Purification and Properties of a Catechol Methyltransferase of the Yeast *Candida tropicalis*

Joseph Veser, Peter Geywitz, and Helmut Thomas

Abteilung Biochemie I der Universität Ulm, Oberer Eselsberg, D-7900 Ulm

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In an effort to investigate catechol methyltransferase activity in sources other than mammalian tissues and cells, a high level of enzyme activity was found in the yeast fungus *Candida tropicalis* CBS 94. Partial purification of the enzyme (approx. 550 fold with a recovery of 7%) could be achieved by using ion-exchange and gel filtration techniques. The molecular weight was estimated at 32,000 ± 2,000 by gel filtration on Sephadex G-100. In isoelectric focusing experiments on Sephadex G-75 the enzyme exhibited a pI-value of 5.0 ± 0.1. In contrast to catechol methyltransferase from various mammalian tissues the enzyme activity was prepared from the pH 5-sediment. The substrate specifity is comparable to other catechol methyltransferases.

Introduction

Catechol methyltransferase (S-adenosyl-L-methionine: catechol-O-methyltransferase, EC 2.1.1.6) [1, 2] catalyzes the transfer of a methyl group from S-adenosyl-methionine to a catechol substrate resulting in the formation of the meta- and para-O-methylated products. This enzyme, which is widely distributed in mammalian tissues [3], plays a primary role in the metabolism of catecholamines. Recent investigations found this enzyme in various species of the yeast *Candida* [4]. A very sensitive method was introduced for the determination of catechol methyltransferase activity, based on the measurement of the intensity of fluorescence of 7-hydroxy-6-methoxycoumarin (scopoletin) enzymatically produced by 6,7-dihydroxycoumarin (aesculetin) in the presence of metal ion Mg^{2+} and methyl donor S-adenosyl-methionine [5]. In this work we report the partial purification and characterization of this enzyme from *Candida tropicalis* using this method.

Materials and Methods

Chemicals:

The following compounds were obtained from the indicated sources: S-adenosyl-L-methionine hydrogensulfate, Combithek (calibration proteins) (Boehringer); S-adenosyl-L-[methyl-^{14}C]methionine (Radiochemical Centre, Amersham); neutral aluminium oxide, activity grade I (Woelm); DEAE-cellulose DE 52, microgranular form (Whatman); DEAE-Sepharose CL-6 B, Sephadex G-75 superfine, Sephadex G-100 (Pharmacia); Ultrogel AcA 44, Ampholine, carrier ampholytes pH 3 – 10 (LKB); Sabouraud dextrose – 2% broth (Merck). All other materials used were of reagent grade. Liquid scintillation counting was performed in a toluene mixture (1 l) containing 3 g PPO and 0.1 g POPOP.

Cultures

The yeast *Candida tropicalis* CBS 94 was cultivated in Sabouraud dextrose – 2% broth in a Lab shaker at 30 °C (120 rpm) for 72 h.

Purification of catechol methyltransferase

All steps were performed at 0 – 5 °C unless specified otherwise.

Preparation of homogenate and 100,000 × g supernatant

The sediment (4,000 × *g*, 20 min) of the yeast culture (1200 ml) was washed 3times with 200 ml ice cold buffer A (10 mM potassium phosphate buffer (pH 7.6), 10 mM $MgCl_2$, 10 mM sucrose and 1 mM EDTA) and finally suspended in 100 ml of this phosphate buffer. The yeast cells were then lysed in a cell homogenizer with glass beads (∅ 0.45 – 0.5 mm, Braun Melsungen) at 4,000 rpm for 4 min. About 95% of the cells were disintegrated. The homogenate was centrifuged at 100,000 × *g* for 1 h. The sediment was discarded.

Reprint requests to Prof. Dr. H. Thomas.

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Differential fractionation with 1 N acetic acid

The 100,000 × *g* supernatant was adjusted to pH 6 with 1 N acetic acid and stirred for 30 min. After centrifugation at 40,000 × *g* the sediment was discarded. The supernatant was then adjusted to pH 5 and stirred for a further 30 min. After centrifuging (40,000 × *g*, 30 min) the sediment (pH 5-sediment) was dissolved in 200 ml buffer A.

