

# Immunological Studies of Catechol Methyltransferase from the Yeast *Candida tropicalis*

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Z. Naturforsch. **35 c**, 712–716 (1980); received April 24/May 27, 1980

Catechol Methyltransferase, Antibodies, Yeast Fungus, *Candida tropicalis* CBS 94

Immunization of rabbits with purified catechol methyltransferase from *Candida tropicalis* yielded a potent antiserum. Ouchterlony double diffusion analysis showed a single precipitin line. There was no cross reactivity with the catechol methyltransferase from rat and bovine liver. Specific antigen-antibody interaction was demonstrated by a potent inhibitory effect of the antibody on the yeast enzyme. Immunological titration and quantitative precipitin reaction of the enzyme showed that the maximum amount of precipitable complex occurred at the equivalence point where enzyme activity was completely inhibited.

## Introduction

Catechol methyltransferase (S-adenosyl-L-methionine: catechol-O-methyltransferase, E.C. 2.1.1.6) [1, 2] catalyses the transfer of a methyl group from S-adenosyl-L-methionine to one of the vicinal hydroxyl groups of various catecholic compounds. This enzyme plays a primary role in the metabolism of catechols [2], catecholamines [2], catechol estrogens [3] and the detoxification of xenobiotic catechol compounds [4, 5]. In addition, this enzyme has a regulatory function in the modulation of the concentration of S-adenosyl-L-methionine. Numerous investigations have demonstrated catechol methyltransferase in a variety of animal tissues and cells with the highest levels present in liver and kidney [5]. Recently we found a catechol methyltransferase in the facultative pathogenic yeast *Candida tropicalis*. Harvesting the yeast cells in the late exponential phase of a batch culture allowed a high purification of this catechol methyltransferase [6].

In this study we report the production of specific antibodies and the immunological characterization of this enzyme. Furthermore attempts were made on answering the question whether there exists chromatographically separable molecular forms of the yeast catechol methyltransferase.

## Materials and Methods

### Chemicals

S-Adenosyl-L-methionine hydrogensulfate was purchased from Boehringer Mannheim GmbH and

S-adenosyl-L-[methyl-<sup>14</sup>C]methionine (50–60 mCi/mmol) was from Radiochemical Centre, Amersham. Complete and incomplete Freund's adjuvant were obtained from Difco Laboratories. Goat anti-rabbit IgG, (IgG fraction, lyophilised) was purchased from Paesel KG. Sephadex G-100 was a product from Pharmacia, and agarose was from Serva. All other reagents were of analytical grade.

Liquid scintillation counting was performed in a toluene-based scintillation solution (3 g of 2,5-diphenyloxazole (PPO) and 0.1 g of 1,4-bis[2-(5-phenyloxazolyl)]benzene (POPOP) in 1 liter of toluene).

### Animals

Rabbits: Chinchilla rabbits, female, 2–2.5 kg, Ivanovas GmbH. Rats: Sprague Dawley (SPF), female, 80–120 g, Süddeutsche Versuchstierfarm GmbH. All animals were allowed free access to food and water.

### Enzymes

Catechol methyltransferase from bovine liver was provided by Paesel KG. Catechol methyltransferase from rat liver was purified according to Axelrod and Tomchick [2]. Catechol methyltransferase from the yeast *Candida tropicalis* CBS 94 was purified by a previously reported procedure [6]. Further purification was achieved by affinity chromatography in a carbodiimide (CDI) coupling reaction by modification of the method of Lefkowitz [7] using  $\beta$ -(3,4-dimethoxyphenyl)-ethylamine as ligand instead of norepinephrine. The elution of enzymatic activity was carried out with a 10 mM potassium phosphate buffer (pH 7.6) (10 mM MgCl<sub>2</sub>, 10 mM sucrose and

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0341-0382/80/0900-0712 \$ 01.00/0



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1 mM EDTA) and a discontinuous salt gradient (0 M, 100 mM, 1 M sodium chloride). Homogeneity of the enzyme was controlled by polyacrylamide gel electrophoresis as described by Davis [8].

