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A Polyphenol-O-transmethylase Isolated from *Streptomyces rimosus*

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An O-methyltransferase with the ability to transfer methyl groups from S-adenosylmethionine to different polyphenols such as resorcinol, catechol, and pyrogallol has been isolated from *Streptomyces rimosus* and purified 100-fold. The pH dependence, heat stability and specificity of the enzyme have been studied as well as effects of ions and SH-reagents, and some kinetic data are reported.

Investigations of O-methylations of catechols have led to the discovery of S-adenosylmethionine O-methyltransferases in various tissues of animals and in plants.[1] In animals, these studies have mainly been undertaken in connection with investigations of the metabolism of catecholamines. The O-methyltransferases found in plants are considered to be of importance in the synthesis of flavonoids and lignin. Many microorganisms, particularly molds and actinomycetes produce phenolic compounds which often carry one or several O-methyl groups. Most of the phenolic compounds of microbial origin as distinguished from plant phenols are of resorcinol type as a consequence of their formation from acetate-polymalonate condensations. *In vivo* experiments have demonstrated the presence of O-methyltransferases in several cases[2] in the mold series by feeding the organism with methionine-$^{14}CH_3$ and subsequent localization of the radioactivity to methoxyl groups of the phenolic compound.

As it could be of interest to study the properties of a resorcinol O-methyltransferase in closer detail some available organisms were tested for their ability to methylate resorcinol with the utilization of methionine in whole cell experiments. Of the tested organisms *Streptomyces rimosus* was found efficiently to perform the reaction giving rise to the monomethylether of resorcinol. *S. rimosus* is an organism that has been thoroughly investigated with regard to its metabolism as well as to its production pattern because of its ability to produce the important antibiotics in the tetracycline series. So far, however, no O-methylated product has been discovered as a natural metabolite in this organism. On the other hand, N-methylation as well as C-methylation have been demonstrated[3] to be reactions that participate in

the formation of tetracyclines. This paper will report the isolation and some properties of the enzyme involved in transferring the methyl group of S-adenosylmethionine to resorcinol, and as will be shown to several other phenolic compounds as well including catechol and pyrogallol.

MATERIAL AND METHODS

S-Adenosylmethionine-^{14}C (spec.act. 13.78 μC/μmole) was purchased from Tracerlab Inc. The *phenols* listed in Tables 3—6 are all commercially available except for 3-methylresacetophenone, 5-methylresacetophenone, 6-methylresacetophenone, 3,5-dimethylresacetophenone, 4-methylresorcinol, 4-ethylresorcinol, 4-propylresorcinol, pyrogallol-1-methylether, and pyrogallol-2-methylether, which were synthesized according to methods described in the literature.

Streptomyces rimosus was grown in 500 ml Erlenmeyer flasks containing 150 ml synthetic medium ($(NH_4)_2SO_4$, 6 g; $MgSO_4 \cdot 7H_2O$, 0.25 g; KH_2PO_4, 0.15 g; $ZnSO_4 \cdot 7\,H_2O$, 0.04 g; $MnSO_4 \cdot 7\,H_2O$, 0.01 g; citric acid (H_2O) 12.8 g; glucose, 40 g, and distilled water to 1000 ml; 1.6 g of $CaCO_3$ was added to each flask) on a shaketable (250 rpm inch stroke) at 28°C for 48 h.

