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Affinity Chromatography of Mushroom Tyrosinase

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ABSTRACT

The purification by column chromatography of a phenol-oxidizing enzyme, mushroom tyrosinase, was investigated using solid phase adsorbents designed to have specific affinity for the enzyme. Sepharose 4B, aminophenyl-bearing porous glass, and p-aminobenzylcellulose were chemically modified to introduce phenolic, catecholic, or benzoic groups on the polymer surface. The resulting preparations were tested for their effectiveness in separating tyrosinase from an impure protein mixture. The phenolic and benzoic polymers displayed no specific affinity for tyrosinase. Aminophenyl glass, with or without an attached phenolic group, adsorbed appreciable quantities of protein nonspecifically, thus complicating studies of its tyrosinase affinity properties. Dopamine, a dihydroxyphenyl derivative, was bound to Sepharose and was found to be effective in retaining tyrosinase at pH 5.5; elution of the enzyme by washing at pH 8.8 resulted in its purification by a factor of 10 to 14. Enzymatic oxidation of the adsorbent limited the number of purification cycles which could be carried out on a single column.

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INTRODUCTION

"Affinity chromatography" (AC) is the term which has been coined to describe a separation on a special solid phase containing a ligand which binds the molecule to be purified. The process is usually applied to biological materials and to interactions such as those between antigens and antibodies, nucleic acids and nucleotides, and proteins and protein-binding substances. As applied to enzyme purification, the method utilizes the specific binding between an enzyme and an inhibitor, a cofactor, or a substrate analog as a means of selectively removing an enzyme from a complex protein mixture. Suitable polymeric materials are chemically modified to introduce pendant groups with enzyme-binding properties; often an intermediary molecule or "spacer" is required between the polymeric backbone and the ligand group to avoid steric hindrance to enzyme-ligand interaction.

The modified polymer then can be used under suitable conditions in the form of a packed column for enzyme retention. Enzyme recovery, and thus column regeneration, is brought about by washing with an appropriate solution (with, e.g., altered pH, altered salt concentration, or added substrate) so that the enzyme is eluted. Although the theory of AC is far from being well developed, previous investigators have established several practical guidelines for the support phase: 1) It should contain functional groups which can react readily with a wide range of ligands, 2) it should exhibit good flow properties (low compaction) in a packed bed, 3) it should have very few ionic or other groups which can adsorb protein nonspecifically, and 4) it should provide free access to the bulky enzyme molecules by containing large pores and by having a high fractional porosity.

OUR WORK

Our efforts have centered around phenol-oxidizing enzymes in general and mushroom tyrosinase in particular. We were interested both in uncovering basic information on affinity chromatography as a process and in devising efficient methods for purifying phenol-oxidizing enzymes. The latter goal is a consequence of our involvement in a large-scale effort directed toward the detection and degradation by enzymatic methods of phenol in waste waters. We recognized that tyrosinase was not an ideal enzyme for such applications because of its well-known "suicidal" behavior [1]. A molecule of enzyme will catalyze only a certain amount of reaction before undergoing spontaneous irreversible loss of activity. Two reactions are catalyzed, the ortho-hydroxylation of monophenols and the conversion of ortho-diphenols (catechols) to ortho-quinones [2]. Despite the complexity of

this enzyme's kinetics and function, we felt that an AC column which was effective with tyrosinase might well be suitable for other phenol-oxidizing enzymes, which we are also in the process of isolating.

In addition to this specific goal, we wished to study the effect of varying the ligand-enzyme binding constant. Present practice suggests that the interaction should be strong enough to absorb enzyme under conditions where impurities wash through the column. The affinity must not be so strong, however, as to necessitate harsh desorption procedures which can inactivate the enzyme during elution. We also wished to compare porous glass, cellulose, and agarose as support phases. There are undoubtedly further principles to be uncovered also, since AC may fail even when one adheres to all of the recognized empirical rules. The simplicity of AC and the sometimes spectacular success it achieves (*e.g.*, a single-pass purification factor of 100 to 1000) make such efforts well worthwhile.