#### *Immunological procedures*

Purified catechol methyltransferase (200 µg) dissolved in 0.5 ml 10 mM potassium phosphate buffer (pH 7.6), containing 10 mM sucrose, 1 mM EDTA and 1 mM mercaptoethanol was emulsified with an equal volume of complete Freund's adjuvant. The emulsified mixture was injected subcutaneously into the back of each rabbit. Subsequent injections in Freund's incomplete adjuvant were made at 14-day intervals after the initial injection. The rabbits were bled 7 days after each injection. Blood samples (30 to 40 ml) were allowed to clot for 4 h at room temperature, followed by 4 h at 4 °C, and the serum was separated from the blood clot by centrifugation. The IgG fraction of the antisera was purified by precipitation with 40% saturated ammonium sulfate followed by chromatography on DEAE-Sephadex CL-6B [9]. The appearance of antibodies directed against yeast catechol methyltransferase was monitored by Ouchterlony immunodiffusion. Control sera were obtained from the rabbits prior to inoculation.

#### *Ouchterlony double diffusion analysis*

Immunodiffusion experiments were performed on 1.5% agarose gels containing 50 mM potassium phosphate buffer (pH 7.6), 150 mM sodium chloride, and 0.01% sodium azide. Immunodiffusion plates (Miles Lab., Inc.) were developed at 4 °C for 24 h. After washing with cold distilled water, the plates were fixed in 140 mM sodium chloride.

#### *Immunological titration and quantitative precipitin reaction of catechol methyltransferase*

Inhibition of catechol methyltransferase activity by the antibody was demonstrated by titration against the IgG fraction. Increasing amounts of  $\gamma$ -globulin were added to the enzyme solutions containing 20 milliunits of catechol methyltransferase activity in a final volume of 0.2 ml. After a preincubation period of 30 min at 37 °C 50 µl goat anti-rabbit immunoglobulin (140 µg) were pipetted. Incubation was continued overnight at 4 °C. Follow-

ing completion of precipitin reaction, the samples were centrifuged at  $5000\times g$  at 4 °C for 15 min (Multex centrifuge, MSE) and supernatants were then carefully decanted. The pellets were washed 3 times with cold 50 mM potassium phosphate buffer (pH 7.6) and suspended in the same buffer for protein determination according to Lowry [10].

#### *Enzyme assay*

Testing catechol methyltransferase activity, the preincubation mixtures contained in addition final concentrations of 50 mM potassium phosphate buffer (pH 7.6), 10 mM  $MgCl_2$ , 2 mM L-cysteine, 0.6 mM S-adenosyl-methionine and  $4\times 10^{-6}$  M 6,7-dihydroxycoumarin (aesculetin) in a total of 0.5 ml. After an incubation at 37 °C for 30 min, the reaction was stopped by heat denaturation. The soformed product scopoletin [6] was determined according to a previously described fluorometric method [11].

One unit of catechol methyltransferase activity is defined as the amount of enzyme catalysing the O-methylation of 1 nmol of aesculetin in 1 min at 37 °C under the standard assay conditions.

#### *Comparison of aesculetin, 3,4-dihydroxybenzaldehyde, and quercetin-methylating activities across a Sephadex G-100 elution profile*

To investigate the distribution of catechol methyltransferase from *Candida* across a Sephadex G-100 elution profile aesculetin-, 3,4-dihydroxybenzaldehyde-, and quercetin-methylating activities were determined [12].