Isolation of the enzyme. The mycelium from 24 flasks was harvested by decanting the culture fluid. After washing twice with distilled water and once with 0.15 M KCl, 0.001 M EDTA solution, the mycelium was centrifuged at 5000 *g* for 5 min to get it as dry as possible. The cell mass (*ca.* 700 g) was disintegrated by grinding in a ball mill containing 3 cm porcelain balls and 25 g of sand at 4° for 75 min. The homogenate was centrifuged at 10 000 *g* at 4° for 10 min and the precipitate discarded. The soluble supernatant fraction (250 ml) was adjusted to pH 6.3 with diluted HCl. To 250 ml of the supernatant fraction were added 63 g of ammonium sulfate to 38 % saturation. The precipitate was discarded and 32 g of ammonium sulfate (38 to 54 % saturation) were added to the supernatant fraction. After centrifugation the precipitate was dissolved in 12 ml of 0.005 M phosphate buffer (pH 6.8). The solution was passed through a column of Sephadex G 25 (2 × 15 cm) equilibrated with 0.005 M phosphate buffer (pH 6.8) to remove remaining low molecular material. In the next step the enzyme solution was purified by negative adsorption on a CM-cellulose column (1 × 8 cm) treated with the same phosphate buffer. After adjusting the pH of the enzyme fraction to 6.2 with 1 M HCl the enzyme was adsorbed on a column of calcium phosphate gel (2 × 8 cm). The calcium phosphate gel was then washed with 50 ml of 0.005 M phosphate solution (pH 6.2) followed by elution of the enzyme with 0.1 M phosphate buffer (pH 6.2) yielding 50—60 ml of enzyme solution. In all procedures described in the purification of the enzyme the temperature was kept at 4°. The enzyme

Table 1. Purification of O-methyltransferase.

	Total volume ml	Total protein mg	Specific activity units */mg	Total units	Recovery %
Supernatant 10 000 *g*	275	797	0.32	255	100
Ammonium sulfate precipitation	16	40	1.8	72	28
CM-cellulose	30	30	4.4	132	52
Calcium phosphate adsorption and elution	60	7	30.1	217	85

* 1 unit = 1 mμmole of methylated product formed under the conditions described in assay method.

solution can be stored at 4° for two weeks with only minor loss of activity. The preparation obtained by this procedure was purified about 100-fold. The purification and recovery of the enzyme in the individual steps are listed in Table 1.

Assay method. 0.4 ml of enzyme solution (0.08 mg of protein) with pH adjusted to 7.2 was incubated with 0.025 μl (0.05 μC, 0.004 μmole) of S-adenosylmethionine-$^{14}CH_3$ and 50 μl (4.5 μmole) of an aqueous solution of resorcinol; the final volume was adjusted to 1 ml with 0.2 M phosphate buffer (pH 7.3). After 1 h incubation at 35° the reaction was stopped by adding 0.1 ml of 5 M HCl. The acid mixture was extracted once with 3 ml of toluene and an 1 ml aliquot of the toluene phase was withdrawn for determination of radioactivity. The measurement of radioactivity was performed in a liquid scintillation counter using 5 ml of 0.5 % diphenyloxazole in toluene. The obtained figures, corrected for a slight radioactivity from S-adenosylmethionine appearing in the toluene phase, is a measure on the amount of the methylated phenol.

The found monomethylether of resorcinol has been identified by mixing the radioactive products with synthetic monomethyl ether of resorcinol followed by coupling with *p*-nitrobenzoyl chloride in alkali. The obtained *p*-nitrobenzoate was recrystallized to constant specific radioactivity.

RESULTS AND DISCUSSION

Properties of the purified enzyme. The pH dependence of the reaction was tested over a range from pH 3.9 to 10.0 at 35° using various buffer systems (pH 3.9—5.5, 0.2 M acetate; pH 6.0—7.5, 0.2 M phosphate; pH 7.5—8.9, 0.2 M Tris; pH 9.0—10.0, 0.2 M glycine). The pH-optimum was found to be between 7.1 and 7.5 (Fig. 1). The reaction was also found to proceed at optimal velocity at 32—35° (Fig. 2) and the rate of methylation being linear over a period of 20 min (Fig. 3). The enzyme did not show any loss of activity by 5 min treatment at different temperatures up to 41° prior to incubations (Fig. 4). K_m for resorcinol was found to be 2.0×10^{-3} M in presence of 7.0×10^{-4} M S-adenosylmethionine by using Lineweaver-Burk plot [6] (Fig. 5). The enzyme activity was inhibited by preincubation with SH-reagents such as *p*-chloromercuriben-

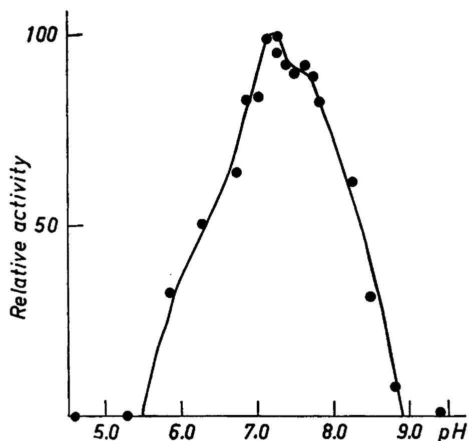

Fig. 1. Influence of pH on enzyme activity.

