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Fluorimetric Assay for Catechol-O-methyltransferase

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A sensitive fluorimetric method for the assay of catechol-O-methyltransferase in a sample solution prepared from rat liver is described. Vanillin formed from the substrate, 3,4-dihydroxybenzaldehyde, under the optimal conditions for the enzyme reaction, is determined after the removal of unreacted substrate by adsorption on alumina. Vanillin is measured by the established fluorimetric method for the determination of aromatic aldehydes with 2,2′-dithiobis(1-aminonaphthalene). The limit of determination for vanillin formed is 300 pmol. The method is readily performed with good precision and is suitable for the rapid assay of catechol-O-methyltransferase in a small amount of sample solution.

Keywords——catechol-O-methyltransferase; rat liver; 3,4-dihydroxybenzaldehyde; S-adenosylmethionine; vanillin; fluorimetry; 2,2′-dithiobis(1-aminonaphthalene)

Catechol-O-methyltransferase (COMT; EC 2.1.1.6) occurs widely in mammalian tissues and catalyzes the transfer of the methyl group of S-adenosyl-L-methionine (SAM) to one of the phenolic groups of catechol or substituted catechol in the presence of a divalent metal cation such as Mg^{2+}.[1]

Many methods have been reported for the assay of COMT in sample solutions from tissue homogenates. Methods using radiochemical,[2–7] fluorimetric[1,8–10] and spectrophotometric[11–14] techniques seem to be most effective. Among these, radiochemical assays appear to be most sensitive, but radioactive substrates or SAM are expensive and not easy to handle. In other methods, pulse polarographic,[15] high-performance liquid chromatographic (with electrochemical[16] and spectrophotometric[17] detection), thin-layer chromatographic[18] and gas chromatographic[19] techniques were also used.

Many kinds of catechols have been used as substrates; *e.g.* catecholamines (norepinephrine,[1,4,15,16] epinephrine[1,2,7,12] and dopamine[1]), catechol acids (3,4-dihydroxyphenylacetic acid[8,9] and 3,4-dihydroxybenzoic acid[3,6,17]), bi- and polycyclic catechols (6,7-dihydroxycoumarin[10] and 2-hydroxyestrone[5]), catechols having un-ionized substituents (3,4-dihydroxyacetophenone[14] and nitrocatechol[13]) and many other catechols.[18]

We have developed a fluorimetric method for the assay of COMT in sample solutions from tissue homogenates. 3,4-Dihydroxybenzaldehyde is used as the substrate and vanillin formed under the optimum conditions for the enzyme reaction is determined by the previously reported method for selective and sensitive determination of aromatic aldehydes with 2,2′-dithiobis-(1-aminonaphthalene)[20,21] (DTAN), after removal of unreacted substrate by adsorption on alumina. A COMT preparation from rat liver was employed to establish the assay procedure.

Experimental

Reagents and Solutions——All chemicals were of reagent grade, unless otherwise noted. Deionized and distilled water was used. DTAN was prepared as described previously.[20] 3,4-Dihydroxybenzaldehyde was recrystallized from toluene. A COMT preparation from porcine liver (1000—2000 units per mg protein) and Chelex-100 (a chelating cation-exchange resin; Na^+ form, 200—400 mesh) were obtained from Sigma (St. Louis, Mo., U.S.A.) and Bio-Rad (Richmond, Calif., U.S.A.), respectively.

DTAN Solution: DTAN (40 mg) was dissolved in 6.0 ml of methanol, then 0.1 ml of tri-*n*-butylphosphine was added. The solution became clear on standing for several min, then 25.0 ml of 30% (v/v) H_2SO_4 was added, and the mixture was diluted with water to 100 ml. The solution was stable for at least 1 week

when stored at 4°.

2-Mercaptoethanol Solution: 2-Mercaptoethanol (5.0 ml) was added to 33.5 ml of 30% (v/v) H_2SO_4 and diluted with water to 50 ml. The solution was usable for 3 days.

