A Differential Determination Procedure for Spermine and Spermidine with Beef Plasma Amine Oxidase

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An enzymatic procedure for the differential determination of polyamines, spermine and spermidine, has been established using beef plasma amine oxidase. This method was specific for these polyamines and required only one reaction system. Small amounts of polyamines (10 μM to 80 μM of spermine and 10 μM to 100 μM of spermidine) were assayed by solving two simultaneous equations obtained from the rate assay method and the end point assay method. The calculated values were in good agreement with those obtained by other method.

The polyamines, spermine and spermidine, which are ubiquitous in living organisms, vary in the amount with the type and physiological state of cells.^{1,2)} The increase of polyamines and the change of these ratio in rapidly growing tissues might accompany the increase of cell death rate in malignancy. Since Russell³⁾ first reported that polyamine content in urine of patients with malignancy showed the increased level compared with that of normal volunteers, there have been many reports on increased polyamines in urine and blood of patients with cancer.^{4~9)} The decrease of polyamine after successful treatment of various types of malignancy was also reported.^{10,11)} Chemical methods to determine polyamines require at least two steps which are the transformation into suitable derivatives followed by the separation with gas chromatography¹²⁾ or ion-exchange liquid chromatography.⁹⁾ On the other hand, biochemical methods might allow to assay these without any separation step. However, the difficulties to distinguish between spermine and spermidine, and to prepare an enzyme have not given much attention to the enzymatic method. Only a few systems such as *Serratia marcescens* enzyme—beef plasma amine oxidase system¹³⁾ and pea amine

oxidase—maize amine oxidase system have been reported.¹⁴⁾

We attempted to establish a rapid, simple and efficient method to determine polyamines for the clinical studies especially in the diagnosis and therapy of cancer. In this paper is described a differential determination procedure for spermine and spermidine using beef plasma amine oxidase by the combining the rate assay method and the end point assay method.

MATERIALS AND METHODS

Chemicals. Spermine tetrahydrochloride, spermidine trihydrochloride and all other chemicals were obtained from commercial sources and used without further purification.

Enzyme preparation. Beef plasma amine oxidase, which was crystallized according to the procedure of Yamada and Yasunobu¹⁵⁾ with a slight modification and had a specific activity of above 300 units/mg, was used in this study. The enzyme activity was assayed with a spectrophotometrical method using benzylamine as the substrate as described by Tabor *et al.*¹⁶⁾ One unit was defined as the amount of enzyme catalyzing a change of 0.001 absorbance unit per min under the assay conditions.¹⁶⁾

Standard assay for spermine and spermidine. Spermine and spermidine were assayed by measuring the amount of quinonimine dye based on the formation of H_2O_2 . The standard reaction mixture (2.0 ml)

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containing 0.0125 μmol to 0.80 μmol of spermine and/or spermidine, 0.74 μmol of 4-aminoantipyrine, 0.034 μmol of phenol, and 7.5 units of peroxidase in 0.1 M potassium phosphate buffer (pH 7.2) was preincubated for 3 min at 37°C. Then, the enzyme solution (0.5 ml) was added and the reaction was allowed to proceed at 37°C. The velocity of quinonimine dye formation was followed by measuring the absorbance change at 505nm in a cuvette of 1-cm light path with a Shimadzu model UV 200 spectrophotometer. In the rate assay method the velocity of quinonimine dye formation per initial one min was measured. The end point assay method was based on quinonimine dye formation after 90-min incubation.

RESULTS AND DISCUSSION

Oxidation of various substrates

Various monoamines, diamines, and polyamines were tested as the substrate for beef plasma amine oxidase with the rate assay method (Table I). Spermine and spermidine were rapidly oxidized, but monoamines and diamines were not significantly oxidized under

TABLE I. RELATIVE RATE OF OXIDATION OF VARIOUS AMINES BY BEEF PLASMA AMINE OXIDASE

The enzyme activity was determined by the rate assay method under the standard assay conditions with 0.125 μmol of the substrate and 0.25 mg of the enzyme in 2.5 ml.

Substrate	Relative rate (%)
Monoamine	
Methylamine	1.6
Ethylamine	0.5
Propylamine	1.3
Butylamine	2.9
Amylamine	3.1
Phenethylamine	2.1
Tyramine	0.8
Dopamine	4.7
Tryptamine	0.8
Serotonin	0.0
Benzylamine	4.9
Histamine	0.5
Agmatine	0.5
Diamine	
Cadaverine	0.0
Putrescine	0.8
Polyamine	
Spermidine	20.3
Spermine	100

the assay conditions employed. These values were considerably different from those of Yamada *et al.*¹⁵⁾ The oxidation rate of spermidine to spermine in the present study was 20/100, while that of the former work was 10/10. The difference may be mainly due to that of the reaction condition, e. g. the substrate concentration and the incubation time. The former values were obtained by measuring the oxygen consumption after 10-min incubation with 6 μmol of each substrate in 2.5 ml.

