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SEPARATION OF CATALASE FROM PHENYLALANINE HYDROXYLASE: USE OF A SEPHAROSE ANTI-CATALASE IgG AFFINITY MATRIX

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SUMMARY: A recently described purification of rat-liver phenylalanine hydroxylase (S. Webber and J. M. Whiteley (1980) Anal. Biochem. 106, 63-72) required 0.04 mg/ml catalase to protect the enzyme during purification. To determine certain physical properties of the hydroxylase it was necessary to remove the catalase, therefore this report describes the synthesis of a rabbit IgG anti-catalase Sepharose matrix containing 34 mg IgG/g dry Sepharose, which was used for the efficient extraction of the catalase contaminant. Enzymatic activity was retained after catalase removal by employing 0.01 M tris(hydroxymethyl)methylaminopropane sulfonic acid, 0.1 M sodium chloride, 0.1% Triton X-100, 50 μM EDTA, and 15% glycerol at pH 8.5 as the storage buffer.

INTRODUCTION

A recent report from this laboratory (1) described the quantitative isolation of phenylalanine hydroxylase (EC 1.14.16.1) (2,3) from rat liver by a 5-step procedure which included passage through a tetrahydropteridine affinity chromatographic matrix to secure the high degree of purification observed in the final product. Although the enzyme could regularly be recovered with high specific activity (3-6 units/mg), in yields approaching 100%, the retention of activity in the final stages of purification depended on the addition of catalase to the buffer solutions used during the affinity chromatography. The presence of this second enzyme, although conferring stability on the hydroxylase, severely restricted the determination of essential physical characteristics such as subunit composition, iron content, and spectral properties. Therefore it was necessary to develop a technique to extract the catalase yet retain enzyme viability.

ABBREVIATIONS: TAPS, tris(hydroxymethyl)methylaminopropane sulfonic acid; DTE, dithioerythritol; EDTA, (ethylenedinitrilo)tetraacetic acid tetrasodium salt; $PtH_4.2HCl$, 2-amino-4-hydroxy-6,7-dimethyl-5,6,7,8-tetrahydropteridine dihydrochloride; SDS, sodium dodecyl sulfate.

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In this report, a simple chromatographic method which requires the synthesis of a second affinity matrix containing anti-catalase IgG bound to Sepharose is described for the removal of the protecting enzyme from samples of the purified hydroxylase. An outline of the preparation of the matrix is presented and the conditions necessary to retain the catalytic activity of the enzyme when isolated in the absence of catalase are defined.

MATERIALS AND METHODS

Chemicals were obtained from the following commercial sources: Bovine liver catalase, L-phenylalanine, Trizma and Trizma-HCl (Tris, Tris-HCl), TAPS, DTE, cyanogen bromide activated Sepharose 6MB [Sigma]; EDTA, Triton X-100 [J.T. Baker]; glycerol [Eastman]; $PtH_4.2HCl$ [Calbiochem]; Freunds incomplete and complete adjuvants [Miles].

Phenylalanine Hydroxylase. The enzyme was isolated quantitatively from rat liver [Pel-Freez Biologicals, Inc.] as described previously (1) using the following purification sequence: (a) acetic acid extraction, (b) ethanol and (c) ammmonium sulfate precipitation, (d) filtration through Sephadex G-100 and (e) affinity chromatography on a matrix containing $PtH_4.2HCl$ bound to Sepharose via a butanediol diglycidyl ether linkage. The last two steps were carried out in 0.01 M TAPS buffer pH 8.5 containing 0.1 M sodium chloride and 0.001 M DTE, to which bovine liver catalase was added (0.04 mg/ml) in step (e).

Antibody Production and Isolation. Antibodies were raised in New Zealand white rabbits by six weekly injections of 1 mg bovine catalase emulsified in 0.75 ml Freund's complete (first injection) or incomplete (subsequent injections) adjuvant, and 0.75 ml saline. The emulsion was injected subcutaneously at multiple sites on the animal's back. After exsanguination, the blood was allowed to clot at 4° overnight and a crude IgG fraction was obtained from the serum by treatment with 30% ammonium sulfate. The precipitate which formed was then isolated by centrifugation at 12,500 x g, dissolved in a minimal volume of 0.9% saline and dialyzed exhaustively against 0.9% saline before coupling.

Preparation of IgG Affinity Matrix. Crude IgG (4.0 ml, 53 mg) was added to cyanogen bromide activated Sepharose (1 g dry wt.), [pretreated and washed with 1 mM HCl (200 ml), then suspended in 10 ml of 0.1 M $NaHCO_3$, 0.5 M NaCl, pH 8.5], and the mixture was stirred gently overnight at 4°. After filtration and resuspension in fresh $NaHCO_3$/NaCl buffer, to which ethanolamine (1 ml) had been added to block any remaining active groups, the mixture was adjusted to pH 9.5 with 5 N HCl and stirred for a further two hours at room temperature. The gel was then washed successively with 150 ml each of 0.1 M $NaHCO_3$, 0.5 M NaCl, pH 8.5; 0.1 M NaAc, 0.5 M NaCl, pH 4.2, and a further quantity of 0.1 M $NaHCO_3$, 0.5 M NaCl, pH 8.5. The resulting matrix contained 34 mg IgG per g dry Sepharose; a coupling efficiency of 65%, of which 1.85% (0.64 mg) IgG was catalase specific when determined by immunotitration.