COMT Preparation from Rat Liver: Supernatant from rat liver homogenate was used as a COMT preparation; it was prepared by the method of Parvez and Gripois[22] with a minor modification as follows. Adult (9- to 10-week-old) Donryu male rats were stunned and exsanguinated, and the livers were immediately removed and chilled on ice. All further procedures were carried out at 0—5°. The livers were trimmed free of fat and weighed. The liver (36 g) was homogenized with 144 ml of 1.15% (w/v) KCl solution in a Potter-Elvehjem homogenizer. The homogenate was centrifuged at 5500×**g** for 30 min. The protein concentration was adjusted to 80 μg or less/20 μl with 1.15% (w/v) KCl solution, and measured by the method of Lowry *et al.*[23] with bovine serum albumin as a standard protein.

Apparatus——Fluorescence spectra and intensities were measured with a Hitachi MPF-4 spectrofluorimeter using quartz cells of 1×1-cm optical pathlengths. The slit widths in terms of wavelength were set at 10 nm in both the exciter and analyzer. The fluorescence spectra are uncorrected. Centrifugation was carried out using a Sakuma 45-CFS or a Hitachi 05 PR-22 refrigerated centrifuge.

Procedure——Substrate-cofactor solution consisted of 20 μl of 500 mM phosphate buffer (pH 6.6), 10 μl of 150 mM $MgCl_2$ solution, and 50 μl of 750 μM 3,4-dihydroxybenzaldehyde solution. To this solution, 20 μl of the COMT preparation was added. The mixture was preincubated at 37° for 5 min, then incubated again at 37° for 30 min after addition of 50 μl of 900 μM SAM solution. The reaction was stopped by heating the mixture at 100° for 5 min, then 2.0 ml of 50 mM Tris-HCl buffer (pH 8.5) was added. The mixture was centrifuged at *ca.* 800×**g** for 10 min. The supernatant (*ca.* 2.0 ml) was poured onto a glass column (130 mm×4 mm I.D.) packed with 300 mg of neutral alumina (AG 7, 100—200 mesh: Bio-Rad). The column could not be re-used. To 1.0 ml of the eluate, 2.0 ml of DTAN solution and 500 μl each of 0.05% (w/v) Na_2SO_3 solution and 15% (w/v) $Na_2HPO_3 \cdot 5H_2O$ solution (both freshly prepared; accelerators of the fluorescence reaction) were successively added. The mixture was allowed to stand at 37° for 30 min to develop the fluorescence. 2-Mercaptoethanol solution (1.0 ml) was added to stop the fluorescence reaction. For the blank, the same procedure was carried out except that SAM solution was added just before heating the incubated mixture. To obtain a standard curve, 50 μl of SAM solution was replaced with 50 μl of vanillin standard solution (2—10 nmol/50 μl). The fluorescence intensities were measured at 460 nm with excitation at 380 nm.

Results and Discussion

The optimum pH for the enzyme reaction was at 6.6, and the maximum activity was attained in phosphate buffer at a concentration of 67 mM in the incubation mixture, for both the rat and porcine liver COMT preparations (Fig. 1). However, Bade *et al.*[24] reported that the enzyme was most active at pH 8.0 in 100 mM phosphate buffer when the same substrate, 3,4-dihydroxybenzaldehyde, was used. The reason for the discrepancy between the pH optima remains unclear.

The Michaelis constant (K_m) for 3,4-dihydroxybenzaldehyde was obtained as 23 μM, so 250 μM in the incubation mixture was used as a saturating concentration for the enzyme reaction. This K_m value is different from those described by Bade *et al.* (4 μM in 100 mM phosphate buffer of pH 7.9)[24] and Creveling *et al.* (1 mM in 100 mM phosphate buffer of pH 8.0).[18] Maximum activity was obtained in the presence of 300 μM SAM with the observed K_m value at 50 μM, and inhibition occurred at 350 μM (Fig. 2); thus, 300 μM in the incubation mixture was used in our procedure. Mg^{2+} provided maximum activity at 8 mM in the incubation mixture (Fig. 3). Weinshilboum and Raymond[25] reported that the activation of rat liver COMT by Mg^{2+} is reduced in the presence of Ca^{2+}. However, the removal of Ca^{2+} from the enzyme preparation with Chelex-100[26,27] has no effect on the enzyme activity. Aldehyde dehydrogenase, if present in the enzyme preparation, may cause loss of 3,4-dihydroxybenzaldehyde and vanillin during incubation. The COMT activity, however, does not change even when measured after addition of the aldehyde dehydrogenase inhibitor, tetraethylthiuram disulfide, to the incubation mixture.

