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PURIFICATION OF ORTHODIPHENOL OXIDOREDUCTASE FROM POTATOES BY AFFINITY CHROMATOGRAPHY ON COIR PITH LIGNIN

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Orthodiphenol oxidoreductase, ODOR (E.C.1.10.3.1) from potato tubers (*Solanum tuberosum*) has been purified on coir pith lignin by affinity chromatography. All isozymes get adsorbed and can be eluted with 70% recovery. The enzyme shows high purity, as judged by specific activity and polyacrylamide gel electrophoresis.

INTRODUCTION

Affinity chromatography makes use of specific interactions of biologically active compounds. Many supports have been prepared to serve as specific affiants (1).

Mostly, the method involves the use of modified natural or synthetic supports. There are very few reports of naturally available supports in unmodified form serving as affiants (1). These rare examples include amylases on starch and acetylglucosaminidase and lysozyme on chitin. The reason for the rarity of such supports is perhaps that the naturally available supporting material, as such, cannot usually conform to the design of a specific need of affinity chromatography.

Lignins, which have not yet found a place among column supporting materials, are abundantly found in wood. Further, lignin columns have good flow rates and do not become choked up. Lignins isolated from coir pith, with its aromatic hydroxyl groups, show good affinity toward ODOR from potato tubers. Lignins can be employed without modification as a column support for affinity chromatography of this enzyme. The description of the

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method and its advantages over the earlier methods forms the subject matter of this paper.

METHODOLOGY

Lignin (Affiant) Preparation

The lignin was prepared essentially by a modified sulfuric acid method (2,3), with slight further modifications as follows. Coconut coir pith (courtesy Canara Coir Industries, Honawar, Karnataka) was ground to 80–100 mesh and dried at 100°C in an oven. Ethanol benzene (in the ratio of 1 : 2 v/v) extractables were removed using a soxhlet apparatus. The residue was washed with ethanol to remove benzene, catechol, and tannins, followed by cold water to remove ethanol, sugars, and other water soluble components. The final residue was again dried to a constant weight.

To 10 g of preextracted oven dried sample, obtained as above, 150 ml of chilled 72% (v/v) sulfuric acid was added. The mixture was stirred for 2 h. The sample was then diluted with water to obtain 3% sulfuric acid concentration and refluxed for 4 h. The insoluble lignin was filtered and washed with hot water (40–50°C). This lignin preparation was used as an affiant.

Column Preparation

Ten g of lignin prepared as above, when packed into a column of 2.2 cm diam, acquired a height of 21.0 cm. The column was extensively washed with distilled water and was equilibrated with 0.05 *M* phosphate citrate buffer (pH 4.6).

Extraction of the Enzyme

Peeled potatoes (300 g), cut into slices of approximately 1 mm thickness, were rinsed with tap water and then with distilled water. The moistened slices were aged by keeping them at 25°C for 48 h. It is known that the ODOR activity increases several times on aging (4). The enzyme was extracted in a 200-ml grinding medium consisting of 0.05 *M* sodium phosphate buffer, pH 7.0, containing 2.0 ml of 0.01 *M* ascorbate and 10.0 ml of 0.001 *M* EDTA, in a Waring blender at 4°C. The homogenate was filtered through cheese cloth and centrifuged at 7000 *g* for 10 min.

Ethanol Treatment

To 150 ml of the above supernatant, 50 ml of 50% chilled ethanol was added. The mixture was kept at 4°C for an hour and centrifuged at 7000 *g* for 10 min. The precipitate was discarded.

Ammonium Sulfate Precipitation

To the ethanol supernatant, solid ammonium sulfate was added to 40% saturation. The mixture was allowed to remain overnight at 4°C, and precipitate was collected by centrifugation at 7000 *g* for 10 min. The precipitate was dissolved in 35 ml of 0.1 *M* phosphate citrate buffer, pH 4.6, and was dialyzed against the same buffer at 4°C.

Affinity Chromatography

Thirty ml of the above dialyzed enzyme was loaded onto the pre-equilibrated lignin column at a flow rate of 20 ml/h. The column was washed with the phosphate citrate buffer of pH 4.6 (0.05 *M*) at a faster flow rate of 60 ml/h until no protein came into the washings. About 300 ml of the buffer washings was found to be adequate. The elution of ODOR was affected by phosphate citrate buffer of pH 4.6 (0.1 *M*) at a flow rate of 20 ml/h. Twenty-five fractions of 15 ml each were collected.