DEAE-cellulose chromatography

100 ml of pH 5-sediment were applied to a K 16/40 column (Pharmacia) of DEAE-cellulose DE 52 equilibrated with buffer A. The elution was carried out with buffer A and a discontinuous salt gradient (0 M, 140 mM, 1 M NaCl).The catechol methyltransferase containing fractions were combined and dialysed against buffer A.

DEAE-Sepharose CL-6 B fractionation

The dialysed active protein fraction was applied to a K 16/40 column of DEAE-Sepharose CL-6 B. The column was developed with buffer A. The elution was carried out with the same buffer and a discontinuous salt gradient (0 M, 100 mM, 1 M NaCl). The active eluate fractions were pooled and dialysed against buffer B (100 mM potassium phosphate buffer (pH 7.6), 10 mM $MgCl_2$, 10 mM sucrose and 1 mM EDTA).

Ultrogel AcA 44 filtration

For gel filtration Ultrogel AcA 44 was equilibrated with buffer B and poured into a column K 16/40. The elution was carried out with the same buffer system. The combined active fractions were dialysed against buffer A and frozen in 1 ml portions.

Enzyme assay

The enzyme activity was determined using aesculetin and S-adenosylmethionine as substrates according to a previously described fluorometric assay [5]. The specific activity was defined as nmol scopoletin produced in 30 min per mg protein. The methylation reaction of aesculetin to scopoletin was linear under standard conditions for up to 60 min. Protein concentrations were determined by the Lowry method [6] using bovine serum albumin as standard.

Disc electrophoresis

Polyacrylamide gel electrophoresis was performed according to the method of Davis [7] by using 6% buffer, 2-mercaptoethanol was added (100 mM). An electrophoretic prerun was made for 1 h. The gels were stained with 0.1% Coomassie Brillant Blue G-250 and scanned in a Zeiss PM Q II recording densitometer at 550 nm. The localization of enzyme activity in the gel was carried out as previously described [8].

Isoelectric focusing

The isoelectric focusing experiments were performed in granulated gels of Sephadex G-75 according to Winter [9]. After establishing the pH gradient by isoelectric focusing over 2 h, the pH 5-sediment (5 ml) was applied as a zone in the pH range 7.2 – 7.8. The separation was complete after running at 8 W constant power at 4 °C for 14 – 16 h. The gel fractions were eluted 2times with 2.5 ml 500 mM potassium phosphate buffer (pH 7.6). The eluate was tested for enzymatic activity by the enzyme assay [5].

Molecular weight determination by gel filtration

Molecular weight estimation was performed at 4 °C using a Sephadex G-100 column (K 9/60, Pharmacia) previously equilibrated with 10 mM potassium phosphate buffer (pH 7.6). The column's void volume was determined using ferritin. A sample (0.5 ml) of the enzyme preparation (pH 5-sediment) was applied to the Sephadex G-100 column and the column eluted with 10 mM potassium phosphate buffer (pH 7.6) at a flow rate of 8 – 10 ml/h collecting 0.6 ml fractions. Estimation of molecular weight was made from a calibration curve of molecular weights vs. elution volumes for standard proteins (*e. g.* cytochrome c, M_r 12,500; chymotrypsinogen A, M_r 25,000; albumin (hen egg) M_r 45,000; albumin (bovine serum) M_r 67,000) [10].