EXPERIMENTAL METHODS

It is interesting to note that Lerman [3] in one of the first reports of the technique now known as affinity chromatography also studied tyrosinase. We therefore expected that our job of finding a suitable adsorbent would not be especially difficult and that we could concentrate on some of these more interesting questions. Lerman reported successful adsorption on cellulose-containing phenolic hydroxyl groups but not on cellulose with attached benzoic groups, despite the fact that benzoic acid itself is an inhibitor of the enzyme. Our plan therefore was to concentrate on phenolic derivatives. A commerical preparation of mushroom tyrosinase (Nutritional Biochemical Corp.) was used as the semipure enzyme mixture. Tyrosinase activity was determined by the chronometric method [4] using catechol as substrate and ascorbic acid to prevent initial production of quinone. All activities were determined in malonate buffer (0.05 M, pH 5.5) at room temperature.

COLUMN MATERIALS

Aminophenyl-bearing glass prepared by Corning Glass Works according to Weetall's method [5], Sepharose 4B (Pharmacia, Uppsala, Sweden), and p-aminobenzyl cellulose (Bio-Rad Laboratories, Richmond, California) were used as the support materials. Specific inhibitory groups or substrate analog groups were created on the supports through a series of reactions. The final products are illustrated in Fig. 1. Phenolic glass derivatives were produced through diazotization of aminophenyl glass by treating with nitrous acid at 4°C. The diazotized

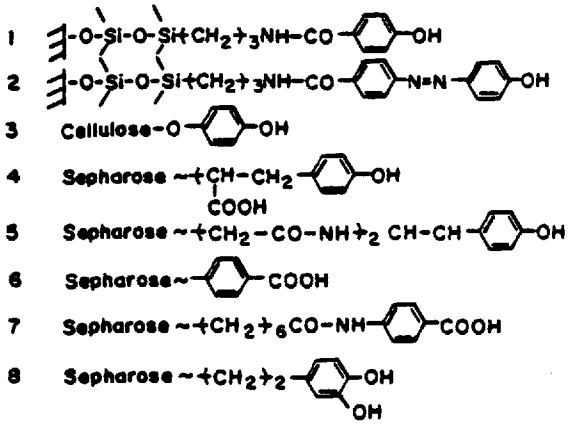

FIG. 1. Affinity adsorbents tested for tyrosinase purification.

product either was hydrolyzed by warming to room temperature (Preparation 1) or was reacted with phenol (Preparation 2). In Preparation 2 the phenolic group is located at a greater distance from the glass surface than in Preparation 1. In a similar manner, p-aminobenz cellulose was diazotized and hydrolyzed to form phenolic end groups (Preparation 3). A commercial preparation of agarose-1-tyrosine (Miles Laboratories, Elkhart, Indiana) was tested without further modif cation (Preparation 4). The agarose is "activated" by cyanogen bromid according to a technique described by Axes and Porath [6] in order to couple amino-bearing ligands. The exact reaction has not been established firmly, but is thought to involve imino carbonic acid esters or isourea derivitives [7]. We prepared a similar derivative with a longer link between the agarose and the end phenolic group (Preparatio 5) by reacting glycylglycyl-1 tyrosine with "activated" agarose (Sepharose 4B). Benzoic acid derivatives of Sepharose 4B were prepar by reacting p-aminobenzoic acid directly with "activated" Sepharose (Preparation 6) and by reaction of aminocaproic acid with "activated" Sepharose followed by coupling of the free acid group of the bound capro chain with the amino group of p-aminobenzoic acid using N,N-dicyclohexylcarbodiimide (Preparation 7). A somewhat different type of ligand resembling catechol rather than phenol was prepared from dopamine (3,4-dihydroxyphenylethylamine). Activated Sepharose was reacted with this compound to give a column packing which was somewhat prone to autoxidation (Preparation 8).