Enzyme Assay

ODOR activity was determined manometrically in the Warburg apparatus (5) at 35°C. In a typical experiment, 2.0 ml of sodium phosphate buffer at pH 6.3 (0.1 *M*), 0.5 ml of ascorbate (0.1 *M*), and 0.5 ml of enzyme sample were used with 0.5 ml of 4-methyl catechol (0.1 *M*) as a substrate. Oxygen uptake for 15 min was recorded.

Enzyme Unit and Specific Activity Determination

One unit of enzyme activity is defined as the amount of enzyme that causes an uptake of 1 μmol of oxygen in 1 min under the above experimental conditions. Protein estimation was carried out by the method of Lowry et al. (6) using bovine serum albumin as the standard. Specific activity of the enzyme is expressed as the units of the enzyme per mg of protein.

Disk Electrophoresis

The method used was essentially that of Reisfeld et al. (7). The electrophoresis was carried out with tris-glycine buffer (pH 8.3), using bromophenol blue as an indicator. Enzyme activity was localized in the gels by a staining procedure (8), wherein freshly extruded gels were immersed in a solution of 4-methyl catechol (1.0 m*M*) and L-proline (1.0 m*M*) in 0.1 *M* sodium phosphate buffer at pH 6.5. Purple color developed at the site of activity in the gel within 2 min. Inactive protein bands were not stained. Protein bands were located by staining with Coomassie Brilliant Blue for 30–90 min (9).

RESULTS

A typical affinity elution profile of ODOR from the lignin column is depicted in Fig. 1. The purification data, along with ODOR activities and protein concentrations at the various stages, are given in Table 1.

In the column operation, almost all the protein comes into the effluents and washings, and these fractions do not show any enzymic activity. Apparently ODOR, which constitutes a very small fraction of the total proteins in the potato extract, is completely adsorbed by the lignin. Subsequently, the ODOR could be desorbed from the lignin by buffer of

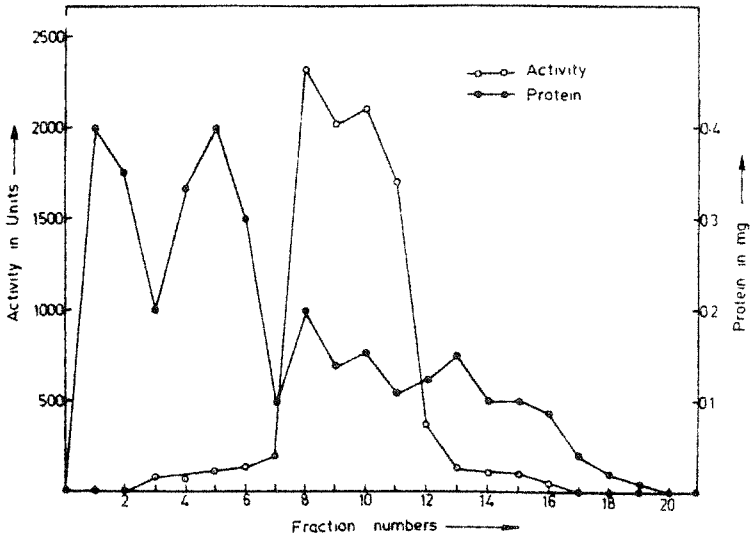

FIG. 1. Typical elution profile of ODOR on lignin column.