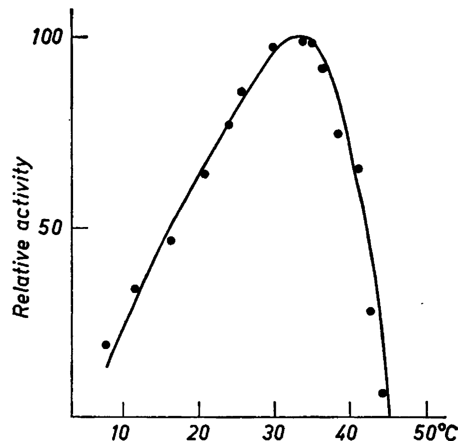

Fig. 2. Influence of temperature on enzyme activity.

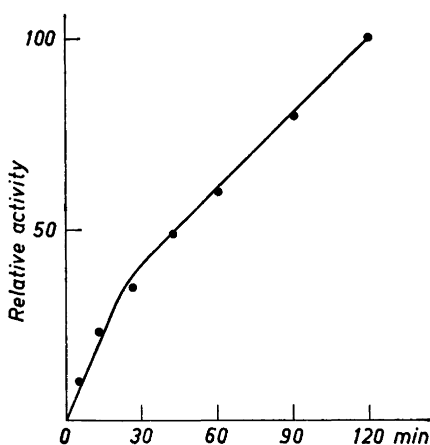

Fig. 3. Influence of time on the enzyme reaction.

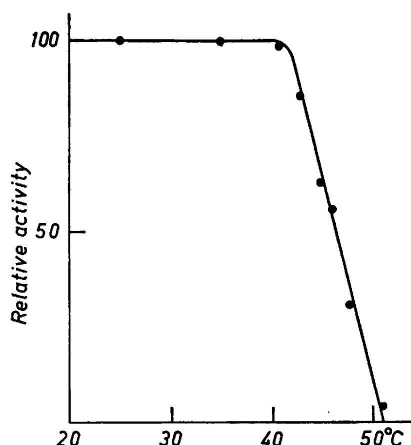

Fig. 4. Heat stability of the enzyme.

zoate, phenylmercuriacetate, N-ethylmaleimide, and iodoacetamide. In all cases the inhibition was counteracted by adding glutathione before the preincubation (Table 2).

Table 2. Effects of SH-reagents on enzyme activity with resorcinol used as substrate.

Inhibitor	Concentration of the inhibitor M	GSH* added before the preincubation M	GSH added after the preincubation M	% Inhibition
p-Chloro-	10^{-6}			0
mercuribenzoate	10^{-5}			75
	10^{-5}	10^{-4}		0
	10^{-5}		10^{-4}	50
Phenylmercuri-	10^{-6}			70
acetate	10^{-5}			100
	10^{-5}	10^{-4}		40
	10^{-5}		10^{-4}	95
N-Ethylmaleimide	10^{-5}			0
	10^{-4}			65
	10^{-4}	10^{-4}		0
	10^{-4}		10^{-4}	20
Iodoacetamide	10^{-5}			0
	10^{-4}			10
	10^{-3}			55
	10^{-3}	10^{-3}		0
	10^{-3}		10^{-3}	0
Cysteine	10^{-3}			0
	10^{-2}			0

* GSH = glutathione

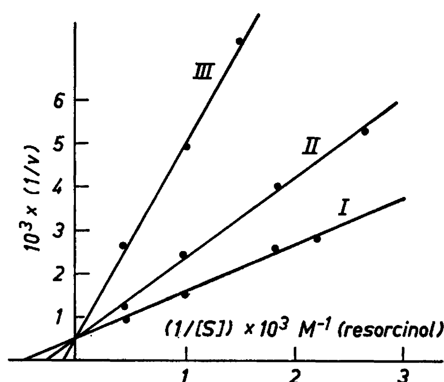

Fig. 5. Determination of K_m for resorcinol and inhibition of the reaction by β-resorcylic acid. I. without inhibitor present; II. with 0.9×10^{-3} M β-resorcylic acid; III. with 3.0×10^{-3} M β-resorcylic acid, $K_i = 1.0 \times 10^{-3}$ M.