COLUMN PREPARATION AND OPERATION

A glass column (10 × 1 cm diam) was filled to about 75% capacity with one of these ligand-support materials and washed thoroughly with malonate buffer (pH 5.5, 0.05 M). Small volumes (1-3) of impure enzyme in malonate buffer (7 to 14 mg/ml) were applied to the column and washed with malonate buffer. Eluate fractions were collected and tested for protein content by their absorption of light at 280 nm and tested for catecholase activity by the technique mentioned previously. After no further protein could be detected, an additional 10 volumes of buffer were passed through the column to ensure that no further removal of protein was occurring. The solution fed to the column was then changed to a higher pH value (pyrophosphate buffer, pH 8.8) or to malonate buffer containing the substrate catechol to provide suitable conditions for elution of bound tyrosinase. Eluate samples were collected and tested as before. Flow rates were in the range 1-30 ml/hr. Sepharose had a hydraulic permeability which placed it at the low end of this range when the column was eluted at 4°C, and glass was considerably more permeable. At room temperature, flow rates at the high end of this range were possible with glass.

RESULTS AND DISCUSSION

Phenolic and benzoic derivatives were found to be incapable of purifying tyrosinase although adsorption and desorption were possible in some cases. With end-groups attached to the support material through an intermediate link (Preparations 1, 2, 3, 5, 7) rather than directly, a large percentage (40-60%) of the protein material added to the column was retained on the support at pH 5.5. This protein was released upon washing with buffer at pH 8.8. However, the protein that eluted directly through the column at pH 5.5 possessed the same catecholase activity as the preparation added to the column. The protein which washed off at high pH also possessed catecholase activity, but at a reduced level relative to the initial feed. Derivatives with ligands attached to Sepharose via short links displayed no retardation properties and gave column elution patterns similar to that obtained using unsubstituted Sepharose. Thus, while Sepharose-glycylglycyl-l-tyrosine retained about 50% of the protein added, Sepharose-l-tyrosine did not retain or retard any protein. These results are illustrated in Fig. 2. Similarly, Sepharose-caproic-benzoic acid retained some protein (~30%), while Sepharose-benzoic acid did not.

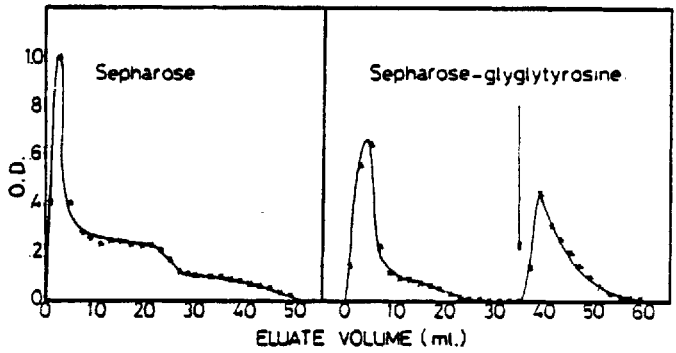

FIG. 2. Protein elution patterns for unmodified Sepharose at pH 5.5 and Sepharose—glycylglycyl-l-tyrosine at pH 5.5 followed by pH 8.8 (buffer changed at arrow). No protein was eluted from plain Sepharose by a pH change.

A further indication of interaction between enzyme and column material was the appearance of a black band at the top of the column when an enzyme sample was passed through. This occurred with all phenolic derivatives (Preparations 1, 2, 3, 5) except where the distance between support material and the phenolic groups was very short (Preparation 4) and indicated reaction between bound ligand and tyrosinase. The color is believed to be due to the polymerization of quinones into melanin-like structures. Nonspecific adsorption effects at pH 5-7.5 were found to be significant with glass columns. As much as 2 mg of protein could be adsorbed per gram of aminophenyl glass, with or without an attached ligand. Protein which could be adsorbed by a glass column thus became a significant fraction of the protein concentration used in the present study. It was decided to avoid this complicating effect by carrying out further work with agarose columns which developed negligible adsorption effects. A further disadvantage of glass was an apparent hydrolysis at high pH of the covalent bonds joining the ligand groups and the glass support phase.