Kinetics of the enzyme reaction

The progress of enzyme reaction was linear with respect to the time under the conditions used, and the rate (the slope of the progress curve) was proportional to the enzyme concentration. The apparent Michaelis constants for spermine and spermidine were calculated to be 9.8×10^{-6} M and 4.85×10^{-4} M, respectively, under the aerobic conditions, and relative maximum velocities for both spermine and spermidine were 2.62×10^{-4} M (H_2O_2)/min/mg of protein.

Stoichiometry of polyamine oxidation

The complete oxidation of spermine (5~160 μM ; 0.0125~0.40 μmol per 2.5 ml reaction mixture) and spermidine (5~320 μM ; 0.0125~0.80 μmol per 2.5 ml reaction mixture)

FIG. 1. Stoichiometry of Spermine and Spermidine Oxidation.

The reaction was completed with 0.25 mg of enzyme in 2.5 ml of the reaction mixture for 90 min under the standard conditions described in MATERIALS AND METHODS except for the substrate concentration. (A), spermine; (B), spermidine.

SCHEME 1. Oxidation of Polyamines by Beef Plasma Amine Oxidase.

and the formation of corresponding amounts of H_2O_2 were obtained after 90-min incubation (the reaction is completed.) using 40 units (per 2.5 ml) of enzyme (Fig. 1). In this case, 1 mol of spermine and spermidine gave rise to 2 mol and 1 mol of H_2O_2 , respectively as shown in Scheme 1,¹⁷⁾ when spermine and spermidine were oxidized by the beef plasma amine oxidase. The molar absorption for quinonimine dye was $12.5 \times 10^3 \text{ M}^{-1}\text{cm}^{-1}$ for the oxidation of spermine, and $6.25 \times 10^3 \text{ M}^{-1}\text{cm}^{-1}$ for that of spermidine, respectively.

Effect of amines on spermine oxidation

As shown in Table II, the appreciable interference on the oxidation of spermine with beef plasma amine oxidase was observed when an equimolar amount of tyramine, dopamine, tryptamine or serotonin was added in the reaction mixture. But the coexistence of these does not give any significant effect on the determination of spermine, since the amounts of these amines were scarce (less than one tenth) compared with that of the polyamines in all tissues.¹⁸⁾

Differentiation of spermine and spermidine

Beef plasma amine oxidase oxidized spermine and spermidine. The optical densities of spermine and spermidine in the rate assay method were in the ratio of 5 to 1 (Table I). The 5/1 ratio is most reasonably brought about from the difference in K_m values of spermine and spermidine. On the other hand, the optical densities of spermine and spermidine in the end point assay method were in the ratio of 2 to 1 (Fig. 1). The 2/1 ratio undoubtedly comes from the difference of the stoichiometry.

TABLE II. EFFECT OF VARIOUS SUBSTRATES ON SPERMINE OXIDATION.

Each amine (0.125 μmol) was added to the standard reaction mixture containing 0.125 μmol of spermine in 2.5 ml.

Substrate	Relative rate (%)	
	Rate assay	End point assay
Monoamine		
Methylamine	94	100
Ethylamine	100	100
Propylamine	96	99
Butylamine	98	100
Amylamine	96	100
Phenethylamine	88	99
Tyramine	78	93
Dopamine	30	26
Tryptamine	55	99
Serotonin	34	53
Histamine	96	100
Agmatine	85	98
Benzylamine	95	98
Diamine		
Cadaverine	99	100
Putrescine	100	100
Polyamine		
Spermidine	83	100
None	100	100

* Relative rate

$$= \frac{[\text{SPM} + \text{Amine}]_{805} - [\text{Amine}]_{805}}{[\text{SPM}]_{805}} \times 100$$

We tried to differentiate between spermine and spermidine by using these two different assay methods. The addition of control serum to the reaction mixture expanded the range of linearity of spermine oxidation into 80 μM in the rate assay method (linearity without control serum was below 30 μM .) and did not affect on the rate of spermine oxidation even in the presence of an equimolar amount of spermidine (the inhibition percent without control serum was about 17%). Moreover, the oxidation ratio (5/1 or 2/1) between spermine and spermidine did not change. Therefore, experiments thereafter were carried out in the presence of control serum.