Specificity of the enzyme. By using the above described assay method with resorcinol substituted with equivalent amounts of various phenols the specificity of the enzyme was investigated. When the phenol used was of pyrogallol type the method was modified by replacing toluene with ether as extracting solvent and the ether withdrawn for radioactive determination evaporated to dryness in the vial before adding the phosphorus solution. As is seen from Tables 3—6 the enzyme has a wide ability to methylate different phenolic compounds. This ability is not restricted to resorcinolic phenols but catechol and pyrogallol are even better substrates for the enzyme than resorcinol. In the resorcinol series 4-chlororesorcinol surpassed resorcinol as substrate. Other different substituents tested in the resorcinol molecule caused a reduced activity relative to resorcinol. It is obvious that the "active methyl group" is acting as an electrophilic reagent in this type of reaction. Consequently one would expect that an increased electron density at a certain position in the phenol would favour methylation. The charge distributions were calculated for some resorcinols by a modified ω-method [5] (Table 7). The differences in π-electron charges comparing the corresponding oxygen atoms in the investigated resorcinols are too small to give rise to conclusions.

Table 3. Specificity determinations of various phenols and N compounds.

Substrate	Relative activity %
Phenol	5
Catechol	140
Resorcinol	100
Hydroquinone	5
Pyrogallol	200
Hydroxyhydroquinone	10
Phloroglucinol	5
Histamine	0
Nicotinic amide	0

Table 4. Specificity determinations of resorcinols.

Substrate	Relative activity %
Resorcinol	100
1-O-Methylresorcinol	30
2-Methylresorcinol	50
4-Methylresorcinol	15
5-Methylresorcinol	70
4-Ethylresorcinol	20
4-Propylresorcinol	10
4-Formylresorcinol	30
2-Carboxyresorcinol	15
4-Carboxyresorcinol	5
4-Chlororesorcinol	120
Resacetophenone	50
3-Methylresacetophenone	65
5-Methylresacetophenone	30
6-Methylresacetophenone	65
3,5-Dimethylresacetophenone	30
2,6-Dihydroxyacetophenone	35
Orsellinic acid	25
Dihydroresorcinol	5

Table 5. Specificity determinations of catechols.

Substrate	Relative activity %
Catechol	100
3-Methylcatechol	70
4-Methylcatechol	15
3,4-Dihydroxybenzaldehyde	175
3,4-Dihydroxybenzoic acid	15
Caffeic acid	5

Table 6. Specificity determinations of pyrogallols.

Substrate	Relative activity %
Pyrogallol	100
1-O-Methylpyrogallol	70
2-O-Methylpyrogallol	250
2,3,4-Trihydroxybenzoic acid	20
3,4,5-Trihydroxybenzoic acid	10
4-Acetylpyrogallol	95
5-Ethylpyrogallol	65
4,6-Dimethyl-5-ethylpyrogallol	40

On the other hand, the LCAO coefficient for the oxygen atom in the highest occupied orbital, expressing the nucleophilic reactivity of the position, may indicate the position for methylation in the molecule. The compounds substituted in position 4 listed in Table 7 would then be methylated on the oxygen in 1 position. Resacetophenone was found to be methylated almost exclusively in *para* position. The determination was performed by recrystallization to constant specific radioactivity with known amounts of nonlabelled synthetic products.

Of the various catechols tested 3,4-dihydroxybenzaldehyde proved to be the most efficient substrate. By using the method described for resacetophenone the reaction mixture was shown to consist of 22 % 3-methoxy-4-hydroxybenzalde-

Table 7. Charge distributions in some resorcinols.