Substrate specifity of catechol methyltransferase

The incubation mixture was similar to that outlined in the enzyme assay [5], except that 5 μCi S-adenosyl-L-[methyl-^{14}C]methionine was added to the mixture of total volume 5 ml. The final concentration of the catechol substrates (see below) was 10^{-3} M. After incubation of the enzyme with protocatechualdehyde, protocatechuic acid, homoprotocatechuic acid, *p*-hydroxybenzoic acid and caffeic acid the reaction mixture was acidified with 1.0 ml 1.0 N HCl and extracted with 20 ml ethyl acetate 3times. Using aesculetin as substrate the acidified mixture was extracted as above, but with chloroform. After

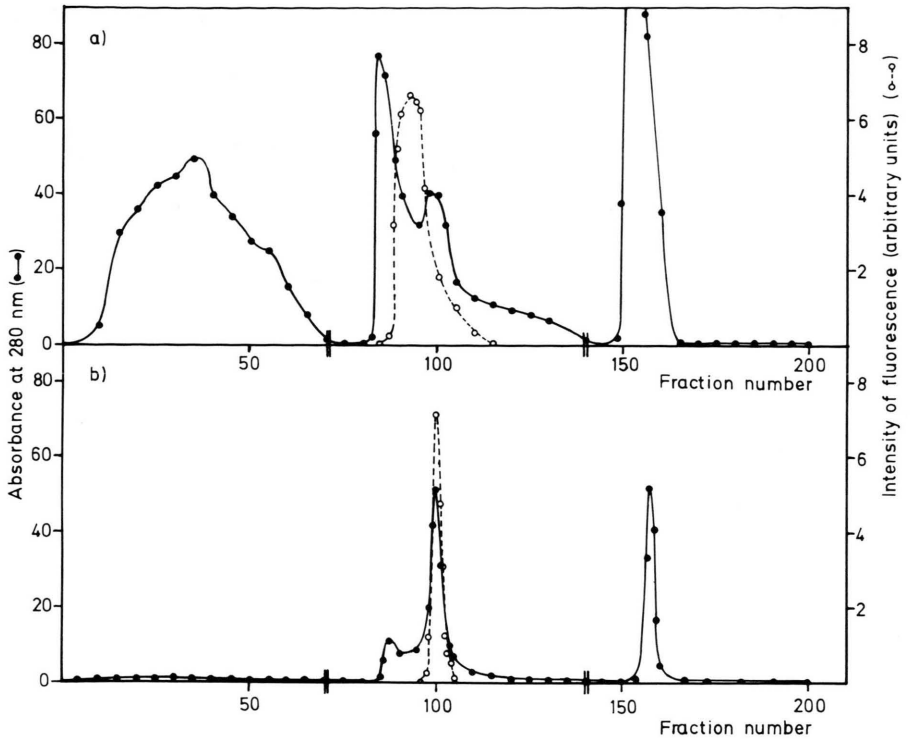

Fig. 1. Elution profile of catechol methyltransferase following DEAE-cellulose chromatography. For elution buffer A and a discontinuous NaCl-gradient (0 M, 140 mM, 1 M) was used. Fractions (3 ml) were collected (a). The catechol methyltransferase containing fractions (88 – 107) with maximal specific activity in fraction 93 were pooled and subjected to chromatography on DEAE-Sepharose CL-6 B. The elution was carried out with buffer A and a discontinuous NaCl-gradient (0 M, 100 mM, 1 M) (b).

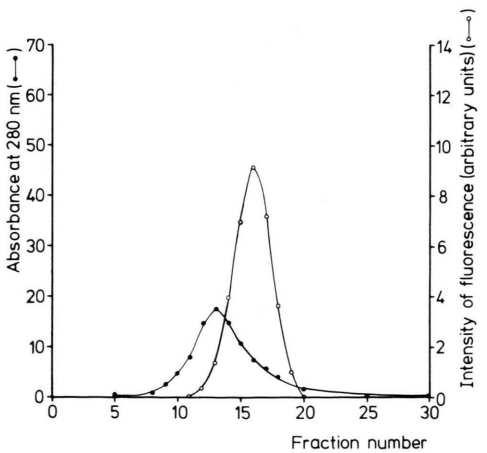

Fig. 2. Ultrogel AcA 44 filtration. The active DEAE-Sepharose CL-6 B eluate fractions (99 – 102) with maximal specific activity in fraction 100 were combined and subjected to gel filtration on Ultrogel AcA 44. Fractions (3 ml) were collected (see methods for details).