As shown in Fig. 2, the optical density values of spermine and spermidine in the rate assay method were linear to 80 and 100 μM , respectively. When 10 to 100 μM of spermidine was added to the reaction mixture containing 10 to

FIG. 2. Colorimetric Assay for Spermine and Spermidine Based on the Rate Assay Method.

The reaction was carried out with 70 units of enzyme per 2.5 ml under the standard conditions except for the substrate concentration. (A), spermine; (B), spermidine; -----, spermine+spermidine.

80 μM of spermine, the slope was parallel to that of spermidine alone. Thus, the optical density values (O. D. per min) of spermine (y_M^R) and spermidine (y_D^R) in the rate assay method (the enzyme concentration, 70 units per 2.5 ml) could be defined by eqs. 1 and 2, respectively.

$$y_M^R = \frac{0.110}{100} [\text{SPM}] \quad (1)$$

$$y_D^R = \frac{0.022}{100} [\text{SPD}] \quad (2)$$

where, [SPM] and [SPD] represent the concentrations of spermine and spermidine, respectively, in μM units. The optical density value (Y_{M+D}^R) of spermine plus spermidine in the rate assay method with the mixture of spermine and spermidine was defined by eq. 3.

$$\begin{aligned} Y_{M+D}^R &= y_M^R + y_D^R \\ &= \frac{0.110}{100} [\text{SPM}] + \frac{0.022}{100} [\text{SPD}] \end{aligned} \quad (3)$$

As shown in Fig. 3, the optical density of spermine and spermidine in the end point assay method was linear at least below the absorbance of 2.0 at 505 nm under the standard assay conditions employed here. When 10 to 100 μM of spermidine was added to the reaction mixture containing 10 to 80 μM of spermine, the optical density of spermine plus spermidine was obtained. Thus, the optical

FIG. 3. Colorimetric Assay for Spermine and Spermidine Based on the End Point Assay Method.

The reaction mixture used in Fig. 2 was further incubated for 90 min.

(A), spermine; (B), spermidine; -----, spermine+spermidine.

densities of spermine (y_M^E) and spermidine (y_D^E) in the end point assay method were defined by eqs. 4 and 5, respectively.

$$y_M^E = \frac{1.08}{100} [\text{SPM}] \quad (4)$$

$$y_D^E = \frac{0.54}{100} [\text{SPD}] \quad (5)$$

The optical density (Y_{M+D}^E) of spermine plus spermidine in the end point assay method was defined by eq. 6.

$$\begin{aligned} Y_{M+D}^E &= y_M^E + y_D^E \\ &= \frac{1.08}{100} [\text{SPM}] + \frac{0.54}{100} [\text{SPD}] \end{aligned} \quad (6)$$

Therefore, the concentrations of spermine and of spermidine could be calculated by solving the simultaneous equations, (3) and (6). Equations 4~6 for the end point assay method were always unchanged regardless of the enzyme concentration, as would be expected. The oxidation ratio between spermine and spermidine was constant regardless of the enzyme concentration. However, the coefficients of eqs. 1~3 for the rate assay method are of course directly proportional to the enzyme concentration. For example, when $Y_{M+D}^R = 0.013$ (O.D. per min) for the rate assay method and $Y_{M+D}^E = 0.160$ (O.D.) for end point assay method were

SCHEME 2. Oxidation of Polyamines by Maize Amine Oxidase.

obtained with this method, the concentrations of spermine and spermidine were calculated as $9.8 \mu\text{M}$ ($=0.0245 \mu\text{mol}$ per 2.5 ml) and $10.0 \mu\text{M}$ ($=0.025 \mu\text{mol}$ per 2.5 ml), respectively. The result obtained with this method was in good agreement with that obtained with the other procedure; Maize amine oxidase gave rise to 1 mol of H_2O_2 from 1 mol of spermine or spermidine in the end point assay method (Scheme 2),¹⁹ in contrast to the beef plasma amine oxidase which gives 2 mol and 1 mol of H_2O_2 for spermine and spermidine, respectively. Therefore, concentrations of spermine and spermidine can be calculated by solving simultaneous equations for the end point assay method with maize and beef plasma amine oxidase. This procedure gave the result; $9.6 \mu\text{M}$ for spermine and $10.3 \mu\text{M}$ for spermidine, respectively, for the same sample as that described above.

This enzymatic procedure based on the combination of the rate assay method and the end point assay method described here is a simple and useful means for the rapid determination of spermine and/or spermidine in biological materials. The present method is more useful and convenient than other enzymatic procedures which use two amine oxidase,^{13,14} since it requires only one enzyme reaction system.

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