Substance	Rel.enz. activity	Oxygen-1			Oxygen-3		
		q_o*	c_h**	c_h^2	q_o	c_h	c_h^2
Resorcinol	100	1.9775	0.1426	0.0203	1.9775	0.1426	0.0203
4-Methyl resorcinol	14	1.9782	0.1535	0.0236	1.9783	0.1238	0.0153
5-Methyl »	68	1.9777	0.1424	0.0203	1.9777	0.1424	0.0203
4-Chloro »	120	1.9777	0.1507	0.0227	1.9778	0.1297	0.0168
4-Formyl »	28	1.9745	0.1279	0.0164	1.9739	0.0942	0.0089

* π-electrone charge
** LCAO coefficient

Table 8. Effects of ions on enzyme activity.

Salt	Substrate	% inhibition at salt concentrations of 10^{-2} M	10^{-3} M	10^{-4} M
$CuCl_2$	Catechol		100	75
	Resorcinol		90	25
	Pyrogallol		90	55
$NiCl_2$	Catechol	100	75	15
	Resorcinol	100	65	0
	Pyrogallol	100	95	20
$Zn(Ac)_2$	Catechol	100	95	70
	Resorcinol	100	100	55
	Pyrogallol	100	100	90
$CoCl_2$	Catechol	100	95	15
	Resorcinol	100	40	0
	Pyrogallol	100	100	20
$MnSO_4$	Catechol	90	65	
	Resorcinol	45	0	
	Pyrogallol	100	75	
$MgCl_2$	Catechol	−5*	0	
	Resorcinol	−5*	0	
	Pyrogallol	38	0	
$CaCl_2$	Catechol	0	0	
	Resorcinol	0	0	
	Pyrogallol	38	−20*	
KCN	Catechol		29	
	Resorcinol		0	
	Pyrogallol		65	

* The reaction is activated.

hyde and 78 % 4-methoxy-3-hydroxybenzaldehyde. The predominating methylation in *para* position is in accordance with the property of the methyltransferase isolated by Mann *et al.*[6] but distinguished from the catechol-O-transmethylase reported by Axelrod and Thomchick [7] which mainly methylates in *meta* position. These enzymes have in no case been found to act on resorcinol. K_m for catechol was found to be 3.0×10^{-3} M in presence of 7.0×10^{-6} M S-adenosylmethionine. As has already been mentioned pyrogallol as well as several of its derivatives can serve as substrate for the enzyme, the most efficient one being pyrogallol-2-methyl ether. In the methylation of pyrogallol the main product is pyrogallol-1-methyl ether constituting 80 % of the formed mixture. The content of pyrogallol-2-methylether is 16 % and the remaining 4 % probably being pyrogallol-1,2-dimethyl ether. In the methylation of pyrogallol there is a further difference in the specificity towards the positions of the hydroxyl groups compared to the catechol-O-transmethylases reported as they mainly methylate position 2 of pyrogallol. Perault and Pullman [8] have computed the net charge of the oxygen atoms in pyrogallol and found position 2 to be the favourable one for methylation. Apparently the charge distribution in the molecule is not always the predominating factor for directing the enzymic reaction. K_m for pyrogallol was determined to be 0.6×10^{-3} M at a concentration of 7.0×10^{-6} M S-adenosylmethionine.

In all series of phenols tested carboxylic acids have been found to be very poor substrates for the enzyme. Furthermore, β-resorcylic acid proved to be a competitive inhibitor for the methylation of resorcinol as shown with the Lineweaver and Burk [9] plot in Fig. 5.

Ion effects on the enzyme. Axelrod and Thomchick found that the catechol-O-methyltransferase isolated from rat liver required the presence of Mg^{2+} or some other divalent metal ions for being active. The plant enzyme isolated from *Nerine bowdenii*,[5] on the other hand, showed no sensitivity to metal ions. Investigation of the ion effects on the enzyme from *S. rimosus* established no requirement of metal ions but pronounced inhibition was obtained in presence of different ions such as Cu^{2+}, Ni^{2+}, Zn^{2+}, Co^{2+}, Mn^{2+}, and CN^{+} (Table 8).

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