Columns of Sepharose 4B-dopamine (Preparation 8) were capable of completely retaining all catecholose activity from impure samples while permitting passage of most of the inactive protein (Fig. 3). A ten to fourteenfold purification based on the ratio of enzymatic activity to protein concentration was achieved. Approximately 80% of the enzymatic activity originally applied to the column was recovered in this purified fraction. These columns were found to be somewhat unstable, however, in that oxidation of the bound dopamine, as evidenced by darkening of the column material, was brought about

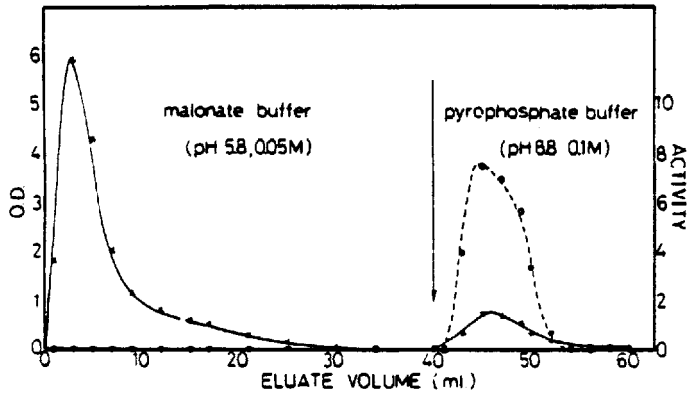

FIG. 3. Affinity chromatography on Sepharose-dopamine (Preparation 8). Enzyme activity (○) and protein concentration (×) in optical density (O.D.) units.

1) by enzymatic oxidation (as with the phenolic columns a black band formed), and 2) by spontaneous oxidation due to prolonged passage of high pH buffer through the column. As a result of these two effects, the usefulness of the column for retardation of tyrosinase decreased with repeated usage (Fig. 4). Improved column stability was obtained by operation at lower temperature (4°C) rather than at room temperature. This reduced but did not eliminate enzymatic oxidation of the bound ligand. Tyrosinase elution by substrate (catechol) at pH 5.5 avoided oxidation due to high pH.

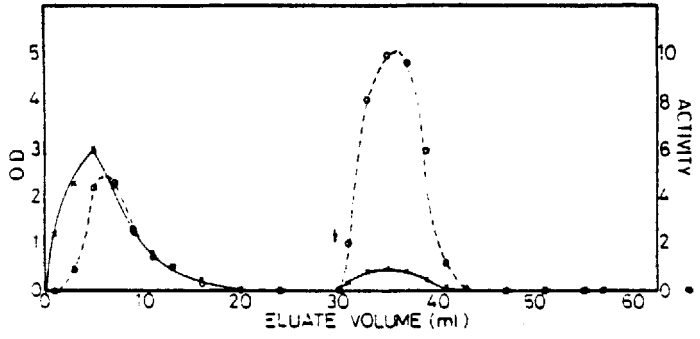

FIG. 4. Affinity chromatography on old Sepharose-dopamine column. Note the appearance of activity in the first protein peak, unlike Fig. 3.

CONCLUSIONS

These preliminary results indicate that dopamine, a dihydroxyphenyl derivative, is a much more effective affinity chromatography adsorbent for tyrosinase than are the monohydroxy derivatives. Benzoic and phenolic polymers will adsorb the enzyme but contaminating protein is also adsorbed and the enzyme apparently is inactivated while adsorbed on the column, since the eluate activity is low. This result is puzzling and somewhat at variance with Lerman's report that benzoic groups were ineffective adsorbants but that phenolic groups were effective in producing a 61-fold enrichment. The cause for this discrepency has not yet been settled, although Lerman's crude enzyme source and enzymatic activity assay were quite different from ours. Our data show quite convincingly that with tyrosinase it is essential to separate the ligand from the polymer support by a spacer molecule. This requirement is shared by a variety of other enzyme-ligand combinations [8].

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REFERENCES

[1] D. Kertesz and R. Zito, in Oxygenases (O. Hayaishi, ed.), Academic, New York, 1962, p. 325.
[2] Ref. 1, pp. 323, 328.
[3] L. S. Lerman, Proc. Nat. Acad. Sci., U.S., 39, 232 (1953).
[4] J. M. Nelson and C. R. Dawson, Advan. Enzymol., 4, 99 (1944).
[5] H. H. Weetall, Nature, 223, 959 (1969).
[6] R. Axen and J. Porath, Ibid., 210, 367 (1966).
[7] J. Porath, Ibid., 218, 834 (1968).
[8] P. Cuatrecases and C. B. Anfinsen, Ann. Rev. Biochem., 40, 259 (1971).

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