For the gel filtration Sephadex G-100 was equilibrated with 10 mM potassium phosphate buffer (pH 7.6) containing 10 mM  $MgCl_2$ , 10 mM sucrose, and 1 mM EDTA, and poured into a column K 16/40 (Pharmacia). In a typical experiment, 5 ml pH-5-sediment [6] were applied to the column and 3 ml fractions were collected. The O-methylating reaction was started as previously described [11], except that 0.05 µCi S-adenosyl-L-[methyl- $^{14}C$ ]methionine were added instead of the unlabeled methyl donor. The molarity of the substrates was  $10^{-3}$  M. The reaction was terminated by heat denaturation. The reaction mixture was acidified with 0.5 ml 1 N HCl and extracted with 2 ml ethylacetate (chloroform in the case of aesculetin) by blending on a Cyclo mixer (Vortex, Clay-Adams, Inc.) vigorously for 2 min. After centrifugation for 5 min in a laboratory centri-

fuge (Multex, MSE) an aliquot (0.5 ml) of the organic phase of the extract was dried by evaporation. The dry residue was dissolved in 1 ml methyl alcohol in each case. After addition of 10 ml scintillation fluid, the [ $^{14}\text{C}$ ]radioactivity was measured by liquid scintillation counting (Tricarb, Packard).

## Results

Following the purification procedure of the yeast enzyme as previously described the active eluate yielded three protein bands after disc electrophoresis on polyacrylamide gel [6]. Further inactive protein material could be separated by the affinity chromatography step on Sepharose 4B coupled with 3,4-dimethoxyphenylethylamine. The result of an electrophoretic analysis of an aliquot of the active fraction is shown in Fig. 1.

Immunization of rabbits with purified catechol methyltransferase yields a potent antibody. The antigen-antibody interaction was demonstrated by means of specific precipitin reactions using both immunochemical and enzymatic methods. Ouchterlony double diffusion patterns of antibody and



Fig. 1. Disc electrophoresis of purified catechol methyltransferase on 6% polyacrylamide gel [8]. 50  $\mu\text{g}$  of the purified yeast catechol methyltransferase were layered on the top of the gel. Intensity of current: 4 mA/tube. Time of electrophoresis: 2 h. The gel was stained with 0.1% Coomassie Brilliant Blue G-250 solution.

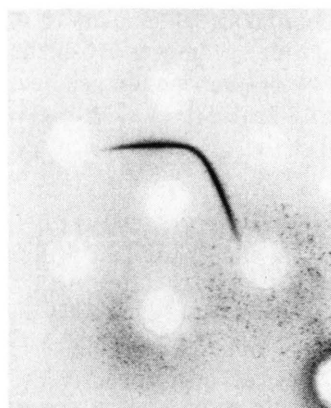


Fig. 2. Agar gel double immunodiffusion reaction of anti-yeast catechol methyltransferase  $\gamma$ -globulin with purified catechol methyltransferase from the yeast *Candida tropicalis*. Agar gels (1.5%) containing 50 mM potassium phosphate (pH 7.6), 0.15 M sodium chloride, and 0.01% sodium azide were used for making the diffusion plates. The center well contained 10  $\mu\text{l}$  of  $\gamma$ -globulin solution and the peripheral wells going clockwise from 12 o'clock were filled in pairs with catechol methyltransferase from *Candida tropicalis*, rat liver and bovine liver respectively.

purified yeast catechol methyltransferase show a single connecting precipitin line, indicating that the enzyme preparation employed as the antigen was homogeneous and specific to the catechol methyltransferase (Fig. 2). The antibody was tested further to determine whether there was any cross reactivity with the catechol methyltransferase from mammalian tissues. By double diffusion analysis of the antibody with catechol methyltransferase from rat liver and bovine liver no precipitation bands could be observed (Fig. 2). Specific antigen-antibody interaction was also demonstrated by a potent inhibitory effect of the antibody on the catechol methyltransferase activity from the yeast. The antibody capability was illustrated by inhibiting the enzymatic reaction (Fig. 3). When varying amounts of the antibody were added to enzyme solutions with constant enzymatic activity (20 milliunits), an increasing amount of inhibition of activity was observed. The inhibitory effect was complete with 20  $\mu\text{l}$  antibody. The results of the quantitative precipitin reaction involving purified catechol methyltransferase from *Candida* and the antibody are also shown in Fig. 3.