The enzyme reaction was linear with time up to at least 90 min, when a mixture containing 80 μg or less protein was incubated at 37° (Fig. 4); 80 μg or less of protein per 20 μl of the enzyme preparation was incubated for 30 min in the recommended procedure.

Optimal conditions for the fluorescence reaction of vanillin with DTAN were identical with those for *p*-hydroxybenzaldehyde.[20] The fluorescence excitation (maximum, 380 nm)

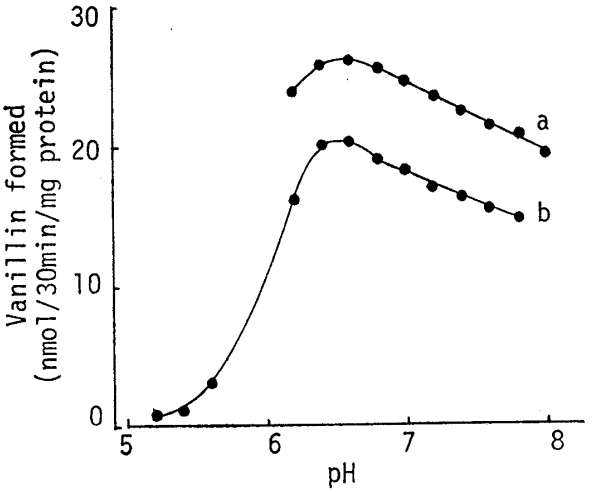

Fig. 1. Effect of pH on COMT Activity

Portions (20 μl) of COMT preparations were treated by the standard procedure but with 500 mM phosphate buffer of various pHs. Mean values of duplicate determinations were plotted.

a: The COMT preparation from rat liver (protein concentration, 80 μg/20 μl); b: COMT preparation from porcine liver (protein concentration, *ca.* 1 μg/20 μl; 1.15% (w/v) potassium chloride solution).

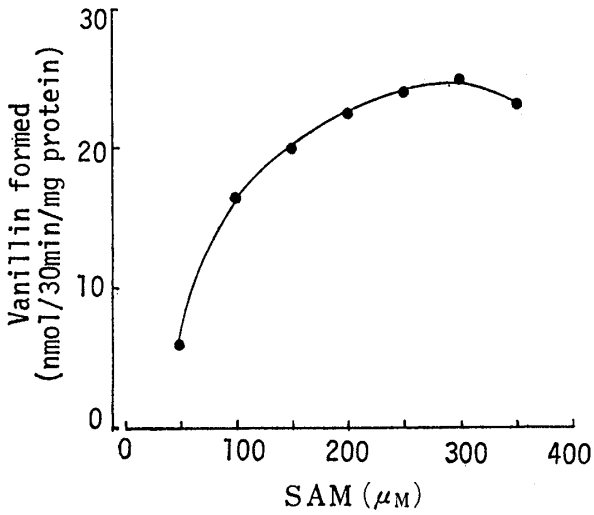

Fig. 2. Effect of SAM Concentration on COMT Activity

Portions (20 μl) of the COMT preparation from rat liver (protein concentration, 80 μg/20 μl) were treated by the standard procedure but with various concentrations of SAM. Mean values of duplicate determinations were plotted.

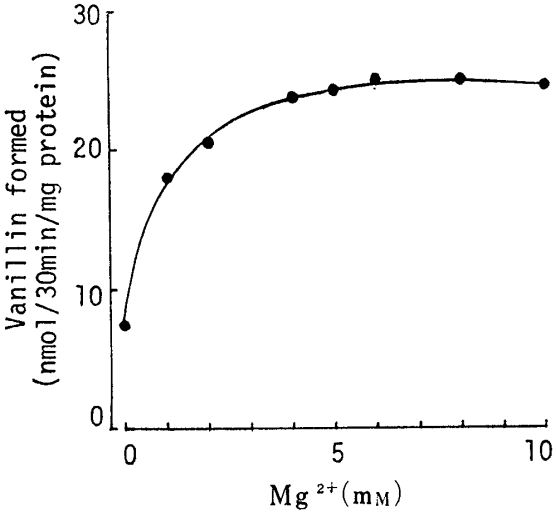

Fig. 3. Effect of Mg^{2+} Concentration on COMT Activity

Portions (20 μl) of the COMT preparation from rat liver (protein concentration, 80 μg/20 μl) were treated by the standard procedure but with various concentrations of $MgCl_2$. Mean values of duplicate determinations were plotted.