TABLE 1. **Typical Preparation of *o*-Diphenol Oxidoreductase**

Sr. no.	Purification stage	Volume (ml)	Total activity (U)	Total protein (mg)	Specific activity (U/mg protein)	Fold purification
1.	Original extract (300 g potatoes)	150	780	200	3.9	
2.	Supernatant after alcohol treatment	190	987	155	6.37	1.63
3.	40% ammonium sulfate precipitation	30	737	105	7.02	1.8
4.	Pooled peak from lignin column (fractions 8–11)	60	543	0.595	912.60	234

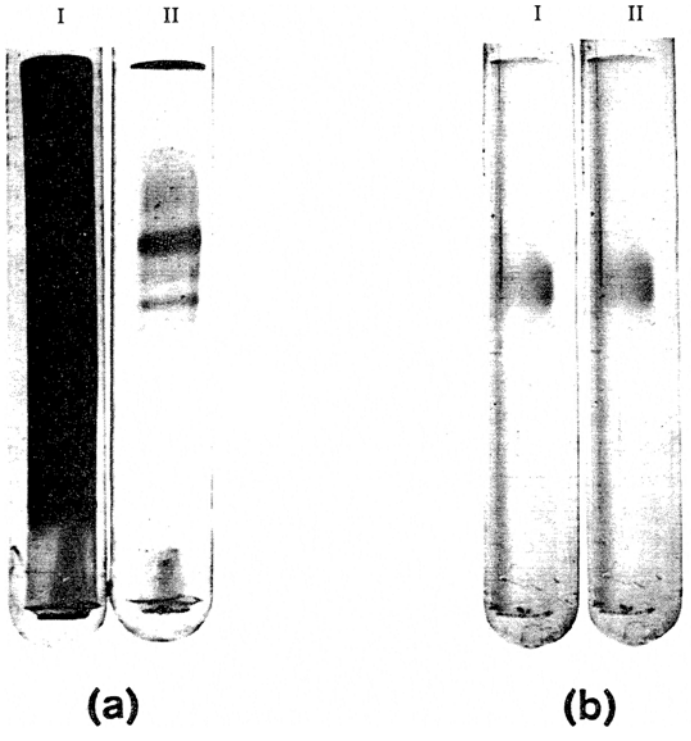

FIG. 2. Electrophoresis of ODOR. (a) Protein bands; (b) activity staining (I crude; II purified).

higher molarity. Fractions 8–11, when pooled together, accounted for 70% of the enzyme activity loaded on the column. However, when subjected to disk electrophoresis, the pooled fractions show four distinct protein bands (Fig. 2a), and when these are subjected to activity staining, all of them become stained (Fig. 2b). Obviously, all four bands constitute the isozymes of the ODOR. Four isozymes of ODOR in potatoes have been reported by earlier workers (10,11). It seems, therefore, that the four isozymes do not have distinctive enough affinities toward lignin to make it possible to resolve them from each other under the above conditions of the experiment. It is quite possible that under some other conditions, the isozymes of ODOR may be resolvable, or the pooled active fractions from the lignin column might be subjected to isozyme separation by a Bio-Gel P-300 column, as described by Balasingam and Ferdinand (10).

DISCUSSION

One of the main problems encountered in the isolation of polyphenol oxidases is that the enzyme may get autooxidized at tyrosyl residues. Polyphenol oxidases have been purified from many higher plant tissues, e.g., potatoes (5,10–12), apples (13,14), mushrooms (15–17), olives (18), pears (19), peaches (20,21), sugar canes (22), spinach chloroplast (23), grapes (24), and cherries (25) by earlier workers.

None of these workers used an affinity procedure. So we are reporting for the first time on the use of affinity chromatography for polyphenol oxidase. Further, the use of lignin is novel, as it is an abundantly available natural material. Moreover, no chemical modification of the affiant was found to be necessary.

Although we have here applied the method to ODOR from potatoes, we have seen in our preliminary experiments that ODOR from grapes, for example, can also be purified by this affinity chromatographic procedure. Thus it seems likely that the method would be useful in the isolation of ODOR from other sources as well. We have observed that the lignin columns can be repeatedly used for affinity chromatography of ODOR at least for six months, without any apparent change in capacity to bind for ODOR.

SHORT DESCRIPTION OF THE METHOD AND ITS APPLICATIONS

Lignins are quite stable aromatic polymers, which give good flow rates when packed into the columns. Because of the presence of hydroxyl groups,

the lignins show affinity toward phenol oxidases. All isozymes of ODOR from potato have been successfully isolated in good yield by affinity chromatography. The method is simple and gives the enzyme in higher yields and in fewer steps than previous methods. The lignin columns can be repeatedly used without any treatment. The method with slight modifications may be applicable for the isolation of phenol oxidases from other sources.

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