incubation with epinephrine, norepinephrine and dopamine, 10 ml of 0.5 M borate buffer (pH 10) were added to the incubation mixture. The extraction was carried out with 10 ml isoamyl alcohol, reextraction was performed 3times with 20 ml 0.1 N HCl. After evaporation the dry residue was dissolved in 0.5 ml methyl alcohol in each case. Aliquots of the dissolved dry residues obtained after incubation with aesculetin or the substituted phenolic compounds were separated on TLC silica gel 60 plates and on TLC silica gel 60 F-254 plates (Merck) respectively in the solvent system benzene/glacial acetic acid/H_2O (2/2/1, upper phase) (system I). Using dopamine as substrate TLC cellulose chromatoplates (Merck) were developed in the solvent system *n*-butanol, saturated with 3 N HCl (system II). After incubation of epinephrine and norepinephrine the extract was treated with sodium metaperiodate as described by Pi-

sano *et al.* [11]. The oxidation products vanillin and isovanillin were extracted 3times with 20 ml toluene and subjected to chromatography on TLC plates silica gel 60 F-254 in system I. The radiochromatograms were scanned with a thin layer radiochromatograph (LB 2723, Berthold). The reference compounds were visualized with ultraviolet light or by spraying with diazotized sulfanilic acid [12]. For determination of the *meta/para*-methylation ratio of the formed hydroxymethoxyphenyl compounds and the ratio of 6-O-/7-O-methyl ethers the areas of the chromatographically separated radioactive products were scraped off and transferred to a counting vial containing 1 ml methyl alcohol and 10 ml scintillation fluid. The [^{14}C]radioactivity was measured by liquid scintillation counting (Tri-Carb, Packard). The identification of the methylation products were performed not only by thin layer chromatography but also by the reversed isotope dilution technique [13].

Results and Discussion

Ninety to 95% of the catechol-methylating enzyme activity was detected in the 100,000 × *g* supernatant of the homogenate from the yeast cells. It could not be found in the culture filtrate. In contrast to earlier

Table I. Purification procedure of catechol methyltransferase of *Candida tropicalis*.

	Enzyme preparation	Protein [mg/ml]	Total protein [mg]	Total activity: nmol scopoletin produced in 30 min	Specific activity: nmol scopoletin produced in 30 min per mg protein	Yield Purification	
						[%]	(-fold)
I	Homogenate	120	24,000	1,550	0.06	100	
II	100,000 × *g* Supernatant	30	6,000	1,430	0.24	92.3	4.0
III	pH 5-Sediment	7	1,400	1,360	0.97	87.7	16.1
IV	DEAE-Cellulose eluate	0.5	60	822	13.7	53	228
V	DEAE-Sepharose CL-6 B eluate	0.75	18	271.2	15.1	17.5	251
VI	Ultrogel AcA 44 filtrate	0.12	3.58	113	31.6	7.3	526

The different enzyme fractions were incubated 30 min with 6,7-dihydroxycoumarin (aesculetin) and S-adenosylmethionine (see methods for details).

Table II. Substrate specificity of catechol methyltransferase of *Candida tropicalis*.

Substrate	methyl ether (total volume: 5 ml)		3-O-(6-O-) methyl ether	4-O-(7-O-) methyl ether	*m/p*-(6-O-/7-O-) methylation
	[nmol]	[%]	[nmol]	[nmol]	ratio
Aesculetin	18.1	100	16.47	1.62	10.1 : 1
Protocatechualdehyde	12.03	66	11.14	0.89	12.5 : 1
Protocatechuic acid	9.97	55	5.99	3.98	1.5 : 1
Homoprotocatechuic acid [a]	1.14	6	–	–	–
p-Hydroxybenzoic acid	0	0	0	0	0
Caffeic acid	14.72	81	8.58	6.15	1.4 : 1
Epinephrine bitartrate [b]	0.92	5	0.33	0.59	0.6 : 1
Norepinephrine bitartrate [b]	0.56	3	0.14	0.42	0.3 : 1
Dopamine hydrochloride [a]	1.16	6	–	–	–