Determination of precipitated protein from the antigen-antibody complex showed that the maximum amount of precipitable complex occurred at

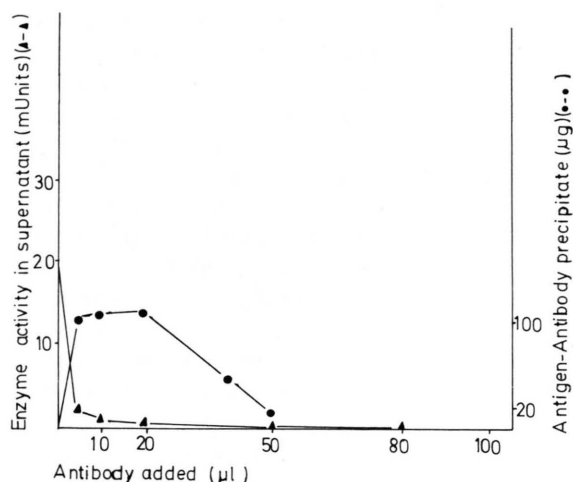


Fig. 3. Immunological titration and quantitative precipitin reaction of the yeast catechol methyltransferase. Inhibition of yeast catechol methyltransferase activity (20 milliunits) by the antibody was demonstrated by titration against the  $\gamma$ -globulin solution. Following completion of quantitative precipitin reaction, the samples were centrifuged at  $5000\times g$  in the cold. The pellets were washed 3 times with cold 50 mM potassium phosphate buffer (pH 7.6) and suspended in the same buffer for protein determination. Catechol methyltransferase activity was measured by fluorometric determination of scopoletin formed from aesculetin and S-adenosyl-methionine. (For details see "Materials and Methods".)

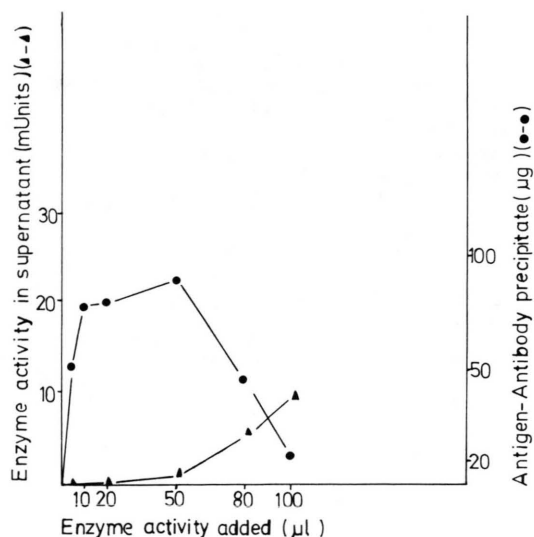


Fig. 4. Quantitative precipitin reaction of the yeast catechol methyltransferase. Increasing amounts of the enzyme solution were added to the antibody. The enzyme activity remaining in supernatant was assayed fluorometrically after completion of incubation of the reaction mixture. (For details see "Materials and Methods".)