Removal of Catalase by the IgG Affinity Matrix. In a series of experiments, 1.0 ml samples of phenylalanine hydroxylase (0.64 units/ml, specific activity 4.8 units/mg) containing 0.04 mg/ml catalase were applied to a 0.6 x 6.0 cm column of the affinity gel pre-equilibrated and eluted with each of the buffer solutions listed in Table 1. Elution was carried out with a flow rate

TABLE 1. *The recovery and long-term stability of phenylalanine hydroxylase activity after chromatography on a Sepharose anti-catalase IgG matrix.*

For each experiment the matrix was pre-equilibrated and eluted with one of the buffer solutions listed, according to the procedure in MATERIALS AND METHODS. In every case the recovery of hydroxylase protein was 100%.

Buffer solution[a]	Phenylalanine hydroxylase activity after elution[b]		
	0 h	4 h	12 h
0.01 M TAPS, 0.1 M NaCl, 0.001 M DTE pH 8.5	48	2	0
+ 15 mM phenylalanine	46	20	7
+ 3,5-di-*tert*-butyl-4-hydroxybenzyl alcohol	62	28	5
+ 50 mM ascorbate	5	0	0
0.01 M Tris-HCl, 0.1 M NaCl, 0.001 M DTE			
pH 5.6	33		0
pH 6.5	37		0
pH 7.4	41		0
pH 8.5	49		0
pH 9.4	30		0
0.01 M TAPS, 0.1 M NaCl, 0.1% Triton X-100, 50 µM EDTA, pH 8.5	51	48	32
+ 0.001 M DTE	59	53	20
+ 15% glycerol	98	97	70
+ 15% glycerol, 0.001 M DTE	100	89	45

[a] All buffer solutions were deaerated with argon prior to use.

[b] The activity is expressed as a percentage of that applied to the matrix.

of 30 ml/hour and 1.2 ml fractions were collected into argon-filled tubes. Each fraction was assayed within 10 minutes of collection for phenylalanine hydroxylase activity, catalase activity, and protein concentration. Samples were then subjected to polyacrylamide gel electrophoresis in the presence of SDS using the method of Weber and Osborn (4).

Enzyme Assays. Phenylalanine hydroxylase activity was measured by determining tyrosine formation at pH 7.4 and 27° using the nitrosonapthol fluorometric method (5) described previously (1). The assay mixture contained Tris-HCl (50 µmole), phenylalanine (1 µmole), DTE (1 µmole), $PtH_4.2HCl$ (0.2 µmole), catalase (0.4 mg) and hydroxylase in a final volume of 0.5 ml. Catalase was assayed by the method of Beers and Sizer (6) in which the disappearance of peroxide is monitored spectrophotometrically at 240 nm, pH 7.0 and 25°.

RESULTS AND DISCUSSION

The results obtained after chromatography of 1.0 ml phenylalanine hydroxylase samples, each containing 0.04 mg catalase, on the anti-catalase IgG affinity matrix using a variety of buffer solutions, are summarized in Table 1. The column effluents showed no catalase activity, and SDS polyacrylamide gel electrophoresis confirmed the absence of this enzyme in the eluted samples. In every case, quantitative recovery of applied phenylalanine hydroxylase protein was achieved, however, enzymatic activity and stability varied with the composition of the eluent. For example, using deaerated 0.01 M TAPS pH 8.5 containing 0.1 M NaCl and 0.001 M DTE, a buffer system previously found to confer stability on the hydroxylase in the early stages of purification (1), 48% recovery of activity was obtained, unfortunately this decayed rapidly to zero after 4-hour storage at 4°. That catalase possessed a unique ability to confer stability on the hydroxylase in this buffer system was demonstrated in experiments where catalase was reintroduced after chromatography. When column fractions were collected into tubes containing catalase (final concentration 0.05 mg/ml), hydroxylase recovery increased from 48% to 67% and the stability after 4-hour storage at 4° increased from 2% to 100%. In the absence of catalase, phenylalanine hydroxylase stability was slightly enhanced in the presence of 15 mM phenylalanine or the radical scavenger 3,5-di-*tert*-butyl-4-hydroxybenzyl alcohol, however 50 mM ascorbate was disadvantageous. When the eluent pH was varied between 5.6 and 9.4 using 0.01 M Tris-HCl buffers containing 0.1 M NaCl and 0.001 M DTE, enzyme recoveries varied between 30% and 49% with the maximum occurring at pH 8.5.

Consistently high enzyme yields and enhanced stability were achieved, however, when the TAPS buffer solutions were supplemented with a mixture of reagents introduced previously by Shiman *et al.* (7) for the isolation of phenylalanine hydroxylase by hydrophobic chromatography. The most effective buffer mixture contained deaerated 0.01 M TAPS pH 8.5 containing 0.1 M NaCl, 0.1%

Triton X-100, 50 μM EDTA and 15% glycerol. Omission of glycerol reduced the yield from 100 to ∿ 50% and the addition of 0.001 M DTE while increasing the yield by 10% in the absence of glycerol, reduced enzyme stability to storage at 4° (both with and without glycerol).

When the above purification step is added to the previously published isolation procedure (1) essentially quantitative recovery of the hydroxylase from rat liver homogenates can be achieved. The anti-catalase IgG matrix is stable to repeated use and bound catalase can be removed by periodic washing with 0.1 M acetic acid. In routine preparations of the hydroxylase in this laboratory, the tetrahydropteridine affinity matrix step continues to be run in the presence of catalase, primarily because the hydroxylase shows greater stability to storage under liquid nitrogen in the presence of the second enzyme. However, the catalase removal step described in this report is rapid and efficient and its introduction as a final purification step allows the major part of the isolation procedure to be done under conditions which retain enzymatic activity but which are without the difficulties associated with the presence of glycerol and Triton X-100 in the eluting buffer solutions.

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