[a] From the isomeric methylethers of homoprotocatechuic acid and of dopamine the total radioactivity was determined.
[b] Using epinephrine and norepinephrine as substrates the radioactivity of the oxidation products vanillin and isovanillin was measured.
For further details compare Materials and Methods.

described procedures for the isolation of the extensively studied catechol methyltransferase from mammalian tissues [2, 14 – 19] (for review see ref. [3]), the enzyme of *Candida tropicalis* was purified from the pH 5-sediment. A summary of a typical purification experiment is shown in Table I. As can be seen, this procedure resulted in about 530-fold purification of catechol methyltransferase over the homogenate with a recovery of 7%. Using a DEAE-Sepharose CL-6 B column for rechromatography of the ac-

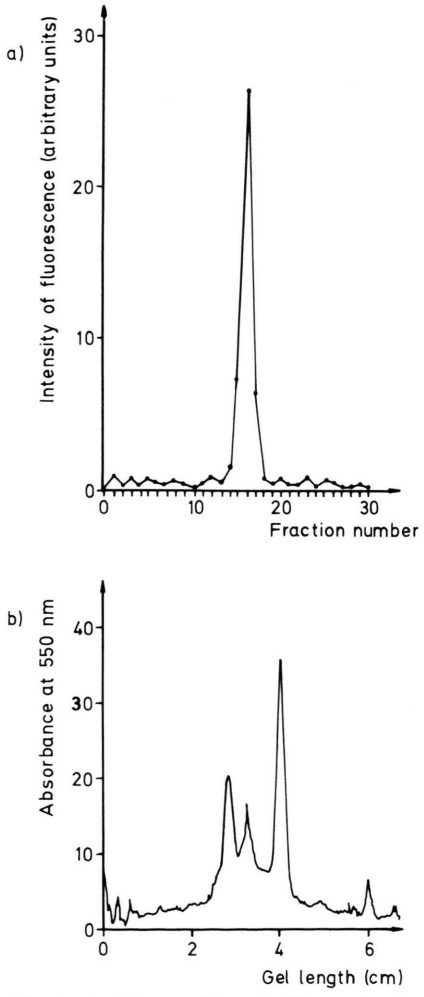

Fig. 3. Activity profile and 550 nm densitometric scan analysis after disc electrophoresis on polyacrylamide gel. The gels were loaded with 100 µl active AcA 44 eluate (fraction Nr. 17; 75 µg protein/ml). a) Gel system: pH 8.9; 6% [7]; to the electrode buffer 2-mercaptoethanol was added (100 mM). Intensity of current: 4 mA/tube. Time of electrophoresis: 2 h. Distance: 60 mm (30 fractions, each 2 mm). b) The gel was stained with 0.1% Coomassie Brillant Blue G-250 solution (see methods for details).

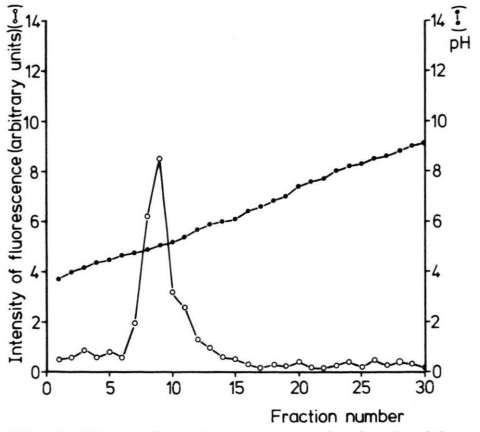

Fig. 4. Electrofocusing pattern obtained with catechol methyltransferase (pH 5-sediment) using a pH 3.0 – 10.0 gradient (2% Ampholine).

