

RESULTS

The results of the affinity chromatography of purified mushroom tyrosinase are given in Fig. 1. These results represent the findings of one experiment; two other experiments gave closely similar results. These results show that 0.84 mg protein (34% of the total added to the column) was recovered in the fractions eluted with the PO_4 buffer. On the other hand, only negligible amounts of tyrosinase activity (approximately 1%) were measurable in these fractions. The

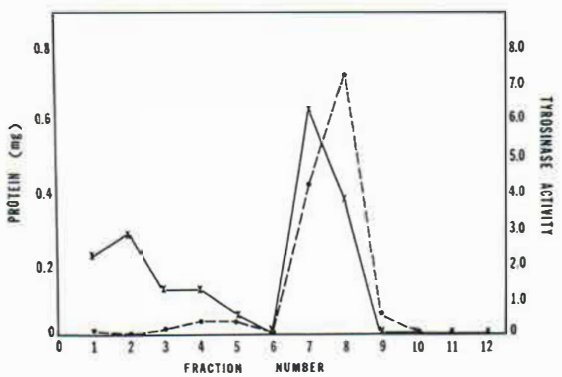

Fig. 1.

fractions eluted with the tyrosine solution contained 1.01 mg protein (40% of the total added) and had approximately 80% of the total tyrosinase activity.

Attempts were made to purify the tyrosinases from B16 melanoma, using the above technique. Preliminary results using conditions similar to those employed for purified mushroom tyrosinase showed almost no purification of tyrosinase.

A modified type of Sepharose containing the iodotyrosine attached to the Sepharose beads through a ligand (AH-Sepharose 4B) was next used. This preparation was found to yield better results, although the purification of tyrosinase was considerably less than might have been expected from the results with the purified mushroom tyrosinase. A wide range of conditions were explored, viz., change of pH of the buffer between 4 and 10, and addition of NaCl at various concentrations ranging from 0.025 to 1.0 M to the buffer containing the enzyme and to that employed for elution. These studies resulted in a partial purification of the enzyme and separation of two or possibly three forms of tyrosinase. Preliminary purification of tyrosinase from melanoma by means of ammonium sulfate fractionation and column chromatography using Sephadex G100 did not considerably improve the purification of the enzyme by affinity chromatography. When crude extracts of fresh mushrooms were employed, only a partial purification of tyrosinase was obtained, as in the case of crude extracts of the melanoma.

DISCUSSION

The results presented in this paper indicate that affinity chromatography could possibly be employed for the purification of tyrosinase. The method was most effective when highly purified samples of the enzyme were employed, e.g., commercially available purified mushroom tyrosinase. Attempts to use similar conditions for the purification of crude extracts from B16 melanoma or fresh mushrooms were not equally successful. It would therefore appear that affinity chromatography could be employed for the purification of tyrosinase, probably during the final stages of the purification procedures.

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