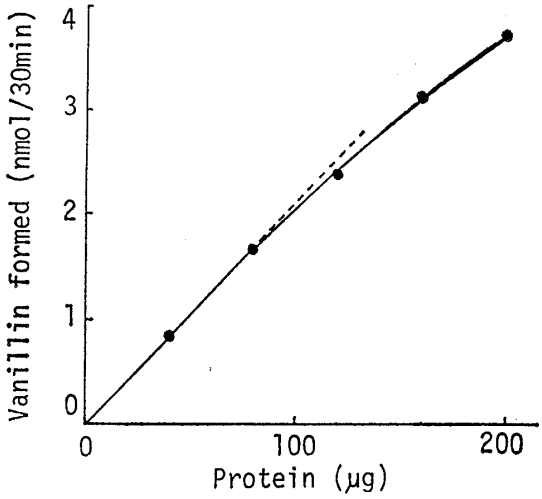

Fig. 4. Effect of Protein Concentration on the Amount of Vanillin Formed

Portions (20 μl) of the COMT preparation from rat liver with various concentrations of proteins were treated by the standard procedure with an incubation time of 30 min. Mean values of duplicate determinations were plotted.

and emission (maximum, 460 nm) spectra for the final reaction mixture are identical with those for the reaction mixture of vanillin with DTAN. The standard curve was linear up to at least 10 nmol of vanillin, and passed through the origin. Isovanillin, the *p*-O-methylation product of the substrate,[5,18] gives a weak fluorescence with an excitation maximum at 380 nm and emission maximum at 460 nm; the intensity is only 1.6% of that given by vanilin at equimolar concentration. Therefore, the present procedure practically measures only vanillin.

3,4-Dihydroxybenzaldehyde fluoresces under the conditions of the fluorescence reaction. The fluorescence, with excitation and emission maxima at 380 and 460 nm, respectively, is

almost 1/5 as intense as that given by vanillin, at equimolar concentration. Therefore, unreacted 3,4-dihydroxybenzaldehyde should be removed from the incubated enzyme reaction mixture, which can be done by adsorption on alumina in an alkaline medium. A constant adsorption rate of 3,4-dihydroxybenzaldehyde on alumina was attained in Tris–HCl buffer of pH 8.0—9.0 or in ammonium hydroxide-ammonium chloride buffer of pH 8.5—10.0, but not in borate buffer. Ammonium hydroxide-ammonium chloride buffer gave less complete adsorption and so Tris–HCl buffer was used. Its concentration affected the adsorption, and concentrations ranging from 25—150 mM gave maximum adsorption. Thus, 50 mM Tris–HCl buffer of pH 8.5 was selected as the optimum. Although amounts of alumina higher than 75 mg gave constant adsorption of the aldehyde, the fluorescence intensity of the blank was minimum when 300 mg of alumina was used; thus, 300 mg of alumina was used in the procedure. The adsorption of the aldehyde (2×10^{-4} M) under the conditions recommended was complete (99% or more).

The recovery of vanillin (2, 4, 6, 8 and 10 nmol) added to the incubated enzyme reaction mixture was 96±2% (mean±SD, $n=3$ each). The lower limit of determination for vanillin was 300 pmol. The limit was defined as the amount which gave a fluorescence intensity of twice the blank value. This sensitivity may also permit the assay of COMT in preparations obtained from other tissues, *e.g.* kidney, spleen, small intestine, lung, brain and heart muscle, but is not sufficient to assay the enzyme in preparations from erythrocytes, which usually have low activity. The precision was also established as regards repeatability. The coefficient of variation was 2.3% ($n=10$) for a mean activity of 26.1 nmol per 30 min per mg protein.

This study provides the first fluorimetric method for the assay of COMT by measuring the amount of vanillin formed enzymatically from 3,4-dihydroxybenzaldehyde. The entire procedure takes less than 2 hr and more than 20 samples can be assayed simultaneously. Therefore, this method should be useful for biological investigations.

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