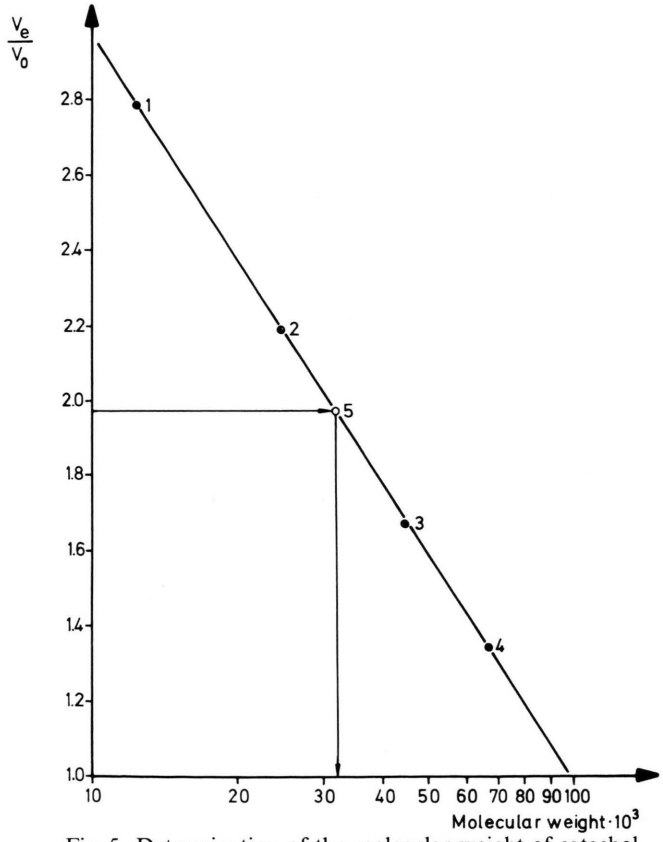

Fig. 5. Determination of the molecular weight of catechol methyltransferase on Sephadex G-100. 0.5 ml catechol methyltransferase (pH 5-sediment) were applied to a column K 9/60. Fractions of 0.6 ml were eluted with 10 mM potassium phosphate buffer (pH 7.6); the void volume v_0 was determined from the elution volume of ferritin. Molecular weights were estimated from standard plots of log molecular weights *versus* v_e/v_0. Molecular standards were: cytochrome c (1), chymotrypsinogen A (2), albumin (hen egg) (3) and albumin (bovine serum) (4).

tive DEAE-cellulose eluate, further inactive protein material could be separated (Fig. 1). After chromatography on DEAE-cellulose, DEAE-Sepharose CL-6 B and Ultrogel AcA 44 filtration (Fig. 2), only one peak of enzymatic activity was found in the eluate. Therefore, it was concluded that there was no evidence to suggest the existence of more than one form of the enzyme. The fluorometric localization of the enzyme activity after polyacrylamide gel electrophoresis is illustrated in Fig. 3 a. The corresponding scan analysis (O. D. 550 nm) is shown in Fig. 3 b. In isoelectric focusing experiments in granulated gels of Sephadex G-75 the purified enzyme exhibited a pI-value of 5.0 ± 0.1 (Fig. 4). The molecular weight of the catechol methyltransferase was estimated to be $32{,}000 \pm 2{,}000$ by gelfiltration on Sephadex G-100 (Fig. 5). The range of pH-optimum was between pH 7.6 and 7.8. The results of experiments referring to substrate specificity of the enzyme are summarized in Table II. All catechols examined, regardless of the side chain were O-methylated. On the other hand the monophenol compound *p*-hydroxybenzoic acid did not serve as substrate. 3,4-Dimethoxyphenyl compounds and 6,7-dimethoxycoumarin could not be detected as products. From the substances tested in our laboratory, homoprotocatechuic acid is a natural substrate for the yeast [20]. It is of interest, that endogenously occurring catecholamines in animal species such as epinephrine, norepinephrine and dopamine can also serve as substrates. The stability of catechol methyltransferase of *Candida tropicalis* was dependent on the degree of purification of the enzyme preparation. Whereas cytosol and pH 5-sediment showed no loss of activity at 4 °C in a week of storage, the activity of the AcA 44 filtrate lost about 60% of its initial activity under the same conditions.

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