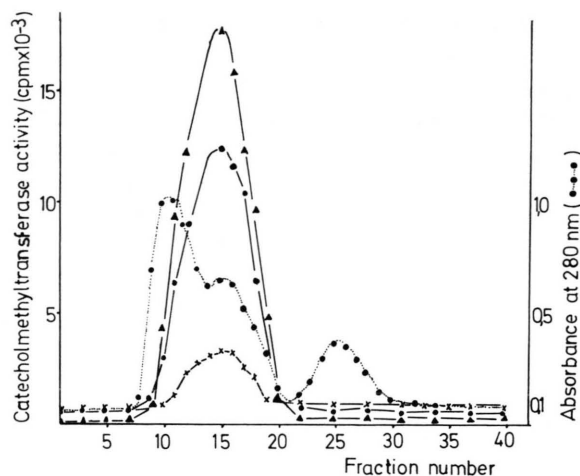


Fig. 5. Comparison of aesculetin-, 3,4-dihydroxybenzaldehyde- and quercetin-methylating activities across the Sephadex G-100 elution profile. Aliquots of each fraction were assayed for catechol methyltransferase with the methyl acceptors  $\Delta$ - $\Delta$ - $\Delta$  aesculetin,  $\bullet$ - $\bullet$ - $\bullet$  3,4-dihydroxybenzaldehyde, and  $\times$ - $\times$ - $\times$  quercetin. Substrate concentration was  $10^{-3}$  M in each case.

the equivalence point, where enzyme activity was completely inhibited. In Fig. 4 the results of titration of the antibody with increasing amounts of enzyme are illustrated. Again, at the equivalence point where enzymatic activity began to appear in the supernatant, the amount of antigen-antibody complex was at a maximum.

Whereas the purified yeast catechol methyltransferase was inhibited by the antibody to the yeast enzyme completely, no neutralisation could be demonstrated against the mammalian liver catechol methyltransferase from rat and bovine. The yeast enzyme is proved to be a different protein with specific antigenic properties. Antisera obtained during 6 months gave a single precipitin line on immunodiffusion and we could not find a heterogeneous response due to the presence of more molecular species of the enzyme.

In addition to immunological homogeneity the yeast enzyme activity could not be resolved into distinct molecular activities by chromatography on DEAE-cellulose, DEAE-Sephadex CL-6B, gel filtration on Ultrogel AcA 44 and isoelectric focusing techniques in granulated gels of Sephadex G-75 as described previously [6]. The fact, that Friedhoff and coworkers [12, 13] were able to separate catechol methyltransferase from mammalian tissues by gel



filtration on Sephadex G-100 into two different molecular forms, a predominant form with a smaller molecular weight, and the other, a minor form with a larger molecular weight led to further investigation under the experimental conditions employed by these investigators [12]. Thus, subjecting the yeast enzyme (pH-5-sediment) to gel filtration on Sephadex G-100 only one activity peak could be shown (Fig. 5). Using different substrates for the enzyme [14], the highest enzymatic activity was always found in the same fraction.

## Discussion

Because of the ubiquitous occurrence and relevance of catechol methyltransferase in animal tissues and cells, it was of interest, to investigate the immunological behaviour of the yeast catechol methyltransferase and to compare it with that of the enzyme from mammalian sources [15–18, for review see ref. 5]. The failure of interspecies cross reaction indicated that the catechol methyltransferase from rat and bovine liver possess no common antigenic groups recognized by the antibody prepared against the yeast enzyme.

Immunological congeniality of yeast and mammalian catechol methyltransferase could therefore

be excluded. In addition, no neutralisation effects on the activity of mammalian enzymes could be demonstrated, so we were unable to show similar immunological identities. The lack of cross reactivity may imply an early divergence of the genetic and evolutionary mechanism of the enzyme.

Booster injections during 6 months exhibited antisera which yield only a single precipitin line on immunodiffusion experiments and there was no gradual appearance of additional enzyme related antibodies during the immunization procedure as found by Creveling *et al.* with purified catechol methyltransferase from rat liver [16]. Catechol methyltransferase of mammalian tissues has been shown to exist (in the soluble fraction of various mammalian tissues) as two distinct molecular forms which are separable by gel filtration of the soluble fraction on Sephadex G-100. We were unable to detect two variant forms to the catechol methyltransferase from *Candida*: chromatography of crude fraction of the enzyme on Sephadex G-100 yielded only one peak of activity.

## Acknowledgements

The authors gratefully acknowledge the technical assistance of Mrs. S. Oza. Special thanks are due to Mrs. M. Baumgärtner for typing the manuscript.

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